Investigations into the biology, behaviour and phylogeny of a potential crop pollinator: the Australian stingless bee, *Austroplebeia australis*

by

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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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Megan Therese Halcroft

March 2012

Dedication

I dedicate this thesis to my family. To Steven and Elen Ruttley, for their patience and genuine interest in my project; most of the time!

My sincere thanks to my daughter Elen, for her support over the many, many years in my endeavours to learn more ‘stuff...’ and for patience during the arduous period of writing up. Also for her occasionally feigned interest in my projects.

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Table of Contents

List of Figures

List of Tables

List of Appendices

List of Figures and Tables in Appendices

Unless otherwise stated, the photographs and images within this dissertation were produced by Megan Halcroft.

Abbreviations

CT	controlled temperature.
ID	internal diameter of tube.
OATH	Original Australian *Trigona* Hive.
OD	outer diameter of tube.
OP	observation platform.
POP	provisioning and oviposition process. Provisioning of a prepared brood cell, with brood food, and the subsequent laying of a single egg by the queen onto the food.
RZA	Rijk Zwaan Australia.
S	pertaining to the sternal (ventral) segments of the metasoma, e.g., S4, S5.
T	pertaining to the tergum (dorsal) segments of the metasoma, e.g., T6, T7.
UWS	University of Western Sydney.

Glossary

Batumen	made of cerumen, resin, and sometimes vegetable material. Plates that form the walls and lining of nests. Mostly seen in *Trigona* spp. and less common in *Austroplebeia* spp.
Brood	nursery for rearing young.
Callow	newly emerged adult, up to one week old and distinguishable by its lighter body colour.
Cerumen	wax and resin mix.
Cloudy day	a cloudy day is recorded when the mean of the 09:00 and 15:00 cloud observations is greater than or equal to 6 oktas (see below).
Endotherm	an animal that is dependent on, or capable of, the internal generation of heat. Often contrasted with ectotherm.
Ectotherm	an animal that is dependent on external sources for body heat. Often contrasted with endotherm. Compare with poikilotherm.

Feeder-float	vessel used to supplement carbohydrate source for colonies. A full description is contained in Chapter 3, Section 3.2 .
Gyne	reproductive, unmated queen. Virgin queen.
Indo-Pacific	comprising the tropical waters of the Indian Ocean, the western and central Pacific Ocean, and the seas connecting the two in the general area of Indonesia, including Australia.
Involucrum	multiple layers of cerumen around brood chamber.
Imago	final developmental stage prior to emergence as an adult. Imagoes, imagines plural.
Monandrous	only mating with one male. Stingless bee species.
Neotropic	the biogeographic region of the New World that stretches southward from the Tropic of Cancer and includes southern Mexico, Central and South America, and the West Indies. Regions in which stingless bees are naturally found.
Oktas	cloud cover observations, are measured in oktas (eighths). The sky is visually inspected to produce an estimate of the number of eighths of the dome of the sky covered by cloud. A completely clear sky is recorded as zero okta, while a totally overcast sky is 8 oktas.
Ontogeny	the development of an individual from oviposition to emerging adult.
Paleotropical	comprising Africa, tropical Asia, New Guinea, and many Pacific islands (excluding Australia and New Zealand).
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.
Pollen trays	a vessel for providing protein supplement to colonies. A full description is contained in Chapter 3, Section 3.2 .
Polyandrous	referring to a mating system in which a female mates with several males during one breeding season.
Potted pollen	*Au. australis* collected pollen, stored by bees in cerumen pot. A full description is contained in Chapter 3, Section 3.2 .
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.

Queen-right	a colony is queen-right if it contains a physogastric queen which has been accepted by the colony and is laying viable eggs.
Requeen	the introduction of a queen cell or live queen in an effort to complete the colony.
Trophallaxis	the mutual exchange of regurgitated liquids between adult social insects or between them and their larvae.

Thesis summary

This research was conducted with a view to increasing knowledge about a little known Australian stingless bee, *Austroplebeia australis* Friese (Hymenoptera: Apidae, Meliponinae). Bees are known to be superior pollinators due to the fact that they actively collect pollen and nectar in large amounts for the purpose of raising their young. There has been limited research into the utilisation of alternative pollinators to the European honey bee (*Apis mellifera*) in Australian horticultural crops. *Au. australis* is a eusocial bee that can be managed in transportable artificial hives and has, therefore, the potential to be managed as a crop pollinator. During this project I investigated aspects of the biology, behaviour and phylogeny of *Au. australis*, with a view to better understanding this species, as well as to determine whether it could be utilised as a greenhouse and / or field crop pollinator.

To develop a context for my studies, an online survey was conducted to assess the current status of the Australian stingless bee industry and its recent development. This was a follow up survey conducted one decade after the first study, by Heard and Dollin in 1998. It showed that the Australian industry had grown over the past ten years but is still underdeveloped. The majority of stingless bee keepers are hobbyists, who own only one colony. There is a high demand for Australian stingless bee colonies and their honey, but with less than 250 bee keepers currently propagating colonies, and many of them on a small scale, it is difficult to meet this demand. Pollination services are provided by a small number of stingless bee keepers; however, there is an urgent need for research into this as well as many other aspects of stingless beekeeping in Australia. Public education is also needed to increase community awareness and, ultimately, to improve efforts in conserving these native species.

Pollination studies were initially conducted using *Au. australis* in vegetable seed crops within an experimental insectary and greenhouse, at the University of Western Sydney, NSW. *Au. australis* colonies acclimated to the greenhouse enclosures and visited crops, with seed set in celery being significantly higher (76.25%) than the control (no pollinator) (0.02%). As a result, further investigations were undertaken with *Au. australis* in commercial field and greenhouse vegetable seed crops, located

at Musk, Vic. These studies, were less successful, due to the cool climatic conditions experienced in this area (Central Victoria), even during summer. These latter results suggest that *Au. australis* colonies do not adapt well to cool climate areas and low ambient and in-hive temperatures negatively influence their flight activity.

The above hypothesis was tested in a subsequent investigation, which assessed the influence of abiotic climatic factors on flight activity of *Au. australis*. The entrance activity of five colonies was monitored and compared to the prevailing weather factors: ambient temperature, in-hive temperature, relative humidity, light intensity and cloud cover. Entrance activity commenced when in-hive temperatures were ≥ 18.6°C and the corresponding ambient temperature was ≥ 20°C. Optimal flight activity was observed when ambient temperatures were ≥ 26°C. Light intensity did not influence flight activity as much as temperature, and relative humidity had a negative impact. Heavy cloud cover and rain periods resulted in *Au. australis* foragers returning to their colonies and ceasing flight activity.

It was unclear whether *Au. australis* was able to thermoregulate its nest, as reported in some other bee species. *Au. australis* colonies naturally occur in northern regions of Australia but some populations experience extreme temperature ranges, including sub-zero temperatures. Thermoregulation enables social insects to produce offspring throughout the year, thus giving them an advantage over solitary insects. To investigate this research question, the in-hive temperatures and brood temperatures of six *Au. australis* colonies were monitored over 12 months. Brood production and development was assessed, along with visible signs of pre-winter preparation. This study showed that colonies of *Au. australis* demonstrated ectothermic characteristics and were unable to warm the brood chamber when temperatures were < 15°C. Brood production, however, continued throughout the cold seasons and developing offspring survived and emerged even after exposure to prolonged periods of sub-zero temperatures, as well as to temperatures > 37°C. *Au. australis*, thus, shows a remarkable ability to survive temperature extremes.

Adaptation to unique environmental characteristics may enable one species to survive over another. To investigate this, comparative studies on the foraging behaviour of three species of Australian stingless bee were conducted. The aim was

to better understand the mechanisms by which *Au. australis* survives during prolonged periods of drought, when floral resources become scarce. The foraging behaviours of *Au. symei, Au. australis* and *Trigona carbonaria* were observed within the confines of an experimental greenhouse, and then compared. Fresh cut floral resources were used instead of artificial feeders, to allow observation of the foragers' behaviour within natural floral structures. Both *Austroplebeia* species were significantly more efficient than *T. carbonaria.* Hovering time was significantly lower in *Austroplebeia* (~ 8%) compared to *Trigona carbonaria* (> 30%). The number of flowers visited by *Austroplebeia* foragers, during a set time period, was greater than for *T. carbonaria. Austroplebeia* foragers demonstrated solitary foraging strategies, while *T. carbonaria* foraged in groups. There were no significant differences between the two *Austroplebeia* species. The different foraging strategies demonstrated by the two different stingless bee genera illustrate adaptive behaviours that may have evolved under different environmental conditions.

Until the current study, little was known about the biology and lifecycle of *Au. australis*. The ontogenic times for developing offspring, as well as the longevity of adults, drive the overall life cycle of a eusocial colony. The developmental times for brood within cluster-building species, such as *Au. australis,* were yet to be reported. In order to observe developing *Au. australis* brood cells for investigations, it was necessary to separate sections of brood from the main brood cluster. A technique was developed where 'donor' brood cells were 'grafted' into hive annexes. This allowed colony members to access the brood 'grafts' for maintenance, while enabling observation of their development. The mean ontogenic time for *Au. australis* workers, maintained at temperatures ~ 26°C, was 55 days, similar to that reported for other stingless bee species. This extended time, compared to *A. mellifera*, may be linked to lower brood incubation temperatures.

In order to determine the longevity of *Au. australis* workers, bees within a single 'closed' colony, as well as six 'foraging' colonies, were marked and observed. 'Foraging' colonies were able to freely access external resources, thus being exposed to a high 'rate of living' and the risk of predation. Workers within both the 'closed' and 'foraging' colonies demonstrated extended longevity, compared to *A. mellifera*. The mean maximum longevity of workers was used to determine the longevity of

cohorts within the foraging colonies. *Au. australis* workers are small (~ 4mm) and cannot be marked with an individual-identification colour scheme. As a result, cohorts of workers within each colony were colour marked and the longest lived bee within each cohort was recorded as the last individual remaining. The maximum longevity of each cohort was used to estimate the mean maximum longevity of all of the cohorts, within the six foraging colonies. The mean maximum longevity of workers from the 'foraging' colonies was 161 days, with the longest lived bee being 240 days old. Within the 'closed' colony, over 20% of the cohorts lived for at least 200 days and the longest lived bee reached 289 days old. The combination of long ontogenic period and extended longevity indicates that individual bees within colonies of *Au. australis* may live as long as seven months.

Natural nest density of social bees is dictated by the availability of both nest substrate and floral resources. A focus survey was conducted to ascertain the nest density of *Au. australis* within an area of south-east Queensland that was known to support substantial bee populations. It was found that nest densities, which ranged from 0.1 to 3.0 nests / ha (mean 0.6 nests / ha) were low, compared to some others reported social bee species. The nest characteristics of *Au. australis* showed that this species prefers narrow cavities within dead trees but does not discriminate between tree taxa. Brood populations range from 2,000 to 13,000 (mean 5,000) in natural nests, within its native range. Colonies under threat of destruction can be easily extracted from their natural cavity and adapt well to artificial hive boxes. *Au. australis* are well adapted to the arid environment of south-east Qld.

During my studies, it became apparent that the current descriptions for the species within the genus *Austroplebeia* are inadequate as a tool for the identification of specimens in either the field or the laboratory. As a result, a triangulated approach was undertaken in an attempt to better delimit morphologically identified groups within *Austroplebeia*. First, morphological data, based on worker bee colour and size were analysed. Drones collected from nests representing morphologically similar groups were dissected and their genitalia were imaged using scanning electron microscopy. Next, data for the geometric morphometric analysis of worker wing venations were obtained. Finally, molecular analysis, using mitochondrial rDNA segment 16S, was conducted on workers from representative nests for each group

which displayed morphological similarities. Data from the four datasets were compared, resulting in the separation of two distinct species, with a large unresolved species complex.

My research results contribute to the previously limited pool of knowledge on *Austroplebeia* spp. in general and *Au. australis* in particular. Its conservative recruitment of foragers for low level floral resources, combined with the efficiency with which foragers harvest these resources, reduces worker mortality within a colony. In the presence of abundant floral resources, forager recruitment is high and resource harvesting and storage is likely to be greater than the energy expenditure of the colony as a whole. Low energy expenditure within the colony, through thermoconformity, reduced nest architecture and efficient foraging strategies enable colonies to conserve precious resources. This combination of behaviours has enabled this species to survive, in harsh environmental conditions.

CHAPTER 1

Literature review, scope and aims of thesis

1.1 General introduction

Worldwide, over 200,000 plant species depend on more than 100,000 species of pollinators. The importance of these pollinators, with regard to agricultural outputs, is finally gaining recognition (Loughlin 2008). In 2000, pollination services provided by the European honey bee were estimated to be worth $A1.7 billion / yr, for the 35 most responsive crops in Australia (Gordon & Davis 2003). However, if the flow-on effects to all Australian horticultural and agricultural industries are taken into account, this figure expands to $4 – 6 billion / yr (Thomson 2007). Unfortunately, globally, the honey bee industry is in crisis, due to pest and disease-induced colony collapse. Humanity's reliance on this industry demonstrates the importance of exploration into and utilization of alternative insect pollinators, such as native bees.

Pollination is essential for fruit and seed production, with most horticultural and many agricultural crops being dependent upon insect vectors. Plants producing scented and coloured floral structures attract insect pollinators, thus enabling or enhancing pollination (Free 1970). Incidental pollination by nectar feeding insects can add to overall pollination; however, pollen collecting insects, such as bees, contribute the highest quality pollination service. They are also capable of carrying pollen from one flower to another, thus facilitating cross-pollination. While insect visitation may not be essential for seed set in some crops, the presence of large numbers of pollinators can improve yield and quality as well as produce earlier and more uniform crops (Free 1970; McGregor 1976; Delaplane & Mayer 2000). Many vegetable seed crops are hybrid cultivars and require insect pollinators for effective cross-pollination and seed set (Banga & Banga 1998).

Bees belong to in the order Hymenoptera and make up a large component of the superfamily Apoidea (Michener 2000). They depend on pollen as a protein source to raise their young (brood) and the evolutionary development of dense, branched, electrostatic hairs increases pollen attraction and facilitate its collection while bees

forage on flowers (Thorp 1979; Michener 2000). These features make bees, by far, the best pollinators of cultivated crops (Free 1970; Delaplane & Mayer 2000; Michener 2000). As foragers move among the flowers, collecting pollen and nectar, pollen grains are attracted to the hairs and transferred to the stigmatic surface. This facilitates self-pollination or cross-pollination within the crop. There are over 18,000 described species of bee worldwide (Michener 2000) but the European honey bee, *Apis mellifera* Linnaeus (Hymenoptera: Apidae), is the most well understood and most important bee for pollination of cultivated crops (Delaplane & Mayer 2000).

The native range of *A. mellifera* extends from northern Europe, down through the Middle East and throughout the arable regions of Africa (Ruttner 1988). As European colonists spread throughout the world, beginning in the early 17th century, so too did the honey bee (Delaplane & Mayer 2000). Since the successful introduction of honey bees into Australia in 1822 (Hopkins 1886) feral populations have spread. Oldroyd et al. (1994; 1997) reports up to 150 colonies per km^2 in some nectar-rich native forests. All of these feral colonies contain foragers that collect nectar and pollen from a variety of floral species, including native and agricultural plants, pollinating as they visit. The pollination services these bees provide are free, abundant and available to any farmer in the area.

Bradbear (1988) published a comprehensive document listing the ten major honey bee pests and diseases and their distribution throughout the world. This list was updated by Ellis and Munn (2005) and of the named threats, only the bee louse, *Braula coeca* Nitzsch (Diptera: Braulidae), and the three parasitic mites: tracheal mite, *Acarapis woodi* Rennie (Acari:Tarsonemidae), varroa mite, *Varroa destructor* Anderson and Trueman (Acari: Paraditidae), and tropilaelaps mite, *Tropilaelaps clareae* Delfinado and Baker (Acari: Laelapidae), had not reached Australian shores. Ellis and Munn (2005) broadened their studies to include viruses that affect honey bees and of the 12 viral diseases suffered by *A. mellifera* only three were absent from Australia. The loss of valuable pollinators, including feral populations, through increasing pressure from introduced pests and diseases is predicted to have devastating effects on world food supplies and a major impact on Australian agricultural and horticultural industries (Allen-Wardell et al. 1998; Anderson & Lawrence 2007; RIRDC 2007). For example, African small hive beetle (*Aethina*

tumida Murray (Coleoptera: Nitidulidae) infestations caused the loss of > 4,500 managed colonies in New South Wales (NSW) between 2002 and 2006 (Rhodes & McCorkell 2007) and > 10,000 colonies in Queensland (Qld) in 2009 and 2010 (Williams 2011).

The most immediate threat comes from the varroa mite, as it occurs in all of Australia's neighbouring countries and has caused incalculable losses in pollination services (van Engelsdorp et al. 2008). Anderson and Lawrence (2007) consider that it is only a matter of time before this destructive pest breaches Australian quarantine borders. This is especially concerning with the recent incursions of Asian honey bees (*Apis cerana* Fabricius (Hymenoptera: Apidae)), a natural host of varroa mite, into Australia (PIF 2012). Continued reliance on honey bees for crop pollination is risky when the threat of population decline is imminent (Somerville 2005; Holden 2006).

Currently, Australian honey bee keepers have the capacity to manage approximately 500,000 colonies. Only 100,000 of these are used for paid pollination services, as feral honey bee populations freely supply services to many growers (RIRDC 2009). Demand for pollination services in the booming almond industry will see approximately 100,000 managed honey bees being migrated south to the almond orchards (RIRDC 2012). It has been estimated that if varroa mite kills all the feral honey bee colonies, which would take less than four years from its initial incursion (Anderson & Lawrence 2007), the demand for managed honey bee colonies would increase to 480,000. This number would be required at the beginning of the season, i.e., September, and would peak at 750,000. This is beyond the current capacity of the Australian honey bee keepers (RIRDC 2010a). To provide essential pollination services for the horticultural and agricultural industries, one option is that other species of insect pollinators be recruited.

1.2 Alternative bee pollinators to *A. mellifera*

Animal pollinators contribute to the production of one-third of the world's dietary input (McGregor 1976; Richards 1993; Klein et al. 2007), including nuts, fruit, seed oil, vegetables, crop seed, meats and dairy (Losey & Vaughan 2006), with non-*Apis* bee species being included in this group. The management and utilization of

alternative pollinators for crop pollination is practised worldwide (reviewed by Bohart 1972; Heard 1999; Ruz 2002). This is, however, not the case in Australia.

Some bee species are intentionally introduced into regions for their pollination services, while others are introduced accidentally, through the transport of building materials, utilised as nesting media by solitary bee species (Bohart 1972). Many non-*Apis* species are effective pollinators but providing these bees in large quantities is extremely difficult (Bohart 1957). Except for bumblebees (described later in this chapter), the most commercially reared alternative pollinators are solitary bees that can nest in man-made materials, thus enabling mass rearing (Bosch & Kemp 2004). A list of some managed bee species is contained in Table 1.1.

The alkali bee, *Nomia melanderia* Cockerell (Halictidae), is a solitary bee which nests gregariously in the ground and is utilised in alfalfa (lucerne), *Medicago sativa* Linnaeus (Fabales: Fabaceae), pollination (Bohart 1972; Richards 1993). Although native to North America its assisted spread by man has had varying degrees of success. It is difficult to transfer nesting material (blocks of soil) any great distance and success is limited by the new conditions presented to the over-wintering larvae. The alkali bee has several predators and parasites and its management can be time consuming and expensive. It is, however, a very effective pollinator of alfalfa crops that occur nearby nesting sites (Bohart 1972).

The alfalfa leaf-cutter bee, *Megachile rotundata* Fabricius (Megachilidae), is a more manageable solitary bee and nests gregariously in naturally occurring and man-made cavities. It can be encouraged to nest in large drilled boards, which provide 2,000 nests / board (Bohart 1972). Management varies from provision of drilled boards only to cocoon harvesting, incubation and storage. This also includes sanitization of nesting material. Management methods have been devised to reduce pests and predators as well as to manipulate bee emergence times through incubation (Bohart 1972). Attempts to introduce this bee into Australia have been minimally successful, although further research is planned by the Rural Industries Research and Development Corporation (RIRDC) and Lucerne Australia (Clarke 2008; D. Anderson, CSIRO, pers. comm., 2008).

Table 1.1 Managed bee species used in crop pollination.

	A. mellifera	***N. melanderi***	***M. rotundata***	***Osmia* spp.**	***Bombus* spp.**	***Meliponini* spp.**
Common name	Honey bee	Alkali bee	Alfalfa leafcutter bee	Mason, Orchard bee	Bumble bee	Stingless bee
Social / solitary	Social	Solitary	Solitary	Solitary	Social	Social
Nest formation	Perennial colony	Ground / aggregations	Borer holes or manmade nests	Borer holes or manmade nests	Annual colony	Perennial colony
Diapause period	None	Winter – mid-Summer	Autumn - Spring	Autumn - Spring	Winter – Spring	None
No workers / aggregation or colony	Up to 50,000	Up to 2,000 / m^2	Up to 2,000 / nesting board	750-1000 / nesting board	100 to 400	500-10,000
Pest/parasite	SHB*, mites, wax moth, bee louse, Nosema	Parasitic wasps	Parasitic wasps	Mites	Mites, beetles, flies, nematodes	Syrphid & phorid flies
Diseases	Chalk and sac brood, AFB*, EFB*, numerous viruses	Brood virus	Chalk brood	Unknown	Chalk brood	Unknown
Native country	Europe	USA	Worldwide	Japan, USA	Europe, USA	Neotropics
Commercial introductions	World wide	New Zealand	Worldwide	Northern hemisphere & New Zealand	Worldwide except Africa & Australian mainland	Neotropics

Data compiled from Holm 1966; Wille 1983; Greer 1999; Richards & Kevan 2002; Spiewok & Neumann 2006; Huntzinger et al. 2008; NBII 2008

*SHB: Small hive beetle, AFB: American foul brood, EFB: European foul brood

Osmia spp. Panzer (Megachilidae) pollinate apple, *Malus* spp. Miller (Rosales: Rosaceae); pear, *Pyrus* spp. Linnaeus (Rosales: Rosaceae); almond, *Prunus dulcis* Miller (Rosales: Rosaceae); plum and cherry, *Prunus* spp. Linnaeus; as well as blueberry and cranberry, *Vaccinium* spp. Linnaeus (Ericales: Ericaceae) blossoms in North America, Japan and Spain (Bohart 1972; Richards 1993; Stubbs & Drummond 1997; Greer 1999; Vicens & Bosch 2000). The bees are managed and maintained in open-ended boxes filled with hollow reeds or straws. These are distributed throughout the orchard to maximize pollination and fruit set (Richards 1993).

Bombus spp. Latreille (Apidae) have been domesticated and utilised in numerous crops for many years (Velthuis and van Doorn 2007). In 1985 bumblebees were found to be effective buzz pollinators of greenhouse tomato, *Solanum lycopersicon* Miller (Solanales: Solanaceae) crops. Since then, *Bombus* spp. have been introduced into other solanaceous crops with good success (Velthuis & van Doorn 2007). *Bombus* spp. are also utilized as pollinators of stock fodder crops in regions where large populations naturally occur (Holm 1966). *Bombus* have a large number of pests including parasitic nematodes, fly maggots, hive beetles and mammals (Holm 1966; Macfarlane et al. 1995; Antonelli & Glass 2004; Spiewok & Neumann 2006).

Stingless bees (Apidae) are important pollinators of many tropical crops including: choko, *Sechium edule* (Jacquin) Swartz (Violales: Cucurbitaceae); coconut, *Cocos nucifera* Linnaeus (Arecales: Arecaceae); mango, *Mangifera indica* Linnaeus (Sapindales: Anacardiaceae); carambola, *Averrhoa carambola* Linnaeus (Geraniales: Oxalidaceae) (Heard 1999); coffee, *Coffea* spp. Linnaeus (Rubiales: Rubiaceae) (Klein et al. 2003) and macadamia nut, *Macadamia integrifolia* Maiden & Betche (Proteales: Proteaceae) (Heard 1993). Coffee plantations located near tropical forests benefit from visiting stingless bees, resulting in higher crop yields (Klein 2009). Stingless bees have also been successfully introduced into greenhouse enclosures for pollination of crops such as strawberries, *Fragaria* spp. Linnaeus (Rosales: Rosaceae) (Kakutani et al. 1993; Slaa et al. 2000; Malagodi-Braga & Kleinert 2004; Roselino et al. 2009), tomatoes (Cauich et al. 2004; Del Sarto et al. 2005) and capsicum, *Capsicum* spp. Linnaeus (Solanales: Solanaceae) (Cruz et al. 2005; Cauich et al. 2006; Greco et al. 2011). They have a variety of natural enemies, depending

upon their native ranges. More detailed information on all aspects of stingless bees will be discussed later in this chapter.

In Australia, investigation of alternative pollinators to *A. mellifera* has been limited. Blue banded bees, *Amegilla* spp. Friese (Hymenoptera: Apidae), and carpenter bees, *Xylocopa* spp. Latreille (Hymenoptera: Apidae), have been investigated for their potential as greenhouse crop 'buzz' pollinators. The use of native bees is preferable to introducing the invasive bumblebee, *Bombus terrestris* Linnaeus (Hymenoptera: Apidae), (Hingston & McQuillan 1999). Buzz pollinators are able to sonicate the poricidal anthers of tomatoes, promoting the release of pollen, and resulting in increased fruit quality and yield (Hogendoorn 2000; Bell et al. 2006; Hogendoorn et al. 2007). Problems are still being encountered with regard to perfecting nesting substrates (K. Hogendoorn, pers. comm., 2010). Stingless bees have been used in macadamia nut crops with good results (Heard 1994).

1.3 Stingless Bees

There is much needed change regarding classification of the genus / subgenus group name of *Trigona* (*Heterotrigona*), which includes a portion of the native Australian Meliponini. At present, according to Michener (1990) species of *Trigona* that occur in the Indoaustralian regions are of the subgenus *Heterotrigona*. Recent molecular studies, and also morphology, suggest this taxonomic classification is incorrect and that Australian species previously named *Trigona* (subgenus *Heterotrigona*) should be changed to the genus *Tetragonula* Moure, 1961 (Rasmussen and Cameron, 2007; Rasmussen and Cameron, 2010). There are many species and subgenera to consider in Asia and Australia, with 15 species in Australian comprising two genera. I do not, therefore, believe it is my place to make such changes when referring to this genus. As such, I have chosen to abide by the rules of nomenclature set down by the International Commission on Zoological Nomenclature (ICZN 1999) and refer to the group name as *Trigona* (*Heterotrigona*) in this dissertation.

The greatest diversity of stingless bees is found in the Neotropical regions of South America, with 412 described species (Camargo & Pedro 2012). Subtropical and tropical regions in Africa, Madagascar, Asia, New Guinea and Australia are also home to many species of stingless bee (Michener 1979). This compares with six to

eight species of *Apis* Linnaeus (Hymenoptera: Apidae) worldwide (Michener 2000). All stingless bees are in the order Hymenoptera and family Apidae[1]. They are then classified into the subfamily Meliponinae which is comprised of two tribes: Meliponini and Trigonini (Wille 1979). These tribes are characterised by morphological differences, and reinforced by some biological and nesting characteristics. Meliponini are more robust in body shape and size than Trigonini and have shorter wings and more dense pubescence (Wille 1983; Michener 1990). Most stingless bees are smaller than honey bees and their wings are reduced in size and venation (Winston & Michener 1977). Stingless bees have a vestigial, functionless sting (Rayment 1935; Wille 1983; Michener 2000); however, they have substantial mandibles, connected to comparatively larger muscles than in *Apis* (Sakagami 1982), that are effectively used for defence. There is a penicillum (long, stiff bristle) on the outer apical margin of their hind tibiae (Wille 1983) and wax glands are located on the dorsal side of the abdomen in stingless bees (Sakagami 1982), whereas they are ventrally located in *Apis* (Snodgrass 1956). The most extensive description of South American Meliponinae species is contained within Moure's Catalog of Neotropical Bees (Camargo & Pedro 2012).

Stingless bees live in social, perennial colonies comprised of a single queen (except in *Melipona bicolor* Lepeletier (Velthuis et al. 2006) and *Melipona quadrifasciata* Lepeletier (Alves et al. 2011)), a variable number of males (drones) and hundreds to thousands of female workers. Colony populations vary greatly in stingless bees; Lindauer and Kerr (1960, cited in Michener 1974) estimate that *M. quadrifasciata* populations are 300 to 400; *Trigona capitata* Smith 1,000 to 1,500 and *Trigona spinipes* Fabricius from 5,000 to 180,000 workers.

Stingless bee nests contain elaborate structures made from cerumen, a mixture of collected plant resin and wax produced by the bees (Wille & Michener 1973). The most common brood structures are horizontal combs (Wille 1983) and these are covered with thin sheets of involucrum, as an insulative layer against temperature extremes (Michener 2000). Cerumen pillars support resin pots in which honey and pollen are stored. The strength of the supports and the colour of these structures are

[1] Within this dissertation, reference will be made to a large number of stingless bee species. In order to assist the flow of text, the order and family (Hymenoptera: Apidae) will not be included with each new species reference.

governed by the proportion of resin to wax. The more resin in the mixture, the harder, darker and stronger is the structure (Michener 2000). Some Meliponini species incorporate mud and plant debris into the nest structure (Wille 1983). Resins have varying levels of flavonoid compounds, depending on their botanical origin, and have been shown to possess antimicrobial properties (Burdock 1998; Kujumgiev et al. 1999). The resins' antimicrobial properties remain active, as part of the cerumen, within the nest (Velikova et al. 2000; Patricio et al. 2002). Some stingless bee honeys possess similar flavonoid profiles to extracts of plants found in the same geographical region as their nests (Vit & Tomás-Barberán 1998). These compounds may aid in the preservation of honey and pollen within pots constructed of cerumen.

1.4 Meliponiculture

Meliponiculture is the practice by which bee keepers reproduce stingless bee colonies, of various species, for profit. This profit may be in the form of honey, cerumen, resin and nucleus colonies (Heard & Dollin 2000). Meliponiculture is practiced by communities throughout South America, Central America and Mexico, as well as in small areas of northern Australia (Cortopassi-Laurino et al. 2006). The ancient Mayans considered the stingless bees worthy of a place in their religious worship, including a god of honey in their ensemble of deities (Cortopassi-Laurino et al. 2006). Stingless bees have been highly regarded by indigenous northern Australians for many centuries and their hive products played an important part in their culture (Akerman 1979; Souza et al. 2006). Today, the aboriginal people manage bees in artificial hives and sustainably harvest products such as honey, 'sugarbag', and cerumen. These products are sold to tourism centres, gift shops, health food stores and restaurants. There has been an increasing awareness and demand for 'bush tucker' and stingless bee honey is one of the prized foodstuffs (Cortopassi-Laurino et al. 2006; Souza et al. 2006). Currently, only a few of these enterprises are run by indigenous communities.

Stingless bee honey is used medicinally in the treatment of gastro-intestinal upsets, ocular complaints, ulcers and wounds and coughs (Vit & Tomás-Barberán 1998; Vit et al. 2004; Cortopassi-Laurino et al. 2006). The honey has a higher moisture content (20 – 42%) (Souza et al. 2006; Persano Oddo et al. 2008) than *A. mellifera* honey (18

– 20%) (Bijlsma et al. 2006) and usually requires pasteurisation or refrigeration to avoid fermentation (Cortopassi-Laurino et al. 2006; R. Zabel, pers. comm., 2006). Moisture content and viscosity of Australian stingless bee honey is reported to be variable (R. Zabel; T. Heard; R. Raymond, pers. comm., 2008) and is dependent on the honey's entomological, botanical and geographical origins (Vit et al. 1997). Limited research has been conducted on Australian honeys; however, *Trigona carbonaria* Smith honey has been shown to have similar composition to those of other Meliponine honeys (Persano Oddo et al. 2008). Preliminary studies on its antioxidant and antimicrobial activities show some promise for nutritional and pharmaceutical uses (Irish et al. 2008; Persano Oddo et al. 2008; Boorn et al. 2010). Worldwide, limited research has been carried out on the chemical composition of the honey, pollen and yeasts contained within stingless bee colonies (Sommeijer et al. 1983; Fernandes-da-Silva & Serrao 2000; Cortopassi-Laurino et al. 2006).

1.4.1 Native plant and agricultural crop pollination by stingless bees

As with all members of the corbiculate Apidae family, stingless bees have morphological adaptations which enable them to gather and safely store pollen whilst foraging on flowers (Rayment 1935; Snodgrass 1956; Michener 2000). The corbiculae enable bees to forage and collect large loads of pollen at a time. A small amount of nectar is added to the pollen mix to aid in its packing (Rayment 1935). Stingless bees also use the pollen basket to transport resin, for use in nest structures (Rayment 1935; Patricio et al. 2002). In *Austroplebeia* Moure, the inner surface of the hind tibial keirotrichiate area is broad and almost reaches the upper margin of the tibia; this is not so for *Trigona* Jurine (Michener 1990; 2000).

Stingless bees perform pollination services, transferring pollen grains to the stigmas, while foraging amongst a wide variety of native and exotic flowers (Sommeijer et al. 1983; Adams & Lawson 1993; Heard & Exley 1994; Heard 1999; Cruz et al. 2005; Cortopassi-Laurino et al. 2006). They can be seen foraging high atop rainforest trees and their contribution to forest biodiversity is considerable within their native ranges (Wille 1983).

Of the 1,000 or more plant species cultivated in the tropics, approximately 250 are compatible with stingless bee pollination (Heard 1999). Pollination services provided

by stingless bees help increase the yield of a number of cultivated crops (Section 1.2). Many economically significant, cultivated crops originate from regions where *A. mellifera* do not naturally occur (Heard 1999).

Stingless bees have the ability to visit and pollinate a large variety of plants, with high floral constancy (Heard & Hendrikz 1993; Ramalho et al. 1994; Hilário et al. 2000; White et al. 2001). This, together with their low susceptibility to European honey bee pests and diseases (Delfinado-Baker et al. 1989), makes the exploitation of these bees an attractive agricultural and horticultural activity. Artificial hives containing *T. carbonaria* or *Trigona hockingsi* Cockerell have been introduced into Australian macadamia orchards, with good results (Heard 1999). The fact that eusocial stingless bees are perennial (Wille 1983) ensures the presence of pollinators throughout the year, while environmental conditions are favourable (Heard & Hendrikz 1993). This is particularly important in year-round greenhouse crop production (Amano 2004; Malagodi-Braga & Kleinert 2004). They are successful foragers within the confines of a greenhouse (Kakutani et al. 1993; Slaa et al. 2000; Malagodi-Braga & Kleinert 2004; Roselino et al. 2009) and, being stingless, they are also less harmful to the humans tending the crops (Heard 1999).

Although some stingless bee species are capable of 1.5 km flight distances (Roubik & Aluja 1983), *Trigona* and *Austroplebeia* are thought to forage only one to two hundred metres from their nests (Dollin 2010a), to a maximum of 500 m (Bartareau 1996). This is advantageous for crop pollination, as stingless bees are more likely to forage within the crop than venture further afield in search of more attractive floral resources, as is the case with honey bees (Graham 1992). When located in macadamia crops, *T. carbonaria* usually forage a mere 25 to 30 m from their hive (F. Adcock, pers. comm., 2008). As a result, hive placement is important and 15 to 20 hives / ha (compared to honey bees' 7 hives / ha) are placed throughout the crop, especially when cross-pollination is required (Heard & Dollin 1998; F. Adcock, pers. comm., 2008; T. Carter, pers. comm., 2010). The distance a stingless bee is prepared to fly depends on how attractive the resource is (Heard 1999) and the relative size of the bee (Michener 1974).

The Australian stingless bee pollination industry had its beginnings in the late 1980s when it was found that yields of macadamia nut, *M. integrifolia* grown near remnant

native vegetation were noticeably higher than for those crops situated near cleared land (Heard 1988a; Heard & Exley 1994). The main pollinators of macadamia are honey bees and stingless bees (Vithanage & Ironside 1986) and the presence of these insects is extremely important for maximum nut set (Wallace et al. 1996). Although the temperature threshold for *T. carbonaria* flight activity is ≥18°C (Heard & Hendrikz 1993), resulting in shorter foraging days compared to honey bees (7 h vs. 10 h / day) (Heard & Exley 1994), *Trigona* are efficacious pollinators of macadamia flowers (Heard 1994; T. Carter, pers. comm., 2009). Their small bodies are able to make more intimate contact with the stigmas while they collect pollen (Heard 1994), optimising pollen transfer. Heard (1987) also demonstrated that *Trigona* foragers returned to hives with 100% macadamia pollen, compared to honey bees with only 24%. Interestingly, *Trigona* prefer warm flowers (Norgate et al. 2010) and this is demonstrated by their attraction to flowers on outer, sunny racemes (Heard & Exley 1994). Macadamia also benefits from varietal interplanting for cross-pollination (Rhodes 1986) as their flowers are mostly self-incompatible and protandrous (Sedgley et al. 1985).

Crops other than macadamia can also benefit from stingless bee pollination. Anderson et al. (1982) showed stingless bees to be effective pollinators of mango (*M. indica*) and anecdotal accounts of increased crop quality and yield have been reported for other crops such as lychee, *Litchi chinensis* Sonnerat (Sapindales: Sapindaceae); avocado, *Persea americana* Miller (Laurales: Lauraceae) and watermelon, *Citrullus lanatus* (Thunberg) Matsumura & Nakai (Violales: Cucurbitaceae) (T. Carter, pers. comm., 2009). Although no scientific studies have been conducted on the effectiveness of stingless bees as pollinators in field crops in Australia other than macadamia and mango, improved crop yields have been reported by one bee keeper and his associated growers (T. Carter, pers. comm., 2009). Stingless bees have also been introduced into blueberry, *Vaccinium corymbosum* Linnaeus (Ericales: Ericaceae), crops and are able to collect pollen and nectar more efficiently than honey bees (F. Adcock, S. Maginnity, M. Grosskopf, pers. comm., 2010) because blueberry flowers are small, with a deep corolla and narrow terminal orifice (Rhodes 2006). Unfortunately, there are no experimental designs or statistical analyses associated with these trials. Although the role of stingless bees in pollination of native flora is well documented, their efficacy in

horticultural and agricultural crops of Australia needs further study (Heard 1999; Slaa et al. 2006).

1.4.2 Meliponiculture in Australia

The practice of meliponiculture in Australia was almost non-existent in 1984. However, a survey conducted in 1998 / 99 showed considerable growth in its popularity to that date. It was predicted that meliponiculture would steadily increase over the next 20 to 30 years (Heard & Dollin 2000). Since then, interest in stingless bee keeping has increased with conservation groups being established, especially along the eastern regions of the country.

More recently, it was estimated that the number of professional service providers was probably six. Even so, pollination service fees were only a secondary income, and some of these providers were orchardists, keeping colonies for pollination of their own crops (Cortopassi-Laurino et al. 2006). A small number of colony producers transferred and split colonies for sale to enthusiasts, honey producers and pollination service providers (Cortopassi-Laurino et al. 2006).

The production of honey and cerumen for niche markets has been only very small in Australia. In 1998 and 2005, it was estimated that production of 'sugarbag' honey was < 100 kg / yr. Prices for the honey increased from $A40 / kg in 1998 to $A50 / kg in 2005 (Heard & Dollin 2000; Cortopassi-Laurino et al. 2006). However, these prices were less than with inflation, representing an actual depreciation in the value of the product. Production was low and costs were high and, as a result, it was thought that in order to commercialise the industry commodity honey prices needed to increase (Cortopassi-Laurino et al. 2006).

Stingless bees are harmless and are an attractive tool which can be used to demonstrate the wonders of nature. These include sociality, pollination and, of course, entomology (Cortopassi-Laurino et al. 2006). Schools, gardens, universities and museums have started to utilise these fascinating creatures in this way. Workshops on stingless bee keeping are often run by enthusiasts in many areas and websites have been set up to help the community increase their knowledge in this area. The Australian Native Bee Research Centre has a website

(www.aussiebee.com) and the Australian native bee interest group (www.australiannativebees.com) is well populated.

To date, while there has been little organised research conducted on Australian stingless bees, the wealth of knowledge held by stingless bee enthusiasts is invaluable. Further scientific studies are needed to support these bee keepers and to help improve techniques in colony propagation, queen rearing, drone rearing and, possibly, artificial insemination. The reported successes in pollination services provided by stingless bees overseas (see Section 1.2) have yet to be realised in Australia.

Colony propagation is the driving factor for the stingless bee industry. Large numbers of colonies are required for honey production and pollination services. A strong colony can provide only 1 to 1.5 kg of honey / yr and three times as many colonies as honey bees are required to pollinate the same area. Other aspects requiring research include honey preservation post-harvest, education of farmers to reduce bee losses through pesticide use and collation of research material to enable efficient whole-community education and industry training (Cortopassi-Laurino et al. 2006).

1.5 Australian stingless bee classification

The stingless bees of Australia belong to two genera, *Trigona* (Jurine 1807) and *Austroplebeia* (Moure 1961) and are in the sub-tribe Trigonini (Wille 1979). They were first described by Hockings (1883) by their Aboriginal names, "Karbi" and "Kootchar", respectively, The genus *Trigona* contains over 100 species worldwide and is divided into ten subgenera. All of the Australian *Trigona* spp. are currently classified in the subgenus *Heterotrigona*. The *Austroplebeia* spp. were also previously placed in the genus *Trigona* until Michener revised the family Apidae in 1990. The bees within both genera are small (< 4.5 mm) and black; however, *Austroplebeia* can be distinguished from *Trigona* by its coloured body markings, thoracic shape and nest architecture. *Austroplebeia* has small creamy, yellow markings on the scutellum and axillae of the thorax and the face, whereas *Trigona* is completely black (Michener 1990; Klumpp 2007). The dorsal rim of the thorax in *Trigona* is more angular than in *Austroplebeia* (Dollin 2010b). Below is the key to

the genera of the Meliponini of Australia and PNG, taken from Michener's (2000) 'The Bees of the World':

> 1. Scutellum and usually face and scutum with well-developed yellow markings; inner surface of hind tibia with keirotrichiate area broad, nearly reaching upper margin of tibia ……*Austroplebeia*
>
> _. Head and thorax without distinct yellow markings; inner surface of hind tibia with strong longitudinal keirotrichiate ridge above which is a broad depressed, shining marginal area ……*Trigona*

1.5.1 *Trigona*

Australia is home to six species of *Trigona*; however, the most commonly domesticated and studied species are *T. carbonaria* and *T. hockingsi.* The drones of *Trigona* are difficult to identify within the nest, without the aid of a magnifying glass, as they have no defining markings (Dollin 2010b). They frequently form drone swarms outside colonies and aggregate on foliage or other structures at night. These aggregations and swarms can be seen for a number of days when seasonal conditions are favourable (Klumpp 2007).

1.5.1.1 Species description

The currently described Australian *Trigona* are classified into three species-groups, namely:

iridipennis group (Sakagami 1978)

Trigona (*Heterotrigona) clypearis* Friese 1908

laeviceps group (Sakagami 1978)

Trigona (*Heterotrigona) sapiens* Cockerell 1911

carbonaria group (Dollin et al. 1997)

Trigona (*Heterotrigona) carbonaria* Smith 1854

Trigona (*Heterotrigona) hockingsi* Cockerell 1929

Trigona (*Heterotrigona) mellipes* Friese 1898

Trigona (*Heterotrigona) davenporti* Franck 2004

(Dollin et al. 1997; J. Klumpp, A. Dollin, pers. comm., 2010).

Identification of Australian *Trigona* spp. is very difficult in the field and some species, especially *T. carbonaria*, can vary considerably in size according to their geographic location (Dollin et al. 1997). The largest Australian *Trigona* is *T. hockingsi*, measuring ~ 4.5 mm in length, while the smallest is *T. clypearis*, at 3.5 mm (Klumpp 2007). Species within the *carbonaria* species-group are difficult to separate on their body size or morphology. Thus, nest architecture is an invaluable tool in the accurate identification of species within the *Trigona* genus (Dollin et al. 1997).

1.5.1.2 Nest architecture

Tree cavities are the most commonly chosen nest substrate for *Trigona* spp. They can also be found inside water meter boxes, stone walls, beneath concrete foot paths and within wall cavities (Dollin et al. 1997). *T. mellipes* has also been reported to nest within termite mounds (R. Zabel, pers. comm., 2007). Nest entrance modifications vary, depending on species although environmental factors, such as weather and predators, can also influence these structures (Dollin et al. 1997). *T. carbonaria* often daub the area around the entrance with substantial amounts of resin, whereas *T. hockingsi* and *T. davenporti* generally leave their entrances unadorned (Dollin 2010b). *T. mellipes, T. sapiens* and *T. clypearis* are capable of building entrance tubes of varying sizes (Table 1.2), although they do not always do so (Dollin et al. 1997).

Table 1.2 Comparative description of *Trigona* spp. (Dollin et al. 1997; Klumpp 2007) and *Austroplebeia* spp. (Michener 1961).

Species	Entrance characteristics & tube length	Mean nest cavity dia.	Brood structure
T. hockingsi	None. Seldom smears entrance with resin	145 mm	Horizontal steps/terraces. Hexagonal comb.
T. carbonaria	None. Usually smears entrance with resin	198 mm	Flat spiral, single layer. Hexagonal comb.
T. mellipes	16 mm (mean)	82 mm	Similar to *T. hockingsi* but smaller.
T. sapiens	6 mm (mean)	58 mm	Irregular, horizontal or diagonal layers. No hexagonal comb.
T. clypearis	28 mm (mean)	78 mm	Roughly arranged in diagonal rows. No hexagonal comb.
Austroplebeia except *Au. cincta*	3 – 10 mm	65 mm	Clustered.
Au. cincta (PNG)	20 – 80 mm	45 mm	Irregular concentric layers of one cell thickness.

1.5.1.3 Colony population and brood structure

It has been estimated that a strong colony of *T. carbonaria* has a population of approximately 11,000 workers (Hoffmann, unpublished data). Brood volume can vary from 940 to 3,535 mL in *T. carbonaria* and from 1,100 to 2,550 mL in *T. hockingsi* (Dollin et al. 1997); however, *T. hockingsi* is able to build much larger nests if provided with the appropriate nest cavity (A. Dollin, pers. comm., 2010). Both *T. davenporti* and *T. hockingsi* build brood areas with similar structure; however, *T. davenporti* has a smaller adult population. *T. mellipes, T. sapiens* and *T. clypearis* have much smaller nests and average brood volumes measure 595 mL, 224 mL and 464 mL, respectively (Dollin et al. 1997).

Most stingless bee species build regular, horizontal brood comb, which is located near the centre of the nest (Wille 1983). All Australian *Trigona* build elongated, vertically-oriented brood cells in regular, or nearly-regular, structures (Figure 1.1) (Dollin et al. 1997). There are distinguishing features within these structures that can aid in species identification (Table 1.2). *T. carbonaria* builds single layers of hexagonal comb, arranged in a horizontal spiral. Brood cells are constructed on the

outer rim of up to three spirals at a time. *T. hockingsi* builds a regular, horizontal brood structure with hexagonal comb, which is best described as terraced or stepped and is not in a single layer. Both *T. davenporti* and *T. mellipes* build similar brood comb to that of *T. hockingsi* but the brood comb area of *T. mellipes* is considerably smaller (J. Klumpp, pers. com., 2010). Neither *T. sapiens* nor *T. clypearis* have a hexagonal comb structure and individual cells are arranged in irregular horizontal or diagonal layers (Dollin et al. 1997).

Figure 1.1 Typical *Trigona* horizontal brood structure (*T. carbonaria*).

1.5.2 *Austroplebeia*

1.5.2.1 Species description

There are nine species of *Austroplebeia* listed in the Zoological Catalogue of Australia (Cardale 1993). Only one of these, *Austroplebeia cincta* Friese, occurs outside Australia, in PNG. Current classification of the species within this genus is based mainly on variations in body markings. Mature adult bees are black, with varying levels of cream-coloured markings on the scutum of their thorax and on their face (Michener 2000). There is considerable confusion with regard to the description of each of the nine species within this genus. There are no clear defining

characteristics to delimit these species (A. Dollin, pers. comm., 2008). These issues will be discussed in depth in Chapter 4.

1.5.2.2 Nest architecture

Similar to *Trigona*, *Austroplebeia* choose tree hollows as their most common nest site; however, the cavity diameter is usually smaller. Some colonies of *Austroplebeia australis* Friese have been found in narrow tree limbs up to 6 m in length (R. Zabel, pers. comm., 2008). They are commonly found in steel hand rails and door cavities in residential areas (A. Beil, pers. comm., 2009). No nest characteristics have been described for the species within this genus.

1.5.2.3 Colony brood structure

As previously stated, most stingless bees construct horizontal brood comb within the middle of the nest structure. There are a few species, however, that construct irregular clusters of brood cells which are arranged to fit into the narrow, irregular cavities of small trees or large limbs. Overseas, cluster-building stingless bees include the species within the genera *Lestrimelitta* Friese, *Plebeia* Schwarz, *Tetragona* Lepeletier & Serville, *Scaura* Schwarz and *Trigona* (*Hypotrigona*) Cockerell (Michener 1961). All *Austroplebeia* spp. construct spherical brood cells and, with the exception of *Au. cincta* (Table 1.2), arrange them into simple, primitive clusters (Michener 1961; Dollin 2010b) (Figure 1.2). Open cells face outwards from the leading edge of the cluster, in irregular directions.

Figure 1.2 Typical, healthy *Austroplebeia* brood cluster (*Au. australis*).

1.6 Colony life cycle

Most species of stingless bees reside in warm climates where workers are able to forage all year round, supplying the colony with food resources continuously. Others inhabit areas of south-eastern Brazil and Argentina that experience cool-cold winter seasons (6 – 20°C) or dry seasons with reduced floral resources (Camargo & Pedro 2012). These factors limit the opportunities for foragers to collect food resources for the colony. According to Roubik (1989), it is not so much the extremes in temperature that limit the distribution of stingless bees, but the limited foraging opportunities.

Some species have evolved mechanism to help them cope with these fluctuating environmental conditions. *Plebeia remota* Holmberg cease brood production with the onset of cool weather (van Benthem et al. 1995; Ribeiro 2002), much like *A. mellifera*. During periods of severe dearth of floral resources, colony workers of *Melipona favosa* Fabricius and *Melipona fulva* Lepeletier cannibalize young larvae and their provisions. Brood production does not completely cease but becomes extremely conservative. Both species also demonstrate conservative foraging behaviours (Roubik 1982).

A. mellifera produces drones only when the colony can support them, usually spring to late summer (Graham 1992). Drones are unable to feed themselves and have only one function – to mate. Most die of old age or are ejected from the nest before they attain their life's goal (Winston 1991). In some stingless bee species (including *Scaptotrigona postica* Latreille) drone populations are maintained throughout the year and some are self-sufficient through nectar and pollen collection (Kerr et al. 1962; Roubik 1989). Drones are produced in periodic clusters within individual colonies of *Trigona* (*Lepidotrigona*) *ventralis flavibasis* Cockerell; however, they are present throughout the year at the local population level. Queens are produced in low numbers all year round but the proportion of sexual offspring produced in a *T. ventralis* colony is positively correlated with the amount of stored food (Chinh & Sommeijer 2005). In *Trigona pectoralis panamensis* Cockerell, drones can account for up to half of the colony population (Roubik 1983). *Melipona beecheii* Bennet produce drones and queens throughout the year yet, during times of reduced food resources, sexual offspring production is reduced (Moo-Valle et al. 2001). In most species of stingless bees, the drones are driven from the colony once they reach maturity (Roubik 1989).

Little is known about the drones of Australian stingless bees. The morphological characteristics of drones have been described for *Trigona* (Dollin et al. 1997), but have not been described for *Austroplebeia*. Some behavioural characteristics have been described for *Trigona* drone swarms (Klumpp 2007), but there are few bee keepers who have ever seen drones of *Au. australis.* The seasonal cycle of *Au. australis* colonies has not been studied. It is unknown whether brood production is continuous or how colonies react to periods of floral dearth. The times of the year for production of reproductive offspring throughout the year is unknown and the effects of reduced food stores on their production is also unstudied.

1.7 Geographic origin, distribution and density of stingless bees

Stingless bees are thought to be of late Gondwanan origin (~ 80 million years ago (mya)) with migration into the Afrotropical and Indo-Malayan / Australasian regions occurring approximately 50 to 60 mya. The greatest diversity resulted in the New

World, occurring around 30 to 40 mya (Rasmussen & Cameron 2010). In South America, there are currently 412 described species (Camargo & Pedro 2012). This is in contrast to 32 species in Africa, 42 in Asia and 20 in Australia, New Guinea and the Solomon Islands (Kerr & Maule 1964). This restricted dispersal may have resulted from the limited land bridges combined with the short flight (1 km) and reproductive swarming (50 m) ranges of the Meliponinae (Kerr & Maule 1964). Australia's *Austroplebeia* is thought to be one of the most recently dispersed taxa (Rasmussen & Cameron 2010).

Under climatically suitable conditions, the factors limiting colony distribution and density include the availability of suitable nest substrates, nest predation or destruction and, most importantly, food resource availability and diversity (Hubbell & Johnson 1977; Eltz et al. 2002). Suitable nest substrate is specific to bee species. Generally, smaller bees require tree cavities of a smaller diameter while large bees, which build larger colonies, require bigger cavities (Hubbell & Johnson 1977). The taxa of trees does not appear to be a limiting factor in the choice of nest site. Nest trees can be living or dead; however, nests in live trees may be under greater threat in areas where trees are harvested for timber. Because Meliponinae colonies are long lived and have low fecundity, land clearing for agricultural development or for timber is likely to have a detrimental effect on stingless bee populations (Eltz et al. 2003). Nest destruction is a major problem worldwide (Cortopassi-Laurino et al. 2006) and land clearing and deforestation is driving some bee species to extinction. While it is not yet serious in Australia, populations are under threat and the pressures are unknown and unstudied (Batley & Hogendoorn 2009).

Diversity in diet is beneficial and nest distribution and density is positively correlated with food resource availability (Hubbell & Johnson 1977). Eltz et al. (2002) found 148 pollen morphotypes in stingless bee nests and areas with the greatest nest density were located near various, non-forest plant species. Overall geographic distribution of stingless bees is very much dependent on the amount of food colonies are able to collect and store. If an area is rich in resources but the weather conditions are not conducive for flight, food cannot be accessed and colonies will starve (Roubik 1989).

The highest rainfall areas within Australia occur in the northern, eastern and far south-western coasts, resulting in tropical, subtropical and temperate forest and

woodland vegetation, respectively. The natural range for Australian stingless bees is in the tropical and subtropical regions of northern Australia, with the exception of *T. carbonaria*, which has, by far, the most southerly distribution. The temperature threshold for flight activity in *T. carbonaria* (Heard & Hendrikz 1993) is ≥ 18°C which means that the foraging time for colonies in the most southerly range of their distribution is substantially reduced, compared with those in more northern locations.

1.7.1.1 Natural distribution of Australian stingless bees

Trigona

Dollin et al. (1997) reported that *T. clypearis* and *T. sapiens* are restricted to the Cape York Peninsula in northern Queensland (Qld) (18°0'S – 10°56'S) compared to the *carbonaria* species-group which is distributed throughout northern and eastern Australia. The recently described *T. davenporti*, was discovered by Peter Davenport, a local bee keeper who helped pioneer stingless beekeeping in Australia (Klumpp 2007; Dollin 2010e). So far, this species has only been reported within a restricted area around the Gold Coast in south-eastern Qld (A. Dollin, pers. com., 2010). As previously stated, *T. carbonaria* is the most widely distributed species, occurring along much of the east coast of Australia. It is found as far north as the Atherton Tableland in Qld (17°15'S) and as far south as Bega in NSW (36°40'S).
T. carbonaria chooses large tree cavities which may provide superior insulative properties against the weather extremes experienced in its most southerly distribution.

Austroplebeia

Dollin (2010c) reported that *Austroplebeia* is distributed throughout the northern regions of Australia and that *Au. australis* and *Austroplebeia symei* Rayment have the widest distributions. Specimens currently considered to be *Au. symei* have been collected along the east coast from the top of Cape York (11°04'S) to Kilcoy in Qld (26°57'S), as well as the northern areas of the Northern Territory (NT). *Au. australis* has been found as far south as Dungog, in New South Wales (32°24'S) and also occurs in arid regions of inland Qld. The remaining species are found mainly in northern parts of Qld, NT and Western Australia (WA), with *Austroplebeia percincta*

Cockerell originally described from an arid region of central Australia (A. Dollin, pers. comm., 2009).

While *Trigona* is commonly found in areas of high rainfall, many *Austroplebeia* spp. thrive in areas that experience low annual rainfall (300 – 600 mm) and wide temperature ranges (-3 – 40.5°C) (A. Dollin, pers. com., 2009; Bureau of Meteorology 2009). Currently, there is little data available pertaining to the nest density and distribution of Australian stingless bees.

1.8 Stingless bee biology

Unlike *A. mellifera*, Meliponinae queens normally mate only once (Kerr et al. 1962; Michener 1974), returning to the nest with the male genitalia still caught in the vagina (Michener 1974). Incidence of low frequency polyandry has been reported in *M. beecheii* and *S. postica* (Paxton et al. 1999); however, it is thought that most stingless bees are monandrous, including the Australian species (Drumond et al. 2000a; Green & Oldroyd 2002). The queen is easily identified by her distended, egg-filled abdomen and short wings (Figure 1.3) (Klumpp 2007). Sperm is stored in the spermatheca. A diploid female, a worker, is produced when a sperm cell is released to fertilise the egg as it passes through the oviduct. If sperm is not released, the egg is not fertilised and a haploid male is produced (Michener 2000). While drones are predominantly produced by the queen (Lacerda et al. 2010; Velthuis et al. 2005), laying workers have been reported in some Brazilian species of *Melipona* Illiger (Koedam et al. 2005; Koedam et al. 2007). Although this is rare in Australian stingless bees (Michener 1974; Drumond et al. 1999; Tóth et al. 2004), *Au. australis* and *Au. symei* workers have been observed laying small numbers of trophic eggs in queenright colonies. However, on all recorded occasions the queen consumed these eggs (Drumond et al. 1999). Microsatellite analysis determined that workers were not responsible for drone production in queenright colonies of *Au. australis, Au. symei* or *T. carbonaria* (Drumond et al. 2000a; Gloag et al. 2007). Drone production has been observed in some queenless colonies; however, this has not been scientifically studied (Klumpp 2007).

Figure 1.3 *Au. australis* queen, easily identified on the brood cluster.

There have been very few studies conducted on stingless bee drone biology. Kerr et al. (1962) found that the number of drones in a mating swarm could range from a few dozen in *S. postica* to 3,000 in *Trigona jaty* Smith. Sperm counts for *M. quadrifasciata* are approximately 1,150,000 compared to an average of 6,000,000 for *A. mellifera*. Some stingless bee species have been reported to produce drones in 'seasonal cycles' (van Benthem et al. 1995) and some *Melipona* produce drones in 'batches' or 'male-producing periods' (MPP) (reviewed by Velthuis et al. 2005) , with drones being present only periodically in a single colony.

No studies have been conducted on Australian stingless bee drones, including aspects such as sperm counts and motility, mass rearing of drones, drone behaviour or drone maturity and longevity.

1.8.1 Ontogeny

In the Meliponini, brood production is an elaborate procedure and involves a sequence of interactions between the queen and a group of workers (Sakagami et al. 1973; Sakagami 1982). This temporal sequence is termed the 'provisioning and ovipositing process' or "POP" (Sakagami SF and Zucchi R, 1963; Michener, 1974; Wittmann et al. 1991). Cells are mass provisioned with a mixture of honey, pollen

and protein-rich secretions from the hypopharyngeal glands (Michener 1974; Silva de Moraes et al. 1996). Some species provision cells successively while others provision synchronously (Sommeijer & Bruijn 1984). Once a cell is provisioned, the queen oviposits and a worker moves in to seal the cell (operculation) (Drumond et al. 1999). *T. carbonaria* constructs and provisions brood cells synchronously and the queen oviposits in batches (Yamane et al. 1995). *Au. australis* and *Au. symei* construct and provision brood cells in a successive pattern and the queen does not oviposit in batches (Drumond et al. 1999). The stages of development in *Melipona* bees are the same as for the honey bee; however, the developmental times differ (Table 1.3). In Trigonini, the larger queens have a longer ontogenic period (Imperatriz-Fonseca & Zucchi 1995). Ontogenic times and the effect of temperature on development have not yet been determined for any Australian stingless bee species.

Table 1.3 Developmental stages and their length of time (in days) in some *Melipona* species (Venturieri 2008) and *A. mellifera* (Winston 1991).

Stages	***Melipona***	***A. mellifera***
Egg hatches	5 days	3 days
Larval	12 – 13 days	8 days
Pupal (cell capped)	18 – 19 days	16 days
Emerging adult	40 – 52 days	21 days

1.8.2 Longevity

Once the callow (immature bee) worker emerges, she is soon employed within the brood chamber (Wille 1983; Winston 1991). Her pupal case is rolled into a small ball and removed from the nest by one of the workers (Klumpp 2007). Division of labour within stingless bee colonies is similar to that in honey bees (Wille 1983), but can vary according to the needs of the colony and the environmental conditions (Simões & Bego 1991). Age polyethism ensures colonies utilise workers for maximum efficiency. Brood rearing tasks are performed by young workers, whose wax glands are active and hypopharyngeal glands are producing proteins, lipids and vitamins (Winston 1991). As they age, workers are employed in the construction of honey and pollen pots, receive and dehydrate nectar, clean the nest and remove debris (Wille

1983). Toward the end of their lives, workers move into high-risk tasks such as nest defence and foraging. This utilisation of age polyethism ensures workers reach maximum longevity.

The longevity of colony members varies in stingless bees. The mean longevity ranges from 21 days in *Tetragonisca angustula angustula* Latreille (Grosso & Bego 2002) to 51 days in *M. beecheii* (Biesmeijer & Tóth 1998). The longevity of workers has not been determined for any Australian stingless bee species. In addition, the mortality rate has not been studied nor have any life tables been compiled.

1.8.3 Colony reproduction in stingless bees

Social bees are often considered to be 'super-organisms' (Moritz & Southwick 1992) and reproduction relates to the duplication of the colony rather than the reproduction of individuals within a colony (Queller & Strassmann 2002; Seehuus 2006). *A. mellifera* reproduce by swarming, which coincides with colony crowding and / or the occurrence of favourable environmental conditions (Michener 1974; Seeley 1985). Workers initiate the construction of queen cells along the bottom edge of the brood comb and the queen lays a fertilised egg in each cell. The hatching larvae are exclusively fed secretions from the hypopharyngeal and mandibular glands of young nursery workers, known as royal jelly (Lercker et al. 1981), to ensure the larvae develop into queens. While the daughter queens develop the mother queen is gradually starved and workers begin to continuously harass her. This results in a reduction in egg production and a dramatic weight loss – up to 25% of her body weight. Finally, the workers gorge themselves with a supply of stored honey and they, along with the 'trimmed' queen, take flight as a reproductive swarm. This 'prime swarm' is guided by scouts to a suitable new nesting site (Seeley 2010). The parental colony, containing the ready-to-emerge virgin queens, is left behind with at least 50% of the colony's workers. The two colonies become completely independent of each other as soon as swarming is initiated (Michener 1974).

Stingless bee colony reproduction is achieved by more complex methods and the new colony remains dependent upon the mother colony for several weeks or even months after it is initially established (Michener 1974; Wille 1983; Inoue et al. 1984; van Veen & Sommeijer 2000). When environmental and nest conditions are

conducive, scout bees leave the mother colony in search of suitable nest sites. Once located, workers commute between the two nest sites, cleaning the new cavity, sealing cracks and constructing an entrance. Materials used in the renovation are sourced from the environment as well as from the mother colony (Michener 1974; van Veen & Sommeijer 2000). Later, pollen and honey stores are also brought in from the mother colony (Wille 1983). Nest sites are usually short distances apart, due to the prolonged dependence upon the mother colony (van Veen & Sommeijer 2000). Once the new nest is established, a newly emerged virgin queen (gyne) and a swarm of workers leave the mother colony. The swarm arrives at the new nest site two to 13 days after the initial nest preparations commence (van Veen and Sommeijer 2000). Reported worker numbers recruited to the new colony vary from 10% (van Veen and Sommeijer 2000) to 30% (Inoue et al. 1984) of the original population. Soon after the arrival of the reproductive swarm, a drone swarm appears outside the nest and the virgin queen takes flight to mate (Michener 1974; Wille 1983; van Veen & Sommeijer 2000). Stingless bee queens may take two to seven days after mating before egg laying commences (van Veen & Sommeijer 2000).

1.9 Stingless bee behaviour

1.9.1 Nest dynamics

Unlike *Apis* spp., stingless bees are able to defecate in their nests without the risk of bee dysentery (Rayment 1935), caused by *Nosema apis* Zander (Dissociodihaplophasida: Nosematidae) (Czekonska 2000). Both *Trigona* and *Austroplebeia* create small stockpiles of debris within their nests which act as ‘rubbish dumps’. The components of these dumps include bee body-parts (both adult and juvenile), foreign matter (such as sawdust or seeds), compressed pupal casings and other, unidentified material (Klumpp 2007; Dollin 2010a). The dumps are located close to the nest entrance during inclement weather and their contents are removed when the weather is suitable for flight. The rubbish is removed by workers of various ages and its removal is essential for the control of disease within the colony (Rayment 1935). Other methods of disease control include the evaporation of nectar into more concentrated honey and the use of antimicrobial resins in nest structures (Velikova et al. 2000; Fernandes et al. 2001).

1.10 Temperature regulation

Most stingless bee species do not thermoregulate and rely on the insulative properties of the nest substrate (tree trunk), nest structures (batumen and involucrum) and the warmth generated by the developing brood (Michener 1974; Sung et al. 2008). Some species (e.g., *Trigona nebulata* Smith) build their nests within termite mounds to take advantage of the heat produced by their neighbours (Darchen 1973). Others, such as *T. spinipes* and *S. postica*, demonstrate an ability to regulate the brood chamber at similar temperatures to *A. mellifera* (Zucchi and Sakagami 1972, cited in Michener 1974; Engels et al. 1995). It has not yet been determined if these species are able to regulate brood temperature by endothermic means or by simply increasing the numbers of nestmates in and around the brood chamber.

Some species belonging to the genera *Frieseomelitta* Ihering and *Leurotrigona* Moure survive temperature extremes and have no obvious ability to thermoregulate the brood (Zucchi and Sakagami 1972, cited in Engels et al. 1995). The ability to thermoregulate may be one of the most important factors limiting the distribution of stingless bees (Wille 1983; Roubik 1989; Engels et al. 1995). Fluctuating brood temperatures may also account for the longer developmental times reported by Venturieri (2008) (Table 1.3). No thermoregulation studies have been reported for Australian stingless bee species to date.

1.11 Foraging and communication

Communicating information about the location of food and nest resources is essential for colony survival. Like *Apis*, stingless bees communicate in many ways. The most primitive communication involves pilot flights by scouts which directly lead new foraging recruits to the resource (Esch et al. 1965; Michener 1974). *S. postica*, *Melipona panamica* Cockerell and *Melipona seminigra* Friese use scent trails leading from the nest to the resource (Esch et al. 1965; Wille 1983; Kerr 1994; Nieh & Roubik 1995; Nieh 1998; Nieh et al. 2000; Hrncir et al. 2004). While sound is used to communicate resource quality in *M. quadrifasciata*, *Trigona* (*Axestotrigona*) *tescorum* Cockerell (Esch et al. 1965) and direction in *Melipona costaricensis* Cockerell, *Plebeia tica* Wille and *T. angustula* (Aguilar & Briceño 2002; Aguilar et al. 2005). *Trigona corvina* Cockerell can communicate distance and direction of a

resource (Aguilar et al. 2005) and *M. panamica* can include the height (Nieh & Roubik 1998).

Few studies have been carried out on Australian stingless bee communication; however, Bartareau (1996) has shown that *T. carbonaria* foragers leave a marker of glandular secretions near the food resource. Nest activities, such as trophallaxis and antennal tapping, communicate resource type and quality (Sommeijer et al. 1983). No studies on *Au. australis* forager recruitment or communication have been reported to date.

1.12 The general scope and aims of this thesis

The use of alternative pollinators has been poorly explored in Australia. Of the 1,500 species of native bees in this country (Dollin et al. 2007), only three or four species have been investigated for their efficacy as crop pollinators. Australia has 15 species of highly eusocial stingless bees, some of which have the potential to be managed and utilised as crop pollinators.

The management of stingless bees in artificial hives has been practiced in Australia since the 1980s. *Au. australis* and *Au. symei* are domesticable species within the genus *Austroplebeia* and have colony populations within the thousands. *Au. australis* has been mostly kept as a 'pet' for its novelty value and has not been investigated as a crop pollinator. The flight range of stingless bees is short, which is advantageous in the management of greenhouse and intensive field-grown horticultural crops. There have been few scientific studies conducted in the area of stingless bees as pollinators, and this thesis aims to address some of the questions related to Australian stingless bees as potential crop pollinators. It is hoped that the information generated will assist in increasing opportunities to provide a sustainable alternative source of pollination services which will help to fill some of the current and potential future gaps occurring in the honey bee industry. The purpose of my studies is to better understand the development of the Australian stingless bee industry, the phylogenetic placement, biology and behaviour of *Au. australis* and to assess its ability to effectively pollinate crops in both the greenhouse and the field.

My key research questions are:

How has the Australian stingless bee industry changed over the last decade?

A survey conducted in 1998 showed that the Australian stingless bee industry was very much in its infancy; however, it had an optimistic future (Heard & Dollin 2000). No subsequent surveys have been conducted in this area. It is important to understand the how the industry has developed and in what areas. The work reported in this thesis looks at how the industry has developed and how the people working within the industry may be supported by research and development (Chapter 2).

Is Au. australis *a potential crop pollinator? Can colonies acclimate to greenhouse enclosures? Will foragers visit trial crop flowers? Will the presence of* Au. australis *colonies increase the crop yield compared to no pollinator presence?*

Au. australis colonies collect pollen and nectar to raise their young and forage on flowers for these resources. Colonies have also been managed in transportable artificial hives for many years. The potential of *Au. australis* as a greenhouse and field crop pollinator is investigated. Chapter 3 reports on flower visitation and foraging behaviour.

What is the phylogenetic placement of Au. australis *within the genus* Austroplebeia*?*

Austroplebeia is reported to have nine species within the genus; however, there is some confusion with the identification and classification of these species. This is likely to have implications for knowledge about their distribution, as well as differences in biology and behaviour. Analysis of morphological, molecular and geometric morphometric data are investigated and the status of species in this genus is reported in Chapter 4 of this thesis.

What is the ontogenic period and longevity of Au. australis *worker bees?*

Developing an understanding of the basic biology and behaviour of Australian stingless bees, in this case *Au. australis*, will enable better evaluation of their suitability in a role as a crop pollinator. Understanding the lifecycle and nestmate turnover is important for colony management. Chapter 5 reports on the ontogenic period and the longevity of *Au. australis* workers.

What is the natural distribution of Au. australis*? What factors influence this distribution? Can colonies be managed outside their native range?*

Au. australis has been reported to naturally occur only north of Dungog, in central-east NSW (Dollin 2010b). The factors limiting the distribution of *Au. australis* may be related to climate, food source or nest substrate type and availability. Chapter 6 reports on a focus study conducted in an area of south-east Qld, where *Au. australis* is known to naturally occur. This study provides a better understanding of the natural distribution and nest density of *Au. australis* and the nest characteristics were also assessed and reported. This chapter also reports on the climatic factors influencing flight activity as well as comparative foraging behaviour of *Au. australis*, as well as on some nest dynamics of *Au. australis* colonies.

CHAPTER 2

The Australian stingless bee industry

2.1 Introduction

As discussed in Chapter 1, meliponiculture is the practice by which bee keepers reproduce stingless bee colonies for profit; and the profit may come in many forms. These include nucleus colonies, hive products such as honey and propolis, pollination services, education and even the mere pleasure of keeping stingless bees. The level of development of meliponiculture industries varies around the world, with it being most advanced in South American countries (Cortopassi-Laurino et al. 2006).

When Dr Tim Heard, from the Commonwealth Scientific and Industrial Research Organisation (CSIRO), Division of Entomology, Qld, first began his work with *T. carbonaria*, in 1984, the Australian stingless bee industry was almost non-existent (Heard & Dollin 2000). Since then, interest in stingless bee keeping has increased, and conservation groups have been established, especially along the eastern regions of the country. One group in Brisbane requested information pertaining to land clearing and sought permission to remove and relocate nests under threat. These naturally occurring colonies were transferred to artificial hives and later used for production of domesticated colonies. Husbandry techniques developed by Heard (1988b) and others helped to facilitate the growth of this industry.

In 1998 / 99 Dr Heard, and Dr Anne Dollin, of the Australian Native Bee Research Centre (ANBRC), NSW, conducted a survey to ascertain the status of the industry at that time. They found that it was very much in its infancy, but was growing and had potential for expansion over the next few decades. The survey concluded that 62 bee keepers used their 317 hives for crop pollination (Heard & Dollin 2000), but it was not clear how many of those bee keepers provided those services professionally, charging service fees. Heard and Dollin (2000) predicted that 24,000 colonies would be domesticated by the year 2010. No further research had been conducted in this area and it was unclear as to whether the Australian stingless bee industry had developed over the last decade.

In this chapter I report on a follow up survey; one decade on, which was conducted to elucidate how the industry had changed and in what ways. Information obtained from the new survey conducted in 2010 allowed for a comparison between it and data obtained in 1998 / 99. One of the key intended outcomes of the survey was to identify areas within the Australian stingless bee industry that required research support for its further development.

2.2 Materials and methods

In collaboration with Dr Tim Heard and Dr Anne Dollin, the authors of the original survey, a follow up survey was designed (Appendix 11). The original 1998 / 99 survey consisted of 11 multiple choice or short answer questions, and these formed the basis of the follow-up survey. Additional questions were included in the 2010 survey in an effort to optimise the data comparisons. Therefore, the first question asked if the participant had completed the 1998 / 99 survey. One additional question regarding honey production and 15 additional questions pertaining to pollination were also included, to gain a more detailed picture of the services provided by stingless bee keepers in these areas at this point in time. Ethics approval, to conduct the survey, was sought and granted through the UWS Human Research Ethics Committee (Protocol Number H7677).

An online survey was designed and produced through 'Survey Monkey' (www.surveymonkey.com). Drs Heard and Dollin, along with other willing colleagues, completed several test runs of the survey to identify any technical glitches. Several ongoing problems were encountered and the form was modified until the problems were minimised. This process took approximately four weeks. One problem that could not be completely resolved was that of 'aggressive' firewalls. Participants with aggressive firewalls were only able to access and complete the first page of the survey. We endeavoured to remove the pagination so this problem would no longer occur; however, that led to complete failure of information download, so this activity was abandoned.

The first page of the survey generated basic, useful data which helped to obtain an overview of the industry; such as stingless bee species, location and hive numbers. It was not possible to overcome the download problem, but this initial information was

of high quality. I continued with the survey, knowing that a small percentage of respondents would be unable to complete the entire survey.

Dr Dollin kindly composed an attractive article titled "Stingless bees around the world". This article was used as a 'reward' to participants for completing the survey. A website (www.beesbusiness.com.au) was set up where participants could click a link to the survey. Once they had completed the survey they were taken back to 'beesbusiness' and were able to download the reward article. Again, firewalls proved to be a problem and some participants were unable to download the article. To overcome this problem, a link to my private email address was provided and any problems encountered by participants were communicated to me personally. Problems were addressed and rectified as soon as possible.

On several occasions, an electronic version of the survey was forwarded to the participant or a hard copy was mailed to their home address. The 'mail-out package' for hard copy postage contained a survey form, consent form, 'survey' envelope to provide anonymity, instructions, a reward article and a stamped return addressed envelope. Anonymity was maintained throughout the survey process; however, on many occasions participants contacted me personally to share their beekeeping experiences. At no time did I attach a name to any survey response.

The online survey was made available for public access from 4 July to 31 August 2010. A press release, prepared with the assistance of the University of Western Sydney (UWS) media unit, resulted in the advertisement of the survey in over ten rural and metropolitan newspapers or magazines throughout NSW, Qld and the Northern Territory (NT) during the survey period. An on-air interview with 702 ABC Sydney radio's Simon Marnie, on 17th April 2010, increased the profile of the survey noticeably. The survey was also advertised through the Australian Native Bee Research Centre's (ANBRC) *Aussie Bee Email Update* newsletter (www.aussiebee.com.au/emailupdate.html), which is sent to over 2,000 recipients, as well as the Australian Native Bees Yahoo Group (www.australiannativebees.com/). Email notification containing the online survey link and an electronic version of the survey were sent to known bee keepers. The advertised information provided details of the website, with its on-line survey link, as well as a contact telephone number where people could leave a message with their contact details. Over 80 'mail-out

packages' were posted to bee keepers who were not internet users. All surveys remained anonymous and data obtained from returned postal survey data were manually entered into the on-line survey. Survey data were downloaded weekly. The final data, download on 31 August 2010, was used in the comparative analysis of industry growth.

2.3 Results

2.3.1 Overall comparison between surveys

The first page of the survey included questions pertaining to previous survey participation, bee species and nest numbers. The total number of respondents (n = 637) completing the first page showed that over the last decade stingless bee keeper numbers had increased 2.5-fold, from 298 to 637, and the number of nests they kept had increased almost 3.5-fold, from 1,429 to 4,935 (Figure 2.1).

The most commonly 'kept' bee species were *T. carbonaria*, *T. hockingsi* and *Au. australis*. Of these, *T. carbonaria* (61.5% of hives) was the most popular species, which was kept by 74% of the survey respondents, with *Au. australis* (22.9%) the second most popular, followed by *T. hockingsi* (8.8%). The remaining 6.8% was made up of *T. clypearis*, *T. sapiens*, *T. davenporti*, *Au. symei* and unknown species (Figure 2.2). Only 15% of bee keepers (n = 98) kept ≥ 2 bee species.

In 1998 / 99, 10% of bee keepers owned *Au. australis* colonies and these accounted for only 9% of the total number of hives. In 2010, however, 16% of bee keepers owned *Au. australis* colonies. The number of hives kept by bee keepers ranged from 1 to 476, and single-hive ownership accounted for 57% of respondents (n = 361). Only 5.5% of the bee keepers (n = 35) owned more than 20 hives (Figure 2.3), four bee keepers owned between 100 and 200, while five bee keepers owned more than 200 hives each.

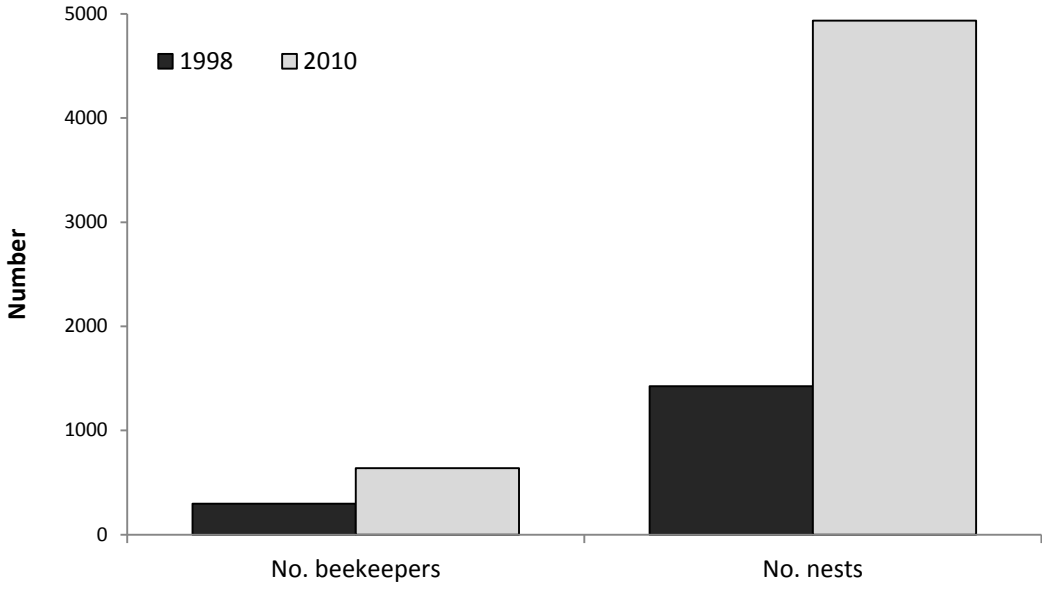


Figure 2.1 Increase in bee keepers and their nests between 1998 / 99 and 2010.

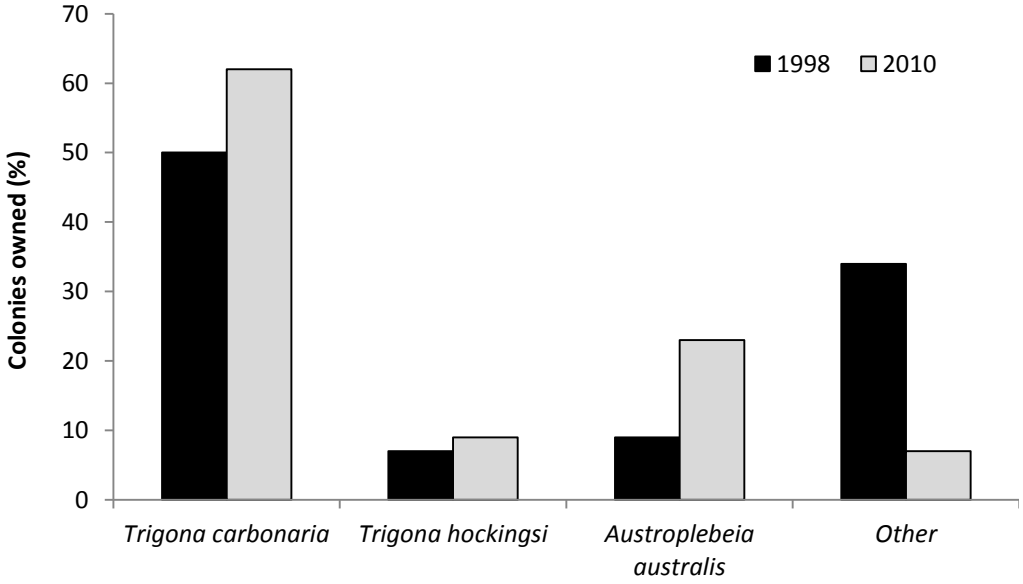


Figure 2.2 The total percentage of stingless bee species owned by surveyed bee keepers.

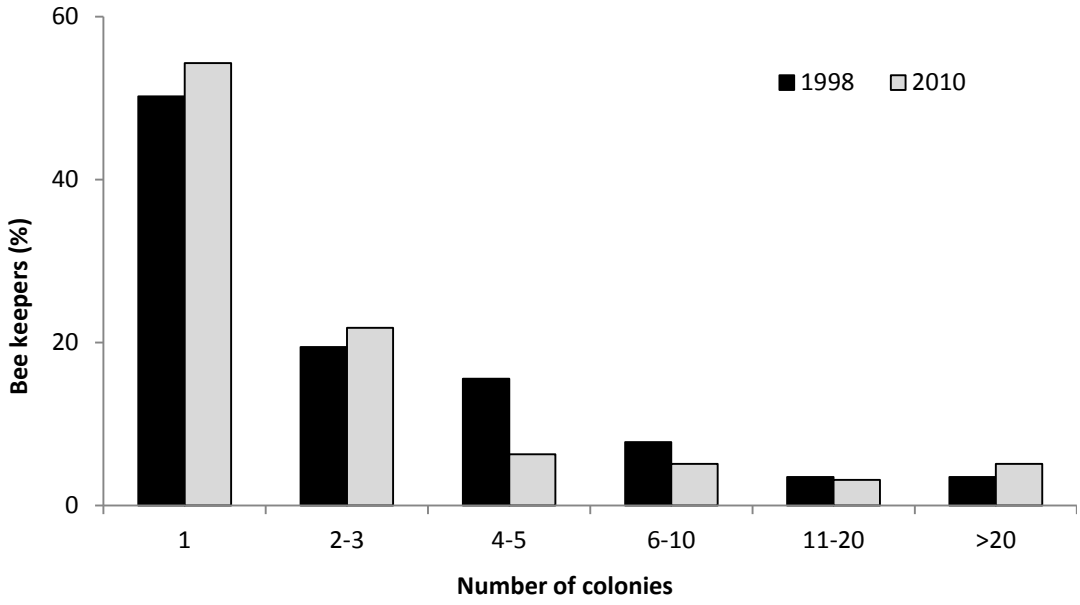


Figure 2.3 Number of colonies owned by bee keepers in 1998 / 9 and 2010.

2.3.2 Detailed analysis and comparisons

Eighty-nine percent (n = 568) of respondents were able to progress past page 1, and these respondents owned 4,086 stingless bee hives. Detailed data from these respondents were compared to the data from the previous 1998 / 99 survey (see summary in Appendix 11). All but five survey respondents resided in NSW or Qld. Three of these lived in the NT and two resided overseas. Data from the overseas respondents were not included in the analysis.

2.3.2.1 Summary of bee keepers and their stingless bee colonies

The following information pertains to the comparison of data collected in the 1998 / 99 and 2010 surveys. The number of bee keepers increased in both Qld (208 to 346) and NSW (83 to 215); however, there was an almost 10% shift, with NSW bee keepers increasing from 29% to 38% of the overall number of bee keepers (Figure 2.4). Nest numbers also demonstrated an increasing shift to NSW, but only by 7% (Figure 2.4).

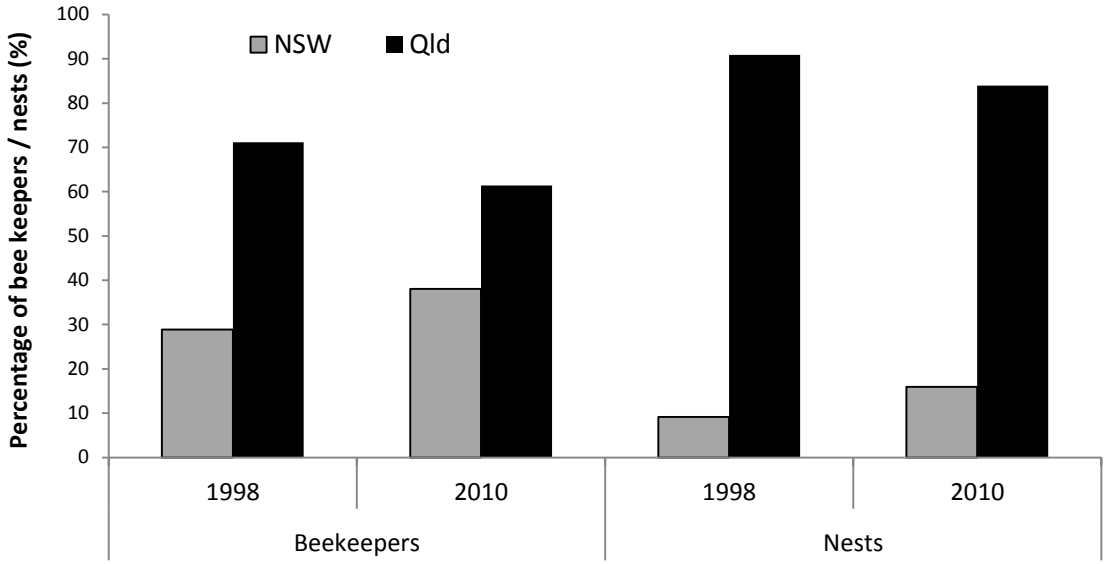


Figure 2.4 Bee keepers residing in NSW and Qld in 1998 / 99 and 2010. Less than 0.5% of bee keepers resided in the NT during both survey periods.

Enjoyment and conservation were, by far, the most popular reasons for keeping stingless bees (Table 2.1) and 80% of single-hive owners kept their hive for these purposes. Over two-thirds of bee keepers maintained their hives on suburban blocks (Figure 2.5), although 41% of these also lived within half a kilometre of some form of native vegetation (Postcodes were used to locate residential areas on Google Earth and remnant bushland was identified).

Table 2.1 Reasons for keeping stingless bees.

Reasons for keeping stingless bees	1998	2010
	(%)	(%)
Enjoyment	81	78
Conservation	68	67
Pollinate bushland	27	29
Pollinate crops	24	24
Honey production	8	11
Hives sales	5	3
Education	5	12
Research	2	4
Other hive products (resin, wax)	2	2
Professional crop pollination services	---	1

Newcomers, those bee keepers with < 1 year of experience, accounted for 25% of respondents and those with < 3 years experience accounted for 40% of bee keepers

(Figure 2.6). Fifty-seven percent of the bee keepers owned just one hive and a quarter of those had < 3 years experience. Only 8% of respondents reported that their nests remained in the original cavity, compared to 18% in 1998 / 99.

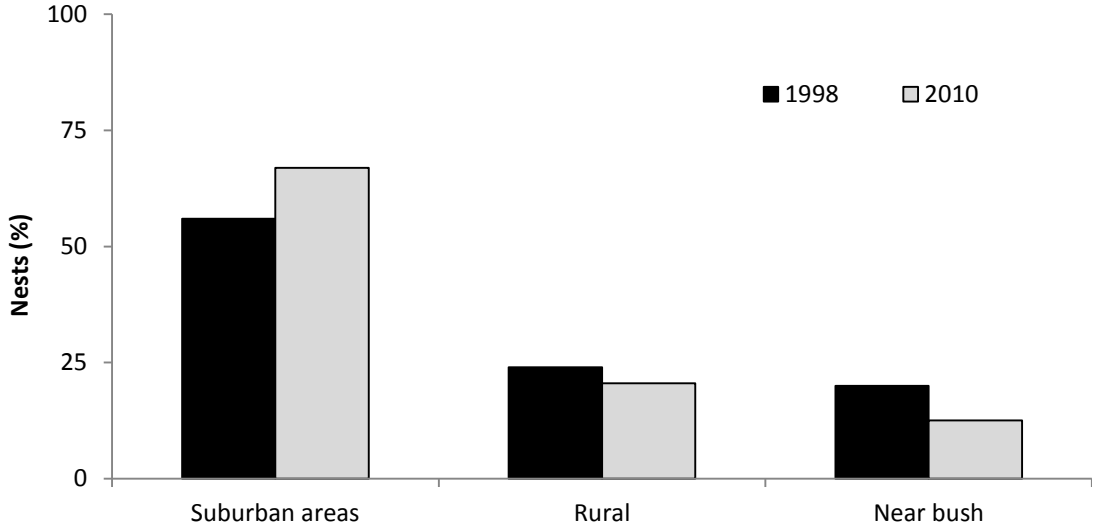


Figure 2.5 Areas where nests were located in 1998 and 2010.

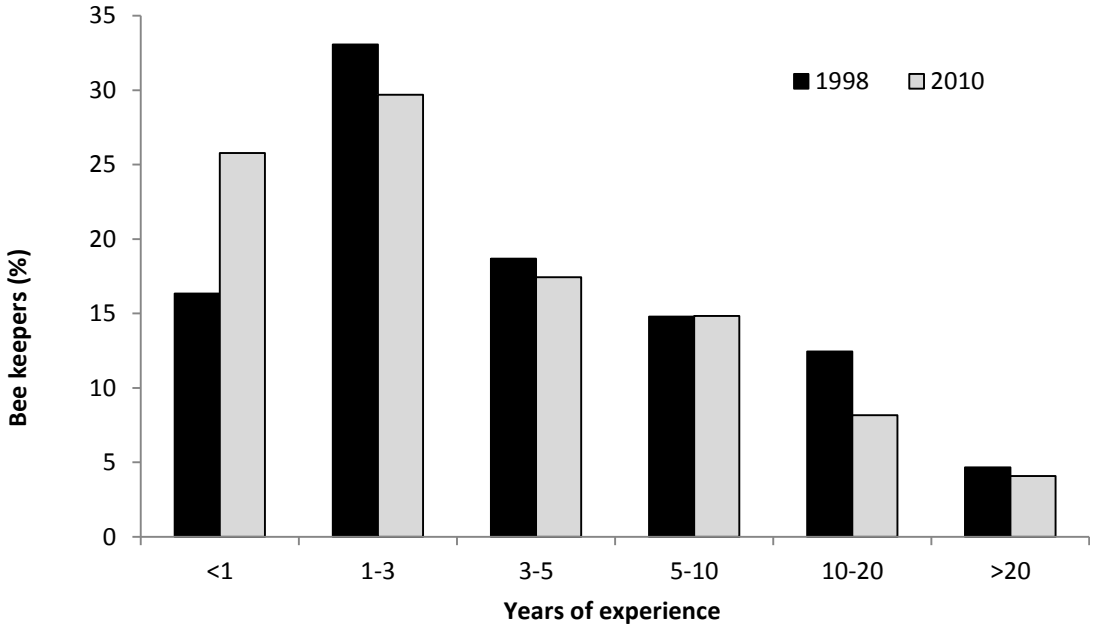


Figure 2.6 Number of years of stingless beekeeping experience of respondents.

2.3.2.2 Honey production

The number of bee keepers harvesting honey had more than doubled (26 to 63), resulting in an almost three-fold increase in annual honey production (90 to 254 kg / yr) (Figure 2.7). Of the 63 bee keepers who stated they harvested honey, only five reported selling their product, and this group accounted for approximately half of the overall production. Those who on-sold their product distributed it through local markets, restaurants and via the internet, and two producers exported to Japan.

