University of Western Sydney

Tetragonula carbonaria and disease: Behavioural and antimicrobial defences used by colonies to limit brood pathogens

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Our treasure lies in the beehive of our knowledge.

We are perpetually on the way thither,

being by nature winged insects and honey gatherers of the mind.

Friedrich Nietzsche (1844 – 1900)

Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, whether in full or in part, for a degree at this or any other institution

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Jenny Shanks

July 2015

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What a journey. We made it.

Preface

I have presented this thesis as a series of four experimental chapters in a traditional dissertation format. This thesis includes an introductory chapter, including literature review and aims and scope sections (Chapter 1). The thesis finishes with a concluding chapter (Chapter 6) that discusses key findings, their significance and applications, and outlines prospects for future research. The study was conceptualised by myself and the supervisory panel, Assoc. Prof Robert Spooner-Hart (Principal), Dr Tony Haigh, and Assoc. Prof Markus Riegler. The supervisory panel and I discussed and decided the aims, hypotheses, and experimental design of the chapters. I have undertaken the experimental work; analysed the data; and written the chapters, with input and feedback by the supervisory panel, and external persons where acknowledged in text. The structure of this thesis is given below. Any supplementary tables and figures of each chapter were presented in Appendices, as detailed below:

Chapter 1: **Introductory chapter**

Chapter 2: **Hive management and investigations into nest thermoregulation**. Supplementary video is provided in Appendix 1

Chapter 3: **Hygienic behaviour expression in *Tetragonula carbonaria*.** Supplementary videos, tables, and figures are provided in Appendix 2

Chapter 4**: Suppression of insect pathogens by the antimicrobial activity of stingless bee nest products.** Supplementary tables and figures are provided in Appendix 3

Chapter 5: **First documented brood pathogen in a stingless bee species, *Tetragonula carbonaria*.** Supplementary tables and figures are provided in Appendix 4

Chapter 6: **Thesis Discussion**

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Unless otherwise stated, the photographs, images and videos within this dissertation were produced by Jenny Shanks.

Abbreviations

AEDT	Australian eastern daylight time
AEST	Australian eastern standard time
AFB	American foulbrood
asl	Above sea level
bp	Base pair(s)
CFU	Colony forming units
d	Day(s)
DAT	Days after treatment
DMSO	Dimethyl sulfoxide
EBP	Estimated brood population
EFB	European foulbrood
h	Hour(s)
HAT	Hours after treatment
IZ	Inhibitory zone
LAB	Lactic acid bacteria
LC-MS	Liquid chromatography-mass spectrometry
exoTSAP	Exonuclease I, Thermosensitive alkaline phosphatase mixture
MEGA	Molecular Evolutionary Genetics Analysis
MLST	Multi Locus Sequencing Typing
MRS	de Man, Rogosa and Sharpe agar
NA	Nutrient agar
NGS	Next Generation Sequencing
NSW	New South Wales
OP	Observation Platform
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
QLD	Queensland
rDNA	Ribosomal DNA
SBA	Sheep blood agar with nalidixic acid
SCU	Southern Cross University
SDA	Sabouraud dextrose agar
SE	Standard error

STDEV	Standard deviation
SDA	Sabouraud dextrose agar
STE	Sodium chloride- Tris-EDTA buffer
TEM	Transmission electron microscopy
UWS	University of Western Sydney

Glossary

Batumen	a layer of hardened cerumen at the top and bottom of a nest space, with holes to allow for ventilation
Brood	nursery for rearing young
Callow	newly emerged adult, up to one week old and distinguishable by its lighter body colour
Cerumen	propolis mixed with wax secreted from the dorsal surface of worker abdomens, and used as the building material in stingless bee colonies
Colony	a unit of bees cooperating to rear young
Drone	honey bee male
Endotherm	an animal that is dependent on, or capable of, the internal generation of heat
Ectotherm	an animal that is dependent on external sources for body heat
Eurytherm	an organism which is adapted to functioning at a wide range of temperatures. Is the opposite of stenothermic
Gyne	reproductive, unmated queen; a virgin queen
Hive	a nest housed inside either an artificial managed box, or a natural feature such as a hollow tree
Incidence	the first occurrence of a brood disease in a stingless bee colony
Involucrum	multiple layers of cerumen around brood chamber
Nest	a colony, with all internal structures, and food stores
Physogastric queen	a mated queen
Poikilotherm	an organism that cannot regulate its body temperature except by behavioural means such as basking or burrowing. Dependent upon the temperature of its environment
Propolis	plant resins mixed with bee salivary gland secretions

Queenless	a queenless colony that does not have a functional queen. This includes a colony with no physogastric queen, one with only a virgin queen or one with a queen which is not laying viable eggs
Resin	sticky sap secreted by plant wounds, young leaves, buds, fruits and plant inflorescences. Collected resin is used to create propolis
Scales	the dried remains of *Paenibacillus larvae*-infected larva in brood cells
Splitting	one type of hive propagation, where a managed artificially boxed hive is split horizontally into two, to form two new separate hives
Stenothermic	a living organisms capable of surviving within a narrow temperature range. Is the opposite of eurytherm
Trophallaxis	the mutual exchange of regurgitated liquids between adult social insects or between them and their larvae

Species referred to in this thesis

As a large number of bees, and pathogens will be referred to in this dissertation; a species list has been compiled. In order to assist text flow, only scientific names will be included.

Current scientific name	Authority	Common name
HONEY BEES		
Apis mellifera caucasia	Pollmann, 1889	Caucasian honey bee
Apis cerana	Fabricius, 1793	Asian honey bee
Apis cerana indica	Fabricius, 1798	
Apis cerana japonica	Radoszkowski, 1887	
Apis dorsata	Fabricius, 1793	Giant honey bee
Apis florea	Fabricius, 1787	Dwarf honey bee
Apis mellifera	Linnaeus, 1758	European honey bee
Apis mellifera carnica	Pollmann, 1879	Carniolan honey bee
Apis mellifera intermissa	Maa, 1953	
Apis mellifera lamarckii	Cockerell, 1906	Lamarck's honey bee
Apis mellifera ligustica	Spinola, 1806	Italian honey bee
Apis mellifera scutellata	Lepeletier, 1836	African honey bee
Apis m. scutellata x A. m ligustica		Africanised honey bee
BUMBLE BEES		
Bombus impatiens	Cresson, 1863	Eastern bumble bee
Bombus terrestris	Linnaeus, 1758	Buff-tail bumble bee
Bombus wilmattae	Cockerell, 1912	
Bombus vosnesenskii	Radoszkowski, 1862	Yellow-faced bumble bee
STINGLESS BEES		
Austroplebeia australis	Friese, 1898	kootchar
Cretpotrigona prisca	Michener & Grimaldi, 1988	
Frieseomelitta schrottkyi	Friese, 1900	
Frieseomelitta silvestrii	Friese, 1920	
Frieseomelitta varia	Lepeletier, 1836	
Leurotrigona muelleri	Friese, 1900	lambe-olhos
Melipona beecheii	Bennet, 1831	colecab
Melipona bicolor bicolor	Lepeletier, 1836	
Melipona compressipes fasciculata	Smith, 1854	
Melipona colimama	Ayala, 1999	

Melipona panamica	Cockerell, 1912	
Melipona compressipes	Fabricius, 1804	tiúba
Melipona quadrifasciata	Lepeletier, 1836	mandaçaia
Melipona seminigra	Friese, 1903	
Melipona rufiventris	Lepeletier, 1836	
Plebeia droryana	Friese, 1900	lambeojitos
Plebeia remota	Holmberg, 1903	
Proplebeia dominicana	Wille & Chandler, 1964	
Scaptotrigona depilis	Moure, 1942	torce cabelos
Scaptotrigona pectoralis	Dalla Torre, 1896	magua canche
Scaptotrigona postica	Latreille, 1807	
Tetragonisca angustula	Latreille, 1811	jataí
Tetragonisca angustula fiebrigi	Schwarz, 1938	
Tetragonula carbonaria	Smith, 1854	karbi
Tetragonula hockingsi	Cockerell, 1929	
Trigona denoiti	Vachal, 1903	
Trigona (Frieseomelitta) nigra paupera	Moure, 1963	
Trigona spinipes	Fabricius, 1793	
Trigona weyrauchi	Schwarz, 1943	
Trigona ventralis hoozana	Strand, 1913	
PATHOGENS		
Ascosphaera apis	Massen ex. Claussen, Olive & Spiltoir, 1955	Chalkbrood
Lysinibacillus sphaericus	Ahmed *et al.* 2007	
Metarhizium anisopliae	Driver & Milner, 2000	Green muscardine disease
Melissococcus plutonius	White, 1912, Bailey & Collins 1983	European foulbrood
Paenibacillus alvei	Cheshire & Cheyne, 1885	
Paenibacillus larvae	White 1906, Ash et al. 1994	American foulbrood

Thesis summary

The honey bee, *Apis mellifera*, is suffering heavily from the impacts from intensive management. Pests and diseases contribute to the population losses experienced globally. Brood disease is of concern for the apiculture industry because of the direct effects it has on population numbers and despite control measures; resistance to antibiotics and pesticides are common. Alternative pollinators such as stingless bees, including *Tetragonula carbonaria*, appear to be less impacted by brood diseases. However, there is very little information regarding why this is so. Prior to this study, there are only a few indications about a possible bacterial brood disease in Brazilian stingless bees (Kerr 1948, Nogueira-Neto 1997), with no follow up investigations, and no cases of brood disease losses in Australian stingless bees. As a result, this study presents information on the behavioural and antimicrobial defences of *T. carbonaria* colonies as mechanisms to limit the development of brood pathogens. In addition to these aims and objectives, this study also introduces and documents the first disease causing brood pathogen in Australian stingless bees. Therefore, the interaction of the defence mechanisms and the identified brood pathogen was also explored.

Suitable nest conditions need to exist to sustain pathogen growth and development. *Apis mellifera* pathogens such as *Paenibacillus larvae* and *Ascosphaera apis* utilise nest conditions, especially in the brood area for growth. The limited number of stingless bee pathogens may be related to brood temperature. Thermoregulation behaviour has been investigated in a number of stingless bee species; however, Australian studies are limited to *Austroplebeia australis* (Halcroft et al. 2013b) and greenhouse maintained *T. carbonaria* colonies (Amano et al. 2000, Amano 2004, A. Tse, pers. comm., 2011), with outcomes applied to their pollination servicing. This study (Chapter 2) investigated *T. carbonaria* thermoregulation behaviours during fluctuating ambient temperatures and the influence these have on brood production. Over the 13-month study, *T. carbonaria* was able to maintain brood temperatures between 15–31ºC, despite ambient temperatures ranging from 0–37°C. The recorded brood temperatures resulted in colonies maintaining yearlong brood development, which would suggest that this could provide a suitable resource for pathogen development year-round. However, pathogen occurrences are rare, it is speculated

that the greater brood temperature range which is tolerated by colonies, is ultimately unsuitable for brood pathogen development, especially the lower winter temperatures.

Worker behaviours have been acknowledged to play an important role in controlling the development and spread of brood diseases. The earliest identification of hygienic bees (Park, 1935) paved the way for many studies over the last 80 years. Studies have investigated the expression of hygienic behaviours in different populations and species, relationships with pests and disease establishment, influences of environmental factors and genetic control of expression in colonies. As a result, superior hygienic populations of *A. mellifera* have been selected and maintained. More recently, stingless bees have been tested for their hygienic behaviour, though these studies are limited to only five neotropical species (Tenório 1996, Medina et al. 2009, Nunes-Silva et al. 2009). This study (Chapter 3) therefore investigated the hygienic behaviour of *T. carbonaria* colonies by challenging workers with pin-killed pupae. *Tetragonula carbonaria* appears to be highly superior to other bees. Colonies on average took 7 h to remove 99% of the dead pupae, compared to other stingless bee species which remove between 1–99% in 48 h, and hygienic *A. mellifera* colonies removing 99% in 48 h. Factors including weather conditions, population number and nest entrance accessibility had little influence on hygienic behaviour expression. *Tetragonula carbonaria* colonies showed short-term learning, with colonies displaying more efficient behaviours after initial exposure to dead pupae. Results from this study suggest the low pathogen incidences in *T. carbonaria* may be related to these excellent hygienic behaviours, however as there are differences in expression within and between stingless bee species hygienic behaviour alone must not be the sole mechanisms for limiting brood pathogen growth.

Bee products such as pollen, honey, and propolis have long been known for their health benefits and therapeutic uses against human pathogens. Many studies have investigated *A. mellifera* nest products against known bee brood pathogens. Despite evidence of excellent antimicrobial properties for stingless bee nest products used in human health, no studies have tested the products against bee pathogens. Therefore,

this study (Chapter 4) tested *T. carbonaria* pollen, honey, and propolis against insect pathogens (*P. larvae, A. apis, Metarhizium anisopliae*). Additionally, *Weissella hellenica*, a type of lactic acid bacteria which are known for their inhibitory activities against pathogens, was isolated from *T. carbonaria* digestive system and included in inhibitory assays. Compared to *A. mellifera*, *T. carbonaria* propolis has a greater inhibitory activity against *P. larvae* and *A. apis* mycelial growth, and reduced the area of sporulation of *M. anisopliae*. *Tetragonula carbonaria* pollen was better at reducing bacterial and fungal growth while *T. carbonaria* honey was better at inhibiting fungal pathogens, compared to equivalent *A. mellifera* nest products. *Weissella hellenica* displayed no inhibitory activities to insect pathogens.

Unsurprisingly, propolis (stingless bee and honey bee) had the greatest inhibitory activities against these insect pathogens. Propolis is a mixture of plant resins and bee salivary gland secretions and is utilised throughout nests as a medium to seal and block airflow, as well as being incorporated into the entire internal stingless bee hive structure, which includes the brood cells. It is with this in mind that further studies were conducted to investigate the chemical composition of the propolis extracts, as well as freshly removed brood comb, to understand their connection. Both *T. carbonaria* and *A. mellifera* propolis contained flavanones. The identity of flavanones (by ion mass and retention time) differed between the samples. This could explain some of the difference in inhibitory activity of propolis against the insect pathogens. As the chemical profile of *T. carbonaria* propolis is highly similar to the profile of the brood comb, this supports the claim that stingless bees incorporate propolis into their nest structures. As honey bees do not, this may help to explain the lower incidence of stingless bee brood disease compared to *A. mellifera*.

An important discovery from this thesis was the identification and documentation of a bacterial brood pathogen in *T. carbonaria* colonies (Chapter 5). From 2012, extensive investigations were performed to study brood losses in managed *T. carbonaria* colonies at UWS. These studies followed Koch's postulates guidelines to isolate and identify the bacterium and determine its pathogenicity. *Lysinibacillus sphaericus* was isolated from symptomatic larvae, contaminated cell provisions, and

honey stores. The disease took 22 d from infection to first appearance of symptoms, which included brown larvae. At the late stages of infection, larvae changed to a thick brown fluid in the cells. The brood cell provisions were thick and dark yellow-green in colour. The characteristic spiral brood structure of *T. carbonaria* was disrupted and the appearance of nest structures changed, including the colour and texture of the involucrum. Infection resulted in a significant drop in brood population, and adult workers became lethargic and unresponsive. Eventually, the nest deteriorated and the colony died. Investigations into *L. sphaericus* mode of action showed, without being conclusive, the possibility of toxic genes and toxin producing crystals. As a result of this study, an outline of brood disease symptoms, suggested protocols to control disease spread, and hive sanitisation practises were developed.

My research contributes greatly to the previously limited knowledge regarding disease control in stingless bees in Australia, particularly for the most widely managed species, *T. carbonaria*. The previously undocumented thermoregulation ability, and hygienic behaviour expression, is an interesting insight into the colony behaviours to control their internal environments and limit brood pathogen development. In the presence of plant resinous materials, *T. carbonaria* utilises these resources to create an internal nest environment where all nest structures are able to suppress superficial microorganism growth. It is their behaviour of incorporating propolis into brood cells, which seems to provide stingless bees with the advantageous adaptation to reduce brood pathogens. It is the combination of these defence mechanisms, which have contributed to the conclusion that, despite the identification of a brood pathogen, stingless bee brood disease is a rarity.

These findings open the doors for opportunities to develop industry strategies and management practices to assist the survival and utilisation of stingless bees in Australia, with limited effects of brood diseases.

Manuscripts prepared for submission

1. Identification of the first bacterial brood pathogen isolated from the stingless bee; *Tetragonula carbonaria* in Australia
   (Intended journal: *Journal of Invertebrate Pathology*)
2. Expression of hygienic behaviour by nursery workers of Australian stingless bee; *Tetragonula carbonaria*
   (Intended journal: *Journal of Insect Behaviour*)
3. Nest temperature regulation of temperate located *Tetragonula carbonaria* colonies exposed to yearly temperature fluctuations
   (Intended journal: *Insectes Sociaux*)
4. Antimicrobial activities of the nest products from the stingless bee, *Tetragonula carbonaria* against brood pathogens of *Apis mellifera* (Intended journal: *PloS Pathogens*)

Chapter 1

Literature review, scope and aims of thesis

Losses of European honey bee, *Apis mellifera*, populations in Australia and other countries, mainly as a result of pests and diseases, impact crop pollination, and food production. Despite a wide range of control measures being employed against bee pathogens and parasites, pesticide resistance is now common, and a number of other commercial beekeeping practices exacerbate the problem. This has led to increasing interest in alternative pollinators to the honey bee.

However, little is known about Australian native social bees; particularly their ability to defend themselves against pathogenic infections. There is limited information regarding their hygienic and thermoregulation behaviours, and the antimicrobial activity of hive materials and digestive microbes which may limit bee pathogen growth which may play significant roles in defence.

This project aimed to investigate and elucidate how Australian native stingless bees, represented by *Tetragonula carbonaria*, are able to defend against invading brood pathogens, compared to *Apis mellifera*.

1.1 Introduction

Bee health has become an increasing concern, as the declines in honey bee populations are seen on an international scale. Today, there are over 30 recorded pests and diseases that affect honey bee colonies (Morse & Flottum 1997, Ellis & Munn 2005). Commercial honey bee colonies in the US have declined from 5.9 million in 1940s, to 4.3 million in 1985 and 2.7 million in the 1990s, with the decline brought on by pests and diseases (Southwick & Southwick 1992, Neumann & Carreck 2010).

In Australia, the honey bee industry has a total estimated economic annual value of $90 million (Rural Industries Research and Development Corporation 2012). The pollination services provided by *Apis mellifera* and other species is estimated to provide around 65% of Australia's commercial pollination for 35 commodities including almonds and pome fruit, along with pasture crops (lucerne and clover) (Gordon & Davis 2003). Australia is currently not experiencing the large declines in populations as seen overseas, however the combination of pests, parasites, and pathogenic infections, and the over exploitation of agricultural chemicals might change this. This especially centres on the associated effects that are brought on by varroa mite *Varroa destructor* Anderson & Trueman 2000 (Acari, Varroidae) (currently not present in Australia), especially regarding brood pathogens. To overcome the impact of the predicted losses of *A. mellifera* in Australia, investigations into alternative pollinators are needed; this includes not only solitary bee species, but also other eusocial species as outlined in a recent report (DAFF 2011).

Bees belong to the order Hymenoptera, which is comprised of three main bee families: Colletidae (solitary bees), Megachilidae (mostly solitary bees) and Apidae (eusocial bees, the largest family) (O'Toole & Raw 2004). My project will focus on bees from the subfamily Apinae (Family Apidae), which is comprised of three tribes: Apini, the true honey bees, Bombini, the bumble bees, and Meliponini, the stingless bees (Michener 2007). Species of stingless bees, principally *Tetragonula carbonaria,* will be the primary focus of my studies.

Australian stingless bees are highly eusocial. Those in the genus *Tetragonula* Jurine, have been reported to be comparable pollinators to honey bees (Dollin et al. 1997, Slaa et al. 2006) and have the potential to be used as alternative commercial pollinators for a number of crops.

1.2 European honey bee compared with stingless bees

1.2.1 Historical perspective

Honey bees, stingless bees, and their products have been important to civilisations since ancient times (Crane 1999), as food and drink, medicine, for ceremonial rituals, hunting and other practical uses (Voorhies et al. 1933, Rayment 1935, Schwarz 1948, Ransom 2004, Klumpp 2007, dos Santos & Antonini 2008, Jones 2013, Rosales 2013). Depictions of honey hunting and use of bee products from hieroglyphics and tomb exploration in Egypt indicate some of the earliest records of *A. mellifera* management (Crane 1999, Ransom 2004, Jones 2013). *Apis mellifera* populations expanded rapidly with European colonisation, and today populations have been successfully introduced in many other areas, including New Zealand (Bailey & Ball 1991), and Australia during the early 1800s (Crane 1999). Today there are approximately 80 million managed honey bee colonies worldwide (FAO 2013), with approximately 572,000 hives in Australia, managed by10,000 registered apiarists (Crooks 2008).

In comparison, stingless bees are found throughout the tropic and sub-tropic regions; with approximately 600 species located in Central and South America, Africa, Asia. Two genera, *Tetragonula* and *Austroplebeia*, comprised of 12–15 species are found in Australia (O'Toole & Raw 2004, Halcroft et al. 2013a). Stingless bees have been used in ancient religious ceremonies as symbols of cosmology in Mayan culture dating from 2000 BC–900 AD (Klumpp 2007, Jones 2013). The first observations, by explorers of stingless bee management occurred around 1536 in South America, and of honey hunting by Australian aborigines in 1648 (Jones 2013). Beekeeper management of Australian stingless bee *T. carbonaria* did not begin until very recently (1980s) (Heard 1988b, a). In 2013, of the 4,935 managed stingless bee

hives, *T. carbonaria* was the most widely distributed and commonly kept species (62% of managed hives) (Heard & Dollin 2000, Halcroft et al. 2013c).

There have been a number of published studies on the biology, ecology and management of stingless bees, especially *T. carbonaria* (Wille 1983, Dollin et al. 1997, Heard & Dollin 2000). *Tetragonula carbonaria* is an efficient species for crop pollination, due to its generalist foraging behaviour (Slaa et al. 2006). Its nest structure, food storage and larger brood volume than other species favour survival in cooler climates (Dollin et al. 1997), giving it the opportunity to be used alongside *A. mellifera* during peak pollination periods (Heard & Dollin 2000). *Tetragonula carbonaria* is distributed from the warm tropical areas of coastal Queensland (Cape York, 16º S, 145º E) to the temperate areas of southern New South Wales (Bega, 36º 40.27' S, 149º 50.34' E), and has a flight temperature range between 18–35ºC (Rayment 1935, Halcroft 2007, Klumpp 2007).

1.2.2 Nest architecture

Cavity nesting species such as *A. mellifera*, in nature, build their nests in caves and hollow trees. The vertical cavity space is normally a somewhat cylindrical shape and between 20–100 L in volume (Seeley 1977) with the nest entrance located at the bottom. The space is normally lined with propolis (a mixture of wax and plant resins), with combs attached to the top of the cavity and the side walls and with space passages between them for bee movement (Seeley & Morse 1976). Honey bees form hexagonally arranged, cylindrical-shaped cells constructed from body wax, which either store honey or pollen, or used to raise the brood (Darwin 1859).

In comparison, stingless bees may nest in tree cavities or in the ground. *Tetragonula carbonaria* nests naturally in tree cavities within rainforests, eucalypt forests and woodlands (Australian Museum 2009). The nest is enclosed at the top and bottom with batumen (a hardened layer of cerumen), and an entrance tube connects the colony to the outside (Rayment 1935, Michener 1961, Wille 1983). Worker bees collect plant resins in their corbiculae (Figure 1.1), and mix it with bee salivary gland secretions to create propolis (Simone-Finstrom & Spivak 2010), which is used throughout the nest similar to honey bee colonies for protecting and sealing gaps, and

as additional defensive barriers against pests by trapping ants and beetles (Rayment 1935).

Figure 1.1 *Tetragonula carbonaria* worker with the collected plant resin stored in her corbicula (yellow arrow) for transport back to the nest, used to make propolis (©Duncan 2015).

Cerumen (propolis mixed with wax secreted from the dorsal surface of worker abdomens) (Heard 1996, Klumpp 2007, Michener 2013), is used for nest structures such as brood cells, involucrum (multiple insulating layers around the brood chamber), supporting pillars and storage pots (Michener 1961, Wille 1983) (Figure 1.2, Figure 1.5). The queen and the cells in which to rear her offspring are located in the brood chamber, in the centre of the nest. Cylindrical *T. carbonaria* brood cells form overlapping sheets or spirals, with a leading edge where newly laid cells can be located (Michener 1961, Wille 1983, Brito et al. 2012) (Figure 1.3). The brood chamber is surrounded by layers of involucrum (Michener 1961, Roubik 1979). Surrounding the brood chamber outside the involucrum layers is the storage area (Wille 1983), that consists of large globoid pots filled with nectar and pollen (Roubik 1979, Dollin et al. 1997) (Figure 1.4).

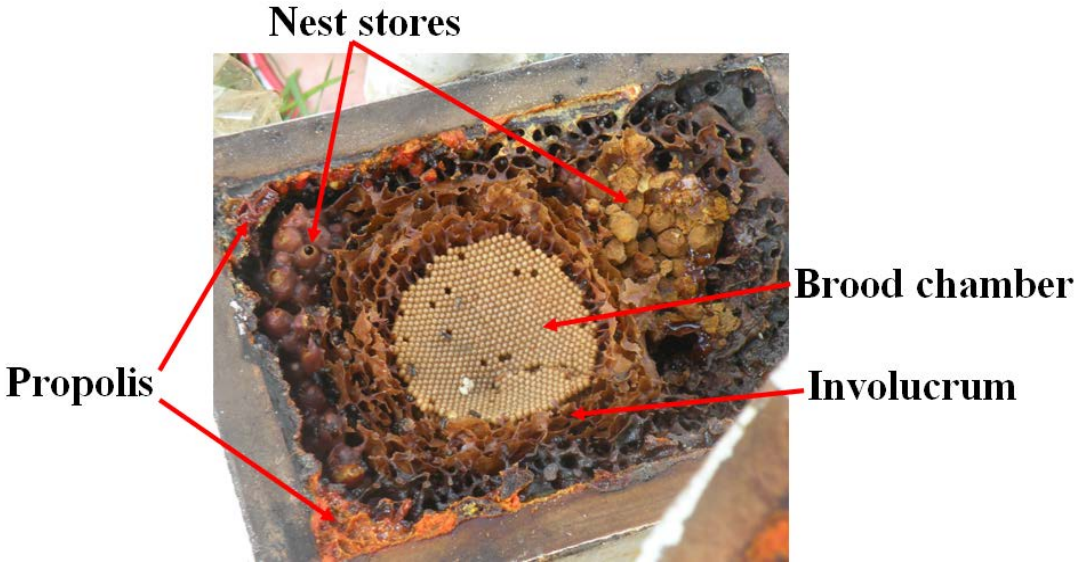

Figure 1.2 *Tetragonula carbonaria* colony which has been opened to display the internal architecture and design. The brood chamber is centrally located and encapsulated by layers of involucrum; the surrounding area is packed with pollen and honey nest stores. The brood chamber, involucrum and nest stores are constructed of cerumen. Propolis is deposited along the edge of the nest and can vary in colour, depending on plant resin sourced.

Figure 1.3 The characteristic spiral structure of *T. carbonaria* brood chamber. The chamber consists of cylindrical brood cells made from cerumen; it has a leading edge where new cells are laid into.

Figure 1.4 *Tetragonula carbonaria* nest stores consist of a) pollen pots and b) honey pots. These large globoid pots made of cerumen, are located surrounding the brood chamber.

1.2.3 Biology and social life

Honey bees and stingless bees have similar colony social structures and castes; with colonies headed by a single reproductive queen, small numbers of males which may not always be present throughout the year, and hundreds of sterile female workers, upon whom the queen is completely dependent (Roubik 1989, Winston 1991, Michener 2007) (Figure 1.5).

Castes and their tasks

Both the honey bee and stingless bee queen are easily distinguished by having a smaller head and thorax and a larger abdomen (Winston 1991, Dollin & Dollin 2010). The queen's responsibility lies in continual egg laying while she is cared for by her workers for colony survival (Bassindale 1955). Upon emergence, a young adult (callow or immature bee) initially works within the brood chamber. The tasks a worker can perform as she ages include; cleaning, debris disposal, cell preparation, construction of storage cells, and dehydration of nectar. Waste removal in stingless bees is suggested to be a key factor in eliminating potential harmful pathogens, and has been shown to be a task partitioned activity in *M. beecheii* (Medina-Medina et al. 2014). Towards the end of her life, a worker is involved in nest entrance activities such as fanning, guarding and finally, foraging (Bassindale 1955, Wille 1983, Winston 1991, Klumpp 2007). The purpose of drones (often referred to as males in stingless bees) within a colony is for mating. The males can be cared for, and solicit food from the workers (via trophallaxis) at early stages of their adulthood; however

as they age they can be observed feeding from honey stores (Free 1957, Imperatriz-Fronseca & Zucchi 1995, van Veen et al. 1997).

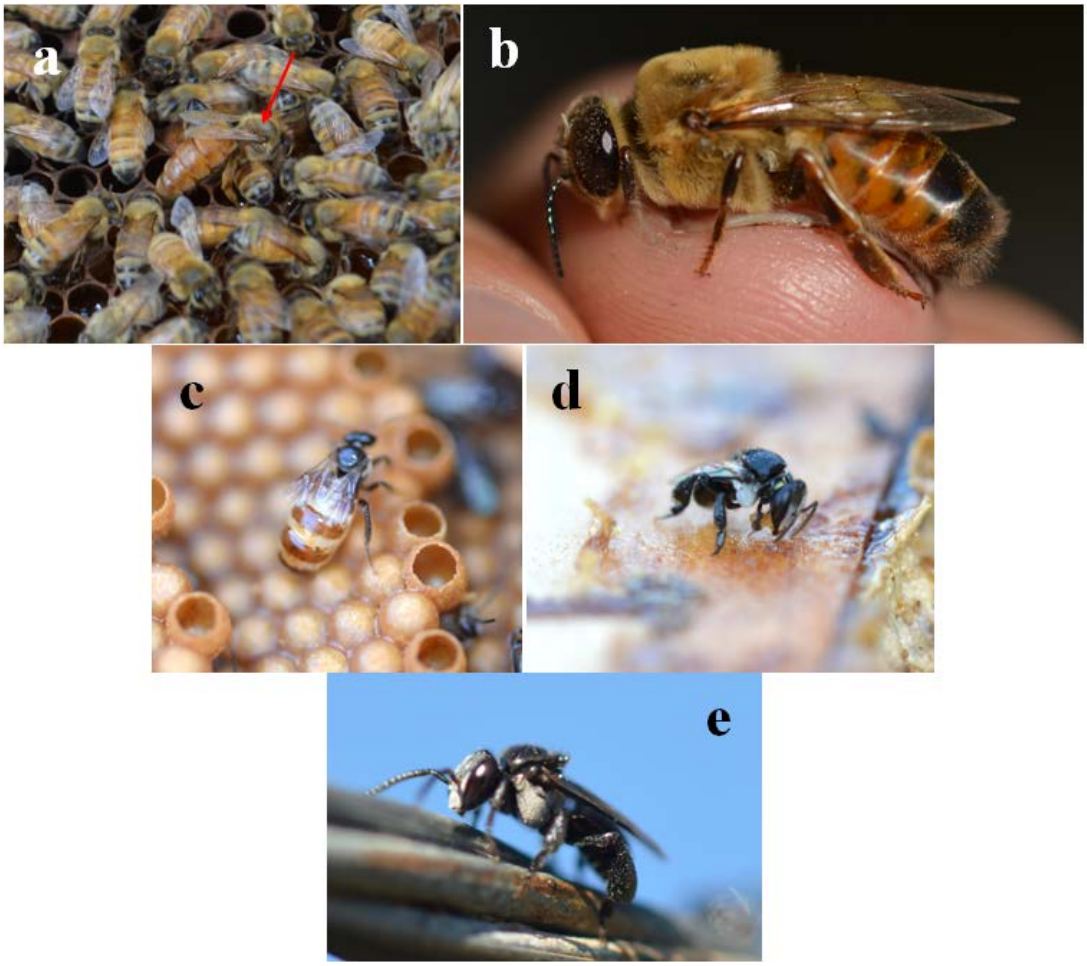

Figure 1.5 Bee hive castes. *Apis mellifera* a) queen (red arrow) surrounded by her female workers (10–15 mm long) and b) drone, compared with hive members of *T. carbonaria* c) queen, d) 4 mm long female worker and e) male.

Reproduction

In *A. mellifera* colonies, the daughter (or gyne) remains behind in the existing nest, while half of the worker population leaves with the old queen in search for a new nesting site (Klumpp 2007, Seeley 2010). The daughter gyne takes her nuptial flight in a congregation of more than 10,000 drones (Koeniger et al. 2005), mating with between 7–17 of the strongest drones (Kerr et al. 1962, Kolmes et al. 1989). In contrast, the stingless bee daughter leaves with half of the workers leaving behind the original queen (Roubik 2006). The daughter gyne takes her nuptial flight within a male congregation of 400–3,000 individuals, depending on species (Kerr et al. 1962), and mates with a single male (Kerr et al. 1962, Green & Oldroyd 2002).

1.3 *Apis mellifera* pests and pathogens

Honey bees are attacked by a number of pests such as parasitic mites and scavengers, as well as brood pathogenic infections caused by fungi, bacteria or viruses (Genersch et al. 2010).

Pests

There are a considerable number of pests that are of concern to the honey bee industry, including *Tropilaelaps clareae* Tropilaelaps mite, Delfinado & Baker 1961 (Arachnida: Laelapidae), *Acarapis woodi* Tracheal mite, Rennie 1921 (Arachnida: Tarsonemidae), *Braula coeca* Braula fly, Nitzsch 1818 (Diptera, Braulidae), and *Aethina tumida* Small hive beetle, Murray 1867 (Coleoptera, Nitidulidae). However, the biggest pest which is a concern for the honey bee industry currently is varroa mite, *Varroa destructor* (a notifiable pest under the *Animal Diseases and Animal Pests* (*Emergency Outbreaks*) *Act 1991*), particularly as it has been associated in increasing the prevalence of honey bee viruses (Rosenkranz et al. 2010) such as: Kashmir bee virus (KBV), Sacbrood virus (SBV), Acute bee paralysis virus (ABPV), deformed-wing virus (DWV), and Israeli acute paralysis virus (IAPV) (Martin 2001). The rapid, unexplained loss of *A. mellifera* colonies, known as colony collapse disorder (CCD) (Cox-Foster et al. 2007), is generally observed in colonies with higher varroa mite infestations, and bacterial, viral and other brood diseases (Martin et al. 1998, Martin 2001, Cox-Foster et al. 2007, vanEngelsdorp et al. 2009, Ratnieks & Carreck 2010).

Suggested research into population losses include investigating beekeeping management practices to limit pests and pathogen transmission between hives, improved pesticide usage, and breeding for behavioural defences (including grooming and hygienic behaviours) (Ratnieks & Carreck 2010, Rosenkranz et al. 2010).

Brood diseases

There are a number of brood diseases of significance for the industry, such as bacterial infections by *Paenibacillus larvae* (American foulbrood) and

Melissococcus plutonius (European foulbrood), and fungal infections by *Ascosphaera apis* (Chalkbrood).

1.3.1 *Paenibacillus larvae* – American foulbrood

The bacterium *Paenibacillus larvae*, causes one of the most serious honey bee brood diseases. It occurs throughout the world where bee keeping practices take place, including the temperate and sub-tropical regions of Australia (Bailey & Ball 1991). This bacterium is highly heat and antibiotic resistant with spores remaining infective and surviving in bee products and the environment for at least 35 years (Haseman 1961). American foulbrood (AFB) is a notifiable disease under the *NSW Apiaries Act 1985 No.16*.

Paenibacillus larvae is a slender, motile, rod-shaped, gram-negative, spore-forming bacterium 2.5–5 µm x 0.5–0.8 µm, appearing in chains. The pathogen can be transferred between worker bees, queens and drones. Developing larvae are most at risk. It takes on average 12.5 days after infection for bee larvae to show signs of disease (Hornitzky & Anderson 2003). The capping of an infected cell becomes dark, moist and concave, the larva turns brown and gives off a distinct odour (Bailey & Ball 1991, Hornitzky & Anderson 2003). Diagnosis in the field is usually made by placing a match stick into the infected cell and observing a brown, semi-fluid thread as the match is slowly removed (Shimanuki & Knox 1991, Hornitzky & Anderson 2003) (Figure 1.6). Approximately 2.5 billion spores are produced in each infected larva (Bailey & Ball 1991, Shimanuki & Knox 1991). It appears that faster growing strains of *P. larvae* can infect and kill larvae before the capping stage (Genersch et al. 2005); thus, the larvae can be detected and removed by nurse bees earlier than other infected larvae with slower growing strains.

Transmission to the larvae occurs by the ingestion of spores from contaminated food sources, with the spores germinating in the gut (Bailey & Ball 1991, Hornitzky & Anderson 2003). Bee hive tasks, such as cleaning and dismantling infected cells, help to transmit the disease. As larvae continue to die, the infected hive weakens, and robbing of these infected hives by other colonies may result in the robbing bees coming into contact with viable spores and infecting their own hives (Hornitzky & Anderson 2003), thus spreading the disease throughout an area.

In many countries, some *P. larvae* strains have shown resistance to oxytetracycline and tetracycline (Spivak & Gilliam 1998b, Alippi 2000, Miyagi et al. 2000, Murray & Aronstein 2006, Alippi et al. 2014). On mainland Australia, antibiotic treatment is not permitted, as antibiotics do not kill the spores, and antibiotic residues can be detected in honey after official withholding periods (Bogdanov 2006). Consequently, preferred methods for *P. larvae* management in Australia involve eradication measures such as destroying all infected colonies by burning of bees, diseased combs and other hive components in large ground pits, or the sterilisation of salvaged hive components by gamma-irradiation (Bailey & Ball 1991, Shimanuki & Knox 1991, Hornitzky 2003, Somerville 2012a).

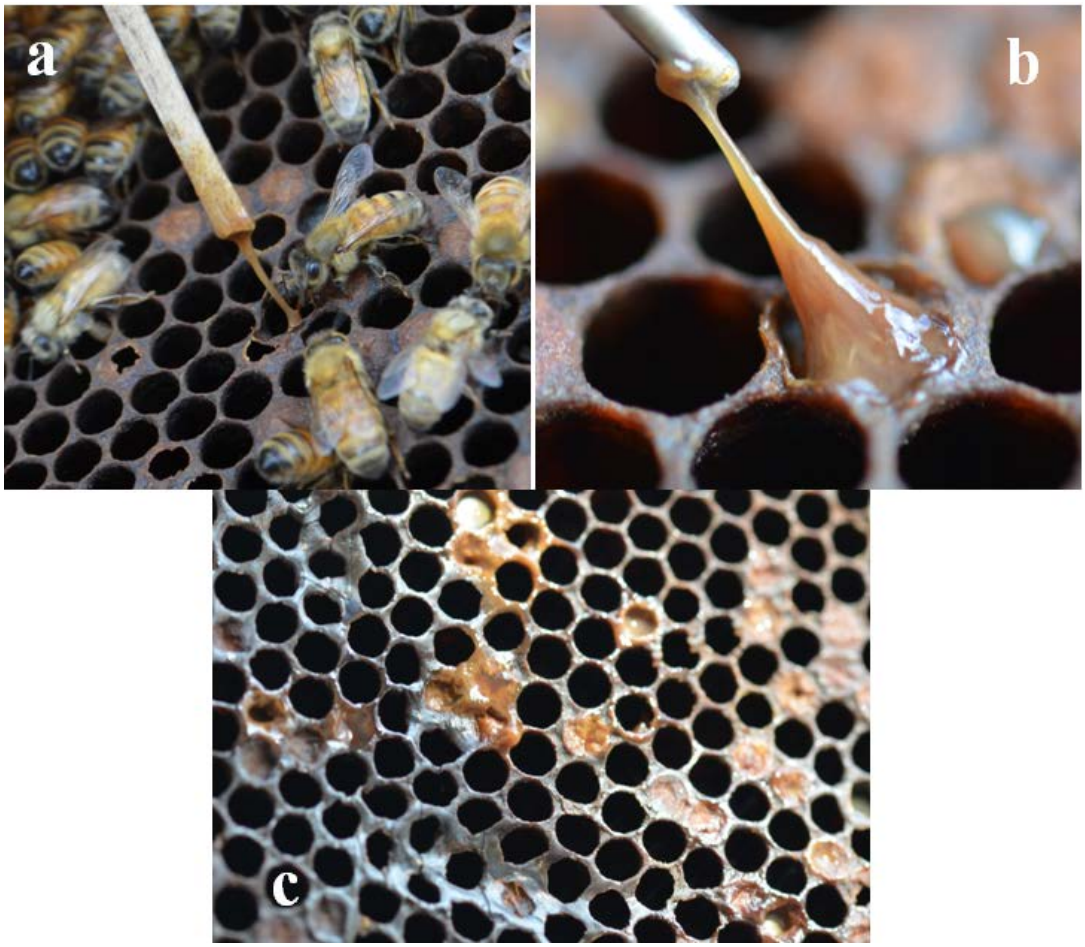

Figure 1.6 Signs of *P. larvae* infection in *A. mellifera* colonies. Field diagnostic tests include the matchstick (a, b) where a probe is inserted into a cell and a brown fluid (degraded larva contents) is pulled from the brood cell. Brood cells become sunken and the brood comb becomes degraded, a distinct foul odour is characteristic of this infection.

Melissococcus plutonius – European foulbrood (EFB)

Spores of *Melissococcus plutonius* are transmitted around the hive by contaminated faeces and adults, but affect developing larvae after spore germination in the midgut (Bailey & Ball 1991). The developing larvae either turn brown and decompose, or survive but produce underweight pupae and undersized adults (Bailey & Ball 1991, Hornitzky & Anderson 2003, Hornitzky 2010). Unlike American foulbrood, destruction of *M. plutonius* infected colonies is an ineffective and uneconomical method, as some colonies are able to readily recover. Good hive nutrition, antibiotic use, and the ability of nurse bees to remove infected larvae all play key roles in reducing outbreaks and hive losses (Hornitzky 2010). European foulbrood (EFB) is a notifiable disease under the *NSW Stock Diseases Act 1923*.

1.3.2 *Ascosphaera apis* – Chalkbrood

Ascosphaera apis is a heterothallic fungus that develops spore cysts when compatible strains are fused; cysts are 60 µm in diameter and enclose the 12 µm diameter spore balls which contain the spores (Hornitzky & Anderson 2003). All larval stages within the brood cell can potentially be infected with *A. apis* spores via food provisions (spores can remain infectious for 15 years or more (Hornitzky 2010)). The spores germinate in the lumen of the gut eight to nine days after infection (Invernizzi et al. 2011). Infected larvae become overgrown by mycelia and swell to the size of the cell, and the larvae dry into hard, shrunken, white chalk 'mummies' (Hornitzky & Anderson 2003, Hornitzky 2010, Invernizzi et al. 2011) (Figure 1.7). The larval mycelia subsequently produce spore cysts, which turn the mummies black in appearance (Shimanuki & Knox 1991). Each larva that is killed by *A. apis* produces one or more fruiting bodies consisting of 10^8–10^9 spores (Bailey & Ball 1991). The optimal temperature for growth and formation of fruiting bodies of *A. apis* is 30ºC (Bailey & Ball 1991). Larval death occurs once cells have been capped, and the infected larvae die within two days or at the pre-pupal stage (Bailey & Ball 1991).

As well as infection from contaminated food and brood comb, spores can be transmitted by unclean hive management practices (Bailey & Ball 1991), forcefully removing adult bees, giving hives extra brood to rear, placing new sealed brood in outer parts of the hive, dividing colonies as a means of swarm prevention or by

contaminated tools (Hornitzky & Anderson 2003). There are no effective fungicides to control *A. apis*; thus, selecting colonies with effective hygienic behaviour is of great importance in management of this disease (Invernizzi et al. 2011). Chalkbrood is a notifiable disease under the *NSW Stock Diseases Act 1923*.

Figure 1.7 *Ascosphaera apis* mummies (red arrow) removed from an infected *A. mellifera* colony. The characteristic appearance of mycelial growth from the larvae explains the common name (chalkbrood) for this infection.

The two pathogenic brood infections; American foulbrood (*Paenibacillus larvae*) and Chalkbrood (*Ascosphaera apis*), are of particular relevance to this thesis.

1.4 Stingless bee pathogens

Pests

Similar to honey bee colonies, stingless bee colonies can also be affected by predating or scavenging pests, however stingless bees are unaffected by varroa mite and to date no viruses have been detected. Pests of importance include Syrphid fly *Ceriana ornata australis*, Saunders 1845 (Diptera, Syrphidae), Phorid fly

Dohrniphora trigonae, Disney 1995 (Diptera, Phoridae), Scuttle fly *Megaselia scalaris*, Loew 1866 (Diptera, Phoridae), Mantisflies *Plega hagenella* Westwood, 1867) (Neuroptera: Mantispidae) (Maia-Silva et al. 2013), Sand wasp *Bembix musca*, Handlirsch 1894 (Hymenoptera, Crabronidae), Braconid wasp *Syntretus trigonaphagus*, Gloag, Shaw & Burwell 2009 (Hymenoptera, Braconidae), grain itch mite *Pyemotes tritici* LaGrèze-Fossat and Montagné 1851 (Acari, Pyemotidae) (Menezes et al. 2009) and also small hive beetle *A. tumida*.

Brood diseases

In contrast to honey bees, there is almost no documentation of brood disease in stingless bees. In fact, the only credible report is that of Kerr who observed possible diseased pupae in *Melipona quadrifasciata* and *M. bicolor bicolor* (Kerr 1948, Nogueira-Neto 1997). However, at the time only microscopic investigations were performed and the presence of bacterial spores was reported. It was not until 1957, that the identity of the bacterium, *Bacillus para-alvei*, was reported in communication between Kerr and Prof. Paulo Nogueira-Neto (Nogueira-Neto 1997). Apart from this single reference, there is no published information regarding the pathogenicity, signs or symptoms of the infection.

However, interestingly, there is some information regarding a relationship between *B. para-alvei* and *A. mellifera*. This bacterium was first reported in 1932 in honey bee colonies solely located in North and South Carolina, Georgia and Florida (Burnside 1932, Burnside & Foster 1935), with no other foulbroods identified in the colonies, but has since not been reported outside this area. Research conducted by Burnside and colleagues to identify the morphological and pathogenic characteristics of *B. para-alvei*, referred to it as causing parafoulbrood (Burnside & Foster 1935). It appears that parafoulbrood (at the time one of three bacterial brood diseases, including AFB and EFB) was only listed as a disease of *A. mellifera* between 1930s and 1950s (Burnside 1932, Burnside et al. 1949, Eckert & Bess 1952, Steinhaus 1952, Eckert 1955). This may be because of its initial misidentification as *B. para-alvei* rather than *B. alvei* (now *Paenibacillus alvei*). A number of other studies, around the same time, suggest that these two bacteria were identical (Root 1980). This conclusion was based on morphological characteristics, spore motility, and nutritional requirements (Burnside 1934, Clark 1939, Katznelson & Lochhead 1946),

however there may be differences between the fermentation characteristics (Tarr 1935), and antibiotic sensitivity (Katznelson 1950). If, indeed, these bacteria are the same, this might explain why parafoulbrood appeared to have limited distribution and is no longer referenced as a disease of honey bees. Either way, parafoulbrood has similar symptoms and characteristics to AFB and EFB, and can be transmitted between hives by robbing, drifting, and exchange of brood or honey from infected colonies (Foster & Burnside 1933). Today, most honey bee diagnostic services, which test infected brood for the presence of AFB or EFB, also include testing for *P. alvei* and not *B. para-alvei*.

Further complicating the situation is the historical identification of EFB pathogens, as outlined by Steinhaus (1946). *Paenibacillus alvei* was originally described as the cause of EFB (Cheshire & Cheyne, 1885); however, this was changed to include both *Streptococcus apis* and *B. alvei* being required to cause EFB (Maaseen, 1907/1908). It was not until 1912, when White identified *Bacillus pluton* (now *Melissococcus plutonius*) as the causal organism, though there was still references to *B. para-alvei* (and other bacteria), required to induce EFB disease (Tarr 1937). It is now accepted that *B. alvei* is a secondary saprophytic bacterium associated with EFB (Steinhaus 1946). This saga highlights the confused taxonomic and nomenclature problems associated with the genus *Bacillus* (Steinhaus 1946), especially as the early description and classification systems utilised culture-based studies.

1.5 Mechanisms for suppression of brood pathogens in eusocial bees

1.5.1 Colony behaviours

Colony behaviour responses to reduce the growth and development of brood pathogens can include individual worker response to the presence of dead brood such as hygienic behaviour, or whole colony-responses such as regulation of nest temperatures.

Hygienic behaviour

Hygienic behaviour: the detection, uncapping and removal of infected brood comb, is a social mechanism initially recorded in *A. mellifera* colonies (Park 1935). For colonies to be confirmed hygienic, workers need to remove 95% of dead or diseased brood within 48 hours (Spivak & Downey 1998). The early detection and removal of diseased larvae or pupae from the brood comb, within such a short period restricts overall disease incidence (Woodrow & Holst 1942, Spivak & Reuter 2001b, Invernizzi et al. 2011). Hygienic behaviour can be tested in the field by subjecting colonies to either freeze-killed (Milne 1982, Spivak & Reuter 1998, Kamel et al. 2003, Waite et al. 2003, Woyke et al. 2012), or pin-killed brood sections (Danka & Villa 1994, Gramacho & Gonçalves 2001, Nedić et al. 2005, Invernizzi 2012), and recording the time taken for colonies to remove all dead cells.

Hygienic behaviour is a known heritable trait in honey bee colonies (Rothenbuhler 1964a, Moritz & Crewe 1988, Oxley et al. 2010). Several quantitative loci influence workers to engage in hygienic behaviour, with a specific locus initiating workers to either uncap or remove only infected brood cells (Lapidge et al. 2002, Oxley et al. 2010). These desirable behaviours can be bred for, by using queens from selected superior colonies, to assist in management of *P. larvae* and *A. apis*, as well as parasites (Gilliam 1997, Spivak & Reuter 1998, Stanimirović et al. 2008). Colonies bred from hygienic breeding programs are reported to have fewer varroa mites (i.e., lower levels of infestation) than colonies not bred for such behaviour (Spivak & Reuter 2001a).

Bee species and races tested include: *A. mellifera* (Milne 1982, Spivak & Gilliam 1993, Arathi et al. 2000, Waite et al. 2003, Palacio et al. 2005, Espinosa-Montaño et al. 2008, Palacio et al. 2010), *Apis mellifera carnica* (Gramacho & Gonçalves 2001, Stanimirovic et al. 2001, Nedić et al. 2005, Gramacho & Gonçalves 2009b), *A. m. intermissa* (Adjlane & Haddad 2014), *A. m. lamarckii* (Kamel et al. 2003), *A. m. ligustica* (Spivak & Reuter 1998, Palacio et al. 2000, Spivak & Reuter 2001a), *A. m. scutellata* (Danka & Villa 1994, Gramacho & Gonçalves 2009a, Pereira et al. 2013), *A. cerana indica* (Arthreya & Reddy 2013), and *A. florea* (Woyke et al. 2012). Most of these species and races displayed some signs of hygienic behaviour, however the most efficient removed between 90-100% infected or dead brood (*A. mellifera, A. m.*

intermissa, A. m. ligustica, A. m. scutellata, A. florea). The results depended on the selected mating of the queen and the expression of traits in her workers. The differing rates of efficiency within and between races, supports what was previously known about the heritability of hygienic behaviour.

Stingless bee hygienic behaviour

There have been few hygienic behaviour investigations in stingless bees species: *Plebeia remota* (Nunes-Silva et al. 2009), *M. beecheii* and *Scaptotrigona pectoralis* Medina et al. 2009), and *M. quadrifasciata* and *T. angustula* (Tenório 1996), with hygienic behaviour efficiencies differing greatly between species (i.e., 1–97% removal in 48 h).

Despite hygienic behaviour having been extensively studied in *Apis* spp., and a limited number of studies testing tropical stingless bees, there have been no studies investigating the hygienic behaviour of Australian stingless bees. As hygienic behaviour plays a significant role in disease suppression in a number of other bee species, the lower level of pathogenic infections observed for *T. carbonaria* could be the result of the expression of efficient hygienic behaviours.

Thermoregulation behaviour

Thermoregulation has been investigated in bumble bees, honey bees, and stingless bees, with *A. mellifera* the most advanced in maintaining their nests at optimal temperature range between 33–35ºC (Gates 1914, Simpson 1961, Fahrenholz et al. 1989). Regulation of stable nest temperatures is important to facilitate year-long brood production, with adequate incubation to reduce mortality and developmental abnormalities such as stunted growth and wing defects (Heinrich 2004). Studies have shown when pupae develop at lower nest temperatures (≤32ºC) (Tautz et al. 2003), there is a reduction in short-term learning (Fukuda & Sakagami 1968, Tautz et al. 2003, Groh et al. 2004, Jones et al. 2004, Jones et al. 2005), forager activity and waggle dance performance (Tautz et al. 2003).

Maintaining optimal nest temperatures may also be important in disease control. During periods of stress, *A. mellifera* brood temperatures may drop below 33ºC which is advantageous for the germination of the heat-sensitive fungal brood pathogen *A. apis*. Detection and worker response to the drop in brood temperature by thermoregulation behaviours, can reduce the germination of spores by raising brood temperature back to optimal ranges (Starks et al. 2000, Simone-Finstrom et al. 2014).

There are two types of thermoregulation: passive (associated with nest site, orientation, and nest architecture) and active (involving behavioural activities of nest members). Cavity dwelling bee species build colonies which rely upon the cavity space for adequate flow of air, insulation and protection from ambient temperature fluctuations (Seeley & Morse 1978). *Apis mellifera* and *T. carbonaria* architecturally design colonies with the brood centrally located (Seeley & Morse 1976); this results in the brood being insulated by surrounding nest structures and stores. The metabolic heat from the brood and workers is either retained by a layer of wax as in honey bees (Jones & Oldroyd 2006), or by multiple layers of involucrum as for stingless bees (Michener 1974, Engels et al. 1995, Roubik 2006, Sung et al. 2008, Torres et al. 2009, Barbosa et al. 2013). Some stingless bee species also control air flow through the colony (Engels et al. 1995, Halcroft et al. 2013b, Jenny Shanks, personal observations) by closing or partially closing nest entrances with resin and cerumen (Chinh et al. 2005), while both stingless bees and honey bees use propolis along cracks and crevices to reduce unwanted airflow (Seeley & Morse 1976, Ghisalberti 1979).

Active thermoregulation by colony members involves behaviours such as: wing fanning to draw warm air away from the nest (Jones & Oldroyd 2006), collection of water and the depositing of the water droplets throughout nest to aid in evaporative cooling (Lindauer 1954 cited in Southwick & Moritz 1987), and heat shielding by workers positioned on internal nest walls to shield brood from external heat (Starks & Gilley 1999). During cooler periods workers may cluster together on or near the brood to maintain more constant temperatures (Kronenberg & Heller 1982) by vibrating their flight muscles (Stabentheiner et al. 2003), referred to as shivering *en masse* (Kronenberg & Heller 1982, O'Toole & Raw 2004). Workers can also consume honey and pollen, to elevate their thoracic temperatures (Kronenberg &

Heller 1982, Stabentheiner 2001, Nieh & *S*ánchez 2005, Nieh et al. 2006, Macías-Macías et al. 2011).

Thermoregulation has been investigated in a number of stingless bee species (Table 1.1), with many displaying cooling and warming behaviours: *Scaptotrigona postica* (Engels et al. 1995), *Trigona denoiti* (Fletcher & Crewe 1981), *S. postica* (Engels et al. 1995), *Melipona compressipes fasciculata* (Pacheco & Kerr 1989). *Tetragonisca angustula angustula* (Proni & Hebling 1996), *T. nigra pauper* (Torres et al. 2009), and *T. ventralis hoozana* (Sung et al. 2008). However, unlike honey bees, only *Scaptotrigona depilis* has been observed collecting water for evaporative cooling (Vollet-Neto et al. 2015).

Table 1.1 Species of social bees reported to regulate nest temperatures.

Species	Ambient temp (ºC)	Brood temp (ºC)	Source
Apis mellifera	-10–40	33–37	Gates 1914, Simpson 1961, Fahrenholz et al. 1989
Austroplebeia australis	-1.4–37.9*	-0.4–37.7	Halcroft et al. 2013b
Melipona beecheii	18. –>34	25.4–34.0	Moo-Valle et al. 2000
Melipona seminigra *Melipona rufiventris*	22.7–29.6	31.9–32	Roubik & Peralta 1983
Scaptotrigona postica	21–30	30–35	Engels et al. 1995
Tetragonisca angustula	14.5–24.4	25.4–30.3	Torres et al. 2007
Tetragonisca angustula fiebrigi	10.5–36.3	26.0–32.0	Proni & Hebling 1996
Tetragonula carbonaria	10–25	24–29	Amano et al. 2000, Amano 2004
Trigona denoiti	15.4–31.0	29.6–32.0	Fletcher & Crewe 1981
Trigona (Frieseomelitta) nigra paupera	17–22	25–30	Torres et al. 2009
Trigona ventralis hoozana	15.5–25.5	24.0–31.0	Sung et al. 2008
Frieseomelitta varia *Leurotrigona muelleri* *Melipona quadrifasciata* *Plebeia droryana* *Scaptotrigona depilis* *Trigona spinipes*	15.5–28.0	19.0–29.0 19.0–28.0 25.0–31.0 20.0–29.0 25.0–32.0 34.0–36.0	Sakagami 1982

*cavity temperatures

The first published thermoregulation study on Australian stingless bee species exposed to fluctuating ambient temperatures was for *Au. australis* (Halcroft et al. 2013b). This species was able to survive ambient temperatures between -4.0 and 37.7ºC, with workers involved in wing fanning at higher temperatures, but entering a chill coma below 12ºC (Halcroft et al. 2013b). A result of these lower temperatures, colonies experienced a reduction in brood development. In comparison, *T. carbonaria* has only been studied for thermoregulation under glasshouse conditions (Amano et al. 2000, Amano 2004, A. Tse, pers. comm., 2011). Colonies could sustain brood development by maintaining temperatures between 24–29ºC by wing fanning or clustering over the brood area and raising the internal hive temperature by 1–2 ºC above the ambient temperature (A. Tse, pers. comm. 2011), with no chill comb experienced.

However, no studies have investigated *T. carbonaria* temperature regulation under fluctuating environmental conditions, between different seasons and how this may influence colony management, brood production, and the incidence of brood diseases.

1.5.2 Nest products and their antimicrobial activities

The shift towards pesticide low/free honey bee products worldwide, together with increasing incidence of resistant strains of bee pests and diseases, has caused research to focus on developing alternative methods to pesticides for pest and disease control. This includes selection and breeding of hygienic bee colonies, as well as identifying and exploiting the role of nest products and their antimicrobial activities for pathogen suppression.

Pollen and honey

Pollen and honey not only play a major role in bee nutrition (protein and carbohydrate, respectively), but may also assist in the suppression of brood pathogens in developing larvae.

Antimicrobial activity of honey bee pollen extracts has been reported against a number of human pathogens such as *Aspergillus* spp. Micheli, 1792 (Eurotiales),

Bacillus cereus Frankland & Frankland, 1887, (Bacillales), *B. subtilis* Cohn, 1872 (Bacillales), *Escherichia coli* Castellani & Chalmers, 1919 (Enterobacteriales), *Klebsiella* spp. Trevisan, 1885 (Enterobacteriales), *Pseudomonas aeruginosa* Migula, 1990 (Pseudomonadales), *Salmonella typhi* Eberth, 1880 (Enterbacteriales), *Staphylococcus aureus* Rosenbach, 1884 (Bacillales), *Streptococcus pyogenes* Rosenbach, 1884 (Lactobacillales) and yeast *Candida magnolia* Mey & Yarrow, 1978 (Saccharomycetales) (Carpes et al. 2007, Abouda et al. 2011, Morais et al. 2011, Kačániová et al. 2012, Cabrera & Montenegro 2013), as well as plant pathogens such as *Alternaria alternata* Keissler, 1912 (Pleosporales), *Botrytis cinerea* Persoon, 1794 (Helotiales), and *Fusarium oxysporum* Schlechtendal & Hansen (Hypocreales) (Özcan et al. 2004, Basim et al. 2006, Cabrera & Montenegro 2013). Several studies have reported the inhibitory activity of *A. mellifera* pollen extracts against bee pathogens, including *P. larvae* (Lavie 1960, Crailsheim & Riessberger-Gallé 2001) and *A. apis* (Gilliam et al. 1988).

The medicinal uses of *A. mellifera* honey is well researched for assisting in wound healing, and reducing swelling and redness associated with many bacterial infections of humans (Wellford et al. 1978, Snowdon & Cliver 1996, Cooper et al. 2002, Al-Waili 2004, Mandal & Mandal 2011, Hammond & Donkor 2013, Schneider et al. 2013, Sultanbawa et al. 2015), with the active ingredients of medicinal honeys such as Manuka, influenced by their botanical source (Allen et al. 1991, Adams et al. 2009), and the phytochemicals produced (Mavric et al. 2008, Adams et al. 2009, Atrott & Henle 2009, Windsor et al. 2012, Alvarez-Suarez et al. 2014, Sultanbawa et al. 2015). Recent studies have investigated the antimicrobial activity of stingless bee honey; however, this focus is predominantly on human pathogens (Garedew et al. 2003, Miorin et al. 2003, DeMera & Angert 2004, Temaru et al. 2007, Irish et al. 2008, Kimoto-Nira & Amano 2008, Boorn et al. 2010, Ewnetu et al. 2013, Kwapong et al. 2013, Mercês et al. 2013, da Cruz et al. 2014, Massaro et al. 2014b).

The antimicrobial activity of bee pollen extracts has been attributed to the activity of phenolic compounds (Abouda et al. 2011, Morais et al. 2011, Basuny et al. 2013), and the changes in the stored products due to microbial farming via the addition of

microbes such as lactic acid bacteria (Lavie 1960, Gilliam 1978, Gilliam et al. 1984, Gilliam 1997). As for the antimicrobial activity of honey, physical and chemical properties, like pH, osmolarity, sugar content, and hydrogen peroxide production by the production of enzyme glucose oxidase are attributed to the effects (Molan 1992, Mundo et al. 2004, Mandal & Mandal 2011, Kwakman & Zaat 2012, reviewed by White et al. 1963). However, in some cases, pollen and honey activity may also be associated with the phytochemical component (Allen et al. 1991, Mavric et al. 2008, Adams et al. 2009, Atrott & Henle 2009, Windsor et al. 2012, Alvarez-Suarez et al. 2014, Sultanbawa et al. 2015). Hydrogen peroxide production (Allen et al. 1991, Irish et al. 2008, Irish et al. 2011), as well as high flavonoid content (Tomás-Barberán et al. 2013, Massaro et al. 2014b) is suggested to be responsible for the antimicrobial effects in *T. carbonaria* honey.

Propolis and cerumen

Propolis is known for its antibacterial, antifungal, antiviral, antitumor, anti-inflammatory, and antioxidant properties (Grange & Davey 1990, Dobrowolski et al. 1991, Kujumgiev et al. 1999, Fernandes et al. 2001, Bastos et al. 2008, Massaro et al. 2011). More than 300 active chemical compounds have been identified in propolis (Abu-Mellal et al. 2012, Wilson et al. 2013, Huang et al. 2014). However, the major components such as: flavonones, phenolic acids, esters (Markham et al. 1996, Kujumgiev et al. 1999, Velikova et al. 2000, Midorikawa et al. 2001, Popova et al. 2009, Massaro et al. 2011, Salatino et al. 2011, Massaro et al. 2014a, Massaro et al. 2015), and terpenes (Pereira et al. 2003, Leonhardt et al. 2009), are influenced by the geographical location of the bee species and the access to floral resources within the foraging range (Wallace & Trueman 1995, Bankova 2005, Bastos et al. 2008, Wallace et al. 2008, Leonhardt et al. 2009, Wallace & Lee 2010, Pereira et al. 2013, Drescher et al. 2014, Massaro et al. 2014a).

Honey bee propolis has been extensively studied for its antimicrobial activity, not only against human pathogens (reviewed by Marcucci 1995, Lotfy 2006), but also against the bee brood pathogens *P. larvae* (Lavie 1960, Lindenfelser 1968, Mlagan & Sulimanovic 1982, Antúnez et al. 2008, Bastos et al. 2008, Simone 2010, Mihai et al.

2012, Kamel et al. 2013, Wilson et al. 2013, Wilson et al. 2014), and *A. apis* (Chorbiński 2009, Senka et al. 2011, Ali & Abd El-Ghafar undated). A number of studies have investigated the activity of stingless bee propolis, although similar to stingless bee pollen and honey, studies have focused primarily against human pathogens (Velikova et al. 2000, Fernandes et al. 2001, Miorin et al. 2003, Manrique & Santana 2008, Farnesi et al. 2009, Umthong et al. 2009, Campos et al. 2011, Dota et al. 2011, Liberio et al. 2011, Massaro et al. 2011, Massaro et al. 2013, Drescher et al. 2014, Massaro et al. 2014a, Campos et al. 2015, Massaro et al. 2015).

Propolis is the major constituent of cerumen in stingless bee colonies and is used to construct brood cells and storage pots. Its antimicrobial properties may therefore play a role in suppressing brood pathogens, either by direct contact in brood cells, and/or by increasing the antimicrobial activity of stored pollen and honey which are used for cell provisioning.

1.5.3 Role of microbial symbionts in the digestive system

A recent review paper by Anderson et al. (2011) provides detailed discussion of the past and present research on microbial flora and fauna and their roles in honey bees. Gut microorganisms play an important role in bee digestion, nutrition, and health (Audisio & Benítez-Ahrendts 2011, Martinson et al. 2012, Vásquez et al. 2012, Crotti et al. 2013, Engel et al. 2013, Olofsson et al. 2014), and the suppression of potentially harmful pathogens (Gilliam 1997, Evans & Armstrong 2006, Audisio & Benítez-Ahrendts 2011, Audisio et al. 2011, Hamdi et al. 2011).

To date, a number of studies, primarily employing inhibition assays on microbial plate cultures, have focussed on the antagonistic activity of gut bacterial isolates to *P. larvae* and *A. apis* (Olofsson & Vásquez 2008, Yoshiyama & Kimura 2009, Forsgren et al. 2010, Omar et al. 2014). A number of *Bacillus*, *Brevibacillus* and *Lactobacillus* species have been shown to inhibit *P. larvae* (Evans & Armstrong 2005, Alippi & Reynaldi 2006, Evans & Armstrong 2006, Sabaté et al. 2009, Mudroňová et al. 2011). Lactic acid bacteria (such as *Lactobacillus*) not only inhibit the growth of *P. larvae*, but also appears to induce an immune responses in honey bee workers (Evans

& Lopez 2004, Yoshiyama et al. 2013), suggesting potential usefulness as probiotics to increase bee health.

Most studies have investigated honey bee-isolated gut bacteria and their inhibitory activities against bee brood pathogens; despite more recent identification and classification of stingless bee gut microbiota of three Australian native stingless bee (Leonhardt & Kaltenpoth 2014), as yet, no studies have investigated the role stingless bees microbiota may play in bee pathogen suppression.

1.6 Research scope and aims

My thesis focusses on the behavioural and microbial defence mechanisms of the Australian stingless bee, *Tetragonula carbonaria*, and their role(s) in brood pathogen suppression. In doing so, I hope to elucidate why stingless bee colonies experience lower levels of pathogens than European honey bee, *Apis mellifera*, colonies in Australia.

Brood temperature is likely to be an important factor in determining the frequency and extent of brood diseases. *Apis mellifera* brood is constantly maintained around 35-37ºC, the optimal temperature for its brood bacterial pathogen, *P. larvae* (Alvarado et al. 2013), although the optimum temperature for the fungal brood pathogen *A. apis* is 33ºC (Flores et al. 1996, Vojvodic et al. 2011). Colony temperature is maintained through active or passive thermoregulation (Simpson 1961, Fahrenholz et al. 1989). There have been a number of studies investigating thermoregulation in stingless bees (as outlined above) most commonly in tropical environments. However, there have been few studies on Australian stingless bees, especially under sub-tropical or temperate conditions. In the most detailed study *Au. australis*, despite displaying thermoregulation behaviour, brood temperature fluctuated greatly (Halcroft et al. 2013b). Two smaller studies investigated temperature regulation in *T. carbonaria*, but only under glasshouse conditions (Amano et al. 2000, A. Tse, pers. comm., 2011).

If *T. carbonaria*, like *Au. australis*, can produce brood over a much wider temperature range than *A. mellifera*, and particularly at lower temperatures, this may, in part explain the lack of observed brood pathogens. Thus, a study was undertaken to investigate brood temperature and thermoregulation in *T. carbonaria* (Chapter 2), that aimed to:

- Record nest temperatures of field managed *T. carbonaria* colonies in a temperate region (Sydney Basin) over a period of 13 months.
- Observe and record any colony thermoregulation behaviours associated with seasonal changes in ambient temperatures.

