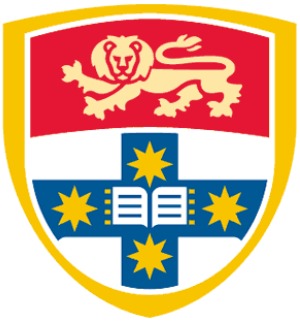
Native bees as alternative crop pollinators: Reproductive behaviour of *Tetragonula carbonaria*

Francisco Garcia Bulle Bueno



THE UNIVERSITY OF SYDNEY

School of Life and Environmental Sciences

New South Wales, 2006, Australia

2021

A thesis submitted in fulfilment of the requirements of the degree of Doctor of Philosophy

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The work contained in the body of this thesis, except otherwise acknowledged, is the result of my own investigations.

Chapter 2 is being prepared for submission. Francisco Garcia Bulle Bueno (FGBB) collected and analysed the data. The co-authors of this study are Liam Kendall (LK), Denise Alves (DA), Tanya Latty (TL), Tim Heard (TH), Manuel Lequerica (ML), Ben P. Oldroyd (BPO) and Rosalyn Gloag (RG). All co-authors contributed to the study design. LK, DA and ML contributed to collection of the data. RG, LK, TH and DA provided valuable feedback and editing.

Chapter 3 is being prepared for submission. FGBB designed and conducted the experiment. The co-authors of this study are Bernardo Garcia Bulle Bueno (BGBB), Gabriele Buchmann (GB), TH, TL, BPO, Anette Hosoi, (AO) and RG. BGBB assisted in coding and data analyses. GB assisted in the lab work. RG assisted in the experimental design. All authors provided valuable feedback and editing.

Chapter 4 is published in Journal of Experimental Biology. FGBB designed and conducted the experiment. The co-authors of this study are Isobel Ronai (IR), TL and RG. All authors provided valuable feedback and editing.

Chapter 5 is being prepared for submission. FGBB designed and conducted the experiment. The co-authors of this study are Rabia Hajjar (RJ), Théotime Colin (TC), GB, TL, TH, BPO and RG. RJ assisted in the experimental design and the collection of data from the virgin queens. TC assisted in coding and data analyses. GB assisted in the lab work. All authors provided valuable feedback and editing.

Chapter 6 is being prepared for submission. FGBB designed and conducted the experiment. The co-authors of this study are Malcolm Possell (MP), Guillaume Kerdoncuff (GK), GB, TL, TH, and RG. MP assisted in the experimental design and chemical data analyses. GK assisted with the data collection. GB assisted in the lab work. All authors provided valuable feedback and editing.

Preface

The chapters presented in this thesis are written in a format for publication in peer-reviewed, scientific journals.

For organisation purposes, all references have been compiled at the end of this thesis for easier cross-referencing.

This thesis has been saved in a PDF format file and has been submitted digitally. I have made hyperlinks throughout the document for the table of contents, chapters, figures and tables. The links have been coloured in **green**. Clicking on any section will automatically bring the reader to the relevant chapter, figure or table.

Hereafter, I wish these small details will be useful for the reader to better navigate this thesis.

Statement of Authorship

This thesis has not been submitted for any other degree or purposes. I certify that the research described in this thesis is the original work of the author, and that all the assistance received in preparing this thesis and sources have been acknowledged.

Francisco Garcia Bulle Bueno | 20 December, 2020

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Ros Gloag | 20 December, 2020

Acknowledgements

This thesis is the product of an amazing support and guidance from very talented and dedicated people which I will acknowledge hereafter.

I want to thank my main supervisor **Rosalyn Gloag** for all her guidance, support and mentorship throughout my PhD. I could not have asked for a better supervisor. Thank you for sharing your passion and enthusiasm for bees and biology, it is contagious.

Tim Heard. Thank you for your guidance, support, generosity and help. You opened the door to me of the marvellous world of Australian stingless bees to me and I could not be more grateful for that. I could not have asked for a better conference travel partner for Brazil and the Philippines. I deeply enjoyed our science chats when hiking in the tropics.

Tanya Latty. Thank you for the encouragement, cheerful personality and optimism, especially when the experiments did not go as expected.

Ben Oldroyd. Thank you for letting me be part of the BEE LAB, the chats about bees and feedback on this work.

Gabrielle Buchmann. Thank you for training me and helping me in the lab and the molecular sections of my research. I will always remember the dinners and the chats outside of the lab, as well as your kindness and generosity.

Julianne Lim. Thank you for your personal approach and always checking on the PhD students and asking about our life and personal relationships plus the support and help in the fieldwork preparation.

Eliza Middleton. Thank you for your unconditional help, friendship and support.

To my fabulous co-authors and volunteers, specially to my brother **Bernardo Garcia Bulle Bueno**, **Isobel Ronai**, **Théotime Colin** and **Liam Kendall**. Thank you for your time investment and dedication to this thesis, your valuable input in the data analyses and providing excellent feedback to the manuscripts.

Liz Gibson, Peter Clark, Lindy Williams and **Alex Austin** for facilitating the use of colonies of stingless bees from Ku Ring Gai for my fieldwork and training.

BEE Lab and the Invertebrate Behaviour and Ecology Lab colleagues. Amanda Norton, Patsavee Utaipanon, Manuel Lequerica, Arisa Hosokawa, Thomas Gillard, Thomas Hagan, Sarah Aamidor, Michael Holmes, Inez Vlasich, Jules Smith and Riley Fergusson. I am very happy and grateful to have walked this PhD path together, for your help and support during times of stress but mostly for the laughs and chats in the student office.

Giovanna Lescale for being there in the last months of my PhD, for her love and unconditional support. To my friends and family, the ones in Mexico and the ones in Australia (**Daniela Méndez**, **Rebeca Durán**, **Isabella Mangos** and **Grace Barrand**). Thank you for the shared memories during my PhD and for making my time in Australia such a joy.

Financial support and funding. This thesis would not have been possible if it was not for the University for Sydney and Agrifutures funding.

Finally, this thesis is dedicated to **my parents** for their unconditional support since the moment I was born.

Abstract

Bee pollination benefits the productivity of a wide variety of vegetable and fruit crops worldwide. Although the western honey bee *Apis mellifera* is the dominant pollinator of most crops, global instability in honey bee populations has led to calls to diversify the world's pollination services by enlisting other bee species as alternative pollinators. The stingless bees (Meliponini) are top candidates for this role. Like honey bees, they are highly eusocial, and can be managed and propagated in wooden hives. They have a large native distribution covering the tropics and subtropics of the world and are already known to be effective pollinators of many tropical fruits. Knowledge of how to maintain stingless bees in agricultural landscapes will also have substantial benefits for their conservation in the wild, where they provide key ecosystem services to native plants. An important step towards better utilising these bees as crop pollinators is to advance our understanding of their reproductive and foraging biology. In this thesis, I review the plants visited by stingless bees around the world, to uncover broad patterns in their floral visitation (**Chapter 2**). I then investigate the reproductive biology of the Australian endemic stingless bee *Tetragonula carbonaria* to: determine the distance males travel between their natal nest and mating aggregations, and assess the viability of using male dispersal behaviour to estimate the colony density of a region (**Chapter 3**); describe the reproductive anatomy of queens and workers, and confirm that workers are irreversibly sterile as adults (**Chapter 4**); document the early phase of a queen's life, and rear and mate queens under controlled conditions (**Chapter 5**); describe the volatiles produced by virgin queens, males and queenless colonies, and assess their effect on the attraction of rival colonies (**Chapter 6**). Together, these new insights improve our understanding of the biology of *T. carbonaria* and other stingless bees, and bring us closer to the goal of utilizing stingless bees as alternative crop pollinators in Australia.

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CHAPTER 1

General Introduction

1. Bees as pollinators

The importance of pollinators

Pollination is one of the main ecosystem services provided to humanity (Klein et al., 2007, Kremen et al., 2002). Animals pollinate 35%-66% of all the crops worldwide, and 30% of the world's fruit and vegetables consumed by humans come from bee-pollinated crops (Klein et al., 2007, Roubik, 1995, Kearns and Inouye, 1997). The global economic value of animal pollination was estimated to be US$235-$577 billion in 2015 (Potts et al., 2016a).

Globally, a high proportion of crops is pollinated by a single species: The Western honey bee (*Apis mellifera*) (Berenbaum et al., 2007, Morse and Calderone, 2000, Potts et al., 2010, Goulson, 2003) although in several crops the integration of other wild pollinator insects enhances fruit yield regardless of the abundance of honeybees (Garibaldi et al., 2013). Honey bees are widely used in the pollination industry because they are easy to manage, breed and transport. Each colony has several thousand foragers making pollination services very efficient (Kearns and Inouye, 1997). Native to Europe and Africa, they have been introduced to every other inhabited region of the world, where they are now naturalized (Huryn, 1997)**.** Thus, in addition to the millions of colonies worldwide kept in hives, wild *A. mellifera* populations in the landscape often provide valuable free pollination services to the agricultural industries (Breeze et al., 2011, Cunningham and Le Feuvre, 2013, Gill, 1990). For instance, the pollination service honey bees provide in Australia is estimated to be worth an average economic value of $A 14.2 billion annually (Karasinski, 2017). However, this strong dependence on a single pollinator represents a significant risk to food security; that is, pollination services are at the mercy of the parasites and diseases of honey bees, which can trigger dramatic fluctuations in honey bee populations (Winfree et al., 2007).

Risks to honey bee population health

These first decades of the 21st Century have seen significant instability in *A. mellifera* populations in several parts of the world. For example, the arrival of a novel honey bee pathogen, the ectoparasitic mite *Varroa destructor*, in the U.S.A. was followed by a dramatic 59% decrease in the United States hived population in the years following the introduction of *V. destructor* in (Berenbaum et al., 2007, Bretagnolle and Gaba, 2015, Richards, 1993). This *Varroa* epidemic decreased honey bee populations in several other parts of the western honey bee's global range (Goulson, 2003). In addition, beekeepers in Europe and North America reported spates of extremely high colony mortality, in a phenomenon that became known as Colony Collapse Disorder (CCD) (Kluser et al., 2010). Multiple interacting factors have since been associated with the CCD syndrome, including pathogens, parasites, pesticides, and immune system disorders (Stokstad, 2007, vanEngelsdorp et al., 2017).

Because of their key role as pollinators, the recent instability in honey bee populations has important implications for global food security (Garibaldi et al., 2011, Sammataro et al., 2000, Kluser et al., 2010). Indeed, in the same decade as problems such as Varroa and CCD were first documented, the volume of total crops depending on pollinators increased more than 300% worldwide due to global demand (Aizen and Harder, 2009). Fortunately, however, honey bees (*Apis.* sp) share very few of their pests and diseases with the many thousands of other bee species in the world (Berenbaum et al., 2007). For instance, *Varroa destructor* is a host specific parasite which only parasitizes species from the *Apis* genus (Potts et al., 2010, Berenbaum et al., 2007) comprising 7-9 species in the world, most of them distributed in Asia (Oldroyd and Wongsiri, 2009). This has led to calls to enlist other bee species as alternative pollinators of our crops (Ormond et al., 1984, Potts et al., 2010, Aizen and Harder, 2009, Slaa et al., 2006).

Stingless bees as alternative crop pollinators

The bee fauna native to a given location represents an insurance policy against a decrease in honey bee populations (Nabhan and Buchmann, 1997, Parker et al., 1987, Kremen et al., 2002). At least 17000 species of bees have been described worldwide (Michener, 2000) (**Figure 1**), the vast majority of which are solitary bees. Few solitary bees are actively managed for pollination, as most present significant challenges for propagation (Bosch and Kemp, 2002). Exceptions include several species in the genera *Osmia*, *Nomia* and *Megachile* which are used

for specific crops in North America (Cane, 2008, Kemp and Bosch, 2000, Vicens and Bosch, 2000). Many regions of the world, however, are home to at least some species of native social bees from the three clades of the social Apidae: honey bees, *Apis* sp. (7 species native to Asia); bumble bees, *Bombus* sp. (>250 species native to temperate regions of the world) and stingless bees; Tribe Meliponini (>600 species in 60 genera, native to tropical and subtropical regions of the world) (Rasmussen and Cameron, 2007, Rasmussen and Cameron, 2010).

Figure 1. Some bee species from Australia. There are approximately 2000 species of bee native to Australia. Here we show some of the most well-known and charismatic ones. From top left to right: The green carpenter bee (*Xylocopa aerata*), the neon cuckoo bee (*Thyreus nitidulus*) and the teddy bear bee (*Amegilla bombiformis)*. Bottom left blue banded bee (*Amegilla cingulata*). Bottom right: the sugarbag stingless bee (*Tetragonula carbonaria*). The western honey bee (*Apis mellifera*) is shown for comparison centre bottom row; it has been naturalized in Australia since being introduced in the 1820s. Drawings by Giovanna Lescale Cano and Francisco Garcia Bulle Bueno.

Across the tropics of the world, the stingless bees represent an excellent candidate for managed crop pollinators because, just like honey bees, they are highly eusocial, living in perennial colonies with a single reproductive queen and hundreds or thousands of workers (Wille, 1983, Nogueira Neto, 1997) (**Figure 2**). They are a highly diverse group of social bees (Michener, 2000), meaning that inter-specific differences allow for selection of the most appropriate species for a given crop. As they are native to the tropics, they can tolerate relatively high temperatures and be active all year round on crops grown in these regions. Furthermore, some species are already kept in hives for honey production and recreational beekeeping.

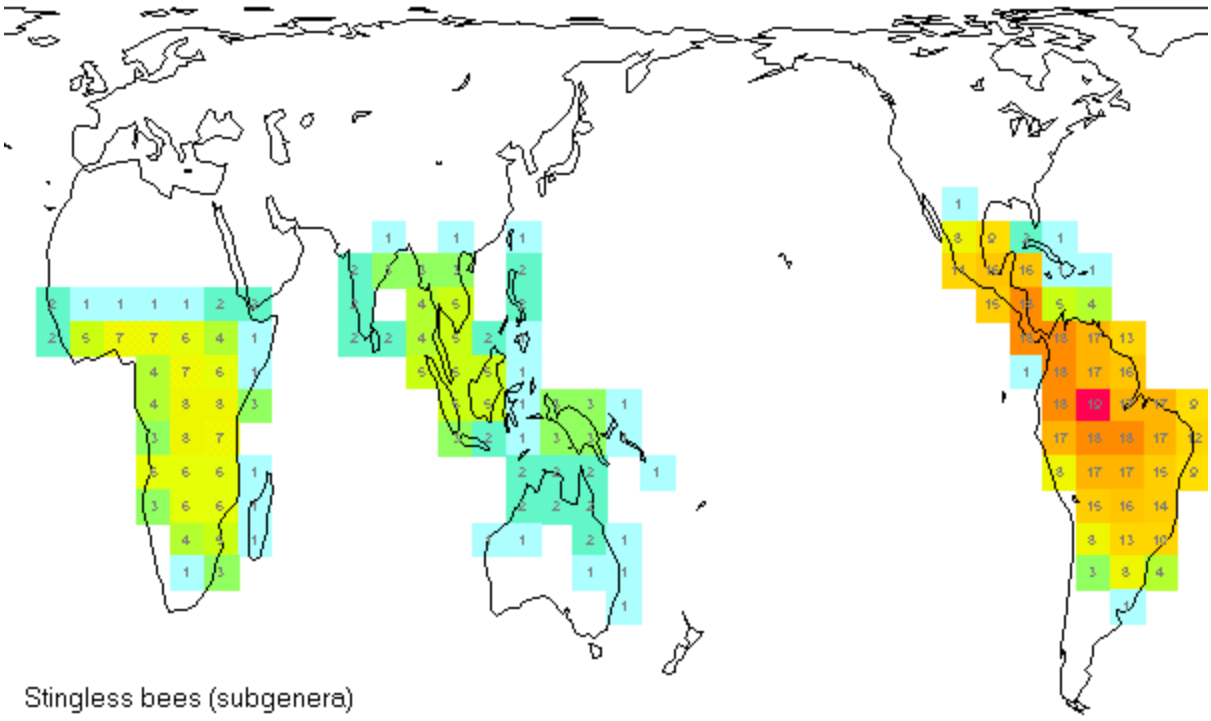


Figure 2. Global distribution of stingless bee subgenera across the tropics and subtropics of the world. Numbers in squares indicate the number of genera. The highest diversity is located in the Amazon. Three different areas of distribution align broadly with three monophyletic clades: Africa, India/Asia/Australia and the Neotropics. Source: The UK Natural History Museum.

Stingless bees are among the most common flowers visitors of flowering plants in the tropics. Therefore, they play a key role as pollinators of both native plants and crops across the pantropics, including Australia, South-East Asia, India, Africa and Central and South America (Heard, 1999). Thus far, they have been shown to be effective pollinators for a great variety of crops species **(**41 crops, **Table 1)** and make contributions to pollination of several other crops (Giannini et al., 2015, Heard, 1999, Slaa et al., 2006, Ramírez et al., 2018). Unlike honey bees, some stingless bees will even forage on crops in closed greenhouses (Sánchez et al., 2000, Greco et al., 2011).

Table 1. Crops on which stingless bees are confirmed to be efficient pollinators.

Plant common name	Plant genus	Plant species	Stingless bee species	Reference
Carambola	*Averrhoa*	*carambola*	Tt	1
Annato	*Bixa*	*orellana*	Mm, Mf, Mr, Hp, Tri, Mfu	1,2,3,4
Murici pitanga	*Byrsonima*	*chrysophylla*	Mfl, Tfu	3
Sweet pepper	*Capsicum*	*annuum*	Tl, Tc, Np, Fv, Ta, Ts, Msu, Mfa, Mf, Ms, Mqa, Msu	2,3,4
Sweet pepper	*Capsicum*	*sp*	Mc	*3*
Habanero Pepper	*Capsicum*	*chinense*	Mf	3
Malagueta pepper	*Capsicum*	*frutescens*	Mqa	3
Sweet pepper	*Caspicum*	*annuum var. cascadura Ikeda*	Ta	3
Sweet orange	*Citrus*	*sinensis*	Ts	3
Coconut	*Cocos*	*nucifera*	M	1,4
Coffee	*Coffea*	*arabica*	Lt	2
Coffee	*Coffea*	*arabica var. bourbon*	Mqa, Pl, Tf	3
Coffee	*Coffea*	*canephora*	Lt	2
Melon	*Cucumis*	*sativus*	Sd, Nt	2
Pumpkin	*Cucumis*	*sativus (Hokushin, Yoshinari and Soudai crop)*	Nt, Ta	3
Pumpkin	*Cucurbita*	*maxima*	Ts	3
Pumpkin	*Cucurbita*	*moschata*	Pb, Ts	4
Pumpkin	*Cucurbita*	*moschata var. Menina Brasileira*	Mqa, Tru	3
Pumpkin	*Cucurbita*	*pepo var. melanopepo*	Ts	3
Carrot	*Daucus*	*carota Cultivar Brasília*	Sb, Ta, Ts	3
Acai Palm	*Euterpe*	*oleracea*	Mf, Mfl, Tp	3
Strawberry	*Fragaria*	*x ananassa*	Pt, Ta, Ts, Nt, Pn, S, Tm	2,3,4
Sunflower	*Helianthus*	*annuus*	Nt, Ts	3
Tomato	*Lycopersicum*	*esculentum*	Mqa, Mf	2,3
Macadamia	*Macadamia*	*integrifolia*	Tri, Te	4

Plant common name	Plant genus	Plant species	Stingless bee species	
Acerola	*Malpighia*	*emarginata*	Tri	3
Acerola	*Malpighia*	*glabra*	Pc, Pl, Tri, Ts, Nt	3
Apple	*Malus*	*domestica*	Mqa	4
Mango	*Mangifera*	*indica*	Ta, Ts	3,4
Camu-camu	*Myrciaria*	*dubia*	Mf, Np, P, Pc, Tb, Tp, Tr, Mfl, Msp, M, Spo	1,3
Rambutan	*Nephelium*	*lappaceum*	Sm, Ta	2
Avocado	*Persea*	*americana*	Fn, Np, Ga, Tn, Pb, Sp, Sm, Tfu, Pf	2
Guava	*Psidium*	*guajava*	Mq	3
Pomegranate	*Punica*	*granatum*	Ts	3
Salvia	*Salvia*	*farinacea*	Np, Ta, Nt	2
Choko	*Sechium*	*edule*	Ts, Pc, Tco	1,3
Eggplant	*Solanum*	*melongena*	Mf	3,4
Hog plum	*Spondias*	*mombin*	St, Mf, Mfl, Mse, P, Tfs, T, Tfu, Tp,Tra	3
Imbu	*Spondias*	*tuberosa*	Spo, Sf, Tfs	3
Rose apple	*Syzigium*	*malaccense*	Mb, Msa	3
Cupuacu	*Theobroma*	*grandiflorum*	Pm, P, T, Ta, Tfu, Pp	1,3
Acapu	*Vouacapoua*	*americana*	Pm, Ta, Tp, Tb, Tfu	3

Abbreviations: Frieseomelitta nigra = Fn, Frieseomelitta varia = Fv, Geotrigona acapulconis = Ga, Hypotrigona pothieri = Hp, Lepidotrigona terminata = Lt, Melipona brachychaeta = Mb, Melipona compressipes = Mc, Melipona fasciculata = Mf, Melipona favosa = Mfa, Melipona flavolineata = Mfl, Melipona melanoventer = Mm, Melipona quadrifasciata = Mq, Melipona quadrifasciata anthidioides = Mqa, Melipona rufiventris = Mr, Melipona scutellaris = Ms, Melipona seminigra = Mse, Melipona seminigra abunensis = Msa, Melipona seminigra pernigra = Msp, Melipona subnitida = Msu, Melipona = M, Melipona fuliginosa = Mfu, Nannotrigona perilampoides = Np, Nannotrigona punctata = Npu, Nannotrigona testaceicornis = Nt, Paratrigona peltata = Pp, Partamona aff. Cupira = Pc, Partamona bilineata = Pb, Partamona cupira = Pc, Partamona = P, Plebeia minima = Pm, Plebeia nigriceps = Pn, Plebeia tobagoensis = Pt, Plebeia = Pl, Plebeia frontalis = Pf, Scaptotrigona aff. Tubiba = St, Scaptotrigona bipunctata = Sb, Scaptotrigona depilis = Sd, Scaptotrigona flavisetis = Sf, Scaptotrigona mexicana = Sm, Scaptotrigona pectoralis = Sp, Scaptotrigona postica = Spo, Scaptotrigona = S, Tetragona = T, Tetragonisca angustula = Ta, Tetragonisca fiebrigi = Tf, Tetragonula carbonaria = Tc, Tetragonula laeviceps = Tl, Tetragonula minangkabau = Tm, Tetragonula = Te, Trigona branneri = Tb, Trigona corvina = Tco, Trigona fulviventris = Tfu, Trigona fuscipennis = Tfs, Trigona nigerrima = Tn, Trigona pallens = Tp, Trigona recursa = Tr, Trigona ruficrus = Tru, Trigona spinipes = Ts, Trigona thoracica = Tt, Trigona = Tri, Trigonisca = Tra. **Information for references comes from**: 1 (Heard, 1999), 2 (Slaa et al., 2006), 3(Giannini et al., 2015) and 4 (Ramírez et al., 2018).

Human use of stingless bees

Stingless bees have captivated local communities for centuries, mainly because all species produce honey and cerumen (a mix of plant resin and wax produced by the bees) (Cortopassi-Laurino et al., 2006). The cultivation of stingless bees is called meliponiculture and it varies in management depending on the local community as well as the regional and traditional techniques where the different species are found (Cortopassi-Laurino et al., 2006). For example, the earliest evidence of meliponiculture is in Mexico, where bees have been cultivated by indigenous communities in Yucatan (Southern Mexico) since pre-Colombian times (Quezada-Euán et al., 2001, Crane, 1992) (**Figure 3**, left). Apart from having an economic use they were also linked to traditional and religious practices. The Mayans developed the first managed hives of stingless bees, in which they utilised hollow logs closed from both ends by pieces of wood or stone, with a hole in the upper middle part, mostly to keep *Melipona beecheii* (Cruz Bojórquez, 1992). In other parts of Mexico (Puebla and Veracruz), a different ethnic prehispanic culture (Nahua and Totonaca) also kept bees. They mainly kept *Scaptotrigona mexicana*, propagated in clay pots united at the rims to form a cavity, with the lower pot containing the brood and the top one the honey and pollen pots (Quezada-Euán et al., 2001). In other areas of the world, stingless bees were not actively cultivated but nevertheless had an important place in traditional societies. For example, indigenous communities in Northern Australia have exploited stingless bees for wax and honey for thousands of years ago (Fijn, 2014) (**Figure 3**, right). The honey was not only eaten but also played a significant part in religious practices, art, mythology, and medicine (Heard, 2016). Propolis was, on the other hand, used to enhance hunting tools such as spears and mouthpieces for the didgeridoo, a traditional Australian music instrument (Jones, 2013).

Nowadays stingless bee keeping is most advanced and widespread throughout South America (Cortopassi-Laurino et al., 2006) where it represents a major source of income for many communities (de Carvalho et al., 2014, Quezada-Euán et al., 2018) and where a majority of the species are found (c. 70% of all known species; (Michener, 2000, Pedro, 2013). Stingless bee industries have grown, however, over the past decade in several other parts of the world, including parts of Africa, India, Southeast Asia and Australia (Halcroft et al., 2013b, Chuttong et al., 2014, Macharia et al., 2007, Aidoo et al., 2011, Bafo, 2019) **(Figure 4).** This growing interest stems in part from the potential for honey production and recreation sales, but largely from their potential as commercial crop pollinators (Cortopassi-Laurino et al., 2006).

Modern meliponiculture tends to involve keeping the bees in wooden boxes comprised of two sections like some examples in Brazil (Caixas INPA, National Institute of Amazon Researches; Oliveira, 2000) and Australia (OATH, Original Australian *Tetragonula* Hive; Heard, 2016). This technique is gaining popularity across the natural distribution of stingless bees, in large part because boxes can easily be divided into two and facilitate the process of colony propagation by "splitting" (Heard, 1988). Another, slower method for propagation is by eduction or budding, which consists in attaching a new empty box to a full-size colony; eventually the original colony extends into the new box and adds a new queen that starts producing new brood (Heard, 2016). Once this happens the attached box is separated.

Figure 3. Representations of stingless bees around the world. (left) Representations of the Mayan bee god *Ah-Mucen-kab* holding the bees, the hives (in orange and yellow), the brood and the storage pots (in yellow). From pages 104 (left) and 108 (centre) of the Madrid Codex (c. 9001521 AD). Source: http://www.famsi.org/ mayawriting/codices *in* Hrncir, M., Jarau, S., & Barth, F. G. (2016) (Hrncir et al., 2016). (right) Native Bee hunting in Australia. Traditional techniques from an indigenous community in Australia chasing bees to get to the colony and extract the honey and propolis. Source Native Australian bee-hunters from Arthur, J.K., Kangaroo and Kauri: Sketches and anecdotes of Australia and New Zealand (London, Sampson Low, Marston and Company, 1894) (Arthur, 1894).

Figure 4. Some common and traditional management techniques of stingless bees around the world. From left to right: Management of *Melipona* sp.in long wooden boxes in Para State, Brazil. Management of *Tetragonula biroi* in the Philippines known as the *cocotech,* where they are kept in in coconut shells. Extraction of *Tetragonula carbonaria* in Australia from a log.

2. Gaps in our knowledge of stingless bee biology

Despite growing recognition of the potential of stingless bees as crop pollinators, many aspects of the basic biology and ecology of stingless bees remain poorly understood. Furthermore, the management of stingless bee colonies in hives is far less advanced than that of honey bees (Cortopassi-Laurino et al., 2006). With the exception of Brazil, in most countries meliponaries remain small-scale practices principally developed for the harvest of hive products, and not for commercial pollination services (Cortopassi-Laurino et al., 2006). There are still several key knowledge gaps that must be filled to ensure we benefit sustainably from stingless bee pollination of crops. Two of these broad knowledge gaps are outlined below.

Conserving free pollination services by wild stingless bees

Naturally-occurring stingless bees nesting in remnant vegetation around crops provide valuable free pollination services, yet we have a limited knowledge of the population dynamics of wild stingless bees. For example, a better understanding of their floral preferences would allow us to better frame bee management and conservation programs, and to understand more about the pollination biology of both wild flora and crops. We also lack an effective protocol for estimating the number of wild colonies in an area. Colony density estimates are an important baseline for knowing how to best conserve natural populations, including in agricultural

regions; for example, orchards could be positioned within crop-growing regions that have a high population density of stingless bees (Arundel et al., 2012). In the honey bee, the genetic analysis of wild-caught males has been used to indirectly estimate colony densities and track changes in population sizes over time (Utaipanon et al., 2019a). An equivalent protocol for stingless bees would advance efforts to conserve naturally-occurring colonies.

Propagating stingless bees in hives

The reproductive biology of most of the 600 stingless bees worldwide remain poorly known (Imperatriz-Fonseca and Zucchi, 1995, Engels and Imperatriz-Fonseca, 1990, Smith, 2019). Stingless bees share several basic features of their reproductive ecology. Colonies contain three castes: workers, queens and males. Most species have only one egg-laying queen (a few species have more; Jarau et al., 2009). In most species, queens are reared in special and bigger royal cells and their caste fate is determined by nutrition (with the exception of species in the genus *Melipona*; Imperatriz-Fonseca and Zucchi, 1995, Baptistella et al., 2014, dos Santos et al., 2016a, Hartfelder et al., 2006). If a new queen is needed, the colony selects her from among the available virgin queens in the colony, or rears a new one from available brood (Engels and Imperatriz-Fonseca, 1990, Imperatriz-Fonseca and Zucchi, 1995). Males congregate outside colonies with virgin queens ready to mate. Queens typically mate with a single male and store his sperm for the duration of their lifetime, using it to fertilize all subsequent eggs laid. And during the mating, the male loses his genitalia, leaving it attached as a "mating plug" to the queen's genital chamber (de Camargo, 1972, Imperatriz-Fonseca et al., 1998, Imperatriz-Fonseca and Zucchi, 1995, Da Silva et al., 1972, Kerr et al., 1962, Green and Oldroyd, 2002, Peters et al., 1999, Smith, 2019, Engels and Imperatriz-Fonseca, 1990).

Yet many of the finer aspects of the life cycles of queens and males are undocumented (dos Santos et al., 2014, Sommeijer et al., 2004, Van Veen et al., 1997, Engels and Engels, 1988, Carvalho-Zilse and Kerr, 2004, Imperatriz-Fonseca et al., 1998, Engels and Imperatriz-Fonseca, 1990, Imperatriz-Fonseca and Zucchi, 1995). An understanding of stingless bee reproduction is key to developing better strategies for mass rearing of colonies in hives (Menezes et al., 2013). For example, a limiting factor of stingless bee colony propagation is the small number of queens found in colonies at any given time, because queens are needed to head the new colonies produced (Menezes et al., 2013, Imperatriz-Fonseca and Zucchi, 1995, Jaffé et al., 2015). A critical first step in developing better propagation techniques is a better

understanding of the natural behaviours associated with rearing and choosing new queens, male attraction, queen mating and egg-laying.

3. Stingless bees in Australia

Sugarbag beekeeping

Australia is home to 11 species of stingless bee in two genera: *Tetragonula* and *Austroplebeia* (Heard, 2016). Also, known as sugarbag bees, all Australian stingless bees distributed across different regions between Northern and Eastern Australia (depending on the species). All species are reasonably cryptic in appearance, being less than 4mm long and having bodies that are all or mostly black in colour. They nest primarily in hollow trees and build their nests from secreted wax mixed with tree resin (Halcroft et al., 2013a, Dollin and Dollin, 1997) (**Figure 5**, a).

Australian stingless bees forage on a wide range of wild flora, as well as agricultural crops in regions where they naturally occur. They are especially beneficial for rural tropical and subtropical landscapes and crops, where they can be managed and transported for pollination (Roubik et al., 2018) (**Figure 5**, b). Currently in Australia, stingless bees have been reported to visit many wild plants belonging to the families Myrtaceae (Corymbia, Eucalyptus and Melaleuca spp.), Poaceae, Asteraceae, Fabaceae and Proteaceae (Wilson et al., 2021). They are also known to pollinate crops such as avocados (*Persea americana*), coconuts (*Cocos nucifera*), macadamia (*Macadamia integrifolia*), strawberries (*Fragaria X anasssa*), mangoes (*Mangifera indica*), raspberries (*Rubus sp.)* and blueberries (*Vaccinium* spp.) (Heard and Dollin, 2000, Kendall et al., 2020). Furthermore, their small size and short flight range (Smith et al., 2017) make them excellent candidates for pollination in greenhouses (Houston, 2018) which is currently under investigation.

Australian stingless beekeeping has gained significant momentum in recent years, with the number of managed hives increasing at 12% per year every year in the last decade (Nunes et al., 2015, Halcroft et al., 2013b). This industry's main hub is located on the coast of Queensland where the most commonly kept species are *T. carbonaria* and *T. hockingsi.* An example of how much the industry has grown over the last decades is seen in the increase in the demand and

price of a stingless bee hive, from $A 200 (Heard and Dollin, 2000) in 2000 to $A 550 in 2020 (Heard, 2020).

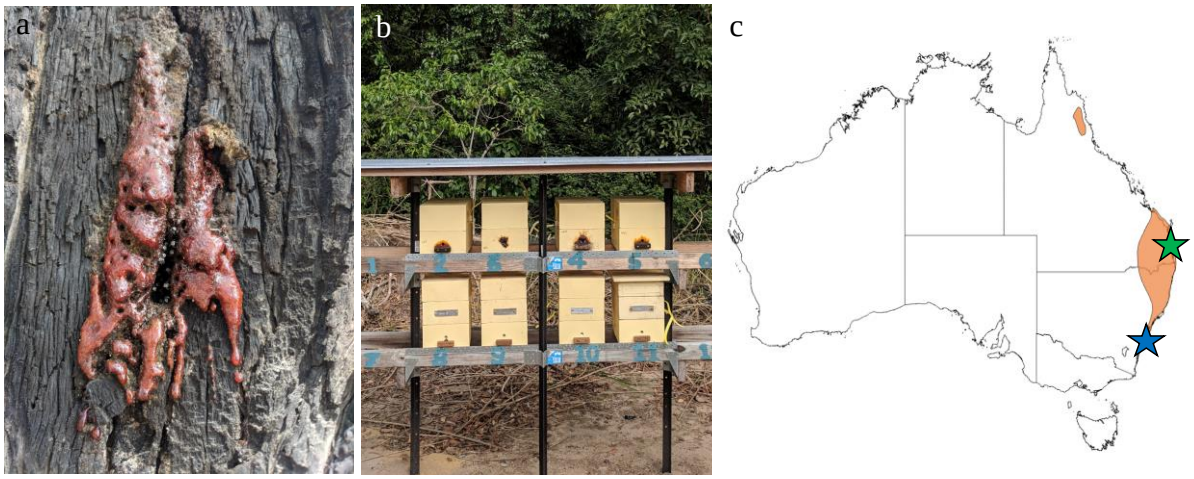


Figure 5. From left to right: **a**. Nest entrance of a natural colony of *T. carbonaria,* recognized by the resin bits surrounding the entrance holes. **b**. Managed wooden box hives of *T. carbonaria* used to enhance pollination in an agricultural landscape in Queensland **c**. Distribution map of *Tetragonula carbonaria* in Australia with the stars showing the capitals of the states where *T. carbonaria* is found, Sydney (blue) and Brisbane (green).

Tetragonula carbonaria

In this thesis, I focus my research on the endemic Australian species *T. carbonaria.* This species is distributed continuously from South-East Queensland to the NSW south coast, and also has several isolated remnant populations in Northern Queensland. As it has the most southern natural distribution of any stingless bee in Australia, its natural range includes major urban centres including Brisbane and Sydney (**Figure 5**, c). One of the main differences with the rest of the species in Australia is the spiral shape of their brood (**Figure 6**). It is also not very aggressive and readily kept in wooden hives. There has already been some research suggesting that *T. carbonaria* has the potential to pollinate important Australian tropical and subtropical fruit crops, including lychee, coconut, carambola, macadamia and mangoes (Heard, 1999). Importantly, it is native to the areas of Australia where these crops are grown (Amano et al., 2000, Wille, 1983). It is important to fill the gaps in knowledge of *T. carbonaria* basic biology in order to harness their full potential as alternative pollinators in Australia. In the process, I will advance understanding of the role of these bees in Australia's natural ecosystems.

Figure 6. Different views of a *Tetragonula carbonaria* colony. From left to right. 1. The queen and the workers walking on top of the brood comb of a colony. 2.View of the brood also known as the advancing front (youngest part of the brood) recognised by the cells made of wax and the uncapped cells on the edge. 3. View of the oldest part of the brood recognised by the lack of wax around the pupae ready to hatch soon.

4. Thesis Aims

My thesis aims to facilitate the use of stingless bees as crop pollinators, specifically *T. carbonaria* in Australia, in two ways. First, I review floral visitation of stingless bees around the world with the aim of showing their importance for the environment and encourage the conservation of wild populations (**Chapter 2**). Second, I investigate aspects of *T. carbonaria* reproductive biology with the aim of both protecting the "free pollination services" of naturally-occurring colonies, and improving our ability to propagate this species in hives. This second aim includes: (i) study of the males' reproductive behavior to estimate the density of colonies at a landscape scale (**Chapter 3**), (ii) study of queen-rearing behavior and anatomy to improve propagation of colonies (**Chapters 4** and **5**), and (iii) study of the chemical ecology of colonies during the requeening process (**Chapter 6**). Specifically, I aim to address the following questions:

Chapter 2. What plants are visited by stingless bees across the global tropics and subtropics?

Here, I collect and analyse records of stingless bee floral visitation for 287 species of stingless bee in ecosystems across the Neotropics, Africa, Asia, India and Australia.

Chapter 3. How far do *T. carbonaria* males travel from their natal nests to join mating aggregations? Can the genetic diversity of male aggregations provide colony density estimates for an area?

Here, I manipulate hived colonies to generate male aggregations, genotype these males and then apply a mathematical model to estimate male dispersal distances.

Chapter 4. Can we rescue the reproductive capacity of adult workers in *T. carbonaria*?

Here, I dissect and describe the reproductive anatomy of mated queens, virgin queens and female workers and then test the effect of social and nutritional environment on worker ovary development.

Chapter 5. Can we keep *T. carbonaria* queens alive in closed micro-colonies until mating age? Can we mate these queens in semi-controlled conditions?

Here, I collect queen cells from *T. carbonaria* colonies and raise them in micro-colonies under controlled conditions. I record and describe the behaviour of queens from eclosion until egg-laying and devise a technique to mate them in semi-controlled conditions.

Chapter 6. What volatiles are associated with virgin queens and re-queening colonies in *T. carbonaria*? Is the risk of nest usurpation higher for colonies that are forced to re-queen after a hive-split?

Here, I describe for the first time the volatiles associated with virgin queens and the colony re-queening process for an Australian stingless bee. I also test the hypothesis that colonies are more vulnerable to attack from conspecific colonies during a re-queening event.

Finally, in **Chapter 7** I provide a general discussion of my thesis findings, and suggest directions for future research in these areas.

CHAPTER 2

Stingless bee floral visitation in the global tropics and subtropics

Tetragonula biroi visiting a native plant commonly known as jade vine (*Strongylodon macrobotrys*) for the emerald colour flowers in Los Banos, the Philippines.

Abstract

Bees play a key role in maintaining healthy terrestrial ecosystems by pollinating plants. Stingless bees (Apidae: Meliponini) are a diverse clade of social bees (>600 species) with a pantropical distribution spanning South and Central America, Africa, India and Australasia. They are garnering increasing attention as commercially-beneficial pollinators of some crops, yet their contribution to the pollination of native plants in the tropics and subtropics remains poorly understood. Here, we conduct for the first time a global review of the plants visited by stingless bees. We compile a database of reported associations (flower visits) between stingless bees and plants, from studies that have made either direct observations of foraging bees or analysed the pollen stored in nests. Worldwide, we find stingless bees have been reported to visit the flowers of plants from at least 215 different families and 1434 genera, with frequently reported interactions for many of the tropics most species-diverse plant families including Fabaceae, Asteraceae, Rubiaceae, Poaceae, Euphorbiaceae, Myrtaceae, Malvaceae, Arecaceae, Solanaceae, and Anacardiaceae. The stingless bee fauna of each of three major biogeographic regions (Neotropical, Afrotropical and Indo-Malayan-Australasian) were frequent visitors of many of the same plant families, however we detected differences in the proportional use of these families by the stingless bees of the Indo-Malayan-Australasian and Neotropical regions, likely reflecting differences in the available flora of those regions. Stingless bees in all regions visit a range of exotic species in their preferred plant families (crops, ornamental plants and weeds), in addition to native plants. Although most reports of floral visitation on wild plants do not confirm effective pollen transfer, it is likely that stingless bees make at least some contribution to pollination for the majority of plants they visit. In all, our database supports the view that stingless bees play an important role in the ecosystems of the global tropics and subtropics as pollinators of an exceptionally large and diverse number of plants. This database also highlights important gaps in our knowledge of stingless bee resource use and should benefit future efforts to understand stingless bee-plant interactions.

Introduction

Animal pollination is critical to ecosystem functioning and service provisioning in terrestrial ecosystems globally (Klein et al., 2006, Fontaine et al., 2005, Potts et al., 2016b). A diverse range of vertebrates and arthropods may pollinate plants, but the majority of plant species (65-80%) rely on insects as their primary pollinators (Buchmann and Nabhan, 2012, Ollerton et al., 2011). There is growing evidence of widespread declines in insect pollinator populations over recent decades, in some cases causing synergistic declines in pollinator-dependent plants (Biesmeijer et al., 2006, Potts et al., 2016b). The plants of the species-rich tropics and subtropics may be particularly vulnerable to changes in insect populations, as the flora of these regions are more dependent on pollinators than those of temperate-zones (94% of all tropical plants are estimated to rely on animal-pollination (Ollerton et al., 2011). In addition, insects in the tropics are predicted to be the least capable of rapidly adapting to changing climates, and thus the most at risk of extinction (Kellermann et al., 2012). In order to mitigate the impacts of pollinator declines in the tropics and subtropics, we first require a greater understanding of pollinator ecology in these regions (Vanbergen and Initiative, 2013), and the relationship between floral resources and pollinators at landscape scales (Vanbergen and Initiative, 2013, Kleijn and Raemakers, 2008).

The stingless bees (Hymenoptera: Apoidea: Anthophila: Meliponini) are social corbiculate bees native to the world's tropical and subtropical regions (Michener, 2000, Michener, 1979, Roubik, 1992a). Like other social bees (e.g. honey bees, *Apis* sp.), stingless bees are abundant flower visitors in the ecosystems they inhabit, because each colony contains thousands to tens of thousands of workers (Nogueira Neto, 1997, Hepburn and Radloff, 2011). There are an estimated 516 species of stingless bees in 60 genera (Schuh, 2010, Rasmussen and Cameron, 2007, Rasmussen and Cameron, 2010). These are divided between three monophyletic clades that diverged from each other 50-70 million years ago and that correspond to three major biogeographic regions: Neotropical, African and Indo-Malayan-Australasian (Rasmussen and Cameron, 2010). The Neotropics harbours the greatest stingless bee diversity, with 417 known species, c. 80% of global described species (J. S. Moure & A. Dal Molin, 2012).

The high global species diversity of stingless bees is also reflected in their morphological and behavioural diversity (Michener, 2000). They range in body size from just 2mm (some

Trigonisca spp.; Roubik, 2018) to 15 mm (*Melipona fuliginosa;* Camargo and Pedro, 2008). They may nest in tree cavities, termite nests, or underground (Roubik, 1983). In the absence of an effective sting, they have evolved varied defence mechanisms including acid discharge (Roubik et al., 1987), suicidal biting (Shackleton et al., 2015) and sticking resin (Lehmberg et al., 2008). And they show a variety of social structures, whereby colonies may have multiple queens or single queens (Velthuis et al., 2006, Alves et al., 2011), workers may lay eggs regularly or be completely sterile (Sommeijer et al., 1999, Garcia Bulle Bueno et al., 2020), and the workers of some species include a "solider caste" (Baudier et al., 2019, Hammel et al., 2016, Grüter et al., 2012). All stingless bees, however, share a need to visit flowers for nectar for food, and almost all also collect pollen to provision their offspring (excluding a handful of Neotropical species that feed their offspring carrion or are cleptoparasites of other stingless bees; Sakagami et al., 1993, Mateus and Noll, 2004). Stingless bees therefore contribute to pollinating the native flora of vegetation throughout the tropics and subtropics (Gill et al., 2016).

Stingless bees have attracted significant research as pollinators of crops in many of the countries where they naturally occur (Giannini et al., 2015, Ish-Am et al., 1999, Nates Parra, 2016). Similar to honeybees, stingless bees can be readily kept and transported in hives (Wille, 1983, Nogueira Neto, 1997) meaning they can be introduced to orchards when in flower and then reallocated (Giannini et al., 2020). They are effective pollinators of a variety of tropical and subtropical crops species, including açaí palm (*Euterpe oleracea*) coconut (*Cocos nucifera*), coffee (*Coffea arabica*), macadamia (*Macadamia* spp.) and rambutan (*Nephelium lappaceum*) (Giannini et al., 2015, Heard, 1999, Slaa et al., 2006). They are also adapted to the local conditions in many regions where these crops are grown (Jaffé et al., 2015), with wild stingless bees providing valuable free pollination services. For example, wild stingless bees (*Tetragonula carbonaria*) are as effective as managed honeybees at pollinating blueberry crops (*Vaccinium* spp.) in Australia (Kendall et al., 2020). In most parts of the world, however, our understanding of the relationship between stingless bees and native flora is comparatively incomplete (Roubik, 1995, Giannini et al., 2020). Stingless bees actively forage on diverse floral resources throughout the year (Roubik, 1982, Roubik, 1992b, Kleinert et al., 2012), but are proposed to have stronger interactions with some groups of native plants than others (e.g. Asteraceae, Euphorbiaceae, Sapindaceae, Rubiaceae, Fabaceae, Melastomataceae and Myrtaceae; (Grüter, 2020). Such preferences might confer a benefit on stingless bees via a reduction in interspecific competition (i.e. they focus on resources neglected by *Apis* sp.), or

they may simply select those flowers that produce nectar and pollen in most abundance (Antonini et al., 2006, Ramalho et al., 1989). At least some genera (e.g. *Melipona* spp. in the Neotropics) show a clear preference for some floral resources over others, irrespective of availability (Vossler, 2013, Ramalho et al., 1991, Ramalho et al., 1990, Kleinert et al., 2012, Antonini et al., 2006).

Here, we aim to consolidate current knowledge of stingless bee floral visitation of native plants at the regional and global scale, by creating a database of reported floral visitation by stingless bees. From this database, we assess: (i) the diversity of plant families and genera used as food sources by stingless bees in each of three major biogeographical regions: Neotropical, Afrotropical and Indo-Malayan-Australasian; (ii) the most frequently-used plant families (according to number of genera visited), as a proxy for candidate floral preferences of stingless bees (we acknowledge that this proxy might have problems due to not being able to differentiate whether the bees visit the plants due to their abundance or active selectivity, further discussion); and (iii) the type of growth (Herb/Shrub/Tree/Liana/Vine), endemism to a particular region, and native or exotic status of plants commonly used as forage by stingless bees. We also provide a reference list of those plants for which stingless bees have been experimentally confirmed to be pollinators (both crops and wild plants). Our database is intended as a first step towards a richer understanding of how stingless bees contribute to ecosystem functioning in the tropics and subtropics, and provides an online resource for further studies on plant-pollinator interactions.

Methods

Database of floral visitation by stingless bees

To build a database of reported interactions between stingless bees and flowering plants, we conducted a search of peer review journals, books, student theses and conference abstracts. We used the keywords Meliponini, pollination, floral preferences and stingless bees to search Scopus, Web of Science and Google Scholar (first 200 references per search; May 2020). We likewise searched unpublished literature in Spanish and Portuguese from library databases and the University repositories of The National University of Colombia, The University of Costa Rica and The University of Brazil (Catálogo de Teses e Dissertações Literature Teses CAPES,

from 2013 to now; CAPES, 2016), plus the Conference abstracts from the annual Brazilian Bee Meeting "Encontro sobre Abelhas" (1994 to 2018). Finally, we included reported interactions from Brazil's online index of Bee-Plant Interactions (A.B.E.L.H.A., 2017), and from three books: 'Pot Pollen' (Vit et al., 2018), 'Atlas of Pollen and Plants used by Bees' (da Silva et al., 2020) and 'Pollination of cultivated plants in the tropics' (Roubik, 1995). We only included literature that was available online.

From each source, we collated the following information for each stingless bee species-flower interaction: biogeographic region (Neotropical, Afrotropical, Indo-Malayan-Australasian) and country where the interaction was reported, plant species, genus and family, bee species and type of interaction (either floral visitation or pollination). Floral visitation included cases where the researcher directly observed bees visiting flowers, or where visitation was inferred from palynological study (i.e. pollen resources collected from colonies or off the legs of returning foragers). An interaction was scored as pollination only if the study confirmed the bee pollinated the plant. We did not consider pollination efficiency (i.e. single visit efficiency of pollen deposition, fruit set, seed set, etc.; (King et al., 2013) as only a handful of studies in our database reported such detail. Finally, for the 15 plant families with the greatest number of genera visited by stingless bees, we retrieved the native distribution of each genus and the growth type (Herb/Shrub/Tree/Liana/Vine) using Plants of the World Online (POWO, 2019) and noted whether the genus was documented as introduced in the region of the reported interaction according to the Centre for Agriculture and Bioscience International (CABI, 2020).

We considered commonly used plant families to be those in which stingless bees were reported to visit the most genera within the family. For the Neotropical region, we also accessed data on the total number of genera per plant family known to occur in the region via Neotropikey (Milliken, 2009), an online key developed to identify and inform about the flowering plants in the Neotropical region. This allowed us to account for high richness of genera in some plant families, by considering the proportion of locally-occurring genera (In the Neotropics) in a family that were visited by stingless bees.

To confirm our database used current scientific names of all stingless bees, we checked names against the "Catalogue of Bees (Hymenoptera, Apoidea) in the Neotropics" (J. S. Moure & A. Dal Molin, 2012), the "Catalogue of Afrotropical bees (Hymenoptera: Apoidea: Apiformes)" (Eardley and Urban, 2010) and the "Catalogue of the Indo-Malayan/Australasian stingless bees

(Hymenoptera: Apidae: Meliponini)" (Rasmussen, 2008). For plant species, we cross-checked names against those listed on the Missouri Botanical Garden's Tropicos website (Missouri Botanical Garden, 2020), The Plant List (The Plant List, 2010), Plants of the World Online (POWO, 2019) and the R package *taxize* v0.9.98 (Chamberlain and Szöcs, 2013).

Analyses

We visualised plant ~ pollinator networks for the top ten most abundant bee genera and plant families using chord diagrams (*circlize* package v0.4.1; Gu et al., 2014). We also provide example flower types for each plant family in chord diagrams, based on (Simpson, 2010).

We tested whether the plant families visited by stingless bees varied between biogeographical regions (Neotropical, Afrotropical and Indo-Malayan-Australasian) using a two-step approach. First, we transformed our databases to interaction matrices of bee genera (rows) ~ plant family (columns, count of the number of visited genera) and then calculated the Bray-Curtis dissimilarity between bee genera using the *vegan* package v2.5-6 (Oksanen et al., 2013). Second, we compared compositional differences in plant use (at family level) between biogeographical regions using a pairwise PERMANOVA (Anderson, 2001, Martinez Arbizu, 2017). We clustered the bee genera from each of the three regions and compare the composition of reported plant families visited between each of the regions. We adjusted *P*-values using the false discovery rate (FDR) method to account for multiple comparisons (Benjamini and Hochberg, 1995). We visualised differences in the interactions between stingless bees and plant families in two-dimensional space with non-metric multidimensional scaling (nMDS) ordination. We conducted all data analyses in R v4.0.2 (R Core Team, 2013).

Results

Our database includes 19,770 bee-flower interactions reported in 541 studies (**Table S1**). In all, 287 species of stingless bees were represented by at least one reported interaction in the database; this spanned 52% (219/417) of Neotropical stingless bee species, 68% (22/32) of Afrotropical species and 51% (46/89) of Indo-Malayan-Australasian species. The great majority of reported interactions were floral visitation records (19,006 interactions, 96%), with the remainder (764 interactions, 4%) confirming that bee visitation resulted in pollination.

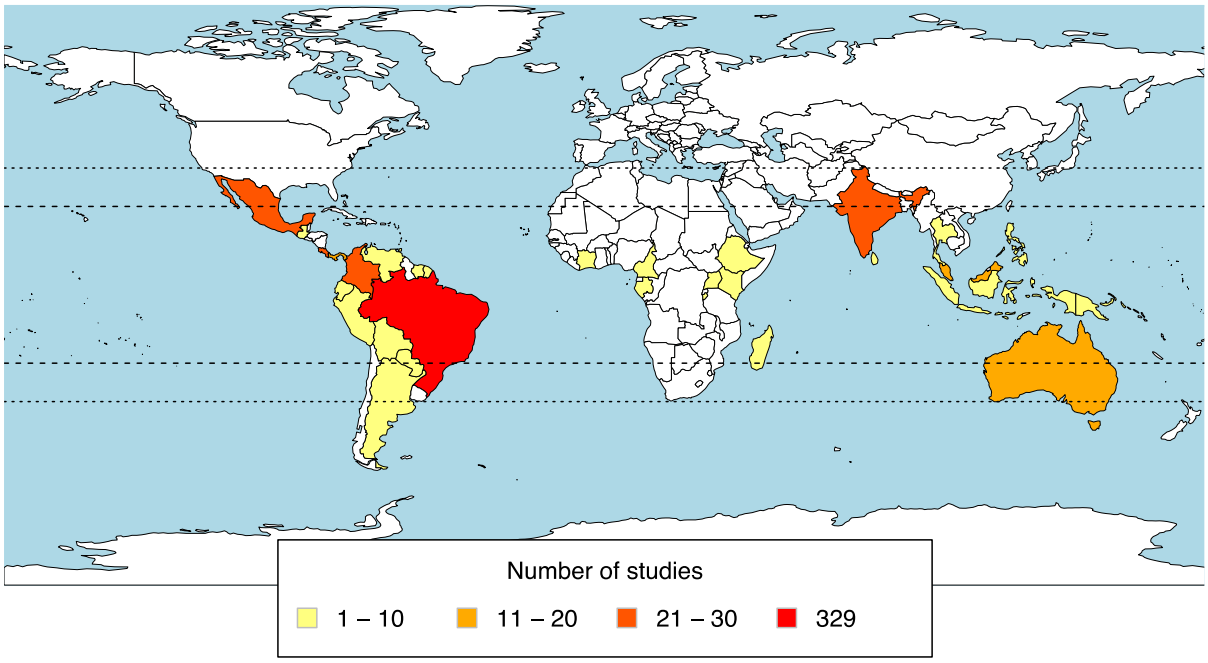


Figure 1. To visualise bias in reported stingless bee-plant interactions in the available literature, we plotted the number of studies that recorded interactions between stingless bees and flowering plants at a country level per region included in our database using *rworldmap* (v.1.3-6, South 2011). Dashed and dotted lines indicate the latitudinal range of the Tropics and Subtropics respectively.

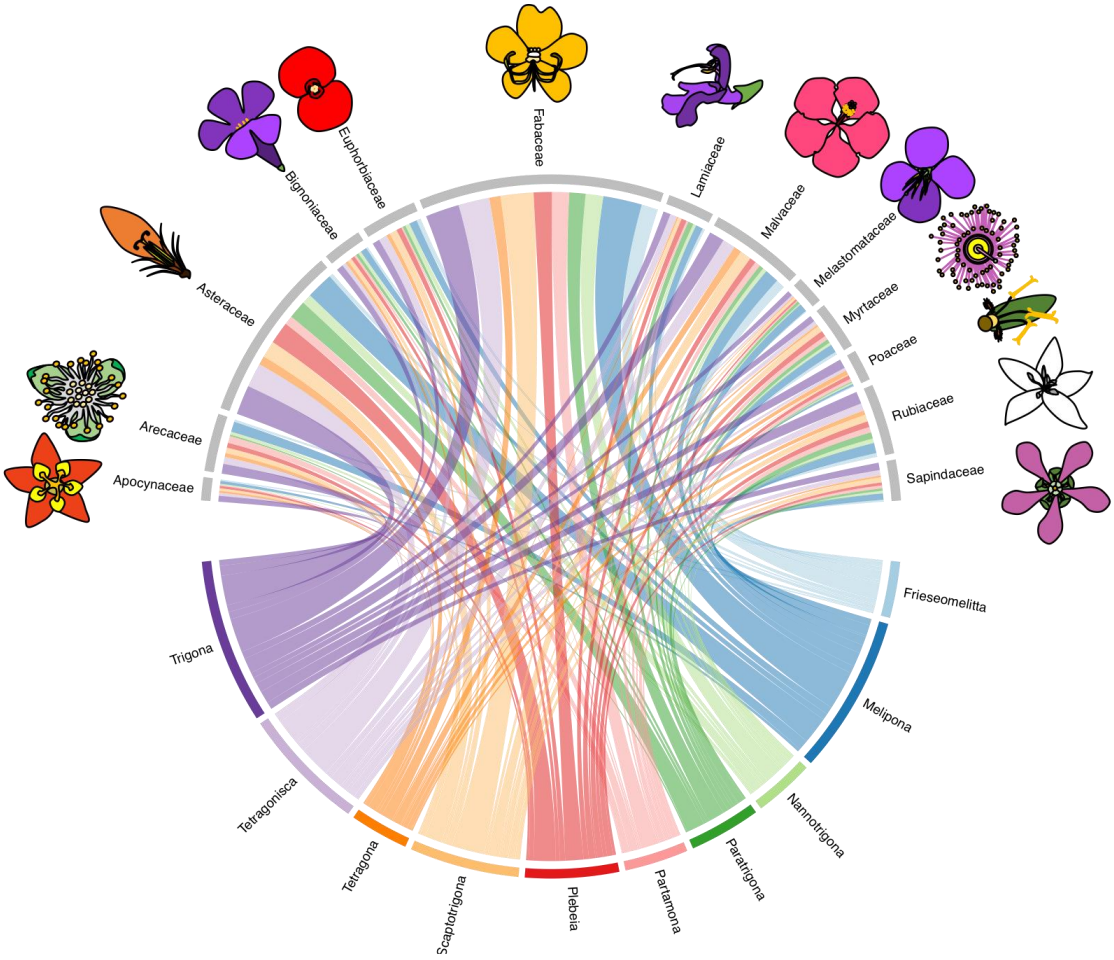


Figure 2A. A visitation network of stingless bees to flowering plants in the Neotropical region, showing the 10 stingless bee genera and 10 plant families with the most reported interactions in the literature. Example flower types for each plant family are shown. Details of all interactions are given in supplementary material, **Table S1**.