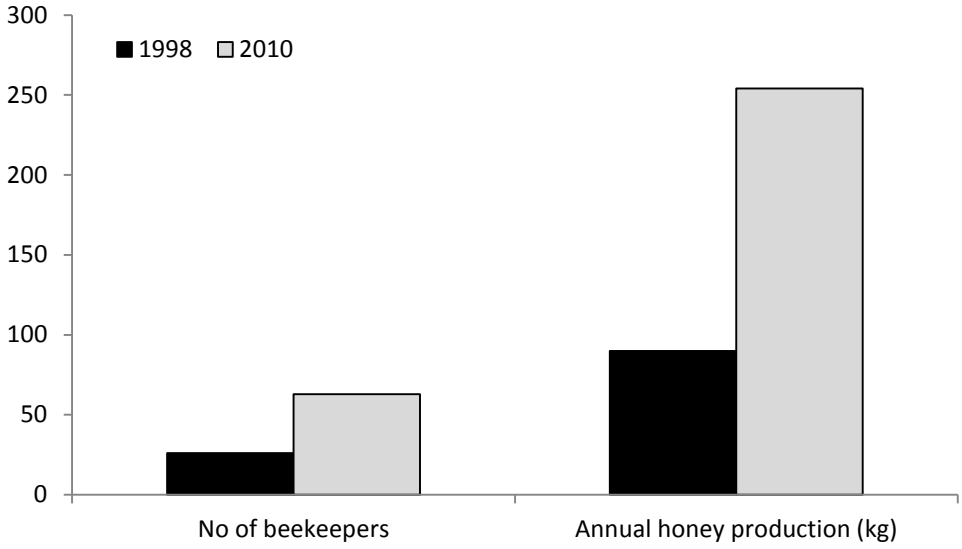


Figure 2.7 Number of bee keepers producing honey and total annual honey production.

2.3.3 Colony propagation

Since 1998 / 99, the number of bee keepers involved in hive manipulation had doubled (119 to 238); however, the number of bee keepers practicing colony splits had an eight-fold increase (17 to 139) (Figure 2.8). In 2010, 238 bee keepers reported they had produced a total of 11,421 hives through nest / hive manipulation. Fifty-five percent of these hives were produced by splitting (Figure 2.9), compared to 29% in 1998 / 99.

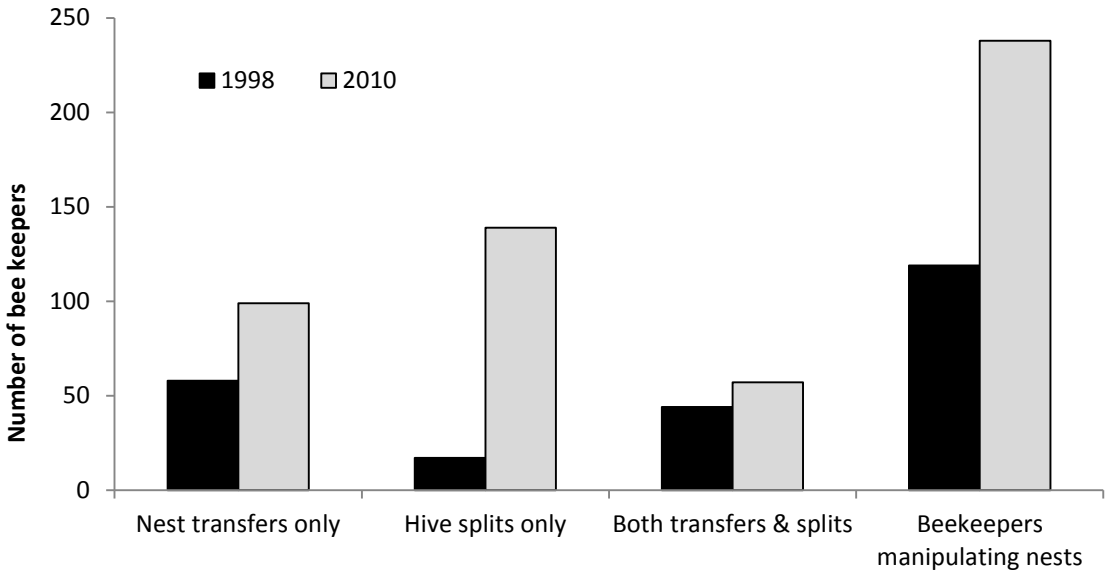


Figure 2.8 Number of bee keepers participating in colony propagation, including nest transfer and hive splits, during the years 1998 and 2010.

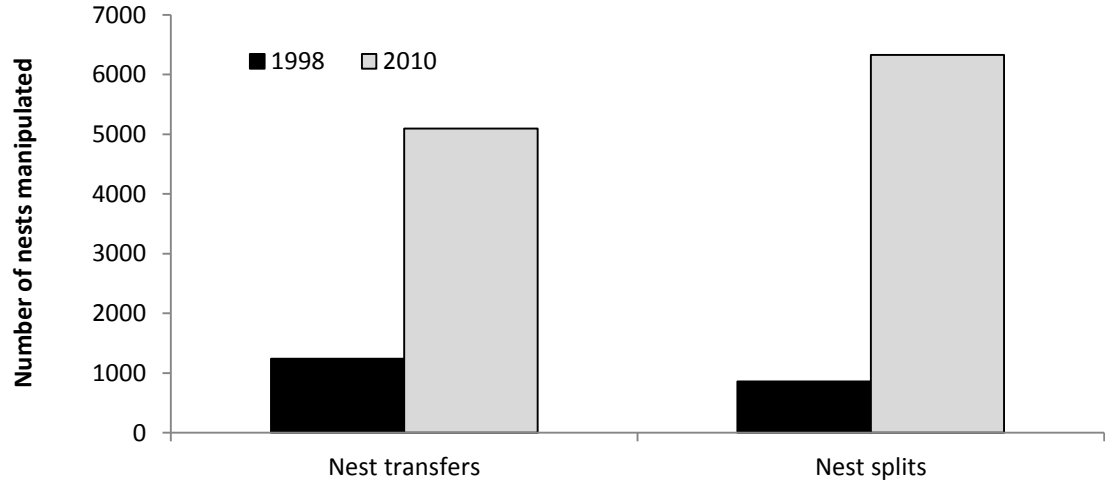


Figure 2.9 Number of nests manipulated, through nest transfer or splits, by bee keepers in 1998 and 2010.

Over the last decade, the number of bee keepers selling hives more than doubled (10 to 25); however, the number of hives sold each year had more than quadrupled (103 to > 460 / yr). Approximately 18% of bee keepers made their own nest boxes in 2010; most of them being based on the OATH design or similar, with a 7 to 10 L internal capacity.

The overall annualised growth, through colony propagation, was 13% / yr (i.e., 1,429 in 1998 / 99 to 4,935 in 2010). In Question 1 of the 2010 survey, respondents were asked if they had participated in the original 1998 / 99 survey and 28 answered "yes" to this question. Based on the hive ownership (n = 1,757) of these 28 bee keepers, the following information was used to estimate annual colony propagation by some of the original survey respondents. In 1998 / 99, 84 bee keepers predicted a 15% / yr growth in hive numbers over ten years. These 84 bee keepers owned an average of 9 hives each. In 2010, the 28 bee keepers who completed the original survey owned an average of 63 hives each (1,757 / 28). Thus, these bee keepers actually increased their hive ownership from 9 hives each to 63 hives each, an increase of 21% / yr.

2.3.4 Pollination

In 2010, eight bee keepers reported that they provided pollination services on a professional basis; and of those, only four charged a service fee. One bee keeper charged $AU10 / hive while the other three charged from $AU35 to 40 / hive. Pollination service providers reported a variety of crops which benefited from the introduction of stingless bees or which were undergoing trials with stingless bee pollinators (Table 2.2).

Table 2.2 Reported crops which benefit from stingless bee pollination services.

Common name	**Genus and species**	**Pollination service areas**
Macadamia	*Macadamia tetraphylla* L.A.S Jonhson & *Macadamia integrifolia*	Coastal NSW & southern / central Qld
Lychee	*Litchi chinensis*	Coastal NSW & southern / central Qld
Watermelon	*Citrullus lanatus*	Coastal NSW, southern / central Qld & Darling Downs
Avocado	*Persea americana*	Coastal NSW & southern / central Qld
Blueberry	*Vaccinium* spp. (under trial)	NSW mid north coast & northern rivers
Mango	*Mangifera indica*	Coastal NSW & southern / central Qld

Question 27 of the 2010 survey was an open-ended question which asked participants to nominate areas where they thought the industry needed most research and development support. Four of the respondents felt there was a great need for research into queen breeding, hive multiplication, hive stocking rates for pollination

and swarm capture. They also identified a need for education in all of these areas. Many of the respondents reported that demand was high and that they were unable to keep up with the demand for hives or for honey.

2.4 Discussion

Although the Australian stingless bee industry is still extremely small, it is clear that it has grown over the past decade. The bee keepers have already developed effective methods for managing perennial colonies in artificial, portable hives. Hives containing thousands of workers can be transported into crops for pollination, utilised for sugarbag honey production or just be a pleasure to own. Overall, the industry has grown, with reported bee keeper numbers increasing by 114% since 1998. This is compared to the Australian honey bee industry which increased membership by only 3% between 2000 and 2006. Stingless bee hive numbers have increased by 245% whereas honey bee hive numbers have increased by 17% for the same period (Rodriguez et al. 2003; Crooks 2008).

2.4.1 Hive ownership for enjoyment

Single-hive ownership accounts for over half of the domesticated colonies and a quarter of those bee keepers have less than three years' experience. Additionally, the fact that 80% of single-hive owners obtained their hive for the purpose of conservation or enjoyment demonstrates that there is still a high novelty value in the industry (Heard & Dollin 2000). Similarly, of the 9,900 registered honey bee keepers in Australia, 8,200 (83%) are hobbyists (Crooks 2008). The surge in interest in stingless bees in Australia for their use as 'pets' is apparent with respondents' comments such as "we love our bees…", "we have our bees for fun…, help educate the kids…" and "they are the perfect pet…". This is supported by an increase in *Au. australis* colony ownership. This species is not utilised in crop pollination, and is seldom used in honey production, as are *T. carbonaria* or *T. hockingsi*; however, there has been an increased interest in it as a 'pet'. *Austroplebeia* colonies do not store as much resin in their hives and are less aggressive than *Trigona* species. As a result, it is possible to more easily observe in-hive activities and gain greater enjoyment. Stingless bees as 'pets' is a market which could be well exploited by hive producers. In ten years, the retail value of a strong stingless bee hive in Australia has

increased from $AU200 to between $AU325 and 450 / hive (R. Zabel, T. Carter, pers. comm., 2010). Taking into account the 327 single-hive owners in this survey, that is over $AU100,000 in revenue from hobbyists alone.

Many of the survey respondents listed conservation as a major reason for keeping their hives. There has been a boost in conservationist bee keeper numbers, probably as a result of awareness programmes. These programmes increase understanding of wildlife and encourage conservation of various native species. Ku-ring-gai Municipal Council, for example, has initiated the *WildThings* programme (www.kmc.nsw.gov.au/www/1190-wildthings.asp) which allows local residents to acquire stingless bee hives, free of charge, in an effort to increase the native bee populations within their national park areas. Through this programme, the council has supplied 185 *T. carbonaria* hives to local residents over the last six years (P. Clarke, pers. comm., 2010). It is interesting to note that of the survey participants who resided in the Ku-ring-gai council wards, 74% of these respondents reported that they received their hives through the *WildThings* programme. Ku-ring-gai council has extended the programme to include another six Sydney councils, who pay an annual $1,000 fee each to fund a regional coordinator. More programmes such as this would be of great benefit in improving stingless bee species conservation. Land clearing due to the spread of metropolitan development is ongoing and is accompanied by the loss of wild nests and a reduction in species populations. Rescued nests from areas destined for clearing could be used to maintain a population and, simultaneously, provide residents with great enjoyment.

2.4.2 Colony propagation

Based on overall hive ownership (i.e., 637 bee keepers with 4,939 hives) the Australian stingless bee industry has experienced a growth rate of 13% / yr, which is close to that predicted (15%) by bee keepers who took part in the 1998 / 99 survey (Heard & Dollin 2000). However, if figures were derived from survey participants who took part in both the 1998 / 99 and the 2010 studies (i.e., 28 bee keepers with 1,757 hives), increased colony propagation achieved by those bee keepers is 21% / yr. This figure is closer to that predicted (30%) by Heard and Dollin (2000), who themselves actively manipulated nests for colony propagation. No matter how the growth is calculated, there has clearly been an increase in colony propagation

over the last decade. None-the-less, this industry is tiny compared to the Australian honey bee industry, whose 1,702 commercial bee keepers (excluding the large number of amateur bee keepers) own 571,968 hives (Crooks 2008).

As previously stated, surveyed bee keepers owned 4,939 hives; however, this number conflicts with the reported number of hives propagated (11,421) by the 238 bee keepers who manipulate nests. The reasons for this conflict could be many and varied. Whilst every effort was made to obtain responses from as many stingless bee keepers as possible, there is no doubt that many would have been missed. It is clear that a substantial proportion of stingless bee keepers did not take part in this survey. In Australia, the stingless bee industry is not regulated and several bee keepers reported concerns regarding possible government intervention. I also received direct communication from some bee keepers who did not wish to take part in the survey due to concerns regarding taxation (Anon., pers. comm., 2010). It was reported to me, during the time I was trying to recruit survey participants, that several major stakeholders chose not to participate in this survey. Another explanation for the discrepancy may be that some of the hive producers on-sell their hives to crop growers (T. Carter; F. Adcock, pers. comm., 2010). These farmers and orchardists may not have taken part in the survey, as this was not their primary area of interest: they merely use the hives in the management of their crops.

Another reason for the conflict in colony propagation vs. ownership may be due to colony death. In the 2010 survey, 13 bee keepers reported colony losses due to pesticide poisoning, starvation, hive splitting, honey extraction or for reasons unknown. Similar losses are frequently reported by members of the Australian Native Bees Yahoo Group. Discrepancies between colony production and colony ownership were also reported in the 1998 / 99 survey (Heard & Dollin 2000). There will, of course, always be gaps in such studies as it is not possible to elicit participation from every stakeholder in an industry. Never-the-less, the information gleaned from this survey provides a good, general overview of the industry as it stands in 2010.

Despite the described shortcomings, this survey was useful in obtaining an overview of the industry as a whole, as well as gaining information on specific areas of meliponiculture. The number of bee keepers involved in hive manipulation has

doubled since 1998 / 99, and the practice of hive splitting has increased eight-fold. Hives that were produced through splitting accounted for 55% of production. This has ramifications for the overall population levels of stingless bee colonies. Splits enable colonies to be actively multiplied, whereas colony transfers merely move populations from a natural substrate to an artificial one. The ability to safely and successfully split hives enables a bee keeper to increase his / her hive numbers in a sustainable manner. Nest transfers are performed to remove colonies from possible destruction during land clearing or natural disaster.

2.4.3 Honey production

T. carbonaria, Au. australis and *T. hockingsi* are the most popular species of stingless bees kept in Australia. Bee keepers producing honey use mostly *T. carbonaria*, although a small number use *T. hockingsi* or *Au. australis*. Eleven percent of bee keepers reported that they produce honey; however, only five producers on-sell their product. All bee keepers who are able to harvest ≥ 1kg of honey / yr reside in Qld. This supports the view that commercial production of sugarbag honey is only possible in the warm northern regions of Australia (Dollin & Heard 2010), where colonies are able to forage for most of the year. Sugarbag honey has become popular as a 'bush tucker' and caters to a niche market in Australia. The wholesale price has increased from $AU40 / kg in 1999 to $AU70 / kg in 2010 (taking inflation into account, this is an increase of $AU20 / kg, http://www.rba.gov.au/calculator/) but the retail price remains the same, at approximately $AU160 / kg (Heard 2010) (a decrease in value of $AU42.50 / kg). This is a high price when compared to *A. mellifera* honey, which sells for only $AU6.50 / kg (Shaw 2010), the price is indicative of rarity. Total stingless bee honey production is < 300 kg / yr, compared to the Australian honey bee industry's 27,800 tonne. Of this, 62% is produced by only 2.6% of honey bee keepers. In 2007 / 2008, the Australian honey bee industry was estimated to have produced a gross value of honey and wax of $AU75 million (Crooks 2008).

Sugarbag honey has a niche market in Australia and overseas but annual production of < 300 kg is well below that produced by meliponiculture industries overseas. For example, one cooperative in Mexico produces 1,500 kg / yr using *Scaptotrigona mexicana* Guérin-Méneville (P. Vit, pers. comm., 2011) and a group of ten bee

keepers, using *Melipona fasciculata* Smith in the Amazonian region of South America, expect to harvest 500 kg / yr from 2012 (G. Venturieri, pers. com, 2011). Given the right infrastructure, the Australian sugarbag honey industry could also be developed to a more commercial level. A greater number of bee keepers are utilising honey 'supers' on their hives for ease of harvest (Dollin 2002), which may result in increased production. Improved methods of harvesting honey and postharvest storage are areas that would also benefit from further research (Cortopassi-Laurino et al. 2006).

2.4.4 Pollination services

In both the 1998 / 99 and 2010 surveys, pollination of nearby vegetable and flower gardens, as well as bushland, was reported to be of considerable benefit. In 2010, only eight respondents reported they provided pollination services on a professional basis. The number of hives owned by these service providers ranged from 10 to 130 each, and totalled only 423. Compared to 102,000 honey bee colonies used for paid pollination (RIRDC 2009), this is a tiny proportion of Australia's managed pollinators. While these stingless bee hive numbers are extremely low, correspondence with a small number of bee keepers following the conclusion of the survey has located at least four additional bee keepers that own over 200 hives each and also provide pollination services (T. Heard; R. Luttrell; T. Carter, pers. comm., 2010). It is unfortunate that these additional bee keepers could not be incorporated into the survey data; however, it demonstrates that several of the major stakeholders chose not to participate, for one reason or another.

According to McGregor (1976) most cultivated crops are pollinator limited and an increase in pollinator populations may increase horticultural and agricultural production (Roubik 1995). Pollination service providers reported good crop pollination by stingless bees in a variety of sub / tropical crops and one service provider reported increased yields in lychee, avocado and watermelon (T. Carter, pers. comm., 2009). However, this information was generally anecdotal, with no supporting quantitative data. Compared to honey bees (Heard 1994; Heard & Exley 1994), stingless bees have been proven to be efficient pollinators of macadamia and may be more suited to pollinating tropical plants with which they have evolved.

Further research in this area is needed if there is to be any likelihood of expanding pollination services within the Australian stingless bee industry.

Stingless bee keepers reported obtaining up to $AU40 / hive for pollination services. In Australia, honey bee keepers are paid pollination fees of $AU25 to 35 / hive in inland NSW (Gibbs & Muirhead 1998). Honey bee keepers in WA are asking as high as $AU65 to 75 / hive, due to the isolation and low colony numbers in that state (Manning 2002). Paid honey bee pollination services were estimated to be worth $AU3.3 million in 2001 (Rodriguez et al. 2003).With dwindling honey bee populations in the US, almond growers had to pay as much as $AU180 / hive in 2007 (RIRDC 2010b). This is indicative of how scarce honey bee colonies have become in countries that have suffered massive losses through pests and diseases. It is probably only a matter of time before Australia suffers an incursion of varroa mite, resulting in large reductions in honey bee populations. Increased scarcity of colonies will see honey bee keepers being drawn to the highest bidder (RIRDC 2007). The highest bidders will not be located in the sub / tropical areas growing macadamia, mango, lychee, watermelon and avocado; they will be located in the almond groves of south-eastern Australia (Somerville 2007, cited in RIRDC 2010).

Australia is home to over 1,500 species of native bee (Dollin et al. 2007). Little research has been carried out on most of these species; however, four species of stingless bees are currently managed by Australian stingless bee keepers. Manageable, transportable colonies of stingless bee have the potential to provide pollination services to a number of crops grown in the sub / tropics, thus helping to fill a gap in the pollination service industry within those areas. The only truly scientific studies carried out on pollination by Australian stingless bees have been in macadamia (Heard & Exley 1994; Heard & Dollin 1998) and mango (Anderson et al. 1982). Their efficacy as pollinators of a variety of other crops warrants further investigation.

2.4.5 Bee keepers and the future of the industry

The stingless bee and the honey bee industries, although substantially different in size, have similar structures. A small number of key players dominate the commercial market and many of these key players are part of an ageing population. The average age of honey bee keepers is over 54 years old (Rodriguez et al. 2003)

and many large scale stingless bee producers are close to or past retirement age. Unfortunately, some of the most experienced stingless bee keepers have down-sized their meliponaries due to their advancing age (T. Carter, R. Luttrell, pers. comm., 2010). It is important that the knowledge held by these experienced bee keepers is not lost and that education in stingless bee husbandry is shared and developed for the next generation. Many bee keepers believe there is a need for research into queen rearing, as queen availability is the major limiting factor for hive multiplication. Queen breeding and artificial insemination have been practiced with honey bees for decades (Woyke 1960). Limited research has been conducted in this area of meliponiculture overseas, except for Menezes and Imperatriz-Fonseca (2010), and none has been conducted in Australia. More information pertaining to *Au. australis* queens see presented in Appendix 9.

Some bee keepers suggested that research could be conducted in conjunction with research institutes (such as universities, state departments of agriculture and CSIRO) and be funded through grants, low interest financing and tax breaks. Initially, infrastructure and mother colonies would require the greatest financial outlay, but this will be necessary if the Australian stingless bee industry is to grow at a more rapid rate. Colony multiplication is the backbone of the sustainable development of meliponiculture and there are many economic possibilities for this industry. As one bee keeper stated... "Research is needed in order to make stingless beekeeping a commercial reality". For further information on management intervention in *Au. australis* colonies see Appendix 10 'Brood production and overwintering'.

2.5 Key findings

- The Australian stingless bee industry has grown over the past ten years but is still very underdeveloped.
- Most colonies are kept singly, by hobbyists, for enjoyment.
- Demand for colonies is high and supplies are low.
- Education of the public, on all levels, is necessary to increase awareness of Australian stingless bees and their conservation.
- Conservation of these species is dependent upon increased education and cooperation with government bodies.

- Colony propagation is slow and is carried out by only a small number of bee keepers.
- Little is known about the biology and behaviour of Australian stingless bees.
- Honey production caters to niche markets and is in high demand but in low supply.
- Pollination services are provided by a small number of stingless bee keepers, whose services are in high demand but who have limited resources.
- Minimal research has been conducted on stingless bees as potential crop pollinators.
- Australian stingless bee keepers are an ageing population and their legacy appears not to being passed on to a younger generation.
- Minimal research has been conducted in the area of colony propagation in Australian stingless bees.
- There is limited research and development support provided to this industry in Australia.

CHAPTER 3

Studies on the pollination efficacy of *Au. australis* in greenhouse research chambers and the field

3.1 Introduction

Flower visitation by insects is a priority in many vegetable seed crops and the associated pollination maximises seed set and, thus, seed yield and quality. While many insects are capable of pollinating vegetable crops, honey bees are the most effective (Free 1970). Gordon and Davis (2003) estimated that honey bee pollination was worth over $AU750 million to the Australian vegetable production industry. Plant breeding programmes incorporating cross-pollination require many seed crops to be managed within greenhouse enclosures. It is, therefore, necessary to find pollinators that are able to adapt to working under these conditions.

Vegetable seed production has become much more sophisticated over the last half century, and plant breeders utilise specific biological mechanisms to ensure they obtain unique hybrid seeds (Edwardson 1956; 1970; McGregor 1976). The use of cytoplasmic male-sterility and genetic male-sterility, as well as the presence of protandry and sticky pollen, make breeding many crops totally dependent on insect vectors for pollination. Cytoplasmic inheritance has been intensively studied since its discovery in the early 1900s (Mogensen 1996), and the inheritance of male-sterility has been utilised by plant breeders for decades (Edwardson 1956; 1970). Hybridisation has been made easier through the use of cytoplasmic male-sterility, making heterosis more attainable, resulting in improved off-spring traits (Edwardson 1956).

In Australia, recent studies have been conducted to investigate the efficacy of a variety of native solitary bees within vegetable crops, as alternatives to *A. mellifera*. Natural populations are provided with nest substrate, positioned close to the crops, which encourages the bees to collect floral resources from the crop (Hogendoorn 2011; 2012).

The current study included an industry partner, Rijk Zwaan Australia (RZA), which is involved in research, development and production of seeds for over 800 varieties of 20 vegetable crop species. They are part of the global network of Rijk Zwaan Seeds, which is headquartered in The Netherlands. Rijk Zwaan has a financial turnover of €150 million (~ $AU260 million) p. a. (2009) and produces seed at breeding stations worldwide, including a branch in Musk, Victoria. Many of the breeding programmes are conducted within the confines of greenhouses or poly-tunnels to reduce out-crossing with undesirable types (RZA 2009). Yet, horticultural enclosures create pollination problems due to an absence of natural pollinators. Managed pollinators, such as honey bees, are introduced into the enclosures to address this problem, generally with good success. However, the world 'pollination crisis' (see Chapter 1, Section 1.1) highlights the need to investigate alternatives to honey bees. Studies conducted and described in this chapter were, therefore, undertaken to investigate the potential of *Au. australis* as an alternative pollinator to honey bees for some greenhouse-grown vegetable seed crops.

3.1.1 Trial crops

Crops used in this pollination study were carrot: *Daucus carota* subsp. *sativus* Hoffman (Apiales: Apiaceae); celery, *Apium graveolens* Linnaeus var. *dulce* (Miller) Pers. (Apiaceae: Apiales); leek, *Allium porrum* Linnaeus (Asparagales: Alliaceae) and lettuce, *Lactuca sativa* Linnaeus (Asterales: Asteraceae).

3.1.1.1 Carrot

Carrot is one of the top ten economically important vegetable crops on a world market scale (Prohens & Nuez 2008b). A great deal of research has been invested in improving cultivar qualities such as yield, colour and root uniformity (reviewed by Stein and Nothnagel 1995) as well as disease resistance (Gabelman et al. 1994), vernalisation requirements (Alessandro & Galmarini 2007) and hybridization (Linke et al. 1999; Nothnagel et al. 2000; Magnussen & Hauser 2007). For seed production, carrots are grown over a two year period and root vernalisation is required to initiate flowering (McGregor 1976). Flowers are arranged in umbels and the nectar is easily accessed by a range of insects. Carrot flowers are not particularly attractive to honey bees but, with sufficient numbers, honey bees can pollinate carrots effectively (Jones

& Harper 1981; George 1999; Delaplane & Mayer 2000). The presence of abundant insect pollinators within a self-fertile carrot crop can aid seed uniformity, seed yield, maturation time and germination success (Hawthorn et al. 1956, cited in McGregor 1976). However, breeding lines requiring cross-pollination are totally dependent upon insect vectors. The breeding lines used for this trial included self-fertile (male) plants and cytoplasmic male-sterile (female) plants that required cross pollination.

3.1.1.2 Celery

The stalks and leaves of celery can be eaten as a salad vegetable or cooked. Worldwide, as a fresh vegetable, it is not produced in large enough quantities to warrant a Food and Agriculture Organisation crop ranking (FAOSTAT 2010). However, celery cultivation for seed production is increasing as the value of the seeds' essential oils (especially for medicinal use) is becoming more widely recognised (Falzari & Menary 2005).

Celery is grown as an annual if marketed as a vegetable, but for seed production it is a biennial plant. During the first year, stems develop, then vernalisation is required to promote bolting and flower initiation (George 1999). For seed production, propagation is undertaken by cuttings (L. Hannah, RZA, pers. comm., 2008). Celery flowers are protandrous but self-fertile, and individual flowers release pollen before the stigma is receptive (McGregor 1976). The nectar of celery is attractive to bees and cross-pollination within the umbels of the crop is made possible by transfer of pollen grains during foraging activity (McGregor 1976; Quiros et al. 1986).

3.1.1.3 Leek

Leek flowers are arranged in a single umbel that can measure up to 12 cm in diameter (McGregor 1976). As with onion, male-fertile leek flowers are protandrous but acceptable seed set is achievable with an ample supply of pollinators, such as bees or flies (McGregor 1976; George 1999). Limited literature is available on leek pollination; however, Bohart et al. (1970) report that honey bees over-worked onion flowers, resulting in low seed yields. Honey bees were, nevertheless, more successful than blowflies at cross-pollinating onion crops (Ali 1982, cited in Currah and Ockendon 1984). Onion (and presumably leek) nectar is unattractive to bees (Gary et

al. 1972, cited in McGregor 1976) and produces poor quality honey (Hoff 1995). The breeding lines used in the current trial included self-fertile (male) plants and genetically male-sterile (female) plants, which require cross-pollination. Breeding lines requiring cross-pollination are totally dependent on insect vectors for pollen transfer from male to female lines.

3.1.1.4 Lettuce

Lettuce is a major fresh summer vegetable crop, and in 2005 world production was in excess of 22 million tonnes (Prohens & Nuez 2008a). Up to 24 florets develop in a capitulate arrangement along the length of the stem (George 1999). 'Bolting' may be stimulated with the addition of lighting in the greenhouse (Prohens & Nuez 2008a) and, although naturally self-fertile, some cultivated lettuce hybrids require cross-pollination for seed production (George 1999). Lettuce flowers appear to be unattractive to honey bees (Watts 1958) although visitation by pollen-gathering honey bees, maintained within horticultural enclosures, has been observed (L. Hannah, pers. comm., 2009). Goubara and Takasaki (2003) report many different insect pollinators in their field of lettuce; however, honey bees were not amongst them. The pollen of lettuce is sticky and cannot be carried on the wind, so many breeding programmes require hand pollination or introduced insect vectors (Nagata 1992; Prohens & Nuez 2008a).

3.1.2 Trial aims

The initial trial was carried out at the University of Western Sydney (UWS), Hawkesbury campus, Richmond, NSW. This trial was intended as a preliminary study only and, as such, a single colony of stingless bees was used within a mixture of seed crop types. This initial study was undertaken in cooperation with RZA and was conducted in order to ascertain whether *Au. australis* would:

- adapt to the confines of a greenhouse enclosure
- visit the crop flowers
- forage for pollen or nectar on the trial crops
- cross-pollinate breeding lines and
- increase seed set compared to a no-bee control.

The ability of *Au. australis* to achieve any or all of the above pollination requirements would determine the course of action for further studies. If the UWS trial proved to be successful, further investigations were planned to be carried out in a commercial greenhouse environment, located at RZA's site at Musk, Victoria.

For ease of understanding and flow of information, this chapter is set out in two main sections. The first is the initial trial, conducted at the UWS facility in 2008 / 2009, where a series of small, disparate studies were conducted. This was done because the small enclosure provided an ideal environment to observe different bee behaviours. Each study will be reported as a discrete study, with materials and methods, immediately followed by the results. A general discussion will summarise the main findings within these disparate studies and comment on their implications. These studies were conducted in conjunction with the main pollination trial, over a short period of time and were designed to minimise interference to the crop pollination tasks of the colonies.

The second trial, conducted at Musk, Vic, in December 2009, contained two sites, a field site and a commercial greenhouse site. Behaviour of the *Au. australis* colonies is reported for both sites, within different seed crops. The materials and methods, followed by results, will be reported for each site. A general discussion will follow. Key findings from both the UWS and RZA trials will be discussed at the conclusion of the chapter.

3.2 General materials and methods

The information below covers the general materials and methods used throughout this PhD research project. Terms are presented in bold: they will be referred to throughout this dissertation.

The bee shed – stingless bee colonies were maintained in controlled temperature (CT) rooms, within a large Colorbond™ shed, at UWS, Hawkesbury campus. Each room was insulated with a polystyrene lining and temperature control was achieved through the use of a reverse cycle air conditioner, (ASTA09LC and AOTR09LCC, Fujitsu General Australia, www.fujitsugeneral.com.au). Thermostats were set to control temperature between 22 and 26°C and monitored using 'Tinytag' temperature

data loggers (Hastings Data Loggers, Port Macquarie NSW, 2444, Australia). Rooms were furnished with particle board shelves along the walls, where the hives were positioned. Unless otherwise stated, all colonies had access to external foraging via a clear vinyl tubing (0.5 m x 8 mm ID) through the external wall (Figure 3.1). The area around each external entrance hole was painted with a variety of colours to aid in colony orientation (Figure 3.2).

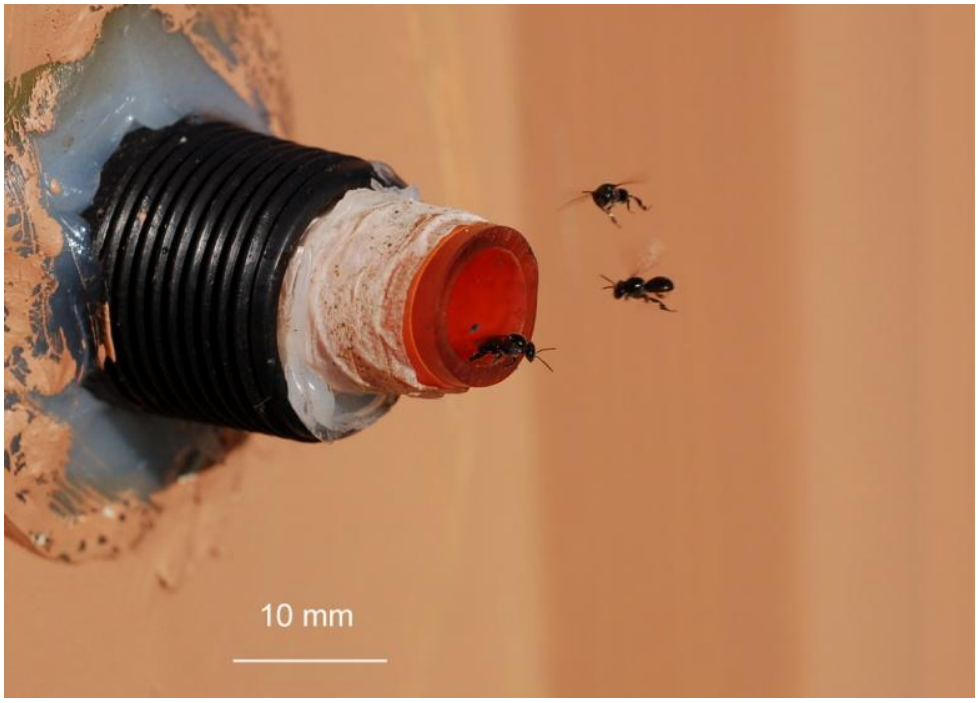


Figure 3.1 External entrance tube to a hive housed in the bee shed.

Figure 3.2 Hive entrances protruding from the bee shed. Entrances were marked with a variety of colours to aid in colony orientation.

When **supplementary feeding** was necessary, a honey **feeder-float** was used to provide a carbohydrate source. The floats consisted of a 30 mL container with an upturned lid, fitted with a handle for easy removal (Figure 3.3a). The mixture of water and *A. mellifera* honey (water : honey, 1:5 (v/v)) was poured into the container and the float was placed on top. The feeder-float was then either placed in an external feeding station (described below) or directly into the hive. A protein source was provided as freeze dried, irradiated *A. mellifera*-collected pollen (Pender Beekeeping Supplies, info@penders.net.au) or trapped *Au. australis*-collected pollen (described below). The **pollen trays** consisted of ground pollen powder (½ tsp, ~ 2 g) placed in a bottle cap. This was placed in the external feeding stations or directly into the hive.

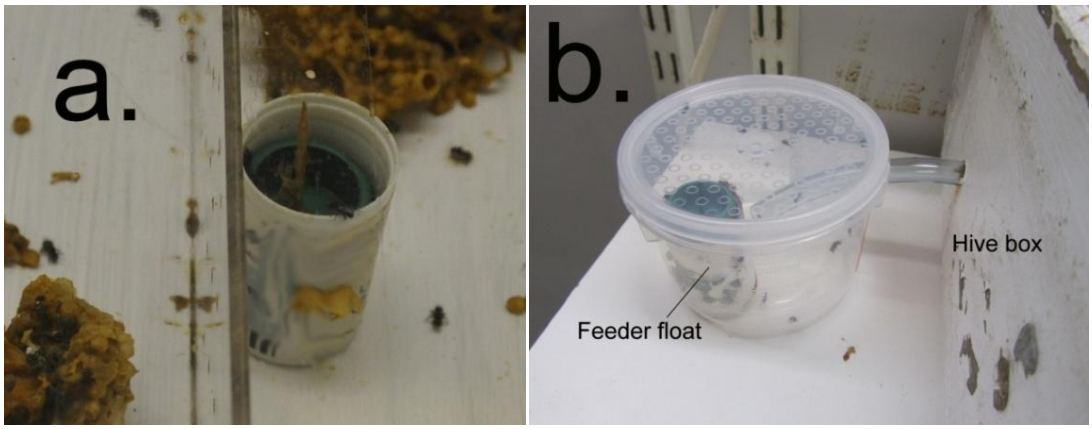


Figure 3.3 Feeder-float inside hive (a) and float within an external feeder (b).

External feeding stations were used to reduce colony disruption, as this meant that hives did not need to be opened. A 10 mm hole was drilled into the side of a 350 mL plastic container (Tellfresh®Round, 350 mL, 71 mm x 103 mm diam., The Decor Corporation, Scoresby, Vic). A 100 mm x 8 mm (ID) piece of silicone tubing was inserted into the hole and the other end was inserted into a pre-drilled hole in the side of the bee hive. Workers were able to access the container, and therefore the honey and pollen, via the tubing (Figure 3.3b). Food resources were harvested and transferred to cerumen pots by workers within three to four days. Feeding regimes varied according to experimental needs.

Observation platforms (OP) (Halcroft et al. 2008) were utilised in some experimental designs during this project. The internal diameter of the silicone tubing used in these studies was 8 mm. All joins between tubing and the OP openings were sealed with reusable adhesive (Blu Tack®, Bostick Australia, Thomastown, Vic., Australia). ***Au. australis* pollen traps** – OPs were connected between the entrance tube and the hive entrance. Foragers unloaded their pollen pellets directly into pots constructed within the OP. During a good season, with large levels of incoming floral resources, the OPs could fill up over a few months (Figure 3.4). This pollen could then be collected and stored in the freezer for use as a supplementary food source.

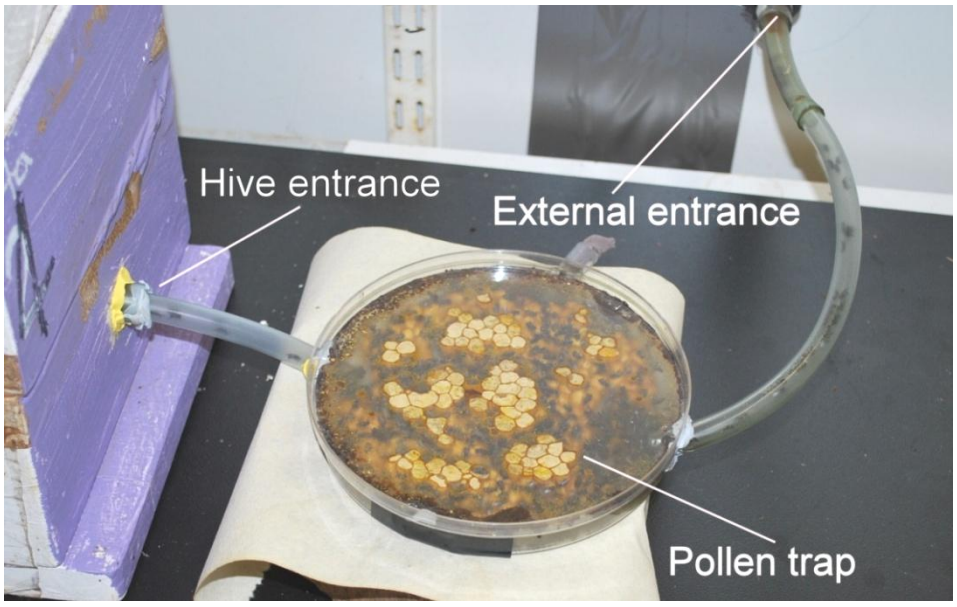


Figure 3.4 OP connected between *Au. australis* hive and external entrance. Note the large amount of potted pollen and bees in the OP.

Beil hives – These hives were designed by Allan Beil (see Chapter 6, Section 6.4) and they housed several of the experimental colonies. The boxes were constructed of 20 mm thick cypress pine, 320 mm x 180 mm x 110 mm, with a capacity of 7 L. Clear acrylic lids, cut into three sections and connected with transparent sticky tape, were fitted to the tops of the box. A cypress pine lid, insulated with 10 mm thick polystyrene sheeting, was fitted over the top of the observation lid. Entrance holes measured 10 mm diam.

OATH hives – The Original Australian Trigona Hive (Dollin & Heard 1999) was first used by Dr Tim Heard (Heard 1988b) and the design is used by many Australian stingless bee keepers, or as a basis for their own box designs. They housed several of the experimental colonies. Hives containing *Au. australis* were always fitted with a 2-section clear acrylic lid, to allow for observation of the colony.

A strong colony – Based on information obtained from experienced stingless bee keepers (A. Beil, pers. comm., 2008) and visual assessment, colony strength could be evaluated. An *Au. australis* colony was considered to be 'strong' when the hive / nest contained a brood volume of at least 500 mL (~100 mm diam. in an artificial hive), and pollen and honey stores measured at least 500 mL each.

3.2.1 General observations pertaining to *Au. australis* colonies

Throughout my PhD project there were situations where I observed biology and behaviour within the colonies, which were incidental to the study I was conducting at the time. Also, as a result of studies reported in Chapter 5, I attempted to conduct a division of labour study, to investigate the task partitioning. Unfortunately, unseasonal weather conditions resulted in abnormal behaviours within the colonies and, after almost five months the study was terminated. The observations made during this time and throughout the project were not worthy of a chapter in themselves; however, the behaviours seen here that have not as yet been reported for *Au. australis.* These observations are reported in a number of appendices;

- Appendix 2 reports on the use of hive weights as a tool to assess stingless bee colony health.
- Appendix 3 reports on drone populations and possible maturity.
- Appendix 6 reports on improved marking techniques for *Au. australis* nestmates.
- Appendix 7 reports on some age related worker behaviours.
- Appendix 8 reports on failed queens or brood disease.
- Appendix 9 reports on gyne production, introduction and imprisonment.
- Appendix 10 reports on brood production and overwintering.

A much improved technique for marking *Au. australis* workers, drones and queens was also developed, resulting in lower mortality due to chilling injury. This technique (described below) could be utilised in further studies.

3.3 Greenhouse chamber pollination study

3.3.1 Modifications to the stingless bee comparative study

The experimental design for this pollination study originally included two pollinators, *Au. australis* and *T. carbonaria,* as well as a no pollinator control. As previous studies have shown *T. carbonaria* is a successful pollinator of some crops (see Chapter 1, Section 1.4.2, also Heard (1987), Heard & Exley (1994), Greco et al. (2011)), a single *T. carbonaria* colony was used as a comparison against the single *Au. australis* colony.

It was possible to monitor the queen status of *Austroplebeia* colonies within artificial hives by including a transparent acrylic lid on the top of the hive. This was not possible in *Trigona* colonies as the workers covered the acrylic lid with resin and obscured the nest structures from view. Opening the top of the hive did not enable the queen to be located, as she is usually found within the brood chamber, which is covered with a layer of involucrum. To check the queen status of a *Trigona* colony, the hive is opened at the centre join, and brood chamber inspected. This process can be quite destructive and there is always a risk of killing the queen (Dollin & Heard 1999). As I was not very familiar with *Trigona* husbandry, I was not confident enough to open the hive for fear of causing damage. Therefore, the queen status for the experimental *Trigona* colony was not visually confirmed. To help assess colony health (Dollin 2002), hives were weighed before, during and after the trial and activity of workers at the hive entrance was monitored. Colony health is briefly discussed in Appendix 2.

Hive entrance and in-crop activity were compared during the trial period. The *T. carbonaria* colony was usually observed to be more active than the *Au. australis* colony. Most of the returning foragers from both colonies carried pollen, which is indicative of brood production (Ribbands 1953). The two hives were returned to the bee shed at the conclusion of the greenhouse trial and colonies were able to access natural, external foraging. Entrance activity for both colonies was monitored for several days, but by now the activity for the *T. carbonaria* colony was markedly lower than that of *Au. australis*. The *T. carbonaria* hive was opened, inspected and found to have only a small number (< 10) of workers present. There was no queen and the colony was broodless. It was apparent that the colony had been queenless for a long period of time and may have never re-queened after initially being split (i.e. prior to receiving the hive).

The behaviour of this queenless, broodless colony and its foragers is likely to have been very different to that of a healthy, queenright colony. The data produced from a single, unreplicated colony could only be used as a preliminary comparison (as this was the first phase of a larger trial) and the fact that it was not a healthy queenright colony made the data unreliable. As a result, the data for *T. carbonaria* have not been included in this chapter. However, hive entrance activity and forager behaviour were

observed for both *T. carbonaria* and *Au. australis* throughout this trial and information gleaned from these observations prompted the undertaking of the paralleling study, which is described in Chapter 6, Section 6.3 .

3.3.2 Materials and methods

The greenhouse chambers were located at UWS, Hawkesbury campus (33°36'S, 150°44'E, elevation 28 m), and contained within a climate-controlled quarantine bioassay insectary (Croudace Greenhouse International, Box Hill, NSW, Australia). The walls and ceilings were constructed from 6 mm twin-well polycarbonate sheeting, on an anodised aluminium frame and had concrete floors. Each chamber measured 5 m x 3 m, with a sloping ceiling with a minimum height of 3 m, reaching a maximum height of 4 m. Controlled environment conditions were maintained with APAC reverse cycle air conditioners (Carrier Air Conditioning, Silverwater, NSW, Australia) and Braemar ducted gas heaters (Seeley International, Lonsdale, SA, Australia) and thermostats were set to control temperature between 22 and 26ºC, with no added humidification. The water supply was delivered at 15 – 23 L / min via a Davey pressure pumping system (Davey Products, Scoresby, NSW, Australia). Potted plants were elevated on 230 mm high galvanized steel mesh benches.

Three greenhouse chambers (Ch) were used for this trial: Ch 1 *Au. australis,* Ch 2 *T. carbonaria* (see amended experimental design, Section 3.3.1) and Ch 3 control (no pollinator). On 20 August 2008, the industry partner representative, Lea Hannah, assisted with the trial setup. The following crops were potted up into 12 L (260 mm diam.) plastic pots, using a premium garden soil mix, and placed in each of the three chambers:

- potted cuttings of self-fertile celery (x 6)
- mature, bare-rooted, male leeks (x 5)
- immature, potted, female leeks (x 10)
- bare-rooted, male carrots (x 5) and
- bare-rooted, female carrots (x 6).

Plants were positioned equidistant, in species groups on the benches (Figure 3.5) and fertilized with 30 g of complete, slow release fertiliser (Osmocote® Exact, Scotts Australia Pty Ltd, Baulkham Hills, NSW, Australia). Two bamboo stakes (1.5 m) were set into the pots for ongoing plant support, and a drip irrigation system was attached to automatic timers ('Garden Mate' Easy Program, Automatic Tap Timers, RIS Irrigation Systems, Beverly, SA, Australia). Irrigation was initially programmed for 2 min, twice a day. As the plants developed and their water requirements increased, the irrigation regime was adjusted to suit. On 24 October 2008, eight potted lettuce seedlings were placed in each chamber, as described above.

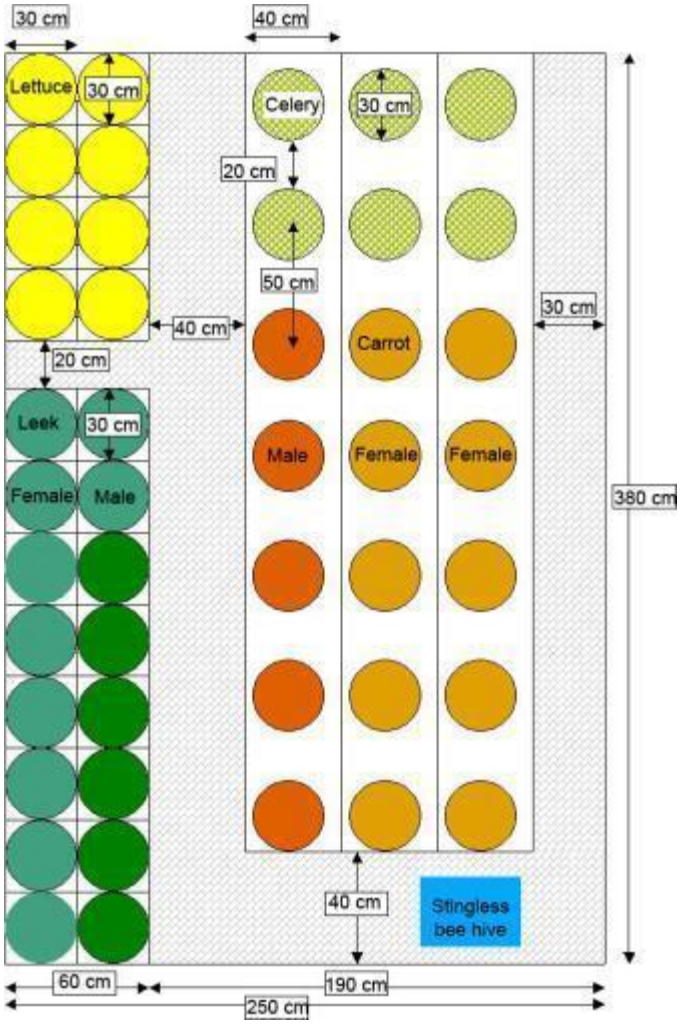


Figure 3.5 Layout of vegetable seed plants in each greenhouse chamber at UWS.

Integrated pest management (IPM) is the ideal form of pest management systems for crops requiring insect pollinators (Johansen 1977). This ensures the pollinators are

not exposed to damaging chemicals, which, in turn, promotes colony health and optimal pollination efficacy.

On 30 August 2008, IPM strategies for the UWS trial were discussed with Andy Ryland (The Beneficial Bug Company, Richmond, NSW, Australia), resulting in the implementation of an IPM programme for the control of several insect pests. Table 3.1 shows the biological control agents employed and their target pests. The release of beneficials was carried out every one to two weeks.

Table 3.1 Biological control agents used in the crops.

Host plant	Pest	Bio control agent	Mode of control
Celery *A. graveolens*	Greenhouse whitefly *Trialeurodes vaporariorum* Westwood (Hemiptera: Aleyrodidae)	*Encarsia formosa* Gahan (Hymenoptera: Aphelinidae)	Parasitic wasp
Celery *A. graveolens*	Green peach aphid *Myzus persicae* Sulzer (Hemiptera: Aphididae)	*Aphidius colemani* Viereck (Hymenoptera: Braconidae)	Parasitic wasp
Leek *A. porrum*	Thrips (especially western flower thrips (*Frankliniella occindentalis* Pergande and onion thrips, *Thrips tabaci* Lindeman) (Thysanoptera: Thripidae)	*Typhlodromips montdorensis* Schicha *Neoseiulus cucumeris* Oudemans *Hypoaspis aculeifer* Canestrini (Acarina: Laelapidae),	Predatory mites

(Biological Services 2008a; b; c; d; Ryland 2008)

3.3.3 Orientation to the greenhouse chamber

It is common for honey bee colonies to lose many (thousands) foragers in the first week after introduction into an enclosure (Graham 1992). Landmarks have been shown to help foragers orient to new environments (Ribbands 1953; von Frisch 1971), thus reducing losses.

3.3.3.1 Materials and methods

The *Au. australis* colony was housed in the bee shed until the greenhouse chambers were prepared. On 10 September 2008, it was transferred into a greenhouse chamber. The hive front was painted with the corresponding colour of its bee shed entrance and coloured cardboard shapes were attached to the chamber walls. Forager losses

were monitored, where possible, and dead bees were collected from the floors and window sills each day, for the first three days. A ten-day period was set aside to allow the bees to orient to the new, artificial environment. This was to ensure that once the trial crops started flowering, the bees would have adapted to the enclosure and be able to forage naturally. As well as being provided with landmarks the bees were supplied with attractive foraging resources to assist their 'settling-in' to the chamber. Pots of *Westringia longifolia* R. Br. (Lamiales :Lamiaceae) and *Leptospermum* sp. Forster (Myrtales: Myrtaceae) were placed within 1.5 to 2 m of the hive entrance. In addition, a bucket of water containing cut branches of flowering Callery pear (*Pyrus calleryana* Decne. (Rosales: Rosaceae)), peach (*Prunus persica* Decne. (Rosales: Rosaceae)) and *Buddleia* sp. L. (Lamiales: Scrophulariaceae). The buckets for the cut flowers were enclosed in a plastic bag to prevent bees from drowning. Cut flowers were replaced each day. As soon as the trial crops started to flower, the supplementary foraging resources were removed from the chamber.

3.3.3.2 Results

Bees were observed for several hours after the colony was introduced into the greenhouse. Numerous workers flew to the ceiling and walls where they buzzed frantically, until they died. The following day, a mechanical aspirator (Australian Entomological Supplies, Bangalow, NSW, Australia) was used to collect the bodies of as many dead bees as practicable. Most accumulated along the window sills and floor near the walls of the chambers. The number of dead *Au. australis* collected over the first two days of orientation into the chambers was > 500. Bee losses greatly decreased from Day 3 onwards and no further collection was carried out. During the orientation period, the bees foraged on the cut and potted flowers. Once the crops commenced flowering the supplementary forage was removed and foragers moved to the flowers of the crops, although in smaller numbers.

3.3.4 Forager activity and flower visiting behaviour

The time spent moving over and between flowers can impact on the quantity and quality of pollination. Pollination occurs mostly while bees are collecting pollen; however, incidental pollination can occur while foragers collect nectar (Heinrich 1975). As bees move over the flower(s), pollen adheres to their body hairs; this in

turn is transferred to other flowers. Incidental pollination is of major importance for crops that require cross-pollination.

3.3.4.1 Materials and methods

Forager behaviour was mostly observed while bees worked within the celery crop. This was because the celery was the first crop to flower, it had a relatively high numbers of flowers, the umbels were quite large and bees were easily observed. Attempts to mark individual foragers with acrylic paint pens for ease of tracking were unsuccessful.

The hive entrance was blocked with a piece of tissue paper to prevent foragers from leaving the hive. The crop was inspected for foragers and those present were removed with a mechanical aspirator. Collected foragers were placed in a cool, darkened area until the study period was completed. The tissue paper was then removed from the hive entrance, one forager was allowed to depart and the entrance was blocked again. Activities noted were: length of the foraging trip, number of plants visited and number of exchanges between secondary umbels. The number of exchanges between secondary umbels was the number of times the forager moved from one umbel to another, thus increasing the potential for pollen transfer between different flowers. Observation continued until the forager returned to the hive, hovering near the entrance. The tissue paper was removed to allow the forager to enter the hive, after which the entrance was, again, blocked. This process was repeated nine more times, to produce data for ten foraging bees.

The accumulation of pollen on the body of the returning foragers was observed while bees worked within the celery and leek crops. Nectar- and pollen-collecting foragers were removed from flowers using a mechanical aspirator. Each bee, within the aspirator vial, was immobilised by chilling and then imaged using a Leica MZIZ stereomicroscope (Leica Microsystems, North Ryde, Australia) 20 X magnifications. The bees later were warmed and released. Celery flowers were also collected and imaged using a Leica MZIZ stereomicroscope to confirm pollen transfer to the stigma.

3.3.4.2 Results

Individual foragers spent a considerable amount of time within the celery flowers and foraging trips lasted from 22 to 47 min (mean 35 ± 2.61 min). During a single trip, foragers were observed mostly walking (Figure 3.6), and moved between up to 20 primary umbels, on four plants. Movement between plants was quick and concise with minimal time spent in flight, and hovering was seldom observed. Pollen grains were found on the body hair of both nectar- and pollen-collectors as well as on the stigmas of the celery flowers (Figure 3.7).

Figure 3.6 *Au. australis* forager walking over the celery flowers. Note the large amount of pollen on her body.

Figure 3.7 Pollen grains attached to the body hair of *Au. australis* nectar forager (left) and on a celery flower stigma (right).

3.3.5 Stingless bee behavioural observations

3.3.5.1 Materials and methods

Forager activity was observed within the crop, with foraging tasks (nectar or pollen collector) and numbers being recorded. Throughout the pollination studies, the number of bees entering the hive was recorded for a 2 min period, as well as the number of bees carrying pollen loads. Entrance activity was initially monitored four times per day, every second day, from the commencement of the orientation period (11 September 2008) into the first week of crop bloom (26 September 2008). This was to ascertain the most active flight periods. Light intensity (photosyntheticly active radiation (PAR)) was also recorded, several times per day, using a LI-COR 250A light meter with (LI-COR® Biosciences, Millenium Science, Surrey Hills, Vic, Australia).

Brood production was assessed by monitoring incoming corbicular pollen loads (Graham 1992) and removal of pupal casings by workers, as well as visually assessing the size of the brood cluster. These activities were monitored at varying times during the study, depending on the availability of floral resources.

3.3.5.2 Results

Entrance activity alone was not a good measure of the *Au. australis* colony's activity within the UWS greenhouse chamber. Throughout the study, forager numbers were

low and workers appeared to be recruited in response to floral resource availability. Higher activity was observed during the orientation period (when large amounts of floral resources were brought into the chamber), during the first ten days of the celery flush and once the male carrots flowered (Figure 3.8). Forager activity reduced as the celery began to set seed and flowering declined. Large numbers of foragers were observed on both male and female carrot plants, but it was not possible to track foragers moving between lines. It was considered more important to allow the bees to have free access to the plants than to close the hive and allow only one out at a time. Movement between male and female plants, and thus pollen transfer, would be apparent with seed set. No foragers were observed on the female leeks.

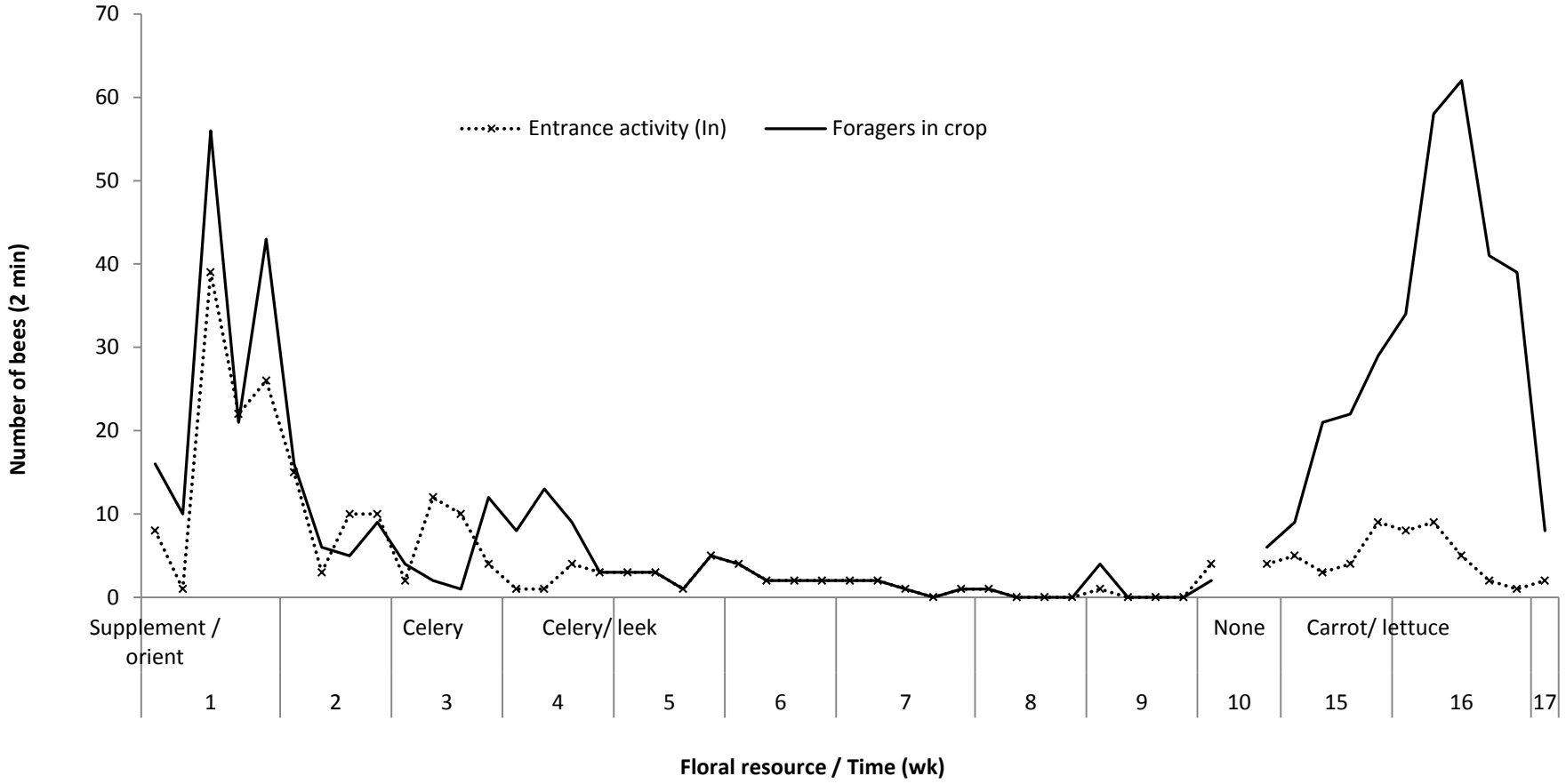


Figure 3.8 Number of *Au. australis* workers entering the hive (In) for 2 min and those working in the crop for the same 2 min during the UWS trial. Supplement food was provided during the first ten days, until the celery commenced flowering, from weeks 3 - 9. Leek flowered from weeks 4 – 9. No floral resources were available from weeks 10 – 15, when the carrot and lettuce commenced flowering, until week 17 when the trial was terminated.

Au. australis hive entrance activity commenced at first light, 05:30 – 06:30 Eastern Australian Daylight Savings Time (EADST), and ceased before 16:00 (i.e., 3 to 4 hours before sunset). Between these times, the time of day did not appear to influence the number of foragers working within the crop so a convenient time was chosen to monitor this activity, two to five times a week (depending on available floral resources at the time).

Workers began to fly at light intensities as low as 2.8 µmols / m^2 / s and activity did not otherwise appear to be influenced by light intensity (Figure 3.9). On several occasions, foragers were observed exiting the hive entrance while the resin curtain was still being dismantled by guard bees. The light intensity within the polycarbonate-clad greenhouse chambers was measured over several days during the initial setup period. Throughout the day, light intensity did not exceed 294 µmols / m^2 / s. For the purposes of this study, flight activity was the combined number of bees returning to the hive entrance plus the number of bees foraging within the crop at the time of observation. This gave a more realistic picture of worker bee activity than by simply monitoring returning workers.

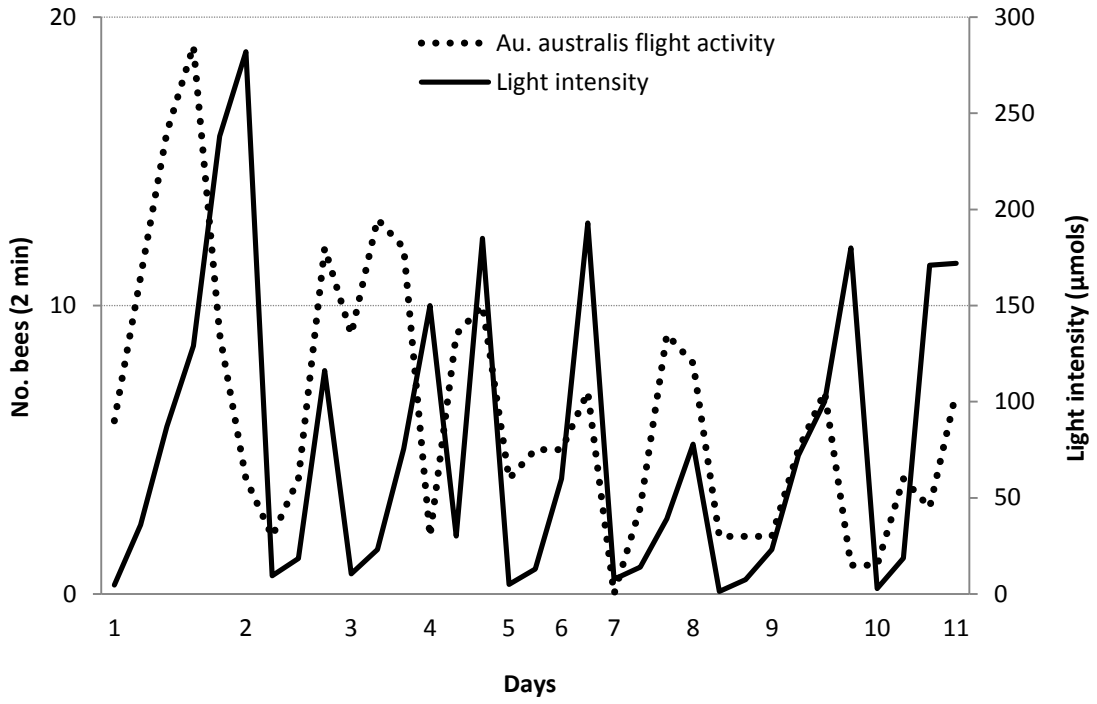


Figure 3.9 Number of active *Au. australis* (combined number of bees returning to the entrance (in 2 min) and bees active in the celery crop (in 2 min)) and its relationship to light intensity in the UWS greenhouse.

3.3.6 Crop yield

3.3.6.1 Materials and methods

Pollination efficacy in vegetable seed crops is determined by seed set and yield, as assessed by seed number and seed weight. Crops trialled at UWS Richmond were hand harvested and seed set was determined by seed count. Seed quality was not assessed here as it was beyond the scope of this trial. Activities associated with seed harvest and seed set calculation are set out in chronological order of harvest.

3.3.6.1.1 Leek

On 19 October 2008, leek heads were harvested and hung to dry. Flower counting and seed harvest took place from 19 to 21 November 2008. Flowers containing seeds were placed in a mortar bowl and gently pounded with a pestle until all seeds were free from floral tissue. The seeds were counted for each flower head, then bagged and tagged. As leek flowers contain three ovaries, there was potential for each flower to set three seeds. Potential seed set was thus calculated as [No. of flowers x 3]. Female and male plants were calculated separately.

3.3.6.1.2 Celery

Seed harvest commenced on 17 December 2008 with individual plants being cut off at the base, placed in a bag and removed from the chambers, as required, to reduce seed drop. One celery plant was prepared in the following way. A secondary umbel was removed from the terminal end of the plant and then the number of primary umbels was counted on the secondary umbel. The number of flower pedicels on each primary umbel was counted (Figure 3.10). This procedure was repeated for every secondary umbel on the plant. Seeds were removed from each umbel, counted and placed in a plastic beaker. The bag containing the plant was emptied of all loose seed. Care was taken not to include large pieces of foreign matter such as leaf or stem. The plastic beaker was covered with a coarse mesh. Fine foreign matter was sieved out of the beaker, leaving the clean seeds behind. Finally, the seeds were bagged, tagged and later weighed.

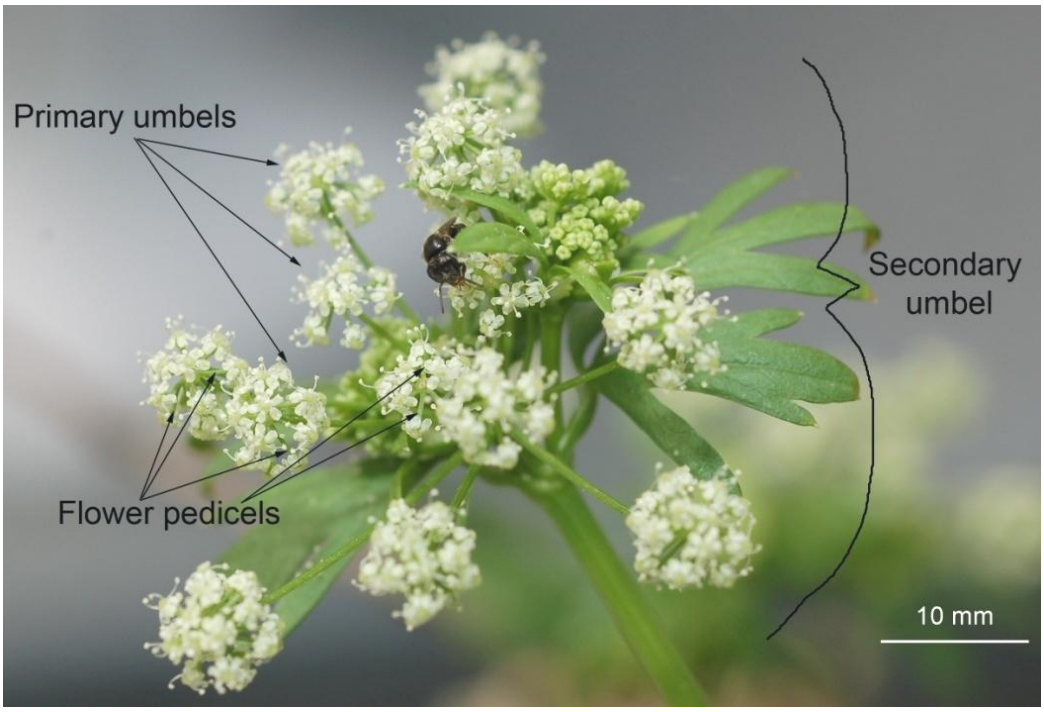


Figure 3.10 Celery flowers showing primary and secondary umbels, as well as pedicels.

The process described above took five days for one plant. As it was too time-consuming to count all flower pedicels for all of the plants, estimates of the possible number of flowers for each plant treatment were made. An estimate of the possible seed set was calculated by the following method. Two plants in each treatment had their entire flower pedicels counted. This was to ensure an accurate estimation, based on the average of the two plants. The number of flowers produced by the plant varied with pollination success. The higher the pollination success, the lower the number of flowers produced. From the sampled plants (n = 6) it was possible to calculate the average number of flower pedicels / primary umbel (n = 21,114) and the average number of primary umbels / secondary umbel (n = 1,631). The remaining plants merely had the number of primary umbels / secondary umbel and the number of secondary umbels / plant counted.

Celery flowers contain two ovaries, potentially setting two seeds / flower. Finally, it was possible to estimate the potential seed set for each plant, as [(No. pedicels x No. primary umbels) x 2]. Plants that set only a small number of seeds had individual seeds counted at harvest. Those plants with high seed set were processed in the following manner: each treatment had 10 x 100 seeds counted and weighed; this was

averaged to obtain a mean weight for 100 seeds. Bulk seed was then weighed and seed set was estimated as:

Seeds / plant = (seed weight ÷ mean weight of 100 seeds) x 100

Celery seed set % = [No. seeds / plant ÷ ((potential No. of flowers / primary umbel) x 2)] x 100

3.3.6.1.3 Carrot

During the process of counting flower pedicels for the celery plants, the older plant parts had become dry and brittle, making them difficult to count. As a result, to estimate the potential number of carrot flowers per umbel, harvesting and counting of the flowers took place while the flowers were still supple and easy to count. This was done while pruning, prior to flowering of the male lines (see Section 3.3.6.2.1, management problems). Random samples of secondary umbels were collected from the plants within each treatment (n = 12). The number of flowers (n = 4,298) / primary umbel (n = 423) was counted for each of the secondary umbel samples. The average number of flowers per primary umbel was calculated. Carrots have two ovaries; therefore, potential seed set is two seeds per flower. Seed set was low, so seeds were harvested and counted for each plant. Percentage of seed set in each treatment was calculated as:

Carrot seed set % = [No. seeds / plant ÷ ((potential No. of flowers / primary umbel) x 2)] x 100

3.3.6.1.4 Lettuce

On 10 February 2009, 48 randomly chosen newly-opened flowers were removed from the lettuce plants of each treatment. The compound flowers (n = 144) were dissected and the number of ovaries (simple flowers) was counted (n = 2,360). The average number of simple flowers per compound flower was calculated.

Seed harvest commenced on 20 February 2009. It was important to remove as much of the fluffy pappus from the seed cluster, as this becomes a mass of fluff from which the individual seeds are extremely difficult to remove. Bulk seed was collected,

cleaned of as much debris as possible, bagged and tagged. Seed weights were calculated in the same way as for celery and this was used to estimate the total seed numbers produced for each plant, as:

Seed No. = (seed weight ÷ weight of 100 seeds) x 100.

Percentage seed set was calculated as:

Lettuce seed set % = [No. seeds / plant ÷ (flowers x compound flowers / plant)] x 100.

3.3.6.1.5 Data analysis

Data were tested for homogeneity of variance using Levene's test. The data were analysed by one-way ANOVA and means were compared by Tukey's HSD test in SPSS 17 (IBM Company, Chicago, Illinois). The setting of significance was $\alpha = 0.05$.

3.3.6.2 Results

3.3.6.2.1 Overview of pest and crop management problems at UWS site

Beneficial invertebrates were released on a regular basis but unfortunately the success of the IPM programme was poor. Humidity within the chambers was kept to a minimum to reduce plant disease development; however, this low humidity was not favourable for the introduced beneficial species. Pest numbers grew at an alarming rate and plant health was affected. Leek seeds were harvested early so that the thrips-infested plants could be removed from the greenhouse. Celery plant health and resulting seed set were threatened by heavy aphid infestations. Plants were removed from the chambers prematurely, in order to spray infestations on these plants with insecticides. Once sprayed, all the celery plants were relocated and managed in the 'control' chamber (where there were no bees), to allow the seeds to set and mature. Late blooming flowers were pruned off to remove them from the final yield calculations. Lettuce plants were infested with both whitefly and thrips but they did not appear to suffer any ill effects.

The air conditioning units were inadequate for managing the cool-climate vegetable crops. Plant stress and reduced health were brought on by overheating, and the male carrot plants de-vernalised, which prevented them flowering. Newly-pulled male plants were used to replace the de-vernalised ones; however, they suffered transplant shock and most of them died. The flowers on the female plants were pruned off while waiting for the male plants to flowers. This, in turn, caused nutritional stress and reduced flower quality.

3.3.6.2.2 Leek

Bees were never observed on the female leeks and seed set was so poor that the data could not be statistically analysed. There was no seed set for the control treatment and only 1.22% for the *Au. australis* treatment. The male line was very attractive to the bees; however, seed set in a healthy self-fertile crop is expected to be close to 100% (L. Hannah, RZA, pers. comm., 2008), and it was only 18.31% in the *Au. australis* treatment, compared to 1.77% for the control.

3.3.6.2.3 Celery

Au. australis foragers moved into the celery crop as soon as it began to flower; however, the maximum number observed on the flowers at any one time was 13. There was a significant difference ($F_{1, 11} = 358.845$, $p < 0.001$) between treatments, with the seed set for *Au. australis* being 76.25% and for the no pollinator control, 0.02%.

3.3.6.2.4 Carrot

After four days of foraging on the male carrot line, *Au. australis* moved onto the female line also, after which some fruit development was seen (Figure 3.11). The male plants flowered for another three weeks, at which time the trial was terminated due to pest infestations. There was a significant difference ($p = 0.015$) between treatments, with final seed set in the *Au. australis* treatment being 8.35%, compared to < 0.01% in the control.

3.3.6.2.5 Lettuce

There were only three occasions where *Au. australis* were observed foraging on the lettuce flowers. As a result, no useful data were collected regarding forager activity within the lettuce crop. Lettuce seed set for the *Au. australis* and the control treatments were very similar, at 24.41% and 23.94% respectively.

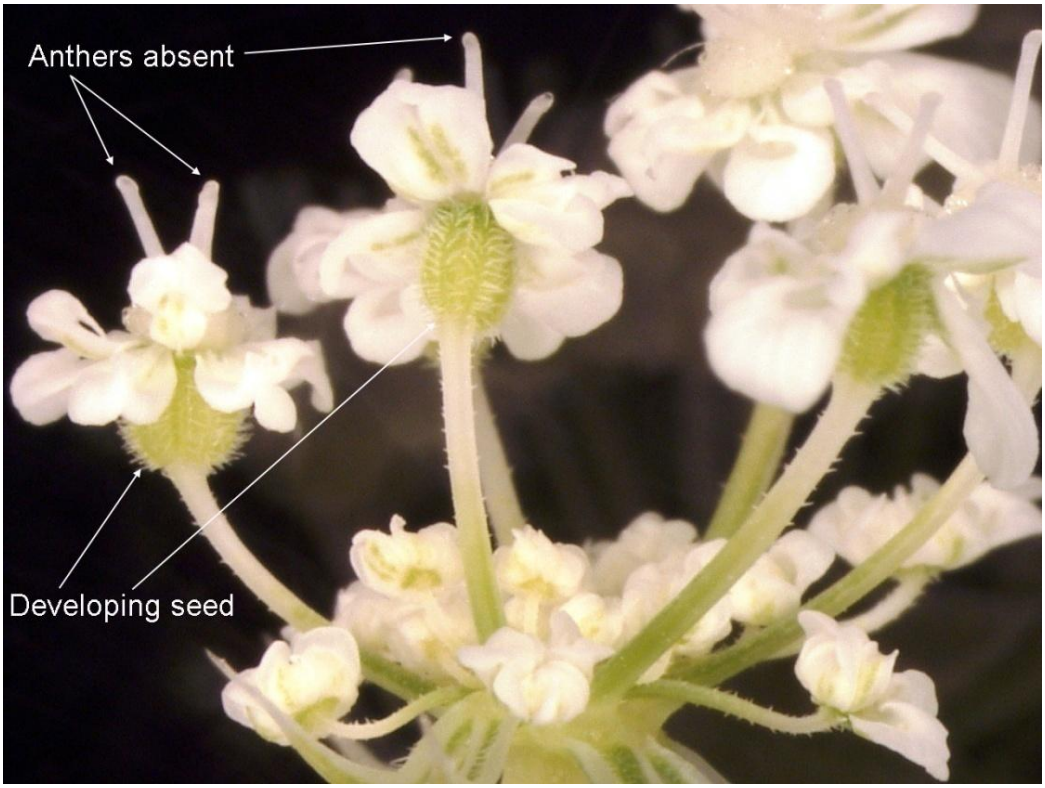


Figure 3.11 Developing fruit in the male sterile flowers of the cross-pollinated female line of carrot.

3.4 Key findings in UWS pollination study

- The *Au. australis* colony adapted to the confines of the greenhouse chamber.
- Foragers visited the crop flowers.
- Foragers collected both nectar and pollen.
- Foragers moved between plant lines and cross-pollinated the carrot crop.
- Foragers significantly increased the seed set in the celery and carrot crops, compared to the no-bee control treatment.

- As a result of the above findings, it was appropriate to conduct further studies, investigating the potential of *Au. australis* as a crop pollinator, within a commercial environment.

3.5 Greenhouse and field crop pollination studies

The commercial greenhouse and field crop trials were carried out at RZA, Musk, Victoria (37°22'S, 144°12'E, elevation 677 m). It was predicted that monitoring of individual bees would be more difficult in this environment, compared to the chamber study (UWS site); consequently, entrance activity and flower visitation were considered the most important observations to be carried out.

3.5.1 Crop layout and pollinator setup

3.5.1.1 Materials and methods

The RZA commercial greenhouses were constructed from clear plain, light-diffusing plastic sheeting which had been painted with white Reduheat® wash (Mardenkro, Geerstraat, The Netherlands). This resulted in a barrier which allowed 0% UVB, 15% UVA and 86% PAR light into the enclosure. The greenhouse was prepared by RZA staff prior to installation of the bee hives. Leeks were planted in eight 3.8 m long beds. Each bed had four rows of male-sterile 'females' planted in the middle of the bed, and one to two rows of 'males' on each side of the female rows. Plants were supported with metal stakes and plastic mesh.

The RZA greenhouse (80 x 20 m) was divided in half to create two areas (40 x 20 m) and two *A. mellifera* colonies were placed in each area, one week prior to my arrival. The area at the west end of the greenhouse was equipped with four platforms, consisting of a galvanised-steel frame and wooden floor (540 x 360 mm), attached to greenhouse uprights (1.2 m high) located 8 m apart (Figure 3.12).

On 13 December 2009, four *Au. australis* colonies, managed in Beil hives, were installed on the platforms. Three of the four platforms were furnished with a thermostatically controlled heat mat (30 watts Flexi Pad, C & M Innovations, Ingleburn, NSW, Australia) with the hive placed on top. The thermostat probe was

inserted through a pre-drilled hole (6 mm diam.) in the side of the hive and secured with reusable adhesive (Blu Tack®, Bostick Australia, Pty Ltd, Thomastown, Vic, Australia) and tape (Figure 3.12). A fridge / freezer thermometer (Jaycar Electronics, Rydalmere, NSW, Australia) was fitted to one of the three hives to monitor in-hive and ambient temperatures could be monitored directly. The mat thermostats were adjusted to maintain colony temperature at ~18°C. A Tinytag Plus 2 data logger (Tinytag Plus 2 TGP-4020, Hastings Data Loggers, Port Macquarie, NSW, Australia) was inserted through a pre-drilled hole in each hive to record temperature. Greenhouse temperature was recorded using a single data logger which was placed beside the hive that was closest to the centre of the greenhouse. Hive entrances closures were removed at 15:00 EADST.

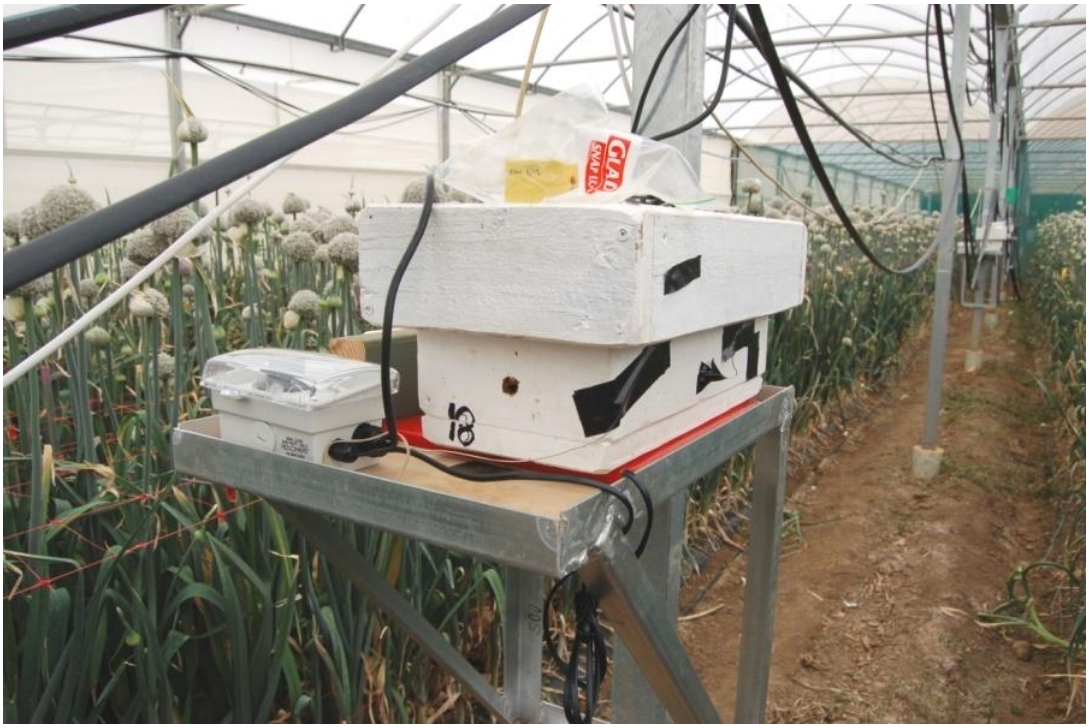


Figure 3.12 Hives set up on steel platforms attached to uprights in greenhouse. Temperature probes were inserted through holes in the hive walls. Heat mat (red) was positioned beneath, with thermostat control next to the hive.

It was not possible to collect bees from the earth floor of the commercial greenhouse. Therefore, to estimate worker losses during the orientation period, a camcorder (JVC Hard Disk Camcorder 'Everio', GZ-MG26AA, Victor Company of Japan, Ltd, Kanagawa, Japan) was set up outside the entrance of one of the heated hives. This was left to record for ~ 4 hours per day from 14 to 22 December 2009. Footage was

downloaded each evening and saved on DVD. Footage was later viewed using Nero 7 Premium software, Show Time (Softpedia, Bucharest, Romania).

The field crops consisted of eight beds of carrot and two beds of leek. Each carrot bed was 50 m long and contained two rows of male or female plants. The male:female ratio was 1:3, with a male bed being planted between the female rows (i.e., F, M, F, F, leek, leek, F, F, M, F). Once the male plants finished flowering, they were removed. Two 50 m beds of leek were planted in the same field, with male and female ratios as described for the greenhouse. All crops at were managed by RZA staff, including pest management.

The field crop also contained four colonies of honey bees, installed one week prior to my arrival. On 13 December 2009, four OATHs containing *Au. australis* colonies were fitted with a piece of PVC pipe (200 mm long x 50 mm ID), secured to the side of the box with a 10 mm long screw and a 60 mm long screw through the full width of the top of the pipe. A star picket was driven into the ground and the PVC pipe was slipped over the top, the long screw holding the box at the top of the picket. The hives were positioned 1 m off the ground and 10 m apart along the west side of the field crop. The hive entrances were exposed to full morning sun and were protected from afternoon sun by a 70% shade cloth wind break (Figure 3.13). Each hive was fitted with a data logger temperature probe, as described above. A data logger was secured underneath one hive, in the shade, to monitor ambient temperatures. None of the field hives were fitted with heating units. Hive entrance closures were removed at 13:00 EADST.

Both the greenhouse and field crops were well established and flowering had commenced when the stingless bee hives were introduced into the commercial crops. This removed the opportunity for an orientation period.

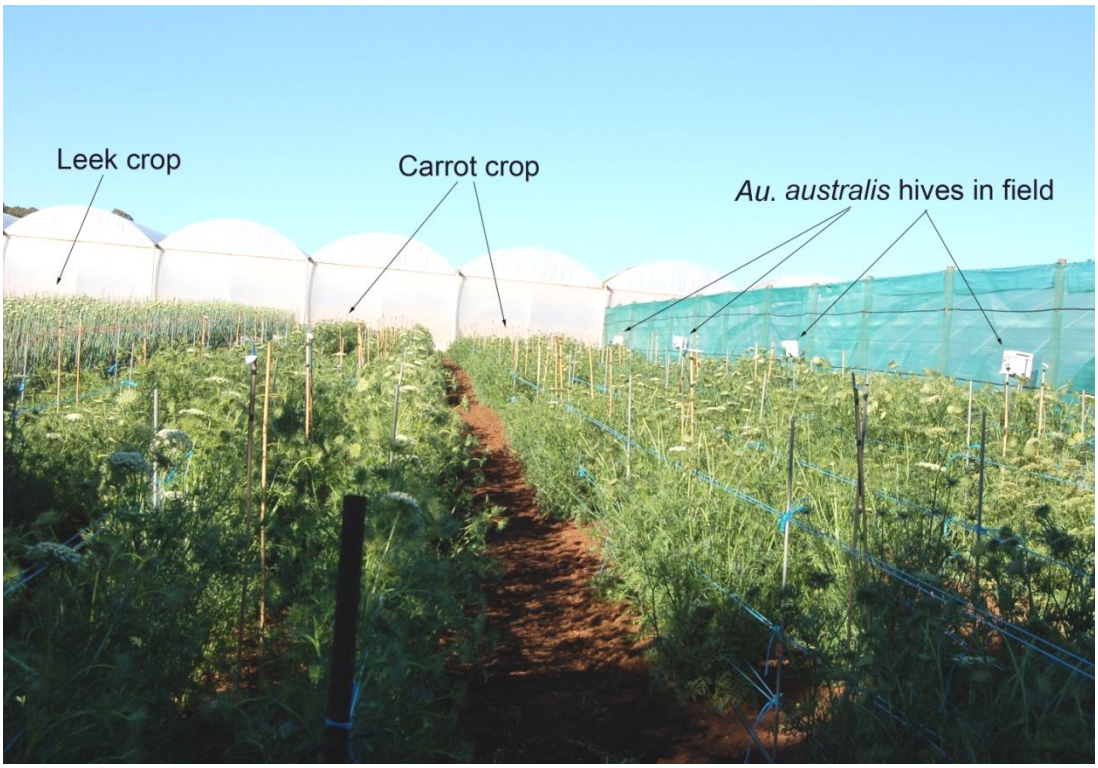


Figure 3.13 OATH boxes attached to star pickets and positioned on the west side of the field crops.

3.5.2 Pollinator behaviour

The timing for the introduction of colonies of both honey bees and stingless bees into the crops was dictated by the crops' flower development. Ideally, pollinators should be introduced into the crops just prior to bud burst; however, due to technical and logistical problems, introduction of the stingless bee hives was delayed until 14 December 2009. The 'period of observations' (viz., 14 – 22 December 2009) was the time where I was able to physically monitor hive entrances and forager activity within the greenhouse and field crops. Although the study was terminated with the maturation of the crop and the removal of the colonies for the greenhouse (January 2010), the 'period of observations' was the time where the bees' behaviour was monitored in relation to the environmental conditions of both the greenhouse and the field.

3.5.2.1 Materials and methods

On 14 December 2009 (Day 1) entrance activity for all *Au. australis* hives (i.e., greenhouse and field, n = 8) was monitored every one to two hours (> 6 times during the day). During this time, light levels and temperatures were also recorded. Monitoring commenced at 07:30 EADST, two hours after sunrise, and ceased at 19:30, just prior to sunset. This day was a fine, sunny day which provided optimum conditions for foraging.

Forager activity within the crops was monitored once a day for both *Au. australis* and *A. mellifera* from 14 to 22 December 2009. There were no reports by the RZ staff of *Au. australis* foraging in the greenhouse crops and very little activity was observed in the field crops during this time (viz., after 23 December 2009).

By 22 January 2010, some hive entrance activity within the greenhouse was observed by RZA staff, with foragers returning with pollen loads (L. Hannah, pers. comm., 2010). However, the male leeks had almost finished flowering and the weather was predicted to be unfavourable. Male leeks were harvested and removed from the greenhouse one week later, and the honey bee and *Au. australis* hives were removed from the greenhouse soon after (4 February 2010) and placed in the field until they could be collected (2 March 2010).

As there was no recorded foraging activity by the *Au. australis* greenhouse colonies during the 'period of observations', more defined parameters of activity were recorded. Entrance activity (Table 3.2) was subsequently correlated with in-hive and ambient temperatures as well as with light intensity. From 16 to 21 December 2009, entrance activity was monitored more than three times per day, beginning before ambient temperature reached approximately 18°C (this is the temperature-threshold for flight required by *T. carbonaria* (Heard & Hendrikz 1993)) (08:30 EADST) and concluding before end of business (17:00 EADST) (see Safety Issues, Section 3.3.5.2).

Hive entrance activity was monitored for the honey bee colonies within both greenhouse enclosures. Hive entrances were monitored morning and afternoon for the west end enclosure (*Au. australis* greenhouse) and daily for the east end.

Table 3.2 Rating of *Au. australis* hive entrance activity within the greenhouse and field

Activity rating	Observation
0	Nil – entrance closed, no audible sounds coming from within the hive
1	Audible – entrance closed but with audible hum from within the hive
2	Opening – entrance in the process of being opened but no external activity
3	Open – entrance fully open, guards present in entrance hole but no external activity
4	Active – active at and outside entrance, including guarding and removing debris
5	Foraging – all of the above activities plus exiting and entering for foraging

Au. australis entrance activity of hives in the field (n = 4) was monitored in between the greenhouse observations (n = 4), beginning late morning as ambient temperatures approached ~ 18°C (10:00), mid afternoon (14:00) and late afternoon (17:30). Honey bee entrance activity in the field (n = 1) and in the greenhouse (east and west, n = 2) was monitored once a day, at 14:00, and the crop was inspected between 12:00 and 14:00 to assess foraging activity of both honey bees and stingless bees.

3.5.2.2 Results

Honey bee hives were placed in the commercial greenhouse one week prior to the commencement of the study. On Day 2 at 15:00, the small number (10 – 20) of honey bees that were observed to be airborne, grew to between 600 and 1,000 bees. Within 10 min, the greenhouse contained approximately 3,000 honey bees, most of them buzzing the west wall of the greenhouse (the wall that contained the exit door). The situation became quite dangerous, and the study was terminated for the remainder of the day.

It became apparent that the greenhouse walls contained several holes which enabled the honey bees to escape the confines of the enclosure. However, they were unable to return to their colonies and became increasingly aggressive. The following days saw incidences of honey bees buzzing and chasing me as well as RZA staff, whenever we

were outside the greenhouse. This continued until Day 5, when staff were able to repair all obvious holes in the greenhouse wall. Due to the increasing threat of bee stings and thus, for Occupational Health and Safety reasons, I chose to conduct activities in or around the RZA greenhouse only during normal working hours (i.e., until 17:00) while staff were present in the area. Protective clothing was donned prior to approaching and whilst inside the greenhouse enclosure.

In contrast, at no stage were large numbers of stingless bees observed leaving the hive or buzzing the ceiling or walls of the commercial greenhouse. Some orientation and debris-disposal flights were observed on Days 2 and 3, with exiting worker numbers correlating with entering numbers. Real time monitoring of hive entrances, between 10:00 and 17:00, showed very little flight or entrance activity from Day 4. Entrances were opened when ambient temperatures reached ~18°C and guard bees patrolled the area around and within the entrance hole of the hive. At no time were *Au. australis* workers observed on the baits of pollen, honey or resin. The main flight activity observed was that of drones leaving or being expelled from the entrances (see below). Video footage of the single colony showed an increase in worker activity during the afternoon of the final day of the 'period of observations'. From 13:45, an average of five workers per 2 min were observed removing debris, departing the hive and returning within five to ten seconds. From 14:40 to 16:33, an average of 12 workers per 2 min departed the hive. Thirty percent of these workers did not remove debris and spent more time away from the hive, returning more than 20 seconds after their departure.

A small number of drones were observed exiting the greenhouse hives, in real time and on video footage, during the first two days of the trial. These did not return to the hive and were seen buzzing the ceiling of the enclosure. Video footage, recorded during the 'period of observations', 14 – 21 December 2009, showed a considerable amount of activity in the monitored hive on the afternoon of the last day (Day 8) (Figure 3.14). It also showed small numbers of drones leaving the hive on Day 2 and a surge in the drone exodus on Days 7 and 8. Close observation of the video footage showed some drones being forcibly pushed out of the entrance. No drones returned to the hive.