Hygienic behaviour is another factor likely to significantly impact the presence of brood disease in eusocial bees. Studies that have compared managed and wild honey bee hives have suggested that intensive selection for other desirable characteristics has reduced these behaviours in managed bees. As a result, a number of recent studies have investigated the genetic basis of hygienic behaviour; and how to select for this trait in managed honey bees. Hygienic behaviour has been reported in a number of Brazilian stingless bee species, whose level of selection for production traits has been substantially less than *A. mellifera*. (Tenório 1996), (Medina et al. 2009, Nunes-Silva et al. 2009). Thus, a study was undertaken to investigate hygienic behaviour in *T. carbonaria* (Chapter 3) that aimed to:

- Observe and identify hygienic behaviours of *T. carbonaria* individuals to potentially invading pathogens. This will be the first such study conducted on Australian stingless bees.
- Investigate several in-hive and external factors that could influence the expression of hygienic behaviour in nursery workers.
- Compare the similarities and/or differences in the hygienic behaviour of *T. carbonaria* against that published for other stingless bee species, and particularly *A. mellifera*.

A third factor likely to impact the incidence and extent of brood diseases in bees is the antimicrobial activity of the nest contents and structures. Both honey bee and

stingless bee nest products; such as pollen, honey and propolis have been shown to possess antimicrobial activity, including against human and some plant pathogens. While a few studies have confirmed the inhibitory activity of honey bee nest products to important honey bee pathogens, none have tested stingless bee nest products. Determining the antimicrobial activity of *T. carbonaria* nest products and comparing with those of *A. mellifera* from the same location, could help to explain the lower brood pathogen levels in stingless bee colonies.

Thus, a study was undertaken to compare the activity of *T. carbonaria* and *A. mellifera* nest products obtained from co-located colonies against honey bee brood pathogens (Chapter 4), that aimed to:

- Assess the inhibitory activity of *T. carbonaria* nest products from colonies managed under temperate conditions on the growth of the honey bee pathogens *P. larvae* and *A. apis*, and the entomopathogen, *Metarhizium anisopliae* var. *acridum*.
- Compare the inhibitory activity of *T. carbonaria* nest products with that of nest products obtained from *A. mellifera* colonies in the same location.
- Compare the chemical composition of propolis and brood comb from *T. carbonaria* and *A. mellifera* colonies.
- Identify key chemical groups and compounds which may contribute to any differences in inhibition activities between *T. carbonaria* and *A. mellifera* propolis.
- Examine the similarities and differences in the composition of propolis and brood comb for each bee species, and in doing so, postulate the role stingless bee propolis or brood comb chemistry may play in brood pathogen suppression.

Chapter 2

Hive management and investigation of nest thermoregulation

2.1 Abstract

The information provided in this chapter describes the general materials and methods used throughout this PhD research project to maintain and monitor *T. carbonaria* colonies. This included: maintaining colonies in the field and in purpose-built temperature controlled rooms, splitting colonies for propagation and maintaining nest populations, supplementary feeding of colonies during cooler periods, and the monitoring of nest and ambient temperatures. As part of the hive management program, ambient and internal nest temperatures of four *T. carbonaria* colonies were recorded while they were exposed to fluctuating external temperatures in the Hawkesbury region. During the 13-month study, worker behaviours were also monitored. *Tetragonula carbonaria* colonies were able to survive throughout the year, and have continuous brood production, by maintaining brood above 10ºC and below 35ºC, whilst ambient temperatures ranged between 0–37ºC. To maintain this temperature range, workers in colonies were observed undertaking both active and passive thermoregulation behaviours. Colonies produced involucrum to insulate the brood chamber, while workers performed activities such as honey consumption to generate heat in cooler temperatures, and wing fanning to cool the nest in warmer periods. These findings show that *T. carbonaria* survive and produce brood during cooler winters and milder summers experienced in a temperate location. The temperatures experienced in the brood chamber may impact the incidence of brood diseases, with lower temperatures and possibly even fluctuating temperatures suppressing pathogen growth and development.

2.2 Management of *T. carbonaria* hives

2.2.1 Bee shed set-up

Permanent *T. carbonaria* colonies, unless otherwise stated, were sourced from Russell Zabel (Australian stingless native bees, Hatton Vale, Queensland (QLD). Colonies were located at the University of Western Sydney (UWS) Hawkesbury campus, Richmond NSW (33º 36.42’ S, 150º 44.44’ E), 20 m asl. Richmond has a sub-humid temperature climate with a mean annual temperature of 17ºC, a mean maximum of the hottest month (January) of 30ºC, and the mean minimum of the coldest month (July) of 3.6ºC. Frost occurs on average 13 times a year. The long-

term mean annual rainfall is 801 mm, with summer months being wet and winter being dry (Australian Bureau of Meteorology, http://www.bom.gov.au).

Tetragonula carbonaria colonies were maintained in the OATH (Original Australian *Trigona* Hive) (28 × 21 × 21 cm), which are suited for tropical and subtropical conditions (Heard 1988a). The hives positioned in the apiary grounds at UWS foraged on floral resources from managed gardens and mixed horticultural crops, with flowering throughout the year. Hives were either located in the field or in a temperature-controlled building. The majority of colonies were maintained inside a large Colorbond™ steel clad shed (hereafter referred to as the bee shed) on the grounds of the apiary, Hawkesbury campus, Richmond. Each room was insulated with polystyrene lining and temperature was controlled via reverse cycle air conditioning (Fujitsu General Australia, www.fujitsugeneral.com.au). Thermostats were set to 26 ºC. Inside each room, particle board shelves were placed along the walls where hives were positioned. All colony workers could exit the shed for foraging via a clear silicone tube (0.5 m × 8 mm diam.) through the external wall of the shed (Figure 2.1, Figure 2.2). Around each entrance/exit hole, the external wall was painted with a selection of colours, which aided bees to recognise the correct entrance (Figure 2.1).

Colonies inside the bee shed also had observation platforms (OP) (Halcroft et al. 2008) which were attached to the nest entrance and external shed entrance via 8 mm diam. silicone tubing. To enable internal nest observations, 3 mm thick clear acrylic lids (Cain's Glass Service Pty. Ltd., Penrith, NSW) were attached over the top of the nest underneath the hive lid. Masking tape (3M, Maplewood, Minnesota, US) was used to securely attach the acrylic lids and seal any gaps to prevent bees from escaping.

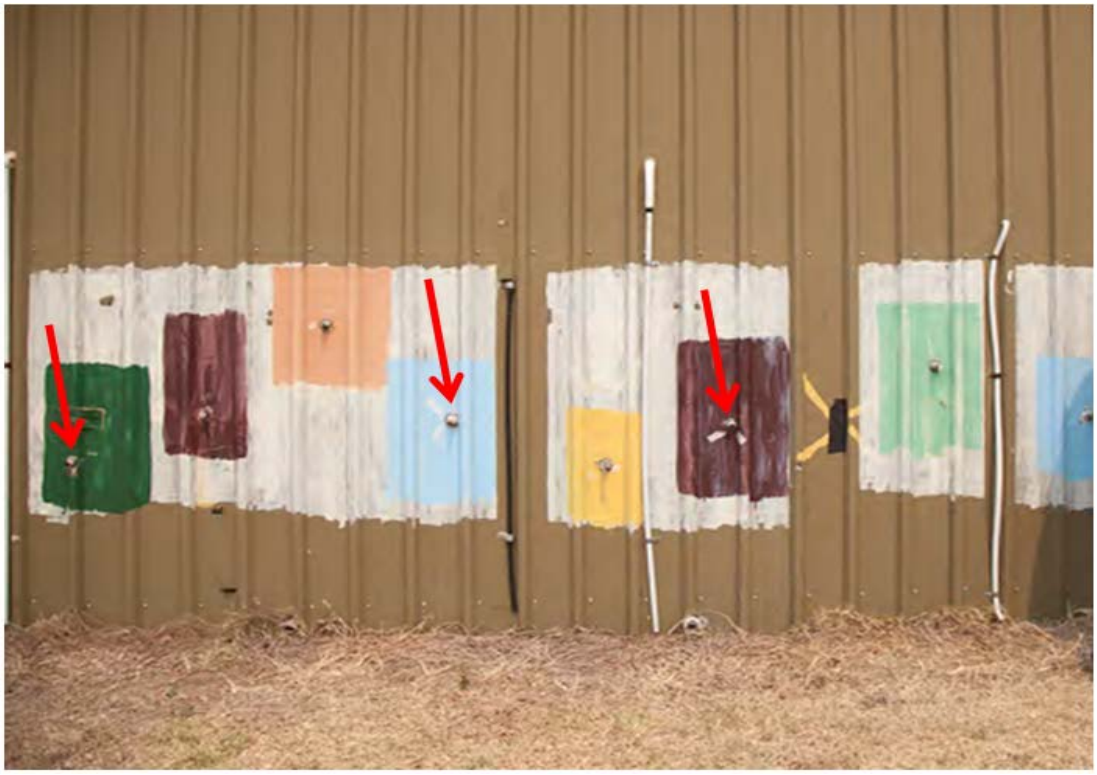

Figure 2.1 The hive entrances extending from the bee shed (red arrow). The exterior of the bee shed was painted at each nest entrance to aid in bee orientation.

Figure 2.2 Returning workers entering the external tubing entrance/exit, which was connected to a *T. carbonaria* hive inside the bee shed.

2.2.2 Field set-up

Four *T. carbonaria* colonies were set-up within the grounds surrounding the apiary. Hives were positioned at least 5 m apart and held above the ground on star-picket posts. Two steel star-picket posts (135 cm long black Ultrapost, Bunnings Warehouse, Rouse Hill, NSW) per hive box were placed 25 cm apart from the centre of each star picket; approximately 60 cm of the picket was driven into the ground. On the lateral sides of each of the colony boxes, two 45 mm length pieces of PVC piping (Holman, 50 mm diam., PVC DWV pipe, Bunnings Warehouse, Rouse Hill, NSW) were securely attached, along with an additional screw at the top of the PVC tube to act as a support point when placed over the star-picket post (Figure 2.3). The colony entrances were 30–70 cm above ground level. A 25 mm wide ring of Tanglefoot® Tangle-Trap™ Insect Trap Coating Paste (Australian Entomological Supplies Pty. Ltd http://www.entosupplies.com.au) was applied once, mid-height on the star-pickets to aid in preventing ants from accessing the colonies.

Figure 2.3 Field set-up of *T. carbonaria* colonies. A colony positioned in a sheltered, sunny location and securely supported by two star pickets.

2.2.3 Hive supplementary feeding

During the winter period (June to August), supplementary feeding was undertaken to assist colony survival. A honey feeder was used to provide the carbohydrate source for colonies maintained in the bee shed as well as those located outside. The honey feeders used were those previously described by Halcroft (2012), and consisted of a 30 mL container fitted with a smaller floating modified lid which acted as platform for workers to stand on while collecting the honey mixture (Figure 2.4 a). The honey mixture was provided in the form of 1:5 v/v water: *A. mellifera* honey solution. Observation platforms located between the nest entrance and external shed entrance had a third connection point made with 100 mm × 8 mm diam. silicone tubing connecting the OP to a 350 mL plastic container (Tellfresh®Round, 71 mm × 103 mm diam., The Décor Corporation, Scoresby, Victoria) that contained the honey feeder (Figure 2.4 c). Supplementary pollen as a protein source was dispensed into 35 mm × 20 mm diam. clean plastic bottle lids containing 10–15 mL of ground irradiated *Eucalyptus* spp. pollen collected by *A. mellifera* (Pender Beekeeping Supplies, Cardiff, NSW) (Figure 2.4 b). Pollen feeders were placed along with the honey feeders into the plastic containers.

For hives located in the field, the feeders were attached to the front of the nest box, adjacent to the nest entrance. These modified feeders with attached lids and drilled access holes (5 mm diam.), were attached to the boxes by two picture hooks and connecting wire (Figure 2.4 c).

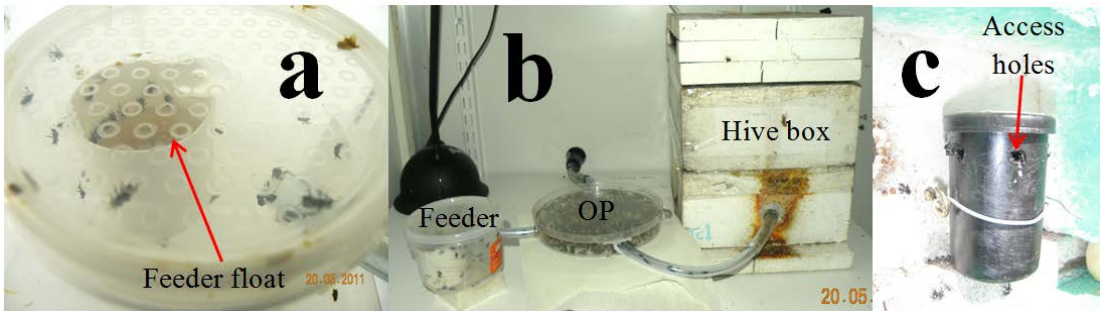

Figure 2.4 Supplementary feeding designs for colonies during cooler periods. Consisted of either a) a honey and pollen feeder, b) connected to nest boxes via the OP for colonies maintained in the bed shed, or c) in a closed container attached via wire to the hive box for field colonies.

2.2.4 Colony propagation

During the warmer periods of the year, stingless beekeepers commonly undertake a propagation technique called “splitting” in order to manage nest volume within hives, to prevent colonies moving out of managed boxes and to increase overall colony numbers (Heard 1988b). A healthy, strong colony from one box is split to form two separate box sections (two halves), thus forming two new colonies from one initial colony (Figure 2.5). During an ideal split, the nest is divided equally with regard to honey and pollen stores as well as brood. If the brood chamber is not equally split between the box halves, brood discs from the larger half are transferred to the smaller half until brood is equalised. Once a colony is split into the two separate halves, an empty half box is then added to each of these halves to form two new complete boxes. The two halves are secured together by 48 mm masking tape (3M, Maplewood, Minnesota, US) or a hive lock (Pender Beekeeping Supplies, Cardiff, NSW). One of the newly formed colonies is repositioned more than 500 m away (which is further than the foraging distance of *T. carbonaria*) to assist with the establishment of two independent new colonies.

During the PhD research project, colonies were propagated using the splitting technique to manage nest numbers and to produce replicates for experimental work. During late October to late November (2012 and 2013), colonies were split to enable successful establishment of the colonies over summer.

Figure 2.5 Splitting technique used for *T. carbonaria* hive propagation. A successful split is achieved when equal brood and nest stores are observed across both box halves. New empty boxes are attached to the halves and sealed.

2.3 Nest temperature and thermoregulation activity within *T. carbonaria* colonies

2.3.1 Introduction

Stingless bees mostly occur in tropical regions of the world (Sakagami 1982), with Australian stingless bees also naturally occurring in tropical/subtropical regions. Australian stingless bees can be located from the far northern parts of Western Australian, Northern Territory and Queensland, tropical and or arid regions, as well as along eastern Australia with one species, *Tetragonula carbonaria*, present as far south as Bega, NSW (36º 40.27’ S, 149º 50.34’ E) (Dollin et al. 1997, Halcroft 2012). The southernmost locations are in a temperate climate.

Nest thermoregulation is an important mechanism performed by some social insects to maintain temperatures within optimal ranges to facilitate brood incubation, year-round brood production and worker development, flight initiation and forager activity, and to prevent neural abnormalities associated with temperature extremes, such as short-term memory loss in workers (Fukuda & Sakagami 1968, Tautz et al.

2003, Groh et al. 2004, Jones et al. 2004, Jones et al. 2005). Low nest temperatures have also been linked to the development of some brood diseases such as chalkbrood, caused by *Ascosphaera apis* (Starks et al. 2000). This fungus which germinates in the gut, has an optimum spore germination temperature between 30–32ºC (Bailey & Ball 1991, Flores et al. 1996, Vojvodic et al. 2011). *Apis mellifera* have been reported to actively raise the temperature of brood when it fell below 33ºC, thus limiting the germination of *A. apis* spores (Simone-Finstrom et al. 2014).

Cavity dwelling honey bee colonies are the most advanced insects with regard to thermoregulation (Simpson 1961, Jones & Oldroyd 2006). While this has been extensively studied in *Apis* spp., it also occurs in a number of stingless bee species including *Melipona beecheii* (Moo-Valle et al. 2000), *Melipona seminigra* and *Melipona rufiventris* (Roubik & Peralta 1983), *Scaptotrigona postica* (Engels et al. 1995), *Tetragonisca angustula* and *Tetragonisca angustula fiebrigi* (Proni & Hebling 1996, Torres et al. 2007), *Tetragonisca weyrauchi* (Cortopassi-Laurino & Nogueira-Neto 2003), *Trigona nigra pauper* (Torres et al. 2009) and *Trigona ventralis hoozana* (Sung et al. 2008).

To regulate nest temperatures, both ‘passive’ thermoregulation (nest site, orientation and nest architecture) and ‘active’ thermoregulation involving nest members (wing fanning to cool and activation of thoracic muscles to warm the nest) are employed. In some stingless bee species, passive thermoregulation is achieved through construction of involucrum, an important insulator. For example, *Melipona rufiventris* and *M. seminigra* retain heat from the developing brood cells (31–32ºC) and heat from the thoraces of workers (26–36ºC) (Roubik & Peralta 1983) by constructing involucrum around the brood chamber, and *Scaptotrigona postica* which can have up to five layers of involucrum which helps to maintain brood nest temperatures between 29–35ºC, even if night temperatures are 2–4ºC (Engels et al. 1995). Other stingless bee species, such as *Frieseomelitta silvestrii* and *Frieseomelitta schrottkyi* however, do not use involucrum in their nests (Sakagami 1982).

During cool conditions, active warming of the nest may also be undertaken. Individual workers can contract their thoracic muscles to contribute heat and raise the temperature of the brood chamber. *Melipona beecheii* is able to maintain brood between 25.4–34.0ºC, whilst the ambient temperature fluctuated from 18.2ºC >34ºC (Moo-Valle et al. 2000). Similarly, *M. compressipes fasciulata* workers contribute to nest temperatures by raising their thoracic temperature by between 1.0–3.4ºC (Pacheco & Kerr 1989). Mass recruitment of *S. postica* workers to the brood chamber, forming up to two layers of slow moving bees, occurred when nest temperatures dropped to below 20ºC (Engels et al. 1995). This behavioural response has only been reported in this species.

During warmer conditions, active cooling can also occur in Brazilian stingless bees, *Tetragonisca angustula angustula*, and *T. a. fiebrigi* maintained brood chamber temperatures between 29–34ºC and 26–32ºC respectively, during summer, when the ambient temperature varied between 20–36ºC (Proni & Hebling 1996). Activities such as wing fanning contribute to temperature stability. Active cooling by wing fanning was observed for *S. postica* when ambient temperatures exceeded 35ºC (Engels et al. 1995). In an attempt to reduce heat generation, other general hive tasks (such as building and cleaning) ceased and nursery bees, brood workers, and the queen vacated the brood chamber, workers left the nest and regular foraging stopped (Engels et al. 1995). Water collection for evaporative cooling, until recently, was only linked with *Apis* (Lindauer 1954, Southwick 1987, Dyer 1991, Schmaranzer 2000) and *Bombus* species (Ferry & Corbet 1996, Weidenmüller 2004, Westhus et al. 2013). However, *Scaptotrigona depilis* has been observed to collect and use water to cool nests (Vollet-Neto et al. 2015). Most other stingless bee species have rarely (Cauich et al. 2004), or never been observed collecting water (Roubik & Peralta 1983, Engels et al. 1995, Proni & Hebling 1996, Halcroft et al. 2013b).

Most stingless bees that have been studied thermoregulate nests between 24ºC and 35ºC (as summarised in Table 1.1, Chapter 1, section 1.5.1). Studies on thermoregulation activity in Australian stingless bees are limited. *Austroplebeia australis* was able to raise brood temperature during spring but less so at other times of the year, never-the-less, brood could survive ambient temperatures fluctuating

between -1.4–37.9ºC (Table 1.1) (Halcroft et al. 2013b). Brood survival at such low temperatures has rarely been reported, as most stingless bees occur in tropical or subtropical climates. A number of exceptions to this have been reported in stingless bee species. *Plebeia remota* has been reported to cease cell production during the cooler period, to only begin normal cell production once ambient temperatures increase (van Benthem et al. 1995), *Trigona ventralis hoozana* can survive ambient temperatures as low as 8ºC, whilst the brood was maintained 20ºC warmer (Sung et al. 2008), *Scaptotrigona postica* is reported to have LT_{50} at -4ºC (Macieira and Proni 2004), and *Au. australis* survives and continues brood production during the cold season, despite low temperatures exposures (-0.4ºC) (Halcroft et al. 2013a).

There is a paucity of information on nest temperature regulation in the widely distributed Australian stingless bee, *Tetragonula carbonaria*. In a preliminary experiment undertaken at UWS, *T. carbonaria* colonies were observed to survive external ambient temperatures as low as 10°C, by workers clustering over the brood area and raising the internal hive temperature by 1–2 ºC above external ambient temperatures (A. Tse, pers. comm. 2011). In contrast, a study investigating the introduction of *T. carbonaria* as a greenhouse pollinator in Japan, reported that *T. carbonaria* did not cluster on the brood and could not regulate temperatures as successfully as *Apis* species (Amano et al. 2000).

A constantly active nest with developing workers may be an important factor influencing the development of brood pathogens in this species, as cooler or warmer nest temperatures throughout the year may directly influence pathogen growth and development, as well as continuous provision of susceptible brood. A study was therefore conducted as part of the hive management program to record the internal nest temperature of *T. carbonaria* associated with fluctuating external conditions over a period of 13 months. In addition, colonies were monitored throughout the investigation period for worker behaviours likely to be associated with active thermoregulation.

2.3.2 Methods and materials

Four *T. carbonaria* colonies previously established in the field for at least six months, in locations that allowed for direct sunlight in winter and shade in summer, were used to monitor the brood and hive cavity temperatures for a further 13 months from December 2011 to December 2012. The four selected colonies had either one or two holes (8 mm diam.) drilled into the side of the nest box. These holes aligned either with an empty cavity section (totalling two data sets from two colonies, n = 2) or were within the brood chamber (totalling four data sets, one from each colony, n = 4).

Each hive hole had a 10 cm long probe inserted and which was attached to a 'Tinytag' temperature data logger (Hastings Data Loggers, Port Macquarie, NSW, 2444, Australia) to record internal temperatures. Two additional data loggers were also placed within close vicinity (hanging from the neighbouring tree or post) and which were also protected from direct rain and sunlight, to record ambient temperatures (n = 2). A further data logger was placed inside an empty *T. carbonaria* box (n = 1), which was placed in the same location as the other positioned hives, to record internal hive temperatures in the absence of bees or nest structures. The Tinytags were set to log temperature every 30 mins, for a period of 13-months.

Recorded worker behaviour of wing fanning for nest ventilation from the thermoregulation study, is provided as mp3 formatted videos in the accompanying USB drive (Appendix 1). Data obtained from temperature loggers were examined using Microsoft Office Excel 2007 (Microsoft Corporation, ver. 12.0.6).

2.3.3 Results

The period from May to September was colder than the long-term average, with lower rainfall from May to December. There was higher rainfall between January and April, which resulted in the total being slightly above average rainfall for the year (Figure 2.6).

Ambient temperatures, as well as brood and cavity temperature data, were successfully collected from hives containing colonies during the period 1 December 2011 to 31 December 2012. The data logger in the 'empty box' malfunctioned on 5 March 2012, 96 days into the experiment, although this was not detected until 6 November 2012. Prior to its malfunction, the cavity temperature data tracked closely to ambient temperature (Figure 2.7) with an average difference between mean ambient temperature (n = 2) and the empty box (n = 1) being 0.13 ± 0.04ºC. These results indicated that the empty box had limited insulation properties, and thus ambient temperature could be used as a surrogate for internal cavity temperatures in a hive without a colony and its associated structures.

Based on average monthly data, there was a consistent temperature difference between the hive and ambient temperatures throughout the 13 month study (Figure 2.8). Brood chamber temperature was consistently higher than the cavity, and both brood and cavity temperatures remained higher than ambient temperatures. However, the brood temperature did not remain constant throughout the year and did cool during cooler periods. The mean difference between brood chamber and ambient temperatures during this period (total mean data points per hive (n = 17615) was 7.0ºC. The brood chamber was maintained between 4.8ºC (February) to 10.5ºC (July) warmer than the ambient temperatures, whereas the hive cavity space was 1.1 ºC (November) to 4.3ºC (July) degrees warmer than ambient temperature. Thus, within the nest, the brood chamber was, on average, 1.0ºC (December 2011) to 5.3ºC (September) warmer than the cavity space.

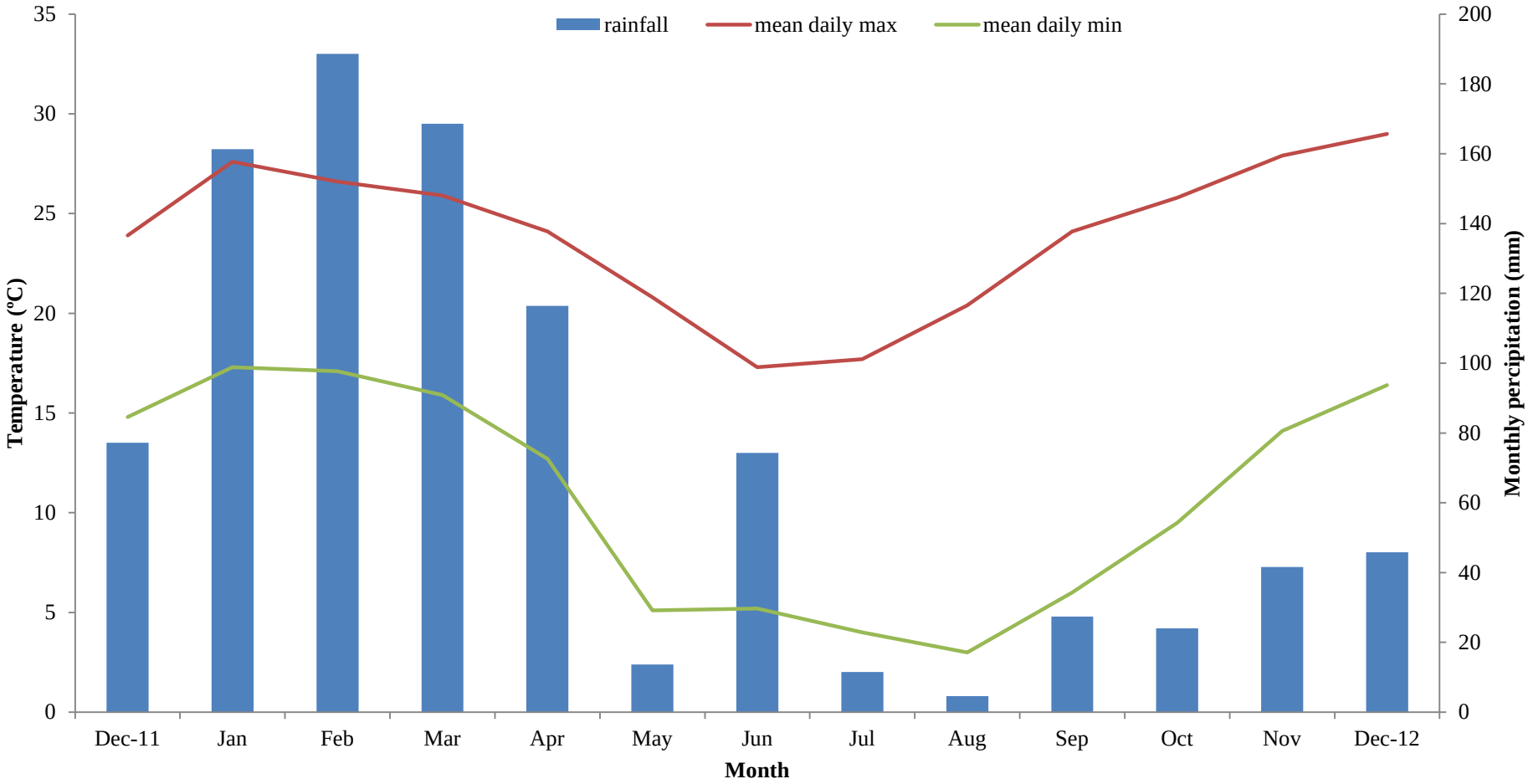

Figure 2.6 Monthly rainfall (mm), mean daily maximum, and mean daily minimum temperatures (ºC) for each month recorded for Richmond, NSW 1 December 2011 to 31 December 2012.

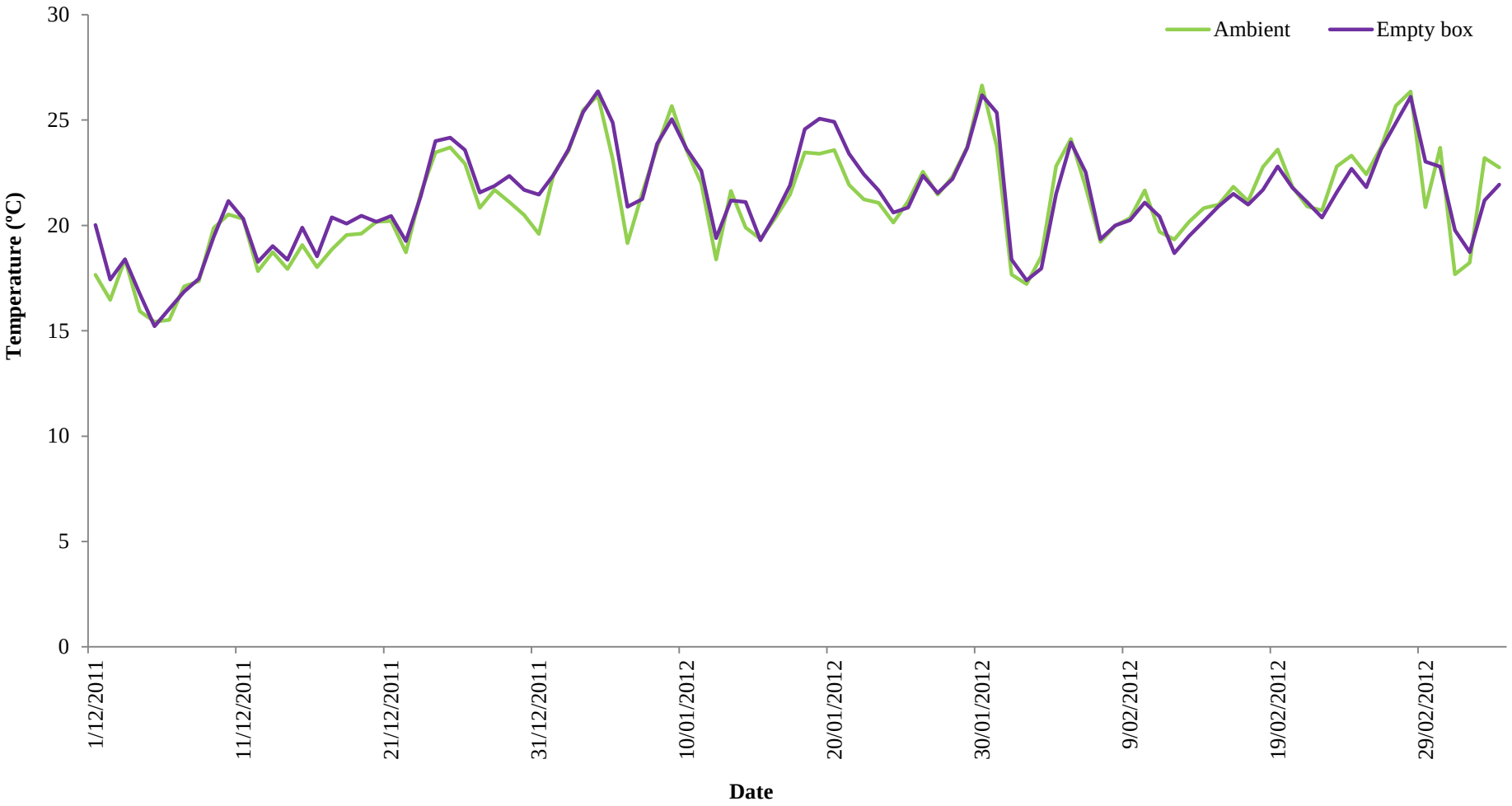

Figure 2.7 Ambient (n=2) and Empty box (n =1) temperature measurements from 1 December 2011 to 1 March 2012.

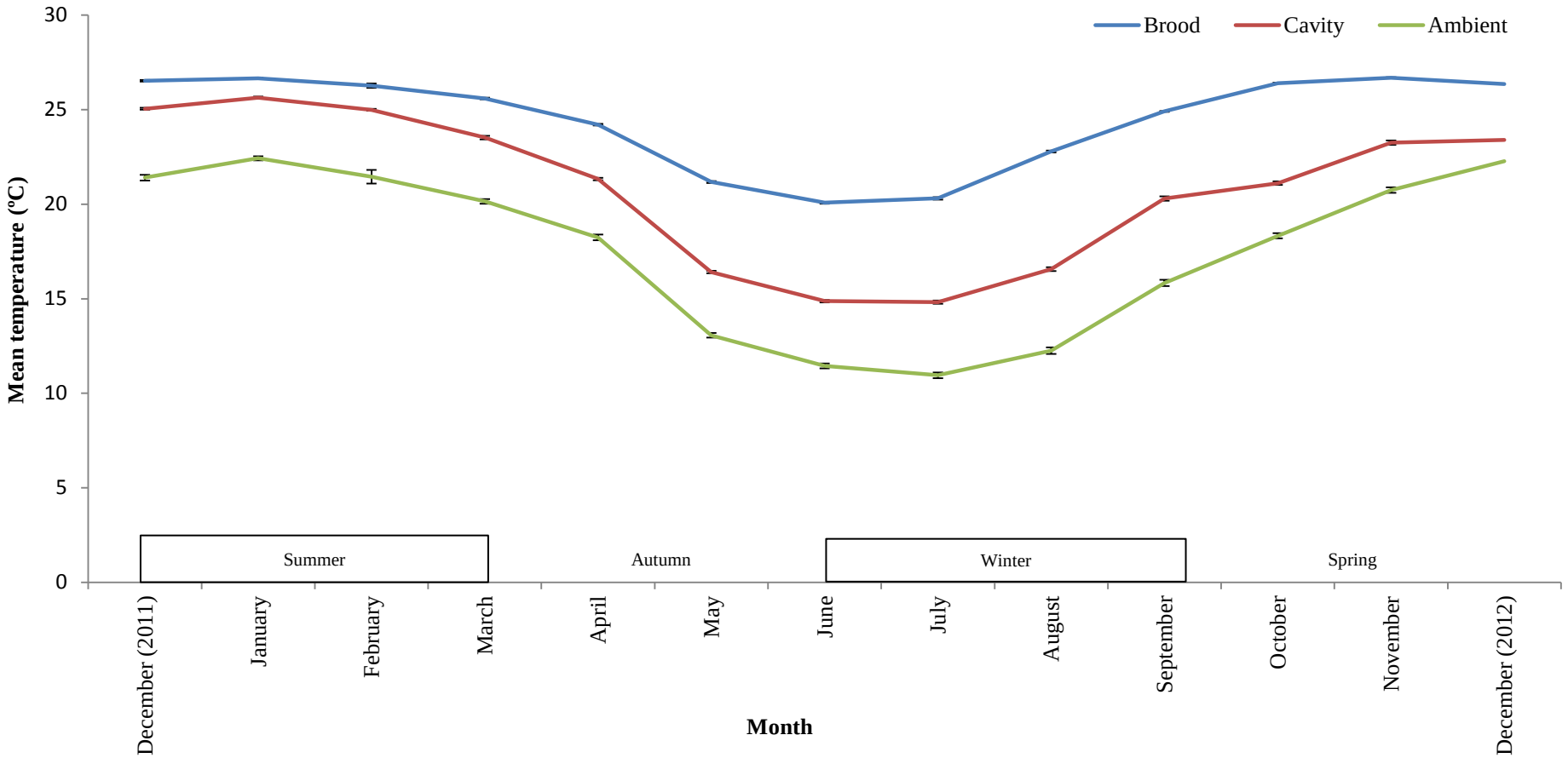

Figure 2.8 Mean half hourly temperature for each month within the brood (n = 4) and cavity (n = 2) of the chambers of four *T. carbonaria* field hives, compared to ambient temperature (n = 2) over a 13-month period. Error bars = standard error of the means.

Nest readings tracked similar daily fluctuation patterns to ambient temperature (Figure 2.9). During this study period, ambient temperatures ranged from 1.5–36.5ºC whereas within the hive, the temperatures for the nest cavity ranged from 8.0–35.4ºC, and the brood chamber ranged from 10.3–34.4ºC (Figure 2.9 a, b).

In winter, the brood chamber was up to 16.9ºC warmer than the ambient temperature (Figure 2.9 b). At the minimum mean winter ambient temperature of 1.5ºC, the brood was 13.5ºC warmer (08/07/2012). The warmest mean maximum ambient temperature recorded in winter was 30.7ºC, while on the same day (28/08/2012) the brood was 27.5ºC.

The cavity was consistently warmer than the ambient temperature during winter, however remained cooler than the brood chamber. During spring and summer, the mean maximum daily ambient temperature fluctuations were greater, while the *T. carbonaria* brood chamber remained more constant in temperature (Figure 2.9 b, c, Figure 2.10).

During the most active months for brood development (October and November), the brood was up to 17.6ºC warmer than ambient temperatures. During spring (Figure 2.10), the brood chamber temperature remained more constant (19.0–34.3ºC) compared to the fluctuations of the ambient temperature (4.4–36.8ºC).

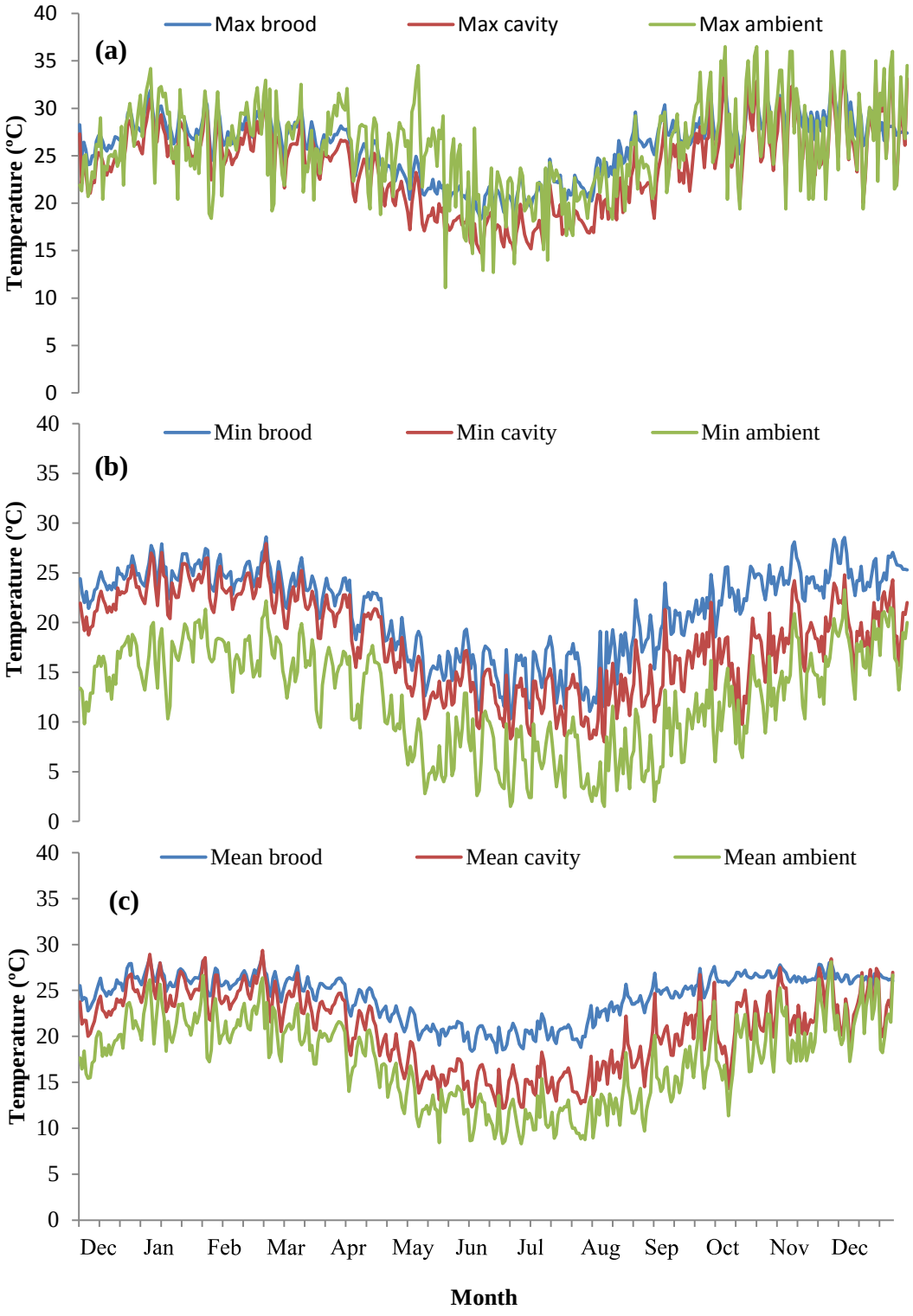

Figure 2.9 Temperature based on the (a) mean daily maximum, (b) daily minimum, and (c) daily average for *T. carbonaria* brood chamber (n = 4), hive cavity (n = 2) and ambient (n = 2) over 13 months.

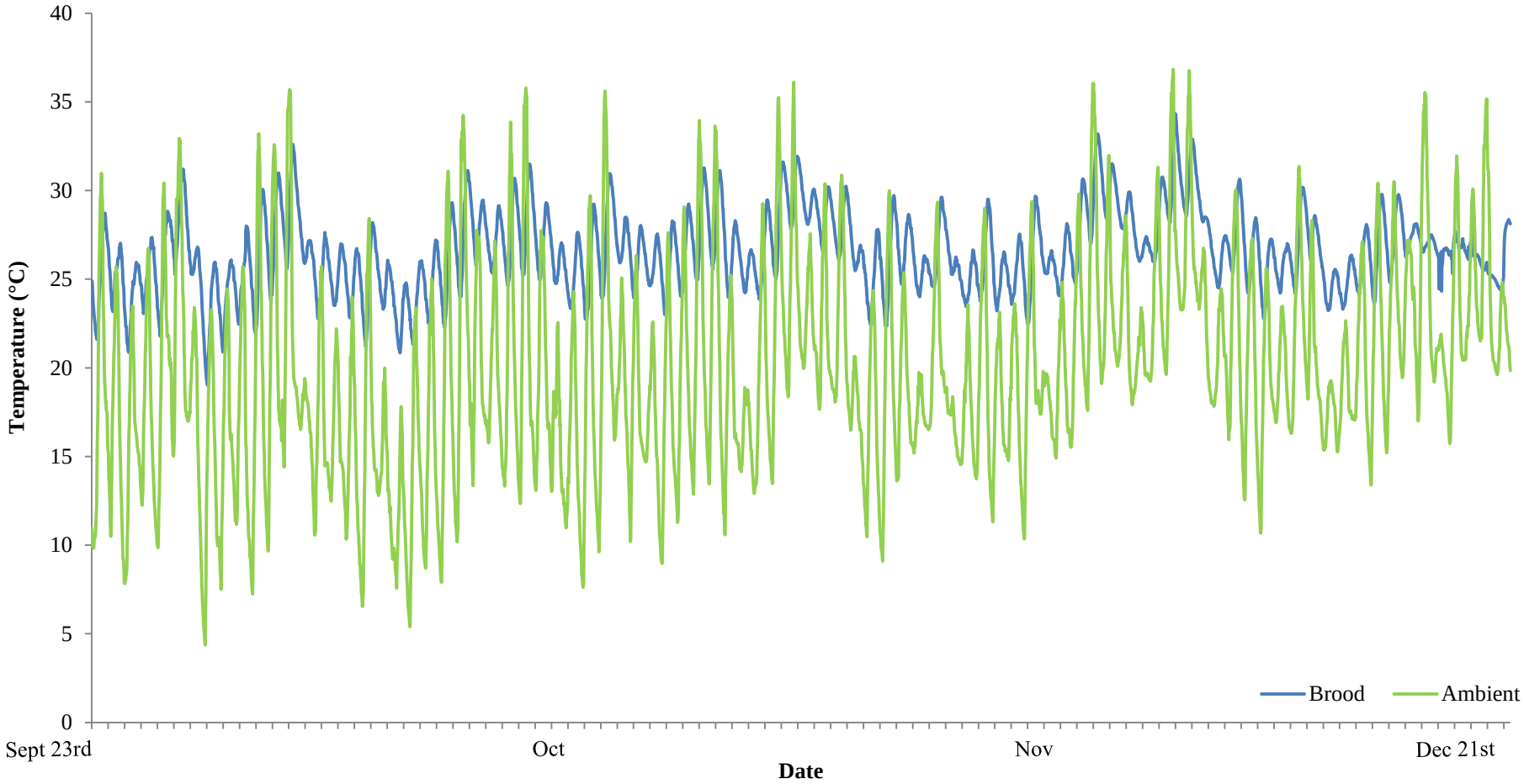

Figure 2.10 Half hourly temperature fluctuations during spring 2012 for *T. carbonaria* brood chamber (n = 4), and ambient (n = 2). Brood chamber temperature was relatively constant throughout the season compared to ambient temperature fluctuations.

During summer, daily minima temperature for ambient ranged between 10.3–22.2ºC, while the brood chamber daily minima ranged between 21.4–28.6ºC, and was at most 12.5ºC warmer than ambient daily minima (Figure 2.11 a). The maximum brood chamber temperature recorded during summer was 3.8ºC cooler compared with the maximum ambient temperature (31.4ºC). At the lowest summer ambient temperature maximum, the brood chamber was 8.7ºC warmer. The brood chamber maintained a summer temperature range between 21.4–31.9ºC, while the ambient temperature fluctuated between 10.3–34.2ºC (Figure 2.11 b).

During winter, daily minima temperature recorded for the brood chamber ranged between 10.3–24.0ºC, while the ambient daily minima was as low as 1.5ºC and up to 21.3ºC (Figure 2.12 a). At the lowest ambient temperature of 1.5ºC, the brood chamber was 13.1ºC warmer (at 14.6ºC). The largest difference between ambient and brood chamber daily minima was 16.9ºC, when the ambient was 2.4ºC. The brood winter daily maxima ranged between 10.3–30.4ºC, whilst the ambient was slightly warmer with a range of 13.6–30.7ºC (Figure 2.12 b). At the highest temperature of 30.7ºC, the brood chamber was at 27.5ºC, cooler than the ambient temperature. The largest difference between brood chamber and ambient daily maximum temperature was on 1 September 2012, when the brood chamber was 25.1ºC, 6.7ºC warmer than ambient temperature.

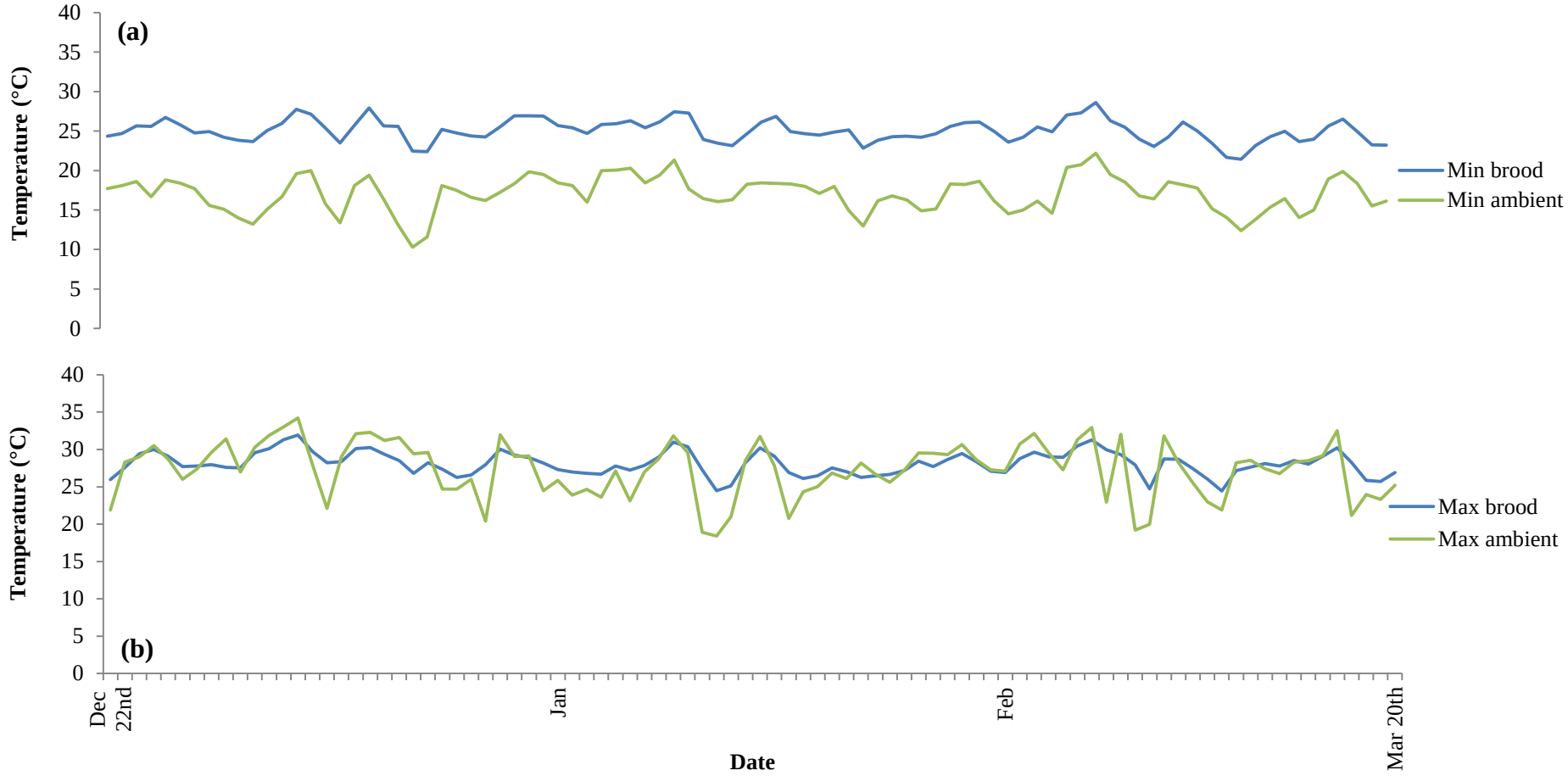

Figure 2.11 Daily (a) minimum and (b) maximum brood (n = 4) and hive ambient (n = 2) temperatures during summer, 2011-2012.

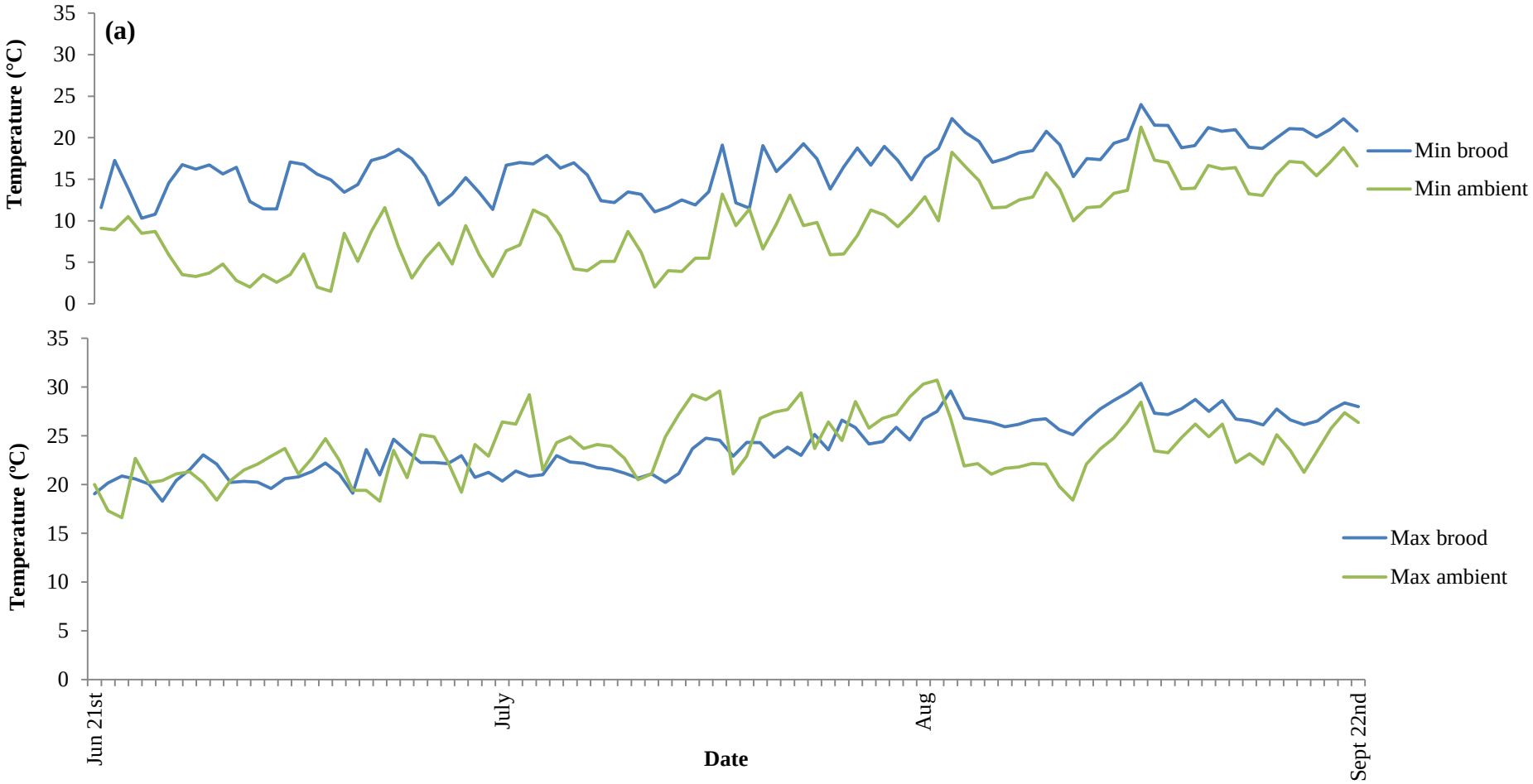

Figure 2.12 Daily (a) minimum and (b) maximum brood (n = 4) and hive ambient (n = 2) temperatures during winter, 2012.

Four different seasonal time points were examined to understand the temperature fluctuation changes between the nest and the ambient temperatures. Three relatively similar, consecutive days representing average conditions for each for the four seasons for 2012 were chosen.

In summer, the temperatures within the brood chamber and hive cavity remained constant. During summer mornings, ambient temperature increased from 04:00 and peaked between 12:00 noon and 13:00 (AEDT). In comparison, the brood chamber and cavity temperatures increased later in the morning from 07:00 and peaked around 19:00 (Figure 2.13 a). Brood chamber temperature remained reasonably constant, ranging from 26.1–29.9ºC, while the ambient temperature ranged from 15.6–30.7ºC. (Figure 2.11 b, Figure 2.13 a).

In autumn, the temperatures in the brood (19.2–24.6ºC) and cavity (16.4–24.2ºC) fluctuated more than in summer (Figure 2.13 b). The cavity reached peak temperature at16:00, later than ambient (13:00) and before brood temperature peak (21:00). Ambient temperature reached a peak of 26.3ºC, while cavity and brood were below this (24.2 and 24.6ºC, respectively).

In winter, there were larger fluctuations in the brood chamber temperature than in autumn and summer (Figure 2.13 c). The brood chamber was 5.6 ± 0.27ºC warmer than the cavity temperatures, with cavity temperatures tracking brood temperatures. During winter mornings, the ambient temperature increased from approximately 05:30 and 06.00 (AEST), whereas the cavity and brood chamber temperatures increased approximately 2 h after, from 08:30 and 09:00 (AEST), (Figure 2.13 c).

In spring, the brood chamber temperature (23.1–31.0ºC) was more constant than in winter (Figure 2.13). During spring, the cavity experienced greater temperature fluctuations (15.2–30.4ºC) compared to brood temperatures; however, ambient temperatures showed the greatest fluctuation, 8.9–33.2ºC (Figure 2.13 d, Figure 2.10).

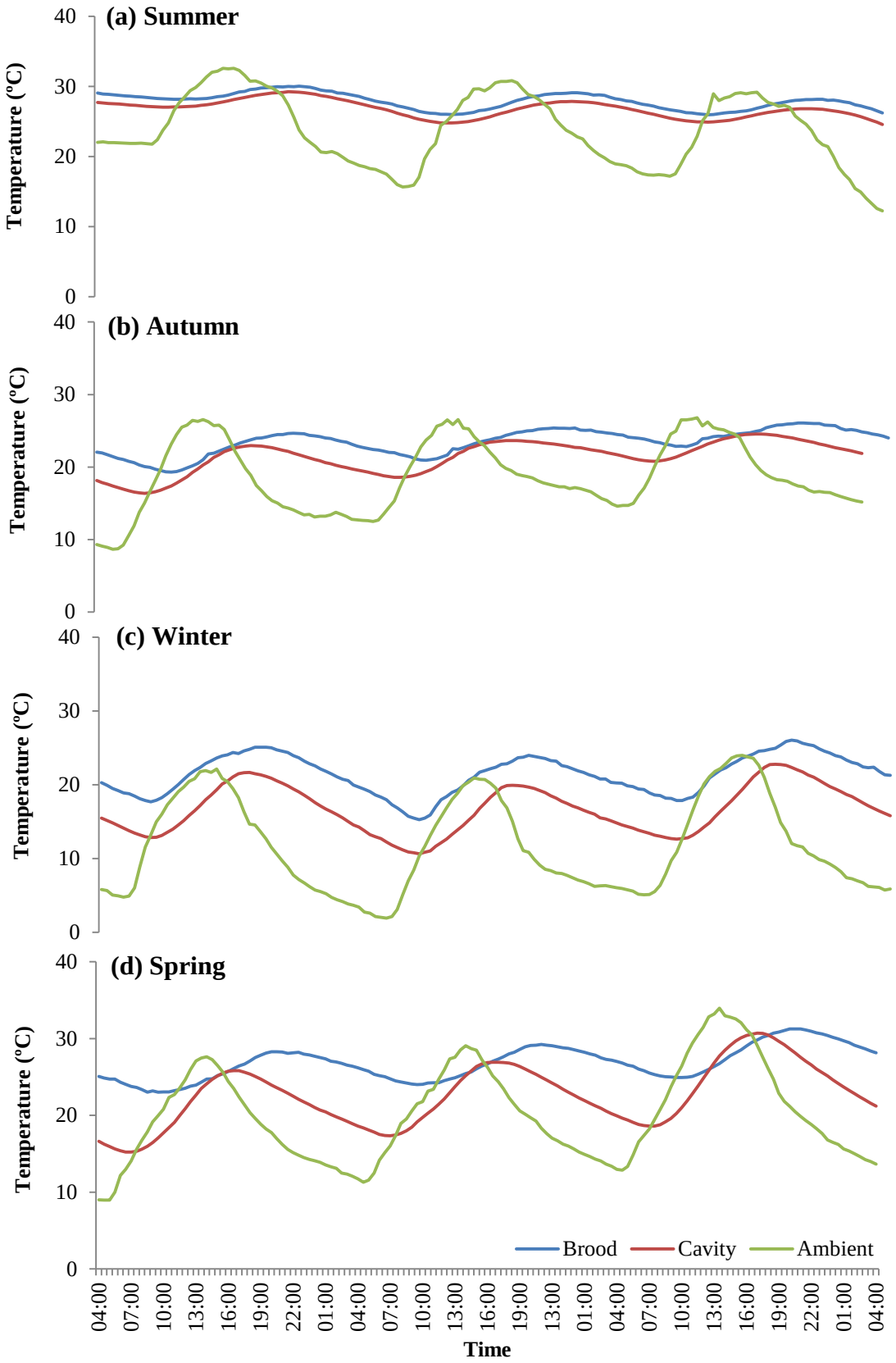

Figure 2.13 Brood (n = 4), cavity (n = 2), and ambient (n = 2) temperature fluctuations during three consecutive (a) summer, (b) autumn, (c) winter and (d) spring days.

2.3.4 Discussion

Tetragonula carbonaria was able to regulate its brood chamber temperature mostly between 15–31ºC, so the brood cells were warmer in cool ambient conditions, and cooler in warm ambient temperatures. The brood experienced temperatures between 10–34ºC, with no nest mortality, and maintained continuous brood production. *Tetragonula carbonaria* was able to maintain more consistent brood temperatures in summer, with greater temperature fluctuations occurring in winter. *Tetragonula carbonaria* is therefore not strongly stenothermic and its brood can tolerate considerable temperature fluctuations, unlike *A. mellifera* which is highly stenothermic and depends on close regulation of brood temperatures for development (Stabentheiner et al. 2010).

Thermoregulation has been investigated in a number of other stingless bee species, but in tropical and subtropical climates. Most of these species regulate their nest temperatures above ambient temperature, and maintain their brood temperatures at 25–36ºC (Table 1.1). Nest temperatures in previously studied stingless bee colonies have been rarely tested at ambient temperatures below 15ºC, with the brood chamber not falling below 19ºC (Table 1.1). Most tropical stingless bee species do not actively generate heat due to their environment. It was previously concluded that *T. carbonaria* were suited to tropical and subtropical locations due to their effective temperature requirements being 16–39ºC (Amano et al. 2000). However, *T. carbonaria* colonies at Richmond, a temperate location within their natural distribution, experienced brood temperatures ranging from a low of 10ºC to a high of 34ºC. The natural distribution of *T. carbonaria* is from coastal QLD (Cape York, 16º S, 145º E) to southern New South Wales (Bega, 36º S, 149º E) (Rayment 1935, Dollin et al. 1997, Klumpp 2007). Bega is 1ºC to 2ºC cooler in winter compared with Richmond (Australian Bureau of Meteorology, http://www.bom.gov.au). This suggests that *T. carbonaria* located in NSW can survive and develop brood during cooler winters and milder summers than those experienced in tropical and sub-tropical locations. The lethal temperature (9ºC) identified by Amano et al. (2000) was below the minimum temperature recorded in brood at Richmond.

Tetragonula carbonaria colonies located at Richmond were exposed to continuous fluctuating daily ambient temperatures, and they adapted to the cooler temperatures by passive thermoregulation mechanisms, such as building a number of involucrum layers around the brood chamber. *Scaptotrigona postica* (Engels et al. 1995), and *Au. australis* (Halcroft et al. 2013b) similarly produce involucrum during cooler weather. Heat generated from developing brood and worker thoraces can be retained within the chamber by the insulating properties of the involucrum (Michener 1961, Sakagami 1982, Engels et al. 1995). Involucrum was built around the brood chamber throughout the year; however, there were more layers during winter and less in summer. There was no evidence of workers clustering in the brood chamber or forming layers of slowly moving bees.

To maintain heat production during cooler periods, bees would be expected to consume stores of honey particularly when opportunities for foraging are limited; this was observed in *T. carbonaria*. In *A. mellifera* colonies, the consumption of honey for generation of warmth can result in their nest temperatures being maintained between 18–32ºC (Kronenberg & Heller 1982). *Melipona panamica* thoracic temperatures can increase up to 5ºC higher than other body parts following consumption of honey (Nieh & *S*ánchez 2005).

Newly emerged *A. mellifera* adults have little ability to generate their own warmth and require heat from the surrounding brood; they remain in the brood chamber normally for 2 d for self-warming, activation of flight muscles and ensuring proper adult development (Stabentheiner et al. 2010). *Tetragonula carbonaria* worker callows were observed to huddle in groups in the brood layers after emergence, after which they engaged in hive tasks. *Apis mellifera* drones from 8 d old contribute to nest temperatures by heating their thoraces by 1ºC above their abdominal temperature (Kovac et al. 2009). If *T. carbonaria* males are present during winter they may contribute to raising temperatures, as well as contributing to the higher, less variable spring brood temperatures. However, *T. carbonaria* males cannot be readily identified, and their contribution to nest temperatures is unknown.

During warmer seasonal periods, *T. carbonaria* brood had greater temperature consistency compared to the cavity and ambient temperature fluctuations. During the summer period, ambient temperatures were not recorded above 37ºC, while the brood and cavity temperatures did not exceed 35ºC. As internal temperatures were only slightly lower than ambient temperatures (by 2ºC), it suggests that colonies may be more at risk of overheating than cooling (as temperatures were kept at least 5ºC above ambient during cooler periods). Previously, *T. carbonaria* colonies at Richmond have experienced ambient temperatures exceeding 40ºC, with losses recorded at temperatures above this (Jenny Shanks, personal observations), supporting reports of *T. carbonaria* death at 46ºC (Amano et al. 2000). The observed responses by *T. carbonaria* colonies to cooling the nest in extreme temperatures included stopping entrance activity and increasing internal locomotion activities such as wing fanning (Jenny Shanks, personal observations; Appendix 1, Video 1). During these warm periods, *T. carbonaria* workers lined up at nest entrances, facing away from the nest (Jenny Shanks, personal observations, Amano et al. 2000) and/or singly on the brood chamber (Jenny Shanks, personal observation) and fanned their wings, aiding in nest ventilation. This worker behaviour has previously been documented to maintain the brood chamber between 28–32ºC under glasshouse conditions (A. Tse, pers. com. 2011). A similar response has been reported in *S. postica*, where at temperatures above 35ºC workers began wing fanning within the nest, on the involucrum and at nest entrances, probably assisting in ventilation (Engels et al. 1995). Similarly, *S. depilis* increased wing fanning at brood temperatures above 32ºC, and water collection from 29ºC, helping to lower the brood temperature 2.5ºC below ambient temperature (Vollet-Neto et al. 2015)

As brood and cavity temperatures over the three consecutive day periods never dropped as low as ambient temperature during the evening, the increase in heat in the nest is most likely associated with returning foragers and the amount of metabolic heat produced by workers and brood (Stabentheiner et al. 2010). As the brood temperatures were always warmer than cavity temperatures, especially during the cooler weather, this may be due to the immigration of workers to the brood (Stabentheiner et al. 2010), though this was not observed. The brood was always above the lowest ambient temperature, while the cavity space was below the highest

ambient temperatures; this has been similarly observed in *M. beecheii* colonies (Moo-Valle et al. 2000).

High temperatures above 40ºC are known to be lethal to *T. carbonaria* hives, because of the melting point of internal resinous structures. As the ambient temperatures rise the resin and nest structures soften and the hive "slumps" (Klumpp 2007). The, incorporation of different plant resins in the nest structures which have similar melting points may contribute to hive slumping (Halcroft et al. 2013a).While plant resin presence in hives is a concern for colony survival during hot summer conditions, *C. torelliana* resin is reported to have a high antimicrobial activity, thereby otherwise benefiting the hive (Massaro et al. 2014a). However, no temperatures >40ºC occurred during the investigation period.

Brood temperature and pathogen development

Apis mellifera maintains its brood development in a narrow temperature range; if brood temperatures drop below optimal, the incidence of fungal infections can increase. *Ascosphaera apis* is a heat-sensitive fungus, with optimum spore germination in the gut of larvae if they are chilled for a minimum of 24 h between 30–32ºC (Bailey & Ball 1991, Flores et al. 1996, Vojvodic et al. 2011). When brood temperature drops, *A. mellifera* workers respond by increasing the temperature by thermoregulation behaviours (Starks et al. 2000), thereby reducing the spore germination (Simone-Finstrom et al. 2014). On the other hand, bacterial brood infections caused by pathogen *P. larvae* and *M. plutonius* require optimum temperatures of 35–37ºC (max. 40ºC) for maximum spore germination (Ludwig et al. 2009a, Alvarado et al. 2013), which falls within the optimum temperature range for *A. mellifera* larval development (Simpson 1961, Fahrenholz et al. 1989). Unsurprisingly therefore, there are no published data on temperature regulation by *A. mellifera* workers in response to bacterial brood infections.

In the current study, the brood temperatures in *T. carbonaria* colonies fell as low as 10ºC and peaked at 34ºC, with continual brood production throughout the year. The temperatures experienced in the brood chamber may impact the incidence of brood

diseases, with lower temperatures and possibly even fluctuating temperatures suppressing pathogen growth and development.

Chapter 3

Hygienic behaviour in *Tetragonula carbonaria*

3.1 Abstract

The ability of a number of eusocial bee species, to control brood pathogens is associated with the hygienic behaviour of their workers, to detect and remove infected brood cells. *Tetragonula carbonaria* has little or no recorded brood pathogens, and it was hypothesised that hygienic behaviour may play a significant role. This study therefore investigated the hygienic behaviour of *T. carbonaria* colonies for the first time, under different experimental conditions, such as different seasons and with open or closed nest entrances. Colonies were also assessed for their short-term learning ability and also whether worker hygienic behaviour was passed on to fellow nest members. Worker behaviour was video recorded and assessments were made hourly until all killed pupae had been detected, their cells uncapped and the pupae removed. Results were compared to published data for *A. mellifera* and five Neotropical stingless bees. *Tetragonula carbonaria* was shown to possess superior hygienic behaviour to all these species taking a total of 420 min ± 27 min to complete hygienic behaviour activities. Uniquely, colonies completely dismantled and removed damaged or diseased brood cells, possibly a method that could further reduce potential disease transmission. During winter, colonies took longer to detect dead pupae, while increasing worker populations inside a nest by restricting entrance access did not influence worker expression of hygienic behaviour. Interestingly, there was an indication that short-term learning of hygienic behaviour occurred; however, there was no passing on of learned hygienic behaviour between brood workers over time. These highly efficient hygienic behaviours may, at least in part, explain why there are fewer brood diseases in *T. carbonaria* colonies than in co-located honey bee colonies.