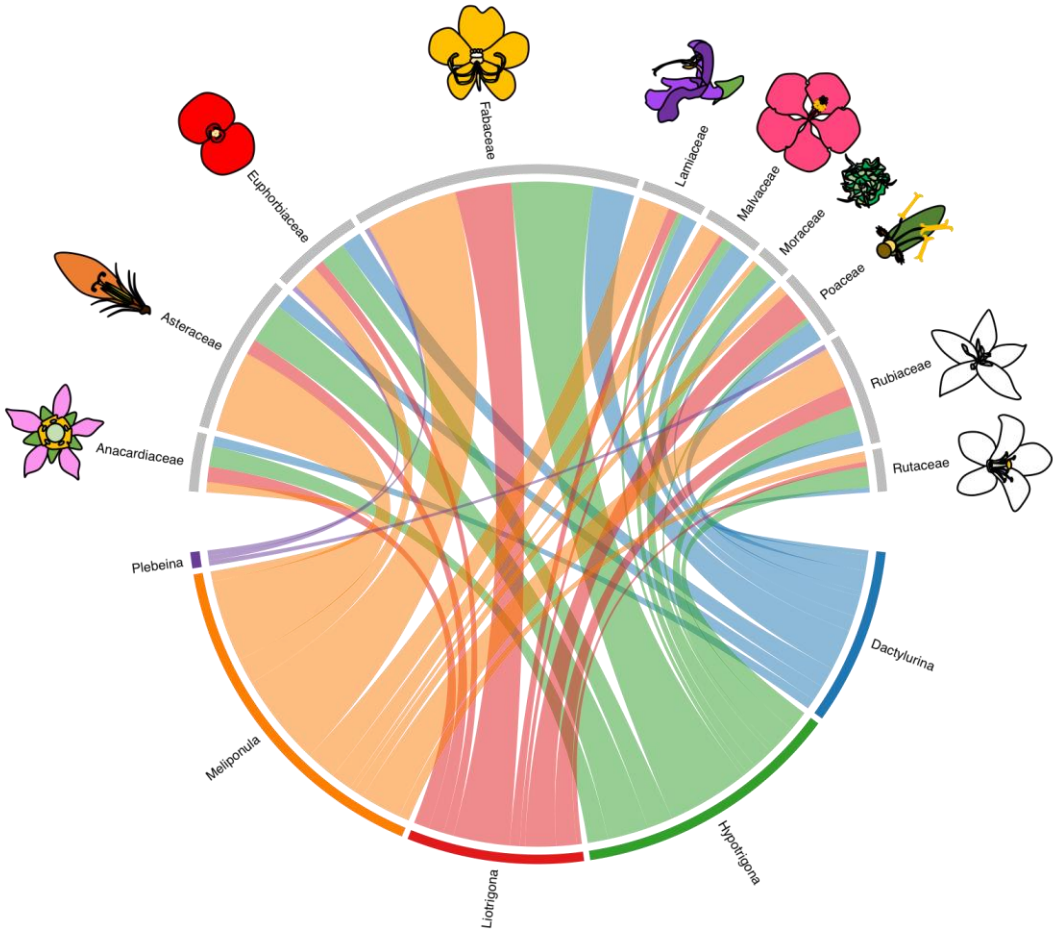


Figure 2B. A visitation network of stingless bees to flowering plants in the Afrotropical region, showing the 5 stingless bee genera and 10 plant families with the most reported interactions in the literature. Example flower types for each plant family are shown. Details of all interactions are given in supplementary material, **Table S1**.

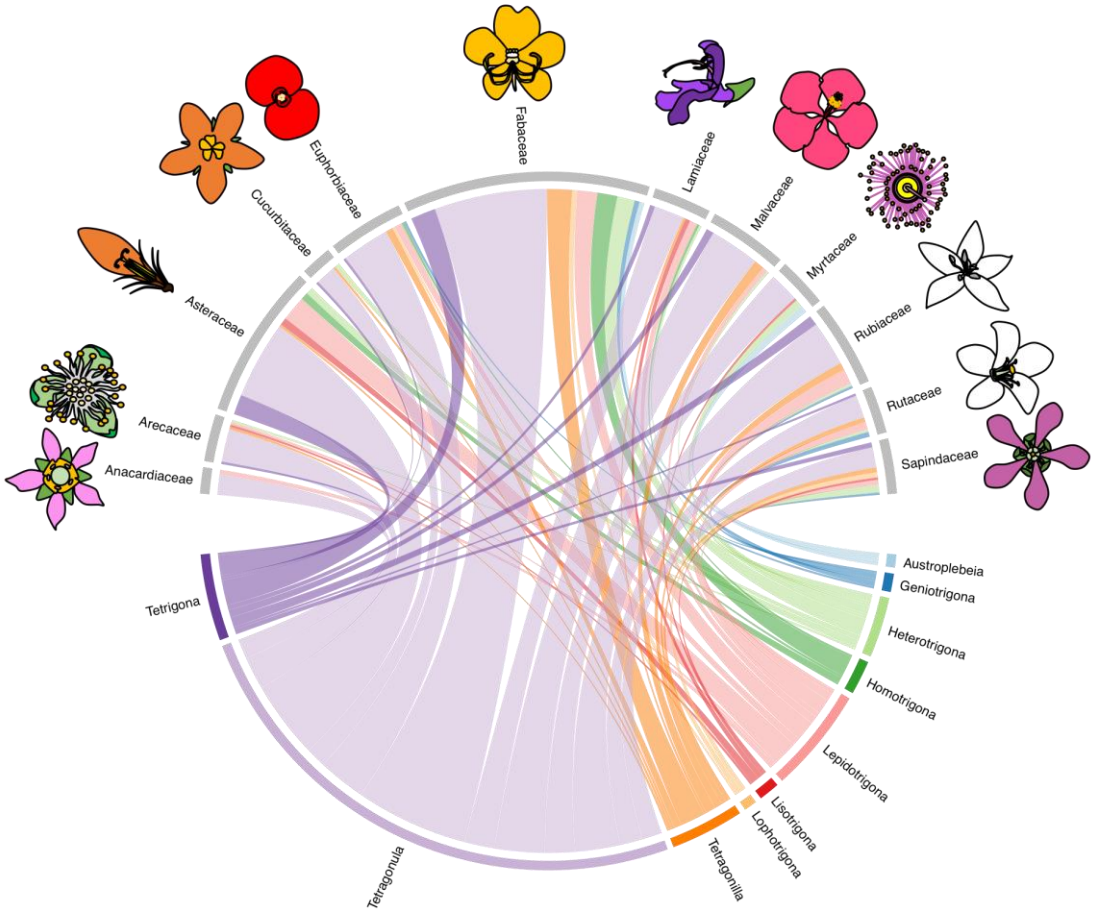


Figure 2C. A visitation network of stingless bees to flowering plants in the Indo-Malayan-Australasian region, showing the 10 stingless bee genera and 10 plant families with the most reported interactions in the literature. Example flower types for each plant family are shown. Details of all interactions are given in supplementary material, **Table S1.**

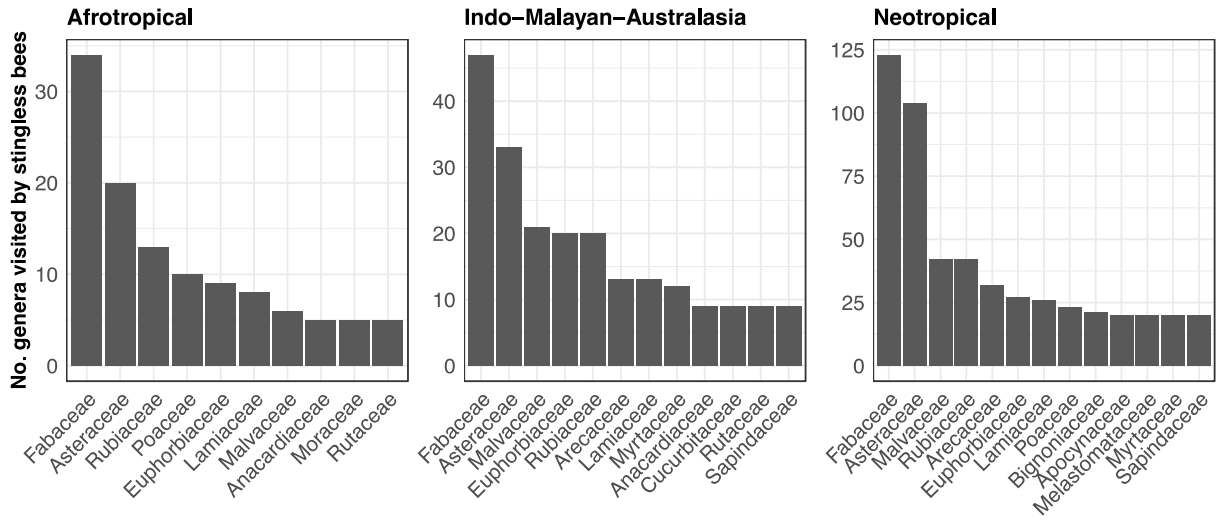


Figure 3. The number of genera visited by stingless bees of the Neotropical, Afrotropical and Indo-Malayan-Australasian regions, in each of the 10 plant families with the highest number of genera visited, according to reported interactions in the literature (>10 families shown where multiple families had the same rank).

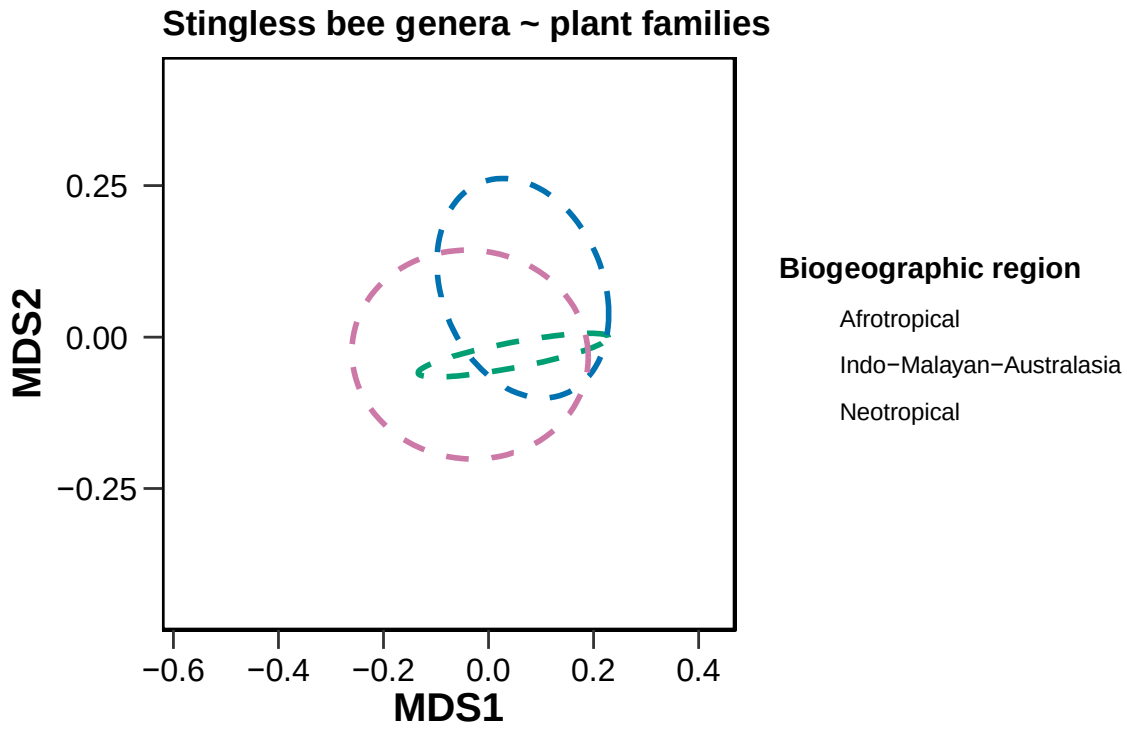


Figure 4. NMDS ordination of stingless bee genera ~ plant family composition in each biogeographic region. Each point represents a bee genera's floral associations at the plant family level (Afrotropical in green, N= 5 bee genera; Indo-Malayan-Australasian in blue, N= 13; Neotropical in pink, N= 31). Dashed circles represent the 95% confidence ellipses for each biogeographic region mean (group centroid).

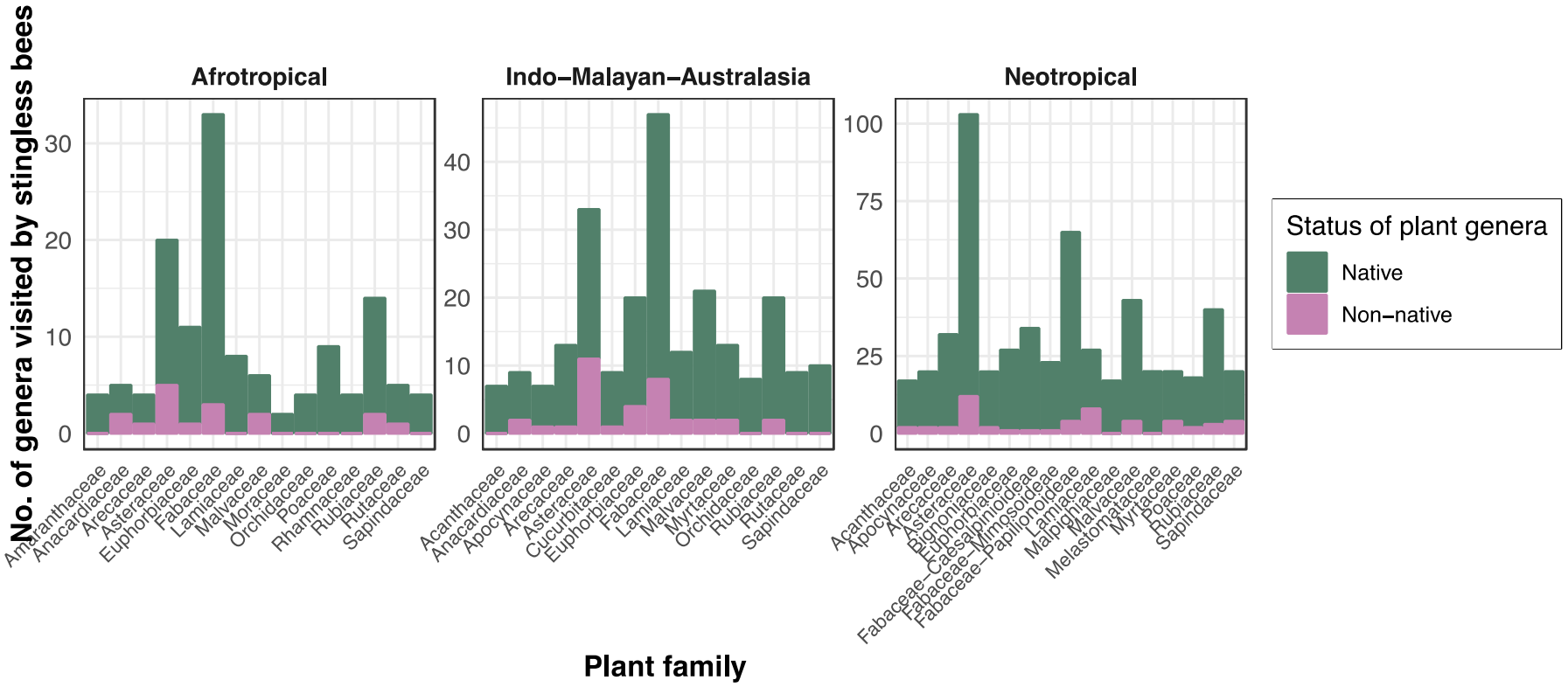


Figure 5. The number of genera visited by stingless bees in the 10 most visited plant families coded by native status, for each of three biogeographical regions: Afrotropical, Indo-Malayan-Australasian and Neotropical (>10 families shown where multiple families had the same rank). Most reported flower visits are for plant genera native to that region (green) but a minority are plant genera introduced to that region (pink). For the Neotropics, Fabaceae is shown here divided into its subfamilies (Caesalpinioideae, Mimosoideae and Papilionoideae) due to the large number of visited genera in that region.

The majority of reviewed studies were carried out in the Neotropics (16900 interactions reported in 424 studies; 85% of all interactions), and particularly Brazil (13619 interactions); **Figure 1**. This reflects in part the higher diversity of stingless bees in this region (e.g. half of the world's stingless bee genera are found in Brazil), and in part the intensity of research to date on the Neotropical stingless bee fauna, relative to that of Afrotropics (1068 interactions reported in 17 studies; 5% of database interactions), and Indo-Malayan-Australasian (1803 interactions reported in 57 studies; 10% database interactions); **Figure 1**. Although our database included some "grey literature" (conference abstracts, student theses, etc) from the Neotropics but not from other regions, the proportion of total interactions reported from the Neotropics was similarly high even if we included only bee visitations that were published in international journals.

Plant genera and families visited by stingless bees

Stingless bees worldwide are reported to forage from the flowers of 1435 genera of plants in 215 families worldwide (62% of all angiosperm families; 205 families in the Neotropics, 82 in the Afrotropics and 137 in the Indo-Malayan-Australasian region); (Supplementary Material, **Tables S2** and **S3)**. Individual stingless bee species were reported to forage on 30 ± 56 (SD) plant genera (range: 1 – 535; *Trigona spinipes*).

The ten plant families with the largest number of genera visited by stingless bees were Fabaceae (legumes; N=153), Asteraceae (daisies; N=121), Rubiaceae (madders; N=63), Malvaceae (mallows; N=52), Euphorbiaceae (spurges; N=42), Arecaceae (palms; N=41), Lamiaceae (mints; N=34), Poaceae (grasses; N=33), Myrtaceae (myrtles; N = 26), Apocynaceae (dogbanes), Bignoniaceae (bignonias), Melastomataceae (melastomes), Orchidaceae (orchids) and Sapindaceae (soapberries) (equal 10th, N=25 each); **Figures 2A-C, Figure 3,** Supplementary Material, **Figure S1)**. All these families are highly diverse in number of genera and species, and all have pantropical distributions (Bramley and Utteridge, 2014).

In the Neotropics, the plant families for which the greatest proportion of total genera occurring in the region are visited by stingless bees were Myrtaceae (stingless bees are reported to visit 55% of all genera described for the Neotropics), Arecaceae (44%) and Bignoniaceae (43%) (Supplementary Material, **Table S4**).

Several plant families are reported to be commonly used by the stingless bees of all geographic regions (e.g. Fabaceae, Asteraceae, Euphorbiaceae); **Figure 3**. Nevertheless, there were differences in the composition of reported plants (at family level) visited by the stingless bees of each biogeographical region (PERMANOVA: $P = 0.01$, $R^2 = 0.07$, **Figure 4,** Supplementary Material, **Table S5**). This trend was driven particularly by differences between the floral use of Neotropical and Indo-Malayan-Australasian stingless bee fauna, presumably reflecting differences in the plant communities available to bees in these two regions ($P = 0.015$, R2=0.053). Afrotropical stingless bee floral use (at plant family level) did not differ significantly from either other region (Indo-Malayan-Australasian: $P = 0.064$, R2=0.091; Neotropical: $P = 0.072$, R2=0.043).

Traits of highly visited plant genera

Across all visited plants, stingless bees visited genera of plants spanning a variety of common type of growth (herbs, trees, shrubs, vines, lianas) and including economically important plants such as crops, timber, fibres, medicinal and ornamental use (Supplementary Material, **Table S3**).

In addition to native flora, stingless bees were reported to visit non-native plants (**Figure 5**). We identified 109 genera of plants that are not native to the region where the bee-plant interaction was reported (52 in the Neotropics, 17 in the Afrotropics and 39 in the Indo-Malayan-Australasian region; Supplementary Material, **Table S6**). Of these 19 (18%) were common edible crops, such as rambutan (*Nephelium* sp.), lychee (*Litchi* sp.) and coffee (*Coffea* sp.) in the Neotropics, mango (*Mangifera* sp.), hog-plum (*Spondias* sp.) and corn (*Zea* sp.) in the Afrotropics, and oil-Palm (*Elaeis* sp.), tamarind (*Tamarindus* sp.) and guava (*Psidium* sp) in the Indo-Malayan-Australasian region. The remaining genera were non-native garden and ornamental plants, or have been documented as weeds (CABI, 2020); these included numerous genera of Lamiaceae (mint, sage etc.) and Asteraceae (daysies, dandelions, whiteweed, etc).

Discussion

We consolidated records of floral visitation of wild plants by stingless bees around the world. These records highlight the wide variety of plants used as forage by individual stingless bee

species (as many 535 plant genera for *Trigona spinipes*), and by the Meliponini in general. Worldwide, stingless bees visit over 1435 genera from 250 families of flowering plants, around 62% of all angiosperm families (The Plant List, 2010). This summary of floral use therefore supports the view that stingless bees play an important role in ecosystem functioning throughout their native range, as pollinators of many tropical and subtropical plants.

Plants used by stingless bees

Globally, stingless bees forage from the flowers of 25 or more genera from each of 13 major tropical plant families: Fabaceae, Asteraceae, Rubiaceae, Malvaceae, Euphorbiaceae, Arecaceae, Lamiaceae, Poaceae, Bignoniaceae, Myrtaceae, Sapindaceae, Apocynaceae and Melastomataceae. The frequent use of several of these plant families has been previously reported for various stingless bee species of the Neotropics, which commonly use Fabaceae, Asteraceae, Myrtaceae, Malvaceae and Arecaceae (Antonini et al., 2006, Ramalho, 1990, Miranda et al., 2015, Ramalho et al., 1989, Cortopassi-Laurino and Ramalho, 1988, Faria et al., 2012, Aleixo et al., 2013, Ramalho et al., 1985, Guibu et al., 1988). We find that most of the plant families reported to be commonly visited by stingless bees are found in every tropical region (Americas, Africa, India, Austral-Asia), suggesting that broad foraging preferences are shared across each of the three major stingless bee clades. However, based on available reported floral visitations, the *relative* use of different plant families differs between the stingless bees from the Indo-Malayan-Australasian and Neotropical regions. This likely indicates, at least in part, differences in the abundance and availability of each plant family in the different regions. For example, bignonias (Bignoniaceae) appear more frequently in visitation records of Neotropical stingless bees than those of other regions, and the greatest diversity of bignonias is also in the Neotropics (Gentry, 1980, Gentry, 1992). It remains to be investigated whether some differences in floral use between regions also reflect different foraging preferences of the stingless bees in each clade, or different coevolutionary histories between the bees and flora of each region.

Floral morphology regulates the accessibility to floral resources for animal visitors. Many of the plant families commonly visited by stingless bees have floral traits that have evolved to favour animal visitation, and bee visitation in particular. For instance, the flowers of Myrtaceae, Arecaceae, Asteraceae, Malvaceae and Fabaceae (Subfamily: Mimosoideae) have open corollas with many stamens and longitudinally opened anthers, which facilitates the

acquisition of pollen and nectar by bees (Lewis, 2005, Torres and Galetto, 2002). It is possible that most of the plant genera visited by stingless bees are themselves "generalists" with respect to pollinators and all attract other pollinating insects or vertebrates." This should however be confirmed with more pollination studies on these plant genera. Notably, stingless bees do not visit some flower types, such as those with long and narrow corollas (e.g. some species from Lamiaceae) that have instead specialized on one or several species of long-tongued visitors (e.g. Euglossini (orchid bees) (Rodríguez-Gironés and Santamaría, 2006, Borrell, 2005). This morphological match between the traits of flowers and the mouthparts of insects plays a major role in floral resource partitioning (Nagamitsu and Inoue, 2005). Even within a single bee clade such as stingless bees (short tongued bees), some variation in tongue length and tongue shapes may affect their floral preferences (Nagamitsu and Inoue, 2005) and help to avoid competition with sympatric short-tongued bees (Inouye, 1978).

For all three biogeographical regions, Fabaceae and Asteraceae had the most genera visited by stingless bees. These families are also the two most speciose angiosperm families in the world (Christenhusz and Byng, 2016). Asteraceae and Fabaceae tend to dominate as bee-forage plants in tropical areas with open vegetation, including where forests have been cleared for human activities (Ramalho et al., 1990). For example, plants in these families may be the first ones to appear after forest disturbance and clearance (Valdez-Hernández et al., 2014, Citadini-Zanette et al., 2017). The ready use of these plants by stingless bees may thus help to explain their resilience in disturbed habitats, when other sources of forage are not available (Aizen et al., 2012).

Evidence for floral preferences in stingless bees

Stingless bees are generalist foragers of pollen and nectar (Aleixo et al., 2013, Faria et al., 2012, Absy et al., 1984). However, the tropics hold a vast range of angiosperm plant species flowering at the same time, making it a diverse and competitive marketplace for the plants. Floral rewards for pollinators differ strongly between plant species, and also vary over time (Willmer and Stone, 2004, Heinrich, 2004), encouraging some level of specialisation even among foragers that use a wide variety of plants. Each pollinator thus becomes receptive to particular flower traits in their search for food, such as flower color, morphology, scent, and temperature (Heinrich, 2004, Menzel, 1985, Dyer et al., 2006). Consistent with this, past

studies done in the Neotropics have indicated that stingless bees are not indiscriminate generalists, but rather that at least some neotropical genera (*Scaptotrigona* and *Melipona*) show preferences for certain families of plants such as Sapindaceae, Arecaceae, Solanaceae, Myrtaceae, Asteraceae, Melastomataceae Fabaceae and Convolvulaceae (Antonini et al., 2006, Wilms and Wiechers, 1997, Ramalho et al., 1989, da Luz et al., 2019). There is little current information on regions outside the Neotropics, nor on how these floral preferences are induced in stingless bees in their natural habitats. As they have perennial nests with overlapping generations, stingless bees may simply favour the most predictable floral resources in their particular locale. For example, in the Neotropics, the most frequently visited species belong to families that flower all year-round (Antonini et al., 2006).

Bee body size may also be a key predictor of floral preferences. Body size is related to foraging distance (Araújo et al., 2004, Greenleaf et al., 2007) and determines the spatial scale at which species are able to visit flowering plants and tolerate spatial and temporal changes in floral resource availability (Borges et al., 2020). In theory, larger bees might therefore have the scope to be choosier when it comes to floral resources, while smaller species may be more constrained to forage on the plants close to their nest. The Neotropical genera *Melipona* and *Trigona* include some of the largest stingless bees, and these genera also provide some of the clearest evidence for specialisation in floral preferences (Antonini et al., 2006, Nagamitsu and Inoue, 2005, Ramalho et al., 1989). In a study in Brazil, *Melipona* only visited 21% out of all the plants in flower within their area of foraging. Both body size and colony size (i.e. number of foragers) might also shape floral preferences via competition. For example, species that are small in size, or have few foragers, may choose to exploit flowers less frequently visited by larger, more aggressive bees or colonies (Hubbell and Johnson, 1978, Johnson and Hubbell, 1974, Johnson and Hubbell, 1975, Sommeijer et al., 1983) which tend to monopolize rich resources (Nagamitsu et al., 1999).

Visitation of non-native plants by stingless bees

Stingless bees are reported to visit many plant genera that are not native to their region, even in areas where native vegetation is preserved (da Luz et al., 2019). Stingless bees are thus capable of facilitating the pollination and spread of non-native species (Marvier et al., 2004, Levine et al., 2004, Chytrý et al., 2008). For example, stingless bees in every region visited the genus *Eucalyptus*, which are trees with abundant flowering events that produce high amounts

of nectar and pollen. *Eucalyptus* is native to Australia and cultivated in most tropical regions of the world (Doughty, 2000). Plantations of Eucalypt for timber production in many parts of the world cause habitat fragmentation and loss of biodiversity (Williams, 2015). However, these and other introduced plants may also provide a safe source of food for bees in degraded ecosystems (Hilgert-Moreira et al., 2014). In addition to Eucalypt, Neotropical stingless bees also forage on at least eight genera of Lamiaceae that are not native to their continent, and the stingless bees of Indo-Malayan-Australasia forage on at least 11 introduced genera of Asteraceae. Many species in these two families are considered invasive weeds. For instance, *Sonchus oleraceus* (Asteraceae) and *Leonorus sibiricus* (Lamiaceae) are catalogued as some of the worst to control weeds due to their quick life cycles and production of highly dispersive seeds (Peerzada et al., 2019, Kwon et al., 2016, Holm et al., 1977). Yet it is this same willingness to use novel resources that makes stingless bees good pollinators of some crops. Thus, stingless bees also visit and pollinate a diverse range of economically important plants that are not native to their continent. Across all regions, they visited economically important crops with mass-blooming phenology such as *Coffea* (coffee), *Psidium* (guava), *Mangifera* (mango), *Spondias* (hog-plum), *Tamarindus* (tamarind), among others.

Gaps in our knowledge of stingless bee-plant interactions

Our database includes reported floral visitation by 53% of all stingless bee species (287 species). Thus, the foraging habits of the remaining half of the world's stingless bee fauna remain particularly poorly known. Even the Neotropical stingless bee fauna, for which floral use has been most intensively documented, includes many species for which data is limited. For example, while decades of research in Brazil have pioneered knowledge of stingless bee behaviour and ecology (Giannini et al., 2020), much of Brazil's rich stingless bee diversity still remains little-studied (Campbell et al., 2019).

Data on stingless bee floral use from the Indo-Malayan-Australasian and Afrotropical regions is particularly sparse. With the exception of Australia and New Guinea, honey bees (*Apis* sp) are also native to these regions and are often the focus of traditional beekeeping practices. For example, the Asian hive bee *Apis cerana* is widely kept for honey and pollination throughout Asia and India (Oldroyd and Wongsiri, 2009), while the Western honey bee *Apis mellifera* is native throughout Africa. Perhaps for this reason, research interest in stingless bees as pollinators (of both crops and native plants) has lagged behind South and Central America in

recent decades. It is now steadily increasing in some countries, such as India and Australia (Heard and Dollin, 2000), and knowledge of the bees of these regions is certain to advance in coming years. Many tropical regions in which stingless bee diversity is highest, however, face significant long-term challenges to conserving their ecosystems and pollination services, including high levels of poverty and habitat degradation (Bradshaw et al., 2009). In addition, many stingless bee species in Africa, India, Asia and Australia are cryptic in their morphology, and thus difficult to ID reliably in the field. For example, in Australia, three common species with overlapping distributions are identical in forager appearance, though each are genetically distinct and build unique nest structures (*Tetragonula* sp. of the "carbonaria complex"; (Heard, 2016).

Another key knowledge gap is the extent to which stingless bees contribute to the reproduction of the many plants they visit, including how habitat composition impacts the pollination of wild plants by stingless bees at the landscape level. Not all floral visitation results in pollination, and not all pollinators are equally efficient on a per visit basis (King et al., 2013). Pollination studies are time-intensive and difficult in the field, and most confirmed pollination by stingless bees is for crop species (Slaa et al., 2006). Where pollination of wild plants is considered, it is often in regions adjacent to crops. Yet the presence of mass-flowering crops can jeopardize the fitness of concurrently flowering wild plants by diverting pollinators, even where mass-flowering crops enhance overall abundances of generalist pollinators (Holzschuh et al., 2011). Whether stingless bees impact plant reproduction in other ways is also poorly understood. There is evidence that at least three species of stingless bees are seed dispersers for plants (mellitochory) which have evolved seeds that offer resin rewards to the bees (Wallace and Trueman, 1995, Garcia et al., 1992).

Conclusion

Tropical regions support many biodiversity hotspots (Myers et al., 2000), including a high diversity and endemism of both stingless bees and flowering plants (Hawkins et al., 2011, Antonelli et al., 2015). Just as stingless bees rely on plants as food sources for pollen and nectar, plants rely on stingless bees and other pollinators for reproduction. Here, we consider for the first time stingless plant-bee interactions at a broad, global scale, focusing on patterns at the level of bee genera and plant families. Ultimately, a rich understanding of stingless bee floral

use will require continued study at local levels for the many and diverse ecosystems of the tropics and subtropics. Our database aims to provide an easy-reference and helpful initial resource for such future studies on the interactions between the wild endemic, endangered or invasive plant species and their stingless bee visitors.

Supplementary information

Table S1. Database of reported interactions between stingless bees and flowering plants. This database can be accessed here:

https://datadryad.org/stash/share/XDmAqN_sxG2qf91qiK040SOHlRP4IKrsWsmoX00UxRA.

Table S2. The 15 plant families with the most reported genera visited by stingless bees worldwide, and for each of three regions (Neotropical, Afrotropical and Indo-Malayan-Australasian); the total genera reported per family worldwide (The Plant List, 2010), the total visited and the proportion of all genera in the plant family known to be visited by stingless bees based on reported interactions in the literature.

		Genera		
Region	Plant Family	Total	Visited	Proportion (%)
All regions	Bignoniaceae	80	33	41
All regions	Anacardiaceae	79	21	27
All regions	Arecaceae	182	43	24
All regions	Malvaceae	250	56	22
All regions	Fabaceae	768	166	22
All regions	Myrtaceae	130	28	22
All regions	Euphorbiaceae	227	45	20
All regions	Sapindaceae	142	27	19
All regions	Lamiaceae	236	35	15
All regions	Melastomataceae	174	25	14
All regions	Rubiaceae	601	66	11
All regions	Asteraceae	1,679	126	8
All regions	Apocynaceae	359	26	7
All regions	Poaceae	756	35	5
All regions	Orchidaceae	735	25	3
Neotropical	Bignoniaceae	80	20	25
Neotropical	Malpighiaceae	76	17	22
Neotropical	Arecaceae	182	32	18
Neotropical	Malvaceae	250	43	17
Neotropical	Myrtaceae	130	20	15
Neotropical	Fabaceae	768	118	15
Neotropical	Sapindaceae	142	20	14
Neotropical	Euphorbiaceae	227	27	12
Neotropical	Melastomataceae	174	20	11

Neotropical	Lamiaceae	236	27	11
Neotropical	Acanthaceae	207	17	8
Neotropical	Rubiaceae	601	40	7
Neotropical	Asteraceae	1,679	103	6
Neotropical	Apocynaceae	359	20	6
Neotropical	Poaceae	756	18	2
Indo-Malayan-Australasian	Anacardiaceae	79	9	11
Indo-Malayan-Australasian	Myrtaceae	130	13	10
Indo-Malayan-Australasian	Cucurbitaceae	101	9	9
Indo-Malayan-Australasian	Euphorbiaceae	227	20	9
Indo-Malayan-Australasian	Malvaceae	250	21	8
Indo-Malayan-Australasian	Arecaceae	182	13	7
Indo-Malayan-Australasian	Sapindaceae	142	10	7
Indo-Malayan-Australasian	Fabaceae	768	47	6
Indo-Malayan-Australasian	Rutaceae	150	9	6
Indo-Malayan-Australasian	Lamiaceae	236	12	5
Indo-Malayan-Australasian	Acanthaceae	207	7	3
Indo-Malayan-Australasian	Rubiaceae	601	20	3
Indo-Malayan-Australasian	Apocynaceae	359	8	2
Indo-Malayan-Australasian	Asteraceae	1,679	33	2
Indo-Malayan-Australasian	Orchidaceae	735	8	1
Afrotropical	Rhamnaceae	61	4	7
Afrotropical	Anacardiaceae	79	5	6
Afrotropical	Euphorbiaceae	227	11	5
Afrotropical	Fabaceae	768	33	4
Afrotropical	Lamiaceae	236	8	3
Afrotropical	Rutaceae	150	5	3
Afrotropical	Sapindaceae	142	4	3
Afrotropical	Malvaceae	250	6	2
Afrotropical	Rubiaceae	601	14	2
Afrotropical	Arecaceae	182	4	2
Afrotropical	Amaranthaceae	187	4	2
Afrotropical	Poaceae	756	10	1
Afrotropical	Asteraceae	1,679	20	1
Afrotropical	Orchidaceae	735	4	1

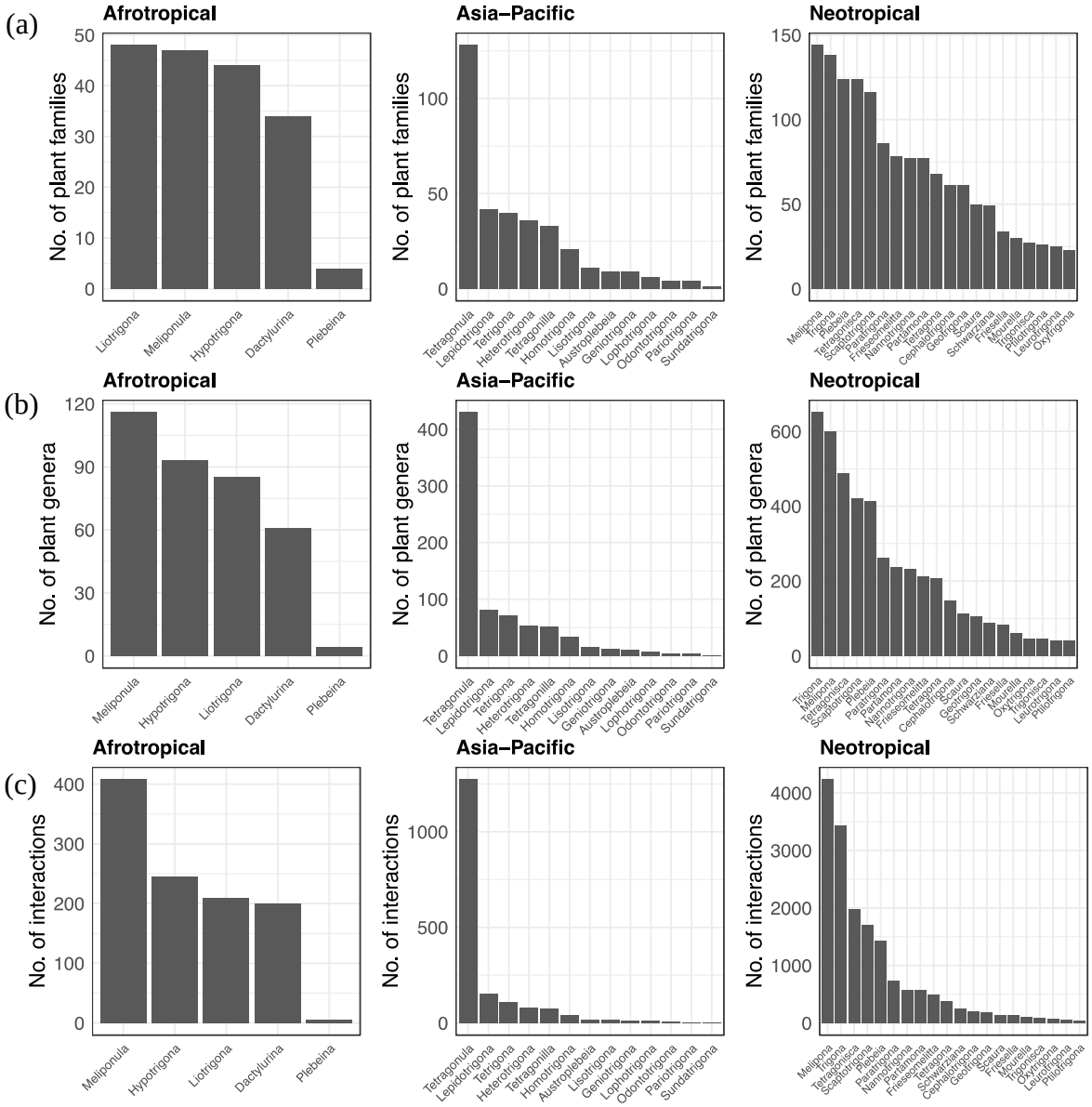


Figure S1. Histograms with the counts a of plant families visited by stingless bee genera per geographical region. b) of plant genera visited by stingless bee genera per geographical region c) with the total number of plant and stingless bee interactions ordered by stingless bee genera.

Table S3. Plant genera with the most recorded visitations by stingless bees in three biogeographical regions (POWO, 2019). * IMA region = Indo-Malayan-Australasian

Region*	Family	Genera	Distribution	Growth type	Crop name	Other use
Afrotropical	Fabaceae	Acacia	Native	Shrub/Tree		Timber and sap
IMA	Fabaceae	Acacia	Native	Shrub/Tree		Timber and sap
Neotropical	Fabaceae	Acacia	Native	Shrub/Tree		Timber and sap
IMA	Asteraceae	Acmella	Native	Herb		
Afrotropical	Passifloraceae	Adenia	Native	Herb/Shrub/Tree/Liana/Vine		
Afrotropical	Asteraceae	Ageratum	Non-native	Herb/Shrub		Ornamental and medicinal
IMA	Asteraceae	Ageratum	Non-native	Herb/Shrub		Ornamental and medicinal
Neotropical	Euphorbiaceae	Alchornea	Native	Shrub/Tree		
IMA	Amaryllidaceae	Allium	Native	Herb	Onion, garlic, scallion, shallot, leek, and chives.	Medicinal
Afrotropical	Amaranthaceae	Amaranthus	Native	Herb	Grains	
Afrotropical	Annonaceae	Annona	Native		Custard apple, soursop	Medicinal
IMA	Phyllanthaceae	Antidesma	Native	Shrub/Tree		
Afrotropical	Polygonaceae	Antigonon	Non-native	Vines		
IMA	Primulaceae	Ardisia	Native	Shrub/Tree	Drupe fruits	Medicinal
Afrotropical	Acanthaceae	Asystasia	Native	Herb		Ornamental and food
IMA	Acanthaceae	Asystasia	Native	Herb		Ornamental and food
Afrotropical	Oxalidaceae	Averrhoa	Non-Native	Shrub/Tree	Star-fruit	
IMA	Oxalidaceae	Averrhoa	Native	Shrub/Tree	Star-fruit	
IMA	Meliaceae	Azadirachta	Native	Tree	Neem oil	Medicinal
Neotropical	Asteraceae	Baccharis	Native	Shrub		
Neotropical	Fabaceae	Bauhinia	Native	Herb/Shrub/Tree/Liana		Ornamental and food
Afrotropical	Asteraceae	Bidens	Native	Herb		
IMA	Asteraceae	Bidens	Native	Herb		

Neotropical	Bixaceae	Bixa	Native	Shrub	Annatto	
IMA	Euphorbiaceae	Blumeodendron	Native	Tree		
Afrotropical	Arecaceae	Borassus	Native	Tree		Leaves for crafts and sweet sap
Afrotropical	Rubiaceae	Borreria	Native	Herb		Medicinal
Neotropical	Rubiaceae	Borreria	Native	Herb		Medicinal
IMA	Brassicaceae	Brassica	Native	Herb/Shrub	Canola, brown mustard, Chinese cabbage, turnip, cabbage, cauliflower, broccoli, etc.	
Afrotropical	Asteraceae	Brenandendron	Native	Herb		
Afrotropical	Phyllanthaceae	Bridelia	Native	Shrub/Tree		Medicinal
Neotropical	Malpighiaceae	Byrsonima	Native	Shrub/Tree	Nance	
Afrotropical	Fabaceae	Caesalpinia	Native	Tree/Shrub/Liana		Ornamental
Neotropical	Fabaceae	Caesalpinia	Native	Tree/Shrub/Liana		Ornamental
Afrotropical	Fabaceae	Calliandra	Native	Shrub/Tree		Ornamental
IMA	Myrtaceae	Callistemon	Native	Shrub/Tree		Ornamental
IMA	Solanaceae	Capsicum	Non-native	Herb/Shrub	Chilli	
Neotropical	Salicaceae	Casearia	Native	Shrub/Tree	Chilli	Medicinal
Afrotropical	Fabaceae	Cassia	Native	Tree/Shrub		Ornamental and use in reforestation projects
IMA	Fabaceae	Cassia	Native	Tree/Shrub		Ornamental and use in reforestation projects
Neotropical	Fabaceae	Cassia	Native	Tree/Shrub		Ornamental and use in reforestation projects
Neotropical	Urticaceae	Cecropia	Native	Tree		Ornamental and use in reforestation projects
Neotropical	Fabaceae	Chamaecrista	Native	Tree/Shrub		Ornamental and use in reforestation projects
Afrotropical	Vitaceae	Cissus	Native	Liana		Ornamental/Medicinal
Afrotropical	Rutaceae	Citrus	Non-native	Tree/Shrub	Citrus fruits	Ornamental
IMA	Rutaceae	Citrus	Native	Tree/Shrub	Citrus fruits	Ornamental
Neotropical	Rutaceae	Citrus	Non-native	Tree/Shrub	Citrus fruits	Ornamental

IMA	Cleomaceae	Cleome	Native	Herb/Shrub		
Neotropical	Polygonaceae	Coccoloba	Native	Shrub/Tree/Liana		
Afrotropical	Bixaceae	Cochlospermum	Native	Shrub/Tree		
IMA	Arecaceae	Cocos	Native	Tree	Coconut	Ornamental
Neotropical	Arecaceae	Cocos	Native	Tree	Coconut	Ornamental
Afrotropical	Rubiaceae	Coffea	Native	Shrub	Coffee	
IMA	Rubiaceae	Coffea	Native	Shrub	Coffee	
Neotropical	Rubiaceae	Coffea	Non-native	Shrub	Coffee	
Afrotropical	Combretaceae	Combretum	Native	Shrub/Tree		Medicinal
Neotropical	Boraginaceae	Cordia	Native	Shrub/Tree		Medicinal, Timber and sap
Afrotropical	Rubiaceae	Crossopteryx	Native	Shrub/Tree		Medicinal
Afrotropical	Fabaceae	Crotalaria	Native	Herb/Shrub	Mitoo	Ornamental
IMA	Fabaceae	Crotalaria	Native	Herb/Shrub	Mitoo	Ornamental
IMA	Euphorbiaceae	Croton	Native	Herb/Shrub/Tree/Liana		Ornamental and use of bark
Neotropical	Euphorbiaceae	Croton	Native	Herb/Shrub/Tree/Liana		Ornamental and use of bark
Neotropical	Cucurbitaceae	Cucurbita	Native	Herb/Vine	Squash, pumpkin, cucumber, etc.	
Neotropical	Sapindaceae	Cupania	Native	Shrub/Tree		
Afrotropical	Araliaceae	Cussonia	Native	Shrub/Tree		Medicinal
Afrotropical	Cyperaceae	Cyperus	Native	Herb		
Afrotropical	Burseraceae	Dacryodes	Native	Shrub/Tree		
IMA	Orchidaceae	Dendrobium	Native	Herb		Ornamental
Afrotropical	Euphorbiaceae	Dichostemma	Native	Shrub/Tree		
IMA	Dilleniaceae	Dillenia	Native	Shrub/Tree		
IMA	Ebenaceae	Diospyros	Native	Shrub/Tree	Persimmon	Ornamental and use of bark
Neotropical	Verbenaceae	Duranta	Native	Shrub/Tree		Ornamental

Afrotropical	Asteraceae	Elephantopus	Native	Herb		Medicinal
Afrotropical	Fabaceae	Entada	Native	Shrub/Tree/Liana		Medicinal
Afrotropical	Myrtaceae	Eucalyptus	Non-native	Shrub/Tree		Ornamental and medicinal
IMA	Myrtaceae	Eucalyptus	Native	Shrub/Tree		Ornamental and medicinal
Neotropical	Myrtaceae	Eucalyptus	Non-native	Shrub/Tree		Ornamental and medicinal
IMA	Myrtaceae	Eugenia	Native	Shrub/Tree	Edible fruit	Ornamental
Neotropical	Myrtaceae	Eugenia	Native	Shrub/Tree	Edible fruit	Ornamental
Afrotropical	Euphorbiaceae	Euphorbia	Native	Herb/Shrub/Tree		Ornamental
Neotropical	Euphorbiaceae	Euphorbia	Native	Herb/Shrub/Tree		Ornamental
Neotropical	Arecaceae	Euterpe	Native	Tree	Açai berry	
IMA	Gentianaceae	Fagraea	Native	Shrub/Tree		Ornamental and medicinal
IMA	Moraceae	Ficus	Native	Tree/Shrub/Vine		Ornamental
Neotropical	Rosaceae	Fragaria	Native	Herb	Strawberry	
Afrotropical	Rhamnaceae	Gouania	Native	Shrub/Liana		
IMA	Tiliaceae	Grewia	Native	Shrub/Tree		Medicinal
Afrotropical	Asteraceae	Guizotia	Native	Herb	Oil and edible seeds	
Afrotropical	Hypericaceae)	Harungana	Native	Shrub/Tree		Medicinal
IMA	Asteraceae	Helianthus	Non-native	Herb	Edible seeds and oil	Ornamental
Neotropical	Asteraceae	Helianthus	Native	Herb	Edible seeds and oil	Ornamental
IMA	Malvaceae	Hibiscus	Native	Herb/Shrub/Tree	Edible flowers	Ornamental and fibres
Neotropical	Lamiaceae	Hyptis	Native	Herb/Shrub/Tree		Medicinal
Afrotropical	Fabaceae	Indigofera	Native	Herb/Shrub/Tree		Ornamental
Neotropical	Fabaceae	Inga	Native	Shrub/Tree		Ornamental
Afrotropical	Convolvulaceae	Ipomoea	Native	Herb/Shrub/Tree/Liana		Ornamental, sweet potato and water spinach

IMA	Convolvulaceae	Ipomoea	Native	Herb/Shrub/Tree/liana		Ornamental, sweet potato and water spinach
IMA	Rubiaceae	Ixora	Native	Shrub/Tree		Ornamental
IMA	Euphorbiaceae	Jatropha	Native	Shrub/Tree		Fibres
IMA	Lythraceae	Lagerstroemia	Native	Shrub/Tree		Ornamental
Afrotropical	Fabaceae	Leucaena	Non-native	Tree/Shrub	Edible fruits	Timber and reforestation
IMA	Fabaceae	Leucaena	Non-native	Tree/Shrub	Edible fruit	Timber and reforestation
Afrotropical	Anacardiaceae	Mangifera	Non-native	Shrub/Tree	Mango	
IMA	Anacardiaceae	Mangifera	Native	Shrub/Tree	Mango	
Afrotropical	Bignoniaceae	Markhamia	Native	Shrub/Tree		Medicinal
IMA	Melastomataceae	Melastoma	Native	Shrub		Ornamental
IMA	Convolvulaceae	Merremia	Native	Herb		Ornamental and medicinal
Neotropical	Melastomataceae	Miconia	Native	Shrub/Tree		Timber
Neotropical	Asteraceae	Mikania	Native	Herb		Medicinal
IMA	Fabaceae	Millettia	Native	Shrub/Tree		
IMA	Fabaceae	Mimosa	Native	Herb/Shrub		Ornamental, pioneer trees and Timber
Neotropical	Fabaceae	Mimosa	Native	Herb/Shrub		Ornamental, pioneer trees and Timber
IMA	Muntingiaceae	Muntingia	Non-native	Shrub/Tree	Edible fruit	Medicinal
IMA	Commelineae	Murdannia	Native	Herb		
Afrotropical	Musaceae	Musa	Non-native	Herb	Banana	
Neotropical	Myrtaceae	Myrcia	Native	Shrub/Tree		
IMA	Sapindaceae	Nephelium	Native	Shrub/Tree	Litchi	
Neotropical	Lauraceae	Ocotea	Native			Timber
Afrotropical	Passifloraceae	Passiflora	Native	Herb/Shrub/Vine	Passion fruit	Ornamental
IMA	Passifloraceae	Passiflora	Native	Herb/Shrub/Vine	Passion fruit	Ornamental
Neotropical	Passifloraceae	Passiflora	Native	Herb/Shrub/Vine	Passion fruit	Ornamental

IMA	Fabaceae	Peltophorum	Native	Shrub/Tree		Medicinal and Timber
Neotropical	Lauraceae	Persea	Native	Shrub/Tree	Avocado	
IMA	Urticaceae	Pipturus	Native	Shrub/Tree		Medicinal
IMA	Rosaceae	Prunus	Native		Apricot, plum, peach, etc.	Ornamental and Timber
Afrotropical	Myrtaceae	Psidium	Non-native	Tree/Shrub	Guava	
IMA	Myrtaceae	Psidium	Non-native	Tree/Shrub	Guava	
Neotropical	Myrtaceae	Psidium	Native	Tree/Shrub	Guava	
IMA	Rosaceae	Rubus	Native	Herb/Shrub	Raspberry	
IMA	Burseraceae	Santiria	Native	Shrub/Tree		
Neotropical	Araliaceae	Schefflera	Native	Shrub/Tree		
Neotropical	Anacardiaceae	Schinus	Native	Shrub/Tree		Medicinal
IMA	Fabaceae	Senna	Native	Herb/Shrub/Tree		Ornamental
Neotropical	Fabaceae	Senna	Native	Herb/Shrub/Tree		Ornamental
Neotropical	Sapindaceae	Serjania	Native	Liana/Vine		
Afrotropical	Pedaliaceae	Sesamum	Native	Herb	Sesame seeds	
IMA	Solanaceae	Solanum	Native	Herb/Tree/Shrub/Vine	Tomato, Eggplant, Potato	Ornamental
Neotropical	Solanaceae	Solanum	Native	Herb/Tree/Shrub/Vine	Tomato, Eggplant, Potato	Ornamental
Afrotropical	Lamiaceae	Solenostemon	Native	Herb	Tuber crop	
Afrotropical	Rubiaceae	Spermacoce	Native	Herb		Medicinal
Neotropical	Anacardiaceae	Spondias	Native	Shrub/Tree	Hog plum	
Afrotropical	Verbenaceae	Stachytarpheta	Native	Herb/Tree/Shrub/Vine		
Neotropical	Loranthaceae	Struthanthus	Native	Shrub		Medicinal
Neotropical	Arecaceae	Syagrus	Native	Tree	Edible fruit	
IMA	Asteraceae	Synotis	Native	Herb		
Afrotropical	Myrtaceae	Syzygium	Native	Shrub/Tree	Roseapple	
IMA	Myrtaceae	Syzygium	Native	Shrub/Tree	Roseapple	
IMA	Lamiaceae	Tectona	Native	Shrub/Tree		Teak timber
IMA	Combretaceae	Terminalia	Native	Shrub/Tree		Medicinal

Neotropical	Malvaceae	Theobroma	Native	Shrub/Tree	Cacao	
IMA	Acanthaceae	Thunbergia	Native	Herb/Shrub		
Neotropical	Melastomataceae	Tibouchina	Native	Herb/Shrub/Tree		
IMA	Asteraceae	Tridax	Non-native	Herb		Medicinal
Afrotropical	Malvaceae	Urena	Native	Shrub/Tree		Medicinal
Afrotropical	Asteraceae	Vernonia	Native	Herb/Shrub/Tree/Liana		Medicinal, edible leaves, ornamental
Neotropical	Asteraceae	Vernonia	Native	Herb/Shrub/Tree/Liana		Medicinal, edible leaves, ornamental
IMA	Sapindaceae	Xerospermum	Native	Shrub/Tree		
Afrotropical	Poaceae	Zea	Non-native	Herb	Corn	
Neotropical	Burseraceae	Protium	Native	Shrub/Tree	Edible fruit	Medicinal and Timber
Neotropical	Rubiaceae	Psychotria	Native	Herb/Shrub/Tree		
Neotropical	Malvaceae	Sida	Native	Herb/Shrub/Tree		

Table S4. The 15 plant families with the most reported genera visited by stingless bees in the Neotropics; and the proportion of all genera reported for the Neotropics (Milliken, 2009) in the plant family known to be visited by stingless bees, based on reported interactions in the literature.

	Genera	Genera visited in the Neotropics			
Plant Family	Worldwide	Neotropics	Native	Non-native	Proportion (%)
Acanthaceae	207	NA	15	2	NA
Apocynaceae	359	50	18	2	36
Arecaceae	182	68	30	2	44*
Asteraceae	1,680	589	91	12	15
Bignoniaceae	80	77	18	2	43
Euphorbiaceae	227	82	26	1	32
Fabaceae	767	314	112	6	36
Lamiaceae	236	65	19	8	29
Malpighiaceae	76	59	17	0	29
Malvaceae	250	129	39	4	30
Melastomataceae	174	107	20	0	19
Myrtaceae	130	29	16	4	55*
Poaceae	756	288	16	2	6
Rubiaceae	601	220	37	3	17
Sapindaceae	142	38	16	4	42*

Table S5. PERMANOVA (Permutational Multivariate Analysis of Variance) results comparing the differences in floral preferences (plant family) between the three biogeographical subclades of stingless bees (Afrotropical, Indo-Malayan-Australasian and Neotropics), based on reported bee-plant interactions in the literature.

	F.Model	R^2	*P* (adjusted)
Afrotropical vs Indo-Malayan-Australasian	1.585	0.091	0.0645
Afrotropical vs Neotropical	1.522	0.043	0.072
Indo-Malayan-Australasian vs Neotropical	2.374	0.0535	*0.015

Table S6. List of invasive plant genera (POWO, 2019) visited by the stingless bees from the three biogeographical regions. The common edible crop names are at the top of each region.