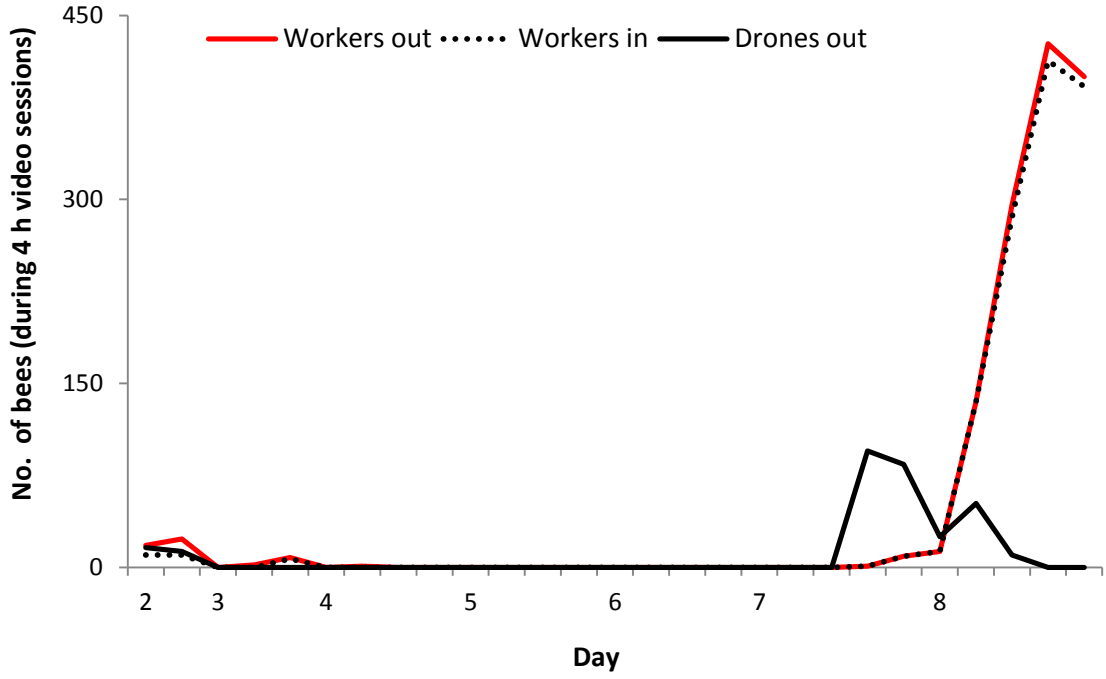


Figure 3.14 Entrance activity of worker and drone bees during the 4 h video sessions which monitored a single *Au australis* hive entrance, RZA greenhouse site.

Colonies installed in the field oriented as normal and workers were observed undertaking short flights from the hive entrance. Soon after these flights, small numbers of foragers were observed returning with pollen loads. However, due to the low night temperatures entrance activity did not commence until quite late in the day, if at all.

Ambient and in-hive temperatures varied widely in both the greenhouse and field, and appeared to affect the entrance and flight activity for the *Au. australis* colonies. By monitoring the thermometer, it was possible to set the heat mat thermostats to optimal temperatures, which meant in-hive temperatures were maintained at or above 18°C (Figure 3.15). In comparison, the minimum in-hive temperature for the unheated greenhouse hive fell to 10.9°C, resulting in delayed hive entrance activity the following morning.

The ambient temperatures within the RZA greenhouse ranged from 6.2 to 39.1ºC during the trial period. The *Au. australis* colonies did not open their hive entrances until 11:30 on the first morning (Day 1). The first day was used to monitor in-hive temperatures and set heat mat thermostats for optimal hive management. From

Day 2, heated colonies began opening hive entrances before 08:30, when in-hive and ambient temperatures reached ≥ 18.8°C and ≥ 19.1°C, respectively. The colony within the unheated hive began opening its hive entrance when the in-hive temperatures reached 15.5°C and the ambient temperature reached 26°C, up to three hours after the colonies in the heated hives had opened theirs (Figure 3.16).

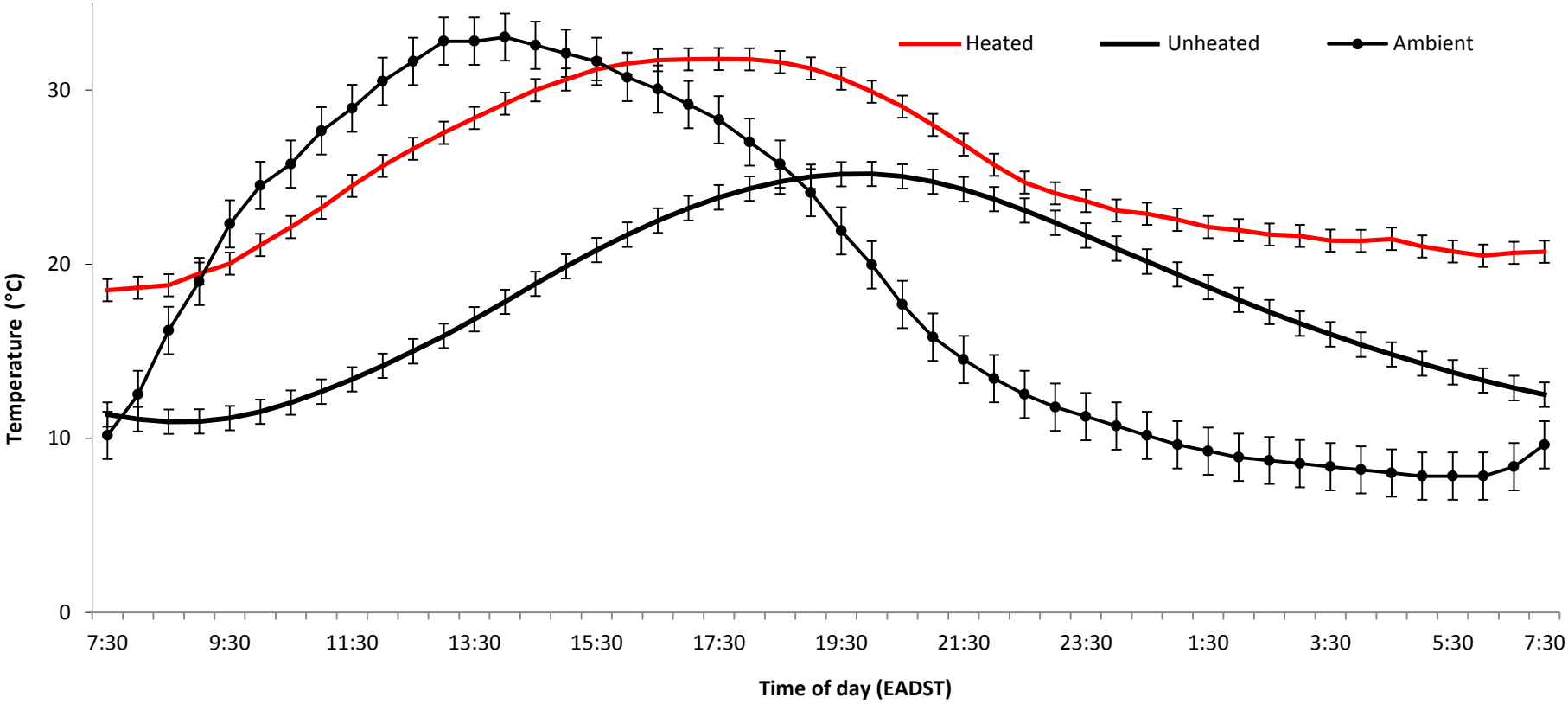


Figure 3.15 Ambient temperature compared to in-hive temperatures of heated (mean, n = 3) and one unheated hive in the RZA commercial greenhouse, over 24 h.

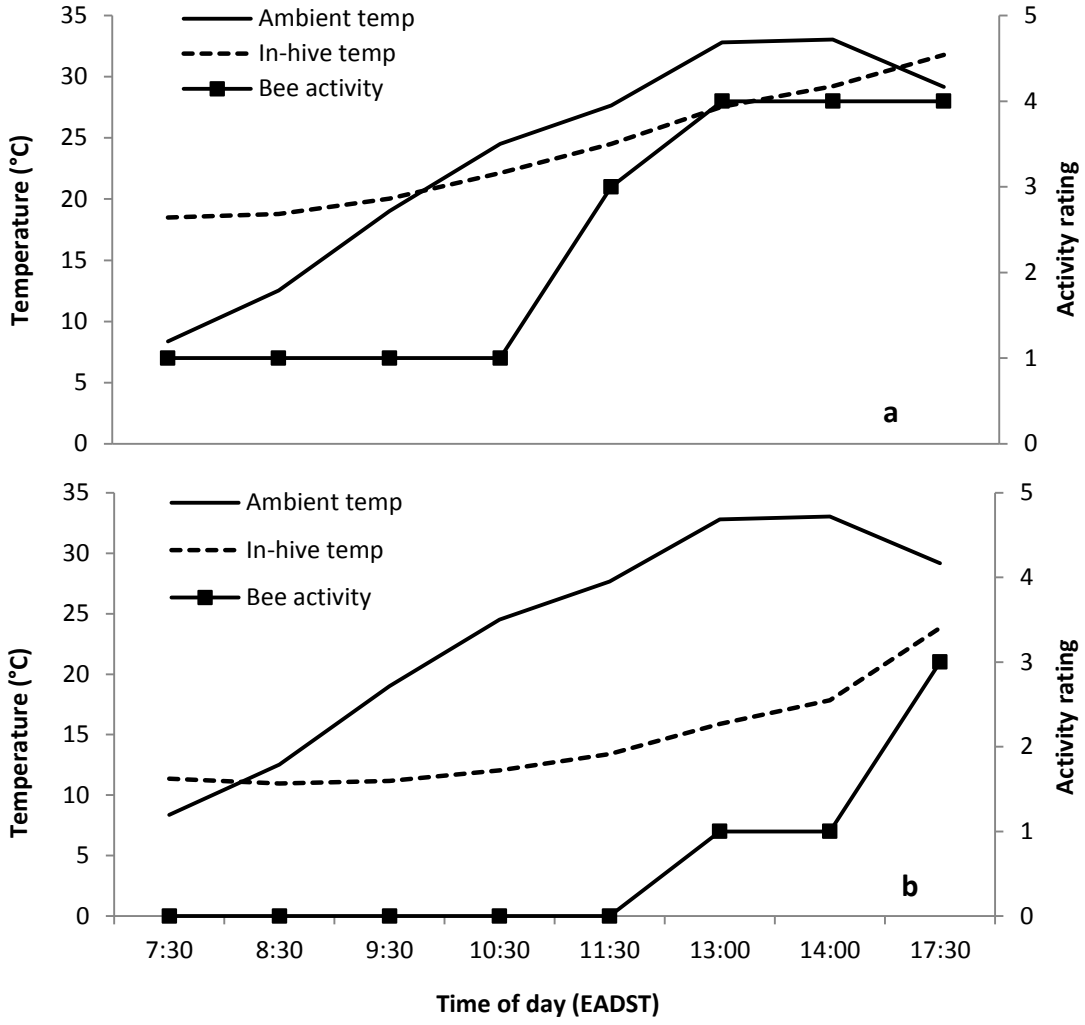


Figure 3.16 Hive entrance activity compared to in-hive and ambient temperatures, within the heated hives (n = 3) (a) and the unheated hive (b) in the RZA greenhouse. Activity ratings are taken from Table 3.2, (Section 3.5.2) and data was collected on a single, sunny day.

Hives in the field were not heated. The overnight temperatures were low and ambient temperatures in the field ranged from 4.6 to 38.5ºC during the study period. Once the ambient temperature reached ~ 18°C, it took at least 1 h for the in-hive temperatures to reach this temperature threshold. On days following a long, cold night (e.g., 4.6 – 7.0°C for >7 h), it took up to 5 h for the in-hive temperatures to reach ~ 18°C. As shown in Figure 3.17a, on Day 1, by the time the in-hive temperature reached ~ 18°C, it was too late in the day (17:00) for the bees to commence any foraging activity. Days following milder nights (≥ 8°C) resulted in the in-hive temperatures increasing more quickly and reaching ~ 18°C by late morning (Figure 3.17b). This resulted in an increase in entrance and flight activity for these colonies.

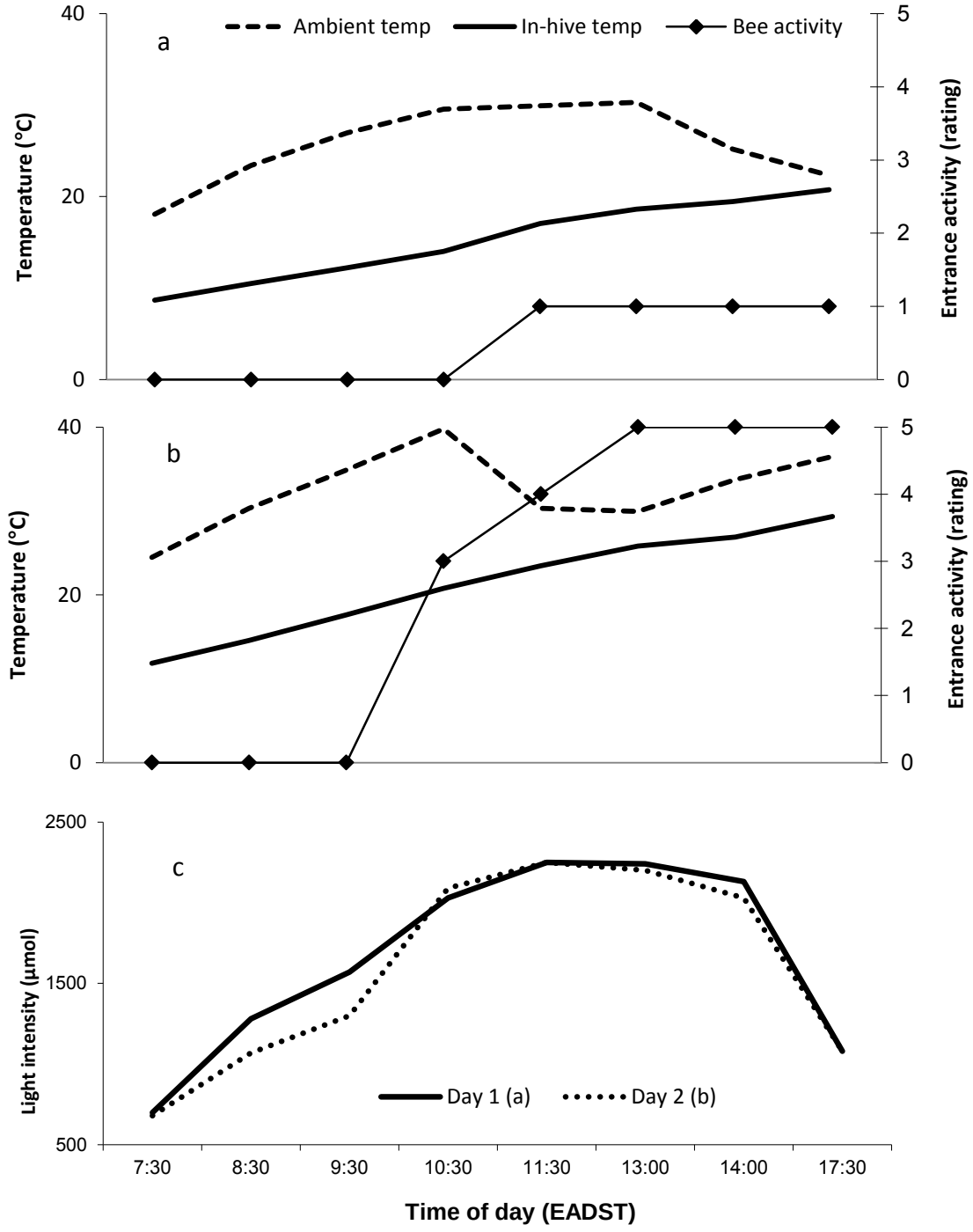


Figure 3.17 Entrance activity (see Table 3.2, Section 3.5.2.1) correlated with in-hive and ambient temperatures. (a) On a cold, sunny day with delayed in-hive temperature increases and (b) on a warm, sunny day, with steadily increasing in-hive temperatures. (c) Mean light intensity for the two days.

Light intensity did not appear to affect entrance or flight activity as much as temperature. For example, Figure 3.17 shows entrance activity of the field hives on two different days. One (a) was a cold, sunny day and the other (b) was a warm,

sunny day, but both days had similar light intensity levels. Entrance activity and eventual flight activity commenced when the in-hive and ambient temperatures were ≥ 20°C. There was little or no entrance activity while in-hive temperature remain below 20ºC, even though the ambient temperature was above 20ºC for most of the day (Figure 3.17a). There was an increase in entrance activity at approximately 10:30 (Figure 3.17b), while the light intensity (Figure 3.17c) was still increasing. As the light intensity peaked at 2248 µmol / m / s at 11:30, entrance activity was still increasing and remained high as the light intensity fell to 1080 µmol / m / s at 17:30. As with the greenhouse hives, colony activities were rated to determine the progress of hive entrance activities as temperatures increased.

Entrance activity of the honey bee colonies in both halves of the greenhouse was much lower than the activity of the field colonies (Figure 3.18). Honey bee foraging activity within the greenhouse and field crops was low. Ninety-five percent of field foragers sourced yellow pollen from outside the crop (which was not carrot, as its pollen is white).

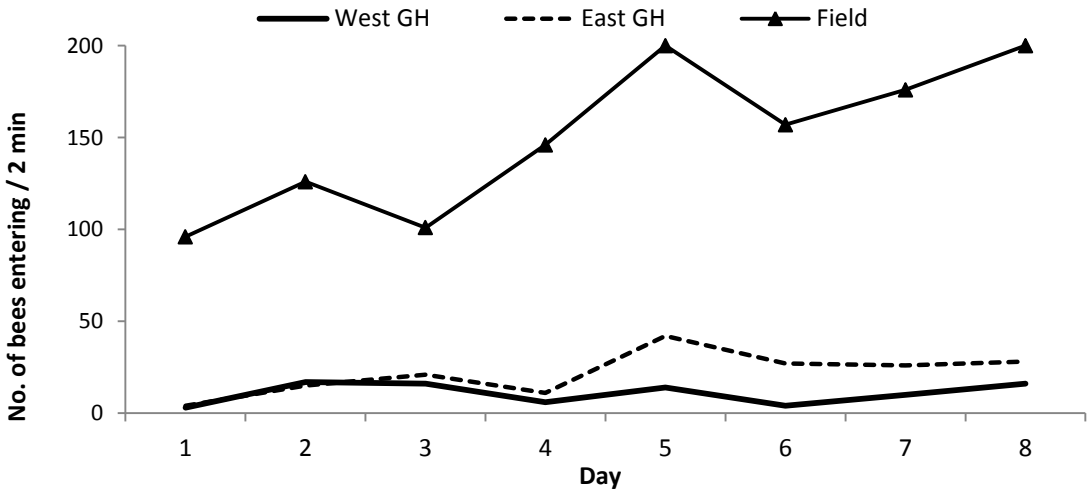


Figure 3.18 Honey bee hive entrance activity. Number of bees entering hive in 2 min.

At no time during the 'period of observations' were *Au. australis* foragers observed on the greenhouse crop. Video footage showed an increase in entrance activity and possible signs of orientation flights on 21 December 2009 (Day 8, the final day of observations). Unfortunately, video footage of this day was not viewed for several weeks after my return from the Musk site. In hind-sight, it may have been advantageous to have stayed for several more days to observe the hives; however, the

lack of stingless bee activity and time of year (viz., Christmas) and new experimental commitments made this decision impractical. A maximum of 45 honey bees were observed in the greenhouse leek crop during the 'period of observations'. Lea Hannah reported that on Day 38 a small number (< 10 / 2 min) of *Au. australis* foragers were observed carrying pollen loads to the hives and some were observed in the crop. Honey bees were working the crop well by this time. After a lengthy discussion with Lea, we decided that a return trip to RZA would not be feasible as the crop was almost depleted and the weather was predicted to turn very cold.

During the 'period of observations', field crops were inspected daily and activity of both pollinator species within the crop was monitored. On two occasions, a small number of *Au. australis* foragers (< 5) was observed in the carrot crop and honey bees were also observed in equally low numbers. Two *Au. australis* and ten honey bees were observed foraging on the first blooms of the field leeks on the last day of the 'period of observations'. Forager numbers for both species subsequently increased as the leek flowering increased, with > 20 *Au. australis* / m^2 observed during January 2010 (L. Hannah, pers. comm., 2010).

3.6 Key findings in greenhouse and field studies

- Signs of orientation flights were observed toward the end of the 'period of observations'.
- Heating mats successfully maintained in-hive temperatures above 17°C.
- In the greenhouse, the colonies within the heated hives commenced entrance activity earlier than the colony in the unheated hive.
- Ambient temperatures in the field were mostly too low to stimulate flight activity in the *Au. australis* colonies.
- Small numbers of *Au. australis* foragers were observed in the field crops, when ambient temperatures were conducive to flight activity.
- Ambient and in-hive temperatures in both the field and greenhouse appeared to greatly affect the entrance and flight activity of the *Au. australis* colonies.
- The greenhouse leek crop did not appear to be attractive to the *Au. australis* colonies.

3.7 Discussion

The *Au. australis* colony foraged on all flowers of all four seed crops at the UWS site. Individual foragers moved extensively throughout the crops and appeared to favour walking rather than hovering. Both nectar and pollen foragers carried pollen grains on their body hairs, thus facilitating pollen transfer between flowers as they walked amongst them. Good pollination was achieved in the celery crop, resulting in significantly higher seed set (76%) compared to the no-pollinator control treatment. Movement between plants was observed in the celery crop and cross-pollination was evident in the carrot crop, with a small percentage of seed set being attained. Forager recruitment within the UWS greenhouse chambers, seen as entrance and forager activity, was very low; especially when compared to favourable external foraging conditions, (> 100 entering / 2 min). Still, this level of recruitment did achieve a satisfactory level of pollination in the celery crop. The apparently efficient behaviour with which the foragers moved over and between the flowers prompted further studies, which are described in Chapter 6, Section 6.3.

As a social bee which is manageable in small, transportable colonies, it was important to investigate the potential of *Au. australis* as a crop pollinator. The experimental opportunities presented during these trials were enlightening, but far from ideal for investigating pollination efficacy. The UWS experimental greenhouse chambers enabled the detailed observations of foraging behaviour which resulted in pollination of the celery crop. The follow-up studies carried out in the RZA commercial greenhouse and field demonstrated that is was possible to introduce *Au. australis* colonies into these environments and that they would forage on the crops, although only to a very limited degree. The cool-climate conditions in the field were far from ideal for this sub / tropical bee species.

While light intensity clearly influenced the commencement and cessation of flight activity of *Au. australis*, it did not have as much influence on the level of flight activity as temperature or the presence of floral resources. At the UWS site, where temperatures remained above 22°C, flight activity commenced at light levels as low as 2.8 µmol / m^2 / s (dawn). Forager recruitment has not been successfully studied in *Au. australis* (A. Tse, pers. comm., 2011); however, Bartareau (1996) reports that

T. carbonaria, another Australian stingless bee, uses primitive recruitment strategies such as scent markers near the floral resource. Nieh et al. (2000) were able to train *T. carbonaria* foragers to feeders, of which they preferred the closest; however, successful resource location depended on wind direction. This could indicate that, for Australian stingless bees, light may play a less important role in resource location than it does for honey bees.

Temperature appears to be the most influential climatic factor driving *Au. australis* flight activity. It is speculated that *Au. australis* may have ambient temperature-thresholds for flight activity similar to other Australian stingless bees (Heard & Hendrikz 1993) of around 20°C. In the RZA greenhouse, when ambient temperatures reached 20°C, colonies within the heated hives commenced entrance activities (i.e., rating 3). It took a further three hours for the in-hive temperature of the unheated hive to reach the temperature threshold for flight activity. The same behaviour was seen in the field, where fight activity was delayed due to low in-hive temperatures. Some stingless bee species do not thermoregulate, and if this is the case in *Au. australis* it may explain the delay in flight activity. The role of temperature is explored more fully in Chapter 6, Sections 6.1 and 6.2.

The colonies foraged on both of the field crops at the Musk site, although they appeared to favour the leek crop over the carrot. Activity in the field crops was, however, limited by the temperatures experienced in the area. The commercial greenhouse crop was not visited by *Au. australis* foragers during the 'period of observations', although environmental conditions were conducive to hive entrance activity. The timing of their introduction into the greenhouse could have been earlier. The incorporation of an orientation period may improve foraging activity once the crop is in full bloom, thus improving pollination opportunities. It may be feasible to manage greenhouse enclosures with one to two beds of flowering plants on the outer edges of the crop. Beds could be prepared so that foraging resources are available to bees for up to three weeks prior to crop bloom. Once the crop begins to bloom and flower numbers increase the supplementary crop could be ploughed in, cut off or pulled out, depending on the species. Further studies in this area would be helpful in greenhouse crop management with both stingless bee species and honey bees.

Conditions were not ideal to fully assess the potential of *Au. australis* colonies to pollinate crops at the UWS or Musk sites. Further assessment of *Au. australis* as a potential crop pollinator would be better conducted in areas where ambient temperatures regularly exceed 20°C. Horticultural and agricultural activities are carried out in areas within the native range of *Au. australis* and would be better suited for further pollination trials.

3.8 Key findings

- *Au. australis* colonies will acclimate to a greenhouse enclosure and appear to benefit from an orientation period. (Also see Chapter 6, Section 6.3).
- *Au. australis* colonies will forage for nectar and pollen in vegetable seed crops which are managed in the field or in a greenhouse enclosure.
- They will move between plant lines within an enclosure, but it is not clear as to whether foragers will perform this task in the field.
- *Au. australis* colonies appear to be conservative in their foraging activity and in their forager recruitment.
- The flight activity of *Au. australis* colonies appears to be strongly affected by ambient and in-hive temperature.
- Colonies of *Au. australis* do not adapt well to cool climate areas.
- Ambient temperature, as well as in-hive temperature, has the greatest influence on the flight activity in *Au. australis.*
- Large numbers of drone bees were observed leaving at least one of the colonies over a short period of time. This prompts the question as to whether *Au. australis* produce drones in ‘batches’. Further observations pertaining to *Au. australis* drones is contained in Appendix 3.

CHAPTER 4

Phylogenetic placement of *Au. australis* within the genus *Austroplebeia*

4.1 Introduction

Worldwide, there is a lack of taxonomic information about insect pollinators (Dias et al. 1999; Imperatriz-Fonseca 2010). Many Australian bee species are undescribed, with over half of the named taxa requiring revision (Batley & Hogendoorn 2009). The genus *Austroplebeia* Moure is amongst this group (Dollin 2010b).

Species within *Austroplebeia* were described as *Trigona* until Moure (1961) proposed a name change for some of the Trigonini from Australia and Papua New Guinea (PNG), which closely resembled the Neotropical *Plebeia*. He proposed they be placed as a subgenus of *Plebeia.* A similar subgenus classification (e.g. *Trigona* (*Plebeia*) *australis*) was utilised by Michener in his classification of bees of the Australian and South Pacific regions (1965). Classification of *Trigona* (*Plebeia*) to *Austroplebeia* was undertaken by Michener (1990) in his revision of the family Apidae. He found that this genus was more robust than other Trigonini and had a "distinct yellow area on the scutellum and axillae, usually along the lateral margins of the scutum and on the face." (Michener 1990).

At present, the genus *Austroplebeia* has nine named species (Cardale 1993). Table 4.1 presents these species together with the holotype collection locations. These species were described from specimens collected and classified between 1898 and 1932. The taxonomic classifications were based on a small number of pinned specimens, many of which were collected from flowers (Cockerell 1905; 1910).

Table 4.1 Abbreviated comparative descriptions of currently named *Austroplebeia* species. Sources listed in table. Only the commonly used characteristics or comparative terms are listed here. Locations of holotype collection are shown in Figure 4.1.

Current species name & source	Holotype location	Differentiating characters
Au. symei Rayment 1932	North Qld, Australia	4.5 mm long; black; labrum amber; clypeus two minute cream dots anterior margin; subclypeal crescentic cream mark; scutellum two cream dots, long hair.
Au. australis Friese 1898	Central Australia & Bundaberg, Qld	4 mm long; brown/black; frons yellow fleck; clypeus red/brown; scutellum yellow edges; metanotum thickly punctured.
Au. cassiae Cockerell 1910	Mackay, Qld	Very like *T. carbonaria*, perhaps the sp. recorded from Mackay by Friese as *carbonaria*; black; scutellum prominent with broad transverse dusky yellow bands; mesothorax smooth; face narrower below than *carbonaria.*
Au. cockerelli Rayment 1930	Borroloola, NT, Australia	4.5 mm long; black; face dull yellow spot orbital margin; labrum dull white; frons transverse dull white median band; supraclypeal yellow spots laterally, dull white crescentic mark; scutellum large cream dot laterally; mesothorax narrow cream stripe laterally.
Au. essingtoni Cockerell 1905	Port Essington, NT	4 mm long; black; labrum yellow marks; clypeus yellow marks; scutellum yellow; mesothorax narrow stripe each side, extend to axillae.
Au. ornata (Rayment 1935)	Cape York, Qld	4.5 mm long; colour black; labrum dull yellow; clypeus yellow spots; mesothorax narrow dull cream margins laterally; apical half of scutellum with interrupted yellow band.
Au. percincta (Cockerell 1929)	Finke River, NT	A very small species with light face markings; scutellum pale; mesothorax pale band along each side; species as described from New Guinea, but an Australian race, appreciably larger (up to 5mm instead of 3.5).
Au. websteri Rayment 1932	Wyndham, Nth WA, Australia	5 mm long; black; face dull white markings; labrum dull white patches; clypeus light amber median spot, triangular dull white mark laterally; scutellum creamy yellow except bracket shaped median area; narrow, creamy line bordering lateral margins of prothorax.
Au. cincta Mocsary 1898 (in Friese1898)	(Papua) New Guinea	3.5 mm long; black; border of the eyes, scutum, antennal scape, 'shoulders', side of thorax, scutellum, glossy elevated and its lobes lateral yellow (translated from Latin). Similar to *Au. australis*, but more elegant. Easy to recognise.

The published descriptions of species within *Austroplebeia* are inadequate. Described characters for the holotypes are inconsistent and insufficient for use in accurate identification of specimens. For example, Friese (1898) described *Au. cincta* by a black shiny bee with yellow markings on the border of the eyes, scutum, antennal scape, shoulders, sides of the thorax and scutellum. He also commented that it was similar to *Au. australis*, but more elegant, and that the species was easily recognised. *Au. cincta percincta* was described by Cockerell (1929) as having a pale face and scutellum, with a pale band along each side of the mesothorax; and he stated that it was the same as *Au. cincta* except larger, but he did not mention yellow marking bordering the eyes. *Au. cassiae* was said to be similar to *T. carbonaria* (Cockerell 1910) (Table 4.1).

These shortcomings were recognised by Moure when he attempted to classify some old world Meliponine bees (1961), using holotypes. Moure (1961) elaborated on the species descriptions of *Trigona cassiae* (Moure 1961, pp. 195 – 196) but found conflicting information given in Cockerell's descriptions, compared to information he obtained by examining of the holotypes himself: "Cockerell ...omits reference to yellow markings on the terga, although these marks are very conspicuous on the type specimen." Moure also stated that "...apparently *T. symei* Rayment, 1932, *T. cockerelli* Rayment, 1930 and its variety *ornata* Rayment, 1935 and *T. websteri* Rayment, 1932, are of these groups, and probably synonyms of older species." (Moure 1961, pg 197). Cockerell (1929) described *Trigona cincta percincta* as "... an Australian race (of *T. cincta*), appreciably larger (up to 5mm instead of 3.5 mm) occurs at Hermannsburg, Finke River". More recently, Rasmussen (2008) made the comment that "no workable key exists for *Austroplebeia*."

According to the name structure, both *Trigona cockerelli ornata* (Rayment 1935) and *T. cincta percincta* (Cockerell 1929) were subspecies and Moure (1961) classified them as such in his classification of old world bees. Later, Michener (1965) elevated *T. cincta percincta* and *T. cockerelli ornata* from subspecies to species level; listing them as *T. percincta* and *T. ornata.* These classifications endured and they were listed in Cardale's Zoological Catalogue (1993) as *Austroplebeia percincta*

(Cockerell 1929) and *Austroplebeia ornata* (Rayment 1935). Cardale (1993) is the authoritative text which is followed today.

The species within *Austroplebeia* require redescription; however, this was a task outside the scope of this project. The broad aims of this study were to identify specimens of *Austroplebeia* workers into groups, with the ultimate objective of delimiting these groups into species. This would provide a framework to redescribe the species within the genus *Austroplebeia*. A number of reliable diagnostic techniques were used, including morphological characteristics such as size and colour, wing vein morphometrics, drone morphology and molecular analysis.

The studies reported in this chapter took the form of several discrete experiments. As such, the structure of this chapter is set out for ease of understanding, describing each experiment in isolation, with materials and methods immediately followed by the results. The key findings and overall discussion are placed at the end of the chapter.

4.1.1 Collection of specimens

In order to achieve an accurate description of species, it is important to adequately sample the population, incorporating a large geographic range. The use of small sample groups, from a limited geographic range, will result in incomplete taxonomic representation of the genus (Vogler & Monaghan 2007). Once a biogeographically diverse sampling strategy has been completed, the methods of analysis can be chosen and employed.

4.1.1.1 Materials and methods

Between 1980 and 1998, Dr Anne Dollin and her husband Les Dollin conducted nine field trips to investigate the Australian species of *Trigona* and *Austroplebeia*. Over this time, they visited the areas where holotype specimens had been collected (Figure 4.1), sampling over 300 stingless bee nests (Dollin 1997b; c; Dollin 1997d; e; 1998a; b; 1999a; b; c; d; 2000c; a; b; 2001). Of the 300 sampled nests, 106 were *Austroplebeia* nests located in WA, NT, Qld and NSW (Figure 4.2). Mostly, workers were collected from nest entrances but occasionally nests were opened, with nest structures and characteristics being recorded. Where possible, drones as well as

workers were collected from opened nests. Bees were killed in ethyl acetate killing jars and either pinned on site or dry-stored in specimen jars with tissue paper (A. Dollin, pers. comm., 2009). One group of nests (allocated nest codes beginning with 'S', see Appendix 4) were collected from an area near Kilcoy, in Qld (26°56' S, 152°33' E). The worker populations from these nests contained bees that had undefined low-grade thoracic markings.

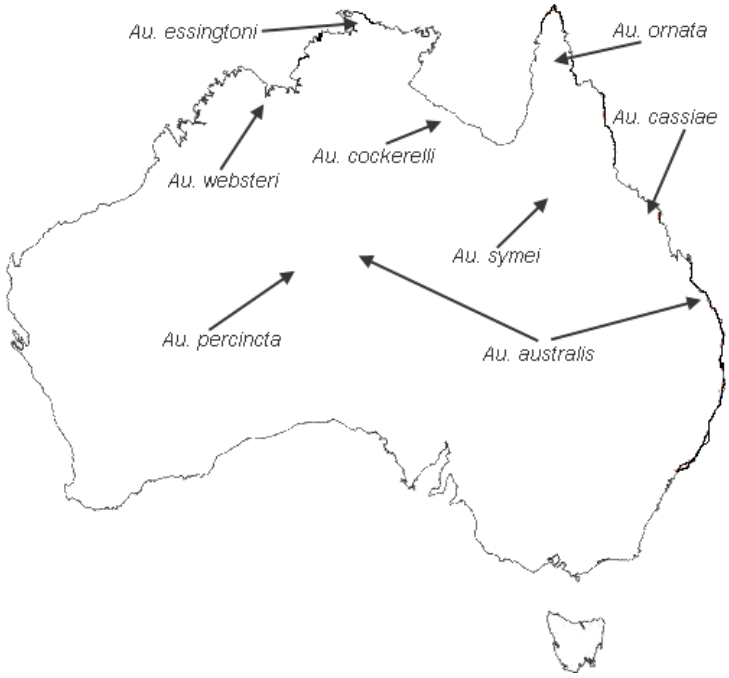


Figure 4.1 Locations of holotype specimen collection, between 1898 and 1935.

Five pinned specimens of PNG *Au. cincta* workers were given to Dr Dollin by Professor Shôichi F. Sakagami (Hokkaido University, Sapporo, Japan) and these were included in her *Austroplebeia* collection. At the time of the field sampling, it was thought that *Au. cincta* only occurred in PNG; however, in late 2009, the existence of an unusual bee, found in the Daintree area of Qld, was brought to Dr Dollin's attention. She noted that this bee had similar markings to *Au. cincta*, and subsequently arranged with the property owner, the naturalist Lewis Roberts, to collect a series of bee specimens and data from six nests. The distinctive markings and hair patterns on the faces and thoraces of the specimens matched those of the PNG *Au. cincta*. The specimens were, therefore, tentatively identified as *Au. cincta* (A. Dollin, pers. comm., 2010); however, they will be referred to as the 'cincta' group in this chapter.

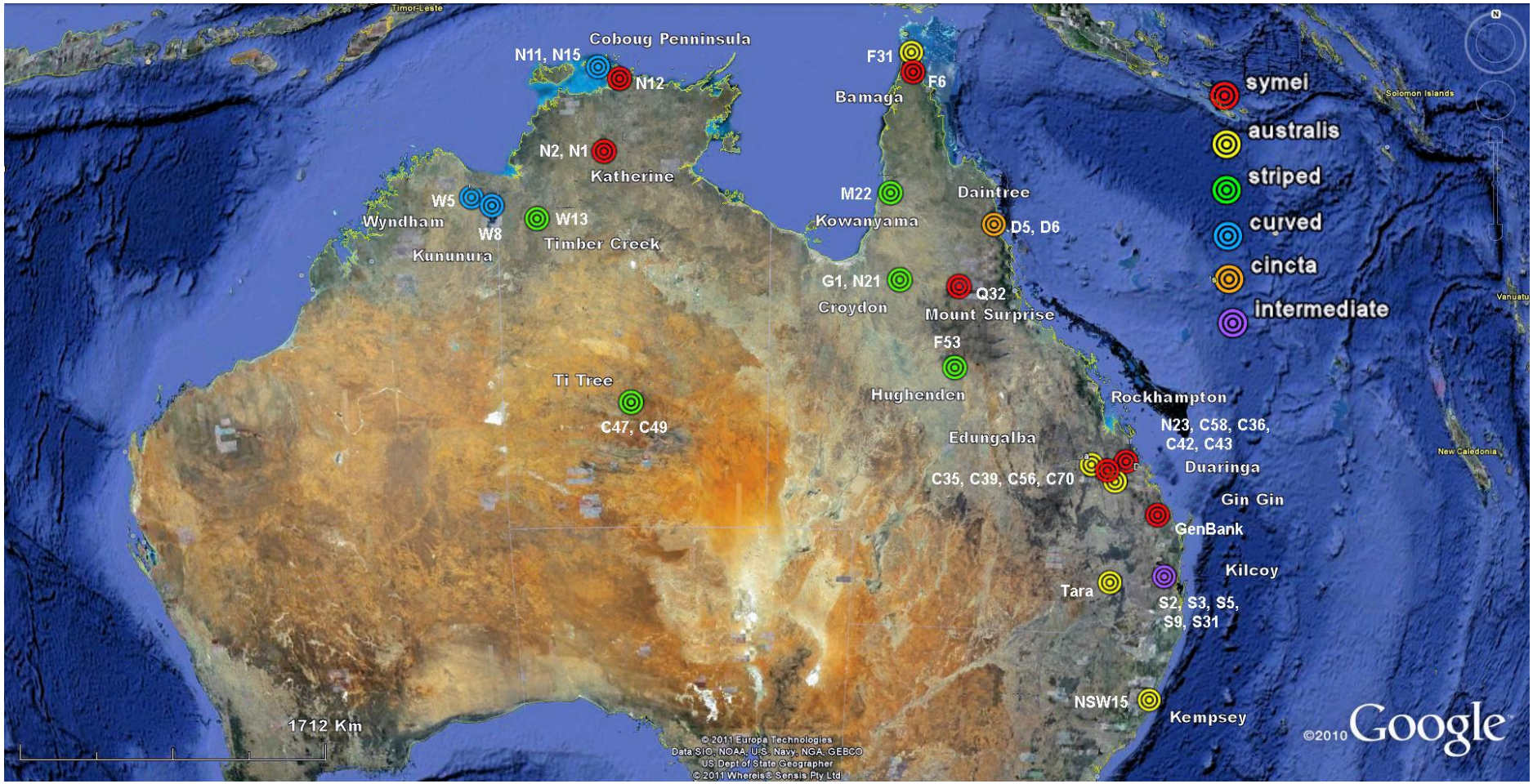


Figure 4.2 Distribution of collected *Austroplebeia* specimens. Colours indicate the groups which were later allocated to the collection. Nest codes included.

4.1.2 Head width and colouration analysis

Traditionally, numerous combinations of measurements of various body parts were, and still are, utilised by insect taxonomists (Daly et al. 1982; Weeks et al. 1999). Among the many characters used by taxonomists are the size and colour markings of individual specimens (Camargo et al. 2000). Problems that can be encountered when trying to identify insect species are numerous. Inappropriate storage methods can cause deformation of specimens. For example, storage in ethanol causes the eyes to collapse, rendering head-width measurements inaccurate or unusable as a diagnostic measurement. Ethanol can also cause discolouration of body pigments, whilst dry storage, on the other hand, can cause shrinkage of the abdomen, affecting body length measurements. Long-term dry storage can also lead to degradation of the DNA, which is particularly pertinent with the increasing use of molecular techniques for identification and phylogenetic studies (Hebert et al. 2003; Hebert & Gregory 2005). Adding to the already difficult task of species identification is the lack of trained taxonomists available to carry out this work.

Preliminary studies on the identification and revision of species within the genus *Austroplebeia* were conducted by Prof. Sakagami, in collaboration with Dr Dollin. He found that "Probably there are little structural differences among 'species' except for between *Au. cincta* of (Papua) New Guinea and all Australian forms but coloration could be useful to a degree." (unpublished communication, to A. Dollin, 20 November 1993). It seemed that head width (HW) and body markings may be the most useful taxonomic characters in delimiting the species within *Austroplebeia.* Initial studies were conducted in the 1990s by Dr Dollin, and HW and colour markings were analysed. The data obtained during these studies showed some degree of difference in these characters but the outcomes were inadequate to delimit the species. The methods used by Dr Dollin in these studies are documented below. In 2009 a collaborative relationship developed between myself and Dr Dollin. The aim was to delimit the species of *Austroplebeia* by means of traditional and modern taxonomic techniques.

4.1.2.1 Materials and methods

A sample (n = 10 – 20) of workers was available from 70 of the 106 sampled nests. These specimens were used in the analysis of body colouration and HW measurement (Appendix 5). In the late 1990s, a dissecting microscope (40 X magnifications) fitted with an eyepiece graticule was used to measure the HW of at least ten workers from each of the 70 nests. Measurements were taken of the maximum width of the head and were recorded to the nearest 0.01 mm (Figure 4.3). An average HW score was calculated for each nest (A. Dollin, pers. comm., 2009).

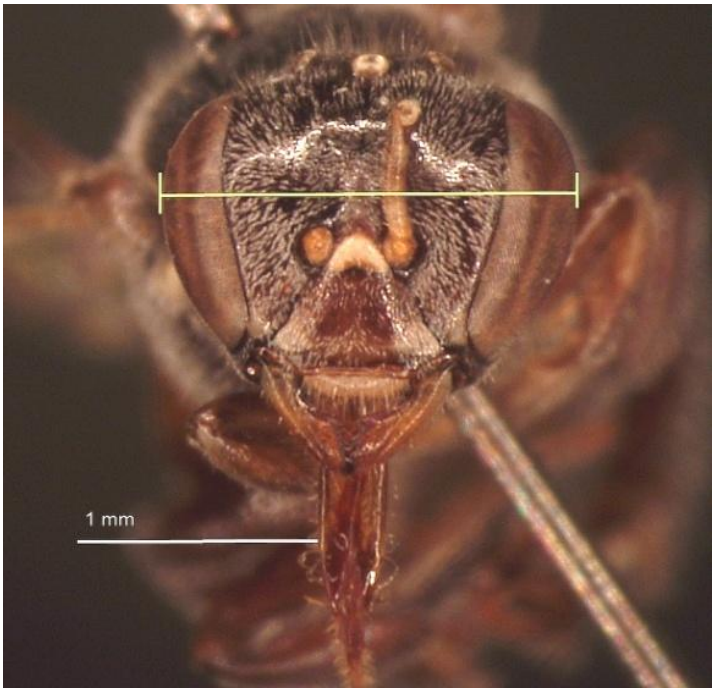


Figure 4.3 Maximum head width measurements, used in scoring calculations.

As stated by Prof. Sakagami, the most obvious differences between the specimens were the colour markings on the workers' thoraces and faces; however, the thoracic markings were more easily defined and categorised than were the facial markings. In the 1990s, the initial studies included the analysis of thoracic and facial colour markings in 10 – 20 workers from each nest (n = 70). Attempts were made to quantitatively analyse the level of thoracic colour markings within the sampled groups, with these groups being separated into 11 colour grades. These grades were not, however, precise enough to delimit the nests or groups (A. Dollin, pers. comm., 2009).

Between 30 September 2009 and 8 February 2010, as part of the current studies, digital imaging of thoracic markings was carried out on pinned specimens, using a Leica MZIZ stereomicroscope at 20 X magnification. Photographic records were obtained with a JVC digital camera. Using Image-Pro Plus 5.1 software (MediaCybernetics, Bethesda, Maryland), the total area of the colour markings and the total area of the thorax of each of the previously measured bee were outlined and measured. The proportion of the thorax with colour markings for each 'typical' colour grade was calculated as:

Proportion of marking (%) = (Total area of colour / Total area of thorax) x 100

These final scores were then used to calculate an average colour grade for each nest (n = 70). As a result, each bee that had been classed as a grade 1 was allocated a colour score of 1.2%, a grade 2 bee was allocated a colour score of 3.6%, grade 3 was 6.5% and so forth. The colour score of 10 – 20 bees in each nest was analysed and an average score was allocated to the nest.

For each nest (n = 70) the mean HW was plotted against the mean colour score. The analyses did not include the 'cincta' group. Five groups were identified within the scatter plot and the mean group characteristics were analysed by one-way ANOVA and means were compared by Tukey's HSD test in SPSS 17. The setting of significance was $\alpha = 0.05$. Data for both HW and colour score were tested for homogeneity of variance using Levene's test.

4.1.2.2 Results

Five groups were separated in the scatter plot (Figure 4.4). These were 'symei' (n = 27), 'australis' (n = 14), 'striped' (n = 13), 'curved' (n = 9) and 'intermediate' (n = 7). All groups were separated out according to HW and colour; however, 'intermediate' was also separated out based on geographical distribution (this is discussed further in Section 4.2).

The proportion of thoracic marking present in individual workers ranges between 1.2 and 20.2% of the total thorax (Table 4.2). Thirteen colour grades were separated by the proportion of thoracic colour marking within the 70 *Austroplebeia* nests (Table 4.3). The extent of colour marking present on the thorax (%) for all groups was

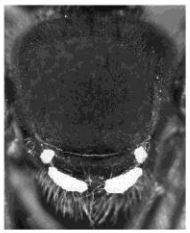
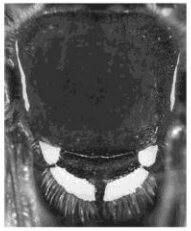
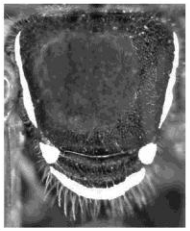
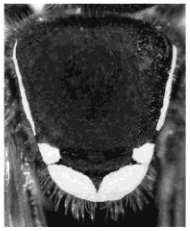
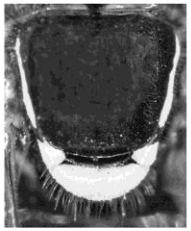
significantly different from each other ($F_{4, 69} = 266.683$, $p < 0.001$). For HW, there was a significant difference among groups ($F_{4, 69} = 72.902$, $p < 0.001$); however, there was no significant difference between 'australis' and 'symei' ($p = 0.086$). There was also no significant difference between the 'intermediate' group and 'australis' ($p = 0.783$) or 'symei' ($p = 0.966$).

Nest populations contained a range of colour grades and most contained three different grades, with some having up to five. When the mean HW and mean colour scores (Table 4.2) for each nest were plotted against each other, four distinct clusters became apparent (Figure 4.4). The clusters were allocated a group name either according to their morphological characteristics or their closest species name. The average colour grades for the four groups are represented in Figure 4.5. The scatter plot in Figure 4.4 shows a sparsely populated area labeled 'intermediate' (grey shading), and this group contained the nests located near Kilcoy, Qld.

Table 4.2 Allocated colour grades and their colour percentage based on grades illustrated in Table 4.3.

Colour grade	Percentage of thorax with colour markings (%)
1	1.2
2	3.6
3	6.5
4a	8.8
4b	10.6
4c	6.7
5a	12.9
5b	12.6
6a	10.4
6b	15.8
6c	16.7
7	15.1
8	20.2

Table 4.3 Thorax colour grading system for identification of *Austroplebeia* groups within the sampled nest, using the proportion of markings on the thorax.

	Grade 1	Grade 2	Grade 3	Grades 4 a, b and c	Grades 5 a and b	Grades 6 a, b and c	Grade 7	Grade 8
No Lateral Stripes								
Incomplete Lateral Stripes								
Well-Defined Lateral Stripes								

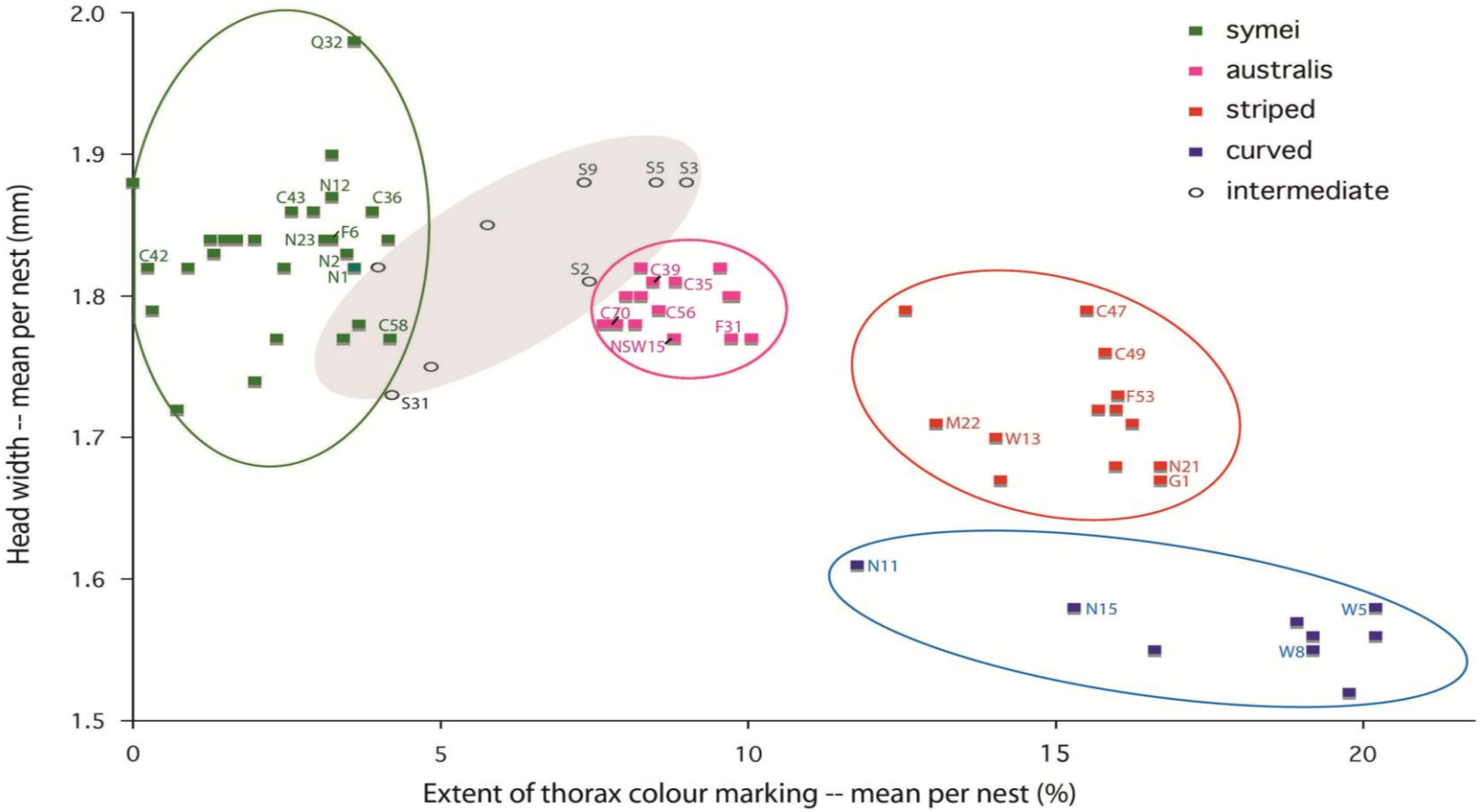


Figure 4.4 Scatter graph showing the clustering of the *Austroplebeia* groups in relation to head width vs. thorax colour. (Labelled data points represent nests from which samples were successfully sequenced for mitochondrial rDNA segment 16S, Section 4.1.7.).

Figure 4.5 Thoracic markings of 'symei' (left), 'australis' (centre) and 'striped' / 'curved' (right). The proportion of colour on the thorax of 'symei' and 'striped' is obviously different and they can be easily distinguished from each other.

A large sympatric population of ‘symei’ and ‘australis’ nests was found at Duaringa, Qld. Figure 4.6 shows the colouration and HW data of 14 nests from this location. Despite their close proximity, a clear difference in thorax colouration was observed between the ‘symei’ and ‘australis’ nests.

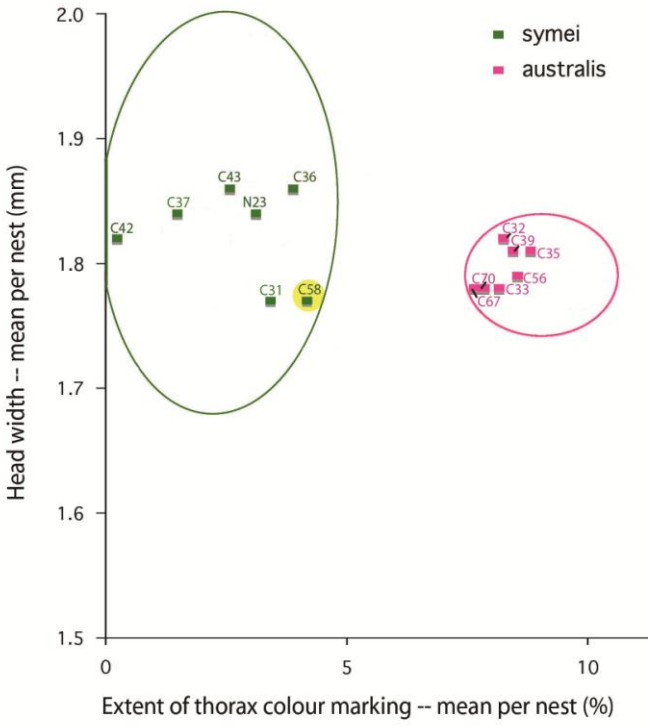


Figure 4.6 Scatter graph showing the grouping of 14 *Austroplebeia* nests from Duaringa, Qld, in relation to head width vs. thorax colour.

As a result of this study, six groups can now be allocated names. These names are used when referring to the nests represented by these groups in the remainder of this chapter. As previously stated, the groups are named either according to their morphological characteristics (e.g. for ‘curved’, see section 4.1.4.2) or their closest species name. The groups are ‘symei’, ‘intermediate’, ‘australis’, ‘striped’, ‘curved’ and ‘cincta’. Further studies were carried out with the aim of delimiting the species within *Austroplebeia* but, for ease of understanding and continuity, this list of names is used throughout. Specimens from representative nests of these groups were used in the studies that follow.

4.1.3 *Au. cincta* morphology

4.1.3.1 Materials and methods

The morphological differences between *Au. cincta* and the other *Austroplebeia* species had been previously documented (Friese 1898). Pinned specimens of PNG *Au. cincta*, obtained from and identified by Prof. Sakagami, were imaged at 20 X magnification, using a Leica MZIZ stereomicroscope. Photographic records were obtained with a JVC digital camera. Images were also obtained for the new 'cincta' specimens, from the Daintree, Qld. These two groups were compared for differences in morphological characteristics. They were also compared with the other *Austroplebeia* groups.

4.1.3.2 Results

The morphological characteristics of workers from the 'cincta' group from Qld matched those of specimens of *Au. cincta* from PNG. They were clearly distinct from those of other Australian *Austroplebeia* groups, in both colour and pilosity. On the thorax, 'cincta' group workers had a yellow / cream marking on the lateral surface of the metepisternum (Figure 4.7), whereas in all other groups this area had no marking. On their face, 'cincta' workers had distinct dull to bright yellow markings, whereas other groups had only diffuse dull, cream markings (Figure 4.8).

Pilosity was quite different between the 'cincta' group, where workers' thoracic mesepisternum and hypoepimeral areas appeared shiny with short bristles, and all other groups had thick plumose hair in these areas. 'Cincta' workers' faces appeared relatively shiny, as most of the hair covering was fine and sparse. In contrast, in all other groups the face was covered with thick white hair including well developed plumose hair on the paraocular and suprantennal areas (Figure 4.8).

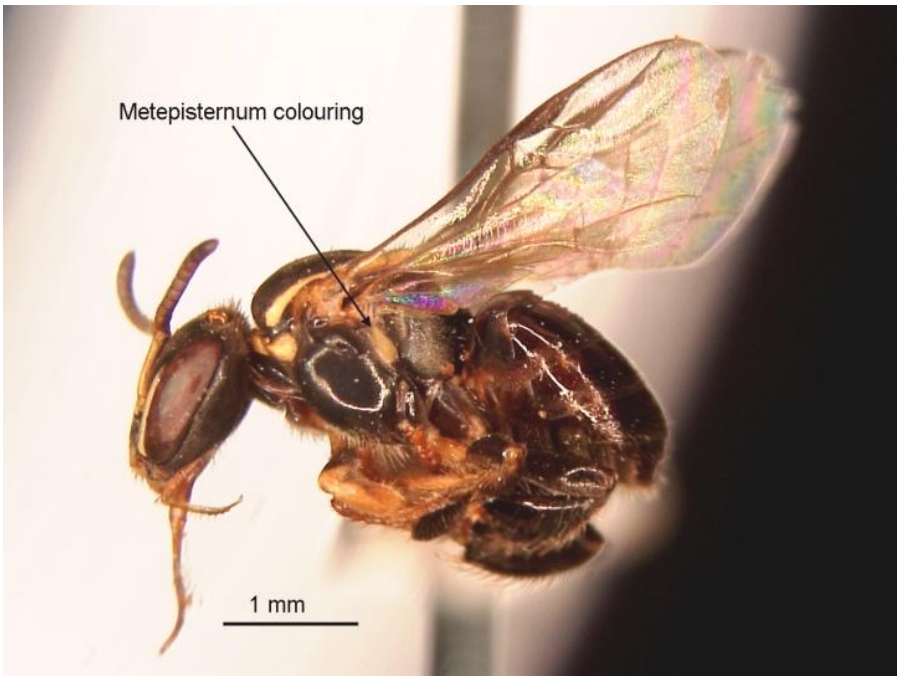


Figure 4.7 'Cincta' marking on the metepisternum, unlike the other *Austroplebeia* groups.

Figure 4.8 'Symei' (left), 'striped' (centre) and 'cincta' (right) facial markings. 'Cincta' has distinct facial markings unlike the other *Austroplebeia* groups.

4.1.4 Drone morphology

Where male specimens are available, dissection and identification of taxonomic characters within the segments of the metasomal apex, as well as the genital structures, are reliable delimiting methods (Camargo et al. 1967; Dollin et al. 1997; Michener 2000). In order for this technique to be utilised, male specimens need to be collected from nests in association with the workers earmarked for identification (Camargo et al. 2000). Opening nests for scientific data collection usually results in the destruction of the colony and this practice should be carried out conservatively. Metasomal sterna (S) and terga (T) dissected from drones are commonly used in the

identification of stingless bee species (Camargo et al. 1967). As drone genitalia are usually partially or completely retracted in dead specimens, it is recommended the drone genitalia, as well as S6 and S7, be dissected for analysis of morphological differences (Michener 2000). Dollin et al. (1997) demonstrated taxonomic differences in Australian *Trigona* spp. through the dissection and comparison of terminal segments of male specimens. Differences can be seen in segments S4 – S7 as well as T7 and the genitalia. Dissection of individuals results in the destruction of specimens, rendering them unavailable for further investigation. The use of potassium hydroxide as a clearing agent during dissection renders the specimens unavailable for DNA analysis.

4.1.4.1 Materials and methods

No *Au. cincta* or 'cincta' specimens were available for the drone morphology studies but drones were available for all other groups.

All metasomal segments were dissected and imaged using scanning electron microscopy (SEM), to ensure no differences, including hair patterns, were overlooked. The techniques described below were used to prepare specimens for SEM. Fresh drones, earmarked for 'intact' imaging, were collected from colonies managed at UWS Hawkesbury campus, and killed by freezing overnight. They were then placed in Davidson fixative solution (2.5% formaldehyde + 0.1 M phosphate buffer + sodium chloride) for 24 h. They were dehydrated in the following way: 50% ethanol for 48 h, then 70% ethanol for 1 h, then rinsed three times in 99.5% ethanol. Finally, they were transferred to 100% ethanol and rinsed three times. Specimens were transferred to a 'jig' basket, containing 100% ethanol, and placed in a critical point drier (Polaron E3000, Quorum Technologies Ltd., UK). Dried specimens were mounted on a self-adhesive carbon coated, 25 mm diam. x 5 mm high, with internal thread, lathe finished, anodised aluminium mount (ProSciTech, Australia, Thuringowa, Qld). Once mounted, specimens were sputter coated with gold under a 7 kpa vacuum and argon gas for 40 seconds (Polaron E5100, Quorum Technologies Ltd., UK) (Figure 4.9). Specimens were stored in a desiccator. Images were captured using a JSM-6510LV scanning electron microscope (JEOL, Japan). A high resolution of 3.0 nm was achieved at high vacuum mode 30 kV, GUI interface.

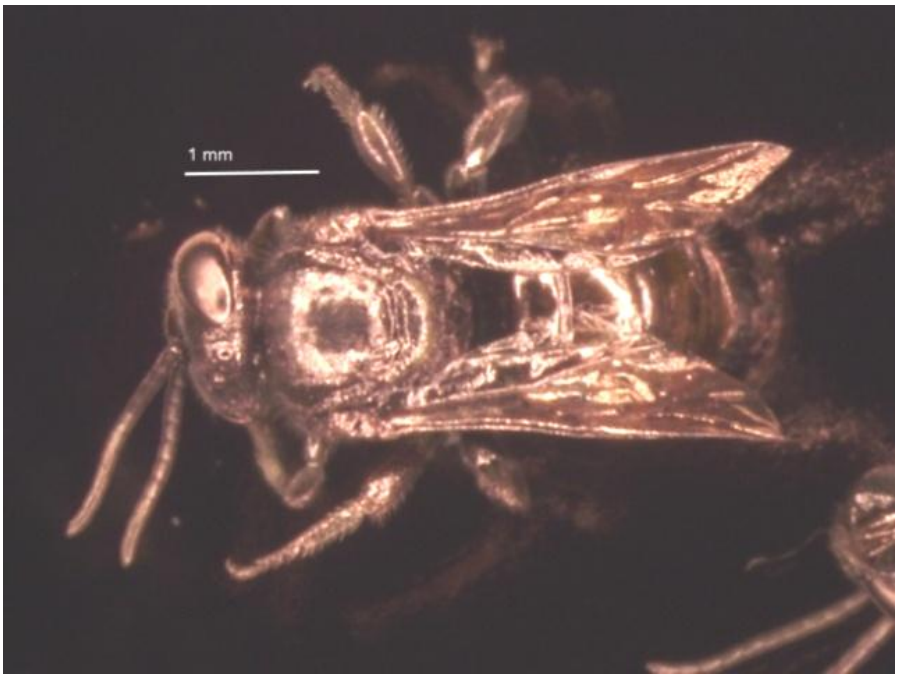


Figure 4.9 Ventral view of dehydrated whole bee. Specimens were sputter coated with gold.

Two to four drones from each group (n = 19) had their abdomens removed and cleared in 1% potassium hydroxide for 48 h. Metasomal segments and genitalia were separated under a Wild M5 dissecting microscope (http://www.wild-heerbrugg.com), at 32 X magnification, using a straight needle probe, straight jeweller's forceps (0.1 mm) and sable hair brush (Australian Entomological Supplies Pty. Ltd., Bangalow, Australia) (Figure 4.10). Specimens were dried, mounted and gold coated. After carrying out some preliminary imaging studies, it was determined that critical point drying was not necessary for specimens which consisted of only chitin. Images were not perfect, but they were of high enough quality for analysis of morphological differences. This way, the metasomal segments and genitalia could be mounted directly into the SEM stub, ensuring the structures were mounted in order of their anatomical position (Figure 4.11).

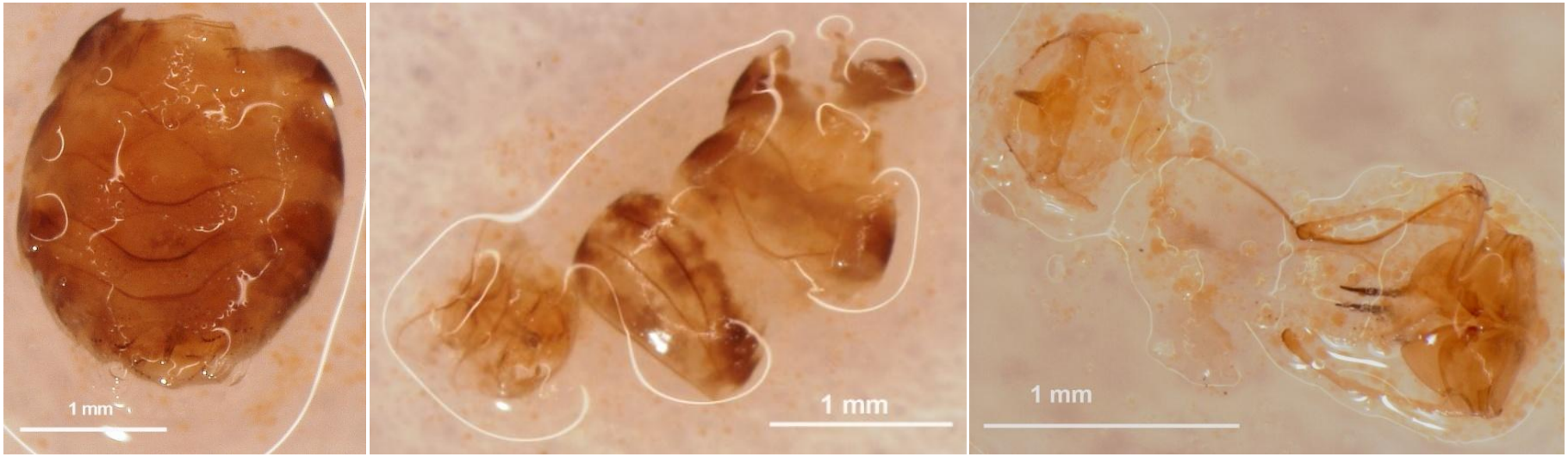


Figure 4.10 KOH-treated drone abdomen (left) showing ventral (tegumen) surface; dissected metasomal segments (centre) and apical segments and genitalia (right) of an *Austroplebeia* drone.

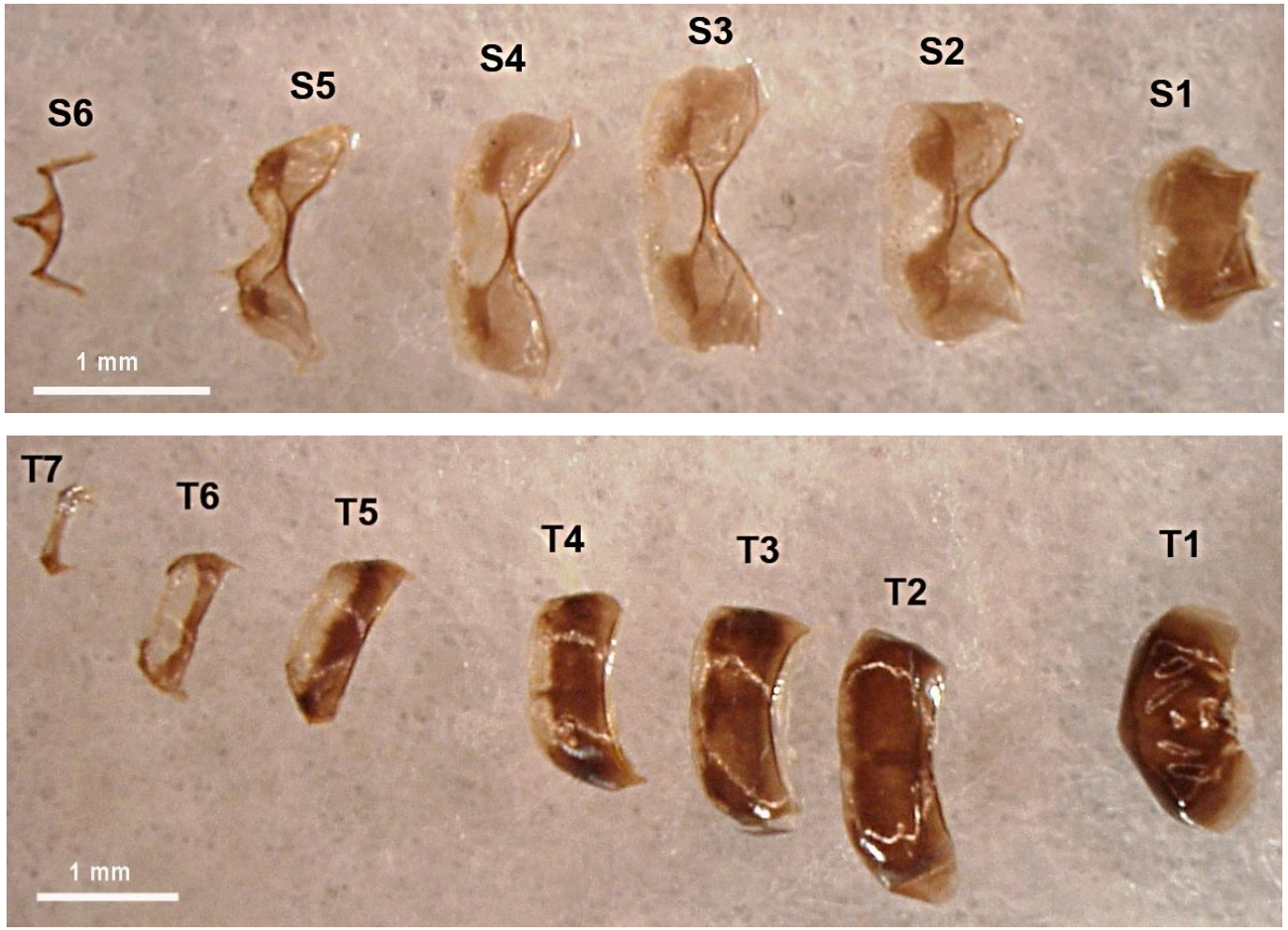


Figure 4.11 Sternal segments (top) and tergal segments (bottom) mounted in anatomical order.

Genital structures as well as S6 (Figure 4.12) were measured using JEOL software. Measurements included gonocoxite width, gonostylus length and penis valve length. Dissection of S7 from T7 was only possible in the fresh specimens. Imaging under SEM of intact segments was inconclusive, as S7 structures were uninformative due to mounting problems (Figure 4.13). Further studies on these structures were, therefore, carried out using an Olympus BX60 compound light microscope (Olympus Australia, Waverly, Victoria) and a Jenoptik ProgRes® C14 digital camera (SciTech Pty Ltd., Preston, Victoria) (see below). Differences detected in the genitalia prompted stereomicroscopic study of both dissected and *in situ* structures (see below).

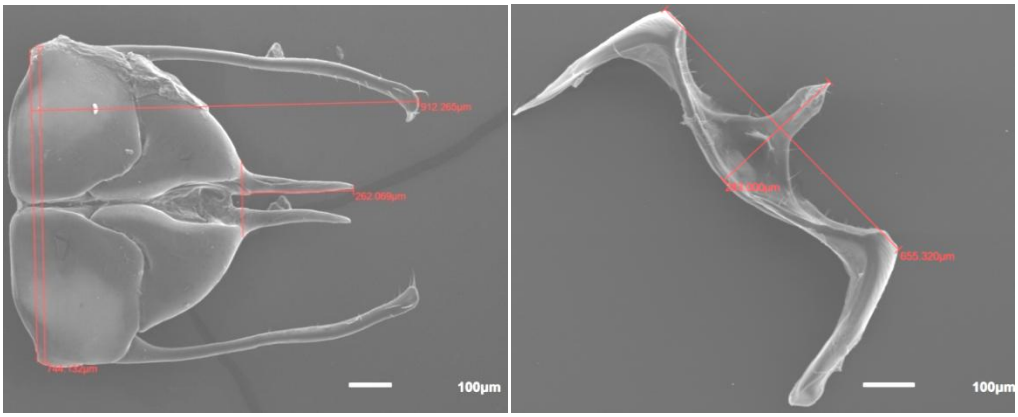


Figure 4.12 Drone genitalia (left) and sternal segment, S6 (right) showing some structural measurements.

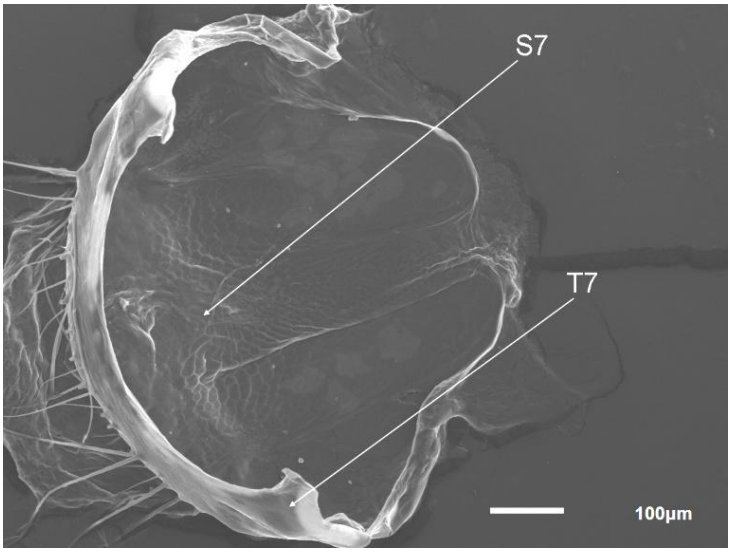


Figure 4.13 SEM images of intact metasomal segments S7 and T7 were uninformative due to mounting problems. Note how S7 structures are undefined and distorted.

Measurements of the S6 segments, obtained using SEM, were found to be unreliable due to the lack of depth-of-field associated with this technique. As a result, two specimens from each group were dissected and the S6 and S7 were analysed using compound light microscopy. The metasomal segments T7 and S7 (Figure 4.14) were difficult to separate without damaging S7. Manual dissection of S7 from T7 in fresh specimens was successful (Figure 4.14); however, it was unsuccessful in many of the old, dried specimens. A less destructive technique, using a laser dissecting microscope (Zeiss PALM microbeam, Carl Zeiss MicroImaging, GMBH, Germany. http://www.zeiss.com), successfully separated the S7 segment from T7.

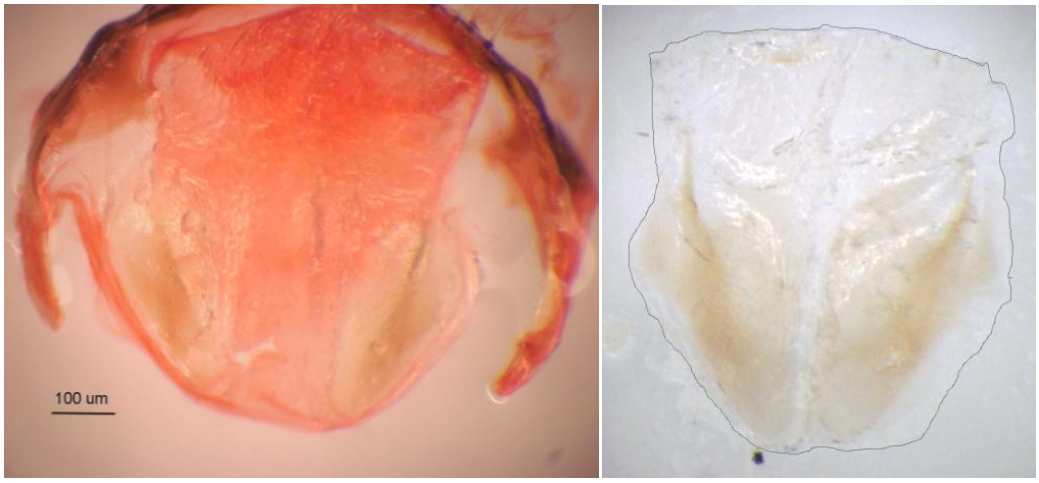


Figure 4.14 Intact T7, S7 segments of a fresh 'australis', stained with Congo Red to show edges of S7, (left) and laser-dissected S7 segment, outlined to show tissue edges (right).

Dissected S7 segments were mounted on slides with glycerol and imaged using an compound light microscope and digital camera. Where possible, measurements of potentially defining features were recorded using Image-Pro 6.2 software. The fragility of some of the old specimens made mounting the segments too difficult and as a result, they were unable to be measured. Others were impaired by extensive pollen contamination.

Metasomal segments S6 were also mounted on slides with glycerol and a cover slip. The cover slip flattened the segment into a two-dimensional structure, thus removing the problem of depth-of-field. Measurements of the compressed segment were taken using Image-Pro 6.2 software. Again, some of the old specimens did not dissect well and segments were damaged during mounting.

Fresh dissection of drones was also carried out and genital structures were mounted on glycerol slides. Because the specimens had not been dried to critical point, it was possible to resurrect some of the rarer specimens from the SEM mount, using KOH. Pinned drone specimens which showed some protruding genital structures were selected from nest groups, and intact genitalia were imaged using stereomicroscopy. Images of both dissected and *in situ* genitalia were compared for structural differences.

4.1.4.2 Results

The SEM image of a drone's metasomal apex (Figure 4.15) illustrates the difficulty associated with identifying intact structures. Also, genitalia become distorted when everted, possibly giving misleading information (Figure 4.16). The images of everted drone genitalia illustrate the features which may contribute to the fact that stingless bees mate only once (Kerr et al. 1962; Michener 1974; Green & Oldroyd 2002). The penis valve of the drone is sharply curved in the everted state, probably contributing to the degree of difficulty the mated queen has in removing the male genitalia (Naves Da Silva et al. 1972). Images of dissected, non-everted genitalia provided the most informative comparisons.

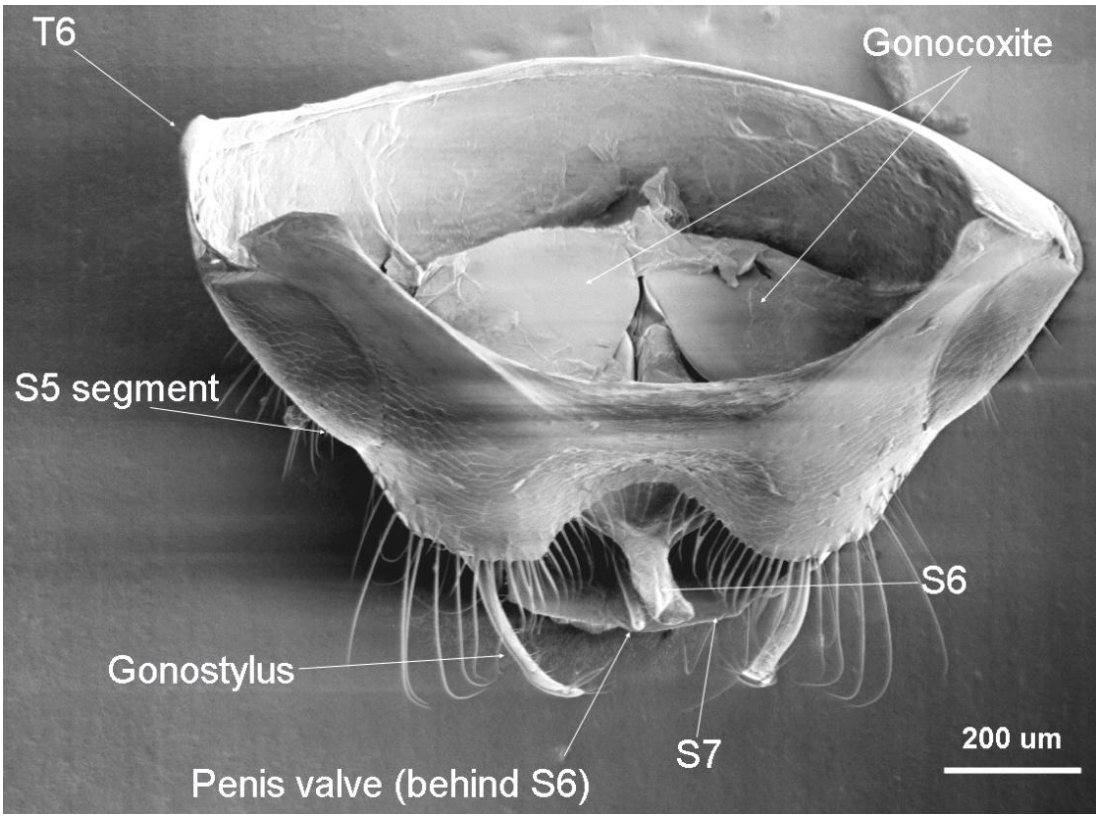


Figure 4.15 Drone metasomal apex with genitalia *in situ*.

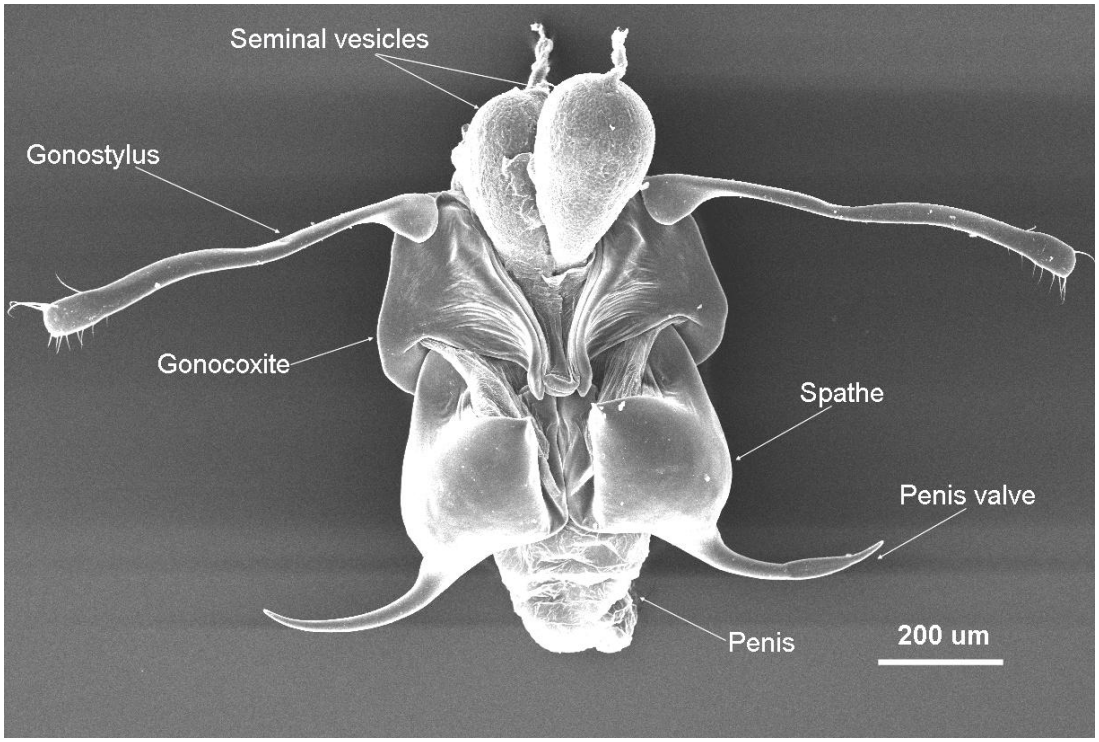


Figure 4.16 Drone genitalia showing distortion associated with eversion.

SEM images of the metasomal segments S4, S5, S6 and S7 as well as T7 and the genitalia were obtained for individual drones, from representative nests of each group. Hair patterns were easily distinguished (Figure 4.17) and no differences were observed in the S4 or S5 segments between groups. SEM images of intact S7 and T7 were uninformative. Data obtained from measurement of these structures under compound light microscopy showed no differences between groups. Data obtained from measurement of S6 landmarks, using SEM images, of were conflicting. This was due to the difficulties encountered when trying to uniformly mount the structures on the SEM stub (Figure 4.18). There was also no depth-of-field in these images, which resulted in misleading information. Subsequent measurements of S6 landmarks under compound light microscopy showed no differences between groups.

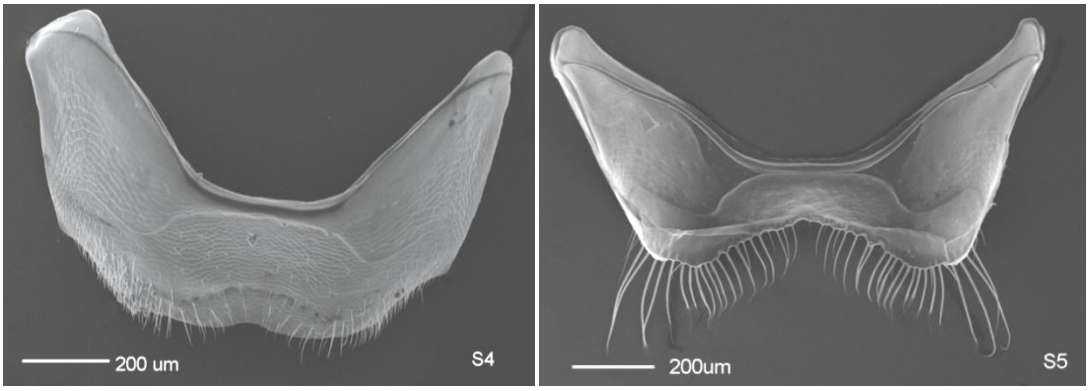


Figure 4.17 Metasomal segment morphology and hair patterns on S4 (left) and S5 (right) were found to be the same for all groups.

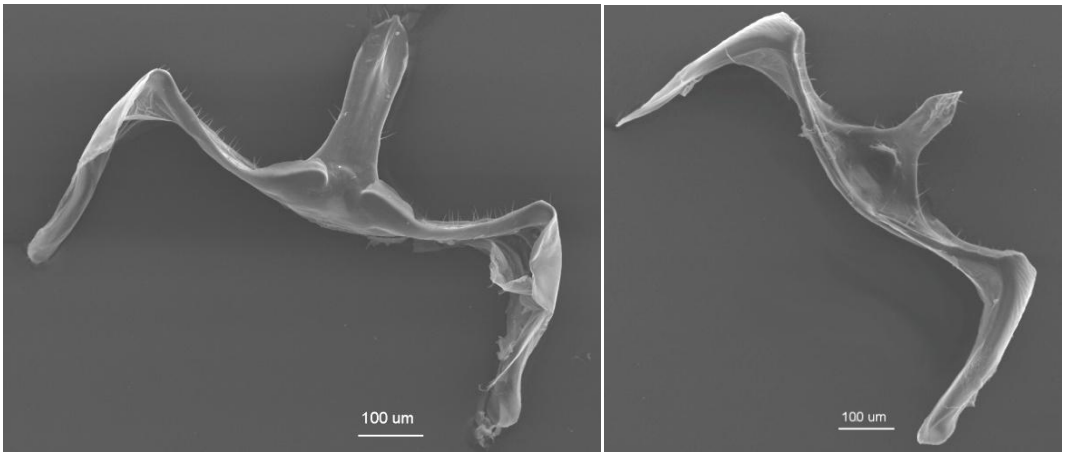


Figure 4.18 SEM images of S6 segments from two 'australis' drones. Mounting angles produce conflicting data.

SEM images of dissected genitalia provided the most informative comparisons. Drones collected from WA and from the Cobourg Peninsula in NT were found to have gonostyli which were curved at the tip, while all the other groups had straight tipped gonostyli (Figure 4.19). Stereomicroscope images of dissected genitalia clearly showed differences between the WA / NT ('curved') specimens and the other groups (Figure 4.20).

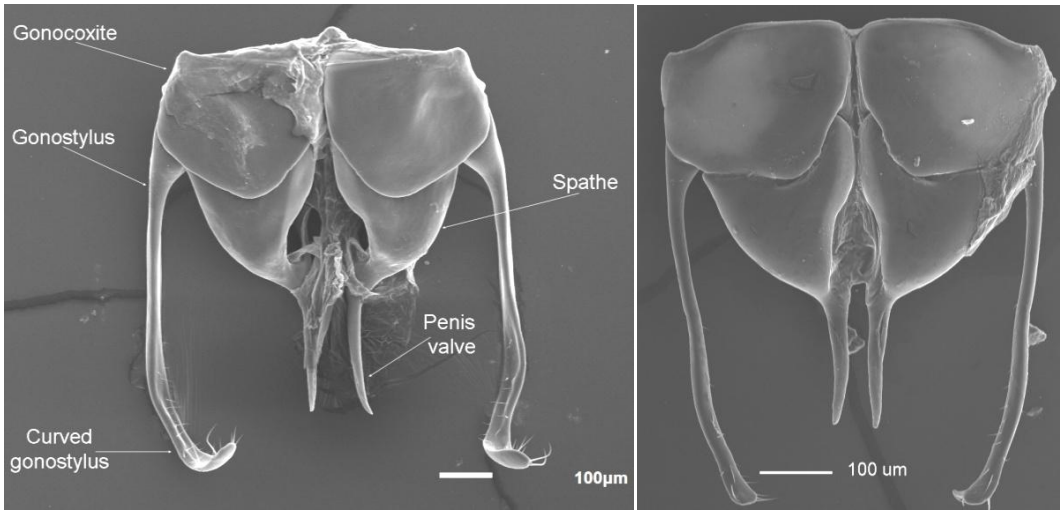


Figure 4.19 Curved gonostylus of drones collected from WA and Cobourg Peninsula NT (left) compared to the straight gonostylus of 'australis' (right).

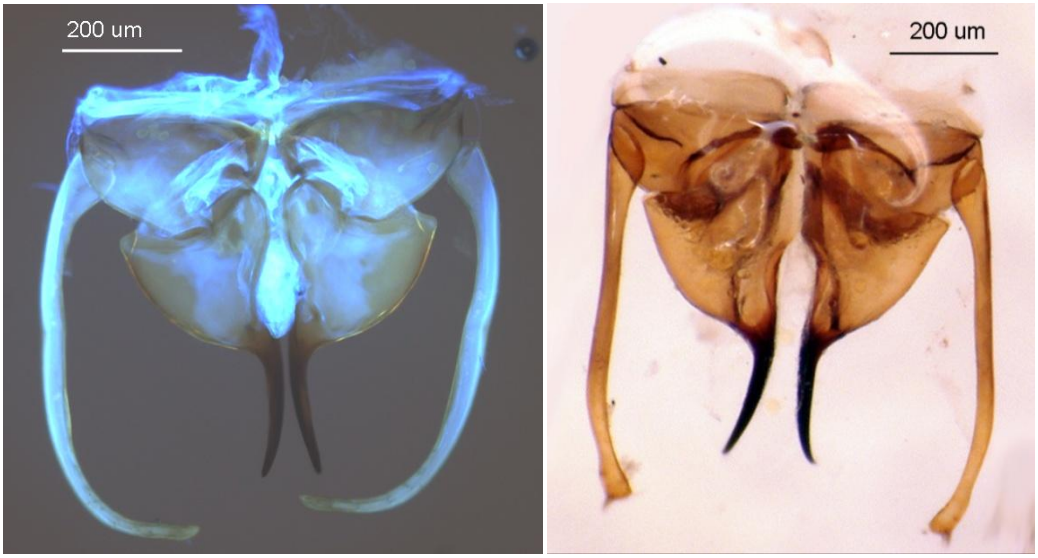


Figure 4.20 Freshly dissected genitalia of a drone from the 'curved' group (left) (imaged under polarised light) demonstrating the large degree of gonostylus tip curvature. This is compared to the straight gonostylus of the 'australis' group (right), representative of all of the other groups.

The intact genitalia of the pinned drone specimens showed curvature of the gonostylus tip in the ‘curved’ group and no curvature in the other groups (Figure 4.21). It was, therefore, possible to separate the ‘curved’ group from the other groups: ‘symei’, ‘intermediate’, ‘australis’ and ‘striped’, using distinct morphological character differences in the male genitalia.

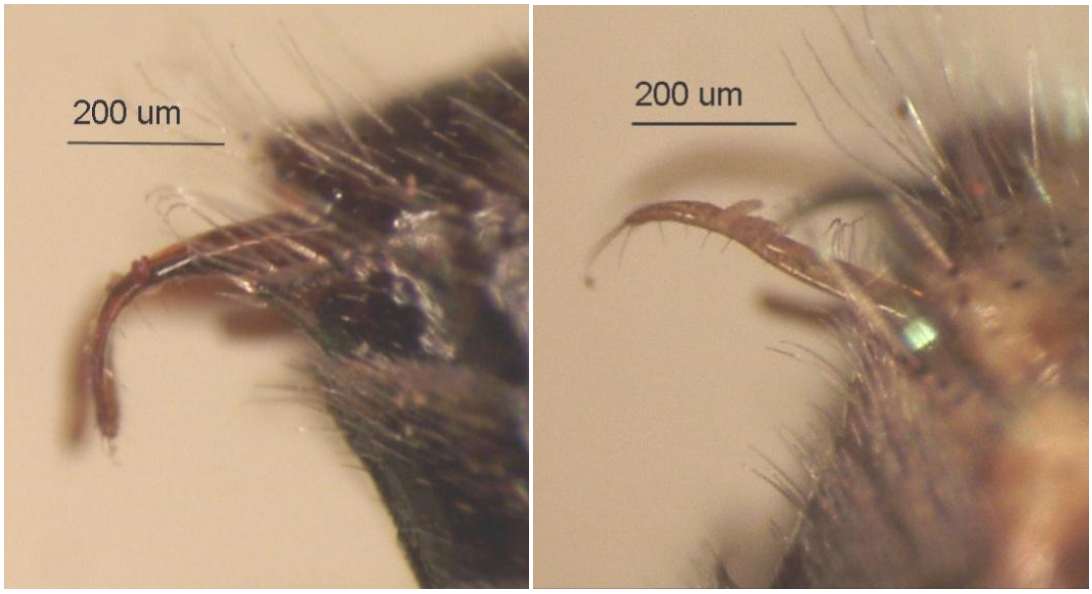


Figure 4.21 Pinned drone specimens showing intact gonostyli. Note the curved angle of the 'curved' group (left) and the straight tip of the 'symei' group (right).