3.2 Introduction

Hygienic behaviour in eusocial bees is the detection, uncapping and removal of infected and/or dead pupae by workers. A colony which can detect early and remove diseased pupae from the brood comb before the pathogen reaches the highly infectious reproductive stage can restrict overall disease incidence within it (Woodrow & Holst 1942, Spivak & Reuter 2001b, Invernizzi et al. 2011). Times taken for colonies to detect and uncap cells, rather than the removal time, are

important parameters that differ between hygienic and non-hygienic colonies. (Invernizzi 2000, Invernizzi et al. 2011).

Hygienic behaviour has been investigated for honey bee nests infected with *Paenibacillus larvae, Ascosphaera apis* and, most recently, for varroa mite (Boecking & Drescher 1992). These infections disrupt brood development. Most studies have reported that *Apis* spp. will remove 90–100% of infected or dead pupae within 48 h; *Apis mellifera* Linnaeus (Milne 1982, Spivak & Gilliam 1993, Spivak & Downey 1998, Waite et al. 2003, Kavinseksan et al. 2004, Palacio et al. 2005, Espinosa-Montaño et al. 2008, Palacio et al. 2010), *Apis mellifera carnica* (Gramacho & Gonçalves 2001, Stanimirovic et al. 2001, Nedić et al. 2005, Gramacho & Gonçalves 2009b), *A. m. intermissa* (Adjlane & Haddad 2014), *A.m. lamarckii* (Kamel et al. 2003), *A. m. ligustica* (Spivak & Reuter 1998, Palacio et al. 2000, Spivak & Reuter 2001a), African honey bee *A.m. scutellata* (Danka & Villa 1994, Gramacho & Gonçalves 2009a, Pereira et al. 2013), *A. cerana indica* (Arthreya & Reddy 2013), and *A. florea* (Woyke et al. 2012).

Cannibalism occurs in many honey bee colonies as a means to recycle nutrients by the consumption of larvae (Fukuda & Sakagami 1968, Woyke 1977, 1980, Webster & Peng 1988, Schmickl & Crailsheim 2001), but also when varroa mite infested (Rath & Drescher 1990), or infected with brood pathogens such as *A. apis* (Invernizzi 2000, Invernizzi et al. 2011). During brood disease development, workers have been observed to detect and cannibalise the brood before the infected pupae show signs of disease i.e. the mummification of *A. apis* infections (Milne 1983, Invernizzi et al. 2011). During hygienic behaviour studies, cannibalism can be observed by either partial remains of pupae in uncapped cells (Gramacho & Gonçalves 2001, 2009a, b) or the complete removal and cannibalism of pupae before uncapping is completed (Invernizzi 2012). Unfortunately, the consumption of spore infected young aids in the transmission of the disease by the adult workers. To date, all tested stingless bees have been observed to not cannibalise dead larvae or pupae (Tenório 1996, Medina et al. 2009, Nunes-Silva et al. 2009).

Hygienic behaviour expression

Hygienic behaviour in honey bees is a heritable trait (Rothenbuhler 1964a, Moritz & Crewe 1988, Oxley et al. 2010), where workers in a population carrying these traits can be stimulated to remove dead brood. Odours and volatiles released from brood cells (Masterman et al. 2001) can initiate hygienic behaviour responses such as uncapping (Gramacho & Spivak 2003). Workers performing uncapping are more sensitive to lower concentrations of volatiles emitted from dead brood than the workers removing it (Masterman et al. 2000, Masterman et al. 2001, Gramacho & Spivak 2003, Spivak et al. 2003), indicating separation of workers based on task specialisation (Gramacho & Gonçalves 2009a). In hygienic colonies, when a worker commenced the uncapping stage, that worker continued this stage until finished (Palacio et al. 2010); however, in non-hygienic colonies workers sometimes recapped dead pupae (Spivak & Gilliam 1993), indicating that these workers lacked the ability to detect the difference between dead and live brood (Swanson et al. 2009). Contact with excreted bodily fluids (haemolymph and other material) from brood cells can also stimulate hygienic behaviour in *A. mellifera* (Spivak & Downey 1998, Gramacho et al. 1999, Espinosa-Montaño et al. 2008), and *A. dorsata* (Woyke et al. 2004).

Apart from the genetic control, a colony's hygienic behaviour can also be affected by environmental factors, with blossom availability and higher nectar flows influencing its expression. It has been suggested that faster brood removal occurs during good nectar flows, as the colonies require more cells for nectar storage (Thompson 1964, Momot & Rothenbuhler 1971, deGuzman et al. 2002, Adjlane & Haddad 2014, Uzunov et al. 2014). For example, hygienic *A. mellifera* colonies removed more dead brood (94.2%) in 48 h compared to commercial colonies (82.3%) during high nectar flows, with higher honey production/storage in the hygienic colonies compared to the commercial colonies (40.5 kg and 30.1 kg, respectively) (Spivak & Reuter 1998). Furthermore, *A. mellifera* colonies removed 91% of dead brood in 48 h during nectar flow compared to 81% during nectar dearth (deGuzman et al. 2002), while *Apis m. intermissa* removed 91.5% of dead brood after 24 h in higher nectar flow during spring, compared to 83.6% in autumn (Adjlane & Haddad 2014). Conversely, during

low nectar flows, removal of diseased larvae takes longer (Rothenbuhler 1964b, Thompson 1964, Momot & Rothenbuhler 1971, Kavinseksan et al. 2004). However, a number of other studies have reported no relationship between nectar flow and hygienic behaviour (Boecking et al. 2000, Panasiuk et al. 2009, Bigio et al. 2013, Güler & Toy 2013). Interestingly, hygienic behaviour had a higher correlation with honey production in *A. m. scutellata* than with propolis production (Garcia et al. 2013).

Another factor reported to affect expression of hygienic behaviour is colony size and composition. When a full-sized *A. mellifera* hive (10-frame hive) was reduced to a two-frame observation hive, the associated decrease in population negatively impacted on the expression of hygienic behaviours performed by the workers (Spivak & Gilliam 1993). This may be associated with the proportion of aged brood workers and the genotypic composition of the colony (Arathi & Spivak 2001). A difference in the balance of young and old bees in the population will affect hygienic expression (Momot & Rothenbuhler 1971, Uzunov et al. 2014), with greatest expression in colonies with a range of different age workers (Stanimirović et al. 2001, Stanimirović et al. 2002). Workers younger than four weeks exhibit more efficient hygienic behaviour (Thompson 1964, Arathi et al. 2000, Arathi et al. 2006, Palacio et al. 2010, Panasiuk et al. 2010), when present in colonies with a composition of 50–100% hygienic workers (Arathi & Spivak 2001). Compared to a colony composed of only 25 % hygienic bees, the hygienic workers continued to perform hygienic behaviours past middle-age (mean age 39 d, maximum 56 d) (Arathi & Spivak 2001). The effect of the proportion of workers exhibiting hygienic behaviours in a colony, was also shown when non-hygienic bees were added to a hygienic colony, and reducing the performance of the hygienic workers by taking longer (Spivak & Gilliam 1993, Arathi & Spivak 2001). However, other studies have concluded that the population size of a colony is not correlated to the efficiency of hygienic behaviours performed (Gramacho 1995 cited in Invernizzi et al. 2011, Kavinseksan et al. 2004, Bigio et al. 2013).

Other variables may influence the outcome of the hygienic behaviour evaluation of a colony. Comb age, brood size, cell dimension and capping thickness differences between worker and drone brood appeared to affect hygienic outcomes (Gramacho 1999, Gramacho & Gonçalves 2009a, Pereira et al. 2013); workers uncapped and cleaned worker cells within 24 hours compared with 48 hours for drone brood (Invernizzi 2012), as well as removing brood faster in new comb than old comb, possibly associated with the condition of the cells (Pereira et al. 2013). The time of day is another variable reported to affect hygienic behaviour, being significantly shorter during day time (Pereira et al. 2013).

Hygienic behaviour in stingless bees

Colonies of *M. beecheii* and *S. pectoralis* (Medina et al. 2009), *M. quadrifasciata*, *T. angustula* (Tenório 1996), and *P. remota* (Nunes-Silva et al. 2009) have shown hygienic behaviours similar to those of *Apis* spp. *Plebeia remota* removed 70% of dead pupae in 24 h, and 96% within 48 h (Nunes-Silva et al. 2009). *Scaptotrigona pectoralis* hygienic behaviour was similar to *P. remota*, taking 2.3 ± 0.6 d to remove 100% of the dead pupae (Medina et al. 2009). This is considerably faster than *M. beecheii*, which took 4.4 ± 2.0 d to remove 100% dead brood (Medina et al. 2009), and *T. angustula* removing 30% and *M. quadrifasciata* removing only 1%, in 72 h (Tenório 1996).

Unlike *Apis* spp., cell reuse following removal of diseased brood does not occur in stingless bees, where contaminated cells are dismantled and removed (Tenório 1996, Nogueira-Neto 1997). In contrast, *A. mellifera* responded to *P. larvae* infection by partially dismantling all or most of the infected brood, down to the cell midrib (Newton & Ostasiewski 1986, Spivak & Reuter 2001b); new wax was then added to these cells and rebuilt for raising new brood. This latter response means that re-infection is more likely to occur (Park 1935, Spivak & Reuter 2001b).

No correlations were found between brood comb size, colony strength and the number of bees performing hygienic behaviour in *P. remota* (Nunes-Silva et al. 2009). *Melipona beecheii* workers performed hygienic behaviour tasks at a mean age

of 20.7 ± 5.7 d (Medina et al. 2009) and in *T. angustula* it was between 12–28 d old (Tenório 1996), indicating that it was middle aged workers that performed hygienic behaviours (Arathi et al. 2000). Tenório (1996) made specific observations of worker hygienic behaviour and task recruitment in *M. quadrifasciata* and *T. angustula*. Workers were observed to tap their antennae onto the cells and rub their hind legs as they passed over the brood cells throughout the uncapping and removal stages. Tenório (1996) suggested this may be a form of communication to stimulate hygienic behaviour within the nest. Once a worker was assigned a hygienic task, the task would be completed before the worker moved to the next task (Tenório 1996). Examples of specific task allocation included some workers exclusively removing dead pupae from cells and dumping the corpses on the surrounding brood chamber, while other workers were involved only in removing these corpses from the hive (Tenório 1996).

This study aimed to observe, document and assess hygienic behaviour performed by *T. carbonaria* and compare the results with those reported for other stingless bees and *Apis* spp. It was hypothesised that hygienic behaviour may play a significant role in the low incidence of brood pathogen infections in *T. carbonaria* in Australia, as well as other stingless bee species worldwide.

3.3 Materials and methods

3.3.1 Observing normal behaviour

A single strong colony maintained in the bee shed located at the UWS apiary was monitored over a single day in warm, sunny conditions. To allow for observations of workers and the queen on the brood chamber, the colony was opened by removing the acrylic lid, and carefully pulling open the involucrum sheath covering the brood chamber. During this process, it was noted that storage pots and brood cells were damage, and there was unavoidable killing of some workers.. A clean acrylic lid was placed onto the top of the box, the workers were allowed to forage. Observations of worker and queen activities and behaviours were noted as well as recorded using a

high definition video camcorder (Sony Handycam HDR-SR1 HDD, Sony Corportation, Minato-ku, Tokyo, Japan).

3.3.2 Development of methodology for assessment of hygienic behaviour

A preliminary pin-kill experiment using *T. carbonaria* brood cells was carried out to observe behavioural responses displayed by workers. A colony maintained in the bee shed was opened on a warm, sunny day. The colony was split in two separate halves, exposing the brood chamber. A disc was selected that had a range of developmental stages; these were: recently provisioned and deposited with an egg at developing larval stage, or with pupa at white-eye stage. Seven cells of each stage were selected, then pin-killed using a 38 mm x 0.55 mm entomology pin (Asta black steel entomology pins, Australian Entomological Supplies Pty. Ltd. Coorabell, NSW) through the cell cap. The hive half then had an empty box added to the top and was covered with a clear piece of acrylic for easy observation. The pin-killed cells were left for 24 h; during this time behaviours displayed by workers were recorded and described, using terminology similar to that used for hygienic behaviour in honey bees. Six of the recently provisioned pinned cells had their contents removed and the cells were completely deconstructed. The seventh cell was recapped. This stage of cell development (viz., recently laid eggs) was too young for comparative analyses with other hygienic behaviour assays. In addition, it was not possible to observe the "removal" stage of hygienic behaviour as larvae had not hatched, and the egg was not destroyed by the pinning process. However, worker bees were observed to remove killed larvae and pupae from the other pinned cells. From these observations, the black-eyed pupae stage was selected in preference to white-eyed, as it was easily seen through the cell (Figure 3.1). Therefore, this age was used for all hygienic behaviour assays.

Figure 3.1 *Tetragonula carbonaria* brood disc with pupae at the black-eyed developmental stage that can easily be seen through the cell caps.

Opening, hive set-up and brood disc exposure

To expose the disc with black-eyed pupae, the standard splitting technique was used (Chapter 2, section 2.1.4). If the desired brood disc age was not seen on the initial split, it was found in 1–3 discs above or below the exposed disc; a simple extraction of the age-appropriate disc and its placement on the top layer was adequate to allow for testing. If nests when opened did not have ideal brood discs, appropriately aged brood discs could be removed from neighbouring colonies and placed on the top layer, as described above. Discs from different colonies were readily accepted, as also reported by Spivak & Downey (1998).

As some experiments were running for 2–4 d, and to keep hive splits communicating, the two nest halves were connected via silicone tubing (8 mm diam.) to the OP (Figure 3.2).

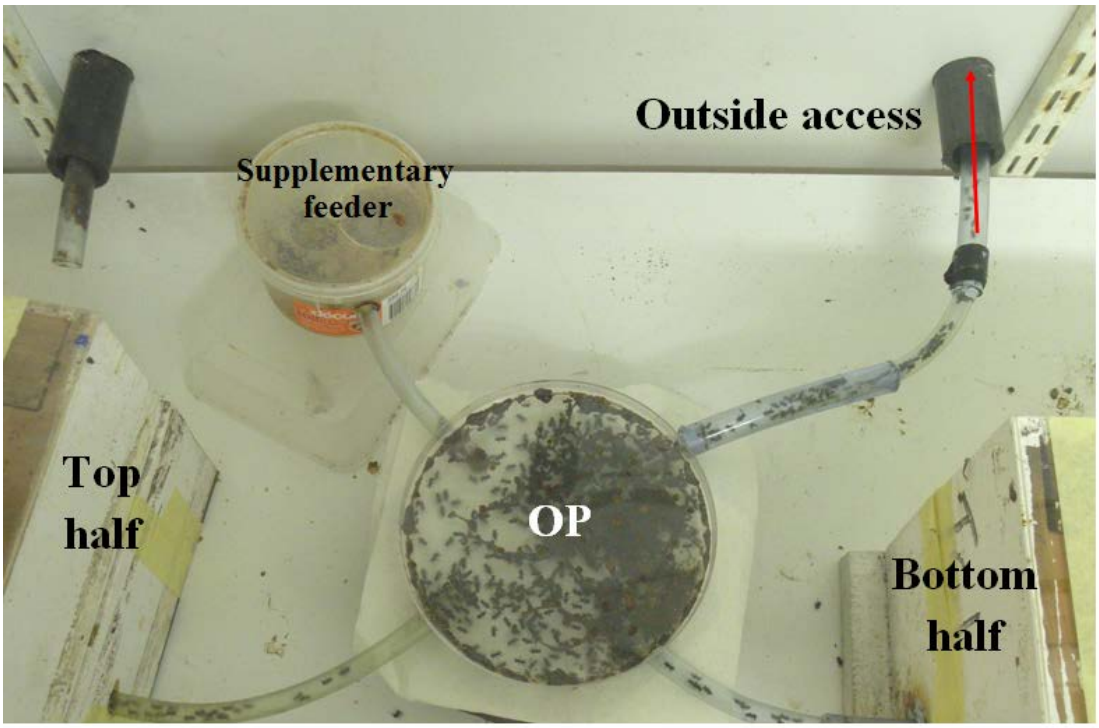

Figure 3.2 The two nest halves can continue to communicate and perform tasks during the hygienic experiments, by connecting box halves with tubing to a central OP, which had access to the environment.

Marking pin-killed brood

Trial studies were designed to perfect the pin-kill marking method for experiments. Marking of brood cells was required to aid in visual observation of the cells and the worker behaviour associated with these cells. It was important that the marker used did not cause abnormal behaviour or emit odours that would alter the worker bees' performance.

A selection of intact brood with black-eyed pupae were marked with either black permanent marker (Sharpie® Super permanent marker, Sanford Manufacturing Corporation, Illinois, US), white acrylic paint pen (Mitsubishi Pencil Co. Ltd., Tokyo, Japan) and orange fluorescent powder (Radiant Colour, Richmond, CA) (Figure 3.3), then a subsection of these cells were pin-killed while the surrounding cells were not (control). The unmarked cells and marked cells which were not pin-killed, were not uncapped by the workers, whereas marked and unmarked pin-killed cells were uncapped equally, confirming that none of the markers interfered with normal worker hygienic behaviour. The white acrylic paint pen was therefore used to

mark the perimeter of the test area and the black permanent marker for the 50 cells tested.

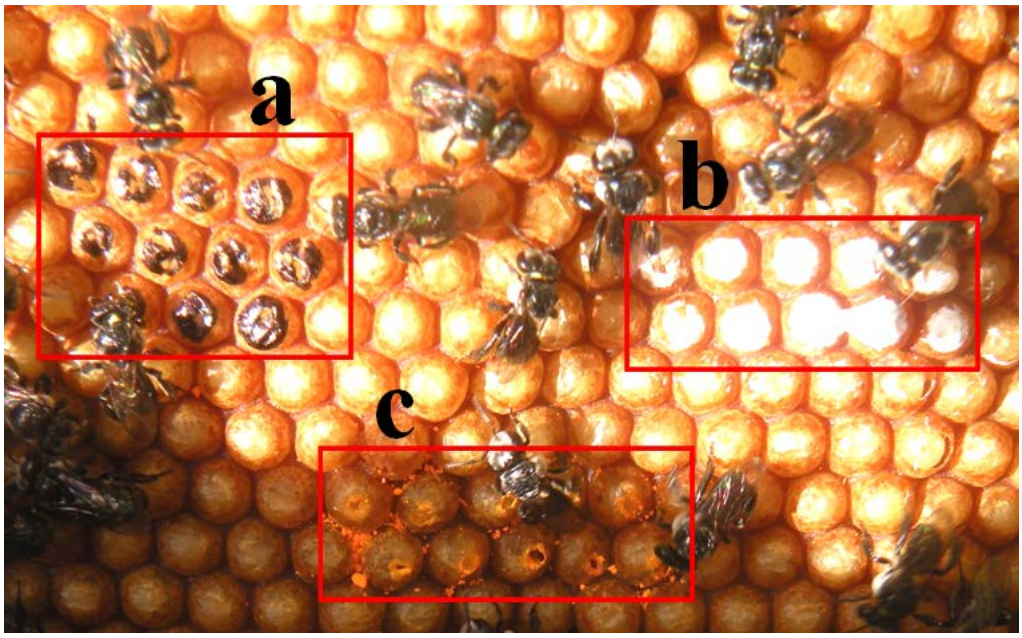

Figure 3.3 Different brood cell marking trials a) black permanent marker b) white acrylic paint pen and c) orange fluorescent powder.

Estimating brood population (EBP)

In a preliminary assessment, a hive was opened, and five discs (ranging from smallest to largest) were selected. Five individual cells from each disc were randomly selected and their diameter measured. The cell diameter was consistent at 2.5 mm (supporting Michener 1961).

Subsequently, for brood population assessments of tested colonies, the hives were opened and the area (the width and length) of five discs (ranging from smallest to largest) was divided by the value of the 25 cells (Roubik 1979) to estimate the population of each disc.

Nest weight

Colony nest weight was measured 61 times between 2012 and 2014, for eight colonies permanently maintained at UWS and which were used for the hygienic behaviour experiments. The weight of a colony: all internal hive structures, stores

and adult population (not including the box weight), was measured by firstly blocking the entrance of the colony before first day foraging and weighing the hive as is. The mean box weight (based on weighing five empty boxes) was then subtracted from the total hive weight.

Entrance worker activity

Worker entrance activity was regularly observed and recorded in colonies located at UWS between 2012 and 2014. Colony entrance counts were conducted weekly, on the same day, between the same times (11:00–13:00 AEST). Activity was recorded by counting both number of bees entering and leaving each hive for a duration of 2 min, once every week. The two counts were used to calculate the mean worker entrance activity for each hive.

3.3.3 General hygienic behaviour

As previously outlined in the preliminary experiments, a *T. carbonaria* colony maintained in the bee shed was opened, and a disc containing black-eyed pupae was selected. Within the disc, 50 cells were selected (test area), with the perimeter of the test area marked with white acrylic paint pen; from these 50 cells, 25 were marked with black permanent marker. The 25 marked cells were pin-killed by inserting the clean entomology pin carefully through the cap and piercing the crown of the pupae. The remaining 25 cells in the test area were left undamaged and formed the control. A high definition video camcorder was set to night-vision mode and worker behaviour was recorded until all pin-killed cells were removed.

For the experiments described in this chapter, standardised recording of the detection stage was determined when one worker was observed chewing at the cerumen cap of a pinned cell for at least 5 sec. The time taken to first uncapping was determined by recording the time from pin-kill to the time when one cell cap was completely removed, but an intact pupa could still be seen inside.

For each colony tested, hourly cell counts were taken for the number of cells (i) still capped, (ii) completely uncapped, (iii) pupae removed, and (iv) the number of cells completely dismantled. For each colony tested, the video recordings were used to calculate the total time taken for workers to complete detection, uncapping and removal stages, as well as record the total time from pin-kill to when all cells were completely dismantled. Each colony was tested on two consecutive days, and video recording and the time taken to perform each hygienic task recorded for both days.

This experimental set-up was used to address several questions about hygienic behaviour of *T. carbonaria*.

Does *T. carbonaria* display hygienic behaviour; and if so, how does it compare with other stingless bee species and honey bees? To test whether *T. carbonaria* exhibited hygienic behaviour, a total of 18 hives were tested using the pin-kill method between October 2011 and April 2014, giving a total of 92 sets of observations. Hives used in the tests were both colonies were those originally located at UWS, as well as hives sourced from S.E. QLD (Dr Tim Heard, Sugarbag, West End, Brisbane). Upon arrival, the colonies sourced from S.E. QLD were placed into the bee shed and allowed one week to adjust to the conditions. All colonies were tested under the normal bee-shed temperatures with open hive entrances allowing worker foraging.

Are there other factors which can affect hygienic behaviour efficacy?

Do seasonal changes affect worker behaviour and their efficiency in conducting hygienic behaviour? To assess the influence of season on hive hygienic behaviour, 17 healthy *T. carbonaria* hives were tested, totalling 40 recordings. For each season: winter, spring, and summer (2012) and autumn (2014), five hives were tested for hygienic behaviour at the same time, on two consecutive days. Hives used in the tests were those originally located at UWS, as well as those sourced from S.E. QLD and were also in the bee-shed temperatures with open-external entrances.

Does the strength of a colony determine the level of hygienic behaviour? The time taken for nests to perform hygienic behaviours was compared between hives, with regard to their EBP (n = 27), nest weight (n = 61), and mean worker entrance activity (n = 124).

Does external access to the environment affect hygienic behaviour? This experiment aimed to determine what happens to hygienic behaviour in a colony when the weather was unsuitable for foraging, and the foraging workers remained inside the hive. Four colonies previously used for hygienic behaviour (16 weeks prior), were tested for their hygienic behaviour when their hive entrances were blocked to simulate non-foraging conditions. Two colonies were tested on two consecutive days, then the two remaining colonies were tested on the next two consecutive days; this provided eight recordings over 4 d (n = 8). This was then compared to 24 previous recordings using the same hives but with open entrances (n = 24)

Can hygienic behaviour be "learned" by a colony? To answer this question, four UWS located hives, were exposed to hygienic experiments from October 2011 to February 2012 as follows. The brood of a single colony was pin killed every day for four consecutive days. After this, the colony was left for six to seven weeks untested, after which the pin-kill test was repeated. The entire test was repeated two more times with one colony. Each of the four colonies was treated using the same method. This provided 48 × single day recordings and 12 × four consecutive day recordings, enabling a comparison between the rates of hygienic behaviour over several consecutive days (short-term memory) and also between periods of one month (long-term memory learning).

Statistical analysis

Data collected on the second consecutive day was used for statistical analysis as there was a change (increase) in expression of hygienic behaviour between the first

and second days, but no further changes thereafter. The time intervals for each hygienic task were analysed using ANOVA (IBM SPSS ver.22 for Window, IBM Corp. 2013). Homogeneity of variance was tested using Levene's test. Means were compared using Tukey HSD for the majority of data, which had homoscedastic variances; however, Welch's t-test was used for data from experiments assessing access to environment, as they had unequal sample size. Pearson's correlation analyses were performed between the parameters EBP, hive weight, worker entrance activity and the time to perform hygienic behaviours (MINITAB.17.1.0, Minitab Inc. 2013).

Supplementary videos

Behaviours of workers before and during the hygienic behaviour experiments are provided as mp3 formatted videos in the accompanying USB drive (Appendix 1), and are cited in the text.

3.4 Results

3.4.1 Normal behaviour in the brood chamber prior to experiments

Workers were highly active in undertaking brood maintenance activities and continuously communicated with other workers. In a healthy, active colony in warm conditions, workers were not observed to be motionless within the brood area; even if a worker was not performing a brood task, she was conducting some other activity such as, preening her antennae and mandibles after working a cell, or antennating with a fellow worker. Trophallaxis was rarely observed within the brood area. There were no aggressive behaviours (e.g., biting and pulling appendages, chasing nest mates, mounting and holding down workers) between workers within the brood chamber. The only time aggressive, defensive behaviours were observed was when an intruder (a worker from a nearby nest) entered (Figure 3.4), or when a virgin queen and an active queen were present at the same time in the brood chamber and fought for dominance.

Figure 3.4 Defensive behaviour displayed by *T. carbonaria* towards an intruder. Workers in teams a) biting and pulling *A. mellifera* worker, and b) using resin balls to hold down an intruder.

Brood chamber workers were observed constructing and provisioning cells, fixing broken cells by chewing and manipulating cerumen. Workers were also seen removing cerumen from cell caps (when pupae were close to emergence). These tasks were performed singly or in small groups of 2-3 workers.

Due to opening of the nests to allow for observations it was noted that damage occurred to storage pots, brood cells and the unavoidable killing of workers. Broken cells were observed either being repaired using cerumen with their contents intact (i.e., left to develop and emerge), or the contents were removed and the cell dismantled. Workers were also involved in constructing pillars and supporting structures (the involucrum) which were attached to surrounding storage pots. If the pots were broken at any time and contents spilt in the brood chamber, workers were seen in large groups collecting the contents and repairing the pot. Pollen foragers were not seen often in or walking through the brood chamber.

Workers were observed to perform different tasks depending on their age; age was determined visually by the level of pigmentation and body size. Newly-emerged callows had very little colouring with no pigmentation on their legs, thorax or head, with minimal antennal darkening, whereas older workers were overall darker in colour (Figure 3.5). There were two types of older workers within the brood area, distinguished by their abdominal size. Workers with swollen abdomens were observed maintaining and provisioning brood cells (Figure 3.6), while the workers

with smaller abdomens worked mainly with cerumen either adding to or removing it from the cells. Newly emerged callows could be observed at times clustered together under the brood discs in groups of >5 workers, and undertook no tasks immediately after emergence; instead they preened themselves and fluttered their wings. As they aged, they commenced to undertake cell cleaning tasks. Callows were not observed to pair or regularly work with older workers, indicating they were not directly instructed by older workers in how to perform their tasks.

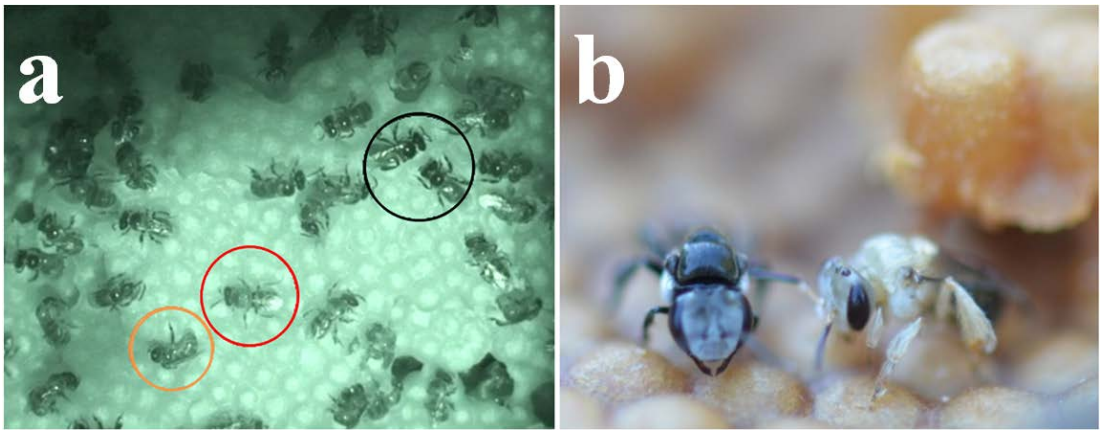

Figure 3.5 Different aged adults within the brood chamber, as indicated by coloured circles (a). Red and orange circles enclose older callows as indicated by leg and thorax colouring. Black circle encloses much older worker as indicated by complete black colouring. Newly emerged callows, showing little pigmentation (b).

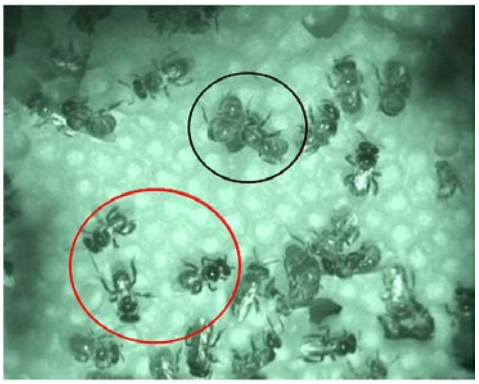

Figure 3.6 General brood behaviours prior to pin-kill experiments. Different coloured and sized workers are observed performing the same task; manipulating cerumen off cells. Red circle encloses darker workers with small abdomens. Black circle encloses workers with larger abdomens as evidenced by expanded abdominal segments.

Worker numbers were observed to increase within the brood chamber at times of cell repairing, cell provisioning, before a queen laid an egg, or when an intruder was present (Figure 3.7). Workers collected cerumen by chewing at cells or the surrounding involucrum using their mandibles; their forelegs were not involved in the manipulation.

An individual or a small group of workers could construct a cell, where workers took turns in attaching and manipulating cerumen, as well as provisioning the cell. Workers were observed to wait at the sides of cells to provision or sometimes appeared agitated while waiting for the previous worker to finish (Figure 3.8). This continued until a cell was provisioned sufficiently to allow an egg to be laid by the queen (Appendix 1, Video 2).

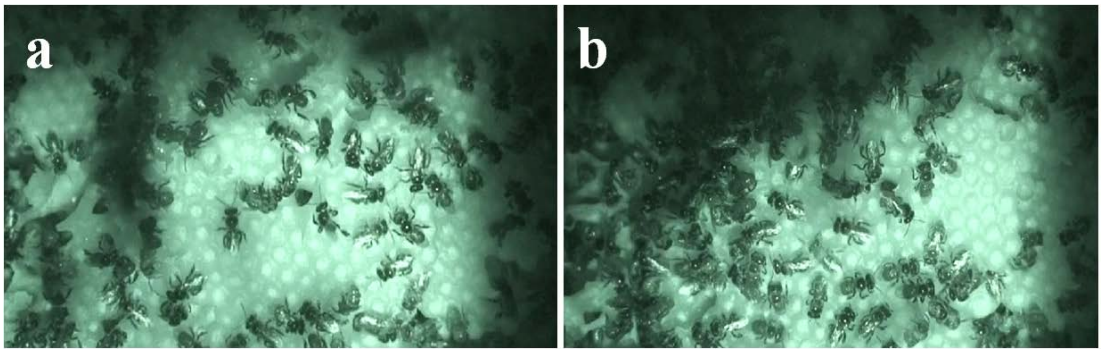

Figure 3.7 Increase in worker numbers within the brood area which has damaged cells. There is an increase in workers from image a) to b) to repair broken brood cells Approximately 15 mins between the two images.

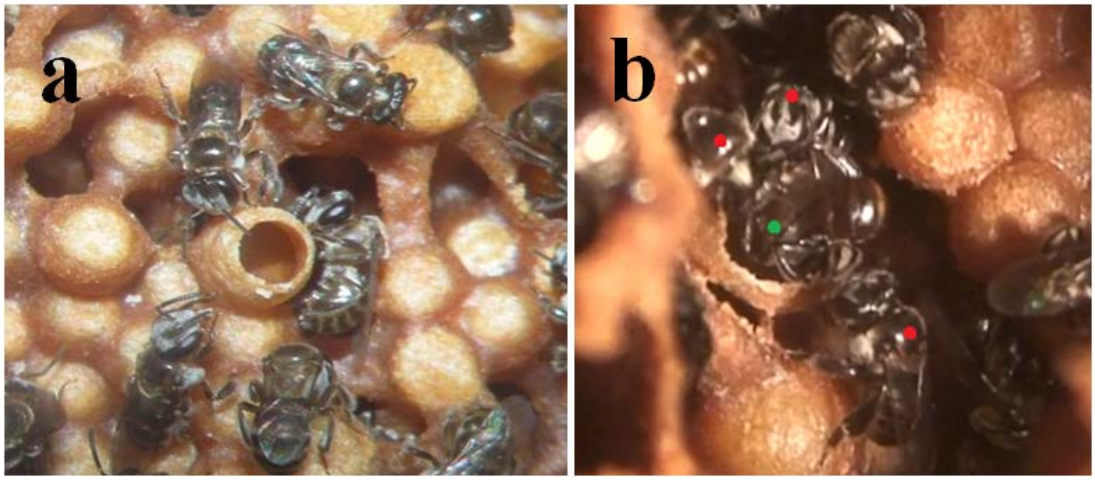

Figure 3.8 Workers constructing a cell (a), and provisioning a cell (b). Green dot shows a bee provisioning the cell and the red dots show other bees waiting to provision.

In a healthy, active colony, the queen was observed walking around her brood chamber alone (Figure 3.9), not harassed by carers. If the queen came near workers in her brood chamber or made contact, the workers would move away quickly in the opposite direction, then return shortly after to resume their tasks once the queen had passed (Appendix 1, Video 3). As a cell approached readiness for laying, the queen was observed making a number of rapid passes over the provisioned cell, fluttering her wings. Associated with this activity, workers became excited and worker numbers and activity increased (Appendix 1, Video 4) (Figure 3.10 a). Once the cell was fully provisioned, the queen laid a single egg (Appendix 1, Video 4), and an individual worker capped the cell (taking 5 to 6 min), during which time the number of workers around the cell decreased (Figure 3.10 b).

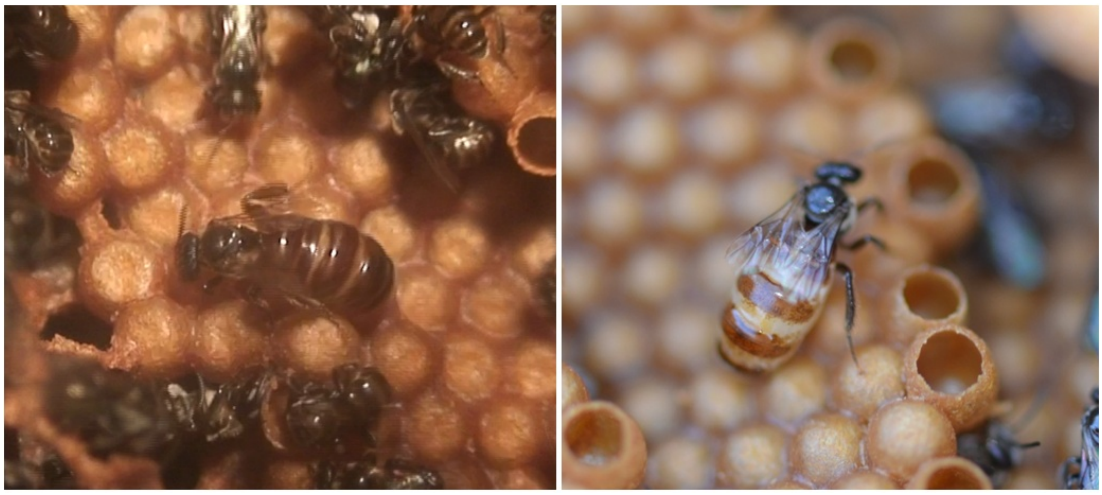

Figure 3.9 An active queen in her brood chamber with new cells nearby; note the queen is alone.

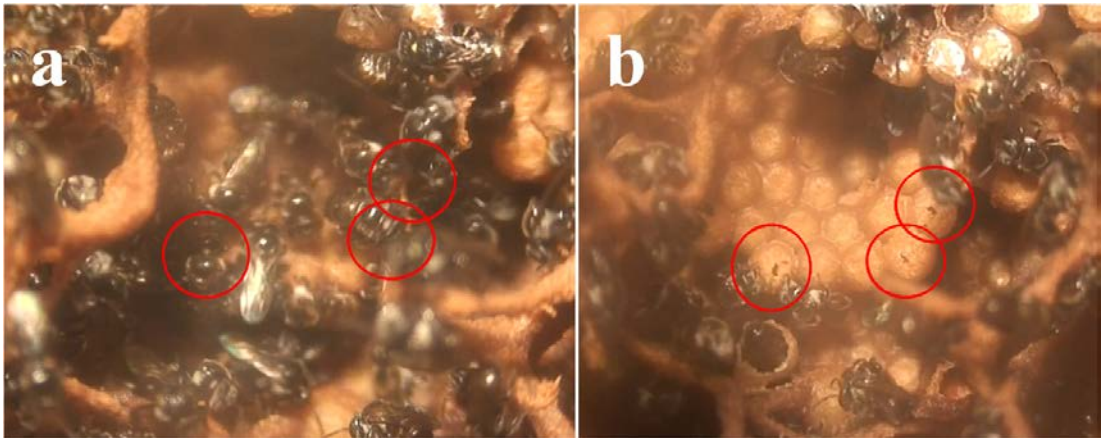

Figure 3.10 a) Increase in worker numbers within the brood area where cells are close to final provisioning and a queen is active in the area. a) Red circles indicate cells being prepared, b) Red circles showing capping of the same cells, and a decrease in worker numbers after eggs have been laid.

3.4.2 Does *T. carbonaria* display hygienic behaviour, and if so, how does it compare with other stingless bee species and honey bees?

The combined data (92 recordings) collected from 18 hives, showed it took on average 23 ± 2 min (mean ± SE) to detect a pin-killed cell, by 70 ± 6 min the first cell was uncapped and by 420 ± 27 min all cells had been removed (Figure 3.11). The longest and shortest times recorded to perform hygienic behaviour tasks within tested colonies are given in Table 3.1.

Table 3.1 Shortest and longest times (min) recorded to perform hygienic behaviour tasks.

Time taken to:	Shortest (min)	Longest (min)
Detection	20 (sec)	95
First uncapping	10	406
All cells removed	126	1620

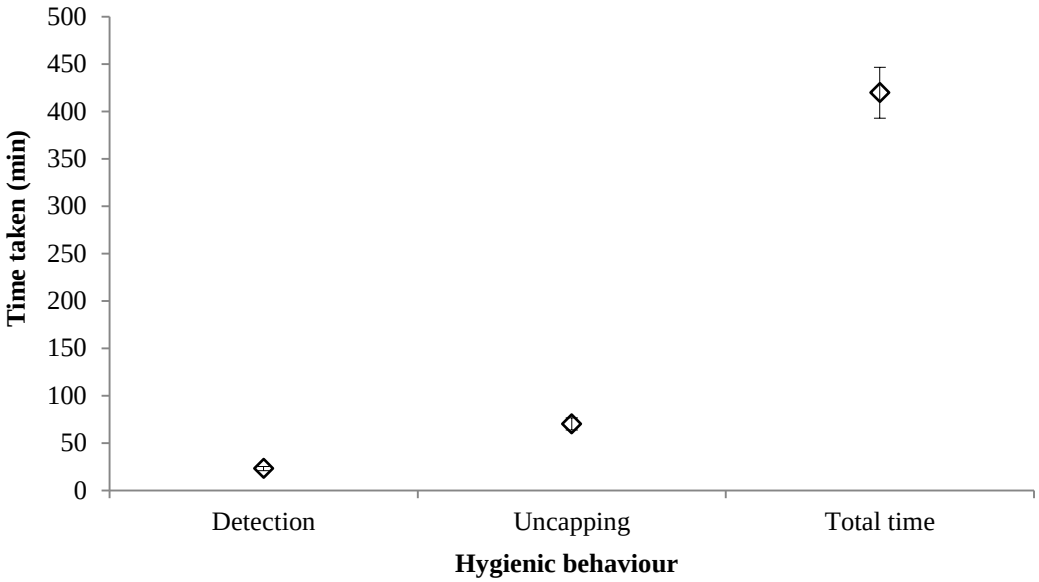

Figure 3.11 Cumulative time taken (min) for *T. carbonaria* to undertake detection (n = 92) and uncapping (n = 92) behaviours, and the total time to detect, uncap and remove cells (n = 92), tested between October 2011 and April 2014. Error bars = SE of means.

3.4.3 General hygienic behaviour of *T. carbonaria*

Initial detection behaviours after pin-kill

On the first day of a pin-kill experiment, brood workers were observed to be agitated and to move in quick "frantic walks" around the test area, but there was no display of aggressive behaviours. Workers antennated the pinned cells and the excreted bodily fluids close to the edge of the test area. Once in contact with this area, workers did not continue further onto the 25 pinned cells; they appeared to clean their antennae immediately after contact was made and avoided making tarsal contact with the cells (being instead stretched across the cells). When presented with a pin-kill test on the second consecutive day, workers appeared at the pinned area in the brood chamber more quickly, within seconds, sometimes while surrounding cells were still being pinned. As the number of workers increased, a number of them were observed collecting the excreted thick and tacky bodily fluids from the cells, forming it into balls and removing it. Initially, some workers placed small resin balls and fragments of cerumen onto the pinned cells. This material did not appear to be being used to repair the damaged cells or to cover the pin holes (Appendix 1, Video 5). This

material remained until it was removed when more workers participated in detection tasks.

Uncapping of cells

Detection was a short-duration stage. Uncapping of cells was undertaken by a single worker or by small groups (2–3 workers), with workers taking turns or working together to remove the cerumen cell caps (Figure 3.12) (Appendix 1, Video 6).

A number of distinct tasks were observed during the uncapping stage. Some workers commenced dismantling cells from underneath the disc; this meant that a pin-killed pupa could be removed from beneath the cell before it was uncapped. In other cases, workers removed pupae via the side of the capped or partially uncapped cells. This occurred in cases when workers were dismantling cells and started to remove the cerumen from the side of neighbouring cells which were still being uncapped or partially uncapped.

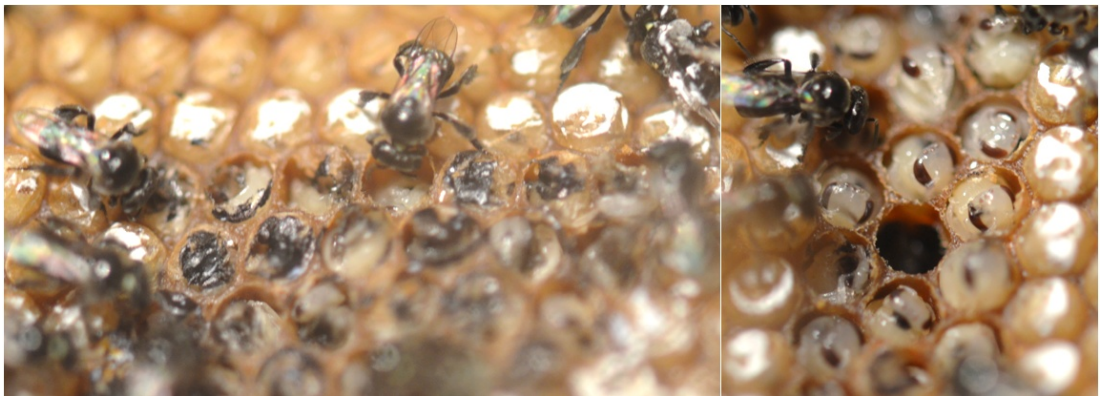

Figure 3.12 After detection, workers begin to chew the cerumen and remove the cell caps, exposing the dead pupae inside.

Removal of dead pupae

The third stage of hygienic behaviour was the removal of dead pupae from the cells (Figure 3.13). On some occasions, workers removed a pupa and dumped the corpse

within the test area, then progressed onto the next cell. This meant that other workers removed the corpse from the test area and from the brood chamber. There was an increase in worker activity when corpses were removed. In particular, as corpses were carried by workers from the test area, other brood workers pulled at the corpse or touched it with their antennae. The worker carrying the pupa increased her pace as she walked, and sometimes made a few circles while holding the dead pupae, before continuing to walk and remove the pupa from the brood chamber. Cannibalism of cell contents (including pupae) was not observed; the removed corpses were observed in the OPs attached to the hives.

Some workers attempted to remove pupae from partially uncapped cells (i.e., with at least 50% cap remaining). This appeared to increase the time for removal as the pupa was caught under the cell cap; however, while this activity occurred other workers continued to uncap the cell.

Figure 3.13 After uncapping, workers begin to remove the dead pupae from the cells, dumping the bodies into internal trash piles or removing them completely from hive.

Complete dismantling of cells

Once all pupae were removed, workers continued removing the cerumen and dismantling the entire cell, leaving a hole in the brood disc (Figure 3.14). After a

period of time (approximately 30 min to 1 h after all cells were dismantled) workers began to fill the space with a cerumen shelf and supporting pillars, ensuring maintenance of the brood disc rigidity. Occasionally, the final remains of dismantled cells were incorporated into this shelf. During the dismantling stage, worker numbers and pace of activity decreased, as only a few workers remained to perform the space filling task.

All four stages of hygienic behaviour (detection, uncapping, removal and dismantling) were recorded being performed in a colony at the same time by workers. This meant that in some instances, cells were completely emptied and dismantled at the same time a worker was detecting another dead pupa. At other times, however, all 25 cells were uncapped before any pupae were removed.

On a number of occasions, the queen was observed to enter the test area; however there was no acknowledgment of the pinned cells by the queen, nor were any new cells available in the test area.

Figure 3.14 The final stage of hygienic behaviour for *Tetragonula carbonaria* was the complete dismantling of the brood cells after contents were removed.

3.4.4 Do seasonal changes affect worker behaviour and their efficiency in conducting hygienic behaviours?

Hygienic behaviour experiments were conducted in winter, spring and summer (2012), and autumn (2014) to assess seasonal effects on worker behaviour.

There were no seasonal differences in total time to perform hygienic behaviour ($F_{3, 18}$ = 0.44, p = 0.726) or uncapping ($F_{3, 18}$ = 1.18, p = 0.344). However, there were significant seasonal differences in detection time ($F_{3, 18}$ = 3.55, p = 0.035), with hives in winter (41.7 ± 5.48 min) taking longer to detect pin-killed pupae than in autumn (14.3 ± 2.4 min, p = 0.037) (Figure 3.15). There were no effects of season on the percentage time devoted to detection ($F_{3, 18}$ = 1.616, p = 0.221), uncapping (χ^2 (3) = 3.45, p = 0.327) or removal ($F_{3, 18}$ = 1.14, p = 0.358) (Figure 3.16).

All hives tested in the different seasonal conditions demonstrated similar hygienic behaviour to that described previously. However, winter hygienic behaviour observations were different to other seasons. Hives in winter still had partially dismantled cells from Day 1 to Day 2. However, by the end of Day 2 all cells pinned over the two day period were completely dismantled. During winter, worker recruitment and activity levels were low, cells were uncapped and the pupae exposed; however there were only up to four workers present at any one time in the test area and these workers were not removing pupae. Eventually, pupae removal occurred, this was not at the pace and efficiency previously observed.

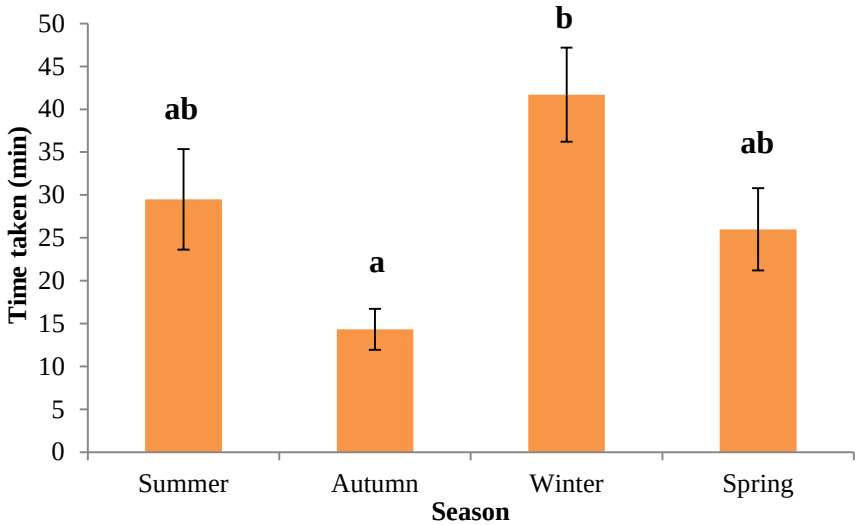

Figure 3.15 Time taken for *T. carbonaria* hives to detect pin-killed pupae during four seasons; summer (n = 4), autumn (n = 3), winter (n = 10) and spring (n = 5) in 2012 and 2014, based on day 2 testing data. Error bars = SE of means, columns headed by different letters are significantly different at p = 0.05.

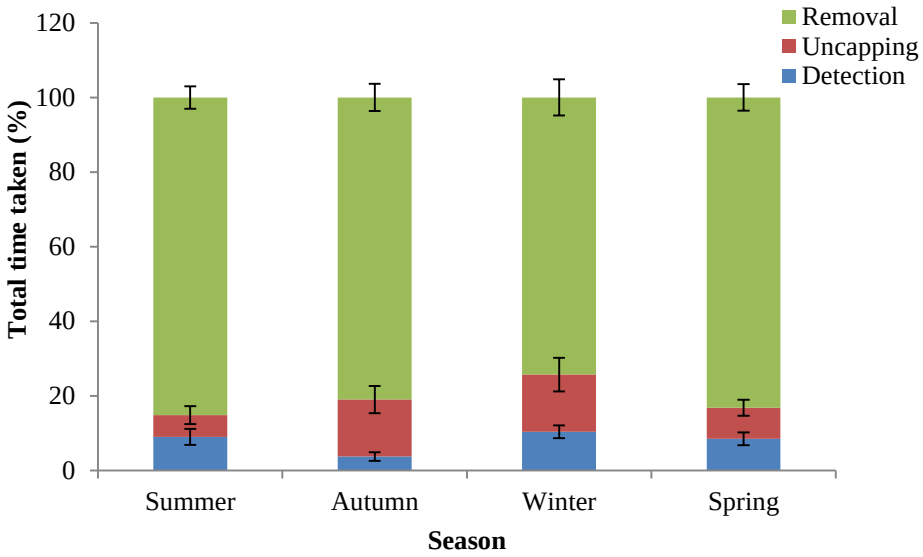

Figure 3.16 Percentage of time for *T. carbonaria* to perform each hygienic behaviour over four seasons. Error bars = SE of means.

There were no correlations between ambient weather conditions (temperature, and solar hours) and the hygienic behaviours displayed by individual hives. For example, mean external ambient temperature on the days when colonies were tested during winter was 14.9 ± 0.4 ºC (n = 192), with mean solar exposure of 3.2 ± 0.9 kWh m^2 (n = 4). On average, hives during winter took 164 ± 32 min (n = 10) to uncap cells and a total time of 506 ± 65 min (n = 10) to perform all three steps. By comparison, a hive tested during summer conditions (mean temperature 21.8 ± 0.4ºC (n = 96), mean solar exposure 7.6 ± 1.8 kWh m^2 (n = 2)), took 52 ± 10 min (n = 8) to uncap the cells and 586 ± 121 min (n = 8) in total to perform all three steps.

3.4.5 Does the strength of a colony determine the success of hygienic behaviour?

The influence of EBP, nest weight, and worker entrance activity on hygienic behaviour of colonies was assessed to determine if colony strength played a role in expression of behaviour. There were correlations between EBP and time to detect (r = -0.809, p = 0.003), and returning entrance activity and time to uncap cells (r = -0.702, p = 0.035). There were no other significant correlations between EBP, hive weight, worker entrance activity and the time taken to perform the hygienic behaviours (all correlation data is provided in Appendix A2.1, Table A2-a).

3.4.6 Does external access to the environment affect hygienic behaviour?

When hives with blocked entrances were examined, the number of workers present on the test section of brood the first day of testing was lower than on the subsequent day. However, even though the number of workers on Day 2 was higher, none of these workers performed hygienic behaviour or other general brood chamber activities.

Comparison of the time taken by the four colonies tested for hygienic behaviour with either opened or closed entrances are shown in Figure 3.17. There was a significant difference between the time taken to detect dead pupae between open (n = 24) (10.1 ± 1.4 min) and closed (29.3 ± 5.4 min) colonies (n = 8) (Welch's $t_{1, 7.93} = 12.015$, $p = 0.009$, as indicated by asterisk in Figure 3.17). However, there were no other differences in time between them for uncapping (54.3 ± 9.2 min and 88.4 ± 21.6 min, respectively) (Welch's $t_{1, 9.69} = 2.094$, $p = 0.179$), and total time taken (365.3 ± 31.9 min and 458.5 ± 97.3 min, respectively) (Welch's $t_{1, 8.56} = 0.0828$, $p = 0.388$).

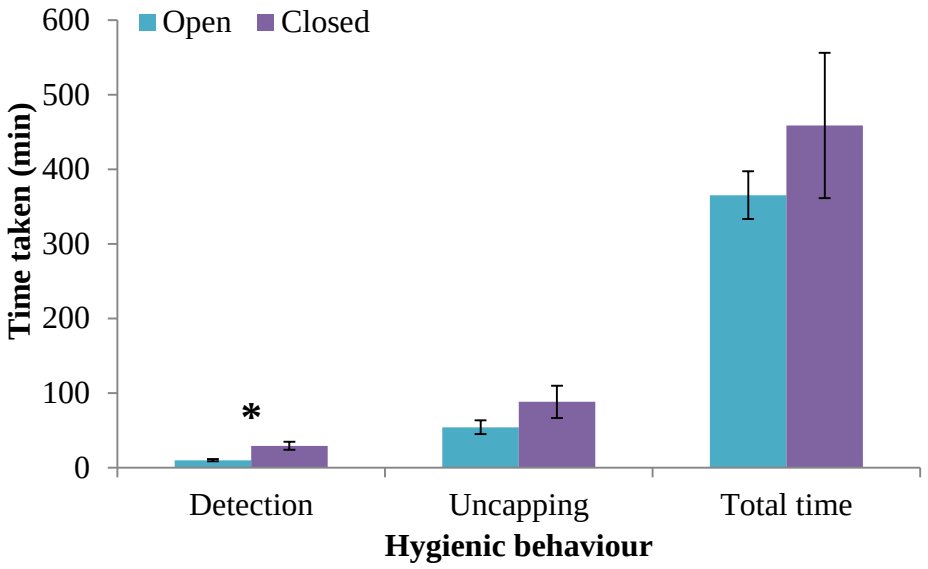

Figure 3.17 Comparison of the mean time taken (min) from four *T. carbonaria* hives for detection, uncapping and the total time taken to complete hygienic behaviour. Comparison includes hives with either open (n = 24) or closed (n = 8) nest entrances. Error bars = SE of means, asterisk (*) indicates statistically significant difference at $p = 0.05$.

When comparing the percentage of time devoted to the detection stage, colonies with closed entrances used significantly more time (7%) to perform this activity Welch's $t_{1, 9.07} = 37.334$, $p < 0.001$, than hives with open entrances (2%), as indicated by asterisk in Figure 3.18. There were no other differences in the percentage of time devoted to uncapping (Welch's $t_{1, 13.49} = 0.025$, $p = 0.877$) and removal (Welch's $t_{1, 29.97} = 0.850$, $p = 0.364$) in comparing open or closed entrance colonies.

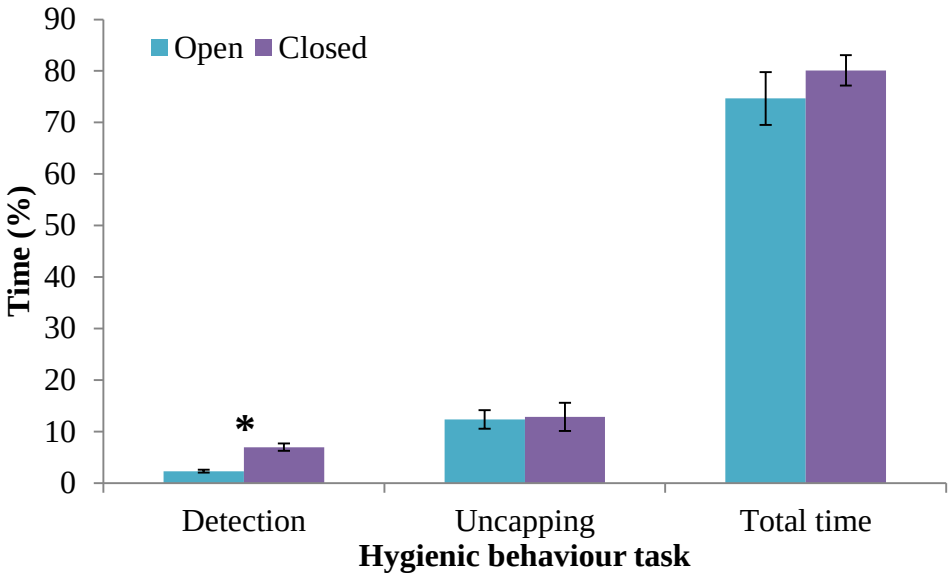

Figure 3.18 Percentage of time devoted to the three hygienic behaviour tasks for *T. carbonaria* colonies with either open (n = 24) or closed (n = 8) hive entrances. Error bars = SE of means, asterisk (*) represents statistical significant difference at $p = 0.05$.

3.4.7 Can hygienic behaviour be "learned" by a colony?

Mean total time for colonies to perform hygienic behaviour tasks was compared over four consecutive days. There were no significant differences in the mean detection time ($F_{3, 44} = 1.29$, $p = 0.290$), or in the total time (i.e. to complete removal) ($F_{3, 44} = 1.71$, $p = 0.179$) between the four consecutive days (Figure 3.19). However, there were significant differences in the mean uncapping time between Day 1 (75.4 ± 16.3 min), Day 2 (33.3 ± 15.6 min), and Day 3 (35.7 min ± 15.7 min) ($p = 0.017$ and $p = 0.027$, respectively), but not Day 4 ($p = 0.099$) (Figure 3.20).

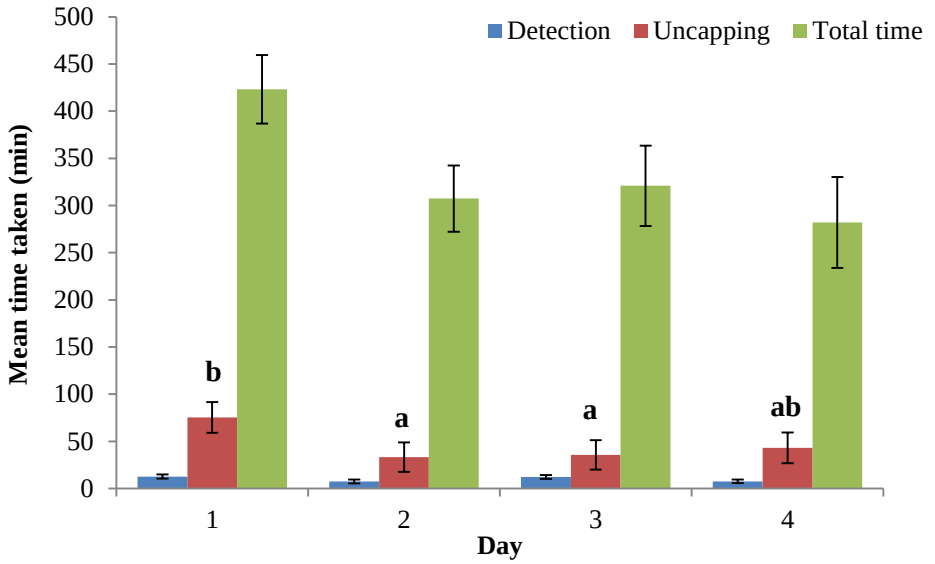

Figure 3.19 Comparison of the mean time taken (min) for *T. carbonaria* colonies to perform the detection (n = 12), uncapping (n = 12) and total (n = 12) stages of hygienic behaviour, over four consecutive days (n = 48). Error bars = SE of means, columns headed by different letters are significantly different at *p* = 0.05 within a stage, where no letters are shown there are no significant differences

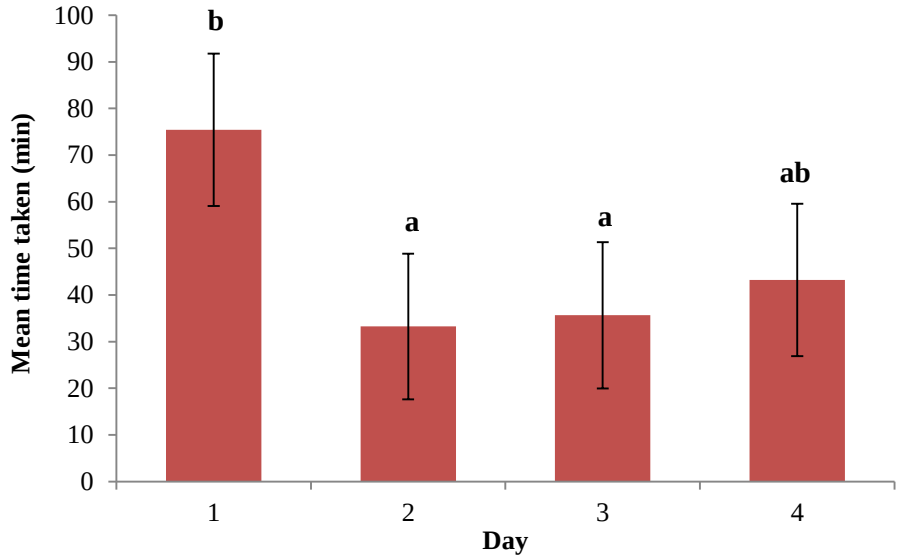

Figure 3.20 Comparison of the mean time taken (min) for *T. carbonaria* colonies to perform uncapping (n = 48), over four consecutive days. Error bars = SE of means, columns headed by different letters are significantly different at *p* = 0.05.

Comparison of the mean total time of four colonies to perform the hygienic behaviour tasks three times (repetitions) with 6–7 weeks between testing is shown in Figure 3.21. There were significant differences in the average time taken for detection of dead brood ($F_{2, 45}$ = 4.69, *p* = 0.014) between repetitions 1 (6.2 min ± 1.1

min) and 3 (12.2 ± 2.7 min) (p = 0.010), but no difference between other repetitions (repetitions 1 and 2) 11.8 ± 5.6 min, p = 0.249; and repetitions 2 and 3, p = 0.327). There were no significant differences between the time taken to uncap cells between the repetitions ($F_{2, 45}$ = 1.01, p = 0.373), but there were significant differences between the total time taken for colonies to complete hygienic behaviour between the three repetitions ($F_{2, 45}$ = 3.78, p = 0.030). There was a steady increase in the total times taken from repetition 1, to repetition 3 (275.1 ± 43.4 min, 351.4 ± 49.4 min, and 469.3 ± 54.9 min, respectively). There was also a significant difference in the total time taken to complete hygienic behaviour between repetition 1 and repetition 3 (p = 0.024), but no other differences between repetitions (repetitions 1 and 2, p = 0.405; and repetitions 2 and 3, p = 0.989) (Figure 3.21).

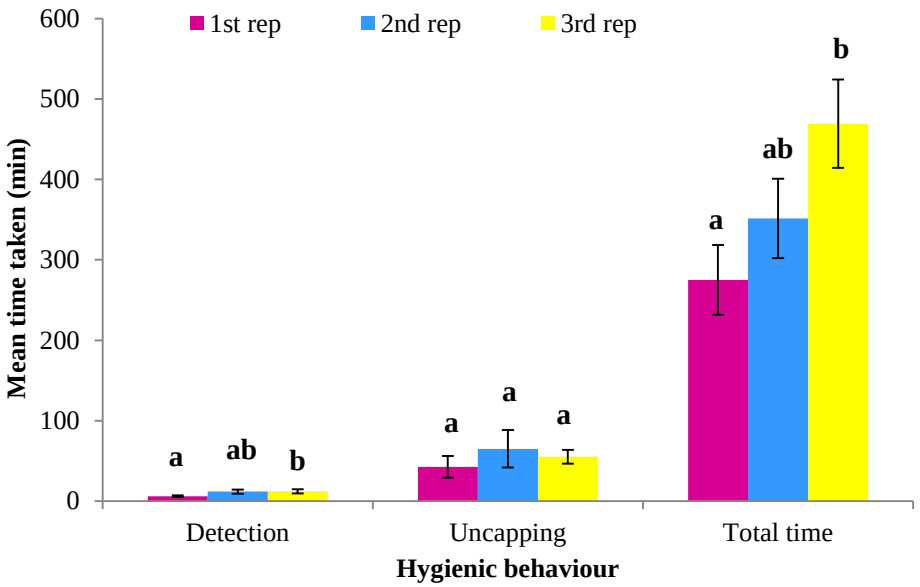

Figure 3.21 Comparison of the mean time taken (min) for *T. carbonaria* colonies to perform detection (n = 16), uncapping (n = 16) and total time taken (n = 16) between three repetitions (maximum 7 weeks part, n = 48). Error bars = SE of mean, columns headed by different letters are significantly different at p = 0.05 within a stage.

Comparison of the percentage of time allocated to the hygienic behaviour tasks is shown in Figures 3.22. There were no significant differences in the percentage of time devoted to detection ($F_{3, 44}$ = 2.154 p = 0.107), uncapping ($F_{3, 44}$ = 2.89, p = 0.046) and removal ($F_{3, 44}$ = 1.05, p = 0.379) over the four consecutive days, and no

significant differences in the time allocated to detection ($F_{2, 45} = 2.87$ $p = 0.067$), uncapping ($F_{2, 45} = 0.47$ $p = 0.627$) or removal ($F_{2, 45} = 2.77$ $p = 0.074$) between the three replications.

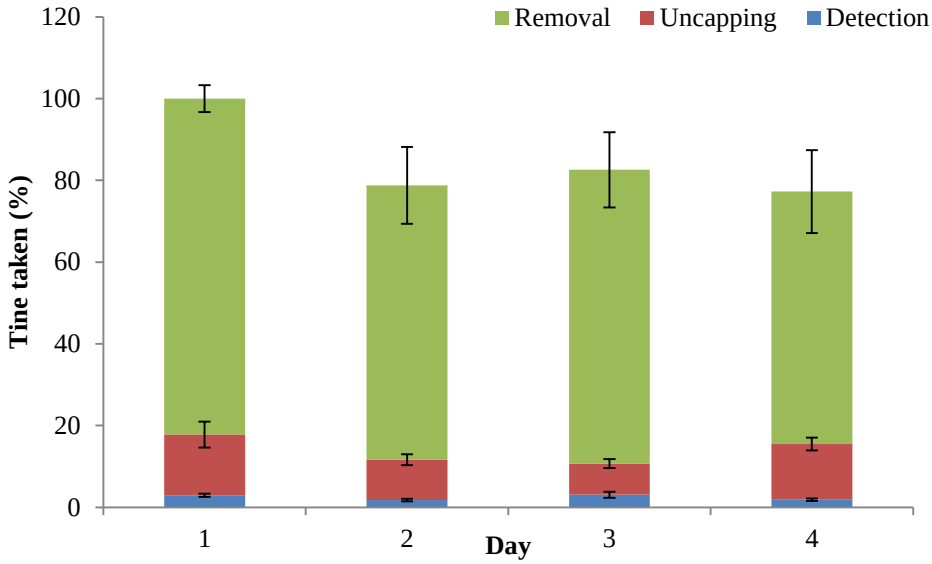

Figure 3.22 Percentage mean time allocated to hygienic behaviour tasks (detection n = 12, uncapping n = 12, and removal n = 12), comparing between four consecutive days (n = 48, maximum 7 weeks apart). Errors bars = SE of mean.