Region	Family	Non-native genera	Crop name
Neotropical	Lamiaceae	*Origanum*	Oregano
Neotropical	Fabaceae	*Lablab*	Hyacinth bean
Neotropical	Asteraceae	*Phoenix*	Date
Neotropical	Sapindaceae	*Litchi*	Litchi
Neotropical	Sapindaceae	*Nephelium*	Rambutan
Neotropical	Poaceae	*Coix*	Job's tears grain
Neotropical	Rubiaceae	*Coffea*	Coffee
Neotropical	Asteraceae	*Helianthus*	Sunflower seeds
Neotropical	Malvaceae	*Abelmoschus*	Okra
Neotropical	Malvaceae	*Bombax*	
Neotropical	Malvaceae	*Dombeya*	
Neotropical	Malvaceae	*Grewia*	
Neotropical	Lamiaceae	*Tectona*	
Neotropical	Lamiaceae	*Congea*	
Neotropical	Lamiaceae	*Holmskioldia*	
Neotropical	Lamiaceae	*Leonurus*	
Neotropical	Lamiaceae	*Melissa*	
Neotropical	Lamiaceae	*Solenostemon*	
Neotropical	Lamiaceae	*Tetradenia*	
Neotropical	Fabaceae	*Adenanthera*	
Neotropical	Fabaceae	*Alysicarpus*	
Neotropical	Fabaceae	*Julbernardia*	
Neotropical	Fabaceae	*Pueraria*	
Neotropical	Fabaceae	*Spartium*	
Neotropical	Arecaceae	*Archontophoenix*	
Neotropical	Euphorbiaceae	*Triadica*	
Neotropical	Sapindaceae	*Harpullia*	
Neotropical	Sapindaceae	*Koelreuteria*	
Neotropical	Myrtaceae	*Callistemon*	
Neotropical	Myrtaceae	*Corymbia*	
Neotropical	Myrtaceae	*Eucalyptus*	
Neotropical	Myrtaceae	*Syzygium*	
Neotropical	Acanthaceae	*Geissomeria*	
Neotropical	Acanthaceae	*Thunbergia*	
Neotropical	Apocynaceae	*Catharanthus*	
Neotropical	Apocynaceae	*Nerium*	
Neotropical	Rubiaceae	*Gardenia*	
Neotropical	Rubiaceae	*Pentas*	
Neotropical	Bignoniaceae	*Podranea*	
Neotropical	Bignoniaceae	*Spathodea*	

Neotropical	Asteraceae	*Aster*	
Neotropical	Asteraceae	*Bellis*	
Neotropical	Asteraceae	*Calendula*	
Neotropical	Asteraceae	*Chrysanthemum*	
Neotropical	Asteraceae	*Cichorium*	
Neotropical	Asteraceae	*Cyanthillium*	
Neotropical	Asteraceae	*Cynara*	
Neotropical	Asteraceae	*Gazania*	
Neotropical	Asteraceae	*Gerbera*	
Neotropical	Asteraceae	*Guizotia*	
Neotropical	Asteraceae	*Sonchus*	
Neotropical	Poaceae	*Schizostachyum*	
Afrotropical	Anacardiaceae	*Mangifera*	Mango
Afrotropical	Anacardiaceae	*Spondias*	Hog-plum
Afrotropical	Poaceae	*Zea*	Corn
Afrotropical	Rutaceae	*Citrus*	Citrus fruit
Afrotropical	Asteraceae	*Ageratum*	
Afrotropical	Asteraceae	*Flaveria*	
Afrotropical	Asteraceae	*Galinsoga*	
Afrotropical	Asteraceae	*Synedrella*	
Afrotropical	Asteraceae	*Vernonanthura*	
Afrotropical	Euphorbiaceae	*Manihot*	
Afrotropical	Fabaceae	*Caesalpinia*	
Afrotropical	Fabaceae	*Calliandra*	
Afrotropical	Fabaceae	*Leucaena*	
Afrotropical	Malvaceae	*Ceiba*	
Afrotropical	Malvaceae	*Pachira*	
Afrotropical	Rubiaceae	*Casasia*	
Afrotropical	Rubiaceae	*Genipa*	
Indo-Malayan-Australasian	Fabaceae	*Phaseolus*	Bean
Indo-Malayan-Australasian	Cucurbitaceae	*Cucurbita*	Cucurbits
Indo-Malayan-Australasian	Myrtaceae	*Psidium*	Guava
Indo-Malayan-Australasian	Arecaceae	*Elaeis*	Oil-Palm
Indo-Malayan-Australasian	Amaranthaceae	*Spinacia*	Spinach
Indo-Malayan-Australasian	Asteraceae	*Helianthus*	Sunflower seeds
Indo-Malayan-Australasian	Fabaceae	*Tamarindus*	Tamarind
Indo-Malayan-Australasian	Amaryllidaceae	*Hippeastrum*	
Indo-Malayan-Australasian	Amaryllidaceae	*Nerine*	
Indo-Malayan-Australasian	Anacardiaceae	*Schinus*	
Indo-Malayan-Australasian	Anacardiaceae	*Tapirira*	
Indo-Malayan-Australasian	Apocynaceae	*Thevetia*	
Indo-Malayan-Australasian	Asteraceae	*Ageratum*	
Indo-Malayan-Australasian	Asteraceae	*Calendula*	

Indo-Malayan-Australasian	Asteraceae	*Chromolaena*
Indo-Malayan-Australasian	Asteraceae	*Cosmos*
Indo-Malayan-Australasian	Asteraceae	*Crassocephalum*
Indo-Malayan-Australasian	Asteraceae	*Gaillardia*
Indo-Malayan-Australasian	Asteraceae	*Guizotia*
Indo-Malayan-Australasian	Asteraceae	*Tagetes*
Indo-Malayan-Australasian	Asteraceae	*Tridax*
Indo-Malayan-Australasian	Asteraceae	*Verbesina*
Indo-Malayan-Australasian	Euphorbiaceae	*Hevea*
Indo-Malayan-Australasian	Euphorbiaceae	*Manihot*
Indo-Malayan-Australasian	Euphorbiaceae	*Ricinus*
Indo-Malayan-Australasian	Euphorbiaceae	*Sapium*
Indo-Malayan-Australasian	Fabaceae	*Caesalpinia*
Indo-Malayan-Australasian	Fabaceae	*Calliandra*
Indo-Malayan-Australasian	Fabaceae	*Delonix*
Indo-Malayan-Australasian	Fabaceae	*Gliricidia*
Indo-Malayan-Australasian	Fabaceae	*Leucaena*
Indo-Malayan-Australasian	Fabaceae	*Psophocarpus*
Indo-Malayan-Australasian	Lamiaceae	*Anisomeles*
Indo-Malayan-Australasian	Lamiaceae	*Hyptis*
Indo-Malayan-Australasian	Malvaceae	*Ceiba*
Indo-Malayan-Australasian	Malvaceae	*Malvastrum*
Indo-Malayan-Australasian	Myrtaceae	*Syncarpia*
Indo-Malayan-Australasian	Rubiaceae	*Hamelia*
Indo-Malayan-Australasian	Rubiaceae	*Vangueria*

CHAPTER 3

Long-distance dispersal of males in a subtropical pollinator, the stingless bee *Tetragonula carbonaria*

Males from *Tetragonula carbonaria*. From left to right: Once the males reach sexual maturity they leave their hives in search of a receptive virgin queen. Once they find a receptive virgin queen, they form aerial aggregations during the day and during cold days or nights they cluster by perching in branches nearby.

Abstract

The conservation of native pollinators is aided by knowledge of their dispersal ability, as dispersal is a key behaviour shaping gene flow and thus population resilience. In eusocial bees of the tribe Meliponini (stingless bees), female dispersal is highly restricted because new queens rely on resources from the parent nest during the founding phase. Males are presumed to be the dispersing sex, but their movements once they leave the natal nest are poorly known. We investigated male dispersal in the Australian stingless bee *Tetragonula carbonaria.* To assess the distance that males disperse, we manipulated hived colonies into re-queening, deposited the colonies at varying distances from each other (1-48 km) and genotyped the males that gathered at mating aggregations outside each colony. Sibship assignment revealed brothers were detected most often in the same or nearby aggregations, but some were sampled from aggregations as much as 20km apart. Simulations of the distribution of male genotypes across our study area produced the best-fit models when males dispersed an average of 1.3-3km, and maximum of 19km, from their natal nests. Mark-recapture of males further supported these estimates, by showing that males are capable of flying 4.5km in 48 hours to join mating aggregations. We conclude that in *T. carbonaria*, limited female dispersal is offset by efficient male dispersal, with males capable of maintaining gene flow over distances 40-times the typical foraging range of female workers. Such male dispersal would alleviate inbreeding, even in fragmented habitats. We also show that the targeted attraction of male stingless bees to 'bait' colonies, as used in this study, can be a tool for estimating the density of stingless bee colonies in a region, and thus population monitoring of these important tropical pollinators.

Introduction

In many animals, offspring actively disperse away from their place of birth. Such dispersal allows an individual to access new resources (Van Valen, 1971), reduce competition for mates (Gompper et al., 1998, Greenwood, 1980, Dobson, 1982), and minimize the risk of breeding with near relatives (Ronce, 2007). How regularly and how far individuals disperse from natal sites also impacts a range of ecological and evolutionary processes at the population level. For example, local persistence, colonization ability, gene flow and local adaptation are all shaped by dispersal (Zavodna et al., 2005, Adams, 1992, Waser and Strobeck, 1998, Dieckmann et al., 1999). These processes in turn are critical for predicting species' vulnerability to environmental change (Estrada et al., 2015)**.**

Eusocial bees of the tribe Meliponini (stingless bees) are key pollinators in tropical and subtropical ecosystems across the globe (Ramalho, 2004, Slaa et al., 2006). They are also economically important pollinators of tropical crops in many countries, with both wild and managed colonies providing pollination services (Heard, 1999, Giannini et al., 2015, Van Nieuwstadt and Iraheta, 1996). Indeed, because stingless bees form long-lived perennial colonies of thousands of workers, are generalist pollinators and can be kept, relocated and propogated in wooden hives, they offer many of the same advantages that honey bees do as managed pollinators (Wille, 1983, Nogueira Neto, 1997, Heard, 2016). Interest in the pollination services of meliponine bees has grown rapidly in recent decades, but so too have threats to their habitat, such as deforestation, habitat degradation, and climate change (Campbell et al., 2018, Brown and de OLIVEIRA, 2014, Kennedy et al., 2013). Effective conservation of these bees in the face of such threats will benefit from knoweldge of the factors that affect their population genetic structure and resilience, including dispersal (López-Uribe et al., 2017, Zayed, 2009).

Natal dispersal in stingless bees is likely to be strongly sex-biased. Females (queens) have limited dispersal potential due to the mode of colony fission. Workers locate a new nest cavity and provision it over many months, before rearing a new queen to inherit it (Wille, 1983, Inoue et al., 1984, Van Veen and Sommeijer, 2000a). New nests must be within ready-flight distance of the parent nest, as workers travel between each many times a day during the provisioning phase (Kerr et al., 1962, Wille, 1983, Inoue et al., 1984). Daughter queens therefore rarely

move far from their mother nest. In contrast, males leave the natal nest at maturity and never return (Vollet-Neto et al., 2018). Researchers typically only observe them again when they congregate to mate outside colonies that contain virgin queens (Van Veen and Sommeijer, 2000b, Sommeijer et al., 2004, Velthuis et al., 2005, dos Santos et al., 2014). In several species, males have been confirmed to travel distances at least similar to worker flight ranges, before joining mating aggregations (e.g. 100m-2km; (dos Santos et al., 2016c, Carvalho-Zilse and Kerr, 2004, Kerr et al., 1962). Indirect evidence, however, suggests that males may be capable of dispersing much further. Male aggregations can contain dozens to hundreds of unrelated individuals (Cameron et al., 2004, Sánchez et al., 2018, Mueller et al., 2012, dos Santos et al., 2016b, Kraus et al., 2008), suggesting that aggregations may draw males from a large catchment area. Furthermore, some populations of Neotropical stingless bees show low genetic differentiation across ranges of 200-500 km, consistent with significant male-mediated gene flow (Francisco et al., 2014, Tavares et al., 2013, Jaffé et al., 2016a).

Male dispersal distances inform the management of wild stingless bee populations in at least two ways. First, the vulnerability of stingless bees to habitat fragmentation or degradation depends in part on their natal dispersal ability. Inbreeding costs are high in stingless bees and many other Hymenoptera due to their genetic system of sex determination (Heimpel and de Boer, 2008, Vollet-Neto et al., 2018). Under this system, homozygosity at just one or a few critical "sex loci" causes diploid embryos to develop as infertile or subfertile diploid males (Cook & Crozier 1995). These diploid males take the place of female workers needed for colony growth and function (Plowright and Pallett, 1979, Cook and Crozier, 1995). High levels of diploid male production can lead small populations to spiral into extinction (Zayed et al., 2004). The extent to which fragmentation disrupts gene flow, and thus increases inbreeding risk, depends on the likelihood of individuals dispersing between fragments (Landaverde-González et al., 2017, López-Uribe et al., 2014, Jaffé et al., 2016a).

Second, male dispersal distance predicts the catchment area that is being sampled around the site of a male aggregation, and thus allows estimates of local colony density and population size (Jaffe et al., 2010, Kraus et al., 2008, Mueller et al., 2012). The colonies of cavity-nesting eusocial bees are cryptic and often high in the canopy, making them extremely challenging to survey by traditional means (Utaipanon et al., 2019b). This problem has been overcome in the western honey bee (*Apis mellifera*) via a protocol that exploits male bees' reproductive biology

(Baudry et al., 1998, Utaipanon et al., 2019b). If the typical distance that males travel from their natal nests to an aggregation is known, then males at an aggregation can be collected, genotyped, assigned to colonies, and used to estimate the number of colonies in the catchment area. This method has been deployed effectively to monitor changes in wild honey bee populations and identify local population declines (Utaipanon et al., 2019a, Arundel et al., 2013, Jaffe et al., 2010). A similar approach may be viable for stingless bees provided that, for a given species, we know both the typical male dispersal distance, and how to reliably attract males to a desired sampling site.

In this study, we investigate the dispersal of males of the endemic Australian stingless bee *Tetragonula carbonaria*. This species is widely-propagated in hives in Australia as pets, and for honey or crop pollination (Heard, 2016). Both males and workers are 4-5mm in length and the maximum foraging range for workers (and thus natal dispersal of queens) has been estimated at around 700m (Smith et al., 2017). To estimate typical and maximum male dispersal distances, we positioned colonies at target sites, manipulated them into attracting male aggregations, genotyped the males that arrived and identified brothers based on genotype. We then considered the distances between aggregations from which brothers were collected and simulated the dispersal distribution that was most likely to produce our observed sibship dataset. Finally, we evaluate the potential for stingless bee mating aggregations to provide estimates of local colony density.

Methods

Sampling and genotyping of males at aggregations

To estimate the average and maximum distance that *T. carbonaria* males travel from their natal nests to mating aggregations (dispersal distance), we collected males from mating aggregations at multiple sites in a target study area and then mapped the distribution of brothers across aggregations. We made three sets of male collections: Set 1 (three sites spanning 15 km; Sunshine Coast 26.6500° S, 153.0667° E, September 2017), Set 2 (seven sites spanning 23 km; Sydney 33.7416° S, 151.1520° E, October-December 2017), Set 3 (13 sites spanning 48 km, Greater Sydney 33.7416° S, 151.1520° E, October- December 2018). We selected these times

of the year because *T. carbonaria* are more active during warmer months (Heard, 2016) (**Figure 1 a-c;** site details in Supplementary Material, **Table S1**).

We lured male aggregations to our sites by stimulating hived colonies to requeen. We made use of a common stingless beekeeping propagation technique, whereby a single colony that is established within two half-boxes is split to produce two hives. Both halves of the original colony (containing brood and food stores) are given a new empty half-box; (Heard, 2016). Following these splits, one resulting hive retains the original queen, while the other is forced to requeen. To confirm which half of a recently-divided colony was in the process of requeening, we placed perspex lids over newly-divided colonies and observed colony activity regularly until a virgin queen was observed (thereby confirming a colony in the requeening process; usually <24 hours after splitting). We kept hive entrances plugged during this period to ensure that virgin queens could not leave the hive to mate. We then positioned these 'bait colonies' at our target sampling sites and monitored them daily for two weeks for male aggregations. The reliability of attracting males to bait colonies is key to understanding their utility as a tool for inferring population density. Therefore, we also noted five variables during the observation period that might affect the presence of a male aggregation: three variables related to temperature (month, mean weekly temperature and no. sunny days post-split), and two variables associated with the hive manipulation (no. days between splitting and unplugging the colony, and whether the colony was split *in situ*, or relocated to a new site directly after the split) (see Supplementary Material, **Table S1** for details). We then used ordinary least squares (OLS) regressions to assess whether these variables were significant predictors of male aggregations forming at our bait colonies using Python (Sanner, 1999).

T. carbonaria males form aerial aggregations (hereafter "male aggregations") close to the requeening colony (Heard, 2016). Males closely resemble workers in size and colour (4-5mm, black), and workers also sometimes swarm at the front of colonies as part of colony defence (Gloag et al., 2008, Stephens et al., 2017). However, we found that the sex of swarming individuals could be readily discriminated in the field with the naked eye by focusing on the facial characteristics of the bees, with males having larger eyes and smaller mandibles than workers (**Figure 2a**).

When males were detected at one of our bait colonies, we collected them by sweeping a net several times through the aggregation. We collected and genotyped at least 200 males for large

aggregations, and every male possible for smaller aggregations (mean: 203±132 males per aggregation; Supplementary Material, **Tables S1** and **S2**). For all bait colonies, we also collected 20 workers prior to hive-splitting, which we used to infer the colony's maternal genotype and thus assess whether males aggregate in front of their natal nest; Supplementary Material, **Table S3**). Additionally, for Set 1 samples only, we also sampled 10 workers per colony from 76 known managed colonies in the area, to determine whether we could verify male travel by matching the genotypes of males collected at mating aggregations to the genotypes of known local colonies (Supplementary Material, **Table S4)**. All samples were preserved in 99% ethanol in the field and stored at -20°C until DNA extraction.

We extracted DNA by grinding whole abdomens in 5% Chelex solution (1mM Tris HCl pH 7.6, 0.1mM EDTA pH 8) and boiling for 15 mins (Walsh et al., 1991). Supernatant containing DNA was diluted 1:1 with distilled water prior to PCR amplification. We genotyped each bee at 7 microsatellite loci using seven primers: Tc3. 155, Tc4. 63, Tc3. 302, Tc7. 13 and Tc4. 287 (Green et al., 2001) and Tang60 and Tang70 (Brito et al., 2009). Primers were fluorescently labelled with one of four dyes (FAM, NED, PET, VIC; Sigma-Aldrich, U.S.A.). PCR amplifications were performed according to (Green and Oldroyd, 2002) and the resulting products were analysed using a 3130xl Genetic Analyser and Genemapper V5 (Applied Biosystems, U.S.A.).

Composition of male aggregations

We used COLONY V2.0.6.4 (Wang, 2004) to estimate the number of colonies contributing males to each aggregation. COLONY uses population allele frequencies to estimate the likelihood of relatedness among haplo-diploid individuals and assign them to families. We input population allele frequencies based on the full sample of males for each collection set. We then took the number of families per swarm to be the number of families COLONY output with an (inclusive) probability of more than *0.95* that all the individuals were siblings. These are conservative estimates of the true number of colonies represented in each swarm for two reasons. First, a minority of males in each swarm were assigned to families with low likelihood scores (inclusive probability <0.95; 19 ± 19 males unassigned per swarm) and represent an uncertain number of additional families. Second, most male aggregations were much larger than our average sample size. To estimate what proportion of total families that were typically

detected from a sample of 200 males, we genotyped additional males for three large aggregations sampled in Set 1 (507±28 males). The cumulative distributions of estimated family number for these aggregations indicated that 200 males captured around 85% of the families contributing to large swarms (Supplementary Material, **Annex 1.1, Figure S1**).

Estimated mean and maximum male dispersal distances

In Set 1, we aimed to assess male flight behaviour across distances of 5-15km by matching males sampled from three aggregations to the genotypes of colonies kept at the same sites (8-56 colonies per site**; Figure 1a)**. While this strategy confirmed that males can travel such distances, the proportion of males in aggregations that matched our "supplier" colonies was extremely low (2%). To make better use of the data from all males sampled in an aggregation, we therefore took a different approach in sample Sets 2 and 3 whereby we: (i) calculated the pairwise probability that any two males sampled at mating aggregations were brothers, (ii) determined the distribution of likely brothers across all aggregations, and (iii) compared this distribution to simulated distributions under different assumptions of mean dispersal distance. A full description of this approach is in Supplementary Material **Annex 2.1-2.3**, and the key aspects are given below. Model and simulations were run in Python (Sanner, 1999).

(i) Pairwise sibship probability

We used a Bayesian model to estimate the probability that two males were brothers, based on their genotypes and the population allele frequencies (Supplementary Material, **Table S2**). This approach is "family-blind", as the probability of sibship of two males is not affected by their respective probabilities of sibship with any other male. It is therefore more suitable for estimating the pairwise sibship likelihood than COLONY's family assignments. Under this model, for any pair of males, the rarer the shared alleles at a given locus, the higher the probability that they are brothers. We computed the pairwise sibship probabilities under scenarios in which the total number of colonies (S) contributing to our sample set ranged from low to high; that is, scenarios in which the prior probability that any given pair are siblings ranged from high to low. The selection of the range of priors was adjusted according to our sample size for each set (Set 1: S = 193, 300, 538, Set 2: S = 200, 380, 475, 570; Set 3: S = 450, 600, 750).

(ii) Distribution of brother pairs across aggregations

We next assessed the relationship between the probability of sibship of each pair of males and the physical distance (km) separating the aggregations in which they were sampled. If we found that sibship probability did not vary by distance in our sample sets, then males must disperse evenly over distances at least as large as our sample area. In contrast, if males with high sibship probabilities were only ever sampled from the same aggregation site, then males must not disperse far from their natal nests. To visualize this relationship between sibship probability and distance, we plotted the cumulative distance flown by pairs of males, with pairs binned by their probability of sibship (**Figure 1 d-f**). The cumulative distribution plot indicated that actual male dispersal was somewhere in-between the two extremes described above, and therefore that our scale of sampling would be informative. That is, pairs with a high probability of sibship were more likely to be collected from nearby sites than pairs with a low probability of sibship, yet sometimes likely-brothers (i.e. sibship probabilities > 0.95) were found at distant aggregations. These cumulative distribution plots (one per set) served as the "observed data" against which our simulations were compared. As an additional check, we also assessed distances between males with identical genotypes (i.e. a highly conservative set of brothers), which gave similar results to our simulations (Supplementary Material, **S3.1** and **Figure S4**).

(iii) Simulated data vs observed data

Finally, we simulated the distribution of males by sibship across aggregations for different distributions of male dispersal distances, and assessed which simulations gave results most similar to our observed data. Simulations assumed that the likelihood a male joins an aggregation decreases exponentially with distance from that male's natal nest (**Figure 3a**). Each simulation generated male-producing colonies at random locations within a virtual catchment area matching our actual study area and sample sites (**Figure 1 b-c**). Each simulated colony was randomly assigned a diploid queen genotype based on population allele frequencies (and thus haploid male genotypes for the queen's sons). Males from each colony dispersed in uniform directions from their natal colony at distances according to:

$$P(x) = \lambda e^{-\lambda x}$$

Where $x = metres\ flown$ and λ = 1/mean dispersal distance. By using an exponential distribution, we assume that the probability of a male surviving to fly an additional unit of distance is fixed, regardless of how far they have already flown.

In the simulation, any male that was within *M* metres of a collection site was added to that collection in the simulation. This process continued until the total number of colonies contributing to the virtual sample set was equal to the number of total colonies used to calculate sibship probability (*S*). If the number of males in a virtual collection exceeded the number collected in our real dataset (n), then n males were randomly retained (a simulated process equivalent to randomly sampling 200 males from a large swarm).

We ran 100 simulations for each of 30 distributions, where mean dispersal distances of the distributions varied from 100m to 5200m, and for each prior (*S*). We then assessed the fit between simulations and our observed data by comparing the average areas between the curves in the cumulative distribution plot of sibship by distance (**Figure 3 b-c**).

Male flight distance per day

To support our estimates of male dispersal distances based on sibship probabilities, we performed four mark and recapture experiments (Sydney; April and December 2018, October and November 2019). In each case, we collected males from one or more aggregations and painted dots on their thoraxes. We kept marked males overnight in plastic containers and fed them sugar solution. We then released them at 9AM the following morning at known distances from a different target male aggregation: 1 km away (N=3 releases), 2 km away (N= 2 releases), 4.5 km (N= 1 release); (1600±535 males marked per release). In each release, males were colour-coded into two batches, with each batch released in the opposite compass direction from the target aggregation. We then collected samples at the target aggregation 24, 36 and 48 hours after release and counted the number of marked males we captured (Supplementary Material, **Table S5**).

Estimating the density of colonies in a region

We used the estimates of typical male dispersal distance generated by our simulations to then estimate the density of *T. carbonaria* colonies in the two broad regions where we sampled

mating aggregations (Sunshine Coast – Set 1, and Sydney – Sets 2 and 3). We calculated colony density as:

$$\text{Colony density} = N_f / \pi r^2$$

Where N_f is the average number of families represented per aggregation calculated by COLONY (for all aggregations N >180), and *r* is the mean dispersal distance of males. That is, the males of an aggregation are drawn from a catchment area around the aggregation of radius *r*. For one aggregation sampled in Sydney, we also had an opportunity to check our estimate against a known density of managed colonies. Sydney's Ku Ring Gai Council runs a *T. carbonaria* breeding program that allocates hives to residents of the area, and in 2019 maintained 700 colonies across an 86 km^2 area. 8 colonies/km^2 (*Pers Comm* P. Clarke and A. Austin, Ku Ring Gai Council). For one large aggregation sampled in this area, we validated our estimate of colony density against this known density of hives.

Colony investment in male production

The use of males from mating aggregations to estimate the density of colonies in an area assumes that, at the time of sampling, most or all colonies in an area are producing males and thus have the potential to be detected in the male population. To test this assumption, we determined the variability in male production between *T. carbonaria* colonies throughout the year. We sampled 100 pupal brood cells per colony from four colonies per month in South-East Queensland (27.4698° S, 153.0251° E) (July 2016-June 2017, excluding January). We uncapped brood cells and sexed pupae based on facial morphology. To determine whether the higher seasonality in southern parts of their distribution affected the variability of male production, we also sampled between 4-6 colonies per month in winter (July, 2018 N=4), spring (November, 2018 N=6), summer (February, 2019 N=4) and autumn (April/May, 2019 N=5) from Sydney (33.7416° S, 151.1520° E). Both Sydney and South-East Queensland have humid subtropical climates, but the annual mean temperature of Sydney is lower (min 13.8-max 21.8) than South-East Queensland (min 15.9-25.5) (Bureau of Meteorology, Australia; BOM, 2020).

We also confirmed: (i) that males in *T. carbonaria* do not return to nests after leaving, by checking the proportion of males among bees entering and exiting colonies throughout the day

(Sydney, October-November, 2018, 7 AM until 2 PM.; N= 71 colonies), and (ii) that all adult males leaving a colony were brothers (i.e. that adult males do enter non-natal colonies), by genotyping a subset of exiting males (Sunshine Coast, September 2017, April 2018, 24 males per colony; N= 47 colonies, Supplementary Material, **Table S6**).

Results

Attracting male aggregations using bait colonies

We attracted a total of 31 male aggregations from 41 attempts with bait colonies (i.e. recently-split colonies in which a virgin queen was confirmed present; Set 1 = 3, Set 2 = 13, and Set 3 = 15 colonies). On average, aggregations formed four days after unplugging the entrance of the bait hive (range 1-11 days, SE=3). The aggregations persisted anywhere from two days to more than three weeks, often even after the colony's new queen became visibly physogastric and thus had already mated.

The remaining 10 bait colonies failed to attract visible male aggregations within the 2-week observation period, but five were later confirmed to have nevertheless re-queened during this period. Thus, queens sometimes located a mate despite the absence of a conspicuous male aggregation. The probability that bait colonies attracted a detectable male aggregation was related to month, with aggregations occurring more reliably in spring months (**Figure 1, b**, OLS Regression; $p<0.05$). Interestingly, whether the bait colony was split *in situ* or relocated post-split was also a significant predictor of whether it attracted a visible male aggregation. *In situ* colonies were more likely to attract aggregations (OLS Regression; $p<0.01$), perhaps indicating that some males locate colonies even before they lose their queen. The number of sunny days post-split, mean weekly temperature (independent of month) and number of days between splitting and unplugging the colony were not predictors of male aggregations ($p>0.05$; Supplementary Material, **Table S1**).

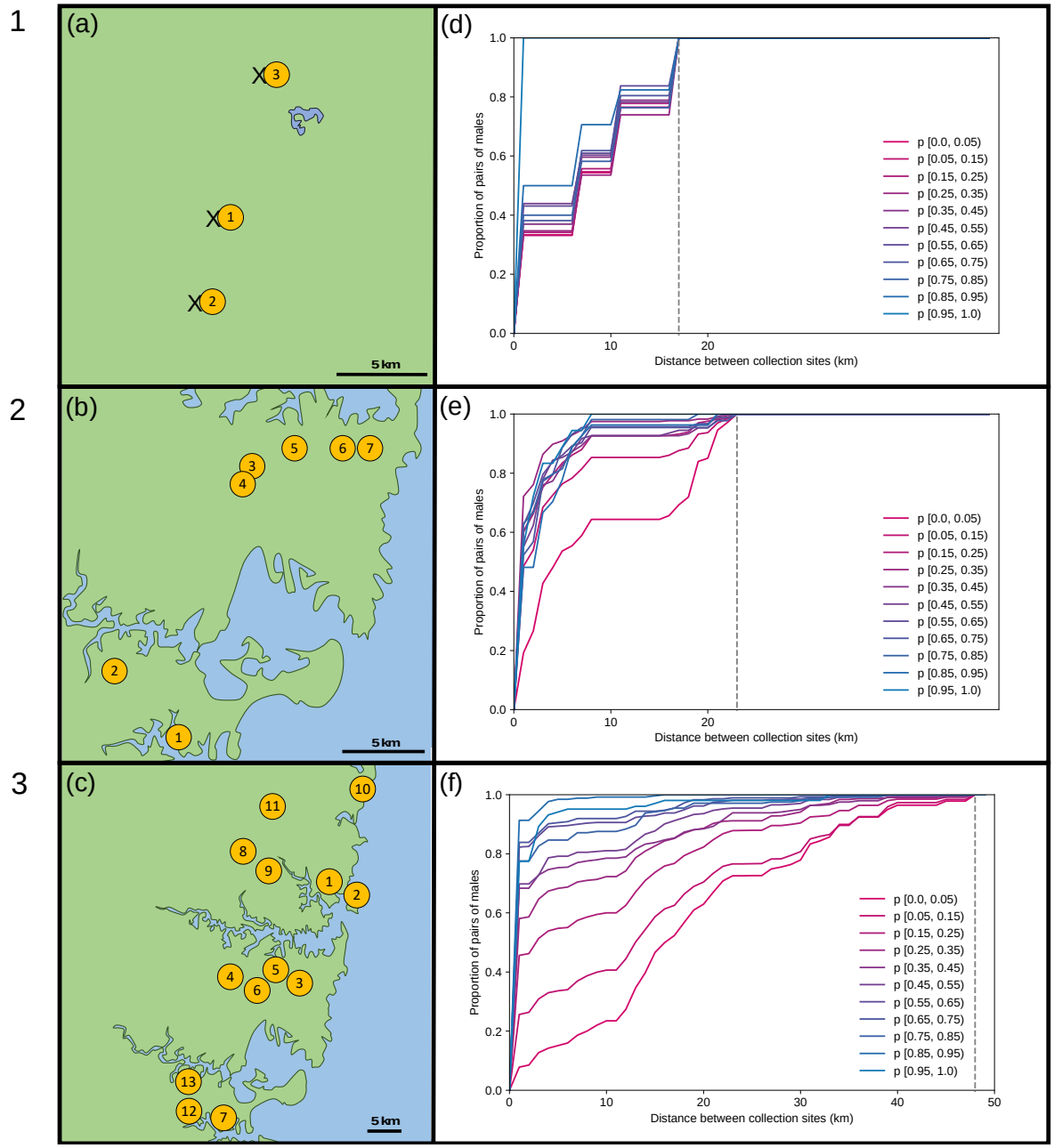


Figure 1. Left: Sites (yellow circles) at which *T. carbonaria* male aggregations were successfully attracted using "bait" re-queening colonies for each of three sample sets: (a) Set 1 (Sunshine Coast), (b) Sets 2 (Sydney), (c) Set 3 (Greater Sydney). In Set 1, we also sampled from hived colonies at each site ("X"). Right: The cumulative distribution plots of the distance between pairs of males binned by their probability of sibship (*p*), for males collected in each of the three Sets 1-3 (d-f). High *p* values for sibship (blue) indicate pairs of males that are likely to be brothers, while low p values (pink) indicate pairs of males that are not related. Distances of 0 indicate pairs of males in the same aggregation, while distances >0 indicate males were sampled from different aggregations. Dotted lines indicate the maximum possible distance separating male pairs for each Set. These cumulative distribution curves served as the observed data against which our simulated sibship data was compared, to estimate best-fit male dispersal distributions.

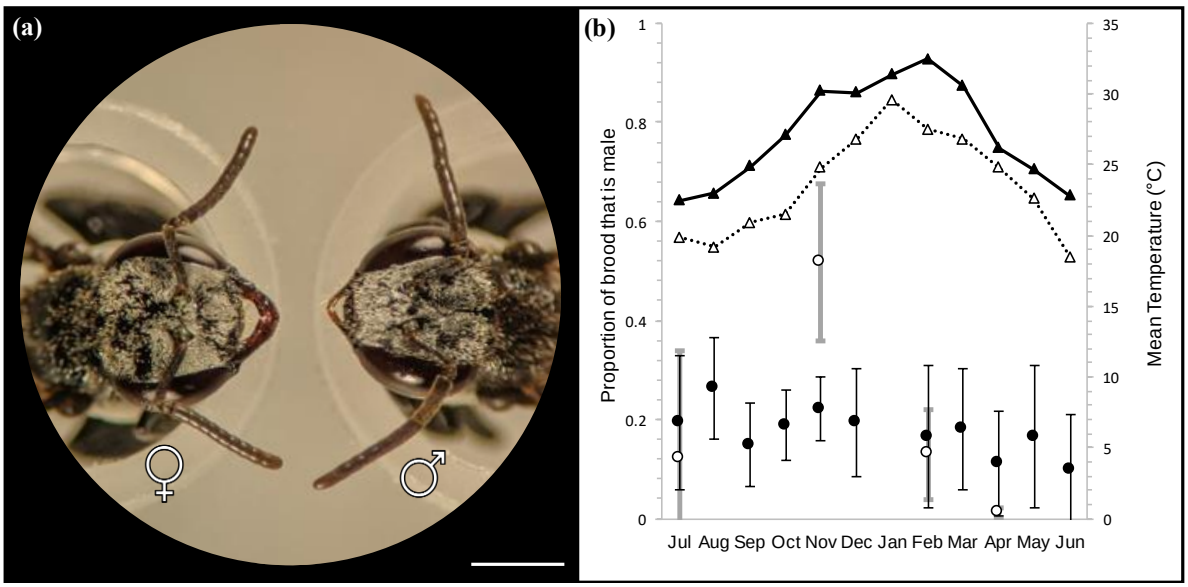


Figure 2. (a) Head morphology of female (left) and male (right) *T. carbonaria.* Females only have 9 antennae segments while males have 10. Females also have eyes spaced more widely and larger mandibles than males. (scale bar: 1mm). **(b)** Annual average male brood investment per month for *T. carbonaria* in Southern Queensland (black circles, N=4 colonies per month, 2016-2017) and Sydney (white circles, N=4 colonies per month, 2018-2019). Triangles represent the Maximum Temperature Mean in Brisbane (black) and Sydney (white) based on data from the Bureau of Meteorology, Australia

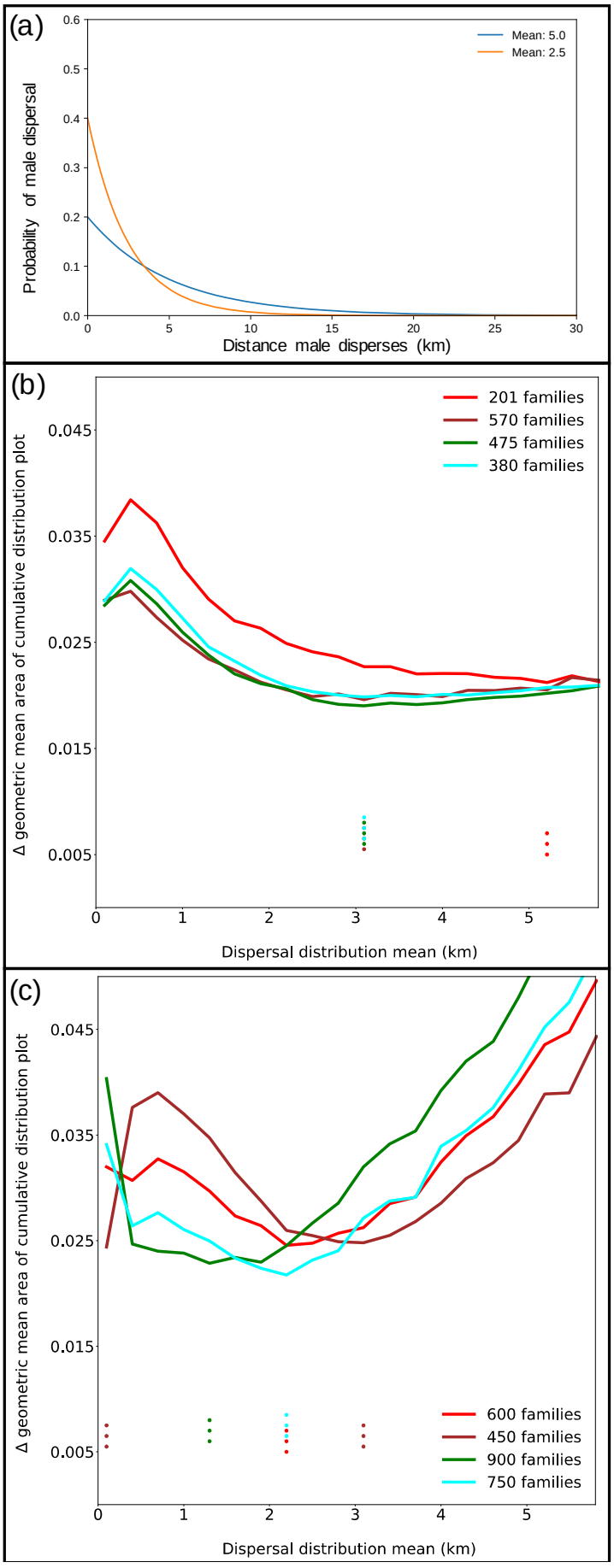

(a)
Mean: 5.0
Mean: 2.5
Probability of male dispersal
Distance male disperses (km)
(b)
201 families
570 families
475 families
380 families
Δ geometric mean area of cumulative distribution plot
Dispersal distribution mean (km)
(c)
600 families
450 families
900 families
750 families
Δ geometric mean area of cumulative distribution plot
Dispersal distribution mean (km)

Figure 3. (a) Examples of the distributions of male dispersal being modeled in simulations, showing mean dispersal values of 2.5 km (yellow) and 5 km (blue). The average difference between simulated data and observed data for Sets 2 **(b)** and Set 3 **(c)** for different values of mean male dispersal distance. Difference between simulated and observed data is measured as the area between the curves of the cumulative distribution plots of each data type. Bold lines indicate the average difference for given values of *S* (the prior estimate of total number of families represented by the males in our datasets). Fine lines represent each of the 100 simulations for a given set of parameters. Dots indicate the mean male dispersal distance (km) at which the difference between simulated data and observed data was the lowest i.e. the mean male dispersal distance that produced the best-fit for our observed data. We included local minimums when there happened to be two local minimums, as was the case in the plot (C) for 450 families.

Composition of male aggregations

Male aggregations comprised males from an average of 80 different colonies (COLONY family assignment, N = 80 ± 90 families per aggregation; or 49 ± 20 families per 100 males sampled). This is consistent with either the catchment area for males being large (i.e. large male dispersal distances), the density of colonies in the study area being high, or both. For example, at colony densities of 1 colony/km^2, an aggregation of males from 80 colonies would need to be attracting males from a catchment radius of 5km (i.e. approx. 80 km^2).

Most male aggregations contained few to no males with genotypes matching the bait colony attracting the aggregation, (4 ± 2%, N=31), consistent with *T. carbonaria* males dispersing away from their natal nest even if it had a virgin queen. However, three aggregations were significant outliers in this respect, with 28-39% of male genotypes matching the bait colony itself (Supplementary Material, **Tables S2** and **S3**).

Male dispersal distance

Brothers (defined as males with pairwise sibship probabilities >0.95) were frequently detected in the same aggregation (N=47 pairs), but also in different aggregations ranging from 1 km to 33 km apart **(**N=32 pairs, **Figure 1 d-f).** Where brothers occurred in different aggregations, they were typically adjacent ones, with only a few at greater distances (<2 km apart, N=53 brother pairs; 2-5 km, N=20; 5-7 km, N=2; 7-10 km, N=1; 10-20 km, N=2; >20 km, N=0). A similar trend was observed if the definition of brothers was relaxed to include all males with sibship probabilities >0.85 (<2km apart, N=144 pairs; 2-5 km, N= 35 pairs; 5-7 km N=7 pairs; 7-10 km, N=3; 10-20 km, N=5; >20km, N=1), or if we defined brothers only as the subset of males with identical genotypes (same aggregation, N=232; aggregations 1-7 km apart, N=18 pairs; aggregations >7 km apart, N=2 pairs; Supplementary Material, **3.1** and **Figure S4**). None of these cases of dispersed brothers could be accounted for by our own colony movements (i.e. brother pairs in different aggregations did not match the genotoypes of colonies we relocated), and it is unlikely that the chance movements of pet hives by other people could produce the observed trend of decreasing male sibship probability with distance. Furthermore, consistent with our observed distribution of brothers among the male aggregations of all sets, a small proportion of total males sampled at aggregations in Set 1 (1%; 22 of 1532 males) had

genotypes matching known colonies in the area, with most of these joining aggregations near to their natal colony (<500m; N=20) but two males having travelled 6km and 16km respectively before joining an aggregation (Supplementary Material, **Table S4**).

Simulations indicated that mean male dispersal distances between 1.3km and 3 km produced distributions of sibship probability that best fit our observed data (Set 2: **Figure 3b**, Set 3: **Figure 3c**). This estimate of mean male dispersal was somewhat higher (5km) if we assumed few total families contributed males to the set (Set 2, S =200; **Figure 3b**). In all cases, these simulations assumed male dispersal followed an exponential distribution, with male survivorship decreasing steadily with distance from the natal nest. Given this function, a mean dispersal distance of 2km corresponds to approximately 40% of a colony's males dispersing 1 km, 8% more than 5 km and 1% more than 10km (**Figure 3a**). That is, this dispersal function closely reflects the distribution of brothers we observed in aggregations, with the great majority found in the same or nearby aggregations, and a minority separated by large distances.

Finally, mark-recapture studies supported our estimates of male dispersal by confirming that males can travel several kilometres per day. Paint marked males were recaptured at target aggregations within 48 hours after release at distances of 1km (32 of 2830 total released), 2km (6 of 2600) and 4.5 km (2 of 1000); (Supplementary Material, **Table S5**).

Estimating the density of colonies in a region

Assuming mean male dispersal distances between 1.3-3km, we estimate the density of colonies in the Sunshine Coast region (a mix of natural bushland and agricultural land) to range from 11-60.18 colonies/km^2 (Nf = 80; 10 aggregations), and in Greater Sydney (urban and remnant bushland) to be 2.8-15.2 colonies/km^2 (Nf = 80; 10 aggregations). For one area of Sydney where the density of hived *T. carbonaria* colonies is known to be particularly high due to a local breeding program (8 colonies/km^2), our estimate closely matched this known density if average male dispersal was assumed to be 2km (Nf = 100; 1 aggregation).

Colony investment in male production

T. carbonaria colonies produced males throughout the year (average 20% total brood ±0.02 S.E., N=62), with male production relatively constant per month in South East Queensland (9.7-26.3%, N=43) and showing a springtime peak in Sydney (1% in winter to 51.63% in spring, N=19 colonies); **Figure 2b**. In colonies of typical size (8000-10,000 workers; (Heard, 2016), this equates to approximately 450 males produced per week, and up to 1000 per week during spring (assuming 300 eggs produced per day; Heard, 2016).

Males were often observed exiting a colony in the first few hours of the morning (32% samples, N=217 males, 71 colonies). Males exiting from the same colony were always brothers (N=15 colonies), and samples of bees re-entering colonies never contained males (480 samples, N=24 colonies), consistent with male *T. carbonaria* never returning to their own or other colonies after leaving the natal nest (Supplementary Material, **Table S6**).

Discussion

We find that in the stingless bee *T. carbonaria*, a large portion of males join mating aggregations within 1km of the natal nest, a distance similar to the maximum flight range of workers (700m) (Smith et al., 2017). Some males, however, cover far greater distances, with an estimated 8% dispersing more than 5km, and a maximum dispersal range estimated at 20 km. This capacity for long-distance dispersal by males supports a central role for males in inbreeding avoidance in stingless bees and, more broadly, in shaping the genetic structure of their populations (dos Santos et al., 2016c, Carvalho-Zilse and Kerr, 2004, Kerr et al., 1962, Kraus et al., 2008). As the dispersal of female reproductives (queens) is constrained by the initial dependence of new nests on their parent nest (Nogueira-Neto, 1954, Heard, 2016), male dispersal may therefore be key to the species' resilience to population decline or fragmentation (Knowlton and Jackson, 1993, Thornhill, 1993, Bengtsson, 1978). In particular, males are likely to maintain gene flow across natural or manmade landscape barriers that females do not traverse.

Strong male-biased dispersal in stingless bees is consistent with the population genetic patterns observed in several previous studies (Chapman et al., 2018, Paxton, 2000, Cameron et al., 2004). In a landscape genetic study of 17 stingless bee species in South and Central America,

gene flow was found to be largely independent of landscape features such as forest fragmentation, rivers or roads (Jaffé et al., 2016b), suggesting that reproductive individuals must be capable of maintaining gene flow across significant distances in heterogeneous landscapes. *T. carbonaria* also show low population differentiation across 900km^2 of their southern range (Chapman et al., 2018). Female-mediated gene flow may contribute in part to these patterns. For example, high rates of colony reproduction and/or the movement of colonies by beekeepers (Jaffé et al., 2016a) will facilitate gene flow, even if natural female dispersal distances are low. In the case of *T. carbonaria*, colonies also sometimes usurp existing colonies rather than provision a new site (Cunningham et al., 2014), which might allow larger leaps in distance between parent and daughter colonies. Even so, it seems likely that the free movement of males makes a greater contribution than females to gene flow. Signatures of such sex-bias in gene flow can be found in mitochondrial genomes, which are maternally-inherited (Melnick and Hoelzer, 1992, Peters et al., 2012, Francisco et al., 2013, Doums et al., 2002). For example, a study of the stingless bee *Plebeia remota* in Brazil found mitochondrial haplotypes were highly site-specific while population structure in the nuclear genome (microsatellites) was low across sites separated by 300-800km. This pattern is consistent with female philopatry but male dispersal (Francisco et al., 2013). Applying the model and simulations used here to male aggregation data of other species would reveal whether the dispersal capacity of *T. carbonaria* males is typical of meliponine bees.

We have assumed that male dispersal in *T. carbonaria* follows a negative exponential dispersal distribution, in which male survival probability decreases steadily over time and males, on average, move further from the natal nest each day. Although alternative dispersal distributions are possible, too little is known about male stingless bee behaviour to make clear predictions for more complex functions. Male movement is presumably the results of both chance and active search behaviour. On the one hand, males are at the mercy of abiotic factors such as wind (Compton, 2002, Tóth et al., 2004). In this sense, they are comparable to the male gametes of other organisms that are produced on mass and thrown to wind or currents, such as the pollen of wind-pollinated plants, or the sperm of some egg-brooding marine invertebrates (Oddou-Muratorio et al., 2001, Garcia et al., 2007, Yund, 1995). In this respect, male reproductive success in stingless bees is largely a numbers game, with low likelihood of success per male, and gene flow decreasing steadily by distance from the parent nest.

On the other hand, there is likely to be strong selection on males for effective strategies to locate mates. Male-biased dispersal is common among eusocial insects (Doums et al., 2002, Hardy et al., 2008, Johnstone et al., 2012, Barth et al., 2013, Holzer et al., 2009, Kuhn et al., 2017), but stingless bees are atypical in that the responsibility for mate finding falls more or less entirely on males (Velthuis et al., 2005) (queens and their daughters remain at or very near their colonies, with the exception of rare cases where young queens fly to parasitize other colonies; Wenseleers et al., 2011). Males are presumably attracted to a pheromone produced by virgin queens (Engels et al., 1997, Verdugo-Dardon et al., 2011), but additional strategies may have evolved to deal with the seemingly impossible task of finding colonies that happen to have virgin queens ready to mate. For example, in several species of hymenopterans, males are also attracted to other males, allowing them to gain safety-in-numbers by roosting together overnight, and possibly increasing their chances of detecting mates (Dos Santos et al., 2015, Kimsey, 1980, Masciocchi et al., 2020, Spradbery, 1973, Starr and Velez, 2009, López and Kraus, 2009). A tendency of *T. carbonaria* males to cue into the signals of other males would help explain why large male aggregations stay large, even after the queen has mated. Interestingly, in this study we also found that male swarms appeared more reliably at bait colonies that had not been recently relocated than those that had. One explanation for this pattern is that some males locate colonies that have resident queens and simply hang around, waiting opportunistically for a requeening event. Further research is needed to determine how males locate virgin colonies and how mate-search behaviour is affected by the environment and other males (dos Santos et al., 2014, von Zuben, 2017). Indeed, the extent to which male dispersal is active rather than passive will further inform stingless bee conservation, because it is likely to affect males' probability of travelling between habitat patches.

Can stingless bee male aggregations be used to estimate colony densities?

Stingless bee colonies are difficult to census by simple transects. Indeed, even workers may be rarely observed in Australian forests, as many forage 20-30m up in the canopy. The habit of males aggregating at colonies with virgin queens thus offers a unique opportunity to quickly collect a 'snapshot' of the genetic diversity of local stingless bee colonies (Sánchez et al., 2018). Furthermore, if average dispersal distances are known, and males can be readily assigned to a given number of natal colonies, then aggregations may also provide an estimate of colony density. This technique has already been developed for honey bees, and subsequently used to track changes in the health of wild and managed honey bee populations (Arundel et al., 2012, Arundel et al., 2013, Arundel et al., 2014, Hinson et al., 2015, Moritz et al., 2008, Baudry

et al., 1998, Jaffe et al., 2010, Utaipanon et al., 2019b). Relative to honey bees, estimating colony density via male aggregations presents some novel challenges in stingless bees. In particular, as stingless bee males do not return to their nests each day (as do honey bee drones), aggregations are likely to contain a small proportion of males that originated far outside the average catchment area. Our data suggests that using average dispersal distances is nevertheless sufficient to give informative estimates for *T. carbonaria* colony density, particularly where the goal is to monitor changes in population size of the same region over time, or to make broad comparisons of colony densities in different regions. For example, our estimates for *T. carbonaria* colony density on the Sunshine Coast gave values around 4-fold that of urban Sydney, consistent with predictions given the larger bush fragments, and large number of managed hives, in Sunshine Coast orchards. Our estimate also accurately reflected hived colony density in a region of Sydney with known colony density. Further truthing of this current protocol in *T. carbonaria* is now needed, to better develop its application to conservation and management goals.

Continued advances in our knowledge of stingless bee behaviour will also help over time to fine-tune this technique for estimating population sizes. In this study, we lured male stingless bees to sampling sites using whole colonies that had been manipulated into re-queening. However, characterization of the pheromones used by virgin queens to attract males might eventually allow males to be lured with a synthetic volatile, such as is used when sampling male honey bees (Taylor Jr, 1984, Williams, 1987). A better understanding of male movement patterns will also inform this protocol; for example, how male dispersal is affected by different landscapes, and the distance at which males must pass within colonies in order to detect them.

Conclusion

In both social and solitary bee species, females have the outsized role as pollinators because they forage not only for themselves but also for the nest. Males in contrast, are focused only on finding mates. This key difference in life history shapes movement patterns (both foraging and dispersal) in sex-specific ways across the bee phylogeny. Progress in bee population genetics and conservation will benefit from further study of dispersal potential of both sexes.

Supplementary information

Annex

1.1 Detection error of the number of colonies contributing to male aggregations

T. carbonaria male aggregations vary in size, but in some cases are very large. In our study, we typically genotyped only 200 males from large aggregations. To estimate what proportion of total colonies (families) contributing males to an aggregation would be detected from a sample of 200 males, we genotyped additional males for three large aggregations sampled in Set 1 (507±28 males genotyped per aggregation). We then calculated the number of families represented in our sample using COLONY (Wang, 2004) for increasing intervals of 100 males and plotted the number of samples vs number of detected families in Python (Sanner, 1999). Based on these plots, 200 males typically detected around 80% of the families contributing to large swarms (**Figure S1**).

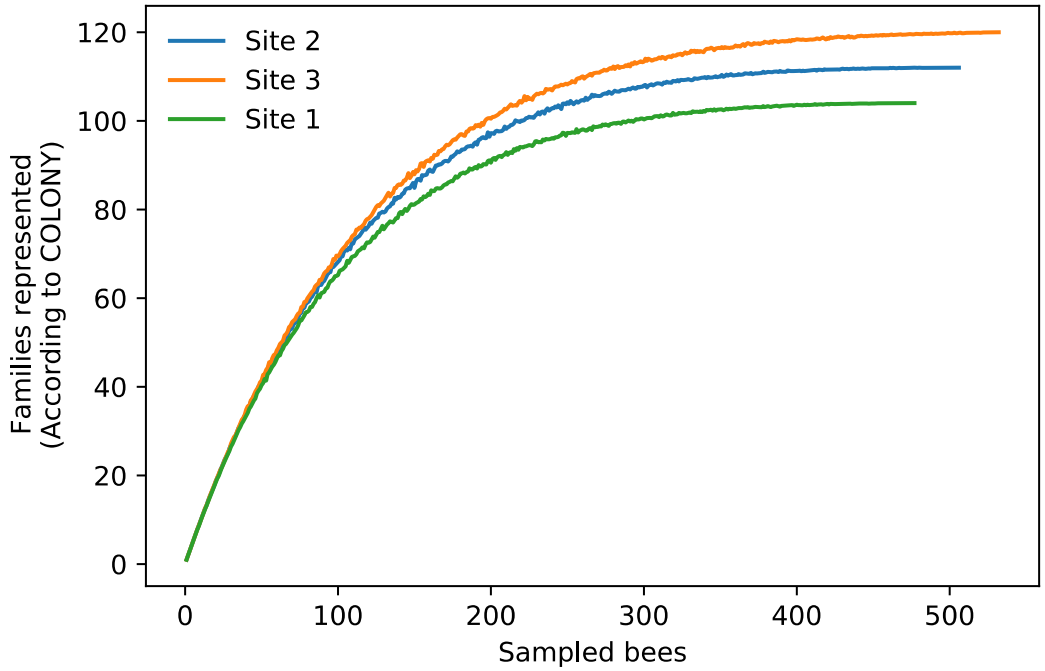


Figure S1 Cumulative sampling distributions of estimated number of families contributing males to a mating aggregation in large swarms (Set 1, Sites 1-3).

2.1 Probability of male sibship

We used a Bayesian model to estimate the probability that two male *Tetragonula carbonaria* in our dataset were brothers, given their alleles at seven microsatellite loci and the allele frequencies of our sampled population. Under this model, pairwise sibship probability is calculated as follows:

$$\boldsymbol{P\left(b_{ij}=1\middle|G_i=g_i,G_j=g_j\right)}$$
$$=\frac{\boldsymbol{P(G_i=g_i,G_j=g_j,b_{ij}=1)}}{\boldsymbol{P(G_i=g_i,G_j=g_j)}} \quad (1)$$

where b_{ij} is a variable indicating that males *i* and *j* are brothers, G_i and G_j are random variables which take values from the set of all possible genotypes for males *i* and *j* respectively, and g_i and g_j are their actual genotypes.

Equation (1) is equal to:

$$=\frac{P\left(G_i=g_i,G_j=g_j\middle|b_{ij}=1\right)*P(b_{ij}=1)}{P\left(G_i=g_i,G_j=g_j\middle|b_{ij}=1\right)*P\left(b_{ij}=1\right)+P\left(G_i=g_i,G_j=g_j\middle|b_{ij}=0\right)*P(b_{ij}=0)}$$

We can then consider each term in this equation in turn. First, the probability that males *i* and *j* have genotypes g_i and g_j if they are brothers can be expressed as:

$$P\left(G_i=g_i,G_j=g_j\middle|b_{ij}=1\right)=\Pi_{k=1}^{7}P\left(G_{ik}=g_{ik},G_{jk}=g_{jk}|b_{ij}=1\right) \quad (2)$$

where G_{ik} is the random variable taking value from the set of all possible alleles at the *k*-th locus for male *i* , $G_{jk}$is the random variable taking value from the set of possible alleles at the *k*-th locus for male *j*, and g_{ik} and g_{jk} are the actual alleles of each male at that locus.

Haploid male bees inherit **one** of their diploid mother's two alleles at each locus. If we imagine a mother's genotype to be A_1A_2, then the probability that brothers inherit the same allele (say, A_1) is 0.5, and we denote the event as $b_{ij}=1$. Likewise, the probability that brothers inherit

different alleles (one brother inherits A_1 and the other A_2) is 0.5 and we denote the event as $b_{ij} = 0$. Therefore:

$$P\left(G_{ik} = g_{ik}, G_{jk} = g_{jk} | b_{ij} = 1\right) = \left(\frac{1}{2}\right) * P\left(G_{ik} = g_{ik}, G_{jk} = g_{jk} | b_{ij} = 1,\ b_{ij}^{k} = 1\right) + \left(\frac{1}{2}\right) * P\left(G_{ik} = g_{ik}, G_{jk} = g_{jk} | b_{ij} = 1,\ b_{ij}^{k} = 0\right) \quad (3)$$

Note that the alleles at the *k*-th locus of brothers may have the same value even under the condition $b_{ij}^{k} = 0$. This happens if the mother is homozygous at that locus (i.e. $A_1 = A_2$).

We then estimate the probability of each condition in equation (3) based on the frequency of alleles in the total population, such that:

$$P\left(G_{ik} = g_{ik}, G_{jk} = g_{jk} | b_{ij} = 1, b_{ij}^{k} = 1\right) = P(G_{ik} = g_{ik})\ if\ g_{ik} = g_{jk}, 0\ otherwise \quad (4)$$

And

$$P\left(G_{ik} = g_{ik}, G_{jk} = g_{jk} | b_{ij} = 1, b_{ij}^{k} = 0\right) = P(G_{ik} = g_{ik}) * P\left(G_{jk} = g_{jk}\right)$$

where the probability of carrying a given allele at the k-th locus (that is, $P(G_{ik} = g_{ik})$) is equal to that allele's frequency in our total sampled population:

$$P(G_{ik} = g_{ik}) = \sum_{l=1}^{n} I_{g_{lk=gik}} / n \quad (5)$$

Where $I_{g_{lk=gik}}$ is an indicator variable taking the value of 1 if $g_{lk=}g_{jk}$, and 0 otherwise.
We similarly use population allele frequencies to calculate the probability of males *i* and *j* carrying their observed genotypes if they are not brothers:

$$P\left(G_i = g_i, G_j = g_j \middle| b_{ij} = 0\right) = P(G_i = g_i) * P(G_j = g_j) \quad (6)$$

where

$$P(G_i = g_i) = \Pi_{k=1}^{7} P(G_{ik} = g_{ik})$$

and likewise, for $P\left(G_j = g_j\right)$.

Finally, we assume that the prior probability of two males being brothers, independent of any genotype information, is proportional to the total number of colonies contributing males to the sample set, *S*. Thus:

$$P(b_{ij} = 1) = 1/S \tag{7}$$

And

$$P(b_{ij} = 0) = 1 - 1/S$$

2.2 Simulations

We used a simulation-based approach to estimate the typical natal dispersal distances of male *T. carbonaria*. Males dispersed from their natal nests according to the exponential function:

$$P(d) = \lambda e^{-\lambda d}$$

where $d =$ metres flown, λ = 1/mean dispersal distance. For each of sample Sets 2 and 3, we ran simulations for 30 values of λ that represented mean male dispersal distances between 500m and 6500m. Each simulation followed these steps:

i. **Location of male-producing colonies**. We generated a colony at a random site within a virtual study area. The virtual study area was a rectangle overlaid on the map of our actual study site, with all boundaries at least 30km away from any collection site (**Figure S2**). Colony locations were determined according to wr, where a random state $wr \in 1,2,3,\ldots,100$. We used onwater.io (https://onwater.io/) to assess whether simulated colonies fell onto water and reassigned them if so.

ii. **Male genotypes.** For each colony, we first assigned a queen genotype with independent random sets of alleles at each of seven loci, based on population allele frequencies. We then generated 3000 males per colony where males were randomly assigned one allele per locus from their mother. This number approximates total males produced by a strong colony in Sydney during spring in one month (see Results, this study).

iii. **Male dispersal**. The distance flown by each male (*d*) was generated according to the distribution *P(d)* above. The final destination of each male was uniformly selected as a random point on the circle of circumference *d*, centred on the natal colony. That is, we assumed males were equally likely to fly away from the colony in all directions. Any males whose final destination was above water were allocated another final destination.

iv. **Male collection**. Any males with final destinations within *M* metres of a collection site was added to that collection (Set 3, *M* = 500m, Set 2, *M* = 300m). A sensitivity analysis of *M* is provided in Supp. Material 1.3)

v. **End collection**. We continued to simulate virtual colonies until the number of represented families in our virtual collection was equal to Nf (the number of males sampled in our actual dataset). If the number of virtually collected bees is larger than

the number collected in the experiment, we randomly keep only a number of bees equal to the collected sample.

vi. **Cumulative Distribution Functions** Finally, we calculated the sibship of each male pair in our simulated collection (as done for actual data above, **Supplementary Material, 2.1**) and obtained the cumulative distribution function, binned by the probability of sibship (0-0.05, 0.05-0.15, 0.15-0.25, ..., 0.85-0.95, 0.95-1). We calculated such CDFs for each of five values of *S*, representing different assumptions about the number of total colonies represented in our sample (Set 2, S = 201, 380, 475, 570; Set 3, S=100, 300, 450, 600, 750, 900).

vii. **Simulations vs Observed data** We assessed which values of λ (i.e which dispersal distributions) gave simulated datasets of male collections that most closely matched our actual datasets. For each λ, we took the geometric average of the area between the simulated and observed CDF curves. The lower the area, the more closely the simulation matched our real data.