4.1.5 Geometric morphometric wing analysis

Morphometric measurements of various body parts, including the wings and legs, were first utilised in the identification of Africanised honey bees (Daly et al. 1982). This technique required precision and skill and was quite time consuming. Schröder et al. (1995) developed the Automated Bee Identification System (ABIS) which used landmarks created by the vein junctions on the wings of the honey bee. The technique was further refined and currently the ABIS programs are capable of automatically identifying and marking the landmarks on digital images of insect wings. Using this method, researchers were able to correctly classify 94% of the subspecies of *A. mellifera*, enabling early detection of Africanized honey bee colonies (Francoy et al. 2009). Stingless bees have reduced venation, thus rendering ABIS ineffective; however, wing analysis using geometric morphometrics (GM) has recently been developed and has proved to be an effective taxonomic tool in insects

with reduced wing venation (Mendes et al. 2007; Francisco et al. 2008; Francoy et al. 2011).

4.1.5.1 Materials and methods

Specimens were prepared for GM analysis. Wings were cut at their bases using entomological scissors to avoid damaging individual specimens. This method removed the need to rehydrate specimens. To avoid unnecessary destruction of specimens, a preliminary study was conducted using specimens from two to three nests from each group. The results clarified the value of this study and from there a larger set of nests was used. The right forewing of five workers from 67 nests was mounted between two microscope slides. Digital images were captured using the compound light microscope and digital camera. Images were enhanced and compressed in Adobe® Photoshop® CS2 (Adobe Systems Inc. www.adobe.com/au).

The following work was completed by Dr Tiago Francoy University of Sáo Paulo (USP), Sáo Paulo, Brazil in his laboratory. Ten homologous landmarks were plotted at the wing vein intersections, within the central part of the wing (Figure 4.22) using tpsDig2. V2.16 software (Rohlf 2005). Images were all scaled to the same size and superimposed, using the centroid of the landmarks distribution and rotated to the best fit of the landmarks. The aligned Cartesian coordinates of the landmarks were used to perform statistical analyses. In order to avoid distortions in the data, instead of individual measures, the mean configuration of bees from the same colony was used in the analysis. A principal component analysis (PCA) of the Cartesian coordinates of each landmark was conducted after alignment. A discriminant analysis was then conducted using forward stepwise analysis (tolerance 0.01; F to enter 1.00) using the same measures to determine the discriminant functions. A canonical analysis and a cross-validation test followed, to evaluate the accuracy of colony identification. Groups were labelled according to their morphological characteristics or original species names (as described in Section 4.1.2.2). The statistical analysis was carried out using Statistica 6.0 (StatSoft 2001). The Mahalanobis distances between the centroids of the groups were also calculated in the discriminant analysis and a dendogram of morphological similarity was constructed based on this data. These were constructed using neighbour-joining methodology (Saitou & Nei 1987) and

MEGA version 4.1 (Tamura et al. 2007). The coefficients of cophenetic covariation, using TreeFit (Kalinowsi 2009), were calculated to determine if the dendogram gave a good representation of these data.

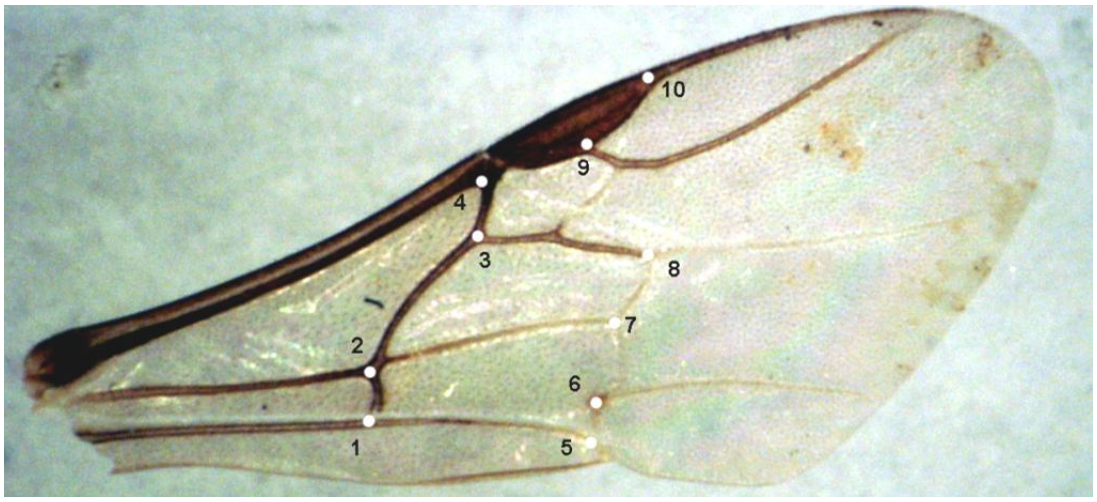


Figure 4.22 Landmarks on wings used in geometric morphometric analysis.

By the time this study was underway, the Qld property owner where the cincta-like bees had been discovered had located five more nests of this type and under Dr Dollin's instruction he collected specimens and data from these nests for the study. Therefore, wings from all six groups were available for this study: 'symei', 'intermediate', 'australis', 'striped', 'curved' and 'cincta'.

4.1.5.2 Results

Five factors with eigenvalues > 1 were extracted in the PCA and explained a total of 74.64% of the total variability of the data. The first principal component explained 22.30% of the data variability and the second principal component explained 20.59% of the variability of the relative positions of the landmarks. The relative position of landmarks that contributed most to the first component were the Cartesian coordinate 'y' in the landmark 10 and the 'x' position in the landmarks 3, 4 and 6. The most informative positions contributing to the second component were the 'y' position in the landmarks 2 and 5 and the 'x' position of the landmarks 7 and 8. Both 'cincta' and 'curved' were placed on the right-hand quadrant. The 'striped' group was placed on the lower quadrant, and along the edge of the distribution (Figure 4.23). An analysis of Mahalanobis distances between the centroids indicated that there were

significant differences ($p < 0.005$) between all groups except 'intermediate' which differed only from 'cincta' (Table 4.4).

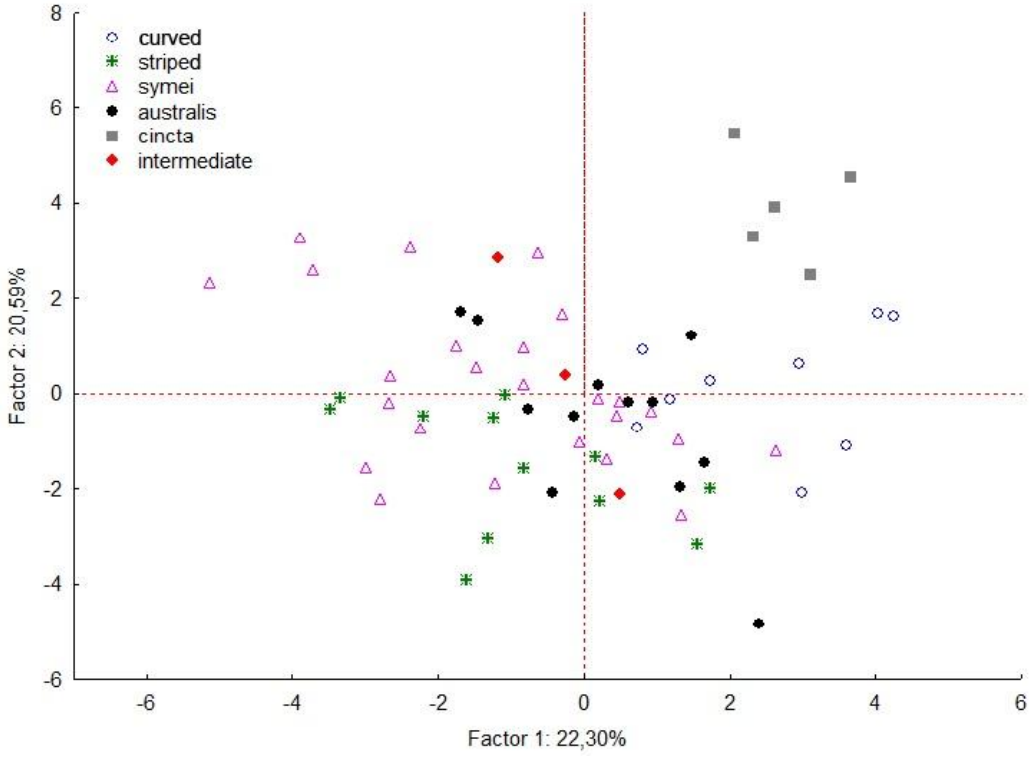


Figure 4.23 Principal component analysis (PCA) of the six *Austroplebeia* groups with Cartesian coordinates of each landmark after alignment.

Table 4.4 The *p* values for centroids differences within the Mahalanobis analysis.

	'striped'	**'symei'**	**'australis'**	**'cincta'**	**'intermediate'**
'curved'	0.000	0.000	0.000	0.000	0.127
'striped'		0.000	0.002	0.000	0.186
'symei'			0.000	0.000	0.368
'australis'				0.000	0.433
'cincta'					0.000

The dendogram of morphological proximity (Figure 4.24) placed 'cincta' in an isolated branch, while the other five groups were split into sub-branches. The coefficient of cophenetic correlation was 0.969, indicating the dendogram was a good two-dimensional representation of the calculated distances (Kalinowsi 2009).

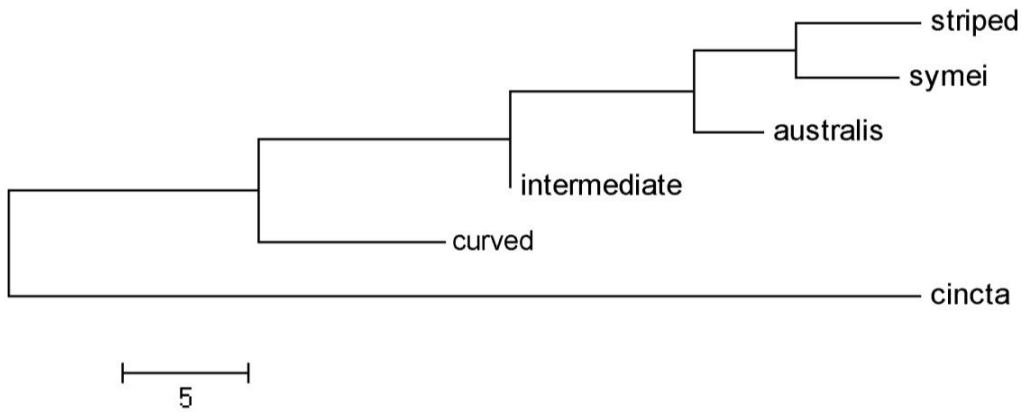


Figure 4.24 Dendogram of morphological proximity of the *Austroplebeia* groups, constructed using neighbour-joining methodology, based on the Mahalanobis square distances between the centroids of the groups.

The discriminant functions 1 and 3 separated the 'groups' more distinctly (Figure 4.25) than the discriminant functions 1 and 2, which superimposed the 'symei' and 'striped' groups (Figure 4.26). The latter analysis did, however, separate out the 'curved' group. In both analyses, the 'cincta' colonies were placed on the right hand side of the quadrant.

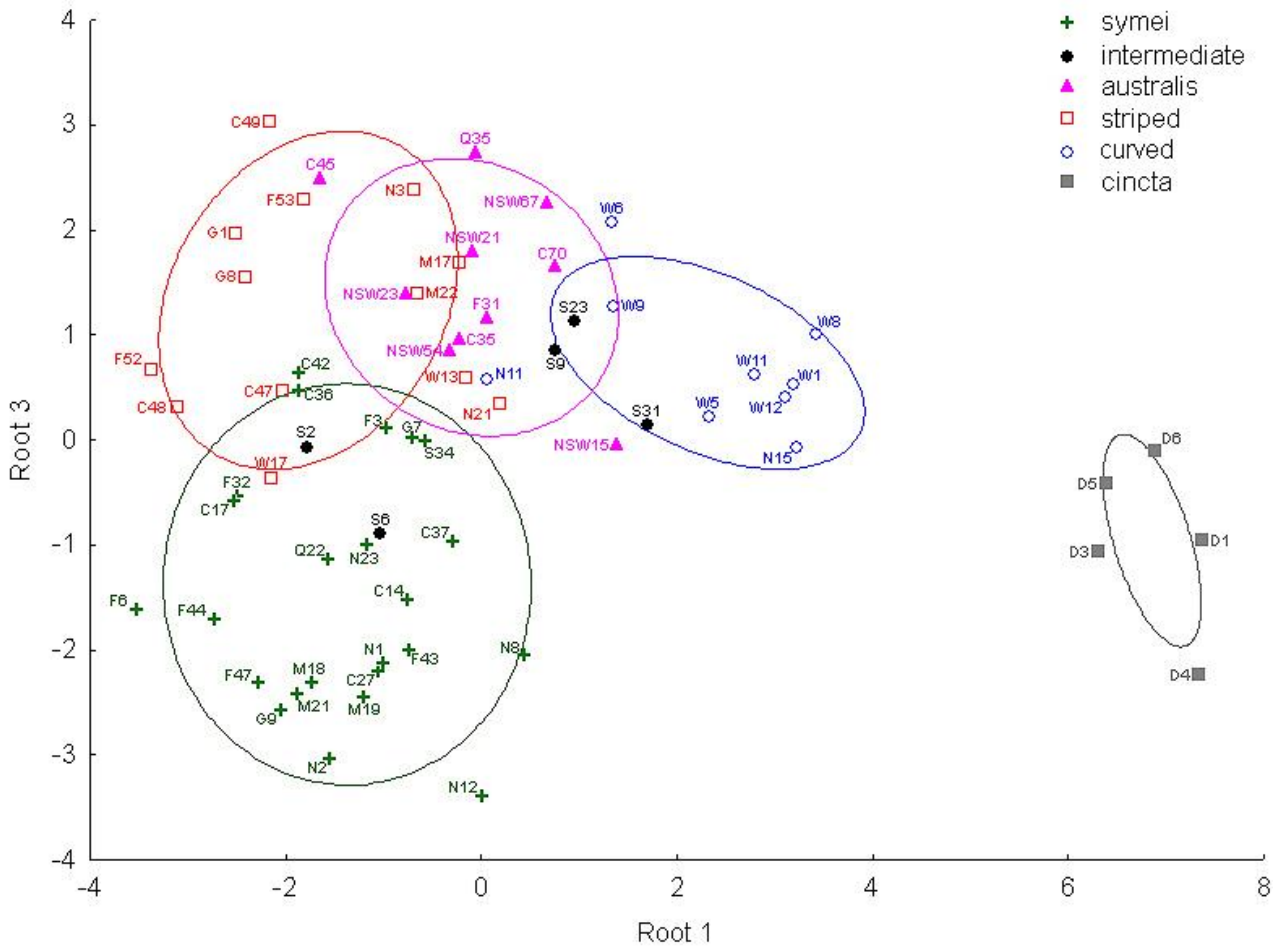


Figure 4.25 Discriminant analysis of *Austroplebeia* groups using discriminant functions 1 and 3.

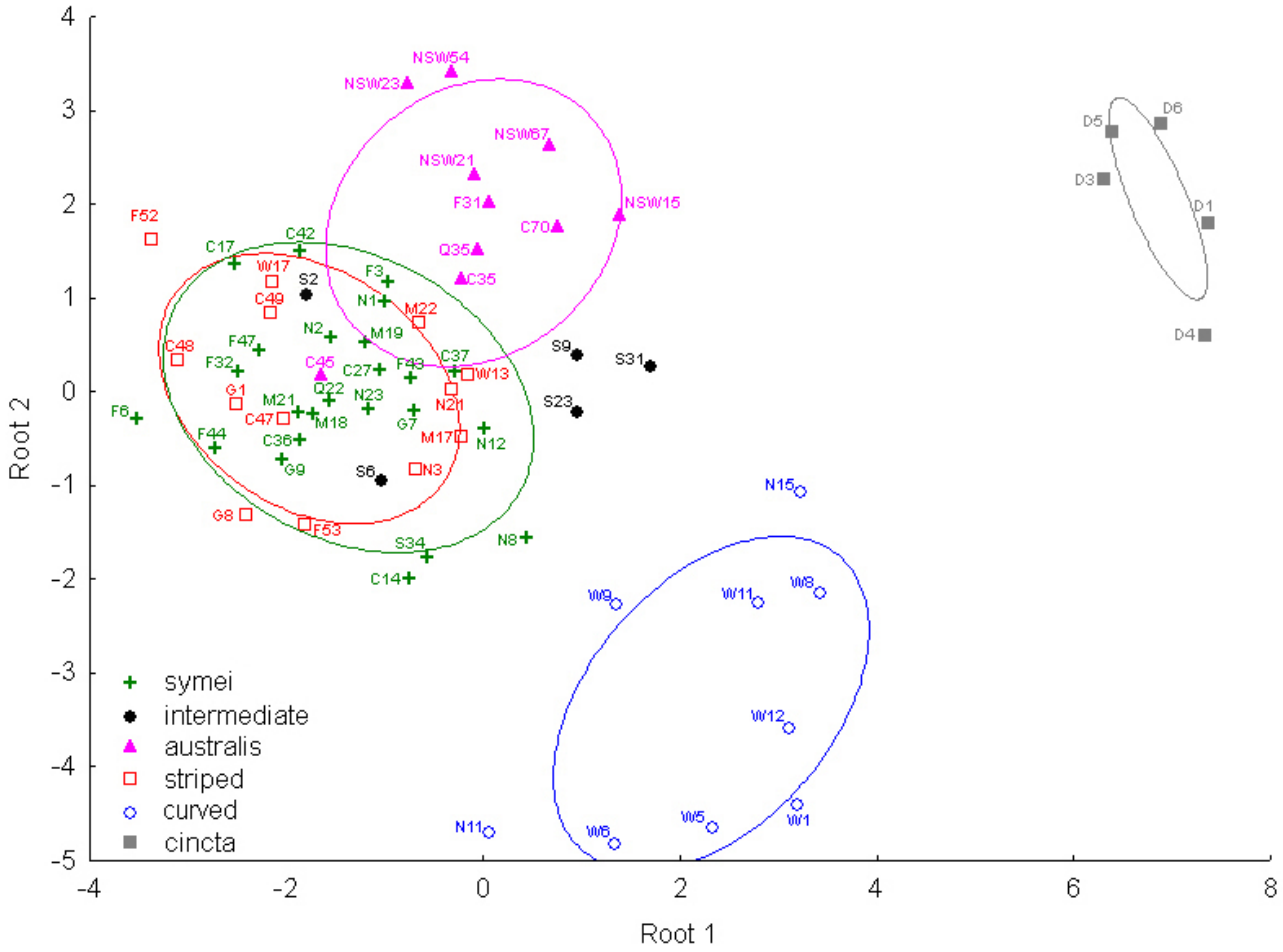


Figure 4.26 Discriminant analysis of *Austroplebeia* groups using discriminant functions 1 and 2.

4.1.6 Molecular analysis

The analysis of mitochondrial DNA (mtDNA) polymorphism (Dowton & Austin 1994; Costa et al. 2003; Marco et al. 2003; Quezada-Euán et al. 2007) is a more recent addition to taxonomy, and phylogenetics have been utilised in species delimitation for the last two decades (Simons et al. 1994; Hebert et al. 2003; Hebert & Gregory 2005; Vogler & Monaghan 2007). Hajibabaei et al. (2006) analysed 521 taxonomically identified species of tropical Lepidoptera, using mtDNA analysis, and found 97.9% of the species possessed distinctive DNA base pair differences. Mitochondrial DNA analysis has been used to successfully delimit several stingless bee species (Rasmussen & Cameron 2007). Choosing suitable gene regions is an important step in successful species discrimination (Hebert et al. 2003; Vogler & Monaghan 2007). The mitochondrial cytochrome oxidase I gene (COI) is considered to be an excellent DNA barcoding gene (Hebert et al. 2003). The universal primers used for this gene are robust and recovery of sequences from this gene are obtainable in most animal phyla (Folmer et al. 1994). Some stingless bee researchers have had good success with the gene that encodes 16S ribosomal RNA (rDNA) (Costa et al. 2003; Marco et al. 2003; Rasmussen & Cameron 2007), although insertions and deletions (indels), that are more likely to occur in 16SrDNA than a protein encoding gene, may complicate sequence alignment (Doyle & Gaut 2000). When undertaking molecular analysis, it is ideal to use either fresh samples, samples preserved in ethanol or samples stored at -80ºC. Specimens which have been killed in ethyl acetate and air dried suffer from DNA degradation and usually yield little or no DNA (Dillon et al. 1996). However, successful sequencing of very old specimens has been achieved by some researchers (DeSalle et al. 1992).

4.1.6.1 Materials and methods

4.1.6.1.1 DNA extraction, amplification and sequencing

Due to the destructive nature of DNA extraction, initial studies were carried out using a small number of workers from nests with large nestmate samples, while still choosing from a range of groups and geographic locations. Further analysis of additional specimens followed, depending on the quality of the sequences obtained.

The age of the specimens and method of storage led to degradation of DNA. In some specimens the DNA yield was insufficient for polymerase chain reaction (PCR) amplification and sequencing. Fresh specimens always yielded more DNA than did the old specimens (fresh specimens ranged from 55.6 to 89.1 ng / µL, collection specimens ranged from 3.7 to 83.6 ng / µL) and a quality of DNA that was not sheared. This enabled amplification of longer stretches of DNA through PCR. Fresh specimens, harvested from the UWS, Richmond campus hives, were first used to perfect the extraction and PCR amplification techniques.

DNA extraction for the first fresh specimens (viz., 'australis', 'symei' and *A. mellifera*) was successfully carried out using DNeasy Blood & Tissue Kit (Qiagen, Doncaster, Victoria. www.qiagen.com). However, in order to maximise DNA harvest, QIAamp DNA Micro Kits (Qiagen Pty Ltd) were used for the older, more degraded specimens. Extraction of DNA from the legs and thoracic tissue was carried out following the manufacturer's protocol, with some modifications: tissue was lysed overnight at 56°C and 300 rpm on an Eppendorf Thermomixer comfort (Eppendorf South Pacific, www.eppendorf.com.au) and elution was with 60 µL of elution buffer. DNA was stored at -20°C. DNA concentration was measured using a Nanodrop spectrophotometer (Thermo Fischer Scientific Inc, Scoresby, Victoria).

Several DNA amplification protocols were used in an attempt to obtain the best templates. Cytochrome oxidase I (COI) primers were chosen because of their robust amplification and phylogenetic information content. The primer set LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-AAACTTCAGGGTGACCAAAAAATCA-3'), using the protocol set out by Hebert et al. (2003), successfully amplified the expected amplicon of 700 bp from fresh specimens of honey bee, 'symei' and 'australis'. This same protocol was used with two 'symei' and two 'australis' specimens from the collection of old specimens; however, amplification of the old 'symei' specimens was unsuccessful. Attempts to improve these results, by amplifying the DNA at gradient temperatures, were also unsuccessful for the 'symei' specimens, indicating that the DNA was degraded to the extent that a fragment of about 700 bp length could not be amplified.

Primers for mitochondrial 16S rDNA, LR13943F (5'-CACCTGTTTATCAAAAACAT-3') and LR13392R (5'-CGTCGATTTGAACTCAAATC-3') using the protocol described by Costa et al. (2003), successfully amplified a 420 bp amplicon for both fresh and old specimens of 'australis' and 'symei', and were therefore utilised for the remaining studies. DNA templates were amplified on a thermocycler (Thermomix comfort, Eppendorf Pty Ltd, North Ryde, NSW) performing 40 amplification cycles (94°C, 1 min; 42°C, 1.5 min; and 64°C, 1.5 min), followed by a final extension step at 72°C for 5 min (Costa et al. 2003).

Reaction components were provided in kit form (GoTaq® PCR core systems, Promega Corporation, www.promega.com) and initial reactions were carried out in a total volume of 20 µL. Ethidium bromide 0.5 µg / ml (AMRESCO 2011) was added to a 1% agarose gel and electrophoresis was carried out, at 100 V for 1 h. Bands were visualised on a Gel Doc 2000 / ER (Bio-Rad Laboratories Ltd, www.bio-rad.com).

PCR products were sent to Macrogen Korea for sequencing with BigDye™ terminator chemistry (Sanger sequencing). Prior to submission to Macrogen, reaction products were purified using ethanol precipitation and run using an ABI Automatic Sequencer 3730XL (Macrogen Inc., Seoul, Korea). Sequences obtained from the first set of DNA fragments of the old specimens were of extremely poor quality, with multiple peaks (Figure 4.27).

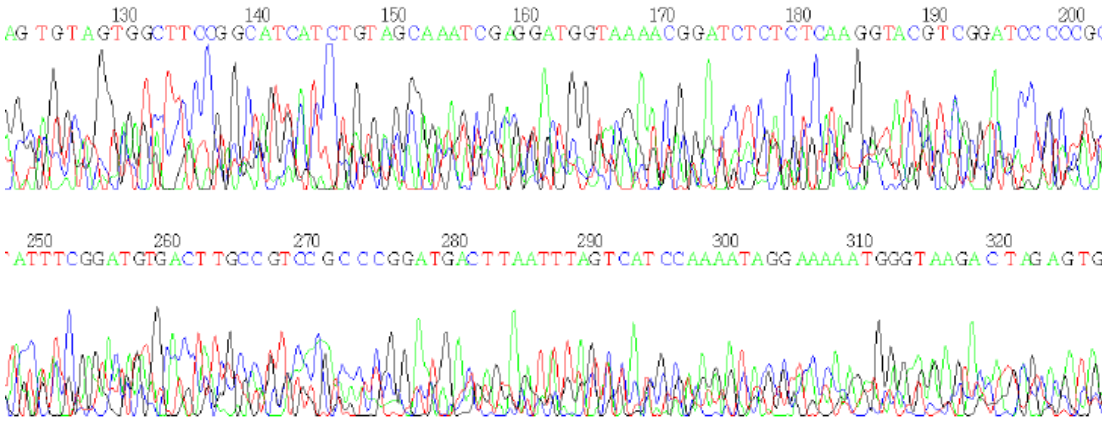


Figure 4.27 First sequencing result for first old specimen of 'australis' using the 16S forward primer, resulted in a very poor quality chromatogram with multiple peaks.

For subsequent reactions, PCR amplifications were carried out in a total reaction volume of 40 µL, using 21.6 µL of PCR water, 8 µL of reaction buffer, 4 µL of $MgCl_2$ (25 mM), 0.8 µL of dNTP (10 mM), 1.6 µL of each primer (2) (10 mM), 0.4 µL of Taq polymerase and 2 µL of DNA. Electrophoresis of the amplified fragments (40 µL) was carried out on a 2% agarose gel in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA / pH 8.0), with ethidium bromide added 0.5 µg / mL, and run at 70 V for a minimum of 2.5 h, and up to 4 h, to obtain maximum resolution (Figure 4.28). Progress of gel bands and images were obtained with a Gel Doc 2000 / ER. A positive and negative control was always included to control for DNA contamination and PCR artefacts during PCR amplification.

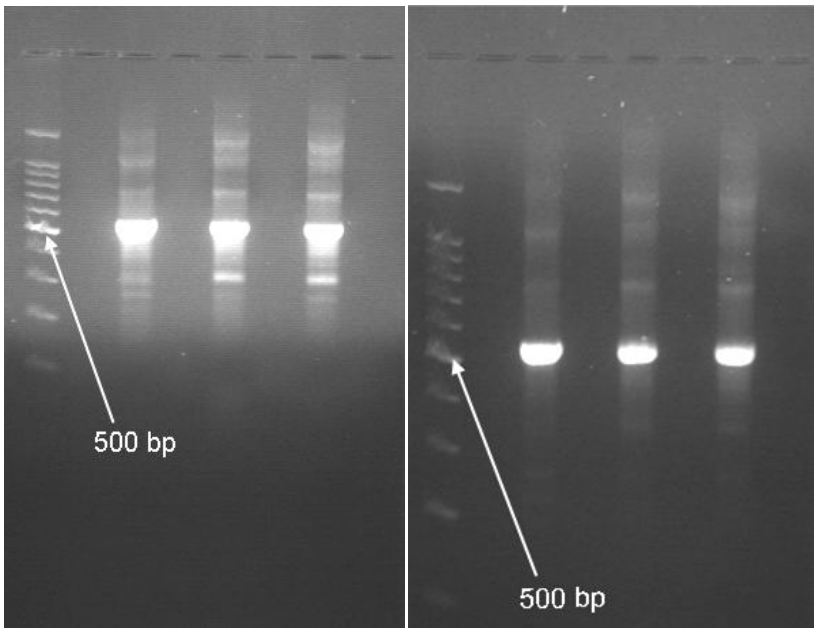


Figure 4.28 Electrophoresis time was extended from 1 h (left) to 2.5 h (right) to 'clean up' the PCR product.

Once the bands were well separated, gels were placed on a UV transilluminator (Cleaver Scientific Ltd, Warwickshire, UK). In order to protect the DNA from the UV light, aluminium foil was placed between the gel and the UV lamp. Individual target bands of the expected fragment size were quickly visualised and the gel segment cut out with a No. 10 scalpel blade. The UV lamp was turned off and the segment was carefully removed from the gel. The segment was briefly illuminated to trim the excess gel from the DNA band. The gel slice was transferred into a pre-

weighed and labelled 1.5 mL Eppendorf tube and sealed. This process was repeated for the bands of expected fragment length.

Amplification of some specimens was unsuccessful (e.g., Figure 4.29, lanes k and l), while others produced faint bands (e.g., Figure 4.29, lane a) or strong multiple bands (e.g., Figure 4.29, lanes d and h). Where multiple bands remained close to each other, the band closest to 500 bp was dissected (e.g., Figure 4.29, lanes c, d, h and j). Multiple attempts to amplify DNA from a single PNG *Au. cincta* worker were unsuccessful. 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 Corporation, www.promega.com). PCR products were sequenced as described above.

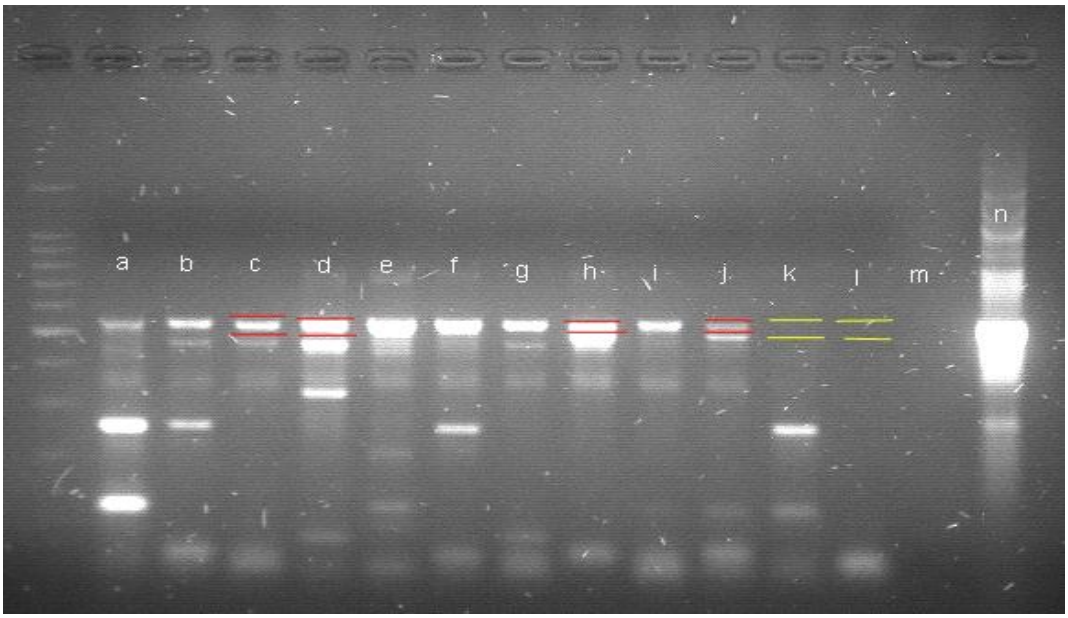


Figure 4.29 Gel bands prior to cutting. While some templates did not amplify (k and l), multiple bands (c, d, h and j) were partially excised. Note bright amplification of fresh 'australis' positive (n) compared to the older specimens.

4.1.6.1.2 Sequence alignment

Raw data were downloaded from the Macrogen web portal in several formats, including the *.ab1 file format that was required for import into DNA analysis software. Sequences were assembled, analysed and manually adjusted using Sequencher® 4.5 (Informer Technologies Inc. www.informer.com). Initially, sequence similarities with previously published data were obtained through an on-

line BLAST® search (Basic Local Alignment Search Tool, http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al. 1990). This confirmed that the sequences were from mitochondrial 16S rDNA of *Austroplebeia*. A total of 53 specimens were used for DNA extraction. In total, 96 amplification attempts from these specimens, ranging from one to six times per specimen were required to obtain 36 readable sequences (Appendix 12).

4.1.6.1.3 Phylogenetic methods and parsimony analysis

The data analysis and resulting phylogenetic tree construction were performed, using 36 sequences. Each nest had previously been allocated to one of six morphological groups ('symei', 'intermediate', 'australis', 'striped', 'curved' and 'cincta'). Each sequence was identified by its nest code, consisting of the morphological group name and the original collection code. Four additional mitochondrial 16S sequences from two species of Australian *Austroplebeia* were downloaded from GenBank for the phylogenetic analyses. A GenBank 16S sequence from the corbiculate bee, *Apis dorsata*, was also selected as an outgroup.

Sequences were aligned in Muscle, integrated in MEGA5 (Tamura et al. 2011). The dataset was analysed for nucleotide composition, pairwise genetic distances (p-distances) and phylogenetic relationships using Neighbor-Joining (NJ), Maximum Parsimony (MP) and Maximum Likelihood (ML) inferences of MEGA5. Firstly, all 40 *Austroplebeia* sequences from this study and from GenBank were analysed together and secondly, consensus sequences from the 'symei', 'intermediate', 'australis' and 'striped' groups were compared.

NJ phylogenies were reconstructed using the Maximum Composite Likelihood model, with transitions and transversions at a uniform rate. For gaps, the pairwise deletion option was chosen. The MP phylogenies used the Close Neighbor Interchange algorithm with Search Level 1. Ten initial trees were obtained with random addition of sequences. For gaps the partial deletions option was selected with a site coverage cutoff set to 95%. For the ML phylogenies, a model and parameters for each phylogenetic reconstruction were selected using Maximum Likelihood estimates computed by MEGA5. The General Time Reversible model was used as it yielded substantially higher Maximum Likelihood values. The phylogenies were

constructed using the Nearest Neighbor Interchange heuristic method with the initial tree made automatically. For gaps, the partial deletion option was selected, with site coverage cutoff set to 95%. All trees were rooted with the *A. dorsata* GenBank sequence as an outgroup and reliability of the nodes was assessed using 1,000 bootstrap replicates.

4.1.6.2 Results

The length of the 16S rDNA amplicons sequenced in this study varied from 416 bp, for the 'cincta' group, 418 bp for the 'intermediate' groups and 420 bp for the remaining groups. The length of the GenBank sequences ranged from 338 to 526 bp. When sequences were compared, they contained 27 (6.4%) polymorphic sites, including eight singletons and 19 parsimony-informative sites. The average nucleotide composition (%) was: T: 39.1; C: 6.6; A: 42.2; G: 12.0. This gave a high (81.3%) A+T content for these sequences.

The mean pairwise genetic distance (p-distance) across all sequences was 1.4%. The mean divergence within groups ranged from 0 to 0.7% with the mean divergence between groups ranging from 0.4 to 3.3% (Table 4.5)

Table 4.5 Pairwise distances (p-distances) between the six groups, based on differences in 16S rDNA nucleotide sequences.

	'symei'	**'intermediate'**	**'australis'**	**'striped'**	**'curved'**
'symei'	-	-	-	-	-
'intermediate'	0.014	-	-	-	-
'australis'	0.012	0.005	-	-	-
'striped'	0.007	0.016	0.013	-	-
'curved'	0.018	0.023	0.021	0.019	-
'cincta'	0.024	0.024	0.023	0.023	0.033

Phylogenetic reconstruction of this dataset by NJ is presented in Figure 4.30. The MP phylogeny had a tree length of 99. The ML phylogeny was computed using the General Time Reversible model with a Gamma Distributed rate. This resulted in a tree with a l-nL score of -929.69.

Figure 4.30 Phylogenetic reconstruction of dataset by Neighbor-Joining. Values shown are bootstrap values.

In the phylogenetic tree, the ‘cincta’ group formed a strongly supported clade (> 90%), well separated from the remaining groups except for a single ‘striped’ nest (G1) (Figure 4.30). The bees sampled from G1 demonstrated morphological characteristics of the ‘striped’ groups, however; the DNA sequence produced from this nest very closely matched the consensus sequence of the ‘cincta’ group, differing by only one base pair. The ‘curved’ group formed a subclade with strong bootstrap support (> 90%); however, this subclade was contained within a polychotomy. The remaining groups were also unresolved and formed weakly supported polychotomous subclades (Figure 4.30). The ‘symei’ group was divided into two separate subclades representing the Qld and NT colonies. The ‘striped’ group formed two closely related subclades, also representing the Qld and NT colonies, and the ‘australis’ and ‘intermediate’ groups formed two subclades. The deeper relationships of the ‘symei’, ‘intermediate’, ‘australis’, ‘striped’ and ‘curved’ groups were variable and weakly supported in these phylogenies.

The *Au. symei* and *Au. australis* sequences, downloaded from GenBank, were very similar or identical to the sequences obtained from my samples and fell within the expected clades in all phylogenies. There were no base pair differences between the three GenBank *Au. symei* sequences and ‘symei-Qld’ consensus sequence. The GenBank *Au. australis* sequence differed from ‘australis’ consensus sequence at two sites. The symeiGB (AF343113) and australisGB (AF343112) were short sequences, only 337 and 401 bp, respectively.

In the NJ phylogenetic tree (Figure 4.30), 11 *Austroplebeia* sequences failed to cluster tightly with their group. Six sequences (symeiN23b, symeiQ32, symeiN12b, australisTara, curvedN11 and curved N15) differed from other sequences in their group by one to three base pairs. Half of these were unique base pair changes.

Two other colonies (symeiF6 and australisF31) were from a geographically isolated area near Bamaga, on the tip of Cape York Peninsula, Qld (Figure 4.1 and Figure 4.30). They showed particularly large divergence from other sequences in the analysis. The symeiF6 sequence was loosely associated with the ‘australis’ clade in the phylogeny. However, it differed from both the ‘symei’ consensus sequence (4 bp changes) and the ‘australis’ consensus sequence (6 bp changes).

The australisF31 sequence was closely associated with the ‘symei’ subclade in the phylogenies. However, it differed from both the ‘symei’ consensus sequence (3 bp changes) and the ‘australis’ consensus sequence (6 bp changes). Three other sequences (symeiC58, stripedM22 and stripedG1) clustered within the subclade of a different morphological group; however, the bees that were sequenced demonstrated the representative colour morphology for their group.

Twenty-five of the 36 colonies had identical sequences within their group. In some cases, identical sequences were obtained from colonies which were separated by hundreds of kilometres (Table 4.6 and Figure 4.2). These 25 sequences contained up to seven autapomorphic sites and Table 4.7 shows the diagnostic 16S sites separating the groups. As seen in Figure 4.30 there is a clear separation of ‘cincta’ and ‘curved’ groups, with the remaining groups demonstrating weak phylogenetic separation.

Table 4.6 Nests (n = 25) and GenBank sequences demonstrating identical 16S sequences within each group and their geographical separation. Figure 4.1 shows the location of each nest listed below.

Group	Nests with identical sequences	Geographic range of collection localities (km)
'symei-QLD'	symeiC36, symeiC42, symeiC43, symeiRock, symeiGB, symeiBG223, symeGB224	250
'symei-NT'	symeiN1, symeiN2	0
'intermediate'	intermediateS2, intermediateS3, intermediateS5, intermediateS9, intermediateS31	50
'australis'	australisC35, australisC39, australisC56, australisC70, australisNSW15	900
'striped-NT'	stripedC47, stripedC49, stripedW13	900
'striped-QLD'	stripedN21, stripedF53	350
'curved-WA'	curvedW5, curvedW8	100
'cincta'	cinctaD5, cinctaD6	0

Table 4.7 Autapomorphic sites within the consensus sequences for *Austroplebeia*.

Autapomorphic base pair sites																					
Group (nests)	**6**	**25**	**49**	**53**	**115**	**117**	**135**	**151**	**194**	**202**	**209**	**254**	**266**	**268**	**269**	**276**	**289**	**291**	**315**	**320**	**418**
symei Qld	A	A	A	A	T	T	A	A	A	A	A	A	A	A	A	T	-	A	T	A	A
symei NT	A	A	A	A	T	C	A	A	A	A	A	T	A	G	A	T	-	A	T	A	A
australis	A	G	A	A	T	C	A	A	T	A	A	A	A	A	T	T	-	A	T	A	A
intermediate	A	G	A	A	T	C	A	A	T	C	A	A	A	A	T	T	-	A	T	A	A
striped Qld	A	A	A	A	T	T	A	A	A	A	A	A	A	A	A	T	-	T	T	A	A
striped NT	A	A	A	A	T	T	A	A	A	A	A	A	A	G	A	T	-	T	T	A	A
curved	G	A	A	A	-	T	A	A	T	A	T	T	A	A	A	A	-	A	C	*	A
cincta	A	G	C	G	-	T	T	T	T	A	A	A	T	G	A	T	A	A	T	A	C
Unique base pair in	curve		cincta	cincta			cincta	cincta		inter	curve		cincta		aust / inter	curve	cincta	stripe	curve	curve	cincta

Nest codes associated with group : ‘symei’ Qld - C36, C42, C43, ROCK, GB223, GB224; ‘symei’ NT – N1, N2; ‘australis’ - C35, C39, C56, C70, NSW15; ‘intermediate’ - S2, S3, S5, S9, S31; ‘striped’ Qld - F53, N21; ‘striped’ NT - C47, C49, W13; ‘curved’ - W5, W8, N11; ‘cincta’ – D5, D6. *Nest N11 sequenced W at BP320, ‘W’ being either A or T.

The ‘symei’, ‘intermediate’, ‘australis’ and ‘striped’ sequences in this study were very closely related. In order to examine the relationships between these groups in more detail, a NJ phylogeny of nine ‘symei’, five ‘intermediate’, five ‘australis’ and six ‘striped’ sequences was constructed (Figure 4.31). Nineteen of the selected sequences were typical for their group, three varied from the typical sequences by only one or two base pairs and two sequences were selected as possible hybrids. The MP phylogeny had a tree length of 80. The ML phylogeny was computed using the General Time Reversible model with a Gamma-Distributed and Invariant-Sites rates, resulting in a l-nL score of -851.56.

The ‘striped-Qld’ and ‘striped-NT’ sequences formed two closely related subclades that were associated with the 'symei-Qld’ subclade. The ‘symei-NT’ sequences formed a separate subclade with low bootstrap support (40%). The ‘intermediate’ and ‘australis’ sequences also formed two separate subclades. The split of the ‘australis’ / ‘intermediate’ subclade from the other subclades was well supported in the phylogeny with bootstrap values of 89%. Almost all clades appeared to be poorly supported in this analysis.

The three sequences with minor base pair differences (symeiQ32, symeiN23b and australisTara) clustered closely with their own morphological group. Again, the two sequences from possible hybrids, clustered with the subclades of different morphological groups; stripedM22 with the ‘symei’ subclade and symeiC58 within the ‘australis’ subclade.

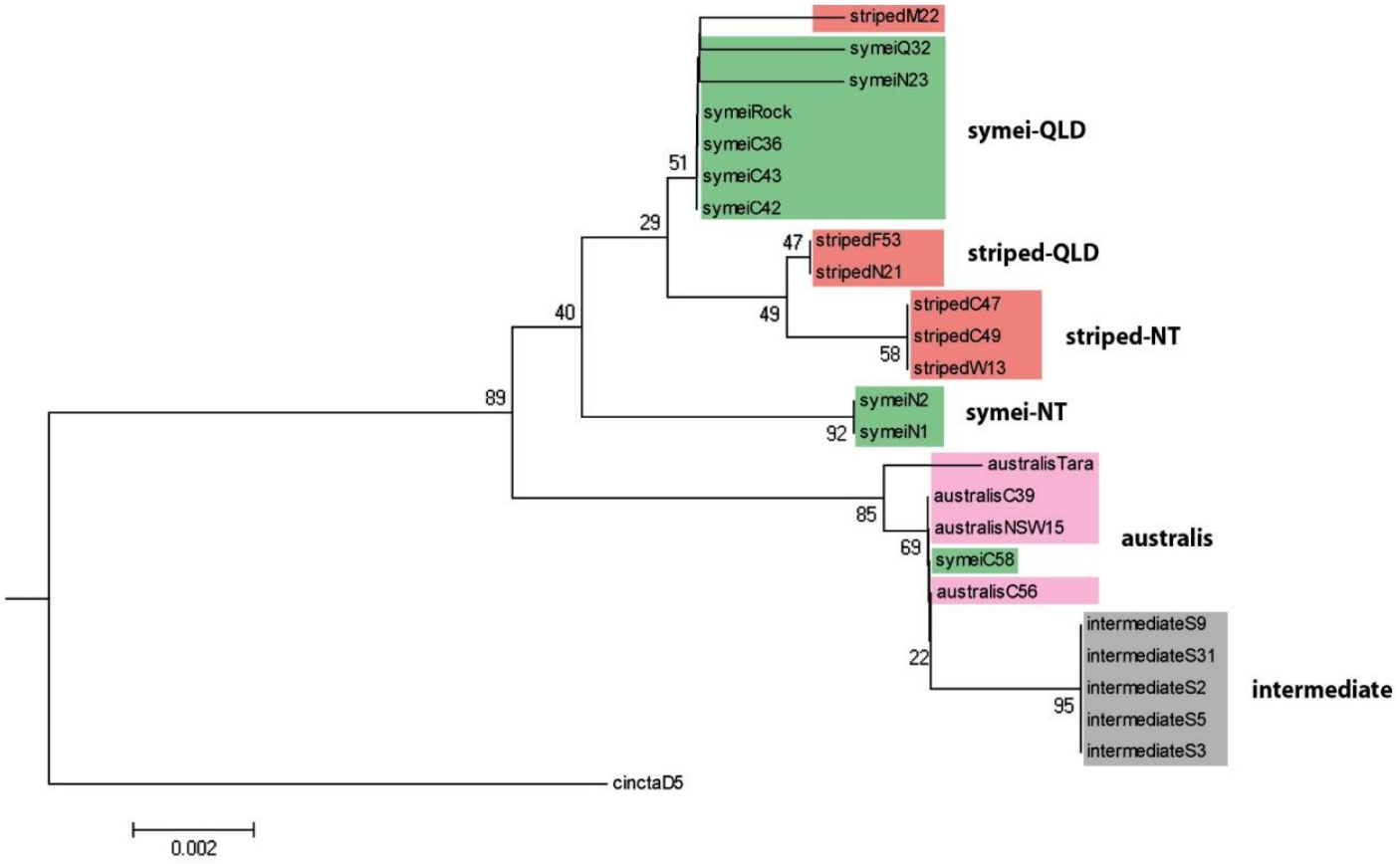


Figure 4.31 Neighbor-Joining phylogenetic tree constructed from typical sequences for the 'symei', 'intermediate', 'australis' and 'striped' groups within the genus *Austroplebeia*.

4.2 Discussion and key findings

The descriptions which identify the species within the genus *Austroplebeia* are inadequate and, as such, may hinder their identification, ecological assessment and management. Delimiting the *Austroplebeia* species has posed a frustrating and long term problem (Michener 1961; Moure 1961; Michener 1990; Rasmussen 2008) and the small amount of research, using *Austroplebeia* spp., has been less than helpful in this area (Drumond et al. 1999; Costa et al. 2003; Rasmussen & Cameron 2007).

The nest architecture is an important tool used in the identification of many stingless bee species (Dollin et al. 1997; Michener 2000; Roubik 2006). However, there are no distinct architectural differences in the nest or nest entrances for colonies of 'symei', 'australis', 'striped' or 'curved'. *Au. cincta*, described from New Guinea, builds brood comb in irregular concentric layers, of one cell thickness, and nest entrances are similar to *Au. australis*, but longer (Michener 1961). The nests of the Australian 'cincta' are, as yet, unstudied.

In the field, morphological characters can aid in species recognition and / or identification. Koch (2010) reports that morphological characters such as body size can be used to successfully separate species of *Liotrigona* and these data are supported by DNA analysis. On the other hand, if these characteristics show no real lines of demarcation, as is the case with the *Austroplebeia* 'groups', they become ineffective as taxonomic tools in the field. The combined HW and colour data weakly support separation of the four groups: 'symei', 'australis', 'striped' and 'curved' for the sampled *Austroplebeia* nests. However, these morphological groupings are not always well supported by the GM wing analysis or the 16S rDNA analysis.

The 'curved' group – the HW and colour analysis enables the 'curved' group to be separated into a single cluster (Figure 4.4). These data are further supported by the distinct morphological differences in the drone genitalia. The GM wing analysis separate 'curved' from the other groups in the PCA and discriminant wing analysis, which is demonstrated in the dendogram of morphological proximity (Figure 4.24). Although placed within a polychotomy, the 16S rDNA phylogenetic analysis strongly support (99%) the partitioning of the 'curved' group into a separate subclade

(Figure 4.30). The combined data from these studies suggest that the 'curved' group warrant the status of a separate species.

The 'symei' group – the HW data alone do not support separation of this group from 'australis'. However, combined with the colour measurements, the 'symei' group is separated into a single group (Figure 4.4). The GM wing analysis does not support the separation of this group, as demonstrated in the dendogram of morphological proximity (Figure 4.24). The 16S rDNA phylogenetic analysis suggests that bees of the 'symei' group may be a paraphyletic group (Figure 4.31). 'Symei' divides into two separate clades representing the Qld and NT nests, with three base pair differences but do not separate morphologically. These geographically separated groups may have evolved differently due to a bottleneck effect (Thorpe & Stenson 2003; Alves et al. 2011). The data do not support the delimitation of the 'symei' group into a separate species.

The 'striped' group – the HW and colour analysis separate the 'striped' group into a separate cluster (Figure 4.4). The GM wing analysis does not support these data, as demonstrated in the dendogram of morphological proximity (Figure 4.24), which groups it with 'symei'. The phylogenetic analysis of the 16S rDNA did not resolve the 'striped' group into a single clade (Figure 4.30 and Figure 4.31). There is only one base pair difference between the 'striped'-Qld and the 'striped'-NT subclades, suggesting there may also be a bottleneck effect due geographic separation of these groups. The delimitation of the 'striped' groups into a separate species is not supported by these data.

The 'australis' group – similar to 'symei', the HW data alone do not support separation of this group. However, combined with the colour measurements, the 'australis' groups separate from the other groups (Figure 4.4). The GM wing analysis data support the separation of this group, as demonstrated in the dendogram of morphological proximity (Figure 4.24). The 16S rDNA phylogenetic analysis separate the 'australis' group from the rest of the groups with a bootstrap of 85%, but did not resolve the samples into a single clade (Figure 4.30 and Figure 4.31).

The 'intermediate' group – the 'intermediate' group is made up of bees collected from nests found within a small geographic area. This group cannot be allocated to

any major cluster in the colouration / size analysis or GM wing analysis. The phylogenetic analysis is unable to resolve the 'intermediate' group into a dichotomy, but suggests that 'australis' and 'intermediate' belong to the same group. The data here do not support the delimitation of either 'australis' or 'intermediate' as separate species or subspecies.

Duaringa, Qld 'australis' and 'symei' – bees collected from 14 nests at Duaringa, Qld, 80 km south-west of Rockhampton, are identified morphologically as 'australis' and 'symei' (Figure 4.6). These nests were found within close proximity of each other but the morphological analysis (HW and colour) place these nests into two distinct groups – 'australis' and 'symei'. Bees from six of these nests were successfully sequenced, and the consensus sequences also form into these two distinct groups, with 4 bp differences. This then raises the question: If 'australis' and 'symei' make up the same species, why do they retain their morphological and phylogenetic characteristics, even within closely located mixed populations? While a bottleneck effect may be responsible for the differences in some of the isolated groups, the same cannot be said for the two groups, 'australis' and 'symei', found in the same farm paddock. The retention of these groups or sub-groups may be due to many things, and the production of species-specific pheromones may be one (Law & Regnier 1971; Ayasse et al. 2001).

The 'cincta' group – the pilosity and metepisternal colour markings of 'cincta' morphologically delimits this group from the four endemic Australian groups. GM analysis of the wings supports this delimitation, with the PCA and discriminant wing analyses separating 'cincta' from all the other groups. The dendogram of morphological proximity places 'cincta' on an isolated branch. These data are further supported by the fact that the 16S rDNA phylogenetic analyses clusters 'cincta' as a genetically distinct group. These results suggest that bees from the 'cincta' group warrant the status of a separate species.

Degradation of the DNA within the specimens limits the length of fragment that can be successfully amplified. Small base pair differences within a fragment of only 420 bp, can result in considerable changes within a phylogenetic tree. The phylogenetic analysis of all the groups, with the exception of 'cincta', demonstrate

polytomies. The 16S rDNA data do not resolve the groups into the morphologically identified groups; ‘symei’, ‘australis’, ‘striped’ and ‘curved’. Further molecular analysis using fresh specimens, utilising larger DNA fragments of genes such as COI or the first internal transcribed spacer (ITS1) region of the ribosomal DNA (May-Itzá et al. 2009; Cruz et al. 2006), is needed to resolve this question.

This data produced during these studies have clearly demonstrated that there are not nine species of *Austroplebeia*. The correct identification of insect species is important for many reasons. Not only does it increase our knowledge (the ultimate scientific goal) regarding insect genera and the species within them; this knowledge can be utilised in other areas. These include studies in insect biodiversity (Danks 1988), floral biodiversity (Coulson & Witter 1984) and in environmental impact assessment (Rosenberg et al. 1986), all of which have ecological and economic importance in our society (Rosenberg et al. 1986). Management of insect fauna within forests and crops relies on the correct identification of pests and beneficials within the environment (Coulson & Witter 1984). This includes insect pollinators such as bees.

4.3 Key findings

- This study indicates there are fewer than nine species of *Austroplebeia*.
- The current studies showed some delimiting of groups within the sampled specimens.
- Further work is recommended, to define the ‘species’ which have been only partially delimited in these studies.

CHAPTER 5

Ontogeny and longevity

5.1 Introduction

The ontogenic period in bees begins at oviposition and progresses through larval development and pupation to emergence as an immature adult (callow) (Winston 1991). In social colonies, the time spent in ontogenesis strongly affects population growth as well as nest member replacement. This influences the colony's capacity to collect and store nectar and pollen resources, thus impacting on the overall health and strength of the colony, together with its long-term survival. Both the ontogenic period and longevity are dependent upon nutritional input (Maurizio 1950; Haydak 1970), and differ between species (Michener 2000). Brood development is also highly dependent upon incubation temperatures (Howe 1967; Fukuda & Sakagami 1968; Winston 1991).

There is little known about the development of stingless bee species because nest and brood comb architecture make it difficult to observe and monitor the developing brood. Stingless bees, unlike *A. mellifera,* do not progressively feed their young, but mass provision brood cells with sufficient food to support larval development. A brood cell is provisioned, a single egg is oviposited on top and the cell is sealed (Wittmann et al. 1991; Drumond et al. 2000b; Michener 2000). The developing larva is, therefore, not easily observed and it is only when the pupal cocoon is complete that the metamorphic process becomes apparent.

A number of stingless bees, including those in the genus *Austroplebeia,* produce brood clusters rather than combs (Michener 1961; Michener 1964; Roubik 1983). This presents a challenge when investigating the development of offspring within the cluster cells. As each layer of cells is built, provisioned, oviposited in and sealed the next layer of cells is soon produced on top, obscuring the developing cells from view. A small number of researchers have successfully studied brood development within the spiral brood comb of *Melipona* and *Trigona* spp. (Kerr 1950; Salmah et al.

1987; Salmah et al. 1996; Moo-Valle et al. 2004); however, data on brood development within cluster-building species have not been reported.

The longevity of individuals is also important in understanding the overall lifecycle of an organism. Adequate nutrition and parental care of juveniles extends the longevity of individuals within a social colony (Winston 1991). Eusociality also ensures emerging callows are fit and well equipped to begin in-hive activities (Carey 2001). Individuals live longer when high-risk tasks, such as colony defence and foraging, are postponed until later in life (O'Donnell & Jeanne 1995). Also, as an individual's 'rate of living' increases, metabolic rates also increase, resulting in molecular damage and morphological decline (Pearl 1928, cited in Brys et al. 2007). Foragers 'wear out' more quickly than nest workers.

Most social insect have evolved with age polyethism (Wilson 1971; Michener 1974; Hartfelder et al. 2006) and as workers get older, they move from low-risk, in-nest tasks to high-risk defence and foraging tasks. This results in low mortality rates during the early stages of life with increasing mortality rates later in life. These data can be utilised in the construction of life tables, which are further used to graphically represent current and predicted survivorship and mortality curves within communities (Chiang 1960).

Since their inception in 1693 (Halley), life tables have become a means of reporting and presenting data by insurance companies, manufacturers, physicists, biologists and zoologists alike (Chiang 1960; Krebs 2001). Life tables have become an important ecological tool and have been utilised in this field since 1921 (Krebs 2001). The data obtained from a life table can then provide a survivorship curve, based on hypothetical curves, and its corresponding mortality rate curve. They can be used to predict life expectancy within certain populations, given different environmental parameters (Visscher & Dukas 1997; Tanner 2001; Peeters et al. 2003).

Pearl (1928, cited in Brys et al. 2007; Krebs 2001) theorised that the 'rate of living' negatively correlated with lifespan. He then described three hypothetical survivorship curves: Type I, Type II and Type III. The Type I, convex survival curve represents a population with low per capita mortality rates for most of its life span,

but then suffers high death rates as the organisms get older. Examples include humans in developed worlds (Krebs 2001) or killer whales (Tanner 2001). The Type II, log-linear curve represents populations with a constant mortality, independent of age. Many bird species demonstrate this type of survivorship curve (Tanner 2001). The final, Type III, concave curve represents a population which suffer high rates of mortality at the early stages of life, such as fish, marine invertebrates (Krebs 2001) and short lived plants such as *Lindera benzoin* (Linnaeus) Blume (Laurales: Lauraceae) (Tanner 2001). Corresponding mortality rates are also termed as Types I, II and III (Krebs 2001).

Understanding the ontogeny, longevity and possible mortality rates of workers within a colony is of great importance when using colonies for pollination services. To provide adequate services, colony populations should be high. Potential forager numbers need to be high at the time of crop flowering. The overall life cycle of workers will impact on these numbers.

The aim of the studies reported here was to document the life cycle of *Au. australis*. To determine the ontogenic times for *Au. australis* brood, it was necessary to design a technique by which developing brood cells could be easily observed, while still being maintained by the colony.

For ease of understanding and flow of information, each section in this chapter is set out with materials and methods, followed immediately by the results. The findings for each study are discussed together at the end of the chapter, with a summary at the end.

5.1.1 Ontogeny

5.1.1.1 Materials and methods

In spring 2008, eight queenright colonies of *Au. australis* were prepared for this study. Six of the hive boxes were a 'Beil' design (see Chapter 3, Section 3.2) and two were OATH boxes. Each colony was fitted with a 3 mm transparent acrylic lid to allow ease of access and monitoring of the colonies, and this was covered with the original timber lid, to exclude light. All colonies were maintained in the bee shed (see Chapter 3, Section 3.2), under conditions of 27.6 ± 1.7ºC and darkness. Each

colony was provided with external, free foraging access via an 8 mm (ID) silicone tube from the hive entrance through the wall of the building (see Chapter 3, Section 3.2).

Each hive box had 2 x 10 mm diam. holes drilled into one side and an observation platform (OP) (Halcroft et al. 2008) was attached to each hole with 8 mm ID silicone tubing (Figure 5.1). OPs were left untouched for at least one month to ensure they were accepted by the colonies, as evidenced by construction of honey pots within the structures (Halcroft et al. 2008).

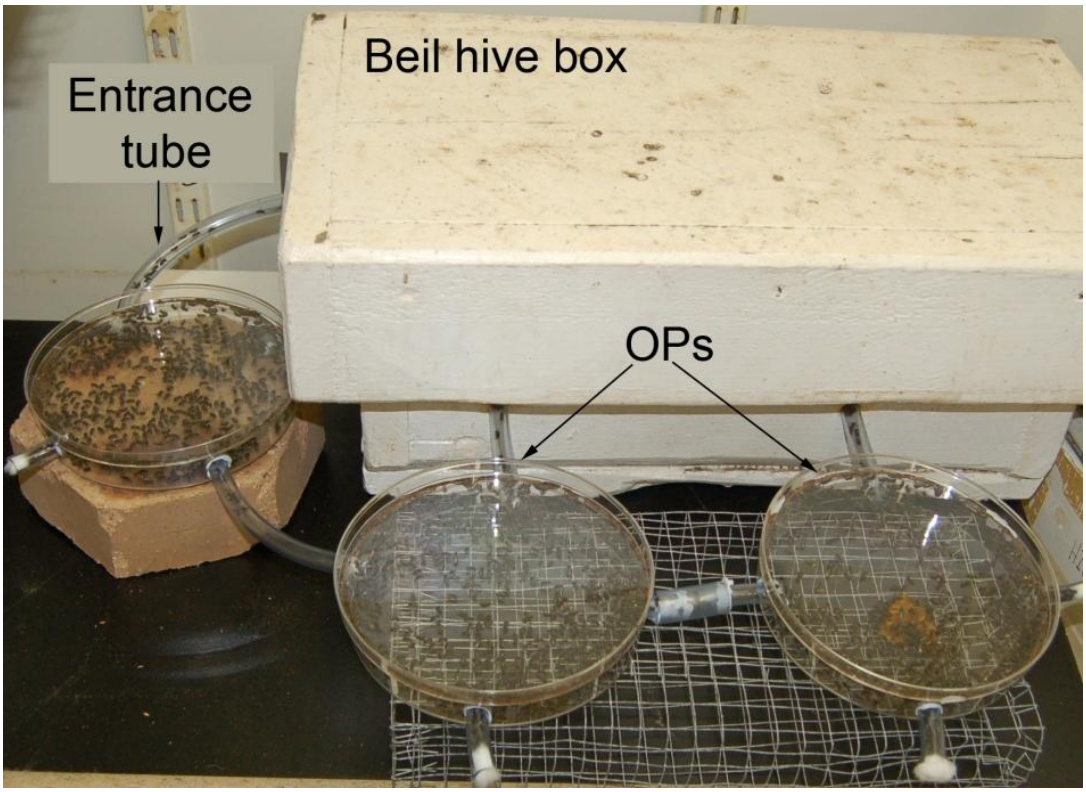


Figure 5.1 Mother colony with OPs attached to house brood grafts.

Forty-six days later, the lid of the each of the hives was removed and a wooden toothpick was placed on the leading edge of the brood cluster (Figure 5.2). The toothpick became the frame upon which the colony built the new brood cells. The lids were closed and the colony was left undisturbed for a further seven days. The hives were then reopened and the toothpicks, along with the attached 'donor' brood cells, were carefully removed from the brood clusters. The toothpicks and harvested cells were transferred (grafted) into one of the attached OPs (Figure 5.3) and the date of transfer recorded. The process of toothpick placement, brood harvest and transfer

was repeated several times over a six week period, with a total of 16 replicates being produced.

The cells within each ‘graft’ were observed for stages of brood development and emergence, with initial and final brood emergence being recorded. For each ‘graft’, brood development times were calculated, using the difference between the day prior to cell harvest and the day of final emergence. Means were compared using analysis of variance in SPSS 17.

Figure 5.2 Tooth pick positioned on the leading edge of the brood cluster. Over seven days, donor cells ‘grew’ over the tooth pick.

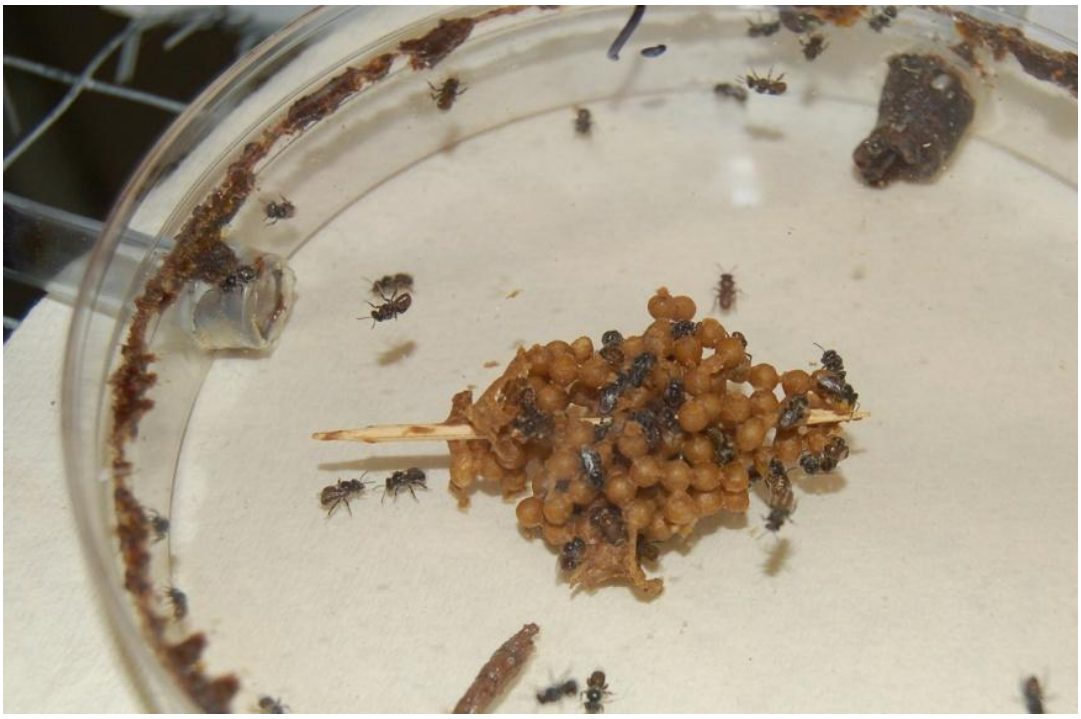


Figure 5.3 Grafted brood cells, attached to tooth pick, in OP.

5.1.1.2 Results

The OPs with 'grafted' brood were accepted by the colonies, even when the brood did not originate from that colony, and workers used cerumen to secure the brood cells to the floor of the OPs within hours of their transfer. However, in most cases they built other nest structures around the brood cells, such as honey and pollen pots, which made it impossible to observe stages of larval or pupal development within the cells. While there were several occasions where eye pigmentation and pupation were noted, these were insufficient to accurately determine time of commencement of the different developmental stages. The mean (n = 16) ontogenic period of *Au. australis* worker brood at 27.6ºC, was 54.8 ± 1.1 days (ranging from 49 to 63 days).

5.1.2 Longevity and life table

5.1.2.1 Materials and methods

The normal clustered brood configuration of *Au. australis* colonies made it difficult to observe emerging imagoes (final development stage). Newly emerged bees are often located deep within the cluster and the callows remain within this area for

many days. When using a fully developed colony it was difficult to obtain adequate numbers of callows to mark and monitor. Marked bees were also extremely difficult to locate within the nest structures. Frequent opening of the hive also interferes with the normal colony behaviour (see Appendix 6 & 7). This study, therefore, necessitated the removal of pupating brood from the mother colony.

Due to the small size of individual *Au. australis* workers (1.5 mm thorax), it was not possible to mark individual bees with a number. A colour combination system could be used to mark bees but this limited the sample size to 28 individuals. To increase the sample size, groups of cohorts were marked with different colour combinations. Utilising a 'closed' colony was the only way of obtaining information regarding worker mortality rates. Although the 'closed' colony did not simulate a real life situation, where workers were exposed to the risks of predation and a 'high rate of living', this study provided the optimum longevity for workers within a non-foraging colony.

5.1.2.1.1 The 'closed' colony and life table study

An initial study (commenced 12 August 2008, late winter) was conducted using a single small hive (280 x 200 x 100 mm). A piece of pupating brood (~ 50 mm x 40 mm x 30 mm), along with an unknown number (but > 50) of adult workers was dissected from the brood cluster of a strong colony. The brood structure and its occupants were placed inside the hive, along with a section (30 mm x 20 mm x 20 mm) of pollen pots. A feeder-float (see Chapter 3, Section 3.2) was placed inside the hive and replenished every two weeks. Ventilation was provided via the hive entrance, which was covered with a square (20 x 20 mm) of 0.8 mm aluminium fly wire. The hive was fitted with a 3 mm thick transparent acrylic lid, hinged at the back edge using 2 cm adhesive tape. A wooden lid was placed on top of the acrylic to exclude light. The hive was stored on a bench under an upturned Styrofoam™ box (570 x 382 x 276 mm). A thermostat probe was fixed to the hive lid and set at 25ºC. The thermostat was connected to a 230 / 240v ~50 Hz, 25 watt 'Brilliant' Reflector lamp (Crompton Lighting Pty Ltd, Chullora, NSW, Australia) and all of this was housed inside the Styrofoam™ box, to maintain hive temperature.

Each day, a 90 mm Petri dish, lined with a filter paper was placed onto a cold pack (Medi-pak Australia, East Brisbane, Qld, Australia) to chill. The hive was opened and callows were carefully removed from the box using a pair of blunt, round-tip metal forceps. Bees were grasped on both sides of the thorax and then dropped onto the pre-chilled Petri dish. They became immobile within five to 15 seconds. The Petri dish of chilled bees was examined under a 230 / 240v ~50 Hz, 20 W, halogen desk lamp (Crompton Lighting Pty Ltd, Chullora, NSW, Australia). Using a fine camel-hair paint brush, the bees were positioned and held in place with their dorsal thorax exposed. A coloured acrylic art pen (Mitsubishi Pencil Co Ltd, Japan) was used to mark one side of the thorax of each bee. A different colour was used to mark the other side of the thorax. Once all the bees in the group were marked, they were returned to the hive. Any dead bees were removed from the hive and placed in a separate Petri dish for later examination. The acrylic hive lid was closed and the halogen lamp was directed over the returned workers. Once all marked bees had revived (3.5 – 4 min), the hive was returned to the warming box. Different colour combinations were used for different days.

This ‘closed’ colony was checked for callows each day; however, new emergences were not always present. A total of 428 bees, within 28 groups, were marked over the 37-day period. Once all of the pupating brood had emerged, debris within each hive was removed with a hand-held aspirator, to allow detection of dead bees on the hive floor. Dead bees were collected and their thoracic colours were recorded daily, until no bees remained in the hive. The experimental period spanned late winter to early the next winter.

5.1.2.1.2 The ‘foraging’ colonies study

The experiment was repeated nine months later (commencing 25 May 2009, early winter) using five hive boxes. Each hive was supplied with brood from a different mother colony. Three of the five brood segments contained at least one queen cell. It was hoped the colonies might re-queen during the study. The ‘closed’ colony study had shown that callows were lighter in colour than the older workers for up to six days after their emergence. Therefore, for this study callows were marked every two or three days, to allow a larger number of bees to be marked within each group. After 19 days the five hives contained 602 marked bees, within 39 cohorts.

Hives were maintained in the bee shed (see Chapter 3, Section 3.2), at 25°C and darkness. Once all callows were marked, each hive was connected to an entrance tube which provided the colony access to external foraging. Thus, these hives were designated as 'foraging' colonies. As the colonies had external access I was unable to collect the dead workers, as they were regularly removed by the colonies workers. The colonies were examined weekly via the acrylic lids and workers' colour markings were recorded. When individuals bearing a particular colour marking were no longer observed in the hive, this date was recorded as the maximum age reached for members of that cohort (maximum longevity).

One of the five colonies successfully re-queened during this study, thus producing one queenright colony and four queenless colonies. All colonies were observed to forage, collect and store honey and pollen, although some colonies were more active than others. The experiment was terminated 249 days after its commencement, when no marked workers remained in any of the hives. The experimental period spanned early winter to mid summer. The maximum longevity for each of the cohorts within each hive was recorded and the mean of these was calculated (mean maximum longevity).

Data were tested for homogeneity of variance using Levene's test. The data were analysed by one-way ANOVA and means were compared by Tukey's HSD test in SPSS 17. The setting of significance was $\alpha = 0.05$.

5.1.2.2 Results

5.1.2.2.1 The 'closed' colony and life table study

In the 'closed' colony, dead workers were removed and examined daily and it was, therefore, possible to estimate the age of marked bees from this data. There was a high mortality rate at the beginning of the study, with 26% of the colony dying between one and eight days old. This was attributed to trauma caused during the marking process (see discussion and also Appendix 6), and these data were removed from the analysis.

Table 5.1 shows a summary of the life table for the 'closed' colony from eight to 289 days old. The columns of the table are assigned the following symbols, which are extensively used in ecological disciplines (Sakagami & Fukuda 1968; Terada et al. 1975; Krebs 2001; Grosso & Bego 2002):

x = age at death

n_x = number alive at age x

l_x = proportion surviving from the start of the life table age x (n_x / n cohort)

d_x = number dying during interval x to x + 10 days

q_x = per capita rate of mortality during the age interval x

Table 5.1 Summary, showing 50 day intervals as well as the last two days, of *Au. australis* cohort life table for the 'closed' colony.

Age in days (x)	Bees remaining alive (nx)	Proportion surviving at age x (lx)	No. dying within age interval (dx)	Rate of mortality (qx)
8	316	1.00	0	0.00
58	250	0.791	24	0.10
108	200	0.633	17	0.09
158	133	0.421	17	0.13
208	65	0.206	13	0.20
258	15	0.047	8	0.53
289	1	0.003	2	2.00
290	0	0.000	1	--

The 'closed' *Au. australis* colony demonstrated a Type II survivorship curve. The survival rate and mortality rate of the workers within the 'closed' colony indicated a steady rate of death (Figure 5.4). Over 20% of the population of the 'closed' colony lived for more than 200 days and the longest lived bee reached 289 days.

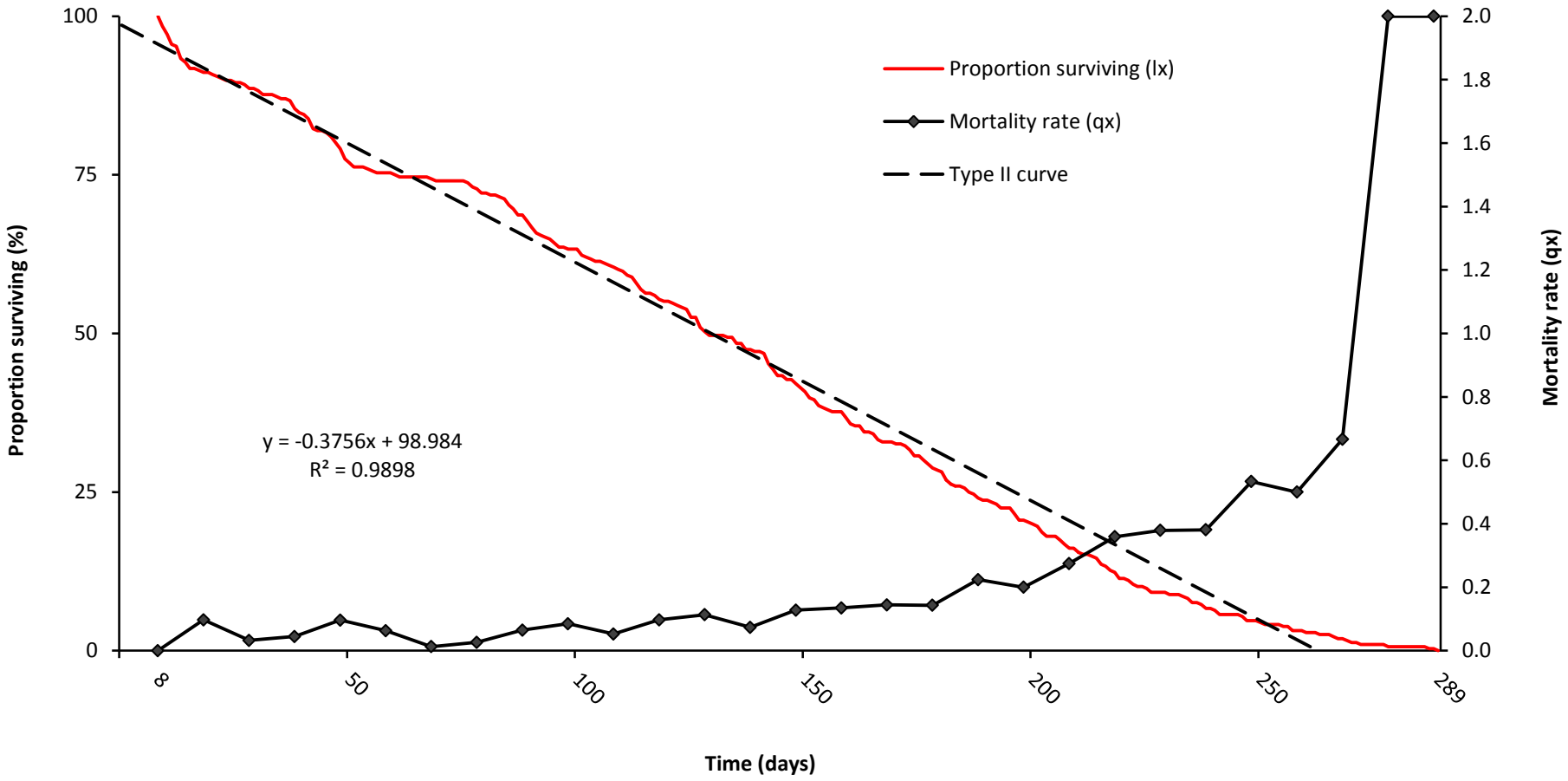


Figure 5.4 Survivorship (red) and mortality (black with marker ♦) rate curves for the *Au. australis* cohort in the 'closed' colony, with regression equation y = -0.3756x + 98.984, plotted against hypothetical Type II survivorship curve (broken line).

5.1.2.2.2 The ‘foraging’ colonies study

The mean maximum longevity of the cohorts within the ‘closed’ colony (235.9 ± 6.7 days) was significantly ($F_{5, 66} = 14.499$, $p < 0.001$) greater than that of the cohorts within the ‘foraging’ colonies (161.4 ± 6.9 days). Maximum longevity of cohorts within the colonies ranged from 119 to 289 days for the ‘closed’ colony (n = 28) and from 100 to 240 days for the ‘foraging’ colonies (n = 39).

There was no correlation between the date of emergence and longevity. There was no significant difference ($p = 0.312$) between the mean maximum longevity of the queenless, broodless colonies (161.7 ± 7.4 days) and the queen-right, brood producing colony (160.5 ± 8.4 days).

At the conclusion of the ‘foraging’ colonies study, each of the four queenless, ‘foraging’ colonies contained 10 to 100 pollen pots and two to six provisioned honey pots. During the first 85 days of the study, these colonies had also constructed between five and 38 brood cells. Of these, 43% of the cells contained viable drones. The queen-right colony had built a brood cluster ~ 70 mm in diameter and had good stores of honey and pollen. The ‘closed’ colony did not, at any stage, construct brood cells.

5.2 Discussion

This study reports on two basic biological characteristics of, *Au. australis*; the ontogenic times and longevity. The ontogenic period for *Au. australis*, of 55 days, is comparable to previously studied stingless bee species (see below) and is 2.6 times longer than that reported for *A. mellifera* (Jay 1963; Winston 1991). This large difference may be attributed to the temperature differences experienced within the brood chamber of stingless bees compared to honey bees. *A. mellifera* incubates its brood at around 35°C and if temperatures fall much below this, brood development times (21 days) are lengthened and survival rates reduced (Fukuda & Sakagami 1968; Winston 1991). Many stingless bee species do not thermoregulate their nest, and brood temperatures fluctuate with ambient temperatures (Wille 1983; Roubik 1989; Engels et al. 1995). This may be a major reason for the extended

developmental times reported here and in some other stingless bee species (see below).

The extended time required for *Au. australis* brood to reach maturity may be, in part, attributable to the low incubation temperature (27.6°C). Lengthy ontogenic periods have also been reported for other stingless bee species, including *M. beecheii* (53 days) (Moo-Valle et al. 2004), *Trigona* (*Heterotrigona*) *itama* Cockerell (46.5 days) (Salmah et al. 1996) and *Trigona* (*Trigonella*) *moorei* Schwarz (46.5 days) (Salmah et al. 1987). These cited studies were conducted at similar temperatures to the current study. There may also be seasonal variations in the ontogenic periods of stingless bee species where brood development can occur outside of a narrow temperature range. In some environments, this may give stingless bees an advantage over temperature-sensitive species. Nest thermoregulation is very energy expensive (Free & Racey 1968) and an *A. mellifera* colony can consume up to 30 kg of honey during winter, just warming the colony (Graham 1992).

The greatest diversity and abundance of stingless bee species is found in warmer climates (18 – 35ºC), between the Tropics of Cancer (23.5ºN) and Capricorn (23.5ºS) (Kerr & Maule 1964). However, several species, including *Au. australis*, can be found in more temperate regions (Michener 1979; Roubik 1989; Dollin et al. 1997; Dollin 2010c). Temperature fluctuations within the brood may lengthen ontogenic times, especially during the colder months of the year. This will impact on colony strength, especially as they come out of winter and begin foraging activity. Further investigations into the influence of temperature on brood development times would be useful in colony management.

The longevity of *Au. australis* workers, within both the ‘closed’ and ‘foraging’ colonies, is substantially greater than that of any other reported bee species (Table 5.2), with the exception of winter honey bees (Sakagami & Fukuda 1968). It could be anticipated that workers within broodless colonies would naturally have a greater longevity, as they are likely to take on the physiological characteristics of wintering bees (Maurizio 1950). There was, however, no significant difference between the mean maximum longevity of bees within the queenright colony and those within the queenless colonies. All colonies participated in foraging activities, collecting both nectar and pollen.

Table 5.2 Longevity (in days) of previously studied social bee species. * Indicates species known to demonstrate a Type I convex survivorship curve.

Species	Mean	Max	Forager age	Source
Apis mellifera (spring / summer bees)*	43	70	25 – 70	Sakagami & Fukuda 1968
Plebeia droryana	42	75	15 – 60	Terada et al. 1975
*Friesella shroffkyi**	30	-	-	Camillo-Atique 1977, cited in Simões & Bego 1991
*Melipona bicolor bicolor**	44	68	20 – 65	Bego 1983
*Melipona favosa**	40	68	20 – 70	Sommeijer 1984
Frieseomlitta languida	33	-	-	Ribeiro 1989, cited in Simões & Bego 1991
*Nannotrigona postica**	33	60	30 – 55	Simões & Bego 1991
*Plebeia remota**	-	90	30 – 90	van Benthem et al. 1995
*Melipona compressipes fasciulata**	43	80	20 - 80	Giannini 1997
Melipona beecheii	51	101	20 – 60	Biesmeijer & Tóth, 1998
*Tetragonisca angustula angustula**	21	60	15 – 55	Grosso & Bego 2002
Austroplebeia australis	Mean max 161	240	~ 40 (see Appendix 7)	Current study

It was not possible to obtain the overall mean longevity within the *Au. australis* 'foraging' cohort, which would, of course, be shorter than the mean maximum longevity. Never-the-less, a mean maximum longevity of 161 days is three times greater than the mean longevity of *M. beecheii* (51 days) the longest lived stingless bee thus-far reported. It is also much greater than the maximum longevity of *M. beecheii*, which is 101 days (Biesmeijer & Tóth 1998).

While the 'closed' colony, experimental design for the life table did not simulate a natural, actively foraging colony in a real-world situation, it was the only way to obtain daily rates of mortality within an *Au. australis* colony. As previously stated, it is not possible to individually mark a large number of *Au. australis* workers, due to their small size. In social bee colonies, bodies are removed from hives soon after death as a part of their hygienic behaviour (Roubik 1989), making it impossible to keep track of individual workers unless they are tagged with a number. In order to obtain general information pertaining to the mortality rate of *Au. australis*, it was

necessary to close the colony to facilitate collection of dead, marked workers each day. *Austroplebeia* colonies often experience extended periods of drought within their native range, where floral resources become scarce. Colonies have been reported to dramatically reduce foraging activity when floral resources are depleted. They have even been observed to close their nest entrance during periods of floral scarcity or under conditions where floral resources cannot be harvested, due to cold or wet weather (A. Beil, pers. comm., 2009; MH personal observation). The 'closed' colony in this study could, therefore, simulate some of the conditions experienced during drought, inclement weather or in winter.

Within the life table study, juvenile life stages were not taken into account. Initially, data were based on the period from emergence as an immature adult to death; however, over a quarter of the cohort died before reaching nine days old. While a high rate of juvenile death is not unusual in fish and marine invertebrates (Deevey 1947; Krebs 2001), it is not commonly observed in eusocial insects (O'Donnell & Jeanne 1995). This abnormally high rate of mortality could reasonably be attributed to trauma caused during the chilling and marking process, and has been reported by other researchers (Free & Spencer-Booth 1959). As a result, data for the first eight days were removed from the analysis and the remaining data were used to construct the life table. Improved marking techniques have been subsequently developed and are outlined in Appendix 6.

The mean (21 – 51 days) as well as the maximum (68 – 101 days) longevities of social bee species, reported in Table 5.2, are considerably shorter than the mean maximum (161 days) longevity of the 'foraging' colonies of *Au. australis*. The life table data for some of these reported species (marked with * in Table 5.2), demonstrate Type I survivorship curves. Mortality rates for these species increased as workers reached an age where they commenced foraging tasks. The mean longevity and the highest mortality rates also correlated with the age at which foraging activity peaks. For example, the highest proportion of workers in *M. bicolor bicolor, M. favosa* and *N. postica* commenced foraging activities at 44, 40 and 38 days old, and the mean longevities for these colonies were 44, 40 and 33 days, respectively (Bego 1983; Sommeijer 1984; Simões & Bego 1991). Sakagami & Fukuda (1968) reported greatly increased mortality rates in *A. mellifera* at ~ 40 days

old, which was attributed to the recruitment of a large number of foragers during the main nectar flow period. Peak foraging age also corresponded to mean longevity, of 43 days old. Lundie (1925, cited in Neukirch 1982) reported the mortality rate in *A. mellifera* workers increased from 2% as house bees to 98% as foraging bees.

The Type II survivorship curve, demonstrated in the 'closed' colony of *Au. australis*, resulted from the lack of exposure to a high 'rate of living' as well as to high-risk foraging tasks. Workers remained within the safe confines of the nest and were provided with a reliable supply of food reserves. A pattern of senescence can be seen in the 'closed' colony (O'Donnell & Jeanne 1995), with over 20% of the cohorts living for at least 200 days. Although this model is not typical of a natural foraging colony, it does demonstrate the potentially attainable life span of workers within this species. Extended longevity is also demonstrated in the 'foraging' colonies with maximum longevity ranging between 100 and 240 days old. Living in environments which regularly experience prolonged periods of drought, with scant or absent floral resources, promotes evolutionary adaptations to safeguard species against dying out. Workers remain within the safe confines of the nest, experiencing only low levels of molecular damage (Brys et al. 2007). Low mortality rates due to senescence can continue for long periods of time. The life table, although modified, demonstrates a constant, low mortality rate for a large proportion of the colony.

Extended longevity is seen in social species, where parental care is prolonged (Carey & Judge 2000; Carey 2001; Carey & Judge 2001) and is also demonstrated in some insects (e.g. cave beetles, orchard bees, monarch butterflies, African locusts (Carey & Judge 2001)) which inhabit areas with unpredictable and scarce resources. Both of these phenomena are experienced by *Au. australis* workers. The extended longevity of individuals ensures that some of the community is still alive when food resources finally become available again. If the age of mortality correlates with the age at which workers take up high-risk, energy-expensive tasks, the extended longevity of cohorts within the 'foraging' colonies suggests that some workers may never leave the nest. This has been reported in other social bees (Biesmeijer & Tóth 1998; Page & Peng 2001). O'Donnell and Jeanne (1995) showed that patterns of senescence in workers of social insect communities can be profoundly affected by delaying the performance of high-risk tasks. The age at which *Au. australis* workers commence

foraging activity is around 40 days old (see Appendix 7). This is quite late compared to other species (Table 5.2) and may contribute to the overall extension of their life span.

5.3 Key findings

- This is the first study that reports the ontogenic period for a cluster-building species of stingless bee.
- The ontogenic period for *Au. australis*, of ~ 55 days, is similar to those reported for other stingless bee species.
- Long ontogenic periods, compared to *A. mellifera*, may be linked to low incubation temperatures.
- Ambient temperatures may affect ontogenic times.
- *Au. australis* workers have an extended longevity, mean maximum of 161 days old, compared to those reported for other social bee species.
- Data from these studies demonstrate the life cycle of *Au. australis* workers, from egg to death, could feasibly extend to as long as one year. This gives colony offspring the opportunity to benefit from available food resources, albeit unreliable, spanning all seasons.

CHAPTER 6

Colony dynamics and forager behaviour

This chapter contains five sections (viz., 6.1 - 6.5). These sections were considered too small to include as separate chapters and have, therefore, been incorporated into one. The sets of information contained within these sections do, however, complement and support each other. An overall discussion and summary pertaining to the entire content is given at the end of the chapter.

6.1 Climatic factors influencing flight activity

6.1.1 Introduction

Endotherms are able to regulate their body temperature independent of ambient temperature (OED 2009). Some insect species are capable of generating body heat when the environmental temperature is low. Pre-flight warm-ups, through activation of thoracic muscles resulting in generation of body heat, have been reported in species of Lepidoptera, Hymenoptera and Orthoptera (Heinrich 1974). Endothermy enables animals to function and forage at low temperatures. Bumblebees and honey bees are capable of flight at temperatures as low as 5°C (Heinrich 1972b) and 13°C (Graham 1992), respectively, by utilising energy generated by warm-up activity. Ectotherms or poikilotherms are unable to generate enough body heat to maintain a constant temperature and are dependent on the temperature around them (Roubik 1989). They become inactive at low temperatures and are usually stimulated at high temperatures (Mellanby 1939). Stingless bees occur mainly in the tropics and subtropics where ambient temperatures seldom fall below 15°C (Roubik 1989). Generation of additional body heat would be unnecessary in these environments. On the other hand *Au. Australis*, occurs in areas with a wide range of climatic conditions and little is known about its environmental requirements. It is important to understand the climatic factors that influence the flight and foraging activity of any potential crop pollinators (Corbet et al. 1993). This is especially important in cases where the colonies are relocated to areas outside their natural range.

This study was set up to assess the abiotic factors which might influence the flight, and therefore foraging activity, of *Au. australis* colonies.

6.1.2 Methods

Five *Au. australis* colonies, housed in OATH boxes (H1 – H5), were used for this study, which was conducted in summer to early autumn (January – March) 2008. All colonies contained strong populations. Each hive was fitted with a 10 cm long temperature probe connected to a Tinytag Plus 2 data logger. The tip of the probe was situated inside the box but not within any hive structures and temperatures were recorded at 30 min intervals. Each hive was positioned on a domestic decking located at Blaxland, NSW, Australia (33°45'S, 150°36'E) facing north, 1.5 m above the ground and 1.2 m apart. Hives were protected from direct rain and sun by a decking roof but were exposed to ambient temperatures and wind during the study. A temperature and relative humidity (RH) data logger was also positioned on the decking, to record ambient conditions. The front entrance of each hive was fitted with a 5 cm long piece of silicone tube (10 mm ID) to aid observation of bee activity at the hive entrance. A digital camcorder (JVC Hard Disk Camcorder 'Everio', Victor Company of Japan, Ltd) was positioned at the entrance of one of the *Au. australis* hives so that the entrance tube could be clearly monitored for bee entry and exit. The entrance activity was recorded for two to three minutes, after which time the camcorder was moved and repositioned to monitor and record the next hive entrance. Repositioning of the camcorder continued until the entrance activity of each hive was recorded for two to three minutes, for that hour. This routine commenced at 09:30 and finishing between 18:00 and 19:30 EADST (17:00 in March). Monitoring and recording of hive entrances was carried out as often as possible; however, the weather conditions during the summer of 2008 were unusually inclement and the number of warm sunny days were minimal.

At the beginning of each hour of observation, the light intensity of the open, unshaded area within the vicinity of the decking was measured using a LI-COR 250A light meter. The recorded video footage was saved to DVD and later viewed using Nero 7 Premium software. The number of bees entering and exiting the hive over the two minute recording periods was counted and recorded. This was repeated

for all recordings. Data pertaining to light intensity, RH, ambient and in-hive temperatures and bees entering and exiting the hive were analysed by correlation and multiple linear regression using SigmaPlot 11 (Systat Software Inc., www.sigmaplot.com).

6.1.3 Results

The strong correlation between the number of bees entering (In) and exiting (Out) the hive (n = 415) was high (0.985), thus supporting the assumption that most of the workers leaving the hive return to the hive. Multiple linear regression weakly supported ($R^2 = 0.405$) the prediction that in-hive temperature, light and RH significantly contribute to entrance activity (Out). 'Out' was considered to be more useful and accurate than 'In', as climatic factors would influence bees more in their decision to leave the hive than those bees which had previously left. In-hive temperature ($p < 0.001$) (Figure 6.1a) and light ($p < 0.001$) (Figure 6.1b) had significantly positive effects on entrance activity, whereas RH ($p = 0.029$) (Figure 6.1c) had a negative influence.