3.5 Discussion

The results reported above overwhelmingly demonstrate that workers of *T. carbonaria* could detect, uncap and remove dead brood from cells. The hygienic behaviour of *T. carbonaria* is substantially superior to honey bees and most other stingless bee species, removing 99% of pupae within seven hours (Table 3.2). The rapid removal of dead pupae recorded by *T. carbonaria* provides a possible explanation for the apparent absence of brood diseases in colonies of this species. Efficient removal of infected pupae is likely to reduce a pathogen's spread (Woodrow & Holst 1942).

Table 3.2 Percentage of dead pupae removed after 24 or 48 h for *T. carbonaria*, other stingless bee species and a hygienic strain of *A. mellifera*.

Species	% pupae removed			Source
	<10h	24h	48h	
A. mellifera			>95	Spivak & Downey 1998
Scaptotrigona pectoralis		97		Medina et al. 2009
Plebeia remota		69	96	Nunes-Silva et al. 2009
Melipona beecheii			66	Medina et al. 2009
Tetragonisca angustula			10	Tenório 1996
Melipona quadrifasciata			1	Tenório 1996
Tetragonula carbonaria	99			This study

Within a *T. carbonaria* colony, workers were often performing all hygienic behaviour tasks at the same time. However, there was task dedication, once a worker commenced a task the worker would continue until the task was completed. A similar observation has been reported from hygienic *A. mellifera* colonies (Arathi & Spivak 2001), and is suggested to be related to the percentage of age-appropriate workers within the population expressing hygienic traits (Arathi et al. 2000, Arathi & Spivak 2001).

The difference in behaviours between *T. carbonaria* colonies may be related to the mating of the queen and her selected male. However, within *T. carbonaria* colonies the worker hygienic behaviour expression would be assumed to be uniform, as genetically the variation between workers is small as the queen only mates once (Kerr et al. 1962, Green & Oldroyd 2002), compared to *A. mellifera* queens who mate with 7-17 drones (Kerr et al. 1962, Kolmes et al. 1989), though in some colonies some of the workers were more efficient at some tasks, or performing additional tasks which were not necessarily efficient. Such as during the initial minutes after pin-kill, some workers placed resin droplets or cerumen onto the pinned cells, but not necessarily to repair them, and they were subsequently removed. Similarly, in non-hygienic *A. mellifera* colonies, workers placed wax pieces onto pin-killed cells but did not progress in their hygienic behaviour (Arathi et al. 2006). The behaviours displayed by *T. carbonaria* workers, may be in response to detecting damaged cells, but not the dead pupae. As only some *T. carbonaria* colonies

performed this task while others did not, this suggests that there may be a range of hygienic behaviour expression between and within colonies.

Tetragonula carbonaria workers performed an additional hygienic behaviour task to *A. mellifera*; namely complete cell dismantling, which has previously been documented in some other stingless bee species as a part of their hygienic behaviour (Tenório 1996). Unlike *A. mellifera* (Park 1935, Newton & Ostasiewski 1986, Spivak & Reuter 2001b), *T. carbonaria* did not rebuild cells connected to the tested brood disc; this strategy is likely to assist in reducing brood disease transmission (Medina et al. 2009). A further mechanism for reducing brood disease transmission in *T. carbonaria* is the absence of cannibalism of dead larvae or pupae, as occurs in honey bees (Fukuda & Sakagami 1968, Woyke 1980, Rath & Drescher 1990, Invernizzi 2000, Schmickl & Crailsheim 2001, Invernizzi et al. 2011). Lack of cannibalism has also been observed in *T. angustula* (Tenório 1996), where, similar to *T. carbonaria*, dead nest members were removed to trash piles or completely out of the hive.

Does season affect behaviour?

Season had little effect on the performance of hygienic behaviour. While detection took longer in winter than in autumn (Figure 3.15), there were no other seasonal effects on hygienic behaviour. This may be because in stingless bees, there are specialised brood cells and storage pots with no overlapping of use. This is unlike *A. mellifera*, which use their cells for various activities (i.e. for brood, honey, or pollen storage), so there is competition for cell availability, especially in periods of high nectar flow (Thompson 1964, Momot & Rothenbuhler 1971, Spivak & Reuter 1998, Kavinseksan et al. 2004, Güler & Toy 2013, Adjlane & Haddad 2014, Uzunov et al. 2014). The longer detection time in winter may be associated with a decrease in nectar resources, signalling workers to reduce brood size to a manageable volume as has been previously observed in *T. carbonaria* colonies (Jenny Shanks, personal observations). Decreased brood size in winter would, in turn, reduce the colony population and thus decrease the number of age-appropriate workers to perform particular activity such as hygienic behaviour.

A further explanation for efficient hygienic behaviours may be associated with the volume of propolis produced. Colonies of Africanised honey bees which were selected to produce significant volumes of propolis also had superior hygienic behaviour, particularly during the uncapping stage (Nicodemo et al. 2013). Stingless bees also produce copious volumes of propolis to create cerumen to build brood cells. As all cells are dismantled, more resin and propolis would be required to rebuild the brood chamber. This involves fixing any neighbouring damaged cells, improving structural rigidity and integration of the brood chamber by building pillars, as well as sometimes creating a layer of cerumen over the hole made from cell removal (Jenny Shanks, personal observations). The connection between propolis production in *T. carbonaria* and superior hygienic behaviour, though not investigated, may be related to plant resin availability during different seasons and the corresponding volume of propolis produced. *Tetragonula carbonaria* has superior hygienic behaviour to other stingless bee species as well as hygienic strains of *A. mellifera* (Table 3.2); although most of the stingless bee species tested to date have far superior hygienic behaviour to non-hygienic strains of *A. mellifera*. The possibility of other nest factors influencing disease control needs further investigation, including the direct role propolis might play in brood disease suppression; this is addressed in Chapter 4.

Does the strength of a colony and ability to access the external environment affect hygienic behaviour?

Expression of *Tetragonula carbonaria* hygienic behaviour was not correlated with colony size, nest weight, or worker entrance activity.

Confining foraging workers in the nest did not change the time taken to perform hygienic stages. Although there was a larger population, this did not alter the number of age-related task workers present in the brood chamber to perform hygienic behaviour, as the additional workers were foragers. These results are generally similar to those reported for *A. mellifera* (Newton & Ostasiewski 1986, Kavinseksan et al. 2004, Bigio et al. 2013), although Spivak & Gilliam (1993) found when colony populations were reduced (10 frames to two frames), hygienic behaviour decreased.

This was most probably due to reduction in numbers of age-appropriate brood workers who express hygienic traits (Trump et al. 1967, Spivak & Gilliam 1993).

Is hygienic behaviour learned?

Hygienic behaviour was tested to investigate whether workers learn in the short term (over several days) and remember these behaviours in the long term (over a number of weeks). There were significant differences only in the uncapping stage between the first (75 min) and second day (33 min) of testing; indicating short-term learning by workers to quickly uncap brood cells. There was no further decrease in total time taken on Days 3 and 4, indicating that hygienic behaviour efficiency was not increased beyond Day 2. When colonies were tested weeks later, within the expected lifespan of these bees, they showed no signs of remembering the hygienic behaviours, therefore, indicating a lack of long-term memory.

Hygienic behaviours are also not learnt by honey bee workers (Trump et al. 1967), but are genetically driven (Rothenbuhler 1964a, Gramacho & Gonçalves 2001, Lapidge et al. 2002) and expressed in proportions of age-related brood workers. To date, there have been no studies investigating the hygienic traits present in stingless bees. Despite young (several days old) *T. carbonaria* workers being next to older adults, there was no indication that the young, inexperienced workers learned by observing the older workers. Only the older brood workers performed detection and uncapping behaviours. Similarly, the addition of hygienic workers to a non-hygienic honey bee colony, did not stimulate non-hygienic workers to perform hygienic behaviours (Trump et al. 1967, Arathi et al. 2006).

Middle-aged honey bee brood workers always performed detection and uncapping rather than removal, suggesting a stronger olfactory sensitivity of workers at this age (Gramacho & Spivak 2003), possibly due to their previous exposure to decay volatiles (Masterman et al. 2000, Masterman et al. 2001, Gramacho & Spivak 2003). There were fewer *T. carbonaria* workers involved in the final removal and

dismantling stages, compared to the larger numbers involved in uncapping. This difference in worker numbers may be due to olfactory cues occurring during the early stages of hygienic behaviour, as previously seen in *A. mellifera* (Palacio et al. 2010). These odours may be detected by more sensitive, age-appropriate workers. It is likely that *T. carbonaria* workers were responding to burst fat cells and haemolymph present in the body fluids excreted from the pin-killed brood cells. Spivak and Downey (1998) found body fluids from a healthy pupa placed onto honey bee capped cells did not initiate uncapping behaviour; but Gramacho et al. (1999) reported that when body fluids were injected under a capped cell, workers began uncapping the brood.

Despite the consistency in hygienic behaviour across all *T. carbonaria* hives tested, occasionally some hives were slower in detection and uncapping. A possible explanation for the difference in hive hygienic behaviour may have been the caste of the pupae selected for testing. In contrast to *A. mellifera*, *T. carbonaria* worker and male brood are indistinguishable; therefore selection of male brood may have occurred. Previous studies have shown that some *A. mellifera* workers are more selective at uncapping and removing worker brood than male brood (Gramacho 1999 cited in Gramacho & Gonçalves 2009a, Invernizzi 2012).

Chapter 4

Suppression of insect pathogens by the antimicrobial activity of stingless bee nest products

4.1 Abstract

Apis mellifera hive products, pollen, honey and propolis have been reported to possess therapeutic benefits, as well as potential use as an inhibitor of *P. larvae*. Several studies have also reported the chemical composition and antimicrobial activity of stingless bee propolis and honey; however, few have investigated their potential as inhibitors of specific bee pathogens. A previous study identified hygienic behaviour in *T. carbonaria* as one mechanism contributing to lower incidence of brood pathogens. As a result the antimicrobial activity of *T. carbonaria* hive nest products were investigated to determine their possible role in contributing to lower incidence of brood pathogens in this species. Disc-diffusion and microtiter plate methodologies were used to test the inhibitory activity of *T. carbonaria* and *A. mellifera* hive products on the growth and development of three insect pathogens: *P. larvae*, *A. apis*, and *Metarhizium anisopliae*. *Tetragonula carbonaria* propolis had the greatest inhibitory effects on *P. larvae* development, mycelial growth of *A. apis*, and reducing the area of *M. anisopliae* sporulation. *Tetragonula carbonaria* and *A. mellifera* honey samples had slightly better inhibition than pollen extracts, whereas pollen extracts were the least inhibitory. *Tetragonula carbonaria* and *A. mellifera* pollen and honey had similar activities in decreasing the area of *M. anisopliae* sporulation, whilst *A. mellifera* propolis initially induced *M. anisopliae* sporulation. Chemical profiles of propolis and newly emerged brood comb from *T. carbonaria* and *A. mellifera* were determined by LC-MS. *Tetragonula carbonaria* and *A. mellifera* propolis extracts were composed of different quantities of flavanones, with key flavanones with masses of 271 and 285 (mass+1). Comparison of *T. carbonaria* propolis and brood comb, showed very similar LC-MS profiles, with similar identified compounds, whereas *A. mellifera* propolis and brood comb had no similarities. These findings support the hypothesis that the incorporation of propolis into brood comb by *T. carbonaria*; may contribute to suppression of brood pathogens.

4.2 Introduction

There is little information regarding brood pathogens of stingless bees (Kerr 1948, Nogueira-Neto 1997). Possible explanations for this may be associated with the

antimicrobial activity of nest products (pollen, honey and propolis) and/or the presence of unique gut flora providing an immune response to invading pathogens (Evans & Lopez 2004, Yoshiyama et al. 2013). It is known that honey bee nest products can suppress the growth of a number of pathogens (Lavie 1960, Lindenfelser 1968, Mlagan & Sulimanovic 1982, Gilliam et al. 1988, Crailsheim & Riessberger-Gallé 2001, Antúnez et al. 2008, Bastos et al. 2008, Chorbiński 2009, Simone 2010, Senka et al. 2011, Mihai et al. 2012, Kamel et al. 2013, Wilson et al. 2013, Erler et al. 2014, Wilson et al. 2014, Ali & Abd El-Ghafar undated) studying how stingless bee colonies utilise nest products, and the antimicrobial properties of these products, may provide an understanding to disease suppression in colonies.

4.2.1 Nest and bee products

Pollen

Pollen is the key source of protein for bees, and feeding pollen to larvae characteristically separates bees from other Hymenoptera (Michener 2007). The antimicrobial activity of stored pollen is largely influenced by season (Anderson et al. 2014), botanical source (Lavie 1960, Campos et al. 1997, Almaraz-Abarca et al. 2004, Cabrera & Montenegro 2013), geographical location (Carpes et al. 2007, Morais et al. 2011), and the biochemical changes occurring after the addition of honey (Herbert & Shimanuki 1978, Anderson et al. 2011) or microbes by worker bees (microbial farming) (Lavie 1960, Gilliam 1997).

Pollen extracts have been reported to have antimicrobial activity against a number of human pathogens, both bacterial and yeast (Carpes et al. 2007, Abouda et al. 2011, Morais et al. 2011, Kačániová et al. 2012, Cabrera & Montenegro 2013), as well as some plant pathogens (Özcan et al. 2004, Basim et al. 2006, Cabrera & Montenegro 2013). Pollen and their extracts have had differing results on the growth and development of the bee pathogen *P. larvae*. Smith et al. (1949), for example, reported that the use of 2% v/v pollen in carrot media increased the germination of *P. larvae* spores, whereas Lavie (1960) reported alcoholic extracts of pollen inhibited its growth, with no effect from the ethanol solvent. More recently, Crailsheim &

Riessberger-Gallé (2001) confirmed Lavie (1960) findings, with pollen extracts of pellets taken from corbiculae and bee bread (stored and preserved pollen) inhibiting *P. larvae* (Crailsheim & Riessberger-Gallé 2001). *Ascosphaera apis* was also inhibited by bee bread, possibly due to antimycotic compounds from the presence of *Rhizopus* sp. and Mucorales in the pollen (Gilliam et al. 1988).

These studies suggest that pollen may not only play a nutritional role, but also assist in the suppression of brood pathogens in developing larvae. There are no studies investigating the antimicrobial activity of stingless bee pollen extracts against bee pathogens.

Honey

Along with a protein source, bees require a source of carbohydrates for energy, and this occurs in the form of honey. Blossom nectar and insect honeydew collected by foragers are transported to the hive, where they are transformed into honey. Transformation is performed by the action of secreted bee salivary enzyme (invertase) and the removal of water (Crane 1990, Olaitan et al. 2007). Honey is a concentrated mixture of sugars (such as fructose, glucose, sucrose and maltose), and other minerals, enzymes and aromatics (Crane 1990, Olaitan et al. 2007).

Application of medical-grade bee honey has assisted wound healing in humans, and reducing the effects of swelling and redness associated with many bacterial infections (Wellford et al. 1978, Cooper et al. 2002, Al-Waili 2004, Mandal & Mandal 2011, Hammond & Donkor 2013, Schneider et al. 2013, Sultanbawa et al. 2015). Recent studies have assessed the inhibitory activities of stingless bee honey (Garedew et al. 2003, Miorin et al. 2003, DeMera & Angert 2004, Temaru et al. 2007, Kimoto-Nira & Amano 2008, Boorn et al. 2010, Ewnetu et al. 2013, Kwapong et al. 2013, Mercês et al. 2013, da Cruz et al. 2014, Massaro et al. 2014b). However, studies have focused predominantly on the inhibition of human bacterial and fungal pathogens (Irish et al. 2008, Boorn et al. 2010, Massaro et al. 2014b).

To date, there have been four studies investigating the activity of *A. mellifera* honey against bee pathogens. Although a few studies have investigated inhibitory activity of honey extracts on the germination and growth of *P. larvae* and *A. apis* (Lavie 1960, Erler et al. 2014), the majority have focused on activity of specific bacteria isolated from honey, particularly *Bacillus* spp. because they are the most abundant intestinal microflora of adult bees (Reynaldi et al. 2004, Alippi & Reynaldi 2006, Sabaté et al. 2009). To date, there have been no studies investigating the suppressive activity of *T. carbonaria* honey against bee pathogens.

Antimicrobial activity of honey may be a result of the physical and chemical properties including pH, osmolarity, sugar content, as well as hydrogen peroxide production (White et al. 1963, Molan 1992, Mundo et al. 2004, Mandal & Mandal 2011, Kwakman & Zaat 2012). Alternatively, non-peroxide producing honeys are dependent on the phytochemical influences, such as the species of plant, the season and growing region (Allen et al. 1991, Adams et al. 2009). Antimicrobial activity of *T. carbonaria* honey has been attributed to production of hydrogen peroxide (Allen et al. 1991, Irish et al. 2008), as well as phytochemicals, particularly flavonoids (Temaru et al. 2007, Tomás-Barberán et al. 2013, Massaro et al. 2014b). Compared to *A. mellifera*, stingless bee honey has a greater opportunity to be in contact with plant-derived compounds resulting from exposure to propolis from storage pots. This interaction may contribute to the antimicrobial properties of the honey (Temaru et al. 2007, Kimoto-Nira & Amano 2008), and result in reduced levels of pathogens in stingless bee colonies.

Propolis

Propolis is a mixture of plant resins and bee salivary gland secretions (Simone-Finstrom & Spivak 2010), and is used in honey bee and stingless bee colonies to seal holes and gaps, narrow entrances, line the walls to control airflow, and for moisture- and water-proofing (Seeley & Morse 1976, Ghisalberti 1979). Stingless bees also use propolis to create cerumen (Michener 2013), which is used for structural components of the nest; including the brood cells and storage pots (Wille 1983). In contrast,

honey bee nests are primarily constructed from wax produced by worker bees' abdominal wax glands (Michener 1974, Ghisalberti 1979).

Propolis is suggested to play an essential role in pest and disease control (Seeley & Morse 1976 cited by Visscher 1980), due to its antimicrobial composition associated with plant derived resins. To date, more than 300 compounds have been identified in propolis (Abu-Mellal et al. 2012, Wilson et al. 2013), with the composition influenced by the geographical distribution of botanical sources (Marcucci 1995, Wallace & Trueman 1995, Bankova et al. 1996, Bastos et al. 2008, Wallace et al. 2008, Wallace & Lee 2010, Drescher et al. 2014, Massaro et al. 2014a, Wilson et al. 2014). It appears both temperate propolis and tropical and sub-tropical propolis contain flavanones and phenolic acids (Markham et al. 1996, Kujumgiev et al. 1999, Velikova et al. 2000, Midorikawa et al. 2001, Bankova 2005, Popova et al. 2009, Massaro et al. 2011, Massaro et al. 2014a, Massaro et al. 2015). However, temperate propolis differs from tropical and sub-tropical by the production of esters in temperate propolis (Markham et al. 1996, Kujumgiev et al. 1999, Salatino et al. 2011) and higher in terpenes in tropical and sub-tropical (Pereira et al. 2003, Leonhardt et al. 2009).

A number of studies have shown the inhibitory activity *A. mellifera* propolis extracts against *P. larvae* (Lavie 1960, Lindenfelser 1968, Mlagan & Sulimanovic 1982, Antúnez et al. 2008, Bastos et al. 2008, Simone 2010, Mihai et al. 2012, Kamel et al. 2013, Wilson et al. 2013, Wilson et al. 2014), and also against the mycelial growth of *A. apis* (Chorbiński 2009, Senka et al. 2011, Ali & Abd El-Ghafar undated). Colonies challenged by *A. apis* infections responded with an increase in resin collection, suggesting a social immune-response (Simone 2010, Simone-Finstrom & Spivak 2012) benefiting colony health (Nicodemo et al. 2013, Nicodemo et al. 2014, Simone-Finstrom & Spivak 2010).

To date, numerous studies have investigated the medical and therapeutic uses of stingless bee propolis, with promising results (Velikova et al. 2000, Fernandes et al. 2001, Miorin et al. 2003, Manrique & Santana 2008, Farnesi et al. 2009, Campos et

al. 2011, Liberio et al. 2011, Massaro et al. 2011, da Cunha et al. 2013, Massaro et al. 2013, Drescher et al. 2014, Massaro et al. 2014a, Massaro et al. 2015). However, there have been no studies investigating the antimicrobial activity of stingless bee propolis against bee pathogens. As propolis is the major constituent of cerumen in stingless bee colonies and used to create brood cells and storage pots, it is hypothesised that its antimicrobial properties may play a role in suppressing brood pathogens. This could either be by direct contact with the developing larvae and the provisions via the brood cells, or by increasing the antimicrobial activity of stored pollen and honey.

Digestive system

The microbial diversity of the *A. mellifera* digestive system has been investigated in several studies (Jeyaprakash et al. 2003, Martinson et al. 2010, Engel et al. 2012, Martinson et al. 2012). It appears that a mutual dependence exists between the digestive system and its microbial gut symbionts (Olofsson & Vásquez 2008, Vásquez et al. 2012), with symbiosis especially with *Bacillus* spp. occurring over evolutionary time (Cano et al. 1994, Cano & Borucki 1995). Most studies have isolated bacteria including *Bacillus* spp., *Lactobacillus* spp., *Bifidobacterium* spp. and *Pseudomonas* spp. and investigated their antimicrobial, probiotic and immunity-inducing properties (Olofsson & Vásquez 2008, Tajabadi et al. 2011, Gerbaldo et al. 2012, Reis et al. 2012, Butler et al. 2013, Wu et al. 2013, Yoshiyama et al. 2013, Olofsson et al. 2014). Lactic acid bacteria (LAB) (such as *Weissella* and *Lactobacillus*) not only inhibit the growth of *P. larvae*, but also appear to induce immune responses in honey bee workers (Evans & Lopez 2004, Yoshiyama et al. 2013), suggesting their potential use as probiotics to increase bee health.

Microbiota of the digestive system of three Australian native stingless bee species have been characterised (Leonhardt & Kaltenpoth 2014). While there were similarities in the types of lactic acid bacteria (LAB) found in the three Australian species compared with *Apis* species (Olofsson & Vásquez 2008, Vásquez et al. 2012) and bumbles bees (Koch & Schmid-Hempel 2011), Leonhardt & Kaltenpoth (2014) identified a host-specific clade of lactic acid bacteria in *Tetragonula* and

Austroplebeia species. It appears that gut microbiota composition, particularly the widely studied LAB, may depend on the bee species (Leonhardt & Kaltenpoth 2014), and on ancestral co-evolution (Olofsson & Vásquez 2008, Vásquez et al. 2009). Despite studies reporting the inhibitory activity of gut bacteria isolated from honey bees on bee pathogens, and the information regarding stingless bee digestive microbes, no studies have investigated stingless bee gut isolates for their pathogen suppression activity.

The aims of the work reported in this chapter were:

1) To compare inhibitory activity of nest products from *T. carbonaria* and *A. mellifera* hives: pollen, honey, propolis, and isolated gut bacteria (*Weissella hellenica*) against bee pathogens *P. larvae* and *A. apis*, and the generalist entomopathogen *Metarhizium anisopliae.*

2) To compare the chemical profiles of propolis and brood comb from *T. carbonaria* and *A. mellifera* to explain any differences in their antimicrobial activity.

4.3 Materials and methods

To address the aims of this study, investigations were carried out in two parts. Firstly, inhibition assays were performed using hive products (pollen, honey and propolis) from *T. carbonaria* and *A. mellifera* colonies located at UWS, including *Weissella hellenica* isolated from *T. carbonaria* digestive systems. The inhibition assays were conducted against three insect pathogens; the honey bee brood diseases *P. larvae* and *A. apis*, and the generalist entomopathogen, *Metarhizium anisopliae*. Secondly, to identify compounds which might be contributing to the different antimicrobial activity of the extracts from nest structures, propolis and brood comb from *T. carbonaria* and *A. mellifera* were analysed using LC-MS and their profiles compared.

Sample preparation

4.3.1 Collection and extraction of hive products

Three hive products; pollen, honey, and propolis were collected from healthy field-based *T. carbonaria* and *A. mellifera* colonies located at UWS, Hawkesbury campus. The *T. carbonaria* colony which had been located at UWS for two years had continuous access to foraging resources. This colony had not previously been used in any experimentation. The *T. carbonaria* colony was opened by splitting the nest on a warm, sunny day (September 2013). Opening the hive broke open and exposed a number of storage pots, making pollen and honey collection easy.

A ten-frame *A. mellifera* colony that had been located at the UWS apiary for over 10 years was opened in early September 2013 with the assistance of the UWS apiarist (M. Duncan). Appropriate beekeeping tools (smoker and hive tools) were used to open the *A. mellifera* colony and collect nest product samples. These samples were handled similarly to those collected from the *T. carbonaria* hive.

Pollen

Pollen was collected from the *T. carbonaria* colony (Figure 4.1) from the exposed pots using a sterilised metal laboratory spoon, and the collected pollen was placed into a clean dry container (Klip IT™ 700 mL, 115 mm x 155 mm x 114 mm, Sistema®, Auckland, New Zealand) and transferred to the laboratory. The *A. mellifera* pollen was collected from the single colony via the use of a pollen trap (30% efficiency supplied by Jones Apiaries, Dubbo, NSW; and similar to O.A.C. trap) (Waller 1980). The bottom board pollen trap fitted the ten-frame size colony (Somerville 2012b), and was removed easily from the colony and the collected pollen was handled similarly to the *T. carbonaria* pollen for transport to the laboratory.

Figure 4.1 *Tetragonula carbonaria* pollen pots. Pollen became exposed when opening a nest, workers can be observed immediately on the opened pots collecting and repairing stores.

Pollen samples were washed twice in sterile water and the water filtered off. The solid product was placed into a pre-weighed sterilised conical flask. The flask and sample was re-weighed and ethanol added at a volume of 6.5 mL 70% ethanol per 0.5 g of sample (Tichy & Novak 2000). The sample was broken up in the ethanol using a sterilised glass rod, the flask was sealed and completely covered with aluminium foil. Samples were rotated continuously for 15 d at 120 rpm on a benchtop platform mixer (OM6 Ratek Instruments Pty. Ltd. Boronia, Victoria). The flask was then removed and the extract was filter separated from remaining sediment by Whatman #1 150mm filter paper (Whatman International, GE Healthcare, Buckinghamshire, United Kingdom). The final solution was centrifuged at 4,000 rpm (Eppendorf Centrifuge 5810R, Eppendorf, Hamburg, Germany) for 17 min; the supernatant was stored at -20ºC until needed.

Propolis

To aid in handling of the sticky material and to avoid contamination, nitrite gloves were worn whilst removing and handling of propolis from the opened *T. carbonaria* colony. Propolis was identified as the sticky material along the edge of the colony boxes (Figure 4.2), and was removed by scraping it off with a sterile hive tool. Propolis samples were washed and treated as previously described for the pollen

samples. Propolis collected from *A. mellifera* colony was collected with the aid of the UWS apiarist.

Figure 4.2 *Tetragonula carbonaria* propolis in a managed hive. Propolis was found deposited around the edge of boxes and was extremely sticky, making hive handling difficult.

Pollen and propolis extracts

Despite a number of previous studies reporting no inhibitory effects when ethanol was used as the extraction solvent/diluent for antimicrobial bioassays (Antúnez et al. 2008, Bastos et al. 2008), preliminary investigations using ethanol as the diluent for pollen and propolis produced false positives. As a result, dimethyl sulfoxide (DMSO) was used to dilute the extracts (Vignes 2000, Bilikova et al. 2013, Netíková et al. 2013). Ethanol was completely evaporated from *T. carbonaria* and *A. mellifera* pollen and propolis samples in a water bath at 50ºC (model NBCT9, Labec Laboratory Equipment Pty. Ltd. Marrickville, NSW, www.labec.com.au), after which the samples were redissolved into 99% DMSO (Ajax FineChem, Thermofisher Scientific, Massachusetts, USA). DMSO produced no inhibitory effects; however, it contributed to water loss during microtiter assays, sometimes resulting in poor fungal growth. Consequently, DMSO was not used as a negative

control in the microtiter assays, and no further effects on mycelial growth and development were attributable to the use of DMSO.

Honey samples

Honey was removed from the opened *T. carbonaria* colony from the exposed pots using a 2 mL syringe; the honey was then dispensed into 15 mL centrifuge tubes (Greiner, Greiner Bio-One, Kremsmünster, Austria) and stored at -20ºC until required. For *A. mellifera* honey samples, the honey was previously extracted from frames (2012) from a number of hives using on-site commercial extraction equipment. Glass jars (250 mL) of *A. mellifera* honey were randomly selected, then stored at -20ºC until required.

4.3.2 Chemical analysis of hive products from *T. carbonaria* and *A. mellifera*

Samples of extracts of the *T. carbonaria* and *A. mellifera* pollen and propolis collected in September 2013, which were used throughout inhibition assays, as well as newly emerged callow brood cells of *T. carbonaria* and *A. mellifera* collected in June 2014, were sent to the Analytical Research Laboratory of Southern Cross Plant Science (Southern Cross University, SCU, Lismore, NSW) for analysis by Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS).

The LC-MS analysis was performed using an Agilent 1290 HPLC (high performance liquid chromatography) coupled to an Agilent 6120 Quadrapole Mass Selective Detector. UV/vis detection was performed using a diode array detector (DAD) scanning between 190 and 600 nm. The HPLC was run on a GraceSmart RP 18 column (100 x 4.6 mm i.d.; 3µ diam.), solvents used were 100% water and 100% acetonitrile (ACN) both containing 0.05% trifluoroacetic acid. The solvent linear gradient started with 90% water, 10% ACN which ramped to 5% water and 95% ACN over 20 min. The flow rate was 0.75 mL/min with the column compartment heated to 40ºC.

The Agilent 6120 quadrapole detector was used in Atmospheric Pressure Chemical Ionisation (APCI) in positive-ion mode with the fragmentor set at 150 V and the mass range at 100 to 1200 amu with the gain set at 1 EMV. The vaporiser was set at 350ºC using a pressure of 35 psig (pounds per square inch gage) with the nitrogen drying gas flow rate set at 5.0 L/min at 350ºC.

Compounds found in the extracts were characterised by their ion mass (mass +1) and retention times (min). Compounds with identical mass spectra and retention times were regarded as the same substances. Using MS and UV spectra profiles, compounds were tentatively characterised. Chemical profiles of the same hive products from *T. carbonaria* and *A. mellifera* were compared, as well as comparisons between different hive products sourced from the same hives.

In February 2015, fresh samples of propolis and newly-emerged callow brood cells from both *T. carbonaria* and *A. mellifera* were again collected (as previously) for a follow-up LC-MS; however, this time, samples remained in the raw state, to enable quantification. Sample extracts were treated in the laboratory by suspending 250 mg of sample in 10 mL of methanol. LC-MS analyses were performed as previously outlined and absolute amounts of compounds present in each sample were determined.

Results presented in this chapter display LC-MS profiles of the identified chemical components with their retention times (min) and the masses (mass+1). Further supporting data are provided in Appendix A3.5.

4.3.3 Digestive system extraction

Adult *T. carbonaria* bees were washed in 1% v/v sodium hypochlorite solution (Super Strength Bleach 4%, Formula Chemicals, West Ryde, NSW) for 2 min, and then rinsed in distilled water three times. Samples were held in sodium chlorite-tris-EDTA (STE buffer) (Sigma-Aldrich, Missouri, USA) during the dissection process.

Under a dissecting light microscope (model M275, Leica Microscopes, Wetzler, Germany) adults were submerged individually in STE buffer located within a concave microscope slide. Using sterile forceps and scalpel while carefully holding the bee, the head was removed. Securely holding the thorax, the seventh abdominal segment was held and slowly pulled in a vertical direction away from the thorax. The entire internal system was then easily exposed, from the thorax and through the abdomen. This abdominal segment (sometimes showing the male or virgin queen reproductive organs) was removed. The entire digestive system (viz., oesophagus, crop, proventriculus, ventriculus, malpighian tubules, rectal pad and rectum) was isolated from other internal organs. The digestive system was treated as below for the isolation and identification of an appropriate bacterial representative for inhibition studies.

Fifty dissected *T. carbonaria* digestive systems were suspended in 1 mL sterile water, then samples of the suspension were lawn plated onto de Man, Rogosa and Sharpe (MRS) plates (Thermofisher Scientific Australia, Scoresby, Victoria). Seven colonies were isolated by this method, and pure cultures obtained. Identity was confirmed using previously described microbiological techniques, by using a Gen III MicroPlate as per the protocol (BiOLOG, Heidelberg Victoria, http:/www.biolog.com/), and by molecular techniques for adequate identification, as outlined below.

Molecular identification of the gut bacterial isolate

Using aseptic techniques, an individual colony of the above isolated bacteria was added to 1 mL of sterile water in a 1.5 mL Eppendorf tube (Eppendorf, Hamburg, Germany). Following the protocol from Isolate II Genomic DNA Kit (Bioline, London, UK) the sample was treated as follows. The bacterial solution was centrifuged for 5 min at 8,000 ×g, after which the supernatant was removed and the pellet resuspended in 180 µL of Lysis Buffer GL, 25 µL Proteinase K solution and 3 µL of RNase solution. The solution was incubated at 56°C overnight, shaking continuously at 500 rpm.

DNA amplification using universal eubacterial primer pairs 530F (5'-GCTCTAGAGCTGACTGACTGAGTGCCAGCMGCCGCGG-3') and 1495R (5'-GCTCTAGAGCTGACTGACTGAGGYTACCTTGTTACGACTT-3') (Madrid 2001). Reactions were made up to 20 µL with components provided from the Velocity™ PCR Kit (Bioline, London, UK). DNA was amplified in a Bio-Rad Dyad Peltier Thermal Cycler (ALS1296, Bio-Rad Laboratories, California, USA) following the protocols of Madrid et al. (2001) with the following modifications: initial denaturing 95ºC, 5 min, 35 cycles (94ºC, 1 min; 50ºC, 30 sec; 72ºC, 1.5 min), followed by a final extension step at 72ºC for 10 min.

Amplicon volumes of 3 µL were run on 0.8% agarose gel with 0.5 µg ethidium bromide (AMRESCO 2011) and electrophoresis was carried out at 70 V for 60 min. Bands were visualised on a Gel Doc 2000/ ER (Bio-Rad Laboratories, California, USA). PCR products were cleaned to remove unused primers and dNTPs by treating with 2 µL of exoTSAP mixture. ExoTSAP mixtures were produced by mixing 0.025 µL of Exonuclease I (New England Biolabs Inc., Massachusetts, USA), 0.25 µL of thermosensitive alkaline phosphatase (Promega, Madison, Winconsin, USA), and 1.725 µL Milli-Q water. PCR products treated with exoTSAP (37ºC for 30 min, then 95ºC for 5 min) were sent to Macrogen (Macrogen Inc., Seoul, Korea) for sequencing.

DNA sequence analysis of digestive isolate

DNA sequences were trimmed and edited in Sequencher 4.0 Gene Codes Corp. Sequence alignment utilised the MUSCLE algorithm within Mega 5 (Tamura et al. 2011), and compared with sequences obtained from NCBI Blast database.

Inhibition assays

4.3.4 Insect pathogens used for assays

Paenibacillus larvae

Fresh samples of *P. larvae* were obtained from infected *A. mellifera* frames located from a colony in Cowra, NSW. The *P. larvae* symptomatic larvae were removed

from the infected colony prior to its disposal via gamma radiation (Steritech, Wetherill Park, NSW), complying with the protocols outlined in the *NSW Apiaries Act 1985 No. 16. Paenibacillus larvae* infection was confirmed by the match-stick test and molecular diagnostic tools (World Organisation for Animal Health 2013).

Following outlined protocols (Alippi 1999, de Graaf et al. 2013, World Organisation for Animal Health 2013), five *P. larvae* scales (the dried larval remains in brood cells) were removed from cells and ground in 10 mL of sterile distilled water in a 15 mL centrifuge tube. The solution was heat treated at 85ºC for 10 min, then streak plated onto sheep blood agar (SBA) plates containing the antibiotic nalidixic acid (3 mg/ mL) (Oxoid, Thermofisher Scientific, Massachusetts, USA). Plates were incubated at 37ºC at 5% CO_2 for 48 h (Sanyo CO_2 Incubator model HCO-20AIC, Panasonic Healthcare co., Ltd., Tokyo, Japan). Pure isolated colonies were confirmed to be *P. larvae* by the previously described molecular techniques.

Aseptic streak plate techniques were followed for maintenance of the cultures and subculturing colonies onto fresh sheep blood and nalidixic acid plates every second week or when fresh isolated colonies were required.

Ascosphaera apis

Following recommended culturing techniques (Aronstein & Murray 2010), mummies of *A. mellifera* were collected from the entrance of an infected hive in the field. The infected colony had been located at the UWS apiary for over 10 years, and was managed for chalkbrood infection by supplementary feeding. The collected mummies were treated in the laboratory first by surface sterilising in 2% v/v sodium hypochlorite for 30 sec. Four mummies were then rinsed three times in sterile water, and cut into four pieces; each piece was placed separately into the centre of a potato dextrose agar (PDA) (Difco™, Bacto Laboratories Ltd. Pty. Mt Pritchard, NSW, Australia) plate. The 16 plates were then incubated at 23ºC and maintained in the dark (Aronstein & Murray 2010). Subculturing was performed every three to four weeks, following aseptic techniques. A 1 cm^2 section of growth was removed from a section of high density sporulating mycelia from each of the plates and inserted into

the centre of four to eight fresh PDA plates where a 1 cm^2 agar section had been previously removed.

Metarhizium anisopliae

Fresh spores of *Metarhizium anisopliae* var. *acridum* Driver and Milner (2000) (GreenGuard® SC biological insecticide) were obtained from Becker Underwood Pty. Ltd. (Somersby, NSW, www.beckerunderwood.com.au) and stored at 4ºC until required. Five grams of spores were initially sprinkled onto four Sabouraud dextrose agar (SDA) (Difco™, Bacto Laboratories Ltd. Pty. Mt Pritchard, NSW, Australia) plates and incubated at 25ºC to establish the culture. Subculturing was undertaken fortnightly or every third week using a similar method to that used for *A. apis*.

4.3.5 Spore suspensions for inhibition assays

Spore suspension of 48 h *P. larvae* culture was created by aseptically removing bacterial colonies and placing them into sterilised 1.5 mL Eppendorf tubes (Eppendorf, Hamburg, Germany) containing 1 mL sterilised water. The suspension was vigorously mixed continuously on a benchtop platform mixer (OM6 Ratek Instruments Pty. Ltd. Boronia, Victoria) 15 min.

Metarhizium anisopliae var. *acridum* and *A. apis* fungal spores were suspended in 100 mL PDB (Fluka Analytical Sigma-Aldrich, Missouri, USA) with 10 µL Tween® 80 (Sigma-Aldrich), along with sterilised glass beads. The suspension was agitated on a benchtop platform mixer (OM6 Ratek Instruments Pty. Ltd. Boronia, Victoria) until all spore clumps were broken up.

The digestive system bacterial isolate was used for the inhibition assays and maintained on MRS media. The spore suspension used for assays followed the same methods used for pathogenic bacteria spore suspensions.

Calculation of colony forming units in the spore suspensions

For all biological spore suspensions, the average number of colony forming units (CFU) was calculated using a haemocytometer slide (Neubauer Improved Bright Line, Laboratory Supply, NSW) (Figure 4.3). A fresh cover slip was placed over the counting grid surface of a clean haemocytometer slide. For fungal counts, a suspension of *A. apis* was prepared with 0.05% v/v aqueous solution of the non-ionic surfactant polysorbate (Tween®80, Sigma-Aldrich), and sterile beads. The suspension was vigorously stirred to break any clusters. A micropipette (Eppendorf, Hamburg, Germany) was used to slowly dispense 10 µL of the suspension at the edge of the coverslip and haemocytometer slide surface. By capillary action the suspension was drawn across the surface of the slide; this ensured that the chambers were not over- or under-filled. A drop of immersion oil was placed on the centre of coverslip, the slide was viewed at 1000× with a Nikon Eclipse E200 light microscope (Nikon Corporation, Tokyo, Japan). CFUs were calculated by counting the number of 'spore balls'. Spore balls are clusters (16–26 µm diam.) of *A. apis* spores (3.5–4.0 µm long × 1.6–2.0 µm wide), released from thin-walled spherical spore cysts. Upon viewing the suspension sample, if the spore ball density was too high to count accurately or clustering occurred, the original suspension was diluted until a uniform distribution was observed. Fungal CFU concentration was estimated by taking the spore ball counts in five (four corners and the centre) of the nine primary 1 mm^2 haemocytometer squares. Spore balls touching the top and left margins of each square were counted and spore balls touching the middle, bottom and right margins were excluded. Once counts were made, the slides were cleaned and the process repeated a further four times, giving 25 counts. Total number of CFUs in the original volume = spore balls per mL × the original volume of the suspension from which spore balls were removed (Blankenship & Campbell 1976).

To calculate the concentration of bacterial CFUs in a prepared spore suspension, the process was similar to that used for fungal suspension, except Tween®80 was not included, and the CFUs were counted by viewing the central square on the haemocytometer slide (containing 25, 1 mm^2 squares, each of which contained a further 16 smaller squares). Bacterial CFU counts were taken in five (four corners

and the centre) of the smaller 25 haemocytometer squares, with CFUs touching the top and left margins of each square counted and CFUs touching the middle, bottom and right margins excluded. This process was performed five times, totalling 25 counts. Total number of CFUs in the original volume = spores per mL × the original volume of the suspension from which spore were removed (Blankenship & Campbell 1976).

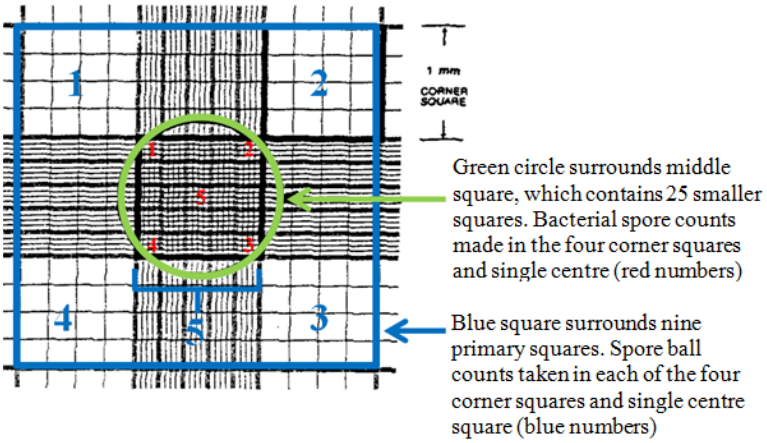

Figure 4.3 Diagram of haemocytometer square used for fungal and bacterial CFU counts. Fungal CFUs were counted using the five larger squares (blue), whilst bacterial counts were undertaken using the smaller central square (green).

4.3.6 Inhibition assay methods

During all pathogen and experimental assays, aseptic techniques were followed and they were performed in a biological safety cabinet class II (Email Air Handling, Minto, NSW).

Disc-diffusion assays

The disc diffusion method was selected, based on previously reported bacterial pathogen inhibition investigations (Antúnez et al. 2008, Forsgren et al. 2010, de Graaf et al. 2013). Three nutrient agar plates (NA) (Thermofisher Scientific Australia, Scoresby, Victoria, Australia) (i.e., replicates) were selected per treatment including the positive control tetracycline hydrochloride (MP Biomedicals, Seven Hills, NSW) and two negative controls. Ten treatments were assessed:

Negative control (*P. larvae* with no treatment)

Negative control (*P. larvae* with DMSO solvent only)

Positive control (*P. larvae* treated with 20 µg/mL tetracycline hydrochloride)

P. larvae treated with *T. carbonaria* propolis extract

P. larvae treated with *T. carbonaria* pollen extract

P. larvae treated with *T. carbonaria* honey

P. larvae treated with *T. carbonaria* gut-isolated *Weissella hellenica*

P. larvae treated with *A. mellifera* propolis extract

P. larvae treated with *A. mellifera* pollen extract

P. larvae treated with *A. mellifera* honey

Fresh NA plates were lawn plated with 300 µL each of the *P. larvae* suspension; this equated to ~230,400 CFU. The plates were allowed to dry for maximum 10 min. Sterile 6 mm discs (Fluka Analytical, Sigma-Aldrich, Missouri, USA) were dipped into the respective treatment and placed singly, onto the centre of the corresponding plates. All plates were then sealed with Parafilm® (Bemis Company, Inc., Neenah, Wisconsin, USA), and placed into the incubator at 37ºC and 5% CO_2. Plates were examined at 24 and 48 h after treatment (HAT). The inhibition halo was recorded by measuring two diameters per plate. The mean diameter per plate was calculated from these two measurements, and the overall treatment mean diameter was calculated from the replicate means.

Assessment of fungal inhibition

Inhibition of *A. apis* was determined by assessing mycelial growth in 96-well microtiter plates. The areas (mm^2) of mycelial inhibition was calculated using image processing and analysis software ImageJ 1.47V (Rasband 1997-2014). However, imaging of *M. anisopliae* var. *acridum* mycelial growth was difficult; therefore, inhibition was assessed by determining the area of sporulation (mm^2) as easily seen by the presence of green-grey spores in the 96-well plates. The area of sporulation was calculated using ImageJ 1.47V software.

Sterile microtiter Corning® Costar® Ultra-Low attachment 96 multiwell plates (Costar® International, Sigma-Aldrich, Missouri, USA), were labelled corresponding to one plate per treatment for both fungal pathogens; this equated to seven plates per pathogen. Working with one pathogen and one treatment at a time to avoid contamination, 100 µL of sterile water was dispensed into column 2 and columns 4–12 of the plates. Into column 4, 10 µL of the corresponding bee treatment was mixed. A 1:2 serial dilution was then performed. From each of the eight wells in column 4, 100 µL of the solution was removed and placed into corresponding column 5 wells and mixed, creating a 5.0% w/v treatment concentration. From column 5 wells, 100 µL was removed and placed into corresponding column 6 wells (creating a 2.5% w/v treatment concentration); this process was repeated for the remaining six columns (eventually with the lowest concentration 0.02% w/v for each treatment). Copper sulphate solution (Bluestone, Yates, Padstow, NSW) in 100 µL aliquots of 2.8% w/v were dispensed into column three (positive control) wells. Finally, 100 µL of the fungal spore suspension was dispensed into each of the 96 wells. Approximately 47 and 42 CFUs per 100 µL were suspended into each well for *A. apis* and *M. anisopliae* treatments, respectively.

All plates were then sealed with Parafilm and placed in an incubator at 27ºC (Performer Incubator, LABEC Laboratory Equipment Pty. Ltd. Marrickville, NSW). Plates were checked daily and any changes in fungal mycelial growth and sporulation recorded. After 21 days, the plates were removed from the incubator and final areas of growth or sporulation were measured.

Statistical analysis of inhibition assays

Statistical analysis on data was performed using IBM SPSS ver. 22 for Windows IBM Corp. 2013. Raw data collected are provided in Appendices A3.2–A3.4.

Data recorded during disc diffusion assays were tested for homogeneity using Levene's test. Differences among the activity of the bee products as measured by the

bacterial zone of inhibition were analysed using one-way ANOVA, followed by Tukey HSD post-hoc test if there were significance differences between means ($P \leq 0.05$).

Data for microtitre plate assays were recorded as area (mm^2) of *A. apis* mycelia inhibition or area (mm^2) of sporulation of *M. anisopliae* var. *acridum*. Inhibition results for each fungus pathogen were compared between each bee species and the corresponding nest product treatment; i.e., *A. apis* mycelial inhibition by *T. carbonaria* propolis vs. inhibition activity by *A. mellifera* propolis, for each the of nine separate treatment concentrations. Data were tested for homogeneity using Levene's test, and means were compared using one-way ANOVA.

4.4 Results

4.4.1 Identification of digestive system micro-organism

The isolated bacterium from *T. carbonaria* digestive system was identified as *Weissella hellenica* Collins et al. (1993) (Bacilli: Lactobacillales). This isolate was subsequently used for inhibition assays as the representative of adult bee gut microbes.

4.4.2 Inhibition of *P. larvae* using disc diffusion

All *T. carbonaria* and *A. mellifera* hive product extracts were shown to inhibit the growth of *P. larvae*, although the positive control, tetracycline hydrochloride, had the largest inhibition zone (IZ) (59.0 ± 0.29 mm) (Figure 4.4).

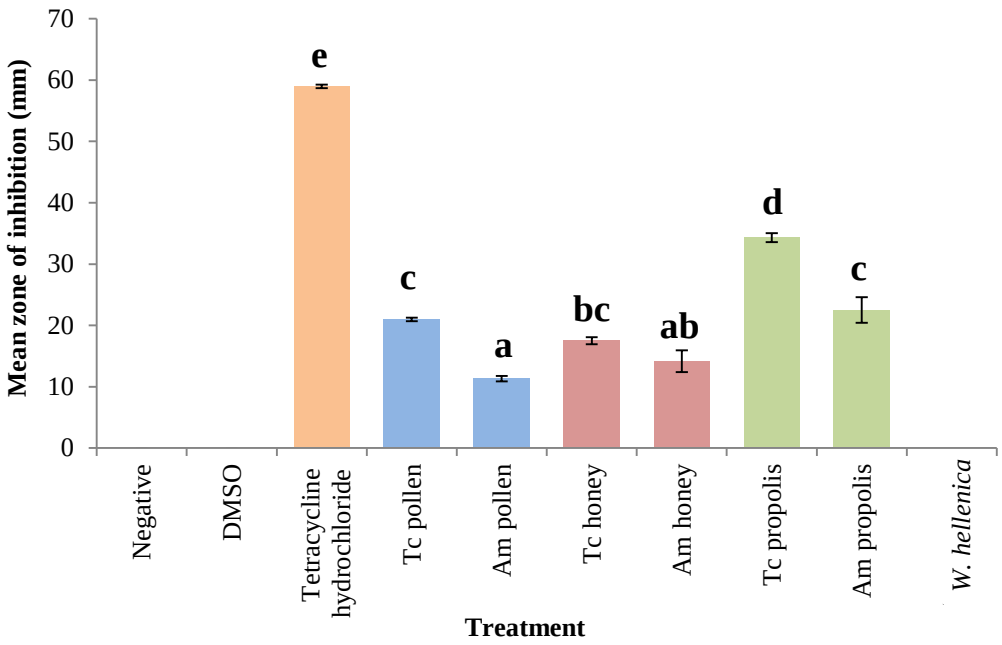

Figure 4.4 Mean diameter (mm) of the inhibition zone for *P. larvae* in response to *T. carbonaria* (Tc) and *A. mellifera* (Am) bee products. Error bars = SE of means, letters indicate statistical significances between means of nest product treatments. Statistical analysis only included hive products, because inclusion of the controls and *Weissella hellenica* resulted in non-homogeneous variances.

There were significant differences in the inhibition zones of *P. larvae* growth between the tested nest products ($F_{5, 17} = 45.9$, $p < 0.001$). *Tetragonula carbonaria* propolis had the largest mean inhibition zone (34.33 ± 0.73 mm), significantly more than all other products tested ($p < 0.001$). *Tetragonula carbonaria* pollen was superior to *A. mellifera* pollen ($p = 0.001$) and *A. mellifera* honey ($p = 0.016$), but not different to *T. carbonaria* honey ($p = 0.362$) or *A. mellifera* propolis ($p = 0.943$). *Apis mellifera* pollen was statistically significant in inhibition to *A. mellifera* propolis ($p < 0.001$) and *T. carbonaria* honey ($p = 0.031$). There were no other significant differences among the inhibition activities of nest products. The *W. hellenica* isolated from *T. carbonaria* gut showed no activity against *P. larvae*, nor did the extract solvent DSMO.

4.4.3 Inhibition of *A. apis* and *M. anisopliae* by nest and bee products

All possible relationships between inhibition of growth of the two fungal pathogens and nest and bee products (based on the methodology described in section 4.2.3) are provided in Appendix A3.3 and A3.4. Only significant relationships with $R^2 \geq 0.5$ are presented in this chapter.

Ascosphaera apis

Ascosphaera apis mycelial growth increased with increasing concentration of *T. carbonaria* pollen extract up to 2.5% w/v, after which mycelial growth greatly decreased with further increasing concentration (Figure 4.5a). The relationship can be represented by the equation $y = 9.5971x^2 - 38.646x + 37.084$ ($R^2 = 0.66$). Maximum inhibition (82% compared to the control) occurred at the highest concentration tested (5.0% w/v), where it was significantly more inhibitory than most lower concentrations (viz. 2.5% w/v ($p < 0.001$), 1.25% w/v ($p < 0.001$). 0.63% w/v ($p < 0.001$), 0.31% w/v ($p < 0.001$), 0.16% w/v ($p < 0.001$) and 0.08% w/v ($p < 0.001$)). *Tetragonula carbonaria* pollen extract concentrations at 2.5% w/v, 1.25% w/v and 0.63% w/v were significantly more inhibitory than at 0.02% w/v ($p < 0.001$). There was no change in mycelial growth with increasing concentration of *A. mellifera* pollen extract and no significant relationship could be determined. There were thus no significant differences between any of the *A. mellifera* extract concentrations tested ($F_{8, 63} = 1.5$, $p = 0.164$). *Tetragonula carbonaria* pollen extract was significantly more inhibitory than *A. mellifera* pollen extract at the highest concentration tested 5.0% w/v ($F_{1, 14} = 6.7$, $p = 0.021$).

Tetragonula carbonaria honey showed increased inhibition with increasing concentration, with a plateau after 0.63% w/v (Figure 4.5b). The relationship can be represented by the equation $y = 12.1441n(x) + 67.898$ ($R^2 = 0.82$). Maximum inhibition (98%) occurred at the highest concentration tested (5.0% w/v), where it was significantly more inhibitory than 0.08% w/v, 0.04% w/v and 0.02% w/v concentrations (all $p < 0.001$). There were no other differences between concentrations. There was no significant relationship displayed in *A. apis* mycelial

inhibition by *A. mellifera* honey, and no significant differences between concentrations tested ($F_{8, 63} = 0.7$, $p = 0.699$), however inhibition reached 76% at 5.0% w/v. There was no significant difference between inhibition activities of these two honey extracts at the two highest concentrations tested, 2.5% w/v and 5.0% w/v ($F_{1, 14} = 0.2$, $p = 0.600$; $F_{1, 14} = 1.7$, $p = 0.214$ respectively).

Tetragonula carbonaria propolis showed an initial decrease in inhibition to 0.63% w/v concentration, after which inhibition increased to 95% at an extract concentration of 2.5% w/v and reached 100% at 5.0% w/v (Figure 4.5c). The relationship (to 2.5% w/v) can be represented by the equation $y = -9.6918x^3 + 4.5888x^2 + 87.18x + 4.586$ ($R^2 = 0.93$). There were significant differences ($p < 0.001$), between extract concentrations, with 5.0% w/v more inhibitory than 1.25% w/v and all lower concentrations ($p < 0.001$); 2.5% w/v was more inhibitory than 1.25% w/v and all lower concentrations ($p < 0.001$); and concentrations 1.25% w/v, 0.31% w/v and 0.08% w/v were more inhibitory than 0.02% w/v ($p < 0.001$).

Apis mellifera propolis showed increased inhibitory activity with increasing concentration, with a plateau after 1.25 % w/v; total inhibition (100%) was recorded at 2.5% and 5.0% w/v concentrations. The relationship (to 2.5% w/v) can be represented by the equation $y = -33.808x^3 + 159.28x^2 – 170.84x + 54.372$ ($R^2 = 0.91$). The three highest concentrations were not significantly different to 0.63% w/v but were significantly superior to all other lower concentrations ($p < 0.001$); 0.63% w/v was not significantly different to 0.31% w/v, but more inhibitory than all lower concentrations (0.16% w/v ($p = 0.001$), 0.08% w/v ($p < 0.001$), 0.04% w/v ($p = 0.001$) and 0.02% w/v ($p = 0.002$)). *Apis mellifera* propolis was significantly more inhibitory than *T. carbonaria* propolis at 0.63% w/v and 1.25% w/v ($F_{1, 14} = 59.7$, $p < 0.001$; $F_{1, 14} = 62.3$, $p < 0.001$, respectively), but not at higher concentrations.

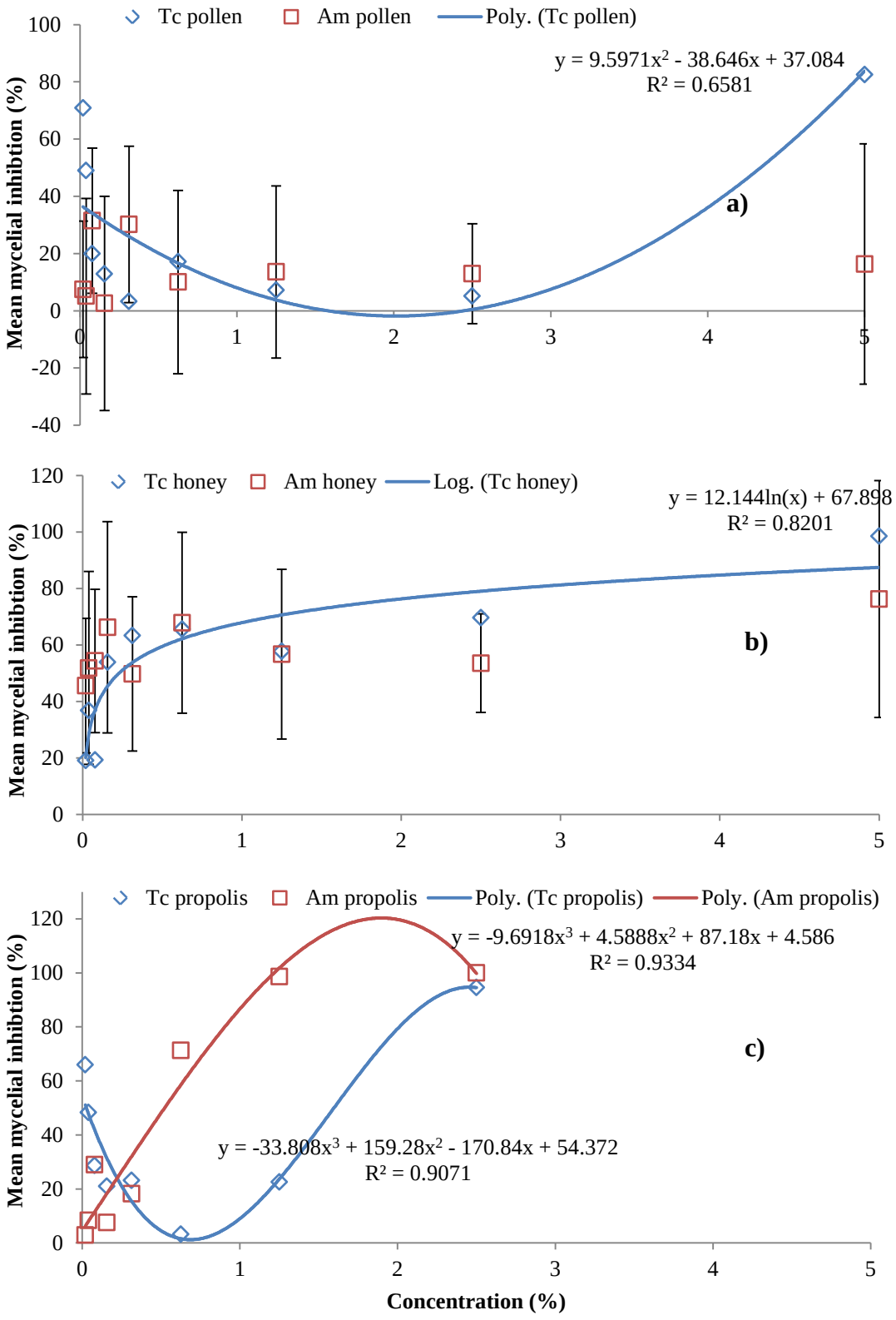

Figure 4.5 Mean *A. apis* mycelial inhibition by bee nest products a) pollen, b) honey and c) propolis collected from *T. carbonaria* and *A. mellifera*. Data points represent mean mycelial inhibition (%) of either *T. carbonaria* (blue) or *A. mellifera* (red) nest products. Trendlines represent significant relationships ($R^2 \geq 0.5$) between growth and nest products either as polynomial (Poly.) or logarithmic (Log.). Error bars = SE of means.

Weissella hellenica spore suspension gave very variable results with no discernible relationship between spore concentration and inhibition of mycelial growth (Figure 4.6). However, despite all spores concentrations inhibiting mycelial growth to some extent, there were no significant differences between the concentrations tested ($F_{8, 63} = 1.9$, $p = 0.082$).

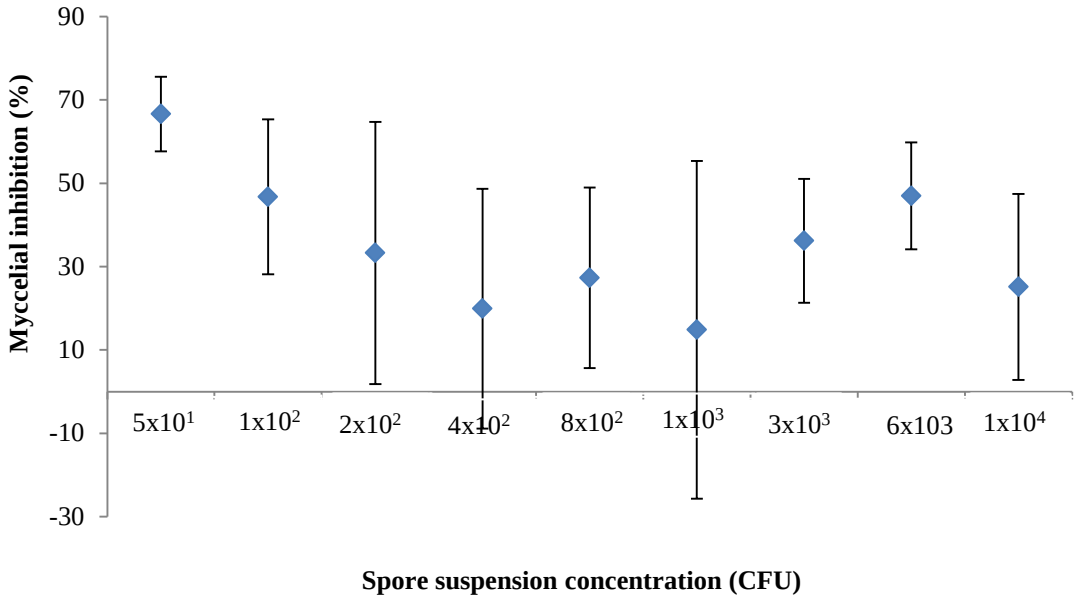

Figure 4.6 Inhibition of *A. apis* mycelial growth (%) by treatment of increasing *Weissella hellenica* spore suspension (CFU). Error bars = SE of means.

Metarhizium anisopliae

As the concentration of *T. carbonaria* pollen extract increased, the area of sporulation decreased, with the minimum area (2.3 mm^2) occurring at the highest concentration (5.0% w/v) (Figure 4.7a). The relationship can be represented by the equation $y = 5.7498x^{-0.406}$ ($R^2 = 0.68$). The area of sporulation was significantly less at the two highest concentrations tested (5.0% w/v and 2.5% w/v) than for 0.63% w/v ($p = 0.027$, $p = 0.047$, respectively). Furthermore, *T. carbonaria* pollen extracts at 5.0% w/v, 2.5% w/v and 1.25% w/v, had less area of sporulation than at 0.31% w/v (all $p < 0.001$), 0.16% w/v (all $p < 0.001$), 0.08% w/v ($p = 0.008$, $p = 0.014$, $p = 0.028$, respectively), 0.04% w/v ($p = 0.002$, $p = 0.004$, $p = 0.008$, respectively) and 0.02% w/v (all $p < 0.001$). Area of sporulation was significantly less at 0.63% w/v than 0.16% w/v ($p < 0.001$) and 0.02% w/v ($p = 0.038$), while 0.31% w/v was significant less than 0.16% w/v ($p = 0.004$) and 0.16% w/v was significant less than

0.08% w/v and 0.04% w/v (both $p < 0001$), and 0.02% w/v ($p = 0.023$). There were no significant differences between any other extract concentrations.

Apis mellifera pollen extract showed a similar trend to *T. carbonaria* pollen extract, with the greatest suppression of sporulation at the higher concentrations (Figure 4.7a). The minimum area of sporulation (0.5 mm^2) (i.e., maximum inhibition of *M. anisopliae* development, 99%) occurred at the highest concentration tested (5.0% w/v). The relationship can be represented by the equation $y = 2.0933x^{-0.795}$ ($R^2 = 0.83$). The area of sporulation was significantly less ($p < 0.001$) in the three highest concentrations tested, 5.0% w/v, 2.5% w/v and 1.5% w/v, than all other lower concentrations from 0.31% w/v. *Apis mellifera* pollen extract applied at a concentration of 0.63% w/v was significantly superior in reducing the area of sporulation compared to 0.31% w/v ($p = 0.025$), 0.08% w/v ($p = 0.016$), 0.04% w/v ($p = 0.001$) and 0.02% w/v ($p < 0.001$). There were no significant differences between other extract concentrations. *Apis mellifera* pollen was significantly more active at 0.16% w/v (area of sporulation 15 mm^2) than *T. carbonaria* (30 mm^2) ($F_{1, 14} = 12.9$, $p = 0.003$). Minimum sporulation occurred at 5.0% w/v and 2.5% w/v for *A. mellifera* pollen extract. At these concentrations, *T. carbonaria* pollen extract was significantly more inhibitory than *A. mellifera* extract at 5.0% w/v ($F_{1, 14} = 10.2$, $p = 0.006$) and 2.5% w/v ($F_{1, 14} = 32.7$, $p < 0.001$).

As the concentration of *T. carbonaria* honey extract increased, the area of sporulation decreased, with the minimum area occurring at the two highest concentrations tested, 5.0% w/v and 2.5% w/v (Figure 4.7b). The relationship can be represented by the equation $y = -4.479\ln(x) + 9.9828$ ($R^2 = 0.66$). The two highest concentrations (5.0% and 2.5% w/v) had significantly less area of sporulation than all lower concentrations; 0.31% w/v ($p = 0.005$), 0.16% w/v ($p < 0.001$), 0.08% w/v ($p < 0.001$), 0.04% w/v ($p = 0.001$) and 0.02% w/v ($p < 0.001$). The third highest extract concentration (1.25% w/v) had greater inhibitory activity compared to lower extract concentrations of 0.16% w/v ($p = 0.006$) and 0.08% w/v ($p < 0.001$). Extract concentrations of 0.63% w/v had significantly less area of sporulation compared to the three most lowest concentrations (0.16% w/v, $p = 0.001$; 0.08% w/v, $p < 0.001$;

and 0.02% w/v, $p = 0.050$); whereas 0.31% w/v, was significantly more inhibitory than 0.08% w/v ($p = 0.001$). There were no other differences between extract concentrations.

For *Apis mellifera* honey extract, there was essentially no change in the area of sporulation, which commenced from a high level, and no significant relationship was discernable, although there were significant differences in the area of sporulation between the highest concentration (5.0% w/v) and 2.5% w/v ($p < 0.001$), 1.25% w/v ($p = 0.026$), 0.63% w/v ($p = 0.002$), 0.31% w/v ($p = 0.023$) and 0.16% w/v ($p = 0.008$), and all other lower concentrations (all $p < 0.001$) (Figure 4.7b). The area of sporulation was significantly less at 2.5% w/v compared to 0.02% w/v ($p = 0.004$), all other lower concentrations from 1.25% w/v were significantly inhibitory compared to 0.02% w/v (all $p < 0.001$). There were significant differences in the area of sporulation at the lower concentrations of two honey extracts (viz. 0.04%, 0.08%, 0.16% and 0.031% w/v), with *T. carbonaria* honey having less area of sporulation than *A. mellifera* honey ($F_{1, 14} = 18.2$, $p = 0.001$; $F_{1, 14} = 306.4$, $p < 0.001$; $F_{1, 14} = 87.3$, $p < 0.001$; $F_{1, 14} = 7.5$, $p = 0.016$, respectively). There was also a significant difference in the area of sporulation between the two honey samples at 2.5% w/v, with the *T. carbonaria* honey treatment having a smaller area than *A. mellifera* honey ($F_{1, 14} = 21.5$, $p < 0.001$).

Despite a decrease in the area of sporulation as the concentration of *T. carbonaria* propolis increased, no satisfactory relationship was able to be discerned (Figure 4.7c). However, minimum area of sporulation was recorded at 5.0% w/v, and the three highest extract concentrations tested (5.0% w/v, 2.5% w/v and 1.25% w/v) had significantly less sporulation than at 0.31% w/v ($p = 0.001$, $p = 0.002$ and $p = 0.006$, respectively) and 0.02% w/v ($p = 0.008$, $p = 0.013$ and $p = 0.040$, respectively). The area of sporulation was significantly less at 0.31% w/v than the lower concentrations 0.08% w/v ($p = 0.055$) and 0.04% w/v ($p = 0.045$). There were no significant differences between other extract concentrations.