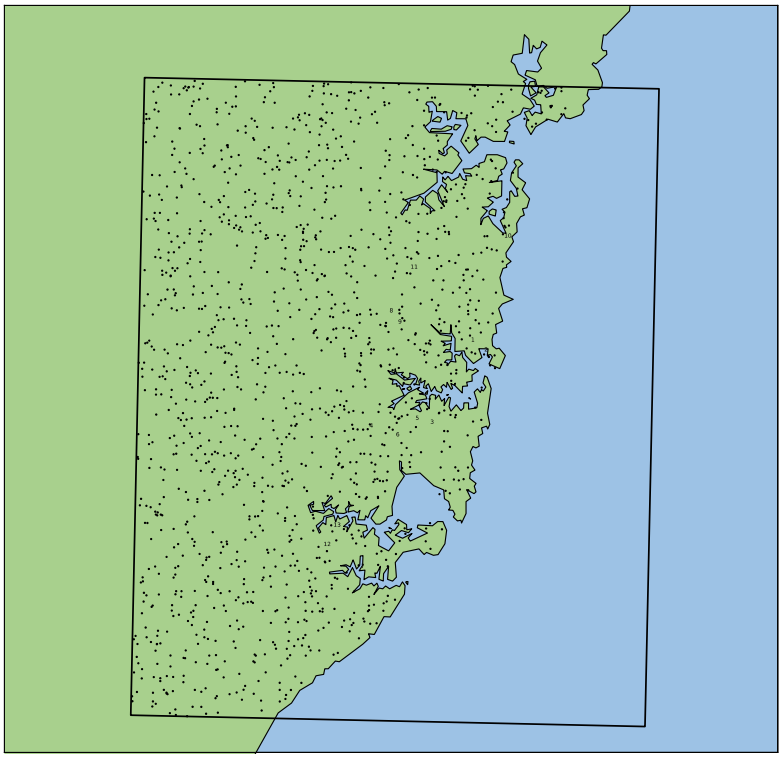


Figure S2. Map of Sydney based on our Set 3 collection with virtual simulated colonies represented by the black dots. The simulated colony locations were determined according to wr, where a random state $wr \in 1,2,3,\ldots,100$. We used onwater.io (https://onwater.io/) to assess whether simulated colonies fell onto water and reassigned them if so.

2.3 Sensitivity analysis of *M*

In our simulations, *M* is the distance that a male must pass within a requeening colony (i.e. a collection site) for the male to be included in our sample. In biological terms, it represents the range at which *T. carbonaria* males can detect a virgin queen's pheromone (or other signal emitted by colonies in the requeening process). In honey bees, this distance has been estimated at 100m (Brockmann et al., 2006), but for stingless bees it is unknown. We chose values ranging between 300 and 500m for Sets 2 and 3 respectively, which represent the largest possible area without causing overlap in the detection radius of our collection sites. To check that these values of *M* did not introduce significant variability in our results, we tested how the results for Set 3 (Sydney 2018) would change if *M* took different values (200m, 300m or 500m); **Figure S3** below. As each value of *M* gave similar mean dispersal distances for males, we conclude that our simulation results are robust within a reasonable range of possible values of *M*.

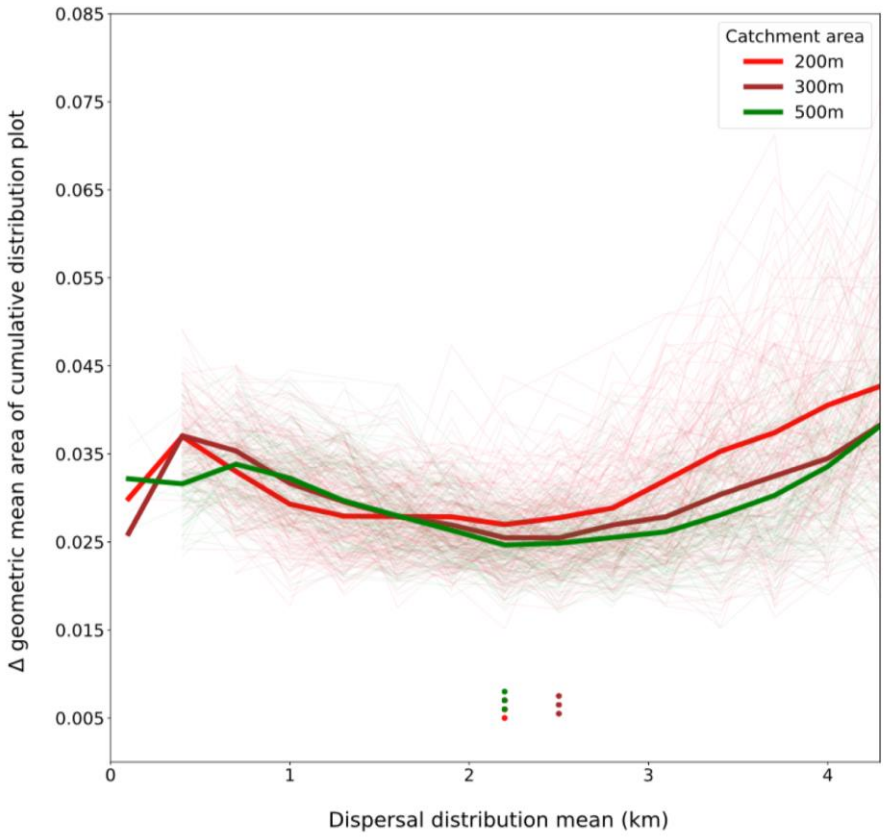


Figure S3. Cumulative distribution plots of difference between simulated data and observed data based on Set 3 collections for three values of *M* (200, 300, 500m). These simulations ran with 600 total families, and minimum and maximum flight means of 100m and 4.2km, respectively.

3.1 Evidence for male dispersal distances based on males with identical genotypes

We manually sorted genotypes of all the males from set 2 and 3 to identify males that shared alleles at all seven loci. For the genotypes of each of these conservative sets of possible brothers, we then calculated the probability that two males in our population would share that genotype by chance, using the formula:

$$p = f_1 * f_2 * f_3 \ldots * f_7$$

where f_1 is the frequency of the observed allele at locus 1, f_2 is the frequency of the observed allele at locus 2, etc. We then calculated the probability that the males' shared a genotype by descent (i.e. were brothers), rather than chance, according to:

$$p_s = (1 - p)^n$$

where *n* is the total sample size of all males sampled. In this way, we identified pairs of males that were carrying rare alleles in combinations that made it highly unlikely that they shared genotypes by chance alone. We considered pairs of males with $p_s > 0.85$ to be likely brothers, and $p_s > 0.95$ to be highly likely brothers. We then plotted the distance separating the sample location of these brother pairs (**Figure S4**). As for our sibship assignment using models and simulations (Supplementary Material, **2.1-2.3**), this estimate revealed that the great majority of likely brothers were collected from the same or nearby aggregations, 0-7km apart ($p_s > 0.85$, N=310; $p_s > 0.95$, N=199), but a small number were sampled at aggregations separated by >10km ($p_s > 0.85$, N=15; $p_s > 0.95$, N=1); Fig S4.

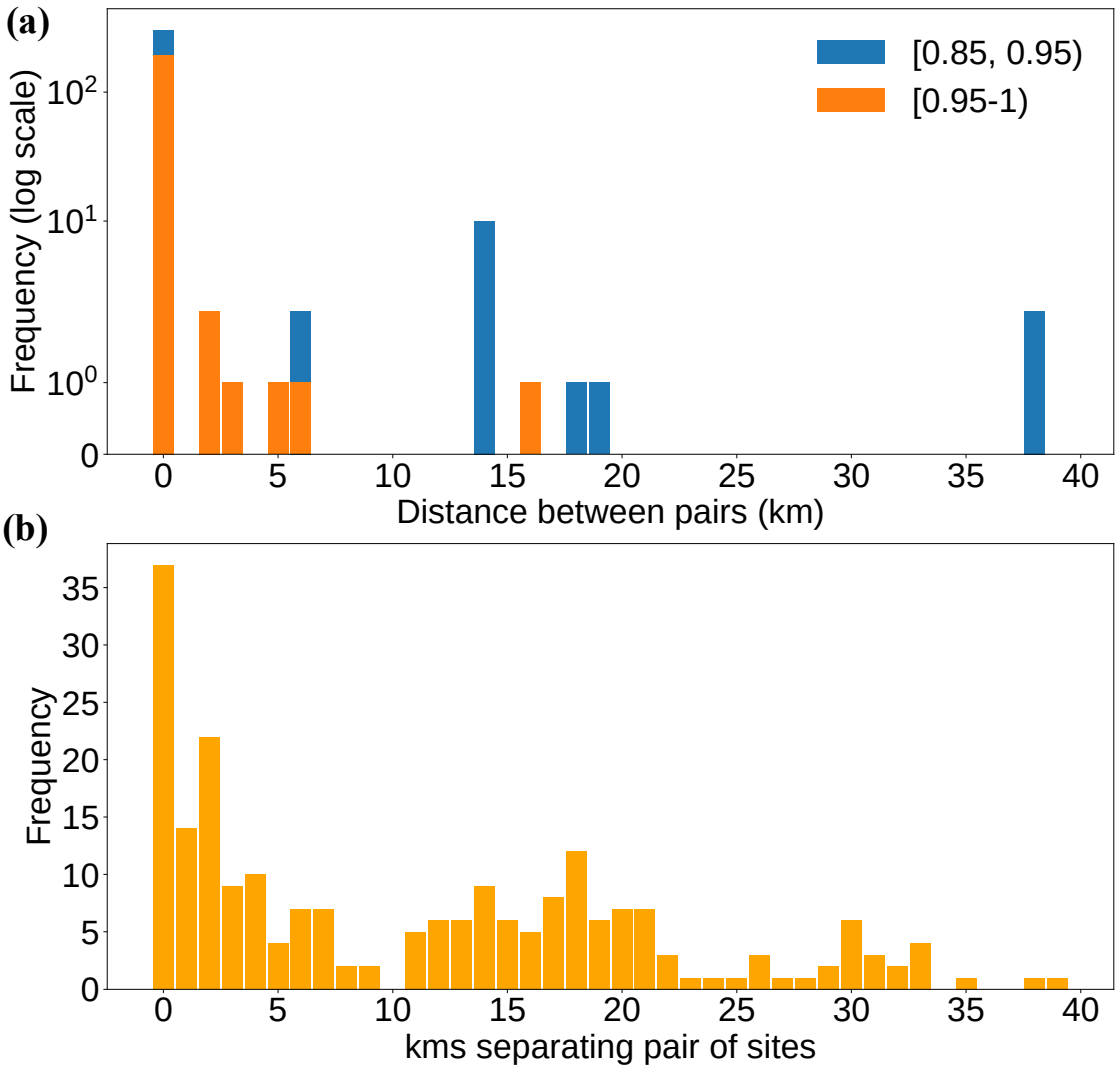


Figure S4. Distances between pairs of males sampled at mating aggregations, for males that share alleles at all seven loci we analysed. (a) The number of these males with high probability of sibship sampled from mating aggregations separated by different distances (p_s > 0.85, blue bars, n=325; p_s> 0.95 (orange bars, n=200). Most of these likely pairs of brothers were collected from the same or nearby aggregations, but a small number were sampled at aggregations separated by >10km ($p_s > 0.85$, N=15; $p_s > 0.95$, N=1). (b) the distance between pairs of sampled aggregations (sites) in our collections (Set 2 and 3).

Table S1. Male aggregation details. BC: Bait colony (Y when we used a bait colony and attracted males, N when the colony was recently split and attracted males and * when we split the colony but did not have visible virgin queens). Location: sampling site. Longitude. Latitude. Split day. Set up date. Sample date. Confirmation of VQ: When the colonies had an accepted virgin queen (no aggression from the workers and trophilaxis with the virgin queen). Requeened successfully: If the virgin queen mated and started laying eggs (Y the new queen was laying eggs). GSE: Global Solar exposure (J/m2) during the day of the sampling of the male aggregation. Temp: Temperature (°C) during the day of the set-up of the bait colony. Max Temp: Average maximum temperature (°C) during the month of sampling. *All Weather data were obtained from the Bureau of Meteorology, Australia (BOM, 2020) using the localities Sydney Observatory Hill and Sunshine Coast, Sunshine Coast Airport since they were the closest to our sampling sites. Size: Size of the aggregation sizes (small: 1-100 males, medium: 100-1000 males, big: >1000males) *The measurements were done to the eye by looking at the aggregation. Duration: Durations of the male aggregation (days)*The maximum number is 14 but some of them persisted for further days. Split location (P: Colonies that were split *in-situ* or NP: relocated pos-split) F: Fighting swarm (Y: if we found fighting pairs of bees around the hives.). No. of fam: Number of families (according to COLONY) and No. of males: Number of males genotyped per site.

Table S1 accessed here:

https://datadryad.org/stash/share/IZn77cU89Ul3Jc87cfkHjjnZ9aq4Z3XURmFrqGiikuE

Table S2. Male genotypes for 7 microsatellite loci for the three sample sets. We present a summary of this table below.

Table S3. Reconstructed mother genotypes of the workers from the bait colonies used for the three replicates

Table S4. Reconstructed mother genotypes of the workers from the 76 known managed colonies in Set 1.

Tables S2, **S3** and **S4** can be accessed here:

https://datadryad.org/stash/share/urjI91-GJ0f9vLlWV95ZC9OebkVLOmbBSaASIc-5yJE

Table S5. Mark recapture of males. Four replicates performed around Sydney during April and December 2018, October and November 2019. n= number of painted males. C: Control, Da: Wind Direction, Db: Opposite Wind Direction. Time of release: 9 AM. Distance released. 24h, 36h and >48h of males that turn out at the target swarm. Site a: 33°53'05.5"S 151°11'18.4"E, Site b: 33°47'33.0"S 151°16'22.1"E, Site c: 33°54'16.0"S 151°09'38.4"E and Site d: 33°53'37.7"S 151°10'19.6"E.* Repicate 3 For the first 2 days after releasing the marked males the weather was cloudy and rainy, possibly explaining why no marked males were found during the first 36 hours. *Replicate 4 had no control release and had multiple target sites with swarms at the same time.

Replicates	n	Site	Males released C/Da/Db	Distance (km)	24h	36h	>48h
1. April 2018	1500	a	260/633/600	0/1/1	34/3/2	13/2/01	10/2/0
2. Dec 2018	1000	b	100/400/400	0/1/1	17/7/4	10/6/03	0/0/0
*3. Oct 2019	2300	a	100/1000/1000	0/2.6/4.5	0/0/0	0/0/0	17/2/2
*4. Nov 2019	1600	c	0/800/800	0/2/1.7	0/0/0	0/1/2	0/0/1
		d	0/800/800	0/0.93/3	0/0/0	0/16/0	0/4/0

Table S6. Time and proportion of males entering and exiting colony entrances for 71 colonies. For 47 colonies at Sunshine Coast, we also genotyped all exiting males at seven microsatellite loci and confirmed they were full brothers indicated by the *.

Col ID	Site	Location	Longitude	Latitude	Date	Time	Ratio of males in exiting bees	Ratio of males in entering bees
48	East Killara	Sydney	33°44'59.7"S	151°10'47.1"E	16/10/18	10:00	0/20	0/20
49	East Killara	Sydney	33°45'37.7"S	151°10'36.4"E	16/10/18	10:30	1/20	0/20
50	East Killara	Sydney	33°45'29.0"S	151°10'26.5"E	16/10/18	11:30	0/20	0/20
51	East Killara	Sydney	33°45'42.1"S	151°10'26.5"E	16/10/18	12:27	0/20	0/20
52	Killara	Sydney	33°45'48.9"S	151°10'10.3"E	16/10/18	13:00	0/20	0/20
53	East Killara	Sydney	33°45'06.6"S	151°11'06.5"E	16/10/18	14:00	0/20	0/20
54	St Ives	Sydney	33°42'21.0"S	151°10'49.7"E	22/10/18	10:45	0/20	0/20
55	Wahroonga	Sydney	33°43'58.4"S	151°06'29.3"E	30/10/18	9:00	0/20	0/20
56	Wahroonga	Sydney	33°42'24.9"S	151°07'16.6"E	30/10/18	9:45	0/20	0/20
57	North Wahroonga	Sydney	33°41'52.3"S	151°07'17.7"E	30/10/18	10:45	2/20	0/20
58	North Wahroonga	Sydney	33°42'07.8"S	151°07'34.8"E	30/10/18	11:30	0/20	0/20
59	Wahroonga	Sydney	33°42'50.6"S	151°08'33.0"E	30/10/18	13:00	0/20	0/20
60	Gordon	Sydney	33°45'43.9"S	151°09'03.0"E	6/11/18	10:10	0/20	0/20
61	Gordon	Sydney	33°45'32.2"S	151°08'42.2"E	6/11/18	11:00	0/20	0/20
62	Gordon	Sydney	33°45'15.2"S	151°08'35.5"E	6/11/18	11:45	0/20	0/20
63	Gordon	Sydney	33°45'27.1"S	151°09'02.8"E	6/11/18	13:30	0/20	0/20
64	Gordon	Sydney	33°44'48.3"S	151°09'42.4"E	6/11/18	14:13	0/20	0/20
65	Gordon	Sydney	33°44'43.3"S	151°09'32.0"E	6/11/18	15:00	0/20	0/20
66	Camperdown	Sydney	33°53'05.5"S	151°11'18.4"E	19/11/18	10:30	2/20	0/20
67	East Lindfield	Sydney	33°45'57.0"S	151°11'23.8"E	21/11/18	9:23	1/20	0/20
68	East Lindfield	Sydney	33°45'42.5"S	151°11'10.4"E	21/11/18	10:37	2/20	0/20

69	East Lindfield	Sydney	33°46'11.9"S	151°10'58.4"E	21/11/18	12:00	0/20	0/20
70	East Lindfield	Sydney	33°45'54.9"S	151°11'08.9"E	21/11/18	13:00	0/20	0/20
71	Camperdown	Sydney	33°53'37.6"S	151°10'18.8"E	13/2/18	7:00	3/20	0/20
1	Mooloolah Valley	Sunshine Coast	26°45'14.1"S	152°59'14.5"E	15/9/17	10:00	0/24	
2	Mooloolah Valley	Sunshine Coast	26°45'14.1"S	152°59'14.5"E	15/9/17	10:00	2/24*	
3	Mooloolah Valley	Sunshine Coast	26°45'14.1"S	152°59'14.5"E	15/9/17	10:00	19/24*	
4	Mooloolah Valley	Sunshine Coast	26°45'14.1"S	152°59'14.5"E	15/9/17	9:30	0/24	
5	Mooloolah Valley	Sunshine Coast	26°45'14.1"S	152°59'14.5"E	15/9/17	9:30	0/24	
6	Mooloolah Valley	Sunshine Coast	26°45'14.1"S	152°59'14.5"E	15/9/17	9:30	0/24	
7	Mooloolah Valley	Sunshine Coast	26°45'14.1"S	152°59'14.5"E	15/9/17	9:30	0/24	
8	Beerwah	Sunshine Coast	26°50'23.4"S	152°57'10.9"E	15/9/17	14:00	0/24	
9	Beerwah	Sunshine Coast	26°50'23.4"S	152°57'10.9"E	15/9/17	14:00	1/24	
10	Beerwah	Sunshine Coast	26°50'23.4"S	152°57'10.9"E	15/9/17	14:00	0/24	
11	Beerwah	Sunshine Coast	26°50'23.4"S	152°57'10.9"E	15/9/17	14:00	0/24	
12	Beerwah	Sunshine Coast	26°50'23.4"S	152°57'10.9"E	15/9/17	14:00	0/24	
13	Beerwah	Sunshine Coast	26°50'23.4"S	152°57'10.9"E	15/9/17	14:00	0/24	
14	Beerwah	Sunshine Coast	26°50'23.4"S	152°57'10.9"E	15/9/17	14:00	0/24	
15	Beerwah	Sunshine Coast	26°50'23.4"S	152°57'10.9"E	15/9/17	14:00	0/24	
16	Beerwah	Sunshine Coast	26°50'23.4"S	152°57'10.9"E	22/4/18	12:00	16/24*	
17	Beerwah	Sunshine Coast	26°50'23.4"S	152°57'10.9"E	22/4/18	12:00	23/24*	
18	Mooloolah Valley	Sunshine Coast	26°45'14.1"S	152°59'14.5"E	22/4/18	10:00	7/24*	
19	Mooloolah Valley	Sunshine Coast	26°45'14.1"S	152°59'14.5"E	20/4/18	10:00	4/24*	
20	Mooloolah Valley	Sunshine Coast	26°45'14.1"S	152°59'14.5"E	21/4/18	10:00	21/24*	
21	Mooloolah Valley	Sunshine Coast	26°45'14.1"S	152°59'14.5"E	21/4/18	10:00	16/24*	
22	Beerwah	Sunshine Coast	26°50'23.4"S	152°57'10.9"E	21/4/18	12:00	21/24*	
23	Beerwah	Sunshine Coast	26°50'23.4"S	152°57'10.9"E	21/4/18	12:00	16/24*	
24	Beerwah	Sunshine Coast	26°50'23.4"S	152°57'10.9"E	21/4/18	12:00	0/24	
25	Beerwah	Sunshine Coast	26°50'23.4"S	152°57'10.9"E	2/5/18	12:00	0/24	

26	Beerwah	Sunshine Coast	26°50'23.4"S	152°57'10.9"E	21/4/18	12:00	0/24
27	Mooloolah Valley	Sunshine Coast	26°45'14.1"S	152°59'14.5"E	21/4/18	10:00	0/24
28	Mooloolah Valley	Sunshine Coast	26°45'14.1"S	152°59'14.5"E	21/4/18	10:00	0/24
29	Mooloolah Valley	Sunshine Coast	26°45'14.1"S	152°59'14.5"E	21/4/18	10:00	0/24
30	Glasshouse Mountains	Sunshine Coast	26°53'20.8"S	152°55'57.0"E	4/10/18	11:00	0/24
31	Glasshouse Mountains	Sunshine Coast	26°53'20.8"S	152°55'57.0"E	4/10/18	11:00	0/24
32	Glasshouse Mountains	Sunshine Coast	26°53'20.8"S	152°55'57.0"E	4/10/18	11:00	0/24
33	Glasshouse Mountains	Sunshine Coast	26°53'20.8"S	152°55'57.0"E	4/10/18	11:00	0/24
34	Glasshouse Mountains	Sunshine Coast	26°53'20.8"S	152°55'57.0"E	4/10/18	11:00	0/24
35	Glasshouse Mountains	Sunshine Coast	26°53'20.8"S	152°55'57.0"E	4/10/18	11:00	0/24
36	Mooloolah Valley	Sunshine Coast	26°45'14.1"S	152°59'14.5"E	4/10/18	10:00	15/24*
37	Mooloolah Valley	Sunshine Coast	26°45'14.1"S	152°59'14.5"E	4/10/18	10:00	10/24*
38	Beerwah	Sunshine Coast	26°50'23.4"S	152°57'10.9"E	4/10/18	11:00	1/24
39	Beerwah	Sunshine Coast	26°50'23.4"S	152°57'10.9"E	4/10/18	11:00	10/24*
40	Beerwah	Sunshine Coast	26°50'23.4"S	152°57'10.9"E	4/10/18	11:00	5/24*
41	Beerwah	Sunshine Coast	26°50'23.4"S	152°57'10.9"E	4/10/18	11:00	8/24*
42	Mooloolah Valley	Sunshine Coast	26°45'14.1"S	152°59'14.5"E	4/10/18	10:00	0/24
43	Mooloolah Valley	Sunshine Coast	26°45'14.1"S	152°59'14.5"E	4/10/18	10:00	0/24
44	Mooloolah Valley	Sunshine Coast	26°45'14.1"S	152°59'14.5"E	4/10/18	10:00	0/24
45	Mooloolah Valley	Sunshine Coast	26°45'14.1"S	152°59'14.5"E	4/10/18	10:00	0/24
46	Beerwah	Sunshine Coast	26°50'23.4"S	152°57'10.9"E	4/10/18	11:00	0/24
47	Beerwah	Sunshine Coast	26°50'23.4"S	152°57'10.9"E	4/10/18	11:00	0/24

CHAPTER 4

Irreversible sterility of workers and high-volume egg production by queens in the stingless bee *Tetragonula carbonaria*

Australian stingless bee (*Tetragonula carbonaria*) workers walking on top of the brood comb of a colony. Typically, the female workers perform all of the manual labour in the colony and are non-reproductive. Photo credit: Francisco Garcia Bulle Bueno. This chapter has been published in the Journal of Experimental Biology and this picture was selected as the cover for the September 2020 Edition.

Abstract

Social insects are characterised by a reproductive division of labour between queens and workers. However, in the majority of social insect species the workers are only facultatively sterile. The Australian stingless bee *Tetragonula carbonaria* is noteworthy as workers never lay eggs. Here, we describe the reproductive anatomy of *T. carbonaria* workers, virgin queens, and mated queens. We then conduct the first experimental test of absolute worker sterility in the social insects. Using a controlled microcolony environment, we investigate whether the reproductive capacity of adult workers can be rescued by manipulating the workers' social environment and diet. The ovaries of *T. carbonaria* workers that are queenless and fed unrestricted, highly nutritious royal jelly remain non-functional, indicating they are irreversibly sterile and that ovary degeneration is fixed prior to adulthood. We suggest that *T. carbonaria* might have evolved absolute worker sterility because colonies are unlikely to ever be queenless.

Introduction

In social hymenopterans (referred in this chapter as social insects) the reproductive labour is divided between queens and workers (Naug and Wenzel, 2006, Hammond and Keller, 2004). The queen is typically the sole reproductive female and her specialised function in the colony is to lay eggs. Whereas, the non-reproductive female workers forage for food, defend the colony against predators and care for the queen's brood (Michener, 1974).

In the majority of social insect species, workers are only facultatively sterile and have the ability to lay unfertilised haploid eggs that develop as males (Wenseleers et al., 2004b, Khila and Abouheif, 2010, Ebie et al., 2015). The contribution of workers to the colony's male output is highly variable across social insect species, and is determined by kinship-mediated cooperation and within-colony conflict over male production (Alves et al., 2009, Tóth et al., 2002, van Veen et al., 1990, Herbers, 1984, Mehdiabadi et al., 2003). Facultative worker sterility means that if the queen is lost and the colony cannot requeen, then workers can secure some reproductive success by producing males (Bourke, 1988).

The key determinants of the reproductive capacity of social insect workers is their nutritional status and social environment (Ronai et al., 2016). First, the presence of the queen, through aggressive interactions or pheromones, triggers programmed cell death in the ovaries of the workers (Luna-Lucena et al., 2018, Ronai et al., 2015); workers may therefore develop activated ovaries only in the queen's absence. Second, ovary development is trophogenic (Lisboa et al., 2005) as the quantity or quality of nutrients has a strong effect on ovary development (Cruz-Landim, 2000, Luna-Lucena et al., 2018, Boleli et al., 2000, Boleli et al., 1999, Jarau et al., 2010, Schwander et al., 2010, Corona et al., 2016). For example, in the honey bee *Apis mellifera*, female workers fed highly nutritious royal jelly as adults have activated ovaries even in the presence of queen pheromone (Kamakura, 2011, Lin and Winston, 1998, Pirk et al., 2010).

Stingless bees (Meliponini) are a species-diverse pantropical clade of social insects, comprising Neotropical, Afrotropical and Indo-Malayan Australasian subclades that diverged 50-60 MYA (Michener, 1974, Roubik, 1995, Rasmussen and Cameron, 2010).The reproductive habits of stingless bee workers have been best studied in the Neotropical subclade, where workers

produce males under queenright conditions (Boleli et al., 2000, Beig et al., 1985, Contel and Kerr, 1976, Vollet-Neto et al., 2018), produce males only when queenless (Cruz-Landim, 2000, Tóth et al., 2004), lay trophic eggs which serve as nourishment for the queen (Wille, 1983), or never lay eggs (Staurengo da Cunha, 1979, Boleli et al., 1999). However, the reproductive habits of workers in the Indo-Malayan Australasian subclade are relatively unknown.

In this study, we describe the reproductive anatomy of mated queens, virgin queens and female workers of the Australian stingless bee *Tetragonula carbonaria*. A colony of this Indo-Malayan Australasian subclade species has one mated queen, thousands of non-reproductive female workers, some males and a few virgin queens (Gloag et al., 2007, Nunes et al., 2015, Heard, 2016). Notably, when the queen is removed from the colony, *T. carbonaria* female workers do not activate their ovaries (Nunes et al., 2015), suggesting they may be irreversibly sterile. To confirm this, we test whether manipulating the nutritional and social environment of age-matched adult female workers in a controlled microcolony environment can rescue their reproductive capacity (i.e. stimulate oogenesis). We hypothesised that if adult *T. carbonaria* workers are irreversibly sterile, then those fed an unrestricted highly nutritious diet in the absence of a queen would show no change in reproductive capacity.

Method

Biological material

Our study was carried out at the University of Sydney (Sydney, Australia) in December-January of 2017 and December-February of 2019. As the source colonies, we used seven *T. carbonaria* colonies (Colony 1–7) housed in wooden Original Australian Tetragonula Hives (Heard, 2016). These colonies were queenright, weighed above average (8–9 kg; Heard, 2016) and had high forager activity at the entrance of the colony (~100 bees exiting per minute).

To obtain age-matched workers we extracted two discs of late stage worker brood comb (approximately 200 bees per disk) from a source colony and placed the discs in a plastic container. The containers were then placed in an incubator (34.5 °C) for approximately 5 days. *T. carbonaria* worker brood comb contains both male and female brood in identical cells. Any males that emerged were removed from the container.

Worker and queen reproductive capacity

To assess the differences in reproductive anatomy between castes under natural conditions, we collected newly emerged workers (*n* = 200, per colony), recognised by their pale colour, from four of the source colonies (Colony 1–4), colour-marked their thorax using POSCA Pens and returned them to their source colony. After 14 days, the majority of marked workers have lost their mark or died, but we collected 10 marked workers from each source colony.

We collected the mated queen (unknown age) and one virgin queen (unknown age) from each of these four source colonies (Colony 1–4). To extract the queens, we set up observation hives with a transparent plastic lid so that we could observe the brood comb. Once the mated queen was collected, we waited 24 h for a virgin queen to emerge on the brood comb and then extracted a virgin queen. These virgin queens were walking on top of the brood comb flapping their wings and were being fed via trophallaxis by the workers. Note we observed male aggregations outside nearby colonies. All collected workers and queens were immediately placed into a -80 °C freezer.

Manipulation of workers in microcolonies

To manipulate the social environment and diet of adult workers we designed laboratory-based microcolonies (Supplementary Material, **Figure S1**) using 500 mL plastic containers. Each microcolony was provided with a 1.5–2 cm (diameter) pellet of propolis (a mix of wax and plant resins that stingless bees use as nest building material; Heard, 2016) and a 250 mg pellet of pollen was scooped out of a pot-pollen (pollen is stored in pots made of propolis and is the main protein source for stingless bees; Heard, 2016), both obtained from a nearby colony. Into each microcolony we placed newly emerged workers (*n* = 50 per microcolony, 8 microcolonies) from one of three source colonies (Colony 5–7). The eight microcolonies contained no queen and also no brood.

Four of the microcolonies were randomly assigned a control diet consisting of stingless bee honey and the other four paired (from the same source colony) microcolonies were assigned a royal jelly diet consisting of 50% stingless bee honey to 50% frozen royal jelly (*A. mellifera* Royal Jelly, Australia). 0.2 mL of each diet was placed onto plastic dishes (1 cm diameter)

using a 3 mL plastic pipette. We established that the workers were ingesting the food by observing the bees feeding and the food containers were emptied. In addition, when dissecting the workers, we observed that their crop was full of food.

Royal jelly is high in protein and contains important fatty acids (Altaye et al., 2010) and is fed to honey bee (*Apis* sp.) queens throughout their lifespan (Howe et al., 1985, Melampy and Jones, 1939, Viuda-Martos et al., 2008). Notably, feeding royal jelly to adult honey bee workers causes them to activate their ovaries (Lin and Winston, 1998, Wang et al., 2014, Yang et al., 2017, Cardoso-Júnior et al., 2020) and feeding it to female fruit flies, crickets and silkworms increases their fecundity (Xin et al., 2016, Kamakura, 2011, Hodin, 2009, Hodin and Riddiford, 2000, Miyashita et al., 2016, Kunugi and Mohammed Ali, 2019, Kayashima et al., 2012). Stingless bees are not known to use royal jelly but a high protein diet increases fertility in stingless bee workers, such as *Melipona flavolineata* (Costa and Venturieri, 2009).

The microcolonies were checked daily. Food, pollen and water were replenished when needed. Any dead workers were removed and recorded (Supplementary Material, **Table S1**). We collected two workers from each microcolony at 0 days of age and all remaining workers at 14 days of age. All collected workers were immediately placed into a -80 °C freezer.

Ovary dissections

We extracted the paired ovaries from each bee collected from both natural colonies and microcolonies. The tiny ovaries of the workers need to be carefully dissected as they are fragile and likely to break. We placed the ovaries in a drop of distilled water on a microscope slide and covered them with a cover slip. The ovaries were imaged under a dissecting microscope (Leica IC80 HD) at 0.67X (mated queens and virgin queens) and 4X (workers) magnification. We analysed the images with ImageJ (Rasband, 1997) while blind to treatment.

We evaluated the reproductive capacity and anatomy of *T. carbonaria* females following the protocols used in our previous studies of honey bee ovaries (Ronai et al., 2017, Ronai et al., 2015). For each bee, we assessed the ovary activation state (deactivated, semi-activated or activated) and counted the number of ovarioles (filaments) in an ovary. We then quantified the length of the ovary as a proxy for the amount of ovary development or degeneration. For

workers, we measured the length of both ovaries (from the tip of the longest ovariole of each ovary to where the two oviducts join; Supplementary Material, **Figure S2a**). For mated queens and virgin queens, it was impossible to measure the length of the ovarioles as they are tightly curled, so we measured the elliptical area of the ovary (area = $a \times b \times \pi$, where a is the major diameter divided by 2 and b is the minor diameter divided by 2; Supplementary Material, **Figure S2b**). If still attached after dissection, we noted the presence and status (semen present or absent) of the spherical spermatheca, the specialised organ used to store the sperm, and measured its diameter if present (Supplementary Material, **Figure S2**).

For all workers, we investigated whether the length of the right and left ovary was correlated using a Pearson correlation so we could then compare the length of the longest ovary in our experimental treatments. To compare the length of the longest ovary of workers fed a Control diet and Royal jelly diet, we conducted a Student t-test (all assumptions were met) using R Studio (Version 1.3.959), (RStudio Team, 2015), with R packages the function "t.test" in the R package *stats v4.0.0* (R Core Team, 2013).

Results

Reproductive morphology of workers in queenright colonies

Queenright 14-day-old *T. carbonaria* workers had deactivated ovaries as no developing oocytes were present inside the ovarioles (**Figure 1**). The number of ovarioles in the ovary of workers varied from four ovarioles to no ovarioles. The mean length of the longest ovary was 0.65 mm ± 0.15 mm (n = 40; Supplementary Material, **Table S2**). The length of the right and left ovary was asymmetric (P =0.098, Table S3). Only 20% of workers (n = 8) had an identifiable, but vestigial spermatheca with a mean diameter of 0.05 mm ± 0.01 mm (**Figure 2a,** Supplementary Material, **Table S2**).

Reproductive morphology of virgin queens and mated queens

T. carbonaria queens had four ovarioles per ovary (**Figure 2**). We observed that the ovaries of *T. carbonaria* are of the meroistic polytrophic type (Martins and Serrão, 2004, Büning, 1994) as is typical for Hymenoptera. As an oocyte moves from the tip of the ovariole to the base it

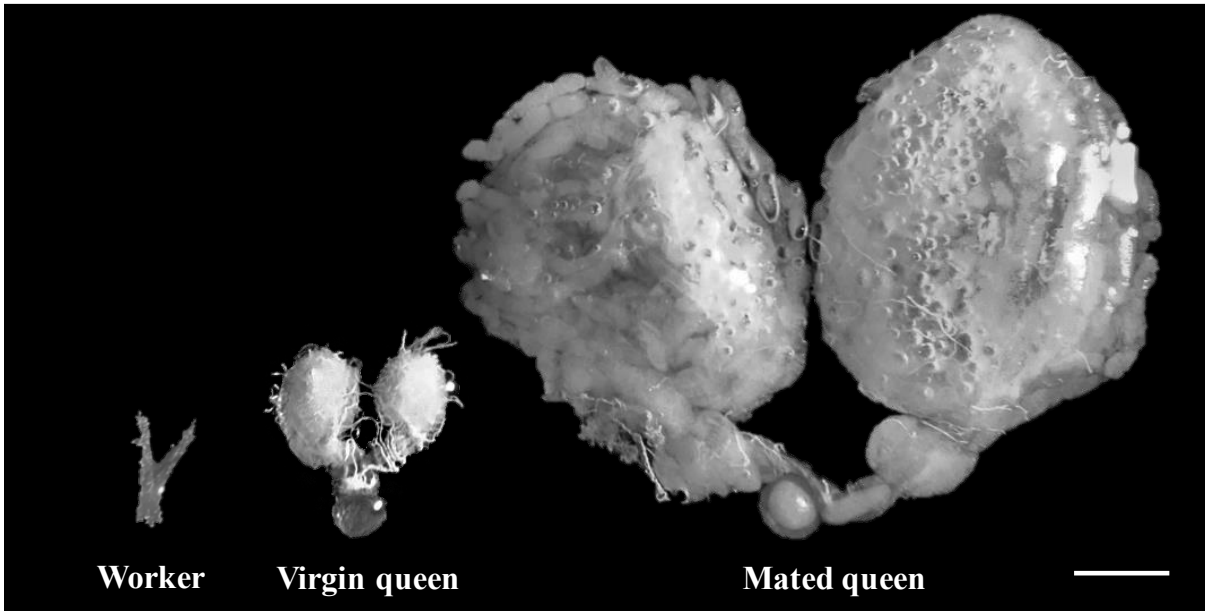


Figure 1. The ovaries of *T. carbonaria* females. Queenright 14-day old workers have deactivated ovaries (no developing oocytes present) with zero to four ovarioles. Virgin queens have semi-activated ovaries (oocytes not mature) with four ovarioles, Mated queens have activated ovaries (mature oocytes present) with four ovarioles. The ovaries of mated and virgin queens are covered by an extensive tracheal network. Image background has been removed. Scale bar 1 mm.

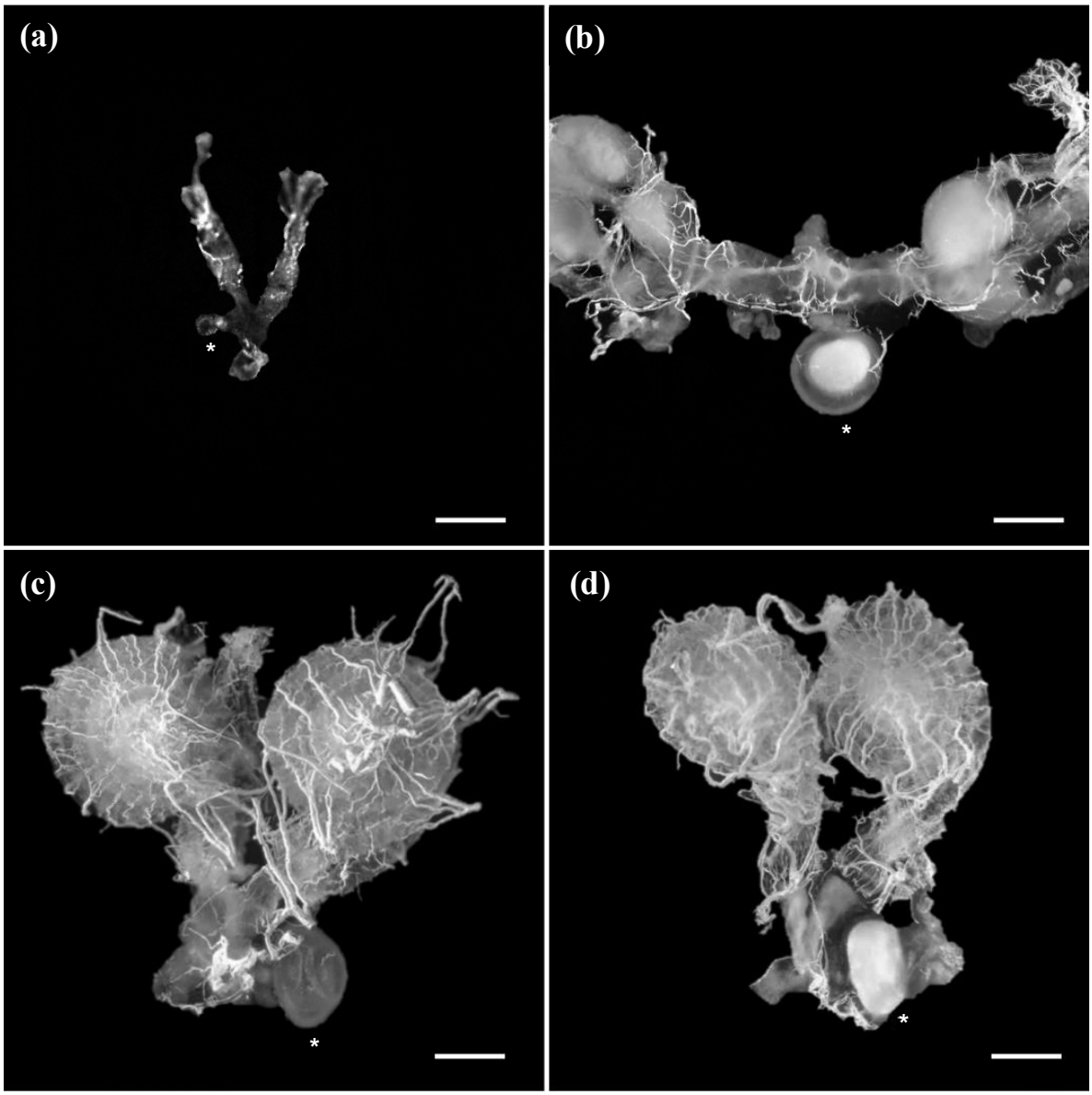


Figure 2. The spermatheca of *T. carbonaria* females. (a) Queenright 14-day old worker with a vestigial spermatheca. (b) Mated queen with a full spermatheca reservoir containing semen. (c) Virgin queen with an empty spermatheca reservoir. (d) Recently mated queen with a full spermatheca reservoir containing semen and semi-activated ovaries, suggesting mating occurred recently. Spermatheca (*). Image background has been removed. Scale bars 0.5 mm.

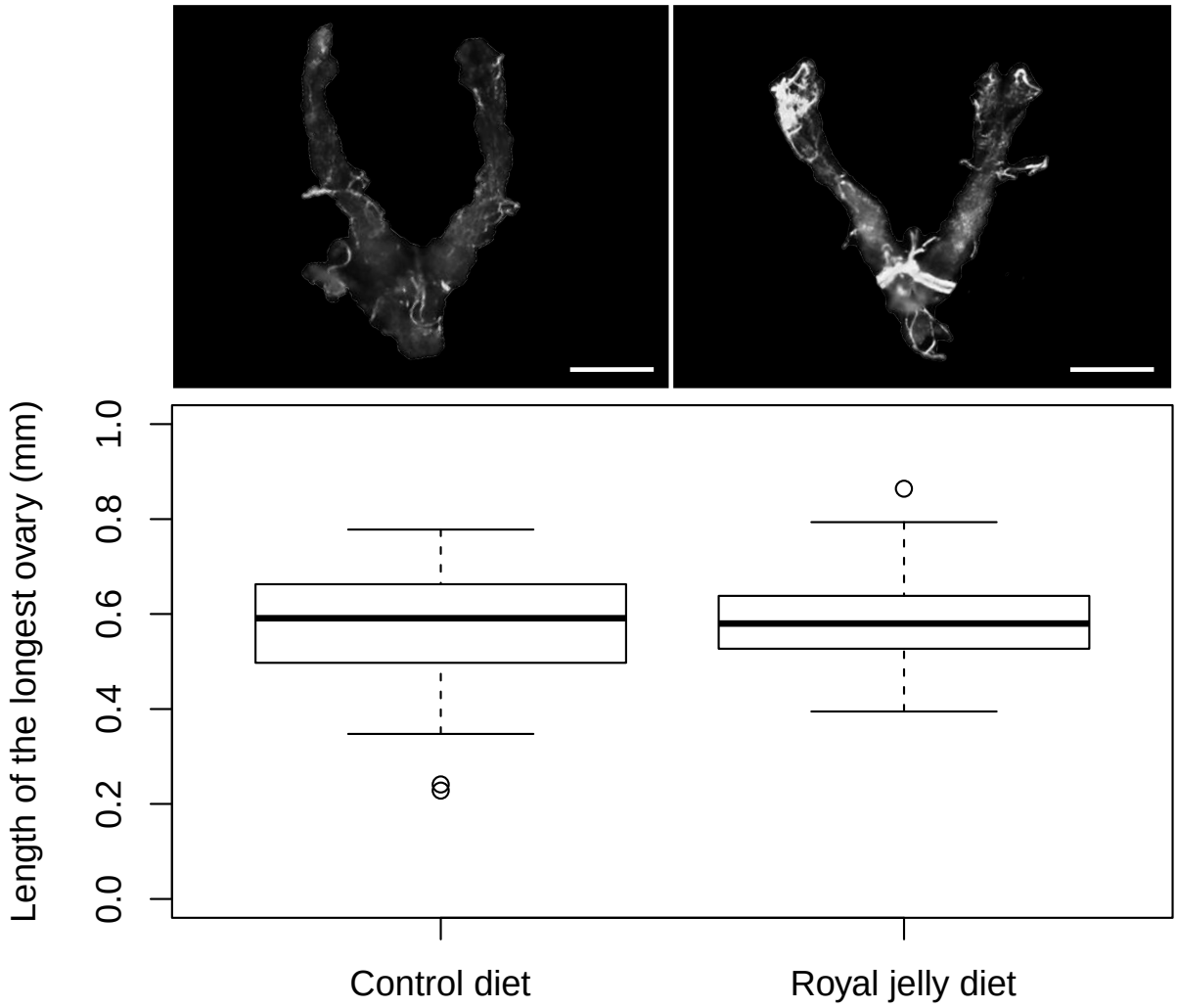


Figure 3. Ovary length of 14-day old queenless *T. carbonaria* workers. The workers were fed a Control diet (*n* = 40, 10 per microcolony) or a Royal jelly diet (*n* = 40, 10 per microcolony). Image background has been removed. Scale bars 0.25 mm. There was no significant difference between the treatment groups.

progresses through stages of development (stem cell, germarium and vitellarium) (Ronai et al., 2015).

Virgin queens had semi-activated ovaries with oogenesis underway (**Figure 1**) and the mean area of the largest ovary was 1.02 mm² ± 0.12 mm² (*n* = 4; Supplementary Material, **Table S4**). The spermatheca had a mean diameter of 0.55 mm ± 0.03 mm (n = 3, Table S4). For two of the virgin queens the spermatheca reservoir was transparent indicating it was empty (**Figure 2c**), however the third virgin queen had a filled spermatheca reservoir indicating that she mated within the 24 hours that there was no mated queen in the colony (**Figure 2d**).

Mated queens had activated ovaries full of mature oocytes (**Figure 1**). Some of the activated ovaries had yellow bodies present, a consequence of previous oviposition (Gobin et al., 1998, Feneron and Billen, 1996). The mean area of the largest ovary was 13.41 mm² ± 2.99 mm² (*n* = 4; Supplementary Material, **Table S4**), approximately thirteen times larger than the virgin queens. In order to fit inside the abdomen (approximately 6.5 mm in length) of the queen the ovaries are curled tightly in a spiral and occupy the majority of the abdominal cavity, which causes the mated queen to become highly physogastric. The spermatheca had a mean diameter of 1.05 mm ± 0.81 mm (*n* = 2; Supplementary Material, **Table S4**) and for both the reservoir was filled with a milky white substance indicating the presence of semen (**Figure 2b**).

Reproductive morphology of queenless workers fed royal jelly diet and control diet

Both royal jelly diet and control diet queenless workers had degenerated and deactivated ovaries. The mean length of the longest ovary of 0-day old workers was 0.56 mm ± 0.1 mm (*n* = 16; Supplementary Material, **Table S2**). The mean length of the longest ovary of 14-day-old workers fed a control diet was 0.57 mm ± 0.14 mm (*n* = 40; Supplementary Material, **Table S2**) and a royal diet was 0.59 mm ± 0.11 mm (*n* = 40; Supplementary Material, **Table S2**). The mean length of the right and left ovary was significantly correlated for both control diet and royal diet workers (*P* = 0.001 and 0.007 respectively; Supplementary Material, **Table S3**). There was no significant difference in the mean length of the longest ovary between the control diet and royal diet treatments after 14 days (*t* = -0.68194, *df* = 75.203, *p* = 0.4974, **Figure 3**).

Discussion

An unrestricted high quality diet and the absence of the queen failed to rescue the reproductive capacity of *T. carbonaria* adult workers, indicating that they are irreversibly sterile. This absolute sterility is consistent with observations from natural nests in which workers never lay eggs, even if queenless (Nunes et al., 2014, Gloag et al., 2007). Absolute sterility of workers is relatively rare among social insect species. Only 13 genera have so far been classified as having truly sterile workers based on observations under natural conditions (Ronai et al., 2016); whether sterility is irreversible in these species has not yet been tested experimentally.

Why have a few genera of social insect evolved absolute worker sterility and forgone the obvious benefits of facultative sterility? It is likely that the life history of some species predisposes them to the evolution of absolute worker sterility. *Tetragonula carbonaria* have a high and constant rate of queen production (Heard, 2016) when compared to other stingless bee species with facultative worker sterility (Van Veen and Sommeijer, 2000a, Grosso et al., 2000, Ribeiro et al., 2003) and also honey bees that have facultative worker sterility (Jay, 1968, Kropáčová and Haslbachová, 1969). In our study, we found a mated 'virgin' queen within 24 hours of the original mated queen being removed. In addition, *T. carbonaria* workers are able to create emergency queen cells from worker cells (Nunes et al., 2015). A *T. carbonaria* colony is therefore likely to always have a queen present and there would never be a need for workers to lay eggs (Engels and Imperatriz-Fonseca, 1990, Michener, 1974). Species that have a constant reservoir of virgin queens from which the workers select the most fecund one or rear new queens very quickly may be more likely to evolve absolute worker sterility (Sommeijer et al., 1994). Another possible explanation for the evolution of absolute worker sterility is the high rate of colony-takeovers in *T. carbonaria* (Cunningham et al., 2014, Gloag et al., 2008). This behaviour reduces the chance that a colony would ever have a prolonged period where it was queenless and that the workers would need to lay eggs.

Alternatively, absolute worker sterility may resolve reproductive conflict within colonies. *Tetragonula carbonaria* is a monogamous species (Green and Oldroyd, 2002). Under monogamous queens, workers in a colony are all full sisters and workers would be more related to their own sons ($r = 0.5$) than the sons of the queen ($r = 0.25$). If workers have the ability to lay eggs, there would be worker-queen conflict for male production in the colony (Trivers and Hare, 1976). The evolution of absolute worker sterility in monogamous species eliminates the

worker-queen conflict to the benefit of the queen. However, many social insects, including most Neotropical sublclade stingless bees, have both monogamous queens and workers that lay eggs (Vollet-Neto et al., 2018).

We find that *T. carbonaria* workers emerge from pupation with non-functional ovaries that contain no germ cells. The degeneration of the ovary in *T. carbonaria* workers must therefore occur prior to adulthood. To date, only one other genera of stingless bee in the Neotropical subclade has been proposed to have absolute worker sterility, *Frieseomelitta* spp. (Boleli et al., 2000, Luna-Lucena et al., 2018, Boleli et al., 1999, Ronai et al., 2016) and their ovaries degenerate during the last pupal stage via apoptosis (Boleli et al., 1999). Further work is needed to establish whether the degeneration of the ovaries in *T. carbonaria* workers is initiated during the embryonic, larval or pupal stage. In *T. carbonaria* the ovary degeneration in workers is likely determined by their nutritional environment pre-emergence as worker-destined larvae provisioned with a smaller quantity of food than queen-destined larvae (Nunes et al., 2015).

Even when the workers of social insects have activated ovaries, they may not have a storage organ for semen. The spermatheca is thus normally absent from workers (Gobin et al., 2008, Hölldobler and Wilson, 1990, Van Eeckhoven and Duncan, 2020, Khila and Abouheif, 2010). However, *T. carbonaria* workers sometimes have a vestigial spermatheca, the first reported case in the Indo-Malayan Australasian subclade of stingless bees. Vestigial spermatheca are present in workers of the one other stingless bee genera with absolute worker sterility (Boleli et al., 2000) and also in honey bee species with facultative worker sterility (Gobin et al., 2006, Gotoh et al., 2013). The presence of a vestigial spermatheca in species where the workers have degenerated ovaries provides evidence that spermatheca degeneration is a separate process to the degeneration of the ovaries.

Our study of *T. carbonaria* ovaries highlights that the exceptionally high reproductive capacity of queens is achieved in different ways across the clades of eusocial bees. Stingless bees and honey bees (*Apis* spp.) each evolved highly eusocial life histories independently, from an ancestral corbiculae apid with more simple sociality (Cardinal and Danforth, 2011). *Tetragonula carbonaria* queens lay approximately 300 eggs per day (Heard, 2016) but have only 8 ovarioles in total; therefore, each ovariole is elongated and produces around 37 eggs per day. In contrast, *A. mellifera* queens lay approximately 2000 eggs per day (Page and Erickson, 1988) but have on average 320 ovarioles in total (Jackson et al., 2011); therefore, each ovariole

is relatively short and produces only around 6 eggs per day. That is, the lower number of ovarioles in stingless bee queens when compared to honey bee queens is compensated by high volume egg production in each ovariole of the ovary.

Conclusion

In summary, our results provide strong evidence that *T. carbonaria* workers are irreversibly sterile as adults. Conducting similar studies of workers in the other *Tetragonula* spp. would establish whether absolute sterility is a characteristic of this genus. Studies of absolute worker sterility, and the factors that favour its evolution, are likely to provide a deeper understanding of the evolution of eusociality.

Supplementary information

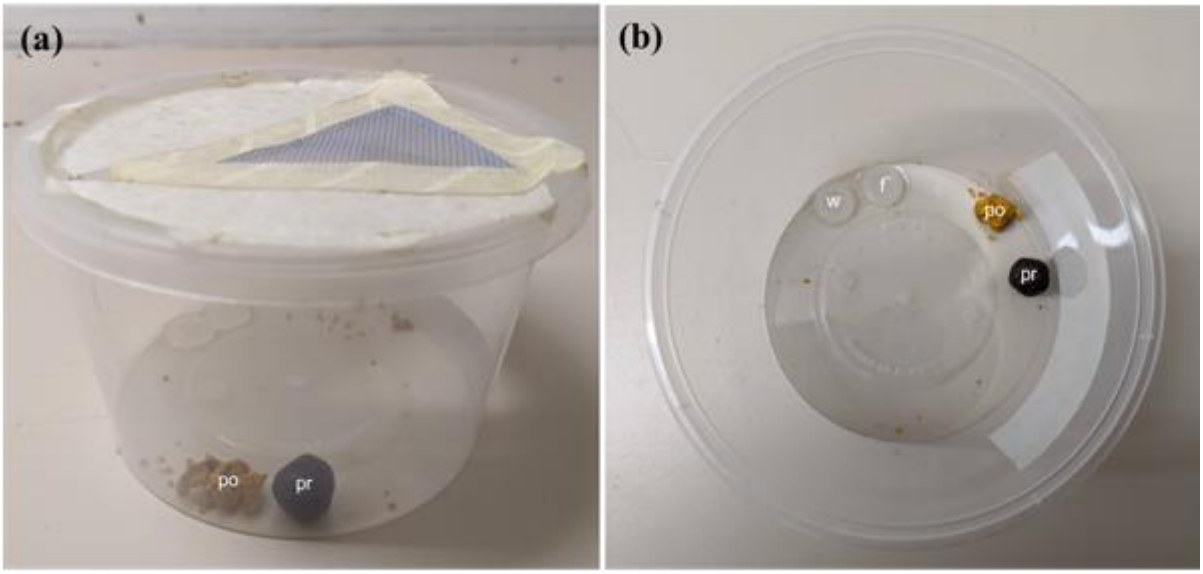


Figure S1. *T. 101arbonária* microcolony setup. A plastic container (diameter 9.5 cm and height 7.3 cm) with a hole covered with mesh. Each microcolony contains a pellet of propolis (pr), a pellet of pollen pot (po), water dish (w) and food dish (f). (a) Side view. (b) Upper view.

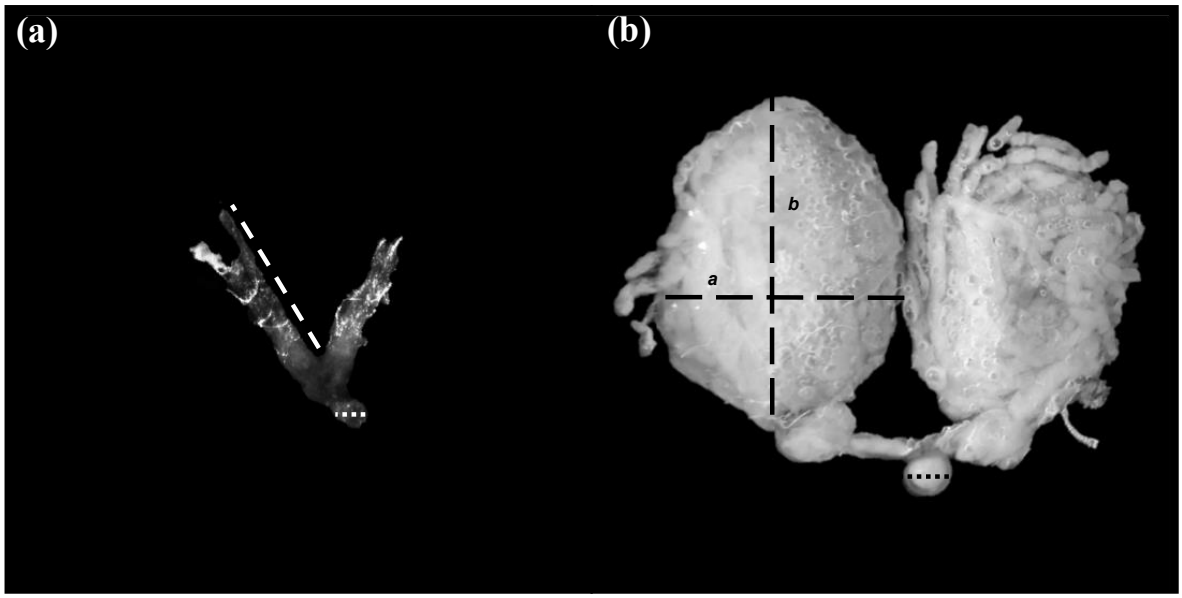


Figure S2. Ovary measurements for *T. carbonaria* workers and queens. A) Length of the worker ovary (dashed line) and diameter of the spermatheca (dotted line). B) Area of the queen ovary (dashed line, major diameter *a* and minor diameter *b*) and diameter of the spermatheca (dotted line).