Entrance activity commenced when in-hive temperatures were ≥ 18.6°C, (see equation for entrance activity and in-hive temperature in Figure 6.1a) and the corresponding ambient temperature was ≥ 20°C (n = 34). Optimal flight activity was recorded during periods where the in-hive temperature was ≥ 21°C and the ambient temperature was ≥ 26°C. Although light influenced entrance activity, optimal flight activity was observed within a wide range of light intensities (512 – 2070 µmol. m^{-2} s^{-1}) (Figure 6.1b). On fine days, flight activity continued as long as the ambient temperature was above 20ºC, until dusk, when light intensity became very low (27 µmol m^{-2} s^{-1}), and then all flight activity ceased. Flight activity did not take place during 'cloudy' (≥ 6 oktas) or rainy periods (n = 24). On one day, entrance activity had begun to increase with ambient and in-hive temperatures; however, activity ceased with a sudden increase in cloud cover, which was followed by a storm. Although the rain stopped within an hour of flight cessation, the overcast conditions continued and flight activity did not recommence for the rest of the day, despite the

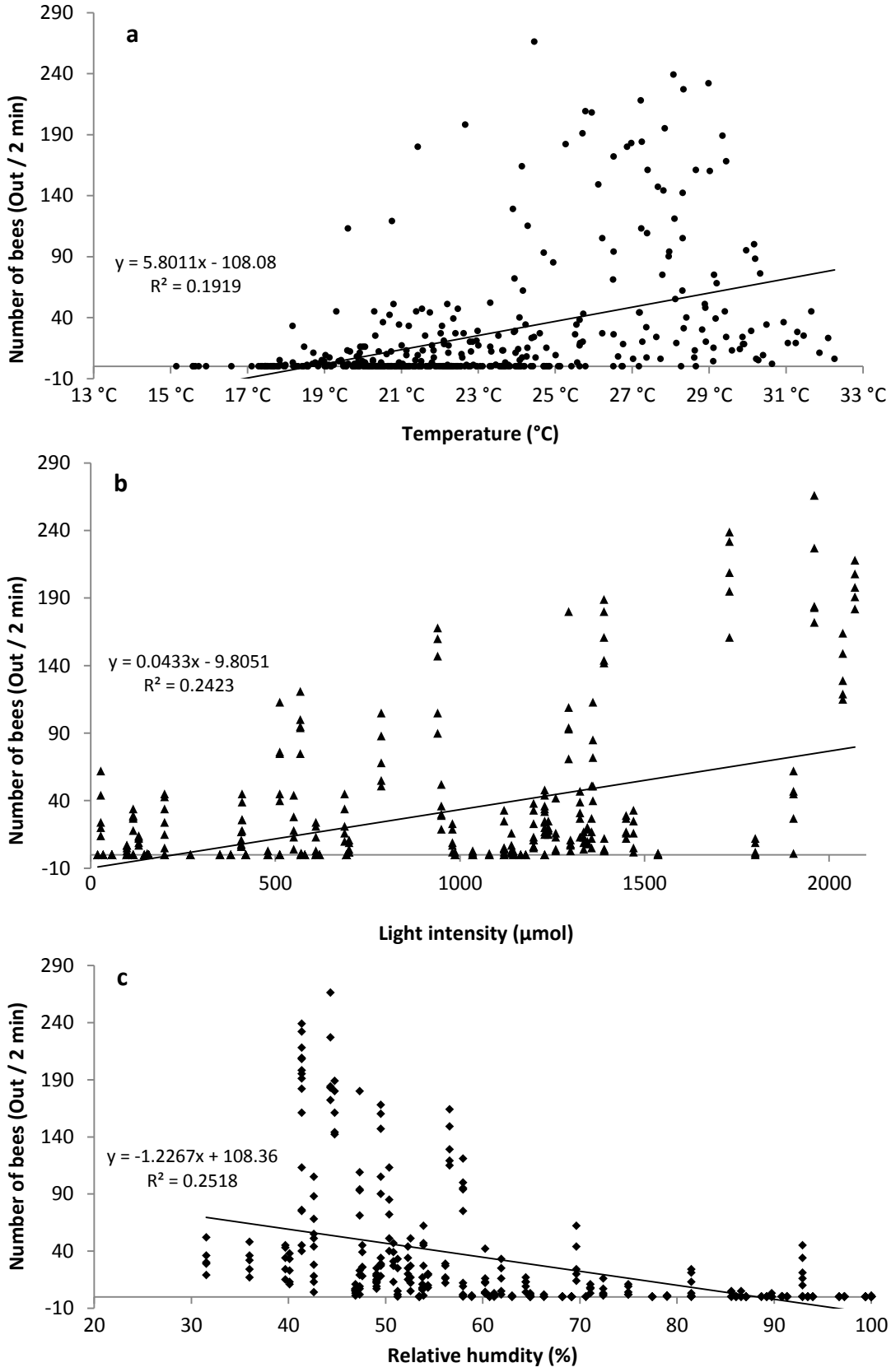


Figure 6.1 Entrance activity (number of bees exiting the hive) compared in-hive temperatures (a), light intensity (b) and relative humidity (c). Temperatures were mostly too low before 09:30 for flight activity to commence.

in-hive and ambient temperatures remaining above the threshold for the commencement of entrance activity.

Pollen collection was observed throughout the day, commencing at 10:30 and ceasing at dusk. Resin collection was seldom observed during this study. Removal of debris from the hive commenced in the early afternoon and continued until dusk.

6.1.4 Discussion

This short study showed that ambient and in-hive temperatures have the greatest influence on flight activity commencement in *Au. australis*. The ambient temperature threshold for flight activity of ≥ 20°C is comparable to the daytime temperatures experienced in much of its native range (BOM 2010a). These temperature thresholds are similar to those reported for other Australian stingless bees in the genus *Trigona* (Heard & Hendrikz 1993). Also, similar to *T. carbonaria*, *Au. australis* continued its flight activity until dusk, so long as ambient temperatures remained above 20°C (Heard & Hendrikz 1993).

Au. australis' temperature threshold for flight activity is also similar to most of the studied *Plebeia* spp., with the exception of *Plebeia pugnax* Moure (15°C) (Table 6.1) (Hilário et al. 2001). These bee species are similar in size (4 – 7 mm long) (Michener 2000) to *Au. australis* (4 mm / 6.5 mg) and *T. carbonaria* (4 mm / 4.5 mg). This is in contrast to the much larger *M. bicolor bicolor* (15 mm / 70 mg, Hilário et al. (2003)) and *A. mellifera* (15 mm / 93 mg, Winston (1991)) which commence flight activity at 11°C and 13°C, respectively. Large, dark bees absorb more solar radiation than small, lighter coloured bees, giving them a clear advantage at the beginning of the day. They also generate more heat during flight and their pubescence aids heat retention (Willmer & Unwin 1981). Some larger bees also utilise pre-flight warm up behaviour (Heinrich 1974).

Although smaller bees may be too cold to fly at the beginning of the day in temperate areas, they are less susceptible to overheating in the middle of the day. Their bodies have a greater surface area to volume ratio, which allows generated heat to dissipate through internal ventilation and evaporation (Digby 1955; Willmer & Unwin 1981). Small, black bees such as *Au. australis* absorb radiated heat and they often bask at

their nest entrance prior to flight, to help warm up at the beginning of their flight period (Heinrich & Esch 1994; MH personal observation). Midday foragers can also take advantage of the increases in nectar concentration as ambient temperatures increase and water evaporates from nectaries, due to vapour pressure deficit increases (Corbet et al. 1979).

Table 6.1 Threshold and optimal ambient temperatures for flight activity of previously studied stingless bee species.

Species	Temperature threshold	Optimal temperature	Source
Plebeia remota	16 – 18°C	22 – 29°C	Imperatriz-Fonseca et al. 1985, cited in Hilário et al. 2001
Plebeia saiqui Friese	16 – 18°C	26 – 32°C	Oliveira 1973, cited in Hilário et al. 2001
Plebeia emerina Friese	16 – 22°C	21 – 27°C	Kleinert-Giovanni 1982
Plebeia droryana Friese	17 – 19°C	22 – 32°C	Oliveira 1973; Hilário et al. 2001
Plebeia pugnax	14 – 15°C	20 – 34°C	Hilário et al. 2001
Trigona carbonaria	18°C	26 – 30°C	Heard & Hendrikz 1993
Melipona bicolour	11°C	17 – 22°C	Hilário et al. 2000
Austroplebeia australis	20°C	≥ 26°C	This study

Au. australis is well adapted to its native environment, where solar radiation is moderate to high (BOM 2011d) and average daytime temperatures range from 19.8 to 26.1ºC (BOM 2012c). It is speculated that freezing temperatures do not limit stingless bee distribution; it is more the duration of low daytime temperatures and food availability that influence this (Roubik 1989). While temperatures remain below the threshold for flight, floral resources, even when present, become unavailable to colonies. During this study, flight activity also ceased with the development of heavy cloud cover and rain, thus making floral resource unavailable for harvest. While temperatures may be conducive to in-hive activities such as brood rearing and general hygienic behaviour, the external climate factors may prevent the colony from collecting resources. This results in a depletion of food stores and may lead to colony starvation.

The stimulus triggering whether bees within the colonies of *Au. australis* commence flight activity is dependent on many factors. These factors and the behaviour associated with them have been seen in this and other studies reported in this dissertation (Chapter 3). If the in-hive temperature is > 18ºC, colonies begin to remove their entrance curtain at sunrise, even though light intensity remains low (Chapter 3, section 3.5.2.2). When ambient temperature reaches 20ºC, bees begin to leave the hive. Flight activity increases with increasing ambient temperatures, with optimal flight levels occurring at temperatures > 26°C. At around mid-afternoon, the number of bees leaving and entering the hive begins to decline, until flight activity ceases at dusk. Cloud formation and increased RH result in a reduction or complete cessation of flight activity.

While the presence of light must influence the commencement and cessation of flight activity, light intensity does not appear to affect the level of activity in *Au. australis* as much as temperature does. Flight activity was low during periods of high RH, but this is more likely to be due to the negative correlations between temperature and RH, during this study. Floral resource availability was not measured, as it was beyond the scope of this study. It is speculated, however, that the presence of resources and level of abundance would also influence the flight activity of *Au. australis* foragers.

This study clearly demonstrated that inclement weather and low temperatures adversely affect *Au. australis* flight activity.

6.2 Nest temperature and colony dynamics

6.2.1 Introduction

Nest thermoregulation in social insects has been widely studied (reviewed by Jones & Oldroyd 2006), and colonies use two main mechanisms to maintain brood temperatures. Passive mechanisms include the orientation and architecture of the nest. These mechanisms provide a buffering effect against environmental extremes and modify the internal nest conditions. Active mechanisms refer to activities performed by colony members which manipulate the nest temperature. These include wing fanning, to cool the nest, and activation of thoracic flight muscles to generate

heat (Heinrich 1974; Heinrich & Esch 1994; Jones & Oldroyd 2006). The ability to cool or warm the nest enables colonies to remain active during periods of ambient temperature extremes. It also enables the colony to maintain brood production, even during the cooler seasons, when most solitary insects are going into hibernation. The brood chamber is the heart of the nest and development of offspring drives the colony's life cycle. Stabilisation of nest temperatures facilitates brood incubation and development throughout the year, thus giving social insects an advantage over many diapausal solitary species.

Some endothermic social insects can utilise the energy generated by thoracic muscle activation to assist in temperature regulation for brood incubation. *Bombus* spp. can maintain the brood clump at between 30 and 32°C while ambient temperatures range from < 10 to 24°C (Heinrich 1974; Michener 1974). *A. mellifera* is renowned for its ability to thermoregulate brood temperatures between 34 and 37°C at ambient temperatures ranging from -40 to 40°C (Simpson 1961; Winston 1991; Graham 1992; Jones & Oldroyd 2006). Both *Bombus* spp. and *A. mellifera* naturally occur in temperate climates, thus necessitating incubation of developing brood.

Stingless bees mostly occur in tropical regions (Sakagami 1982), where average annual temperatures range from 20 to 30°C (Ritter 2006). Stingless bees are not endotherms and are seldom found outside the subtropics (Sakagami 1982). There are, however, a few species that occur in temperate zones, including *Au. australis*. Macías-Macías et al. (2011) reports that *Melipona colimana* Ayala, from the tropical highlands of Mexico (elevation 1,500 m), displays an ability for homeostatic control. When exposed to temperatures as low as 7°C, adult workers consume nectar, which fuels increasing heat generated in the thorax (> 20°C). Most Meliponinae do not actively generate heat and are, therefore, extremely susceptible to the fluctuations of the local climate. The selection of suitable nesting sites is important for nest temperature regulation (Roubik 1989).

Some stingless bee species, e.g. *Trigona eburnensis* Darchen (Darchen 1973), *Trigona cilipes* Fabricius and *Trigona pallens* Fabricius (Roubik 1989), occupy subterranean cavities beside termite or ant nests, taking advantage of the heat generated by the two million-strong colony (Rosengren et al. 1987; Abe et al. 2000).

However, most stingless bees choose large tree cavities in which to construct their nests (Eltz et al. 2003; Sung et al. 2008), which provide considerable insulation against ambient temperature extremes (Greves 1964). Combined with the nest modifications built by the colony, this enhances the intranidal microclimate.

Most species line the nest with batumen sheeting, or at least seal the ends of the cavity with batumen plates (Michener 1974; Roubik 1989). Batumen is a hard shell constructed from resin, wax, plant material and dirt. It seals and fortifies the nest against the weather as well as against intruders (Michener 1974; Roubik 1989). The brood is usually centrally located and constructed in horizontal layers or spirals. These are connected by short cerumen pillars and the stack of combs contain hundreds to thousands of developing, metabolising offspring. This heat source is often surrounded by one or more layers of involucrum, a thin cerumen sheet, providing layers of insulating air pockets. Involucrum is an important part of over-wintering protection for the colonies (Wille & Michener 1973; Engels et al. 1995; Jones & Oldroyd 2006). Outside these layers are the pollen and honey stores, adding more layers of insulative structures (Figure 6.2) (Roubik 1989; Michener 2000). Some species, such as *Melipona rufiventris* Lepeletier and *M. seminigra*, construct the brood chamber at the base of the nest cavity. Metabolic heat generated by developing brood and clustering adults spreads upwards into the rest of the nest, creating a thermal gradient (Roubik & Peralta 1983).

Similar to honey bees, stingless bees store pollen and honey in compartmentalised areas; however, stingless bees will not place their stores in removable frames. Pots of pollen are stacked near the nest entrance and honey pots are constructed in several areas of the nest and are different in shape and size (Figure 6.3). As such, it is difficult to easily estimate the area of stores, by using a brood / stores measuring tool as is done in honey bee management (Pokhrel et al. 2006).

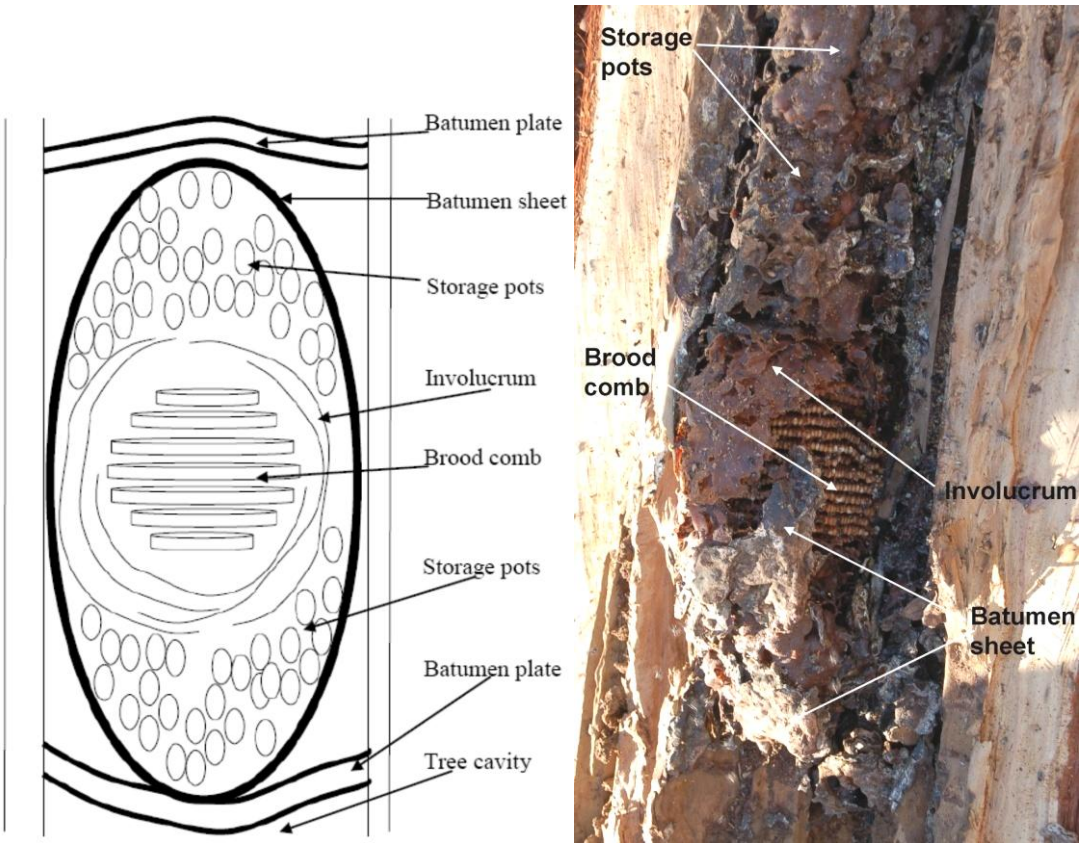


Figure 6.2 Schematic diagram (left) of 'typical' stingless bee nest structures within a tree cavity, (adapted from Michener 2000) and a *T. carbonaria* nest showing similar nest structures (right).

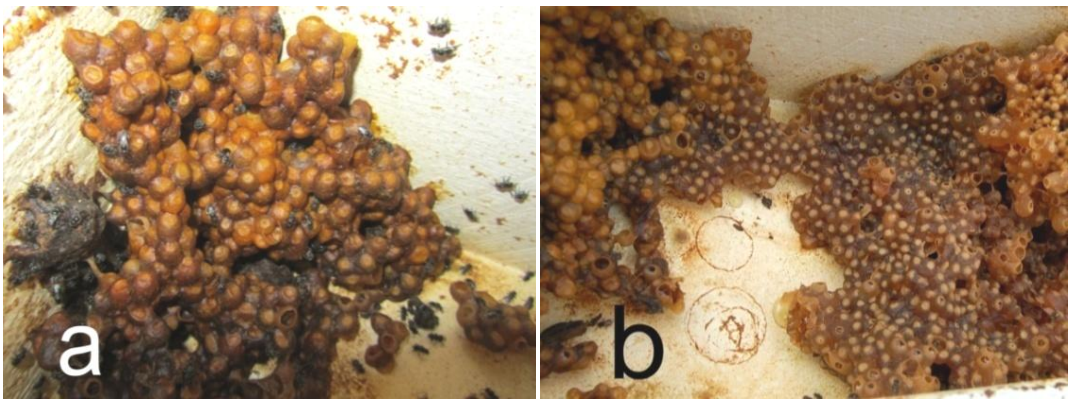


Figure 6.3 Pollen pots (a) stacked near hive entrance and multiple stores of honey (b) within a hive.

Optimal brood temperatures for stingless bees are considered to be between 28 and 36°C (Nieh & Sánchez 2005). Zucchi & Sakagami (1972, cited in Neih & Sánchez 2005) report that *T. spinipes, M. rufiventris, Scaptotrigona depilis* Moure and

Melipona quadrifasciata anthidiodes Lepeletier can maintain brood temperatures within this range. *M. seminigra, M. rufiventris* and *S. postica* are also capable of maintaining the brood chamber above the nest cavity and ambient temperatures (Roubik & Peralta 1983; Engels et al. 1995). Brood temperatures of *M. beecheii* are kept fairly stable, relative to ambient (Moo-Valle et al. 2000); however, ambient temperatures reported in these studies did not drop below 14°C, except for *Trigona ventralis hoozana* Strand (Sung et al. 2008) (Table 6.2).

Table 6.2 Species of social bee reported to regulate nest regulation capabilities. Colony populations are estimates from natural nest capacity.

Species	Colony population	Ambient (°C)	Brood temp (°C)	Source
Apis mellifera	12,000 – 20,000 workers	-40 – 40	30 – 37	Simpson 1961; Fahrenholz et al. 1989
Bombus vosnesenskii Radoszkowski	Incubating queens only	18 – 24	30 – 32	Heinrich 1972
Trigona spinipes *Scaptotrigona depilis* *Melipona quadrifasciata* *Plebeia droryana* *Frieseomelitta varia* Lepeletier *Leurotrigona muelleri* Friese	5,000 – 180,000 - - - - -	15.5 – 28	35 25 – 32 25 – 32 20 – 29 19 – 28 19 – 28	Sakagami 1982
Melipona seminigra	1,200 workers, 5 – 10 L brood (2 – 3,000 cells)	23 – 29	30 – 32	Roubik & Peralta 1983
Melipona rufiventris	1,500 workers, 7 – 14 L brood (3 – 6,000 cells)	23 – 29	30 – 32	Roubik & Peralta 1983
Scaptotrigona postica	20,000 workers	20 – 32	28 – 35	Engels et al. 1995
Melipona beecheii	300 - 750 workers, 600 – 850 mL brood (300 – 750 cells)	18.2 – > 34	25.6 – 34	Moo-Valle et al. 2000
Tetragonisca angustula	5,000 workers, 1,000 mL	14 – 24	25.4 – 30.3	Torres et al. 2007
Trigona ventralis hoozana	10,000 workers, 12,000 cells	8.0	24 – 31	Sung et al. 2008
Trigona nigra pauper Provancher	2,000 workers, 1.4 L	17 – 22	22 – 28	Torres et al. 2007

The native range for *Au. australis* spans the districts of Fitzroy and Central West Qld (23°31'S), through the Darling Downs and into northern NSW (31°04'S). The climate in the most southern range is vastly different to the regions of Central Qld and is considered to be marginal for colony survival (A. Dollin, pers. comm., 2010). *Au. australis* is also thought to be less tolerant of temperature extremes than *T. carbonaria*, which can occur as far south as Bega, NSW (36° 40'S) (Dollin 1997a). Never-the-less, *Au. australis* naturally occurs in areas which experience very cold winters and hot, dry summers (Table 6.3).

Table 6.3 Climate details of areas where *Au. australis* colonies naturally occur (*) and areas to which experimental colonies had been relocated. Weather data for Tara and Blaxland were obtained from the closest weather station. Mean minimum and maximum temperatures are long term average mininum and maxinum temperatures during a calendar month (BOM 2012c).

Town	Location	Mean annual rainfall (mm)	Long term mean min and max temperatures (°C) (20 – 50 yr)		Hottest daily max and coldest daily min (°C) for 2010		Elevation (m)
			Summer (January)	Winter (July)	Max	Min	
Warwick*	28.22° S 152.03°E	690	17.3 – 29.8	3.4 – 17.4	39.2	-4.0	475
Tara* (Dalby)	27.18°S 151.26°E	676	18.5 – 32	4.1 – 18.7	40.6	-2.9	344
Inverell*	29.46°S 151.06°E	760	16.3 – 29.5	3.5 – 15.1	36.5	-2.6	584
Richmond	33.35°S 150.45°E	810	17.4 – 29.6	3.6 – 17.2	43.3	-4.8	28
Blaxland (Springwood)	33.45°S 150.36°E	1090	16.6 – 31.1	4.9 – 16.5	40.6	0.0	229

If *Au. australis* is to be considered as a potential crop pollinator, it is important to understand the conditions under which colonies will survive. This study aimed to increase understanding of the strategies used by *Au. australis* to survive climate extremes. I investigated whether colonies thermoregulate the brood chamber by cooling or warming mechanisms. Brood cluster production or cessation was monitored, as well as the presence of reproductives. Pollen and honey stores were

also monitored. Again, as a potential crop pollinator, it was important to ascertain the ability of the colonies to function in artificial hives during these conditions.

6.2.2 Materials and methods

In October 2009, six colonies of *Au. australis* were transferred from tree hollows into Beil hives (see Chapter 3, Section 3.2) at Tara, Qld, (27°16'S, 150°28'E). Hives were transported to Blaxland, NSW, and positioned on mesh shelving, underneath a north-facing, domestic decking, between 0.5 and 1 m above the ground (Figure 6.4). The northerly aspect of the decking provided the hives with full shade in spring and summer and sun in autumn and winter.

Figure 6.4 *Au. australis* hives, protected from severe weather elements but exposed to ambient conditions.

On 28 November 2009, a 4 mm diam. hole was drilled into the side of each hive box and a 100 mm long 'Fast response thermistor probe' (PB-5002-1M5) was carefully passed through the hole and secured into place with reusable adhesive and tape. The end of the probe was positioned so that the tip sat in the brood cluster. The probe was connected to a Tinytag Plus 2 data logger and the data logger was placed in a snap-lock bag to provide protection against moisture. This process was repeated for the remaining five hives. Three of the hives were also fitted with a 50 mm long probe connected to a Tinytag Ultra 2 (TGU-4020). The tip of these probes was positioned

within the hive cavity, but not within any hive structures. This was to give a comparative hive cavity temperature vs. brood temperature. A single Tinytag data logger was placed in a snap-lock bag and hung from the mesh shelving, in the shade, to record ambient temperatures. Hives were also fitted with 3 mm thick clear acrylic covers, underneath the hive lids, to allow non-invasive observation of colony activities throughout the 12-month study. This also allowed visual inspection of the thermistor probes, to ensure they remained positioned within the brood clusters throughout the study period. Assessment of the colonies was carried out on fine, sunny days whenever possible. This enabled the hive to be opened when necessary to more easily view nest structures and colony populations.

It was not always possible to see the queen within a colony but the presence of a queen was confirmed for all colonies, over the four-week period, prior to commencement of the study. Active brood production was confirmed at the beginning of the study and whenever the brood cluster could be observed. At times, during the coldest months, it was not possible to see the leading edge of the brood due to construction of involucrum layers over the brood chamber (Figure 6.5). The involucrum was not disturbed.

Figure 6.5 Involucrum brood-coverage of 30% (a) (late February) and > 90% (b) (late April).

At the commencement of the study, and at the end of each subsequent month, the diameter of the brood clusters was measured, the involucrum coverage of the brood was estimated and pollen and honey stores were assessed and rated. The size of the

pollen and honey pot clusters was assessed visually. Approximate area and volume were estimated and the stores were given a rating of one to five (Table 6.4). Hives were also checked for the presence of the queen, queen cells, virgin queens, callows and drones.

Table 6.4 Approximations of honey and pollen pot volumes, based on area.

Dimension examples (approximation only)	Volume (mL)	Volume ranges (mL)	Honey / pollen store rating
40 x 40 x 80 mm	128	< 200	1
40 x 80 x 100 mm	320	200 – 350	2
90 x 100 x 50 mm	450	350 – 500	3
200 x 130 x 20 mm	520	500 – 650	4
130 x 130 x 40 mm	676	650 – 800	5

6.2.3 Results

Unfortunately, the 'ambient temperature' data logger malfunctioned on 13 January 2010, and this did not become apparent until the conclusion of the study. There is no weather station at Blaxland, to provide comparative data. None-the-less, there was ambient temperature data available from 27 December 2009 to 12 January 2010. During this time the cavity temperature tracked the ambient temperature almost exactly (Figure 6.6). The mean difference between the cavity and ambient temperatures (n = 768) was 0.43°C. Ambient temperature was 0.32°C above the cavity temperature for 17.32% of the time. Cavity temperature was > 2.0°C above the ambient temperature on three consecutive occasions (0.39%) on 5 January 2010, during which time there was a rapid drop in ambient temperature due to the passage of a cold front through the area. The slight time lag between ambient and cavity temperatures was due to the time taken for the heat to penetrate the insulative hive walls. After analysis of the data it was concluded that, under the circumstances, the hive cavity temperatures were a suitable surrogate for ambient temperature.

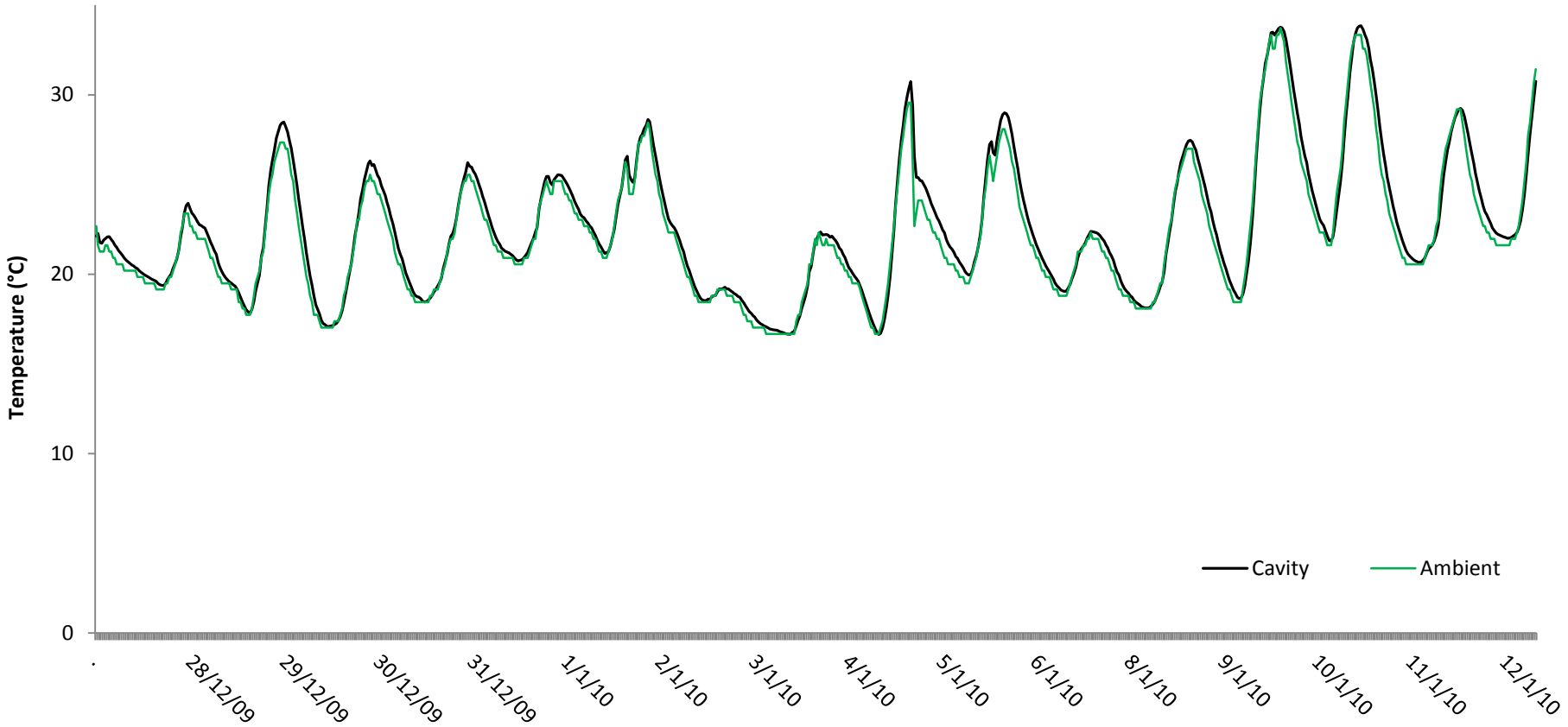


Figure 6.6 Ambient and hive cavity (n = 3) temperatures measured during early stages of study period, from 27 December 2009 – 12 January 2010.

During the 12-month study, hive cavity temperatures ranged between -1.25 and 37.16°C and within the brood chamber they ranged between -0.03°C and 37.7°C. Based on the min / max temperature ranges (Figure 6.7), cavity and brood temperatures closely mirrored each other throughout the year, especially within the lower temperature ranges (Figure 6.7b, Figure 6.8a & Figure 6.9a). Brood temperature remained at least 0.48°C warmer than the cavity. In winter, heat generated by the brood increased substantially (up to 9.69°C warmer) at temperatures ≥ 15°C, compared to temperatures < 15°C (which were only 0.48 – 3.89°C warmer) (Figure 6.8). During the most active months (October / November), brood temperature was up to 15.19°C warmer than the cavity temperature (Figure 6.9 & Figure 6.10).

Within a 24 hour period, the brood and cavity temperatures fluctuated considerably, with brood temperatures lagging 30 minutes to three hours behind the cavity temperatures (Figure 6.11). Evidence of diurnal fluctuations were observed even when cavity temperatures were > 20°C (Figure 6.11a); however, the temperature difference between the brood and the cavity was greatest at night during October and November (Figure 6.10).

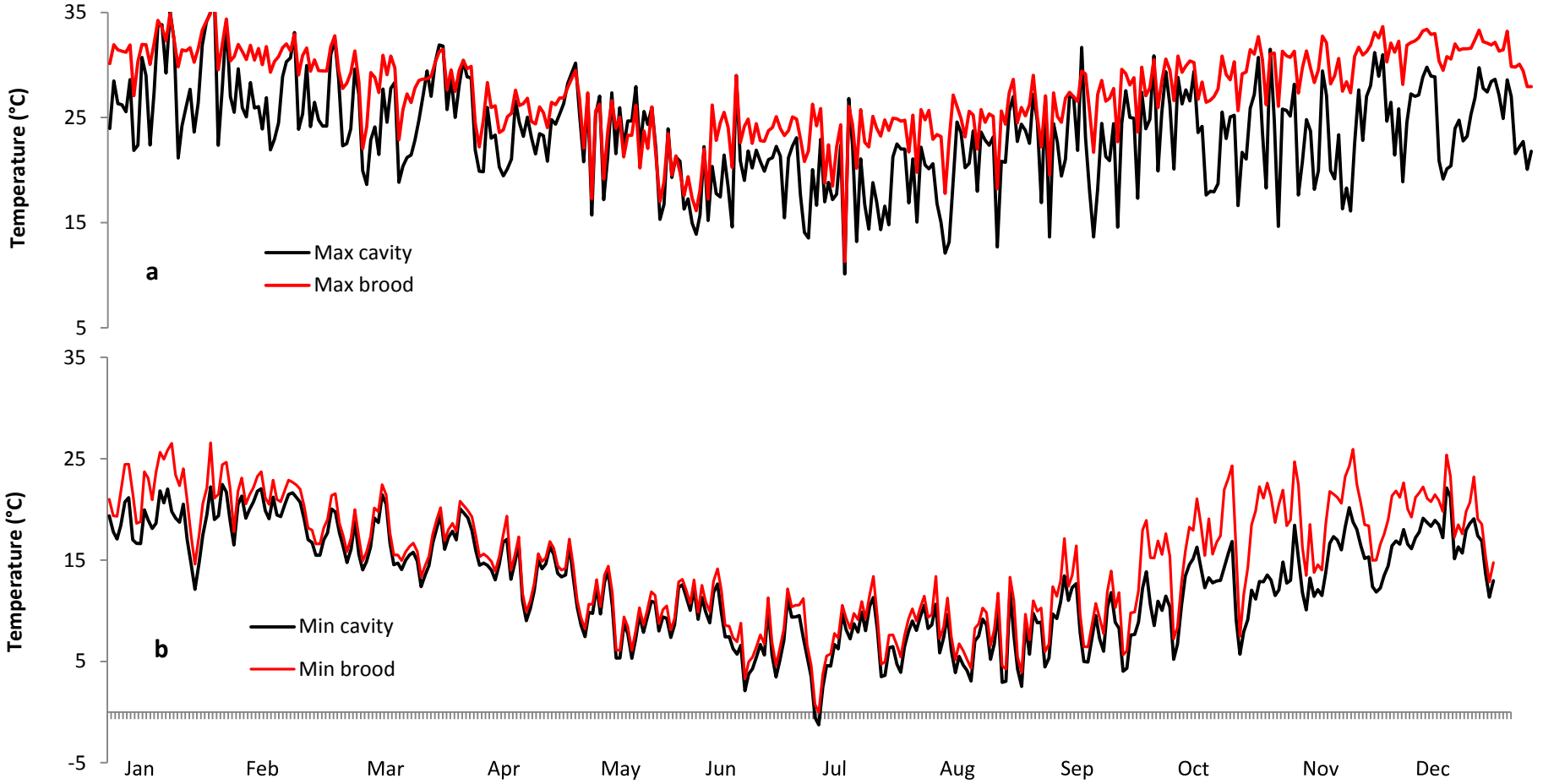


Figure 6.7 Daily minimum and maximum temperatures detected in the *Au. australis* brood (n = 6) (red) and hive cavity (n = 3) (black) over the 12 month period. Brood temperatures closely tracked cavity temperatures within the minimum ranges during March to September (b) but were less similar within the maximum ranges throughout much of the year, 2010 (a).

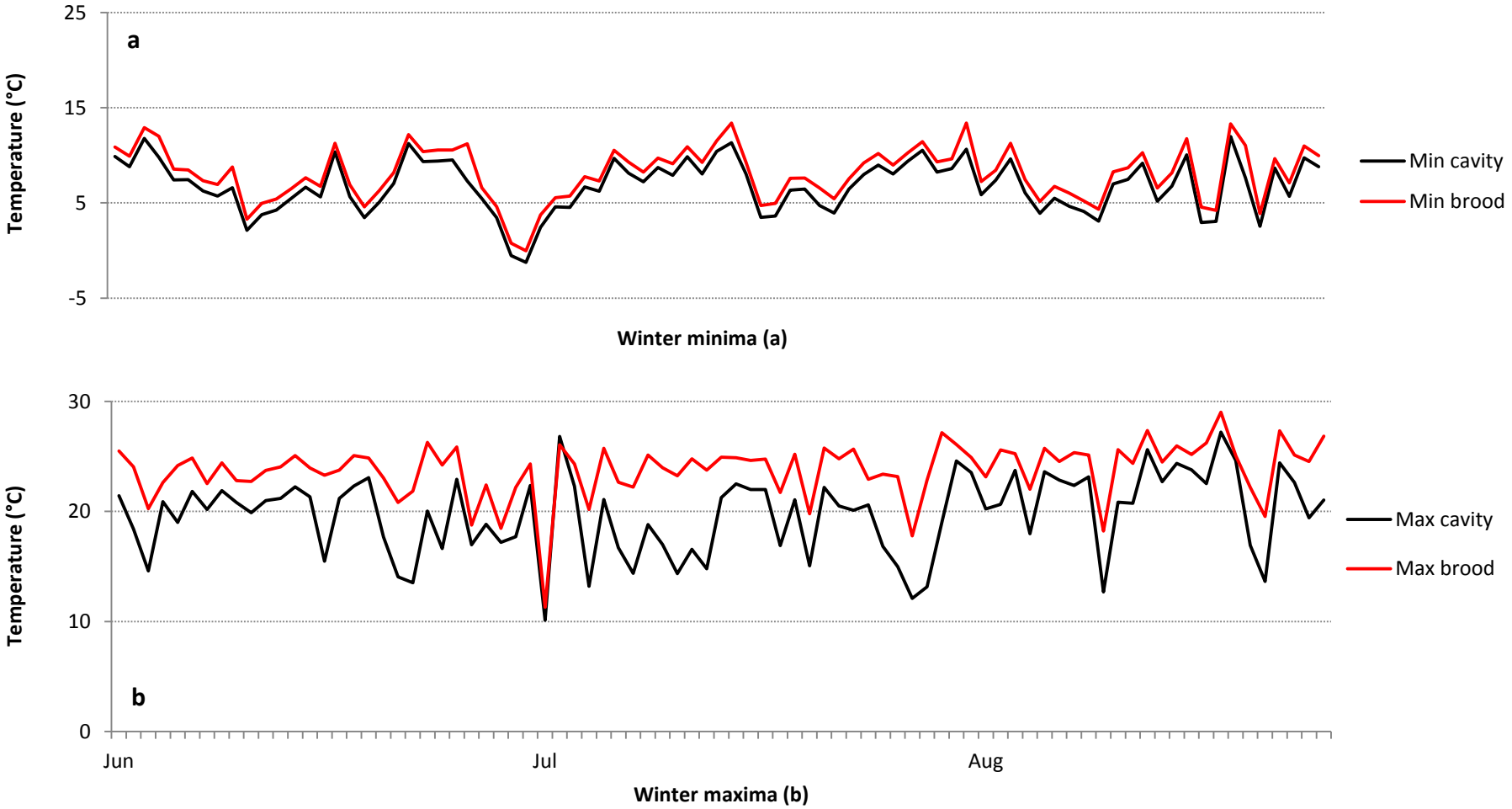


Figure 6.8 Daily minimum (a) and maximum (b) temperatures of the *Au. australis* brood (n = 6) (red) and hive cavity (n = 3) (black) during winter, 2010.

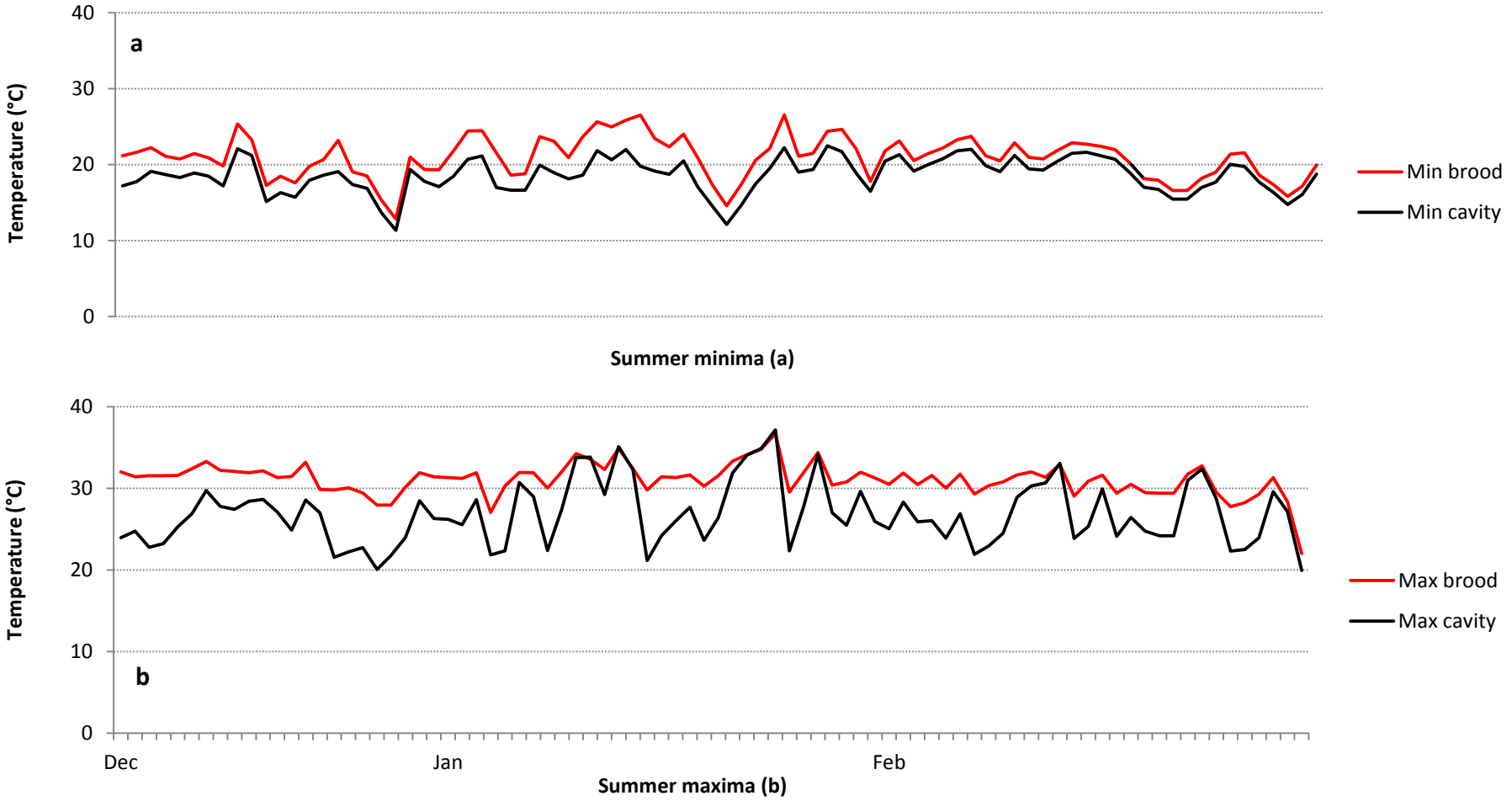


Figure 6.9 Daily minimum (a) and maximum (b) brood (n = 6) and cavity (n = 3) temperatures during summer, 2010.

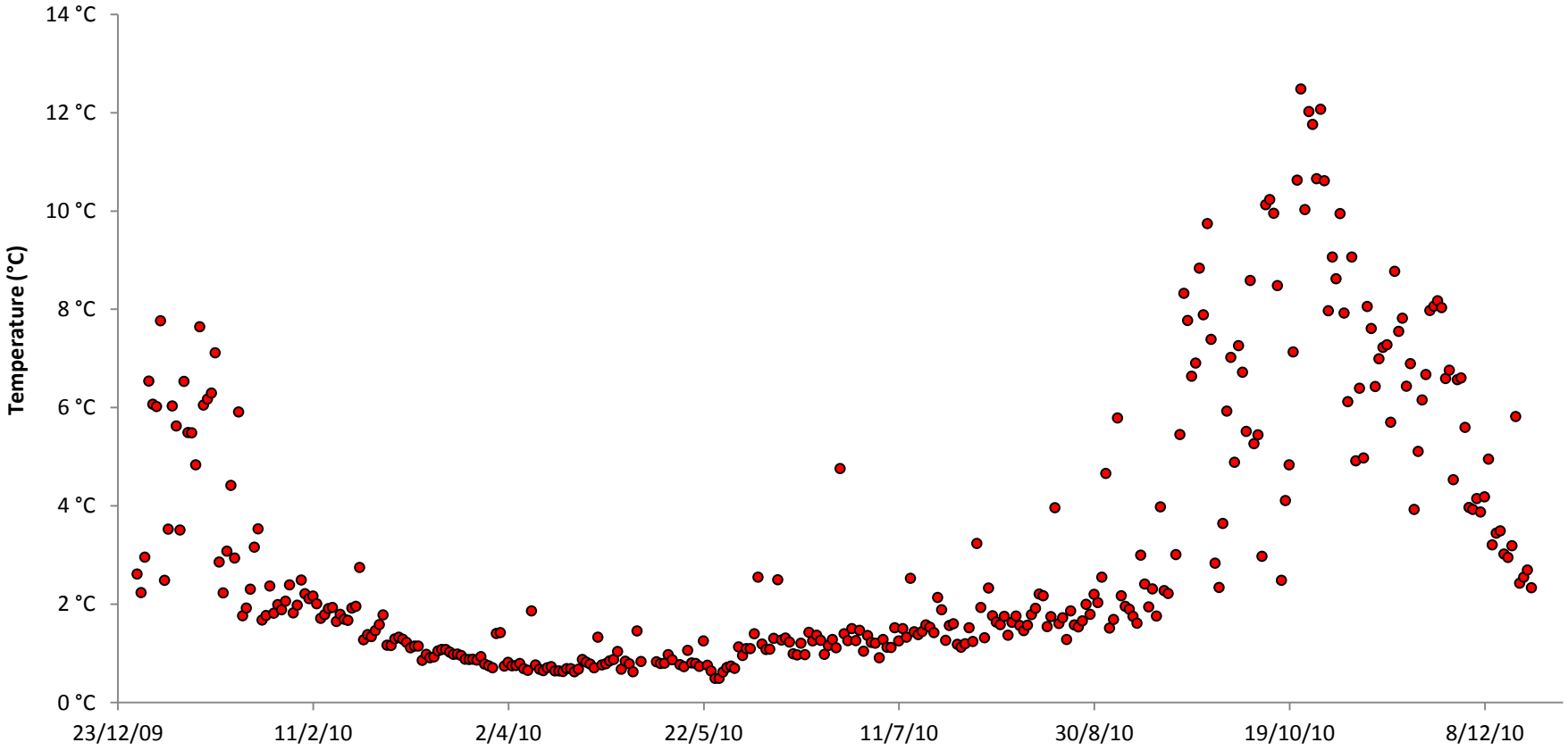


Figure 6.10 Data based on differences (n = 3) between the daily minimum brood temperature and minimum cavity temperature (viz. Brood - Cavity) over the 12 month period, from December 2009 to December 2010.

Figure 6.11 *Au. australis* brood (n = 6) (red) and cavity (n = 3) (black) temperatures, showing diurnal fluctuations during three consecutive summer (a) and winter (b) days.

Throughout the year, the brood clusters contained various stages of developing bees, from egg to pre-emergent imago. During the coldest months, the presence of larval and pupating brood was confirmed. It was not possible to confirm the presence of eggs during the winter months as this would have caused considerable damage to the brood chamber and insulative involucrum layers. It is presumed that since adults within the colonies become visibly inactive at temperatures < 11°C (MH personal observation), brood production would cease at or below this temperature. Colonies did not, however, become broodless during the winter months.

The *Au. australis* colonies survived extremes in temperature with very little evidence of protection against these conditions, other than increased involucrum production. Colonies began extending the layers of involucrum over the brood base by late February and by the end of April involucrum coverage was 100% (Figure 6.12a). Honey pots were also incorporated into these structures. Some of the colonies had constructed the brood cluster on the observation lid and on cold days workers were observed in large numbers (hundreds) crowded inside the bee spaces of the cluster. At no time were workers observed actively vibrating their bodies within the brood cluster. When temperatures dropped to < 11°C, colony members became inactive and there were no audible sounds coming from the hive entrances. Developing brood matured and callows were observed within the hives throughout the year.

Brood diameter began to decline at the end of March, as daytime temperature decreased within both the cavity and the brood, remaining fairly static throughout the coldest months. Brood increased and involucrum coverage decreased as daytime temperatures within the cavity and brood reached ~ 14°C and continued to progress with increasing daytime temperatures. There was a substantial increase in brood production during October (Figure 6.12a).

Pollen and honey stores were also reduced during the colder months. Pollen stores increased, although only slightly, after winter and honey stores continued to decline throughout the remainder of year (Figure 6.12b).

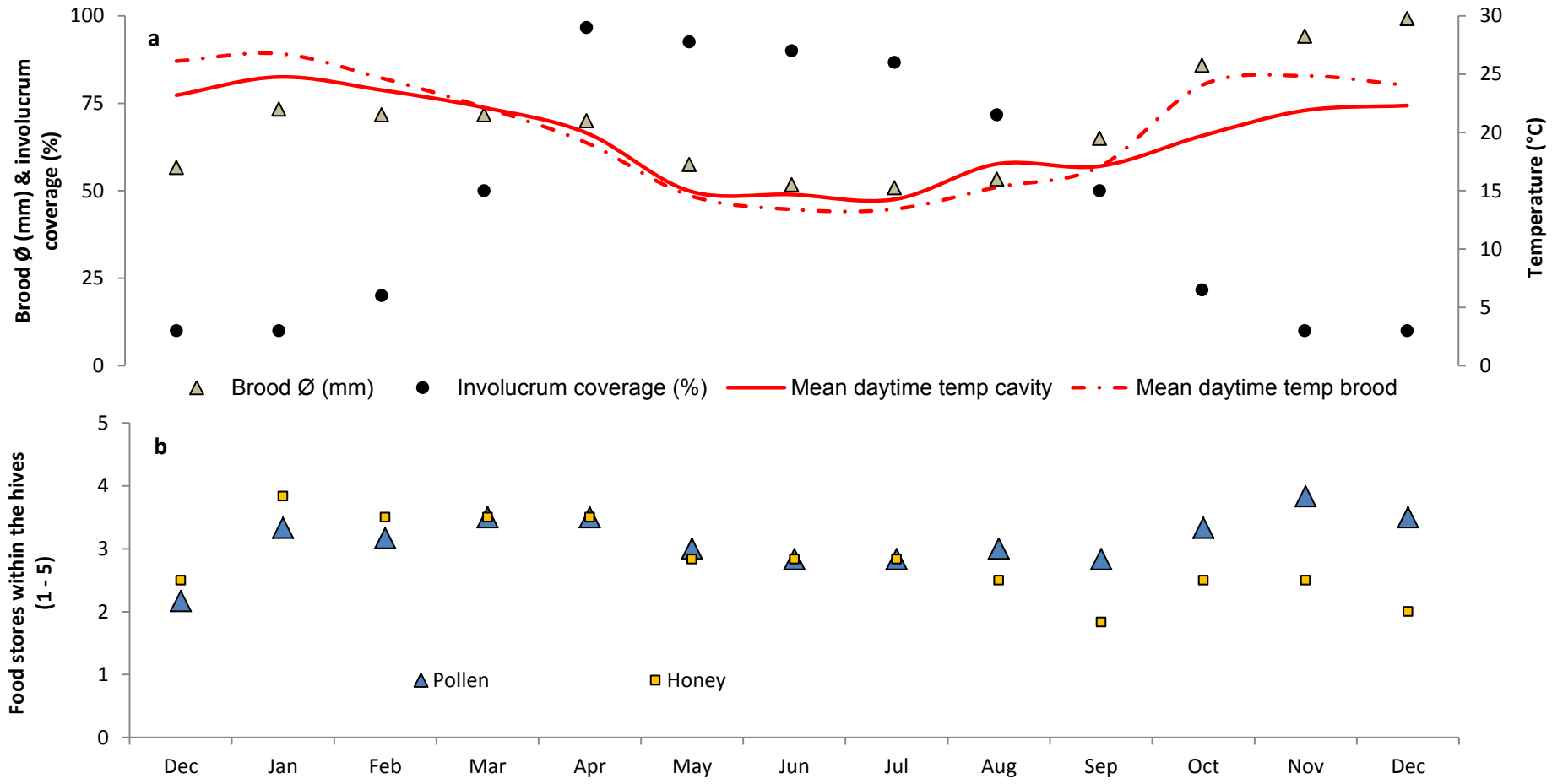


Figure 6.12 Trends for brood growth and involucrum coverage over brood clusters, compared to mean daytime temperatures (a) and trends for stored pollen and honey (b) from December 2009 to December 2010.

Drones were observed within the hives throughout most of the year, including the winter months. Not every hive contained drones all year round but drones were observed within at least one of the hives for nine of the twelve months. The area in which the study was conducted contained ten additional *Au. australis* colonies and when these were taken into account, drones were present all year round within the local population, but not necessarily in any particular colony.

6.2.4 Discussion

Au. australis naturally occurs in areas that experience extremes in temperature (Table 6.3). Like many stingless bee species, *Au. australis* uses passive mechanisms to modify nest temperatures. These include utilising tree cavities for nesting sites and closing the nest entrance at night or during inclement weather. Active nest thermoregulation has been observed on hot days and workers stand in line within the internal nest tube, fanning their wings (MH personal observation). Nest cooling has been reported for many stingless bee species but there have been few reports of them bringing water into the nest to aid in cooling (Cauich et al. 2004). Throughout the time of my studies, I did not observe *Au. australis* bringing water to the hive; however, there have been reports of *Austroplebeia* workers gathering near and collecting water from cattle troughs and dams in arid areas such as central Australia and outback Qld (A. Dollin, pers. comm., 2011).

Artificial hives do not have the same insulative qualities as a cavity in a living tree, as demonstrated by the close tracking of ambient and cavity temperatures during the initial experimental period (27 Dec 2009 – 12 Jan 2010). The insulative quality of a dead tree, the preferred nest site of *Au. australis* (A. Beil, pers. comm., 2008), is much less than that of a living tree (Coombs et al. 2010). It is, therefore, hypothesised that colonies do not rely heavily on live-tree insulative properties for protection against temperature extremes.

Au. australis demonstrates the strict thermoconformity of a poikilotherm (Prosser & Nelson 1981), as shown by the close tracking of brood and cavity temperatures during winter. Colonies appeared to become inactive at ~ 11°C and substantially increased metabolic and physical activity at temperatures ≥ 15°C. Quiescence is a survival strategy brought on by adverse conditions, such as low temperatures. It leads

to a reduction in metabolic activity and oxygen consumption (Wigglesworth 1956). Unlike diapause, quiescent insects can be roused out of inactivity when conditions, such as increasing temperature, become more conducive to activity. Quiescent insects continue to slowly metabolise accumulated fat stores, resulting in low levels of heat production (Storey & Storey 2001), heat as produced by metabolism (Howe 1967). During prolonged cold periods *Au. australis* colonies can generate sufficient heat to maintain the brood at least 0.48°C above that of the cavity.

Eskov (2007) demonstrates that individual members of *A. mellifera* emit different amounts of heat at different stages of development, due to varying levels of metabolism. Young, developing larvae produce more heat than pre-pupal larvae, and pre-emergent brood produce twice as much heat as they fidget in their cocoon. Metabolising brood contributes to the overall heat of the nest and the larger the brood population, the greater the heat production (Eskov 2007). Adults metabolise at an even higher rate, and oxygen consumption within the workers of *M. quadrifasciata* increases with age (Teixeira et al. 2011). Increasing ambient temperature induces greater activity, thus, generating greater heat (Wigglesworth 1956).

Several researchers report that some species of stingless bee thermoregulate above ambient temperatures (Sakagami 1982; Roubik & Peralta 1983; Engels et al. 1995; Moo-Valle et al. 2000; Sung et al. 2008). At ambient temperatures ≥ 15°C, *T. spinipes* can maintain its brood at 35°C (Sakagami 1982) and *M. rufiventris* and *M. seminigra* maintain theirs between 30 and 32°C (Roubik & Peralta 1983). *T. spinipes* has an estimated colony population of 5,000 to 18,000 while *M. seminigra* and *M. rufiventris*' populations are only 4,200 to 7,500. These two *Melipona* spp. have brood populations estimated at five to 10 L, containing 3,000 to 6,000 large brood cells (Sakagami 1982; Roubik & Peralta 1983). For all three above-mentioned species, this equates to a substantial heat producing mass, contributing to the internal energy of the colony. General estimates of colony populations for *Au. australis* can be calculated by measuring brood volume (see this chapter, Section 6.5). The colonies in the current study had mean brood volumes ranging from 70 to 500 mL (350 – 2,500 cells), with peak volume occurring during October. This is small compared to the afore-mentioned South American species. Yet, increasing brood volumes, combined with increasing ambient temperature and

colony activity, produce substantial amounts of heat (up to 15.19°C above ambient) during periods of active brood production.

Brood temperatures in the other studied stingless bee species vary between 2 and 8°C above the ambient temperature. Sung et al. (2008) shows that *T. ventralis hoozana* thermoregulate its brood at between 24 and 31ºC, while ambient temperatures are as low as 8°C. This species has an estimated colony population of 10,000 adults with 12,000 brood cells (Sakagami & Yamane 1984). The colonies are housed in the cavities of large, living trees which are likely to provide excellent insulation, coupled with heat generated from a massive population. It is, therefore, not surprising that they are able to maintain brood temperatures during the cooler seasons. *F. varia* and *L. muelleri*, both cluster-building species, do not increase brood temperatures above ambient temperatures (Sakagami 1982).

With the exception of Sung et al. (2008), all reports pertaining to this research have been conducted on nests that experienced minimum ambient temperatures of at least 15°C (Table 6.II). At temperatures ≥ 15°C, the data show that *Au. australis* is capable of raising brood temperatures by up to 9.7°C above ambient in winter and up to 15.2°C in summer. *Au. australis* could, therefore, be considered capable of thermoregulation at temperatures ≥ 15°C; however, they show no ability to incubate brood at temperatures < 15°C. Therefore, *Au. australis* should not be regarded as a thermoregulating species, but rather, one that is capable of taking advantage of moderate ambient temperatures to generate metabolic heat, as are many other stingless bee species.

The developing brood within the experimental *Au. australis* colonies experienced temperatures ranging from -0.03 to 37.7°C. No brood of any other stingless bee species has been reported to survive such extremes. Solitary bees are able to survive low temperatures in winter by going into a state of diapause. This is a hormonally-driven physiological condition, usually stimulated by photoperiod (Beck 1983; Storey & Storey 2001). Insect development is arrested at various stages, depending on the species, and termination of diapause is only possible when the appropriate combination of environmental conditions occur (Stephen & Osgood 1965; Undurraga & Stephen 1980). The brood of *Au. australis* do not diapause and callows are

observed within the hives at all times of the year. The colonies do, however, demonstrate an ability to resist extremes in temperature.

Insects are also capable of developing a seasonal resistance to high and low temperatures, enabling them to survive the temperature extremes of summer and winter (Wigglesworth 1956). Gradual introduction to rising or falling temperatures promotes acclimatisation to such conditions and improves their chances of survival (Bullock 1955; Colhoun 1960). Acclimatisation is brought about by biochemical restructuring, resulting in increased levels of polyunsaturated phospholipids within the cell membranes, which allow fluidity at low temperatures (Storey & Storey 2001).

Au. australis begins visible preparation for the onset of cold weather by extending the involucrum sheets over the brood cluster. This study demonstrated that colonies commence involucrum extension over the brood by the end of February. This is commonly a very hot month in Australia and may be unlikely to stimulate behavioural reactions for the preparation of the pending cold season. This leads to speculation as to whether daylength may be involved in this process. Just as daylength is involved in the onset of diapause in many solitary insect species (Storey & Storey 2001), it may also be important in thermoconforming, non-diapausing social species. Involucrum expansion increases in February, when daylength is declining, and involucrum coverage begins to reduce in July, subsequent to the shortest day (Figure 6.13). Further investigations into the effect of daylength would help in understanding the biochemical changes thermoconforming social insects undergo as part of their preparation for weather extremes.

Au. australis has evolved in areas that experience hot, dry summers with frequent severe drought (BOM 2010b). The colonies at Blaxland were exposed to summer temperatures of up to 37.7°C and foragers were observed entering and exiting the hives on fine, hot days. Wing fanning at the hive entrance and inside the hive was also observed in *Au. australis* colonies during hot weather. Within the artificial hives, there is only one entrance. Some bee keepers have attempted to include cross-ventilation holes in their artificial hives but these holes are promptly sealed by *Au. australis* colonies (although not so in *T. carbonaria*). During periods of elevated temperatures, the colonies in this study did not appear to be distressed and entrance

activity was high. However, the maximum brood and cavity temperatures showed a synchronous pattern and there was no indication that the colonies were able to cool the brood chamber. Nest temperature experienced in natural cavities of dead poplar trees, a favoured nesting site (see this chapter, Section 6.4.4), at the height of summer in south-east Qld, would be much greater than 37°C. Nest cooling may be more achievable in natural cavities, where perforated batumen plates are used to regulate air flow through the cavity (Roubik 1989).

During this study, pollen and honey stores fluctuated throughout the year but there was no distinct seasonal increase or decrease. The year 2010 was unusually wet in the eastern states of Australia, with above average rainfall from January to March and record rainfall from August to December (BOM 2011a). In 2010, Blaxland experienced 1,094 mm of rain spread over 60 days, with 85% of that occurring during the warmer months, when temperatures were conducive to foraging. While temperatures may have been favourable for foraging, cloud cover and rain periods reduce foraging activity in *Au. australis* (see this chapter, Section 6.1.3). Honey stores became so depleted following this study that the colonies were relocated to the bee shed at UWS and fed over the following winter to avoid starvation.

Drones of many stingless bee species are present in colonies for much of the year (Roubik 1989). *Au. australis* drones were observed within the local population throughout the year, but not in any one colony all year (see Appendix 3 for additional information regarding drone production). This is similar to some species of *Melipona* where drones are periodically present in a single colony during 'male-producing periods' (MPP) (Velthuis et al. 2005) and *P. remota*, which produce drones in 'seasonal cycles' (van Benthem et al. 1995).

6.3 Paralleling study on foraging behaviour

6.3.1 Introduction

Food collecting strategies used by different animals, whether solitary or social insects, reptiles, mammals or humans, determine the probability of that organism's survival. Competition for food resources is fierce, especially during times of resource scarcity or unpredictability. Johnson and Hubbell (1975) demonstrate different

foraging strategies in two social stingless bees in Costa Rica. *Trigona fuscipennis* Friese uses a group-foraging strategy which enable it to monopolise flowers, and rapidly collect high-reward floral resources. *Trigona fulviventris* Guérin, on the other hand, forages as individuals or within small groups. Johnson and Hubbell (1975) conclude that bees using group-foraging strategies are best suited to areas which provide abundant, high-reward floral resources. The solitary foraging behaviour is thought to be an adaptation for areas with low density, low-reward resources. Populations of Australian *Trigona* and *Austroplebeia* are quite different, with *Trigona* being found mostly in areas of high rainfall (A. Dollin, pers. comm., 2010, Figure 6.23), which contain abundant, reliable floral resources.

Areas of eastern Australian began developing arid conditions during the late Miocene period (Martin 2006). Rainforests receded and more sclerophyllous vegetation such as *Eucalyptus* / *Casuarina* woodlands developed in central inland areas. Rainforests remained in the coastal regions but inland Australia became even more arid, with the development of woodlands and shrublands (Martin 2006). *Austroplebeia* is thought to be one of the last taxa to disperse to its current range (Rasmussen & Cameron 2010), indicating that bees inhabiting arid, inland areas of Australia will have evolved with unpredictable resources (Archer et al. 1994).

This study was set up as a result of previous foraging behaviour studies (also reported in this thesis) and its aim was to compare, under experimental conditions, the foraging behaviour of three species of stingless bees. It was hoped to elucidate the foraging strategies used by these different species, and as a result, to better understand how *Au. australis* colonies survive within their harsh natural habitat.

6.3.1.1 Study background

The earlier experimental design for the UWS greenhouse pollination trial (Chapter 3, Section 3.2) originally included *T. carbonaria* as well as *Au. australis*. Unfortunately, the *T. carbonaria* colony was found to be queenless and broodless at the conclusion of the experiment and, for this reason, all data collected regarding *T. carbonaria* was removed from that chapter. The observations made during the pollination trial did, however, uncover some interesting differences between *Au. australis* and *T. carbonaria.* It was these observations that prompted the

development of this current study and *Au. symei* was included as an additional species. *Au. symei* has been domesticated by a small number (< 5) of Qld bee keepers who have successfully used them as pollinators in some crops. Some of the data collected from the UWS pollination trial are included here to give some background information.

During the pollination trial, workers from a single *T. carbonaria* and a single *Au. australis* colony were observed. Entrance and in-crop activity were monitored, including foraging and flight behaviour. During the first two weeks of the trial, activity at the hive entrances showed that *T. carbonaria* (Figure 6.13) spent more unproductive time outside the hive than *Au. australis* (Figure 6.14). *T. carbonaria* moved in and out of the hive entrance; however, they were never observed on the flowers of the crop during this time. Removal of debris and flights resembling orientation flights were observed until mid afternoon each day. In Week 3, *T. carbonaria* started to 'work' the leek crop and much larger numbers of workers moved in and out of the hive entrance, although they were not all foraging on the crop. Also, many foragers spent a great deal of time hovering, before alighting on a flower. Seed set for the celery crop was significantly lower ($p < 0.001$) in *T. carbonaria* (0.08%) compared to *Au. australis* (76.0%).

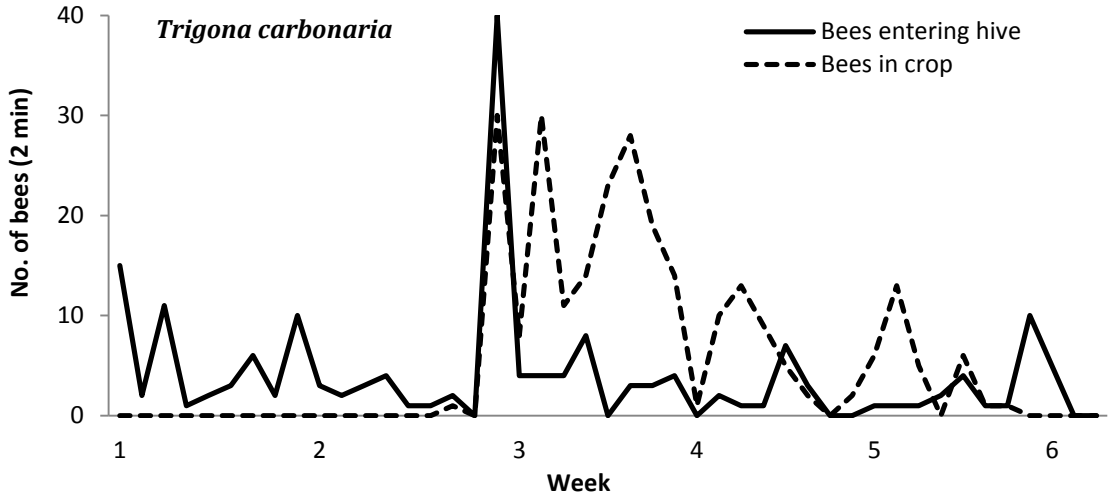


Figure 6.13 *T. carbonaria* flight activity during UWS pollination trial. Hive entrance activity (number of bees entering the hive) compared to activity within the crop. Note the peak around Week 3, when the male leeks commenced flowering.

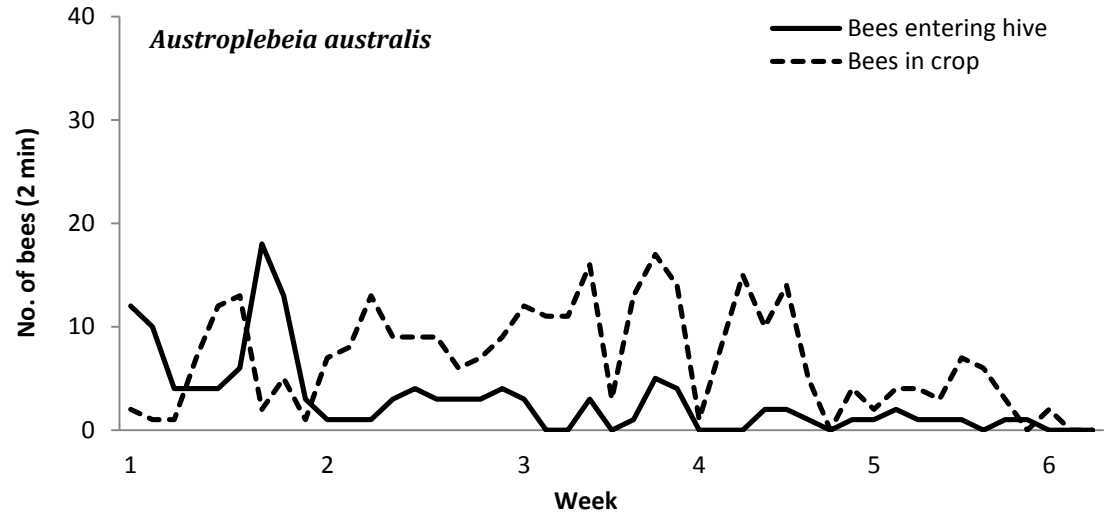


Figure 6.14 *Au. australis* flight activity during UWS pollination trial. Hive entrance activity (number of bees entering the hive) compared to activity within the crop.

A Sony Handycam (Digital HD Video Camera Recorder, HDR-SR1E) was set up during the pollination trial to record forager activity within the crop for four to six hours per day, for five days, for each bee species. Approximately 50 hours of video footage was collected and MTS files were converted to WMV files. Footage was viewed using Nero 7 Premium Showtime. During viewing, I attempted to record 'hover' times and 'alighting' times, but this proved to be very difficult as foragers moved in and out of the viewing frame, making it impossible to keep track of

individual foragers. No conclusive data were obtained from this exercise but it did help me develop an experimental design for the current paralleling study.

6.3.2 Materials and methods

6.3.2.1 Enclosure set up

The experiment was carried out within the confines of experimental greenhouse chambers of the Quarantine Bioassay Insectary (as described in Chapter 3, Section 3.3.2). The pollination trial had shown that bees would forage within the confines of these chambers. The chambers provided an environment that could be maintained at optimal foraging temperatures and facilitate easy observation of confined bees, while being supplied with attractive, disposable foraging material.

Greenhouse chambers were maintained at temperatures ranging between 21.6 and 32.9°C during the observation periods, which ensured that temperature was not a limiting factor for forager behaviour for any of the species. To aid orientation, each chamber's walls were decorated with cardboard cut-outs of blue and green rectangles and yellow triangles (as described in Chapter 3, Section 3.3.2).

The preliminary paralleling study commenced on 1 April 2009. Two greenhouse chambers were each supplied with 20 x 12 L pots of flowering annual and perennial plants (*Dahlia* sp. Cavanilles and *Zinnia* sp. Linnaeus (Asterales: Asteraceae)). These flowering plants were known to be attractive to these stingless bees (MH personal observation). Plants were fertilised with a controlled release fertiliser (40 g Osmocote® Exact, Everris, Baulkham Hills, NSW, Australia) and set up with a drip irrigation system (as described in Chapter 3, Section 3.3.2). On 20 April 2009, once the plants started flowering, a single *T. carbonaria* and an *Au. australis* hive were installed in each chamber. Unfortunately, flower numbers on the plants remained low and powdery mildew, aphids and two-spotted mite began to infest the plants. Attempts to treat the infestations with bee-safe methods were unsuccessful and the trial was terminated on 17 May 2009. At no time were foragers observed on the small number of flowers present.

I, therefore, decided that 'disposable' foraging material would be preferable to potted plants. This would provide large numbers of flowers which would not be adversely

affected by pest and diseases, such as those encountered in this initial trial and the UWS pollination trial (see Chapter 3, Section 3.3.6.2.1).

6.3.2.2 Colony orientation

On 31 July 2009, a total of nine colonies of Australian stingless bees: three each of *T. carbonaria, Au. australis* and *Au. symei,* were prepared for the study. All of the *Austroplebeia* colonies were confirmed to be queenright and it was presumed the *Trigona* colonies were queenright, as entrance activity was very high (~120 / 2 min) and they had not been split for over 12 months. The colonies of each species were introduced into the chambers separately so that their behaviour could be observed during the orientation period. Each chamber was supplied with ~ 20 flowering branches, cut from pesticide-free stone fruit trees, including a variety of *Prunus* spp., *Pyrus* spp. and *Malus* spp., harvested from the university's fruit orchards. These plants were chosen because they were a readily available source of blossom and the inflorescences contained flowers of similar size, thus providing a fairly uniform medium for the bees to forage on. During the bee orientation period (12 days), the chambers were also supplied with feeder-floats (Chapter 3, Section 3.2) as an additional food source.

On 1 August 2009, an *Au. symei* colony was introduced into each of the three chambers. Behaviour was observed and specimens of workers and drones were collected as they buzzed the walls, at the end of each day. The *Au. symei* were allowed to 'settle in' before a *T. carbonaria* colony was introduced into each greenhouse chamber on 4 August 2009. Having only one *Austroplebeia* species in the chamber with the *Trigona* made it easy to distinguish the two species. An *Au. australis* colony was introduced into each chamber on 8 August 2009. The nine colonies, one of each of the three species / chamber, were finally housed and oriented into the greenhouse chambers by 13 August 2009.

For each of the species, dead bees were collected from the window sills and accessible floor space during their orientation period and the bodies were counted, weighed and an average weight of individual worker bees was calculated. Worker losses were graded as 'low' (< 200), 'moderate' (200 – 500) and 'high' (~ 1000) and these numbers were used to establish a guide for estimating losses throughout the

study. It was not possible to collect and count all dead individual bees throughout the study as it was too time-consuming. It was also not possible to sweep the bodies up to weigh them, as the floors were littered with flower petals and nest debris. The floors were, however, cleaned every three or four days and visual estimates of worker losses, based on the previously collected bodies, were used to calculate the number of worker losses. This, plus observation of air traffic within the chambers, enabled an approximation of colony losses throughout the study.

6.3.2.3 Preliminary observations

The experimental design and methodology initially included 'locking-up' the colonies and then releasing only one bee at a time from the hive (as described in Chapter 3, Section 3.3.4). The released bee was then closely observed on the flowers and its behaviour monitored. However, when the entrance was blocked in the *Austroplebeia* colonies, guards and foragers withdrew into the hive for an extended period of time. Also, the number of *T. carbonaria* foragers was extremely high and workers were continuously airborne, making it impossible to collect all *T. carbonaria* foragers. The plan to 'lock up' the hives was, therefore, abandoned.

It was easy to distinguish between *Trigona* and *Austroplebeia* foragers (Figure 6.16), as *T. carbonaria* are smaller than the more robustly built *Austroplebeia*. *T. carbonaria* also have heavier hair patterns on the side of the thorax (Dollin 2010b; MH personal observation). On the other hand, it was more difficult to distinguish between *Au. australis* and *Au. symei*. Both are about the same size (see section 6.3.3.1) and thoracic markings can be difficult to see when bees are foraging. To help distinguish the two species, foragers were marked. *Au. symei* were less disrupted by entrance modifications than were *Au. australis* so, to aid in the identification of foragers on the flowers, the entrance of the *Au. symei* hive was dusted with a fluorescent powder, orange/yellow JS-0y3022 (Radiant Colour, Richmond, CA), at the beginning of each observation session (Figure 6.15). There were no observable behavioural differences between dusted bees and undusted bees.

Preliminary observations also showed that flowers were more heavily attended by the *T. carbonaria* foragers than either of the *Austroplebeia* species. It was extremely difficult to track any of the bees individually, due to the large numbers of

T. carbonaria foragers on the flowers. The entrance of the *T. carbonaria* hive was, therefore, blocked with a piece of tissue paper. Over a period of 10 min the returning foragers were captured using a mechanical aspirator. These foragers were chilled and weighed to estimate their number, which was > 500. I decided, in order to facilitate observation of all bee species, the *T. carbonaria* hive would be closed at the commencement of each observation period to reduce the foraging population. There were still dozens of *T. carbonaria* on the flowers and in the air so observation of this species was still easily carried out.

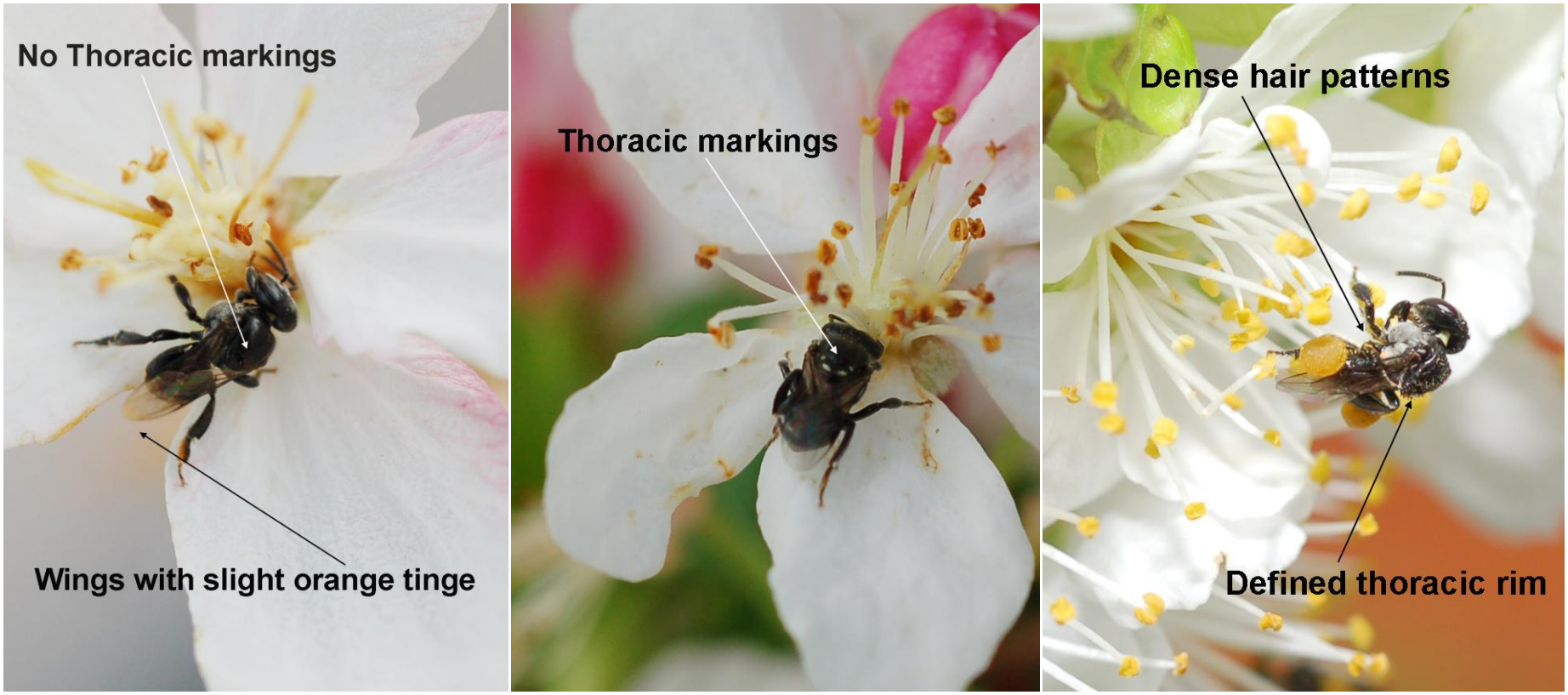


Figure 6.15 ***Au. symei*** **with no obvious markings on her 'robust' thorax and a slight tinge of orange on her wings, from the dusting of fluorescent powder (left),** ***Au. australis*** **with obvious cream / yellow markings on scutellum (centre) and** ***T. carbonaria*** **showing dense thoracic hair patterns and finer, more prominent thoracic rim (right).**

6.3.2.4 Forager observations

Observations commenced on 18 August 2009. Individual bees from each colony were observed three times per day: morning (commencing 08:30), midday (commencing 12:00) and early afternoon (commencing 14:30). Entrance activity was monitored for two minutes each day, at around midday. During the final week of monitoring, I noted that entrance activity varied greatly throughout the day, so entrance activity was monitored four times per day for the remainder of the study.

The schedule for observation periods for each chambers and species was selected at random. Each morning, flowers were replenished in all chambers. At the beginning of each individual observation, an audible alarm timer (Nokia mobile telephone) was set for 60 seconds. When a suitable individual forager was located, the timer was started. When the bee alighted on a flower, a separate stop watch was started and a tally counter was pressed. When the bee left the flower, the stop watch was stopped until the bee alighted on a flower again. The stop watch was restarted and the tally counter pressed. This continued for 60 seconds, after which time the audible alarm would sound and the stop watch was stopped, marking the end of the individual observation. This process recorded the number of seconds spent on flowers, the number of seconds hovering and the number of flowers visited, in a 60 seconds period. Observation periods were conducted three times per day, three to four times per week, until the supply of blossoms was exhausted (viz., 19 days, 464 observations).

Data were tested for homogeneity of variance using Levene's test. The data were analysed by one-way ANOVA and means were compared by Tukey's HSD test in SPSS 17. The setting of significance was $\alpha = 0.05$.

6.3.2.5 Digital imaging

Digital images, both moving and still, were captured throughout the study period, to record and more closely observe the behaviour of foraging bees. Equipment used for this purpose included: a high-speed SportsCam 500, fitted with a 75 mm lens (Measurement and Analysis Camera Systems, Pty Ltd. Wahroonga, NSW, Australia), a Sony Handycam, Digital HD Video Camera Recorder, HDR-SR1E

(Sony Australia, North Ryde, NSW, Australia) and a Nikon D50 SLR camera fitted with either an AF-S Nikkor 18 to 55 mm or an AF Micro Nikkor 60 mm lens (Nikon Australia, Lidcombe, NSW, Australia). The high-speed footage was analysed with the aid of the program Midas Express 4.0 (Xcite, Inc, Cambridge, MA, USA).

At the conclusion of the paralleling study, attempts to estimate wing speed for each species were made using the high-speed SportsCam. A length of cardboard, marked at 1 cm intervals, was attached to the side of each hive and the camera was set up facing the cardboard. Bees were recorded as they entered and exited the hive entrance. *Au. australis* entrance activity declined markedly when the cardboard was attached to the hives and images for this species were not obtained.

6.3.3 Results

6.3.3.1 Worker bee losses

The mean weight of individual worker bees differed only slightly between the *Austroplebeia* spp., with *Au. symei* weighing 6.7 mg, *Au. australis* weighing 6.5 mg. *T. carbonaria* was considerably lighter at 4.5 mg. Based on estimated numbers of dead bees on the floors and window sills, worker losses were between 1,000 (in all three *Au. australis* colonies) and 5,000 (in one of each *T. carbonaria* and *Au. symei* colony) for the duration of the study. The highest losses were suffered by the *Au. symei* colony in chamber 2 and the *T. carbonaria* colony in chamber 3.

6.3.3.2 Hive entrance activity

There was no significant difference between the hive entrance activity of individual colonies within each species (*Au. symei*, $p = 0.229$, *Au. australis*, $p = 0.129$, *T. carbonaria*, $p = 0.883$). However, there was a significant difference between entrance activity of *T. carbonaria* and the two *Austroplebeia* spp. ($F_2 = 111.24$, $p < 0.001$) (Figure 6.16), with consistently much higher numbers of *T. carbonaria* workers returning to hives during each two minute observation period. There was no significant difference between the two *Austroplebeia* spp. ($p = 0.210$).

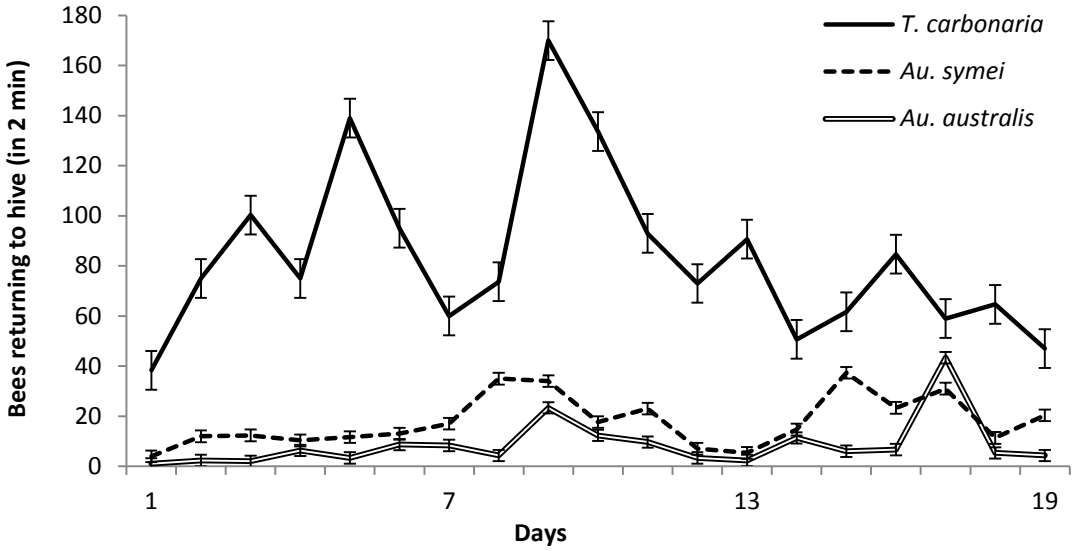


Figure 6.16 Entrance activity (number of bees entering the hive) of *T. carbonaria* was much higher than either *Au. symei* or *Au. australis*. Means and their standard errors are shown.

Foraging resources were replenished daily and the level of forager activity appeared to be affected by the age, or quality, of the floral resources. The progressive, daily entrance activity, monitored during the final week, indicated that the forager recruitment and resulting entrance activity was significantly affected by the availability of suitable foraging resources within the chambers ($F_{57, 143} = 1.60$, $p = 0.024$) (Figure 6.17). Thirty minutes after new floral resources were introduced into the chambers, mean forager numbers for *T. carbonaria* colonies were three and four times greater than *Au. symei* and *Au. australis*, respectively. This difference was even more marked when the floral resources were most depleted (6 h-old, in the mid-afternoon), with *T. carbonaria* numbers being nine times and 40 times greater than *Au. symei* and *Au. australis*, respectively. *T. carbonaria* were significantly more active throughout the day than either of the *Austroplebeia* spp. ($F_{2, 143} = 31.49$, $p = 0.000$), even when the flowers were pollen- and nectar-depleted. There was no significant difference between the two *Austroplebeia* spp. ($p = 0.588$).

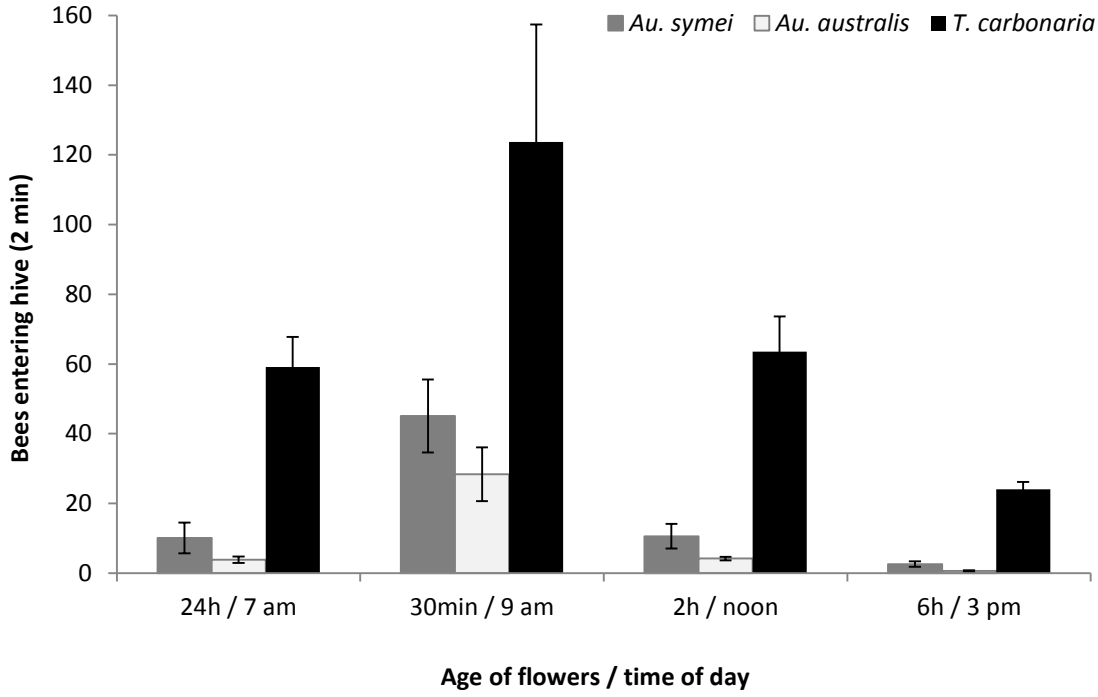


Figure 6.17 Entrance activity (number of bees entering the hive) of *T. carbonaria* was significantly greater than either *Au. symei* or *Au. australis*, even when floral resources were old (6 h) and depleted. As flowers aged and the day progressed, pollen and nectar availability was reduced. Means and their standard errors are shown.

6.3.3.3 Forager behaviour

T. carbonaria spent 31.7% of their foraging time hovering near flowers and only 68.3% of the time attending the flowers. The hovering time of *T. carbonaria* was significantly greater than *Au. symei* (7.7%) or *Au. australis* (8.1%) ($F_{2, 462} = 126.11$, $p < 0.001$) (Figure 6.18). There was no significant difference between the two *Austroplebeia* spp. ($p = 0.942$).

During the 60-second observation period, *T. carbonaria* visited an average of 2.55 flowers, which was significantly less than *Au. symei* (3.53) or *Au. australis* (3.93) ($F_{2, 462} = 32.57$, $p = < 0.001$). Some *T. carbonaria* foragers spent more time on each flower, whereas others spent more time hovering. There was no significant difference between the two *Austroplebeia* spp. ($p = 0.084$).

T. carbonaria foragers were observed attending flowers in relatively large numbers, with up to five foragers working a single flower at one time (Figure 6.19). They were also observed hovering in close proximity to flowers that were already heavily populated (Figure 6.20). No more than two *Austroplebeia* foragers were observed on

a single flower at any one time, and usually only a single forager worked a flower. At no time were *Austroplebeia* foragers observed on flowers already occupied by *T. carbonaria.*

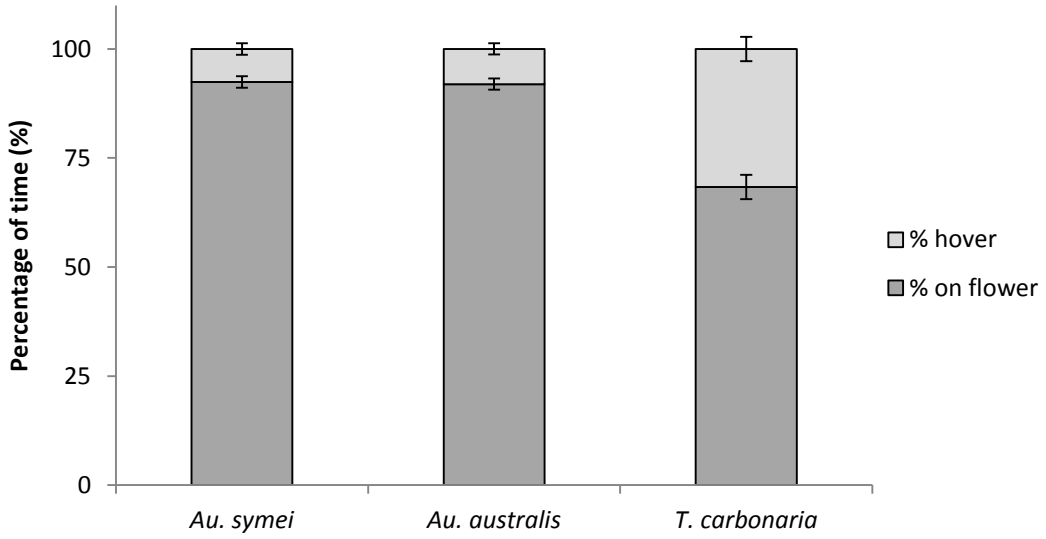


Figure 6.18 Percentage of time the three bee species, *Au. symei, Au. australis* and *T. carbonaria,* spent hovering vs. time on flowers in a 60-second observation period. Bars represent the standard errors of the means.