The area of sporulation decreased as the concentration of *A. mellifera* propolis increased, and the relationship can be represented by the equation $y = -0.0054x^2 - 2.0471x + 19.985$ ($R^2 = 0.82$). *Apis mellifera* propolis at the highest concentration (5.0% w/v) had significantly less area of sporulation than 1.25% w/v ($p = 0.051$), 0.31% ($p = 0.001$), 0.08% w/v ($p < 0.001$), 0.04% w/v ($p = 0.039$) and 0.02% w/v ($p = 0.007$) extracts (Figure 4.7c). There were no other significant differences between extract concentrations. There were significant differences between the two propolis extracts, with *T. carbonaria* extract having consistently significantly less area of sporulation than *A. mellifera* extract (0.02% w/v $F_{1, 14} = 7.0$, $p = 0.019$; 0.04% w/v $F_{1, 14} = 44.5$, $p < 0.001$; 0.08% w/v $F_{1, 14} = 57.0$, $p < 0.001$; 0.16% w/v $F_{1, 14} = 85.0$, $p < 0.001$; 0.31% w/v $F_{1, 14} = 13.1$, $p = 0.003$; 0.63% w/v $F_{1, 14} = 39.2$, $p < 0.001$; 1.25% $F_{1, 14} = 204.4$, $p < 0.001$; 2.5% w/v $F_{1, 14} = 633.4$, $p < 0.001$ and 5.0% w/v $F_{1, 14} = 74.3$, $p < 0.001$).

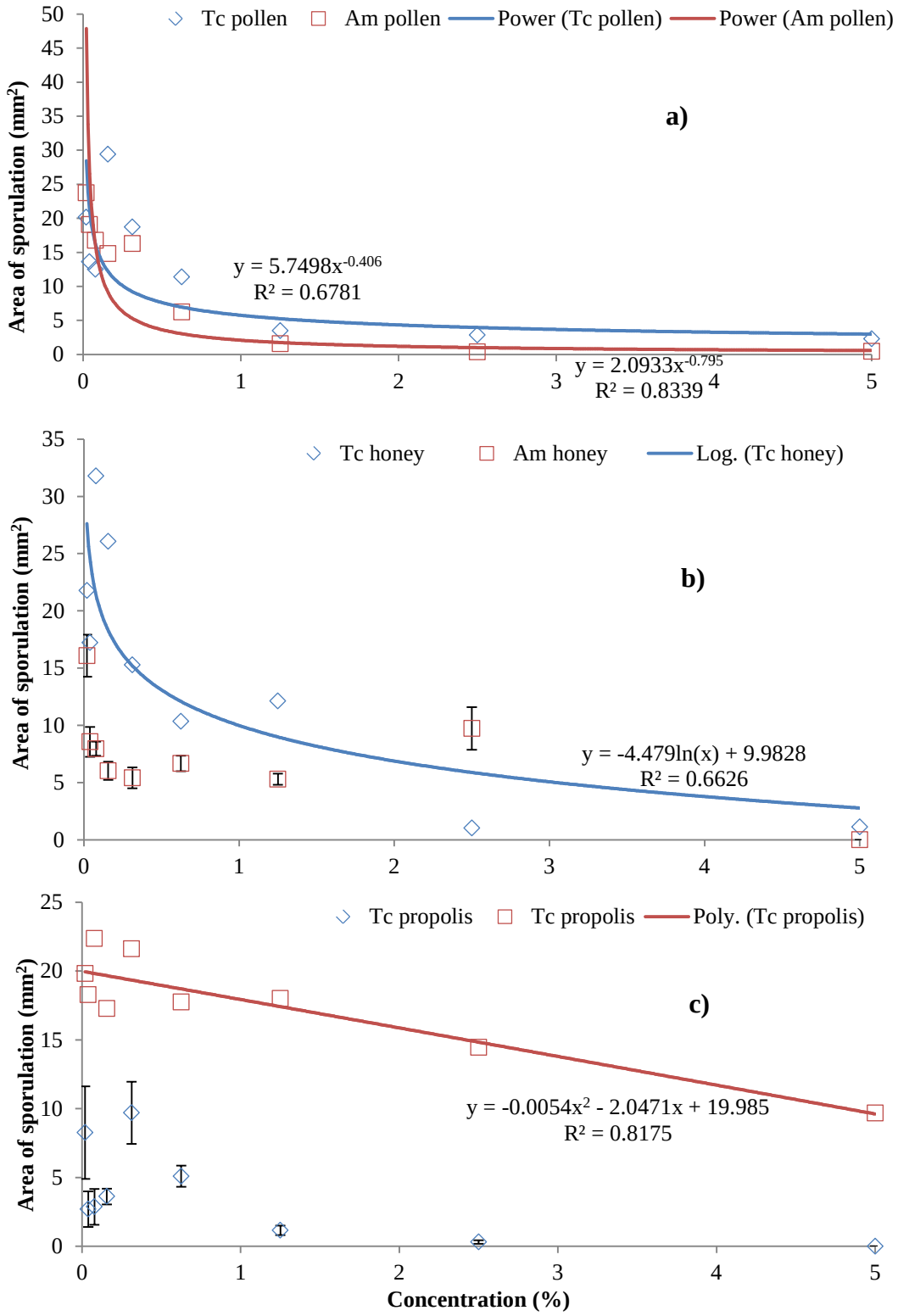

Figure 4.7 Mean area of sporulation (mm^2) of *M. anisopliae* after treatment with bee nest products a) pollen, b) honey and c) propolis collected from *T. carbonaria* and *A. mellifera*. Data points represent mean area of sporulation (mm^2) of either *T. carbonaria* (blue) or *A. mellifera* (red) nest products. Trendlines represent significant relationships ($R^2 \geq 0.5$) between growth and nest products as power, logarithmic (Log.), or polynomial (Poly.) equations as shown. Error bars = SE of means.

There was a trend towards an overall decrease in the area of sporulation as the concentration of *Weissella hellenica* spores increased (Figure 4.8). Maximum inhibition of 94% occurred at the highest concentration tested (1×10^4). The area of sporulation was significantly less at the two higher spore suspension concentrations (1×10^4 and 6×10^3) than at 4×10^2 ($p = 0.002$ and $p = 0.030$, respectively) and 5×10^1 ($p = 0.001$ and $p = 0.016$, respectively). Area of sporulation at 2×10^2 was significantly less than the lowest spore suspension concentration (5×10^1) tested ($p = 0.052$).

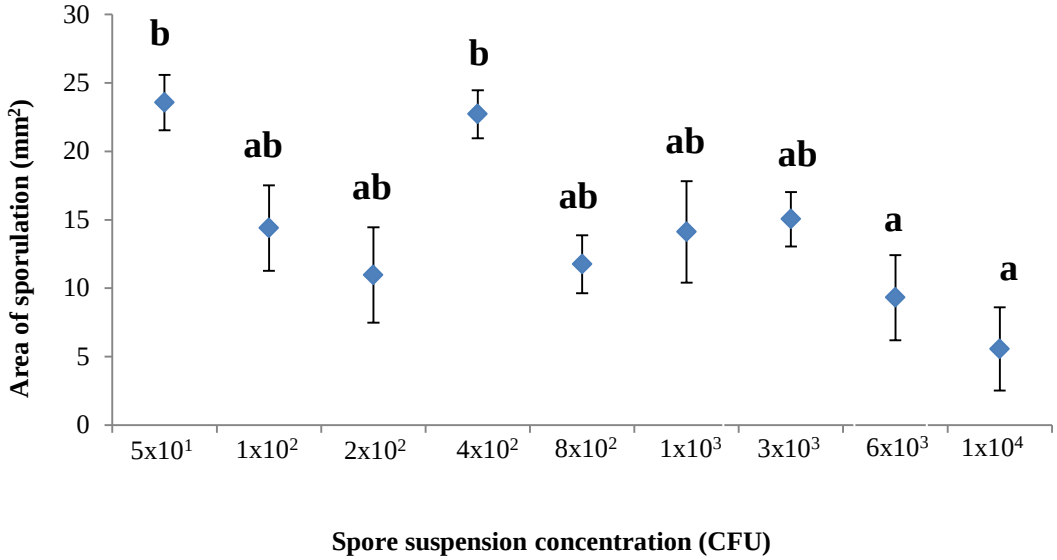

Figure 4.8 Area of sporulation (mm^2) of *M. anisopliae* after treatment with *Weissella hellenica* spore suspension (CFU). Error bars = SE of means, letters indicate statistical significances at $p = 0.05$.

4.4.4 Liquid chromatography mass-spectrometry of nest products

Data presented in this chapter represents LC-MS analysis of bioactive compounds identified in *T. carbonaria* and *A. mellifera* propolis and newly emerged brood comb from 2013 – 2015. Further supporting data are provided in Appendix A3.5.

1. Activity in *T. carbonaria* and *A. mellifera* propolis

Propolis extracts were compared with reported bioactive compounds from the literature and were found to contain compounds with corresponding ion masses (mass+1: m+1) and UV profiles (Table 4.1). A typical LC-MS analysis from *T.*

carbonaria propolis is displayed in Figure 4.9. The compounds found in propolis exhibit chromophores which are typical of flavanones (Appendix A3.5, Figure A3-h). The flavanones described in the literature with ion masses 271 and 285 (m+1) (Table 4.2), were detected in both *T. carbonaria* and *A. mellifera* propolis.

Table 4.1 Detected flavanones with ion masses (271 or 285 m+1) reported in literature from stingless bees and honey bee propolis.

	Name	**References**
Honey bee	flavanone, pinostrobin,	Machado et al. 2007, Falcão et al. 2010, Tukmechi et al. 2010
Stingless bee	flavanone, cryptostrobin, pinostrobin, strobopinin	Massaro et al. 2014a, Nobakht et al. 2014, Massaro et al. 2015

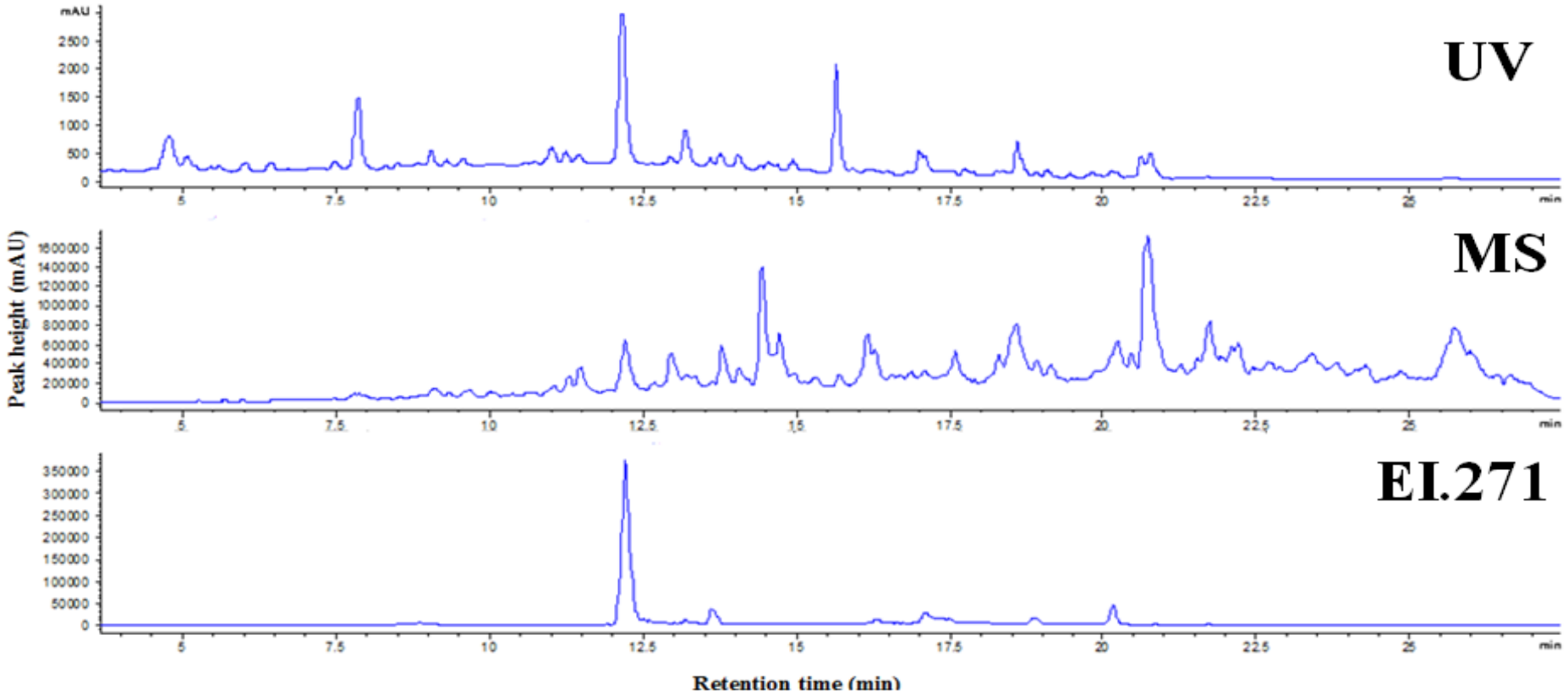

Figure 4.9 LC-MS chemical peaks from *T. carbonaria* propolis. Data are represented as peak height (mAU) against peak retention time (min) in the form of the UV and MS analyses, and identify a compound with an extracted ion mass EI 271.

LC-MS profiles revealed the presence of flavanones with ion masses of 271 and 285 (m+1) from propolis (as summarised in Table 4.2). There was one flavanone identified in *T. carbonaria* propolis with an extracted ion mass of 271 (m+1), and a retention time of 12 min (Figure 4.9). There were three flavanones identified in *A. mellifera* propolis with an extracted ion mass 271 (m+1) with retention times of 10 min, 12 min and 13.5 min (Appendix A3.5, Figure A3-i). *Tetragonula carbonaria* and *A. mellifera* propolis also contained flavanones with an ion mass of 285 (m+1). There were two identified in *T. carbonaria* propolis at 13 min and 15 min, while *Apis mellifera* propolis contained one flavanone with ion mass 285 (m+1) at 10.5 min (Appendix A3.5, Figure A3-j).

Ion masses of the flavanones identified in propolis samples were compared with a standard for pinostrobin from Kava root (*Piper methysticum* Frost) (D. Brushett, pers. comm. 2015). The flavanone from *T. carbonaria*, *A. mellifera*, and Kava had the same ion mass (271 m+1), retention time (12 min) and chromophore as pinostrobin (Appendix A3.5, Figure A3-k).

Table 4.2 Summary of detected flavanones from UWS *A. mellifera* and *T. carbonaria* propolis samples, with ion masses (mass+1), and retention time of detected flavanones, and identified compound name.

Source	Ion mass (m+1)	Name	Retention time (min)
T. carbonaria	271	Pinostrobin	12
T. carbonaria	285 285	Flavanones	13 15
A. mellifera	271	Pinostrobin	12
A. mellifera	271 271 285	Flavanones	10 13.5 10.5

2. Comparison between *A. mellifera* and *T. carbonaria* propolis between 2013 and 2015

Comparison of the LC-MS profiles of propolis (Figure 4.10 and Figure 4.11) collected in 2013, 2014 and 2015 from *T. carbonaria* and *A. mellifera* colonies, showed a similarity in their composition of the flavanones. However, the relative ratios of the compounds somewhat changed between the years. There were more late-running compounds (after 15 min) in 2015 for *T. carbonaria* propolis, though key flavanones were still present in both samples. The three major compounds present in *A. mellifera* propolis at approximately 11.5 min, 13 min, and 15.5 min identified in 2013, were also present in 2015. The late running peaks (after 15 min) in the *T. carbonaria* propolis were not present in *A. mellifera* propolis extracts (Figure 4.11).

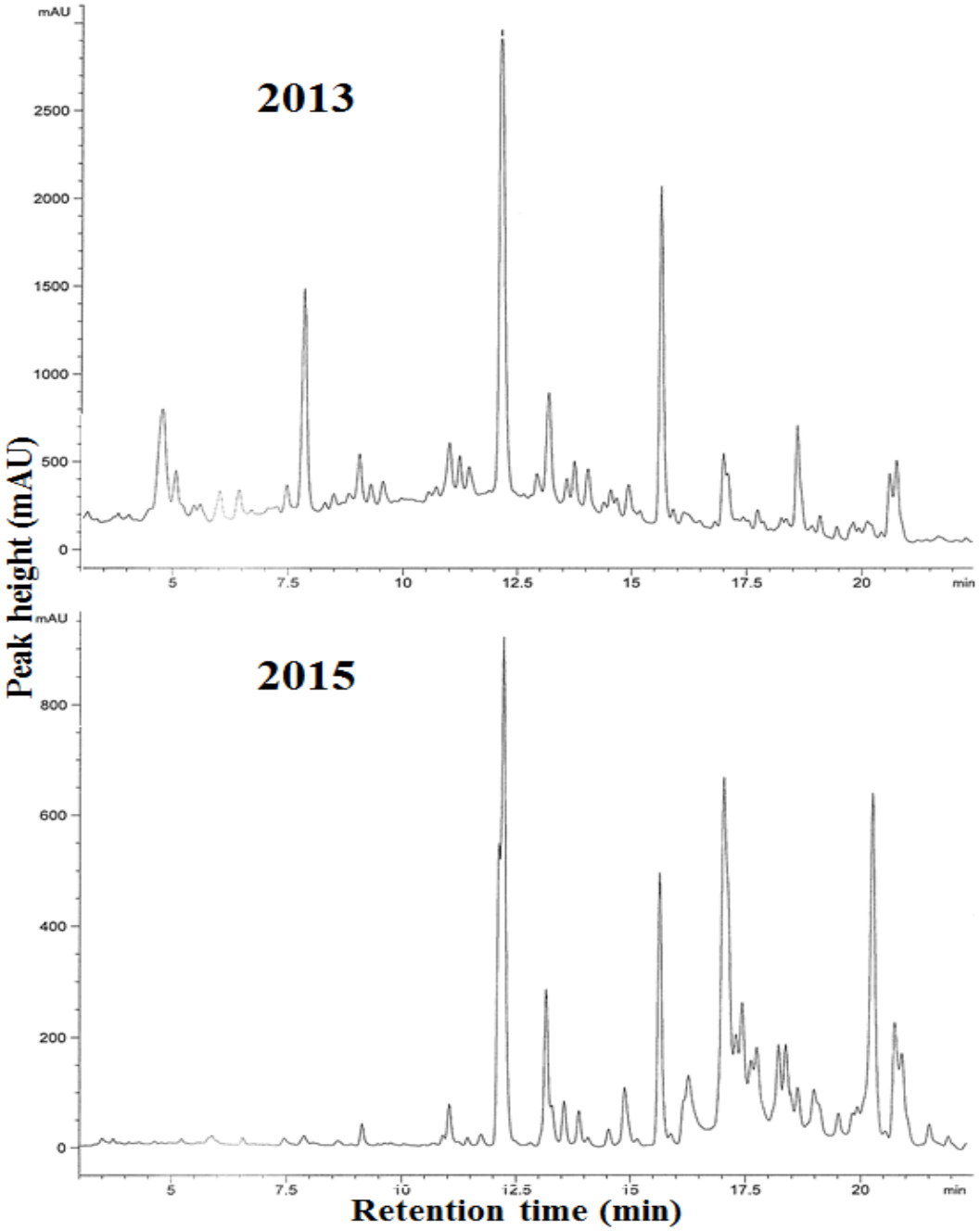

Figure 4.10 Comparison of the chemical compositions between *T. carbonaria* propolis collected in 2013 and 2015. Identified compounds are represented by height (mAU) and retention time (min).

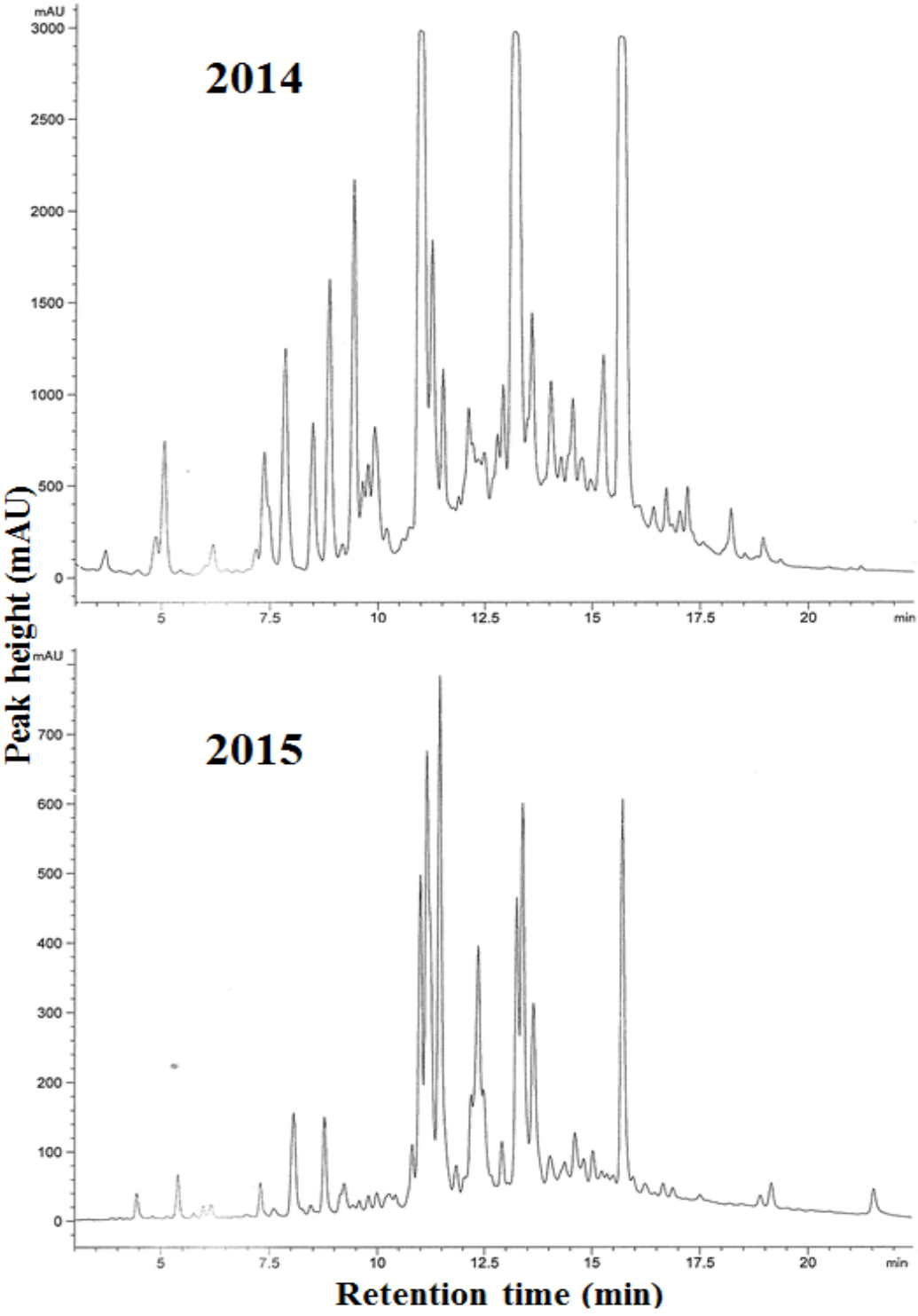

Figure 4.11 Comparison of the chemical compositions between *A. mellifera* propolis collected in 2014 and 2015. Identified compounds are represented by height (mAU) and retention time (min).

3. Comparison of *A. mellifera* and *T. carbonaria* propolis and brood comb (2015)

The LC-MS profile of the propolis and brood comb of *T. carbonaria* are very similar (Figure 4.12). This suggests that the propolis is present in the *T. carbonaria* brood comb. The two samples were made up quantitatively for analysis, and the flavanone concentrations in the brood comb can be observed to be approximately one-third the concentration in the propolis. There were substantially more flavanones in the stingless bee brood comb than what are present in the *A. mellifera* brood comb (Figure 4.14).

The LC-MS profile of the *A. mellifera* brood comb shows that there were essentially no flavanones present (Figure 4.13).

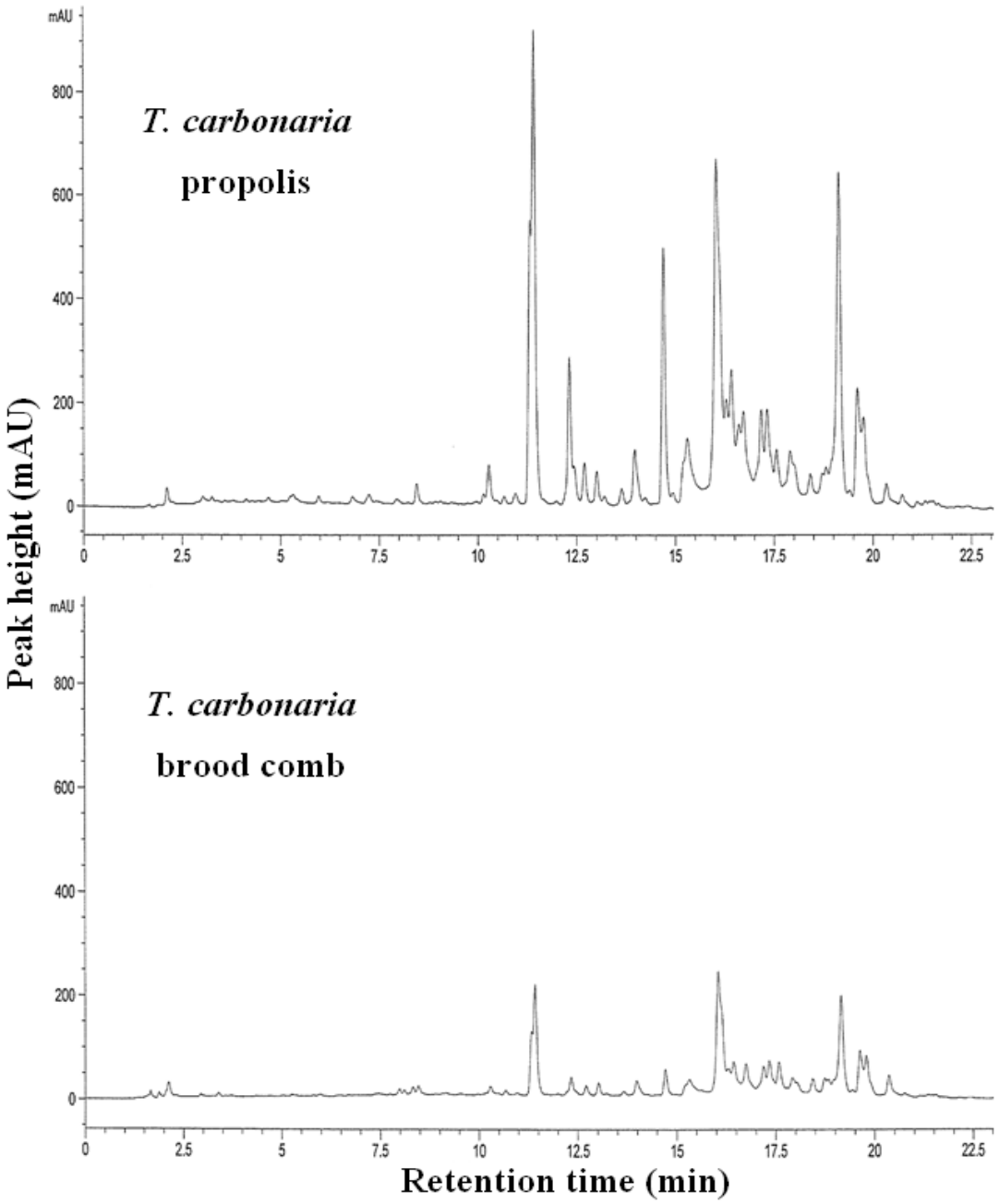

Figure 4.12 Comparison of the compositions between *T. carbonaria* propolis and brood comb, identified compounds are represented by height (mAU) and retention time (min). The three key flavanones were detected in both samples.

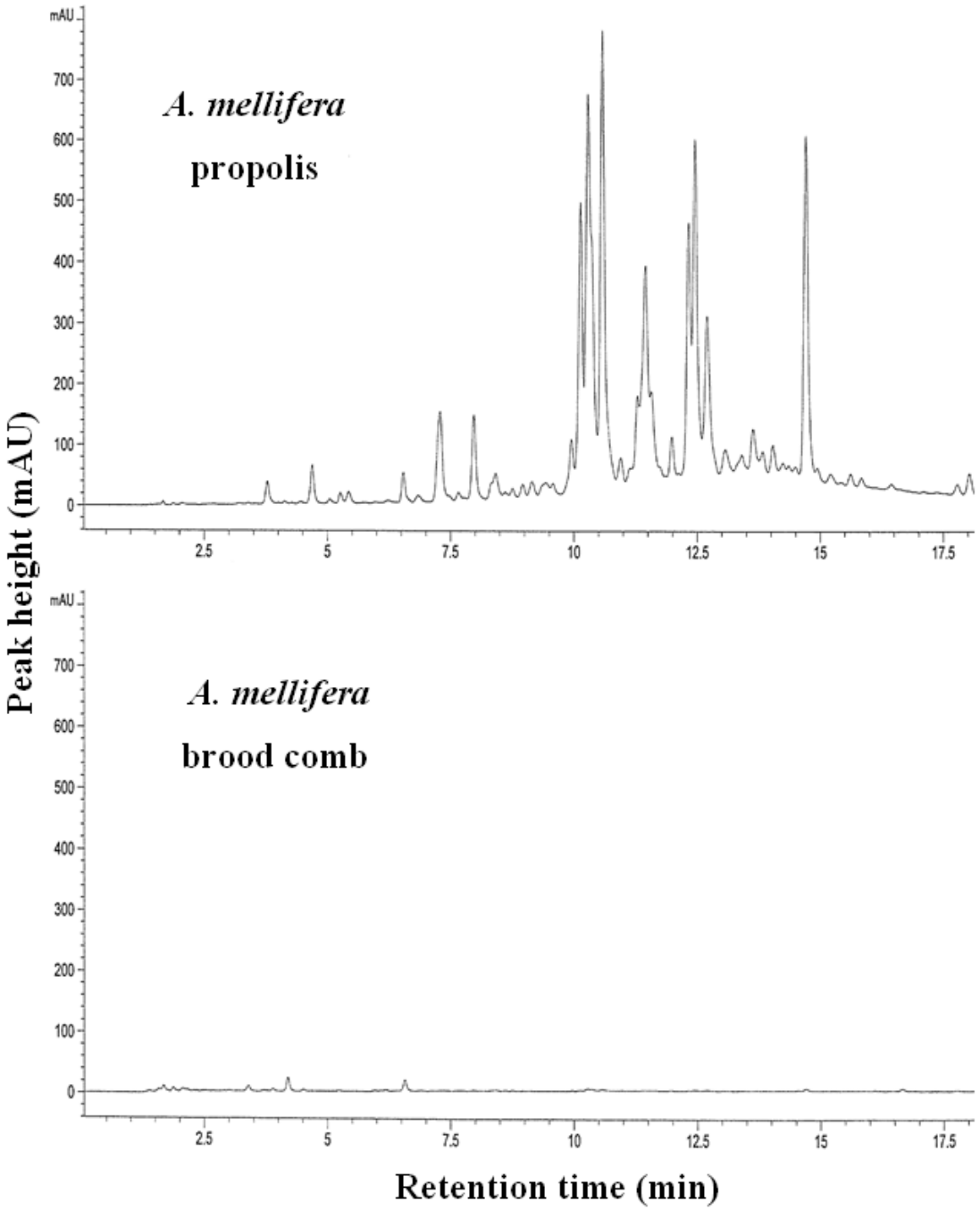

Figure 4.13 Comparison of the chemical compositions between *A. mellifera* propolis and brood comb, identified compounds are represented by height (mAU) and retention time (min). There no similarities between the two samples.

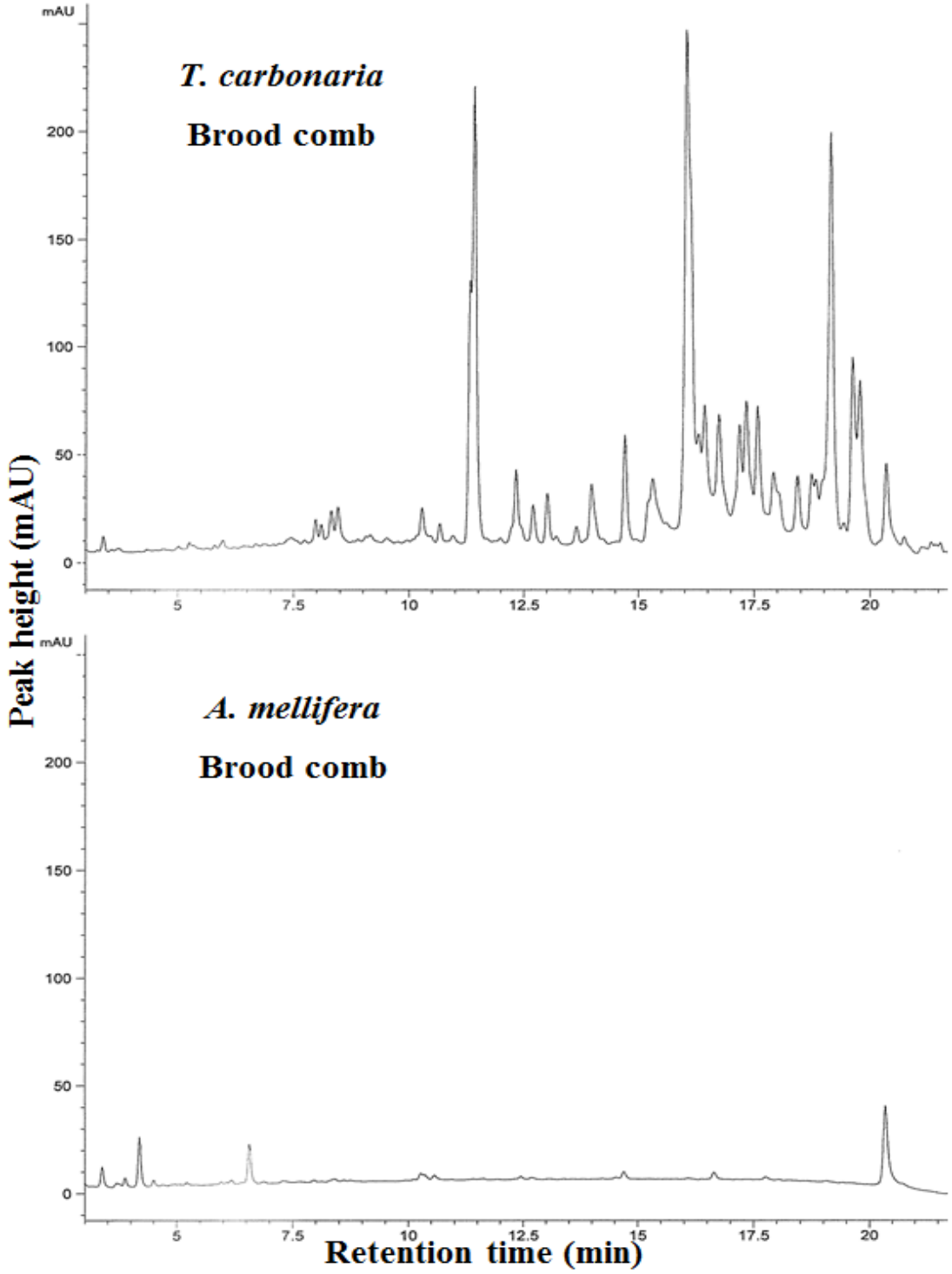

Figure 4.14 Comparison of brood comb composition between *T. carbonaria* and *A. mellifera* collected in 2015. Identified compounds are represented by peak height (mAU) and retention times (min).

4.5 Discussion

The results presented here are the first to test stingless bee pollen and propolis extracts and honey samples against known honey bee and insect pathogens. In general, *T. carbonaria* nest products were superior at inhibiting the tested insect pathogens, especially the bacterial brood pathogen (*P. larvae*), than were *A. mellifera* nest products.

Tetragonula carbonaria pollen extract was superior at inhibiting *P. larvae* and *A. apis* than was *A. mellifera* pollen. Previously, studies have shown similar results using *A. mellifera* pollen in reducing brood pathogen growth (Lavie 1960, Gilliam et al. 1988, Crailsheim & Riessberger-Gallé 2001). However, it should be clarified that the pollen from *T. carbonaria* was obtained from storage pots and would be influenced by the microbes and other substances added by bees for storage (Gilliam et al. 1988, Crailsheim & Riessberger-Gallé 2001). However, *A. mellifera* pollen was collected from pollen traps at the front of the hives and was not influenced by the storage processes which might have affected the results. *Tetragonula carbonaria* honey was superior to *A. mellifera* honey in inhibiting both fungal pathogens, *A. apis* and *M. anisopliae*. This finding supports previous work that showed that stingless bee honey inhibited *Candida albicans* at lower concentrations (30–35% v/v) than did *A. mellifera* honey (≥40% v/v) (da Cruz et al. 2014).

A number of studies have isolated specific microbes from *A. mellifera* honey (Reynaldi et al. 2004, Sabaté et al. 2009), and there is a similarity between gut symbionts and microbes identified in honey and pollen which appear to be added by workers (Gilliam et al. 1988, Crailsheim & Riessberger-Gallé 2001). It was somewhat surprising that *Weissella hellenica* isolated from *T. carbonaria* showed little inhibitory activity against bacterial and fungal entomopathogens. However, other LAB have been shown to inhibit *P. larvae* growth (Evans & Lopez 2004, Yoshiyama et al. 2013) and therefore there is opportunity to further investigate the antimicrobial properties of microorganisms, especially LAB, from stingless bee honey and pollen, and gut symbionts.

Another possible explanation for the difference in the activity of *T. carbonaria* pollen and honey compared to those from *A. mellifera*, may be from the infiltration of antimicrobial compounds from the propolis incorporated in storage pots (Temaru et al. 2007, Kimoto-Nira & Amano 2008), which does not occur in *A. mellifera* colonies where beeswax is used. This hypothesis is supported by the finding in this study that *T. carbonaria* propolis extracts alone greatly inhibited *P. larvae, A. apis* and *M. anisopliae*. The zone of inhibition by *A. mellifera* propolis against *P. larvae* in this study (23 mm) is consistent with other studies (13–35 mm) (Antúnez et al. 2008, Bastos et al. 2008, Simone 2010). *Tetragonula carbonaria* propolis has not been previously tested against *P. larvae*; however, my current finding (32 mm) is similar to the superior inhibition by *A. mellifera* propolis from Brazil (34.7 mm) (Simone 2010). The botanical source of *A. mellifera* Brazilian propolis was *Baccharis dracunculifolia* (Asteraceae), which had better inhibitory activity against *P. larvae*, than propolis derived from an unknown US source (Simone 2010).

The difference between the activity of *T. carbonaria* and *A. mellifera* propolis might be due to the botanical sources foraged by the two species, despite them being co-located, as represented their representative LC-MS profiles, and the number of flavanones detected. There are similarities in the profiles of the propolis extracts, between seasons for each species (e.g. *T. carbonaria* 2013 vs. 2015, and *A. mellifera* 2014 vs. 2015-samples), suggesting similar plant sources were utilised each year by each species. Resin sources for stingless bees in tropical and sub-tropical regions can include *Xanthorrhoea* spp. (Duewell 1965, Ghisalberti et al. 1978), *Araucaria* spp., (Bankova and Popova 2007), many myrtaceous species such as eucalypts (Massaro et al. 2015) and *C. torelliana* (Leonhardt et al. 2011). A number of these plant genera also occur in temperate areas, including the Hawkesbury region of NSW, and were within 500 m of the apiary at UWS. The superior *P. larvae* inhibition activity of *T. carbonaria* propolis appears to be due to the presence of compounds not present in *A. mellifera* propolis, which is reflective of differences in foraging between the two species. Previous work in S.E. Queensland identified the contribution of *Corymbia torelliana* to the chemical composition of *T. carbonaria* propolis (Massaro et al. 2014a, Massaro et al. 2015); resin collection from this source is associated with *T. carbonaria* colonies (Wallace & Trueman 1995, Wallace et al. 2008, Wallace & Lee

2010, Drescher et al. 2014), but not with *A. mellifera*. There were sources of *C. torelliana* within the foraging range of the *T. carbonaria* colony maintained at UWS, and *T. carbonaria* colonies collected *C. torelliana* resin. White resin was deposited in hives (Massaro et al. 2015), workers were observed carrying *C. torelliana* seeds on their corbiculae, and collected seeds accumulated around the nest entrances (Klumpp 2007). The difference between the numbers of identified peaks between the propolis samples may therefore be a result of *C. torelliana* being solely sourced by *T. carbonaria*. These results add to the recent findings on the chemical composition of Australian stingless bee propolis; further studies from other regions should further elucidate chemical composition and resin sources of stingless bee propolis.

Tetragonula carbonaria and *A. mellifera* propolis contained substantial levels of flavanones, polyphenolic flavonoids. This supports previous findings of the presence of phenolic acids and flavanones in *T. carbonaria* (Massaro et al. 2014a, Massaro et al. 2015) and honey bee (Machado et al. 2007, Falcão et al. 2010, Tukmechi et al. 2010, Huang et al. 2014) propolis. The identified flavanone, pinostrobin, has therapeutic uses (Sukardiman et al., Le Bail et al. 2000, Fahey & Stephenson 2002, Wu et al. 2002, Wu et al. 2011). Previous studies found pinostrobin isolated from plant material had no antifungal activities (*Trichophyton mentagrophytes* Priestley, 1917, and *T. rubrum* Sabouraud, 1911 Eurotiomycetes, Onygenales) (Ramirez et al. 2013), but displayed antibacterial activity when extracted from *A. carnica* propolis (Tukmechi et al. 2010). Similarly, pinostrobin from temperate *A. mellifera* propolis inhibited the growth of protistan parasite (*Leishmania* spp.) (Machado et al. 2007). As propolis samples from both *T. carbonaria* and *A. mellifera* contained pinostrobin, this may partially account for the inhibitory activities towards *P. larvae*. However, the superiority of *T. carbonaria* propolis may be associated with other active flavanones (Appendix A3.5, Figure A-3l), or in fact with other classes of compounds. Further studies are needed to identify and isolate the active compounds from *T. carbonaria* propolis and determine their inhibition activity.

The similarities in the propolis and brood cell LC-MS profiles support earlier findings that *T. carbonaria* incorporates propolis into its nest structures (Wille 1983).

The flavanones, for example, were identified in both nest materials. *Tetragonula carbonaria* propolis had superior inhibitory activity against bacterial and fungal bee pathogens. Resin is secreted by many plants in response to wounds and injuries, and as a result reduces colonisation by pathogens and insects (Tippet 1986, Crane 1988 Eyles et al. 2003). It is therefore assumed that the incorporation of plant resins in propolis may also contribute to inhibition of hive pathogens. The incorporation of propolis into brood cells is therefore likely to provide additional protection against brood pathogens for developing larvae and pupae. In comparison, *A. mellifera* only uses wax for nest structures (Michener 1974), which, in contrast to *A. mellifera* propolis and *T. carbonaria* brood comb, is almost devoid of antimicrobial compounds.

Chapter 5

First documented brood pathogen in a stingless bee species

5.1 Abstract

Honey bee brood can be infected by a number of diseases caused by pathogenic fungi, viruses and protozoa, with bacteria as the most contagious. It has long been speculated that *T. carbonaria* have little or no confirmed cases of brood diseases; however, this chapter reports for the first time the isolation and identification of a causal bacterial pathogen, resulting in a brood disease in managed stingless bee colonies. Pathogenicity experiments following Koch's postulates confirmed the causal bacterium, and diagnostic microbiological and molecular tools aided in its isolation and identification. The causative pathogen; *Lysinibacillus sphaericus* (Ahmed *et al.* 2007, comb. nov.) synonym *Bacillus sphaericus* (Meyer and Neide, 1904), (Firmicutes, Bacillaceae), was isolated from worker and queen larvae, brood cell provisions and honey stores of *T. carbonaria*. The bacterium's mode of action was investigated for the presence of toxin genes and toxin producing crystals within spores. Studies to explore disease management of infected colonies were conducted. Detailed descriptions of symptoms of infected *T. carbonaria* brood are provided.

5.2 Introduction

There are three key determinants required for an infectious disease to occur: a pathogen, its transmission and susceptible hosts (Brachman 1996). In addition, favourable biological, chemical and physical conditions for disease development are required (Nelson 1994); for example, ambient temperature, humidity, and water availability within the host's environment (Timmreck 2002). In order to understand the role that microorganisms play in disease development, Robert Koch and Friedrich Loeffler developed guidelines in 1876 (Henle-Koch Postulates, based on earlier concepts described by Jakob Henle, hereafter referred to as Koch's Postulates), to establish the causative relationship between an organism and a disease (Fredricks & Relman 1996). The four guidelines introduced at the Tenth International Congress of Medicine in Berlin 1890 (Koch 1876, Evans 1976) are:

(i) The parasite occurs in every case of the disease in question under circumstances which can account for the pathological changes and clinical course of the disease.

(ii) The parasite occurs in no other disease as a fortuitous and non-pathogenic parasite.

(iii) After being fully isolated from the body and repeatedly grown in pure culture, the parasite can induce the disease anew.

(iv) The organism should be re-isolated from the experimentally infected subject (postulate added after Loeffler) (Walker et al. 2006).

These postulates were not presented as rigid criteria, and concepts of causation are limited by the technologies available at the time (Evans 1976). For example, there have been more recent contributions, particularly with the development of molecular techniques (Falkow 1988, Fredricks & Relman 1996, Falkow 2004).

Similar to other eusocial bees, stingless bee hives possess factors suitable for disease establishment. First, there is a continuous supply of susceptible hosts of appropriate age (as hives have overlapping generations), a store of nutrient-rich materials including honey, pollen, a high density of adults and brood, as well as a social structure which facilitates interaction between colony members for transmission of a pathogen (Keane & Kerr 1995). Second, there is a favourable environment; moisture, temperature and humidity conditions (Keane & Kerr 1995), which may be more constant to sustain pathogen development. However, for a potential disease to establish, a virulent pathogen is also required. There are a number of contagious diseases of honey bees caused by fungi, viruses and protozoa, but the most important brood diseases are caused by bacteria. Pathogenic bacteria possess a number of mechanisms for pathogenicity and overcoming host defences, including methods of adhesion and invasion of host surfaces and tissues, capsule formation, and toxin production (Finlay & Falkow 1997, Wilson et al. 2002). While some bacteria can be pathogenic without toxin production, e.g. through extensive growth, disintegration and septicaemia, other bacteria rely upon the production of toxins for overcoming host defences. Many bacterial pathogens form endospore crystals which contain toxins that impact host cells (de Maagd et al. 2003, Ibrahim et al. 2010). The release of toxins into the host can make the host cell membrane porous, aiding in cellular degradation and the rapid production of bacterial spores (Bravo et al. 2007). There are a number of bacterial toxins identified to be efficient against insect hosts, e.g.,

insecticidal toxin complexes (Tc-toxin), Cytolictic (Cyt) and Crystal (Cry) toxins, and binary toxins (BinAB and PirAB) (Schmitt et al. 1999, Vallet-Gely et al. 2008).

The honey bee pathogen, American foulbrood (*Paenibacillus larvae*), does not produce endospore crystals. However, three novel toxins have been identified in two different genotypes, ERIC I and ERIC II (Poppinga & Genersch 2015). Firstly, two binary AB-toxins (Plx1and Plx2) specifically for genotype ERIC I (Fünfhaus et al. 2013), are the only toxins proven to date to play a role in honey bee pathogenicity (Poppinga & Genersch 2015). Binary toxins consists of two subunits; component A aids in enzymatic activity to produce pores in the host membrane, while component B binds to the host cell surface and transports the toxin to the larval cytoplasm (Fünfhaus et al. 2013, Djukic et al. 2014). Plx1 and Plx2 are suggested to contribute to the destruction of the larval cytoskeleton, resulting in the shedding of dead tissue (Fünfhaus et al. 2013). Secondly, C3larvin, a mono-ADP-ribosyltransferase (mART) toxin, has cellular targeting and enzymatic activity, and was identified in both ERIC I and ERIC II genotypes (Krska et al. 2015, Poppinga & Genersch 2015). Thirdly, S-layer protein (SplA), is expressed only in ERIC II (Fünfhaus & Genersch 2012), however the role SplA plays in pathogenicity is unknown. The browning and degradation of honey bee larvae may also result from the secretion of extracellular proteases produced during the vegetative stage of the bacterium (Holst & Sturtevant 1940, Dancer & Chantawannakul 1997, Genersch 2010).

To date, there have been limited reports of brood diseases in stingless bee nests (Chapter 1). In fact, the premise on which this thesis was initially based was that there were no documented brood diseases in stingless bees. The preceding thesis chapters describe studies to determine the factors that may contribute to the apparent absence of brood diseases in Australian colonies of *T. carbonaria*. These include the lack of narrow temperature thermoregulation in the brood, hygienic behaviour, and antimicrobial activity of nest materials. However, during the course of this research, in December 2012, I observed symptoms consistent with brood disease in one *T. carbonaria* colony. The colony had substantially fewer workers than other hives, as well as changes in the structure of the brood area. Closer inspection of the brood area

revealed dead and decomposing larvae, discolouration of brood provisions, and an odour of ammonia.

Therefore, the aim of the work described in this chapter was to document the first apparent brood disease discovered in a stingless bee colony. My hypotheses were:

1) The disease is caused by a pathogen.

2) If a pathogen is responsible, it is different from that recorded in honey bees.

3) Hive management strategies could assist in controlling the disease.

These were tested by initially following standard protocols to utilise Koch's postulates to confirm presence of a causal organism. Then using microbiological and molecular diagnostic tools for the identification of the pathogen, its mode of action and its pathogenicity were investigated; as well as the undertaking of several disease management studies using extracts of hive products and the manipulation of hives using sanitary techniques and supplementary feeding.

5.3 Materials and methods

5.3.1 *Tetragonula carbonaria* hives

In December 2012, a single field-maintained *T. carbonaria* hive (UWS apiary) which had previously been used for nest thermoregulation monitoring over 13 months, but had not been involved in other investigations, was observed showing symptoms of apparent brood disease.

A further three healthy *T. carbonaria* colonies were used for the Koch's Postulate experiments as outlined below. All four colonies were originally sourced from S.E. QLD (Australian Stingless Native Bees, Hatton Vale, QLD), but had subsequently experienced two years of environmental conditions at the UWS apiary site. When the colonies initially arrived at UWS, they appeared healthy and strong as indicated by

large volumes of internal stores, worker populations, and brood chamber volume, and remained healthy until the presentation of symptoms in one of them.

Throughout the investigations on the putative disease, disposal of any contaminated stingless bee material (viz., colonies, diseased brood, and hive material), as well as treatment of used field and laboratory equipment, complied with protocols outlined in the *NSW Apiaries Act 1985 No. 16* and The Australian Honey Bee Industry Biosecurity Code of Practice (Australian Honey Bee Industry Council 2014) for brood diseases.

5.3.2 Observations of symptoms

Examination of the affected hive included colour, shape, texture of storage pots, involucrum appearance and coverage, size and formation of brood nest, strength of the internal worker population and entrance activity, colony odour and in-hive worker behaviour.

5.3.3 Isolation of a possible causative organism(s)

Isolation and storage of samples for DNA analysis

Upon opening the hive, all visible *T. carbonaria* larvae showing discolouration or fluid appearance were separately placed in approximately equal numbers into 1.5 mL sterile Eppendorf tubes which were either dry or contained 70% v/v ethanol, and retained for future microbiological and molecular investigations. The remaining brood with abnormally flattened caps, cell structure or colour, was removed by gently breaking it off and placing it into dry sterile 50 mL centrifuge tubes (Sigma-Aldrich, Castle Hill, NSW, Australia). The remaining contents of the hive (brood discs, storage pots and involucrum) were separated out and stored dry or in 70% v/v ethanol and placed in either -20ºC or -80ºC, for short- or long-term storage, respectively. The empty colony boxes were prepared for biohazardous material incineration.

Isolation for culturing

The stingless bee samples were processed under aseptic conditions, similar to that reported for diagnosis of *P. larvae*-infected honey bee larvae (Alippi 1999, de Graaf et al. 2013, World Organisation for Animal Health 2013). Five symptomatic *T. carbonaria* larvae were placed together in 5 mL of sterile water in a 10 mL centrifuge tube and heat shocked for 10 min at 80ºC (see Chapter 4, section 4.2.3). The heated suspension was diluted 1:10 and 1:100 with distilled water and samples of each suspension lawn plated onto 4 Petri dishes containing SBA with the antibiotic nalidixic acid (3 mg/ mL), and incubated for 24–48 h at 37ºC in 5% CO_2. A loopful of liquid from symptomatic brood cells was also streak plated onto separate Petri dishes containing various media: 4 SBA plates containing nalidixic acid (3 mg/ mL), 4 PDA plates, and 4 SDA plates, and incubated as above. After incubation, discrete colonies were only observed on all SBA plates, with little difference in bacterial growth between lawn and streak plated SBA (all other media had no colony growth). To produce pure cultures, four isolated colonies were sampled from SBA plates, and re-isolated onto fresh SBA plates, these cultures were subsequently maintained on nutrient agar (NA) at 37ºC in 5% CO_2.

5.3.4 Testing pathogenicity of the isolated bacterium

Based on Koch's postulates, experiments were undertaken to investigate the pathogenicity of the isolated bacterium against *T. carbonaria* brood. In addition, bacteria were examined under transmission electron microscopy (TEM) in order to determine presence of toxin crystals in spores, and also subjected to probing of the bacterial isolate for the presence of toxin genes.

In an initial pathogenicity experiment, a single brood disc with newly laid eggs (1–2 d old) was removed from a healthy field colony not previously used in experiments. The brood disc was placed in a single 1.5 L, 155 × 150 × 80 mm clear plastic container (Klip It™, Sistema Plastics Limited, Auckland, New Zealand), along with approximately 80 workers and honey and pollen pots. The container with brood and workers was left for 24 h to allow the workers to adjust to their surroundings and to

repair any damaged brood cells. After 24 h, the brood disc was partitioned into four sections each containing approximately 50 cells (without actual separation), and the partitions were subjected to one of four treatments:

1. Untreated (control 1).
2. All brood caps perforated, with a single sterile needle syringe that was originally sterile (control 2).
3. All caps perforated with the needle syringe (as per point 2) and injected with 20 µL sterile water only (control 3).
4. All caps perforated with the needle syringe (as per point 2) and 20 µL of bacterial suspension injected into each cell, comprising approximately 10^6 colony forming units (CFU) per mL (calculated using a haemocytometer see Chapter 4, section 4.2.4). This suspension was created by aseptically removing a loopful of isolated bacterial colony from the bacterial culture maintained on NA media, and placing it into a sterilised 1.5 mL Eppendorf tube containing 1 mL of sterile water. The suspension was gently mixed by inverting the tube.

After treatment, the container containing the treated brood disc was placed into an incubator at 27–29ºC (Chapter 2, section 2.1.1).

It was then observed that workers resealed cells treated with water within 24 h (Control 3); while all the bacterium-inoculated cells remained unsealed. Development of clinical symptoms was observed in a few larvae at 17 days after treatment (DAT), as well as in 12% of the untreated control cells. The bacterium was successfully re-isolated from all infected material (treatment and control); this, however, suggests that cross-contamination may have occurred. A subsequent experiment was designed to reflect normal hive conditions by using colonies of *T. carbonaria* maintained in boxes in the bee shed. Testing a complete nest allowed for a colony-level response to infection and observation of worker behaviours in normal nest conditions.

Before sunrise on 22 October 2013, two strong hives with honey-supers attached (i.e., colonies with an additional hive box attached to the top and used for collection of honey), of equal strength with regard to honey and pollen provisions, brood and worker populations, and which had not been previously used in experiments, were selected. The two hives were weighed at the start of the experiment and again at the conclusion of the experiment, as four halves. Estimated brood population (EBP) (Chapter 3, section 3.2) was also calculated at the start and conclusion of the experiment. The two hives were opened and split using standard stingless bee colony-splitting techniques (Chapter 2, section 2.1.4). The first hive split into two halves was used as the uninoculated control. Upon splitting the hive, the brood area, including any damaged cells from the split, and the storage pots (intact or damaged) were exposed, which allowed for easy application of treatments. Both halves were treated with a total of 105 mL of sterile water. Water was applied first using a 10 mL sterile plastic syringe to dispense the water directly into all opened brood cells and any opened storage pots, after which any remaining water was uniformly applied using a 500 mL sterile plastic hand sprayer with fine spray nozzle (Helena Products, Punchbowl, NSW, Australia) over the entire brood chamber, storage pots and nest structures. A 3 mm thick acrylic lid was then placed onto the open side of the lower half split hive and sealed with 48 mm wide masking tape (3M™, Maplewood, Minnesota, USA). This facilitated in-hive observations. The upper half split hive did not allow for daily observations because of the box design; however, it was possible to make observations when the hive was opened during the early morning inspections. Both box halves were placed in the bee shed at 26ºC.

The second hive was split in the same way. A highly concentrated spore suspension (total 105 mL) containing approximately 400 million CFUs of the bacterium, was applied similarly to the sterile water in the control hive. Again, acrylic lids were placed and sealed onto the surface of the lower half. Then both halves were placed in the bee shed at 26ºC on a different bench to the control halves.

In addition, OPs (Chapter 2 section 2.1.1) were attached to all four halves of the two hives to enable observation of debris removal (Figure 5.1). The colonies were not

allowed to forage during the investigation, to reduce possible environmental contamination with the disease; however, in-hive stores were plentiful.

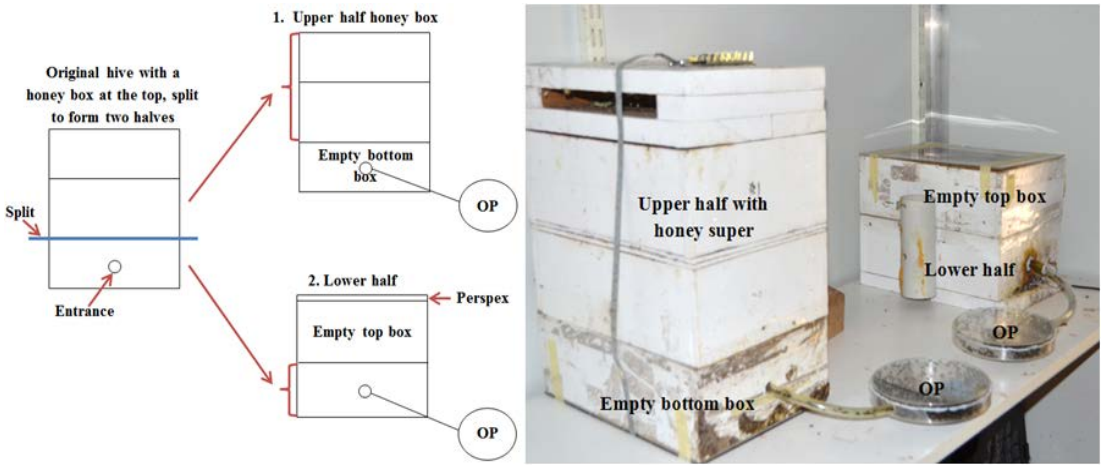

Figure 5.1 Honey super hive after splitting to form two halves used for control treatments. The bottom lower half had an empty box attached to the top and a acrylic lid was attached to the top to allow for viewing in-hive activity. The upper half of the hive, still containing the hive super was placed on top of an empty box. OPs were attached to both entrances and allowed for viewing of entrance activity. The hives could not interact with each other and had no access to the external environment.

Monitoring of worker behaviour

Worker behaviour was monitored daily from the start of the experiment in both the lower halves of inoculated and control hives. Observations were made until the first pathogenic symptoms appeared; thereafter, the colonies were observed weekly until colony death.

Hive opening for sample collection

The pathogenic symptoms of disease (i.e., the presence of brown larvae) were first observed through the acrylic lid at 22 DAT in the inoculated lower hive half. To prevent any cross-contamination during examination and sample collection, the following measures were taken. Nitrite gloves were used throughout the inspection and removed and replaced between samples, and separate tweezers were used to

collect samples from control and inoculated halves. All equipment was bleached and autoclaved after use.

Control hives were opened first and placed on a large white cotton sheet on the ground in an open area near the shed between 04.00–05.00 am AEDT, before sunrise, in November 2013. This procedure aimed to reduce worker loss and prevent dispersal of contaminated workers into the environment. Any workers that had escaped were easily seen on the white sheet and were collected, killed and removed. Samples of brood, callows, adult workers and honey were collected and placed in sterile 1.5 mL Eppendorf tubes or 50 mL centrifuge tubes and were taken to the laboratory and stored at -20 ºC. The halves were placed back into the shed, to continue the experiment.

The inoculated lower hive half, which showed pathogenic symptoms of disease, was removed from the bee shed after examination of control halves. Samples of brood, callows, adult workers and honey were collected for isolation and characterisation of the putative pathogen. The inoculated upper half was also removed and treated the same as the lower half; samples were also collected. The samples were placed in sterile 1.5 mL Eppendorf tubes or 50 mL centrifuge tubes and were taken to the laboratory and stored at -20 ºC until used for isolation and culturing of the putative pathogen, as previously described (see section 5.2).

5.3.5 Microbiological, biochemical, and microscopic identification

All samples collected from the symptomatic hives were used for culturing. Only bacterial colonies were isolated from the diseased hive samples, and were maintained on NA media as previously described (section 5.2). Diagnostic microbiological tests were performed on the cultured colonies as well as on fresh samples collected from hives. The freshly sampled brood cells appeared to be filled with a brown fluid and did not contain larvae with normal body features. A sterile loopful of fluid from a contaminated larval cell, as well as one from previously cultured, isolated bacterial colonies were collected, smeared onto separate microscope slides and Gram stained

(Fluka Analytical, Sigma-Aldrich, Buchs, Switzerland), as previously described (Chapter 4, section 4.2). The dried Gram stained smears were viewed at 1000× magnification with oil immersion using a compound microscope (Nikon Eclipse E200, Nikon Corporation, Tokyo, Japan). For identification of vegetative and reproductive growth including sporulation, wet mounts were prepared by collecting a loopful of a pure, representative bacterial colony, immersed into sterile water and examined using a compound microscope.

Samples were also subjected to biochemical profiling via catalase testing (Reiner 2010), which is commonly used for identification of *P. larvae* (World Organisation for Animal Health 2013). Isolated colonies of the cultured bacterium were scraped off the NA medium using a sterilised inoculation loop and placed as a mass on a clean microscope slide. To test for catalase reaction, 500 µL of a 3% v/v hydrogen peroxide solution (Gold Cross Biotech Pharmaceuticals Pty. Ltd., Laverton, Victoria) was dispensed onto the bacterial samples whilst viewing under an illuminated stereomicroscope (Leica Zoom 2000, model Z454, Leica Microsystems Pty., Ltd., Wetzlar, Germany).

As well as the work conducted at UWS, samples were sent to the State Veterinary Diagnostic Laboratory of the Elizabeth Macarthur Agricultural Institute (EMAI), NSW Department of Primary Industries, that routinely conducts diagnostic work for honey bee and other animal pathogens, for isolation and identification of bacterial organisms.

5.3.6 Molecular characterisation

In addition to the morphological and biochemical investigations, molecular techniques were utilised for further identification of the putative pathogen. Pure culture isolates (Table 5.1) reisolated from inoculated hives in both pathogenicity experiments, were used for bacterial DNA extraction using the Isolate II Genomic DNA Kit (Bioline, London, UK), following the steps for isolating DNA from cultured bacteria as previously described in Chapter 4, section 4.2.2. PCR reactions

(made up to 20 µL) used components from the Velocity™ PCR Kit (Bioline, London, UK), and universal eubacterial 16S rDNA primers 530F and 1495R. DNA amplification was performed on the Bio-Rad Dyad Peltier Thermal Cycler (ALS1296, Bio-Rad Laboratories, California, USA). After amplification and visualisation, molecular cloning using pGEM®–T Easy Vector System I (Promega, Madison, Wisconsin, USA) (Chapter 4, section 4.2.2) was performed, and colony PCR visualised on 0.8% agarose gel.

PCR products were cleaned to remove unused primers and dNTPs by treating with 2 µL of exoTSAP mixture. ExoTSAP mixtures were produced by mixing 0.025 µL of Exonuclease I (20 units/µL) (New England Biolabs Inc., Massachusetts, USA), 0.25 µL of Thermosensitive alkaline phosphatase (1 unit/µL) (Promega, Madison, Wisconsin, USA), and 1.725 µL Mili-Q water. PCR products treated with exoTSAP (37ºC for 30 min, then 95ºC for 5 min) were sent to Macrogen (Macrogen Inc., Seoul, Korea) for sequencing (Table 5.1).

Table 5.1 Number of DNA extracts obtained from single, isolated bacterial colonies previously cultured from the inoculated *T. carbonaria* colonies. Number of PCR products produced, clones transformed and sequences analysed are provided.

Source	No. of DNA extracts	No. of sequences used in phylogenetic analysis
Worker larvae[1]	4	1
Queen larvae[2]	1	1
Queen larvae[2]	1	1
Worker cell provision[1]	3	1
Worker cell provision[1]	2	1
Queen cell provision[2]	2	1
Honey[2]	1	2

[1] Samples from initial pathogenicity experiment, [2] Samples from second pathogenicity experiment

Further characterisation of the cultured bacterial strain utilised multilocus sequence typing (MLST) and results were compared with other known strains, based on the allelic profiles. Protocols were followed using six chromosomally encoded

housekeeping genes: *adk* (*adenylate kinase*), *ccpA* (*catabolite control protein A*), *pycA* (*pyruvate carboxylase*), *glyA* (*serine hydroxylmethyl transferase*), *glcK* (*glucose 6-phosphate kinase*) and *glpF* (*glycerol uptake facilitator protein*) (Ge et al. 2011) (Table 5.2). DNA was amplified on a Bio-Rad Dyad Peltier Thermal Cycler using the Velocity™ PCR Kit. Volumes up to 45 µL were run on a 2% agarose gel for 85 min at 70 V and visualised on a Gel Doc 2000 / ER. Amplified bands of expected length were excised from the agarose gel. Extraction of DNA and purification of PCR products from the gel slices were carried out using Wizard® SV gel and PCR clean-up systems (Promega, Madison, Wisconsin, USA). PCR products were purified using ExoTSAP, and sent to Macrogen Korea (Macrogen Inc., Seoul, Korea) for sequencing (Table 5.3).

Table 5.2 Six MLST primers (designed by Ge et al. 2011) used in this study for further characterisation of bacterial isolates.

Primer	Forward 5'to 3'	Reverse 5'to 3'
adk	CGATATGTTCCGTGCTGCTA	GCTGCGGCTGCTCTGTAAT
ccpA	ATTATTTTWGCCAACTCAGA	GTAATGCAACACTTGTTWGY
pycA	TTCCGTATTTTCGATAGCTTG	ACATAGATTTCAGAGTGTGGGC
glyA	TCGATTATGAGGATGTACG	AAGGGATTGTGTTTTTGTT
glcK	ACAAAATTAGCMACRGCTTTATTCA	AACCTCCGCCTAATACGATGC
glpF	CTAACCATTGCCCTTGCCAC	CCACCAATAATGGGACCTACAAC

Table 5.3 Number of PCR products produced and sequenced from one DNA extract of bacterial colony isolated from symptomatic *T. carbonaria* queen larva. DNA was amplified using the six MLST primers, and four loci were sequenced.

MLST gene	No. of PCR products	No. sequenced	No. of sequences used in phylogenetic analysis
adk	8	4	1
ccpA	2	-	-
pycA	6	4	1
glyA	4	2	multiple peaks
glcK	8	4	1
glpF	8	4	1

Phylogenetic analyses

DNA sequences were trimmed and edited in Sequencher 4.0 (Gene Codes Corp). Sequence alignment utilised the MUSCLE algorithm within Mega 5 (Tamura et al.

2011). Furthermore, four of the seven MLST genes (*adk*, *glcK*, *glpF*, *pycA*) were of high quality for phylogenetic analysis compared to previously published sequence types (ST) ST-2 to ST-9 and ST-17 (Ge et al. 2011). Sequences were aligned and then concatenated, andthe average evolutionary divergence (p-distance) across all sequence pairs was determined. A neighbour-joining phylogenetic tree, based on the Tamura 3-parameter using a discrete Gamma distribution (T92+G), was produced with the inclusion of *B. subtilis* as an outgroup (Ge et al. 2011).

5.3.7 Identification of possible toxin(s) in the isolated bacterium

Production of toxins is one possible explanation for pathogenicity in a number of bacterial pathogens; many of these toxins are produced within a bacterial spore from a crystal inclusion and released into the host cell (de Maagd et al. 2003). In order to investigate whether the isolated bacterium produced toxins, the culture was examined using TEM for possible crystal inclusions, and presence of known toxin genes was also explored using molecular techniques.

Examination of isolated cultures under Transmission Electron Microscopy

Sporulating bacterial cultures of the isolated bacterium and of *P. larvae* were sent to Dr Mukesh Srivastava, Elizabeth Macarthur Agricultural Institute, NSW Department of Primary Industries, for examination under TEM for visual identification of any crystal formations within the spores.