Table S1. Number of dead queenless *T. carbonaria* workers from a total of 50 workers per microcolony.

Microcolony	Replicate	Days 0–7	Days 8–14	Total
Royal jelly diet	1	4	1	5
Royal jelly diet	2	4	0	4
Royal jelly diet	3	7	2	9
Royal jelly diet	4	6	1	7
Control diet	1	4	0	4
Control diet	2	2	1	3
Control diet	3	3	0	3
Control diet	4	7	0	7

Table S2. Ovary length and spermatheca diameter in *T. carbonaria* workers.

Queen state	Age (days)	Treatment	Replicate	Spermatheca diameter (mm)	Left ovary length (mm)	Right ovary length (mm)
Queenright	14	NA	1	0.06	0.52	0.57
Queenright	14	NA	1	0.06	0.58	0.52
Queenright	14	NA	1		0.41	0.22
Queenright	14	NA	1		0.81	0.75
Queenright	14	NA	1		0.41	0.73
Queenright	14	NA	1		0.68	0.29
Queenright	14	NA	1		0.95	0.43
Queenright	14	NA	1		0.4	0.32
Queenright	14	NA	1	0.06	0.83	0.92
Queenright	14	NA	1	0.06	0.97	0.82
Queenright	14	NA	2		0.62	0.59
Queenright	14	NA	2		0.48	0.51
Queenright	14	NA	2		0.71	0.79
Queenright	14	NA	2		0.64	0.49
Queenright	14	NA	2		0.74	0.58
Queenright	14	NA	2		0.48	0.61
Queenright	14	NA	2	0.06	0.85	0.7
Queenright	14	NA	2		0.6	0.64
Queenright	14	NA	2	0.06	0.57	0.55
Queenright	14	NA	2		0.44	0.26
Queenright	14	NA	3		0.54	0.64
Queenright	14	NA	3		0.18	0.4
Queenright	14	NA	3		0.48	0.61
Queenright	14	NA	3		0.51	0.73
Queenright	14	NA	3		0.48	0.44
Queenright	14	NA	3		0.43	0.5
Queenright	14	NA	3		0.5	0.46
Queenright	14	NA	3		0.53	0.53
Queenright	14	NA	3		0.67	0.51
Queenright	14	NA	3		0.77	0.56
Queenright	14	NA	4		0.83	0.72
Queenright	14	NA	4		0.36	0.53
Queenright	14	NA	4		0.2	0.66
Queenright	14	NA	4	0.05	0.66	0.26
Queenright	14	NA	4	0.05	0.67	0.63
Queenright	14	NA	4		0.54	0.65
Queenright	14	NA	4		0.67	0.52
Queenright	14	NA	4		0.73	0.44
Queenright	14	NA	4		0.34	0.86

Queen state	Age (days)	Treatment	Replicate	Spermatheca diameter (mm)	Left ovary length (mm)	Right ovary length (mm)
Queenright	14	NA	4		0.56	0.51
Queenless	0	NA	1		0.52	0.43
Queenless	0	NA	1	0.08	0.48	0.46
Queenless	0	NA	1		0.45	0.42
Queenless	0	NA	1	0.07	0.47	0.51
Queenless	0	NA	2		0.49	0.51
Queenless	0	NA	2		0.52	0.62
Queenless	0	NA	2		0.37	0.4
Queenless	0	NA	2		0.51	0.46
Queenless	0	NA	3		0.5	0.54
Queenless	0	NA	3	0.05	0.55	0.65
Queenless	0	NA	3		0.75	0.63
Queenless	0	NA	3		0.52	0.31
Queenless	0	NA	4		0.7	0.68
Queenless	0	NA	4		0.69	0.59
Queenless	0	NA	4		0.61	0.63
Queenless	0	NA	4		0.56	0.57
Queenless	14	Control diet	1	0.08	0.66	0.51
Queenless	14	Control diet	1		0.53	0.55
Queenless	14	Control diet	1	0.05	0.4	0.29
Queenless	14	Control diet	1	0.05	0.47	0.4
Queenless	14	Control diet	1		0.2	0.23
Queenless	14	Control diet	1		0.52	0.46
Queenless	14	Control diet	1	0.06	0.18	0.35
Queenless	14	Control diet	1		0.67	0.61
Queenless	14	Control diet	1		0.35	0.29
Queenless	14	Control diet	1	0.05	0.24	0.16
Queenless	14	Control diet	2		0.39	0.39
Queenless	14	Control diet	2		0.51	0.49
Queenless	14	Control diet	2		0.59	0.34
Queenless	14	Control diet	2		0.35	0.72
Queenless	14	Control diet	2		0.64	0.59
Queenless	14	Control diet	2	0.05	0.52	0.56
Queenless	14	Control diet	2		0.49	0.42
Queenless	14	Control diet	2		0.73	0.63
Queenless	14	Control diet	2		0.43	0.48
Queenless	14	Control diet	2		0.42	0.66
Queenless	14	Control diet	3		0.7	0.56
Queenless	14	Control diet	3		0.48	0.55
Queenless	14	Control diet	3		0.59	0.56

Queen state	Age (days)	Treatment	Replicate	Spermatheca diameter (mm)	Left ovary length (mm)	Right ovary length (mm)
Queenless	14	Control diet	3		0.5	0.41
Queenless	14	Control diet	3		0.55	0.47
Queenless	14	Control diet	3		0.66	0.49
Queenless	14	Control diet	3		0.41	0.4
Queenless	14	Control diet	3		0.78	0.69
Queenless	14	Control diet	3		0.45	0.64
Queenless	14	Control diet	3		0.75	0.46
Queenless	14	Control diet	4		0.73	0.56
Queenless	14	Control diet	4		0.52	0.57
Queenless	14	Control diet	4		0.51	0.37
Queenless	14	Control diet	4		0.75	0.61
Queenless	14	Control diet	4		0.64	0.35
Queenless	14	Control diet	4	0.06	0.53	0.62
Queenless	14	Control diet	4		0.73	0.48
Queenless	14	Control diet	4		0.64	0.66
Queenless	14	Control diet	4		0.44	0.6
Queenless	14	Control diet	4		0.54	0.65
Queenless	14	Royal jelly diet	1		0.65	0.47
Queenless	14	Royal jelly diet	1		0.58	0.6
Queenless	14	Royal jelly diet	1		0.46	0.36
Queenless	14	Royal jelly diet	1		0.45	0.57
Queenless	14	Royal jelly diet	1		0.52	0.17
Queenless	14	Royal jelly diet	1		0.67	0.62
Queenless	14	Royal jelly diet	1		0.56	0.18
Queenless	14	Royal jelly diet	1		0.4	0.42
Queenless	14	Royal jelly diet	1		0.33	0.44
Queenless	14	Royal jelly diet	1		0.59	0.5
Queenless	14	Royal jelly diet	2		0.63	0.64
Queenless	14	Royal jelly diet	2	0.08	0.59	0.5
Queenless	14	Royal jelly diet	2		0.53	0.36
Queenless	14	Royal jelly diet	2		0.58	0.46
Queenless	14	Royal jelly diet	2		0.45	0.54
Queenless	14	Royal jelly diet	2	0.07	0.75	0.73
Queenless	14	Royal jelly diet	2		0.35	0.45
Queenless	14	Royal jelly diet	2		0.48	0.54
Queenless	14	Royal jelly diet	2		0.62	0.56
Queenless	14	Royal jelly diet	2	0.06	0.39	0.38
Queenless	14	Royal jelly diet	3		0.74	0.78
Queenless	14	Royal jelly diet	3		0.53	0.53
Queenless	14	Royal jelly diet	3		0.45	0.79

Queen state	Age (days)	Treatment	Replicate	Spermatheca diameter (mm)	Left ovary length (mm)	Right ovary length (mm)
Queenless	14	Royal jelly diet	3		0.5	0.54
Queenless	14	Royal jelly diet	3		0.55	0.56
Queenless	14	Royal jelly diet	3		0.32	0.45
Queenless	14	Royal jelly diet	3		0.7	0.76
Queenless	14	Royal jelly diet	3		0.62	0.57
Queenless	14	Royal jelly diet	3		0.48	0.52
Queenless	14	Royal jelly diet	3		0.43	0.39
Queenless	14	Royal jelly diet	4		0.64	0.38
Queenless	14	Royal jelly diet	4		0.54	0.25
Queenless	14	Royal jelly diet	4		0.77	0.72
Queenless	14	Royal jelly diet	4		0.48	0.34
Queenless	14	Royal jelly diet	4		0.55	0.58
Queenless	14	Royal jelly diet	4		0.6	0.62
Queenless	14	Royal jelly diet	4		0.86	0.61
Queenless	14	Royal jelly diet	4		0.77	0.43
Queenless	14	Royal jelly diet	4		0.6	0.59
Queenless	14	Royal jelly diet	4		0.62	0.58

Table S3. Pearson correlation between the right and left ovary of *T. carbonaria* workers.

Treatment group	Coefficient	N	T statistic	DF	P (value)
Queenright 14-day old	0.265	40	1.695	38	0.098
Queenless 0-day old	0.686	16	3.526	14	0.003
Queenless Royal jelly diet	0.422	40	2.867	38	0.007
Queenless Control diet	0.508	40	3.634	38	0.001

Table S4. Ovary area and spermatheca diameter in *T. carbonaria* mated queens and virgin queens (* indicates that the spermatheca had been detached during dissection and not observed).

Mating status	Replicate	Spermatheca diameter (mm)	Spermatheca status	Left ovary length (mm)	Left ovary height (mm)	Left ovary area (mm^2)	Right ovary length (mm)	Right ovary height (mm)	Right ovary area (mm^2)
Mated	1	*		5.04	3.56	14.09	4.68	3.44	12.64
Mated	2	*		4.03	2.63	8.33	4.59	2.50	9.01
Mated	3	1.31	Full	4.59	3.91	14.10	5.03	3.82	15.08
Mated	4	0.79	Full	5.23	3.77	15.46	4.58	3.75	13.50
Virgin	1	0.52	Empty	1.40	0.96	1.06	1.35	0.92	0.97
Virgin	2	*		1.21	1.13	1.07	1.25	1.13	1.11
Virgin	3	0.51	Full	1.03	0.95	0.77	0.90	1.20	0.85
Virgin	4	0.63	Empty	1.15	0.91	0.82	1.38	0.97	1.05

CHAPTER 5

Virgin queen behaviour and mating in the stingless bee *Tetragonula carbonaria*

Paint-marked queen of *Tetragonula carbonaria* on top of the brood.

Abstract

For many stingless bees (Meliponini), the early phase of a queen's life history is poorly known, because virgin queens are elusive and difficult to observe even in hived colonies. A better understanding of virgin queen behaviour is an important step towards improved propagation of stingless bee colonies for crop pollination services. Here, we study the Australian stingless bee *Tetragonula carbonaria* to (i) describe the behaviour of queens from eclosion until egg-laying, (ii) assess whether virgin queens can be kept alive in closed micro-colonies until mating age, and (iii) devise a technique for mating them under constrained conditions. We extracted 60 mature virgin queen cells of *T. carbonaria* from large colonies and reared them in queen maturation boxes. We marked each queen upon emergence and transferred them to small observation colonies containing workers. The interaction of queens and workers followed a typical pattern of three phases; first, a period of high queen activity and wing-flapping on top of the brood (3-7 days of age), then attempts by queens to leave the colony for the nuptial flight (11-15 days of age) and finally oviposition (8-14 days of age). Seven queens were observed to be actively killed by the workers within the first week of life (14%), while a further nine died of unverified causes that might have involved workers. Twenty-one queens (60%) survived to mating age. Of 17 queens that were mated under constrained conditions, dissections revealed that five (30%) had sperm in their spermathecae, indicative of a successful mating. In three cases, where three virgin queens were introduced into the same colony, the first one was always favored by the workers while the other two died within the first week. The observations reported here advance efforts for the controlled rearing and mating of *T. carbonaria* queens. They also suggest that worker aggression is a key factor in the survivorship of queens in the period before and directly after mating.

Introduction

Stingless bees prior to eclosion, the 60 queen cells were randomly placed into one of three identical incubating boxes so we would have virgin queens belonging to different colonies in all the boxes (Meliponini) are a group of highly eusocial insects found in the tropics and subtropics. They are important pollinators of wild plants and some crops, with many stingless bee species propagated in hives for use in crop pollination, honey production and recreational beekeeping (Heard, 1999, Ramalho, 2004, Giannini et al., 2015, Slaa et al., 2006, Kevan and Baker, 1983, Klein et al., 2007, Menezes et al., 2013). Although stingless bees have been kept and propagated for centuries in many parts of their range, recent interest in the commercial use of stingless bees has led to new calls for the largescale breeding of stingless bees (Menezes et al., 2013).

The ability to rear and mate stingless bee queens is key to efforts to accelerate the propagation of managed colonies (Jaffé et al., 2015, Cortopassi-Laurino et al., 2006, Contrera et al., 2011, Menezes et al., 2013). Although queens are produced in low numbers throughout the year in most stingless bees, queen availability can still be a limiting factor during colony propagation (Menezes et al., 2013, Imperatriz-Fonseca and Zucchi, 1995, Jaffé et al., 2015). With the exception of *Melipona*, new queens are a very small proportion of total brood (1–2%)(Prato and Soares, 2013, Kerr, 1969). Additionally, the ability to perform controlled matings in stingless bees would open the door to selection programs that favoured high productivity colonies (Oxley et al., 2010, Plate et al., 2019). The reproductive biology of queens for most of the world's 600 or so stingless bee species, however, remain poorly known (Imperatriz-Fonseca and Zucchi, 1995, Engels and Imperatriz-Fonseca, 1990, Smith, 2019) specifically those species in the African and Indo-Malayan Australasian Clades. Even for those species that are widely propagated, there are often key gaps in our knowledge of queen behaviour.

The stingless bee *Tetragonula carbonaria* is propagated in wooden box hives throughout its range in East Coast Australia. Previous research has suggested that *T. carbonaria* has the potential to pollinate important Australian tropical and subtropical fruit crops, including lychee, coconut, carambola, macadamia and mangoes (Heard, 1999). They are under current investigation for pollination of many more crops, including in greenhouses (Greco et al., 2011). *T. carbonaria* colonies contain a single laying queen. Queens in this species lay all the eggs for the colony, including all males, as workers are entirely sterile and never lay eggs (Gloag et

al., 2007, Garcia Bulle Bueno et al., 2020). Queens are larger than workers and consequently reared in special, larger royal cells **(Figure 1**, a-d**).** Hence, their caste is determined by nutrition (Heard, 2016). The fate of virgin *T. carbonaria* queens when they emerge is unknown, but queenright colonies are believed to retain multiple virgin (non-laying) queens inside of the colony at all times because virgin queens are quick to appear once a resident queen has been removed; sometimes within 30 mins (**Chapter 3**, Garcia Bulle Bueno et al., in prep). As in other stingless bees, these excess virgin queens presumably act as an insurance against death of the mated queen (Sakagami, 1982, Engels and Imperatriz-Fonseca, 1990, Michener, 1974, Nogueira-Ferreira et al., 2009, Garcia Bulle Bueno et al., 2020), ensuring rapid queen replacement. They may also facilitate active queen replacement, such as regicide by workers of queens laying diploid males (Vollet-Neto et al., 2019), though the extent to which workers decide the fate of either virgin or mated queens in *T. carbonaria* remains unknown. Virgin queens also have the opportunity to inherit a colony during natural colony fission, during which workers first locate and provision a new nest cavity, and then escort a virgin queen from the parental nest to the new site (Heard, 2016).

When virgin queens are ready to mate, they fly from their colony to a male aerial aggregation composed of several hundreds to thousands of males only a few metres away from the virgin queen's colony (Heard, 2016) (**Chapter 3**, Garcia Bulle Bueno et al., in prep). Queens then mate with a single male (Green and Oldroyd, 2002), which leaves a mating plug attached to the tip of her abdomen (Smith, 2019) and return to the colony. Presumably this mating plug reduces the chance of further matings, as has been shown in other stingless bees (de Camargo, 1972, Imperatriz-Fonseca et al., 1998, Da Silva et al., 1972, Kerr et al., 1962, Green and Oldroyd, 2002, Smith, 2019).

Once egg-laying, the *T. carbonaria* queens never again leave the colony, storing enough semen in the spermatheca to fertilize eggs throughout her lifetime (Heard, 2016, Kerr et al., 1962). During the mating, the male loses his genitalia (mating plug), leaving them attached to the queen's genital chamber. Previous work suggests that controlled mating of queens could be possible in this species (Smith, 2019). Natural male aggregations are easy to attract, and mature males can also be sampled directly on their exit from colonies (**Chapter 3**, Garcia Bulle Bueno et al., in prep). Furthermore, Smith et al 2019 successfully attracted males from aggregations to mate with tethered, recently-deceased queens. Key aspects of *T. carbonaria* queen mating behaviour, however, remain unknown. There are no reported observations of natural matings,

which presumably happen on the wing in the middle of the male swarms. The age of queens at mating, the duration of the nuptial flight and the behaviour of workers in the nest after queens return remain undescribed for *T. carbonaria*.

In this study, we hatched and observed *T. carbonaria* virgin queens in small observation boxes. We had three aims: (i) to describe in detail for the first time the behaviour of queens from eclosion until egg-laying, (ii) to assess whether queens could be kept alive in closed micro-colonies until mating age, restricted from naturally mating, and (iii) to mate queens in semi-controlled conditions. Together, these efforts are intended as a first step towards efforts to advance propagation techniques for *T. carbonaria*, and in turn their use as managed pollinators for Australian crops.

Method

Biological material

We carried out this work at The University of Sydney, Australia, between October 2019 and January 2020 using colonies from two Sydney meliponaries (Ku Ring Gai Council and The University of Sydney). *T. carbonaria* queen cells are produced continuously throughout the year (Yamane and Sakagami, 1995) and can be readily identified in exposed brood because they are two to three times larger than worker cells and are built at the edge of combs **(Figure 1,** a and b**)**. We extracted 60 mature queen cells from 15 colonies (1-8 per colony). Queen cells were visually estimated to be black/red eyed pupae and recognised by the lack of wax in the late stage pupa (Heard, 2016) To provide workers for our observation micro-colonies, we also collected one disc of mature brood, from a different set of 20 colonies, and used a further six colonies to supply adult workers. As queen cells, brood discs and adult workers were from different colonies, there was no kin relationship between the virgin queens and the callow or adult workers in our boxes. The transferring of brood discs between colonies is a common practice in *T. carbonaria* beekeeping and our previous experience had indicated that colonies regularly accept queens and workers that hatch in their colony, regardless of brood origin.

Incubating boxes

Prior to eclosion, the 60 queen cells were randomly placed into one of three identical incubating boxes so we would have virgin queens belonging to different colonies in all the boxes. Incubating boxes were wooden OATH boxes (23 × 15 × 11 cm, volume 3.8 L) covered with acetate sheets, allowing us to observe the bees' behavior and the emergence of virgin queens. These boxes contained no adult workers, but each was provided with one disc of brood (approximately 1000 brood cells; comprising workers and a few males). To sustain emerging brood, boxes also contained 3-4 pots of pollen (containing approximately 2-3 gr each), 1 mL of *T. carbonaria* honey, and 2 mL of water on a piece of cotton placed in a plastic lid (5 cm in diameter). We also included a 5 cm diameter block of propolis (building material made of wax and plant resins (Heard, 2016). Pollen, honey and propolis were sourced from *T. carbonaria* colonies. The pollen was stored at -20 °C while the propolis was kept under room temperature for future use. The colonies were checked every day and cotton was replaced every 2-3 days to avoid any fungal growth. The food and the pollen were refilled *ad libitum*. All the boxes were kept in darkness and at constant temperature of 28°C.

Queen maturation boxes

Upon emergence in the incubating boxes, the queens were painted for identification with a unique colour code on the thorax using POSCA pens. Each queen was then placed into its own 'maturation box' (another wooden OATH boxes) with identical conditions to the incubating boxes, and a new brood disc of 200-300 mature worker brood cells. Maturation boxes were inspected every day for 30 days or until the queen died. Excretes and dead bees were manually removed every day.

Maturation boxes allowed us to easily observe the behaviour of young queens. These boxes were also intended, however, to assess whether we could keep queens alive in closed micro-colonies until mating age (i.e. rear them until an age suitable for controlled mating, with the certainty that they had not mated naturally). We therefore allocated the callow queens to one of four box treatments (**Table 1**). First, we created "large" microcolonies by adding 800-1000 adult workers to the box, with closed entrance (n=20). Second, to determine if queen survival depended on the number of accompanying workers, we established some boxes with no adult workers (n=5) and with few adult workers (n=10, 50-100 adults). The workers from all boxes were left for 24-48h before the queen introduction for a priori adjustment to the box and the

closed environment. Finally, to determine whether keeping the colonies closed affected queen survival, we established open boxes (n = 4) in the same way as the "large" microcolonies in which queens and workers were allowed to fly free.

Table 1. The 4 set-ups (treatments) for the social environment in the colonies of the virgin queens of *T. carbonaria*.

Treatment	N	Callow workers	Adult workers	Entrance Closed or Open
1- Small/callows	5	50-100	-	C
2- Small	10	50-100	50-100	C
3- Large	20	50-100	800-1000	C
4- Large/open	4	50-100	800-1000	O

Under natural conditions, *T. carbonaria* colonies may have more than one virgin queen at a time. To explore interactions between virgin queens in the same colony, we additionally set up three boxes each with three virgin queens each. The virgin queens added per box were one day apart in age. Each of these "multi-queen" boxes received ~300 brood cells and 50-100 adult workers.

Behavioural observations

We observed maturation boxes twice a day (once in the morning and once in the afternoon) and recorded the behaviours and interactions of the virgin queens. Observations lasted three to five minutes each. When queens were present and not hiding under the brood nest, we noted their behaviour. Any behaviour that was considered novel was filmed (IPhone X and Google Pixel 4). We categorized the behaviour into 11 main categories based on virgin queen behaviour in stingless bees (Kleinert, 2005); 1) *motionless or low activity*, the virgin queen stands motionless or moves very slowly; 2) *trophallaxis*, between virgin queen and workers, or between two virgin queens; 3) *antennal contact between virgin queen and workers*; 4) *walking on the brood*, the queen increases activity and goes on top of the brood, having constant trophallaxis with the workers and wing-flapping behaviour; 5) *court of workers*, the virgin queen is surrounded by inward-facing workers; 6) *worker dominance behaviour,* the worker places her front legs and antennae on a queen, or climbs onto the queen's thorax; 7) *removing mating plug* , the workers remove the mating plug of the queen; 8) *aggression,* workers push, bite and pull a virgin or newly-mated queen; 9) *queen to queen aggression*, as previous, in cases where more than one queen was in the same box; 10) *readiness to mate,* inferred by

observing that queens began to spend time close to the nest entrance. Because most of our observation colonies were closed to prevent queens actually leaving to mate, we determined readiness to mate by putting a small plastic tube at the entrance to the box and waiting to see the queen enter the tube. Studies of neotropical stingless bees indicate that as the day of her nuptial flight approaches, a virgin queen will linger around the entrance until ready to leave (Da Silva et al., 1972). To confirm that this behaviour did reflect queen mating flight age, we took a subset of nesting boxes (n=4) outside and allowed queens to leave; in all cases, presence in the nest tube corresponded to actually leaving the colony. (dos Santos et al., 2016a); and 11) *egg-laying*, we confirmed egg-laying by inspecting the brood and uncapping the cells. For all behavioural categories, we noted the age of queens since eclosion and days since mating.

Constrained Matings

We attempted for the first time a non-lethal constrained mating technique. Once queens entered the plastic tube, indicating readiness for a mating flight, we extracted them and gently inserted them head-first into the cut tip of a plastic pipette (1 mL Livingstone transfer pipette) until their thorax was sufficiently wedged into the tube that the queens couldn't move. This left their abdomen free in the open air **(**Figure 2, a and b**).** The queens were mated in a natural male aggregation that had established at the University of Sydney meliponary. We took the queens outside and put them as close as possible to the male swarm. We then waited until one male landed on top of the queen and started mating. We considered a mating had occurred if we observed the detachment of the male genitalia. We then observed queens under a dissecting microscope to confirm the presence of mating plugs and check the position of the male genitalia in their genital chamber. Each mated queen was then transferred back to their maturation box and observed for the next 1-2 hours.

In most cases, maturation boxes were not suitable for continued observation of queens, because workers in these boxes had not constructed and provisioned brood cells into which queens could lay. We therefore sacrificed queens at 15±20 days post-mating and dissected them under 10x magnification to determine if the spermatheca was filled with sperm, as evidence of a successful mating. A full spermathecae can be readily distinguished from an empty one based on its colour; full spermathecae are milky-pink while empty ones are transparent (Gerula et al., 2012, Garcia Bulle Bueno et al., 2020). In two boxes, workers had produced cells and queens were left alive in these cases and allowed to lay eggs. These two boxes were set outside so the

workers could forage freely outdoors. Males that had mated with these queens were placed into a -20 °C freezer for genetic analyses.

Statistical analyses

We compared survival probabilities by obtaining Kaplan-Meier estimates using the 'survfit' function of the survival library, and pairwise Log-Rank tests were computed between each treatment groups were computed with the 'pairwise_survdiff' function from the survminer library using default parameters (Benjamini-Hochberg adjustment). Queens were considered to have survived if still alive after 120 days.

The proportion of queens that reached mating age was compared using a 3 sample test for equality of proportions without continuity correction with the function 'prop.test' followed by pairwise comparisons of proportions with Holm adjustment with the function 'pairwise.prop.test'.

All analyses were done in R v4.0.0 (R Core Team, 2013) using R Studio v1.3.959 (RStudio Team, 2015). All figures were made using the ggplot2 v3.3.1 library except survival curves which we made using the library survminer v0.4.7 (https://www.r-project.org/).

Genetic analyses

For two queens that were mated under controlled conditions and began to lay eggs while still confined in their closed maturation boxes, we checked whether the brood genotype was consistent with the genotype of the mating male. We extracted 8 pupae from the brood of each queen. We extracted the DNA by grinding whole abdomens in 5% Chelex solution (1mM Tris HCl pH 7.6, 0.1mM EDTA pH 8) and boiling for 15 mins (Walsh et al., 1991). Supernatant containing DNA was diluted 1:1 with distilled water prior to PCR amplification. We genotyped each bee at seven microsatellite loci (Tc3. 155, Tc4. 63, Tc3. 302, Tc7. 13 and Tc4. 287; (Green et al., 2001) and Tang60 and Tang70 (Brito et al., 2009). Primers were fluorescently labelled with one of four dyes (FAM, NED, PET, VIC; Sigma-Aldrich, U.S.A.). PCR amplifications were performed according to (Green and Oldroyd, 2002) and the resulting products were analysed using a 3130xl Genetic Analyser and Genemapper (Applied Biosystems, U.S.A.).

Results

Queen survival in maturation boxes

We reared a total of 48 queens to eclosion in incubating boxes, with the remainder failing to hatch (n=21). From the virgin queens kept in closed maturation boxes (Groups 1-3), 21 queens (60%, n=35) survived until mating age (**Figure 3,** Supplementary Material, **Figure S1** and Supplementary Material, **Table S1**).

The proportion of queens that reached mating age was not significantly different between the three treatment groups (3 sample test for equality of proportions without continuity correction: $\chi 2$= 5.1809, df = 2, p-value = 0.07499, followed by pairwise comparisons of proportions with Holm adjustment: 1v2 p=0.28, 1v3 p=0.48, 2v3 p=0.47). Queens in the three treatment groups died at different rates until the age of mating (log-rank test, $\chi 2$=8.1, df=2, p=0.02). Queens kept alongside a small number of workers of a realistic age range, died at a slower rate than queens kept alongside callow workers only (log-rank test with BH adjustment: p=0.0047). There were no differences in the survival probabilities of queens from the other group comparisons (log-rank tests with BH adjustment; large vs small callows: p=0.1591; large vs small: p=0.0739). However, the high mortality of queens in all closed maturation boxes (Groups 1,2 and 3; 24±32 n=35) was in contrast to the low mortality of four queens reared in open maturation boxes (Group 4; 120±0 n=4), all of which survived.

Behavioural observations of *T. carbonaria* queens from eclosion until mating

Upon emergence, all virgin queens followed a similar trajectory of three key behaviours during the first two weeks after eclosion. 1) Queens emerge from a period of hiding or low activity to become highly and consistently active on top of the brood (age: 5±2 days post-eclosion, n=25). Until this time, callow queens are largely ignored by workers, and activity on top of the brood corresponds to increased interactions with workers, such as trophallaxis. Queens constantly rub their abdomen and flap their wings during this period. 2) Activity in the entrance tube, indicating desire to go outside and mate (age: 13±2, n=21). This usually corresponded to early or mid-afternoon. 3) Onset of oviposition (age: 11±3, n=6); the queen was not visually

physogastric at the time egg-laying was initiated but became enlarged over a period of 10-14 additional days **(Figure 4** and Supplementary Material, **Table S2).**

We additionally observed three other behaviours of note among workers, in response to queens. First, for queens that were mated under controlled conditions, workers removed the mating plug in two cases, by pulling it with their mandibles (**Figure 5**). Mating plug removal in both cases occurred within 20 mins of mating. In a further 18 mated queens, however, the mating plug was not removed and was confirmed to be still present by post-mortem dissections. Second, in ten cases (58% of mated queens), the workers formed a court around the recently mated queen, engaged in trophallaxis, constant antennal contact (**Figure 6**, a and b) and did not allow the queen to move freely. Workers in these courts sometimes engaged in aggression, walking over the queen and biting her (**Figure 6**, b). In the remaining mated queens (42%), the queen hid under the brood immediately such that we were not able to observe further the worker-queen interactions. Third, workers were observed to actively kill eight queens (**Figure 6**, c). All these killings occurred in the first 9 days after eclosion. Workers bit the queen's antennae and legs, and/or attached resin on the queen's body until she was fully immobilized and dead. Active queen-killing occurred in both mated (n=1) and unmated queens (n=7) of various ages (4±3 days of age). A further nine queens were suspected to have been passively killed by workers via starvation (no trophallaxis observed between workers and the queens), although queen killing could not be confirmed in these cases.

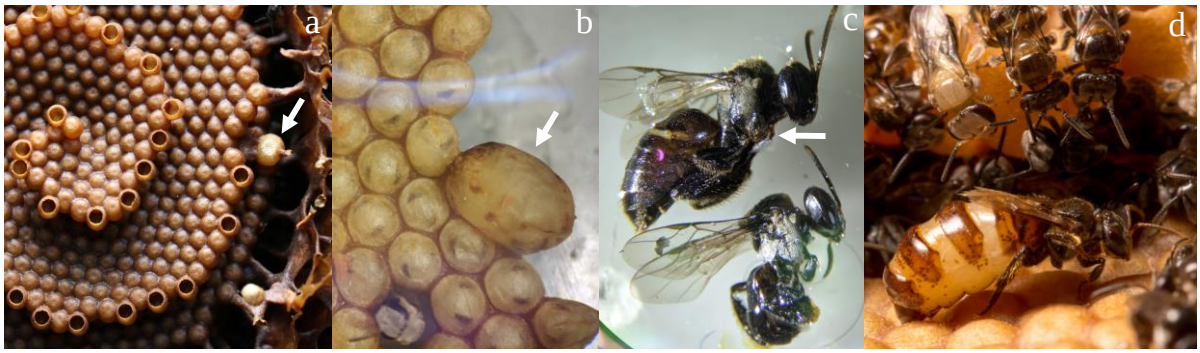


Figure 1. a A queen cell (indicated by arrow) around the brood of a *T. carbonaria* colony. Queen cells are continuously produced throughout the year and always built at the edge of the comb discs (Photo by Glenbo Craig). **b** Queen cells (indicated by arrow) are usually 2-3 times bigger than worker cells. **c** A comparison between a virgin queen (indicated by arrow) and a worker. The virgin queens have a larger abdomen and no curbiculae. **d** A laying queen of *T. carbonaria*. The abdomen enlarges so the tergites get separated giving its typical striped appearance.

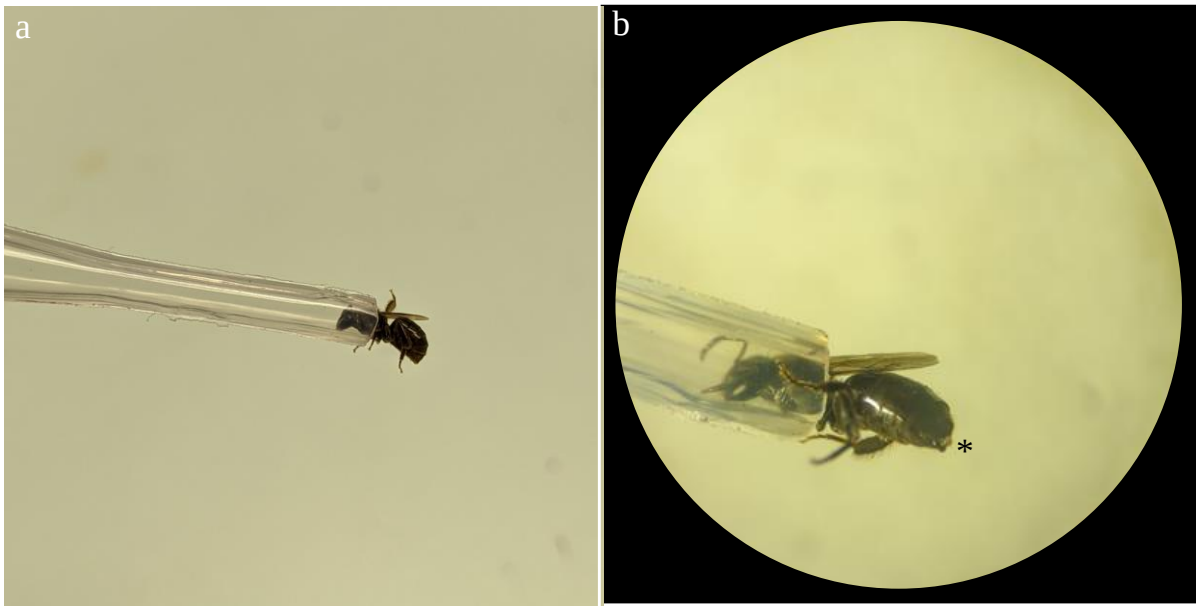


Figure 2. a Virgin queen inserted at the tip of the pipette. **b** Closer look to the virgin queen inserted in the pipette after being mated (the tip of the cream-coloured mating plug is just visible in this image, marked by the *). We immobilised her by inserting the thorax inside the tip and leaving the abdomen open to the air so the males could mate easily.

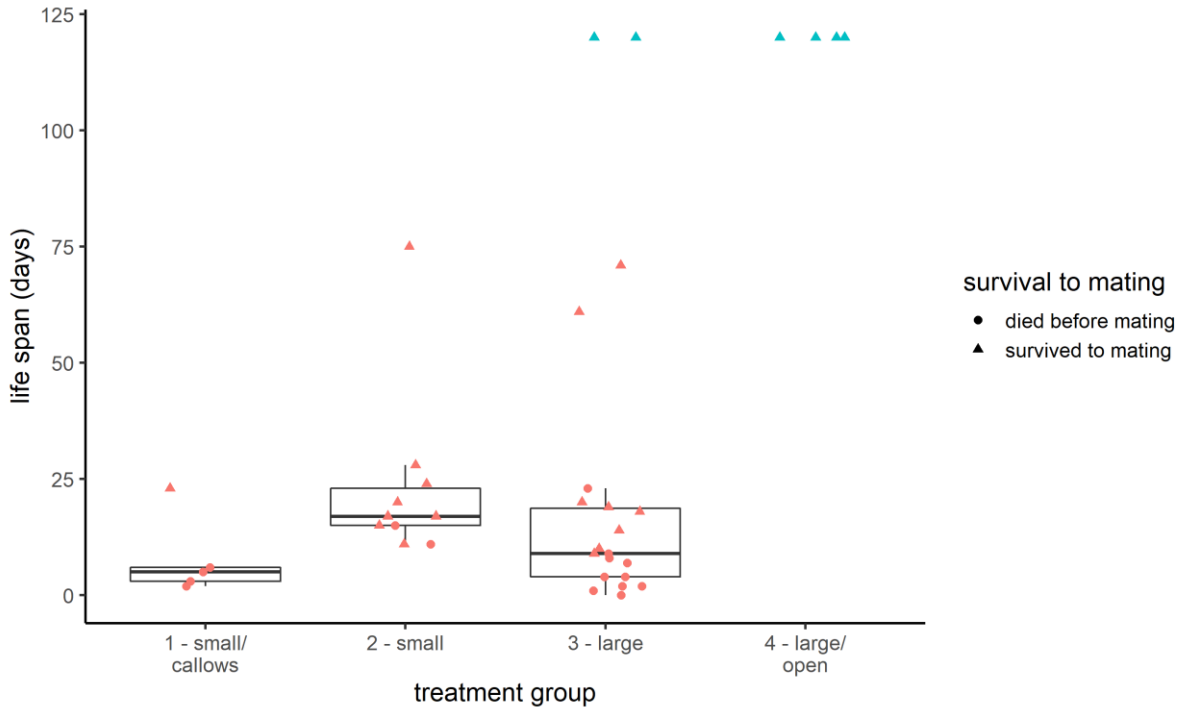


Figure 3. Life span of individual stingless bee queens in four treatment groups: 1 - small population of callow workers in a closed hive, (N=5) 2 - small population of workers from a realistic age distribution in a closed hive (N=X10), 3 - large population of workers from a realistic age distribution in a closed hive (N=20), 4 - large population of workers from a realistic age distribution in an open hive (N=4). Triangles indicate a queen that was observed trying to leave the colony, which we considered a sign they had reached mating age. Circles indicate a queen died before trying to leave the colony/reaching mating age. Blue indicates that the queens were still alive at the end of the experiment, while red indicates the queens died before the end of the experiment (i.e. within 3 months). The proportion of queens reaching mating age was not significantly different between all treatment groups in closed colonies, while all queens in treatment 4 (large, open colonies) survived.

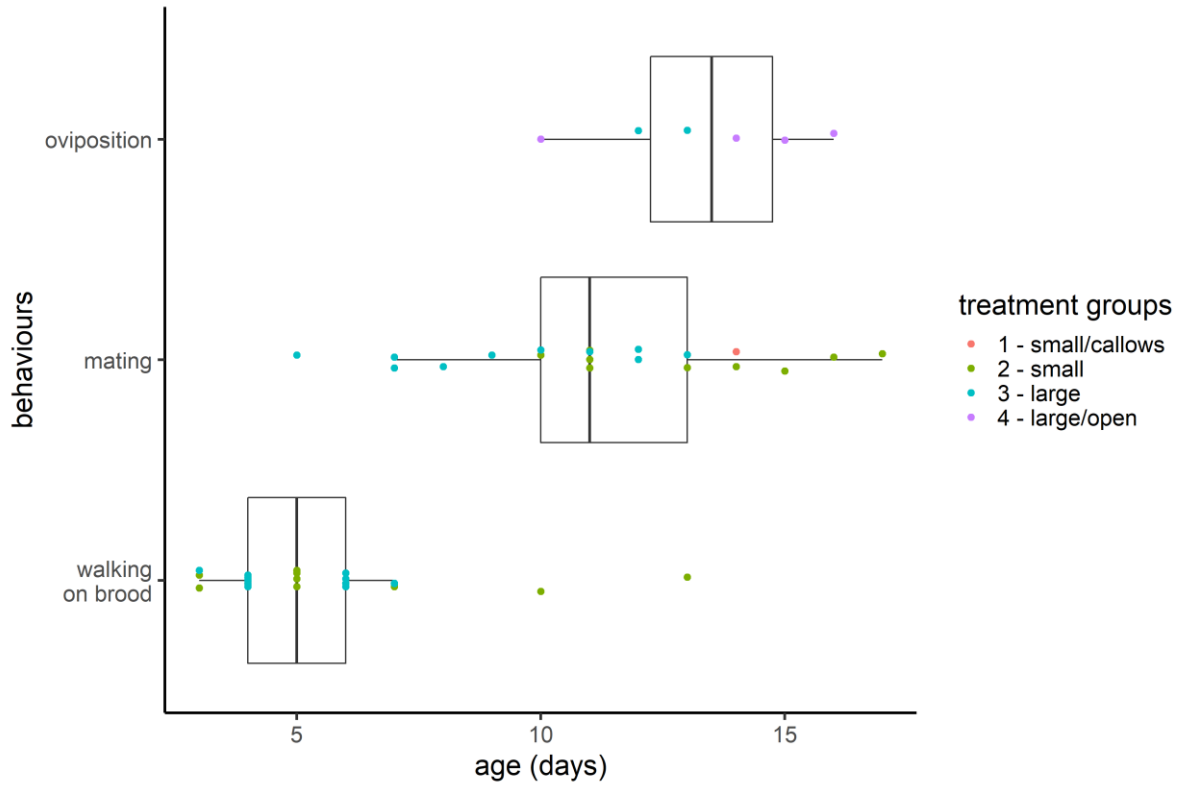


Figure 4. Age at which queens were first observed walking on the brood, mating and oviposition. Colours indicate treatment groups, in group 4 (purple) queens were left undisturbed in open boxes so only the beginning of the oviposition could be observed in this case.

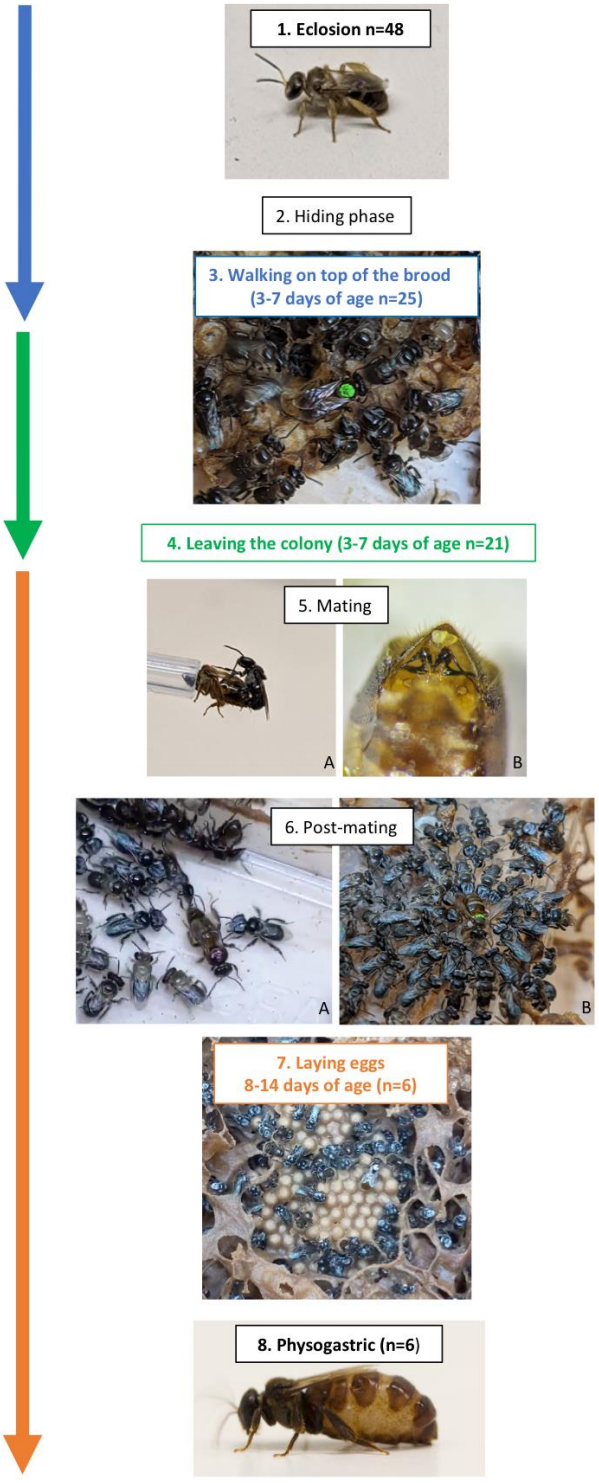

1. Eclosion n=48
2. Hiding phase
3. Walking on top of the brood
(3-7 days of age n=25)
4. Leaving the colony (3-7 days of age n=21)
5. Mating
A
B
6. Post-mating
A
B
7. Laying eggs
8-14 days of age (n=6)
8. Physogastric (n=6)

Figure 5. Behavioural diagram of the queens in *T. carbonaria* mated in captivity. 1. Queen eclosion. 2. Hiding phase. 3. Top of the brood. 4. Mating in captivity. A) Once the females try to go out of the box we mated them by fixing them in the tip of a pipette and took the virgin queen out in a mating swarm so a male could mate with her. B) Mating plug made of the male genitalia and the seminal vesicles attached to the queens' tip of the abdomen. 5. Post mating. A) Mating plug being pulled out by the workers. B) the workers formed an aura around the queen with constant antennal contact and sometimes aggression. 7. Onset of oviposition. 8. Physogastric.

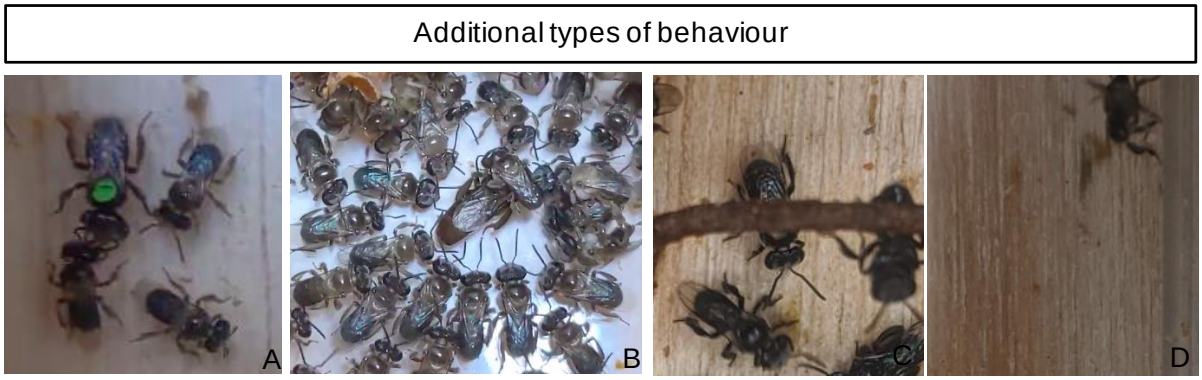


Figure 6. Additional types of behaviour observed in *T. carbonaria* micro-colonies with virgin or recently-mated queens. A. trophallaxis with the workers. B. dominance by workers, where workers crawled over the queen's body. C. killing of the queen, and D. virgin queen loitering around the excretes area.

In boxes in which three newly-eclosed queens were introduced one day apart from each other, workers always engaged most with the queen that was introduced first (i.e. the oldest queen; n=3 boxes). That is, worker-queen trophallaxis and antennal contact was observed only for the first-to-hatch queen. The two younger queens in each box did not interact with workers and instead loitered around the excretes area of the box (**Figure 6**, d) before dying 2-3 days after their introduction. We did not observe aggression between virgin queens in the same box.

Natural Mating flights

In order to confirm the timing and duration of mating flights, we opened the entrances to four of our maturation boxes when we observed the queen showing readiness to mate (i.e. active in the entrance tube). One of the colonies was open at 12:45 PM (7/11/2019) and the queen flew out at 1:15 PM. This queen reappeared near the nest entrance 10 mins later (1:27 PM) and eventually re-entered her colony at 1:35 PM. The post mortem dissection revealed a full spermatheca, indicating her short nuptial flight was successful. The remaining three queens flew at 1:22 PM, 1:45 PM and 4:24PM respectively. None of these queens returned to their boxes.

Constrained Matings

We mated 17 queens under constrained conditions (i.e. a male left a mating plug attached to the queen). Despite the presence of a mating plug, however, only five of these queens (30%) were found to have full spermathecae following dissection, indicating that mating in the remaining cases had failed to transfer sperm.

In most closed maturation boxes, workers did not build and provision brood cells ready for eggs. These boxes were not suitable therefore for assessing the behaviour of mated queens. In two boxes, however, workers did build brood cells and queens in these boxes survived to lay. Both of these queens laid only male eggs (n = 8 pupae genotyped). After being sacrificed and dissected at 110-113 days post-mating, these two queens were found to have empty spermathecae. (Supplementary Material, **Figure S2**). Workers showed no aggression towards these two queens, despite them laying only unfertilized eggs.

Discussion

In this study, we harvested pupal-stage queen cells of *T. carbonaria*, reared them in closed micro-colonies to prevent natural mating, and then mated them under constraint. We draw three key conclusions from these efforts. First, that queen behaviour from eclosion until mating age follows a predictable timeline, with queens showing readiness to mate 10-14 days post-eclosion. Second, that constrained matings are possible, though additional trial-and-error is needed to achieve higher rates of successful sperm transfer, and to maintain queens through to egg-laying. Third, that young queen mortality is high, both before and after mating. We expand on each of these points below, in the context of the broader aim of developing controlled queen rearing and mating in this species.

The behaviour of young *T. carbonaria* queens in the absence of a resident queen is similar to that described for some other stingless bee species. Upon eclosion, callow queens are shy and attract little attention from workers until close to one week of age. Thus, they are born unattractive and non-pigmented (similar to with other South American species such as *Scaptotrigon*a, *Paratrigona*, *Schwarziana* and *Nannotrigona*) (Imperatriz-Fonseca and Zucchi, 1995). A few days after emergence (5-9 days old; de Souza et al., 2017), they start to be noticed by the workers, become active on the brood comb, running excitedly and vibrating their wings intensively in search of trophallaxis with the workers. At this point, it is likely that they begin to produce volatiles which signal their queen status to both nestmate workers and males outside the colony (Imperatriz-Fonseca and Zucchi, 1995, Wilson, 1971). Indeed, in queenless colonies of *T. carbonaria*, if the advancing front of the brood comb is visible, it is easy to observe virgin queens at this stage of their life cycle after just a few minutes of observation (**Chapter 3**, Garcia Bulle Bueno et al., in prep). Under queenright conditions, however, virgin queens presumably continue hiding until the queen dies, the colony reproduces, or they themselves are killed or evicted by workers (Imperatriz-Fonseca and Zucchi, 1995).

Mating flights in *T. carbonaria* occur around 10-14 days. All four queens that we observed to leave the colony for mating flights did so during early- or mid-afternoon. This is when male swarms typically peak in size (**Chapter 3**, Garcia Bulle Bueno et al., in prep). This timing is also consistent with two personal observations of FGBB: a queen on the wing was netted in a male swarm at 15:30 PM and another one was observed outside of a colony surrounded by

males at 14:40 p.m. (Sunshine Coast, Queensland, September 2017). Mating flights may be high risk activities in *T. carbonaria*. Three of the four queens in our observations failed to return to the nest. One possibility could have been that they died and the other one that they try to enter another nest. Regardless, this suggests that mating flights represent the most dangerous period of a queen's life, as is also the case for honey bee queens (Koeniger and Koeniger, 2007).

In order to rear and mate queens under controlled conditions, it is necessary to ensure that queens have not had the opportunity to mate naturally. In this study, we prevented natural mating by keeping queens in small, closed micro-colonies with limited numbers of workers and provisioned them with sugar and pollen. Although we were able to rear 60% of queens to a reproductive age in this way, the high mortality suggests that alternative strategies should be considered in the future. Some queens were killed by workers, but most died soon after emergence from unknown causes. In contrast, four queens reared in open colonies all survived (i.e. colonies in which workers were free to forage). One explanation for this difference is that the provisions available to our closed colonies did not fully replicate those generated by foraging workers, and that something extra is required for queen acceptance and survival. In this case, future studies may consider ways to prevent queen mating while keeping colonies free to forage, such as installing a queen excluder at the entrance of the hive, as is typical for queen-rearing in honeybees, or leaving the colony open while queens are young and closing it only once queens approach mating age (10-14 days of age). Notably though, low survivorship of queens under experimental conditions is reported for other stingless bee species, and might also reflect the high natural mortality rate for young queens (Sommeijer et al., 1994, Da Silva et al., 1972, Kerr, 1950, Kerr et al., 1962, Ribeiro et al., 2003). For example, the year-round production of queens common to most stingless bee species is proposed to have evolved in part due to the high mortality of young queens (Wenseleers et al., 2004a, Prato and Soares, 2013).

We demonstrated that constrained live virgin *T. carbonaria* queens will attract males, and that these males may mate and fill the spermatheca. This represents an exciting step towards artificial breeding programs for this species. Importantly, however, the presence of the mating plug alone, nor even egg laying, were reliable evidence of a successful mating via this technique. Only one third of our queens mated under constraint carried sperm in their spermatheace, and two queens that become physogatric and laid eggs were later found to have an empty spermatheca and be laying only haploid males. This suggests that ovary activation is

not directly linked to successful mating in *T. carbonaria,* but to a mechanical stimulation linked to the attachment of a mating plug, as also reported for the stingless bee *Melipona quadrifasciata* (Melo et al., 2001). Our controlled mating protocol might be improved by reducing the stress of queens during mating via carbon dioxide narcosis, as is commonly used in artificial insemination of *Apis* species (Gillard and Oldroyd, 2020). Other options for controlled matings would be to release queens for free flight into a mating flight cage containing males. Mated queens must then be provided with colonies with provisioned brood cells, to confirm that they lay diploid brood following controlled matings.

Workers play an important role in the survival of young queens among the stingless bees (de Souza et al., 2017, Jarau et al., 2009, Vollet-Neto et al., 2019, Wenseleers et al., 2004a). We report here for the first time that *T. carbonaria* workers do actively kill virgin queens in some cases, a common practice among the Neotropical stingless bee species (Kerr et al., 1962, Sommeijer et al., 1994). In other cases, callow queens seemed to be ignored and may have died from starvation; accepted queens in contrast, enjoyed almost constant trophallaxis with workers. We also observed workers remove the mating plug in some cases, but the importance of this for queen survival is unclear (Melo et al., 2001). In some other stingless bee species, the queen removes the male genitalia herself (de Camargo, 1972, Da Silva et al., 1972), or the male genital capsule remains attached to the queen for several days and detaches on its own (Melo et al., 2001).

When multiple queens were present in the same *T. carbonaria* micro-colony, we observed that the workers determined which queen survived. In three cases, workers showed a preference for the queen introduced first, while the later-to-hatch queens remained in the excretes area and ultimately died. This behaviour has also been observed in other stingless bee species (Imperatriz-Fonseca, 1977, Veiga et al., 2017). Colonies of stingless bees under natural conditions usually have more than one virgin queen and it can be assumed that there is a hierarchy among them (Imperatriz-Fonseca and Zucchi, 1995, Silva, 1972). Not much is known about the interactions between virgin queens in full size colonies, however we have observed two virgin queens in a full-sized queenless hive interacting with the workers on top of the brood at the same time (*pers obs* FGBB). One queen chased the other one and briefly attacked her. On another occasion, we recorded four virgin queens on top of the brood at the same time, with no evidence of any interactions between them. In honeybees, queens have a functional stinger that can be used multiple times, and when new queens eclose in queenless colonies they will

typically kill all the rival queens in the hive (Gilley, 2001, Butz and Dietz, 1994). Contrastingly, in stingless bees, the constant supply of new queens may give more power to the workers. They outnumber the queen and rear the brood, having control over queen production and choosing the best one by killing the rest (Wenseleers et al., 2004a, Koedam, 1995). This strategy comes with the obvious risk that all queens fail, leaving the colony without a queen to produce new brood (Beekman and Ratnieks, 2003). Further work is needed to understand the role of *T. carbonaria* workers in choosing new queens for the colony.

Conclusion

We conclude that virgin queens of *T. carbonaria* can be raised and mated in captivity, and we encourage further studies to develop a viable technique for breeding and genetic improvement programs of this important species. Our view is that success in queen rearing and mating is likely to benefit from a better understanding of queen-worker interactions.

Supplementary information

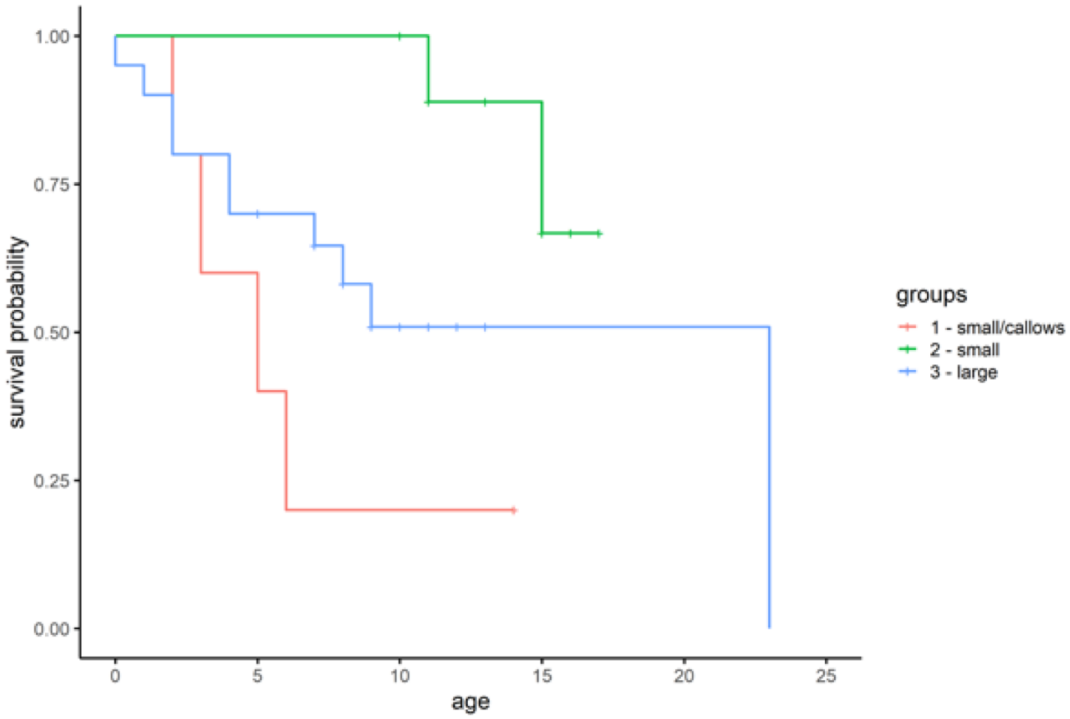


Figure S1. Survival until mating. Lines represent the survival probability of the queens in the different groups, while crosses show queens that were observed surviving until trying to leave the colony, which we consider indicates they have reached mating age. Queens died at a slower rate until mating in the small colonies with a realistic age distribution than in the small colonies with callows only (p=0.0047); there were no significant differences in the survival probability between the other groups.