Figure 6.19 Five *T. carbonaria* foragers attending a single flower.

Figure 6.20 *T. carbonaria* foragers hovering near an already heavily populated flower.

6.3.3.4 Resource collection

A significantly higher proportion of *T. carbonaria* foragers (83.2%) were observed collecting pollen ($F_{2, 6}$ = 18.95, p = 0.003) than either *Au. symei* (7.8%) or *Au. australis* (15.8%), and there was no significant difference between the two *Austroplebeia* spp. (p = 0.836). Conversely, a significantly higher proportion of *Au. symei* (87.6%) and *Au. australis* (73.4%) foragers were observed collecting nectar ($F_{2, 6}$ = 14.61, p = 0.005) than *T. carbonaria* (10.5%) foragers, although, again, there was no significant difference between the *Austroplebeia* spp. (p = 0.623) (Figure 6.21). A small proportion of foragers from all three species was observed collecting both nectar and pollen in a single foraging trip (Figure 6.21 and Figure 6.22); however, there was no significant difference recorded between the species ($F_{2, 6}$ =1.39, p = 0.320) for this behaviour.

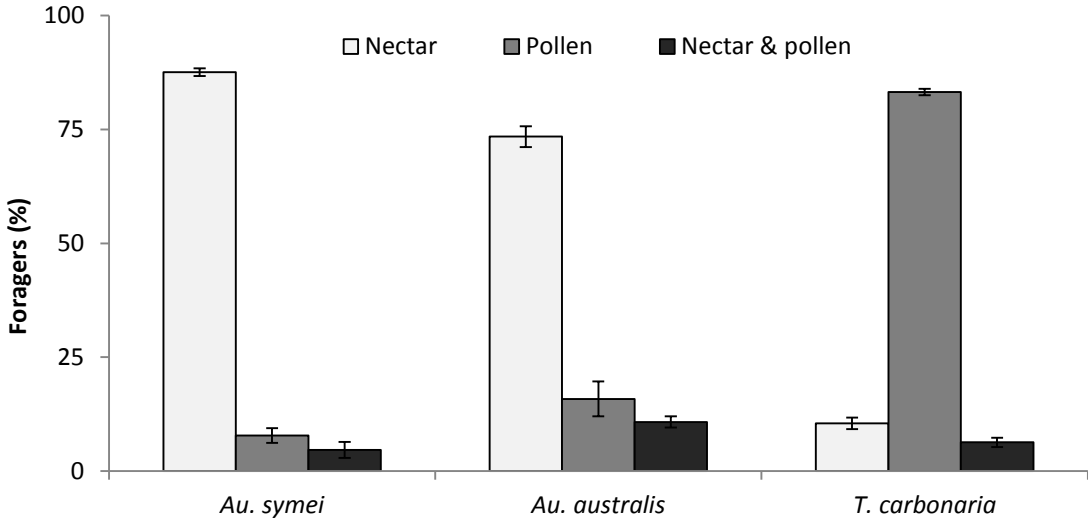


Figure 6.21 Percentage of foragers of *Au. symei, Au. australis and T. carbonaria* collecting nectar and pollen resources. Bars represent standard errors of the means.

Figure 6.22 Pollen forager collecting nectar. Note the pollen loads in her corbiculae.

High speed digital footage of foragers on and around the flowers demonstrated that both *Austroplebeia* spp. were very precise with their flight trajectory and landing compared to *T. carbonaria*. Foragers from the *T. carbonaria* colonies were observed hovering for extended periods of time before alighting on a flower. This hovering was sometimes interspersed with apparent attempts to alight. High speed digital footage recorded one *T. carbonaria* forager that, over a 148 second period, made four unsuccessful attempts to alight on the petal, completely falling away from the flower twice, before successfully alighting on the fifth attempt.

6.3.4 Discussion

The greenhouse chambers of the UWS insectary provided a suitable environment for the observation of stingless bee foraging. The supply of 'disposable' foraging resources proved to be an effective and pest-free medium for this behavioural study. Fresh flowers also provided a more realistic medium for the bees to forage on, rather than artificial flowers, enabling observation of natural foraging behaviour. *Au. symei* foragers appeared to be unaffected by the light dusting of florescent powder and this treatment helped to distinguish *Au. symei* from *Au. australis* during observations. Flight activity commences at ambient temperatures > 18°C in *T. carbonaria* (Heard & Hendrikz 1993) and > 20°C in *Au. australis* (see this chapter, Section 6.1.3). Chamber temperatures were successfully maintained between 21 and 33°C, ensuring that temperature was not a limiting factor for any of the stingless bee species being studied here.

6.3.4.1 Hive entrance activity

Austroplebeia adult population estimates remain, as yet, unstudied. However, thought that *Au. symei* and *Au. australis* have smaller populations than *T. carbonaria*, with ≥ 4,000 adults. Hoffman (unpublished data, 2006) estimates that a strong *T. carbonaria* colony has an adult population of up to 11,000. Large adult and brood populations would account for the higher number of foragers in *T. carbonaria* colonies. Large populations require large resources, which are provided by high forager numbers. Entrance activity in the *T. carbonaria* colonies is significantly greater than either *Austroplebeia* spp.

This study demonstrates that forager recruitment and the associated increase in entrance activity are significantly affected by the availability of floral resources in all three bee species. *T. carbonaria* colonies are more active than the *Austroplebeia*. This may simply be a function of the differences in colony size of *T. carbonaria* and the less populous *Austroplebeia* spp. However, *T. carbonaria* forager numbers are only three to four times higher than *Au. symei* and *Au. australis*, respectively, during peak nectar and pollen availability. This is compared to nine- and 40-fold more foragers leaving the hives, when floral resources are most depleted. Species of *Trigona* in Australia occur mostly in areas of high rainfall (Dollin et al. 1997) and, as

such, *T. carbonaria* has evolved in areas of abundant, reliable floral resources. *T. carbonaria* are opportunistic foragers that use group strategies to search for resources. They then rapidly recruit nestmates once resources are located and harvest them until they are depleted (Bartareau 1996). In the current study, single flowers were visited by up to five *T. carbonaria* foragers at any one time, foraging in groups as described by Bartareau (1996). In contrast, *Au. australis* and *Au. symei* forager numbers were low and they demonstrated solitary foraging strategies.

No forager communication or recruitment studies have been successfully conducted with *Au. australis.* It is speculated that this species may use primitive scent trails, similar to those used by *T. carbonaria* (Nieh et al. 2000), which can be detected from several metres away (Kerr 1959, cited in Johnson & Hubbell 1975). The level of pheromone concentration used for marking resources may influence the resulting level of forager recruitment (Sánchez et al. 2009; Schorkopf et al. 2011). Further studies are required to test this hypothesis.

Forager recruitment in *Au. australis* appeared to be limited during periods of poor floral quality (see Chapter 3, Section 3.3.4). This has been demonstrated in colonies of *M. favosa* and *M. fulva* during floral dearth, at the end of the tropical wet season (Roubik 1982). Entrance activity in the chambers is much lower in both *Au. australis* and *Au. symei* (max 28 returning workers / 2 min and 45 / 2 min, respectively) compared to the entrance activity when colonies have access to external foraging (> 100 returning workers / 2 min) (Halcroft 2007; MH personal observation). This comparison is not as marked in *T. carbonaria*, with entrance activity inside the enclosure (mean 124 returning workers / 2 min) being similar to that reported when colonies have access to external foraging (70 to 100 returning workers / 2min) (J. Shanks, pers. comm., 2011). Bartareau (1996) describes *T. carbonaria* as an opportunistic, facultative group-forager that spreads out from the hive and independently searches for resources. This foraging strategy is demonstrated in the current study, consistent with the strategy predicted by Hubbell and Johnson (1978) in environments where rich floral resources are adequate for a large number of nestmates.

6.3.4.2 Forager behaviour

Roubik (1980) reported aggressive, competitive behaviour between foragers in some South American stingless bee species, e.g., *Trigona hyalinata branneri* Cockerell and *Trigona willana.* Roubik (1980) also found that colony population was positively correlated with the competitive success of foragers, although this was often associated with aggressive behaviour.

Competition between the species in the current study did not appear to be aggressive and it seems that the sheer weight of forager numbers gave *T. carbonaria* a competitive edge over both *Au. symei* and *Au. australis.* It was interesting to note that *Austroplebeia* foragers were never seen alighting on a flower already occupied by a *T. carbonaria* forager. Also, *Austroplebeia* foragers were observed on a few occasions to abandon a flower with the arrival of a *T. carbonaria* forager. Johnson and Hubbell (1974) reported that aggressive behaviour significantly reduced the amount of time a forager could spend on a flower, which consequently reduced the total resources harvested. If aggressive behaviour is more energy expensive than simply moving to a new flower, the observed foraging behaviour of *Austroplebeia* may include forgoing a single resource in preference to losing valuable foraging time at another.

The foraging behaviour of *T. carbonaria* was significantly less efficient than either *Austroplebeia* species. Non-productive hovering accounted for 30% of *T. carbonaria*'s foraging activity. Upon close inspection, many of these foragers appeared to make several attempts at landing on the flower parts before being successful. This faltering behaviour resulted in a substantial proportion of foraging time being spent consuming energy as opposed to collecting energy resources for the colony. It was unclear why the *T. carbonaria* foragers were having difficulty alighting on flowers, but similar behaviour can be observed in their natural environment, within crops and on exotic floral resources (MH personal observation; T. Carter, pers. comm., 2009; J. Shanks, pers. comm., 2011). Once a *T. carbonaria* forager alighted on the flower, she usually worked the flower intensively, sometimes for up to 90 seconds. Some would, however, only alight for three seconds before taking to the air again. *Au. australis* and *Au. symei* foragers spent 92% of their foraging time harvesting nectar and pollen, as well as walking over and between

flowers. Only 8% of their time was spent airborne and this was mainly moving between branches to another flower.

Hovering is the most expensive mode of locomotion in animals (Tucker 1970) and can expend up to 100 times as much energy as walking for the same period of time (Ludwig 1962, cited in Heinrich & Raven 1972). When clusters of flowers can be visited in close succession, walking is far more efficient than flight, resulting in an energy balance in favour of reward over expenditure (Heinrich & Raven 1972). Hovering is also more conspicuous than moving within the floral structures and increase the chances of predation (Sherry & McDade 1982; Jones et al. 2003). Both *Austroplebeia* spp. demonstrated efficient foraging behaviour. Walking was favoured over flight where flowers were clustered; however, precise flight activity was utilised between branches.

Energy gain must exceed 'energy expenditure' or an individual will eventually die. When a forager's energy expenditure is limited, time must be spent during each foraging trip to feed itself, in order to balance the individual's energy budget (Ydenberg et al. 1994). According to Ydenberg et al. (1994), 'provisioning' foragers (that is, foragers that collect and deliver food for sharing or storage) aim to maximise their daily quota of food delivered while still balancing their own energy budget. To do this, they must maximise their foraging efficiency using 'optimal foraging strategies' that maximise energy intake (or collection) per unit of time (Emlen 1966; Pyke 1984). This was demonstrated by individual foragers of the *Austroplebeia* spp. which favoured walking over energy-expensive hovering and also had a precise flight trajectory when alighting on flowers or entering the hive. Thus, they are more energy-efficient than *T. carbonaria*.

While individual *T. carbonaria* foragers would be expected to have limited energy expenditure, especially considering the amount of energy spent hovering, the colonies are unlikely to naturally experience very limited floral resources. *T. carbonaria* have evolved in high rainfall areas which provide plentiful, reliable floral resources. The apparently frivolous behaviour of *T. carbonaria* may be indicative of this evolutionary process.

A small proportion of bees within all three species was observed collecting nectar whilst also carrying pollen loads. It was not possible to ascertain whether these foragers were genuine nectar and pollen collectors, whether they were using the collected nectar to moisten and pack their pollen loads (Thorp 1979), or using nectar as an energy source during foraging trips (Ydenberg et al. 1994). Nectar is the only source of carbohydrate forager bees utilise, so it would seem to be an efficient practice for foragers to 'eat on the go'.

6.4 Nest density, distribution and characteristics

6.4.1 Introduction

Pollinating bees are not only of great economic importance, they also play a significant role in the overall ecological framework of biodiversity (Byrne & Fitzpatrick 2009). There is mounting evidence of a global pollination crisis, with declines in insect pollinators being reported worldwide and data indicate that these declines are worsening (Allen-Wardell et al. 1998; Kevan & Phillips 2001; Biesmeijer et al. 2006; FAO 2009; Gallai et al. 2009).

In 1998, the Brazilian government hosted an international workshop where the International Pollinators Initiative (IPI) was proposed. The IPI was officially formed in 2000 at the 5th Conference of the Parties to the Convention on Biological Diversity, in Nairobi, Kenya. The IPI recommended several courses of action be taken to address the crisis and these were to be coordinated at a global level. Among these, monitoring of pollinator decline and their causes was one (Williams 2003; Byrne & Fitzpatrick 2009).

Researchers have been collecting data on bee communities and their associated habitats for over 30 years in Brazil (Pinheiro-Machado et al. 2002), the United Kingdom, the Netherlands (Biesmeijer et al. 2006) and Europe (Corbet et al. 1992), although some data are thought to be poorly documented (Corbet et al. 1992; Gordon et al. 1998). Paton (2000) has described the impact of habitat loss and fragmentation on Australian nectar-feeding birds and some of the plants they pollinate. However, currently, Australia has no documented data pertaining to native bee populations or

their habitat. The international scientific community is calling for increased research on pollinator decline and Australian researchers need to answer that call.

6.4.1.1 Australian stingless bee habitat

Two genera of stingless bee, *Austroplebeia* and *Trigona*, inhabit the northern regions of Australia (Michener 1961; Dollin 2010d) (Figure 6.23). One small region is near Tara, a small town located in the Darling Downs of south-east Qld. This region is known to support substantial populations of *Austroplebeia* and the region around Chinchilla and Miles, only 60 and 70 km away, respectively, supports mostly *Trigona*. *Austroplebeia* spp. favour long, narrow tree hollows, whereas *Trigona* spp. in this region choose large diameter hollows, to accommodate their larger brood comb (Michener 1961; Dollin et al. 1997; Dollin 2010d). For the purposes of this research a 'focus study site', located near Tara, was used to investigate the nest density, distribution and characteristics of some Australian stingless bees.

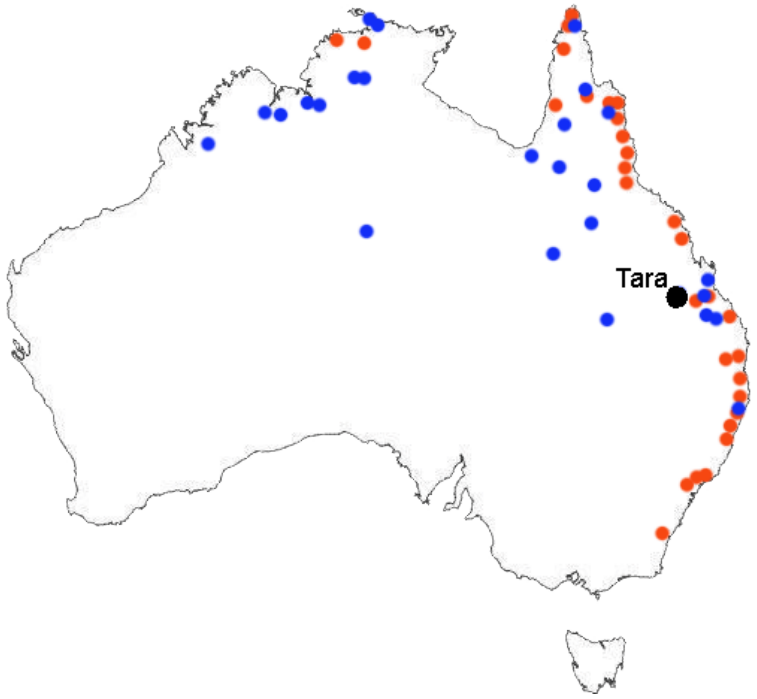


Figure 6.23 Known areas of distribution where *Austroplebeia* (Blue) and *Trigona* (Red) colonies have been reported (A. Dollin, unpublished data). Tara (Black) is in the focus study site.

Understanding the history of a habitat can help explain nest density and distribution. This information may also help to predict the impact of environmental factors and landscape changes on populations of stingless bees and assist in their future

management. The focus study site is located in a region that is subject to long periods of severe drought (BOM 2010b), where floral resources are scarce and unreliable, and occasional major flooding occurs (BOM 2011b). Colonies of *Austroplebeia* appear to thrive in these conditions; however, the land use and flood inundation history of this region indicates that colonies of stingless bees may be under threat in the future.

Cattle grazing was established in the Darling Downs, Qld in the early 1840s (Harrington et al. 1976; Weston et al. 1980; NRW 2006) and tree ringbarking was widely practiced to increase herbage production, thus improving grazing land (Weston et al. 1980; Robertson & Beeston 1981; Roberts 1992). The turn of the 20th century saw a major expansion of rural enterprises and land clearing was carried out in epic proportions (Roberts 1992). By the 1950s land erosion became so serious that thousands of hectares had to be abandoned (NRW 2006). The practice of ringbarking was reintroduced as a form of land improvement for grazing, as it caused less land degradation (Weston et al. 1980). The adage "If it moves shoot it; if it doesn't, ringbark it..." became well known throughout much of Australia (Roberts 1992). This practice continued for decades and rogue cattlemen even ringbarked trees on crown land (now forestry) in an effort to increase their grazing opportunities (Anonymous 1902).

Poplar box, *Eucalyptus populnea* F. Mueller (Myrtales: Myrtaceae), mainly grows in regions that experience annual rainfall of 300 – 750 mm and occurs throughout much of the inland regions of Qld and NSW (Weston et al. 1980), including the Darling Downs. Prior to European settlement, it was reported that poplar box woodlands were far more open than currently, and had understoreys of edible grasses and shrubs (Robertson & Beeston 1981). Poplar box is one of at least half a dozen *Eucalyptus* species that are major hollow-forming trees (Dorricott et al. 1999; QPWS 2011), making them ideal habitat for a myriad of native wildlife, including native bees. It is interesting to note that native bees are not among the listed native wildlife that inhabit these tree hollows (Dorricott et al. 1999; QPWS 2011); an indication of how little is known about these bees.

Today, many of these ringbarked trees still stand and they have become the favoured nesting site of *Austroplebeia* spp. (A. Beil, pers. comm., 2009). Colonies of

Austroplebeia can establish in a variety of tree species, living or dead; however, currently they are more easily discovered in dead trees (A. Beil, pers. comm., 2009). Access to the tree cavity is usually via a hole made by wood boring beetles (Cerambycidae and Buprestidae) or moths (Cossidae and Hepialidae) and these are more prevalent in dead or dying trees. Poplar box can be found in high densities within some regions of the Darling Downs, due to seed dispersal and seedling establishment associated with flood water inundation (Oxley 1987). Poplar box woodlands are not only an important source of nest sites, they are also an important floral resource for bee populations (Weston et al. 1980).

"*Intense pressures on the use and management of land underscore the need for reliable and up-to-date information on the status of native species*." (Keith 2000).

This quote pertains to surveying rare plant species but is equally relevant to native bee populations. Monitoring population levels and their possible declines can only be undertaken if there is a baseline against which to gauge. As yet, there is very little information available on Australian native bee populations, whether social or solitary (Dollin et al. 2007; Dollin 2011). In order to build a baseline, fundamental information is required. This includes natural habitat characteristics, nest characteristics, nest choice, nest site availability, favoured floral resources, available floral resources and nest density and distribution. This study addresses some of these fundamental data. As Keith (2000) points out, there are few guidelines on field survey design or implementation with regard to plant (or, in this case, social bee) populations.

The population density of social insects is very much dictated by the availability of suitable nesting sites. The cluster-type brood structure of *Austroplebeia* colonies may enable them to utilise a variety of nesting cavities and many cluster-building species occupy smaller nest cavities, as is the case with some overseas stingless bee species (See Table 6.8 in the Discussion (Section 6.4.7) for comparative data).

The aims of this study were to:

- locate and sample nests within the focus study site, and identifying sampled colonies to genus level

- to gain skills in nest location in the wild and permanently record their location
- gain fundamental background knowledge on *Austroplebeia* habitat, nest site preferences and nest density.
- provide a basis upon which future surveys may be conducted in order to build an overall picture of the nest density and distribution of Australian stingless bees.

The studies reported in this section took the form of several discrete experiments. As such, the structure of this section (6.4) is set out for ease of understanding, describing each experiment separately, with materials and methods immediately followed by the results. The key findings and overall discussion are placed at the end of this chapter. Photographs within this section were taken by Steven Ruttley.

6.4.2 General materials and methods

Allan Beil is a local Tara resident and enthusiastic stingless bee keeper. Allan is a gifted 'bee hunter' and conservationist who has been 'rescuing' colonies for over 20 years. He is a retired timber cutter and has good knowledge of local vegetation, floral resources and favoured nest sites of stingless bees. In retirement, he enjoys wandering through the bush trying to locate wild stingless bee colonies, using their elusive guards (Figure 6.24a) and foragers (Figure 6.24b) as guides. Allan works in collaboration with personnel from the local office of the Queensland Department of Environment and Resource Management and Tara Shire Council. Nests under threat of destruction are of particular interest to Allan and he marks nests (Figure 6.25) located on public land, to alert council personnel of the presence of a nest. If, for any reason, the tree containing the nest is scheduled for removal, the council notifies Allan and he helps remove and relocate the nest.

Austroplebeia nest entrances are small, concealed and the bees do not congregate around the opening. Occasionally, as a defence against ant predators, a cerumen extension may protrude from the nest entrance (Figure 6.26). This extension makes nest detection much easier. Entrances are located from < 0.5 m to > 6 m above the ground, making nest location more difficult as its height increases (Figure 6.27).

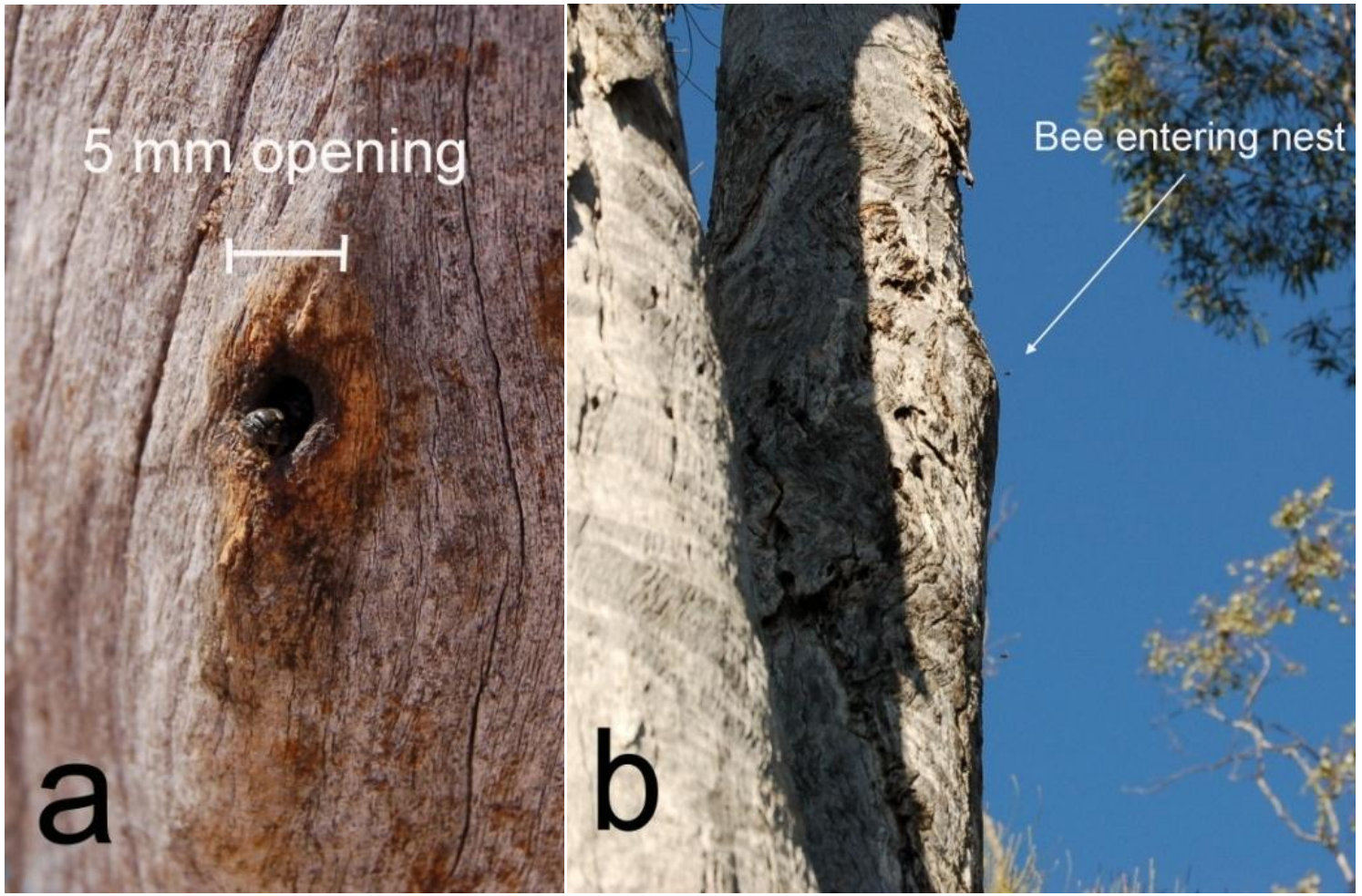


Figure 6.24 A single *Austroplebeia* guard at the nest entrance (a) and a returning forager (b).

Figure 6.25 This tree is located on the roadside and is marked with the base of an aluminium drink can, to notify council personnel of the existing nest inside. Note the nest entrance below the can.

Figure 6.26 *Austroplebeia* nest with a cerumen extension built at the entrance.

Figure 6.27 A 5 mm entrance hole, 5 m up a tree is very difficult to spot as well as access.

Three sets of nest data were collected for these studies. These included:

- GPS coordinates, which were collected for all wild nests. Within the focus study site these were used to estimate the nest density of *Austroplebeia* colonies. These data related to the nest sites in and around the Tara region, which covered ~ 1,500 km^2. Nest density was estimated using 17 locations, which contained 361 nests within the focus study site. Bees were sampled from 40 of these nests and identified.

- Additional GPS coordinates collected outside the focus study site. At the time of this report, Allan had collected coordinates for a total of 549 nest sites. These nest coordinates were used to plot the nest distribution within a wider survey site, based around Allan's fishing trips. This site extended from Inverell to Blackall and covered > 60,000 km^2.
- Detailed information on nest characteristics was also collected. Data pertaining to external nest characteristics were derived from the 549 nest sites and internal characteristics were obtained from 18 'rescued' nests.

6.4.3 Nest density and nest sampling within the focus study site

6.4.3.1 Materials and methods

Most nests had been previously located by Allan Beil and nest sites were accessed by 4-wheel drive or on foot. The nest entrance was located by carefully looking up the length of the tree, until foragers were sighted entering or exiting the entrance (Figure 6.28). Waypoints were recorded for newly discovered nests sites using a hand held Garmin global positioning system (GPS) map 60CSx (Garmin GPS Australia, Seven Hills, NSW, Australia). While standing at the base of the tree, GPS coordinates were recorded, along with entrance height. Tree species was noted and also if the tree was alive or dead.

As part of this study, bees were sampled from 40 nest sites and stored in 100% ethanol for later analysis. Where possible, nest entrances were accessed and a standard mouth-pooter, partially filled with water (1 cm), was used to aspirate bees from the entrance tube (Figure 6.29). Bees were trapped in the water at the bottom of the pooter jar and this immobilised them enough to quickly transfer them to ethanol-filled sample tubes. The waypoint of the nest was recorded on a slip of paper, using lead pencil, and placed inside the tube with the bees. The tube was then sealed and stored in an esky cooler containing ice. Identification of bee species was carried out upon my return.

Figure 6.28 Allan Beil patiently surveying the length of a potential nesting tree.

Figure 6.29 Sampling an easily accessed *Austroplebeia* nest entrance with a pooter.

Nest site data were entered into a CSV Microsoft Excel® file and uploaded to Google Earth® (2010, http://www.google.com/earth/index.html). Once all of the coordinates were identified and labelled, they were entered into Google Earth Pro® (http://www.google.com/enterprise/earthmaps/earth_pro.html). Nest coordinates within the focus study site were plotted in Google Earth Pro® and the area of the focus study site was calculated (Figure 6.30a).

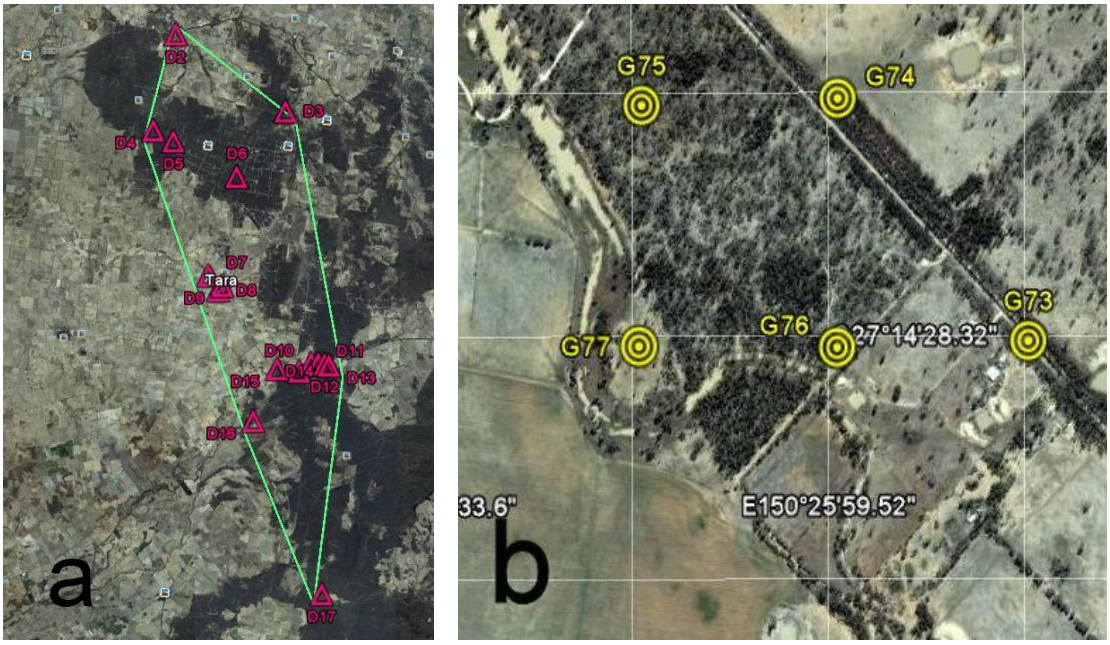


Figure 6.30 Focus study site (green outline) was determined by the nest site coordinates within the areas of density (pink triangles) (a) and Google Earth's 400 m (l) x 350 m (w) grid lines (b) which were used as guides to map out sites of interest within 14 ha areas.

The grid lines provided in Google Earth® were used as a guide to map the areas (Figure 6.30b). The cells created by these grid lines measured 400 m (length) x 350 m (wide), which equated to 14 ha. To estimate the nest density within specific sites, it was necessary to mark out and measure sites of interest and this was done based on parameters set out by the International Union for Conservation of Nature and Natural Resources (IUCN) (Keith 2000; Gaston & Fuller 2009). These sites were:

- the Area Sampled (AS) (Figure 6.31), based on the Google Earth® cells (Figure 6.30b),
- the nest Density site (e.g., D15 in Figure 6.31), which was the site related to a cluster of nests,

- the Extent of Occurrence (EO), which was the area of a polygon created by linking the furthest points within the cluster (Figure 6.31),
- the Areas of Exclusion (AE), which were sections of land that were unsuitable for surveying (e.g., across a river) or were uninhabitable (e.g., where no trees grew) (Figure 6.32),
- the Area of Occupancy (AO), where nests were actually located (Figure 6.33).

Density sites were located in the focus study site and these were used to calculate nest density within the Tara region.

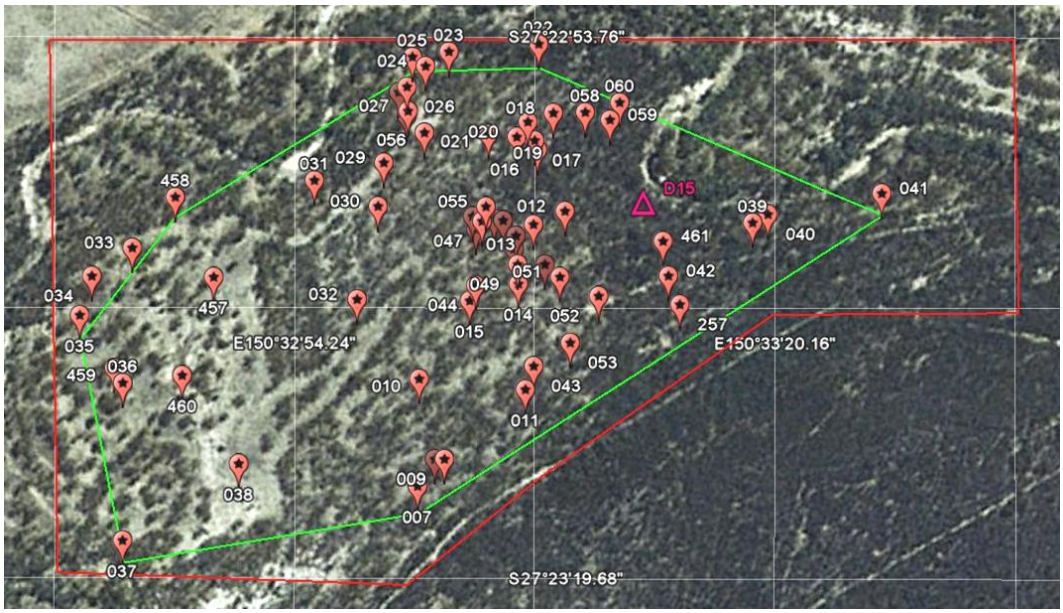


Figure 6.31 Nest Density site D15 (pink triangle), showing the Extent of Occurrence (EO) (green outline) within the Area Sampled (AS) (red outline). Pink balloons represent the nest locations.

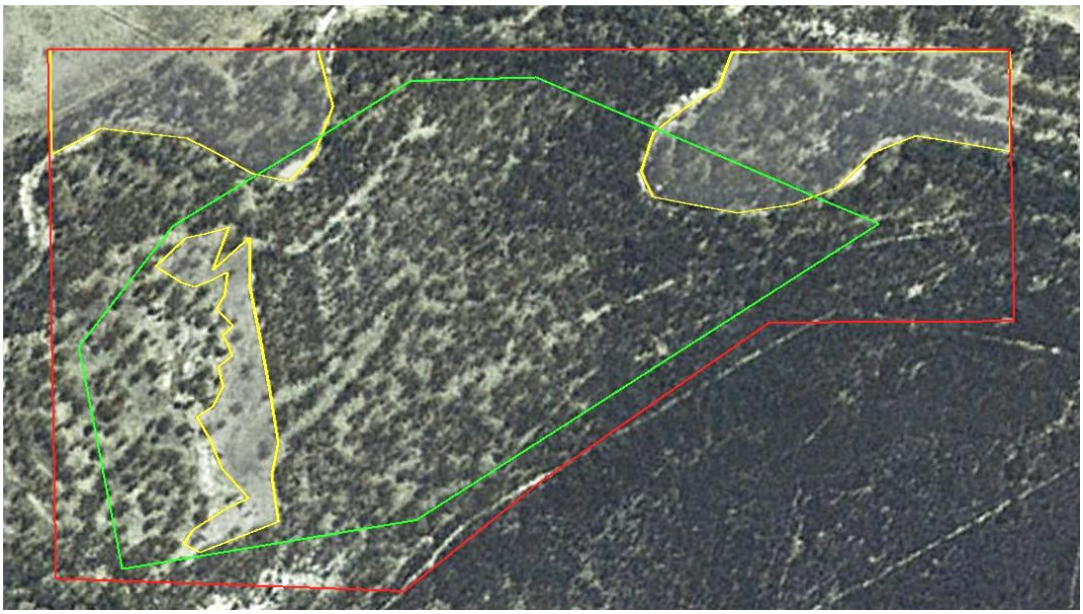


Figure 6.32 The Areas of Exclusion (AE) (yellow outline) were removed from the AS (red outline).

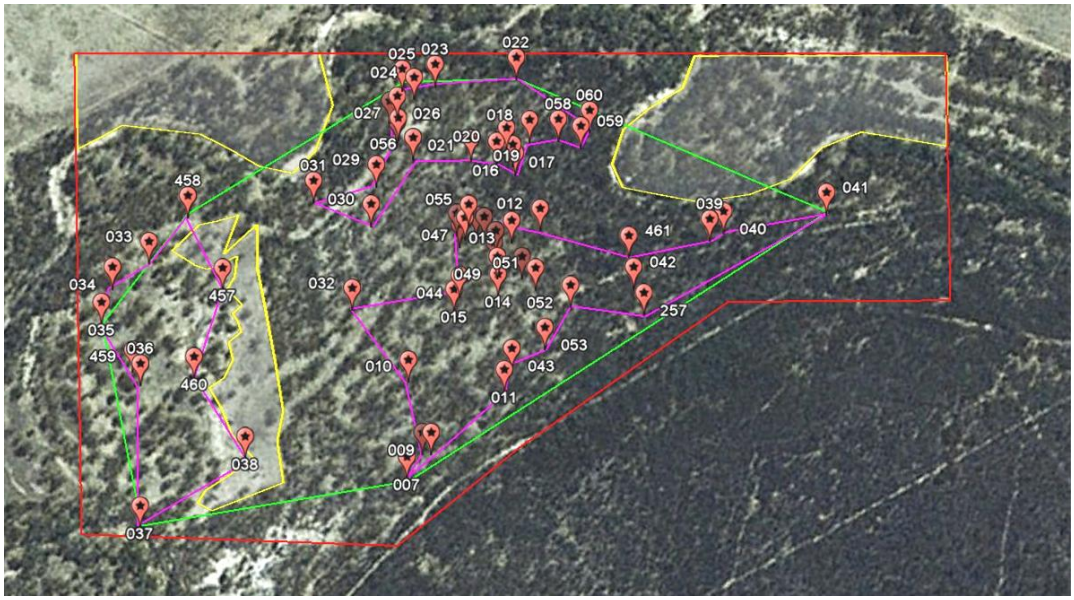


Figure 6.33 Nest sites located within the Areas of Occupancy (AO) (pink outline).

6.4.3.2 Results

Species present in sampled nests were identified by their thoracic colour markings only (see Chapter 4, Section 4.1.1.1). The 40 sampled nests contained either *Au. australis* or *Au. symei*, although it was difficult to definitively identify these species once they had been stored in ethanol (see Chapter 4, Section 4.1.1).

There were 17 Density sites, all varying in size, within the focus study site and they contained 361 *Austroplebeia* nests. The number of nests found within the Density sites ranged from six to 69 nests. Nest density ranged from 0.1 to 3.0 nests / ha, averaging 0.6 nests / ha (Table 6.5).

Table 6.5 Nest density (nests / ha) for each of the 17 Density sites, within the focus study site, as well as site description. The table includes the area sampled (AS) and area of occupancy (AO).

Density site	No nests	Area sampled (km^2) (AS)	Area of occupancy (AO) AS - AE (km^2)	Nest density for AO ha / (AS-AE)	Site description
D1	6	0.05	0.02	3.0	Near creek, mixed uncleared and cleared land
D2	33	1.56	0.72	0.5	Near roadside, mostly uncleared land
D3	17	0.86	0.86	0.2	Near unsealed roadside, forestry
D4	11	0.33	0.33	0.3	Near unsealed roadside, forestry
D5	13	1.69	1.69	0.1	Near unsealed roadside, forestry
D6	69	6.23	6.23	0.1	Near unsealed roadside, forestry
D7	21	0.32	0.32	0.7	Alongside a sealed road
D8	15	0.25	0.18	0.8	Tara golf course
D9	23	1.64	1.64	0.1	Near roadside, mostly uncleared land
D10	9	0.31	0.29	0.3	Forestry
D11	8	0.43	0.43	0.2	Near unsealed roadside, forestry
D12	35	1.25	1.25	0.3	Forestry
D13	10	0.24	0.24	0.4	Near creek, mixed uncleared and cleared land
D14	9	0.16	0.16	0.6	Alongside a sealed road
D15	62	0.89	0.72	0.9	Improved' land
D16	11	0.41	0.41	0.3	Alongside a sealed road
D17	9	0.15	0.13	0.7	Alongside a sealed road
Mean	24	0.99	0.92	0.6	

Based on the means of the cumulative number of nests per cell (based on Google Earth® grid, see Figure 6.30b), the trends of mean nest densities showed that, in order to obtain reliable, consistent data for future sample sets, it would be necessary to sample at least 150 cells / quadrats (Figure 6.34).

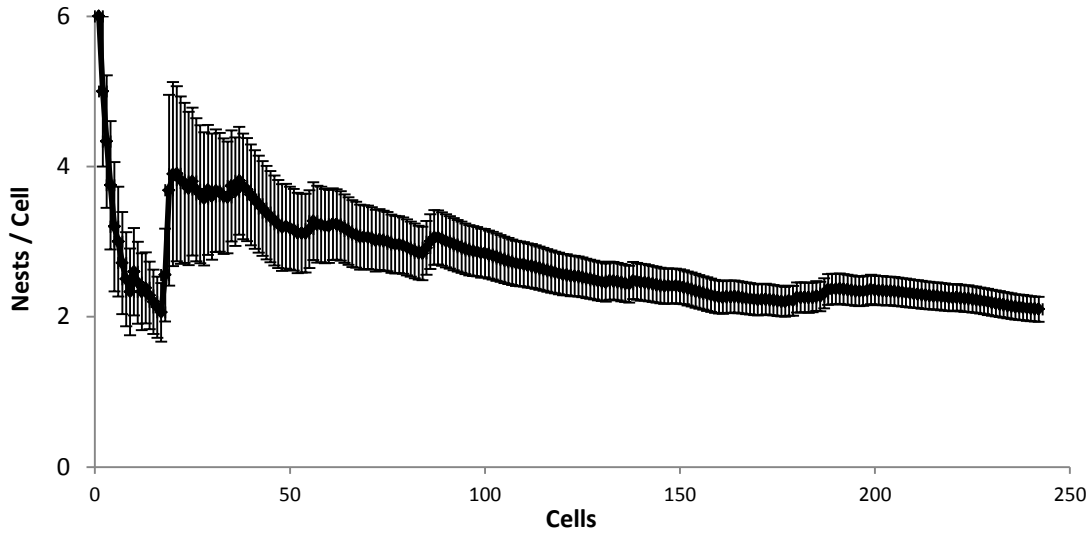


Figure 6.34 Trends in mean and standard error of nest density of entire sample data (n = 564). This graph shows that ≥ 150 cells or quadrats would need to be sampled in order to obtain consistent data.

6.4.4 Nest distribution and nest characteristics

6.4.4.1 Materials and methods

At 60 years of age, Allan decided he would like to document the location of the wild stingless bee nests, for posterity. In January 2008, he was given an old GPS and with this he has gradually revisited previously identified nests and recorded their coordinates. At the time of our visit, in October 2009, Allan had coordinates for over 250 nest sites in the local Tara region. Since my visit to Tara, Allan has recorded the coordinates for an additional 300 nest sites. These sites are more widely spread and cover an area from Inverell in NSW (29°78'S, 151°11'E, elevation 582 m) to Blackall in Qld (24°25'S, 145°27'E, elevation 287 m). Nest sites were discovered on various fishing trips and most sites are located near the roadside or near camping sites.

The nest tree characteristics were determined from data obtained from the nests within the focus study site, plus additional nests outside this site (area of distribution). GPS coordinates for 549 trees, containing 606 nests, were used to plot the nest distribution within the larger survey area of 61,500 km^2 (Figure 6.35). The location of each nest was recorded and colonies within the nest were identified to genus, based on the nest entrance morphology. *T. carbonaria* and *T. hockingsi* naturally occur in southern Qld and have distinctive nest entrance morphology (Dollin et al. 1997), compared to *Austroplebeia* species. Information relating to these nests also included tree species, whether the tree was alive or dead and, in addition, some trees had their diameter at breast height (DBH) and nest entrance height recorded.

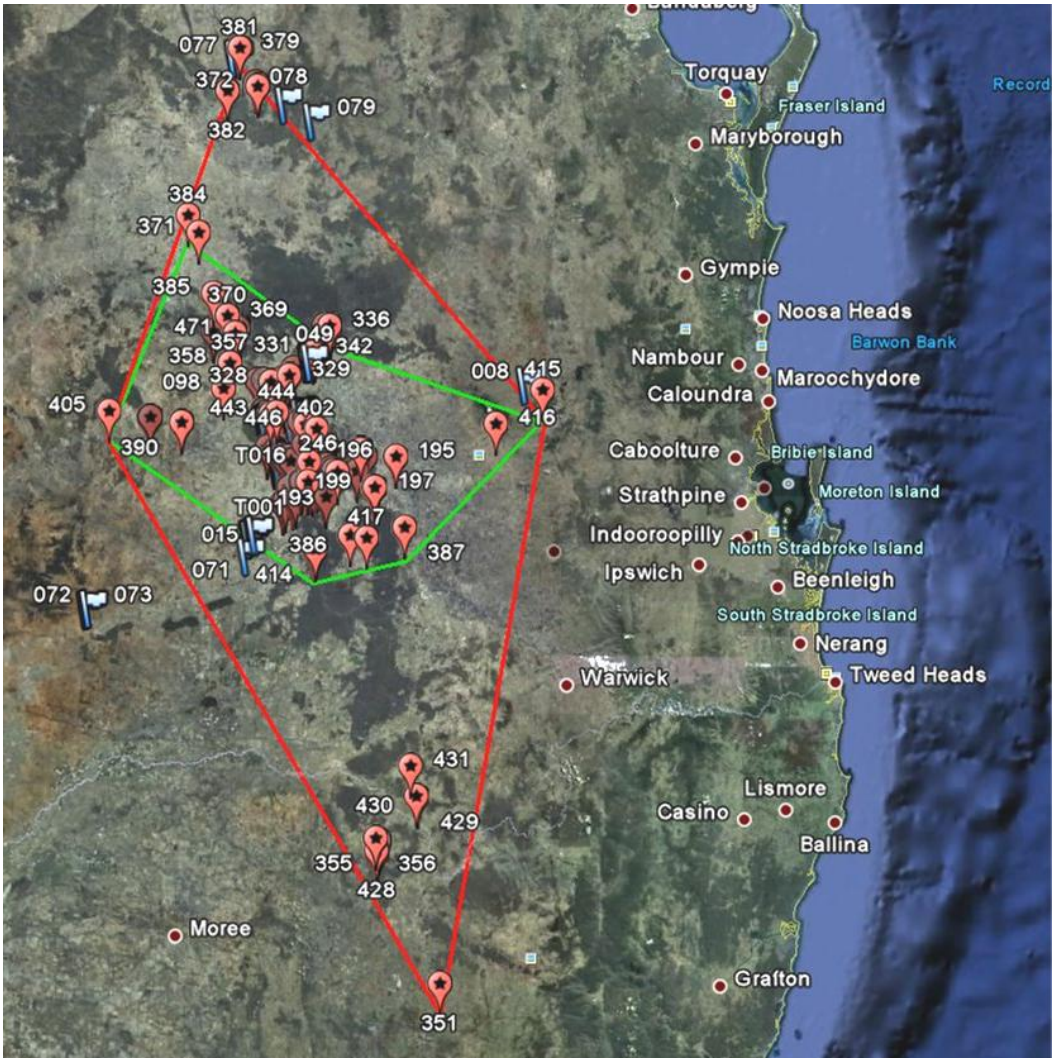


Figure 6.35 Extent of occurrence (EO) for the surveyed site. The area of distribution encompassed the nests within the EO (red outline).

6.4.4.2 Results

All nests were located in pre-formed cavities within either tree trunks or limbs. A total of 549 trees contained 606 nests. Of the 606 nests, 84% were *Austroplebeia* and 16% were *Trigona.* Ten trees contained two *Trigona* nests, 13 trees contained two *Austroplebeia* nests and one tree contained four *Austroplebeia* nests. Nests were located in a variety of living and dead tree species (Table 6.6) and dead trees made up 70% of the nest trees. Data for *Trigona* nesting site preferences were incomplete.

Table 6.6 Tree species used as nesting sites by the stingless bee species. Data for the number of *Trigona* nests is included here, as it was incomplete.

Stingless bee species located in tree cavity	**Tree species (common name)**	**No. of *Austroplebeia* nests**	
		Live tree	**Dead tree**
Austroplebeia	*Lysicarpus angustifolius* Druce (Budgeroo)	2	11
Austroplebeia	*Eucalyptus coolabah* Blakely & Jacobs (Coolibah)	2	5
Austroplebeia & Trigona	*Eucalyptus populnea* (Poplar box)	115	182
Austroplebeia & Trigona	*Eucalyptus* spp. (Ironbark)	0	167
Austroplebeia & Trigona	*Eucalyptus moluccana* Roxb. (Gum top box)	7	1
Trigona	*Callitris intratropica* R. T Baker & H. G. Sm. (Cypress pine)	-	-
Trigona	*Cinnamomum camphora* T. Nees & C. H. Eberm. (Camphor laurel)	-	-

The following data pertain to *Austroplebeia* nests only. A total of 494 trees contained 510 nests and 74% of those trees were dead. Poplar box accounted for 60% of the tree cavities inhabited by *Austroplebeia* and of the 297 poplar box nesting trees, 61% of those were dead (Table 6.7). Trunk diameter at breast height (DBH) of the poplar box nest trees (n = 26) ranged from 162 to 396 mm, averaging 258 mm. Entrance height (n = 118) from the ground ranged from 0.6 to 10.4 m, with a mean height of 4.2 m (see Table 6.8 in Discussion for comparative data).

Table 6.7 *Austroplebeia* nesting site within dead or living Poplar box cavities.

Total number of trees	494
Live trees (%)	26
Dead trees (%)	74
Number of nests	510
Number of multiple nests	14
Trees being Poplar box (%)	60.1
Total number of Poplar box	297
Trees being dead Poplar box (%)	61.3
Trees being live Poplar box (%)	38.7

6.4.5 Nest characteristics

6.4.5.1 Materials and methods

As previously mentioned, Allan ‘rescued’ colonies under threat of destruction. Trees may be removed or pushed over during land clearing for road widening, housing development as well as coal and gas mining activities. Allan obtained permission from the appropriate authorities to remove nest-containing trees from sites under threat. This was usually carried out during the cooler seasons, to avoid shattering the honey pots when the tree was felled. The sections of timber, measuring two to three metres in length, were cut above and below the nest. Logs were then relocated to his home (Figure 6.36). In spring / summer the nest were opened and transferred into a 7 L artificial Beil hive box (Chapter 3, Section 3.2).

Colonies could be left in a log nest for many years and they survive in dead trees for decades. Logs nests were, however, very difficult to manage because of their weight and bulk and could not be easily relocated. They must also be maintained in an upright position as the timber would begin to quickly deteriorate if left in contact with the soil surface. Allan Beil boxed most of his rescued colonies and relocated them to various places to reduce the population density near his home.

Figure 6.36 Rescued nests in logs. Note nest entrances.

Chainsaw extraction was used to remove colonies from the logs. This involved 'ripping' along the length of the log, on both sides. The cut was opened with a block splitter and the two pieces prised apart (Figure 6.37). The two sections were opened onto a clean tarpaulin and sections of brood were extracted from the cavity using a long, thin knife blade (Figure 6.38). Brood was placed at the back of the hive box and pollen at the front, around the entrance hole (Figure 6.39). Where possible, the queen was located and aspirated into a jar with a foam bottom. She was kept aside until the colony transfer was complete, then transferred into the box. Only intact honey stores were transferred to the new hive as spilled honey caused bees to drown.

Figure 6.37 The log was split in two and the pieces pried apart, exposing the nest inside.

Figure 6.38 Brood was extracted with a long, thin knife blade.

Figure 6.39 Brood and pollen stores relocated into the artificial hive box.

Internal nest characteristics were obtained from 18 rescued *Austroplebeia* colonies. Cavity and brood volume were obtained by measuring length and diameter (Chinh et al. 2005), using a digital vernier caliper (Australian Entomological Supplies, http://www.entosupplies.com.au) and a dress-maker's tape measure. It was important to obtain measurements as quickly as possible so as to avoid insect pest invasion. Colonies would also settle into their new hives more quickly if disturbance was kept to a minimum. Honey and pollen stores were given a rating (see Table 6.4, section 6.2.2 of this chapter), as more accurate volume measurements were difficult to obtain. The presence of a queen, queen cells and drones were also noted.

If a colony's stores were very depleted (as seen in the stores in the right hand side of the nest in Figure 6.40), a temporary honey or sugar-water feeder was placed in the hive. The artificial hive box was replaced close to where the nest log entrance was previously located (Figure 6.41) and a piece of resin from the original nest entrance was place on the outside of the new entrance (Figure 6.42). This enabled the workers to locate the entrance more quickly. The hive was sealed with a glass or clear acrylic lid and a heavy wooden lid was placed over that. The extraction process usually took Allan about ten minutes. When Allan had "assistance" from researchers it could take up to 20 min.

As colonies were boxed in spring and summer, there was usually an abundance of queen cells within the brood cluster. If the queen was not successfully relocated to the box, the colonies usually requeen before the end of the season (A. Beil pers. comm., 2009)

Figure 6.40 Honey stores rated 1 on the right side but 2 on the left. Pollen stores rated 3.

Figure 6.41 Relocating newly boxed colony close to original position. The original nest can be seen in Figure 6.36.

Figure 6.42 Workers entering new hive. Note resin around the entrance to improve orientation.

6.4.5.2 Results

The volume of the natural *Austroplebeia* nests (n = 18) ranged from 1.6 to 12.9 L, averaging 4.9 L. The nest cavity diameter ranged from 52 to 109 mm, averaging 75 mm and the log wall thickness ranged from 48 to 127 mm, averaging 85 mm. Nest length ranged from 370 to 1,950 mm, averaging 1,153 mm. The brood volume ranged from 425 to 2,613 mL, averaging 1,087 mL. Estimated stores for both honey and pollen ranged from < 200 to 800 mL and averaged 450 mL (for comparative data with other species see Table 6.8, in the Discussion, Section 6.4.6).

Four nests had large sections of the nest sealed-off from the main cavity, and these sections contained no stores. Sealed-off sections ranged from 17 to 36% of the total nest cavity capacity. Nest storage capacity was far greater than the structures filling the cavity (Figure 6.43). Many nests contained proportionately large sections of empty space in the form of empty storage pots or air spaces between storage pots (Figure 6.44). Involucrum sheets did not encase any of the brood clusters of the rescued colonies. Small sections of involucrum were noted on < 50% of clusters (Figure 6.45).

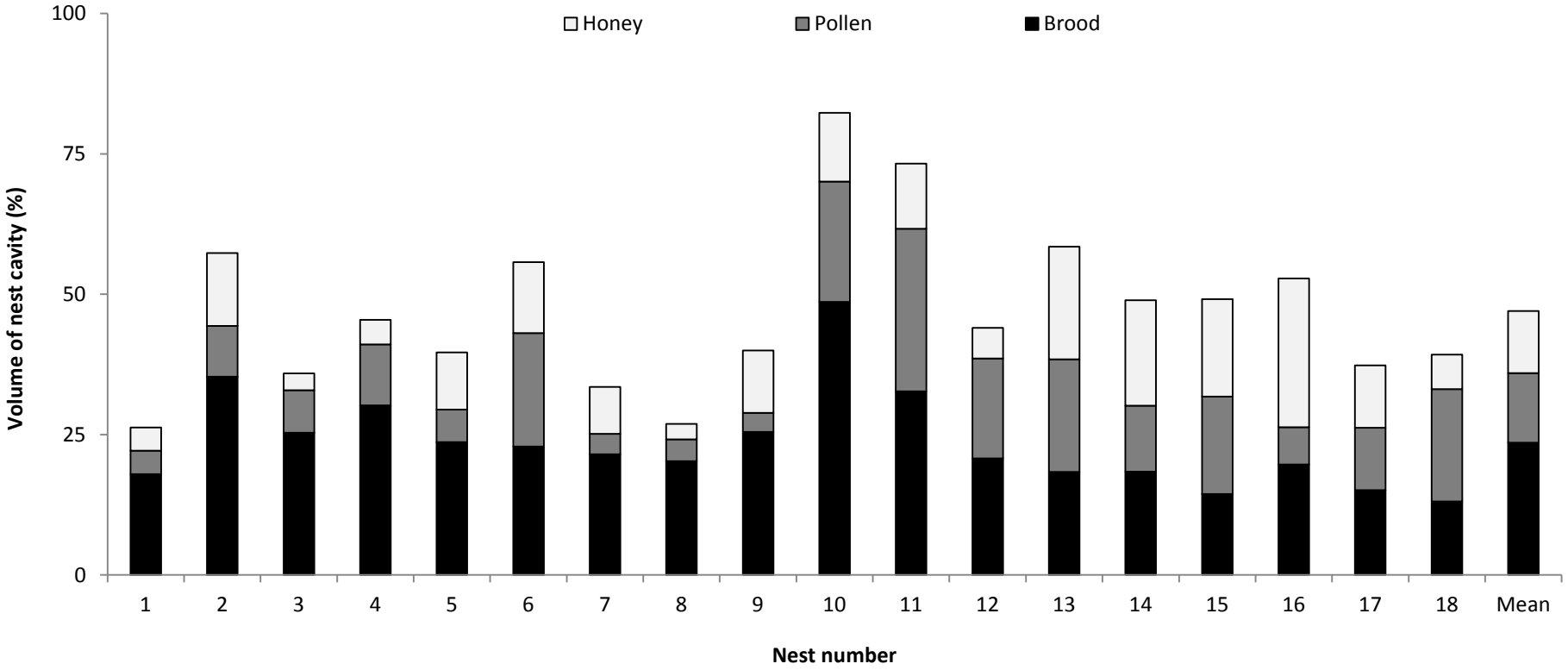


Figure 6.43 Percentage of nest cavities filled with nest structures, including honey (white), pollen (grey) and brood (black). The remaining sections were empty or occupied by empty storage pots.

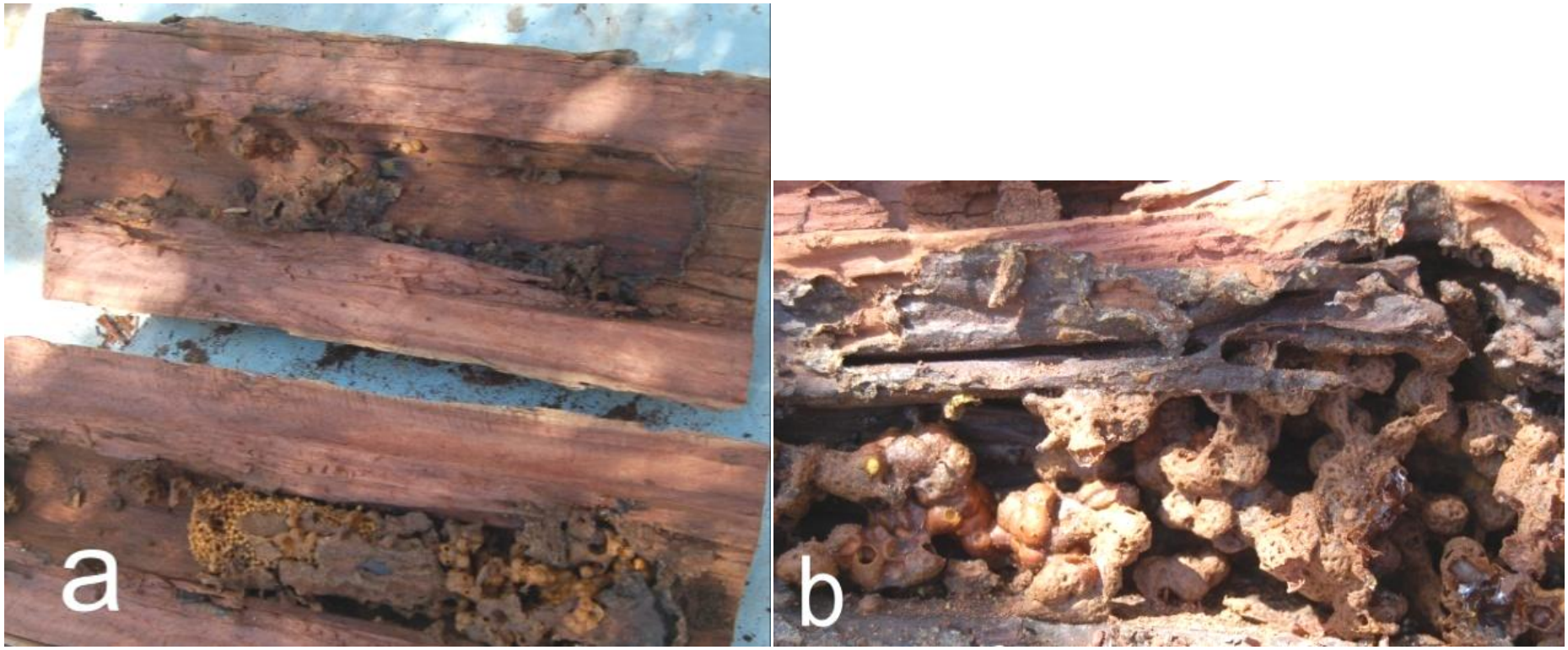


Figure 6.44 ***Austroplebeia*** **nest with severely depleted honey stores (a) and air spaces between pots of stores (b).**

Figure 6.45 Small sections of involucrum over brood surface.

The queen was successfully located and transferred to the new hive in 13 of the 18 nests and queen cells were easily located in 50% of the nests. Drones were observed in 17 of the 18 nests.

6.4.6 Discussion

This study is the first assessment of nest density for Australian stingless bees within a small focus study site. For ease of discovery and to save time, nests in the survey sites are mostly located in places that allowed for vehicular access, or within walking distance of the vehicle. As such, vegetation ranged from long strips of remnant roadside vegetation, sparsely spaced trees around the townships, or forestry vegetation. The focus study site suffers frequent, prolonged droughts and occasional major flooding events, making food resources unreliable and nesting trees, especially dead trees, vulnerable to destruction. Given the harsh environment of their native range, it was hypothesised that this region would contain insufficient resources to support high densities of stingless bee nests.

Austroplebeia nest density within the focus study site averaged 0.6 nests / ha (ranging from 0.1 to 3.0), which is comparable to reports for some other social bee species. Relatively few studies have been conducted in relation to stingless bee nest density. Darchen (1972) reports mixed stingless bee species at densities of 2.5

nests / ha in the savannah-woodland in the Ivory Coast of Africa and Eltz et al. (2002) reported densities ranging between 0 and 2.1, averaging 0.5 nests / ha in continuous forests of Sabah, Malaysia. These densities are considered 'low' compared to the forest fragments, where nest sites border mangroves and plantations, and range from 2.4 to 16, averaging 8.4 nest / ha. Darchen (1972) also reports as many as 22 *Hypotrigona* sp. colonies located within a single tree in the Ivory Coast.

Feral European honey bee populations within the Wyperfeld National Park in Victoria, Australia are considered to be 'very large' when maximum densities reach 1.5 colonies / ha (Oldroyd et al. 1997). Nest density of natural African honey bee populations can vary from 0.04 colonies / ha (McNally & Schneider 1996) to 3.3 colonies / ha, which is also considered to be a 'high' density (Quong 1993, cited in Neumann et al. 2001). The authors of these papers have conflicting opinions of what they consider high or low nest densities. This is probably due to the lack of baseline information regarding the nesting habits of many social bee species.

Hubbell and Johnson (1977) report a nest density of one to 1.4 nests / ha for mixed stingless bee species, within a tropical dry forest of Costa Rica. Nest spacing is uniform for four of the eight studied species, due to intraspecific aggression which prevents new colony establishment. Hubbell and Johnson (1977) demonstrate that nest density is not limited by nest site availability, as numerous suitable nesting sites are identified within the study site. They do, however, show a positive correlation between bee size and nest tree diameter. Large-bodied bees require larger nesting cavities than smaller bees (Hubbell & Johnson 1977) and species that build cluster-type brood require some of the smallest nesting cavities (Roubik 1979; 1983). Nest site availability is restricted for species requiring large nest cavities, especially in districts with young tree populations or trees that suffered premature death.

In the current study, the *Austroplebeia* nests are located in trees with a DBH ranging from 163 to 396 mm, and averaging 258 mm, which is similar to other cluster-building stingless bee species (Table 6.8 below). Poplar box trees can grow to 20 m in height (Harden 2002) with a DBH up to 750 mm (OEH 2003), and could potentially provide substantial hollows for all types of native animals (Dorricott et al. 1999; QPWS 2011), including *Austroplebeia* and *Trigona* colonies. Ring barking was practiced extensively on trees of all ages, causing premature death and reducing

the potential cavity development. These trees, therefore, provide ideal nesting sites for *Austroplebeia*, but are less likely to provide large enough cavities for *T. carbonaria* or *T. hockingsi*.

Food resources appear to be the most limiting factor with regard to colony establishment and ongoing nest density (Hubbell & Johnson 1977; Eltz et al. 2002). With an average density of 0.6 nests / ha, *Austroplebeia* nest density could be considered low. Despite this, several sites shows higher levels of density, such as D15 which contains 62 nests (i.e., 0.9 nests / ha). This section of forestry land appears to have been 'improved' for grazing as it is more sparsely vegetated than the adjacent section (Figure 6.46). The nests furthest from the unimproved vegetation are up to 620 m away and those closest to the boundary are 90 m away. With increasing tree density comes a potential increase in floral resources and nesting trees. It could therefore be expected that if the adjacent section was surveyed, nest density would be higher than D15.

It is thought that Australian stingless bees' home flight range is only 500 m (Heard & Dollin 1998). This increases the importance of sufficient floral resources close to the nest site, as is the case in a more densely forested region. Despite this, vegetation which has been left untouched may not contain the same proportion of dead trees, the apparent favoured nest site for *Austroplebeia*. Based on this study, *Austroplebeia* appear to favour dead trees as nest sites; however, these data may be biased due to the increased ease with which nests are able to be located in dead trees compared to living ones. Further studies on nest site availability, nest site choice and floral resources would be very useful to better understand nest density and distribution of stingless bee colonies.

Figure 6.46 Density site D15 located adjacent to an unimproved section of forest.

The random distribution of nest sites, and the presence of more than one nest / tree in some cases, appear to indicate that there is no intraspecific aggression between colonies of *Austroplebeia* (Hubbell & Johnson 1977). *Au. australis* and *Au. symei* do not forage in aggregations or small groups (see Section 6.3.3.3 of this chapter) and, so far, there is no scientific evidence that pheromone markers are used during foraging or for nest site hunting. However, tree markings have been reported around nest entrance holes and also the holes located nearby (A. Beil, pers. comm., 2009) (Figure 6.47), and it is speculated that these markings may contain pheromones. Pheromone markers are used by more aggressive stingless bee species to ward off rival colonies (Hubbell & Johnson 1977).

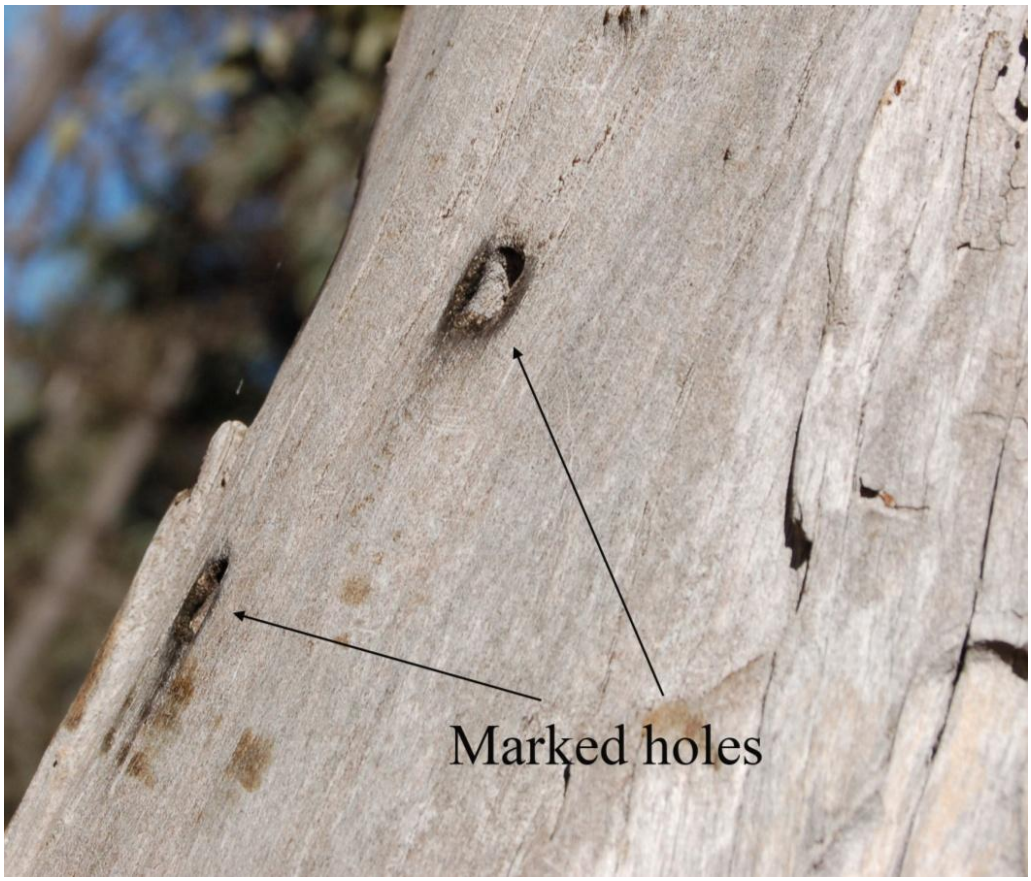


Figure 6.47 Holes which have been 'marked' by *Austroplebeia* workers. One of these is the nest entrance, the other is not.

Austroplebeia spp. appear to prefer long narrow cavities which occur more readily in trees that have suffered premature death. Most stingless bee species nest in living trees (Roubik 1989) and they make up 100% (*Lisotrigona scintillans* Cockerell), 92% (*Trigona collina* Smith), 67% (*Trigona melanocephala* Gribodo, *Trigona rufibasalis* Cockerell) (Samejima et al. 2004), 91.5% (13 *Trigona* species) (Eltz et al. 2003) and 100% (32 Panamanian species) (Roubik 1983) of nest sites, compared to the only 26% for *Austroplebeia* in the current study. In comparison, of the 57 *Trigona* nests located by Allan, 67% of these occupied living trees. It should be noted that this is only a small sample set and further surveys are needed to determine *Trigona*'s preferred nest site.

The data here indicate that *Austroplebeia* colonies are found in the cavities of five different myrtaceous tree species, suggesting that colonies probably do not discriminate between tree species for their nesting. Nests can also be found in fence posts, iron hand rails and architraves (Dollin 2010d). There is, however, an

indication that they may prefer Poplar box. Non-discrimination against tree species provides colonies with a greater chance of locating a nest tree, as long as they have the right cavity characteristics.

Nest cavity and brood volumes of the sampled *Austroplebeia* colonies are within the same size range as *T. nigra pauper* (Roubik 1983); however, other cluster-building species have much smaller nest volumes (Table 6.8). Researchers demonstrate that cluster-building species' nest volumes are substantially smaller than some of the comb-building stingless bees, including the Australian *T. carbonaria* and *T. hockingsi*. Roubik (1983) reports *T. nigra pauper* and *Trigona buyssoni* Friese nesting in trees with a DBH and cavity wall thickness similar to *Austroplebeia* and have various nest entrance heights (Table 6.8). The ability to utilise small diameter cavities is an important advantage in regions where trees have been prematurely killed by ringbarking.

Stores of pollen and honey within the *Austroplebeia* nests were low. Sixteen of the 18 nests contained large, empty spaces equivalent to 26 – 73% of the available cavity space. Four colonies had sealed off an additional 17 – 36% of their nest cavity, presumably to reduce nest upkeep and defence tasks. At the time of my visit (October 2009), Tara shire had been drought-declared since June 2006 (DERM 2011) and depleted *Austroplebeia* colony stores were probably indicative of this lack of floral resources.

With the generous support and teaching by Allan Beil this preliminary study has enabled me to learn how to locate stingless bee nests in their natural environment. It has also given me a basic understanding of how to carry out a nest site survey. This study does not include zero density records. The trend of mean and standard error of nest densities indicates that, using the current technique, it will be necessary to sample at least 150, 14 ha cells. This level of activity seems unrealistic; therefore, a reproducible survey technique needs to be designed.

Table 6.8 Nest tree characteristics for cluster-building (above the heavy line) and comb-building (below the heavy line) stingless bee species. Estimates of brood population in *Austroplebeia* based on 5 cells / mL (see this chapter, Section 6.5).

Species	Entrance height (m)	DBH (mm)	Tree wall thickness (mm)	Cavity diam (mm)	Cavity volume (L)	Brood volume	Reference
Trigona nigra pauper	2 – 8.7	80 – 300	15 – 120	30 – 150	5.6 – 8.7	1280 – 4090 cells	Roubik 1983
Trigona buyssoni	2 –12.0	420	30 – 120	25 – 110	0.2 – 2.9	2400 – 2880 cells	Roubik 1983
Trigona savannensis Roubik	1.5 – 4.5	110	20 – 40	35	2.6	1100 cells	Roubik 1979
Trigona franki Friese	0.2 – 1.6	130	30 – 60	10	0.2 – 0.5	212 – 342 cells	Roubik 1983
Lisotrigona carpenteri Engel	0.9	Nest in crevices	-	-	0.2 – 1.0	< 200 cells	Chinh et al. 2005
Hypotrigona & *Liotrigona* Moure	-	≥ 100	-	-	-	-	Darchen 1972
Austroplebeia	0.6 – 10.4	162 – 396	48 – 127	52 – 109	1.6 – 12.9	425 – 2613 mL	Current study
Trigona carbonaria	-	200 – 1500	-	110 – 550	-	940 – 3535 mL	Dollin et al. 1997
Trigona hockingsi	-	140 – 750	-	70 – 360	-	1100 – 2550 mL	Dollin et al. 1997
Trigona cilipes Fabricius	0 – 3.0	360 – 1500	-	110 – 150	0.8 – 3.5	6000 – 11000 cells	Roubik 1979
Trigona clavipes Fabricius	0.2 – 8.0	330 - 880	-	90 – 280	6 – 67	16000 – 23000 cells	Roubik 1979

To do this, prior knowledge of the survey site will be necessary. Key questions include: are there suitable nest trees in the site? is the entire site accessible? does the site have a history of land clearing or ringbarking? Two survey techniques may be utilised, including ‘random sampling’ (Figure 6.48) of quadrats within the sample site or ‘adaptive sampling’ (Figure 6.49) (Sutherland 2006).

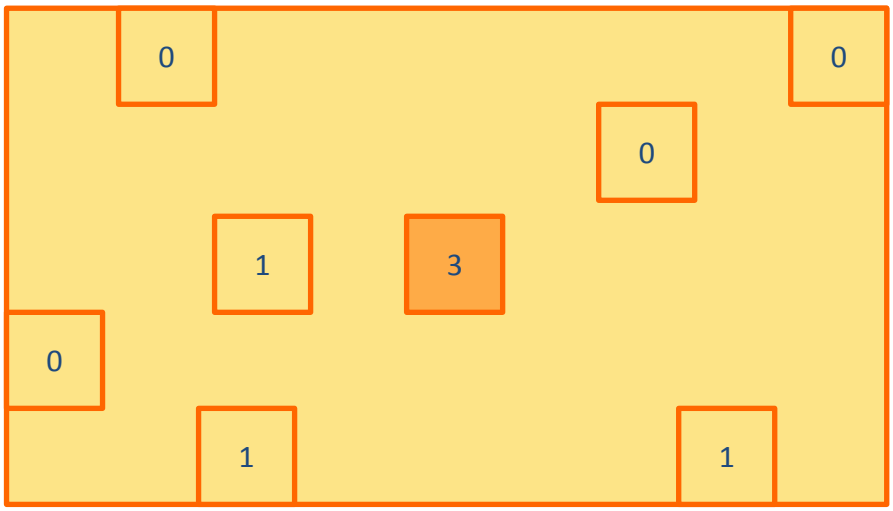


Figure 6.48 Random sampling technique (Sutherland 2000).

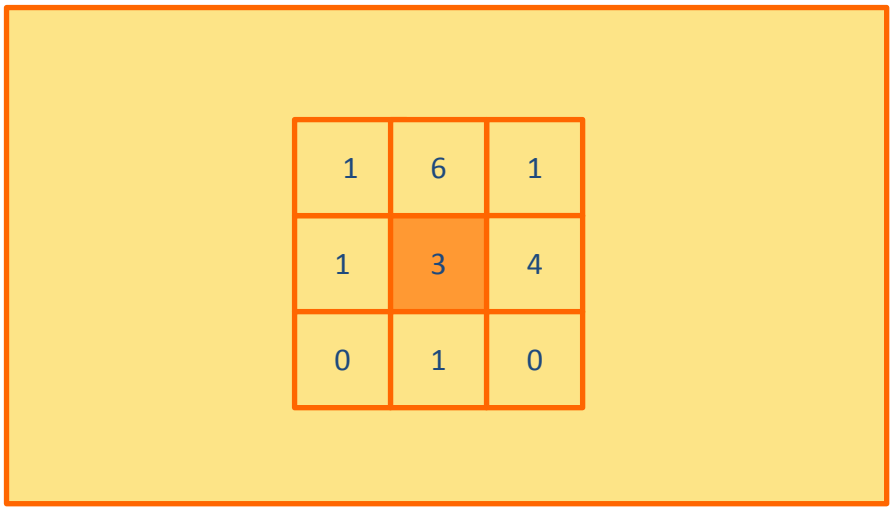


Figure 6.49 Adaptive sampling technique (Sutherland 2000).

One hectare quadrats could be designed around the terrain; where a 100 x 100 m square is used in a site of continuous vegetation, such as managed forestry, or a 1000 x 10 m area could be used for remnant vegetation along a roadside or on farm land.

Areas of occurrence (AO) and exclusion (AE) as well as extent of occurrence (EO) could still be utilised in this technique. Zero density would also be recorded.

6.5 Estimating brood population size

6.5.1 Introduction

As part of colony management, it is important to obtain population estimates of both adults and developing young. Adult populations can give an indication of the colony age and strength (Roubik 1979). The population can only be accurately obtained by killing the colony and counting the individuals (Roubik 1979), a less than practical activity. Some researchers have devised formulae to help estimate colony populations such as Bc = 0.25 Br + 0.5 Be, where Bc = corrected estimate of total adult bees, Br = total brood and Be = field estimate of total adult bees (Roubik 1979). Such a formula requires that the brood population be known.

Estimating the population of developing brood within comb-building colonies can be fairly simply undertaken, by measuring the surface area of the comb, which includes each disc of brood, then measuring 5 x 5 contiguous cells. The total area of all the discs is then divided by the area of 25 cells and the quotient is multiplied by 25 (Roubik 1979). Because comb cells sit tightly beside each other, the average diameter of a cell can be used to estimate the comb area. Cluster-type brood construction, on the other hand, can only be estimated destructively, by counting individual cells (Roubik 1979). Again, this is an impractical management tactic.

In cluster-type broods, knowing the average cell diameter will not give an accurate estimate of the population, as the cells are arranged in a loose cluster configuration. This allows room for normally cell expansion with larval growth and metamorphosis. There are no reported methods for the non-destructive estimation of brood populations for cluster-building species, including *Austroplebeia*.

Colonies of *Au. australis*, which had sustained brood damage due to ‘failing’ queens (see Appendix 8), contained areas of desiccated, but intact, brood clusters. The nature of the brood damage meant that the brood cells were constructed, provisioned, sealed and left to develop normally. However, normal development did not occur,

probably because the egg did not hatch. The cells remained in the position in which they had been constructed, on small pillars of cerumen. Provisions within the cells dehydrated and the operculum collapsed. Cell desiccation produced a skeletal representation of a normally developing brood cluster (Figure 6.50). For reasons unknown, colonies that suffered from this brood damage did not tear down the structure as part of an expected hygienic ritual, but continued to build non-viable cells.

Figure 6.50 A cluster of desiccated, intact brood cells within a 'failing' *Au. australis* colony.

6.5.2 Method

The brood skeleton was used to estimate the number of cells within a given brood volume, thus giving an estimate of brood population / volume. The dimensions of a

large brood cluster within one of the 'failing' colonies were measured while still in the hive, to obtain a volume. The brood cluster was then removed from the hive and cells were individually counted. This method only provided an estimate, but it appears to be the most accurate non-destructive method of calculation found so far.

6.5.3 Results and discussion

There were approximately five cells per mL and from this information it is possible to estimate the brood population of a colony, as long as the brood volume can be measured. From the nest characteristic study (Chapter 6, Section 6.4.4.2) it was found that brood volume ranged from 425 to 2,613 mL, with an average volume of approximately 1,000 mL. This equates to a brood population ranging from 2,000 to 13,000, with an average of 5,000. South American researchers estimate that brood populations in stingless bees are usually three to five times that of the adult population (Roubik 1983). Based on this, it is possible to estimate the total population of a colony; however, it is speculated that the adult population of an *Au. australis* colony may be higher than this estimate, given the longevity of their nestmates as reported in this thesis (Chapter 5).

6.6 Overall discussion

Within their native range, *Au. australis* colonies can experience extreme temperatures; however, the average daytime temperatures range between 19.8 and 26.1°C (BOM 2012). These temperatures are within the ranges of their minimal temperature threshold (~20°C) and optimal temperature threshold (> 26°C) for flight activity (Section 6.1). The optimal temperature is likely to be regularly attained throughout the year. *Au. australis*' preference for colonising narrow cavities within dead trees may be due to an inability to thermoregulate their nests, thus relying on the first morning sun to warm their nest cavity. The relatively thin outer walls of these small trees (mean 85 mm, ranging from 48 to 127 mm) should readily allow solar radiation to warm the long thin cavity within. As the ambient temperatures approached the minimal threshold, and the nest is warm, the colony is prepared for flight activity.

Removing the need to thermoregulate the nest reduces consumption of stored resources. Allowing the brood to cool to the point where its development is minimal will reduce the adult population within the colony in the short term. Unnecessary consumption of stores ensures food reserves remain available for existing and newly emerged adults. Then, as temperatures increase so does brood development, resulting in adult emergence at a time when colony activities demand higher adult populations.

As a result of the studies reported in this chapter it is possible to ascribe the effects of a number of environmental factors to certain *Au. australis* entrance activities. As these factors are not exclusive, and no quantitative data are available, the summary table (Table 6.9) indicates probable activities and the factors that influence them. Further studies should help to develop a more comprehensive understanding of these interactions, and possibly to develop a predictive model.

Table 6.9 Table summarising effects of some environmental factors on *Au. australis* entrance activity. Behaviour may be unchanged (=), increase activity (+) or reduce activity (-).

Environmental factors		**Behavioural response by bees**
Dawn	In-nest temperature > 18ºC	Open entrance curtain Check outside temperature
Heat	Temperature remains below threshold (< 20ºC)	= No flight activity
	Temperature reaches threshold ≥ 20°C	+ Flight activity
	Temperature reaches optimal > 26°C	+ Increase flight activity as temperatures increase (no known threshold where flight ceases)
Light	Enough light to detect sun's position or locate landmarks	+ Flight activity
	Reducing light or increasing cloud cover	- Reduced flight activity
Rain	Clear but rain developing	Flight activity reduces and bees return to nest
	Currently raining	No flight activity. If heavy rain, may close entrance curtain
	Rain clearing	Dependent on time of day (±): Morning shower + flight activity resumes after rain clears Afternoon shower - flight activity ceases for remainder of day
Humidity	If associated with pending rain	- Flight activity ceases
	If clear (< 6 oktas), with ≥ 20°C	+ Flight activity
Food resources	Foragers collect available floral resources	+ Flight activity increases as resources increase or are located
		+ Flight activity remains high while resources are abundant
		- Flight activity declines as resources decline
Dusk	Bees return to nest prior to dusk	+ Flight activity continues but declines as dusk approaches
		- Returning bee numbers greater than numbers leaving nest
		- Flight activity ceases at dusk
		Entrance curtain closed after dusk

6.6.1 Current distribution of *Au. australis*

Nest distribution reported in Section 6.4, ranged from Blackall in the north to Inverell in the south (Figure 6.35). Until now, it was thought that the major factor limiting the southerly distribution of *Au. australis* was its sensitivity to low winter temperatures. However, the data presented in Section 6.2 suggest that this may not be the case. For example, *Au. australis* colonies located near Inverell are exposed to mean minimum temperatures of -0.2°C (> 100 year data) and temperatures as low as -8°C (1981 – 2010) (BOM 2012b). Some inland regions of south-east Qld in which *Au. australis* naturally occur include Warwick, Qld (28.22°S, 152.03°E, 477 m) and Dalby, Qld (27.18°S, 151.26°E, 344 m), which also experience sub-zero temperatures in winter (Table 6.3) (BOM 2012c). On the other hand, Kempsey, NSW (31.08ºS, 152.82ºE, elevation 10 m) is the most southerly-coastal location (see Chapter 4, Figure 4.2) and has less extreme winter temperatures (mean min 5.7°C, lowest recorded -1.8°C) (BOM 2012b) than Warwick and Dalby, where *Au australis* thrive.

Studies reported in Section 6.1 demonstrate that colonies of *Au. australis* have a minimal threshold of ~ 20°C for the commencement of flight activity, and an optimal threshold of ≥ 26°C. Flight activity is also adversely affected by heavy cloud cover, and ceases at levels ≥ 6 oktas. Studies reported in Section 6.2 demonstrate that colonies generate substantial amounts of metabolic heat at temperatures > 15°C. Ambient temperatures ≥ 20°C are sufficient to warm the nest to > 15°C, resulting in increased metabolic heat production, leading to consumption of energy and food resources.

Based on these data alone, it is possible to predict why *Au. australis*' most southern distribution currently exists. The data presented in Figure 6.51 is based on long term (27 – 100 years) climate data reported by the Bureau of Meteorology, Australia (BOM 2012b). The daytime temperature data can be obtained from the BOM website and report the mean monthly 09:00 and 15:00 temperatures, for the above locations. It also contains data for areas where colonies of *Au. australis* have been transferred and are maintained in artificial hives. Reasons for colony transfer include research

(Richmond) and hobbyist activities (Newcastle and Sydney). Long-term monthly averages were used to calculate:

- Cloudy days = days with ≥ 6 oktas of cloud in the sky (see glossary). *Au. australis* foragers remain within the nest when this level of cloud cover exists.

- Sub-optimal foraging = days when ambient temperature reaches ≥ 20°C but do not exceed 26°C and there is < 6 oktas of cloud in the sky, thus enabling foragers to leave the nest, but not necessarily being able to collect available floral resources at optimal levels. These temperatures also stimulate high metabolic activity and consumption of food stores.
    - **The colony's food consumption ≥ incoming food resources.**

- Optimal foraging = days where ambient temperature reaches ≥ 26°C with < 6 oktas of cloud in the sky, thus enabling foragers to leave the nest and collect available floral resources at optimal levels.
    - **The colony's food consumption < incoming food resources.**

- Heavy cloud cover = days when ambient temperature reaches ≥ 20°C but also have heavy cloud cover (≥ 6 oktas), creating conditions where foragers will not leave the nest. These temperatures also stimulate high metabolic activity and energy expenditure, but conditions see no incoming floral resources to fuel the increased metabolism.
    - **The colony's food consumption > incoming food resources.**

- Quiescence = days when ambient temperature is < 20°C, which would not sufficiently warm the nest or colony enough to stimulate metabolic heat production. Energy consumption would remain low and stored food would be consumed at a very low rate, if at all.
    - **The colony's food consumption = zero incoming food resources.**

Data presented in Figure 6.51 are estimates only.

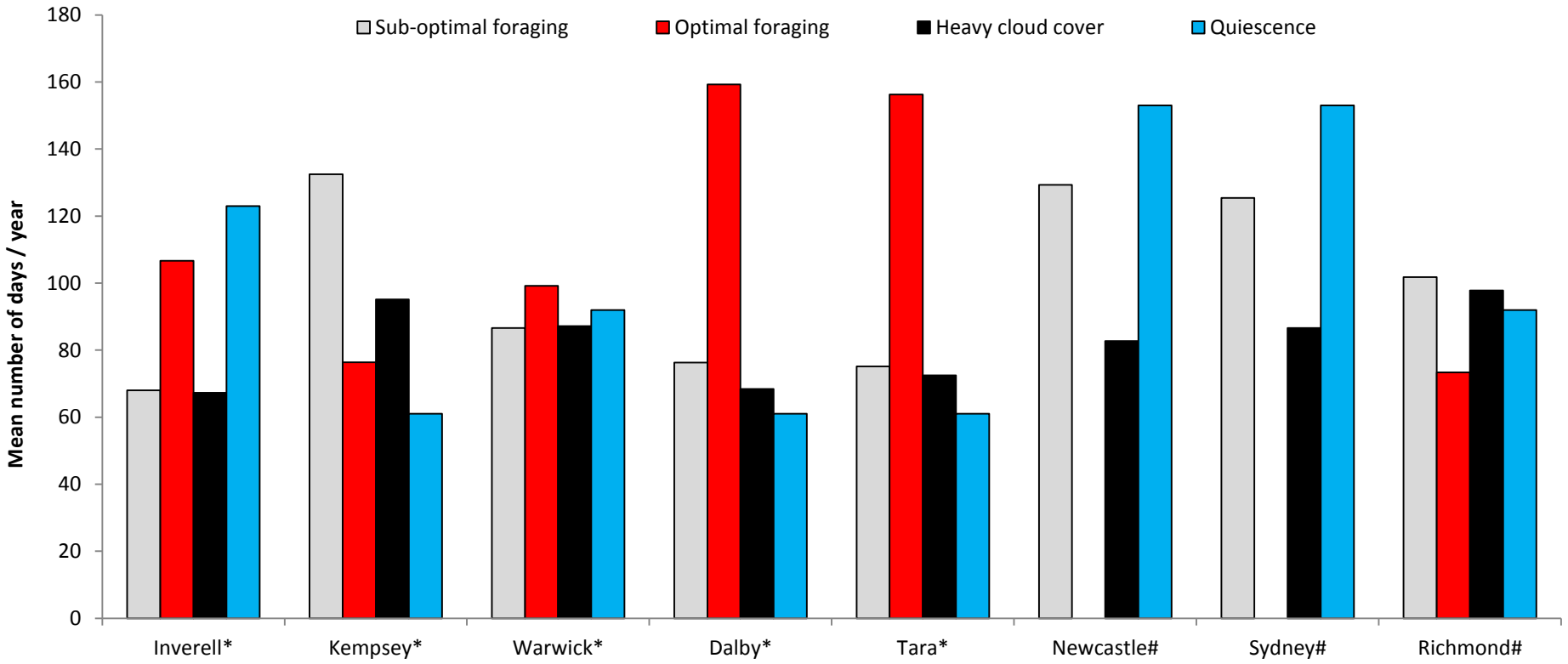


Figure 6.51 Days / year that meet the climatic requirements for *Au. australis* flight activity. Sub-optimal foraging = days where ambient temperature ≥ 20°C but < 26°C, with < 6 oktas of cloud in the sky; Optimal foraging = days where ambient temperature ≥ 26°C, with < 6 oktas of cloud in the sky; Heavy cloud cover = days where ambient temperature ≥20°C, with ≥ 6 oktas of cloud in the sky resulting in foragers being unable to leave the nest; Quiescence = days where ambient temperature < 20°C, thus reducing the colony's metabolism. Symbols * indicate towns within the native range of *Au. australis* and # indicate towns outside the native range. Data are averages for between 27 – 100 years, depending on location (BOM 2012b). Data presented here are estimates only.

Colonies transferred into areas such as Sydney and Newcastle would not experience any 'optimal foraging' days (Figure 6.51). They would be unable to harvest resources at levels sufficient to provide adequate stores to sustain them over periods of time when they cannot leave the nest due to 'heavy cloud cover'. Exposure to consistent conditions when **the colony's energy consumption > incoming resources** would lead to colony death. Even with management intervention, these colonies may still starve.

For a large proportion of the year (26%), foragers within colonies located in Richmond and Kempsey cannot leave the nest due to 'heavy cloud cover'. They are able to forage at 'optimal' levels for 20% of the year. Colonies experience 'sub-optimal foraging' conditions for around half the year (48% and 57%, respectively). This results in **the colony's energy consumption ≥ incoming resources.** These conditions may be considered marginal for colony survival. Natural colonies near Kempsey may be exposed to higher quality floral resources during their limited foraging periods, thus enabling them to survive. Further studies may confirm this.

Locations which are known to support large numbers of strong *Au. australis* colonies, such as Dalby and Tara, experience 'optimal foraging' conditions for 43% of the year. This enables resources to be collected and stored faster than their consumption, resulting in **the colony's energy consumption < incoming resources**. Excess stores increase the likelihood of colony survival during time when foragers cannot leave the colony due to 'heavy cloud cover' (only 17%) or low floral resource availability.

Inverell, *Au. australis*' southernmost location, experiences 'optimal foraging' conditions for only 29% of year, much lower than Tara / Dalby, and bees cannot leave the nest due to 'heavy cloud cover' for 18% of the year, similar to Tara. Time available for resources harvest is proportionately lower in Inverell than in Tara / Dalby. Stores could not sustain the colonies for the reminder of the year except for the 'quiescence' period. Colony quiescence is experienced for 34% of the year, resulting in little or no food consumption. This situation would enable colonies to survive for long periods of time without consuming precious resources. Ultimately, these conditions result in **the colony's energy consumption ≤ incoming resources,** providing conditions which would be marginal colony for survival.