Isolated bacterial samples were prepared for TEM processing as described below (Dr Mukesh Srivastava, pers. comm. 2013). Samples were dissolved in sterilised water and spun at 1500 rpm for 3 min. The supernatant was discarded, and the pellet was re-dissolved in sterile water and centrifuged for a further 3 min; after which the supernatant was removed and the pellet was dissolved in 10% w/v calf serum and centrifuged for 3 min at 1500 rpm. The calf serum was carefully removed without disturbing the pellet. Karnovsky's fixative was slowly added until the specimen was covered by 2 cm of liquid. The sample was stored in a refrigerator (4ºC) for 4–12 h.

The pellet was removed and cut into 1 mm^2 pieces and once the liquid was removed, 2% osmium tetroxide was added for 4 h, after which the sample was washed 3 times for 5 min each in sterilised water. Once washed, 2% uranyl acetate was added and the sample solution was left for 1 h. The specimen was dehydrated in an ethanol series from 50% to 100% for 15–20 min per step, and then finally transferred to dry acetone. The specimen was subsequently transferred to a 50 % Spurr's resin/acetone mixture (Spurr 1969) for 1 h, then transferred and embedded into 100% resin and incubated overnight at 70°C. The following day, the embedded specimen was cut into 70 nm sections, placed on a copper grid (300 mesh) and stained with uranyl acetate and lead citrate for examination under a Philips EM 208 TEM (FEI Australia, ACT, http://www.fei.com/products/tem/tecnai).

To determine the presence of toxin crystal formation in bacterial spores, the TEM scan images were also sent to Dr Colin Berry (Cardiff School of Biosciences, Cardiff University), Dr Jean-François Charles (Department Manager and Image Reprographics, Pasteur Institute) and Associate Professor Hyun-Woo Park (Department of Natural and Mathematical Sciences, California Baptist University), all of whom had previously published work on toxin crystals in entomopathogenic bacteria.

Molecular identification of possible toxin genes in the cultured bacterium

Samples of the isolated bacterial culture were tested, using protocols developed by Ge et al. (2011) which were used to identify toxic and non-toxic bacterial strains, based on the occurrence of toxin genes in isolated bacteria. PCRs were undertaken with primers designed by Ge et al. (2011) for seven toxin genes (Table 5.4). PCR amplicons were visualised on a 2% agarose gel, and PCR products were direct sequenced (without cloning) as previously described.

Table 5.4 Seven primers (designed by Ge et al. 2011) used in this study for identification of possible toxin genes in isolated bacterial samples.

Primer	Forward 5'to 3'	Reverse 5'to 3'
binA	CACTTCCAGAAAACGAGCAATAC	CACTTCCAGAAAACGAGCAATAC
binB	CACTTCCAGAAAACGAGCAATAC	CTGAGTGGTCGTTTGGGATA
mtx1	TGGAACATCAAATACGATAGCA	CCCAAGCCAATGAATAGTTAGG
mtx2	CCCAAGCCAATGAATAGTTAGG	AAATCTGCCCCATGAATTAAGTTA
mtx3	CGAAATGATACCGATAGGGATC	AATCAGGGTTATTGACACTTCTTG
cry48Aa	GTGCTTCCACMAACTTTCAATCAT	TCTTCTTCGGTTAGTAATCGCTCTT
cry49Aa	TACTTTCGCTACTGTCTGCT	AATCCATTTCCTTACGGTCT

5.3.8 Pathological symptoms

A description of hive symptoms to be used in the field for diagnosis of possible stingless bee brood infection was developed (Table 5.5). The following key colony parameters were observed and described: structural formation of the brood, the appearance of brood cells, brood contents and larvae, appearance of pollen and honey stores, hive odour, and vitality of adult workers.

5.3.9 Practices to assist colony recovery from brood infection

Hive manipulation for colony recovery

While pathogenicity experiments were being conducted, three further *T. carbonaria* colonies (labelled A, B, and C) showed symptoms of the disease. This provided the opportunity to investigate hive management practices to assist in colony recovery.

Upon opening these hives, all workers that crawled out were observed to be lethargic. They were easily captured and killed with a synthetic pyrethroid aerosol insecticide and hygienically disposed of. Hives A and B had a population of adult workers, callows and pupating brood similar to a healthy colony, whereas in hive C there was a substantially reduced brood and adult population. As there were newly laid eggs in brood cells in all three hives, it was assumed that a queen was still present and active. The following management practices were applied:

Hive A: All infected material was removed, the hive was resealed and left to recover.

This hive was located in the field; conditions were warm and foraging sources were plentiful (spring conditions), and bees were allowed to forage. Hive entrance activity was monitored weekly; however, hives were not regularly opened for internal observation, to minimise nest damage. In the event of decreased entrance activity, however, the nest was opened for inspection. The removed infected brood sections were stored dry at -20ºC, which also occurred for brood removed from hives B and C. No pollen or honey was removed from the three hives.

Hive B: Infected material was removed, the hive was resealed and placed inside the bee shed at 26°C, and an OP and external honey and pollen feeder were attached to provide supplementary feeding, although the bees were also allowed to forage.

The hive was observed weekly for signs of recovery; such as preparation and provisioning of brood cells with newly laid eggs, complete development of larvae to adult, continual increase in population numbers, and the replenishing of hive stores. Alternatively, signs of hive deterioration were also noted; included eggs not developing, decreased volume of the brood, larvae not developing but showing symptoms of infection, and a reduction in adult population with a lack of foraging workers.

Hive C: Infected material was removed, but two discs of apparently healthy larval cells (determined by bright yellow-orange colour, oval shaped cells with round caps, and lack of odour) were removed from a healthy colony with no disease and placed into the symptomatic hive. The hive was resealed and placed inside the bee shed at 26°C, an OP was attached, and the bees were also allowed to forage. The hive was observed weekly for signs of recovery, as for Hive B.

Activity of T. carbonaria *and* A. mellifera *hive products against the isolated brood bacterium*

The antimicrobial activity of *T. carbonaria* and *A. mellifera* nest components (pollen extracts, honey, propolis extracts and *Weissella hellenica* (Chapter 4) was tested against the newly isolated *T. carbonaria* brood pathogen, similar to the work reported for honey bee pathogens in the previous chapter. This was to determine if the relative activities of these extracts were similar against this novel putative pathogen. Disc-diffusion assays were performed following the same protocol and treatments, with tetracycline hydrochloride used as a positive control (20 µg/mL) as outlined in Chapter 4, section 4.2.4. However, there was one modification; the bacterial spore suspension was made from 48-h-old cultures and 300 µL (approximately 1.92 x 10^6 CFU) of the spore suspension was lawn plated onto NA plates.

Statistical analysis

Statistical analyses of data were performed using IBM SPSS ver. 22 for Windows (IBM Corp. 2013). Data recorded during disc diffusion assays were tested for homogeneity of variance using Levene's test. Differences among the activity of the bee products as measured by the zone of bacterial inhibition were analysed using one-way ANOVA, followed by Tukey's HSD post-hoc test.

5.4 Results

5.4.1 Description of symptoms

The field discovery of a potential diseased hive (December 2012), and the symptoms observed are documented below (Table 5.5).

The hive was initially noted because of its reduced forager activity on an ideal foraging summer day (warm and sunny), low numbers of internal hive workers, and lethargic behaviour of remaining workers. Upon opening the hive, the brood structure was observed to be non-uniform, scattered and with few brood cells,

compared to the normal characteristic spiral formation (Figure 5.2). The nest stores (pollen and honey), however, were plentiful.

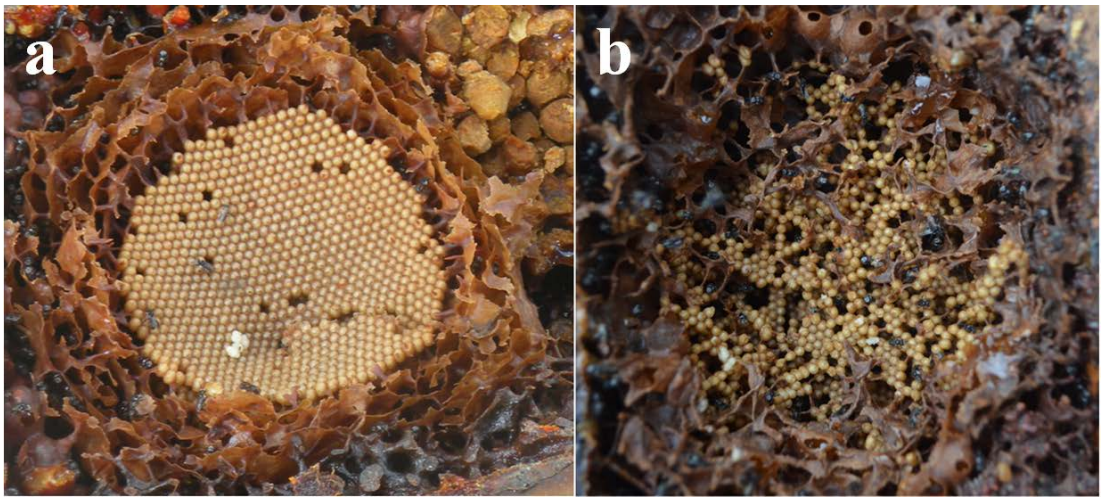

Figure 5.2 Comparison of *T. carbonaria* brood discs. a) Typical healthy nest showing a central circular disc with good structural involucrum surrounding the brood chamber. b) Unhealthy nest, the brood cells are scattered, forming no clear disc structure, and little structural involucrum is present.

Appearance of larvae

Developing larvae of the diseased hive changed in appearance, colour and texture. Many infected larvae had been removed from their cells, presumably by workers, and were located singly or in small groups around the hive, but mainly on the involucrum. These larvae were either half white and half brown (with the posterior end brown) or entirely dark brown to black, at a late stage of disease development (Figure 5.3).

Figure 5.3 Unhealthy *T. carbonaria* larvae displaying varying colour symptoms, depending on stage of disease development. Larvae were occasionally deposited into small groups once they were removed from their brood cells by workers.

Appearance of brood cells

Brood cells were whole and intact, though sometimes with flattened caps. Some cells appeared normal in colour (bright yellow-orange), whereas others appeared brownish. Using this colour differentiation, darker cells were selected and their caps carefully removed. These cells consistently contained discoloured larvae (Figure 5.4).

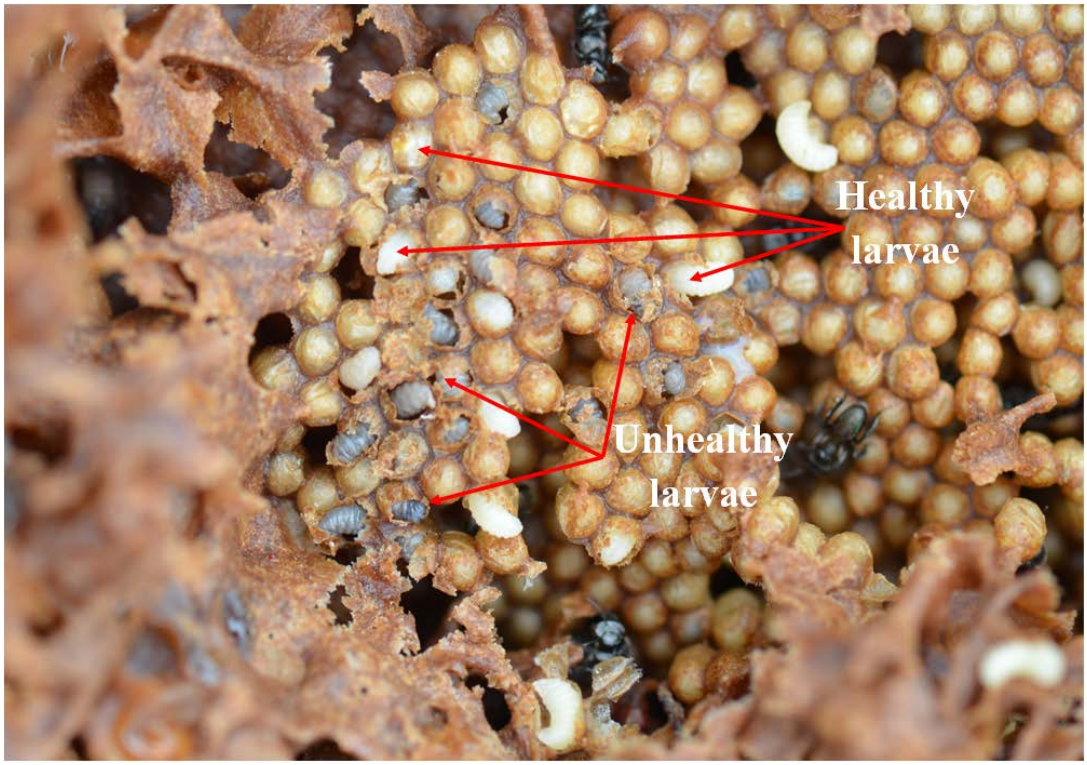

Figure 5.4 Brood with cell caps removed. Darker coloured cell caps contained greyish to brown discoloured larvae; light coloured cells contained white larvae.

Subsequently, the “match-stick test”, commonly used for field diagnosis of AFB in honey bees, and which produces a brown rope-like mass from infected larvae on the stick (Chapter 1, Figure 1.6) (World Organisation for Animal Health 2013), was performed on larval cells containing the brown thick fluid (Figure 5.5). The match-stick test was positive, in that it formed a short ropy mass that connected the brown fluid-filled cell contents to the stick.

Other signs included a prominent ammonia smell in infected brood cells, cell provisions were a greenish-yellow (Figure 5.6), and it appeared that the queen was absent as no newly laid eggs were observed.

Figure 5.5 A brown thick fluid was observed in many unhealthy cells. The cell contents at this stage showed no normal larval morphological features.

Figure 5.6 Greenish-yellow coloured, thick fluid cell provisions of an infected hive.

Table 5.5 Comparison of healthy and unhealthy nest characteristics.

	Healthy	Unhealthy
Colony strength	Large adult population, with strong entrance activity of foragers and cleaners	Adult population reduced, little entrance activity
Odour	Strong smell of plant resins	Pungent, decaying, rotten smell, ammonia smell may be detected
Storage pots	Oval, bright orange brownish, thin, with smooth appearance. Glossy, fermenting honey and fresh, moist pollen	Oval, darker brown, thick and tough appearance. Honey and pollen may be present but do not appear to be newly collected
Involucrum	Orange, soft and malleable, smooth lines and finishes, strong network in nest and multiple (4+) thin layers covering brood chamber	Dark brown, tough and thick network, dry and brittle in advanced stages; may cover the brood chamber but be thick and limited to 1–2 layers
Brood chamber	Large, characteristic spiral formation, with leading edge of newly laid eggs, fresh healthy colour of newly made cerumen	Variety of sizes (depending on infection stage), may lack spiral formation, cells are scattered and may lack leading edge (absence of queen), lack of developing pupae, overall colour variable
Brood cell exterior	Oval, fully formed swollen caps, soft texture, bright yellow-orange colour	Oval or irregular oval with flattened caps, thick dark orange
Cell provision	Smooth, glossy, yellow to orange	Thick, dark yellow to green
Larvae	Glossy, white, soft, solid mass	Half to full brown to black in colour, fluid-like or dry, may form short rope with matchstick test, may be dumped singly or in small groups on nest structures
Worker behaviour	Actively moving (flying, walking, preening), performing hive tasks, will defend nest if opened	Motionless, lethargic, will walk/crawl out of nest, will not aggressively defend nest if opened

5.4.2 Isolation of a possible causative organism(s)

Sheep blood agar plates were dominated by one bacterial species (Figure 5.7). Morphological and biochemical diagnostic tools aided in identification of the bacterium to the order Bacillales (Firmicutes, Bacilli). Table 5.6 shows the comparison of the isolated bacterium from *T. carbonaria* with the honey bee pathogen, *Paenibacillus larvae* (Figure 5.8).

Table 5.6 The comparison of key identifying characters of the bacteria isolated from *T. carbonaria* compared with *Paenibacillus larvae* from *A. mellifera.*

	Isolated bacteria	**American Foulbrood (*Paenibacillus larvae*)[1]**
Time (h) until colony formation	24	48 – 96
Colony morphology at 24 h	0.5–5 mm diam. irregular colonies with lobed edge, flat, opaque and glossy, creamy yellow in colour	1–4 mm diam. irregular colonies with a glossy, butyrous appearance, flat, grey in colour
Gram stain	Gram-positive, terminal spore-forming rods, does not form whips on sporulation	Gram-positive, terminal spore-forming rods, forms whips on sporulation
Catalase test	Positive	Negative
Crystal formation and toxin production	Possible crystal inclusions in spores, inconclusive toxin production	No spore crystal inclusions, production of Plx1 and Plx2[2] toxins

[1]Hornitzky & Clark 1991, Rieg et al. 2010, World Organisation for Animal Health 2013, [2]Fünfhaus et al. 2013.

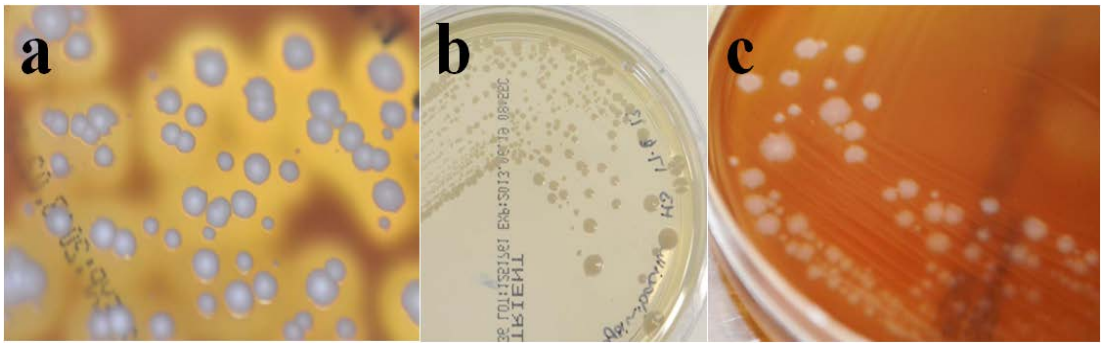

Figure 5.7 Growth of bacteria isolated from diseased *T. carbonaria* brood on a) sheep blood agar and b) nutrient agar compared with c) *P. larvae* on sheep blood agar plates.

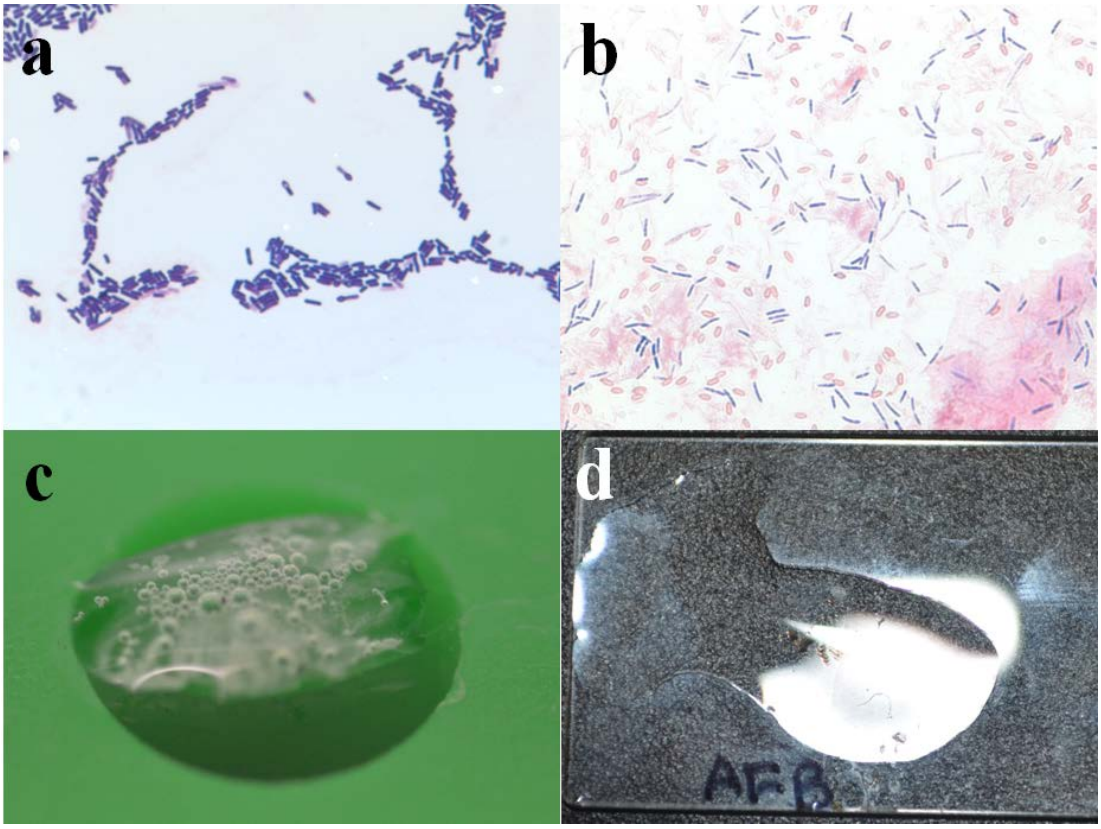

Figure 5.8 Microbiological examination of isolated bacteria compared to *P. larvae*. Gram stain results for a) *T. carbonaria* bacteria (1000× oil) and b) *P. larvae* (1000× oil) showed that both were Gram-positive rods. Catalase reactions for c) isolated *T. carbonaria* bacteria was positive (bubbly appearance) and d) *P. larvae* was negative (no bubble formation).

5.4.3 Testing pathogenicity

The first symptoms of disease in the inoculated hive halves in the Koch's Postulates experiment were observed at 22 DAT. The primary pathological symptom was the presence of discoloured larvae, which had been removed from the brood area and deposited on the surrounding hive structures (Figure 5.9). After these initial recorded observations, all inoculated and control halves were left for a further 14 days to allow for disease development.

Figure 5.9 Unhealthy larvae (indicated by red circle) removed from brood cells and deposited on surrounding structures.

After a further 14 days (i.e., 36 DAT), the hives were re-opened. In the lower inoculated half, there was a distinct difference in colour and structural appearance of the involucrum and cerumen compared to a normal healthy hive. The materials were darker in colour, thick in structure, and hard to move (Figure 5.10). The normal resin aroma could not be detected, but there was a distinct ammonia-like, decaying smell, especially within the brood chamber. Overall, there was a lack of egg development and growth of pupae to adults. The brood chamber became progressively smaller over the experimental period, along with the adult population. No active queen or gyne was observed. The remaining adult workers were lethargic.

The upper half of the inoculated hive showed similar signs of infection to the lower half, such as colour differences in involucrum and cerumen, the presence of an ammonia-like, decaying smell, decreased brood chamber volume and adult population, and absence of an active queen. However, the nest stores of this inoculated half were still plentiful, due to the super box attached.

In comparison, at the start of the experiment the lower half of the control hive had developed a gyne and the overall appearance of the hive structures and materials were an orange colour and malleable. As the experiment continued and the gyne was unable to mate and the EBP decreased, the colour of the hive materials darkened and they became thick and hard in texture. However, no ammonia smell was detected. The upper half of the control hive was healthy in appearance, orange in colour, and the hive structural material was sticky and smelt strongly of plant resins. Brood disc formation was characteristic of *T. carbonaria*, and newly laid eggs were observed with a large number of callows present, indicating the presence of a healthy queen (i.e., a successful hive split), the hive overall looked healthy and highly active.

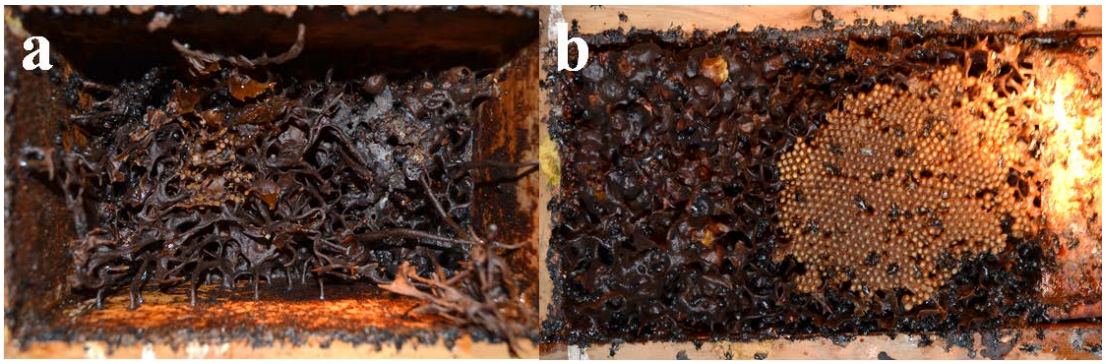

Figure 5.10 Interior view of the a) lower inoculated half and b) upper control half. There is a difference in colour and thickness of hive structures between inoculated and control treatments, with loss of overall hive volume in the inoculated half (a). There is a decrease in the size of the brood chamber between the treatments, with little developing brood in the inoculated half (a). There was a large adult population in control half (as seen in the left side of image (b)), and the large brood chamber volume indicates presence of a queen.

The two inoculated halves had a combined EBP reduction of approximately 99% (Table 5.7). Upon final inspection, both halves contained inactive queen cells within the remaining brood area, and were queen-less. In contrast, the two halves of the control hive had a combined EBP reduction of approximately 43% (Table 5.7), and the upper half contained an active queen. There were no overall differences in the combined hive halve weights between inoculated and control treatments (Table 5.8), with a combined loss of 1.85 kg in the control hive compared to 1.64 kg in the inoculated hive.

Table 5.7 Estimated brood population (EBP) and population reduction (%) of control and inoculated hives. Data represent EBP of colonies at the start before splitting, and halved (upper and lower) mid-way and at the end of the experiment.

Treatment	Start (EBP)	Halves	Mid-way (EBP)	Final (EBP)
Control	9870	Upper	4056	4868
		Lower	1107	791
Inoculated	10199	Upper	2241	29
		Lower	1450	22

Combined (U + L halves)	Final (EBP)	Population reduction (%)
Control	5659	43
Inoculated	51	99

Table 5.8 Weight of intact hives before treatments, compared to the weight (kg) of the treatment halves at the conclusion of the experiment. The treatment halves weights are combined for comparison.

Combined hive starting weight (kg)		
Control	5.36	
Inoculated	4.24	
Final weight		
Halves	**Control**	**Inoculated**
Upper	2.82	1.87
Lower	0.68	0.73
Combined hive final weight		
Control	3.51	
Inoculated	2.60	

Observations of worker behaviour in diseased hives

Observations of worker behaviour and activities were recorded from the start of the experiment. Within the first 10 h of the experiment, workers from both the lower inoculated and control halves were observed performing normal nest activities which occur after a hive split; such as re-building supporting pillars around the brood area and re-forming and attaching involucrum structures, fixing pots and cleaning up spilled stores, re-sealing brood cells and general cleaning-up of the hive. Any dead workers and other hive debris were also identified and dealt with by the workers and were observed being deposited into trash piles in the OPs.

Within 24 DAT, the acrylic lid on the lower half of the control half was completely covered with cerumen and resin deposits. This behaviour is commonly observed and is characteristic of a strong, healthy colony response; however, this meant that subsequent observations became difficult. In contrast, the acrylic lid on the lower half of the queen-less inoculated half had not been covered with cerumen and resin in the same time period, or over the remaining experimental period. The attached OPs associated with both inoculated and control halves were useful to continue monitoring the activity of the cleaners. Workers were very active but with no aggressive behaviours. They engaged in normal interactions with each other, including trophallaxis and antenna touching. Workers moved with purpose and energy, and performed tasks quickly, fixing and cleaning damaged pots, structures, and brood cells and increasing the involucrum network either solely or in small groups, taking turns to contribute to the task.

There was a change in worker behaviour in the inoculated hives at 2 DAT. A number of workers moved rapidly around the nest and across the brood, but with no obvious pattern or routine. They did not conduct normal hive tasks. Other workers were observed chasing the agitated workers, which they caught by a leg or wing, or climbed upon. This behaviour continued for most of the 10 h observation period. This agitated worker behaviour had ceased by 5 DAT. At this time, a small number of workers were observed to be moving very slowly or were motionless, not

performing any tasks in the colony. This lethargic behaviour was recorded for a further 16 days, with progressively less activity. There were a few workers still performing normal nest behaviours, although they were observed to be slower in performance and action.

Laboratory examination of collected samples

There was a change in the overall appearance of brood cells collected from the inoculated halves, where the caps were flattened. The few remaining larvae (all of which were from queen cells) were small in size, and opaque and brown in colour. Healthy brood cell provisions, as were present in the control halves, are a glossy, yellow coloured liquid (Figure 5.11). In contrast, the provisions in the inoculated hive were a dark greenish to brown colour, and formed a thick liquid (Figure 5.12).

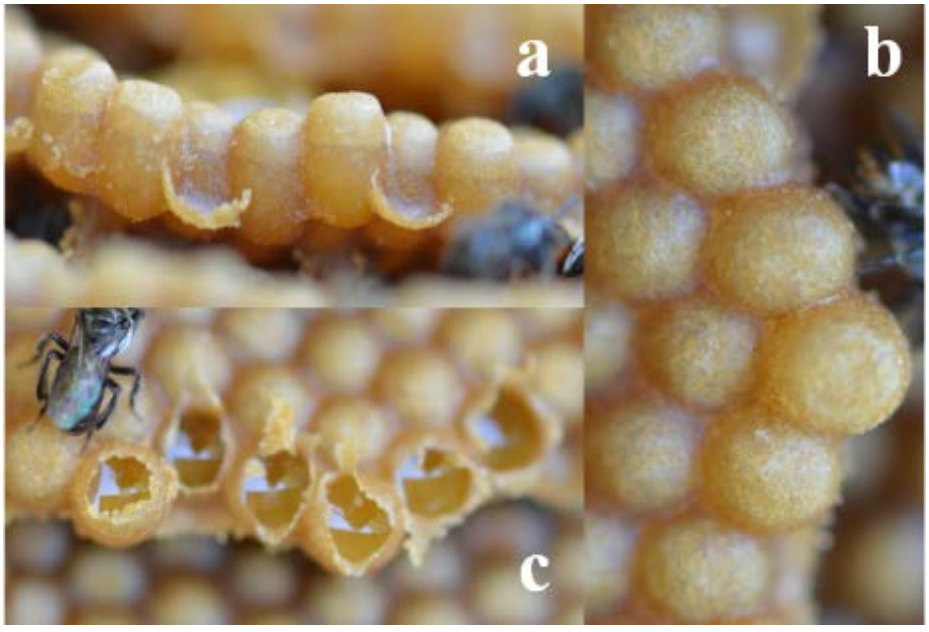

Figure 5.11 Healthy brood cells and provisions. a) Cells are made of thin cerumen and are a bright yellow colour. Cell formation is characteristic for *T. carbonaria* hives, with an oval shape with a domed cell cap (a and b), and c) liquid provisions are glossy and yellow in colour.

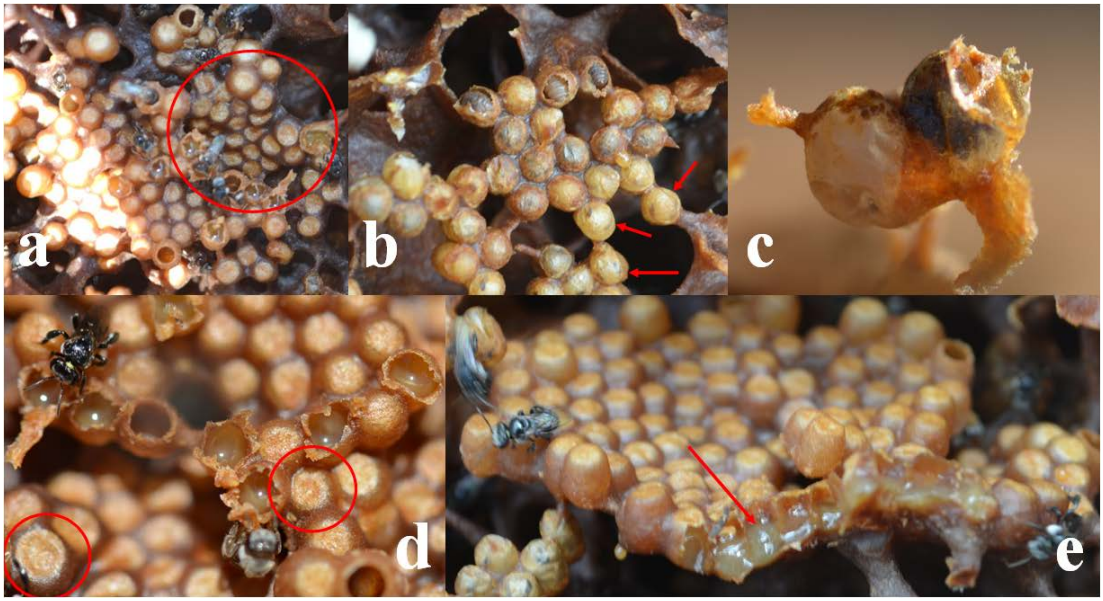

Figure 5.12 Infected brood cells. a) Cell exterior, showing early cell development, with irregular cell formation, flattened caps, and lack of overall circular disc formation. b) Older cells, with discoloured larvae, are next to pink and black eyed pupae (red arrow). c) A dark coloured infected brood cell, next to one containing an uninfected larva (whitish). d) Newer cells may form sunken caps indicating lack of larval development, e) Infected provisions become discoloured taking on a greenish-grey colour and becoming thick (red arrow).

5.4.4 Molecular identification of the causal organism

Bacterial samples collected from the original infected hive were cultured and inoculated into healthy colonies; the inoculated bacterium was re-isolated from the two experiments as part of the confirmation of pathogenicity experiments. Samples collected at the end of the infection experiments were initially analysed using microbiological diagnostic techniques, including biochemical tests (catalase test) and Gram staining. Molecular techniques identified the re-isolated bacterium as *Lysinibacillus sphaericus* (Ahmed *et al.* 2007, comb. nov.) synonym *Bacillus sphaericus* (Meyer and Neide, 1904), (Firmicutes, Bacillaceae). *Lysinibacillus sphaericus* isolated from symptomatic samples were submitted to the NCBI GenBank (also refer to Appendix A4.2) these samples were: worker larvae (KR947300), virgin queen larvae (KR947306 and KR947307), worker cell provisions (KR947301 and KR947304), queen cell provisions (KR947305), and honey samples (KR947302 and KR947303).

The 16S rDNA sequences for samples collected during the Koch's postulates experiments are very similar to those published for *L. sphaericus* (AF169495) (Nakamura 2000), with one single nucleotide polymorphism. The collected samples sent to the Department of Primary Industries, Elizabeth Macarthur Agricultural Institute State Veterinary Diagnostic Laboratory (SVDL), were confirmed to be negative for *P. larvae, Melissococcus plutonius*, and *Paenibacillus alvei* based on microscopy and diagnosic tools. The SVDL report from the 16S rDNA sequencing performed on the samples, also confirmed the identify as *L. sphaericus* (F. Galea pers. comm. 2013, molecular bacteriology, SVDL, Appendix A4.1).

MLST characterisation by sequence analysis

MLST profiling was performed on *L. sphaericus* isolated from a queen larva from the second pathogenicity experiment. Four MLST gene alleles *adk*, *glcK*, *glpF*, and *pycA* were of high quality for concatenation. The four sequences were submitted to the NCBI GenBank, *adk* (KT285615), *glcK* (KT285614), *glpF* (KT285616), and *pycA* (KT285613) (also refer to Appendix A4.2). The isolate was aligned with eight previously identified toxic (ST-2, ST-4, ST-5, ST-9) and non-toxic (ST-3, ST-7, ST-8, ST-17) sequence types (Table 5.9). These sequences were obtained by contacting the authors Ge et al. (2011). The estimates of average evolutionary divergence of all sequence pairs was 23%, with a final dataset of 1,622 bp positions (Tamura et al. 2011). The *T. carbonaria* isolate had strong similarity to the ST-7 isolate from Ge et al. (2011). A neighbour-joining phylogenetic tree based on the Tamura 3-parameter using a discrete Gamma distribution (T92+G) model was constructed for the 1622 bp concatenated sequence of four loci of *L. sphaericus*.

Table 5.9 Estimates of evolutionary divergence between concatenated MLST sequences from *T. carbonaria* and previously published sequence types (ST). Data is represented as percentage difference between bases per site. The analysis involved ten nucleotides with removal of ambiguous positions, a total of 1622 bp positions were in the final dataset, with NC000964 included as out-group (Ge et al. 2011).

	T. carbonaria	ST-2	ST-3	ST-4	ST-5	ST-7	ST-8	ST-9	ST-17
T. carbonaria									
ST-2	15.2								
ST-3	23.4	24.7							
ST-4	14.9	0.5	25.0						
ST-5	14.8	0.5	24.7	0.2					
ST-7	1.6	14.9	23.2	14.7	14.5				
ST-8	14.4	7.9	24.8	7.9	7.8	14.4			
ST-9	14.5	1.8	24.7	2.1	2.1	14.5	6.3		
ST-17	24.1	25.7	6.9	25.9	25.7	23.4	25.1	25.8	
NC000964	56.2	55.3	57.5	55.3	55.1	56.4	56.0	55.9	57.6

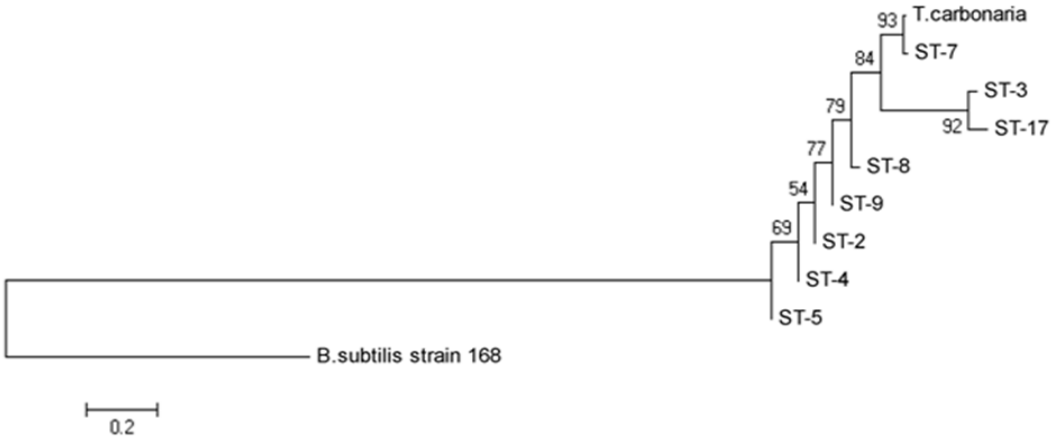

Figure 5.13 Evolutionary history is shown in the Neighbor-Joining tree constructed from concatenated MLST of four loci of *L. sphaericus* isolate from *T. carbonaria* and *L. sphaericus* toxic and non-toxic isolates of Ge et al. (2011) (846 positions). Evolutionary distances were computed using the Tamura 3-parameter and are in the units of the number of base substitutions per site. The rate of variation among sites was modelled with a gamma distribution. All ambiguous positions were removed for each sequence pair. The analysis involved 10 nucleotide sequences, with the tree rooted with *B. subtilis*.

5.4.5 Possible toxin production by *L. sphaericus*

Transmission electron microscopy

There is a marked difference between the TEM images of *L. sphaericus* and *P. larvae* (Figure 5.14). TEM images indicated possible presence of crystals in spores of *L. sphaericus* from 24-h-old cultures, but none in *P. larvae*. One crystal-containing spore was confirmed in *L. sphaericus* by Dr Charles, Associate Professor Park and Dr Berry. However, due to the low resolution of the image, and limited number of suitable spores for examination, further investigations to verify the presence of crystals was advised (J. Charles, H. Park and C. Berry, pers. comm. 2013). This was, unfortunately, beyond the scope of this thesis.

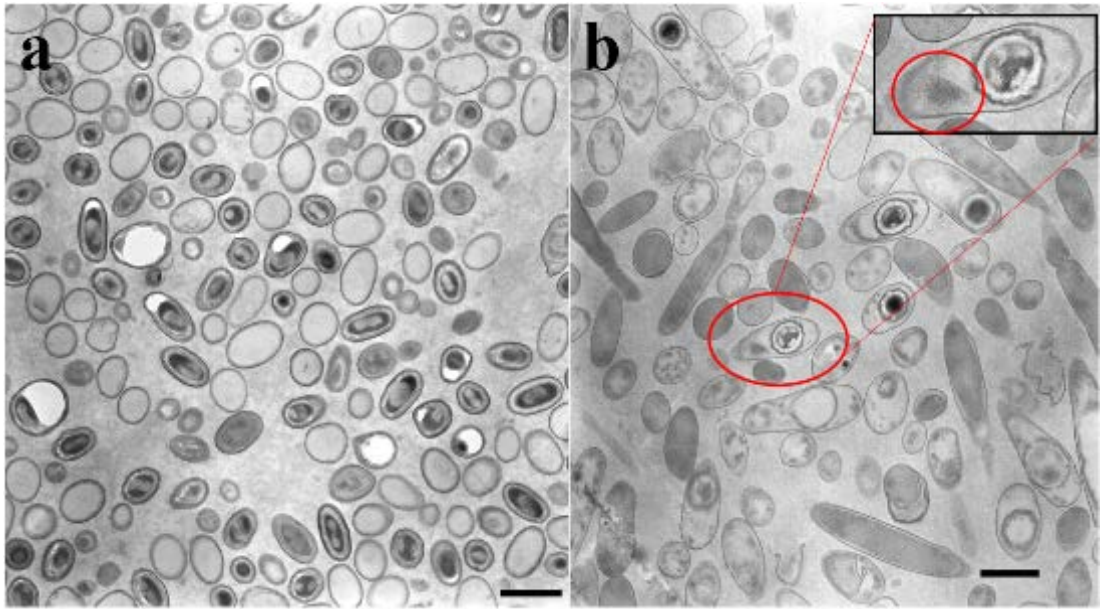

Figure 5.14 Comparison of bacterial spores from a) *P. larvae* and b) *L. sphaericus*. There are differences in the spores between the two images. *P. larvae* lack crystals inclusion, but there is the possibility that the isolated *L. sphaericus* sample produces crystal inclusions (J. Charles, H. Park and C. Berry pers. comm. 2013). This was only observed in one spore (insert image, red circle), and needs to be further confirmed. Scale bar = 1 µm.

***Molecular identification of possible toxin genes of* L. sphaericus**

PCRs were undertaken with primers for a number of toxin genes (*BinA, BinB, Mtx1, Mtx2, Mtx3, Cry48Aa, Cry49Aa*) (Ge et al. 2011). This resulted in negative results for *Mtx1, Mtx2, Mtx3, Cry48Aa, Cry49Aa*, and weak bands for *BinA* and *BinB*. The

sequencing of the weak bands was attempted, but did not deliver any useful sequences. Overall, the results for toxin identification are still inconclusive and require further investigation.

5.4.6 Practices to assist colony recovery from *L. sphaericus* infections

Hive manipulation for colony recovery

Investigations were undertaken to determine whether certain management practices could assist recovery of diseased colonies by brood manipulation, either by (A) removing infected material or (B) removing infected material and providing supplementary feeding, and (C) removing infected material, adding healthy brood from another colony whilst providing supplementary feeding.

Colony entrance activity was monitored for five months in scenario (A); over this time there was a decrease in worker numbers and a distinct absence of the characteristic internal humming sound of a healthy nest. Opening of the nest in autumn revealed the internal structures such as the involucrum were dark in colour and dry. The brood chamber was small in size (30 mm diameter) and also dry and brittle. Very few adult workers were present, and those that remained were lethargic, hardly moving and when they did move, they walked in circles, beating their wings apparently unable to fly despite being exposed to the outside. They appeared smaller in size than typical bees with noticeably smaller abdomens. Symptomatic larvae were found but in very small numbers, and were shrivelled and dehydrated. A very small and lethargic queen was found standing motionless in a mass of dead adult workers. She was unresponsive to tactile stimulation. There were numerous empty pots and very low to non-existent honey stores, despite it being a suitable season for plentiful stores.

For scenario (B), even though the colony was supplementarily fed, this appeared not to assist colony recovery. Upon reopening the colony after 5 months, very little

forager activity was observed, the brood chamber was small, and no queen was apparently present, based on the lack of newly laid eggs.

In scenario (C), the placement of newly laid brood discs from a neighbouring "healthy" colony to boost population numbers of the infected colony was similarly unsuccessful. The remaining adults in the colony did not perform brood cell management, therefore the cells began to dry and larvae did not develop, after 2 months the colony was dead.

Activity of T. carbonaria *and* A. mellifera *hive products against* L. sphaericus

A number of the *T. carbonaria* and *A. mellifera* product extracts inhibited the growth of *L. sphaericus*, although their level of activity differed significantly ($F_{3, 8} = 26.3$, $p < 0.001$) (Figure 5.15). There was no inhibition of *L. sphaericus* by *W. hellenica*, *A. mellifera* pollen or honey and thus have been excluded from analyses.

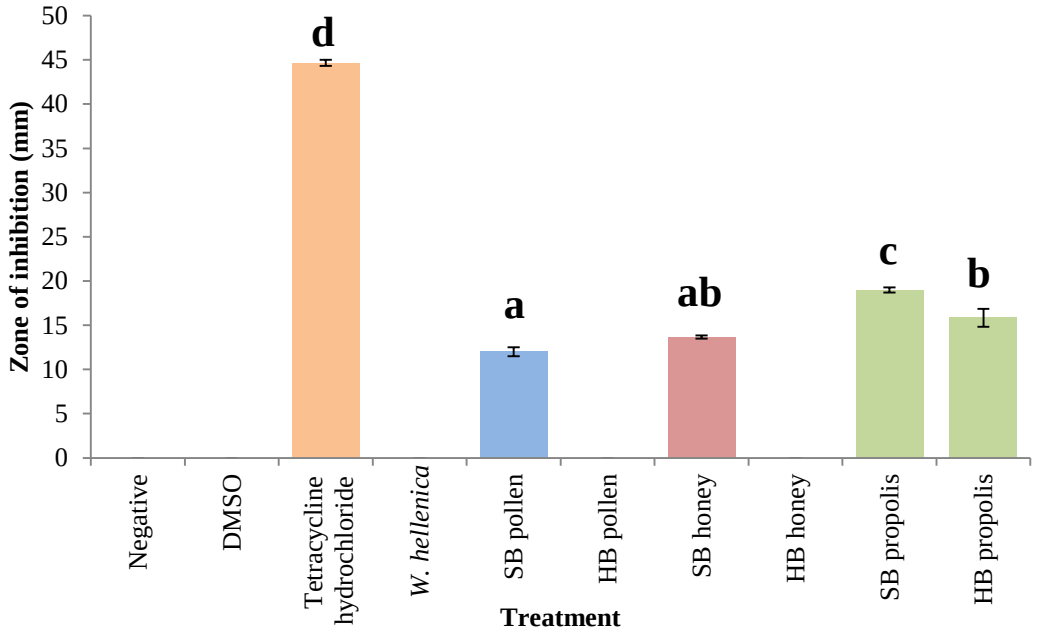

Figure 5.15 Mean diameter (mm) of the inhibition zone for *L. sphaericus* growth using *T. carbonaria* (Tc) and *A. mellifera* (Am) product extracts. Error bars = SE of means letters are indictors of statistical significance between nest product treatments. Statistical analysis only included hive products, as inclusion of the controls and *W. hellenica* resulted in heteroscedasticity of variances.

Tetragonula carbonaria propolis produced the largest inhibition zone for *L. sphaericus* growth (diameter 19 ± 0.29 mm) and was significantly more inhibitory than all other bee products tested; *T. carbonaria* pollen ($p < 0.001$), *T. carbonaria* honey ($p = 0.001$) and *A. mellifera* propolis ($p = 0.22$), but was inferior to the positive control tetracycline hydrochloride. The inhibitory effect of *T. carbonaria* pollen was not different to that of *T. carbonaria* honey ($p = 0.264$); however, *A. mellifera* propolis was superior to *T. carbonaria* pollen ($p = 0.008$). *Apis mellifera* pollen extract and honey extract treatments, as well as the gut isolate *Weissella hellenica* did not inhibit *L. sphaericus* growth. The solvent DMSO used to re-dissolve hive products was also not inhibitory.

5.4.7 Additional observations: Brood disease in *Austroplebeia australis*

Further to the hives already identified to be infected and discussed in this chapter, four *T. carbonaria* colonies, two located at McGraths Hill NSW (33º 61.58’ S, 150º 84.39’ E), one located at UWS apiary, and one located at Richmond lowlands (33º 58.33’ S, 150º 80.00’ E), showed symptomatic signs of *L. sphaericus* infection.

In addition, in November 2013 three *Au. australis* colonies showing similar symptoms of brood disease to those reported in this thesis for *T. carbonaria*, were brought for examination (M. Halcroft, pers. comm.). These colonies, located at Blaxland, Blue Mountains (33.7500º S, 150.6167º E), had not been in contact with the *T. carbonaria* colonies for the previous two years. The three colonies superficially appeared to be strong with a large brood area, ample stores, and an active queen. However, closer examination showed symptoms of disease, such as non-developing brood cells, provisions frequently coloured green and thick, and in some cases dry (Figure 5.16), brood cells with an ammonia-like smell, and lethargic workers. After a period of approximately three months, and with continual disease progression, all three colonies died out.

Using the same methods for bacterial identification of isolates from *T. carbonaria* hives, the *Au. australis* samples were tested for the presence of *Lysinibacillus*. All

three colonies were confirmed to be infected with *L. sphaericus*. One sample was sequenced using 16S rDNA primers (Section 5.2.6); alignment of the isolate was identified to be similar (only single-nucleotide polymorphism) (NCBI GenBank KR947308) to the previously sequenced *L. sphaericus* from the *T. carbonaria* colony.

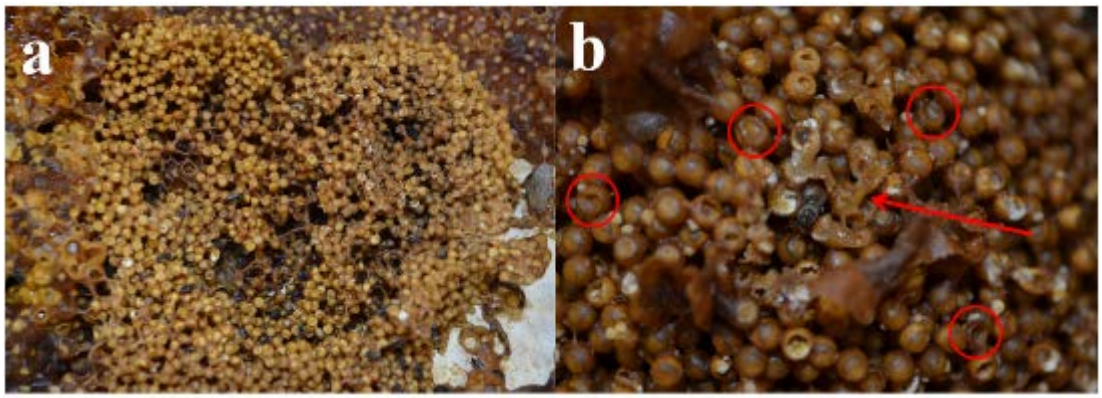

Figure 5.16 Interior view of *Au. australis* nest with possible brood infection. The brood is scattered and does not form a cluster (a), which is characteristic of this species. The brood has similar symptoms to *T. carbonaria* infection, with sunken/flattened cells (red circles) as well as discoloured cell fluids with absence of normal larval development (red arrow).

5.5 Discussion

Native stingless bee colonies may have fewer pathogens than *Apis mellifera*, perhaps because they have been less intensively selected and managed. However, the results reported in this chapter confirm the detection and identification of the first brood disease in managed stingless beehives. Symptoms of disease were observed in at least eleven colonies (eight *T. carbonaria* from total of 15 managed colonies and three *Au. Australis* colonies) over a 20-month period. The pathogen was isolated from worker and queen larvae, cell provisions and honey stores. The pathogen was identified as the bacterium *Lysinibacillus sphaericus* (Ahmed *et al.* 2007, comb. nov.) synonym *Bacillus sphaericus* (Meyer and Neide, 1904) (Firmicutes, Bacillaceae), and was confirmed as the causative organism in pathogenicity experiments following Koch's postulates guidelines.

Apart from strains of *L. sphaericus* reported to range in toxicity to mosquitoes (de Barjac et al. 1985, de Barjac 1990), strains have also been reported having sub-lethal effects, such as retarded growth and decreased fecundity (Berry 2012) in the nematode *Trichostrongylus colubriformus* (Bone & Tinelli 1987), the grass shrimp *Palaemonetes pugio* (Key & Scott 1992), and the water scorpion *Laccotrephes griseus* (Mathavan et al. 1987). There is some evidence that a symbiotic association between *Lysinibacillus* spp. and certain bee species has existed over a long period, with no evidence of any effects on bee larval and pupal development or adult longevity (Davidson et al. 1977, Cantwell & Lehert 1978). A *Bacillus* species closely resembling *L. sphaericus* was isolated from the abdominal contents of a 25–40 million year old extinct stingless bee, *Proplebeia dominicana* (Cano et al. 1994) as well as from brood provisions of a solitary bee, *Crawfordapis flavofasciata* (Gilliam et al. 1990). Another study reported the isolation of isolated *L. sphaericus* from blackened *B. terrestris* larvae with an unpleasant odour (Přidal et al. 1997, Přidal 2001).

Disease establishment

Lysinibacillus sphaericus occurs naturally in soil (Massie et al. 1985, Ahmed et al. 2007) and on plant material (Melnick et al. 2011), and has also been isolated from aquatic habitats and mosquito cadavers (Guerineau et al. 1991, Ludwig et al. 2009b). *Lysinibacillus sphaericus* thrives under aerobic conditions, producing spherical spores within its terminal sporangia (Hu et al. 2008). As the bacterium naturally occurs in the environment, it may be a relatively common organism in stingless bee nests. This may explain the presence of *L. sphaericus* in *T. carbonaria* honey stores. It further suggests that the infections observed may have developed when the colonies were weakened due to external factors (such as environmental stress), and the bacterium subsequently thrived within the colony. The fast, successful establishment of such an introduced bacterium into the colony may contribute to the disorientation and unusual behaviours displayed by workers and the queen, eventually contributing to population decline, queenlessness and colony death (Figure 5.17), possibly associated with its lethal or sub-lethal effects.

Many *Paenibacillus* species are also soil dwelling organisms and the association between *P. larvae* and *A. mellifera* may have also been initially a benign symbiotic association (Qin et al. 2006); however, *P. larvae* has evolved to be highly specialised to utilise conditions within *A. mellifera* colonies, in which the nest enables continual brood production under highly specific temperature regulated conditions (> 35ºC). This association provides an insight into the potential future association between *L. sphaericus* and *T. carbonaria*.

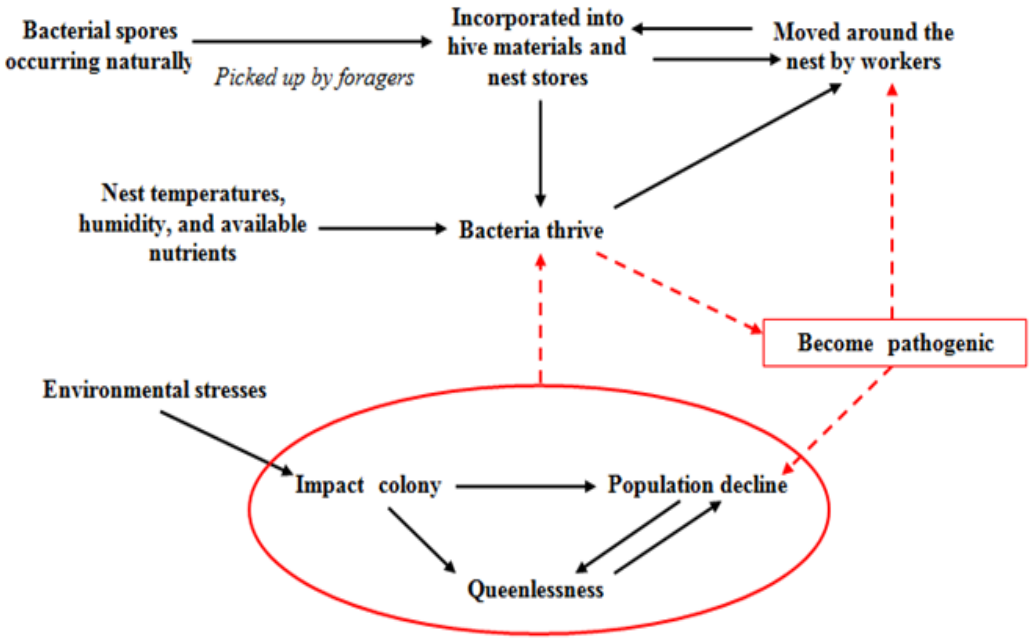

Figure 5.17 Flow-chart showing possible entry route of the bacteria, and at what points the bacteria may encounter workers, nest products and be transported around the nest. It also suggests how the bacteria may move from a symbiotic, benign association, to becoming pathogenic (red), and contributing to colony loss.

Tetragonula carbonaria colony members do not progressively feed their young; once the provisions are added and an egg laid, the cell is capped and left to develop. If cells are provisioned with contaminated food sources, then the young larvae feeding on the contaminated provisions ingest the spores; thereafter, the spores germinate inside the host and cause disease, as occurs with *L. sphaericus* to treat mosquito populations (Davidson 1979, World Health Organisation 1985). This scenario is likely to be similar to *P. larvae*, whereby its spores are only infectious to 12–36 h-old larvae and not adults, and after ingestion by contaminated food (Genersch 2010).

Tetragonula carbonaria workers handling the nest stores (honey and pollen) may also contribute to the movement of *L. sphaericus* spores throughout the nest and contribute to contaminating surrounding storage pots. This may explain why some brood continue to develop to adulthood, as the provisions they were provided during egg laying were uncontaminated.

Temperatures tolerated by different *Lysinibacillus* spp. can be as low as 10–15ºC, with a maximum growth temperature of 30–45ºC (Ludwig et al. 2009b). *Tetragonula carbonaria* can maintain their brood chamber between 15–30ºC throughout the year, with temperatures in the high 20°Cs during peak brood development (Chapter 2, section 2.2). *Tetragonula carbonaria* colonies appear therefore to provide suitable, although at times sub-optimal, conditions for this pathogen, with a continuous supply of active workers aiding in the spread and replication of the bacteria.

Lysinibacillus sphaericus was also isolated from *Au. australis*. However, as *Au. australis* can survive over a wide temperature range, between -4–37ºC, the colonies reduce brood production in cooler weather and are unable to maintain suitable incubation below ambient temperatures of 15ºC (Halcroft et al. 2013b); this may result in these colonies being less suitable hosts. It should be noted that unlike *T. carbonaria* the diseased *Au. australis* colonies were located in an environment outside their natural distribution range, which is likely to add to colony stress.

Mechanism of pathogenicity

The concatenated MLST sequences of the *L. sphaericus* strain from *T. carbonaria* had the strongest match to *L. sphaericus* strain ST-7 (Ge et al. 2011) (Figure 5.13), a strain which falls within a serotyped group (H2) (de Barjac et al. 1985, de Barjac 1990). A group which based on a lack of toxin genes, or contained only *Mtx* genes, shows no mortality towards the mosquito *Culex quinquefasciatus* (Ge et al. 2011), lower pathogenicity against *Anopheles stephensi*, but however has been reported to cause mortality in mosquito larvae of *Culex pipens* (de Barjac et al. 1985, de Barjac

1990). The varying range of pathogenicity of *L. sphaericus* strains of the H2-serotype group (de Barjac et al. 1985), which are less homogenous than other serotype groups such as H5a,5b or H25 (which are highly toxic to both *C. pipens* and *A. stephensi*) (de Barjac et al. 1985, de Barjac 1990), suggests that strains within H2-serotype group may be toxic to one host but not another, and pathogenicity may be by other, non-toxin producing, mechanisms.

There were inconclusive results in the current study with the *L. sphaericus* strain isolated from *T. carbonaria* colonies regarding presence of toxin genes from molecular studies, and toxin crystal inclusions in spores, from TEM studies. Despite amplification of weak *BinA* and *BinB* bands, no useful sequences could be obtained, however, as experienced by Ge et al. (2011), no *Mtx2* genes were identified which suggests that the strain may not be non-toxic to some insects. Further microscopy and molecular work is required to confirm the presence or absence of toxins in the isolated *L. sphaericus* strain. The investigations should also include other possible toxins, such as sphaericolysin, that has been isolated from *L. sphaericus* strains associated with the ant lion, *Myrmeleon bore*, and is toxic to the German cockroach, *Blattella germanica*, although not to mosquitoes (Nishiwaki et al. 2007). S-layer proteins previously identified in *B. anthracis* (Etienne-Toumelin et al. 1995), *B. thuringiensis* (Peña et al. 2006) and *P. larvae* (Fünfhaus & Genersch 2012) have also been identified in some *L. sphaericus* strains which are highly toxic to certain *Culex* species, but have no toxic effects when present in other strains (Lozano et al. 2011). The absence of crystal inclusions in the TEM images may not, in itself, confirm lack of toxins, as recently toxins were identified in stains of *P. larvae*, despite the lack of crystal inclusions in their spores (Fünfhaus & Genersch 2012, Fünfhaus et al. 2013, Krska et al. 2015, Poppinga & Genersch 2015). The findings from the current study suggest that other modes of bacterial pathogenicity including invasiveness, may contribute to colonisation, while there may also be toxins specific to the *L. sphaericus* strain isolated from *T. carbonaria* that are yet to be identified and may not be formed into crystal inclusions.

Strategies for management of brood disease in stingless bee colonies

The investigations reported in this thesis have shown that *T. carbonaria* can undertake activities to reduce potential hive infections, including hygienic behaviour and use of antimicrobial hive materials. In particular, propolis, a major component of hive structures, showed the greatest level of inhibition against *L. sphaericus*, except for the tetracycline hydrochloride control. The *in vitro* studies were conducted on a small scale with highly concentrated, solely propolis-based extracts. In a colony situation, isolated propolis deposits are located along the edges of nests or in gaps, and are therefore less likely to be in direct contact with bacteria in the brood, and to be able to inhibit their growth. Propolis and resins are also present in brood cell construction (cerumen) (Chapter 4, section 4.3.4); however, the levels of propolis compounds are lower due to the addition of other components. As all infected *T. carbonaria* colonies eventually succumbed to the disease, this suggests that there may have been insufficient quantities of antibacterial components in the brood cells to diffuse and suppress a high bacterial spore concentration in the cell contents.

The antibiotic tetracycline hydrochloride showed the greatest inhibition of *L. sphaericus*, and was superior to propolis extract. This is unsurprising, as a similar result was obtained against *P. larvae* (Chapter 4, section 4.3.2). Some *P. larvae* strains have shown resistance to oxytetracycline and tetracycline (Alippi 2000, Miyagi et al. 2000, Murray & Aronstein 2006) because of their extensive use in honey bee hives. Antibiotics do not kill the spores which are able to survive for decades, and can accumulate as residues in hive products such as honey (Bogdanov 2006, Alippi et al. 2014). To date, little resistance has been recorded in Australian strains of *P. larvae* (Wu et al. 2005), and in New South Wales where AFB is a notifiable disease under the *NSW Apiaries Act 1985 No.16*, antibiotics are not recommended for use. Instead, eradication measures to destroy all infected colonies include the burning of bees, diseased combs and other hive components in large ground pits, or the sterilisation of salvaged hive components by gamma-irradiation are required (Somerville 2012a). Naturally occurring resistance to streptomycin has been reported in some pathogenic and non-pathogenic strains of *L. sphaericus* (Burke & McDonald 1983). The problem of antibiotic resistance in *P. larvae* strains

is, therefore, of concern when considering the possible use of antibiotics for the control of stingless bee bacterial infections.

While the laboratory-based inhibition studies showed positive results, *in vivo* field studies need to be conducted to confirm efficacy of propolis and/or antibiotic treatments; however, these will have to align with the *NSW Apiaries Act 1985 No.16* (M. Rankmore, G. Levot, and D. Somerville, NSW DPI, pers. comm., 2012). Interestingly, the Apiaries Act only refers to *Apis mellifera*; there is currently no requirement to register stingless bee hives in Australia, nor to report stingless bee diseases found in managed hives.

Attempts to assist the recovery of infected *T. carbonaria* colonies by hive management practices were not successful. When the majority of symptomatic brood cells were removed and the colonies were supplementary fed, despite there being reasonable numbers of adult workers, callows and pupating brood and newly laid eggs present, the colonies continued to decline and die. As the bacteria were present throughout the hive, including in honey stores, removal of symptomatic bees and brood cells was insufficient to halt the further development of the disease within the colony. As worker bees would likely have been in contact with the contaminated honey, viable spores would have been transferred to pollen stores, cell provisions and spread via trophallaxis within the adult population. Also, as it took 22 days for the first signs of infection to appear following heavy inoculation, removal of symptomatic brood is unlikely to control disease development and spread. The placement of newly laid brood discs from neighbouring apparently healthy colonies into infected colonies to boost population numbers was similarly unsuccessful, most likely due to insufficient nurse bees to care for the brood cells.

Implications of brood disease for the stingless bee industry

Within the Australian stingless bee industry, hives are normally opened for splitting (hive replication) or harvesting honey once or a few times a year, mainly in Queensland, where climatic conditions are more favourable for the bees. As a result, limited monitoring of hives on a scheduled basis is undertaken, especially in NSW.

Most hive monitoring is based initially around forager entrance activity, which may not be a good indicator of hive health; workers may still forage despite development of brood infections. Therefore, any pathogenic brood infections are unlikely to be detected until the hive is severely diseased or has died out. Compared with honey bees which are highly managed and examined routinely following the guidelines of the *NSW Apiaries Act 1985 No. 16*, no legislation or protocols have been developed for hive management within the stingless bee industry. For example, there are no regulatory requirements with regard to hive manipulation techniques, the use of hive equipment between colonies and the movement of colonies interstate. If the hives used in this study were not examined frequently, many activities would likely have been missed and possibly the detection of a brood infection would not have occurred. Despite a minimum of fifteen stingless bee colonies (*T. carbonaria* and *Au. australis*) confirmed to be infected in the last 20 months in the current study, this disease still appears to be rare. However, with increasing interest in stingless bee keeping in Australia, with >4,900 managed stingless bee hives being recorded with 78% of beekeepers hobbyists (Halcroft et al. 2013c), there is increasing opportunity for undetected brood infections to spread. Without widespread dissemination of detailed information about signs and symptoms of infection, it is possible that brood disease in stingless bee colonies could become a serious problem.

This appears to be the first documentation of disease symptoms in managed stingless bees internationally; however, it may not be an isolated occurrence. Brazilian researchers have recently reported colony losses from an unknown cause in managed *Scaptotrigona* colonies. Symptoms include dark coloured larvae and pupae over the combs, with sporulating bacteria present examined microscopically (G. Venturier, pers. comm. 2013). However, no further information about the South American losses is available at this time.

Chapter 6

General discussion

6.1 Introduction

This study set out to elucidate the inherent characteristics whereby stingless bee, *T. carbonaria*, colonies have a lower incidence of brood diseases compared to European honey bee, *A. mellifera* colonies. The investigations described in this thesis identified behavioural mechanisms, such as hygienic behaviour and thermoregulation, as well as antimicrobial effects of nest products as a result of their chemical composition, as likely contributors to the suppression of brood pathogens. Despite these findings and the paucity of information on stingless bee brood diseases prior to my work, this study confirmed that stingless bees are susceptible to brood diseases, by identifying for the first time a bacterial brood disease in managed *T. carbonaria*, and subsequently *Austroplebeia australis*, colonies in NSW, Australia. Therefore, in addition to the research objectives established at the commencement of my study, I also addressed behavioural and antimicrobial defence mechanisms associated with the newly-identified pathogen.

The research presented in this thesis involved *T. carbonaria* colonies located in the field, as well as in a temperature-controlled shed with external foraging access, and employed behavioural, microbiological, chemical, and molecular methodologies to investigate the key research questions. Field colonies were repeatedly monitored over 13 months for hygienic and thermoregulatory studies, whilst extracts of nest products from *T. carbonaria* and *A. mellifera* colonies were assessed for inhibition of known brood pathogens in laboratory assays. The nest products were analysed by LC-MS to identify chemistry likely to be associated with the antimicrobial activity. Over a two-year period, extensive scientific efforts were made to document and identify the disease-causing organism, *L. sphaericus*, in managed *T. carbonaria* colonies.

The outcome of this research is not only central to the further development of Australia's stingless bee industry, but also makes an important contribution to the understanding of the management practices of stingless bees in a global context.