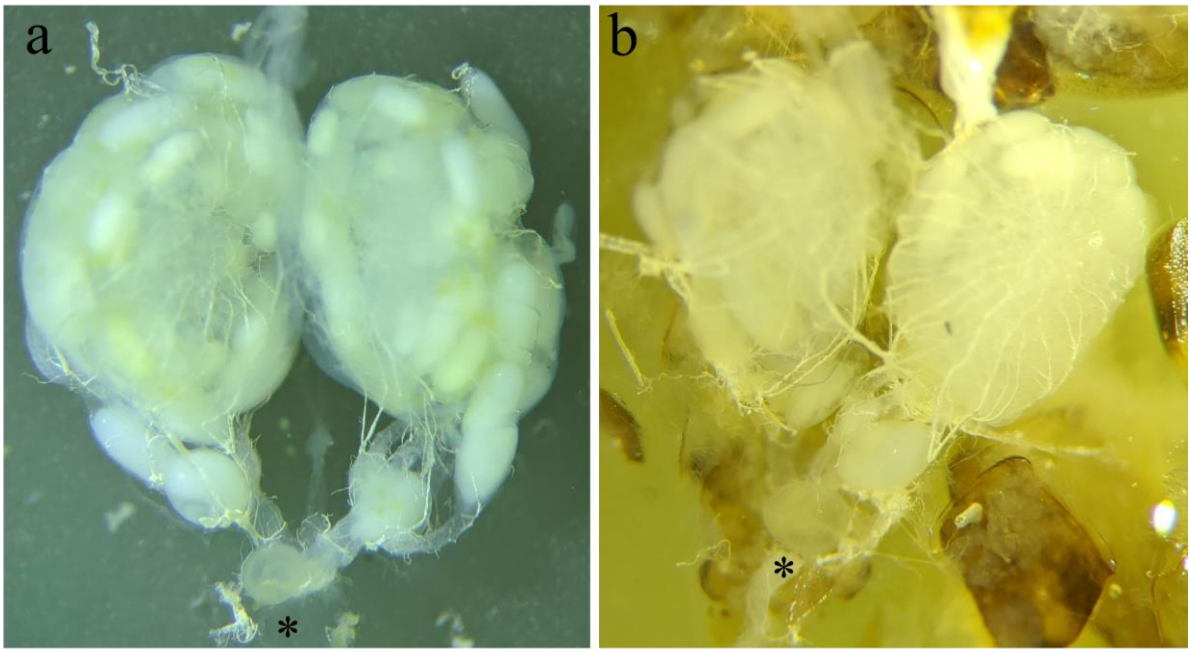


Figure S2. Activated ovaries with empty spermathecae (*) for the two queens (a) and (b) mated under constrained conditions.

Table S1. Sample size, mean age to survival and standard error of the survival probability to mating age (as indicated by queens observed attempting to leave the nest) of *T. carbonaria* queens reared under four types of conditions in maturation boxes.

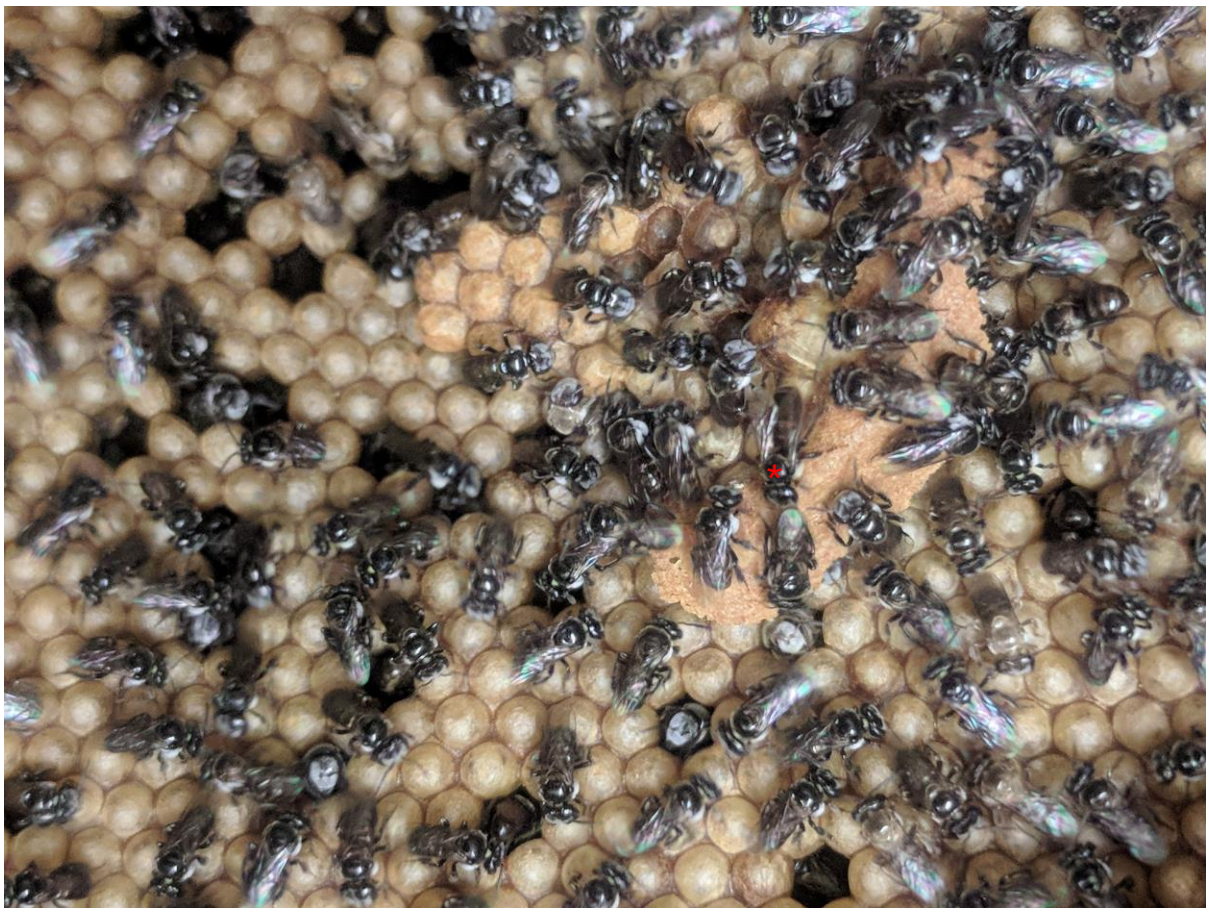
Group	N	Mean	Se
1- Small/callows	5	6.6	2.4
2- Small	10	15.9	0.7
3- Large	20	10.8	1.5
4- Large/open	4	NA	NA

Table S2. Age at which virgin queens of *T. carbonaria* of the five treatments showed different behaviours: a) walking on brood, b) mating (ready to leave the colony) and c) oviposition. Each individual is represented by the lines connecting each dot.

Group	event	Mean (days)	sd	n
1- Small/callows	a	4	0	5
1- Small/callows	b	14	NA	5
1- Small/callows	c	NA	NA	5
2- Small	a	6	3.2	10
2- Small	b	13.1	2.38	10
2- Small	c	NA	NA	10
3- Large	a	5.04	1.4	20
3- Large	b	9.09	3.05	20
3- Large	c	12.5	NA	20
5- Large/open	a	NA	NA	4
5 Large/open	b	NA	NA	4
5 Large/open	c	13.8	2.63	4
All groups	a	5.4	2.25	39
All groups	b	13.3	2.16	39
All groups	c	11.4	3.07	39

CHAPTER 6

Reproductive communication and nest parasitism in an Australian social bee, *Tetragonula carbonaria*



Brood of *Tetragonula carbonaria* exposed after a hive split has rendered the colony queenless (i.e. without a mature egg-laying queen). The virgin queen (shown by the red asterisk) is interacting with the workers on top of the brood.

Abstract

Chemical communication by egg-laying queens is central to the organization and stability of social insect colonies. Young, unmated queens also rely on volatiles to both attract males for mating, and signal their caste to workers. Here, we investigate the volatiles associated with virgin queens and the colony re-queening process for the endemic Australian stingless bee, *Tetragonula carbonaria*. We sampled the volatiles emitted by: (i) individual age-matched virgin queens, males and workers, and (ii) whole colonies in queenright (mature queen present) and queenless (virgin queen only present) conditions. We identified several compounds (pheromones) unique to virgin queens (Benzene, 1,3-bis(1,1-dimethylethyl)-; (2Z)-2-(3,3-Dimethylcyclohexylidene) ethanol; 2-Ethyl-2-propyl-1-hexanol) and queenright colonies (2-Heptanol, 1-Butanol, 3-methyl-). Additionally, we compared the rate at which queenless and queenright colonies were invaded by foreign queens. Nest usurpation by conspecific colonies is common in *T. carbonaria*, and we hypothesized that usurper colonies may locate target colonies by eavesdropping on the volatiles they produce during re-queening. We found that a high proportion of recently-queenless colonies had been usurped by foreign queens (30%, 6 of 20), though not significantly more than colonies that had no confirmed period of queenlessness during the same interval (15%, 3 of 20). We also identified the bodies of three foreign queens dumped outside colonies, which suggests not all nest parasitism in *T. carbonaria* is associated with conspicuous inter-colony fights. Our results provide a first description of the chemical ecology of reproduction in *T. carbonaria* and indicate that queenless colony state may be signalled to males by both the presence of compounds associated with virgin queens and the absence of compounds associated with mated queens. Further investigation is needed to confirm whether these volatiles are associated with the high incidence of nest parasitism in this species.

Introduction

Chemical communication is essential for the organization and reproduction of social insect colonies (Singer, 1998, Howard and Blomquist, 2005, Ayasse et al., 2001). Each individual in the nest has a cuticular chemical profile that signals species, age, caste and sex (Monnin, 2006, Howard and Blomquist, 2005) and also colony of origin (Jungnickel et al., 2004, Dapporto et al., 2006, Châline et al., 2005). Queen pheromone is especially critical to the social order (Engels, 1987, Engels et al., 1990, Engels et al., 1993, Krieger et al., 2006, Van Oystaeyen et al., 2014). For example, in honey bees, the queen indicates her presence to workers by producing volatile pheromones from the mandibular and tergal glands, to which workers respond by inhibiting activation of their ovaries and thus refraining from egg-laying (Wossler and Crewe, 1999a, Wossler and Crewe, 1999b). When this queen pheromone is absent, indicating the death or departure of the queen, workers respond by attempting to rear a new queen (Nunes et al., 2014), and if that fails, laying their own eggs (Oliveira et al., 2015, Oi et al., 2015).

The stingless bees are a large tribe of 600 eusocial bee species that inhabit the tropics and subtropics of the world (Rasmussen and Cameron, 2007, Rasmussen and Cameron, 2010, Kerr and Maule, 1964). They are pollinators of a wide variety of native flora (Nunes et al., 2014) and also some economically valuable crops (Heard, 1999, Slaa et al., 2006, Giannini et al., 2015). Some species in South America, Asia, India and Australia are kept and propagated in wooden or clay hives (Quezada-Euán et al., 2001, Heard, 2016). Some aspects of their reproductive biology are well-documented, while others aspects remain poorly understood. Males leave their natal colony once mature and do not return, in some cases travelling for many kilometres in search of mates (**Chapter 3**, Garcia Bulle Bueno et al., in prep). Males are attracted to colonies with a receptive virgin queen, and aggregations of hundreds or thousands of males may gather close to these colonies waiting for the virgin queen to appear (Paxton, 2000, Paxton, 2005, dos Santos et al., 2016b, dos Santos et al., 2016c). Such “queenless” colonies (i.e. those that lack a resident egg-laying queen, but have a virgin queen ready to mate) occur each time a new colony is established from a parent colony, and when the resident queen of a colony dies and needs to be replaced (Inoue et al., 1984, Van Veen and Sommeijer, 2000a, Wille, 1983). Presumably, queenless colonies emit a chemical signal into the environment that lures the males to the nest, but the chemical identity and source of this signal has not been identified (von Zuben, 2017). Three hypotheses have been suggested to explain male attraction

to nests that are re-queening: (i) virgin queens inside colonies produce a pheromone that wafts out from nest entrances (Engels, 1987, Verdugo-Dardon et al., 2011, Fierro et al., 2011), (ii) all workers inside the queenless nest produce a pheromone that wafts out from nest entrances (i.e. overall nest odour changes when queenless; Engels et al., 1993), or (iii) workers that leave the nest (e.g. foragers) spread a pheromone outside of the nest (Engels et al., 1990, Roubik, 1990, von Zuben, 2017).

Colonies in the queenless state are susceptible to infiltration by unrelated, foreign queens (Wenseleers et al., 2011, Van Oystaeyen et al., 2014, Vergara et al., 1993), or to nest usurpation by another colony, which then installs its own queen (Wagner and Dollin, 1982). At least 60 species of stingless bees are known to engage in heterospecific conflicts over nest resources or nest sites (Sakagami et al., 1993, Cunningham et al., 2014, Nogueira-Neto, 1970). The cues used by parasitic queens or attacking colonies to locate their targets are unknown, but pheromones or other volatiles are likely candidates (Lenoir et al., 2001, Grüter et al., 2016, Van Oystaeyen et al., 2014) . Because re-queening colonies must signal their location to males, any male-attracting volatile could also be detected by unintended receivers. That is, parasitic queens and/or attacker colonies could eavesdrop on the signals produced by colonies to attract mates. Such olfactory eavesdropping is known to occur in other aspects of stingless bee communication. For example, *Lestrimelitta or Trigona hyalinata* eavesdrop on other species food-marking pheromones to gain valuable information about the location of food sources (Grüter et al., 2016, Lichtenberg et al., 2011, Lichtenberg et al., 2014).

In this study, we describe the volatiles associated with virgin queens and queenless colonies of the Australian stingless bee *Tetragonula carbonaria*. This species is kept regularly in hives for use in crop pollination and recreational honey production (Heard, 2016). We also investigate whether *T. carbonaria* colonies that have experienced a recent period of queenlessness are more prone to takeover by foreign queens than those that have not. Australian *Tetragonula* species are known to engage in dramatic inter-colony battles (both within and between species), in which hundreds or thousands of workers fight to the death to gain control of a nest site and resources (Cunningham et al., 2014, Gloag et al., 2008, Grüter et al., 2016). If the attacking colony is successful in usurping the nest site, it installs its own queen and enslaves the developing brood of the host colony (Cunningham et al., 2014). Nest parasitism by lone virgin queens is not yet reported for any Australian *Tetragonula*, though might also occur. We

genotype several dead virgin queens collected opportunistically outside colony entrances to assess whether they are likely to be failed parasites.

Method

Volatiles produced by virgin queens

Queen rearing

To describe the volatiles produced by *T. carbonaria* virgin queens at different ages, we harvested queen cells from colonies and reared them in micro-colonies at The University of Sydney (33°53'05.5"S, 151°11'18.4"E; September 2020). All colonies were local Sydney stock sourced from the meliponary of Ku Ring Gai Council, Sydney. We extracted 14 mature queen cells from seven colonies (two queen cells per colony), visually estimated to be black or red eyed pupae and recognised by the absence of the pupal cocoon (Heard, 2016). These queen cells were incubated alongside worker and male brood cells from the same colony in maturation boxes as described in **Chapter 4** (Garcia Bulle Bueno et al., 2020). We noted the age in days since eclosion of each queen, worker and male by paint-marking their thoraxes with POSCA pen parkers (Water-based paint).

Collection of volatiles

We sampled the volatiles produced by each queen once: less than 24h of age (N=2 queens), 5-14 days of age (N=11 queens), and 23 days of age (N=1 queen). We allocated most of our queens to sampling in the 5-14 day age bracket, because previous evidence indicated that this is the age when queens attempt to mate (Garcia Bulle Bueno et al., 2020). We also sampled the volatiles produced by males and workers of two different ages: less than 24h of age (N=4 workers, N=4 males) and 14 days of age (N=3 workers, N=4 males). Finally, we sampled adult males of unknown age from a mating aggregation at the University of Sydney (N=5 males). We assume that males in mating aggregations are sexually mature males.

We collected volatile samples via stir bar sorptive extraction (SBSE) (Baltussen et al., 1999). We placed one bee per vial into amber glass vials (23 × 75 mm diameter, 20 mL volume) partitioned with wire mesh, and then placed a 10mm stir bar coated with 55µL

polydimethylsiloxane (PDMS; Gerstel, Mülheim an der Ruhr, Germany) adjacent to the mesh and closed the vials with a screw lid. The stir bars were exposed for 2.5-3h before removal. We also collected samples from empty vials to account for background volatile organic compounds present in ambient air.

Due to the timing of eclosion of our queens, sampling was performed in two blocks: Block 1 (16/10/2020) sampled 16 bees (N=2 queens, <24h of age; N=6 queens, 5-14 days of age; N=4 workers, N=4 males, <24h of age). Block 2 (5/11/2020) sampled 18 bees (N=5 queens, 5-14 days of age; N=1 queen, 23 days of age; N=3 workers and N=4 males, 14 days old; N=5 males from mating aggregation).

GC-MS analysis

We analysed the volatiles trapped on stir bars using thermal desorption followed by Gas Chromatography – Mass Spectrometry (GC-MS). Stir bars were placed into glass thermal desorption liners, which were then inserted into a Thermal Desorption Unit (TDU; Gerstel, Mülheim an der Ruhr, Germany). The samples were purged with ultra-high purity helium (BOC Ltd, North Ryde, NSW, Australia) at 35°C for 1 minute to eliminate air from the sample and inlet. Samples were then heated at 12°C s^{-1} to 250 °C with a helium flow of 50 mL min^{-1}. Thermal desorption (TD) products were carried by the helium through to a programmed temperature vaporisation (PTV) inlet (CIS-4; Gerstel) installed in an Agilent 7890 GC (Agilent Technologies Pty Ltd, Mulgrave, Australia), which was used in solvent mode during the TD. The PTV inlet, containing a glass liner filled with Tenax TA, was held at 30°C during the TD using liquid CO_2 (BOC Ltd) as the cryogen. After 5 minutes of TD, the CIS-4 was heated at 12°C s^{-1} to 250 °C and held at that temperature for 3 minutes while the TD products were injected into the GC without splitting. TD products were separated on a HP-5ms capillary column (30 m x 0.25 mm, 0.25 µm film thickness; Agilent), which was connected to a mass selective detector (Model 5975C; Agilent). Ultra-high purity helium was used as carrier gas (flow rate through the HP-5ms column was 2.3 ml min^{-1}). The initial oven temperature of the GC was 40 °C, held for 2 minutes, then heated at a rate of 4 °C min^{-1} to 250 °C. The temperature of the GC-MS interface was 280 °C, the MS ion source 230 °C and the quadrupole 150 °C. The detector, in electron impact mode (70 eV), scanned the range of 35–300 *m*/*z*. Operation of the GC-MS was controlled by Agilent Chemstation (version E.02.01.117) and the TDU by Maestro (version 1.4.36.16; Gerstel).

We removed common contaminating ions (73, 84, 147, 149, 207, 221, 281 and 285 m/z) using the Denoising function in OpenChrom (Wenig and Odermatt, 2010) before processing chromatograms with the MSeasyTkGUI package (Nicolè et al., 2012) in R version 3.6.1 (Team and DC, 2019) to identify putative compounds produced by the bees. For each sample batch, we also subtracted the volatiles collected from our control air-only vials. Tentative identification of compounds associated with each caste and age of bee, was made by comparing mass spectra from the GC-MS and Kovat's non-isothermal retention indexes against those in a NIST mass spectral library (NIST08 in NIST MS Search v.2.0f; NIST, Gaithersburg, MD; match factor threshold=700). Kovat's non-isothermal retention indexes (as described in (Van Den Dool and Kratz, 1963) were calculated from a series of C8 to C20 homologous alkanes (40 mg L^{-1} of each alkane; Sigma-Aldrich, Sydney, Australia) injected onto separate, conditioned stir-bars.

Volatiles produced by queenless colonies

Colony manipulations

To characterize the volatiles produced by queenless colonies, we forced requeening of 10 *T. carbonaria* hived colonies in Sydney (33°53'05.5"S, 151°11'18.4"E, February 2019). All colonies had similar weight (8-9kg) and were kept in OATH hives, which comprise two boxes on top of each other (23 × 15 × 11 cm, volume 3.8 L). Using a common beekeeping propagation technique, we split each hive into its composite two boxes and gave each half (which contains part of the brood and food stores) a new, empty box to produce two hives (Heard, 2016). One hive resulting from each split retains the original queen, while the other is forced to requeen. In most cases, the queenless half will have an adult virgin queen already present and ready to mate as shown in **Chapter 3** (Garcia Bulle Bueno et al., in prep), though sometimes they must wait for a queen to eclose. After splitting, we kept all hives in an observation room in the dark. By placing acetate sheets over the occupied portion of newly-divided hives, we were able to confirm the presence of a physogastric queen in half (n=10) of them and the presence of a virgin queen in the other half (n=10). We then compared the volatiles produced by each hive (queenless vs queenright) in a split-pair using a paired-sample design.

Collection of volatiles

We sampled volatiles in the air inside colonies using a thermal desorption (TD) tube (200 mg Tenax TA; Markes International Ltd, Llantrisant, UK) inserted through a hole in the acetate sheet covering. Tubes collected air at 200 mL min^{-1} for 30 mins per colony, using an Aircheck 2000 sample pump (SKC Inc. Eighty-Four PA, USA). We sampled each hive prior to splitting (N=10) and then each new hive at 24 hours after splitting (N=20). For each hive at each time point, we also sampled the volatiles of the air outside the hive (5m from hive entrance) as a control.

GC-MS analysis

TD tubes were analysed using an automated thermal desorption unit (ULTRA 2 & UNITY 2; Markes International Ltd, Llantrisant, UK) for 6 minutes at 300°C and concentrated on a Tenax TA cold trap held at -30°C before flash heating (300°C) and splitless injection (via a heated transfer line; 150°C) onto a 7890A GC-MS (Agilent Technologies Pty Ltd, Melbourne). The GC-MS was fitted with a BP1 capillary column (60 m x 0.32 mm, 1 µm film thickness; Agilent) with a flow rate of helium set at 2.3 mL min^{-1}. The GC oven was heated at 35°C for 5 minutes, then 4°C min^{-1} to 160°C, then 20°C min^{-1} to 300°C and held for 10 minutes. The GC was coupled to a mass-selective detector (Model 5975C; Agilent) that was set to a scanning range of 35 – 300 amu. We processed GC-MS data using the same procedures described above for individual bee samples, except in this case using Kovat's non-isothermal retention indexes (Van Den Dool and Kratz, 1963) calculated from C8 to C20 homologous alkanes (40 mg L^{-1} each; Sigma-Aldrich, Sydney, Australia) injected onto separate, conditioned TD tubes. We used OLS regression to identify compounds that differed between queenless and queenright pairs of recently-split colonies.

Nest usurpation rates of queenless and queenright colonies

Colony manipulations

To assess whether the re-queening process makes colonies more vulnerable to nest usurpations by foreign queens, we assessed queen turnover in colonies recently propagated by hive-splitting. As above, hive-splitting produces two colonies: one retains the mature queen, while the other will find itself temporarily queenless, and produce a new queen from its stock of

virgin queens or brood (i.e. the colony is inherited by a daughter of the original queen). If attacks from conspecific colonies were either more likely to occur, or more likely to be successful, when colonies were requeening, then we predicted: (1) in pairs of recently-split colonies, the incidence of usurpations will be higher among the half that were forced to requeen than the half that retained the original queen, and (2) the incidence of usurpations will be higher among recently-split colonies than colonies that were not split.

We tested these predictions for hived colonies of *T. carbonaria* kept at farms on the Sunshine Coast, Queensland (26.6500° S, 153.0667° E) during January-June 2019 (N=7 pairs of colonies were split, N=18 colonies were not split), and colonies kept at Ku Ring Gai, Sydney (33.7416° S, 151.1520° E) during October 2019-May 2020 (N =12 pairs of split colonies). Although un-split hives were not available for comparison during the study period in Sydney, we did assess queen turnover in seven natural colonies in tree-cavities during the same period, as an indicator of natural rates of requeening. In all cases, hived colonies were kept in OATH hives of standard dimensions (Heard 2016) and prior to splitting they weighed 8–9 kg (typical of a large colony; Heard, 2016). More details on the hive locations for the two sites are provided in **Supplementary material, Figure S1.**

We inferred queen turnover based on the genotype of workers collected at colony entrances. For all colonies, we sampled eight workers immediately prior to any hive-splitting (Jan 2019 for Sunshine Coast; October 2019 for Sydney) and again six months later (June 2019 for Sunshine Coast; May 2020 for Sydney).

Analyses

For all of the bees collected, we extracted DNA by grinding whole abdomens in 5% Chelex solution (1mM Tris HCl pH 7.6, 0.1mM EDTA pH 8) and boiling for 15 mins (Walsh et al., 1991). Supernatant containing DNA was diluted 1:1 with distilled water prior to PCR amplification. Each individual was genotyped at seven microsatellite loci using five primers designed for *T. carbonaria* (Tc3. 155, Tc4. 63, Tc3. 302, Tc7. 13 and Tc4. 287) (Green et al., 2001) and two designed for *Tetragonula angustula* (Tang60 and Tang70) (Brito et al., 2009). Primers were fluorescently labelled with one of four dyes (FAM, NED, PET, VIC; Sigma-Aldrich, U.S.A.). PCR amplifications were performed according to (Green and Oldroyd, 2002)

and the resulting products were analysed using a 3130xl Genetic Analyser and Genemapper (Applied Biosystems, U.S.A.).

For each colony at each time-point, we manually determined the parental genotype based on worker genotypes. Since *T. carbonaria* is both monandrous and monogynous, workers born from the same mother will all share one paternal allele from their haploid father and one of two possible maternal alleles from their diploid mother (Green and Oldroyd, 2002). To support our assignments, we also input our data using the maximum likelihood sibship reconstruction method of COLONY 2.0 (Wang, 2004). This program uses the population allele frequency (estimated from the sample set) to estimate the genetic relationship among haplodiploid individuals. COLONY reconstructed full sibships for bees from each colony and confirmed that we had correctly inferred all maternal and paternal genotypes.

We categorised queen status after 6 months, relative to the original sample as: (i) retained queen (parental genotypes did not change), or (ii) requeened with daughter queen (the genotype of the queen was consistent with being the daughter of the original queen), or (iii) usurper queen (the genotype of the queen was not consistent with being the daughter of the original queen). The developmental time between egg and forager in *T. carbonaria* is 60-70 days, so it is very unlikely that colonies would have had time for two natural queen turnovers; consistent with this, none of the queens we identified as usurpers had genotypes consistent with being granddaughters of original queens. Importantly, we also cannot rule out that some queen replacement events occurred prior to our original sampling. This is because if a colony had replaced its queen <60 days before our sampling, we would not have inferred the current queen from our forager sample. However, as any such changes should be equally likely to occur in hives of each treatment (split or not split), we assume they do not impact our comparisons. We used chi-square tests to compare the proportion of observed queen usurpations between the pairs of split-colonies, and between all split and non-split colonies (R v4.0.0, R Studio v1.3.959).

Opportunistic sampling of dead virgin queens from outside colonies

In 2019, we came across dead virgin queens on the ground near to the entrances of two hives (<10cm), typical of where workers dump bodies removed from their colony. These hives were not otherwise involved in any of our experiments. At the first hive (“Colony A”, Lindfield,

Sydney; 33°46'14.5"S, 151°09'47.3"E; October 2019) we collected two dead virgin queens and observed a "fighting swarm". These large swarms (thousands of workers) are commonly associated with nest usurpations, and involve pairs of workers slamming together in mid-flight, falling to the ground and then locking onto each other with their mandibles (Gloag et al., 2008). Typically, these pairs wrestle on the ground until both are dead. To determine whether the dead virgin queens belonged to the adjacent (defending) colony or the attacking colony, we sampled workers exiting the hive and 8 pairs of dead workers on the ground that were still locked together in their death grip. At a second hive ("Colony B", Marrickville, Sydney; 33°53'52.8"S, 151°09'54.9"E; October 2019) we collected four dead virgin queens. We did not observe fighting at this colony at the time of collection nor were there any dead bees on the ground indicative of a recent fight, although a large male aggregation was swarming close to the hive, suggesting that it was in the process of requeening.

Results

Volatiles produced by virgin queens

We identified 1022 compounds from volatiles collected from individual *T. carbonaria*. We identified two sets of compounds that we hypothesized were informative of caste. First, we identified those compounds that were present *only* in the virgin queens (52 compounds) (**Figure 1**). Second, we identified compounds that were significantly more likely to be present in virgin queens than other castes (males and workers) based on OLS regressions (70 compounds; $p \leq 0.05$) run in Python (Sanner, 1999). Some compounds were identified by both approaches (34 compounds), such that we identified in total 88 unique candidate compounds. These compounds were mainly mono-sesquiterpenes and alcohols. Of these 88 compounds, 26 could be matched to known compounds. The remainder had poor match factors to known compounds, or they lacked a sufficient retention index value to be properly identified (**Table 1**).

Overall, compounds differed greatly between the two sampling events (batches) (Supplementary material, **Figures S2** and **S3**). This effect likely arose because some ambient background compounds from the sampling room on the two different dates of sampling were not adequately excluded by our ambient air control (Supplementary material, **Figure S2**).

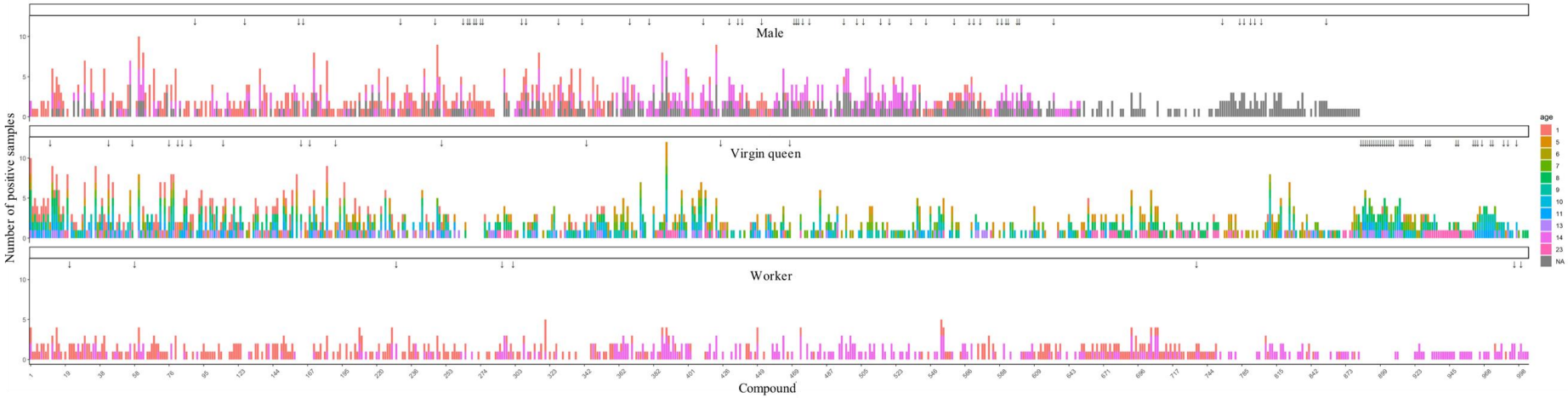


Figure 1. Histogram of chemical compounds (numbered 1-1022) detected in males and two castes of females (workers and virgin queens) of *Tetragonula carbonaria* (n=34). The arrows pointing to the compounds below for each caste designate compounds that were only present in that specific caste (males, n=54; virgin queens, n=52; and workers n=8). The colour of each bar represents the age of the bee at the time of the sampling**.**

Table 1. List of 25 known compounds that were significantly more present in virgin queens vs non-queens (workers and males), or only present in the virgin queens. Compound type is assigned based on the listed references. An asterisk (*) indicates the 3 compounds (1,3-bis(1,1-dimethylethyl)- Benzene, (2Z)-2-(3,3-Dimethylcyclohexylidene) ethanol and 2-Ethyl-2-propyl-1-hexanol, previously identified as insect pheromones (butterflies or beetles; species: Bm = *Bombyx mori, Am = Anthonomus musculus,* Ss =*Sternechus subsignatus* and *Se = Spodoptera exigua*). For the full name of the compounds and more details on the plant related compounds see **Supplementary material, Table S1**.

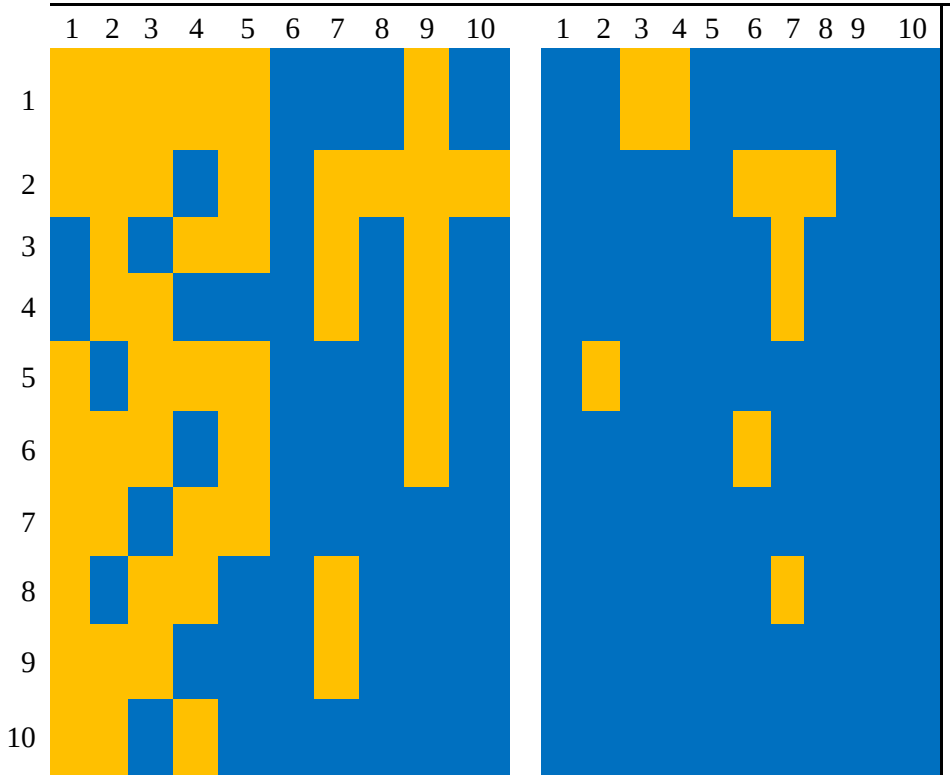
Compound Group	Type	References
2-Ethyl-1-hexanol	Plant	(Jakobsen et al., 1995)
3,3,5-trimethyl-cyclohexanone	Plant	(You and Wang, 2011)
1-(2-Hydroxy-1-methylethyl)-2,2-dimethylpropyl 2-methylpropanoate	Bacteria	(Velázquez-Becerra et al., 2011)
2-Ethyl-1-decanol	Plant	(Martínez et al., 2009)
2-ethylhexyl ester benzoic acid	Plant	(Wei and Yin, 2019)
2-Hydroxybenzaldehyde	Plant	(Thangadurai et al., 2002)
3-Cyclohexene-1-methanol, α,α,4-trimethyl-, (S)-	Plant	(Fayemiwo et al., 2014)
Hexanoic acid, ethyl ester	Yeast	(Benda et al., 2008)
1,2-Benzisothiazole	Biocide	(Uchiyama et al., 1973)
Benzene, 1,3-bis(1,1-dimethylethyl)-*	Ph (Bm)/ Plant	(Arunprasanna et al., 2016, Ruíz-Ramón et al., 2014)
Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	Plant	(Amri et al., 2017)
Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-	Plant	(Ding et al., 2012)
3,7,11-Trimethyl-1-dodecanol	Plant	(LIU et al., 2009)
2-Methyl-1-decanol	Plant	(Yue et al., 2017)
2,3-Dimethyloctane	Plant	(Kaiira et al., 2019)
1,3,3-Trimethylbicyclo[2.2.1]heptan-2-ol	Plant	(Qi and Armstrong, 2007)
Isopinocarveol	Plant	(Zhou et al., 2009)
Bicyclo[3.1.1]heptan-3-ol, 6,6-dimethyl-2-methylene-, [1S-(1α,3α,5α)]-	Plant	(Oso et al., 2018)
(2Z)-2-(3,3-Dimethylcyclohexylidene) ethanol*	Ph (Am, Ss)	(Ambrogi et al., 2012, Szendrei et al., 2011)
4,6-Dimethyldodecane	Plant	(Geethalakshmi and Sarada, 2013)
Geranyl vinyl ether	Plant	(Hossain et al., 2010)
2-(5-Methyl-5-vinyltetrahydro-2-furanyl)-2-propanol	Plant	(Sumangala et al., 2018)
Benzaldehyde	Plant	(Verma et al., 2017)
Isopinocarveol	Plant	(Naidoo et al., 2018)
2-Ethyl-2-propyl-1-hexanol*	Ph (Se)	(Mujiono et al., 2015)

Volatiles produced by queenless colonies

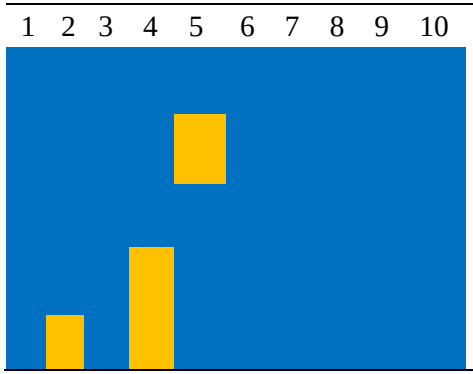
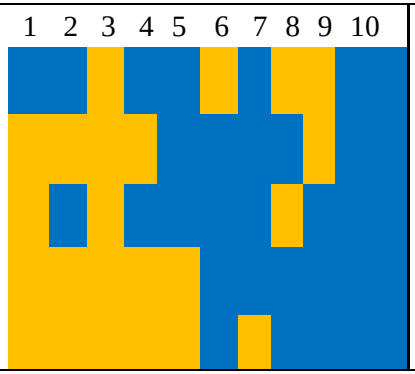
We identified 1005 volatile compounds present in colonies that were recently split (24 hours prior to sampling). We again identified the compounds that differed significantly in the likelihood that they were present between the queenless and queenright colonies based on OLS regressions (33 compounds; $p \leq 0.05$) run in Python (Sanner, 1999). Of these 33 compounds, 15 could be matched to known compounds (**Table 2**); the rest either had poor match factors or they lacked a sufficient retention index value to be properly identified.

Most of the 15 identified compounds (N=12, 86%) were terpenes and alcohols that have been previously associated with plant material (see references **Table 1**), and thus likely to be volatiles from the resins used to build nests. The remaining two compounds (2-Heptanol and 1-Butanol, 3-methyl-) are associated with insects and were both significantly more likely to be present in queenright than queenless colonies, suggesting they may be volatiles produced by mature queens.

Table 2. Presence-absence matrix of the volatiles that were significantly more or less present inside queenright vs queenless colonies 24h after hive-splitting. Yellow squares indicate presence of the volatile; blue squares indicate absence. The compounds are grouped into those that were present significantly more often in queenright than queenless colonies (Group 1) and that were present significantly more often in queenless than queenright colonies (Group 2). Compound type is assigned based on the listed references. An asterisk (*) indicates the two compounds (2-Heptanol and 1-Butanol, 3-methyl-) previously identified from social bees or wasps (species: Scp, *Scaptotrigona postica*, Scm, *S. mexicana*. Am, *Apis mellifera* and Vm, *Vespa mandarina*). For the full name of the compounds and more details on the plant related compounds see **Supplementary material, Table S2.**



Compound Group	Type	References
1		
2-Heptanol*	Ph (Scp, Scm)	(Engels et al., 1993, Grajales-Conesa et al., 2007, Verdugo-Dardon et al., 2011)
α-Cubebene	Plant	(Souza et al., 2018, Patricio et al., 2002)
1,4-Methano-1H-indene	Plant	(Zhang et al., 2010a)
Acetic acid, 1-methylethyl ester	Plant	(Zhang et al., 2009)
Toluene		
Unknown sesquiterpene		
2H-Pyran	Plant	(Jafari and Sani, 2016)
1-Butanol, 3-methyl-*	Ph (Am, Vm)	(Wager and Breed, 2000, Torto et al., 2005, Ono et al., 2003)
Heptane, 2-methyl-		
Unknown monoterpene		

Queenright	Queenless	Compound Group	Type	References
		**2**		
11		Bicyclo[3.1.1]heptan-3-ol	Plant	(Sahi, 2016)
12		2-Phenanthrenol	Plant	(Zhang et al., 2010b)
13		trans-2-Caren-4-ol	Plant	(Aljarah and Hameed, 2018)
14		α-Cubebene	Plant	(Souza et al., 2018, Patricio et al., 2002)
15		1H-Cycloprop[e]azulene	Plant	(Massaro et al., 2018)

Nest usurpation rates of queenless and queenright colonies

Nest usurpation occurred at 11 (19%, N=58) of our experimental hives during our 6-month sampling period, confirming that this behaviour is common in *T. carbonaria* in managed (hived) populations. These nest usurpations were inferred on the basis that workers' genotypes indicated that they were full-sisters but unrelated to the workers sampled from the hive 6-months previously (**Table 3**).

Among all pairs of split colonies, 6 pairs had both halves requeen with daughter queens (6 of 20; 30%). From the remaining pairs, we detected more queen usurpations among the colonies forced to requeen after splits (6 of 14; or 30% of all 20 pairs) than those that retained their original queen (3 of 14; or 15% of all 20 pairs; **Table 3**), though this difference was non-significant (p=0.42; Chi-square test, two-tailed). This comparison assumes that any pairs in which one half requeened with a daughter and the other was usurped (N=2) represent cases of original queens being usurped, so might underestimate the risk queen usurpation during re-queening. Similarly, colonies that had been split, irrespective of requeening, experienced a higher incidence of queen usurpations (N=9 of 31; 29%) than those that were not split (N=2 of 18; 11%), though this difference was not significant (p=0.208; Chi-square test, one-tailed). We did not detect nest usurpations at any of the seven natural nests sampled from tree-cavities.

Table 3. Incidence of queen usurpation in hived *T. carbonaria* colonies, inferred from the genotypes of workers sampled from the hive at two intervals, 6-months apart: Q = original queen (the colony retained the queen from the time of the first sampling), DQ = daughter queen (the colony requeened with a daughter from the original queen), U=usurpation by foreign queen (the colony's new queen did not match the original queen's genotype or a daughter's one).

	Hives split			Hives not split
Colony ID	Split A	Split B	Colony ID	
SC(s)-1	Q	U	SC(ns)-1	DQ
SC(s)-2	Q	DQ	SC(ns)-2	U
SC(s)-3	DQ	DQ	SC(ns)-3	Q
SC(s)-4	Q	DQ	SC(ns)-4	Q
SC(s)-5	DQ	DQ	SC(ns)-5	Q
SC(s)-6	Q	U	SC(ns)-6	DQ
SC(s)-7	DQ	DQ	SC(ns)-7	Q
SC(s)-8	Q	DQ	SC(ns)-8	DQ
S(s)-1	U	DQ	SC(ns)-9	Q
S(s)-2	DQ	DQ	SC(ns)-10	Q
S(s)-3	Q	DQ	SC(ns)-11	Q
S(s)-4	Q	DQ	SC(ns)-12	Q
S(s)-5	DQ	DQ	SC(ns)-13	Q
S(s)-6	DQ	DQ	SC(ns)-14	Q
S(s)-7	Q	DQ	SC(ns)-15	DQ
S(s)-8	U	DQ	SC(ns)-16	DQ
S(s)-9	U	U	SC(ns)-17	Q
S(s)-10	Q	U	SC(ns)-18	U
S(s)-11	Q	U		
S(s)-12	Q	U		
% usurped	15%	30%		11%

Origin of dead virgin queens found outside nest entrances

Colony A (2 dead virgin queens): All workers collected at the colony entrance were full-sisters (N=8). However, neither of the two dead virgin queens collected from outside the colony had genotypes that matched the colony, nor were they sisters (i.e. the two dead queens came from two different foreign colonies). Among 16 bees that formed fighting pairs collected on the ground near to the colony, only four had genotypes consistent with the defending colony, while the remaining 12 had genotypes consistent with originating from three different colonies. One of these "attacking" workers had a genotype consistent with being the sister of one of the dead virgin queens.

Colony B (4 dead virgin queens): All workers collected at the colony entrance were full-sisters (N=8). Three out of the four dead virgin queens retrieved from outside the colony had genotypes indicating they were from the colony, while the fourth had a foreign genotype.

Discussion

Volatiles produced by *T. carbonaria* virgin queens and queenless colonies

We described for the first time some of the volatiles produced by virgin queens of *T. carbonaria*. Three compounds detected in virgin queens were not detected in workers or males. These included one hydrocarbon and two alcohols: Benzene, 1,3-bis(1,1-dimethylethyl)-, 2-Ethyl-2-propyl-1-hexanol and (2Z)-2-(3,3-Dimethylcyclohexylidene)-ethanol. None of these compounds have previously been reported from stingless bees, but they are known to be sex or aggregation pheromones in other insects (Arunprasanna et al., 2016, Ambrogi et al., 2012, Szendrei et al., 2011, Mujiono et al., 2015).

1,3-bis(1,1-dimethylethyl)- Benzene was found previously in head extracts of the lepidopteran *Bombyx mori* (Arunprasanna et al., 2016). 2-Ethyl-2-propyl-1-hexanol was found in sex pheromones glands of virgin females of the lepidopteran *Spodoptera exigua* fed with an artificial diet (Mujiono et al., 2015) and (2Z)-2-(3,3-Dimethylcyclohexylidene) ethanol, a headspace emission from the male beetles *Anthonomus musculus*, is known to act as an aggregation pheromone (Szendrei et al., 2011). Interestingly, this latter pheromone is male-produced and is presumed to function in the sexual behaviour of these beetles. Male-produced

aggregation pheromones have been identified in many insect orders (Schlyter and Birgersson, 1999) and function to attract multiple males to a location (Lacey et al., 2004). In *T. carbonaria*, virgin queens might use aggregation pheromones to attract males over short-ranges, such as when they fly into a male aggregation for their nuptial flight, or longer-ranges, to induce males passing close to nests to gather outside the nest. In beetles, males are known to respond to aggregation pheromones at distances of up to 100m (Lacey et al., 2004).

The vast majority of compounds we detected were shared between workers, males and virgin queens (N=1022). Our approach may have overlooked important caste-specific differences in volatile profiles due to either low sampling size, or because we identified only on those compounds that differed in presence/absence between castes. In other social insects, the compounds that distinguish castes can be qualitatively similar but quantitatively different (Fierro et al., 2011, Grajales-Conesa et al., 2007). Future studies should therefore aim to quantify the compounds emitted by virgin queen of various ages, relative to workers. Likewise, target compounds should be synthesized to confirm whether they have an effect on males' behaviour.

Male stingless bees are predicted to locate queenless colonies on the basis of unique volatiles that these colonies produce. However, our measure of colony-level volatiles from recently-split queenless *T. carbonaria* colonies failed to identify volatiles of insect origin that were present only in queenless colonies. This may indicate that our sampling occurred too early in the re-queening process (24 hours after loss of the queen). However, we observed virgin queens in our queenless colonies at this time, and males are known to arrive rapidly at recently-split colonies (within 1 day), the target volatiles may have been at quantities too low for us to detect.

Alternatively, males may cue into the *absence* of mature queen volatiles when searching for re-queening nests. We identified two insect-origin volatiles that were associated with queenright, but not queenless colonies of *T. carbonaria*, suggesting they may be produced by mated queens. Both of these putative queen-specific compounds are known from other social hymenopterans. In Neotropical stingless bees, 2-Heptanol has been proposed to have varying functions depending on the species. It can act as an alarm pheromone in *Melipona* and *Trigona* (Alavez-Rosas et al., 2019, Johnson et al., 1985), but it is also related to the female reproductive caste in *Scaptotrigona mexicana*, being emitted by both virgin queens and mated queens (Grajales-Conesa et al., 2007). *S. mexicana* queens appear to produce 2-Heptanol in varying

quantities depending on their age and/or egg-laying status, such that amount of volatile, rather than its presence or absence, may signal a queen's reproductive state (Grajales-Conesa et al., 2007). Once a virgin *Scaptotrigona* queen mates, her production of 2-Heptanol increases (Engels et al., 1993, Grajales-Conesa et al., 2007). The idea that physogastric queens produce a volatile that repels males away from queenright colonies has been previously suggested for *Scaptotrigona* species (Verdugo-Dardon et al., 2011), and requires further investigation for *T. carbonaria*. Under this scenario, males searching their environment for re-queening colonies may initially search for all conspecific colonies based on particularly potent volatiles such a nest resins, and then assess each colony's queen status before deciding whether or not to move on.

Outside of stingless bees, 2-Heptanol functions as an alarm pheromone produced by workers in honey bees (*Apis mellifera*) and ants (*Atta texana*) (Collins and Blum, 1983, Free et al., 1983, Moser et al., 1968, Riley et al., 1974). Similarly, 1-Butanol, 3-methyl-, is used as an alarm signal by *A. mellifera* and *Vespa mandarina* (Wager and Breed, 2000, Torto et al., 2005, Ono et al., 2003). Given that we were sampling recently-split colonies, we cannot exclude the possibility that these volatiles also serve an alarm function in *T. carbonaria*, although it is not clear in this case why they were not consistently produced also in queenless colonies.

Nest usurpation at re-queening colonies

Nest usurpation occurred at a high incidence among the colonies used in our experiment (19%), confirming that this behaviour is common among hived populations of *T. carbonaria*. We hypothesized that the release of volatiles by queenless colonies to attract males might also attract rival colonies aiming to take over the nest site. Consistent with this, we observed a higher occurrence of usurpations in colonies that were forced to requeen (35%) than those that did not (10%), however a larger sample of colonies is needed to verify this apparent trend. Importantly, colonies in the re-queening phase might be susceptible to usurpation for reasons unrelated to olfactory eavesdropping. For example, an already queenless colonies may simply be more likely to accept a new queen in place of one of their sisters than a colony that has a resident queen, or worker defences against conspecific attack may be reduced in queenless colonies. Thus, the possible role of re-queening volatiles in nest usurpations remains an open question for *T. carbonaria*.

We identify for the first-time foreign virgin queens rejected from *T. carbonaria* colonies. In one case, the dead virgin queen was found at the time of an active fighting swarm at the colony, and matched the genotype of some attacking workers. This suggests that virgin queens belonging to usurping colonies do not wait until the battle is won, but rather that they fly to the war zone and attempt to infiltrate the target colony during battle. Interestingly, virgin queens from at least two different foreign colonies were found dead near this colony. This is consistent with reports that *Tetragonula* fights often involve multiple colonies (Cunningham et al., 2014, Gloag et al., 2008).

Queen parasitism has been documented in several South American stingless bee species (Wenseleers et al., 2011). In these species, virgin queens are assumed to act solo when infiltrating colonies, rather than being accompanied by cohorts of workers or visible fights. This form of queen parasitism may arise from the fact that, in stingless bees, virgin queens are produced all year round and would otherwise be killed by workers soon after emergence in queenright colonies (Da Silva et al., 1972, Jarau et al., 2009, Vollet-Neto et al., 2019, Wenseleers et al., 2004a). Virgin queens escape this fate by leaving the colony, and take the chance of invading unrelated colonies nearby (Sommeijer et al., 2003). Whether this form of queen parasitism also occurs in *T. carbonaria* remains unclear, but we identified one dead foreign virgin queen outside a colony that was in the process of re-queening but showed no evidence of worker fighting.

More generally, the high incidence of foreign queen takeovers detected in this study suggests that not all *T. carbonaria* nest usurpations are associated with conspicuous fighting. When choosing which colonies to target for usurpation, attacking colonies may adopt a strategy of either maximising gains, or minimising losses. For example, attackers might be expected to attack small colonies, since it is easier to overcome a small defending force (Hölldobler, 1976). These types of nest usurpations may involve little fighting. Large scale warfares with many worker deaths may only occur when large colonies attack other large colonies with rich resources, aiming for a high reward strategy (Pohl and Foitzik, 2011).

Conclusion

In conclusion, our study is the first to describe the chemical profiles associated with colony reproduction in *T. carbonaria*. We found that (i) virgin queens produce specific pheromones, 1,3-bis(1,1-dimethylethyl)- Benzene, (2Z)-2-(3,3-Dimethylcyclohexylidene) ethanol, 2-Ethyl-

2-propyl-1-hexanol, which might be linked to the short attraction range of males, and (ii) 2-Heptanol is a candidate queen-signal pheromone, indicating the presence of a mated laying queen, and thus presumably a repellent pheromone for males. We finally (iii) provide preliminary evidence that queenlessness induced by hive-splitting could increase the rate of foreign queen take-overs in hived colonies, a question that requires further investigation. Finally, we report for the first-time dead virgin queens discarded at the entrance of unrelated colonies of *T. carbonaria*, presumably failed queen parasitism attempts. We recommend further studies to elucidate the complex chemical profiles associated with re-queening colonies and their role in the attraction of both males and conspecific nest usurpers.

Supplementary information

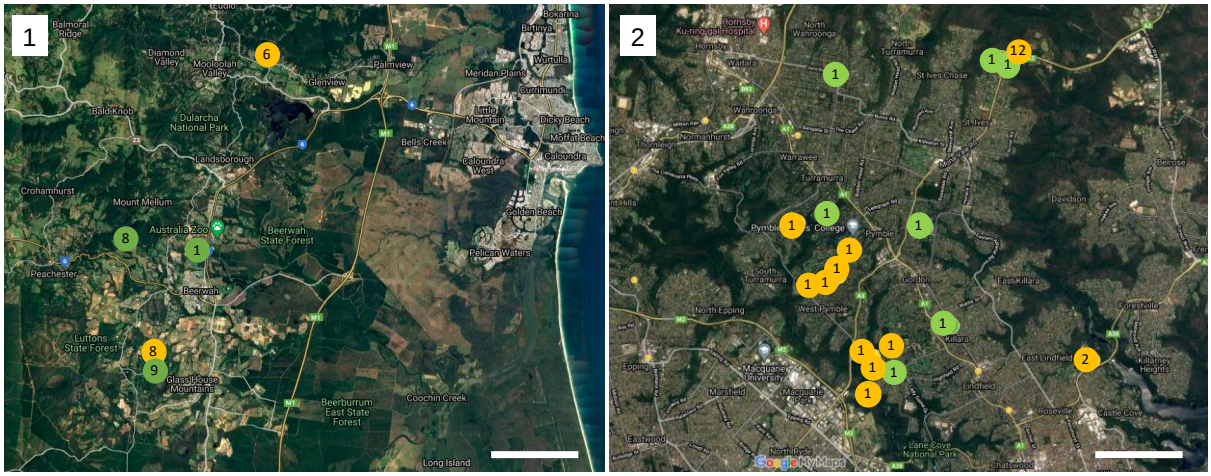


Figure S1. Map of colony locations in Sunshine Coast (1) and Sydney (2) used to assess nest usurpation rates (scale bar: 2km). Each circle is a site; the number inside circles denotes the number of colonies at that site. The colour indicates whether the colony was split (yellow) or not split (green). For Sunshine Coast, all of the split colonies were located into groups of eight and six colonies respectively; once split, all of the split boxes were located in the same site within their cluster. For Sydney, some colonies were clustered at one site (n=10) and the remainder were scattered across a 40 km^2 area. The non-split colonies in Sydney were wild colonies in tree cavities.

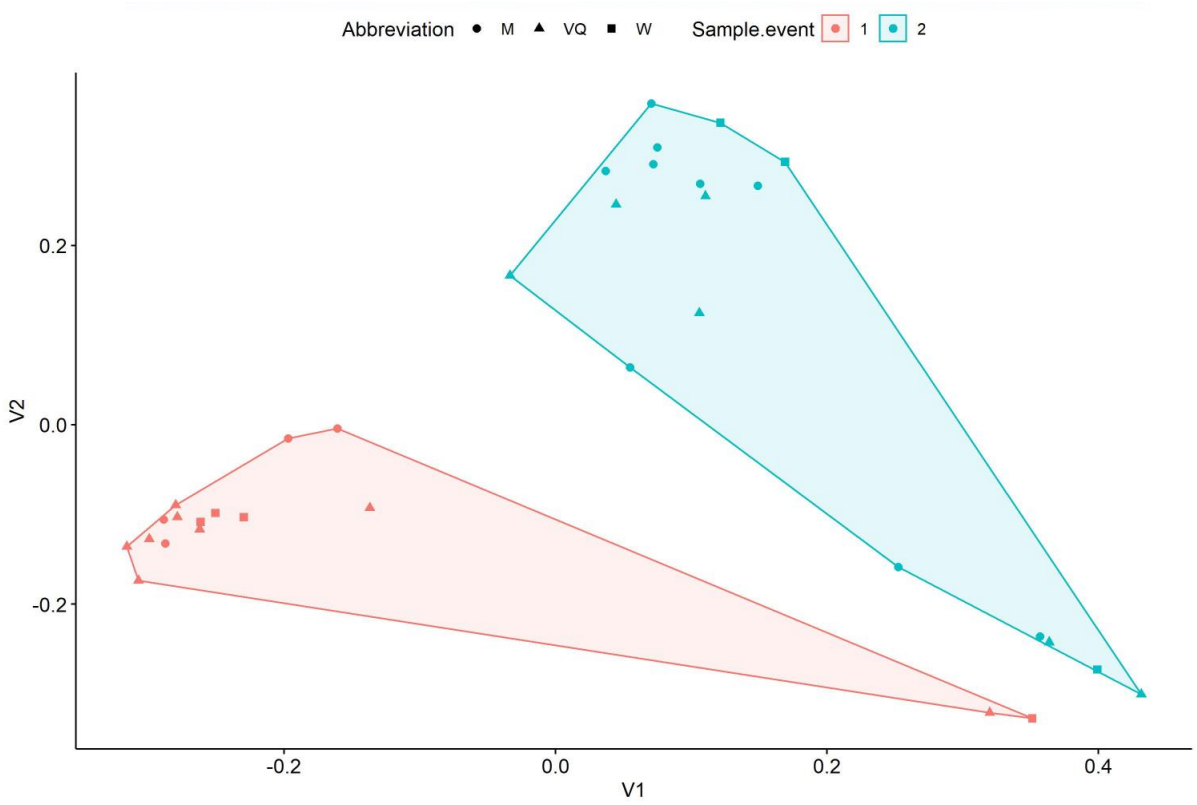


Figure S2. MDS (multidimensional scaling) on the different caste samples that measured the volatiles of the bees (n=35). The MDS is showing the strong effect of the sampling event. Two groups were formed according to the sampling event 1 (in red) and 2 (in blue). Each dot represents a bee (triangle for virgin queens, circles for males and squares for workers). Abbreviations are: M = males, VQ= virgin queens and W = workers.