Thus it appears that, as Roubik (1989) predicted, the limiting factor for stingless bee distribution, including *Au. australis,* is not the direct impact of weather extremes on a colony, but rather the impact of weather patterns on a foragers' ability to leave the nest and collect adequate resources. Colonies may be located in areas where floral resources are abundant but if low temperatures or inclement weather prevents foragers from leaving the nest, colonies are unable to harvest these resources. Colonies of *Au. australis* located at its most southerly boundaries, such as Inverell, are unable to build enough stores to feed a large population. Ontogenic times would be prolonged in association with extended periods at low incubation temperatures (see Chapter 5), inhibiting colony growth. Population levels would seldom produce nest crowding or stimulate reproductive swarming. With limited reproductive swarming, distribution expansion would be very slow.

6.7 Key findings

- *Au. australis* has an ambient temperature threshold for flight activity of 20°, as long as the in-hive temperature is > 18°C.
- The ambient temperature threshold for optimal flight activity is > 26°C, if the corresponding in-hive temperature is ≥21°C.
- *Au. australis* will not fly during rain periods or heavy cloud cover.
- *Au. australis* do not generate enough metabolic heat to maintain the brood cluster at a constant temperature.
- *Au. australis* demonstrate ectothermic qualities and are very dependent on ambient temperatures, and therefore nest cavity temperatures.
- *Au. australis* colonies are able to survive temperature ranges from sub-zero to over 37°C.
- *Au. australis* demonstrated a more efficient foraging behaviour than *T. carbonaria*, with lower forager numbers being recruited and less time wasted on hovering.
- Nest density in south-east Qld is low compared to some reported social bees.
- The preferred nest cavity of *Au. australis* is narrow and within a dead tree.
- The estimated brood population of the sampled colonies ranged from 2,000 to 13,000, averaging 5,000.

CHAPTER 7

General discussion

7.1 Introduction

The exploitation of native bee pollinators in Australia has, to date, been minimal. Only a few bee keepers successfully manage native stingless bees for various commercial activities, with pollination services playing a small role in the industry. The survey of the Australian stingless bee industry indicated that it is still in its infancy (or at best, its childhood) and that it requires further research and development support. The knowledge and experience is held by the large number of hobbyist bee keepers along with the knowledge of researchers, local and overseas. If pooled, these attributes could support substantial growth within the industry, enabling it to become a viable and sustainable economic venture.

At the commencement of my studies, I set out to assess *Au. australis* as a potential pollinator of greenhouse and field crops. As part of this assessment process, I also endeavoured to generate information on the biology and behaviour of this insect. With a better understanding of the biology of *Au. australis*, management programmes for improved health and strength of domesticated colonies can be developed and implemented. Also, being aware of the behavioural strategies of *Au. australis* foragers ensures that colonies are utilised in regions where their behaviour will not hinder their ability as pollinators.

While the pollination studies may have answered some of my original questions, they also generated some more confounding ones. These included: why are the seemingly similar Australian stingless bees, *Au. australis* and *T. carbonaria*, so different in their foraging strategies? Are these strategies associated with their native habitat? What are the characteristics of *Au. australis*' native habitat? What is their distribution and what are the limiting factors for their distribution? How do they survive the climatic extremes experienced in their native habitat? How do they survive in areas with unreliable, often scarce food resources? To answer these new questions the studies reported in Chapters 5 and 6 were conducted.

One of my original aims was to investigate a long standing issue regarding species status within the genus *Austroplebeia.* A triangulated approach was undertaken, through the analysis of morphological characteristics, as well as morphometric and molecular data. Although it was not possible to obtain sufficient data to fully answer the questions raised in Chapter 4, some separation of the groups was obtained. These studies highlighted the geographical diversity of this genus and demonstrated a species complex containing paraphyletic groups which are difficult to separate. Again, these studies seemed to generate more questions than they answered.

7.2 *Au. australis* and its environment

Au. australis' extended ontogenic period (~ 55 days), compared to *A. mellifera,* is probably associated with brood incubation temperatures (Chapter 5). Because colonies do not thermoregulate their nests (Chapter 6, Section 6.2), incubation of the brood cluster is determined by ambient temperatures as well as metabolic heat produced by the colony. Regions in south-east Qld, which are known to support relatively large populations of this species (Chapter 6, Section 6.4.4), experience mean ambient temperatures ranging from 19.5 to 33°C in summer and 3 to 19°C in winter. During the three winter months (June – August) the mean maximum daytime temperatures range from 19 to 21°C (BOM 2012b). *Au. australis* colonies demonstrate an ability to produce substantial amounts of heat when nest temperatures are > 15°C (Chapter 6, Section 6.2). This suggests that colonies located in areas where nest temperatures can reach > 15°C are capable of further increasing their nest temperatures, and subsequently their brood incubation temperatures (Chapter 6, Section 6.2), to above ambient even during the milder months of winter. Therefore, brood production in *Au. australis* could continue throughout the year within its native range, albeit slowly at times.

Au. australis' preference for narrow cavities in dead trees (Chapter 6, Section 6.4.6) may provide an additional form of heating for the colonies. South-east Qld experiences high levels of solar radiation ranging from 12 to 15 MJ / m^2 in winter and 24 to 27 MJ / m^2 in summer, due to the low air moisture content (BOM 2012a). As the sun rises, up to 65% of the heat produced by solar radiation is absorbed by the exposed wood surface (Federer 1971; Baldocchi et al. 1984). As the heat is absorbed, it spreads over the large surface area of exposed tree trunk or limb, penetrating into

the long, narrow colony housed within. The colony gradually warms, with nest temperature soon tracking ambient. As a result, shortly after the temperature threshold for flight activity (≥ 20°C) is reached, the colony is prepared for flight activity.

Although dead trees provide limited insulative protection, they do provide the colony with protection against direct radiant heat. The cavity should, however, be at least as warm as the highest ambient temperatures (Chapter 6, Section 6.2). As *Au. australis*' cerumen is composed of up to 88% bees wax, which has a melting point ranging from 58 to 60°C (Milborrow et al. 1987), nest structures would be unlikely to melt, even during the hottest summer days. The nest architecture reduces the need for large supportive structures, as the column is mostly supported by the walls of the tree cavity and brood is constructed to fit into the remaining space provided (Chapter 6, Section 6.4.6). Pollen and honey pots of *Au. australis* are small (~ 8 mm diam.) compared to *T. carbonaria* (~ 15 mm diam.) (Michener 1961), providing more structural support for the fluid inside, and thus reducing the possibility of collapse during high temperatures. Cerumen is used sparingly in the nests of *Au. australis* and, unlike *T. carbonaria*, colonies do not build a thick batumen sheet or line the walls of the nest cavity. Walls are polished and sealed against intruders but no extraneous cerumen structures are built in the nest. *Austroplebeia* do not collect large quantities of plant resin (Leonhardt 2011; Chapter 6, Section 6.1) whereas *T. carbonaria* foragers have been reported to collect large amounts, especially *C. torelliana* (Klumpp 2007). Low resin requirements mean that plant resin is harvested less frequently, thus reducing overall colony energy consumption and reducing loss of foragers through predation.

7.3 Behavioural adaptations in *Au. australis*

Au. australis is considered by many stingless bee keepers to be a lazy bee because it is 'fussy' about the conditions under which it will recruit foragers. The pollination trials conducted at UWS (Chapter 3) provided the opportunity to observe this phenomenon and the stark differences between *Au. australis* and *T. carbonaria* became quite evident.

Evolution within an arid, unpredictably resourced environment dictates that, for species to survive, they must adapt to prevailing conditions. *Au. australis* foragers demonstrate efficiency in harvesting floral resources. Wasteful hovering behaviour is kept to a minimum, flight trajectories are precise and flight activity is conservative. This is demonstrated during periods of both high and low floral resource availability. Landing precision and minimal hovering time also makes them less conspicuous to predators (Sherry & McDade 1982; Jones et al. 2003). Such strategies are likely to reduce the rate of physical deterioration in individual bees, increasing forager longevity.

Forager recruitment is positively correlated with floral resource availability. Conservative recruitment of foragers during low floral resource availability reduces the number of individuals undertaking high-risk, high-energy activities (Brys et al. 2007). Thus, during times of low resource, the energy expenditure of the colony as a whole is also low. The conservative recruitment tactics used during floral dearth result in a build-up of adults within the colony. The extended longevity of workers ensures that a large number of mature adults can be recruited in response to a sudden high demand for foragers.

The native host range of *Au. australis* consists of a mix of sclerophyllous species, including eucalypts (Martin 2006). These trees often flower in cycles and ecosystems experience waxing and waning of floral resources. *Au. australis* colonies increase forager recruitment with the advent of increasing floral resource availability. A previous extended period of resource scarcity results in a build-up of mature adults, which are now immediately available to leave the nest as foragers. High forager recruitment also results in high bee mortality rates, as more workers take part in risky, energy-expensive tasks (O'Donnell & Jeanne 1995). These worker losses are, however, far outweighed by the gains in colony food storage.

Once again, if the resource cycle moves into floral dearth, surplus foragers are not required for some time, possibly months. A substantial number of mature adults that previously took part in foraging duties have died, resulting in the removal of surplus, food-consuming old workers. The colony now contains young adults which can rear brood and perform in-hive activities, as well as newly emerging immature adults and

developing brood. The large amount of stored resources secures the colony's future brood production and survival.

7.4 Phylogeny of species within the genus *Austroplebeia*

It is important to be able to distinguish between bee species: for research, for ecological assessment and for their management. When commencing this project, I aimed to initiate investigations into the delineation of species within the genus *Austroplebeia*, with the ultimate aim of helping to clarifying the species' classification. As I continued my other studies, resolving this issue was further emphasised.

By using a triangulated diagnostic approach, I attempted to identify different characteristics within the morphologically distinct groups: 'cincta', 'curved', 'symei', 'australis', 'striped' and 'intermediate'. This approach enabled me to delimit two of the six groups. These are 'cincta', which definitively separated in all analyses, and 'curved', which clearly separated in all but the phylogenetic studies.

Even when utilising the triangulated approach the data analyses of groups within the species complex produced consistently confusing results. It is hypothesised that these groups may make up a species complex which is currently going through speciation processes, thus producing consistently confusing genotypic and phenotypic information. Further investigations, including molecular analysis of specimens using a less conservative gene, may help to clarify the situation.

7.5 Possible effects of climate change on *Au. australis* distribution

The work described in my thesis provides insight into the factors that limit the distribution of *Au. australis*. This has long been an issue of interest for Australian researchers and bee keepers. On the basis of my work, it is interesting to postulate what might happen to *Au. australis* distribution in the scenario of future climate change.

The climate change projections described below are relative to the 1990 baseline, and are founded on the 'best estimate' of the 50th percentile and medium emissions

(CSIRO 2007). It is predicted that by 2030 the overall mean annual temperatures for central and southern Qld will be up to 1.5°C higher than they were in 1990. Similar temperature increases are predicted for northern and central NSW, areas which currently contain the southernmost colonies of *Au. australis*, Inverell and Kempsey. It is predicted that by 2050 temperatures will rise by a further 0.5°C and eventually, by 2070, it will be up to 3°C hotter than is was in 1990 (CSIRO 2007). No predicted 'cloud cover' data are available and are based on long term climate averages reported by the Bureau of Meteorology, Australia (BOM 2012b) was used in association with predictions data.

It could be predicted that an overall increase in ambient temperatures may see an extension of the southern distribution of *Au. australis*. Under these conditions, the temperature threshold for 'sub-optimal foraging' activity (≥ 20°C) is reached earlier in the day, resulting in increased foraging opportunities (Figure 7.1). The 'optimal foraging' activity (≥ 26°C) is also achieved earlier in the day, thus increasing opportunities to forage at optimal levels. In spring, a period when a greater variety and abundance of floral resources are likely to be available (Hines & Hendrix 2005), temperatures are predicted to shift from 'sub-optimal' (i.e., 20 – 25°C) to 'optimal foraging' conditions. It is also predicted that there will be a 10% reduction in spring rain in southern Qld and northern NSW, thus increasing opportunities for foraging.

Under this scenario, if floral resources are within proximity, colonies will be able to increase their food storage during this time. Utilisation of increasing stored resources will result in an overall increase in colony populations (Roubik 1982). Brood production will increase, as periods of quiescence and inactivity decrease, and ontogenic times shorten with improved brood incubation, driven by increased ambient temperatures. All of these factors would be expected to result in more frequent nest crowding and increasing incidence of reproductive swarming (Michener 1974). Increasing temperatures may see a gradual broadening of the southern distribution of this species, and probably others.

The scenario described above appears to be true for populations near Kempsey, the most southerly coastal location for *Au. australis*. This region will experience a 13% increase in 'optimal foraging' conditions by 2030 and this will continue to increase to 25% by 2070 (Figure 7.1). While the climate predictions appear to positively

impact colonies of *Au. australis* at Kempsey, the reverse is likely to be true for colonies located in inland areas such as Inverell, the most southerly inland location for *Au. australis*. Conditions predicted for Inverell in 2030 will result in a 6% increase in 'sub-optimal foraging' opportunities, where food consumption exceeds incoming food (see Section 6.6.1), but with no concurrent increase in 'optimal foraging' conditions. This situation is further compounded by a 2% increase in conditions where foragers cannot leave the nest due to 'heavy cloud cover', resulting in more times when food consumption exceeds incoming food.

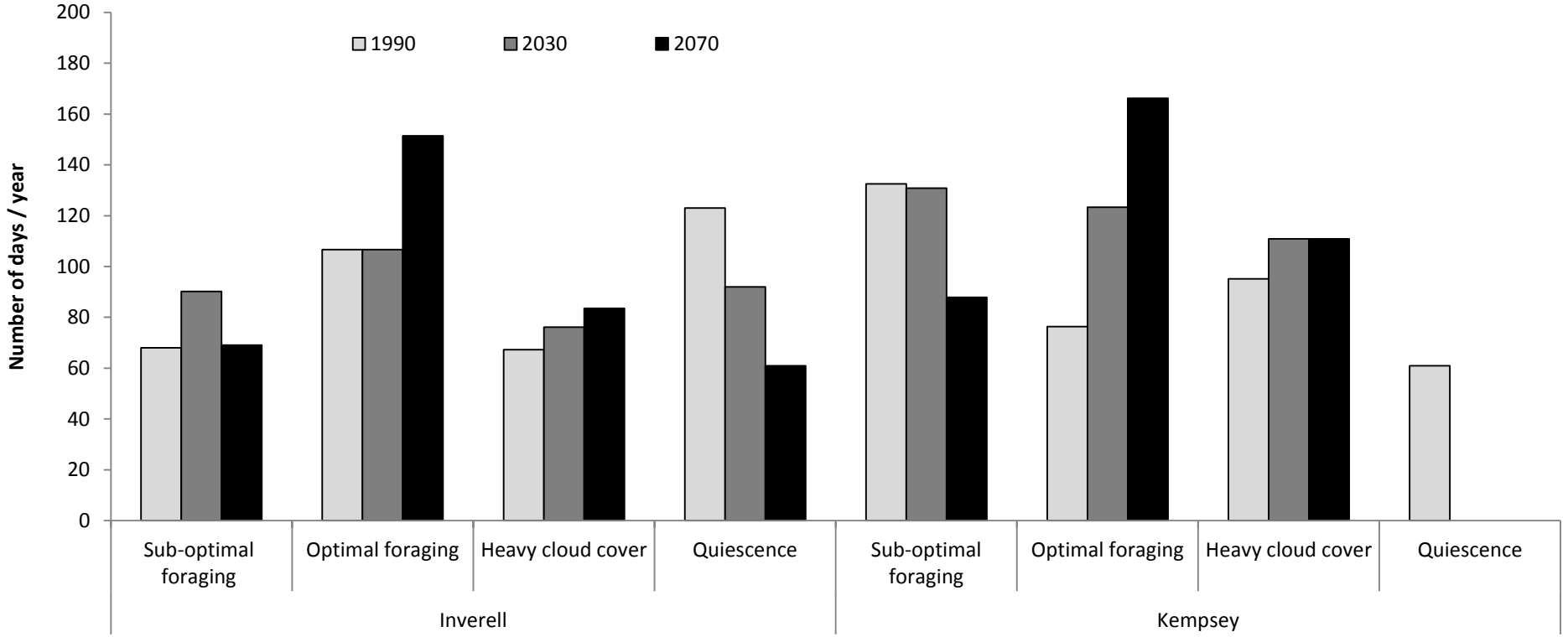


Figure 7.1 Predicted changes in foraging opportunities, between 1990, 2030 and 2070, as a reasult of climate change. Sub-optimal foraging = days where ambient temperature ≥ 20°C but < 26°C, with < 6 oktas of cloud in the sky; Optimal foraging = days where ambient temperature ≥ 26°C, with < 6 oktas of cloud in the sky; Heavy cloud cover = days where ambient temperature ≥20°C, with ≥ 6 oktas of cloud in the sky resulting in foragers being unable to leave the nest; Quiescence = days where ambient temperature < 20°C, thus reducing the colony's metabolism. Predicted temperature increases are based 50th percentile and medium emissions (CSIRO 2007). Cloud cover data are based on averages for over 38 years (BOM 2012b).

Together with an 8% decrease in periods of ‘quiescence’, annual food requirements of colonies will increase, and may not be met under the predicted conditions of decreased foraging. As a result, colonies may fail to thrive, leading to starvation and shrinkage of distribution in this region.

7.6 Implications of my findings for industry

7.6.1 Potential pollinator management

Au. australis demonstrated it can acclimate to a greenhouse enclosure and will visit flowering crops, resulting in pollination and increased yield, at least in some crops such as celery (Chapter 3). However, its conservative forager recruitment strategies may not make it ideally suited for provision of large scale crop pollination services. In comparison, *T. carbonaria* demonstrated constant, high recruitment of foragers and group foraging strategies. In the field, *Au. australis* flight activity was delayed due to low ambient temperatures (i.e., no flight at < 20°C, optimal foraging at ≥ 26°), and foragers demonstrated a reluctance to leave the hive during periods of heavy cloud cover, even when ambient temperatures were suitable for flight activity. These characteristics indicate that *Au. australis* would be unsuitable for use as a crop pollinator in cool climate crops.

Au. australis’ potential as a crop pollinator will depend upon the strategies employed by service providers or crop managers. Using colonies within areas that consistently provide temperatures for optimal flight activity would maximise their foraging opportunities, and thus, their crop pollination potential (Corbet et al. 1993). Regions within south-east Qld (e.g., Condamine, Burnett, Mary and Fitzroy rivers areas) and northern NSW (Northern Rivers) provide such conditions and also produce a variety of important horticultural crops, such as citrus, macadamia nut, avocado, mango, watermelon, capsicum, lychee and strawberries (RDA 2010).

Au. australis is a social bee capable of coping with Australia’s harsh environment. It is well adapted to temperature extremes, is conservative in its foraging strategies when resources are unavailable, as well as being active and efficient when conditions and resources are optimal. Opportunities to utilise *Au. australis* as a crop pollinator in horticulturally important areas may increase in the future, as predicted

temperatures increase, expanding the foraging and pollinating opportunities further south in Australia. On the other hand, the increased incidence of severe weather events that are predicted to accompany climate change may be detrimental to populations of *Au. australis* as well as to the crops they could be pollinating.

7.6.2 *Au. australis* colony management and industry development

My studies have increased knowledge of *Au. australis* biology and behaviour. Data on developmental times and worker longevity should assist in developing management strategies for colonies. Better understanding of the effects of temperature on colony dynamics will help in determining in-hive temperature requirements when managing colonies. For example, by providing hives with additional heat (> 20°C), colony activities such as brood rearing and foraging is enhanced.

Overwintering colonies, with the addition of heat and supplemental food, will significantly increase brood production (Appendix 10). The addition of heat to colonies during cool seasons, where daytime temperatures do not exceed 26°C, may see shorter ontogenic times, resulting in more rapid offspring production. Overall colony growth will facilitate more frequent division, thus increasing colony production. Food supplementation between pollination service periods will help to maintain colony strength.

Au. australis has grown in popularity over the last ten years (2.5-fold increase) (see Chapter 2) and reproduction of colonies for sale as a 'pet' will add to the growth of the Australian stingless bee industry as a whole. Stingless bee colonies are in high demand and producers have reported their inability to keep up with this demand (Chapter 2). Increased multiplication of *Au. australis* colonies, through sound management strategies, would help to fulfil demand. This may include supplementary heating and feeding during the cooler season. The sale of colonies to areas that cannot naturally support their foraging needs should be discouraged. After-sale services, such as providing queens for requeening, providing training in colony division and husbandry and educational workshops, are also an important part of producing a successful product.

Where large managed populations or natural populations of *Au. australis* occur, natural requeening can be achieved. Where colonies are managed and reproduced outside their native range, it is important to ensure an adequate number of founder colonies are present in an area. It is recommended that at least five founder colonies are utilised to guarantee richness of the sex alleles and reduced chances of sterile diploid male production (Alves et al. 2011).

Multiplication of *Au. australis* colonies by indigenous communities could provide "meaningful involvement of Aboriginal people in natural resource management" (QMDC 2012). The Queensland Darling Downs region contains native populations of *Austroplebeia* spp. as well as providing optimal foraging opportunities for colonies. Supplementary feeding could further boost colony growth and increase the opportunities for colony division and sale.

Au. australis colonies appear to cope with temperature extremes. As a result, their management within light-weight, durable, non-wooden, non-porous boxes may be advantageous. Thin-walled, light-weight materials would allow for faster penetration of ambient heat into the cavity, thus facilitating earlier flight activity. Such hives would also make it easier to exclude pests and rain and could be more easily disinfected in the event of pest or disease occurrence. Being light-weight, hives could be moved in and out of crops more easily than OATHs. In addition, monitoring of colony health would be more reliable, as hive weight could be utilised as a tool for assessing colony growth. The wooden OATHs are too heavy relative to colony weight and their porous properties result in unreliable weight measurements, due to moisture content fluctuations (see Appendix 2).

The inability to readily requeen divided colonies is probably the greatest impediment to rapidly increasing colony production (Chapter 2). Without further research into colony multiplication, the growth of the Australian stingless bee industry is destined to remain slow.

7.7 Implementation of my research findings for stingless bee conservation

The greater the biodiversity within an ecosystem, the more malleable and resilient it will be (Ehrlich & Ehrlich 1992). Currently, anthropogenic factors are impacting upon Australian biodiversity, with the removal of nest substrates and food resources through land clearing (Batley & Hogendoorn 2009). While these land use practices are inescapable, the management and protection of wildlife have been recognised as a responsibility that mankind must take seriously (Allen-Wardell et al. 1998). World-wide, conservation policies have been developed and legislated almost *ad infinitum* (Allen-Wardell et al. 1998; Dias et al. 1999; Williams 2003; FBA 2005; Imperatriz-Fonseca et al. 2006; Loughlin 2008; Byrne & Fitzpatrick 2009; FAO 2009; RIRDC 2009). Recommendations are often put forward, yet it is difficult to establish the seriousness with which stakeholders implement these recommendations (RPS 2010).

Land clearing within the mixed forest woodlands results in the removal of important nesting substrate (in *Angophora*, *Corymbia* and *Eucalyptus* spp.) (Wormington et al. 2003) and pollinator food supplies. These environments provide food and shelter for a range of plant and animal species which are important in maintaining ecological balances. In the nest density study (Chapter 6, Section 6.4.4), the highest *Au. australis* densities were recorded in areas located within 'improved' open woodlands, which provided an abundance of dead tree cavities. However, these high density populations were also located adjacent to crown land, mainly managed forests, containing mixed forest woodlands, which provide periodically abundant floral resources. Bee populations are likely to be very reliant on these floral resources. Removal of trees from the adjacent crown land, through land clearing or even selective timber harvest, will result in a substantial reduction in food resources. The resultant increased competition between the stingless bee colonies within the high density population areas may result in large colony losses due to starvation. Conservation of stingless bees, as with other animal species, requires well informed management plans, and information on species population density is essential in formulating such plans. By using the approach described in Chapter 6, Section 6.4 it will be possible to identify areas of high and low nest density.

On a more limited scale, practical steps can be taken to conserve the stingless bee populations within small regions in Australia. The general public appears to be increasingly interested (Chapter 2), and a few municipal councils are aware of the importance of their native flora and fauna (e.g., www.kmc.nsw.gov.au). However, other government agencies appear to have no knowledge of the existence of Australian stingless bees, as they do not list stingless bees amongst the range of 'hollow-dwelling' native animals (Carritt 1999; Gibbons & Lindenmayer 2000; Wormington et al. 2003; OEH 2011).

In Australia, government agencies are responsible for large areas of national parks and reserves, many of which are located near cities and regional towns that are subject to rapid land clearing to meet the demand for housing development. Less than 8% of Australia has been legislated as protected land and in 1999 over 425,000 ha of land were cleared in Queensland for agriculture, mining and urban use, while NSW cleared 30,000 ha (ABS 2002). Education of community members and government officials is a key factor to improving conservation. This is highly relevant for the conservation of native bees.

The development of an education programme, in cooperation with local councils, could be the first step in increasing awareness of native, particularly stingless, bees. Increased public awareness can be achieved in many ways. The most interactive method is to approach the stakeholders directly. In Australia, land developers, tree loppers and residents usually have to apply to their local councils for permission to remove trees of any substantial size. As part of their management strategy, councils ensure that properties are assessed and the need for tree removal is justified. Part of this assessment could include inspection for the presence of stingless bee nests. If nests are found to be present within the tree (s) proposed for removal, a stingless bee rescue service provider could be brought in to advise on the best way to excise the nest segment from the tree. The segment could then be removed and transferred to an artificial hive at a later date (see Chapter 6, Section 6.4.6). The rescue service provider could either pay the council for the privilege of keeping the colony or they could provide consultancy services at a nominal fee. It would be of utmost importance that the rescue service demonstrated that they have the conservation of the species, not profit, as a priority.

7.8 A practical research output from my studies

As a result of the work conducted in collaboration with Allan Beil (see Chapter 6, Section 6.4), a large number of stingless bee nest locations have been recorded. These are currently maintained in a database. A nest location register and interactive map has been established at the website http://tracker1.beesbusiness.com.au/ ("Beetracker"), and is available to community members. Nest location coordinates are entered by the individual and an icon of that location becomes visible on the map (Figure 7.2). Only the person who registered the nest and the data base manager are able to access the coordinate information. The community member obtains positive feedback by being able to see his / her nest on the map. This location is an estimate only, and exact locations cannot be obtained from this site. This is to reduce the possibility of nest poaching. Similar data bases could be registered with local councils or government bodies, and could be set up to monitor the distribution of stingless bee colonies. To broaden the reach of "Beetracker" the database could also be linked with the Atlas of Living Australia (http://www.ala.org.au/).

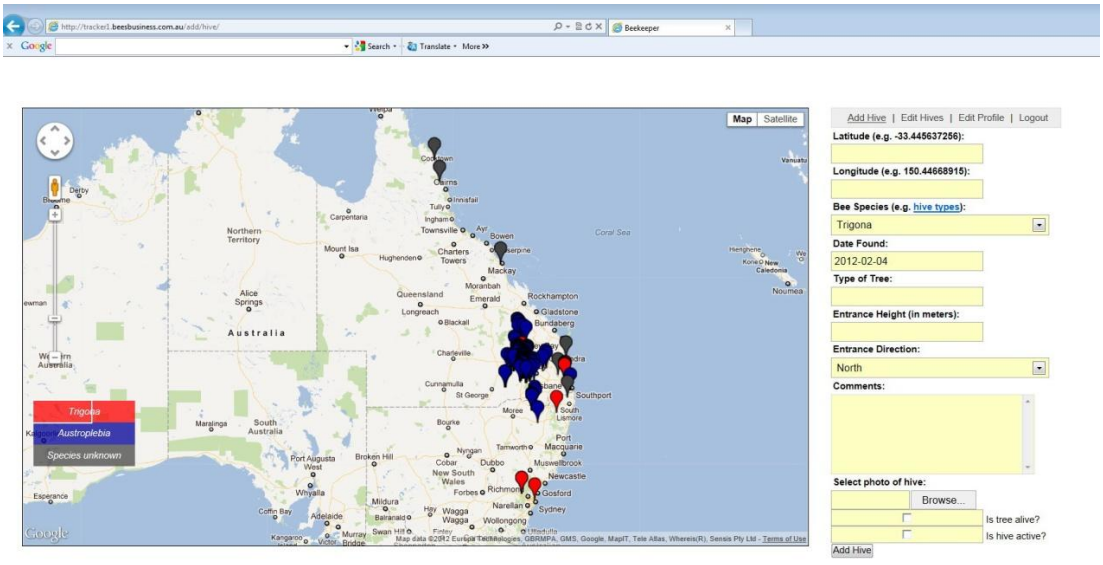


Figure 7.2 Beetracker website with icons representing registered wild nests. Coordinates were entered by myself, on behalf of Allan Beil, or by community members.

The "Beetracker" website is the first of its kind in Australia and has the potential for providing sound baseline data for monitoring the density and distribution of stingless bee colonies. These data may help us better understand the effect of environmental

factors, such as flood or bush fires, as well as anthropogenic factors on the density and distribution of genera in different areas. Tools that aid conservation efforts enable better natural resource management. Biodiversity enables the ecosystem to remain in balance so that mankind can continue to reap the benefits of existing on this earth.

7.9 Recommendations for further research

The findings reported in this thesis have answered a number of questions about the biology and behaviour of *Au. australis*, and my work has gone some way to delimiting the species within the genus *Austroplebeia*. However, further investigations are needed to clarify several issues. Suggested studies include:

Phylogeny

1. Further molecular analysis of fresh specimens of 'australis', 'symei' and 'striped' groups using large fragment-producing nuclear markers, such as COI or ITS1. Results from this work may better delimit species within this complex, or confirm the results reported here.

Pollination

1. Investigate the efficacy of *Au. australis* as a crop pollinator within areas that provide optimal foraging conditions. Locations could include horticulturally active areas such as the Northern Rivers region of NSW, and the Condamine, Fitzroy and Burnett Mary regions of Qld.
2. Investigate whether the provision of supplementary in-hive heating enables colonies to reach the temperature threshold for flight activity at around the same time as ambient temperature reaches this threshold. This work could utilise colonies in pollination trials, to determine whether overall foraging performance increases with supplementary hive heating.
3. Study communication and forager recruitment in *Au. australis*. Investigate the influence of pheromone concentration on forager recruitment.
4. Investigate more fully the environmental factors influencing entrance and flight activity in *Au. australis.*

5. Determine the flight range of foragers.

Colony management

1. Investigate the management of stingless bee colonies in light-weight, non-wooden hives.
2. Develop hive designs which best facilitate colony strength, division and transport.
3. Develop a hive design for ‘pet’ colonies. An attractive container which displays the colony in a more aesthetic fashion, including incorporating a replaceable observation lid. A supplementary feeding station could be included to reduce colony disturbance during this practice.

Conservation

1. Conduct surveys of potential stingless bee nest sites within National Parks using the techniques described in Section 6.4. This could include nest site and floral resources availability.
2. Investigate entrance markings for the presence of pheromone compounds.

Biology

1. Determine how incubation temperature affects the ontogenic times of developing brood and establishing the optimal temperature for rapid, healthy development.
2. Explore colony plasticity in division of labour in situations of plentiful resources vs. scarce resources. This work could be conducted within the confines of an experimental greenhouse.
3. Investigate the temperature tolerance of adult workers.
   Examine the effect of daylength on involucrum extension. Investigate biochemical changes, such as phospholipid composition within the cell membrane, of summer brood compared to winter-preparing and wintering brood (larvae and pupae).

Queen rearing for colony propagation

1. Investigate queen longevity and fecundity.

2. Investigate methods for successfully introducing gynes into queenless colonies.
3. Determine whether colonies will provision artificially made cerumen or wax cells.
4. Investigate the possibility of artificial queen rearing. First, determine the optimal volume of provisions in a naturally constructed queen cell, then determine whether colonies will produce gynes if provided with artificially produced queen cells. If this is successful, determine whether colonies can be stimulated to mass rear gynes.

Artificial insemination

1. Investigate the cues which initiate drone production by workers in queenless colonies. Answer the question: Can orphaned colonies be utilised as drone producing colonies?
2. Investigate factors affecting sperm production and viability in drones produced within orphaned colonies.
3. Improve techniques for sperm harvest in *Au. australis* drones.
4. Assess the possibility of adapting artificial insemination (AI) apparatus used for honey bees for *Au. australis.* Then investigate techniques that may be utilised in the AI of *Au. australis* gynes. Ultimately, determine whether AI can be successfully achieved to produce physogastric *Au. australis* queens.

7.10 Final conclusion

Until the work reported here, little was known about the biology and behaviour of bees within the genus *Austroplebeia.* The phylogenetic studies indicate that there may only be a small number of species within this genus. The information gained through my studies on *Au. australis* may, therefore, be applied to the other 'groups' within *Austroplebeia.*

The small behavioural differences observed between *Au. australis* and *Au. symei* (Section 6.3) indicate that isolated groups, whether subspecies or races, may have evolved as a result of adaptation to their environmental conditions. Nesting habits and temperature conditions under which colonies are found suggest that groups

outside *Au. australis* may be similar in their biological and physiological requirements.

Even if this is so, I speculate that there are likely to be differences in behaviour between colonies located in the wet tropics and those from arid inland areas. Much more can be learned about these fascinating bees, but in the meantime, they will carry on with the important task of contributing to the rich diversity of Australia's ecology.

References

Abe T, Bignell DE, Higashi M, Ed. (2000) Termites: evolution, sociality, symbioses, ecology. Dordrecht, The Netherlands, Kluwer Academic Publishers.

ABS, Australian Bureau of Statistics (2002) Measuring Australia's progress. Retrieved 22 February 2012 Available at http://www.abs.gov.au/ausstats/abs@.nsf/0/1C36C09104A4765ACA256BDC001223FE?Open

Adams P, Lawson S (1993) Pollination in Australian orchids: a critical assessment of the literature 1882-1992. Australian Journal of Botany 41: 553-571.

Aguilar I, Briceño D (2002) Sounds in *Melipona costaricensis* (Apidae: Meliponini): effect of sugar concentration and nectar source distance. Apidologie 33: 375-388.

Aguilar I, Fonseca A, Biesmerjer JC (2005) Recruitment and communication of food source location in three species of stingless bees (Hymenoptera, Apidae, Meliponini). Apidologie 36: 313-324.

Akerman K (1979) Honey in the life of the aboriginals of the Kimberleys. Oceania 49: 169-178.

Alessandro MS, Galmarini CR (2007) Inheritance of vernalization requirement in carrot. Journal of the American Society for Horticultural Science 132: 525-529.

Ali MS (1982) A study of the floral biology of onions and the behaviour of pollinating insects in hybrid seed production. University of Birmingham. Birmingham. MSc thesis.

Allen-Wardell G, Bernhardt P, Bitner R, Burquez A, Buchmann S, Cane JH, Cox PA, Feinsinger P, Ingram M, Inouye DW, Park MD, Jones CE, Kennedy K, Koopowitz H, Medellin-Morales S, Nabhan GP (1998) The potential consequences of pollinator declines on the conservation of biodiversity and stability of food crop yields. Conservation Biology 12: 8-17.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. Journal of Molecular Biology 215: 403-410.

Alves DA, Imperatriz-Fonseca VL, Francoy TM, Santos-Filho PS, Billen J, Wenseleers T (2011) Successful maintenance of a stingless bee population despite a severe genetic bottleneck. Conservation Genetics 12: 647-658.

Alves D, Menezes C, Imperatriz-Fonseca V, Wenseleers T (2011) First discovery of a rare polygyne colony in the stingless bee *Melipona quadrifasciata* (Apidae, Meliponini). Apidologie 42: 211-213.

Amano K (2004) Attempts to introduce stingless bees for the pollination of crops under greenhouse conditions in Japan. Food and Fertilizer Technology Centre for the Asian and Pacific Region. Available at http://www.agnet.org/library/tb/167/

AMRESCO (2011) Ethidium bromide, 0.625 mg / mL. Life Science Research Products and Biochemicals.

Anderson D, Lawrence L (2007) Exotic pests pose pollination threat. Farming Ahead 180: 34-36.

Anderson D, Sedgley M, Short J, Allwood A (1982) Insect pollination of mango in northern Australia. Australian Journal of Agricultural Research 33: 541-548.

Anonymous (1902) General news, land court. The Queenslander. Brisbane, Qld, 4 October 1902: 784.

Antonelli A, Glass JR (2004) Bumble bees. Washington State University PLS-12: June. Available at http://www.puyallup.wsu.edu/plantclinic/resources/pdf/pls12bumblebees.pdf retrieved 8 April 2011

Archer M, Hand SJ, Godthelp H (1994) Patterns in the history of Australia's mammals and inferences about palaeohabitat. 80-103 pp. *In*: History of the Australian Vegetation: Cretaceous to Recent. Eds. Hill RS. Cambridge, Cambridge University Press.

Ayasse M, Paxton RJ, Tengö J (2001) Mating behavior and chemical communication in the order Hymenoptera. Annual Review of Entomology 46: 31-78.

Baldocchi DD, Matt DR, Hutchison BA, McMillen RT (1984) Solar radiation within an oak—hickory forest: an evaluation of the extinction coefficients for several radiation components during fully-leafed and leafless periods. Agricultural and Forest Meteorology 32: 307-322.

Banga SS, Banga SK, Ed. (1998) Hybrid Cultivar Development. New York, Springer-Verlag.

Bartareau T (1996) Foraging behaviour of *Trigona carbonaria* (Hymenoptera: Apidae) at multiple choice feeding stations. Australian Journal of Zoology 44: 143-153.

Batley M, Hogendoorn K (2009) Diversity and conservation status of native Australian bees. Apidologie 40: 347-354.

Beck SD (1983) Insect thermoperiodism. Annual Review of Entomology 28: 91-108.

Bego LR (1983) On some aspects of bionomics in *Melipona bicolor bicolor* Lepeletier (Hymenoptera, Apidae, Meliponinae). Revista Brasileira de Entomologia 27: 211-224.

Bell MC, Spooner-Hart RN, Haigh AM (2006) Pollination of greenhouse tomatoes by the Australian bluebanded bee *Amegilla (Zonamegilla) holmesi* (Hymenoptera: Apidae). Journal of Economic Entomology 99: 437-442.

Biesmeijer JC, Roberts SPM, Reemer M, Ohlemuller R, Edwards M, Peeters T, Schaffers AP, Potts SG, Kleukers R, Thomas CD, Settele J, Kunin WE (2006) Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. Science 313: 351-354.

Biesmeijer JC, Tóth E (1998) Individual foraging, activity level and longevity in the stingless bee *Melipona beecheii* in Costa Rica (Hymenoptera, Apidae, Meliponinae). Insectes Sociaux 45: 427-443.

Bijlsma L, de Bruijn L, Martens E, Sommeijer M (2006) Water content of stingless bee honeys (Apidae, Meliponini): interspecific variation and comparison with honey of *Apis mellifera*. Apidologie 37: 480-486.

Biological Services (2008a) *Aphidius colemani* - aphid parasitoid. Available at http://www.biologicalservices.com.au/ retrieved 25 August 2008.

Biological Services (2008b) *Encarsia formosa* - whitefly parasitoid. Available at http://www.biologicalservices.com.au/ retrieved 25 August 2008.

Biological Services (2008c) *Hypoaspis* (*Stratiolaelaps*) *miles* - fungus gnat predator. Available at http://www.biologicalservices.com.au/ retrieved 25 August 2008.

Biological Services (2008d) *Neoseiulus cucumeris* - thrips predator. Available at http://www.biologicalservices.com.au/ retrieved 25 August 2008.

Bohart GE (1957) Pollination of alfalfa and red clover. Annual Review of Entomology 2: 355-380.

Bohart GE (1972) Management of wild bees for the pollination of crops. Annual Review of Entomology 17: 287-312.

Bohart GE, Nye WP, Hawthorn LR (1970) Onion pollination as affected by different levels of pollinator activity. Bulletin of the Utah Agriculture Experiment Station 482: 3-57.

BOM, Bureau of Meteorology (2010a) Average annual & monthly maximum, minimum, & mean temperature. Available at http://reg.bom.gov.au/jsp/ncc/climate_averages/temperature/index.jsp retrieved 10 March 2010.

BOM, Bureau of Meteorology (2010b) Climate education: drought. Available at http://www.bom.gov.au/lam/climate/levelthree/c20thc/drought.htm retrieved 17 February 2010.

BOM, Bureau of Meteorology (2011a) Annual climate summary 2010. Available at http://www.bom.gov.au/climate/annual_sum/2010/AnClimSum10_LR1.0.pdf retrieved 3 June 2011.

BOM, Bureau of Meteorology (2011b) Flood warning system for the Condamine River to Cotswold. Available at http://www.bom.gov.au/hydro/flood/qld/brochures/condamine_cotswold/condamine_cotswold.shtml retrieved 16 July 2011.

BOM, Bureau of Meteorology (2011c) Flood warning system for the Condamine River to Warwick. Available at http://www.bom.gov.au/hydro/flood/qld/brochures/condamine_warwick/condamine_warwick.shtml retrieved 25 January 2012.

BOM, Bureau of Meteorology (2011d) Twelve-monthly solar exposure for Australia. Available at http://www.bom.gov.au/jsp/awap/solar/index.jsp?colour=colour&time=latest&step=0&map=solarave&period=12month&area=nat retrieved 25 June 2011.

BOM, Bureau of Meteorology (2012a) Average daily solar exposure. Available at http://reg.bom.gov.au/jsp/ncc/climate_averages/solar-exposure/index.jsp?period=dec retrieved 25 January 2012.

BOM, Bureau of Meteorology (2012b) Climate data online. Available at http://www.bom.gov.au/climate/data/index.shtml retrieved 13 February 2012.

BOM, Bureau of Meteorology (2012c) Climate statistics for Australian locations. Available at http://reg.bom.gov.au/climate/data/index.shtml retrieved 4 Jan 2012.

BOM, Bureau of Meteorology (2012d) Queensland rainfall and river conditions. Available at http://www.bom.gov.au/qld/flood/ retrieved 13 February 2012.

Boorn KL, Khor YY, Sweetman E, Tan F, Heard TA, Hammer KA (2010) Antimicrobial activity of honey from the stingless bee *Trigona carbonaria* determined by agar diffusion, agar dilution, broth microdilution and time-kill methodology. Journal of Applied Microbiology 108: 1534-1543.

Bosch J, Kemp WP (2004) Effect of pre-wintering and wintering temperature regimes on weight loss, survival, and emergence time in the mason bee *Osmia cornuta* (Hymenoptera: Megachilidae). Apidologie 35: 469-479.

Bradbear N (1988) World distribution of major honeybee diseases and pests. Bee World 69: 15-39.

Brys K, Vanfleteren JR, Braeckman BP (2007) Testing the rate-of-living / oxidative damage theory of aging in the nematode model *Caenorhabditis elegans*. Experimental Gerontology 42: 845-851.

Bullock TH (1955) Compensation for temperature in the metabolism and activity of poikilotherms. Biological Reviews 30: 311-342.

Burdock GA (1998) Review of the biological properties and toxicity of bee propolis (Propolis). Food and Chemical Toxicology 36: 347-363.

Byrne A, Fitzpatrick Ú (2009) Bee conservation policy at the global, regional and national levels. Apidologie 40: 194-210.

Camargo JMF, Grimaldi DA, Pedro SRM (2000) The extinct fauna of stingless bees (Hymenoptera: Apidae: Meliponini) in Dominican Amber: two new species and redescription of the male of *Proplebeia dominicana* (Wille and Chandler). American Museum Novitates 1-24.

Camargo JMF, Kerr WE, Lopes CR (1967) Morfologia externa de *Melipona (Melipona) marginata* Lepeletier (Hymenoptera, Apoidea). Papéis Avulsos de Zoologia (São Paulo) 20: 229-258.

Camargo JMF, Pedro SRM (2012) Meliponini Lepeletier, 1836. *In* Moure JS, Urban D & Melo GAR (Orgs). Catalogue of Bees (Hymenoptera, Apoidea) in the Neotropical Region - online version. Available at http://www.moure.cria.org.br/catalogue. retrieved 19 January 2012.

Camillo-Atique C (1977) Estudo da variabilidade etolõgica de Friesella incluindo a caracterização de espécies críptica (Hym., Meliponinae). Ribeirão Preto, Fac. de Medicina 203 (Cited in Simões & Bego 1991).

Cardale JC (1993) Hymenoptera: Apoidea. Vol. 10. 318-320 pp. *In*: Zoological Catalogue of Australia. Eds. Houston WWK, GV Maynard. Canberra, Australian Government Publishing Service.

Carey JR (2001) Insect biodemography. Annual Review of Entomology 46: 79-110.

Carey JR, Judge DS (2000) Mortality dynamics of aging. Generations 24: 19-19.

Carey JR, Judge DS (2001) Life span extension in humans is self-reinforcing: a general theory of longevity. Population and Development Review 27: 411-436.

Carritt R (1999) Natural tree hollows. Available at http://www.environment.nsw.gov.au/resources/nature/Factsheet5TreeHollows.pdf retrieved 29 January 2012.

Cauich O, Quezada-Euán JJG, Macias-Macias JO, Reyes-Oregel V, Medina-Peralta S, Parra-Tabla V (2004) Behavior and pollination efficiency of *Nannotrigona perilampoides* (Hymenoptera: Meliponini) on greenhouse tomatoes (*Lycopersicon esculentum*) in subtropical México. Journal of Economic Entomology 97: 475-481.

Cauich O, Quezada-Euán JJG, Meléndez Ramírez V, Valdovinos-Nuñez GR, Moo-Valle H (2006) Pollination of habanero pepper (*Capsicum chinense*) and

production in enclosures using the stingless bee *Nannotrigona perilampoides*. Journal of Apicultural Research 45: 125-130.

Chiang CL (1960) A stochastic study of the life table and its applications: I. Probability distributions of the biometric functions. Biometrics 16: 618-635.

Chinh TX, Sommeijer MJ (2005) Production of sexuals in the stingless bee *Trigona* (*Lepidotrigona*) *ventralis flavibasis* Cockerell (Apidae, Meliponini) in northern Vietnam. Apidologie 36: 493-503.

Chinh TX, Sommeijer MJ, Boot WJ, Michener CD (2005) Nest and colony characteristics of three stingless bee species in Vietnam with the first description of the nest of *Lisotrigona carpenteri* (Hymenoptera: Apidae: Meliponini). Journal of the Kansas Entomological Society 78: 363-372.

Clarke M (2008) Pasture seeds five-year R & D plan 2008-2013. Rural Industries Research and Development Corporation. Australian Government. Available at http://www.rirdc.gov.au/RIRDC/index.cfm?819CD815-F1FE-4F78-A90D-0B3847152259.

Cockerell TDA (1905) Descriptions and records of bees. Annals and Magazine of Natural History 16: 220.

Cockerell TDA (1910) New and little-known bees. Transactions of the American Entomological Society 36: 199-249.

Cockerell TDA (1929) Bees in the Australian Museum collection. Records of the Australian Museum 17: 199-243.

Colhoun EH (1960) Acclimatization to cold in insects. Entomologia Experimentalis et Applicata 3: 27-37.

Coombs AB, Bowman J, Garroway CJ (2010) Thermal properties of tree cavities during winter in a northern hardwood forest. The Journal of Wildlife Management 74: 1875-1881.

Corbet SA, Fussell M, Ake R, Fraser A, Gunson C, Savage A, Smith K (1993) Temperature and the pollinating activity of social bees. Ecological Entomology 18: 17-30.

Corbet SA, Unwin DM, PrŷS-Jones OE (1979) Humidity, nectar and insect visits to flowers, with special reference to Crataegus, Tilia and Echium. Ecological Entomology 4: 9-22.

Corbet SA, Williams IH, Osborne JL (1992) Bees and the pollination of crops and wild flowers in the European community. Apiacta. 4: 1-2. Available at http://www.apimondiafoundation.org/cgi-bin/index.cgi?sid=&zone=cms&action=search&categ_id=189&search_ordine=descriere retrieved 9 July 2011

Cortopassi-Laurino M, Imperatriz-Fonseca VL, Roubik DW, Dollin A, Heard T, Aguilar I, Venturieri G, Eardley C, Nogueira-Neto P (2006) Global meliponiculture: challenges and opportunities. Apidologie 37: 275-292.

Costa MA, Del Lama MA, Melo GAR, Sheppard WS (2003) Molecular phylogeny of the stingless bees (Apidae, Apinae, Meliponini) inferred from mitochondrial 16S rDNA sequences. Apidologie 34: 73-84.

Coulson RN, Witter JA (1984) Forest entomology: ecology and management. New York, John Wiley and Sons, Inc.

Cruz DdO, Freitas BM, Silva LAd, Silva EMSd, Bomfim IGA (2005) Pollination efficiency of the stingless bee *Melipona subnitida* on greenhouse sweet pepper. Pesquisa Agropecuaria Brasileira 40: 1197-1201.

Cruz DdO, Jorge DMdeM, Pereira JOP, Torres DC, Soares CEA, Freitas BM, Grangeiro TB (2006) Intraspecific variation in the first internal transcribed spacer (ITS1) of the nuclear ribosomal DNA in *Melipona subnitida* (Hymenoptera, Apidae), an endemic stingless bee from northeastern Brazil. Apidologie 37: 376-386.

CSIRO, Commonwealth Scientific and Industrial Research Organisation (2007) Climate change in Australia. Available at http://www.climatechangeinaustralia.com.au/index.php retrieved 22 January 2012.

Currah L, Ockendon DJ (1984) Pollination activity by blowflies and honeybees on onions in breeders' cages. Annals of Applied Biology 105: 167-176.

Czekonska K (2000) The influence of *Nosmea apis* on young honeybee queens and transmission of the disease from queens to workers. Apidologie 31: 701-706.

Daly HV, Hoelmer KIM, Norman P, Allen T (1982) Computer-assisted measurement and identification of honey bees (Hymenoptera: Apidae). Annals of the Entomological Society of America 75: 591-594.

Danks HV (1988) Systematics in support of entomology. Annual Review of Entomology 33: 271-294.

Darchen R (1972) Écologie de quelques *Trigones* (*Trigona* sp.) De la savane de Lamto (Cote D'Ivoire). Apidologie 3: 341-367.

Darchen R (1973) La thermorégulation et l'écologie de quelques espèces d'abeilles socials d'Afrique (*Apidae, Trigonini* et *Apis mellifica* var. *Adansonii*). Apidologie 4: 341-370.

Deevey ES, Jr. (1947) Life tables for natural populations of animals. The Quarterly Review of Biology 22: 283-314.

Del Sarto MC, Peruquetti RC, Campos LA (2005) Evaluation of the neotropical stingless bee *Melipona quadrifasciata* (Hymenoptera: Apidae) as pollinator of greenhouse tomatoes. Journal of Economic Entomology 98: 260-266.

Delaplane KS, Mayer D (2000) Crop Pollination by Bees. New York, CABI Publishing.

Delfinado-Baker M, Baker EW, Phoon ACG (1989) Mites (Acari) associated with bees (Apidae) in Asia, with description of a new species. American Bee Journal 129: 609-613.

DERM, Department of Environment and Resources Management (2011) Drought situation reports. Available at http://www.longpaddock.qld.gov.au/queenslanddroughtmonitor/queenslanddroughtreport/2005/Jun1.pdf retrieved 24 July 2011.

DeSalle R, Gatesy J, Wheeler W, Grimaldi D (1992) DNA sequences from a fossil termite in Oligo-Miocene amber and their phylogenetic implications. Science 257: 1933-1936.

Dias BSF, Raw A, Imperatriz-Fonseca VL (1999) International pollinators initiative: The São Paulo declaration on pollinators. Report on the recommendations of the workshop on the conservation and sustainable use of pollinators in agriculture with emphasis on bees. Brazilian Ministry of the Environment. Available at http://www.cbd.int/doc/case-studies/agr/cs-agr-pollinator-rpt.pdf

Digby PSB (1955) Factors affecting the temperature excess of insects in sunshine. Journal of Experimental Biology 32: 279-298.

Dillon N, Austin AD, Bartowsky E (1996) Comparison of preservation techniques for DNA extraction from hymenopterous insects. Insect Molecular Biology 5: 21-24.

Dollin A (1997a) *Austroplebeia australis* - A gentle breed of stingless bee. Aussie Bee 3: 4-5.

Dollin A (1997b) Cape York wilderness - part 1. Aussie Bee Bulletin 1: 12-13.

Dollin A (1997c) Cape York wilderness - part 2. Aussie Bee Bulletin 2: 12-13.

Dollin A (2002) Tips on stingless beekeeping by Australian bee keepers. Native Bees of Australia 2: 1-15.

Dollin A (2010a) Behaviour of Australian stingless bees. Native Bees of Australia 3: 1-14.

Dollin A (2010b) How to recognise the different types of Australian stingless bees. Native Bees of Australia 4: 1-13.

Dollin A (2010c) Introduction to Australian native bees. Native Bees of Australia 1: 1-10.

Dollin A (2010d) Nests of Australian stingless bees. Native Bees of Australia 2: 1-14.

Dollin A (2010e) Stingless beekeeping around the world. Aussie Bee Special 1-5.

Dollin A (2011) Which native bees are in your area? Available at http://www.aussiebee.com.au/beesinyourarea.html retrieved 28 July 2011.

Dollin A, Batley M, Robinson M, Faulkner B (2007) Native Bees of the Sydney Region: a Field Guide. North Richmond, Australian Native Bee Research Centre.

Dollin A, Dollin L, Sakagami SF (1997) Australian stingless bees of the genus *Trigona* (Hymenoptera: Apidae). Invertebrate Taxonomy 11: 861-896.

Dollin A, Heard T (1999) Tips on stingless beekeeping by Australian beekeepers. Native Bees of Australia 1: 1-17.

Dollin A, Heard T (2010) Keeping Australian stingless bees in a log or box. Native Bees of Australia 5: 1-14.

Dollin L (1997d) Exploring Western Australia - part 1. Aussie Bee Bulletin 3: 12-13.

Dollin L (1997e) Exploring Western Australia - part 2. Aussie Bee Bulletin 4: 14-15.

Dollin L (1998a) Bees, brolgas and blown-out tyres. Aussie Bee 7: 14-15.

Dollin L (1998b) In search of the tiny mosquito bee - part 1. Aussie Bee Bulletin 8: 14-15.

Dollin L (1999a) In search of the tiny mosquito bee - part 2. Aussie Bee Bulletin 9: 14-15.

Dollin L (1999b) The mysterious drones of Somerset - part 1. Aussie Bee Bulletin 10: 14-15.

Dollin L (1999c) The mysterious drones of Somerset - part 2. Aussie Bee Bulletin 11: 20-21.

Dollin L (1999d) The mysterious drones of Somerset - part 3. Aussie Bee Bulletin 12: 18-19.

Dollin L (2000a) Overland expedition for an elusive drone - part 1. Aussie Bee 14: 20-21.

Dollin L (2000b) Overland expedition for an elusive drone - part 2. Aussie Bee 15: 8-9.

Dollin L (2000c) Quest for gold in the Gulf country. Aussie Bee 13: 20-21.

Dollin L (2001) The lost *percincta* bees of Baron von Leonhardi in Central Australia. Aussie Bee Bulletin 18: 16-18.

Dorricott K, Voller P, Lawrie B (1999) Balancing production with nature conservation: case studies from southern inland Queensland. Available at http://www.derm.qld.gov.au/services_resources/item_details.php?item_id=201769 retrieved 4 July 2011.

Dowton M, Austin AD (1994) Molecular phylogeny of the insect order Hymenoptera: apocritan relationships. Proceedings of the National Academy of Sciences of the United States of America 91: 9911-9915.

Doyle JJ, Gaut BS (2000) Evolution of genes and taxa: a primer. Plant Molecular Biology 42: 1-23.

Drumond P, Oldroyd B, Dollin A, Dollin L (1999) Oviposition behaviour of two Australian stingless bees, *Austroplebeia symei* Rayment and *Austroplebeia australis* Friese (Hymenoptera: Apidae: Meliponini). Australian Journal of Entomology 38: 234-241.

Drumond PM, Oldroyd BP, Osborne K (2000a) Worker reproduction in *Austroplebeia australis* Friese (Hymenoptera, Apidae, Meliponini). Insectes Sociaux 47: 333-336.

Drumond PM, Zucchi R, Oldroyd BP (2000b) Description of the cell provisioning and oviposition process of seven species of *Plebeia* Schwarz (Apidae, Meliponini), with notes on their phylogeny and taxonomy. Insectes Sociaux 47: 99-112.

Edwardson JR (1956) Cytoplasmic male-sterility. Botanical Review 22: 696-738.

Edwardson JR (1970) Cytoplasmic male sterility. Botanical Review 36: 341-420.

Ehrlich PR, Ehrlich AH (1992) The value of biodiversity. Ambio 21: 219-226.

Ellis JD, Munn PA (2005) The worldwide health status of honey bees. Bee World 86: 88-101.

Eltz T, Brühl C, van der Kaars S, Linsenmair E (2002) Determinants of stingless bee nest density in lowland dipterocarp forests of Sabah, Malaysia. Oecologia 131: 27-34.

Eltz T, Brühl CA, Imiyabir Z, Linsenmair KE (2003) Nesting and nest trees of stingless bees (Apidae: Meliponini) in lowland dipterocarp forests in Sabah, Malaysia, with implications for forest management. Forest Ecology and Management 172: 301-313.

Emlen JM (1966) The role of time and energy in food preference. The American Naturalist 100: 611-617.

Engels W, Rosenkranz P, Engels E (1995) Thermoregulation in the nest of the Neotropical stingless bee *Scaptotrigona postica* and a hypothesis on the evolution of temperature homeostasis in highly eusocial bees. Studies on Neotropical Fauna and Environment 30: 193-205.

Esch H, Esch I, Kerr WE (1965) Sound: an element common to communication of stingless bees and to dances of the honey bee. Science 149: 320-321.

Eskov E (2007) Heat emission by the honey bee *Apis mellifera* L. brood (Hymenoptera, Apidae). Entomological Review 87: 249-252.

Fahrenholz L, Lamprecht I, Schricker B (1989) Thermal investigations of a honey bee colony: thermoregulation of the hive during summer and winter and heat production of members of different bee castes. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 159: 551-560.

Falzari L, Menary R (2005) Development of a celery oil and extract industry. Rural Industries Research and Development Corporation. Available at https://rirdc.infoservices.com.au/items/05-133.

FAO, Food and Agriculture of the United Nations (2009) Global action on pollination services for sustainable agriculture. Available at http://www.internationalpollinatorsinitiative.org/jsp/intpollinitiative.jsp retrieved 3 March 2011.

FAOSTAT (2010) Food and agriculture organisation of the United Nations. Available at http://faostat.fao.org/site/567/default.aspx retrieved 21 March 2011.

FBA, Fitzroy Basin Association (2005) RIS programs at a glance: biodiversity and vegetation. Fact sheet Biodiversity: 1-4.

Federer C (1971) Solar radiation absorbtion by leafless hardwood. Agricultural Meteorology 9: 3-20.

Fernandes-da-Silva P, Serrao J (2000) Nutritive value and apparent digestibility of bee-collected and bee-stored pollen in the stingless bee, *Scaptotrigona postica* Latr. (Hymenoptera, Apidae, Meliponini). Apidologie 31: 39-45.

Fernandes A, Jr., Leomil L, Fernandes AAH, Sforcin JM (2001) The antibacterial activity of propolis produced by *Apis mellifera* L. and Brazilian stingless bees. Journal of Venomous Animals and Toxins 7: 173-182.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3: 294-299.

Francisco FO, Nunes-Silva P, Francoy TM, Wittmann D, Imperatriz-Fonseca VL, Arias MC, Morgan ED (2008) Morphometrical, biochemical and molecular tools for assessing biodiversity. An example in *Plebeia remota* (Holmberg, 1903) (Apidae, Meliponini). Insectes Sociaux 55: 231-237.

Francoy T, Grassi M, Imperatriz-Fonseca V, de Jesús May-Itzá W, Quezada-Euán J (2011) Geometric morphometrics of the wing as a tool for assigning genetic

lineages and geographic origin to *Melipona beecheii* (Hymenoptera: Meliponini). Apidologie 42: 499-507.

Francoy TM, Wittmann D, Steinhage V, Drauschke M, Müller S, Cunha DR, Nascimento AM, Figueiredo VLC, Simões ZLP, De Jong D, Arias MC, Gonçalves LS (2009) Morphometric and genetic changes in a population of *Apis mellifera* after 34 years of Africanization. Genetics and Molecular Research 8: 709-717.

Free JB (1970) Insect Pollination of Crops. London, Academic Press.

Free JB, Racey PA (1968) The effect of the size of honeybee colonies on food consumption, brood rearing and the longevity of the bees during winter. Entomologia Experimentalis et Applicata 11: 241-249.

Free JB, Spencer-Booth Y (1959) The longevity of worker honey bees (*Apis mellifera*). Proceedings of the Royal Entomological Society of London. Series A, General Entomology 34: 141-150.

Friese H (1898) Die *Trigona*-Arten Australiens (The Australian *Trigona*). Termeszet Füzetek (World of Nature Papers) 21: 427-431.

Fukuda H, Sakagami SF (1968) Worker brood survival in honeybees. Researches on Population Ecology (Kyoto) 10: 31-39.

Gabelman WH, Goldman IL, Breitbach DN (1994) Field evaluation and selection for resistance to aster yellows in carrot (*Daucus carota* L.). Journal of the American Society for Horticultural Science 119: 1293-1297.

Gallai N, Salles J-M, Settele J, Vaissière BE (2009) Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. Ecological Economics 68: 810-821.

Gary NE, Witherell PC, Marston J (1972) Foraging range and distribution of honey bees used in carrot and onion pollination. Environmental Entomology 1: 71-78.

Gaston KJ, Fuller RA (2009) The size of species' geographic ranges. Journal of Applied Ecology 46: 1-9.

George RAT (1999) Vegetable Seed Production. New York, CABI Publishing.

Giannini KM (1997) Labor divsion in *Melipona compressipes fasciculata* Smith (Hymenoptera: Apidae: Meliponinae). Anais da Sociedade Entomológica do Brasil 26: 153-162.

Gibbons P, Lindenmayer D (2000) Tree Hollows and Wildlife Conservation in Australia. Collingwood, CSIRO Publishing.

Gibbs DMH, Muirhead IF (1998) The economic value and environmental impact of the Australian beekeeping industry. Australian Honey Bee Industry Council.

Available at http://honeybee.com.au/Library/gibsmuir.html retrieved 2 May 2009.

Gloag RS, Beekman M, Heard TA, Oldroyd BP (2007) No worker reproduction in the Australian stingless bee *Trigona carbonaria* Smith (Hymenoptera, Apidae). Insectes Sociaux 54: 412-417.

Gordon AW, Bernhardt P, Ron B, Burquez A, Buchmann S, Cane J, Cox PA, Dalton V, Feinsinger P, Ingram M, Inouye D, Jones CE, Kennedy K, Kevan P, Koopowitz H, Medellin R, Medellin-Morales S, Nabhan GP, Pavlik B, Tepedino V, Torchio P, Walker S (1998) The potential consequences of pollinator declines on the conservation of biodiversity and stability of food crop yields. Conservation Biology 12: 8-17.

Gordon J, Davis L (2003) Valuing honeybee pollination. Rural Industry Research and Development Corporation. Available at https://rirdc.infoservices.com.au/items/03-077 retrieved 17 August 2008.

Goubara M, Takasaki T (2003) Flower visitors of lettuce under field and enclosure conditions. Applied Entomology and Zoology 38: 571-581.

Graham JM, Ed. (1992) The Hive and the Honey Bee. Revised. Illinois, Dadant & Sons.

Greco M, Hoffmann D, Dollin A, Duncan M, Spooner-Hart R, Neumann P (2010) The alternative Pharaoh approach: stingless bees mummify beetle parasites alive. Naturwissenschaften 97: 319-323.

Greco MK, Spooner-Hart RN, Beattie GAC, Barchia I, Holford P (2011) Australian stingless bees improve greenhouse *Capsicum* production. Journal of Apicultural Research 50: 102-115.

Green CL, Oldroyd BP (2002) Queen mating frequency and maternity of males in the stingless bee *Trigona carbonaria* Smith. Insectes Sociaux 49: 196-202.

Greer L (1999) Alternative pollinators: Native bees. USDA. Available at http://www.attra.org/attra-pub/nativebee.html retrieved 10 May 2008.

Greves T (1964) Temperature studies of termite colonies in living trees. Australian Journal of Zoology 12: 250-262.

Grosso AF, Bego LR (2002) Labor division, average life span, survival curve, and nest architecture of *Tetragonisca angustula angustula* (Hymenoptera, Apinae, Meliponini). Sociobiology 40: 615-638.

Grüter C, Menezes C, Imperatriz-Fonseca VL, Ratnieks FLW (2012) A morphologically specialized soldier caste improves colony defense in a neotropical eusocial bee. Proceedings of the National Academy of Sciences of the United States of America 109: 1182-1186.

Hajibabaei M, Janzen DH, Burns JM, Hallwachs W, Hebert PDN (2006) DNA barcodes distinguish species of tropical Lepidoptera. Proceedings of the National Academy of Sciences of the United States of America 103: 968-971.

Halcroft M (2007) Investigations on the behaviour of the Australian native bee, *Austroplebeia australis*, with particular reference to its defence strategies against the African small hive beetle, *Aethina tumida*. School of Natural Sciences, University of Western Sydney. Richmond, Australia. BSc (Hons) thesis.

Halcroft MT, Spooner-Hart R, Neumann P (2008) A non-invasive and non-destructive method for observing in-hive behaviour of the Australian stingless bee, *Austroplebeia australis*. Journal of Apicultural Research 47: 82-83.

Halley E (1693) An estimate of the degrees of the mortality of mankind, drawn from curious tables of the births and funerals at the city of Breslau; with an attempt to ascertain the price of annuities upon lives. Philosophical Transactions (1683-1775) 17: 596-610.

Harden G, Ed. (2002) Flora of New South Wales. Volume 2. Revised edition. Sydney, Royal Botanic Gardens Sydney.

Harrington G, Oxley R, Tongway D (1976) The effects of European settlement and domestic livestock on the biological system in poplar box (Eucalyptus *populnea*) lands. The Rangeland Journal 1: 271-279.

Hartfelder K, Makert GR, Judice CC, Pereira GAG, Santana WC, Dallacqua R, Bitondi MMG (2006) Physiological and genetic mechanisms underlying caste development, reproduction and division of labor in stingless bees. Apidologie 37: 144-163.

Hawthorn LR, Bohart GE, Toole EH (1956) Carrot seed yield and germination as affected by different levels of insect pollinator. Proceedings of the American Society for Horticultural Science 67: 384-389.

Haydak MH (1970) Honey bee nutrition. Annual Review of Entomology 15: 143-156.

Heard T (1988a) Establishment and propagation of hives of native stingless bees for pollination of macadamia. Australian Macadamia Society News Bulletin 16: 10-13.

Heard T (1988b) Propagation of hives of *Trigona carbonaria* Smith (Hymenoptera: Apidae). Journal of the Australian Entomological Society 27: 303-304.

Heard T (1993) Pollinator requirements and flowering patterns of *Macadamia integrifolia*. Australian Journal of Botany 41: 491-497.

Heard T (1994) Behaviour and pollinator efficiency of stingless bees and honey bees on macadamia flowers. Journal of Apicultural Research 33: 191-198.

Heard T (1999) The role of stingless bees in crop pollination. Annual Review of Entomology 44: 183-206.

Heard T (2010) Sugarbag. Available at www.sugarbag.net retrieved 4 November 2010.

Heard T, Dollin A (1998) Crop pollination with Australian stingless bees. Native Bees of Australia Series 6: 1-17

Heard T, Dollin A (2000) Stingless bee keeping in Australia: snapshot of an infant industry. Bee World 81: 116-125.

Heard T, Exley EM (1994) Diversity, abundance and distribution of insect visitors to macadamia flowers. Environmental Entomology 23: 91-100.

Heard T, Hendrikz J (1993) Factors influencing flight activity of colonies of the stingless bee *Trigona carbonaria* (Hymenoptera: Apidae). Australian Journal of Zoology 41: 341-353.

Heard TA (1987) Preliminary studies on the role of *Trigona* bees in the pollination of macadamia. Proceedings of the second Australian macadamia research workshop, Bangalow, Australia.

Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identifications through DNA barcodes. Proceedings of the Royal Society B: Biological Sciences 270: 313-321.

Hebert PDN, Gregory TR (2005) The promise of DNA barcoding for taxonomy. Systematic Biology 54: 852-859.

Heinrich B (1972a) Physiology of brood incubation in the bumblebee queen, *Bombus vosnesenskii*. Nature 239: 223-225.

Heinrich B (1972b) Temperature regulation in the bumblebee *Bombus vagans*: a field study. Science 175: 185-187.

Heinrich B (1974) Thermoregulation in endothermic insects. Science 185: 747-756.

Heinrich B (1975) Energetics of pollination. Annual Review of Ecology and Systematics 6: 139-170.

Heinrich B, Esch H (1994) Thermoregulation in bees. American Scientist 82: 164-170.

Heinrich B, Raven PH (1972) Energetics and pollination ecology. Science 176: 597-602.

Hilário SD, Gimenes M, Imperatriz-Fonseca VL (2003) The influence of colony size in diel rhythms of flight activity of *Melipona bicolor* Lepeletier (Hymenoptera, Apidae, Meliponini), 191-197 pp. *In*: Apoidea Neotropica: Homenagem aos 90 Anos de Jesus Santiago Moure. Eds. Melo GAR, I Alves-dos-Santos. Criciúma, Editora UNESC.

Hilário SD, Imperatriz-Fonseca VL, Kleinert AdMP (2000) Flight activity and colony strength in the stingless bee *Melipona bicolor bicolor* (Apidae, Meliponinae). Revista Brasileira de Biologia 60: 299-306.

Hilário SD, Imperatriz-Fonseca VL, Kleinert AdMP (2001) Responses to climatic factors by foragers of *Plebeia pugnax* Moure (*in litt.*) (Apidae, Meliponinae). Revista Brasileira de Biologia 61: 191-196.

Hines HM, Hendrix SD (2005) Bumble bee (Hymenoptera: Apidae) diversity and abundance in tallgrass prairie patches: effects of local and landscape floral resources. Environmental Entomology 34: 1477-1484.

Hingston AB, McQuillan PB (1999) Displacement of Tasmanian native megachilid bees by the recently introduced bumblebee *Bombus terrestris* (Linnaeus, 1758) (Hymenoptera : Apidae). Australian Journal of Zoology 47: 59-65.

Hockings HJ (1883) Notes on two Australian species of *Trigona*. Transactions of the Entomological Society of London 9: 149-157.

Hoff FL (1995) Honey: background for 1995 farm legislation. U.S. Department of Agriculture. AER-708. 1-20. Available at http://www.ers.usda.gov/Publications/aer708/aer708a.pdf retrieved 8 July 2011.

Hogendoorn K (2011) Development of native bees as pollinators of vegetable seed crops. Available at http://www.horticulture.com.au/reports/search_final_reports_result.asp?src=&orgid=0&projid=4371&strSearch=&strProjectNo=VG08179&strIndustry=0-All&strSortby=date&strDisplay=titledesc&pageno=1 retrieved 29 January 2012.

Hogendoorn K (2012) The importance of bee-ing earnest. Vegetables Australia January/February: 10-11.

Hogendoorn K, Coventry S, Keller MA (2007) Foraging behaviour of a blue banded bee, *Amegilla chlorocyanea* in greenhouses: implications for use as tomato pollinators. Apidologie 38: 86-92.

Hogendoorn K, Steen Z, Schwarz MP (2000) Native Australian carpenter bees as a potential alternative to introducing bumble bees for tomato pollination in greenhouses. Journal of Apicultural Research 39: 67-74.

Holden C (2006) Report warns of looming pollination crisis in North America. Science 314: 397.

Holm SN (1966) The utilization and management of bumble bees for red clover and alfalfa seed production. Annual Review of Entomology 11: 155-182.

Hopkins I (1886) The Illustrated Australasian Bee Manual and Complete Guide to Modern Bee Culture in the Southern Hemisphere. Auckland, Hopkins.

Howe RW (1967) Temperature effects on embryonic development in insects. Annual Review of Entomology 12: 5-42.

Hrncir M, Jarau S, Zucchi R, Barth FG (2004) On the origin and properties of scent marks deposited at the food source by a stingless bee, *Melipona seminigra*. Apidologie 35: 3-13.

Hubbell SP, Johnson LK (1977) Competition and nest spacing in a tropical stingless bee community. Ecology 58: 950-963.

Hubbell SP, Johnson LK (1978) Comparative foraging behavior of six stingless bee species exploiting a standardized resource. Ecology 59: 1123-1136.

Huntzinger CI, James RR, Bosch J, Kemp WP (2008) Laboratory bioassays to evaluate fungicides for chalkbrood control in larvae of the alfalfa leafcutting bee (Hymenoptera: Megachilidae). Journal of Economic Entomology 101: 660-667.

ICZN, International Commission on Zoological Nomenclature (1999) International Code of Zoological Nomenclature. Available at http://www.nhm.ac.uk/hosted-sites/iczn/code/ retrieved 30 May 2011.

Imperatriz-Fonseca VL (2010) Biodiversity, conservation and sustainable use of pollinators, mainly bees. Proceedings of the IX Encontro sobre abelhas Ribeirão Preto.

Imperatriz-Fonseca VL, Kleinert-Giovannini A, Pires JT (1985) Climate variations influence on the flight activity of *Plebeia remota* Holmberg (Hymenoptera, Apidae, Meliponinae). Revista Brasileira de Entomologia 29: 427-434.

Imperatriz-Fonseca VL, Saraiva AM, de Jong D, Ed. (2006) Bees as pollinators in Brazil: assessing the status and suggesting best practices. Holos Editoria, Ribeirão Preto.

Imperatriz-Fonseca VL, Zucchi R (1995) Virgin queens in stingless bee (Apidae, Meliponinae) colonies: a review. Apidologie 26: 231-244.

Inoue T, Sakagami SF, Salmah S, Yamane S (1984) The process of colony multiplication in the Sumatran stingless bee *Trigona* (*Tetragonula*) *laeviceps*. Biotropica 16: 100-111.

Irish J, Carter DA, Blair SE, Heard TA (2008) Antibacterial activity of honey from the Australian stingless bee *Trigona carbonaria*. International Journal of Antimicrobial Agents 32: 89-90.

Jay SC (1963) The development of honeybees in their cells. Journal of Apicultural Research 2: 117-134.

Johansen CA (1977) Pesticides and pollinators. Annual Review of Entomology 22: 177-192.

Johnson LK, Hubbell SP (1974) Aggression and competition among stingless bees: field studies. Ecology 55: 120-127.

Johnson LK, Hubbell SP (1975) Contrasting foraging strategies and coexistence of two bee species on a single resource. Ecology 56: 1398-1406.

Jones G, Webb PI, Sedgeley JA, O'Donnell CFJ (2003) Mysterious *Mystacina*: how the New Zealand short-tailed bat (*Mystacina tuberculata*) locates insect prey. Journal of Experimental Biology 206: 4209-4216.

Jones JC, Oldroyd BP (2006) Nest thermoregulation in social insects. Advances in Insect Physiology 33: 153-191.

Jones WA, Harper MA (1981) Increase seed-crop returns. Proceedings of Pollination Symposium, Dubbo, NSW, Australia.

Kakutani T, Inoue T, Tezuka T, Maeta Y (1993) Pollination of strawberry by the stingless bee, *Trigona minangkabau*, and the honey bee, *Apis mellifera*: an experimental study of fertilization efficiency Researches on Population Ecology 35: 95-111.

Kalinowsi ST (2009) How well do evolutionary trees describe genetic relationships between populations? Heredity 102: 506-513.

Keith DA (2000) Sampling designs, field techniques and analytical methods for systematic plant population surveys. Ecological Management & Restoration 1: 125-139.

Kerr WE (1950) Genetic determination of castes in the genus *Melipona*. Genetics 35: 143-152.

Kerr WE (1959) Biology of Meliponids VI aspects of food gathering and processing in some stingless bees, 24-31pp. *In* Food Gathering Behaviour of Hymenoptera. Symposium of Entomological Society of America.

Kerr WE (1994) Communication among *Melipona* workers (Hymenoptera: Apidae). Journal of Insect Behavior 7: 123-128.

Kerr WE, Maule V (1964) Geographic distribution of stingless bees and its implications (Hymenoptera: Apidae). Journal of the New York Entomological Society 72: 2-18.

Kerr WE, Zucchi R, Nakadaira JT, Butolo JE (1962) Reproduction in the social bees (Hymenoptera: Apidae). Journal of the New York Entomological Society 70: 265-276.

Kevan PG, Phillips TP (2001) The economic impacts of pollinator declines: an approach to assessing the consequences. Conservation Ecology 5: 8 (online) http://www.ecologyandsociety.org/vol5/iss1/art8/.

Klein AM (2009) Nearby rainforest promotes coffee pollination by increasing spatio-temporal stability in bee species richness. Forest Ecology and Management 258: 1838-1845.

Klein AM, Steffan-Dewenter I, Tscharntke T (2003) Bee pollination and fruit set of *Coffea arabica* and *C. canephora* (Rubiaceae). American Journal of Botany 90: 153-157.

Klein AM, Vaissiére VE, Cane JH, Steffan-Dewenter I, Cunningham SA, Kremen C, Tscharntke T (2007) Importance of pollinators in changing landscapes for world crops. Proceedings of the Royal Society B: Biological Sciences 274: 303-313.

Kleinert-Giovannini A (1982) The influence of climatic factors on flight activity of *Plebeia emerina* Friese (Hymenoptera, Apidae, Meliponinae) in winter. Revista Brasileira de Entomologia 26: 1-13.

Klumpp J (2007) Australian Stingless Bees: A Guide to Sugarbag Beekeeping. Brisbane, Earthling Enterprises Pty; Ltd.

Koch H (2010) Combining morphology and DNA barcoding resolves the taxonomy of Western Malagasy *Liotrigona* Moure, 1961 (Hymenoptera: Apidae: Meliponini). African Invertebrates 51: 413-421.

Koedam D, Cepeda Aponte OI, Imperatriz-Fonseca VL (2007) Egg laying and oophagy by reproductive workers in the polygynous stingless bee *Melipona bicolor* (Hymenoptera, Meliponini). Apidologie 38: 55-55.

Koedam D, Contrera FAL, Fidalgo AO, Imperatriz-Fonseca VL (2005) How queen and workers share in male production in the stingless bee *Melipona subnitida* Ducke (Apidae, Meliponini). Insectes Sociaux 52: 114-121.

Krebs CJ (2001) Ecology: the Experimental Analysis of Distribution and Abundance. New York, Addison Wesley Longman, Inc.

Kujumgiev A, Tsvetkova I, Serkedjieva Y, Bankova V, Christov R, Popov S (1999) Antibacterial, antifungal and antiviral activity of propolis of different geographic origin. Journal of Ethnopharmacology 64: 235-240.

Lacerda LdeM, Paulino Simões Z, Velthuis H (2010) The sharing of male production among workers and queens in *Scaptotrigona depilis* (Moure, 1942) (Apidae, Meliponini). Insectes Sociaux 57: 185-192.

Law JH, Regnier FE (1971) Pheromones. Annual Review of Biochemistry 40: 533-548.

Leonhardt SD, Wallace HM, Schmitt T (2011) The cuticular profiles of Australian stingless bees are shaped by resin of the eucalypt tree *Corymbia torelliana*. Austral Ecology 36: 537-543.

Lercker G, Capella P, Conte L, Ruini F, Giordani G (1981) Components of royal jelly: I. Identification of the organic acids. Lipids 16: 912-919.

Lindauer M, Kerr WE (1960) Communication between the workers of stingless bees. Bee World 41: 29-41, 65-71.

Linke B, Nothnagel T, Börner T (1999) Morphological characterization of modified flower morphology of three novel alloplasmic male sterile carrot sources. Plant Breeding 118: 543-548.

Losey JE, Vaughan M (2006) The economic value of ecological services provided by insects. Bioscience 56: 311-323.

Loughlin C (2008) Rapid assessment of pollinators' status: a contribution to the international initiative for the conservation and sustainable use of pollinators. Available at http://www.internationalpollinatorsinitiative.org retrieved 20 February 2011.

Ludwig HW (1962) Sonderabdruck. Ver. Deut. Zool. Ges. Wien. 30: 355. Cited in Heinrich & Raven 1972.

Lundie AE (1925) The flight activity of the honeybee. US Department of Agriculture Bulletin 1328: 1-37.

Macfarlane RP, Lipa JJ, Liu HJ (1995) Bumble bee pathogens and internal enemies. Bee World 76: 130-148.

Macías-Macías J, Quezada-Euán J, Contreras-Escareño F, Tapia-Gonzalez J, Moo-Valle H, Ayala R (2011) Comparative temperature tolerance in stingless bee species from tropical highlands and lowlands of Mexico and implications for their conservation (Hymenoptera: Apidae: Meliponini). Apidologie 42: 679-689.

Magnussen LS, Hauser TP (2007) Hybrids between cultivated and wild carrots in natural populations in Denmark. Heredity 99: 185-192.

Malagodi-Braga K, Peixoto Kleinert AdM (2004) Could *Tetragonisca angustula* Latreille (Apinae, Meliponini) be effective as strawberry pollinator in greenhouses? Australian Journal of Agricultural Research 55: 771-773.

Manning R (2002) The Beetube - a new honey bee pollination device in Western Australia. Australian Journal of Experimental Agriculture 42: 643-647.

Marco AC, Marco ADL, Gabriel ARM, Walter SS (2003) Molecular phylogeny of the stingless bees (Apidae, Apinae, Meliponini) inferred from mitochondrial 16S rDNA sequences. Apidologie 34: 73-84.

Martin HA (2006) Cenozoic climatic change and the development of the arid vegetation in Australia. Journal of Arid Environments 66: 533-563.

Maurizio A (1950) The influence of pollen feeding and brood rearing on the length of life and physiological condition of the honeybee. Bee World 31: 9-12.

May-Itzá W, Quezada-Euán J, Enriquez E, De La Rúa P (2009) Intraspecific variation in the stingless bee *Melipona beecheii* assessed with PCR-RFLP of the ITS1 ribosomal DNA. Apidologie 40: 549-555.

McGregor SE (1976) Insect Pollination of Cultivated Crop Plants. Washington, United States Department of Agriculture.

McNally LC, Schneider SS (1996) Spatial distribution and nesting biology of colonies of the African honey bee *Apis mellifera scutellata* (Hymenoptera: Apidae) in Botswana, Africa. Environmental Entomology 25: 643-652.

Mellanby K (1939) Low temperature and insect activity. Proceedings of the Royal Society B: Biological Sciences 127: 473-487.

Mendes MFM, Francoy TM, Nunes-Silva P, Menezes C, Imperatriz-Fonseca VL (2007) Intra-populational variability of *Nannotrigona testaceicornis* Lepeletier, 1836 (Hymenoptera, Meliponini) using relative warp analysis. Bioscience Journal, Uberlândia 23: 147-152.

Menezes C, Imperatriz Fonseca VL (2010) Criação in vitro de rainhas de abelhas sem ferrão: Uma ferramenta para a produção racional de colônias. Anais do IX Encortro sobre Abelhas, Ribeirão Preto, São Paulo, Brazil.

Michener CD (1961) Observations on the nests and behavior of *Trigona* in Australia and New Guinea (Hymenoptera, Apidae). American Museum Novitates 2026: 1-46.

Michener CD (1964) Evolution of the nests of bees. American Zoologist 4: 227-239.

Michener CD (1965) A classification of the bees of the Australian and South Pacific regions. Bulletin of the American Museum of Natural History 130: 1-362.

Michener CD (1974) Social Behaviour of the Bees: A Comparative Study. Cambridge, The Belknap Press of Harvard University Press.

Michener CD (1979) Biogeography of the bees. Annals of the Missouri Botanical Garden 66: 277-347.

Michener CD (1990) Classification of the Apidae (Hymenoptera). University of Kansas Science Bulletin 54: 75-164.

Michener CD (2000) Bees of the World. Baltimore, Johns Hopkins University Press.

Milborrow BV, Kennedy JM, Dollin A (1987) Composition of wax made by the Australian stingless bee *Trigona australis*. Australian Journal of Biological Science 40: 15-25.

Mocsáry S (1898) Die *Trigona*-Arten Australiens. Termeszet Füzetek (World of Nature Papers) 21: 427-431.

Mogensen HL (1996) The hows and whys of cytoplasmic inheritance in seed plants. American Journal of Botany 83: 383-404.

Moo-Valle H, Quezada-Euán JJG, Canto-Martín J, Gonzalez-Acereto JA (2004) Caste ontogeny and the distribution of reproductive cells on the combs of *Melipona beecheii* (Apidae: Meliponini). Apidologie 35: 587-594.

Moo-Valle H, Quezada-Euán JJG, Navarro J, Rodriguez-Carvajal LA (2000) Patterns of intranidal temperature fluctuation for *Melipona beecheii* colonies in natural nesting cavities. Journal of Apicultural Research 39: 3-7.

Moo-Valle H, Quezada-Euán JJG, Wenseleers T (2001) The effect of food reserves on the production of sexual offspring in the stingless bee *Melipona beecheii* (Apidae, Meliponini). Insectes Sociaux 48: 398-403.

Moritz RFA, Southwick EE (1992) Bees as Superorganisms: An Evolutionary Reality. Berlin, Germany, Springer Verlag.

Moure JS (1961) A preliminary supra-specific classification of the old world Meliponine bees (Hymenoptera, Apoidea). Studia Entomologica 4: 181-242.

Nagata RT (1992) Clip-and-wash method of emasculation of lettuce. HortScience 27: 907-908.

Naves Da Silva DL, Zucchi R, Kerr WE (1972) Biological and behavioural aspects of the reproduction in some species of *Melipona* (Hymenoptera, Apidae, Meliponinae). Animal Behaviour 20: 123-132.

NBII (2008) Bumble bees: Terrific greenhouse pollinators. Available at http://www.nbii.gov/portal/community/Communities/Ecological_Topics/Pollinators/Pollinator_Species/Invertebrates/Bees_and_Wasps/Bumble_Bees_(Bombus_spp.)/ retrieved 22 August 2008.

Neukirch A (1982) Dependence of the life span of the honeybee (*Apis mellifera*) upon flight performance and energy consumption. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 146: 35-40.

Neumann P, Radloff SE, Moritz RFA, Hepburn HR, Reece SL (2001) Social parasitism by honeybee workers (*Apis mellifera capensis* Escholtz): host finding and resistance of hybrid host colonies. Behavioral Ecology 12: 419-428.

Nieh JC (1998) The role of a scent beacon in the communication of food location in the stingless bee, *Melipona panamica*. Behavioural Ecology and Sociobiology 43: 47-58.

Nieh JC, Roubik DW (1995) A stingless bee (*Melipona panamica*) indicates food location without using a scent trail. Behavioural Ecology and Sociobiology 37: 63-70.

Nieh JC, Roubik DW (1998) Potential mechanisms for the communication of height and distance by a stingless bee, *Melipona panamica*. Behavioural Ecology and Sociobiology 43: 387-399.

Nieh JC, Sánchez D (2005) Effect of food quality, distance and height on thoracic temperature in the stingless bee *Melipona panamica*. The Journal of Experimental Biology 208: 3933-3943.

Nieh JC, Tautz J, Spaethe J, Bartareau T (2000) The communication of food location by a primitive stingless bee, *Trigona carbonaria*. Zoology 102: 239-246.

Norgate M, Boyd-Gerny S, Simonov V, Rosa MGP, Heard TA, Dyer AG (2010) Ambient temperature influences Australian native stingless bee (*Trigona carbonaria*) preference for warm nectar. PLoS ONE 5: 1-8.

Nothnagel T, Straka P, Linke B (2000) Male sterility in populations of *Daucus* and the development of alloplasmic male-sterile lines of carrot. Plant Breeding 119: 145-152.

NRW, Department of Natural Resources and Water (2006) The Condamine catchment. Available at http://www.derm.qld.gov.au/factsheets/pdf/catchments/c1.pdf retrieved 17 June 2011.

O'Donnell S, Jeanne RL (1995) Implications of senescence patterns for the evolution of age polyethism in eusocial insects. Behavioral Ecology 6: 269-273.

OED, Oxford English Dictionary (2009) A dictionary of zoology. Available at http://www.oxfordreference.com/views/BOOK_SEARCH.html?book=t8 retrieved 20 May 2011.

OEH, Office of Environment and Heritage (2003) Report under the NV Act 2003 in relation to the use of more appropriate local data (section 2.4.3. of the Environmental Outcomes Assessment Methodology). Accreditation No. 30628, PVP/DA No. 12327. NSW Government. Available at http://www.environment.nsw.gov.au/resources/nativeveg/CMAdiscretions/20PVP00063MVR12327.pdf

OEH, Office of Environment and Heritage (2011) Tree hollows and wildlife conservation in Australia. Available at http://www.environment.nsw.gov.au/projects/treehollows.htm retrieved 28 January 2012.

Oldroyd BP (2007) What's killing American honey bees? PLoS Biology. 5: 168.

Oldroyd BP, Lawler SH, Crozier RH (1994) Do feral honey bees (*Apis mellifera*) and regent parrots (*Polytelis anthopeplus*) compete for nest sites? Australian Journal of Ecology 19: 444-450.

Oldroyd BP, Thexton EG, Lawler SH, Crozier RH (1997) Population demography of Australian feral bees (*Apis mellifera*). Oecologia 111: 381-387.

Oliveira MAC (1973) Algumas observações sobre a atividade externa de *Plebeia saiqui* e *Plebeia droryana*. Departamento de Zoologia, Instituto de Biociências, University of São Paulo. São Paulo. MSc thesis.

Oxley R (1987) Analysis of historical records of a grazing property in south-western Queensland. 2. Vegetation changes. The Rangeland Journal 9: 30-38.

Page RE, Peng CY-S (2001) Aging and development in social insects with emphasis on the honey bee, *Apis mellifera* L. Experimental Gerontology 36: 695-711.

Paton DC (2000) Disruption of bird-plant pollination systems in southern Australia. Conservation Biology 14: 1232-1234.

Patricio EFLRA, Cruz-Lo´pez L, Maile R, Tentschert J, Jones GR, Morgan ED (2002) The propolis of stingless bees: terpenes from the tibia of three *Frieseomelitta* species. Journal of Insect Physiology 48: 249-254.

Paxton RJ, Weißschuh N, Engels W, Hartfelder K, Quezada-Euan JJG (1999) Not only single mating in stingless bees. Naturwissenschaften 86: 143-146.

Pearl R (1928) The Rate of Living. New York, A. A. Knopf.

Persano Oddo L, Heard TA, Rodríguez-Malaver A, Pérez RA, Fernández-Muiño M, Sancho MT, Sesta G, Lusco L, Vit P (2008) Composition and antioxidant activity of *Trigona carbonaria* honey from Australia. Journal of Medicinal Food 11: 789-794.

Peeters A, Barendregt JJ, Willekens F, Mackenbach JP, Mamun AA, Bonneux L (2003) Obesity in adulthood and its consequences for life expectancy: a life-table analysis. Annals of Internal Medicine 138: 24-32.

PIF, Primary Industries and fisheries (2012) Overview of Asian honeybee. Available at http://www.dpi.qld.gov.au/4790_13530.htm retrieved 19 January 2012.

Pinheiro-Machado C, Alves dos Santos I, Imperatriz-Fonseca VL, Kleinert AdMP, Amaral da Silveira F (2002) Brazilian bee surveys: state of knowledge, conservation and sustainable use. 115-129 pp. *In*: Pollinating Bees - The Conservation Link Between Agriculture and Nature. Eds. Kevan P, VL Imperatriz-Fonseca. Brasilia, Ministry of Environment.

Pokhrel S, Thapa RB, Neupane FP, Shrestha SM (2006) Absconding behavior and management of *Apis cerana* F. honeybee in Chitwan, Nepal. Journal of the Institute of Agriculture and Animal Science 27: 77-86.

Prohens J, Nuez F, Ed. (2008a) Vegetables 1: Asteraceae, Brassicaceae, Chenopodicaceae and Cucurbitaceae: Handbook of Plant Breeding New York, Springer Science+Business Media, LLC.

Prohens J, Nuez F, Ed. (2008b) Vegetables 2: Fabaceae, Liliaceae, Solanaceae and Umbelliferae: Handbook of Plant Breeding New York, Springer Science+Business Media, LLC.

Prosser CL, Nelson DO (1981) The role of nervous systems in temperature adaptation of poikilotherms. Annual Review of Physiology 43: 281-300.

Pyke GH (1984) Optimal foraging theory: a critical review. Annual Review of Ecology and Systematics 15: 523-575.

QMDC, Queensland Murray-Darling Committee (2012) Aboriginal program. Available at http://www.qmdc.org.au/aboriginal-program.html retrieved 2 February 2012.

QPWS, Queensland Parks and Wildlife Service (2011) Poplar box woodland: regional ecosystem. Nature Conservation Extension Unit Southern Region. 11.3.2: 1-2. Available at http://www.derm.qld.gov.au/register/p00599aa.pdf retrieved 2 July 2011

Queller DC, Strassmann JE (2002) The many selves of social insects. Science 296: 311-313.

Quezada-Euán JJG, Paxton RJ, Palmer KA, Itzá WdJM, Tay WT, Oldroyd BP (2007) Morphological and molecular characters reveal differentiation in a Neotropical social bee, *Melipona beecheii* (Apidae: Meliponini). Apidologie 38: 247-258.

Quiros CF, Rugama A, Dong YY, Orton TJ (1986) Cytological and genetical studies of a male sterile celery. Euphytica 35: 867-875.

Quong A (1993) The implications of traditional beekeeping in Tabora region, Tanzania for Miombo woodland conservation. School of International Training, Tanzania. Cited in Neumann et al. 2001.

Ramalho M, Giannini TC, Malagodi-Braga KS, Imperatriz-Fonseca VL (1994) Pollen harvest by stingless bee foragers (Hymenoptera: Apidae: Meliponinae). Grana 33: 239-244.

Rasmussen C (2008) Catalog of the Indo-Malayan/Australasian stingless bees (Hymenoptera: Apidae: Meliponini). Zootaxa 1935: 1-80.