6.2 Key findings and their application

6.2.1 Behaviours expressed by workers which limit the growth and develop of brood pathogens.

Tetragonula carbonaria *displays a high level of hygienic behaviour*

My work showed that *T. carbonaria* is the most efficient species in performing hygienic behaviours (Chapter 3), compared to any previously studied stingless bee or *A. mellifera* colony. Colonies on average took 7 h to remove 99% of the dead pupae; thereafter workers completely dismantled the brood cells, a mechanism that most likely contributes to reduced pathogen transmission. The ability for eusocial workers to detect early and remove dead pupae is highly advantageous for controlling brood diseases. *Apis mellifera* colonies selected for superior hygienic behaviour have fewer cases of *P. larvae* (Rothenbuhler & Thompson 1956, Spivak & Reuter 2001a) and *A. apis* disease (Gilliam et al. 1983). The superior hygienic behaviour of *T. carbonaria* is one explanation of the lower incidence of brood pathogens recorded in this species, as the colonies deal with dead brood before the beekeeper has the chance to observe problems. However, within the *T. carbonaria* colonies tested in the current study, as well as between the five previously tested stingless bee species (Tenório 1996, Medina et al. 2009, Nunes-Silva et al. 2009), there is a wide range of hygienic behaviour efficiency (1–99% removed in 48 h). This variable level of hygienic behaviour within and between species suggests that this trait may be genetically influenced, however further studies are required.

Currently, no studies have reported the genetic hygienic behaviour traits in stingless bee species; however, these are well known in *A. mellifera* (Rothenbuhler 1964a, Spivak & Gilliam 1998a, Spivak & Reuter 1998, Gramacho & Gonçalves 2001, Spivak & Reuter 2001a, Lapidge et al. 2002). Investigation of the genetic basis of stingless bee hygienic behaviours will ultimately provide the opportunity to select and breed superior lines for disease resistance, as previously performed on *A. mellifera* colonies in Tasmania (Gerdts 2014). However, selective breeding for one behaviour (such as hygienic behaviour), should not come at the expense of other

beneficial traits. Some *A. mellifera* races have been bred for lower aggression (such as *A.m. carnica* and *A. m. caucasia* and stock line Midnight) (Tarpy 2011), making handling easier, but these might increase pest (vector) and disease incidence, as highly defensive colonies also appear to be more hygienic (Uzunov et al. 2014 Paleolog 2009). Aggressive behaviours such as mass biting by *T. carbonaria* workers when nests are opened, and capturing and immobilising invading parasites such as small hive beetle by *T. carbonaria* (Greco et al. 2010b) and *Au. australis* (Halcroft et al. 2011) workers, may be advantageous for controlling other vectors. Other traits such as: high resin collection and propolis production (which are generally considered undesirable in honey bees), are critically important in stingless bee colonies for nest architecture and probably for disease suppression. The loss of such traits while breeding for more efficient hygienic behaviours may therefore be counter-productive.

At present, stingless bee colonies are selected and propagated based on their overall strength and appearance upon opening, and as such should indirectly select for hygienic behaviour (i.e., no observed pests or diseases). However, identification and selection of hygienic lines of stingless bees could be undertaken as for *A. mellifera* in Australia and elsewhere, and a breeding program established. This program should involve controlled mating or artificial insemination of queens. While this may be a promising area for future development, particularly as stingless bee queens only mate with a single male, queen rearing and artificial insemination, which is widely practiced with honey bees, has not yet been developed for stingless bees.

Tetragonula carbonaria *can maintain brood development over a wide temperature range*

This study showed that *T. carbonaria* colonies are able to support continual brood development yearlong, by maintaining brood temperatures between 15–31ºC, despite ambient temperatures ranging from 0–37°C. The reason for the wide temperature survival range is that colonies employ both passive thermoregulation behaviours such as involucrum building, and active thermoregulation behaviours like wing

fanning and honey consumption to maintain temperatures. The *T. carbonaria* colonies tested were managed in OATH (Original Australian *Trigona* Hive) (Heard 1988a) hives, which are suited to tropical and subtropical conditions. A more suitable hive design has been suggested for temperate regions (Greco et al. 2010a) which may reduce problems associated with suboptimal ambient temperatures. In addition, no studies have been conducted on the thermoregulation by Australian stingless bees in natural nesting locations, although it is suggested that such natural nesting material provides good insulation for colonies against ambient temperature extremes, compared to managed boxes (Dollin et al. 1997, Sung et al. 2008).

The continuous production of brood suggests that colonies would provide potential brood pathogens with yearlong access to suitable hosts. However, the scant reports of brood pathogens in *T. carbonaria* in Australia indicates that although larval development can occur at low temperatures (to 10ºC) it is not optimal for *L. sphaericus* development. This is confirmed by my results, in which *Lysinibacillus sphaericus* thrived at 37ºC in an incubator, and disease occurrence was only recorded in *T. carbonaria* colonies managed in the bee shed under constant ambient temperatures at 26ºC, or for field colonies during the warmer months. I therefore speculate that low brood temperatures, at least, inhibit the growth of *L. sphaericus* and possibly other brood pathogens in *T. carbonaria* colonies. This can be similarly concluded for *Au. australis* which can survive for extended periods with brood temperatures recorded as low as -0.4ºC (Halcroft et al. 2013b), unsuitable for many bacterial pathogens. This is in stark contrast to *P. larvae*, which is adapted to thrive in the same constant optimum temperatures required by its host, *A. mellifera*, for development (Alvarado et al. 2013).

6.2.2 Antimicrobial properties of nest products suppress the growth of insect pathogens.

In this study, apparently the first assessing activity of stingless bee nest products against insect pathogens, *T. carbonaria* pollen, honey and propolis all suppressed bacterial and fungal bee pathogens. Propolis was the most antimicrobial, and *T. carbonaria* propolis was more efficacious than *A. mellifera* propolis. Interestingly,

the antimicrobial activity of *T. carbonaria* propolis was better against *P. larvae* than against the *T. carbonaria* pathogen, *L. sphaericus*. This may be because *P. larvae* has had no previous exposure to stingless bee nest products, and/or that stingless bee propolis contains active compounds present in higher quantities or not found in, *A. mellifera* propolis. Both *T. carbonaria* and *A. mellifera* propolis contained flavanones; typical of temperate propolis; however, the quality and type of flavanones (ion mass and retention time) differed between the samples, possibly a result of *Corymbia torelliana* sourced by *T. carbonaria* (Wallace & Trueman 1995, Wallace et al. 2008, Wallace & Lee 2010, Drescher et al. 2014), but not by *A. mellifera*. Furthermore, as propolis is a key component of *T. carbonaria* brood cells, any antimicrobial activity it possesses is likely to play a more direct role in management of pathogens than does propolis in *A. mellifera* colonies.

Application of these findings

My results provide encouragement for future investigations on application of *T. carbonaria* propolis for treatment of disease, such as *P. larvae*, in honey bee colonies. However, for this to occur, further research is required to identify the resinous plant sources contributing to the key antimicrobial properties. It should be noted that my studies were conducted in the Hawkesbury district of NSW, and other researchers have reported antimicrobial activity of stingless bee propolis from locations in S.E. Queensland and elsewhere (Brazil and Thailand), presumably with quite different floral resources. Furthermore, for effective application as an end-use product many other factors such as optimising extraction methods, characterisation and standardisation of the extract, determining the concentration and volume required for efficacy, application methods (such as in syrup solutions, pollen cakes, or direct sprays in colonies), and application rate (timing and frequency) as well as possible effects on resident symbiotic microorganisms, all require research and development.

Implications for stingless bee management

The increasing interest in using stingless bees, in particular *T. carbonaria* in Australia, for pollination services in intensively managed crops in field and greenhouse situations may present problems with regard to colony access to plant resins for adequate amounts and quality of propolis production. Monocultures are characterised by limited resources (quality and variety), and for many commercial crop plants, limited availability of plant resins. *Tetragonula carbonaria* selects some different plant resources to *A. mellifera*; and it also has a substantially reduced foraging range (500 m, Heard & Dollin 1998). Thus, if colonies have limited access to suitable plant resources within their foraging range this is likely to contribute to reduced propolis production. In turn, this would negatively affect brood production, and, based on my work, the production of antimicrobial structures within the nest. This deficiency may be overcome by providing supplementary propolis (probably sourced from *A. mellifera*) or migrating hives to areas of natural high resin resources, similar to the management strategies employed in the honey bee industry.

6.2.3 Stingless bees are susceptible to brood disease, despite defence mechanisms deployed by workers.

My study has identified *L. sphaericus* as a disease-causing pathogen. Unlike *A. mellifera* who progressively feed their larvae in open cells for the first 5 d (Page & Peng 2001), and as a result increase the chances of pathogen transfer between nursery workers and larvae, stingless bee nursery workers cap the cell immediately after oviposition (Michener 1974, Yamane et al. 1995, O'Toole & Raw 2004). Therefore, spores of *L. sphaericus* must enter the larvae by ingestion of cell provisions contaminated prior to capping. These spores germinate in the digestive system of the host and the infected larvae change colour from white to brown. In the later stages of infection, the larval bodies degrade to a thick, brown fluid. Infected colonies decrease in population size because few larvae develop. Adults present were lethargic further reducing their ability to perform important nest tasks, such as nest temperature regulation, and hygienic behaviour (Chapter 3). Infections occurred in managed *T. carbonaria* colonies in the Hawkesbury area, and in managed *Au.*

australis colonies located within the Blaxland area (a geodesic distance of approximately 23 km).

My study is the first detailed account of brood disease. The earliest reference to a possible brood disease was from a rather obscure report by Kerr in1948 (Kerr 1948, Nogueira-Neto 1997), who observed a large number of bacterial spores in dead *Melipona quadrifasciata* and *M. bicolor bicolor* pupae. The bacterium was identified as *Bacillus para-alvei* (Parafoulbrood); however this has since been suggested to be the same as *B. alvei* (Clark 1939, Smith et al. 1946, Steinhaus 1946), a secondary bacterium (saprophyte) associated with *Melissococcus plutonius* (European foulbrood). The only other reference to bacterial brood disease is that of Venturier (pers. comm. 2013) in *Scaptotrigona* species in Brazil, with symptoms similar to those described in Chapter 5. Thus, my study provides an important opportunity to collaborate with beekeepers and researchers from Brazil to identify similarities and differences in the disease between the two countries, including predisposing factors and management practices. South America is a key stingless bee "hot spot" with 417 described species (Camargo & Pedro 2013), and a long history of meliponiculture.

There are four possible explanations for the lack of reports of stingless bee brood diseases:

1. Brood infections in stingless bees are genuinely rare. This may be associated with a combination of the colony defence mechanisms reported in this thesis, an inherent resistance to brood diseases, or other, as yet unknown factors. Nevertheless, recent anecdotal evidence suggests that brood diseases may also occur in managed stingless bee colonies in NSW and QLD, and in other locations, such as Brazil.
2. Brood diseases have rarely been investigated or specifically explored in managed stingless bee colonies. With increasing interest in owning and managing stingless bees, there will be increased opportunities to observe and identify nest changes associated with disease. The Australian stingless bee industry is still in its infancy; however, as it develops, along with associated

educational support, it is possible that reports of disease incidence may become more common.

3. Stingless bees globally have been less intensively managed and utilised for nest product harvesting and pollination services than the European honey bee. As a result, there has been less selection pressure in stingless bees away from traits that may impart disease resistance, and generally less stress is placed on their colonies due to fewer hive manipulations.

Factors contributing to colony susceptibility to brood disease

Based on my studies, there a number of stress factors which could contribute to the occurrence of *L. sphaericus* infections, and possibly other pathogens in stingless bee colonies.

Temperature extremes

Brood problems in stingless bee colonies from NSW, QLD, and Brazil have recently been reported to me, and I hypothesise that stressed colonies are likely to be more susceptible. The movement and attempted establishment of *T. carbonaria* and other stingless bee species outside their normal distribution range is likely to result in stressed colonies. As a consequence, despite a of species which have shown to tolerate sub-zero temperatures (van Benthem et al. 1995, Macieria and Proni 2004, Halcroft et al 2013a), low ambient temperatures may result in chilled brood, starvation due to lack of foraging opportunities, and a reduction in populations as a result of limited nest stores. For example Halcroft (2012) showed that *Au. australis* would be unable to adequately forage until in-hive temperatures were ≥ 18.6ºC and ambient temperatures ≥ 20ºC, a reflection of their natural distribution range. An inability to forage on available resources as a result of inappropriate temperatures could result in store depletion and colony starvation (Halcroft 2012).

With a drop in population size and the number of age-task related adults; opportunistic microorganisms may flourish. This scenario could have contributed to the observed *L. sphaericus* infections. Reducing colony stress associated with low

temperatures may assist in maintaining nest conditions and therefore, reduce incidence of brood disease. Despite recommendations of providing artificial heating to reduce temperature stress in stingless bee colonies during winter, and allow for continual brood production (Amano 2004, Vollet-Neto et al. 2011, Klumpp 2012); this might inadvertently provide conditions more suitable for brood disease development.

Extremely high temperatures (>37ºC) may also place severe stress on colonies in some locations. *Tetragonula carbonaria* has nest structures derived from wax and plant resins, which can melt under extreme heat. Although colonies can partially control nest temperatures through their thermoregulation activities (Chapter 2), if they are exposed to very high temperatures for extended periods (e.g. >48 h) no amount of ventilation performed by workers can prevent heat-induced stress.

It should be noted that while this study was conducted in a location where *T. carbonaria* is endemic, the Sydney region (south-east distribution range) is regarded as somewhat marginal (Dollin 2013, Sharpe 2014), indicating that the colonies are more likely to suffer stress from both low and high temperatures than further north in this species' distribution. Furthermore, the *T. carbonaria* colonies used in the study were sourced from suppliers in Queensland, and were thus not of local provenance. Colony stress could be manifested by less forager activity and depletion of nest stores in cool seasons, and the loss of colonies in summer due to nest melting. Similarly, the *Au. australis* colonies detected with disease were located in an area well outside their natural distribution.

Foraging resources

As previously discussed, colony stress can also be associated with limited access to foraging resources, either because temperatures are inadequate for flying, or the colonies are positioned in areas of poor quality floral resources. Beekeepers have reported colony losses due to starvation, hive splitting and honey extraction (Halcroft

2012); therefore stingless beekeepers in NSW, especially around the more southerly distribution of *T. carbonaria*, are advised to limit the number of times a colony is propagated, and not harvest honey (T Heard, pers. comm. 2013). This is because colonies may not adequately recover from these damaging manipulations, and ensuring available time for adequate foraging and replenishing of nest stores before the change in season. In comparison, *T. carbonaria* colonies managed in most of QLD have a longer period of optimum foraging temperatures and available flowering resources, because of the prevailing climatic conditions. Foraging resources are not limited to pollen and nectar requirements, but also access to plant resins used to produce propolis and cerumen.

Other factors likely to induce colony stress in stingless bees

The honey bee industry worldwide is already suffering severe problems associated with rapid loss of adult populations, termed colony collapse disorder (CCD) (Cox-Foster et al. 2007, Oldroyd 2007). There are a number of drivers contributing to colony decline (Potts et al. 2010); these include (but are not limited to), use of neonicotinoid insecticides (Moffat et al. 2015, Rundlöf et al. 2015, Tan et al. 2015), a general cocktail of pesticides and fungicides (Mullin et al. 2010, Pettis et al. 2013, Zhu et al. 2014), pests as vectors of viruses (e.g., varroa mite) (Ratnieks & Carreck 2010, Martin et al. 2012, Francis et al. 2013), and the influence of climate change and loss of floral resources (Schweiger et al. 2010, Bartomeus et al. 2011). A number of these factors are likely to also affect or have the potential to affect stingless bees, and therefore increase their susceptibility to disease. In honey bees, for example, rapidly deteriorating colonies generally have higher virus and other pathogen loads (Martin et al. 1998, Martin 2001, Cox-Foster et al. 2007, vanEngelsdorp et al. 2009).

Beekeeper management inputs

Beekeepers have considerable influence on the development and success of stingless bee colonies. As previously discussed beekeeping practices such as hive propagation, honey harvesting and colony migration/movement appear to be key contributors to

brood disease development. However, beekeeping-breeding programs, which select for desirable traits, may limit other naturally important traits.

Suggested management recommendations

Investigations undertaken in my study showed that supplementary feeding of infected colonies is unsuccessful in assisting their recovery. This is possibly because the disease was too advanced and there were limited numbers of age-appropriate workers to perform tasks which could help in colony recovery. However, it is possible that supplementary feeding at an earlier stage of disease infection may be beneficial. Future work should consider the amount of supplementary feeding required, its quality and particularly the nutritional requirements of the colony.

The following management recommendations are likely to reduce disease incidence in stingless bees:

1. Limit the movement and sale of colonies outside their natural distribution, regardless of the provision of artificial cooling and or heating. This may also mean restricting movement QLD colonies to central or southern NSW (especially if it is shown that there is a difference in climate-responsiveness between different provenances). Apart from the possible increase in temperature related stress problems between the different climatic conditions, this strategy would reduce the risk of pest and disease movement in what is currently an unregulated industry.
2. Colonies should be located where there is an adequate supply of suitable foraging resources including pollen, nectar and resins within their 500 m flight range (Heard & Dollin 1998).
3. The number of times hives are opened, split for propagation or harvested should be restricted in marginal areas. If required, these activities should be scheduled between late October to late November as this timing provides good foraging temperatures and resource availability, while still allowing adequate time for colonies to repair nest structures, replenish nest stores, and increase brood populations before the onset of cooler weather. This is already

recommended for the stingless bee industry (Klumpp & Heard 2005, Klumpp 2007).

4. Undertake sanitary practices, such as cleaning of hive tools and other hive equipment during management activities such as hive health inspections, colony propagation, and honey harvesting. Other sanitary or quarantine practices to reduce disease spread include: limiting robbing of deceased or newly opened colonies by neighbouring colonies, or limiting movement of nest stores (pollen and nectar pots) between colonies for supplementary feeding.

   Currently, any suspected or confirmed *L. sphaericus*-infected *T. carbonaria* and *Au. australis* colonies are recommended to be destroyed, as per the protocol for AFB-infected *A. mellifera* colonies under the *NSW Apiaries Act 1985 No.16*,.

5. Pollination services in field and glasshouse crops should use colonies on a strict rotation, enabling weakened colonies to build up and replacing them with strong, healthy colonies. This should also reduce other problems associated with monocultures; such as monofloral honey and pollen stores and decreased social immunity to diseases (Pasquale et al. 2013, Erler et al. 2014).

   Beekeepers should also consider the type of crop requiring pollination and the time of the year, to determine whether colonies should be used for this purpose, as unsuitable conditions will lead to declined colony strength and vigour.

6. Whenever possible, monocrops for pollination be interplanted, or surrounded by other native vegetation. This has been shown to be beneficial for honey bee health (Park et al. 2015).

6.3 Practical applications and industry communication of my findings

As the infant industry of meliponiculture grows in Australia steps are required now to educate beekeepers, and legislation should also be considered to oversight colony

movement and management practices in this industry, particularly to limit future pest and disease problems. In Australia, each state has a Department of Primary Industries or equivalent, which requires all honey beekeepers to register any managed *A. mellifera* colonies, making the beekeepers accountable for hive monitoring, and recording of any colony movements following, for example, the Beekeeping Code of Practice (2003, NSW). A draft Australian Honey Bee Industry Biosecurity Code of Practice (2015) outlines the requirements of beekeepers to conduct at least two hive inspections per year and record any pests and diseases, especially *P. larvae* which is also a notifiable disease under the *NSW Apiaries Act 1985 No.16*, with infected hives subject to mandatory treatment (hive destruction or irradiation). This action has resulted in successfully reducing the incidence of AFB. The development of a similar program would be an important step to assist in monitoring stingless bee colony movements between and within states (at present, for example, *T. carbonaria* colonies are transported by postal couriers), and enable recording of annual colony health checks. It is further recommended that the institutions providing diagnostic services for honey bee pests and diseases consider including stingless bee disease testing into their assays.

Interestingly, an online database system, 'Beetracker', was established in 2012 (http://tracker1.beesbusiness.com.au/ accessed 2015), for voluntary registration of GPS coordinates of both wild and managed nests. Additionally, the Atlas of Living Australia (http://www.ala.org.au/, accessed 2015) contains information of all known bee species in Australia and is accessible to museums, herbaria, community groups, government departments, universities, and individuals. These two resources could provide a useful base for the recording of managed and wild stingless bee colonies.

A number of workshops on stingless bee management have been conducted in NSW and QLD, and it is intended that future workshops be provided with information on stingless bee brood diseases and their suggested management. It was evident over the course of my study, that the provision of educational resources assisted new as well as experienced beekeepers.

As the Australian stingless beekeeping industry continues to develop and the number of stingless beekeepers increases, so do nest observations. Since my discovery of brood disease, there have been a number of other reports by beekeepers experiencing colony losses from unknown causes, some of which have been linked to a possible bacterial cause. Consequently, there is need for provision of readily available information to assist particularly amateur beekeepers. An initial information sheet 'New Australian stingless bee disease' (Appendix A4.3) was developed and distributed to stingless beekeepers in December 2014 (M. Halcroft and J. Shanks), to provide information about the thesis findings, as well as providing advice on suggested management practices. Australia's stingless bee industry has the potential, in these early stages of its development, to implement good practices that will hopefully limit the disease and the associated problems. It is anticipated that as more brood problems and, probably, other diseases and pests are identified, further support will be required.

6.4 Recommendations for future research

There were a number of limitations associated with the work reported in this thesis. First, the study was conducted in Richmond NSW, which is climatically different to many other areas where *T. carbonaria* is kept. Richmond is regarded as a marginal zone for their distribution range (Sharpe, 2014), and as a result many of the reported behaviours, stresses, and disease-related problems might have been enhanced. In retrospect, it was advantageous that the study was conducted here because the brood disease may have not have been otherwise discovered during this time. Nevertheless, given recent reports of brood disease from more favourable locations, the findings remain highly relevant. There was limited replication of hive numbers for some investigations, for logistical reasons, and as previously discussed, repeated measures on one biological organism (Appendix A2.2) (or in this case a super-organism (Seeley 1989, Moritz & Southwick 1992, Moritz & Fuchs 1998, Tautz 2008) may significantly alter expression of behaviour. However, in comparison to my study, replicate numbers in other studies (e.g. for hygienic behaviour) were much lower (8–10 colonies) (Medina et al. 2009, Nunes-Silva et al. 2009). The nest products used for inhibition assays and chemical compound analysis were only sourced from the

colonies located in Richmond, and as a result are limited in the antimicrobial properties displayed by the botanical sources available at the time, in this location. However, my study is the first to investigate Australian temperate region-collected stingless bee propolis.

While the findings reported in this thesis answered a number of questions about brood disease in *T. carbonaria*, a number of further investigations are recommended to better understand this topic and its implications.

Colony biology

1. Investigate the temperature tolerance of *T. carbonaria* workers, including their lethal threshold temperatures. Determine the heat contribution generated by worker thoracic temperature rises with and without consumption of honey.
2. Compare hygienic behaviour of *T. carbonaria* colonies, comparing freeze-killed with pin-killed assays. Several studies have suggested that pin-killed brood may result in faster hygienic behaviours, due to stimulus created by direct damage of the cell, release of odours, and excretion of bodily fluids (Spivak & Downey 1998, Espinosa-Montaño et al. 2008), which may overestimate hygienic behaviour, as brood disease is rarely associated with bodily fluids or brood cell damage.
3. Conduct hygienic behaviour experiments on other Australian stingless bee species. A preliminary pin-kill investigation was undertaken with *Au. australis* as part of this study (Appendix A2.1), but the different brood structure was more suitable for freeze-kill assay.
4. Investigate the genetics of hygienic behaviour in key stingless bee species. In Australia, the most likely target would be *T. carbonaria*, because of its relatively widespread use. Studies could be based on similar work undertaken for *A. mellifera*. This work is critical if the industry envisages selection and propagation of colonies for hygienic behaviour.

Antimicrobial nest products

1. Further explore the inhibitory activities of stingless bee propolis and honey bee pathogens, this could be conducted alongside the extensive work

regarding antibacterial and antifungal properties of tropical *T. carbonaria* propolis and human pathogens.

Microbial diversity

Despite one published study (Leonhardt & Kaltenpoth 2014) on gut microbial diversity of *T. carbonaria, T. hockingsii* and *Au. australis* from S.E. Queensland using Next Generation Sequencing, little is known about the gut microbial diversity in Australian stingless bees, and their influence on bee health. Data from NGS of *T. carbonaria* gut samples from colonies across their distribution range were collected over the course of my study with the aim of exploring bacterial and fungal diversity and identifying isolates of interest for their inhibitory abilities, such as novel LAB. This extensive data still requires exploration, and future studies should aim to identify gut microbes with potential to inhibit bee pathogens.

Stingless bee diseases

The documentation and identification of the first brood disease pathogen, opens the door for more studies into this stingless bee brood disease. Such work could involve:

1. Further elucidating the pathogen, *Lysinibacillus sphaericus*, including identification of the strain(s) in *T. carbonaria* and *Au. australis*.
2. Determining the pathogenicity of *L. sphaericus* isolated from stingless bee colonies. Studies should further investigate modes of action including but not limited to: toxin genes, toxin crystal production, other toxin producing proteins, and non-toxin pathogenicity. This may also help identify host-pathogen specificity.
3. Explore modes of host entry. Does the bacterium naturally occur in the host's microbial diversity? Examination of NGS gut microbe data from managed and wild populations for the presence of *L. sphaericus* will help in understanding the incidence and pathogenicity of this bacterium.
4. Develop measures for control/management of brood pathogens in stingless bee colonies, alternative to antibiotic treatments, by addressing the following questions. Is developing hygienic colonies important? Is treatment with propolis a useful measure to boost immunity and health of colonies?

Educating beekeepers on sanitary management practices is an important overall aim to reduce disease incidents.

5. Explore the possibility of the implementation of either a state or national stingless bee association which upholds the rules and regulations in the beekeeping practices of hives used for commodity production, pollination services, and selling nests/products. Reviewing the rules and regulations of the honey bee industry may provide insight into how the stingless bee industry could progress.

Biocontrol impacts of *L. sphaericus*

A host-specific, highly toxic strain of *L. sphaericus* has been used to treat water sources in Australia and elsewhere to control mosquitoes. Although this strain is different to the one identified in my study, it nevertheless raises the issue of their potential damage to bee populations, in particular native bee species. While studies on the effects of entomopathogens on non-target pests including honey bees are conducted for registration purposes, these are primarily based on the in-use situation (e.g., crops in bloom). Most ecotoxicology assessments of aquatic biocontrol agents are tested against non-target aquatic organisms such as fish, shrimps, dragonflies, and other aquatic invertebrates (Lacey & Merritt 2003, Brown et al. 2004, Merritt et al. 2005). It is suggested that bee species should also be included in the assessment of entomopathogens, if they are used to treat water sources where direct contact may be made, or uptake by bees from pollen and nectar sources could occur.

6.5 Conclusion

Until the work described in this thesis was undertaken, little information was available about brood diseases in stingless bee colonies and the inherent mechanisms for disease suppression. In fact, unlike *A. mellifera*, there was really no convincing evidence of brood diseases in stingless bees, which initially prompted the study. The behavioural investigations, including the hygienic behaviour work, are the first of their kind in Australia, and make a significant contribution to our knowledge of stingless bees biology and the role this behaviour may play in limiting the incidence of brood diseases, at least in *T. carbonaria*.

The confirmed antimicrobial activity of nest products against honey bee pathogens was useful in understanding the likely disease suppression characteristics of structures in a stingless bee nest compared to a honey bee nest, especially for brood pathogens. Stingless bees' behaviour of collecting plant resins and incorporating them into nest structures, may be a major contributing factor to lower disease incidence compared to *A. mellifera*.

Despite these positive attributes, the confirmation that stingless bees are susceptible to brood disease is a significant and novel contribution to both the stingless bee and honey bee industries. There is now an overwhelming need for research into brood pathogens of stingless bee colonies globally. As the management practices of stingless bees continue to develop, the risk of brood diseases increases. The results presented here provide a base for future work on stingless bee brood diseases and their management.

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Appendices

Appendix 1: Supplementary behavioural videos

Worker wing fanning behaviours for nest ventilation regarding thermoregulation studies, and behaviours of workers before and during hygienic behaviour experiments are provided as mp3 formatted videos in the accompanying USB drive.

Video 1: Worker observed in the tubing connecting nests to the outside, fanning their wings away from the nest (15 sec).

Video 2: Workers taking turns to provision a brood cell prior to egg laying (69 sec).

Video 3: Workers are observed to move quickly away from a queen if she appears in the brood chamber, and if she bumps into workers (11 sec).

Video 4: Cells get closer to being ready for egg laying, worker numbers increase and activity and excitement becomes elevated. The queen is observed laying a single in cells (80 sec).

Video 5: Placement of resin balls in the test area, on the pinned cells. Workers are observed both placing and removing resin balls (179 sec).

Video 6: Uncapping. Workers observed singly or in small groups removing cerumen caps. Cells are observed partially or completely uncapped (149 sec).

Appendix 2: Hygienic behaviour in stingless bees

A2.1 Does colony strength determine the success of hygienic behaviour

Correlation analysis of EBP, hive weight, worker entrance activity and time taken for colonies to complete hygienic behaviour stages are provided for both Day 1 and Day 2 of testing.

Table A-2a Pearson's correlation analysis between hygienic behaviour tasks for day 1 and day 2 and colony strength determinants. Both *r*- and *p*- values are provided, statistically significant results are determined by $p = 0.05$.

	Detection		**Uncapping**		**Removal**	
	Day 1	**Day 2**	**Day 1**	**Day 2**	**Day 1**	**Day 2**
EBP	r = -0.127 *p* = 0.709	r = -0.809 *p* = 0.003	r = 0.204 *p* = 0.547	r = -0.363 *p* = 0.272	r = -0.335 *p* = 0.284	r = 0.001 *p* = 0.998
Weight	r = 0.533 *p* = 0.140	r = -0.530 *p* = 0.142	r = 0.266 *p* = 0.489	r =-0.207 *p* =0.594	r = -0.161 *p* = 0.680	r = 0.237 *p* = 0.539
Entering worker activity	r = -0.197 *p* = 0.612	r = 0.116 *p* = 0.765	r = -0.702 *p* = 0.035	r = -0.415 *p* =0.266	r = -0.203 *p* = 0.601	r = 0.252 *p* = 0.512
Exiting worker activity	r = -0.193 *p* = 0.619	r = -0.007 *p* = 0.987	r = -0.625 *p* = 0.057	r = -0.506 *p* =0.164	r = -0.262 *p* = 0.496	r = 0.297 *p* = 0.438

A2.2 Preliminary experiment to test hygienic behaviours of *Austroplebeia australis*

A preliminary experiment was performed with an *Au. australis* colony using the same methodology used for *T. carbonaria*. *Austroplebeia australis* is a cluster brood builder and this made selecting 50 cells of the same age in one viewing area difficult. Also the strength of the individual cells and the involucrum support structures were

weaker compared to *T. carbonaria* which meant, upon piercing the cell, the entire side of the cell structure and surrounding support structures, collapsed (Figure A-2a). Therefore, appropriate pinning of pupae crown to kill the individual was uncertain. After 24 h, what was believed to be pin-killed cells, had the caps resealed (Figure A-2b). This indicated that pin-killing was not successful in killing the individuals. It is suggested that freeze-kill is used as an alternative for hygienic behaviour testing of a cluster builder. The method would include removing a section of the brood and freezing the section for 24 h at -20ºC or using liquid nitrogen, then re-introducing the brood into the hive for hourly observations.

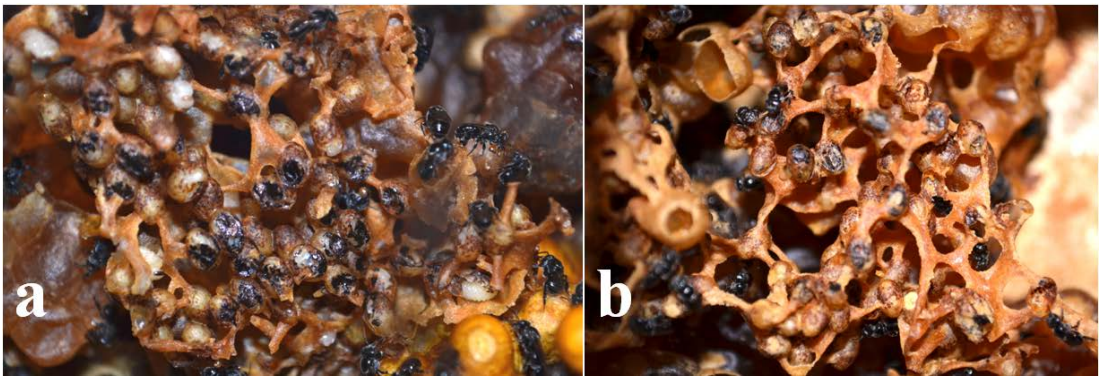

Figure A-2a Testing hygienic behaviour of *Au. australis* using the pin-kill method. A) Permanent black marker was used to mark the 25 pinned cells, difficultly was experienced in selecting 25 cells next to each other at the same age, as well as 25 control cells. B) Pin-killed *Au. australis* cells after 24 h. Cells previously pinned appear fixed and remain in the brood chamber, these pupae may not adequately been killed.

A2.3 Repeated measures with biological organisms and the effects on development and behaviours

A UWS single hive was repeatedly measured (20 readings) for hygienic behaviours over seven months. Data was analysed to evaluate the effects of continuous testing on the colony's performance, and hive development.

There was a significant difference in the time taken for this hive to detect dead brood after being repeatedly tested (χ^2 (6) = 14.28, p = 0.027, Figure A-2b). There were significant differences in the time taken for to uncap the first cell ($F_{6, 13}$ = 4.096, p = 0.016), after the hive was repeatedly measured (Figure A-2b). The time taken to uncap the first cell in January 2012 (25 ± 7.3 min) and February 2012 (38.5 ± 9.4 min), was faster compared to uncapping times in July 2012 (132.5 ± 17.5 min, p = 0.010, and p = 0.026, respectively) (Figure A-2b). There were no other differences in time taken to uncap, and no significant differences in the total time taken to complete hygienic behaviours despite being repeatedly measured (χ^2 (6) = 10.56, p = 0.103).

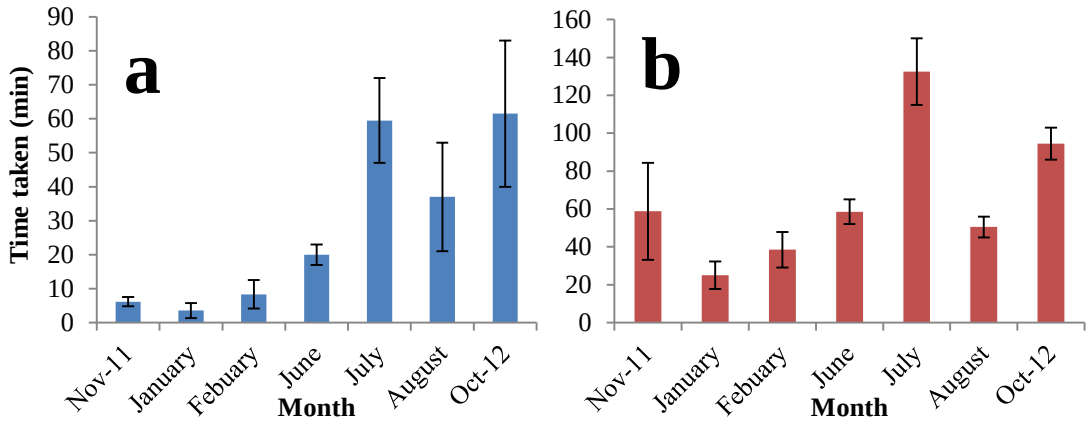

Figure A-2b Mean time taken (min) for a hive repeatedly measured from November 2011 to October 2012, a) detection and b) uncapping stages. Error bars = SE of means.

There were no significant differences in the percentages of time devoted to detection (p = 0.052), uncapping (p = 0.159) and removal (p = 0.062) despite the hive being repeatedly measured Figure A-2c.

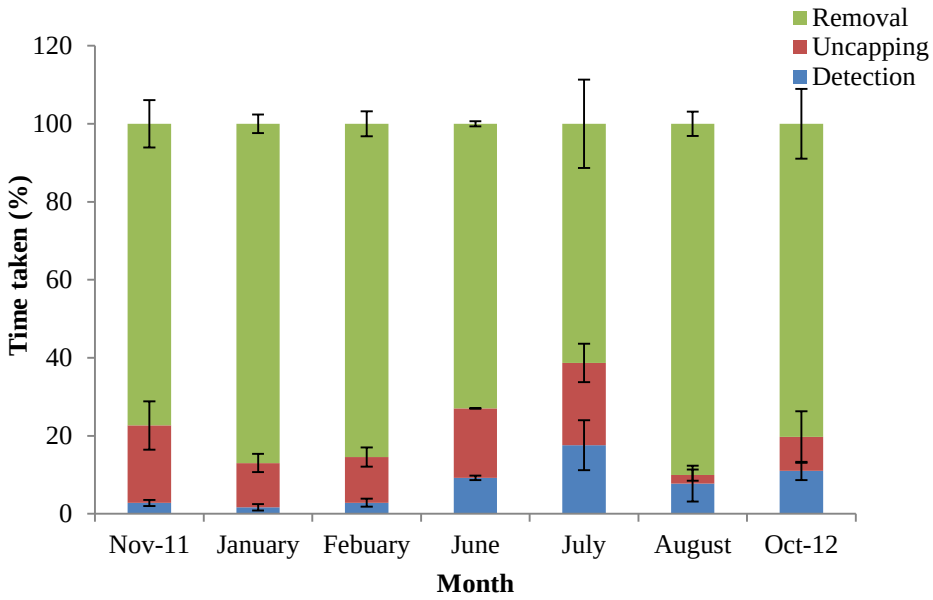

Figure A-2c Percentage of time to perform each hygienic behaviour task, repeatedly measured from November 2011 to October 2012. Error bars = SE of means.

A possible impact on the recorded hygienic behaviour results would likely have been the destructive process of experimentation and assessment. In this hive, in which 20 experiments were deliberately conducted over a seven month period, the fitness of the colony changed, becoming weak, eventually dying out. There were 500 brood cells experimentally destroyed, however this does not include the unavoidable destruction of brood cells and adults during the opening and closing processes of the methodology. Repeatedly opening the nest to test for hygienic behaviours weakened the hive by loss of colony members which affected the population and strength of the nest. The associated reduction in nest population is likely to have impacted on decreased hygienic behaviour recorded over time in this hive The significant increase in uncapping time in July compared to January and February and the changes in the time allocated for each task may be associated with the lack of age appropriate brood workers. The estimated brood population dropped from 2501 in June to 881 in August, this decreased the number of new workers. The workers that remained were aging and moving from the brood chamber.

Nest weight decreased from 1.5kg (June) to 890g (August) and 590g (November). This loss may be associated with worker numbers, but more likely related to reduction in honey and pollen weight. There was a decrease in foraging during the cooler months and colonies rely heavily upon nest stores. During the cooler testing period, colonies would have experienced stress impacted from the hygienic testing, food availability, natural population losses and lack of developing pupae. These would contribute to a decline in nest strength. Opening and closing a hive has the potential to damage a queen which affects laying ability and eventually brood development stops (Klumpp 2007). The hive died in January with no queen, dried non-developing brood and the adult population was less than 10 workers.

Considerations in methodology designs should address the effects of exposing organisms to conditions that affect the growth, development, and behaviour which can affect results, when using biological organisms and repeated replicates for behavioural studies.

Appendix 3: Inhibition of bacterial and fungal pathogens with bee products

A3.1 Digestive system extraction

The isolation method used to extract lactic acid bacteria, *Weissella hellenica* from the digestive system of *T. carbonaria* workers is outlined in Chapter 4, however in order to achieve this outcome, a number of trial methods per performed. Outlined below are four methods that show the development of the protocol used to isolate lactic acid bacteria.

1. One dissected digestive system was placed into 20 µL of sterile water. This gut solution was trialled against *P. larvae* in the disc diffusion assays. No inhibition occurred against bacterial pathogens.
2. Ten adult digestive systems were placed into 40 µL sterile water, and the solution used for disc diffusion as above. Nno inhibition against bacterial pathogens.
3. Fifty adult digestive systems were extracted and placed into 1 mL of sterile water and treated following the Nanosep® Centrifugal Device (Pall Corporation, Surry Hills, NSW) protocol. The digestive system sample spilt into two tubes, each with 500 µL, the tubes were centrifuged for12 min at 12,000 rpm (Microfuge16 microcentrifuge, Beckman Coulter Inc., Brea, California, USA). This extraction method provided two products. A sample from each extraction product was used for the disc diffusion, no inhibition.
4. One dissected gut was diluted (1:10) and lawn plated onto four different media types: potato dextrose agar (PDA), sabouraud dextrose agar (SDA) (Difco™, Bacto Laboratories Ltd. Pty. Mt Pritchard, NSW), nutrient agar (NA) and sheep blood agar (SBA) containing antibiotic nalidixic acid (3 mg/ mL) (Oxoid, Thermofisher Scientific, Massachusetts, USA), to obtain culturable organisms to test for inhibition ability. Six different micro-organisms were isolated, and Gram-stained following manufactures protocol (Fluka Analytical, Sigma-Aldrich, Missouri, USA).

   A small drop from a single isolated bacterial colony was placed in the centre of a glass slide, along with a drop of sterile water. The bacteria and water was mixed together to form a smear and left to dry. Once dry a few drops of

Gram's crystal violet solution were dispensed to cover the smear and set aside to dry for 1 min. After which the excess dye was removed by dropping sterile water across the slide. Gram's iodine solution was next used to promote dye retention by again a few drops to cover the smear. The dye was left for 1 min and removed as previous. The third step used ethanol to decolourise the bacterial dyed smear. Holding the slide on an angle, 1–3 drops of Gram's decolouriser solution (ethanol) were placed at the top of one edge of the slide and allowed to run the length until most of the dye was removed. The decolourised smear was rinsed again in sterile water. Finally a few drops of Gram's safranin solution were dispended to cover the smear and left for 1 min, after which excess dye was removed via sterile water and the slide set aside until completely dry. Once dry, a coverslip was placed on top of the smear, with a drop of immersion oil and the slide was viewed at 400× with a Nikon Eclipse E200 light microscope (Nikon Eclipse E200, Nikon Corporation, Tokyo, Japan). Gram-positive bacteria appeared as purple spores, Gram-negative as pink bacterial spores. Spore suspensions of each cultured microorganism were made (one sterile loop of isolate into 500 µL sterile water) and tested for inhibition ability against *P. larvae*. No inhibition was observed from five tested bacteria and one yeast product.

A3.2 *Paenibacillus larvae* inhibition

Provided in the next sections are the inhibition measurements (including means and standard deviations) from bacterial and fungal assays.

Table A-3a Zone of inhibition for *P. larvae* (mm) with *T. carbonaria* (Tc) and *A. mellifera* (Am) nest products (three replicates per treatment). Two measurements per plate at 24 and 48 h.

		24HOURS				48HOURS			
		Mean diameter (mm)				Mean diameter (mm)			
Treatment	Rep	1	2	MEAN	STDEV	1	2	MEAN	STDEV
Negative	1	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0
DMSO	1	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0

Tetracycline hydrochloride	1	55.00	48.00	51.50	4.95	60.00	57.00	58.50	2.12
	2	54.00	51.00	52.50	2.12	63.00	55.00	59.00	5.66
	3	56.00	53.00	54.50	2.12	60.00	59.00	59.50	0.71
Weissella hellenica	1	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0
Tc pollen	1	16.00	17.00	16.50	0.71	21.00	21.00	21.00	0
	2	15.00	17.00	16.00	1.41	20.00	21.00	20.50	0.71
	3	16.00	16.00	16.00	0.00	22.00	21.00	21.50	0.71
Tc honey	1	16.00	17.00	16.50	0.71	16.00	17.00	16.50	0.71
	2	18.00	19.00	18.50	0.71	18.00	19.00	18.50	0.71
	3	16.00	19.00	17.50	2.12	16.00	19.00	17.50	2.12
Tc propolis	1	24.00	20.00	22.00	2.83	35.00	36.00	35.50	0.71
	2	24.00	27.00	25.50	2.12	35.00	34.00	34.50	0.71
	3	29.00	23.00	26.00	4.24	39.00	27.00	33.00	8.49
Am pollen	1	12.00	11.00	11.50	0.71	12.00	11.00	11.50	0.71
	2	10.00	11.00	10.50	0.71	10.00	11.00	10.50	0.71
	3	12.00	12.00	12.00	0.00	12.00	12.00	12.00	0.0
Am honey	1	12.00	11.00	11.50	0.71	12.00	11.00	11.50	0.71
	2	17.00	18.00	17.50	0.71	17.00	18.00	17.50	0.71
	3	14.00	13.00	13.50	0.71	14.00	13.00	13.50	0.71
Am propolis	1	21.00	21.00	21.00	0.00	25.00	22.00	23.50	2.12
	2	16.00	16.00	16.00	0.0	18.00	19.00	18.50	0.71
	3	18.00	20.00	19.00	1.41	25.00	26.00	25.50	0.71

Table A-3b Mean zone of inhibition (mm) of *P. larvae* after treatment with *T. carbonaria* and *A. mellifera* products. SE = Standard error of the means.

AFB	Negative	DMSO	Positive	*Weissella hellenica*	SB pollen	SB honey	SB propolis	HB pollen	HB honey	HB propolis
Mean (mm) 48h	0	0	59	0	21	17.5	34.3	11.3	14.2	22.5
SE	0	0	0.289	0	0.289	0.577	0.727	0.441	1.764	2.082

A3.3 *Ascosphaera apis* inhibition

Data collected from *A. apis* mycelial inhibition is provided including the means and standard deviation and standard errors. After which, all possible relationships between *A. apis* growth and nest products are provided, these are arranged by nest product.

Table A-3c Area of inhibition (mm^2) of *A. apis* mycelial growth inhibition with *T. carbonaria* and *A. mellifera* nest products. Means, standard deviation (STDEV) and standard error (SE) are given.

Area of *A. apis* mycelial growth (mm^2) per treatment														
	T. carbonaria								*A. mellifera*					
Treatment concentration (% w/v)	**Gut**	**STDEV**	**Pollen**	**STDEV**	**Honey**	**STDEV**	**Propolis**	**STDEV**	**Pollen**	**STDEV**	**Honey**	**STDEV**	**Propolis**	**STDEV**
Negative	20.61	27.93	39.09	20.22	31.15	17.72	35.20	30.66	13.20	23.82	21.13	23.70	27.05	17.60
Negative	21.26	23.26	34.94	21.45	37.20	23.63	39.07	17.24	11.37	16.47	16.74	27.74	12.84	27.41
Negative	9.03	18.31	36.67	25.77	36.84	29.94	37.42	24.38	33.65	15.19	16.66	37.79	29.76	21.18
Negative	13.99	19.58	33.81	22.17	32.26	37.62	42.68	19.53	32.92	11.85	41.62	24.37	12.53	21.23
Negative	38.25	19.39	40.07	25.48	37.00	17.74	39.35	16.62	33.90	24.82	40.98	19.47	19.21	24.52
Negative	40.47	17.75	37.75	27.39	36.53	16.68	41.20	16.40	34.07	16.80	15.97	28.80	35.52	12.93
Negative	36.55	21.43	28.03	21.62	36.38	17.01	40.47	32.17	35.78	25.36	40.06	19.78	33.39	13.33
Negative	14.30	10.91	33.51	28.71	31.43	15.27	36.62	15.91	13.98	19.43	40.84	19.98	20.99	24.16
Mean	24.31		35.48		34.85		39.00		26.11		29.25		23.91	
STDEV	12.35		3.84		2.71		2.49		11.03		12.53		8.88	
SE	4.37		1.36		0.96		0.88		3.90		4.43		3.14	
Copper sulphate	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Copper sulphate	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Copper sulphate	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Copper sulphate	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Copper sulphate	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Copper sulphate	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Copper sulphate	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Copper sulphate	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mean	0.00		0.00		0.00		0.00		0.00		0.00		0.00	
STDEV	0.00		0.00		0.00		0.00		0.00		0.00		0.00	
SE	0.00		0.00		0.00		0.00		0.00		0.00		0.00	

5.00		13.40	23.16	2.21	12.38	0.00	0.00	0.00	0.00	28.65	18.30	0.00	0.00	0.00	0.00
5.00		22.60	10.04	3.57	24.78	4.10	7.27	0.00	0.00	37.10	25.31	0.00	0.00	0.00	0.00
5.00		21.74	8.94	0.00	0.00	0.00	0.00	0.00	0.00	27.33	25.77	0.00	0.00	0.00	0.00
5.00		9.92	13.08	1.59	18.51	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5.00		17.98	18.67	0.00	0.00	0.00	0.00	0.00	0.00	17.68	18.31	0.00	0.00	0.00	0.00
5.00		18.94	21.53	2.77	16.33	0.00	0.00	0.00	0.00	32.32	17.66	0.00	0.00	0.00	0.00
5.00		29.68	11.15	24.84	20.73	0.00	0.00	0.00	0.00	0.00	0.00	17.84	11.86	0.00	0.00
5.00		11.36	23.21	14.72	17.97	0.00	0.00	0.00	0.00	31.66	18.73	37.71	20.31	0.00	0.00
	Mean	18.20		6.21		0.51		0.00		21.84		6.94		0.00	
	STDEV	6.58		8.89		1.45		0.00		14.57		13.91		0.00	
	SE	2.32		3.14		0.51		0.00		5.15		4.92		0.00	
2.50		13.80	22.20	38.68	18.21	6.29	30.15	2.63	12.39	24.17	17.34	0.00	0.00	0.00	0.00
2.50		17.01	9.76	37.59	22.46	8.06	22.99	3.33	17.14	0.00	0.00	18.52	20.96	0.00	0.00
2.50		11.26	22.16	37.97	27.30	7.80	32.12	0.00	0.00	27.59	23.19	0.00	0.00	0.00	0.00
2.50		13.49	8.31	36.03	28.93	11.74	27.19	5.39	11.02	28.41	19.53	23.11	17.36	0.00	0.00
2.50		13.85	11.78	36.89	24.19	11.66	11.62	2.93	12.70	29.74	20.41	0.00	0.00	0.00	0.00
2.50		14.12	25.19	38.48	31.56	15.38	17.51	0.00	0.00	33.09	17.72	23.57	18.65	0.00	0.00
2.50		6.74	20.21	37.16	19.46	23.62	23.28	0.00	0.00	26.59	20.57	5.41	9.68	0.00	0.00
2.50		12.88	16.72	35.67	12.27	0.00	0.00	2.50	10.13	12.22	12.30	38.07	16.94	0.00	0.00
	Mean	12.89		37.31		10.57		2.10		22.73		13.58		0.00	
	STDEV	2.96		1.09		6.97		1.95		11.06		14.32		0.00	
	SE	1.04		0.39		2.46		0.69		3.91		5.06		0.00	
1.25		19.13	26.02	39.28	24.43	15.88	21.67	31.94	21.13	23.82	21.87	38.11	21.00	2.52	10.93
1.25		13.95	17.64	38.50	23.28	3.56	9.28	36.67	22.48	33.77	15.59	5.70	21.58	0.00	0.00
1.25		9.32	17.32	36.84	21.45	24.42	13.51	16.35	11.10	30.19	19.31	9.95	20.43	0.00	0.00
1.25		16.08	19.84	37.22	26.51	11.08	9.11	38.98	22.36	30.34	13.41	8.30	18.37	0.00	0.00
1.25		16.39	6.33	19.72	38.21	24.44	10.45	12.27	10.55	31.74	11.96	8.07	28.94	0.00	0.00

1.25		13.53	19.80	36.32	21.43	6.44	27.07	27.94	23.15	25.01	20.56	7.54	31.96	0.00	0.00
1.25		14.14	5.98	21.87	35.51	23.00	18.89	36.23	19.67	29.09	14.16	8.30	21.95	0.00	0.00
1.25		21.58	16.44	33.73	13.30	8.89	14.53	41.16	22.46	33.30	20.66	15.35	25.42	0.00	0.00
	Mean	15.51		32.93		14.71		30.19		29.66		12.66		0.31	
	STDEV	3.73		7.69		8.44		10.67		3.61		10.66		0.89	
	SE	1.32		2.72		2.98		3.77		1.28		3.77		0.31	
0.63		18.19	19.44	21.29	36.90	9.21	27.04	42.65	19.24	27.47	11.52	28.82	22.35	22.37	9.41
0.63		20.50	10.40	36.65	21.81	24.91	15.94	43.27	19.83	32.28	18.22	1.96	29.82	29.87	30.44
0.63		30.48	15.33	37.71	23.59	9.87	17.65	38.45	22.95	32.96	9.94	0.00	0.00	0.00	0.00
0.63		37.41	15.78	33.17	17.43	18.22	16.81	39.90	38.23	32.10	14.51	8.11	21.58	2.63	15.10
0.63		9.41	25.55	29.82	35.20	10.72	13.38	40.14	23.34	12.51	17.91	15.61	22.37	0.00	0.00
0.63		12.06	17.72	35.91	20.79	5.16	12.92	37.82	20.48	0.00	0.00	10.05	30.46	0.00	0.00
0.63		26.72	15.37	3.61	34.62	4.50	12.84	40.41	28.58	34.60	16.86	2.00	32.90	0.00	0.00
0.63		10.88	19.57	37.08	17.55	13.38	12.66	39.59	23.89	15.94	22.72	8.63	15.14	0.00	0.00
	Mean	20.71		29.40		12.00		40.28		23.48		9.40		6.86	
	STDEV	10.11		11.76		6.81		1.88		12.59		9.39		12.09	
	SE	3.58		4.16		2.41		0.66		4.45		3.32		4.27	
0.31		16.53	23.01	38.52	20.63	18.69	42.57	37.39	24.42	34.93	27.65	17.18	35.38	30.40	15.36
0.31		38.91	20.86	39.43	21.59	38.34	13.63	36.56	20.20	32.11	11.66	30.49	28.11	21.57	10.98
0.31		16.51	18.61	28.85	28.29	4.30	18.70	23.92	24.74	32.88	11.00	10.41	29.63	26.55	18.61
0.31		10.16	30.09	40.28	31.87	14.37	12.75	30.45	26.94	35.14	10.38	11.38	17.77	1.74	10.79
0.31		10.45	21.67	21.79	33.72	6.16	20.80	18.45	31.64	39.12	11.78	17.85	19.97	0.00	0.00
0.31		21.14	30.57	25.64	28.81	7.08	14.86	19.31	25.77	35.42	14.40	10.01	23.32	22.55	14.81
0.31		12.58	30.34	41.03	26.08	0.00	0.00	37.61	27.68	36.37	12.43	6.23	28.69	28.14	10.66
0.31		15.09	24.06	38.95	13.46	13.34	22.28	35.91	13.75	26.04	18.73	14.12	19.88	25.54	23.74
	Mean	17.67		34.31		12.79		29.95		34.00		14.71		19.56	
	STDEV	9.31		7.63		11.97		8.24		3.86		7.45		11.88	

	SE	3.29		2.70		4.23		2.91		1.36		2.63		4.20	
0.16		14.47	19.38	37.98	34.54	27.34	30.18	37.08	36.21	37.21	44.43	13.25	19.33	29.84	19.04
0.16		34.37	15.94	16.28	29.91	13.93	14.93	18.83	25.08	31.53	18.20	0.00	0.00	34.99	17.60
0.16		6.34	10.28	14.98	33.75	11.39	22.93	26.55	22.48	33.82	12.51	16.70	33.89	26.29	18.93
0.16		37.92	19.03	39.23	22.22	4.80	6.76	36.23	20.64	34.76	14.68	9.12	14.95	25.82	22.20
0.16		6.51	31.10	40.08	37.17	29.22	12.72	33.88	32.44	16.50	28.46	17.11	31.70	18.72	15.33
0.16		21.92	7.19	39.50	22.87	23.02	7.95	35.98	24.54	18.98	24.92	8.89	21.41	30.54	19.42
0.16		18.42	7.03	38.29	22.03	6.75	12.45	38.36	25.42	9.24	31.51	0.00	0.00	16.59	19.75
0.16		15.83	10.93	21.01	24.79	12.05	16.67	19.58	21.64	32.27	14.37	13.85	24.61	22.96	22.62
	Mean	19.47		30.92		16.06		30.81		26.79		9.87		25.72	
	STDEV	11.64		11.32		9.29		8.01		10.34		6.79		6.17	
	SE	4.12		4.00		3.29		2.83		3.66		2.40		2.18	
0.08		12.42	22.23	35.61	37.82	32.69	20.22	30.97	38.80	15.34	19.00	12.47	27.60	30.91	19.97
0.08		31.35	13.17	36.54	19.28	33.92	15.01	27.36	20.50	27.88	15.63	7.98	36.94	9.84	21.31
0.08		26.11	8.62	39.94	23.15	32.75	17.61	30.39	18.76	12.44	14.54	13.09	29.77	18.67	30.34
0.08		14.18	13.41	40.47	26.53	35.40	20.93	37.58	28.53	15.17	21.49	13.69	40.03	38.86	16.72
0.08		9.46	29.76	11.00	20.20	38.35	17.74	19.10	20.68	33.99	11.76	10.21	36.53	38.00	15.38
0.08		19.93	6.88	39.00	18.62	11.33	36.31	37.55	18.95	13.08	16.24	11.47	22.94	39.48	16.73
0.08		6.72	12.58	12.06	21.21	24.38	13.26	22.96	17.83	10.86	16.50	36.62	36.89	31.82	10.67
0.08		9.62	14.79	12.67	38.61	16.28	32.57	16.41	22.57	14.42	16.82	1.34	24.70	39.23	18.38
	Mean	16.22		28.41		28.14		27.79		17.90		13.36		30.85	
	STDEV	8.77		13.77		9.78		7.90		8.34		10.20		11.03	
	SE	3.10		4.87		3.46		2.79		2.95		3.61		3.90	
0.04		24.54	42.97	19.31	28.43	12.59	24.86	24.90	20.28	10.29	16.98	12.56	18.19	23.15	20.35
0.04		7.98	8.34	40.95	20.40	12.02	18.13	28.21	21.94	39.87	26.29	12.41	20.77	30.73	12.56
0.04		14.98	8.04	7.56	31.31	32.56	13.72	14.06	31.09	12.96	19.78	20.46	25.78	18.76	17.46
0.04		4.35	11.13	6.87	28.10	16.08	38.32	12.83	32.40	40.23	14.94	9.07	39.80	32.57	12.78

0.04		8.84	20.18	21.53	20.44	39.70	19.53	32.53	25.54	20.51	39.52	10.41	28.41	35.99	25.68
0.04		29.16	11.57	20.43	25.44	33.15	12.19	13.75	30.61	40.83	17.32	13.95	30.63	18.13	23.20
0.04		5.80	15.81	17.73	23.60	21.11	29.18	21.37	23.92	36.62	15.53	6.24	21.13	18.24	23.55
0.04		7.91	10.38	10.35	23.49	8.96	33.50	13.66	27.14	18.21	17.32	27.56	25.67	29.77	17.13
	Mean	12.94		18.09		22.02		20.16		27.44		14.08		25.92	
	STDEV	9.21		10.94		11.61		7.71		13.20		6.84		7.19	
	SE	3.26		3.87		4.10		2.73		4.67		2.42		2.54	
0.02		8.21	15.19	10.83	16.40	11.44	24.01	13.81	18.25	30.13	15.09	21.37	23.28	34.01	15.32
0.02		1.52	43.62	13.91	23.37	34.56	22.43	12.63	29.31	19.00	33.70	13.08	16.17	7.41	9.64
0.02		5.15	25.84	9.65	14.11	34.67	15.28	9.88	13.03	12.21	15.60	21.68	32.14	21.58	12.72
0.02		5.84	13.07	4.78	12.21	36.11	20.70	7.90	12.62	7.21	13.97	8.19	9.13	30.34	10.69
0.02		4.50	13.03	5.43	15.25	26.45	15.28	7.93	17.29	40.02	13.74	20.11	23.99	33.24	13.27
0.02		22.33	24.47	19.69	30.72	36.96	15.88	24.38	31.14	39.23	15.61	18.61	26.76	33.18	11.57
0.02		6.00	16.12	5.69	13.60	32.57	18.98	10.77	14.52	34.84	13.81	6.86	30.39	26.37	10.34
0.02		11.37	20.86	12.74	14.45	12.77	21.61	18.80	18.83	10.57	15.33	17.39	28.32	10.70	19.54
	Mean	8.12		10.34		28.19		13.26		24.15		15.91		24.61	
	STDEV	6.41		5.12		10.44		5.73		13.47		5.85		10.49	
	SE	2.27		1.81		3.69		2.03		4.76		2.07		3.71	

Pollen extracts

Table A-3d Mean *A. apis* mycelial inhibition (%) by *T. carbonaria* (Tc) and *A. mellifera* (Am) pollen extracts. SE = Standard error of the means. Letters represent statistical significant differences between treatment concentrations within each nest product treatment tested either Tc pollen or Am pollen.

Mean *A. apis* mycelial inhibition (%)				
		SE		SE
Treatment concentration (% w/v)	**Tc pollen**	**Tc pollen**	**Am pollen**	**Am pollen**
0.02	70.86 *a*	4.778	7.51 *a*	23.841
0.04	49.02 *ab*	11.038	5.09 *a*	34.142
0.08	19.94 *bc*	13.088	31.45 *a*	25.356
0.16	12.87 *bc*	12.122	2.60 *a*	37.428
0.31	3.31 *c*	10.779	30.22 *a*	27.323
0.63	17.13 *bc*	11.806	10.06 *a*	32.020
1.25	7.19 *bc*	7.209	13.59 *a*	30.078
2.50	5.15 *c*	4.570	12.95 *a*	17.481
5.00	82.50 *a*	11.000	16.33 *a*	41.971

All possible relationships between inhibition of *A. apis* growth and *T. carbonaria* and *A. mellifera* pollen extracts (based on the methodology described in Chapter 4, Section 4.2.3) are provided Figure A-3a. Only significant relationships with a $R^2 \geq 0.5$ are presented in Chapter 4.

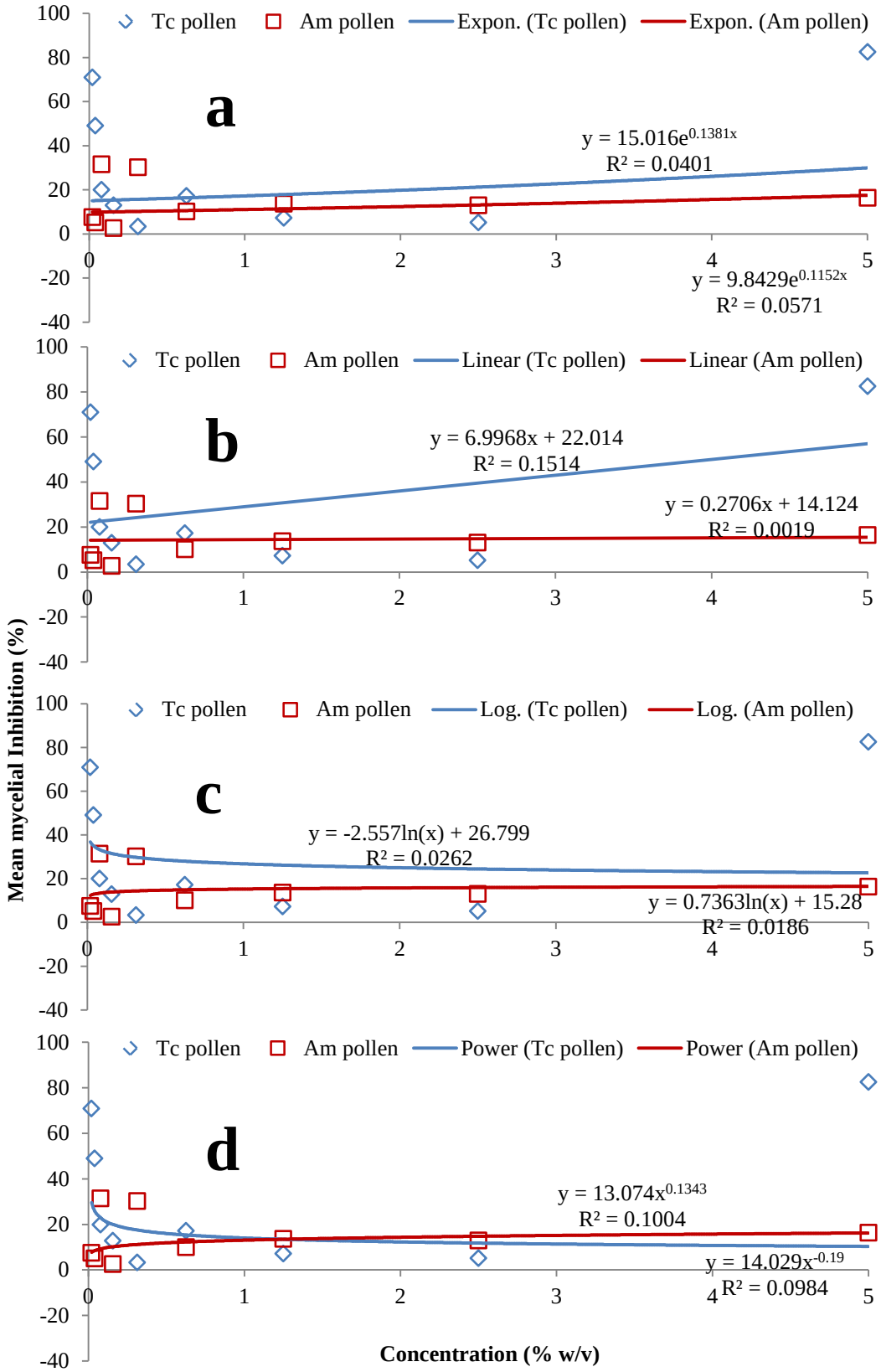

Figure A-3a Mean mycelial inhibition (%) of *A. apis* by *T. carbonaria* (Tc, blue) and *A. mellifera* (Am, red) pollen extracts, with all possible relationship trendlines and equations. a) Exponential, b) Linear, c) Logarithmic, and d) Power.

Honeys

Table A-3e Mean *A. apis* mycelial inhibition (%) by *T. carbonaria* (Tc) and *A. mellifera* (Am) honeys. SE = Standard error of the means. Letters represent statistical significant differences between treatment concentrations within each nest product treatment tested, either Tc honey or Am honey.

Mean *A. apis* mycelial inhibition (%)				
		SE		**SE**
Treatment concentration (% w/v)	**Tc honey**	**Tc honey**	**Am honey**	**Am honey**
0.02	19.10 *c*	9.839	45.60 *a*	15.431
0.04	36.81 *bc*	10.527	51.86 *a*	13.075
0.08	19.26 *c*	10.024	54.33 *a*	10.508
0.16	53.91 *bc*	9.637	66.27 *a*	11.921
0.31	63.31 *ab*	11.740	49.72 *a*	18.535
0.63	65.58 *ab*	6.844	67.88 *a*	15.893
1.25	57.78 *abc*	8.007	56.71 *a*	18.958
2.50	69.68 *ab*	6.753	53.56 *a*	20.647
5.00	98.53 *a*	1.378	76.26 *a*	12.076

All possible relationships between inhibition of *A. apis* growth and *T. carbonaria* and *A. mellifera* honeys (based on the methodology described in Chapter 4, Section 4.2.3) are provided Figure A-3b. Only significant relationships with a $R^2 \geq 0.5$ are presented in Chapter 4.

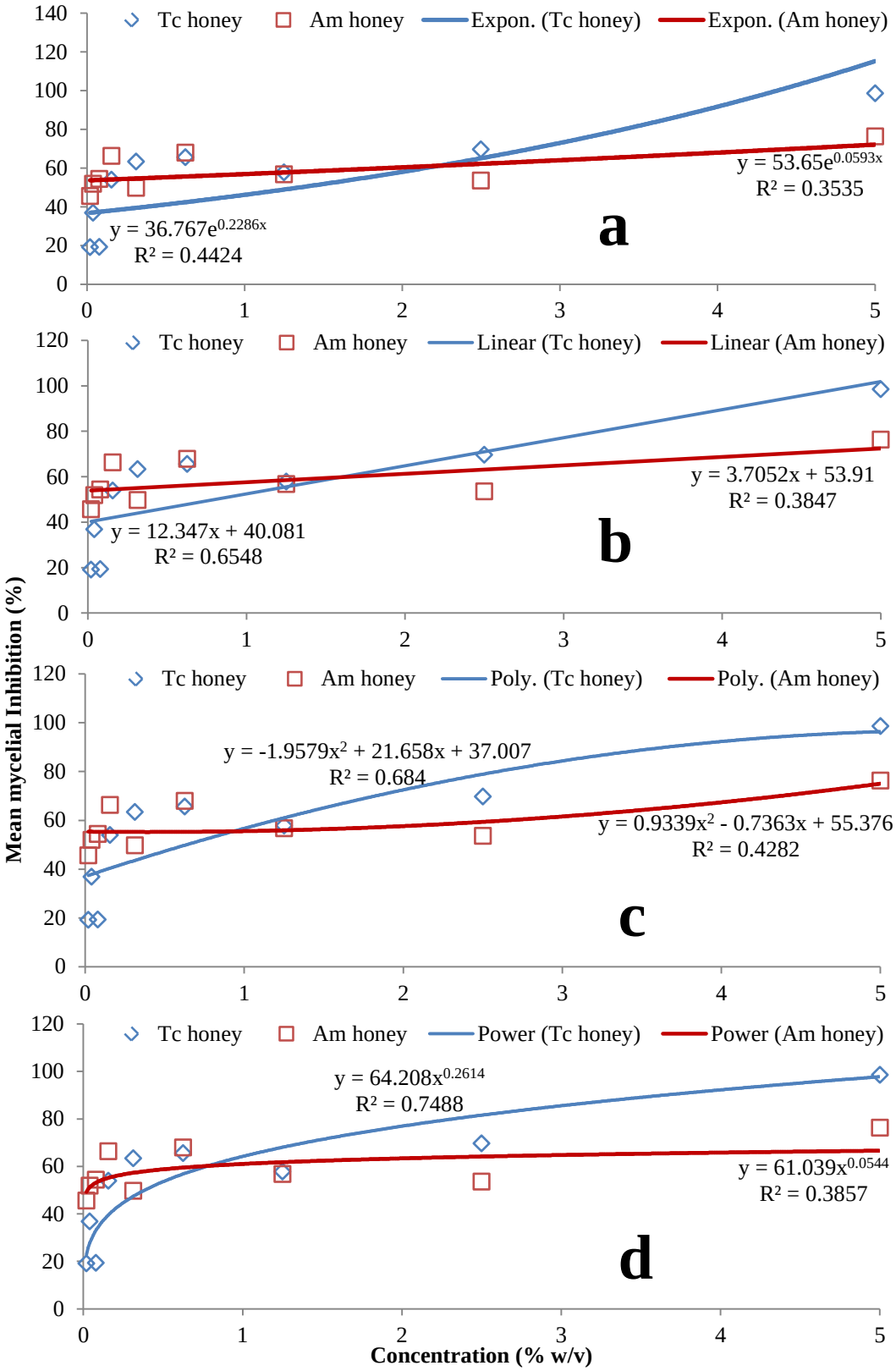

Figure A-3b Mean mycelial inhibition (%) of *A. apis* by *T. carbonaria* (Tc, blue) and *A. mellifera* (Am, red) honeys, with all possible relationship trendlines and equations. a) Exponential, b) Linear, c) Logarithmic, d) Polynomial, and e) Power.