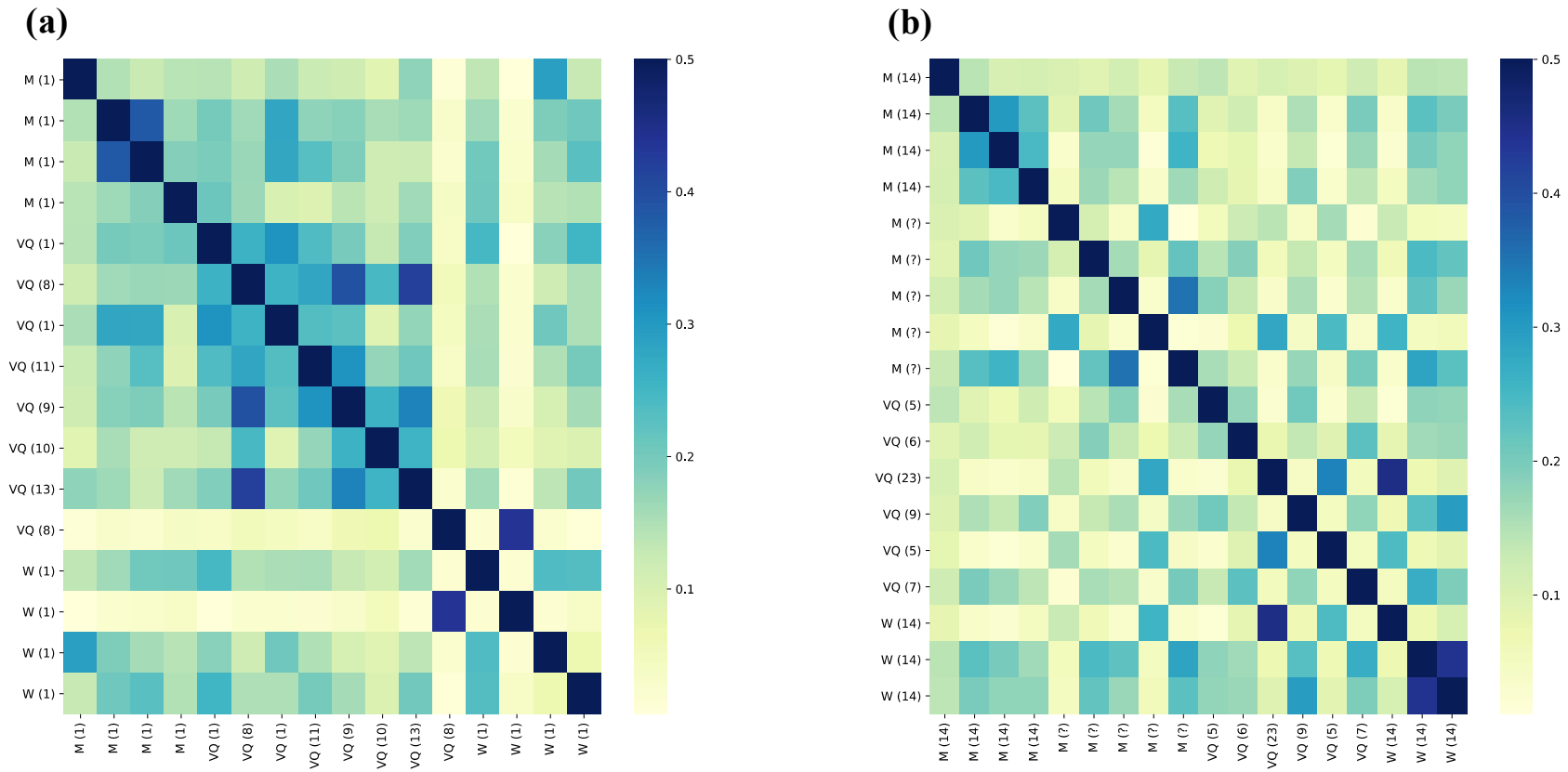


Figure S3. Heat maps representation of a Jaccard similarity between each pair of bees divided by sampling event: 1(a), n=16; 2(b), n=18). M for male, VQ for virgin queen and W for worker. The number in between brackets means the age of the individual, the males with the question mark were the males caught in the mating swarm for which the age was unknown. The map was generated by dividing the number of shared present compounds by the total number of compounds present in between each pair of samples.

Table S1. Full name of all compounds listed in Table 2, and additional details for the compound

Compound name	Comments	Reference
2-Ethyl-1-hexanol	Volatile from *Trifolium repens* and *Brassica napus oleifera*)	(Jakobsen et al., 1995)
3,3,5-trimethyl-cyclohexanone	Plant oil from *Datura Stramonium*	(You and Wang, 2011)
1-(2-Hydroxy-1-methylethyl)-2,2-dimethylpropyl 2-methylpropanoate	Volatile from *Arthrobacter agilis*	(Velázquez-Becerra et al., 2011)
2-Ethyl-1-decanol	Plant oil from *Tagetes patula*	(Martínez et al., 2009)
2-ethylhexyl ester benzoic acid	Plant oil from *Taxus chinensis*	(Wei and Yin, 2019)
2-Hydroxybenzaldehyde	Plant oil from *Decalepis hamiltonii*	(Thangadurai et al., 2002)
3-Cyclohexene-1-methanol, α,α,4-trimethyl-, (S)-	Plant oil from *Pinus sylvestris*	(Fayemiwo et al., 2014)
Hexanoic acid, ethyl ester	Volatile from *Candida* spp.	(Benda et al., 2008)
1,2-Benzisothiazole	Biocide	(Uchiyama et al., 1973)
Benzene, 1,3-bis(1,1-dimethylethyl)-*	Pheromone from *Bombyx mori* and Volatiles from Narcissus	(Arunprasanna et al., 2016, Ruíz-Ramón et al., 2014)
Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	Plant oil from *Teucrium capitatum*	(Amri et al., 2017)
Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-	Volatile from *Zingiber officinale*	(Ding et al., 2012)
3,7,11-Trimethyl-1-dodecanol	Volatile from *Glycine max*	(LIU et al., 2009)
2-Methyl-1-decanol	Plant oil from *Hippophae rhamnoides*	(Yue et al., 2017)

2,3-Dimethyloctane	Volatile from upland rice upland rice, NERICA 1 (S3)	(Kaiira et al., 2019)
1,3,3-Trimethylbicyclo[2.2.1]heptan-2-ol	Plant oil from *Myristica fragrans*	(Qi and Armstrong, 2007)
Isopinocarveol	Plant oil from *Eucalyptus tereticornis*	(Zhou et al., 2009)
Bicyclo[3.1.1]heptan-3-ol, 6,6-dimethyl-2-methylene-, [1S-(1α,3α,5α)]-	Volatile from *Xylopia aethiopica*	(Oso et al., 2018)
(2Z)-2-(3,3-Dimethylcyclohexylidene) ethanol*	Pheromone from *Sternechus subsignatus*	(Ambrogi et al., 2012, Szendrei et al., 2011)
4,6-Dimethyldodecane	Plant oil from *Trianthema decandra*	(Geethalakshmi and Sarada, 2013)
Geranyl vinyl ether	Plant oil from *Stevia rebaudiana*	(Hossain et al., 2010)
2-(5-Methyl-5-vinyltetrahydro-2-furanyl)-2-propanol	Volatile from *Jasminum malabaricum*	(Sumangala et al., 2018)
Benzaldehyde	Volatile from *Prunus persica*	(Verma et al., 2017)
Isopinocarveol	Volatile from *Eucalyptus grandis*	(Naidoo et al., 2018)
2-Ethyl-2-propyl-1-hexanol*	Pheromone from *Spodoptera exigua*	(Mujiono et al., 2015)

Table S2. Full name of all compounds listed in Table 2, and additional details for the compound

Number	Compound name	Comments	Reference
1	2-Heptanol	Pheromone from *S.postica*	(Engels et al., 1993)
1	2-Heptanol	Pheromone from *S.mexicana*	(Verdugo-Dardon et al., 2011)
1	2-Heptanol *S.mexicana*	Pheromone from *S.mexicana*	(Grajales-Conesa et al., 2007)
2	α-Cubebene	Found in the resin of *Frieseomelitta* spp.	(Patricio et al., 2002)
2	α-Cubebene	Found in the resin of *Frieseomelitta* spp.	(Souza et al., 2018)
3	1,4-Methano-1H-indene,octahydro-4-methyl-8-methylene-7-(1-methylethyl)-, [1S-(1α,3aβ,4α,7α,7aβ)]-	Plant oil	(Zhang et al., 2010a)
4	Acetic acid, 1-methylethyl ester		(Zhang et al., 2009)
5	Toluene		
6	Unknown sesquiterpene		
7	2H-Pyran, tetrahydro-4-methyl-2-(2-methyl-1-propenyl)-	Plant oil from *Melissa officinalis*	(Jafari and Sani, 2016)
8	1-Butanol, 3-methyl-	Pheromone from *Apis mellifera*	(Wager and Breed, 2000)
8	1-Butanol, 3-methyl-	Pheromone from *Apis mellifera*	(Torto et al., 2005)
8	1-Butanol, 3-methyl-	Pheromone from *Vespa mandarina*	(Ono et al., 2003)
9	Heptane, 2-methyl-		
10	Unknown monoterpene		

11	Bicyclo[3.1.1]heptan-3-ol, 6,6-dimethyl-2-methylene-, [1S-(1α,3α,5α)]-Eucalyptuscitriodora	Volatile from *Eucalyptus citriodora*	(Sahi, 2016)
12	2-Phenanthrenol, 4b,5,6,7,8,8a,9,10-octahydro-4b,8,8-trimethyl-1-(1-methylethyl)-, (4bS-trans)-	Volatile from *Eucalyptus* spp.	(Zhang et al., 2010b)
13	Trans-2-Caren-4-ol	Compound from Thymus *vulgaris*	(Aljarah and Hameed, 2018)
14	1H-Cycloprop[e]azulene, 1a,2,3,5,6,7,7a,7b-octahydro-1,1,4,7-tetramethyl-, [1aR-(1aα,7α,7aβ,7bα)]-Australianstinglessbeepollen	Found in Australian stingless bee (*Tetragonula* spp.) pollen	(Massaro et al., 2018)

CHAPTER 7

General discussion

This thesis advances our understanding of the foraging and reproductive ecology of stingless bees, with a focus on *Tetragonula carbonaria* (**Figure 1**), a stingless bee species from the Indo-Malay-Australasian clade.

My hope is that the new insights provided here help us to harness the full potential of stingless bees as crop pollinators in Australia and other parts of the world. I have investigated what wild flowers and plant groups are commonly visited by stingless bees to collect food (**Chapter 2**), how far males disperse from natal nests to find mates (**Chapter 3**), whether workers can become reproductive under certain environmental conditions (**Chapter 4**), how to raise and mate queens in captivity (**Chapter 5**) and which chemical volatiles are related to virgin queens and re-queening colonies (**Chapter 6**). Together, these studies inform the conservation of wild stingless bees, and husbandry techniques for *T. carbonaria* (**Figure 2**). Below I briefly recap each chapter's findings. I then discuss some fruitful future research directions.

Tropical Floral Buffet

Stingless bees are found in some of the most florally diverse ecosystems on earth. It is estimated that around two-thirds of all flowering plant species are found in the tropics (Pimm and Joppa, 2015), meaning that a broad menu of food options is available to tropical insect pollinators. In **Chapter 2**, I provide strong support for stingless bees as generalist flower visitors. Based on published reports of 287 species of stingless bees across all the tropics and subtropics of the world (South America, Central America, Africa, Asia and Oceania), stingless bees visit thousands of plant species belonging to 1435 genera grouped in 215 families. Given that there are 405 families of angiosperms in the world, stingless bees therefore visit 62% of all of them (The Plant List, 2010).

Figure 1. View of the Inside of the *T. carbonaria* OATH (Original Australian Tetragonula Hive). The advancing front with its characteristic spiral pattern and a few opened cells on the edge ready for the queen to lay eggs. Photo by Théotime Colin.

Figure 2. One of the most common management practices is the splitting every 1-2 years of the whole hive. The OATH hives comprise a box divided into two half-boxes which can be split to produce two hvies. We used this practice for **Chapters 3** and **6** to obtain queenless halves with transparent lids on top to confirm virgin queens. Photo by Théotime Colin.

Based on the number of genera visited, the families that provide the most important resources to stingless bees are Fabaceae, Asteraceae, Rubiaceae, Malvaceae, Euphorbiaceae, Arecaceae, Lamiaceae, Poaceae, Bignoniaceae, Myrtaceae, Sapindaceae, Apocynaceae and Melastomataceae. All of these families are highly diverse and pantropical. **Chapter 2** also showed that stingless bees visit many introduced plant species, not just crops. The families with the highest number of non-native genera visited by stingless bees were Asteraceae, Lamiaceae and Fabaceae. The foraging generalism of stingless bees therefore may have both benefits (they are able to pollinate crops) and costs (they are liable to spread some weedy invasive species).

Male stingless bees as "sperm with wings"

In **Chapter 3** I focused on the males of *T. carbonaria*. I confirmed that males do not return to their natal nests once they have left, and that colonies produce 5-30% male brood throughout the year. I also estimated the distance travelled by males to find a conspecific mate, using molecular calculations of sibship probability and a mathematical model of dispersal. Most of the males of *T. carbonaria* join mating aggregations within 1km of the natal nest, reaching an average between 800 m and 2 km and an indirect maximum of 20 km. This finding is broadly consistent with previous estimates for stingless bee male dispersal of <2km, but reveals that maximum dispersal is likely much greater than than previously believed.

Why would males fly this far? One likely reason is inbreeding avoidance. Inbreeding has an especially high cost in social hymenopteran insects, because homozygosity at their sex-determining loci leads to infertile or inviable diploid offspring. Stingless bees females tend to disperse only short-distances from natal nests because daughter colonies need to be close to the parent nests that provision them during the early phases of colony budding. Thus male stingless bees are most responsible for ensuring outbreeding (dos Santos et al., 2016c).

In **Chapter 3** I also showed that the genetic diversity of male aggregations can be used to estimate the colony density of *T. carbonaria.* This approach to estimating colony density is modelled on that already used for honey bees, where it has proved useful for monitoring changes in honey bee population sizes over time (Arundel et al., 2012, Arundel et al., 2013, Arundel et al., 2014).

Long live the queen!

Social insects are characterized by a reproductive division of labour between queens and workers, with the queen laying all fertilized eggs. However, in most social insect species, the workers are only facultative sterile and remain capable of laying unfertilized male-destined eggs (Ronai et al., 2016). Moreover, the production of queens is often limited and in most species, only one laying queen is found inside of the colony (Kerr, 1969, Nogueira Neto, 1997, Prato and Soares, 2013). This makes the queens elusive and hard to study, resulting in a knowledge gap about the life and reproductive behaviour of queens in most stingless bee species. The questions I addressed in **Chapters 4**, **5** and **6** were all related to queens, or the effect on the colony of not having a mature queen in the nest.

Chapter 4 provided the first experimental test of whether worker sterility was reversible in *T. carbonaria*, a species in which workers are not known to ever lay eggs. I investigated under controlled experimental conditions whether the fertility of the workers could be rescued by manipulating their nutritional status and social environment (by removing the queen). I found that *T. carbonaria* worker ovaries did not change under these experimental treatments, supporting the hypothesis that *T. carbonaria* adult workers are irreversibly sterile. Unlike the vast majority of social insects, *T. carbonaria* workers therefore cannot lay male eggs and salvage some fitness, even when the queen dies and cannot be replaced, and the colony is doomed. Instead, it seems that *T. carbonaria* invest in forms of insurance against queenlessness, such as producing queen cells in small numbers all year round (Heard, 2016), and creating large numbers of emergency queen cells if the queen dies, by modifying the size of workers' cells (Nunes et al., 2015).

In **Chapter 5** I investigated the behaviour of young queens and attempted to mate them in captivity. Mating under controlled conditions is readily done for honey bees (Koeniger, 1976, Laidlaw, 1987, Nolan, 1932, Watson, 1928) and bumbles bees (Djegham et al., 1994, N Tasei et al., 1998, Röseler, 1985, Jiandong et al., 2001, Frison, 1927, Baer and Schmid-Hempel, 2000) but so far, this technique is not well developed in any stingless bee. In honey bees, the development of queen production techniques (grafting) facilitated efficient breeding and genetic improvement programs (Büchler et al., 2013, Laidlaw and Eckert, 1962, Lodesani and Costa, 2003). In my study (**Chapter 5**) I showed that it is possible to successfully rear *T. carbonaria* virgin queens from pupal stage (where pupae are harvested from colonies) to a

reproductive age in confinement (10 days post-eclosion), the first such success for any stingless bees species in the Indo-Malay-Australasian clade. Most importantly, I show that we can stimulate the virgin queens of *T. carbonaria* to activate their ovaries and lay unfertilized eggs in captivity under semi-controlled conditions (**Figure 3**). Why queen ovaries are activated without any successful sperm transference is an open question in both stingless bees and honey bees. It could indicate that ovary activation is not directly linked to a successful mating but to a mechanical stimulation, such as the attachment of a mating plug (Melo et al., 2001). This is the first study in stingless bees to show that the ovaries of queens may be activated without sperm in the spermatheca. Further work is needed to develop controlled mating protocols for *T. carbonaria* f and confirm that queens mated in captivity will produce fertilized female eggs.

In **Chapters 4** and **5** I also showed that we can create "microcolonies" of *T. carbonaria* (**Figure 4**), in which we can recreate functional colonies with and without a queen. These microcolonies allowed us to closely observe the behaviour of each of the bees inside but most of all to control their environmental conditions such as temperature, queenlessness and food.

In **Chapter 6** I described for the first time some of the volatiles linked to the mating behaviour of *T. carbonaria*. I described the compounds detectable within queenright and queenless colonies shortly after a hive split, and also the volatiles produced by different castes (males, workers and virgin queens), focusing on those volatiles that make a virgin queen distinctive.

The chemical ecology of stingless bees has not been fully addressed yet (von Zuben, 2017), especially for the stingless bees outside the Neotropics. **Chapter 6** identified two strong candidates for pheromones linked to the sexual communication of *T. carbonaria* queens: 2-Heptanol, (2Z)-2-(3,3-Dimethylcyclohexylidene) ethanol and 2-Ethyl-2-propyl-1-hexanol. 2-Heptanol is known to be produced by queens in some other stingless bee species. In contrast, 2-Ethyl-2-propyl-1-hexanol has only been previously described in other insects (butterflies and aggregation pheromone for male beetles) (Rodríguez et al., 2016, Mujiono et al., 2015).

Finally, I found a high overall incidence of nest usurpation in hived *T. carbonaria*, and a possible trend towards higher rates of foreign queen takeovers in colonies that had recently been split and experienced a period of queenlessness. I also confirmed dead foreign queens rejected outside entrances of intraspecific, non-related nests. These behaviours require further

Figure 3 The queens used in **Chapter 5**. This queen was marked in green and her development was closely followed from being a hatchling, mating her in captivity and becoming and reaching a physogastric condition. Photo by Théotime Colin.

Figure 4 One of the outcomes of this thesis was the creation of miniature but fully functional colonies of *T. carbonaria* with less than 1000 workers, compared to the average full-size of 10,000 workers. The pictures showed one of the microcolony set ups for used in **Chapter 5**. The hand is pointing at the *involucrum* which cover the brood and a laying queen inside. Photo by Théotime Colin.

study, but are consistent with evidence from South American stingless bees suggesting that nest or queen parasitism are common (Wenseleers et al., 2011).

Directions for future research

I propose four directions for future research that arise directly from the work in this thesis.

Floral preferences by stingless bees

What flower traits attract stingless bees? Do they have innate preferences for particular plant families or flower traits, or do they simply visit flowers of common and readily available species? **Chapter 2** documented floral visitation by stingless bees, but the extent to which they actively prefer particular plants remains an open question. Apart from flower morphology, some other things that can play a role in flower preferences and selection are the protein content of pollen (Austin and Gilbert, 2018), the concentration of flowers on the. plant (Kleinert et al., 2009, Miranda et al., 2015), the diversity of flowering plant species in the area (Austin et al., 2019), flower syndrome and time of foraging activity (Hubbell and Johnson, 1978). Understanding floral preferences of stingless bees will help to inform which crops they are likely to visit and pollinate most effectively, and also help us conserve native plant communities that best support wild bee populations. Additionally, the database from **Chapter 2** should be a useful reference for future studies on the pollination biology of plant species or genera that are visited by stingless bees, including invasive plants.

Estimating colony densities of wild stingless bee populations

In **Chapter 3**, we saw that developing a protocol for colony density estimation based on the genetic diversity of male aggregations in stingless bees is challenging, but possible. Stingless bee reproductive biology varies in key ways to that of honey bees. What makes the protocol easier in honey bees is that males return each day to their mother colony. Contrastingly, in stingless bees, males never come back to their nest of origin (Vollet-Neto et al., 2018). Therefore, any protocol for colony density estimation in stingless bees must account for the fact that some males fly very long distances before joining mating aggregations, and that the average dispersal distance depends greatly on the dispersal function. It is clear though that this approach can nevertheless provide useful density estimates for many types of questions. In *T.*

carbonaria, additional fine-tuning will improve the protocol; e.g. determining how long males live for once they leave natal nests and whether male dispersal varies with different habitat types. It will then be possible to estimate colony densities in different regions of Australia and begin to track changes in population density over time and space. For example, this technique could be of use for assessing the effect of fires on stingless bee colony densities in National Parks, by sampling before and after these events. Applying the protocol to other stingless bee species will require first estimating male dispersal distances, which are likely to be species-specific and vary with body size.

Artificial selection via controlled queen rearing and mating

In **Chapters 4** and **5** I reveal that it is possible to rear *T. carbonaria* queens to mating age in microcolonies and mate them under semi-controlled conditions. Further development in this area could lead to simple techniques for producing artificial crosses in *T. carbonaria*. It is possible that *T. carbonaria* would even be suitable for an artificial insemination technique, such as widely-used in honey bees (Gillard and Oldroyd, 2020) and some stingless bee species (Da Silva et al., 1972), and the development of queen *in vitro* production (Baptistella et al., 2014, Menezes et al., 2013, dos Santos et al., 2016a). If queens can be reared and mated in captivity, and induced to produce colonies, then the door is open to controlling the genetics of commercial stingless bee populations, as already occurs with honey bees. Managed populations of stingless bees could be artificially selected to favour biological traits that are best suited to crop pollination (e.g. low aggressiveness towards conspecifics, etc). Many novel research questions could also be readily addressed; for example, testing for the effects of hybridization between closely related species (i.e. *T. carbonaria* and *T. hockingsi*) or different conspecific populations, and experimentally manipulating queens to have multiple mates, to test the effects of polyandry on acceptance by workers.

Chemical ecology of stingless bee reproduction

In **Chapter 6** I provide a first insight into the chemical communication and pheromones produced by *T. carbonaria* castes and colonies. This investigation is largely descriptive and I recommend following up this study by combining identification of volatiles with behavioural experiments that aim to tease apart the effects of volatiles in different contexts. For example, which volatiles of virgin queens are produced at the peak of attraction to males? Or what are

the pheromones related to the long-range attraction of males? One way to address these questions would be by sampling queen cells as in **Chapter 5**, raising them in captivity so that their age is known, and then assaying them in petri dishes with males to test their attraction at different ages. Additionally, virgin queen attractiveness could be tested by presenting queens to natural mating swarms and evaluating how many males are attracted to them. Future studies could also investigate whether workers play a role in dispersing volatiles outside the nest that help to attract males. For example, a recent study in Brazil found that queenless colonies of stingless bees attracted less males if the workers were prevented from leaving the nest than if the workers were allowed to fly free (von Zuben, 2017). The importance of general nest odours in attracting males could be tested by assessing whether boxes containing resin and food stores (but no bees) attract males. I have observed *T. carbonaria* males on The University of Sydney campus aggregating at old boxes, suggesting that nest odours play a role.

Concluding remarks

This thesis contributes to a wider ongoing research endeavour in Australia and the world to advance knowledge of stingless bees. In Australia, there has been a notable uptick in awareness of stingless bees in recent decades, both by researchers and by the general public. They have become popular recreational pets across much of Eastern Australia and they are increasingly used and researched in agriculture. With this new market comes both opportunities and risks associated with the exploitation of natural nests and the movement of hives around the country. Now is the time to invest in research of their biology, so that a stingless bee industry in Australia is developed in the most sustainable way possible, for the benefit of both people and the environment.

Like most wild species, stingless bees are susceptible to habitat degradation, human disturbance and deforestation. Because they pollinate many plant species, declines in stingless bee populations may also affect broader ecosystem health. Effective management and conservation of these bees relies on a better understanding of their reproductive biology, population genetics and natural distributions. In particular, efforts to encourage their use in crop pollination must go hand-in-hand with efforts to protect wild populations. For example, educational workshops in local communities can explain the importance of avoiding nest extraction from the wild and the movement of species outside of their natural ranges wherever possible. Ideally, the demand for colonies for crop pollination is achieved via effective

breeding programs of already hived populations, or via free pollination services from wild colonies in natural forest areas surrounding crops. In this way, the commercial use of stingless bees should not negatively impact natural populations, and instead will aid the conservation of these remarkable creatures.

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References

A.B.E.L.H.A. 2017. *Sistema de informação sobre interações abelhas-plantas no Brasil.* [Online]. Available: http://abelhaseplantas.cria.org.br/ [Accessed 4 Nov 2020].

ABSY, M. L., CAMARGO, J. M., KERR, W. E. & MIRANDA, I. P. D. A. 1984. Espécies de plantas visitadas por Meliponinae (Hymenoptera; Apoidea), para coleta de pólen na região do médio Amazonas. *Ver. Brasil. Biol*., *44*, *227-237*.

ADAMS, W. 1992. Gene dispersal within forest tree populations. *New forests,* 6**,** 217-240.

AIDOO, K., KWAPONG, R. C. & KARIKARI, I. A. 2011. Stingless bees in Ghana. *Bees for Development Journal,* 100**,** 10-11.

AIZEN, M. A. & HARDER, L. D. 2009. The global stock of domesticated honey bees is growing slower than agricultural demand for pollination. *Current biology,* 19**,** 915-918.

AIZEN, M. A., SABATINO, M. & TYLIANAKIS, J. M. 2012. Specialization and rarity predict nonrandom loss of interactions from mutualist networks. *Science,* 335**,** 1486-1489.

ALAVEZ-ROSAS, D., SÁNCHEZ-GUILLÉN, D., MALO, E. A. & CRUZ-LÓPEZ, L. 2019. (S)-2-Heptanol, the alarm pheromone of the stingless bee *Melipona solani* (Hymenoptera, Meliponini). *Apidologie,* 50**,** 277-287.

ALEIXO, K. P., DE FARIA, L. B., GARÓFALO, C. A., FONSECA, V. L. I. & DA SILVA, C. I. 2013. Pollen collected and foraging activities of *Frieseomelitta varia* (Lepeletier)(Hymenoptera: Apidae) in an urban landscape. *Sociobiology,* 60**,** 266-276.

ALJARAH, A. K. & HAMEED, I. H. 2018. In vitro anti-diabetic properties of Methanolic extract of *Thymus vulgaris* using α-glucosidase and α-amylase inhibition assay and determination of its bioactive chemical compounds. *Indian Journal of Public Health Research & Development,* 9**,** 388-392.

ALTAYE, S. Z., PIRK, C. W., CREWE, R. M. & NICOLSON, S. W. 2010. Convergence of carbohydrate-biased intake targets in caged worker honeybees fed different protein sources. *Journal of Experimental Biology,* 213**,** 3311-3318.

ALVES, D. A., MENEZES, C., IMPERATRIZ-FONSECA, V. L. & WENSELEERS, T. 2011. First discovery of a rare polygyne colony in the stingless bee *Melipona quadrifasciata* (Apidae, Meliponini). *Apidologie,* 42**,** 211-213.

ALVES, D. D. A., IMPERATRIZ-FONSECA, V. L., FRANCOY, T. M., SANTOS-FILHO, P. D. S., NOGUEIRA-NETO, P., BILLEN, J. & WENSELEERS, T. 2009. The queen is dead—long live the workers: intraspecific parasitism by workers in the stingless bee *Melipona scutellaris*. *Molecular Ecology,* 18**,** 4102-4111.

AMANO, K., NEMOTO, T. & HEARD, T. A. 2000. What are stingless bees, and why and how to use them as crop pollinators?-a review. *Japan Agricultural Research Quarterly,* 34**,** 183-190.

AMBROGI, B. G., CORTÉS, A. M. P. & ZARBIN, P. H. 2012. Identification of male-produced aggregation pheromone of the curculionid beetle *Sternechus subsignatus*. *Journal of chemical ecology,* 38**,** 272-277.

AMRI, J., BADAOUI, K. & HALOUI, Z. 2017. The chemical composition and the antimicrobial properties of the essential oil extracted from the leaves of *Teucrium capitatum* L. *Asian Journal of Pharmaceutical and Clinical Research,* 10**,** 112-115.

ANDERSON, M. J. 2001. A new method for non-parametric multivariate analysis of variance. *Austral ecology,* 26**,** 32-46.

ANTONELLI, A., ZIZKA, A., SILVESTRO, D., SCHARN, R., CASCALES-MIÑANA, B. & BACON, C. D. 2015. An engine for global plant diversity: highest evolutionary turnover and emigration in the American tropics. *Frontiers in Genetics,* 6**,** 130.

ANTONINI, Y., COSTA, R. & MARTINS, R. 2006. Floral preferences of a neotropical stingless bee, *Melipona quadrifasciata* Lepeletier (Apidae: Meliponina) in an urban forest fragment. *Brazilian Journal of Biology,* 66**,** 463-471.

ARAÚJO, E., COSTA, M., CHAUD-NETTO, J. & FOWLER, H. G. 2004. Body size and flight distance in stingless bees (Hymenoptera: Meliponini): inference of flight range and possible ecological implications. *Brazilian Journal of Biology,* 64**,** 563-568.

ARTHUR, J. K. 1894. *Kangaroo and Kauri: sketches and anecdotes of Australia and New Zealand*, Sampson Low, Marston.

ARUNDEL, J., OLDROYD, B. P. & WINTER, S. 2012. Modelling honey bee queen mating as a measure of feral colony density. *Ecological modelling,* 247**,** 48-57.

ARUNDEL, J., OLDROYD, B. P. & WINTER, S. 2013. Modelling estimates of honey bee (*Apis* spp.) colony density from drones. *Ecological modelling,* 267**,** 1-10.

ARUNDEL, J., OXLEY, P. R., FAIZ, A., CRAWFORD, J., WINTER, S. & OLDROYD, B. P. 2014. Remarkable uniformity in the densities of feral honey bee *Apis mellifera* Linnaeus, 1758 (Hymenoptera: Apidae) colonies in South Eastern Australia. *Austral Entomology,* 53**,** 328-336.

ARUNPRASANNA, V., NAGARAJAN KAYALVIZHI, S., ANBALAGAN, N. R., KANNAN, M. & KRISHNAN, M. 2016. A feeding trait study in head space of Silkworm *Bombyx mori* (Lepidoptera: Bombycidae) by GC-MS analysis. *Journal of Entomology and Zoology Studiesg,* 5**,** 476-479.

AUSTIN, A. J. & GILBERT, J. D. 2018. The geometry of dependence: solitary bee larvae prioritize carbohydrate over protein in parentally provided pollen. *bioRxiv***,** 397802.

AUSTIN, M. W., HORACK, P. & DUNLAP, A. S. 2019. Choice in a floral marketplace: the role of complexity in bumble bee decision-making. *Behavioral Ecology,* 30**,** 500-508.

AYASSE, M., PAXTON, R. J. & TENGÖ, J. 2001. Mating behavior and chemical communication in the order Hymenoptera. *Annual review of entomology,* 46**,** 31-78.

BAER, B. & SCHMID-HEMPEL, P. 2000. The artificial insemination of bumblebee queens. *Insectes Sociaux,* 47**,** 183-187.

BAFO, W. 2019. Meliponiculture and physicochemical properties of honey produced by the African stingless bee *Plebeina hildebrandti* Friese in Kalakamati village, Botswana. *Botswana Journal of Agriculture and Applied Sciences,* 13**,** 33-42.

BALTUSSEN, E., SANDRA, P., DAVID, F. & CRAMERS, C. 1999. Stir bar sorptive extraction (SBSE), a novel extraction technique for aqueous samples: theory and principles. *Journal of Microcolumn Separations,* 11**,** 737-747.

BAPTISTELLA, A. R., SOUZA, C. C., SANTANA, W. C. & SOARES, A. E. E. 2014. Techniques for the in vitro production of queens in stingless bees (Apidae, Meliponini). *Sociobiology,* 59**,** 297-310.

BARTH, M. B., MORITZ, R. F. A., PIRK, C. W. W. & KRAUS, F. B. 2013. Male-biased dispersal promotes large scale gene flow in a subterranean army ant, *Dorylus* (Typhlopone) *fulvus*. *Population ecology,* 55**,** 523-533.

BAUDIER, K. M., OSTWALD, M. M., GRÜTER, C., SEGERS, F. H., ROUBIK, D. W., PAVLIC, T. P., PRATT, S. C. & FEWELL, J. H. 2019. Changing of the guard: mixed specialization and flexibility in nest defense (*Tetragonisca angustula*). *Behavioral Ecology,* 30**,** 1041-1049.

BAUDRY, E., SOLIGNAC, M., GARNERY, L., GRIES, M., CORNUET, J. & KOENIGER, N. 1998. Relatedness among honeybees (*Apis mellifera*) of a drone congregation. *Proceedings of the Royal Society of London B: Biological Sciences,* 265**,** 2009-2014.

BEEKMAN, M. & RATNIEKS, F. L. 2003. Power over reproduction in social Hymenoptera. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences,* 358**,** 1741-1753.

BEIG, D., BUENO, O. C. & MULLER, T. 1985. Caracteristicas dos alveolos de cria e postura de operarias em *Melipona quadrifasciata anthidioides* Lep.(Hym., Apidae, Meliponinae). *Naturalia (São José do Rio Preto),* 10**,** 75-81.

BENDA, N. D., BOUCIAS, D., TORTO, B. & TEAL, P. 2008. Detection and characterization of *Kodamaea ohmeri* associated with small hive beetle *Aethina tumida* infesting honey bee hives. *Journal of Apicultural Research,* 47**,** 194-201.

BENGTSSON, B. 1978. Avoiding inbreeding: at what cost? *Journal of Theoretical Biology,* 73**,** 439-444.

BENJAMINI, Y. & HOCHBERG, Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal statistical society: series B (Methodological),* 57**,** 289-300.

BERENBAUM, M., BERNHARDT, P., BUCHMANN, S., CALDERONE, N., GOLDSTEIN, P., INOUYE, D., KEVAN, P., KREMEN, C., MEDELLIN, R. & RICKETTS, T. 2007. Status of pollinators in North America. *Washington, DC: The National Academies Press,* 668569904**,** 9780309102896.

BIESMEIJER, J. C., ROBERTS, S. P., REEMER, M., OHLEMÜLLER, R., EDWARDS, M., PEETERS, T., SCHAFFERS, A., POTTS, S. G., KLEUKERS, R. & THOMAS, C. 2006. Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. *Science,* 313**,** 351-354.

BOLELI, I. C., PAULINO-SIMÕES, Z. L. & BITONDI, M. M. G. 2000. Regression of the lateral oviducts during the larval-adult transformation of the reproductive system of *Melipona quadrifasciata* and *Frieseomelitta varia*. *Journal of morphology,* 243**,** 141-151.

BOLELI, I. C., PAULINO-SIMÕES, Z. L. & GENTILE BITONDI, M. M. 1999. Cell death in ovarioles causes permanent sterility in *Frieseomelitta varia* worker bees. *Journal of morphology,* 242**,** 271-282.

BOM 2020. Bureau of Meteorology.

BORGES, R. C., PADOVANI, K., IMPERATRIZ-FONSECA, V. L. & GIANNINI, T. C. 2020. a dataset of multi-functional ecological traits of Brazilian bees. *Scientific Data,* 7**,** 1-9.

BORRELL, B. J. 2005. Long Tongues and Loose Niches: Evolution of Euglossine Bees and Their Nectar Flowers 1. *Biotropica: The Journal of Biology and Conservation,* 37**,** 664-669.

BOSCH, J. & KEMP, W. 2002. Developing and establishing bee species as crop pollinators: the example of *Osmia* spp.(Hymenoptera: Megachilidae) and fruit trees. *Bulletin of entomological research,* 92**,** 3-16.

BOURKE, A. F. 1988. Worker reproduction in the higher eusocial Hymenoptera. *The Quarterly Review of Biology,* 63**,** 291-311.

BRADSHAW, C. J., SODHI, N. S. & BROOK, B. W. 2009. Tropical turmoil: a biodiversity tragedy in progress. *Frontiers in Ecology and the Environment,* 7**,** 79-87.

BRAMLEY, G. & UTTERIDGE, T. M. 2014. *The Kew tropical plant families identification handbook,* Kew Publishing.

BREEZE, T. D., BAILEY, A. P., BALCOMBE, K. G. & POTTS, S. G. 2011. Pollination services in the UK: How important are honeybees? *Agriculture, Ecosystems & Environment,* 142**,** 137-143.

BRETAGNOLLE, V. & GABA, S. 2015. Weeds for bees? A review. *Agronomy for Sustainable Development,* 35**,** 891-909.

BRITO, R., FRANCISCO, F., DOMINGUES-YAMADA, A., GONÇALVES, P., PIOKER, F., SOARES, A. & ARIAS, M. 2009. Characterization of microsatellite loci of *Tetragonisca angustula* (Hymenoptera, Apidae, Meliponini). *Conservation Genetics Resources,* 1**,** 183.

BROCKMANN, A., DIETZ, D., SPAETHE, J. & TAUTZ, J. 2006. Beyond 9-ODA: sex pheromone communication in the European honey bee *Apis mellifera* L. *Journal of chemical ecology,* 32**,** 657-667.

BROWN, J. C. & DE OLIVEIRA, M. L. 2014. The impact of agricultural colonization and deforestation on stingless bee (Apidae: Meliponini) composition and richness in Rondônia, Brazil. *Apidologie,* 45**,** 172-188.

BÜCHLER, R., ANDONOV, S., BIENEFELD, K., COSTA, C., HATJINA, F., KEZIC, N., KRYGER, P., SPIVAK, M., UZUNOV, A. & WILDE, J. 2013. Standard methods for rearing and selection of *Apis mellifera* queens. *Journal of Apicultural Research,* 52**,** 1-30.

BUCHMANN, S. L. & NABHAN, G. P. 2012. *The forgotten pollinators*, Island Press.

BÜNING, J. 1994. *The insect ovary: ultrastructure, previtellogenic growth and evolution*, Springer Science & Business Media.

BUTZ, V. M. & DIETZ, A. 1994. The mechanism of queen elimination in two-queen honey bee (*Apis mellifera* L.) colonies. *Journal of Apicultural Research,* 33**,** 87-94.

CABI. 2020. *Invasive Species Compendium. Wallingford, UK: CAB International.* www.cabi.org/isc. [Online]. [Accessed 3 Dec 2020].

CAMARGO, J. M. & PEDRO, S. R. 2008. Revision of the species of *Melipona* of the fuliginosa group (Hymenoptera, Apoidea, Apidae, Meliponini). *Revista Brasileira de Entomologia,* 52**,** 411-427.

CAMERON, E., FRANCK, P. & OLDROYD, B. 2004. Genetic structure of nest aggregations and drone congregations of the southeast Asian stingless bee *Trigona collina*. *Molecular Ecology,* 13**,** 2357-2364.

CAMPBELL, A. J., CARVALHEIRO, L. G., GASTAUER, M., ALMEIDA-NETO, M. & GIANNINI, T. C. 2019. Pollinator restoration in Brazilian ecosystems relies on a small but phylogenetically-diverse set of plant families. *Scientific reports,* 9, 17383.

CAMPBELL, A. J., CARVALHEIRO, L. G., MAUÉS, M. M., JAFFÉ, R., GIANNINI, T. C., FREITAS, M. A. B., COELHO, B. W. T. & MENEZES, C. 2018. Anthropogenic disturbance of tropical forests threatens pollination services to açaí palm in the Amazon river delta. *Journal of Applied Ecology,* 55**,** 1725-1736.

CANE, J. H. 2008. A native ground-nesting bee (*Nomia melanderi*) sustainably managed to pollinate alfalfa across an intensively agricultural landscape. *Apidologie,* 39**,** 315-323.

CAPES. 2016. *Catálogo de Teses e Dissertações* [Online]. Available: http://catalogodeteses.capes.gov.br/catalogo-teses/ - !/ [Accessed 4 Nov 2020].

CARDINAL, S. & DANFORTH, B. N. 2011. The antiquity and evolutionary history of social behavior in bees. *PLOS one,* 6, e21086.

CARDOSO-JÚNIOR, C. A. M., OLDROYD, B. P. & RONAI, I. 2020. Vitellogenin expression in the ovaries of adult honeybee workers provides insights into the evolution of reproductive and social traits. *bioRxiv***,** 547760.

CARVALHO-ZILSE, G. A. & KERR, W. E. 2004. Natural substitutions of queens and flight distance of males in tiuba (*Melipona compressipes fasciculata* Smith, 1854) and uruçu (*Melipona scutellaris* Latreille, 1811) (Apidae, Meliponini). *Acta Amazonica,* 34**,** 649-652.

CHÂLINE, N., SANDOZ, J.-C., MARTIN, S. J., RATNIEKS, F. L. & JONES, G. R. 2005. Learning and discrimination of individual cuticular hydrocarbons by honeybees (*Apis mellifera*). *Chemical Senses,* 30**,** 327-335.

CHAMBERLAIN, S. A. & SZÖCS, E. 2013. taxize: taxonomic search and retrieval in R. *F1000Research,* 2.

CHAPMAN, N. C., BYATT, M., COCENZA, R. D. S., NGUYEN, L. M., HEARD, T. A., LATTY, T. & OLDROYD, B. P. 2018. Anthropogenic hive movements are changing the genetic structure of a stingless bee (*Tetragonula carbonaria*) population along the east coast of Australia. *Conservation Genetics,* 19**,** 619-627.

CHRISTENHUSZ, M. J. & BYNG, J. W. 2016. The number of known plants species in the world and its annual increase. *Phytotaxa,* 261**,** 201-217.

CHUTTONG, B., CHANBANG, Y. & BURGETT, M. 2014. Meliponiculture: Stingless bee beekeeping in Thailand. *Bee world,* 91**,** 41-45.

CHYTRÝ, M., MASKELL, L. C., PINO, J., PYŠEK, P., VILÀ, M., FONT, X. & SMART, S. M. 2008. Habitat invasions by alien plants: a quantitative comparison among Mediterranean, subcontinental and oceanic regions of Europe. *Journal of Applied Ecology,* 45**,** 448-458.

CITADINI-ZANETTE, V., NEGRELLE, R. R., LEAL-FILHO, L. S., REMOR, R., ELIAS, G. A. & SANTOS, R. 2017. *Mimosa scabrella* Benth.(Fabaceae) enhances the restoration in coal mining areas in the Atlantic rainforest. *Cerne,* 23**,** 103-114.

COLLINS, A. M. & BLUM, M. S. 1983. Alarm responses caused by newly identified compounds derived from the honeybee sting. *Journal of Chemical Ecology,* 9**,** 57-65.

COMPTON, S. G. 2002. Sailing with the wind: dispersal by small flying insects. *Dispersal ecology***,** Cambridge University Press.

CONTEL, E. & KERR, W. 1976. Origin of males in *Melipona subnitida* estimated from data of an isozymic polymorphic system. *Genetica,* 46**,** 271-277.

CONTRERA, F. A. L., MENEZES, C. & VENTURIERI, G. C. 2011. New horizons on stingless beekeeping (apidae, Meliponini). *Embrapa Amazônia Oriental-Artigo em periódico indexado (ALICE)*.

COOK, J. M. & CROZIER, R. H. 1995. Sex determination and population biology in the Hymenoptera. *Trends in Ecology & Evolution,* 10**,** 281-286.

CORONA, M., LIBBRECHT, R. & WHEELER, D. E. 2016. Molecular mechanisms of phenotypic plasticity in social insects. *Current opinion in insect science,* 13**,** 55-60.

CORTOPASSI-LAURINO, M., IMPERATRIZ-FONSECA, V. L., ROUBIK, D. W., DOLLIN, A., HEARD, T., AGUILAR, I., VENTURIERI, G. C., EARDLEY, C. & NOGUEIRA-NETO, P. 2006. Global meliponiculture: challenges and opportunities. *Apidologie,* 37**,** 275-292.

CORTOPASSI-LAURINO, M. & RAMALHO, M. 1988. Pollen harvest by Africanized *Apis mellifera* and *Trigona spinipes* in São Paulo botanical and ecological views. *Apidologie,* 19**,** 1-24.

COSTA, L. & VENTURIERI, G. C. 2009. Diet impacts on Melipona flavolineata workers (Apidae, Meliponini). *Journal of apicultural research,* 48**,** 38-45.

CRANE, E. 1992. The past and present status of beekeeping with stingless bees. *Bee world,* 73**,** 29-42.

CRUZ-LANDIM, C. D. 2000. Ovarian development in *Meliponine bees* (Hymenoptera: Apidae): the effect of queen presence and food on worker ovary development and egg production. *Genetics and Molecular Biology,* 23**,** 83-88.

CUNNINGHAM, J. P., HEREWARD, J. P., HEARD, T. A., DE BARRO, P. J. & WEST, S. A. 2014. Bees at war: interspecific battles and nest usurpation in stingless bees. *The American Naturalist,* 184**,** 777-786.

CUNNINGHAM, S. A. & LE FEUVRE, D. 2013. Significant yield benefits from honeybee pollination of faba bean (*Vicia faba*) assessed at field scale. *Field Crops Research,* 149**,** 269-275.

DA LUZ, C. F. P., FIDALGO, A. D. O., SILVA, S. A. Y., RODRIGUES, S. D. S. & NOCELLI, R. C. F. 2019. Comparative floral preferences in nectar and pollen foraging by *Scaptotrigona postica* (Latreille 1807) in two different biomes in São Paulo (Brazil). *Grana,* 58**,** 200-226.

DA SILVA, C. I., RADAESKI, J. N., ARENA, M. V. N. & BAUERMANN, S. G. 2020. Atlas of pollen and plants used by bees, CISE.

DA SILVA, D. L. N., ZUCCHI, R. & KERR, W. E. 1972. Biological and behavioural aspects of the reproduction in some species of *Melipona* (Hymenoptera, Apidae, Meliponinae). *Animal Behaviour,* 20**,** 123-132.

DAPPORTO, L., FONDELLI, L. & TURILLAZZI, S. 2006. Nestmate recognition and identification of cuticular hydrocarbons composition in the swarm founding paper wasp *Ropalidia opifex*. *Biochemical systematics and ecology,* 34**,** 617-625.

DE CAMARGO, C. A. 1972. Mating of the social bee Melipona quadrifasciata under controlled conditions (Hymenoptera, Apidae). *Journal of the Kansas Entomological Society***,** 45, 520-523.

DE CARVALHO, R. M. A., MARTINS, C. F. & DA SILVA MOURÃO, J. 2014. Meliponiculture in Quilombola communities of Ipiranga and Gurugi, Paraíba state, Brazil: an ethnoecological approach. *Journal of ethnobiology and ethnomedicine,* 10**,** 3.

DE SOUZA, E. A., TRIGO, J. R., SANTOS, D. E., VIEIRA, C. U. & SERRÃO, J. E. 2017. The relationship between queen execution and cuticular hydrocarbons in stingless bee *Melipona scutellaris* (Hymenoptera: Meliponini). *Chemoecology,* 27**,** 25-32.

DIECKMANN, U., O'HARA, B. & WEISSER, W. 1999. The evolutionary ecology of dispersal. *Trends in Ecology & Evolution,* 14**,** 88-90.

DING, S., AN, K., ZHAO, C., LI, Y., GUO, Y. H. & WANG, Z. 2012. Effect of drying methods on volatiles of Chinese ginger (*Zingiber officinale* Roscoe). *Food and Bioproducts Processing,* 90**,** 515-524.

DJEGHAM, Y., VERHAEGHE, J. & RASMONT, P. 1994. Copulation of *Bombus terrestris* L.(Hymenoptera: Apidae) in captivity. *Journal of apicultural research,* 33**,** 15-20.

DOBSON, F. S. 1982. Competition for mates and predominant juvenile male dispersal in mammals. *Animal behaviour,* 30**,** 1183-1192.

DOLLIN, A. E. & DOLLIN, L. J. 1997. Australian stingless bees of the genus *Trigona* (Hymenoptera: Apidae). *Invertebrate Systematics,* 11**,** 861-896.

DOS SANTOS, C., FERREIRA-CALIMAN, M. & NASCIMENTO, F. 2015. An alien in the group: eusocial male bees sharing nonspecific reproductive aggregations. *Journal of Insect Science,* 15, 157.

DOS SANTOS, C. F., DOS SANTOS, P. D. D. S. & BLOCHTEIN, B. 2016a. In vitro rearing of stingless bee queens and their acceptance rate into colonies. *Apidologie,* 47**,** 539-547.

DOS SANTOS, C. F., FRANCISCO, F. D. O., IMPERATRIZ-FONSECA, V. L. & ARIAS, M. C. 2016b. Eusocial bee male aggregations: spatially and temporally separated but genetically homogenous. *Entomologia Experimentalis et Applicata,* 158**,** 320-326.

DOS SANTOS, C. F., IMPERATRIZ-FONSECA, V. L. & ARIAS, M. C. 2016c. Relatedness and dispersal distance of eusocial bee males on mating swarms. *Entomological Science,* 19**,** 245-254.

DOS SANTOS, C. F., MENEZES, C., VOLLET-NETO, A. & IMPERATRIZ-FONSECA, V. L. 2014. Congregation sites and sleeping roost of male stingless bees (Hymenoptera: Apidae: Meliponini). *Sociobiology,* 61**,** 115-118.

DOUGHTY, R. W. 2000. *The Eucalyptus: a natural and commercial history of the gum tree*, Johns Hopkins University Press.

DOUMS, C., CABRERA, H. & PEETERS, C. 2002. Population genetic structure and male-biased dispersal in the queenless ant *Diacamma cyaneiventre*. *Molecular Ecology,* 11**,** 2251-2264.

DYER, A. G., WHITNEY, H. M., ARNOLD, S. E., GLOVER, B. J. & CHITTKA, L. 2006. Bees associate warmth with floral colour. *Nature,* 442**,** 525-525.

EARDLEY, C. & URBAN, R. 2010. Catalogue of Afrotropical bees (Hymenoptera: Apoidea: Apiformes). *Zootaxa,* 2455**,** 1-548.

EBIE, J. D., HÖLLDOBLER, B. & LIEBIG, J. 2015. Larval regulation of worker reproduction in the polydomous ant *Novomessor cockerelli*. *The Science of Nature,* 102**,** 72.

ENGELS, E. & ENGELS, W. 1988. Age-dependent queen attractiveness for drones and mating in the stingless bee, *Scaptotrigona postica*. *Journal of Apicultural Research,* 27**,** 3-8.

ENGELS, E., ENGELS, W., LÜBKE, G., SCHRÖDER, W. & FRANCKE, W. 1993. Age-related patterns of volatile cephalic constituents in queens of the neotropical stingless bee *Scaptotrigona postica* Latr (Hymenoptera, Apidae). *Apidologie,* 24**,** 539-548.

ENGELS, W. 1987. Pheromones and reproduction in Brazilian stingless bees. *Mem. Inst. Oswaldo Cruz,* 82**,** 35-4.

ENGELS, W., ELISABETH, E. & FRANCKE, W. 1997. Ontogeny of cephalic volatile patterns in queens and mating biology of the neotropical stingless bee, *Scaptotrigona postica*. *Invertebrate Reproduction & Development,* 31**,** 251-256.

ENGELS, W., ENGELS, E., LÜBKE, G., SCHRÖDER, W. & FRANCKE, W. 1990. Volatile cephalic secretions of drones, queens and workers in relation to reproduction in the stingless bee, *Scaptotrigona postica* (Hymenoptera: Apidae: Trigonini). *Entomologia generalis,* 15**,** 91-101.

ENGELS, W. & IMPERATRIZ-FONSECA, V. L. 1990. Caste development, reproductive strategies, and control of fertility in honey bees and stingless bees. *In:* ENGELS, W. (ed.) *Social Insects: An Evolutionary Approach to Castes and Reproduction.* . Berlin/Heidelberg: Springer-Verlag.

ESTRADA, A., MEIRELES, C., MORALES-CASTILLA, I., POSCHLOD, P., VIEITES, D., ARAÚJO, M. B. & EARLY, R. 2015. Species' intrinsic traits inform their range limitations and vulnerability under environmental change. *Global Ecology and Biogeography,* 24**,** 849-858.

FARIA, L. B. D., ALEIXO, K. P., GARÓFALO, C. A., IMPERATRIZ-FONSECA, V. L. & SILVA, C. I. D. 2012. Foraging of *Scaptotrigona* aff. *depilis* (Hymenoptera, Apidae) in an urbanized area: Seasonality in resource availability and visited plants. *Psyche,* 2012.

FAYEMIWO, K. A., ADELEKE, M. A., OKORO, O. P., AWOJIDE, S. H. & AWONIYI, I. O. 2014. Larvicidal efficacies and chemical composition of essential oils of *Pinus sylvestris* and *Syzygium aromaticum* against mosquitoes. *Asian Pacific journal of tropical biomedicine,* 4**,** 30-34.

FENERON, R. & BILLEN, J. 1996. Ovarian cycle in *Ectatomma tuberculatum* workers (Formicidae, Ponerinae). *Invertebrate reproduction & development,* 29**,** 79-85.

FIERRO, M. M., CRUZ-LÓPEZ, L., SÁNCHEZ, D., VILLANUEVA-GUTIÉRREZ, R. & VANDAME, R. 2011. Queen volatiles as a modulator of *Tetragonisca angustula* drone behavior. *Journal of chemical ecology,* 37**,** 1255-1262.

FIJN, N. 2014. Sugarbag Dreaming: the significance of bees to Yolngu in Arnhem Land, Australia. *HU MaN IMALIA,* 6**,** 1-21.

FONTAINE, C., DAJOZ, I., MERIGUET, J. & LOREAU, M. 2005. Functional diversity of plant–pollinator interaction webs enhances the persistence of plant communities. *PLoS biology,* 4**,** e1.

FRANCISCO, F. D. O., SANTIAGO, L. R. & ARIAS, M. C. 2013. Molecular genetic diversity in populations of the stingless bee *Plebeia remota*: A case study. *Genetics and molecular biology,* 36**,** 118-123.

FRANCISCO, F. D. O., SANTIAGO, L. R., BRITO, R. M., OLDROYD, B. P. & ARIAS, M. C. 2014. Hybridization and asymmetric introgression between *Tetragonisca angustula* and *Tetragonisca fiebrigi*. *Apidologie,* 45**,** 1-9.

FREE, J., FERGUSON, A., SIMPKINS, J. R. & AL-SA'AD, B. 1983. Effect of honeybee Nasonov and alarm pheromone components on behaviour at the nest entrance. *Journal of Apicultural Research,* 22**,** 214-223.

FRISON, T. H. 1927. The fertilization and hibernation of queen bumblebees under controlled conditions.(Bremidae: Hym.). *Journal of Economic Entomology,* 20**,** 522-527.

GARCIA BULLE BUENO, F., GLOAG, R., LATTY, T. & RONAI, I. 2020. Irreversible sterility of workers and high-volume egg production by queens in the stingless bee *Tetragonula carbonaria*. *The Journal of Experimental Biology,* 223**,** jeb230599.

GARCIA, C., JORDANO, P. & GODOY, J. A. 2007. Contemporary pollen and seed dispersal in a *Prunus mahaleb* population: patterns in distance and direction. *Molecular Ecology,* 16**,** 1947-1955.

GARCIA, M. V. B., DE OLIVEIRA, M. L. & CAMPOS, L. D. O. 1992. Use of seeds of *Coussapoa asperifolia magnifolia* (Cecropiaceae) by stingless bees in the Central Amazonian forest (Hymenoptera: Apidae: Meliponinae). *Embrapa Amazônia Ocidental-Artigo em periódico indexado (ALICE)*.

GARIBALDI, L. A., AIZEN, M. A., KLEIN, A. M., CUNNINGHAM, S. A. & HARDER, L. D. 2011. Global growth and stability of agricultural yield decrease with pollinator dependence. *Proceedings of the National Academy of Sciences,* 108**,** 5909-5914.

GARIBALDI, L. A., STEFFAN-DEWENTER, I., WINFREE, R., AIZEN, M. A., BOMMARCO, R., CUNNINGHAM, S. A., KREMEN, C., CARVALHEIRO, L. G., HARDER, L. D. & AFIK, O. 2013. Wild pollinators enhance fruit set of crops regardless of honey bee abundance. *science,* 339**,** 1608-1611.

GEETHALAKSHMI, R. & SARADA, D. 2013. Evaluation of antimicrobial and antioxidant activity of essential oil of *Trianthema decandra* L. *journal of pharmacy research,* 6**,** 101-106.

GENTRY, A. H. 1980. Bignoniaceae: part I (Crescentieae and Tourrettieae). *Flora Neotropica,* 25**,** 1-130.

GENTRY, A. H. 1992. Bignoniaceae: part II (tribe Tecomeae). *Flora Neotropica***,** 1-370.

GERULA, D., PANASIUK, B., WĘGRZYNOWICZ, P. & BIEŃKOWSKA, M. 2012. Instrumental insemination of honey bee queens during flight activity predisposition period 2. Number of spermatozoa in spermatheca. *Journal of Apicultural Science,* 56**,** 159-167.

GIANNINI, T., BOFF, S., CORDEIRO, G., CARTOLANO, E., VEIGA, A., IMPERATRIZ-FONSECA, V. & SARAIVA, A. 2015. Crop pollinators in Brazil: a review of reported interactions. *Apidologie,* 46**,** 209-223.

GIANNINI, T. C., ALVES, D. A., ALVES, R., CORDEIRO, G. D., CAMPBELL, A. J., AWADE, M., BENTO, J. M. S., SARAIVA, A. M. & IMPERATRIZ-FONSECA, V. L. 2020. Unveiling the contribution of bee pollinators to Brazilian crops with implications for bee management. *Apidologie***,** 51, 406-421.

GILL, R. A. The value of honeybee pollination to society. VI International Symposium on Pollination 288, 1990. 62-68.