Rasmussen C, Cameron S (2007) A molecular phylogeny of the Old World stingless bees (Hymenoptera: Apidae: Meliponini) and the non-monophyly of the large genus *Trigona*. Systematic Entomology 32: 26-39.

Rasmussen C, Cameron SA (2010) Global stingless bee phylogeny supports ancient divergence, vicariance, and long distance dispersal. Biological Journal of the Linnean Society 99: 206-232.

Rayment T (1929) Bees in the Australian Museum collection. Records of the Australian Museum 17: 199-243.

Rayment T (1930) New and remarkable bees. Proceedings from the Royal Society of Victoria 43: 42-61.

Rayment T (1932) The stingless bees of Australia: 6. The finding of a new species. Victorian Naturalist 49: 104-107.

Rayment T (1935) A Cluster of Bees. Sydney, Endeavour Press.

RDA, Regional Development Australia (2010) Connecting the Northern Rivers to opportunities: horticulture. Available at http://www.rdanorthernrivers.org.au/page/Content?&select=List&rowid=195 retrieved 1 February 2012.

Rhodes J (1986) Macadamia pollination. Australian Macadamia Society News Bulletin 13: 1-4.

Rhodes J (2006) Honey bee pollination of blueberries. Profitable and sustainable primary industries. New South Wales Department of Primary Industry. Primefact 157. 1-2. Available at http://www.dpi.nsw.gov.au/__data/assets/pdf_file/0017/84410/pf157-honeybee-pollination-of-blueberries.pdf retrieved 10 March 2011.

Rhodes J, McCorkell B (2007) Small hive beetle *Aethina tumida* in New South Wales apiaries 2002-6: survey results 2006. New South Wales Department of Primary Industry. 1-32. Available at http://www.dpi.nsw.gov.au/__data/assets/pdf_file/0005/167378/Small-Hive-Beetle-Aethina-tumida-in-New-South-Wales-Apiaries-2002-6-Survey-Results-2006.pdf retrieved 10 December 2010.

Ribbands CR (1953) Behaviour and Social Life of Honeybees. New York, Dover Publications, Inc.

Ribeiro MdF. (2002) Does the queen of *Plebeia remota* (Hymenoptera, Apidae, Meliponini) stimulate her workers to start brood cell construction after winter? Insectes Sociaux 49: 38-40.

Ribeiro MdF (1989) Estudos ecoetolôgicos em *Frieseomelitta latiguida*, Moure, e Camargo, in litt. (Hym., Apidae, Meliponinae). São Paulo, Depto. Ecologia IBUSP 129 (Cited in Simões & Bego 1991).

Richards KW (1993) Non-*Apis* bees as crop pollinators. Revue Suisse de Zoologie 100: 807-822.

Richards KW, Kevan PG (2002) Aspects of bee biodiversity, crop pollination, and conservation in Canada pp.77-94. *In*: Pollinating Bees - The Conservation Link Between Agriculture and Nature. Eds. Kevan PG, VL Imperatriz-Fonseca. Ministry of Environment / Brasília.

RIRDC, Rural Industries Research and Development Corporation (2007) Pollination Australia. Report No. 07/ 180: 1-8. Available at http://www.rirdc.gov.au retrieved 14 March 2011

RIRDC, Rural Industries Resear.ch and Development Corporation (2009) Pollination five year R&D plan: 2009 - 2014. Available at https://rirdc.infoservices.com.au/downloads/09-125.pdf retrieved 20 February 2011.

RIRDC, Rural Industries Research and Development Corporation (2010a) Pollination aware: the real value of pollination. Available at http://www.honeybee.org.au/pdf/PollinationAwareFactSheet.pdf retrieved 10 March 2011.

RIRDC, Rural Industries Research and Development Corporation (2010b) Pollination: almond. Available at http://www.rirdc.gov.au/programs/established-rural-industries/pollination/pollination_home.cfm retrieved 14 November 2010.

RIRDC, Rural Industries Research and Development Corporation (2012) Honeybee RD&E plan: 2012 to 2017. No: 12/049: 1-33. Available at https://rirdc.infoservices.com.au/items/12-049 retrieved 5 September 2012

Ritter ME (2006) The physical environment: an introduction to physical geography. Available at http://www.uwsp.edu/geo/faculty/ritter/geog101/textbook/title_page.html retrieved 11 June 2011.

Roberts BR (1992) Land Care Manual. Sydney, New South Wales University Press.

Robertson J, Beeston G (1981) Methods of clearing and woody weed control in the Poplar Box (*Eucalyptus populinea*) lands. The Rangeland Journal 3: 39-44.

Rodriguez VB, Riley C, Shafron W, Lindsay R (2003) Honeybee industry survey. A report for the Rural Industries Research and Development Corporation by the Australian Bureau of Agricultural and Resource Economics. RIRDC Publication No 03/039, Project No ABA-15A. 1-47. Available at https://rirdc.infoservices.com.au/items/03-039 retrieved 11 July 2011.

Rohlf FJ (2005) tpsDig2, version 2.04. Department of Ecology and Evolution, State University of New York, Stony Brook.

Roselino AC, Santos SB, Hrncir M, Bego LR (2009) Differences between the quality of strawberries (*Fragaria x ananassa*) pollinated by the stingless bees *Scaptotrigona* aff. *depilis* and *Nannotrigona testaceicornis*. Genetics and Molecular Research 8: 539-545.

Rosenberg DM, Danks HV, Lehmkuhl DM (1986) Importance of insects in environmental impact assessment. Environmental Management 10: 773-783.

Rosengren R, Fortelius W, Lindstrom K, Luther A (1987) Phenology and causation of nest heating and thermoregulation in red wood ants of the *Formica rufa* group studied in coniferous forest habitats in southern Finland. Annales Zoologici Fennici 24: 147-155.

Roubik DW (1979) Nest and colony characteristics of stingless bees from French Guiana (Hymenoptera: Apidae). Journal of the Kansas Entomological Society 52: 443-470.

Roubik DW (1980) Foraging behavior of competing Africanized honeybees and stingless bees. Ecology 61: 836-845.

Roubik DW (1982) Seasonality in colony food storage, brood production and adult survivorship: studies of *Melipona* in tropical forest (Hymenoptera: Apidae). Journal of the Kansas Entomological Society 55: 789-800.

Roubik DW (1983) Nest and colony characteristics of stingless bees from Panamá (Hymenoptera: Apidae). Journal of the Kansas Entomological Society 56: 327-355.

Roubik DW (1989) Ecology and Natural History of Tropical Bees. Cambridge University Press, Cambridge.

Roubik DW, Ed. (1995) Pollination of Cultivated Plants in the Tropics. Rome, Food and Agriculture Organization of the United Nations.

Roubik DW, Aluja M (1983) Flight ranges of *Melipona* and *Trigona* in tropical forest. Journal of the Kansas Entomological Society 56: 217-222.

Roubik DW, Peralta FJA (1983) Thermodynamics in nests of two *Melipona* species in Brasil. Acta Amazonica 13: 453-466.

Roubik DW, Smith BH, Carlson RG (1987) Formic acid in caustic cephalic secretions of stingless bee, *Oxytrigona* (Hymenoptera: Apidae). Journal of Chemical Ecology 13: 1079-1086.

RPS, Australia East Pty Ltd. (2010) The benefits of soil conservation works in the Moonie (Back Ck) and Upper Boogera clusters of the Talwood planning group. Prepared for the Queensland Murray-Darling Committee. Available at http://www.qmdc.org.au/publications/download/772/website-pdfs/sub-catchment-planning/the-benefits-of-soil-conservation-works-in-the-moonie-back-ck-and-upper-boogera-clusters-of-the-ta.pdf retrieved 6 February 2012.

Ruttner F (1988) Biogeography and Taxonomy of Honeybees. Berlin, Springer-Verlag.

Ruz L (2002) Bee pollinators introduced to Chile: a review. 155-167 pp. *In*: Pollinating Bees - The Conservation Link Between Agriculture and Nature. Eds. Kevan P, VL Imperatriz-Fonseca. Ministry of Environment, Brasilia.

Ryland A (2008) *Montdorensis*: thrips predator. Richmond, Beneficial Bug Company.

RZA, Rijk Zwaan Australia (2009) Rijk Zwaan. Available at http://www.rijkzwaan.com/ retrieved 5 August 2008.

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4: 406-425.

Sakagami SF (1982) Stingless bees. 362-421 pp. *In*: Social Insects Eds. Hermann HR. New York, Academic Press.

Sakagami SF, Fukuda H (1968) Life tables for worker honeybees. Researches on Population Ecology (Kyoto) 10: 127-139.

Sakagami SF, Yamane S (1984) Notes on taxonomy and nest architecture of the Taiwanese stingless bee *Trigona (Lepidotrigona) ventralis hoozana*. Bulletin of the Faculty of Education Ibaraki University 33: 37-48.

Salmah S, Inoue T, Mardius P, Sakagami SF (1987) Incubation period and post-emergence pigmentation in the Sumatran stingless bee, *Trigona* (*Trigonella*) *moorei*. Japanese Journal of Entomology 55: 383-390.

Salmah S, Inoue T, Sakagami SF (1996) Incubation period and post-emergence pigmentation in the Sumatran stingless bee *Trigona* (*Heterotrigona*) *itama* (Apidae, Meliponinae) Japanese Journal of Entomology 64: 401-411.

Samejima H, Marzuki M, Nagamitsu T, Nakasizuka T (2004) The effects of human disturbance on a stingless bee community in a tropical rainforest. Biological Conservation 120: 577-587.

Sánchez D, Nieh JC, León A, Vandame R (2009) Food recruitment information can spatially redirect employed stingless bee foragers. Ethology 115: 1175-1181.

Schorkopf D, Morawetz L, Bento J, Zucchi R, Barth F (2011) Pheromone paths attached to the substrate in meliponine bees: helpful but not obligatory for recruitment success. Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology 197: 755-764.

Schröder S, Drescher W, Steinhage V, Kastenholz B (1995) An automated method for the identification of bee species (Hymenoptera: Apoidea). Proceedings from the International Symposium on Conserving Europe's Bees 6-7.

Sedgley M, Blesing MA, Vithanage V (1985) A developmental study of the structure and pollen receptivity of the macadamia pistil in relation to protandry and self-incompatibility. Botanical Gazette 146: 6-14.

Seehuus SC (2006) Animal model on social terms: the honey bee (*Apis mellifera*). Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences. Oslo. PhD thesis.

Seeley TD (1985) Honeybee Ecology. New Jersey, Princeton University Press.

Seeley TD (2010) Honeybee Democracy. New Jersey, Princeton University Press.

Shaw R (2010) Shaw's honey. Available at www.shawshoney.com.au retrieved 4 November 2010.

Sherry TW, McDade LA (1982) Prey selection and handling in two neotropical hover-gleaning birds. Ecology 63: 1016-1028.

Silva de Moraes RLM, Brochetto-Braga MR, Azevedo A (1996) Electrophoretical studies of proteins of the hypopharyngeal glands and of the larval food of *Melipona quadrifasciata anthidioides* Lep. (Hymenoptera, Meliponinae). Insectes Sociaux 43: 183-188.

Simões D, Bego LR (1991) Division of labor, average life span and life table in *Nannotrigona* (*Scaptotrigona*) *postica* Latreille (Hymenoptera, Apidae, Melipininae). Naturalia, São Paulo 16: 81-97.

Simons C, Frati F, Beckenbach A, Crespi B, Liu H, Floors P (1994) Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Annals of the Entomological Society of America 87: 651-701.

Simpson J (1961) Nest climate regulation in honey bee colonies. Science 133: 1327-1333.

Slaa EJ, Sánchez Chaves LA, Malagodi-Braga KS, Hofstede FE (2006) Stingless bees in applied pollination: practice and perspectives. Apidologie 37: 293-315.

Slaa EJ, Sánchez Chaves LA, Sandi M, Salazar W (2000) A scientific note on the use of stingless bees for commercial pollination in enclosures. Apidologie 31: 141-142.

Snodgrass RE (1956) Anatomy of the Honey Bee. New York, Cornell University.

Somerville D (2005) Almonds go nuts with bees. Available at http://www.dpi.nsw.gov.au/archive/archive-good-news/almonds-go-nuts-with-bees retrieved 14 April 2011.

Somerville D (2007) Review of supply of honeybees for the pollination of almonds. TimberCorp. Cited in RIRDC 2010.

Sommeijer MJ (1984) Distribution of labour among workers of *Melipona favosa* F. age-polyethism and worker oviposition. Insectes Sociaux 31: 171-184.

Sommeijer MJ, Bruijn LLMD (1984) Social behaviour of stingless bees: "bee-dances" by workers of the royal court and the rhythmicity of brood cell provisioning and oviposition behaviour. Behaviour 89: 299-315.

Sommeijer MJ, de Rooy GA, Punt W, de Bruijn LLM (1983) A comparative study of foraging behavior and pollen resources of various stingless bees (Hym., Meliponinae) and honeybees (Hym., Apinae) in Trinidad, West-Indies. Apidologie 14: 205-224.

Souza B, Roubik D, Barth O, Heard T, Enriquez E, Carvalho C, Villas-Boas J, Marchini L, Locatelli J, Persano-Oddo L, Almeida-Muradian L, Bogdanov S, Vit P (2006) Composition of stingless bee honey: setting quality standards. Interciencia 31 12, 867-875.

Spiewok S, Neumann P (2006) Infestation of commercial bumblebee (*Bombus impatiens*) field colonies by small hive beetles (*Aethina tumida*). Ecological Entomology 31 623-628.

Stein M, Nothnagel T (1995) Some remarks on carrot breeding (*Daucus carota sativus* Hoffm.). Plant Breeding 114: 1-11.

Stephen WP, Osgood CE (1965) The induction of emergence in the leaf-cutter bee *Megachile rotundata*, an important pollinator of alfalfa. Journal of Economic Entomology 58: 284-286.

Storey KB, Storey JM (2001) Hibernation: poikilotherms. Encyclopedia of Life Sciences. Available at http://dx.doi.org/10.1038/npg.els.0003214 retrieved 15 December 2010.

Stubbs CS, Drummond FA (1997) Blueberry and cranberry (*Vaccinium* spp.) pollination: a comparison of managed and native bee foraging behavior. Proceedings, 7th International Pollination Symposium. Acta Horticulturae 437: 341-344.

Sung IH, Yamane S, Honzumi S (2008) Thermal characteristics of nests of the Taiwanese stingless bee *Trigona ventralis hoozana* (Hymenoptera: Apidae). Zoological Studies 47: 417-428.

Sutherland WJ, Ed. (2006) Ecological Census Techniques. 2nd Edition. Cambridge, Cambridge University Press.

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA 4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596 - 1599.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution 28: 2731-2739.

Tanner JE (2001) The influence of clonality on demography: patterns in expected longevity and survivorship. Ecology 82: 1971-1981.

Teixeira L, Waterhouse J, Marques M (2011) Respiratory rhythms in stingless bee workers: circadian and ultradian components throughout adult development. Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology 197: 361-372.

Terada Y, Garofalo CA, Sakagami SF (1975) Age-survival curves for workers of two eusocial bees (*Apis mellifera* and *Plebeia droryana*) in a subtropical climate, with notes on worker polyethism in *P. droryana*. Journal of Apicultural Research 14: 161-170.

Thomson M (2007) Transcript of Evidence, 8 August 2007, p6. to the House of Representatives Standing Committee Inquiry into the Future Development of

the Australian Honeybee Industry. Available at http://www.aph.gov.au/house/committee/pir/honeybee/report/fullreport.pdf retrieved 15 July 2011.

Thorp RW (1979) Structural, behavioral and physiological adaptations of bees (Apoidea) for collecting pollen. Annals of the Missouri Botanical Garden 66: 788-812.

Thorpe RS, Stenson AG (2003) Phylogeny, paraphyly and ecological adaptation of the colour and pattern in the *Anolis roquet* complex on Martinique. Molecular Ecology 12: 117-132.

Torres A, Hoffmann W, Lamprecht I (2007) Thermal investigations of a nest of the stingless bee *Tetragonisca angustula* Illiger in Colombia. Thermochimica Acta 458: 118-123.

Torres A, Hoffmann W, Lamprecht I (2009) Thermal investigations of a nest of the stingless bee *Trigona* (*Frieseomelitta*) *nigra paupera* Provancher in Colombia. Journal of Thermal Analysis and Calorimetry 95: 737-741.

Tóth E, Queller DC, Dollin A, Strassmann JE (2004) Conflict over male parentage in stingless bees. Insectes Sociaux 51: 1-11.

Tucker VA (1970) Energetic cost of locomotion in animals. Comparative Biochemistry and Physiology 34: 841-846.

Undurraga JM, Stephen WP (1980) Effect of temperature on development and survival in post-diapausing leafcutting bee pupae (*Megachile rotundata* (F.)). II. Low temperature. Journal of the Kansas Entomological Society 53: 677-682.

van Benthem FDJ, Imperatriz-Fonseca VL, Velthuis HHW (1995) Biology of the stingless bee *Plebeia remota* (Holmberg): observations and evolutionary implications. Insectes Sociaux 42: 71-87.

van Engelsdorp D, Hayes J, Jr., Underwood RM, Pettis J (2008) A survey of honey bee colony losses in the U.S., fall 2007 to spring 2008. PLoS ONE 3: e4071.

van Veen J, Sommeijer M (2000) Colony reproduction in *Tetragonisca angustula* (Apidae, Meliponini). Insectes Sociaux 47: 70-75.

Velikova M, Bankova V, Tsvetkova I, Kujumgiev A, Marcucci MC (2000) Antibacterial *ent*-kaurene from Brazilian propolis of native stingless bees. Fitoterapia 71: 693-696.

Velthuis HHW, Koedam D, Imperatriz-Fonseca VL (2005) The males of *Melipona* and other stingless bees, and their mothers. Apidologie 36: 169-185.

Velthuis HHW, van Doorn A (2007) A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination. Apidologie 37: 421-451.

Velthuis HHW, Vries de H, Imperatriz-Fonseca VL (2006) The polygyny of *Melipona bicolor*: Scramble competition among queens. Apidologie 37: 222-239.

Venturieri GC (2008) Criacao de abelhas indigenas sem ferrao. Embrapa Amazonia Oriental, Belem, PA.

Vicens N, Bosch J (2000) Pollinating efficacy of *Osmia cornuta* and *Apis mellifera* (Hymenoptera: Megachilidae, Apidae) on 'red delicious' apple. Environmental Entomology 29: 235-240.

Visscher PK, Dukas R (1997) Survivorship of foraging honey bees. Insectes Sociaux 44: 1-5.

Vit P, Medina M, Enriquez M (2004) Quality standards for medicinal uses of Meliponinae honey in Guatemala, Mexico and Venezuela. Bee World 85: 2-5.

Vit P, Soler C, Tomás-Barberán FA (1997) Profiles of phenolic compounds of *Apis mellifera* and *Melipona* spp. honeys from Venezuela. Zeitschrift für Lebensmitteluntersuchung und -Forschung A 204: 43-47.

Vit P, Tomás-Barberán FA (1998) Flavonoids in Meliponinae honeys from Venezuela related to their botanical, geographical and entomological origin to assess their putative anticataract activity. Zeitschrift fur Lebensmittel - Untersuchung und -Forschung 206: 288-293.

Vithanage V, Ironside DA (1986) The insect pollinators of macadamia and their relative importance. Journal of the Australian Institute of Agricultural Science 52: 155-160.

Vogler AP, Monaghan MT (2007) Recent advances in DNA taxonomy. Journal of Zoological Systematics and Evolutionary Research 45: 1-10.

von Frisch K (1971) Bees: Their Vision, Chemical Senses and Language. London, Cornell University.

Wallace H, Trueman S (1995) Dispersal of *Eucalyptus torelliana* seeds by the resin-collecting stingless bee, *Trigona carbonaria*. Oecologia 104: 12-16.

Watts LE (1958) Natural cross-pollination in lettuce, *Lactuca sativa* L. Nature 181: 1084-1084.

Weeks PJD, O'Neill MA, Gaston KJ, Gauld ID (1999) Automating insect identification: exploring the limitations of a prototype system. Journal of Applied Entomology 123: 1-8.

Weston E, Thompson D, Scott B (1980) Current land use in the poplar box (*Eucalyptus populnea*) lands. The Rangeland Journal 2: 31-40.

White D, Cribb BW, Heard T (2001) Flower constancy of the stingless bee *Trigona carbonaria* Smith (Hymenoptera: Apidae: Meliponini). Australian Journal of Entomology 40: 61-64.

Wigglesworth VB (1956) Insect Physiology. New York, John Wiley & Sons Inc.

Wille A (1979) Phylogeny and relationships among the genera and subgenera of the stingless bees (Meliponinae) of the world. Revista de Biologia Tropical 27: 241-277.

Wille A (1983) Biology of the stingless bees. Annual Review of Entomology 28: 41-64.

Wille A, Michener CD (1973) The nest architecture of stingless bees with special reference to those of Costa Rica. Revista de Biología Tropical 21: 1-278.

Williams IH (2003) The convention on biological diversity adopts the international pollinator initiative. Bee World 84: 27-31.

Williams R (2011) Small hive beetles - threat to honey and agriculture. Australian Broadcasting Corporation. Available at http://www.abc.net.au/rn/scienceshow/stories/2011/3161667.htm retrieved 18 March 2011.

Willmer PG, Unwin DM (1981) Field analyses of insect heat budgets: reflectance, size and heating rates. Oecologia 50: 250-255.

Wilson EO (1971) The Insect Societies. Cambridge, Harvard University Press.

Winston ML (1991) The Biology of the Honey Bee. Cambridge, Harvard University Press.

Winston ML, Michener CD (1977) Dual origin of highly social behavior among bees. Proceedings of the National Academy of Sciences of the United States of America 74: 1135-1137.

Wittmann D (1985) Aerial defense of the nest by workers of the stingless bee *Trigona* (*Tetragonisca) angustula* (Latreille) (Hymenoptera: Apidae). Behavioral Ecology and Sociobiology 16: 111-114.

Wittmann D, Bego LR, Zucchi R, Sakagami SF (1991) Oviposition behavior and related aspects of the stingless bees XIV. *Plebeia* (*Mourella*) *caerulea*, with comparative notes on the evolution of the oviposition patterns (Apidae, Meliponinae). Japanese Journal of Entomology 59: 793-809.

Wittmann D, Radtke R, Zeil J, Lübke G, Francke W (1990) Robber bees (*Lestrimelitta limao*) and their host: chemical and visual cues in nest defense by *Trigona* (*Tetragonisca*) *angustula* (Apidae: Meliponinae). Journal of Chemical Ecology 16: 631-641.

Wormington KR, Lamb D, McCallum HI, Moloney DJ (2003) The characteristics of six species of living hollow-bearing trees and their importance for arboreal

marsupials in the dry sclerophyll forests of southeast Queensland, Australia. Forest Ecology and Management 182: 75-92.

Woyke J (1960) Natural and artificial insemination of queen honeybees (Translated at http://jerzy_woyke.users.sggw.pl/1962_nat_artins.pdf) Pszczel. Zes. Nauk. (Polish Journal of Natural Sciences) 4: 183-275.

Yamane S, Heard T, Sakagami SF (1995) Oviposition behavior of the stingless bees (Apidae, Meliponinae) XVI. *Trigona* (*Tetragonula*) *carbonaria* endemic to Australia, with a highly integrated oviposition process. Japanese Journal of Entomology 63: 275-296.

Ydenberg RC, Welham CVJ, Schmid-Hempel R, Schmid-Hempel P, Beauchamp G (1994) Time and energy constraints and the relationships between currencies in foraging theory. Behavioral Ecology 5: 28-34.

Zucchi R, Sakagami SF (1972) Capacidade termoreguladora em *Trigona spinipes* e em algumas outras espécies de abelhas sem ferrão. *In*: Zucchi R (ed) Homenagem à Warwick E. Kerr. 301-309. UNESP, Rio Claro.

Appendices

Appendix 1: Summary and of the responses to the 1998 and 2010 surveys

Table A-1 Summary and of the responses to the 1998 and 2010 surveys.

Activity	**1998**	**2010**
No of bee keepers (n)	257	637
No of nests (n)	1429	4935
Percentage bee keepers with only one hive	50%	54%
Most popular bee species kept	*T. carbonaria* (69%) *T. hockingsi* (20%)	*T. carbonaria* (61.5%) *A. australis* (23%)
More detailed analysis based on 566 (89%) of respondents		
Activity	1998	2010
Colonies kept in artificial hives	82%	92%
Nest remaining in original cavity and location	14%	8%
Bee keepers with > 20 hives	3.5%	5.5%
Nest locations Suburban Remote bush Rural	 56% 24% 20%	 69% 16% 15%
Reasons for keeping stingless bees Enjoyment Conservation Pollinate bushland Pollinate crops Honey production Hives sales Education Research Other hive products (resin, wax) Professional crops pollination services	(%) 81 68 27 24 8 5 5 2 2 ---	(%) 78 67 29 24 11 3 12 4 2 1
Bee keepers with < 1 yr experience	16%	26%
NSW bee keepers Qld bee keepers	29% 71%	38% 62%
Nests managed in NSW Nests managed in Qld	9% 91%	16% 84%
Honey production		
Bee keepers producing honey (n)	26	63
Hives used in honey production (n)	542	1725
Honey produced (kg/yr)	90	254
Hive production		

Bee keepers producing hives	119 (46%)	238 (42%)
Nest transfer (n)	58	99
Hive splits (n)	17	139
Both transfer + split (n)	44	57
Number of nests produced		
Nests transferred (n)	1906	5095
Hives split (n)	884	6328
Total hive produced (n)	2790	11421
Total No of hives produced since 1998	--	8631

Appendix 2: The use of hive weights as a management tool to assess stingless bee colony health

All of the *Au. australis* colonies were checked for queen-right status and the hives were weighed before and after each of the pollination trials (see Chapter 3). Hive weight is commonly used to assess the health and strength of honey bee colonies. This is an effective form of evaluating colony growth, as the plastic hive box makes up only 34% of the total hive weight (MH personal observation). Hive weight is also used in the management of stingless bees for honey production and has been recommended as a means of assessing brood volume and colony health. It is assumed that hive weight fluctuations are a result of colony changes; however, using the weight of a thick, porous, wooden box, which houses a small stingless bee colony, as a management tool is questionable. The weight of a healthy *Au. australis* or *T. carbonaria* hive (OATH) is around 7 – 8 kg with up to 84% of this weight being the box, depending on the hive design (MH personal observation). The quality and craftsmanship of OATH boxes vary between producers and some materials are more vulnerable to the effects of weathering than others. The woods' moisture content can affect its weight and, therefore, the hive weight. Fluctuations in the moisture content of the wooden hive may mask any fluctuations occurring within colony, thus giving false information pertaining to health status of the colony. The following study was set up to assess the weight fluctuations of empty OATH hives that were exposed to weathering.

Six empty OATH boxes, which had been stored within the air conditioned CT rooms (dry environment) (see Chapter 3) for three months, were sealed and weighed. The boxes were placed on a table outside the apiary and each box was placed on a paving brick to ensure it did not sit in pooled water. The boxes were exposed to the effects of the weather for five weeks. They were weighed after one and five weeks.

The group of empty OATH boxes (n = 6) showed a slight weight loss in the first week of exposure to natural weathering conditions, with a mean loss of 6 g. All boxes increased in weight by Week 5, ranging between 109 and 142 g, with a mean increase of 130 g.

Appendix 3: Drone populations and possible maturity

From 22 October – 31 December 2010, during the attempted division of labour study (see Appendix 7), Hive 1 produced 296 drones and Hive 2 produced none. Drones in Hive 1 accounted for 11 – 45% of the emerging callows during this time (Figure A-3a).

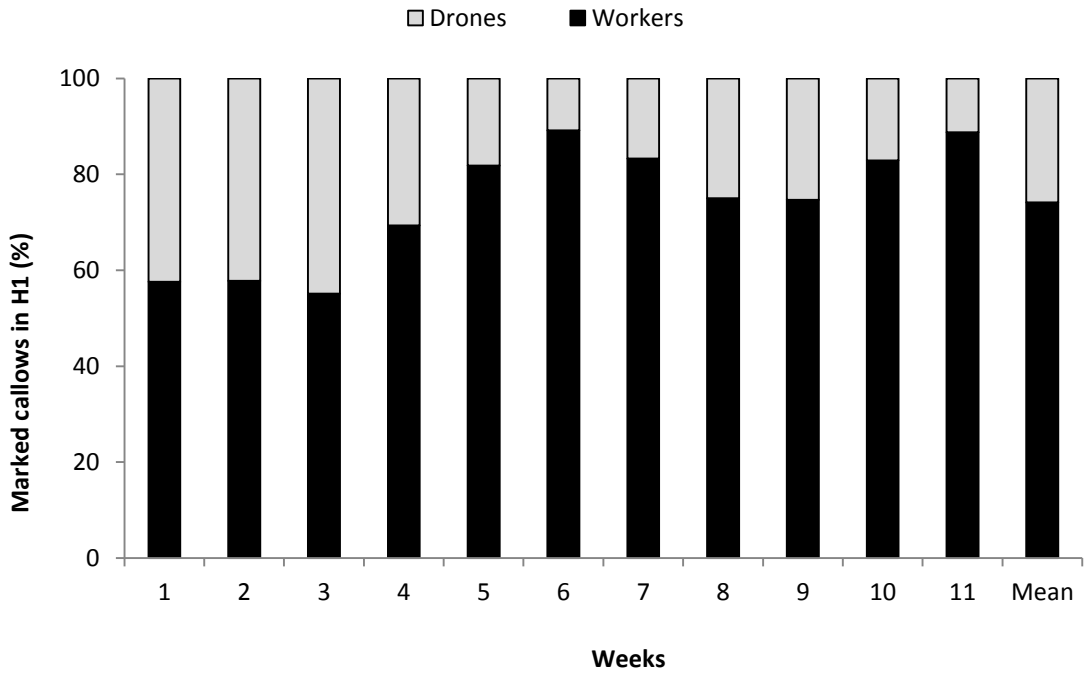


Figure A-3a Proportion of drones to workers emerging in Hive 1 during the division of labour study.

Au. australis colonies appear to produce drones in 'batches' or 'male-producing periods' (MPP), with drones being present only periodically in a single colony. These data are supported by the occurrence of large numbers of drones which were collected during the paralleling study (Chapter 6). From 21 – 25 April 2009, 203 dead and live drones were collected outside the *Au. australis* hive, which was housed in the greenhouse chamber. Drones were easily observed congregating in the OP (Figure A-3b), after leaving the hive box. Most of these drones left the OP entrance without conflict (Figure A-3c); however, some appeared to be being forced out by workers. This was similar to drone expulsion during the pollination trial at Musk (Chapter 3). Monthly observations of colonies within the 'colony dynamics' section of Chapter 6 showed the presence of drones within different colonies, but at varying times of the year. The production of drones in 'batches' supports the hypothesis that

drones of *Au. australis* are present within a population throughout most of the year, but not in individual nests.

Figure A-3b Drones congregating within the OP. Drones are fairly easily distinguished by their longer antennae and cream-marked legs.

Figure A-3c Drone at hive entrance, just prior to leaving the nest.

The fact that stingless bee drones leave the nest and do not return would indicate that sexual maturity should correlate with the time at which they leave the nest. Marked *Au. australis* drones were observed in the hive from 0 to 5 weeks old; however, the most common age for them to disappear from the hive was ~ 3 weeks old. Older drones were only sighted in the hive when there were extended periods of inclement weather.

A congregation of *Au. australis* drones was observed on one occasion, as a result of bringing in multiple hives from Qld. On returning from Tara in October 2009, several of the newly boxed colonies had been recorded as containing queen cells. The drone congregation was first noted at 14:00 on 18 November 2009, when ambient temperature was approximately 28ºC. The swarm consisted of only one to two hundred bees, was loose in configuration and there was no conflict observed between individuals. At times, individual males would settle on structures near the hives and digital images were captured to confirm the caste (Figure A-3d). The swarm dispersed as darkness approached; however, no aggregations of drones could be found that night. The next day the swarm returned but no matings were observed. By the end of January 2010, all of the new colonies from Tara contained mated queens.

Figure A-3d Single drone, easily distinguished by his cream-coloured markings, resting on a structure outside newly relocated *Au. australis* colonies.

Worker laid drones

A small number of workers laid drones were observed in the longevity trial replicates (Chapter 5), illustrating that *Au. australis* will produce offspring in a queenless colony. When setting up the division of labour study, two colonies had their entire brood cluster and queen removed from the hive. The brood, queen and hundreds of workers were transferred to observation hives. A large number of workers remained behind in the orphaned, broodless colonies.

On 18 August 2010, the two colonies of *Au. australis* became orphaned and broodless. Colonies were maintained in the bee shed (Chapter 3) and had access to external foraging. The large number of mature workers within these colonies continued to collect floral resources and on 7 January 2011 the colonies were examined. One colony remained broodless but still contained a small number of workers (> 100). The other colony contained a larger number of workers (> 300) as well as brood cluster of 50 mm diam. The colony was sampled, with > 50 workers, > 50 adult drones and the entire brood cluster being removed for further studies (M. Holmes, Sydney University). The workers within these colonies were at least 126 days old.

Materials and methods

Sperm harvesting and counts

As mentioned above, there were occasions when batches of drones were observed leaving hives. This occurred during the first attempts at the paralleling study, 21 – 25 April 2009 (Chapter 6). Live drones were collected from the greenhouse chamber walls with an aspirator and transferred to a 'holding container'. This was furnished with some cerumen, as a perching substrate, and a honey feeder. This unusual situation provided an opportunity to carry out some preliminary studies to assess the sperm harvesting, staining and counting techniques in *Au. australis.* It was found that chilling the drones could cause spontaneous eversion, so drones used in this experiment were maintained at > 20ºC and were not chilled at any time.

Live drones were manually everted by putting downward pressure on the lower abdomen. Drones were immediately killed after eversion. The penis was placed in a

1.5ml Eppendorf tube with 4 µL of Tris buffer, at room temperature. The penis was squashed and rolled with the end of a blunt probe, rupturing the membranes of the seminal vesicles. The mixture was gently stirred with the probe and then aspirated into a 0.05 – 0.25 mL Exmire microsyringe (ITO corporation, Fuji, Japan). With the assistance of a Wild M5 dissecting microscope, the content of the syringe was placed on a microscope slide along with 1 µL each of SYBR® 14 and propidium iodide dye. The mix was gently combined and then covered with a cover slip. The slide was immediately examined under an Olympus BX60 compound light microscope at 200 X magnification, using blue light. Images of sperm were captured using a Jenoptik ProgRes® C14 digital camera.

As this was only a preliminary study and drones were in short supply, only ten drones were used. Ten fields of view were photographed for each slide. Images were viewed in Microsoft PowerPoint software (Microsoft Corporation, www.microsoft.com) live and dead sperm were manually counted and the percentage of live sperm estimated.

Results and discussion

The mean percentage of live sperm counted within the surveyed fields of view for each of the ten drones, along with the overall mean (> 60%) are shown in Figure A-3e. During examination, dissection and staining, under stereomicroscopy, there was no sign of sperm motility in any of the samples.

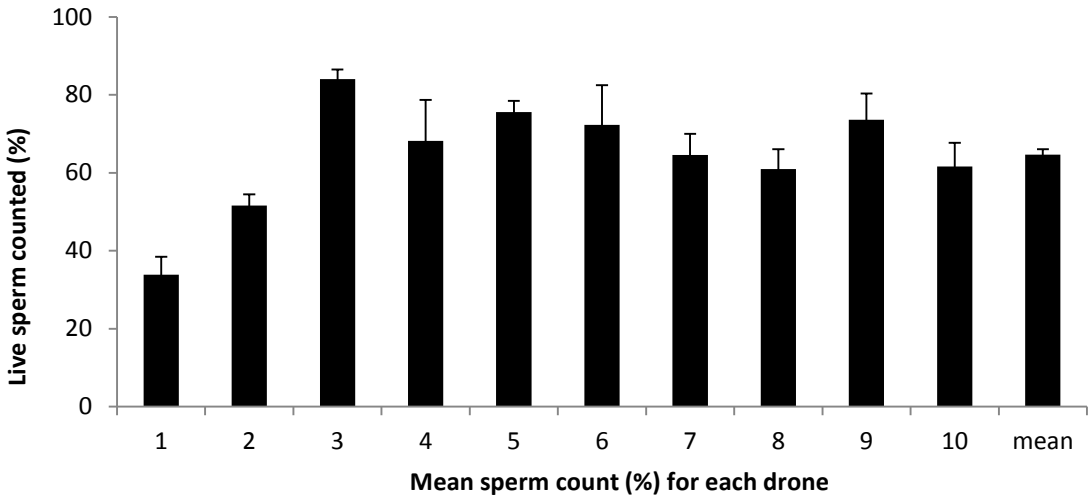


Figure A-3e Mean sperm counts for each drone as well as the overall mean of the samples. Means and their standard errors are shown.

Some slides showed a large amount of damaged sperm, and this was probably due to operator inexperience. Rather than squashing the seminal vesicles, it may be less damaging to puncture or cut the membranes in order to expel the semen. Large areas of massed sperm were also observed on some slides and this may be improved by increasing the dilution of the buffer fluid plus briefly vortexing the tube. The morphology of the sperm cells was quite different to that of honey bee sperm. The heads of the *Au. australis* were quite elongated compared to the honey bee (Figure A-3f).

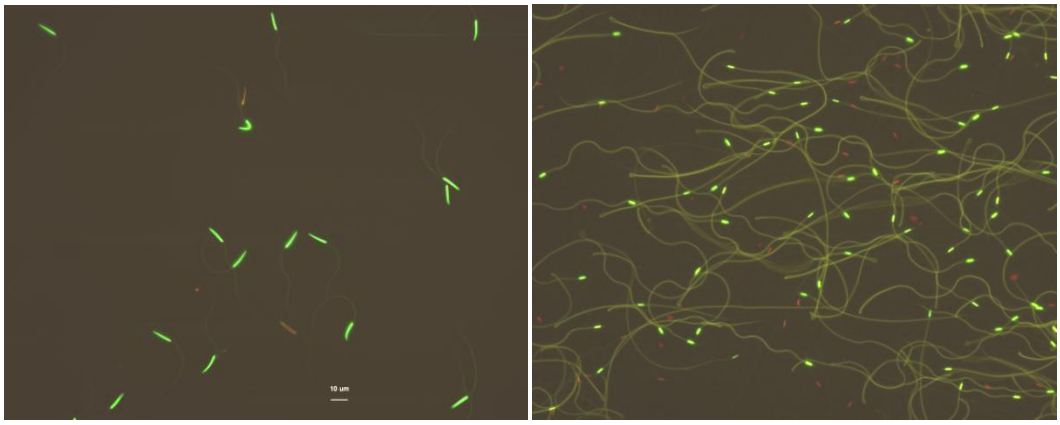


Figure A-3f *Au. australis* sperm (left) compared to *A. mellifera* sperm (right). Green heads are alive and red heads are dead. (*A. mellifera* sperm photo by J. Rhodes).

Au. australis drones, were able to feed themselves (Figure A-3g) and could be maintained in 'holding containers' for up to four weeks. Unlike honey bee drones, it was not necessary to include a cohort of workers to feed them. Lack of sperm motility may have been caused by inappropriate storage of the donor drones. 'Holding containers' were provisioned with a perching substrate and honey feeders; however, they were not provided with pollen. It is not known how *Au. australis* drones obtain their protein source, but those dissected for the SEM imaging (Chapter 4) were often found to have a large number of pollen grains in their abdominal contents. Most of these bees had been collected directly from opened hives (A. Dollin, pers. comm., 2010) and may not have reached reproductive maturity.

Figure A-3g Drone feeding himself while in captivity.

Within the observation hive, occasional antennation between workers and drones was observed, followed by mild aggression towards the drone, which quickly moved away. At no stage during this study were drones observed performing in-hive tasks, thus indicating that *Au. australis* drones, like *A. mellifera* and most other species of stingless bee, may play no in-hive role.

Appendix 4: Austroplebeia nest codes, their locations and the associated holotype for that location

Table A-4 Austroplebeia nest codes, their locations and the associated holotype for that location.

Nest Code	Nest Location	Holotype associated with this location
symeiC36	Duaringa, QLD	*Au. cassiae*
symeiC42	Duaringa, QLD	*Au. cassiae*
symeiC43	Duaringa, QLD	*Au. cassiae*
symeiC58	Duaringa, QLD	*Au. cassiae*
symeiN23b	Duaringa, QLD	*Au. cassiae*
symeiRock	Rockhampton, QLD	*Au. cassiae*
symeiQ32	Mt Surprise, QLD	*Au. symei*
symeiF6	Bamaga, QLD	*Au. ornata*
symeiN1	Katherine, NT	
symeiN2	Katherine, NT	
symeiN12b	Cobourg Peninsula, NT	
intermediateS2	Kilcoy, QLD	
intermediateS3	Kilcoy, QLD	
intermediateS5	Kilcoy, QLD	
intermediateS9	Kilcoy, QLD	
intermediateS31	Kilcoy, QLD	
australisC35	Duaringa, QLD	
australisC39	Duaringa, QLD	
australisC56	Duaringa, QLD	
australisC70	Duaringa, QLD	
australisNSW15	Kempsey, NSW	
australisTara	Tara, QLD	
australisF31	Bamaga, QLD	*Au. ornata*
stripedC47	Ti Tree, NT	*Au. percincta* (300 km Nth)
stripedC49	Ti Tree, NT	*Au. percincta*
stripedW13	Timber Creek, NT	
stripedF53	Hughenden, QLD	
stripedM22	Kowanyama, QLD	
stripedN21	Croydon, QLD	
stripedG1	Croydon, QLD	
curvedW5	Wyndham, WA	*Au. websteri*
curvedW8	Kununurra, WA	*Au. websteri*

curvedN11	Cobourg Peninsula, NT	
curvedN15	Cobourg Peninsula, NT	
cinctaD5	Daintree, QLD	
cinctaD6	Daintree, QLD	

Appendix 5: Full list of the sampled nests (n = 72) used for colour, HW analysis

Table A-5 Full list of the sampled nests (n = 72) used for colour, HW analysis.

Nest #	Location	Av colour %	Av hw mm	Group
M18	QU koolatah stn	0.7	1.72	1
M19	QU koolatah stn	2.0	1.74	1
Q22	QU laura	2.3	1.77	1
C31	QU duaringa	3.4	1.77	1
C58	QU duaringa	4.2	1.77	1
M21	QU koolatah stn	3.7	1.78	1
G09	QU normanton	0.3	1.79	1
C42	QU duaringa	0.2	1.82	1
G07	QU normanton	0.9	1.82	1
F47	QU coen	2.5	1.82	1
N01	NT katherine	3.6	1.82	1
F43	QU jardine river	1.3	1.83	1
N02	NT katherine	3.5	1.83	1
F32	QU cape york	1.3	1.84	1
C37	QU duaringa	1.5	1.84	1
N08	NT cobourg pen	1.7	1.84	1
F44	QU jardine river	2.0	1.84	1
N23	QU duaringa	3.1	1.84	1
F06	QU bamaga	3.2	1.84	1
C27	QU rockhampton	4.2	1.84	1
C17	QU rockhampton	2.6	1.86	1
C43	QU duaringa	2.9	1.86	1
C36	QU duaringa	3.9	1.86	1
N12	NT cobourg pen	3.2	1.87	1
F03	QU cape york	0.0	1.88	1
C14	QU rockhampton	3.2	1.90	1
Q32	QU mt surprise	3.6	1.98	1
NSW15	NSW kempsey	8.8	1.77	2
F31	QU cape york	9.7	1.77	2
C45	QU willows	10.1	1.77	2
C67	QU duaringa	7.7	1.78	2
C70	QU duaringa	7.9	1.78	2
C33	QU duaringa	8.2	1.78	2
C56	QU duaringa	8.5	1.79	2
NSW54	NSW kempsey	8.0	1.80	2

NSW23	NSW kempsey	8.3	1.80	2
Q35	QU eulo	9.7	1.80	2
C75	QU princhester	9.8	1.80	2
C39	QU duaringa	8.5	1.81	2
C35	QU duaringa	8.8	1.81	2
C32	QU duaringa	8.3	1.82	2
NSW67	NSW kempsey	9.6	1.82	2
M17	QU koolatah stn	14.1	1.67	3
G01	QU croyden	16.7	1.67	3
F52	QU hughenden	16.0	1.68	3
N21	QU croyden	16.7	1.68	3
W13	NT auvergne stn	14.0	1.70	3
M22	QU koolatah stn	13.1	1.71	3
G08	QU normanton	16.3	1.71	3
W17	NT auvergne stn	15.7	1.72	3
C48	NT aileron stn	16.0	1.72	3
F53	QU hughenden	16.0	1.73	3
C49	NT aileron stn	15.8	1.76	3
N03	NT katherine	12.6	1.79	3
C47	NT aileron stn	15.5	1.79	3
W09	WA middle springs	19.8	1.52	4
W01	WA windjana	16.6	1.55	4
W08	WA middle springs	19.2	1.55	4
W06	WA home valley	19.2	1.56	4
W11	WA middle springs	20.2	1.56	4
W12	NT auvergne stn	18.9	1.57	4
N15	NT cobourg pen	15.3	1.58	4
W05	WA home valley	20.2	1.58	4
N11	NT cobourg pen	11.8	1.61	4
S02	QU kilcoy	7.4	1.81	5
S06	QU kilcoy	5.8	1.85	5
S09	QU kilcoy	7.3	1.88	5
S23	QU winton	4.8	1.75	5
S31	QU blackbutt	4.2	1.73	5
S34	QU kilcoy	4.0	1.82	5
S03	QU kilcoy	9.0	1.88	5
S05	QU kilcoy	8.5	1.88	5
Groups: 1 – symei, 2 – australis, 3 – striped, 4 – curved, 5 - intermediate				

Appendix 6: Improved marking techniques for *Au. australis*

Callows were aspirated into a cushioned (a piece of foam placed in the bottom of the jar) pooter, to reduce injury to the bees. Marking was carried out in batches of six to ten bees at a time. Collected bees were shaken onto a pre-chilled, filter paper-lined Petri dish (90 mm). The chilling procedure was more effective when the Petri dish was placed in a shallow ice slurry rather than on an ice block. The bees chilled more quickly than previous methods (Chapter 5); they were chilled only to the point of cessation of movement and then the dish was removed from the ice. Marking in small batches helped to reduce individual's chilling time, thus keeping injury to a minimum. Two colours were placed on the thorax, the marked bees were warmed under a halogen light and then returned to the hive. Mortality of callows did not appear to be as high as the mortality rate in the longevity studies (Chapter 5).

Appendix 7: Age related worker behaviour

The extended longevity of *Au. australis* (Chapter 5) led to the question of colony plasticity and whether the division of labour within *Au. australis* colonies would be substantially different from that described in other stingless bees, as well as honey bees. It was hypothesised that there would be a delay in the movement from one in-hive task to the next, and from in-hive to foraging duties.

The following experiment was set up to study the division of labour, in relation to worker age, within two *Au. australis* colonies. Several factors contributed to the failure of this study; however, some interesting information was obtained from this attempt and the results could form the basis for further research.

Two observation hives (300 x 300 x 60 mm cypress pine with Perspex 3mm lid) were housed in the bee shed and given access to external foraging. On 25 August 2010 a brood cluster (~ 30 mm diam.), together with an unknown number of workers and a marked physogastric queen, was dissected from an established colony and placed in each observation hive. Each hive was provided with feeder-float and potted pollen ($4cm^2$) (see Chapter 3).

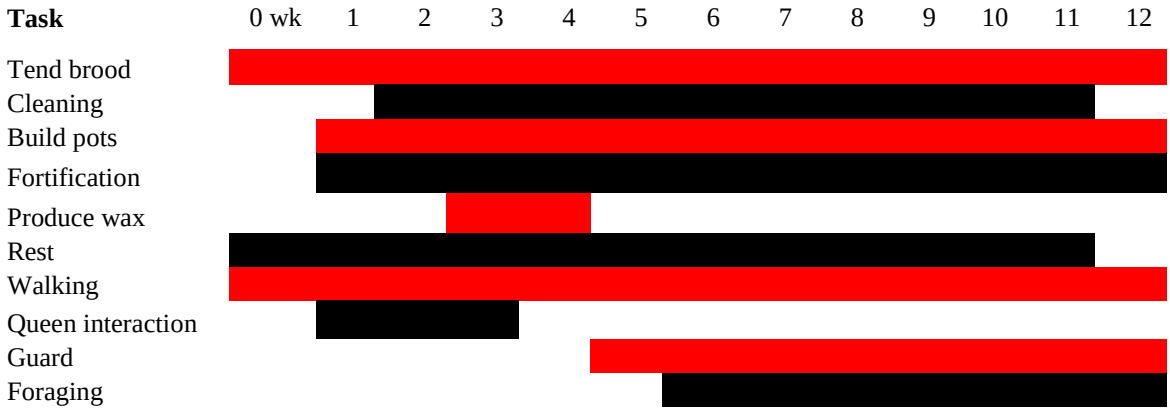
Prior to colony transfer, Perspex partitions had been installed in the centre and along the entrance corridor of each hive, based on Sakagami's design (1966). The initial planned method was to introduce a large number of pupating cells into one side of the hive, the one without the queen and brood cluster. As callows emerged, they would be marked and transferred to the 'colony' section, similar to that described by Biesmeijer and Tóth (1998). Attempts were made to accomplish this; however, the supply of pupating brood was low in all of the other colonies housed in the bee shed. As a result, the partitions were removed. Further investigations into stimulation of brood production resulted from this mishap (see Appendix 12).

Results

Throughout this study there was some variation on worker size but no soldier castes (Grüter et al. 2012) were observed within either of the colonies. Although *Au. australis* workers from one to 12 weeks old were observed moving over or tending the brood cells the most common age for nursery work was from 0 – 3 weeks old. Workers between one

and 12 weeks were observed carrying out most in-hive tasks. The most common age at which workers were no longer observed in the hive was from six to eight weeks old, indicating this may be the most likely age that workers moved from in-hive tasks to foraging. The table below (Table A-7) shows some of the tasks and ages at which *Au. australis* workers were observed. This is by no means a complete picture of the division of labour behaviour in this species; however, it does indicate that there is a large degree of plasticity within the colonies, especially compared to some other bee species.

Table A-7 In-hive tasks performed by the different age groups of workers within the two *Au. australis* colonies used in the 'division of labour' study.



Extreme colony plasticity was also demonstrated in the 'poorly performing' colonies (Appendix 10), where workers within these colonies were approximately six months old. The same was observed in the orphaned colonies which produced drone brood (Appendix 4). There was, of course, no queen interaction, but these orphaned colonies collected excess nectar and pollen as well as constructing cells and rearing brood. They were able to perform all of the above tasks, with the possible exception of wax production. Whilst observing the construction of brood cells, no workers were seen with wax scales protruding from the intersegmental space on the dorsal surface of the abdomen. It is unclear as to whether these workers did produce unobserved wax scales or simply recycled cerumen for cell construction. These 'poor' colonies did not grow in overall size and surplus cerumen would have been readily available.

Appendix 8: Failed queen or brood disease?

During the four years of this PhD project, there were six instances where brood cells became abnormal in seemingly healthy, queen-right colonies. It was first brought to my attention by Dr Anne Dollin, who owned a hive that she thought had suffered from brood chill. At the time, it was thought that *Au. australis* could not tolerate low temperatures and that the brood would die when exposed to temperatures below 5ºC (R. Zabel, pers. comm., 2007). The brood cells looked similar to colonies that had suffered 'brood chill', (seen in colonies which are inappropriately prepared for cold conditions) but the cell operculum did not appear sunken as in 'chilled brood' (Figures A-8a & A-8b). The brood cells were undeveloped and desiccated. Anne gave me this hive to keep in the bee shed, in an effort to bring it back to health. It was maintained at 22 – 26ºC for nine months and was also provided with supplementary feeding over the winter. The queen was infrequently observed ovipositing, she was seen off the brood often and the brood cluster did not expand, remaining at ~ 40 mm diam. Food stores increased while the colony was kept in the bee shed but the worker numbers dwindled. Additional damaged cells were observed within the brood cluster, similar to the 'chilled' brood. The colony was eventually euthanized and the brood cluster examined. Brood cells were examined under a stereomicroscope (Figure A-8c) and no fungal spores were found. The cell contents were plated onto agar medium and incubated at 25ºC for one week. Saprophytic fungi were cultured but no potentially pathogenic organisms were noted.

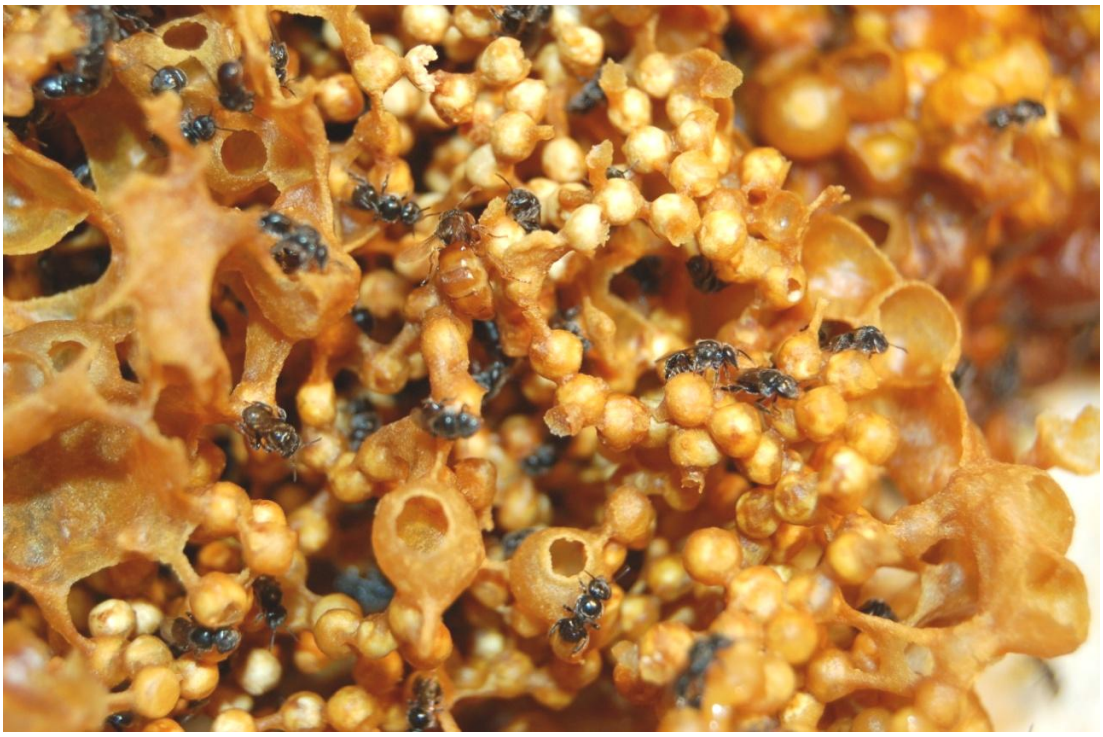


Figure A-8a Damaged brood cells due to chill injury caused by the failure of the heating unit. Note physogastric queen in centre of image. Also note the cells are simply shriveled and the operculum has not sunken in most of the cells.

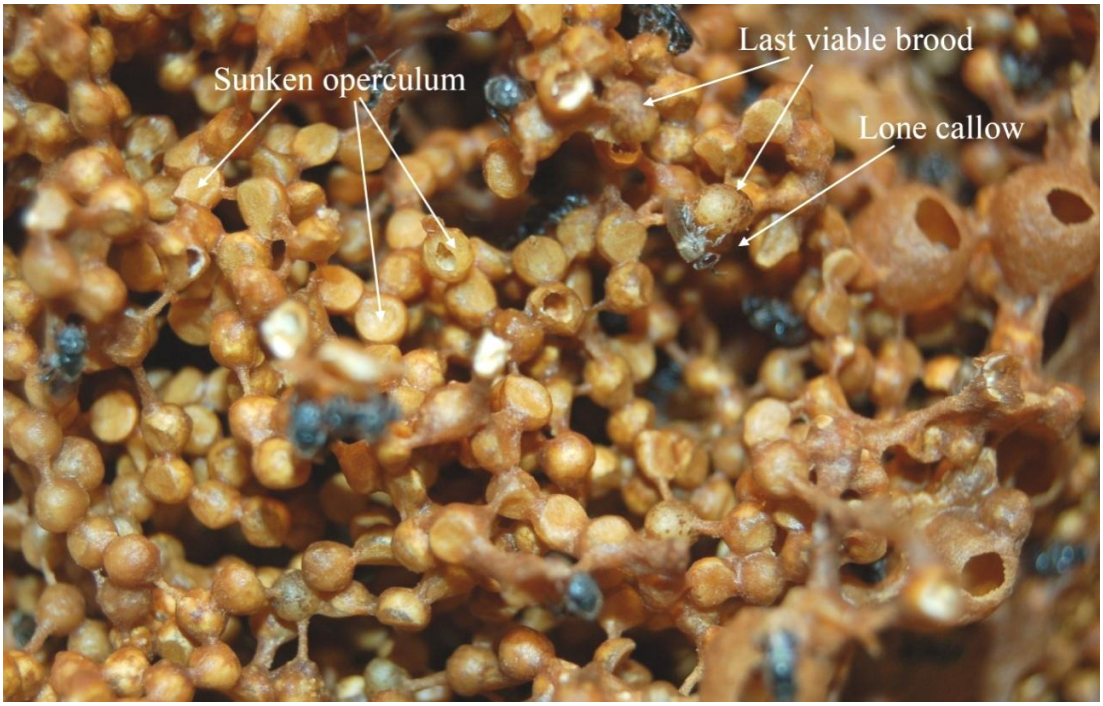


Figure A-8b Damaged brood from possible queen failure. Note the sunken operculum of most cells.

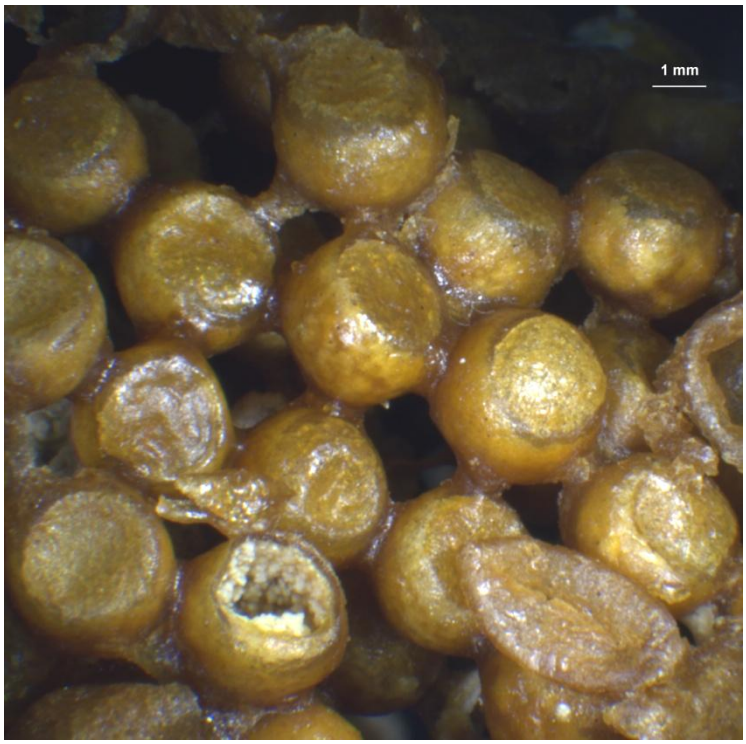


Figure A-8c Desiccated brood cells from *Au. australis* colony.

Occasionally, this symptom of brood death became evident in other colonies, but the occurrence was seldom and intermittent. I became concerned about the possibility of brood disease soon after the overwintering study was set up (Appendix 12). One month after the colonies were placed in the bee shed I noticed three of the colonies had developed brood damage. I was worried that some form of brood disease was being transmitted from one colony to the other as a result of cross contamination during the feeding process. Dr. Michael Hornitzky, a bee pathologist with the NSW Department of Primary Industries, was contacted. I was very fortunate to have the support of Michael's expertise in honey bee brood disease and his laboratory services. Both healthy and damaged brood were sampled and cultured by Michael. The samples tested negative for American foulbrood, European foulbrood and chalkbrood. Microscopic examination of both healthy and damaged brood revealed mixed bacterial flora, but nothing unusual was found in the damaged brood cells. Bacterial and fungal cultures did not yield any significant growth. Michael concluded that it was unlikely that the damaged brood resulted from bacterial or fungal infection.

The 'poorly performing' colonies all contained a physogastric queen (Figure A-8d) which was frequently observed off the brood and seldom observed ovipositing. On

one occasion, a brood cell was observed fully provisioned, but completely unattended by workers or queen (Figure A-8e). On another occasion, the queen was observed resting in front of an open, provisioned cell. She inspected the cell, as described by Drummond et al. (1999), and then rested back again. Instead of constantly vibrating her wings, as described by Drummond et al. (1999), she made small, pulsating wing movements. Antennation was unhurried and random; tapping the edges of the cell or simply moving them up and down. Upon initial observation of this behaviour, the workers appeared excited; antennating the area in front of the queen, wing fanning and moving their heads forward and backward. The process continued for over an hour, with the degree of excitement displayed by the workers reducing to almost motionless resting. The cell was eventually sealed by a worker; however, oviposition by the queen or workers was not observed.

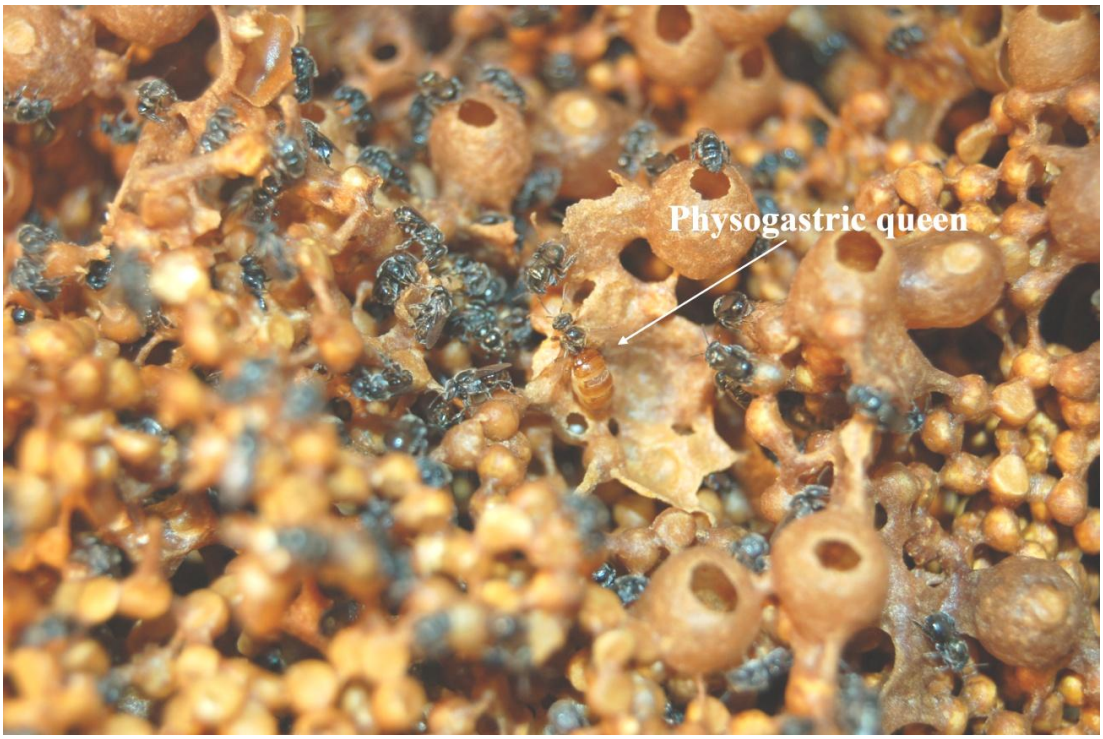


Figure A-8d Physogastric queen present in 'poorly performing' colony.

Figure A-8e Provisioned, unattended brood cell within a 'poorly performing' *Au. symei* colony. Note there is no developing brood visible.

If the queens in these 'poorly performing' *Au. australis* colonies had run out of sperm, there would be a notable increase in drone production. Also, if the queen was failing, it could be expected that workers within the colony would start to develop functional ovaries, as seen in the orphaned colonies, and drone production would increase. This was not the case in any of the colonies. Once desiccated brood cells were discovered, most of the viable brood had already emerged. From this time there was little or no evidence of callow emergence, therefore neither the queen nor the workers were ovipositing viable eggs.

In an effort to combat this poor brood production, a pupating queen cell, harvested from a healthy colony, was introduced into the 'poorly performing' colonies. On all occasions the colonies killed the gyne, leaving the 'poor' queen in residence. These colonies gradually declined in health and were eventually euthanazed.

In hindsight, I should have killed the ‘poor’ queens and introduced pupating queen cells. However, these incidences occurred so sporadically and unexpectedly, I could not bring myself to kill a physogastric queen. With the accumulated information obtained from the six ‘poor’ colonies, over the last four years, treatment by forced supersedure would seem to be appropriate. These incidences may be attributed to the fact that the colonies were being managed outside their native range and that this unusual behaviour is due to unnatural circumstances. An autopsy may have answered some of these questions also. Investigations into the possible presence of a viral disease may be warranted.

Appendix 9: Gyne production, introduction and imprisonment

Queen cells were observed on many occasions within the queen-right *Au. australis* colonies, at various times of the year. Unlike comb-building species, where queen cells are constructed on the edges of the brood comb, the cluster-type brood of *Au. australis* makes it difficult to see queen cells and sightings are random. These cells usually become most obvious as the drones and workers in the adjacent cells begin to emerge. The larger Trigonini queens have a longer ontogenic period (Imperatriz-Fonseca & Zucchi 1995) and remain in view as the brood is dismantled around them (Figure A-9a). As reported by Imperatriz-Fonseca and Zucchi (1995) gynes within the Trigonini tribe are usually larger than the workers, and this is true for *Au. australis.*

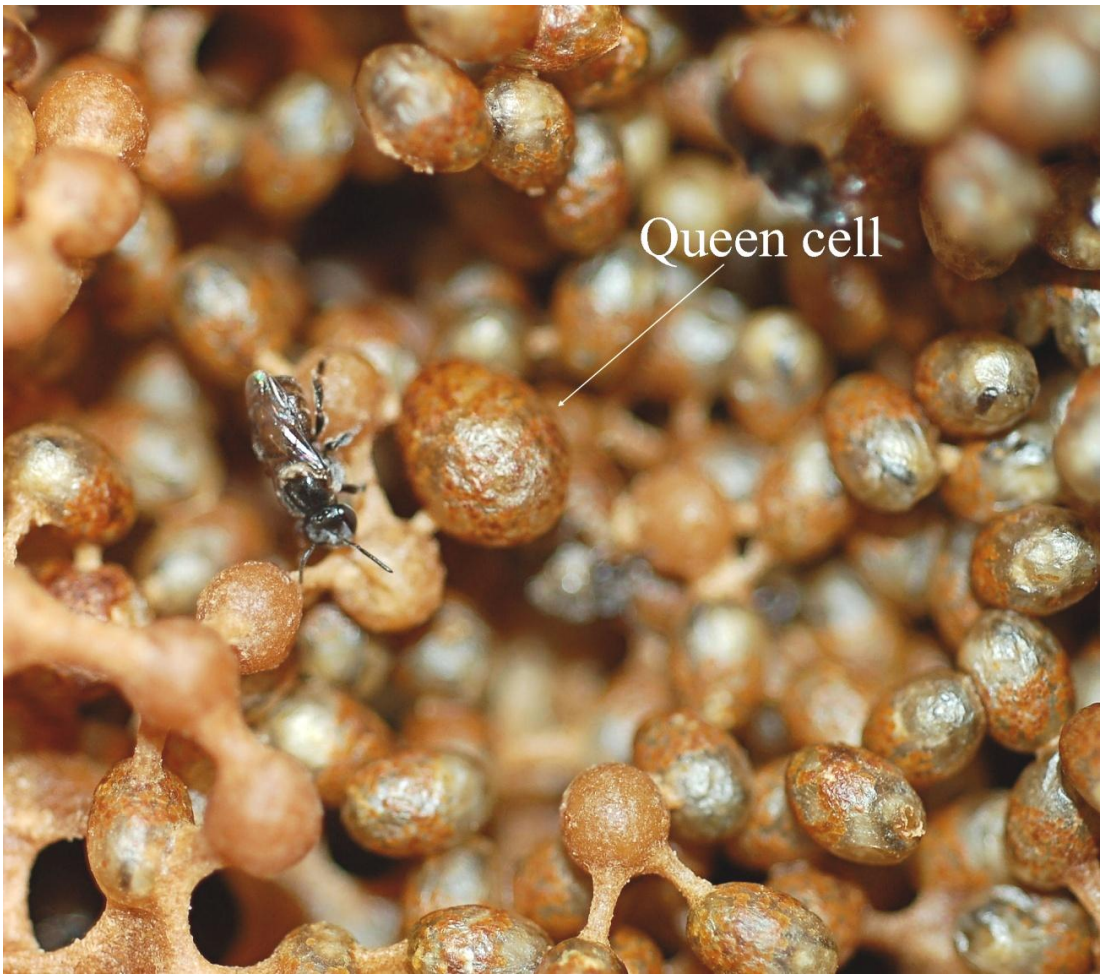


Figure A-9a Pupating queen and worker cells.

Gyne imprisonment within ‘royal chambers’ has been reported in some South American stingless bee species; however, royal chambers are considerably larger than a royal cell (~1 cm diam.) (Figures A-9b).

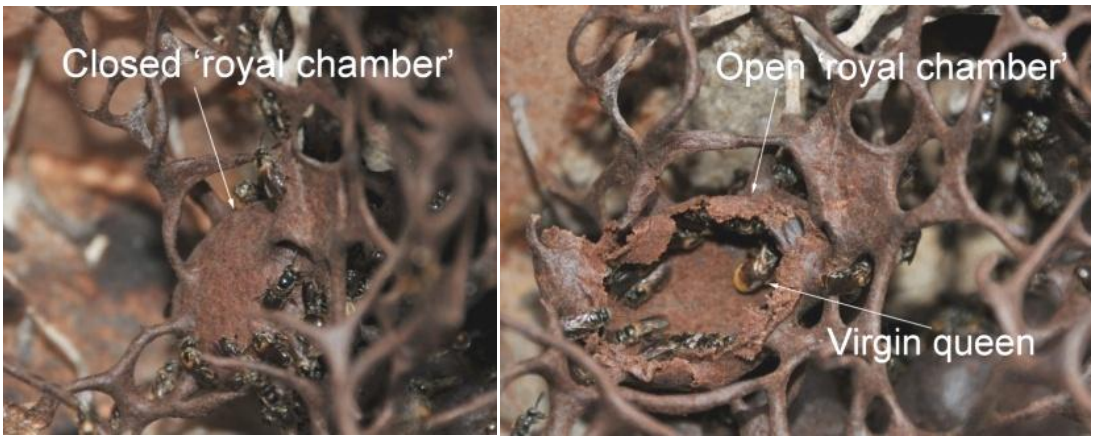


Figure A-9b *Plebeia* remota royal chamber still closed (left) and chamber opened to show virgin queen inside (right).

Gynes observed in queen-right *Au. australis* colonies always disappeared from the hive and it is concluded that they were probably expelled or killed by the workers. Outside their native range, these colonies were never provided with the appropriate cues to stimulate reproductive swarms, therefore no gynes would have left the hives as part of a swarm. One gyne, which was marked and returned to the queen-right hive, remained unharmed within the hive for 12 days before being killed. When a gyne was observed on the peripheries of the nest, the workers appeared to regard her with irritation rather than aggression. Although physogastric queens within my *Au. australis* colonies were not observed to be aggressive toward gynes, it cannot be concluded that they never show aggression towards new comers.

Gyne ‘imprisonment’

On 27 October 2008, a queen-right colony contained three pupating queen cells within close proximity of each other. One of these cells was partially opened and the head of a gyne was protruding from the opening (Figure A-9c). It was not known how long she had been partially emerged; however, she remained *in situ* for a further 45 hours. When the hive was checked the following day, the cell had been dismantled and there was no sign of the gyne.

Figure A-9c Partially emerged gyne. Note the orange colouring of the front legs and antennae.

On 16 February 2009 an ‘imprisoned’ gyne was sighted in another hive. Again, the gyne was only partially emerged, with her head protruding from a small opening in the top of her pupal casing. A camcorder was set up to record the interaction of the gyne and workers. The recording lasted 5.5 hours, the storage time of the recorder. During viewing of the recording, the following interactions were noted;

- The gyne was attended by one to three workers at any one time, including callows and mature workers.
- Interactions included antennation and trophallaxis. At times it appeared that the gyne was being aggressively harassed, but mostly she seemed calm within her cell.
- Workers were observed checking the opening to the royal cell and appeared to frequently secure the edge of the orifice. Occasionally the gyne looked to be close to being able to squeeze out of the cell, with the top half of her thorax visible outside the cell, but immediately a worker would harass her and secure the opening again.
- The gyne was left unattended for a maximum of 45 seconds at a time.
- Occasionally, the gyne would withdraw completely into the cell and, again, a worker would come to check on her.

It is unclear as to whether this behaviour is repeated for every emerging gyne but there have been previous anecdotal accounts of gyne 'imprisonment' (A. Beil, pers. comm., 2009).

Attempts to requeen queenless colonies

Throughout the duration of my studies, I sighted and removed several gynes from various queen-right hives. The supplies were never regular enough to set up and conduct a formal study; however, opportunities occasionally arose to conduct a one-off experiment. During the greenhouse pollination experiments at UWS there were several occasions where a gyne was sighted on the greenhouse chamber floor or inside the queen-right hive. A total of 14 gynes, dead or alive, were collected over a six week period in spring. Over one 15 day period, five live gynes were collected from the hive and used in the following trials.

One gyne was maintained in small 'holding jars', along with a small number (< 10) of workers, for six weeks. Another was captured and maintained alone, for three days, in a jar with a honey-soaked cotton wool feeder, indicating she was capable of self-feeding. On four occasions, I attempted to introduce a live gyne into a strong, well populated queenless colony, with an *A. mellifera* queen-cage being utilised on three of the four occasions. One end of the cage was closed with queen candy, the gyne was placed inside and the other end was closed with a layer of cerumen. A drop of honey was placed on the mesh wall. When the cage was introduced into the colony, trophallaxis and antennation were immediately observed between workers and the gyne (Figure A-9d). After approximately six hours, the workers had removed the cerumen and were observed dragging the gyne out of the cage and down into the colony's structure. She was not sighted again and no further introductions were successful.

Figure A-9d Gyne inside queen cage, introduced into queenless colony.

It was recommended to me that I try a small, nucleus colony, rather than a large one, to increase the chances of gyne acceptance (C. Menezes, pers. comm., 2010), so a small orphaned colony, containing < 300 workers and a small amount of brood was trialled. The gyne was place in a plastic jar which was closed with a piece of tissue paper and an elastic rubber band. The jar was provided with a small honey pot and five workers from the gyne's colony of origin. More than 24 h later, the tissue paper had been chewed away and the gyne was observed in the hive box. Previously described behaviours (Jarau et al. 2010) of aggression by the gyne toward the workers were observed, with frequent incidences of body contact and antennation. This aggressive behaviour of the gyne toward the workers is not seen in honey bees (Winston 1991). The gyne appeared to be accepted by the colony over a few days; however, she disappeared from the colony approximately two weeks after her introduction. It is speculated that she left the colony on her nuptial flight but did not return.

Queenless *Au. australis* colonies were successfully requeened by the introduction of a small section of pupating brood, containing one to three queen cells. There was, however, a high incidence of gynes disappearing from the colonies, presumably as a result of unsuccessful nuptial flights. Successful queening of newly-split or orphaned colonies is one of the most problematic aspects of colony propagation of stingless bees in Australia (see Chapter 2).

Colonies' attempts to rear emergency queens

Colonies that were orphaned through hive manipulation were observed to partake in an unusual behaviour. Within 24 h of queen loss, gathering workers completely covered the surface of the brood cluster, so as to obscure the cells from view (Figure A-9e). This 'gathering' of workers would continue for weeks. In order to observe the behaviour it was necessary to move the workers off the brood. Occasionally, workers could be persuaded to move off the brood by shining a halogen light on the area. This could only be done for short bursts of time, to avoid overheating the brood. The upper-most, newest cells within the cluster had become misshapen, with a domed extension built on the operculum. The cell walls also appeared to become thickened over a period of time. On two of many occasions, recently orphaned colonies became queen-right within two months without my intervention. This behaviour has been anecdotally reported (A. Beil, pers. comm., 2009) and it is speculated that the 'gathering' and operculum extension may be part of an emergency queen rearing process. The behaviour described above was observed many times in colonies that became queenless, for unknown or experimental reasons. The 'gathering' behaviour became an indication that a colony had probably become queenless and this sign could be used when managing *Au. australis* colonies. At times, subsequent queens appeared to be smaller than usual, possibly as a result of receiving less larval provisions.

Figure A-9e *Au. australis* workers gather over the brood cells and blanket it so as to obscure for view their possible manipulation of the cells.

In colonies that were made broodless as well as being orphaned, workers begn to construct brood cells. Some continued with only a very small number (< 5) of mostly empty cells, while others produced large numbers of viable drones. One of the two colonies which was orphaned for the division of labour study (Appendix 9) produced a brood cluster ~ 80 mm in diameter in five months. Adult and pupating drones, as well as adult workers, were collected for further studies (Michael Holland, University of Sydney).

Appendix 10: Brood production and overwintering

The colonies in my care experienced some unseasonably cool, wet weather (even for Richmond and Blaxland), thus reducing their opportunities to freely forage and build up food stores. As a result the colonies' health dramatically declined and management intervention became necessary if the colonies were to survive. One colony, acquired in October 2009, starved due to the depleted honey stores (Figure A-10a). The remaining colonies were moved to the bee shed for overwintering and an additional study was set up to investigate some of the requirements needed for increased brood production.

Figure A-10a Empty honey pots from starved *Au. australis* colony.

In 2006, during my honours project, four *Au. australis* colonies had been maintained in the bee shed over the winter period. The colonies were very weak when I received them and supplementary feeding enabled the colonies to build up their stores and brood, in preparation for experimental studies. In April 2010, colonies designated for the division of labour study (n = 6) were inspected and found to have excellent levels of food stores. These colonies were housed in the bee shed. Unfortunately, by August 2010, it was found that the brood clusters of all of the colonies were ≤ 30 mm diam., with only a small proportion at the pupal stage (~ 10%). This indicated that colonies may require incoming food resources to trigger high levels of brood production. This information would be helpful in planning colony management during the winter

period, in preparation for spring pollination services or for increased forager activity and honey production.

Materials and methods

All of the colonies (n = 18) had suffered from low incoming food resources over the previous spring / summer. Four of the colonies still contained good levels of food stores, collected in previous seasons, and 14 colonies were very low on food stores, requiring supplementary feeding.

On 19 May 2011, colonies were moved from ambient conditions at Blaxland to the bee shed. All hive entrances were closed with a fly wire mesh, thus removing their access to the external environment. Shed temperatures were set at 24°C. Honey feeder-floats and pollen trays (Chapter 3) were utilised for the supplementary feeding regime. The 'external supplement' colonies (n = 7) were fitted with external feeding stations (Chapter 3), furnished with a feeder-float and pollen tray; the 'internal supplement' colonies (n = 7) had feeder-floats and pollen trays placed directly inside the hive box and the 'no supplement' colonies (n = 4) were not supplemented. The hive lids were opened and the diameter of the brood clusters was measured, and the life stages of the brood were recorded. Honey and pollen were replenished twice a week.

On 5 September 2011 (Week 15), the brood clusters were measured and the colonies were relocated to Blaxland. Brood diameter was compared between pre-wintering and post-wintering.

Results and discussion

This study showed that the provision of supplementary food to colonies of *Au. australis* can increase the overall production of brood. There was a significant difference between the two supplement treatments and the 'no supplement' treatment (p = 0.001, $F_{2, 17}$ = 12.551), but no significant difference between the 'internal' and 'external' treatments (p = 0.970) (Figure A-10b).

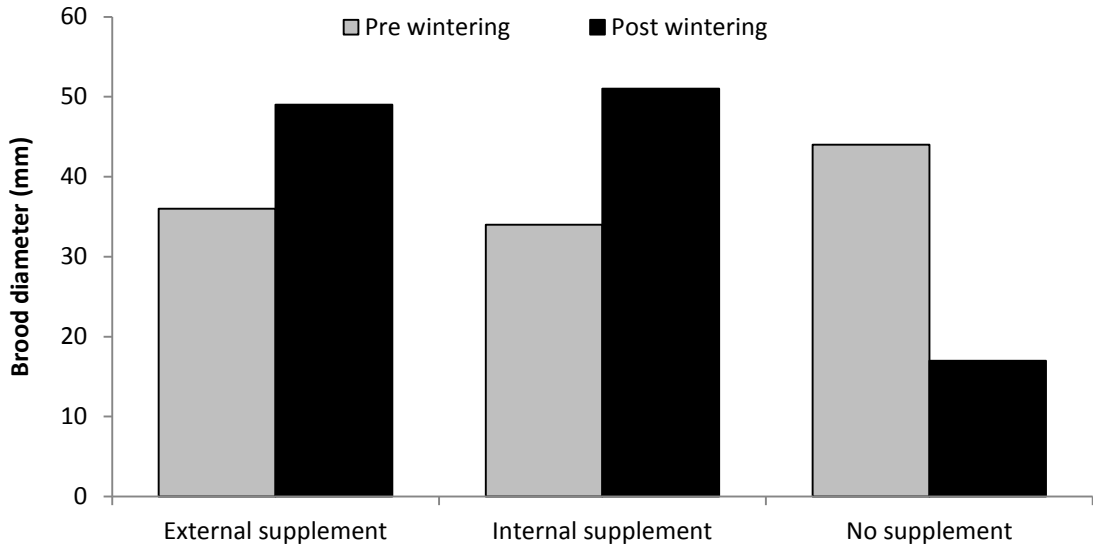


Figure A-10b Mean brood diameter (mm) for each treatment of the feeding regime.

These results indicate that colonies can be successfully overwintered (with heating provided) or supplement fed during adverse environmental conditions (heavy rain or drought) to enhance brood production, thus building forager strength in preparation for pollination services. They also demonstrate the need for incoming resources to ensure continued colony strengthening. It would be advisable to supplement colonies between pollination services, through migration to plentiful foraging resources or, during extended periods of inclement weather, through artificial feeders.

Appendix 11: A follow-up survey on the Australian stingless bee industry, one decade on

A follow-up study on the Australian stingless bee industry, one decade on

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Questions for all stingless bee keepers

1. Did you complete the survey conducted by Anne Dollin, of Aussie Bees, and Tim Heard about 10 years ago?

- ◯ Yes
- ◯ No
- ◯ Don't know

2. How many nests of each species do you have?

Trigona carbonaria	
Trigona hockingsi	
Trigona clypearis	
Unknown species of Trigona	
Austroplebeia australis	
Austroplebeia symei	
Unknown species of Austroplebeia	
Other (please specify)	
Species not known	

3. Where is the nest(s) located? Please enter the number of hives. If there are multiple locations please enter the information in the additional box

City / Suburb	
State	
Postcode	
Multiple hive information please use space below	

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4. In what sort of area is the nest(s) kept?

- [ ] A suburban area
- [ ] Near bushland
- [ ] On a farm

Other (please specify)

5. How many nests of stingless social native bees do you have? Please enter the number of nests in the spaces below

In their original location?(eg: in a tree, fence post, house cavity or wall)	
Brought in from elsewhere but still in the original cavity? (eg: in a cut log)	
In an artificial hive box?	
Other? (please specify)	

6. How did you obtain your nests? (Please indicate the number of nests for each option)

Bought from another person	
Gift from another person	
Found in their natural location	
Split from other nest	
Developed from a swarm	
Budded off from their original nest (soft spilt or eduction)	
Other (please specify)	

7. How long have you been keeping stingless bees?

- [ ] Less than 1 year
- [ ] 1 to 3 years
- [ ] 3 to 5 years
- [ ] 5 to 10 years
- [ ] 10 to 20 years
- [ ] More than 20 years (please specify)

8. Have you tried nest transfers and nest splitting? (If so please write the number of nests in the spaces below)

Number of nests transferred into boxes successfully	
Number of nests transferred unsuccessfully	
Number of nests split successfully	
Number of nests split unsuccessfully	

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9. How many nests do you expect to have in the future?(Please enter the number of nests for each option)

Number of nests in 5 years	
Number of nests in 10 years	

10. What do you use your nests for at present? (Please enter the number of hives in each category and add detail as required

Just for the enjoyment of watching bees	
To help conserve the bees	
Pollination of bushland	
Pollination of a crop	
Honey production (Also, see next section)	
Production of other hive products (e.g. resin, wax or other)	
Hive sales (Please estimate number per year)	
Education (Please specify)	
Research	
Other (Please specify)	

11. If you make and / or design your own hive boxes, could you provide a description of the box design you like best? Please state the

Approximate dimensions	
Wall thickness	
Do you add a foam cover or similar? Please describe using the space below	
Do you have a special roof design? Please describe using the space below	

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12. If you produce honey from your stingless bees

Approximately how much per year?	
Which species (if known) is the best producer of honey in your collection of hives?	
Do you sell the product?	
If so, through which kinds of channels e.g. Health food outlets, local fairs, craft shows, at educational workshops, other (please specify using the space below)	

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Crop pollination

* If you use your Australian stingless bees for crop pollination, please answer the following questions
* If you are not doing crop pollination, please go straight to the end of this page

13. How long have you been a pollination service provider?

14. Do you

- [ ] Use them in your own crops
- [ ] Provide pollination services fro other growers

15. What crops have you attempted to pollinate with your stingless bees?

Successfully

Unsuccessfully

16. Of the total number of hives you own, how many do you use for crop pollination?

17. Do you use different species for different crops? If so please explain, using the space below

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18. What stocking rate do you use (hives per hectare)? Please use the space below

19. Have you &/or the grower noticed a marked difference in fruit/nut set since using stingless bees? Please estimate the increase if applicable, using the space below.

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20. Are your stingless bee hives used as pollinators instead of or together with managed honeybees (not including feral honeybees)?

- [ ] Instead of honeybees
- [ ] As well as honeybees

21. Do you charge a service fee?

- [ ] Yes
- [ ] No
- [ ] Sometimes

22. If you charge a service fee, how much do you charge per hive? (Remember this survey is totally anonymous)

23. If you don't charge a service fee, please explain why, using the space below

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24. Do you expect to continue being a pollination service provider in the future?

- [ ] Yes
- [ ] No

25. Are you able to keep up with the demand from growers with the number of hives you currently have?

- ◯ Yes
- ◯ No
- ◯ Other

Other (please explain)

26. What problems have you faced in using stingless bees for crop pollination? e.g.

- [ ] Swarming (reproductive)
- [ ] Fighting swarms
- [ ] Insecticide poisoning
- [ ] Theft
- [ ] Other

Other (please explain)

27. In what ways can stingless bee pollination service providers be supported? e.g. By research institutes, government bodies, growers or other. Please use the page below

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28. Please feel free to add any further comments that you think may be of value, using the page below

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Appendix 12: Sequences produced from the 36 successfully amplified specimens

CLUSTAL 2.1 MULTIPLE SEQUENCE ALIGNMENT

File: H:/PhD work/Studies/Morphology of Austroplebeia/Molecular work/downloads Austroplebeia COI_seqs final OS spp ren **Date: Tue Sep 27 14:08:52 2011**


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CLUSTAL 2.1 MULTIPLE SEQUENCE ALIGNMENT


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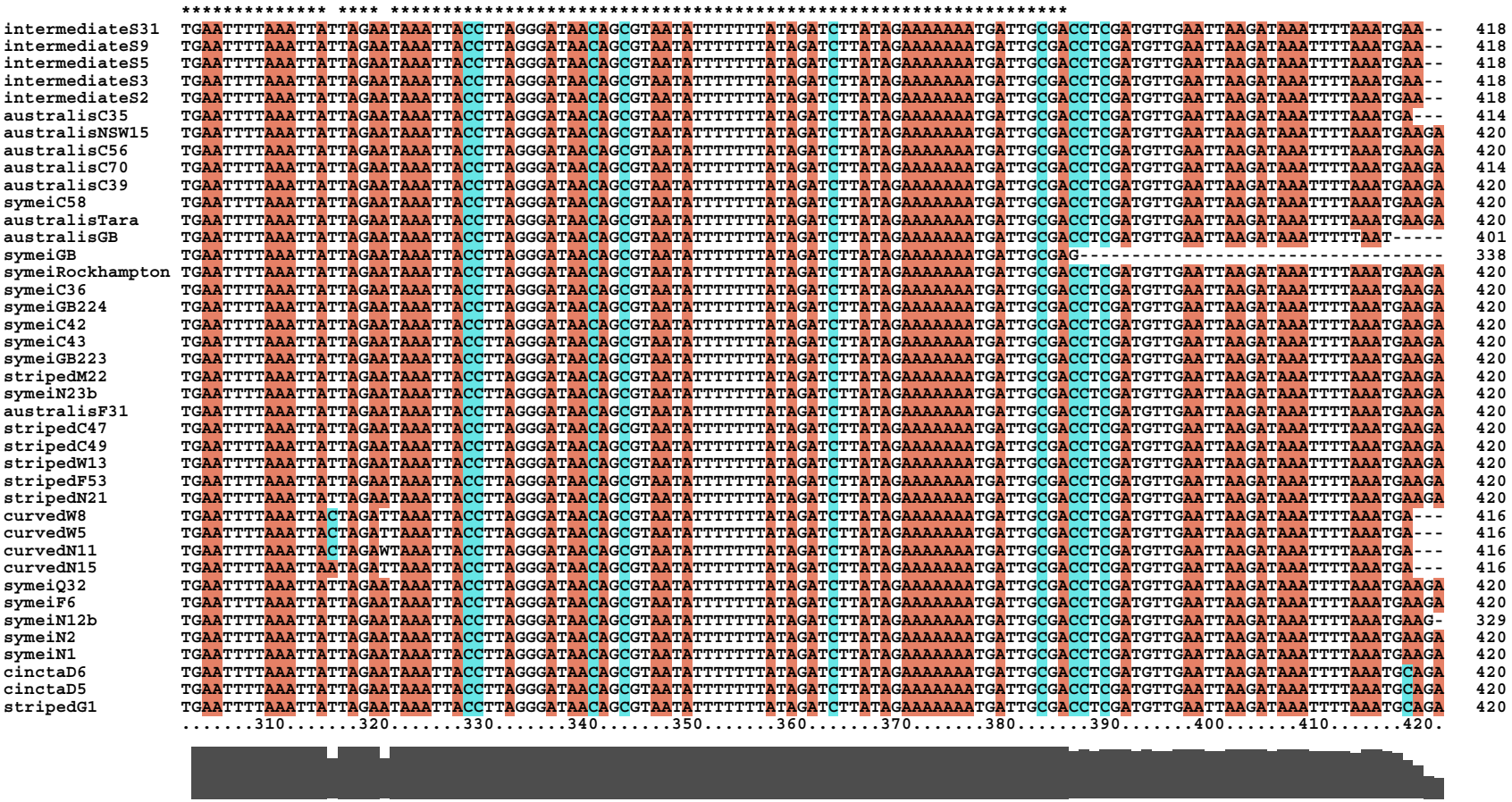
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intermediateS9 TGAATTTTAAATTATTAGAATAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGAA-- 418
intermediateS5 TGAATTTTAAATTATTAGAATAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGAA-- 418
intermediateS3 TGAATTTTAAATTATTAGAATAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGAA-- 418
intermediateS2 TGAATTTTAAATTATTAGAATAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGAA-- 418
australisC35 TGAATTTTAAATTATTAGAATAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGA--- 414
australisNSW15 TGAATTTTAAATTATTAGAATAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGAAGA 420
australisC56 TGAATTTTAAATTATTAGAATAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGAAGA 420
australisC70 TGAATTTTAAATTATTAGAATAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGAAGA 414
australisC39 TGAATTTTAAATTATTAGAATAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGAAGA 420
symeiC58 TGAATTTTAAATTATTAGAATAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGAAGA 420
australisTara TGAATTTTAAATTATTAGAATAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGAAGA 420
australisGB TGAATTTTAAATTATTAGAATAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTTAAT----- 401
symeiGB TGAATTTTAAATTATTAGAATAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGAG---------- 338
symeiRockhampton TGAATTTTAAATTATTAGAATAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGAAGA 420
symeiC36 TGAATTTTAAATTATTAGAATAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGAAGA 420
symeiGB224 TGAATTTTAAATTATTAGAATAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGAAGA 420
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stripedM22 TGAATTTTAAATTATTAGAATAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGAAGA 420
symeiN23b TGAATTTTAAATTATTAGAATAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGAAGA 420
australisF31 TGAATTTTAAATTATTAGAATAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGAAGA 420
stripedC47 TGAATTTTAAATTATTAGAATAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGAAGA 420
stripedC49 TGAATTTTAAATTATTAGAATAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGAAGA 420
stripedW13 TGAATTTTAAATTATTAGAATAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGAAGA 420
stripedF53 TGAATTTTAAATTATTAGAATAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGAAGA 420
stripedN21 TGAATTTTAAATTATTAGAATAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGAAGA 420
curvedW8 TGAATTTTAAATTACTAGATTAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGA--- 416
curvedW5 TGAATTTTAAATTACTAGATTAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGA--- 416
curvedN11 TGAATTTTAAATTACTAGAWTAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGA--- 416
curvedN15 TGAATTTTAAATTAATAGATTAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGA--- 416
symeiQ32 TGAATTTTAAATTATTAGAATAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGAAGA 420
symeiF6 TGAATTTTAAATTATTAGAATAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGAAGA 420
symeiN12b TGAATTTTAAATTATTAGAATAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGAAG- 329
symeiN2 TGAATTTTAAATTATTAGAATAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGAAGA 420
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stripedG1 TGAATTTTAAATTATTAGAATAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCTTATAGAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAATGCAGA 420
.....310.....320.....330.....340.....350.....360.....370.....380.....390.....400.....410.....420.