Propolis extracts

Table A-3f Mean *A. apis* mycelial inhibition (%) by *T. carbonaria* (Tc) and *A. mellifera* (Am) propolis extracts. SE = Standard error of the means. Letters represent statistical significant differences between treatment concentrations within each nest product treatment tested, either Tc propolis or Am propolis.

Mean *A. apis* mycelial inhibition (%)				
		SE		**SE**
Treatment concentration (% w/v)	**Tc propolis**	**Tc propolis**	**Am propolis**	**Am propolis**
0.02	65.99 *b*	5.190	2.90 *c*	26.862
0.04	48.30 *bc*	7.260	8.39 *c*	33.655
0.08	28.74 *c*	6.662	29.02 *c*	29.233
0.16	20.99 *cd*	7.022	7.55 *c*	26.056
0.31	23.21 *cd*	8.285	18.20 *bc*	19.658
0.63	3.28 *cd*	3.378	71.32 *ab*	29.039
1.25	22.58 *cd*	9.802	98.69 *a*	1.163
2.50	94.62 *a*	1.691	100 *a*	0.000
5.00	100 *a*	0.000	100 *a*	0.000

All possible relationships between inhibition of *A. apis* growth and *T. carbonaria* and *A. mellifera* propolis extracts (based on the methodology described in Chapter 4, Section 4.2.3) are provided Figure A-3c. Only significant relationships with a $R^2 \geq 0.5$ are presented in Chapter 4.

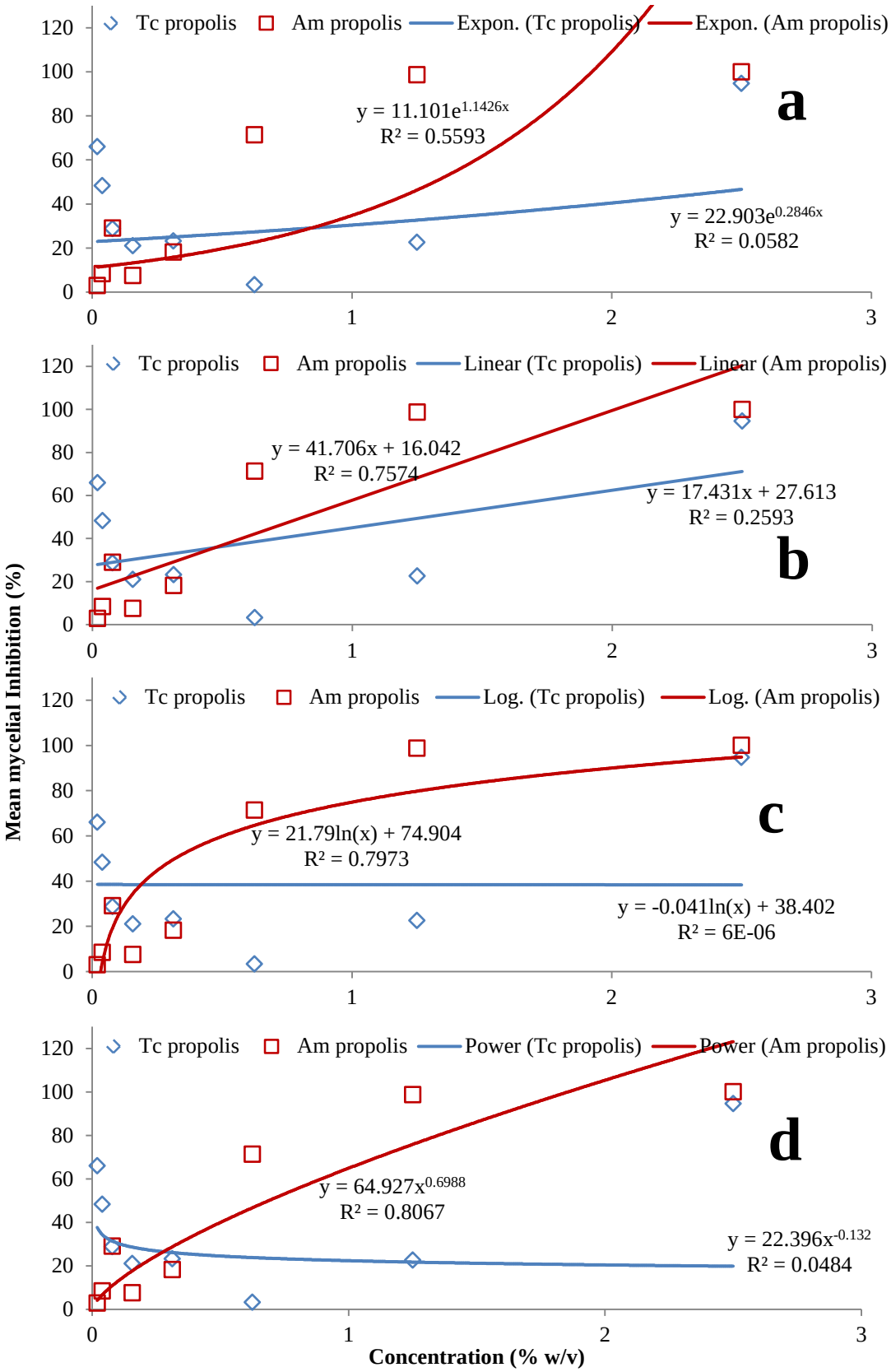

Figure A-3c Mean mycelial inhibition (%) of *A. apis* by *T. carbonaria* (Tc, blue) and *A. mellifera* (Am, red) propolis extracts, with all possible relationship trendlines and equations.) Exponential, b) Linear, c) Logarithmic, and d) Power.

A3.4 *Metarhizium anisopliae* inhibition

Data collected from *A. apis* mycelial inhibition is provided including the means and standard deviation and standard errors. After which, all possible relationships between *A. apis* growth and nest products are provided, these are arranged by nest product.

Table A-3g Mean area of sporulation (mm²) of *M. anisopliae* after the treatment with *T. carbonaria* and *A. mellifera* products.

Area of *M. anisopliae* sporulation (mm²)														
Treatment concentration (% w/v)		***T. carbonaria***								***A. mellifera***					
		Gut	**STDEV**	**Pollen**	**STDEV**	**Honey**	**STDEV**	**Propolis**	**STDEV**	**Pollen**	**STDEV**	**Honey**	**STDEV**	**Propolis**	**STDEV**
Negative		32.88	20.50	17.01	15.49	27.34	15.47	5.80	12.59	16.42	19.55	1.45	12.79	13.62	25.29
Negative		1.60	6.84	2.35	13.25	1.95	10.93	0.66	12.80	22.53	15.21	1.73	13.84	0.00	0.00
Negative		1.37	9.96	0.93	7.72	5.16	6.77	1.13	11.33	7.32	16.70	0.10	12.83	0.00	0.00
Negative		5.63	11.35	1.73	10.88	6.17	13.14	0.84	10.35	14.99	21.19	0.92	4.87	3.52	11.91
Negative		30.26	18.00	8.68	11.90	15.50	15.61	1.22	12.83	20.50	18.10	0.40	6.01	0.00	0.00
Negative		0.80	19.51	0.28	6.96	7.88	18.15	0.32	10.93	8.93	13.16	7.28	17.12	0.00	0.00
Negative		10.09	11.51	0.35	6.36	0.00	0.00	0.00	0.00	3.04	11.17	23.24	20.10	0.00	0.00
Negative		18.99	16.21	0.93	9.34	29.82	16.07	4.48	12.24	18.51	21.67	10.50	14.41	25.18	17.75
	Mean	12.70		4.03		11.73		1.81		14.03		5.70		5.29	
	STDEV	13.12		5.92		11.38		2.12		6.89		8.01		9.32	
	SE	4.64		2.09		4.03		0.75		2.44		2.83		3.30	
Copper sulphate		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Copper sulphate		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Copper sulphate		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Copper sulphate		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Copper sulphate		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Copper sulphate		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Copper sulphate		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Copper sulphate		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Mean	0.00		0.00		0.00		0.00		0.00		0.00		0.00	

	STDEV	0.00		0.00		0.00		0.00		0.00		0.00		0.00	
	SE	0.00		0.00		0.00		0.00		0.00		0.00		0.00	
5.0		13.30	14.27	3.76	18.86	0.00	0.00	0.00	0.00	1.53	10.07	0.00	0.00	9.77	25.06
5.0		0.00	0.00	3.19	16.55	0.00	0.00	0.00	0.00	0.22	4.64	0.00	0.00	12.92	20.68
5.0		2.42	17.52	4.57	23.86	5.39	19.80	0.00	0.00	0.56	6.27	0.00	0.00	8.67	14.58
5.0		0.34	13.92	2.91	14.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	13.34	13.21
5.0		0.44	19.69	1.34	24.25	1.34	24.08	0.00	0.00	0.00	0.00	0.00	0.00	11.11	20.40
5.0		0.00	0.00	0.85	6.07	0.00	0.00	0.00	0.00	1.34	16.89	0.00	0.00	9.63	14.32
5.0		4.34	18.26	1.45	12.55	2.28	25.19	0.00	0.00	0.00	0.00	0.00	0.00	8.86	21.36
5.0		23.66	21.23	0.38	7.34	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.12	15.20
	Mean	5.56		2.30		1.13		0.00		0.46		0.00		9.68	
	STDEV	8.57		1.51		1.92		0.00		0.64		0.00		3.18	
	SE	3.03		0.53		0.68		0.00		0.22		0.00		1.12	
2.5		18.34	19.81	2.97	18.49	0.00	0.00	0.00	0.00	1.84	12.08	20.12	17.36	14.60	28.07
2.5		3.08	14.84	3.18	24.38	0.00	0.00	0.07	6.01	0.17	27.59	9.57	23.80	15.14	29.86
2.5		0.81	22.90	3.62	24.19	1.78	23.32	0.35	4.50	0.66	7.57	5.76	27.08	15.89	28.39
2.5		0.84	11.19	3.14	20.91	0.22	7.37	0.56	7.79	0.00	0.00	8.43	29.55	14.71	26.71
2.5		0.37	12.95	3.96	22.81	2.14	26.02	0.17	6.49	0.44	12.24	3.93	23.30	16.15	25.61
2.5		13.40	15.32	2.77	23.55	1.16	17.61	0.31	3.99	0.00	0.00	7.75	26.68	14.84	23.67
2.5		18.64	18.33	2.39	22.40	1.35	17.88	0.00	0.00	0.00	0.00	7.56	21.56	12.80	29.15
2.5		18.99	20.60	0.59	17.72	1.45	12.36	1.05	9.73	0.00	0.00	14.75	19.82	11.55	25.76
	Mean	9.31		2.83		1.01		0.31		0.39		9.73		14.46	
	STDEV	8.80		1.03		0.83		0.35		0.64		5.25		1.55	
	SE	3.11		0.36		0.30		0.13		0.22		1.86		0.55	
1.25		18.40	17.33	3.17	21.10	31.31	21.00	2.62	10.34	3.88	12.09	7.71	24.50	20.03	20.41

1.25		9.67	13.85	4.88	15.69	1.44	15.82	2.72	17.38	0.94	12.53	3.45	11.25	19.36	27.18
1.25		14.81	18.33	5.79	15.62	10.15	13.90	0.37	18.90	1.10	11.89	5.29	12.63	17.58	21.24
1.25		10.26	16.43	3.28	21.52	2.14	16.60	1.08	18.48	0.00	0.00	6.08	20.15	18.98	21.45
1.25		13.22	19.21	4.29	23.26	5.54	12.68	0.94	19.96	6.42	12.83	4.69	16.78	22.93	23.85
1.25		13.43	17.32	1.81	24.68	10.57	17.70	0.97	13.15	0.33	6.33	5.54	17.25	17.79	24.76
1.25		13.26	20.18	1.62	22.22	2.96	19.24	0.56	20.26	0.00	0.00	3.74	12.33	12.66	26.81
1.25		27.28	14.09	2.88	14.67	32.84	27.20	0.00	0.00	0.00	0.00	5.77	16.76	14.72	30.83
	Mean	15.04		3.46		12.12		1.16		1.58		5.28		18.01	
	STDEV	5.63		1.45		12.79		1.00		2.34		1.36		3.18	
	SE	1.99		0.51		4.52		0.35		0.83		0.48		1.12	
0.63		27.24	21.51	23.97	25.01	4.67	19.79	2.75	9.85	3.58	13.50	7.75	27.70	23.23	23.29
0.63		6.54	16.77	10.61	24.94	2.55	22.94	7.57	17.17	3.87	14.82	7.35	18.98	14.92	22.52
0.63		0.95	20.17	20.64	20.01	5.47	13.27	5.42	20.89	8.05	16.34	3.37	20.77	15.65	25.69
0.63		22.95	20.27	9.78	20.67	19.30	12.90	4.78	21.08	5.75	17.41	4.80	20.81	21.15	26.33
0.63		5.32	15.99	6.34	23.92	9.44	24.68	7.77	24.10	9.33	17.45	7.94	19.12	14.57	26.10
0.63		5.91	21.10	10.38	21.62	9.42	25.03	6.84	23.76	13.76	14.46	8.68	22.95	12.61	19.06
0.63		19.08	20.52	3.29	15.68	6.14	21.41	2.22	14.22	3.35	16.21	5.28	28.02	13.02	22.86
0.63		24.96	25.49	6.05	19.14	25.76	25.83	3.37	24.79	2.11	9.75	8.17	18.45	26.85	24.19
	Mean	14.12		11.38		10.34		5.09		6.22		6.67		17.75	
	STDEV	10.47		7.25		8.05		2.18		3.92		1.92		5.29	
	SE	3.70		2.56		2.85		0.77		1.39		0.68		1.87	
0.31		22.38	19.15	24.87	22.35	31.57	28.31	12.86	20.31	28.20	15.21	1.72	17.29	27.71	27.38
0.31		5.66	18.24	28.46	20.04	10.16	21.45	2.30	17.45	16.58	20.37	9.34	20.70	23.19	22.47
0.31		8.18	22.09	9.33	12.51	7.47	18.28	14.23	22.33	8.89	15.68	4.15	15.99	22.41	25.00
0.31		15.58	23.55	12.03	24.92	12.37	24.31	20.13	23.27	7.84	15.31	4.95	25.83	30.86	26.24

0.31		10.22	19.83	8.68	19.65	11.29	26.64	12.04	21.20	8.68	16.49	7.24	30.43	14.45	24.19
0.31		14.49	22.04	5.96	17.23	11.14	24.88	8.72	13.49	21.89	19.48	7.75	25.29	13.72	25.89
0.31		3.91	21.92	31.48	15.29	7.74	18.02	1.19	7.16	10.39	22.45	5.46	27.29	14.05	24.15
0.31		13.54	25.08	28.77	14.46	30.52	21.24	6.13	17.18	27.92	15.41	2.66	16.16	26.58	22.01
	Mean	11.75		18.70		15.28		9.70		16.30		5.41		21.62	
	STDEV	6.00		10.64		9.88		6.39		8.68		2.60		6.78	
	SE	2.12		3.76		3.49		2.26		3.07		0.92		2.40	
0.16		32.05	18.64	20.39	23.13	22.28	21.06	2.58	14.19	13.19	12.69	5.88	19.70	24.66	29.75
0.16		18.58	18.90	20.35	19.73	28.15	19.04	4.63	21.32	9.85	9.47	6.41	25.46	21.90	21.71
0.16		26.25	24.44	30.82	19.89	17.97	20.21	1.18	13.35	4.81	10.94	6.39	22.56	15.82	27.47
0.16		22.50	19.00	32.24	17.93	27.26	23.36	5.16	23.48	3.64	15.98	5.76	24.03	15.35	24.04
0.16		17.28	27.30	35.73	19.15	25.98	16.97	4.55	17.69	19.35	15.17	6.98	26.78	14.89	24.31
0.16		18.31	22.65	34.88	22.60	20.37	24.98	2.71	17.11	11.08	10.72	9.10	17.92	16.20	25.57
0.16		21.83	19.89	28.78	18.63	33.69	17.17	2.28	23.19	24.37	16.30	6.65	21.91	13.58	23.05
0.16		24.94	27.97	32.00	24.78	32.84	20.53	5.82	15.41	32.05	14.46	1.10	26.47	15.85	28.56
	Mean	22.72		29.40		26.07		3.61		14.79		6.04		17.28	
	STDEV	4.95		5.98		5.63		1.64		9.82		2.25		3.86	
	SE	1.75		2.12		1.99		0.58		3.47		0.79		1.36	
0.08		13.15	10.02	13.68	20.85	34.51	23.65	2.09	18.17	34.16	20.51	6.62	19.06	31.85	25.09
0.08		7.92	11.89	16.71	14.18	30.97	23.47	0.38	7.48	19.27	16.29	7.72	25.67	31.95	22.42
0.08		7.42	13.64	3.87	11.16	29.65	20.76	0.00	0.00	8.08	17.24	7.61	18.79	17.00	23.98
0.08		7.62	12.83	12.13	12.04	27.61	13.64	5.49	10.37	11.86	15.53	7.58	30.32	20.18	20.51
0.08		20.07	13.27	16.93	17.46	28.10	17.96	10.46	20.82	10.96	19.33	10.94	21.40	16.71	23.04
0.08		0.39	13.47	12.08	18.58	35.62	17.92	0.09	11.45	12.82	18.50	5.19	22.21	23.66	22.22
0.08		1.36	14.82	15.55	18.83	36.70	18.22	0.30	7.02	12.84	18.86	8.94	24.55	19.90	23.90

0.08		29.80	27.75	9.11	19.39	31.12	24.26	4.13	27.62	24.00	16.61	9.04	22.93	17.61	28.00
	Mean	10.97		12.51		31.78		2.87		16.75		7.95		22.36	
	STDEV	9.85		4.38		3.44		3.69		8.66		1.72		6.30	
	SE	3.48		1.55		1.22		1.31		3.06		0.61		2.23	
0.04		25.18	18.54	13.07	22.37	20.31	28.12	4.16	25.14	20.08	20.41	8.69	21.54	30.00	21.50
0.04		10.56	15.09	8.20	12.45	19.79	25.78	2.34	17.98	10.85	17.00	5.52	22.96	20.85	25.60
0.04		9.46	24.26	12.68	16.23	13.49	28.38	0.00	0.00	17.03	17.66	5.98	18.95	12.07	25.70
0.04		2.34	15.12	12.45	14.68	21.17	21.85	0.00	0.00	20.87	14.21	6.70	21.67	14.49	25.02
0.04		5.55	15.66	14.71	20.32	20.59	19.76	0.00	0.00	24.75	21.66	7.04	24.23	15.52	26.59
0.04		22.60	21.62	16.23	18.70	13.66	15.63	10.35	10.23	23.57	24.91	6.18	23.30	18.92	24.62
0.04		15.01	27.45	19.43	16.49	9.39	20.60	0.00	0.00	13.86	19.43	12.60	25.17	18.57	26.79
0.04		24.37	28.90	12.08	17.53	19.40	21.13	4.70	13.40	21.67	21.56	15.74	22.42	15.76	32.28
	Mean	14.38		13.60		17.22		2.69		19.09		8.56		18.27	
	STDEV	8.83		3.30		4.41		3.66		4.82		3.69		5.50	
	SE	3.12		1.17		1.56		1.30		1.70		1.30		1.94	
0.02		31.11	23.89	21.18	25.67	24.65	23.81	7.92	13.13	24.30	21.08	18.67	24.85	32.70	37.68
0.02		22.61	23.60	21.82	30.34	30.90	24.59	6.52	18.48	22.18	18.45	14.34	26.28	27.91	28.22
0.02		20.68	24.54	18.94	30.60	32.91	18.51	3.63	12.63	24.52	18.46	15.03	30.48	18.51	21.80
0.02		24.85	20.31	23.10	26.19	10.13	25.25	6.12	11.61	18.13	17.88	9.61	24.12	9.05	30.09
0.02		26.20	26.10	11.76	23.80	13.62	27.16	0.00	0.00	28.40	16.30	14.18	25.82	24.27	24.23
0.02		22.42	29.67	18.88	26.36	22.70	24.00	3.00	11.66	19.57	20.62	10.51	29.74	16.76	31.03
0.02		12.26	21.73	23.83	25.81	21.20	22.44	8.06	19.88	24.89	18.07	23.68	29.17	13.63	29.32
0.02		28.39	25.82	21.63	25.70	18.08	28.50	30.78	12.69	27.79	19.50	22.69	29.56	15.77	32.24
	Mean	23.57		20.14		21.77		8.25		23.72		16.09		19.82	
	STDEV	5.70		3.81		7.86		9.50		3.62		5.19		7.87	
	SE	2.01		1.35		2.78		3.36		1.28		1.84		2.78	

Pollen extracts

Table A-3h Mean *M. anisopliae* area of sporulation (%) after treatment with *T. carbonaria* (Tc) and *A. mellifera* (Am) pollen extracts. SE = Standard error of the means. Letters represent statistical significant differences between treatment concentrations within each nest product treatment tested either Tc pollen or Am pollen.

Mean area of *M. anisopliae* sporulation (%)				
		SE		**SE**
Treatment concentration (% w/v)	**Tc pollen**	**Tc pollen**	**Am pollen**	**Am pollen**
0.02	2.30 *a*	0.532	0.46 *a*	0.225
0.04	2.83 *ab*	0.362	0.39 *a*	0.225
0.08	3.47 *bc*	0.513	1.58 *a*	0.828
0.16	11.38 *bc*	2.562	6.22 *a*	1.387
0.31	18.70 *c*	3.763	16.30 *a*	3.069
0.63	29.40 *bc*	2.115	14.72 *a*	3.473
1.25	12.51 *bc*	1.548	16.75 *a*	3.063
2.50	13.60 *c*	1.165	19.09 *a*	1.703
5.00	20.14 *a*	1.347	23.72 *a*	1.279

All possible relationships between area of *M. anisopliae* sporulation and *T. carbonaria* and *A. mellifera* pollen extracts (based on the methodology described in Chapter 4, Section 4.2.3) are provided Figure A-3d. Only significant relationships with a $R^2 \geq 0.5$ are presented in chapter 4.

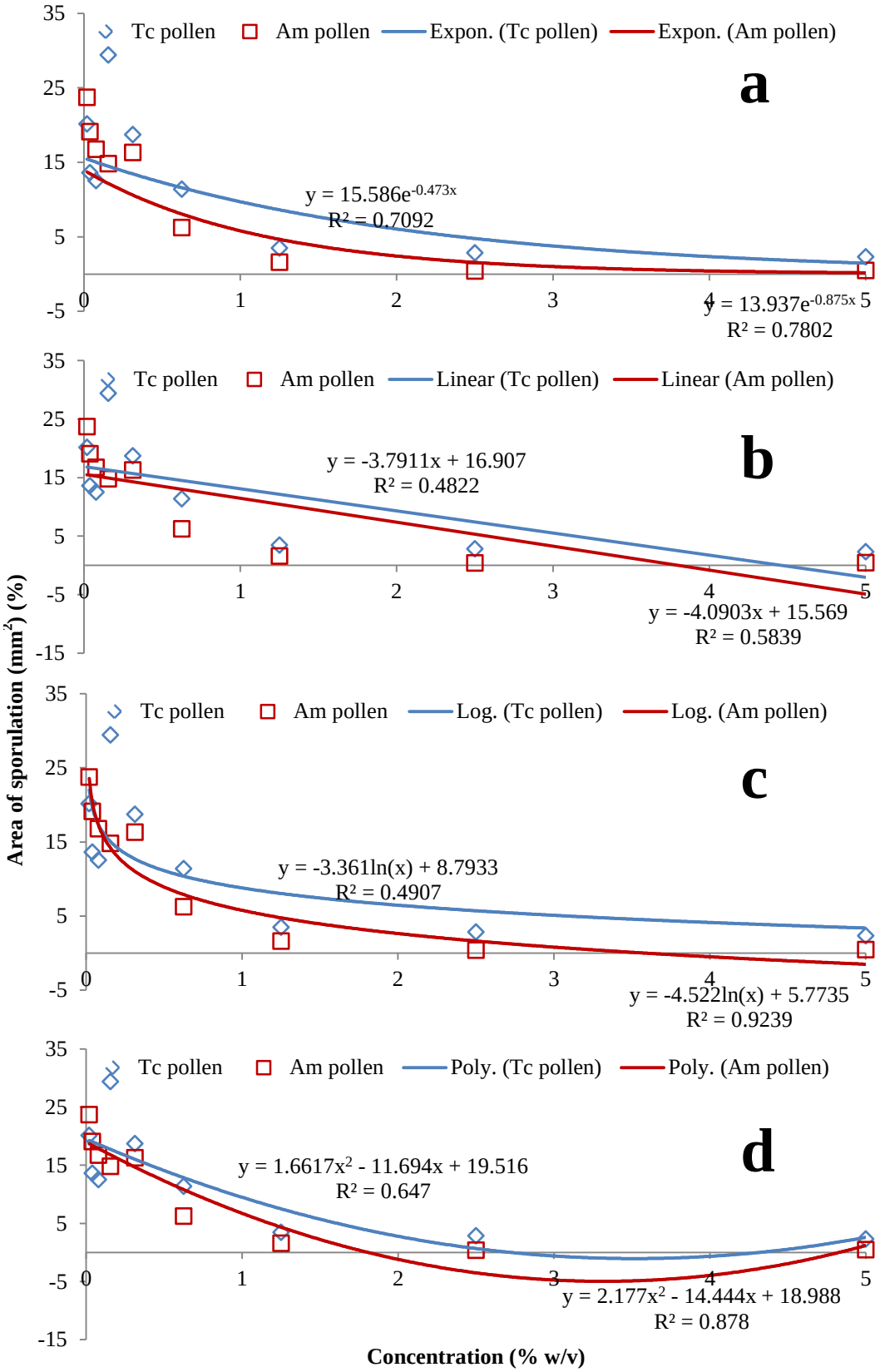

Figure A-3d Area of sporulation (mm^2) of *M. anisopliae* after treatment with *T. carbonaria* (Tc, blue) and *A. mellifera* (Am, red) pollen extracts, with all possible relationship trendlines and equations. a) Exponential, b) Linear, c) Logarithmic, and d) Polynomial.

Honeys

Table A-3i Mean *M. anisopliae* area of sporulation (%) by *T. carbonaria* (Tc) and *A. mellifera* (Am) honeys. SE = Standard error are of the means. Letters represent statistical significant differences between treatment concentrations within each nest product treatment tested either Tc honey or Am honey.

Mean area of *M. anisopliae* sporulation (%)				
		SE		**SE**
Treatment concentration (% w/v)	**Tc honey**	**Tc honey**	**Am honey**	**Am honey**
0.02	1.13 *c*	0.680	0.00 *a*	0.000
0.04	1.01 *bc*	0.295	9.73 *a*	1.856
0.08	12.12 *c*	4.521	5.28 *a*	0.481
0.16	10.34 *bc*	2.848	6.67 *a*	0.680
0.31	15.28 *ab*	3.493	5.41 *a*	0.919
0.63	26.07 *ab*	1.992	6.04 *a*	0.795
1.25	31.78 *abc*	1.217	7.95 *a*	0.609
2.50	17.23 *ab*	1.558	8.56 *a*	1.304
5.00	21.77 *a*	2.779	16.09 *a*	1.836

All possible relationships between area of *M. anisopliae* sporulation and *T. carbonaria* and *A. mellifera* honeys (based on the methodology described in Chapter 4, Section 4.2.3) are provided Figure A-3e. Only significant relationships with a $R^2 \geq 0.5$ are presented in Chapter 4.

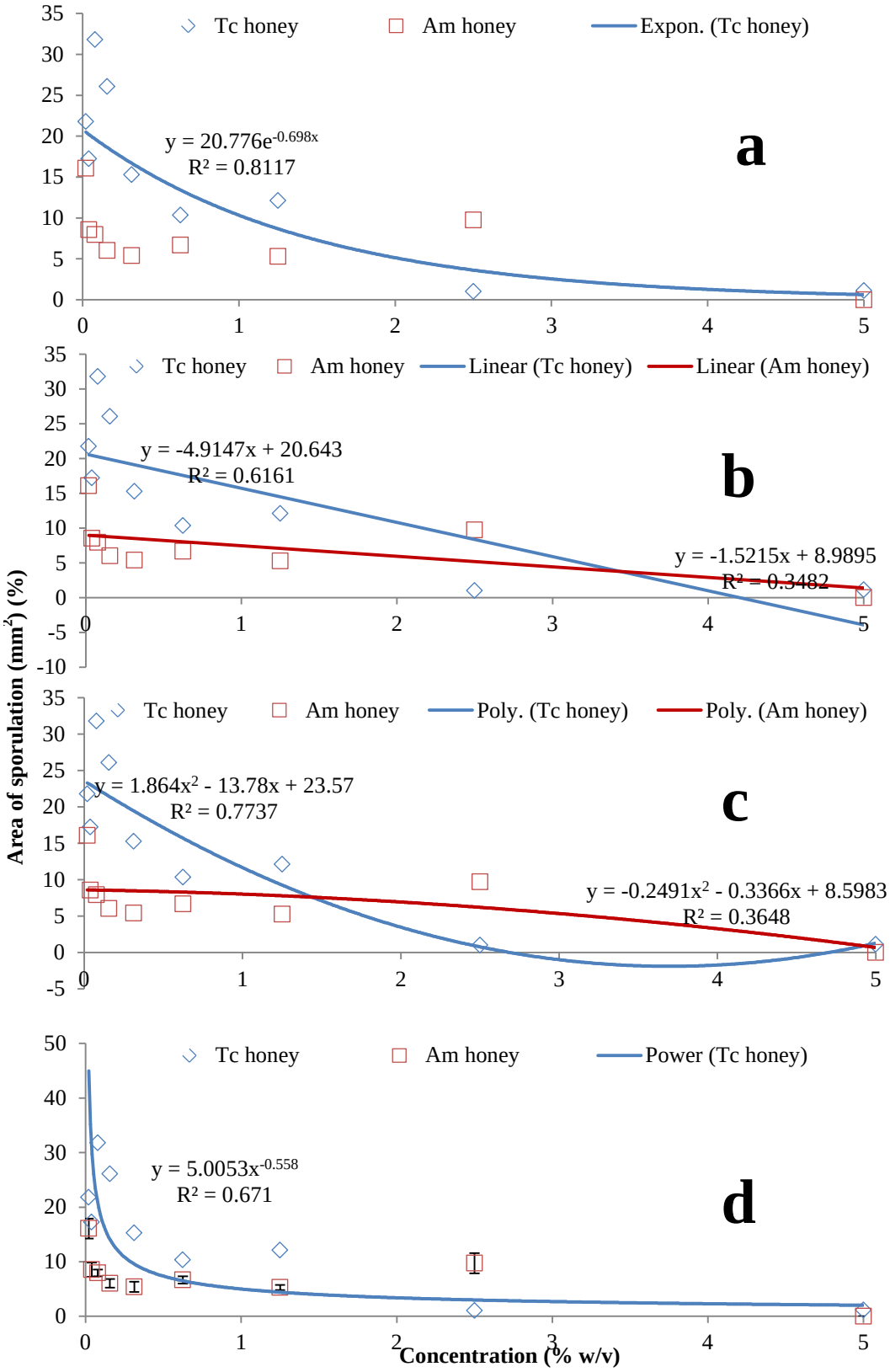

Figure A-3e Area of sporulation (mm^2) of *M. anisopliae* after treatment with *T. carbonaria* (Tc, blue) and *A. mellifera* (Am, red) honey, with all possible relationship trendlines and equations. a) Exponential, b) Linear, c) Polynomial, and d) Power.

Propolis extracts

Table A-3j Mean *M. anisopliae* area of sporulation (%) by *T. carbonaria* (SB) and *A. mellifera* (HB) propolis extracts. SE = Standard error of the means. Letters represent statistical significant differences between treatment concentrations within each nest product treatment tested either Tc propolis or Am propolis.

Mean area of *M. anisopliae* sporulation (%)				
		SE		**SE**
Treatment concentration (% w/v)	**SB propolis**	**SB propolis**	**HB propolis**	**HB propolis**
0.02	1.13 *b*	0.680	0.00 *c*	0.000
0.04	1.01 *bc*	0.295	9.73 *c*	1.856
0.08	12.12 *c*	4.521	5.28 *c*	0.481
0.16	10.34 *cd*	2.848	6.67 *c*	0.680
0.31	15.28 *cd*	3.493	5.41 *bc*	0.919
0.63	26.07 *cd*	1.992	6.04 *ab*	0.795
1.25	31.78 *cd*	1.217	7.95 *a*	0.609
2.50	17.23 *a*	1.558	8.56 *a*	1.304
5.00	21.77 *a*	2.779	16.09 *a*	1.836

All possible relationships between area of *M. anisopliae* sporulation and *T. carbonaria* and *A. mellifera* propolis extracts (based on the methodology described in Chapter 4. Section 4.2.3) are provided Figure A-3f. Only significant relationships with a $R^2 \geq 0.5$ are presented in Chapter 4.

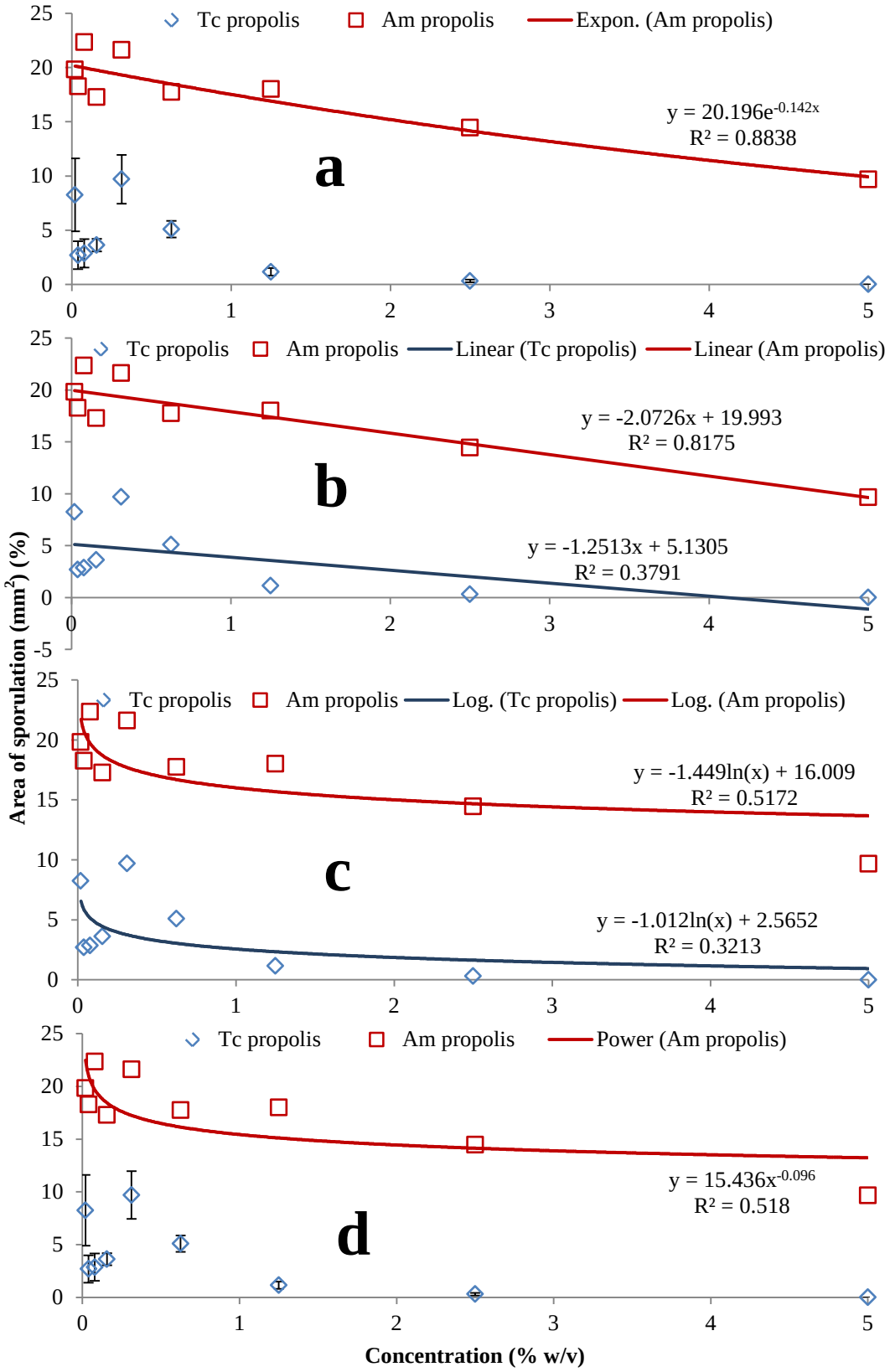

Figure A-3f Area of sporulation (mm^2) of *M. anisopliae* after treatment with *T. carbonaria* (Tc, blue) and *A. mellifera* (Am, red) propolis extracts, with all possible relationship trendlines and equations. a) Exponential, b) Linear, c) Logarithmic, and d) Power.

A3.5 Liquid Chromatography Mass-Spectrometry of nest products

This section contains supporting graphical data from LC-MS analysis. The key chemical composition of nest materials, propolis and newly emerged brood comb, were displayed and further discussed in Chapter 4.

The following sections are divided as follows. Firstly, an overview of the chemical composition in propolis and brood comb from *T. carbonaria* and *A. mellifera* colonies is displayed. Secondly, data compares key flavanones identified in the nest materials between the two bee species, thirdly nests materials chemical compositions is compared between years tested, and finally chemical composition of nest materials is compared to each within each bee species.

1. **Activity in *T. carbonaria* and *A. mellifera* propolis and newly emerged brood comb**

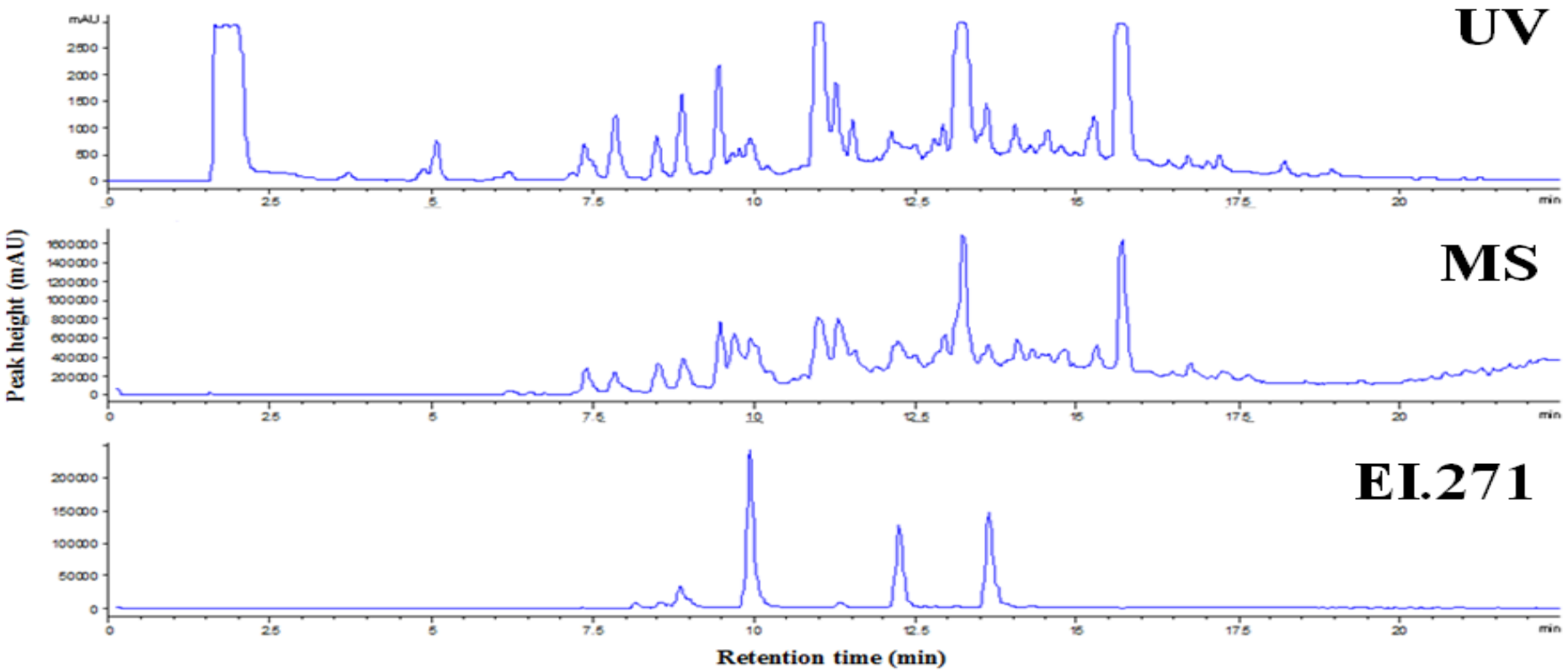

Figure A-3g LC-MS for chemical compounds identified in *A. mellifera* propolis, with extracted ion mass EI.271. Data is represented as peak height (mAU) against peak retention time (min) in the form of UV, MS profiles and flavanone extracted ion mass EI. 271.

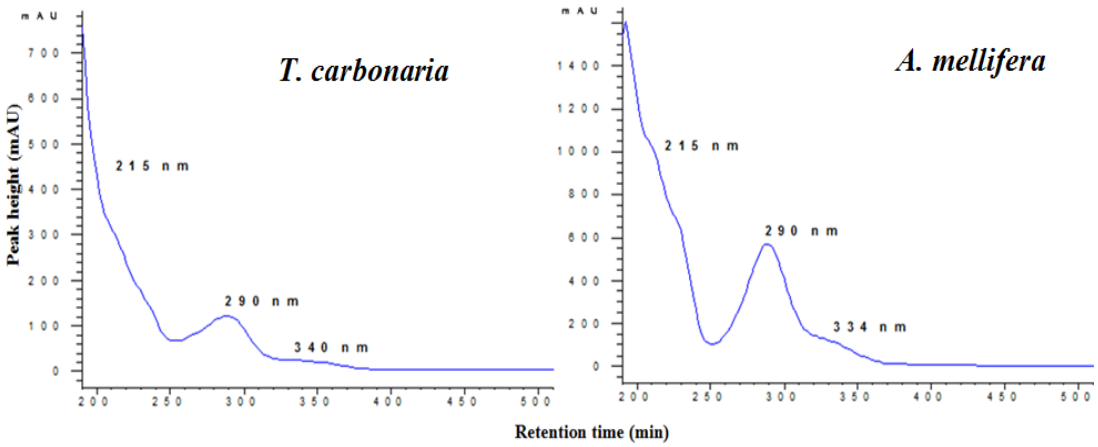

Figure A-3h Typical flavanone profiles with an ion mass 285 (m+1) from *T. carbonaria* and *A. mellifera* propolis. Data displays peak height (mAU) and retention time (min).

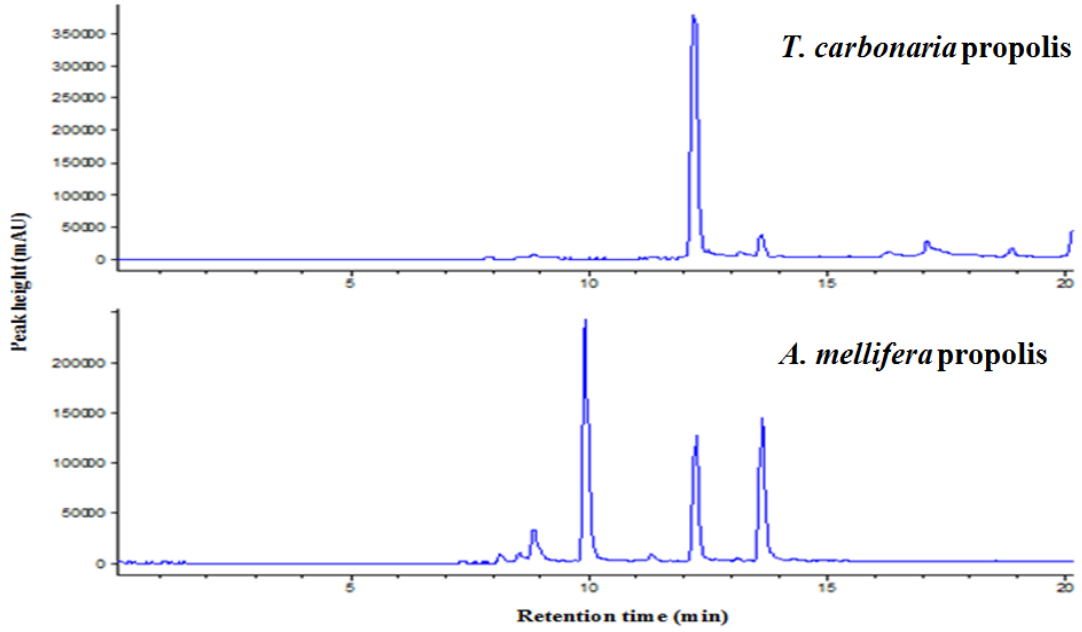

Figure A-3i LC-MS profile for identified flavanones with extracted ion mass EI.271 from *T. carbonaria* and *A. mellifera* propolis. Data displays the comparison of three compounds by peak height (mAU) and retention time (min).

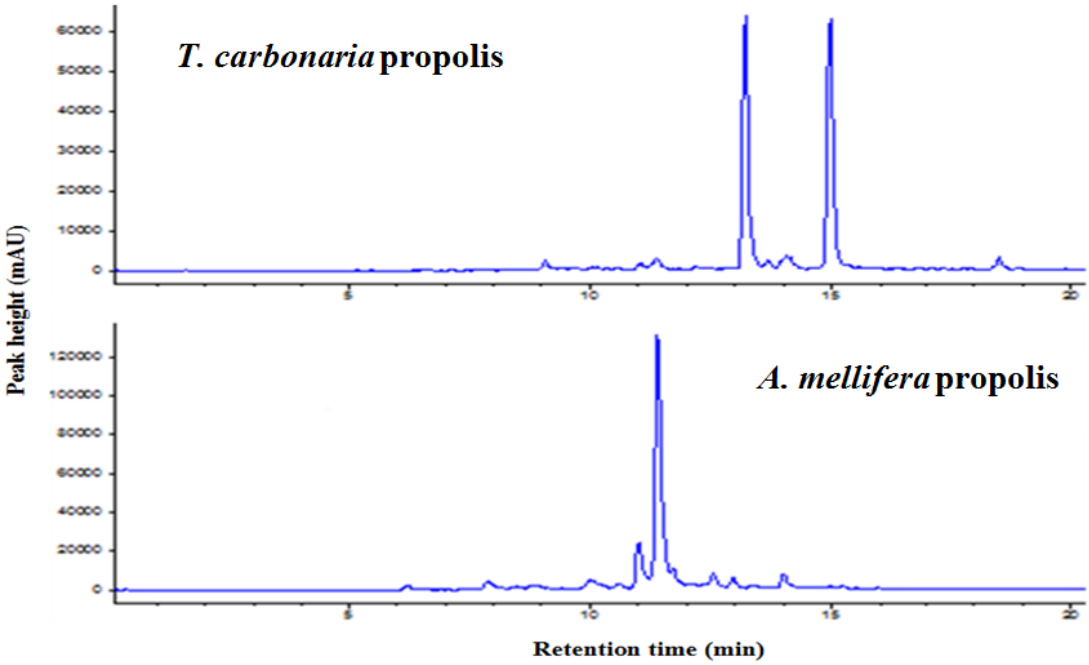

Figure A-3j LC-MS profile for identified flavanones with extracted ion mass EI.285 from *T. carbonaria* and *A. mellifera* propolis. Data displays the comparison of three compounds by peak height (mAU) and retention time (min).

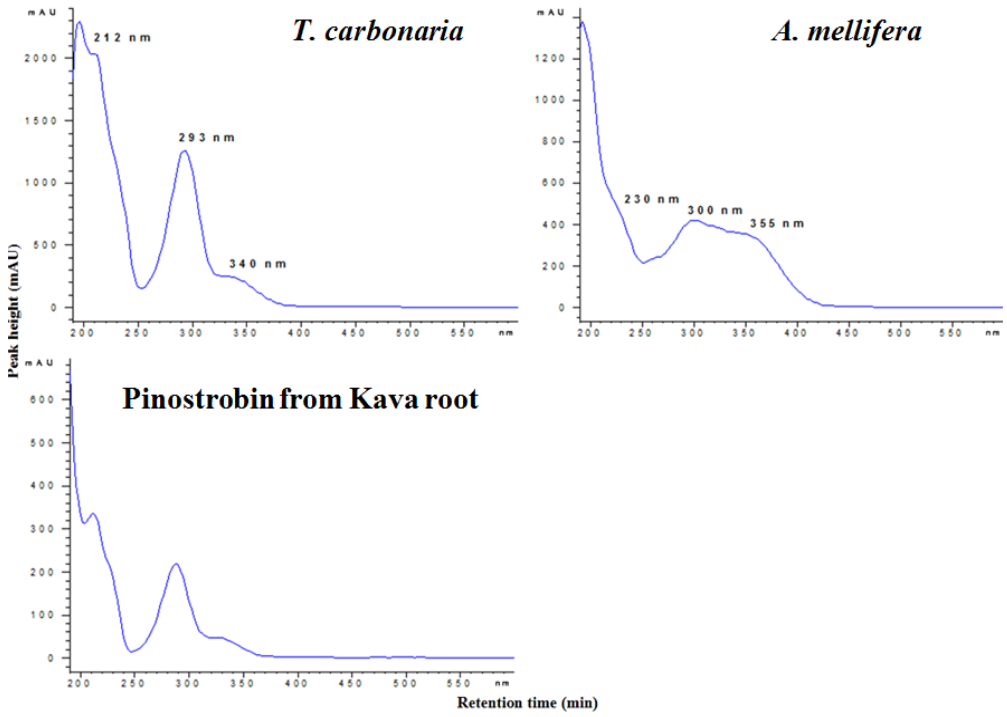

Figure A-3k Typical flavanone profile with an ion mass 271 (m+1), for *T. carbonaria* and *A. mellifera* compared with Pinostrobin from Kava root. Data displayed by chemical peak height (mAU) and a retention time of 12 min.

1. Comparison of *A. mellifera* and *T. carbonaria* propolis (2015)

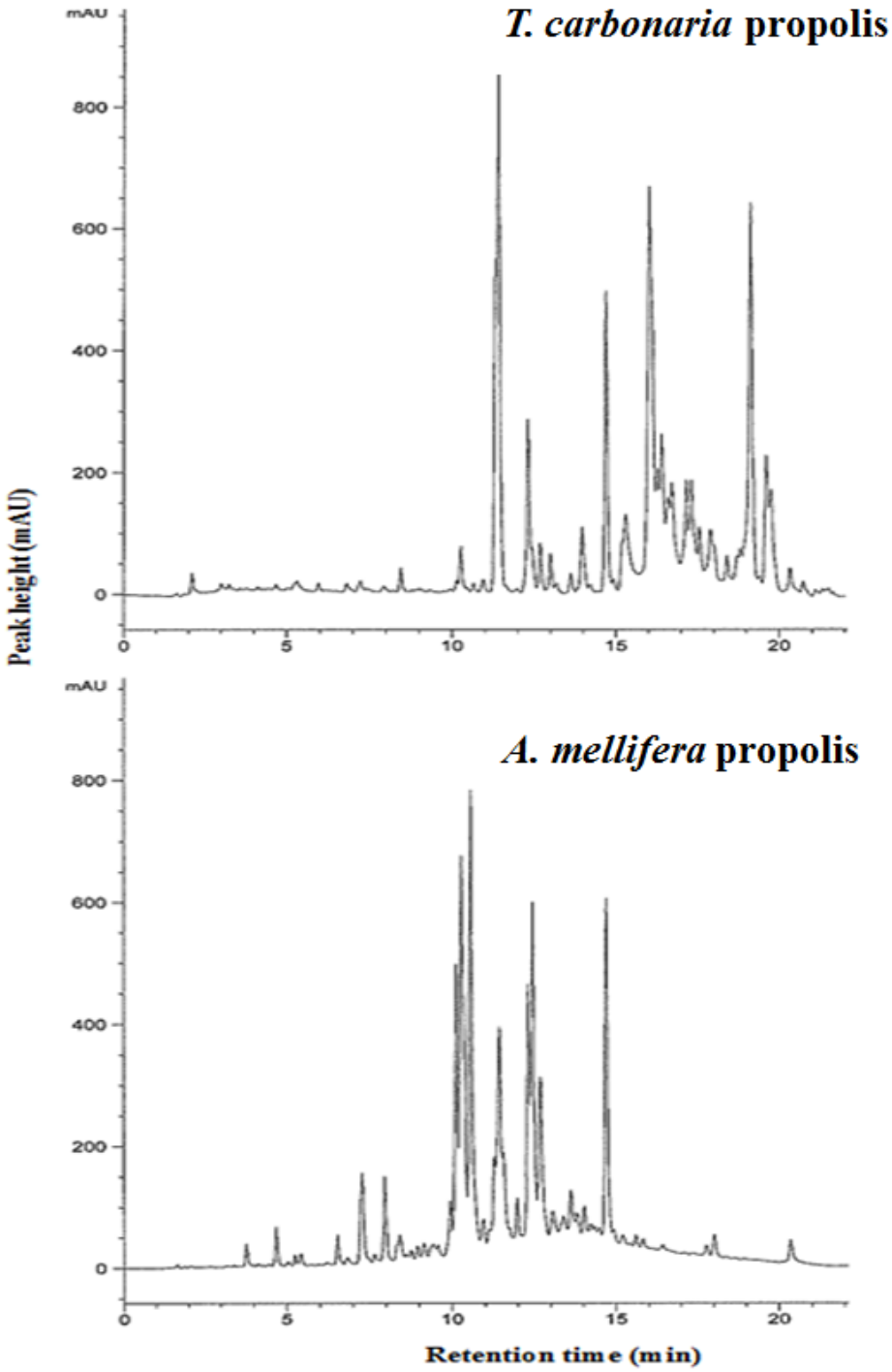

Figure A-3l Comparison of the chemical compositions between *T. carbonaria* and *A. mellifera* propolis collected in 2015. Identified compounds are represented by height (mAU) and retention time (min).

Appendix 4: First documentation of a stingless bee brood pathogen

A4.1 Department of Primary Industries reports

Provided here are the DPI reports of *T. carbonaria* symptomatic brood samples which were sent for diagnosis of infection.

Date Issued: **19 Feb 2013** **Final Report** Report Number: **M13-02524-F-V1**

NSW GOVERNMENT Department of Primary Industries
Elizabeth Macarthur Agricultural Institute
Woodbridge Rd Menangle

Our Ref: M13-02524
Your Ref:
Prev. Ref:
Laboratory Enquiries: 1800 675 623
Invoice Enquiries: 1300 720 773

LABORATORY REPORT

To: UNIVERSITY OF WESTERN SYDNEY
BUILDING 512 ORCHARD ROAD
RICHMOND
2753 NSW AUSTRALIA
Attn: JENNY SHANKS
Fax: 02 4570 1122

Owner: UWS - RICHMOND
Property: BLD 512 ORCHARD ROAD
RICHMOND 2753

Job Type: Bees & honey
Honey bee

Job Manager: Patrick Staples
Date Sampled: 18 Jan 2013
Date Sent: 12 Feb 2013
Date Received: 13 Feb 2013

Submitter Subject: BACTERIAL
Samples Received: 1 X BROOD SAMPLE
History
Age: **Sex:** Not Specified **No. at Risk:** **No. Sick:** **No. Dead:**

Conclusion

Negative for AFB and EFB.

Erika Bunker
Veterinary Pathologist

NATA

NATA Accreditation Numbers
14173 Environmental Laboratory Wollongbar
14488 Orange Agricultural Institute
14495 Elizabeth Macarthur Agricultural Institute
14949 Wagga Wagga Chemistry Services Laboratory

Template V02

Page 1 of 2

EMAI Bacteriology

Microscopy for bee diseases

		Analysis	Paenibacillus alvei like spores	American foulbrood (AFB)	European foulbrood (EFB)
Lab No.	**Sample ID**	**Sample Desc**			
0001		Bee brood	Negative	Negative	Negative

Copies
BRONWYN HENDRY (email: bronwyn.hendry@industry.nsw.gov.au)
NICK ANNAND (email: nicholas.annand@industry.nsw.gov.au)
MICK RANKMORE (email: michael.rankmore@industry.nsw.gov.au)
DOUG SOMERVILLE (email: doug.somerville@industry.nsw.gov.au)

NSW GOVERNMENT Department of Primary Industries
Elizabeth Macarthur Agricultural Institute
Woodbridge Rd Menangle

Our Ref: M13-02524
Your Ref:
Prev. Ref:
Laboratory Enquiries: 1800 675 623
Invoice Enquiries: 1300 720 773

LABORATORY REPORT

To: UNIVERSITY OF WESTERN SYDNEY
BUILDING 512 ORCHARD ROAD
RICHMOND
2753 NSW AUSTRALIA
Attn: JENNY SHANKS
Fax: 02 4570 1122

Job Type: Bees & honey
Native bee

Owner: UWS - RICHMOND
Property: BLD 512 ORCHARD ROAD
RICHMOND 2753

Job Manager: Patrick Staples
Date Sampled: 18 Jan 2013
Date Sent: 12 Feb 2013
Date Received: 13 Feb 2013

Submitter Subject: BACTERIAL
Samples Received: 1 X BROOD SAMPLE
History
Age: **Sex:** Not Specified **No. at Risk:** **No. Sick:** **No. Dead:**

Conclusion

Negative for AFB and EFB.
Lysinibacillus sphaericus

Comments

5/3/2013: Jenny, we will do 16s rRNA sequencing to determine the identity of this bacterium. It might take a while because our molecular testing section is very busy at present. PS.

16/4/13: *Lysinibacillus sphaericus* is a naturally occurring soil bacterium capable of producing insecticidal toxin. EL

Effie Lee
Veterinary Pathologist

EMAI Bacteriology

Microscopy for bee diseases

		Analysis	Paenibacillus alvei like spores	American foulbrood (AFB)	European foulbrood (EFB)
Lab No.	**Sample ID**	**Sample Desc**			
0001		Bee brood	Negative	Negative	Negative

Lab No.	Sample ID	Sample Desc	*16sRNA Sequencing PCR	Culture - routine	Isolate 1(Presumed ID)
0001		Bee brood	Lysinibacillus sphaericus	Profuse predominant growth	Bacillus sp.

Copies
BRONWYN HENDRY (email: bronwyn.hendry@industry.nsw.gov.au)
NICK ANNAND (email: nicholas.annand@industry.nsw.gov.au)
MICK RANKMORE (email: michael.rankmore@industry.nsw.gov.au)
DOUG SOMERVILLE (email: doug.somerville@industry.nsw.gov.au)

* NATA Accreditation does not cover the performance of this service

A4.2 Symptomatic sources of which the material was identified as *Lysinibacillus sphaericus*

Nine sequences isolated from cultured bacterial samples obtained from symptomatic material were cleaned of chimeric sequences using DECIPHER (Wright et al. 2012) (Table A-4a) before submission to NCBI GenBank (Table A-4b). Sequences uploaded into GenBank for toxin protein production are provided along with accession numbers (Table A-4c)

Table A-4a Chimera analysis of sequences obtained from cultured bacterial samples of symptomatic hive material. No chimeras were detected in sequences.

Name	Classification	Group	Nucleotides	Result	Chimeric Region
T. carbonaria worker larva	*Lysinibacillus*	Planococcaceae	450	Not deciphered to be a chimera	NA
T. carbonaria queen larva	*Lysinibacillus*	Planococcaceae	450	Not deciphered to be a chimera	NA
T. carbonaria queen larva	*Lysinibacillus*	Planococcaceae	404	Not deciphered to be a chimera	NA
T. carbonaria worker cell provision	*Lysinibacillus*	Planococcaceae	450	Not deciphered to be a chimera	NA
T. carbonaria worker cell provision	*Lysinibacillus*	Planococcaceae	298	Not deciphered to be a chimera	NA
T. carbonaria queen cell provision	*Lysinibacillus*	Planococcaceae	376	Not deciphered to be a chimera	NA
T. carbonaria honey	*Lysinibacillus*	Planococcaceae	450	Not deciphered to be a chimera	NA
T. carbonaria honey	*Lysinibacillus*	Planococcaceae	337	Not deciphered to be a chimera	NA
Au. australis worker larva	*Lysinibacillus*	Planococcaceae	457	Not deciphered to be a chimera	NA

Table A-4b Sample sequences obtained from symptomatic hive material and their NCBI Genbank accession numbers.

Source	No. of DNA extracts	PCR products	No. of bacterial clones transformed	No. of colony PCRs sequenced	No. of sequences used in phylogenetic analysis	Strain identifier	Accession Number
T. carbonaria worker larva	4	11	16	7	1	HAWK1	KR947300
T. carbonaria queen larva	1	1	3	3	1	HAWK7	KR947306
T. carbonaria queen larva	1	1	4	4	1	HAWK8	KR947307
T. carbonaria worker cell provision	1	8	7	3	1	HAWK2	KR947301
T. carbonaria worker cell provision	4	4	14	5	1	HAWK5	KR947304
T. carbonaria queen cell provision	2	2	6	2	1	HAWK6	KR947305
T. carbonaria honey	1	1	3	2	1	HAWK3	KR947302
T. carbonaria honey	1	1	3	1	1	HAWK4	KR947303
Au. australis worker larva	3	3	8	4	1	HAWK9	KR947308

Table A-4c Four MLST sequences from symptomatic *T. carbonaria* queen larva, used for toxin gene identification, NCBI Genbank accession number are given.

Source	Strain identifier	Toxin protein name	Protein code	Accession Number
T. carbonaria queen larva	Hawk10a	*pyruvate carboxylase*	*pycA*	KT285613
T. carbonaria queen larva	Hawk10b	*glucose 6-phosphate kinase*	*glcK*	KT285614
T. carbonaria queen larva	Hawk10c	*adenylate kinase*	*adk*	KT285615
T. carbonaria queen larva	Hawk10d	*glycerol uptake facilitator protein*	*glpF*	KT285616

A4.3 Information factsheet sent to stingless beekeepers

Provided in this section is the formation sheet sent to stingless beekeepers regarding brood losses to possible bacterial infections. Management and handling protocols suggested to minimise contamination to neighbouring colonies is outlined.

New Australian stingless bee disease

This is a notification to the Australian stingless bee industry stakeholders that there have been several reported incidences of brood disease and colony deaths in the last two years. Reports of increased incidences have come to light in recent times. As a result of these reports, Jenny Shanks, from the University of Western Sydney, has released the following report based on her research work in this area.

Please be aware that this report is an interim notification to industry stakeholders in an effort to alert beekeepers of the need for caution and improved hygienic behaviour when working with stingless bees.

The development and submission of a short communication in a peer reviewed journal is planned, however, in the interest of informing stingless beekeepers in the immediate future, this communication is being shared.

Stingless bee bacterial infection

The isolated bacteria, sampled from symptomatic *Tetragonula carbonaria* and *Austroplebeia australis* hives, is **speculated** to be causing brood infection and colony deaths. The bacteria is a subspecies of the *Bacillus* group, a group that includes *Paenibacillus larvae* (American Foulbrood - AFB). It is stressed that this bacterial infection is **not** AFB, however symptoms are similar.

The suspect bacteria has been cultured from the majority of nest materials and therefore colony-level transmission is high.

Signs of disease are as follows

- Infected brood does not develop, cells can form sunken caps
- Infected brood becomes discoloured, first turning brown from the last segments of the abdomen eventually turning dark brown all over
- Larvae that have been removed from cells can be found singly or groups throughout hive
- Infected brood eventually degrades to a watery consistency
- Cells become darker in appearance as contents degrade
- Cell provisions discolouration of and/or dry
- Ammonia or decaying smell from infected cells
- Brood formation becomes scattered instead of in organised spiral discs (for T. carbonaria)
- Worker behaviour can initially be frenetic and disorganised, becoming lethargic over time
- These field symptoms are mainly for *T. carbonaria* hives, as Jenny has not had occasion to observed *A. australis* prior to advance infection. Symptoms in *A. australis* include a dried chalky-like brood cell / provision, and this bacteria may be a different strain or subspecies.

Figure 1 Brood cells showing discoloured larvae dispersed amongst normal larvae (Photo J. Shanks)

Figure 2 Discoloured larvae that have been removed by workers (Photo J. Shanks)

Figure 3 Diseased larva within a brood cell (Photo J. Shanks)

While many beekeepers may be of the opinion that they can manage such problems please be aware that attempts to manage this condition have been made. These include the removal of infected brood and supplementary feed; however, since the bacteria can reside in the honey stores, spores may be transmitted by adults. Therefore, this action is not an appropriate control measure, and the colony will most probably die. The addition of pupating brood cells, in the hope that emerging workers may assist in overcoming infection, does not work.

Since discovering the bacterial infections, and losing many of her own colonies, Jenny has devised a strategy to minimise the spread of the infection. The following guidelines are recommended to all stingless bee beekeepers who manipulate their nests. It is stressed here that if it is not necessary to open your colonies, DON'T.

The current recommended strategies for control of this new bacterial infection are as follows

- Isolate the nest and workers. That is, block the hive entrance when all nest mates have returned
- Burn the entire colony and box. This is a drastic measure, however, is performed with highly infected colonies
- Minimise chances of cross contamination between colonies. Beekeepers should **sterilise equipment** with bleach (1:100 solution of normal household bleach:water). Sterilisation should take place between working with boxes and when sampling (including being vigilant with all hive tools and the reuse of boxes). If reusing non-diseased boxes, it is recommended that you soak the box for at least an hour in a strong solution of bleach (1:10) and then rinse and sun-dry before reuse.
- Spore longevity is unknown thus it is recommended to large-scale colony producers that they consider the radiation of equipment, as is the case with AFB infection in *Apis* colonies
- DO NOT leave boxes or stores out for other colonies to rob. This increases the chances of infection into neighbouring colonies
- The transfer of brood between colonies, to aid in population or recovery, is NOT RECOMMENDED. The bacteria takes 22 days from infection to first observed symptoms and the spores would have to be present in the provision before egg is laid and cell capped. Therefore the transfer of "healthy" young brood may still be infected and not showing symptoms. Infected larvae do not progress to pupal stage, therefore, to date it is believed that older developing brood should be okay.
- DO NOT supplementary feed with *Apis* honey.

Ongoing actions

In light of this information and concerns beekeepers may have, it is suggested that any questions or queries be formerly directed to Bob Luttrell. Bob is currently assisting stakeholders with potentially infected colonies and with information provided for field identification. Bob may be able to assist in the identification of suspect brood. If, when you open a hive, you become concerned about your colony please obtain **good quality photographs** which show developing larvae. Check for queen-right status if possible. Bob is unable to take brood specimens for lab analysis, but can help with identification through photographic evidence. You can contact Bob at (robertb.luttrell@bigpond.com). Please provide the location of your hive and number of hives in your meliponary with the photo.

Important

DO NOT OPEN YOUR HIVES JUST TO CHECK FOR THE DISEASE. If you have no symptoms, such as discoloured larvae being removed from the nest, reduced worker activity at the nest entrance or other, do not open the hive.

Don't panic

It is important that stingless beekeepers don't start to panic in light of this information. If you manipulate your hives please follow the recommended protocols listed above. Also, keep records of what is happening with your colonies.