GILL, R. J., BALDOCK, K. C., BROWN, M. J., CRESSWELL, J. E., DICKS, L. V., FOUNTAIN, M. T., GARRATT, M. P., GOUGH, L. A., HEARD, M. S. & HOLLAND, J. M. 2016. Protecting an ecosystem service: approaches to understanding

and mitigating threats to wild insect pollinators. *Advances in Ecological Research.* Elsevier.
GILLARD, T. L. & OLDROYD, B. P. 2020. Controlled reproduction in the honey bee (*Apis mellifera*) via artificial insemination. *Advances in Insect Physiolgy,* 59, 1-42.
GILLEY, D. C. 2001. The behavior of honey bees (*Apis mellifera ligustica*) during queen duels. *Ethology,* 107**,** 601-622.
GLOAG, R., BEEKMAN, M., HEARD, T. & OLDROYD, B. 2007. No worker reproduction in the Australian stingless bee *Trigona carbonaria* Smith (Hymenoptera, Apidae). *Insectes Sociaux,* 54**,** 412-417.
GLOAG, R., HEARD, T., BEEKMAN, M. & OLDROYD, B. 2008. Nest defence in a stingless bee: What causes fighting swarms in *Trigona carbonaria* (Hymenoptera, Meliponini)? *Insectes sociaux,* 55**,** 387-391.
GOBIN, B., ITO, F., BILLEN, J. & PEETERS, C. 2008. Degeneration of sperm reservoir and the loss of mating ability in worker ants. *Naturwissenschaften,* 95**,** 1041-1048.
GOBIN, B., ITO, F., PEETERS, C. & BILLEN, J. 2006. Queen-worker differences in spermatheca reservoir of phylogenetically basal ants. *Cell and tissue research,* 326**,** 169-178.
GOBIN, B., PEETERS, C. & BILLEN, J. 1998. Production of trophic eggs by virgin workers in the ponerine ant *Gnamptogenys menadensis*. *Physiological entomology,* 23**,** 329-336.
GOMPPER, M. E., GITTLEMAN, J. L. & WAYNE, R. K. 1998. Dispersal, philopatry, and genetic relatedness in a social carnivore: comparing males and females. *Molecular Ecology,* 7**,** 157-163.
GOTOH, A., ITO, F. & BILLEN, J. 2013. Vestigial spermatheca morphology in honeybee workers, *Apis cerana* and *Apis mellifera,* from Japan. *Apidologie,* 44**,** 133-143.
GOULSON, D. 2003. Conserving wild bees for crop pollination. *Journal of Food Agriculture and Environment,* 1**,** 142-144.
GRAJALES-CONESA, J., ROJAS, J. C., GUZMÁN-DÍAZ, M., RINCÓN-RABANALES, M. & CRUZ-LÓPEZ, L. 2007. Cephalic and Dufour gland secretions of *Scaptotrigona mexicana* queens: Chemical composition and biological activity. *Apidologie,* 38**,** 38-46.
GRECO, M. K., SPOONER-HART, R. N., BEATTIE, A. G., BARCHIA, I. & HOLFORD, P. 2011. Australian stingless bees improve greenhouse *Capsicum* production. *Journal of Apicultural Research,* 50**,** 102-115.
GREEN, C. & OLDROYD, B. 2002. Queen mating frequency and maternity of males in the stingless bee *Trigona carbonaria* Smith. *Insectes Sociaux,* 49**,** 196-202.
GREEN, C. L., FRANCK, P. & OLDROYD, B. P. 2001. Characterization of microsatellite loci for *Trigona carbonaria*, a stingless bee endemic to Australia. *Molecular Ecology Resources,* 1**,** 89-92.
GREENLEAF, S. S., WILLIAMS, N. M., WINFREE, R. & KREMEN, C. 2007. Bee foraging ranges and their relationship to body size. *Oecologia,* 153**,** 589-596.
GREENWOOD, P. J. 1980. Mating systems, philopatry and dispersal in birds and mammals. *Animal behaviour,* 28**,** 1140-1162.
GROSSO, A. F., BEGO, L. R. & MARTINEZ, A. S. 2000. The production of males in queenright colonies of *Tetragonisca angustula angustula* (Hymenoptera, Meliponinae). *Sociobiology,* 35**,** 475-485.
GRÜTER, C. 2020. Stingless Bees: An Overview. *Stingless Bees***,** 1-42.
GRÜTER, C., MENEZES, C., IMPERATRIZ-FONSECA, V. L. & RATNIEKS, F. L. 2012. A morphologically specialized soldier caste improves colony defense in a neotropical eusocial bee. *Proceedings of the National Academy of Sciences,* 109**,** 1182-1186.

GRÜTER, C., VON ZUBEN, L., SEGERS, F. & CUNNINGHAM, J. 2016. Warfare in stingless bees. *Insectes sociaux,* 63**,** 223-236.
GU, Z., GU, L., EILS, R., SCHLESNER, M. & BRORS, B. 2014. circlize implements and enhances circular visualization in R. *Bioinformatics,* 30**,** 2811-2812.
GUIBU, L., RAMALHO, M., KLEINERT-GIOVANNINI, A. & IMPERATRIZ-FONSECA, V. 1988. Exploração dos recursos florais por colônias de *Melipona quadrifasciata* (Apidae, Meliponinae). *Revista Brasileira de Biologia,* 48**,** 299-305.
HALCROFT, M., SPOONER-HART, R. & DOLLIN, L. A. 2013a. Australian stingless bees. *Pot-Honey.* Springer.
HALCROFT, M. T., SPOONER-HART, R., HAIGH, A. M., HEARD, T. A. & DOLLIN, A. 2013b. The Australian stingless bee industry: a follow-up survey, one decade on. *Journal of Apicultural Research,* 52**,** 1-7.
HAMMEL, B., VOLLET-NETO, A., MENEZES, C., NASCIMENTO, F. S., ENGELS, W. & GRÜTER, C. 2016. Soldiers in a stingless bee: work rate and task repertoire suggest they are an elite force. *The American Naturalist,* 187**,** 120-129.
HAMMOND, R. L. & KELLER, L. 2004. Conflict over male parentage in social insects. *PLoS biology,* 2.
HARDY, O. J., PEARCY, M. & ARON, S. 2008. Small-scale spatial genetic structure in an ant species with sex-biased dispersal. *Biological Journal of the Linnean Society,* 93**,** 465-473.
HARTFELDER, K., MAKERT, G. R., JUDICE, C. C., PEREIRA, G. A., SANTANA, W. C., DALLACQUA, R. & BITONDI, M. M. 2006. Physiological and genetic mechanisms underlying caste development, reproduction and division of labor in stingless bees. *Apidologie,* 37**,** 144-163.
HAWKINS, B. A., RODRÍGUEZ, M. Á. & WELLER, S. G. 2011. Global angiosperm family richness revisited: linking ecology and evolution to climate. *Journal of Biogeography,* 38**,** 1253-1266.
HEARD, T. A. 1988. Propagation of hives of *Trigona carbonaria* SMITH (Hymenoptera: Apidae). *Australian Journal of Entomology,* 27**,** 303-304.
HEARD, T. A. 1999. The role of stingless bees in crop pollination. *Annual review of entomology,* 44**,** 183-206.
HEARD, T. A. 2016. *The Australian Native Bee Book: keeping stingless bee hives for pets, pollination and sugarbag honey*, Sugarbag Bees.
HEARD, T. A. 2020. *Sugarbag Bees* [Online]. Available: https://sugarbag.net/about [Accessed 10 Dec 2020].
HEARD, T. A. & DOLLIN, A. E. 2000. Stingless bee keeping in Australia: snapshot of an infant industry. *Bee World,* 81**,** 116-125.
HEIMPEL, G. E. & DE BOER, J. G. 2008. Sex determination in the Hymenoptera. *Annu. Rev. Entomol.,* 53**,** 209-230.
HEINRICH, B. 2004. *Bumblebee economics*, Harvard University Press.
HEPBURN, H. R. & RADLOFF, S. E. 2011. *Honeybees of Asia*, Springer Science & Business Media.
HERBERS, J. M. 1984. Queen-worker conflict and eusocial evolution in a polygynous ant species. *Evolution***,** 631-643.
HILGERT-MOREIRA, S. B., FERNANDES, M. Z., MARCHETT, C. A. & BLOCHTEIN, B. 2014. Do different landscapes influence the response of native and non-native bee species in the *Eucalyptus* pollen foraging, in southern Brazil? *Forest Ecology and Management,* 313**,** 153-160.

HINSON, E. M., DUNCAN, M., LIM, J., ARUNDEL, J. & OLDROYD, B. P. 2015. The density of feral honey bee (*Apis mellifera*) colonies in South East Australia is greater in undisturbed than in disturbed habitats. *Apidologie,* 46**,** 403-413.

HODIN, J. 2009. She shapes events as they come: plasticity in female insect reproduction. *In:* WHITMAN, D. W. & ANANTHAKRISHNAN, T. N. (eds.) *Phenotypic plasticity of insects: mechanisms and consequences.* U.S.A.: Science publishers.

HODIN, J. & RIDDIFORD, L. M. 2000. Different mechanisms underlie phenotypic plasticity and interspecific variation for a reproductive character in drosophilids (Insecta: Diptera). *Evolution,* 54**,** 1638-1653.

HÖLLDOBLER, B. 1976. Tournaments and slavery in a desert ant. *Science,* 192**,** 912-914.

HÖLLDOBLER, B. & WILSON, E. O. 1990. *The ants*, Harvard University Press.

HOLM, L. G., PLUCKNETT, D. L., PANCHO, J. V. & HERBERGER, J. P. 1977. *The world's worst weeds. Distribution and biology,* University Press of Hawaii.

HOLZER, B., KELLER, L. & CHAPUISAT, M. 2009. Genetic clusters and sex-biased gene flow in a unicolonial Formica ant. *BMC Evolutionary Biology,* 9**,** 69.

HOLZSCHUH, A., DORMANN, C. F., TSCHARNTKE, T. & STEFFAN-DEWENTER, I. 2011. Expansion of mass-flowering crops leads to transient pollinator dilution and reduced wild plant pollination. *Proceedings of the Royal Society B: Biological Sciences,* 278**,** 3444-3451.

HOSSAIN, M. A., SIDDIQUE, A., RAHMAN, S. M. & HOSSAIN, M. 2010. Chemical composition of the essential oils of *Stevia rebaudiana* Bertoni leaves. *Asian J. Tradit. Med,* 5**,** 56-61.

HOUSTON, T. 2018. *A guide to native bees of Australia*, CSIRO PUBLISHING.

HOWARD, R. W. & BLOMQUIST, G. J. 2005. Ecological, behavioral, and biochemical aspects of insect hydrocarbons. *Annual review of entomology,* 50, 371-393.

HOWE, S., DIMICK, P. & BENTON, A. 1985. Composition of freshly harvested and commercial royal jelly. *Journal of Apicultural Research,* 24**,** 52-61.

HRNCIR, M., JARAU, S. & BARTH, F. G. 2016. Stingless bees (Meliponini): senses and behavior. Springer.

HUBBELL, S. P. & JOHNSON, L. K. 1978. Comparative foraging behavior of six stingless bee species exploiting a standardized resource. *Ecology,* 59**,** 1123-1136.

HURYN, V. M. B. 1997. Ecological impacts of introduced honey bees. *The quarterly review of biology,* 72**,** 275-297.

IMPERATRIZ-FONSECA, V., MATOS, E., FERREIRA, F. & VELTHUIS, H. 1998. A case of multiple mating in stingless bees (Meliponinae). *Insectes sociaux,* 45**,** 231-233.

IMPERATRIZ-FONSECA, V. & ZUCCHI, R. 1995. Virgin queens in stingless bee (Apidae, Meliponinae) colonies: a review. *Apidologie,* 26**,** 231-244.

IMPERATRIZ-FONSECA, V. L. 1977. Studies on *Paratrigona subnuda* (Moure)(Hymenoptera, Apidae, Meliponinae)—II. Behaviour of the virgin queen. *Boletim de Zoologia, Universidade de São Paulo,* 2**,** 169-182.

INOUE, T., SAKAGAMI, S. F., SALMAH, S. & YAMANE, S. 1984. The process of colony multiplication in the Sumatran stingless bee *Trigona* (*Tetragonula*) *laeviceps*. *Biotropica***,** 16, 100-111.

INOUYE, D. W. 1978. Resource partitioning in bumblebees: experimental studies of foraging behavior. *Ecology,* 59**,** 672-678.

ISH-AM, G., BARRIENTOS-PRIEGO, F., CASTAÑEDA-VILDOZOLA, A. & GAZIT, S. 1999. Avocado (*Persea americana* Mill.) pollinators in its region of origin. *Revista Chapingo Serie Horticultura,* 5**,** 137-143.

J. S. MOURE & A. DAL MOLIN. 2012. *Calliopsini Robertson, 1922. In Moure, J. S., Urban, D. & Melo, G. A. R. (Orgs). Catalogue of Bees (Hymenoptera, Apoidea) in the*

Neotropical Region - online version. Available at http://www.moure.cria.org.br/catalogue. [Online]. [Accessed 3 Dec 2020].
JACKSON, J. T., TARPY, D. R. & FAHRBACH, S. E. 2011. Histological estimates of ovariole number in honey bee queens, *Apis mellifera*, reveal lack of correlation with other queen quality measures. *Journal of Insect Science,* 11**,** 82.
JAFARI, N. K. & SANI, A. M. 2016. Chemical composition and antibacterial activity of essential oil from *Melissa officinalis* leaves. *Res J Agric & Biol Sci,* 11**,** 367-372.
JAFFÉ, R., CASTILLA, A., POPE, N., IMPERATRIZ-FONSECA, V. L., METZGER, J. P., ARIAS, M. C. & JHA, S. 2016a. Landscape genetics of a tropical rescue pollinator. *Conservation Genetics,* 17**,** 267-278.
JAFFE, R., DIETEMANN, V., ALLSOPP, M. H., COSTA, C., CREWE, R. M., DALL'OLIO, R., DE LA RÚA, P., EL-NIWEIRI, M. A., FRIES, I. & KEZIC, N. 2010. Estimating the density of honeybee colonies across their natural range to fill the gap in pollinator decline censuses. *Conservation biology,* 24**,** 583-593.
JAFFÉ, R., POPE, N., ACOSTA, A. L., ALVES, D. A., ARIAS, M. C., DE LA RÚA, P., FRANCISCO, F. O., GIANNINI, T. C., GONZÁLEZ-CHAVES, A. & IMPERATRIZ-FONSECA, V. L. 2016b. Beekeeping practices and geographic distance, not land use, drive gene flow across tropical bees. *Molecular Ecology,* 25**,** 5345-5358.
JAFFÉ, R., POPE, N., CARVALHO, A. T., MAIA, U. M., BLOCHTEIN, B., DE CARVALHO, C. A. L., CARVALHO-ZILSE, G. A., FREITAS, B. M., MENEZES, C. & DE FÁTIMA RIBEIRO, M. 2015. Bees for development: Brazilian survey reveals how to optimize stingless beekeeping. *PLoS One,* 10, e0121157.
JAKOBSEN, H., KRISTJANSSON, K., ROHDE, B., TERKILDSEN, M. & OLSEN, C. 1995. Can social bees be influenced to choose a specific feeding station by adding the scent of the station to the hive air? *Journal of chemical ecology,* 21**,** 1635-1648.
JARAU, S., VAN VEEN, J. W., AGUILAR, I. & AYASSE, M. 2009. Virgin queen execution in the stingless bee *Melipona beecheii*: the sign stimulus for worker attacks. *Apidologie,* 40**,** 496-507.
JARAU, S., VAN VEEN, J. W., TWELE, R., REICHLE, C., GONZALES, E. H., AGUILAR, I., FRANCKE, W. & AYASSE, M. 2010. Workers make the queens in *Melipona* bees: identification of geraniol as a caste determining compound from labial glands of nurse bees. *Journal of chemical ecology,* 36**,** 565-569.
JAY, S. 1968. Factors influencing ovary development of worker honeybees under natural conditions. *Canadian journal of zoology,* 46**,** 345-347.
JIANDONG, A., WENGJUN, P., SHIKUI, L. & JIE, W. 2001. The character of Bumble bees (*Bombus* spp.) for pollination and its breeding in capitivity [J]. *Apiculture of China,* 3.
JOHNSON, L., HAYNES, L., CARLSON, M., FORTNUM, H. & GORGAS, D. 1985. Alarm substances of the stingless bee, *Trigona silvestriana*. *Journal of chemical ecology,* 11**,** 409-416.
JOHNSON, L. K. & HUBBELL, S. P. 1974. Aggression and competition among stingless bees: field studies. *Ecology,* 55**,** 120-127.
JOHNSON, L. K. & HUBBELL, S. P. 1975. Contrasting foraging strategies and coexistence of two bee species on a single resource. *Ecology,* 56**,** 1398-1406.
JOHNSTONE, R. A., CANT, M. A. & FIELD, J. 2012. Sex-biased dispersal, haplodiploidy and the evolution of helping in social insects. *Proceedings of the Royal Society B: Biological Sciences,* 279**,** 787-793.
JONES, R. 2013. Stingless bees: a historical perspective. *Pot-Honey.* Springer.
JUNGNICKEL, H., DA COSTA, A., TENTSCHERT, J., PATRICIO, E. F. L., IMPERATRIZ-FONSECA, V., DRIJFHOUT, F. & MORGAN, E. 2004. Chemical basis for inter-

colonial aggression in the stingless bee *Scaptotrigona bipunctata* (Hymenoptera: Apidae). *Journal of insect physiology,* 50**,** 761-766.
KAIIRA, M. G., CHEMINING'WA, G. N., AYUKE, F., BAGUMA, Y. & NGANGA, F. 2019. Profiles of compounds in root exudates of rice, *Cymbopogon, Desmodium, Mucuna* and maize. *Journal of Agricultural Sciences, Belgrade,* 64**,** 399-412.
KAMAKURA, M. 2011. Royalactin induces queen differentiation in honeybees. *Nature,* 473**,** 478-483.
KARASINSKI, J. 2017. The Economic Valuation of Australian Managed and Wild Honeybee Pollinators. *Presentation presented at* at Agricultural Lecture Theatre, G013 North Wing, Agricultural Building University Western Australia Institute of Agriculture.
KAYASHIMA, Y., YAMANASHI, K., SATO, A., KUMAZAWA, S. & YAMAKAWA-KOBAYASHI, K. 2012. Freeze-dried royal jelly maintains its developmental and physiological bioactivity in Drosophila melanogaster. *Bioscience, biotechnology, and biochemistry,* 76, 2107-2111.
KEARNS, C. A. & INOUYE, D. W. 1997. Pollinators, flowering plants, and conservation biology. *Bioscience,* 47**,** 297-307.
KELLERMANN, V., OVERGAARD, J., HOFFMANN, A. A., FLØJGAARD, C., SVENNING, J.-C. & LOESCHCKE, V. 2012. Upper thermal limits of *Drosophila* are linked to species distributions and strongly constrained phylogenetically. *Proceedings of the National Academy of Sciences,* 109**,** 16228-16233.
KEMP, W. & BOSCH, J. 2000. Development and emergence of the alfalfa pollinator *Megachile rotundata* (Hymenoptera: Megachilidae). *Annals of the Entomological Society of America,* 93**,** 904-911.
KENDALL, L. K., GAGIC, V., EVANS, L. J., CUTTING, B. T., SCALZO, J., HANUSCH, Y., JONES, J., ROCCHETTI, M., SONTER, C. & KEIR, M. 2020. Self-compatible blueberry cultivars require fewer floral visits to maximize fruit production than a partially self-incompatible cultivar. *Journal of Applied Ecology, 57, 2454-2462*.
KENNEDY, C. M., LONSDORF, E., NEEL, M. C., WILLIAMS, N. M., RICKETTS, T. H., WINFREE, R., BOMMARCO, R., BRITTAIN, C., BURLEY, A. L. & CARIVEAU, D. 2013. A global quantitative synthesis of local and landscape effects on wild bee pollinators in agroecosystems. *Ecology letters,* 16**,** 584-599.
KERR, W. E. 1950. Genetic determination of castes in the genus Melipona. *Genetics,* 35**,** 143.
KERR, W. E. 1969. *Some aspects of the evolution of social bees (Apidae),* Appleton-Century-Crofts.
KERR, W. E. & MAULE, V. 1964. Geographic distribution of stingless bees and its implications (Hymenoptera: Apidae). *Journal of the New York Entomological Society,* 72, 2-18.
KERR, W. E., ZUCCHI, R., NAKADAIRA, J. T. & BUTOLO, J. E. 1962. Reproduction in the social bees (Hymenoptera: Apidae). *Journal of the New York Entomological Society,* 70, 265-276.
KEVAN, P. & BAKER, H. 1983. Insects as flower visitors and pollinators. *Annual review of entomology,* 28**,** 407-453.
KHILA, A. & ABOUHEIF, E. 2010. Evaluating the role of reproductive constraints in ant social evolution. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 365**,** 617-630.
KIMSEY, L. S. 1980. The behaviour of male orchid bees (Apidae, Hymenoptera, Insecta) and the question of leks. *Animal Behaviour,* 28**,** 996-1004.
KING, C., BALLANTYNE, G. & WILLMER, P. G. 2013. Why flower visitation is a poor proxy for pollination: measuring single-visit pollen deposition, with implications for pollination networks and conservation. *Methods in Ecology and Evolution,* 4**,** 811-818.

KLEIJN, D. & RAEMAKERS, I. 2008. A retrospective analysis of pollen host plant use by stable and declining bumble bee species. *Ecology,* 89**,** 1811-1823.
KLEIN, A.-M., VAISSIERE, B. E., CANE, J. H., STEFFAN-DEWENTER, I., CUNNINGHAM, S. A., KREMEN, C. & TSCHARNTKE, T. 2006. Importance of pollinators in changing landscapes for world crops. *Proceedings of the royal society B: biological sciences,* 274**,** 303-313.
KLEIN, A.-M., VAISSIERE, B. E., CANE, J. H., STEFFAN-DEWENTER, I., CUNNINGHAM, S. A., KREMEN, C. & TSCHARNTKE, T. 2007. Importance of pollinators in changing landscapes for world crops. *Proceedings of the Royal Society of London B: Biological Sciences,* 274**,** 303-313.
KLEINERT, A. 2005. Colony strength and queen replacement in Melipona marginata (Apidae: Meliponini). *Brazilian Journal of Biology,* 65**,** 469-476.
KLEINERT, A. M., RAMALHO, M., CORTOPASSI-LAURINO, M., RIBEIRO, M. D. F. & IMPERATRIZ-FONSECA, V. L. 2009. Abelhas sociais (Bombini, Apini, Meliponini). *Embrapa Semiárido-Capítulo em livro técnico (INFOTECA-E).*
KLEINERT, A. M., RAMALHO, M., CORTOPASSI-LAURINO, M., RIBEIRO, M. F. & IMPERATRIZ-FONSECA, V. L. 2012. Social bees (Bombini, Apini, Meliponini). *Insect Bioecology and Nutrition for Integrated Pest Management***,** CRC press**,** 237-271.
KLUSER, S., NEUMANN, P., CHAUZAT, M.-P., PETTIS, J. S., PEDUZZI, P., WITT, R., FERNANDEZ, N. & THEURI, M. 2010. Global honey bee colony disorders and other threats to insect pollinators. United Nations Environment Program: Emerging Issues Report. ONON, Nairobi, Kenya.
KNOWLTON, N. & JACKSON, J. B. 1993. Inbreeding and Outbreeding in. *The natural history of inbreeding and outbreeding: theoretical and empirical perspectives***,** University of Chicago Press.
KOEDAM, D. 1995. *Behavioural and physiological implications of queen dominance in stingless bees.* Ph.D. Thesis, Utrecht University, The Netherlands.
KOENIGER, G. 1976. Einfluss der Kopulation auf den Beginn der Eiablage bei der Bienenkönigin (Apis mellifica L.). *Apidologie,* 7**,** 343-355.
KOENIGER, N. & KOENIGER, G. 2007. Mating flight duration of Apis mellifera queens: As short as possible, as long as necessary. *Apidologie,* 38**,** 606-611.
KRAUS, F., WEINHOLD, S. & MORITZ, R. 2008. Genetic structure of drone congregations of the stingless bee *Scaptotrigona mexicana*. *Insectes Sociaux,* 55**,** 22-27.
KREMEN, C., WILLIAMS, N. M. & THORP, R. W. 2002. Crop pollination from native bees at risk from agricultural intensification. *Proceedings of the National Academy of Sciences,* 99**,** 16812-16816.
KRIEGER, G. M., DUCHATEAU, M.-J., VAN DOORN, A., IBARRA, F., FRANCKE, W. & AYASSE, M. 2006. Identification of queen sex pheromone components of the bumblebee Bombus terrestris. *Journal of chemical ecology,* 32**,** 453.
KROPÁČOVÁ, S. & HASLBACHOVÁ, H. 1969. The development of ovaries in worker honeybees in a queenright colony. *Journal of Apicultural Research,* 8**,** 57-64.
KUHN, A., BAUMAN, D., DARRAS, H. & ARON, S. 2017. Sex-biased dispersal creates spatial genetic structure in a parthenogenetic ant with a dependent-lineage reproductive system. *Heredity,* 119**,** 207-213.
KUNUGI, H. & MOHAMMED ALI, A. 2019. Royal Jelly and Its Components Promote Healthy Aging and Longevity: From Animal Models to Humans. *International journal of molecular sciences,* 20**,** 4662.
KWON, S.-J., CHOI, G.-S., YOON, J.-Y., SEO, J.-K. & CHOI, H.-S. 2016. Identification of *Leonurus sibiricus* as a weed reservoir for three pepper-infecting viruses. *The Plant Pathology Journal,* 32**,** 65.

LACEY, E. S., GINZEL, M. D., MILLAR, J. G. & HANKS, L. M. 2004. Male-produced aggregation pheromone of the cerambycid beetle Neoclytus acuminatus acuminatus. *Journal of Chemical Ecology,* 30**,** 1493-1507.

LAIDLAW, H. H. 1987. Instrumental insemination of honeybee queens: its origin and development. *Bee World,* 68**,** 17-36.

LAIDLAW, H. H. & ECKERT, J. E. 1962. *Queen rearing,* University of California Press.

LANDAVERDE-GONZÁLEZ, P., ENRÍQUEZ, E., ARIZA, M. A., MURRAY, T., PAXTON, R. J. & HUSEMANN, M. 2017. Fragmentation in the clouds? The population genetics of the native bee *Partamona bilineata* (Hymenoptera: Apidae: Meliponini) in the cloud forests of Guatemala. *Conservation Genetics,* 18**,** 631-643.

LEHMBERG, L., DWORSCHAK, K. & BLÜTHGEN, N. 2008. Defensive behavior and chemical deterrence against ants in the stingless bee genus *Trigona* (Apidae, Meliponini). *Journal of Apicultural Research,* 47**,** 17-21.

LENOIR, A., D'ETTORRE, P., ERRARD, C. & HEFETZ, A. 2001. Chemical ecology and social parasitism in ants. *Annual review of entomology,* 46**,** 573-599.

LEVINE, J. M., ADLER, P. B. & YELENIK, S. G. 2004. A meta-analysis of biotic resistance to exotic plant invasions. *Ecology letters,* 7**,** 975-989.

LEWIS, G. P. 2005. *Legumes of the World*, Royal Botanic Gardens Kew.

LICHTENBERG, E. M., HRNCIR, M., TURATTI, I. C. & NIEH, J. C. 2011. Olfactory eavesdropping between two competing stingless bee species. *Behavioral ecology and sociobiology,* 65**,** 763-774.

LICHTENBERG, E. M., ZIVIN, J. G., HRNCIR, M. & NIEH, J. C. 2014. Eavesdropping selects for conspicuous signals. *Current Biology,* 24**,** R598-R599.

LIN, H. & WINSTON, M. L. 1998. The role of nutrition and temperature in the ovarian development of the worker honey bee (*Apis mellifera*). *The Canadian Entomologist,* 130**,** 883-891.

LISBOA, L. C. O., SERRÃO, J. E., CRUZ-LANDIM, C. & CAMPOS, L. A. O. 2005. Effect of Larval Food Amount on Ovariole Development in Queens of *Trigona spinipes* (Hymenoptera, Apinae). *Anatomia, Histologia, Embryologia,* 34**,** 179-184.

LIU, J., MA, F.-M. & ZHAO, K.-J. 2009. Component analysis of volatile compounds released from soybean. *Soybean Science,* 28, 719– 722.

LODESANI, M. & COSTA, C. 2003. Bee breeding and genetics in Europe. *Bee World,* 84**,** 69-85.

LÓPEZ, J. C. G. & KRAUS, F. B. 2009. Cherchez la femme? Site choice of drone congregations in the stingless bee *Scaptotrigona mexicana*. *Animal Behaviour,* 77**,** 1247-1252.

LÓPEZ-URIBE, M. M., SORO, A. & JHA, S. 2017. Conservation genetics of bees: advances in the application of molecular tools to guide bee pollinator conservation. Springer.

LÓPEZ-URIBE, M. M., ZAMUDIO, K. R., CARDOSO, C. F. & DANFORTH, B. N. 2014. Climate, physiological tolerance and sex-biased dispersal shape genetic structure of Neotropical orchid bees. *Molecular Ecology,* 23**,** 1874-1890.

LUNA-LUCENA, D., RABICO, F. & SIMOES, Z. L. 2018. Reproductive capacity and castes in eusocial stingless bees (Hymenoptera: Apidae). *Current Opinion in Insect Science,* 31**,** 20-28.

MACHARIA, J. K., RAINA, S. K. & MULI, M. stingless beekeeping: an incentive for rain forest conservation in Kenya. Ecosystem based management: beyond boundaries. Proceedings of the Sixth International Conference of Science and the Management of Protected Areas, 2007. 21-26.

MARTINEZ ARBIZU, P. 2017. pairwiseAdonis: Pairwise multilevel comparison using adonis. *R package version 0.0,* 1.

MARTÍNEZ, R., DIAZ, B., VÁSQUEZ, L., COMPAGNONE, R. S., TILLETT, S., CANELÓN, D. J., TORRICO, F. & SUÁREZ, A. I. 2009. Chemical composition of essential oils and toxicological evaluation of Tagetes erecta and Tagetes patula from Venezuela. *Journal of Essential Oil Bearing Plants,* 12**,** 476-481.

MARTINS, G. F. & SERRÃO, J. E. 2004. A comparative study of the ovaries in some Brazilian bees (Hymenoptera; Apoidea). *Papéis Avulsos de Zoologia (São Paulo),* 44**,** 45-53.

MARVIER, M., KAREIVA, P. & NEUBERT, M. G. 2004. Habitat destruction, fragmentation, and disturbance promote invasion by habitat generalists in a multispecies metapopulation. *Risk Analysis: An International Journal,* 24**,** 869-878.

MASCIOCCHI, M., ANGELETTI, B., CORLEY, J. C. & MARTÍNEZ, A. S. 2020. Drone aggregation behavior in the social wasp *Vespula germanica* (Hymenoptera: Vespidae): Effect of kinship and density. *Scientific Reports,* 10**,** 1-7.

MASSARO, C. F., VILLA, T. F. & HAUXWELL, C. 2018. Metabolomics analysis of pot-pollen from three species of Australian stingless bees (Meliponini). In *Pot-Pollen in Stingless Bee Melittolog.* Springer.

MATEUS, S. & NOLL, F. B. 2004. Predatory behavior in a necrophagous bee *Trigona hypogea* (Hymenoptera; Apidae, Meliponini). *Naturwissenschaften,* 91**,** 94-96.

MEHDIABADI, N. J., REEVE, H. K. & MUELLER, U. G. 2003. Queens versus workers: sex-ratio conflict in eusocial Hymenoptera. *Trends in Ecology & Evolution,* 18**,** 88-93.

MELAMPY, R. & JONES, D. B. 1939. Chemical Composition and Vitamin Content of Royal Jelly. *Proceedings of the Society for Experimental Biology and Medicine,* 41**,** 382-388.

MELNICK, D. J. & HOELZER, G. A. 1992. Differences in male and female macaque dispersal lead to contrasting distributions of nuclear and mitochondrial DNA variation. *International Journal of Primatology,* 13**,** 379-393.

MELO, G. A., BUSCHINI, M. L. T. & CAMPOS, L. A. 2001. Ovarian activation in *Melipona quadrifasciata* queens triggered by mating plug stimulation (Hymenoptera, Apidae). *Apidologie,* 32**,** 355-361.

MENEZES, C., VOLLET-NETO, A. & FONSECA, V. L. I. 2013. An advance in the in vitro rearing of stingless bee queens. *Apidologie,* 44**,** 491-500.

MENZEL, R. 1985. Learning in honey bees in an ecological and behavioral context. *Fortschritte der Zoologie (Stuttgart),* 31**,** 55-74.

MICHENER, C. D. 1974. *The social behavior of the bees: a comparative study*, Harvard University Press.

MICHENER, C. D. 1979. Biogeography of the bees. *Annals of the Missouri botanical Garden***,** 277-347.

MICHENER, C. D. 2000. *The bees of the world*, JHU press.

MILLIKEN, W., KLITGÅRD, B. & BARACAT, A. EDS,. 2009. *Neotropikey - Interactive key and information resources for flowering plants of the Neotropics.* www.kew.org/neotropikey [Online]. [Accessed 3 Dec 2020].

MIRANDA, E., CARVALHO, A., ANDRADE-SILVA, A., SILVA, C. & DEL LAMA, M. 2015. Natural history and biogeography of *Partamona rustica*, an endemic bee in dry forests of Brazil. *Insectes sociaux,* 62**,** 255-263.

MISSOURI BOTANICAL GARDEN. 2020. *Tropicos.org. Missouri Botanical Garden.* <http://www.tropicos.org/> [Online]. [Accessed 3 Dec 2020].

MIYASHITA, A., KIZAKI, H., SEKIMIZU, K. & KAITO, C. 2016. Body-enlarging effect of royal jelly in a non-holometabolous insect species, Gryllus bimaculatus. *Biology open,* 5**,** 770-776.

MONNIN, T. Chemical recognition of reproductive status in social insects. *Annales Zoologici Fennici*, 43, 515-530.

MORITZ, R. F., DIETEMANN, V. & CREWE, R. 2008. Determining colony densities in wild honeybee populations (*Apis mellifera*) with linked microsatellite DNA markers. *Journal of Insect Conservation,* 12**,** 455-459.
MORSE, R. A. & CALDERONE, N. W. 2000. The value of honey bees as pollinators of US crops in 2000. *Bee culture,* 128**,** 1-15.
MOSER, J. C., BROWNLEE, R. & SILVERSTEIN, R. 1968. Alarm pheromones of the ant *Atta texana*. *Journal of Insect Physiology,* 14**,** 529-535.
MUELLER, M. Y., MORITZ, R. F. & KRAUS, F. B. 2012. Outbreeding and lack of temporal genetic structure in a drone congregation of the neotropical stingless bee *Scaptotrigona mexicana*. *Ecology and evolution,* 2**,** 1304-1311.
MUJIONO, K., WITJAKSONO, W. & PUTRA, N. S. 2015. The sex pheromone content of the *Spodoptera exigua* (Hubner) under artificial and natural diets. *International Journal of Science and Engineering,* 8**,** 146-150.
MYERS, N., MITTERMEIER, R. A., MITTERMEIER, C. G., DA FONSECA, G. A. & KENT, J. 2000. Biodiversity hotspots for conservation priorities. *Nature,* 403**,** 853-858.
N TASEI, J., MOINARD, C., MOREAU, L., HIMPENS, B. & GUYONNAUD, S. 1998. Relationship between aging, mating and sperm production in captive Bombus terrestris. *Journal of Apicultural Research,* 37**,** 107-113.
NABHAN, G. P. & BUCHMANN, S. L. 1997. Services provided by pollinators. *Nature's Services: societal dependence on natural ecosystems*, Island Press.
NAGAMITSU, T. & INOUE, T. 2005. Floral resource utilization by stingless bees (Apidae, Meliponini) In Pollination ecology and the rain forest (pp. 73-88). Springer, New York, NY.
NAGAMITSU, T., MOMOSE, K., INOUE, T. & ROUBIK, D. W. 1999. Preference in flower visits and partitioning in pollen diets of stingless bees in an Asian tropical rain forest. *Population Ecology,* 41**,** 195-202.
NAIDOO, S., CHRISTIE, N., ACOSTA, J. J., MPHAHLELE, M. M., PAYN, K. G., MYBURG, A. A. & KÜLHEIM, C. 2018. Terpenes associated with resistance against the gall wasp, *Leptocybe invasa*, in *Eucalyptus grandis*. *Plant, Cell & Environment,* 41**,** 1840-1851.
NATES PARRA, G. 2016. *Iniciativa Colombiana de Polinizadores Capítulo Abejas,* Universidad Nacional de Colombia, Bogotá, Colombia.
NAUG, D. & WENZEL, J. 2006. Constraints on foraging success due to resource ecology limit colony productivity in social insects. *Behavioral ecology and sociobiology,* 60**,** 62-68.
NICOLÈ, F., GUITTON, Y., COURTOIS, E. A., MOJA, S., LEGENDRE, L. & HOSSAERT-MCKEY, M. 2012. MSeasy: unsupervised and untargeted GC-MS data processing. *Bioinformatics,* 28**,** 2278-2280.
NOGUEIRA NETO, P. 1997. *Vida e criação de abelhas indígenas sem ferrão*, Nogueirapis.
NOGUEIRA-FERREIRA, F., SILVA-MATOS, E. & ZUCCHI, R. 2009. Interaction and behavior of virgin and physogastric queens in three Meliponini species (Hymenoptera, Apidae). *Genetics and Molecular Research,* 8**,** 703-708.
NOGUEIRA-NETO, P. 1954. Notas bionômicas sobre meliponíneos: III–Sobre a enxameagem. *Arquivos do Museu Nacional,* 42**,** 419-451.
NOGUEIRA-NETO, P. 1970. Behavior problems related to the pillages made by some parasitic stingless bees (Meliponinae, Apidae). In: Development and evolution of behavior: essays in memory of TC Schneirla. W. H. Freeman, San Francisco, 416-434.
NOLAN, W. J. 1932. *Breeding the honeybee under controlled conditions*, US Department of Agriculture.

NUNES, T. M., HEARD, T. A., VENTURIERI, G. C. & OLDROYD, B. P. 2015. Emergency queens in *Tetragonula carbonaria* (Smith, 1854)(Hymenoptera: Apidae: Meliponini). *Austral Entomology,* 54**,** 154-158.

NUNES, T. M., MATEUS, S., FAVARIS, A. P., AMARAL, M. F., VON ZUBEN, L. G., CLOSOSKI, G. C., BENTO, J. M., OLDROYD, B. P., SILVA, R. & ZUCCHI, R. 2014. Queen signals in a stingless bee: suppression of worker ovary activation and spatial distribution of active compounds. *Scientific reports,* 4**,** 7449.

ODDOU-MURATORIO, S., PETIT, R., LE GUERROUE, B., GUESNET, D. & DEMESURE, B. 2001. Pollen-versus seed-mediated gene flow in a scattered forest tree species. *Evolution,* 55**,** 1123-1135.

OI, C. A., VAN ZWEDEN, J. S., OLIVEIRA, R. C., VAN OYSTAEYEN, A., NASCIMENTO, F. S. & WENSELEERS, T. 2015. The origin and evolution of social insect queen pheromones: novel hypotheses and outstanding problems. *BioEssays,* 37**,** 808-821.

OKSANEN, J., BLANCHET, F. G., KINDT, R., LEGENDRE, P., MINCHIN, P. R., O'HARA, R., SIMPSON, G. L., SOLYMOS, P., STEVENS, M. H. H. & WAGNER, H. 2013. Package 'vegan'. *Community ecology package, version,* 2**,** 1-295.

OLDROYD, B. P. & WONGSIRI, S. 2009. *Asian honey bees: biology, conservation, and human interactions*, Harvard University Press.

OLIVEIRA, F. 2000. *Divisão de uma colônia de jupará (Melipona compressipes manaosessis) usando-se uma colmeia eo método de Fernando Oliveira*, INPA.

OLIVEIRA, R. C., OI, C. A., DO NASCIMENTO, M. M. C., VOLLET-NETO, A., ALVES, D. A., CAMPOS, M. C., NASCIMENTO, F. & WENSELEERS, T. 2015. The origin and evolution of queen and fertility signals in Corbiculate bees. *BMC evolutionary biology,* 15**,** 254.

OLLERTON, J., WINFREE, R. & TARRANT, S. 2011. How many flowering plants are pollinated by animals? *Oikos,* 120**,** 321-326.

ONO, M., TERABE, H., HORI, H. & SASAKI, M. 2003. Components of giant hornet alarm pheromone. *Nature,* 424**,** 637-638.

ORMOND, W., PINHEIRO, M. & CASTELLS, A. D. 1984. Contribuição ao estudo da reprodução e biologia floral de *Jatropha gossypifolia* L.(Euphorbiaceae). *Rev. Brasil. Biol,* 44**,** 159-167.

OSO, B., BOLIGON, A. & OLADIJI, A. 2018. Metabolomic profiling of ethanolic extracts of the fruit of *Xylopia aethiopica* (Dunal) a. rich using gas chromatography and high-performance liquid chromatography techniques. *J. Pharmacogn. Phytochem,* 7**,** 2083-2090.

OXLEY, P. R., HINHUMPATCH, P., GLOAG, R. & OLDROYD, B. P. 2010. Genetic evaluation of a novel system for controlled mating of the honeybee, *Apis mellifera*. *Journal of heredity,* 101**,** 334-338.

PAGE, R. & ERICKSON, E. 1988. Reproduction by worker honey bees (*Apis mellifera* L.). *Behavioral Ecology and Sociobiology,* 23**,** 117-126.

PARKER, F. D., BATRA, S. & TEPEDINO, V. J. 1987. New pollinators for our crops. *Agric. Zool. Rev.* 2, 279–304.

PATRICIO, E., CRUZ-LÓPEZ, L., MAILE, R., TENTSCHERT, J., JONES, G. R. & MORGAN, E. D. 2002. The propolis of stingless bees: terpenes from the tibia of three *Frieseomelitta* species. *Journal of Insect Physiology,* 48**,** 249-254.

PAXTON, R. J. 2000. Genetic structure of colonies and a male aggregation in the stingless bee *Scaptotrigona postica*, as revealed by microsatellite analysis. *Insectes Sociaux,* 47**,** 63-69.

PAXTON, R. J. 2005. Male mating behaviour and mating systems of bees: an overview. *Apidologie,* 36**,** 145-156.

PEDRO, M. F. C. S. R. M. 2013. *Meliponini Lepeletier, 1836. In Moure, J. S., Urban, D. & Melo, G. A. R. (Orgs). Catalogue of Bees (Hymenoptera, Apoidea) in the Neotropical Region - online version.* [Online]. Available: http://www.moure.cria.org.br/catalogue [Accessed 4 Dec 2020].

PEERZADA, A. M., O'DONNELL, C. & ADKINS, S. 2019. Biology, impact, and management of common sowthistle (*Sonchus oleraceus* L.). *Acta Physiologiae Plantarum,* 41**,** 136.

PETERS, J. L., BOLENDER, K. A. & PEARCE, J. M. 2012. Behavioural vs. molecular sources of conflict between nuclear and mitochondrial DNA: the role of male-biased dispersal in a Holarctic sea duck. *Molecular Ecology,* 21**,** 3562-3575.

PETERS, J. M., QUELLER, D. C., IMPERATRIZ–FONSECA, V. L., ROUBIK, D. W. & STRASSMANN, J. E. 1999. Mate number, kin selection and social conflicts in stingless bees and honeybees. *Proceedings of the Royal Society of London. Series B: Biological Sciences,* 266**,** 379-384.

PIMM, S. L. & JOPPA, L. N. 2015. How many plant species are there, where are they, and at what rate are they going extinct? *Annals of the Missouri Botanical Garden,* 100**,** 170-176.

PIRK, C. W., BOODHOO, C., HUMAN, H. & NICOLSON, S. W. 2010. The importance of protein type and protein to carbohydrate ratio for survival and ovarian activation of caged honeybees (*Apis mellifera scutellata*). *Apidologie,* 41**,** 62-72.

PLATE, M., BERNSTEIN, R., HOPPE, A. & BIENEFELD, K. 2019. The importance of controlled mating in honeybee breeding. *Genetics Selection Evolution,* 51**,** 74.

PLOWRIGHT, R. & PALLETT, M. 1979. Worker-male conflict and inbreeding in bumble bees (Hymenoptera: Apidae). *The Canadian Entomologist,* 111**,** 289-294.

POHL, S. & FOITZIK, S. 2011. Slave-making ants prefer larger, better defended host colonies. *Animal behaviour,* 81**,** 61-68.

POTTS, S. G., BIESMEIJER, J. C., KREMEN, C., NEUMANN, P., SCHWEIGER, O. & KUNIN, W. E. 2010. Global pollinator declines: trends, impacts and drivers. *Trends in ecology & evolution,* 25**,** 345-353.

POTTS, S. G., IMPERATRIZ-FONSECA, V., NGO, H., BIESMEIJER, J., BREEZE, T. D., DICKS, L., GARIBALDI, L., HILL, R., SETTELE, J. & VANBERGEN, A. 2016a. Summary for policymakers of the assessment report of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services on pollinators, pollination and food production. (No. hal-01946814).

POTTS, S. G., IMPERATRIZ-FONSECA, V., NGO, H. T., AIZEN, M. A., BIESMEIJER, J. C., BREEZE, T. D., DICKS, L. V., GARIBALDI, L. A., HILL, R. & SETTELE, J. 2016b. Safeguarding pollinators and their values to human well-being. *Nature,* 540**,** 220-229.

POWO. 2019. *Plants of the World Online. Facilitated by the Royal Botanic Gardens, Kew. Published on the Internet;* http://www.plantsoftheworldonline.org/ [Online]. [Accessed 3 Dec 2020].

PRATO, M. & SOARES, A. 2013. Production of sexuals and mating frequency in the stingless bee *Tetragonisca angustula* (Latreille)(Hymenoptera, Apidae). *Neotropical entomology,* 42**,** 474-482.

QI, M. & ARMSTRONG, D. W. 2007. Dicationic ionic liquid stationary phase for GC-MS analysis of volatile compounds in herbal plants. *Analytical and bioanalytical chemistry,* 388**,** 889-899.

QUEZADA-EUÁN, J. J. G., DE JESÚS MAY-ITZÁ, W. & GONZÁLEZ-ACERETO, J. A. 2001. Meliponiculture in México: problems and perspective for development. *Bee World,* 82**,** 160-167.

QUEZADA-EUÁN, J. J. G., NATES-PARRA, G., MAUÉS, M. M., ROUBIK, D. W. & IMPERATRIZ-FONSECA, V. L. 2018. The economic and cultural values of stingless bees (Hymenoptera: Meliponini) among ethnic groups of tropical America. *Sociobiology,* 65**,** 534-557.

R CORE TEAM 2013. R: A language and environment for statistical computing. R foundation for statistical computing Vienna, Austria. Available from: http://www.R-project.org/ [Online].

RAMALHO, M. 1990. Foraging by stingless bees of the genus, *Scaptotrigona* (Apidae, Meliponinae). *Journal of Apicultural Research,* 29**,** 61-67.

RAMALHO, M. 2004. Stingless bees and mass flowering trees in the canopy of Atlantic Forest: a tight relationship. *Acta Botanica Brasilica,* 18**,** 37-47.

RAMALHO, M., IMPERATRIZ-FONSECA, V., KLEINEKT-GIOVANNINI, A. & CORTOPASSL-LAURINO, M. 1985. Exploitation of floral resources by *Plebeia remota* Holmberg (Apidae, Meliponinae). *Apidologie,* 16**,** 307-330.

RAMALHO, M., IMPERATRIZ-FONSECA, V. & KLEINERT-GIOVANNINI, A. 1991. Ecologia nutricional de abelhas sociais. *Ecologia nutricional de insetos e suas implicações no manejo de pragas,* 4**,** 1983.

RAMALHO, M., KLEINERT-GIOVANNINI, A. & IMPERATRIZ-FONSECA, V. 1989. Utilization of floral resources by species of *Melipona* (Apidae, Meliponinae): floral preferences. *Apidologie,* 20**,** 185-195.

RAMALHO, M., KLEINERT-GIOVANNINI, A. & IMPERATRIZ-FONSECA, V. L. 1990. Important bee plants for stingless bees (*Melipona* and Trigonini) and Africanized honeybees (*Apis mellifera*) in neotropical habitats: a review. *Apidologie,* 21**,** 469-488.

RAMÍREZ, V. M., AYALA, R. & GONZÁLEZ, H. D. 2018. Crop Pollination by Stingless Bees. In *Pot-Pollen in Stingless Bee Melittolog.* Springer.

RASBAND, W. S. 1997. ImageJ. Bethesda, MD. Available from: https://imagej.nih.gov/ij/ [Online].

RASMUSSEN, C. 2008. *Catalog of the Indo-Malayan/Australasian stingless bees (Hymenoptera: Apidae: Meliponini),* Citeseer.

RASMUSSEN, C. & CAMERON, S. A. 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, S. A. 2010. Global stingless bee phylogeny supports ancient divergence, vicariance, and long distance dispersal. *Biological Journal of the Linnean Society,* 99**,** 206-232.

RIBEIRO, M. F., IMPERATRIZ-FONSECA, V. L. & SANTOS FILHO, P. S. 2003. Exceptional high queen production in the Brazilian stingless bee *Plebeia remota*. *Studies on Neotropical Fauna and Environment,* 38**,** 111-114.

RICHARDS, K. W. 1993. Non-*Apis* bees as crop pollinators. *Revue suisse de Zoologie,* 100**,** 807-822.

RILEY, R., SILVERSTEIN, R. & MOSER, J. C. 1974. Isolation, identification, synthesis and biological activity of volatile compounds from the heads of *Atta* ants. *Journal of Insect Physiology,* 20**,** 1629-1637.

RODRÍGUEZ, S. A., PÉREZ, M. L. D. P. & NAZARENO, M. A. 2016. Identification of male-produced aggregation pheromone of the curculionid beetle *Acrotomopus atropunctellus*. *Bulletin of entomological research,* 106**,** 494.

RODRÍGUEZ-GIRONÉS, M. A. & SANTAMARÍA, L. 2006. Models of optimal foraging and resource partitioning: deep corollas for long tongues. *Behavioral Ecology,* 17**,** 905-910.
RONAI, I., ALLSOPP, M. H., TAN, K., DONG, S., LIU, X., VERGOZ, V. & OLDROYD, B. P. 2017. The dynamic association between ovariole loss and sterility in adult honeybee workers. *Proc. R. Soc. B,* 284**,** 20162693.
RONAI, I., BARTON, D. A., OLDROYD, B. P. & VERGOZ, V. 2015. Regulation of oogenesis in honey bee workers via programed cell death. *Journal of Insect Physiology,* 81**,** 36-41.
RONAI, I., VERGOZ, V. & OLDROYD, B. 2016. The mechanistic, genetic, and evolutionary basis of worker sterility in the social Hymenoptera. *In:* MARC NAGUIB, J. C. M., LEIGH W. SIMMONS, LOUISE BARRETT, SUE HEALY, MARLENE ZUK (ed.) *Advances in the Study of Behavior.* Cambridge, US: Academic Press.
RONCE, O. 2007. How does it feel to be like a rolling stone? Ten questions about dispersal evolution. *Annu. Rev. Ecol. Evol. Syst.,* 38**,** 231-253.
RÖSELER, P.-F. 1985. A technique for year-round rearing of *Bombus terrestris* (Apidae, Bombini) colonies in captivity. *Apidologie,* 16**,** 165-170.
ROUBIK, D. 1992a. Stingless bees: a guide to Panamanian and Mesoamerican species and their nests (Hymenoptera: Apidae: Meliponinae). In Insects of Panamá and Mesoamerica, Oxford University Press, Oxford, UK, pp. 495–524.
ROUBIK, D., HEARD, T. & KWAPONG, P. 2018. Stingless bee colonies and pollination. *The pollination of cultivated plants: A compendium for practitioners,* 2**,** 39-64.
ROUBIK, D., SMITH, B. & CARLSON, R. 1987. Formic acid in caustic cephalic secretions of stingless bee, *Oxytrigona* (Hymenoptera: Apidae). *Journal of chemical ecology,* 13**,** 1079-1086.
ROUBIK, D. W. 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, D. W. 1983. Nest and colony characteristics of stingless bees from Panama (Hymenoptera: Apidae). *Journal of the Kansas Entomological Society***,** 56, 327-355.
ROUBIK, D. W. 1990. Mate location and mate competition in males of stingless bees (Hymenoptera: Apidae: Meliponinae). *Entomologia generalis,* 15**,** 115-120.
ROUBIK, D. W. 1992b. *Ecology and natural history of tropical bees*, Cambridge University Press.
ROUBIK, D. W. 1995. *Pollination of cultivated plants in the tropics*, Food & Agriculture Org.
ROUBIK, D. W. 2018. 100 Species of Meliponines (Apidae: Meliponini) In *Pot-Pollen in Stingless Bee Melittology*. Springer.
RSTUDIO TEAM 2015. RStudio: integrated development for R. *RStudio, Inc., Boston, MA URL* http://www/. *rstudio. com,* 42**,** 14.
RUÍZ-RAMÓN, F., ÁGUILA, D. J., EGEA-CORTINES, M. & WEISS, J. 2014. Optimization of fragrance extraction: Daytime and flower age affect scent emission in simple and double narcissi. *Industrial Crops and Products,* 52**,** 671-678.
SAHI, N. M. 2016. Evaluation of insecticidal activity of bioactive compounds from *Eucalyptus citriodora* against *Tribolium castaneum*. *International Journal of Pharmacognosy and Phytochemical Research,* 8**,** 1256-1270.
SAKAGAMI, S. 1982. Stingless bees In Hermann HR, editor.(Ed.) Social insects (Vol. III, pp. 361–423). London, UK: Academic Press.
SAKAGAMI, S. F., ROUBIK, D. W. & ZUCCHI, R. 1993. Ethology of the robber stingless bee, *Lestrimelitta limao* (Hymenoptera: Apidae). *Sociobiology*. 21, 237-277.
SAMMATARO, D., GERSON, U. & NEEDHAM, G. 2000. Parasitic mites of honey bees: life history, implications, and impact. *Annual review of entomology,* 45**,** 519-548.

SÁNCHEZ, D., VANDAME, R. & KRAUS, F. B. 2018. Genetic analysis of wild drone congregations of the stingless bee *Scaptotrigona mexicana* (Hymenoptera: Apidae) reveals a high number of colonies in a natural protected area in Southern Mexico. *Revista Mexicana de Biodiversidad*, 89**,** 226-231.

SÁNCHEZ, L. A., SLAA, E. J., SANDÍ, M. & SALAZAR, W. Use of stingless bees for commercial pollination in enclosures: a promise for the future. VIII International Symposium on Pollination-Pollination: Integrator of Crops and Native Plant Systems 561, 2000. 219-223.

SANNER, M. F. 1999. Python: a programming language for software integration and development. *J Mol Graph Model,* 17**,** 57-61.

SCHLYTER, F. & BIRGERSSON, G. 1999. Forest beetles. *Pheromones of non-lepidopteran insects associated with agricultural plants. CAB International, Wallingford, UK***,** 113-148.

SCHUH, R. T., S. HEWSON-SMITH, AND J.S. ASCHER., 2010. Specimen databases: A case study in entomology using web-based software. *American Entomologist,* 56, 206-216.

SCHWANDER, T., LO, N., BEEKMAN, M., OLDROYD, B. P. & KELLER, L. 2010. Nature versus nurture in social insect caste differentiation. *Trends in ecology & evolution,* 25**,** 275-282.

SHACKLETON, K., AL TOUFAILIA, H., BALFOUR, N. J., NASCIMENTO, F. S., ALVES, D. A. & RATNIEKS, F. L. 2015. Appetite for self-destruction: suicidal biting as a nest defense strategy in *Trigona* stingless bees. *Behavioral ecology and sociobiology,* 69**,** 273-281.

SILVA, D. D. 1972. Considerações em torno de um caso de substituição de rainha em *Plebeia* (*Plebeia*) *droryana*. *Homenagem a WE Kerr (C Cruz-Landim, ed), Ribeirão Preto, Brazil*.

SIMPSON, M. G. 2010. Diversity and classification of flowering plants: eudicots. *Plant Systematics (Second Edition), Academic Press, San Diego***,** 275-448.

SINGER, T. L. 1998. Roles of hydrocarbons in the recognition systems of insects. *American Zoologist,* 38**,** 394-405.

SLAA, E. J., CHAVES, L. A. S., MALAGODI-BRAGA, K. S. & HOFSTEDE, F. E. 2006. Stingless bees in applied pollination: practice and perspectives. *Apidologie,* 37**,** 293-315.

SMITH, J. P., HEARD, T. A., BEEKMAN, M. & GLOAG, R. 2017. Flight range of the Australian stingless bee *Tetragonula carbonaria* (Hymenoptera: Apidae). *Austral Entomology,* 56**,** 50-53.

SMITH, T. 2019. Evidence for male genitalia detachment and female mate choice in the Australian stingless bee *Tetragonula carbonaria*. *Insectes Sociaux***,** 67, 189-193.

SOMMEIJER, M., CHINH, T. & MEEUWSEN, F. 1999. Behavioural data on the production of males by workers in the stingless bee *Melipona favosa* (Apidae, Meliponinae). *Insectes Sociaux,* 46**,** 92-93.

SOMMEIJER, M., DE BRUIJN, L. & MEEUWSEN, F. 2004. Behaviour of males, gynes and workers at drone congregation sites of the stingless bee *Melipona favosa* (Apidae: Meliponini). *ENTOMOLOGISCHE BERICHTEN-NEDERLANDSCHE ENTOMOLOGISCHE VEREENIGUNG,* 64**,** 10-15.

SOMMEIJER, M., DE ROOY, G., PUNT, W. & DE BRUIJN, L. 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.

SOMMEIJER, M., KOEDAM, D. & MONGE, I. A. 1994. Social Interactions of Gynes and Their Longevity in Queenright Colonies of *Melipona favosa* (Apidae: Meliponinae). *Netherlands Journal of Zoology,* 45**,** 480-494.

SOMMEIJER, M. J., DE BRUIJN, L. L. & MEEUWSEN, F. 2003. Reproductive behaviour of stingless bees: solitary gynes of *Melipona favosa* (Hymenoptera: Apidae, Meliponini) can penetrate existing nests. *ENTOMOLOGISCHE BERICHTEN-NEDERLANDSCHE ENTOMOLOGISCHE VEREENIGUNG,* 63**,** 31-35.

SOUZA, E. C. A. D., SILVA, E. J. G. D., CORDEIRO, H. K. C., LAGE FILHO, N. M., SILVA, F., REIS, D. L. S. D., PORTO, C., PILAU, E. J., COSTA, L. A. & DE SOUZA, A. D. 2018. Chemical compositions and antioxidant and antimicrobial activities of propolis produced by *Frieseomelitta longipes* and *Apis mellifera* bees. *Química Nova,* 41**,** 485-491.

SPRADBERY, J. P. 1973. *Wasps. An account of the biology and natural history of social and solitary wasps, with particular reference to those of the British Isles*. Sedgwick and Jackson, London

STARR, C. K. & VELEZ, D. 2009. A dense daytime aggregation of solitary bees (Hymenoptera: Apidae: Centridini) in the Lesser Antilles. *Journal of Hymenoptera Research,* 18**,** 175-177.

STAURENGO DA CUNHA, M. A. 1979. Ovarian development in *Scaptotrigona postica* Latr. 1807 (Hym.: Apidae) II. A quantitative study. *Insectes Sociaux,* 26**,** 196-203.

STEPHENS, R. E., BEEKMAN, M. & GLOAG, R. 2017. The upside of recognition error? Artificially aggregated colonies of the stingless bee *Tetragonula carbonaria* tolerate high rates of worker drift. *Biological Journal of the Linnean Society,* 121**,** 258-266.

STOKSTAD, E. 2007. Puzzling decline of US bees linked to virus from Australia. *Science,* 317**,** 1304-1305.

SUMANGALA, H., RAO, V. & ROY, K. S. T. K. 2018. High concrete and ester containing Jasmine species (*Jasminum malabaricum* Wight). *IJCS,* 6**,** 3008-3013.

SZENDREI, Z., AVERILL, A., ALBORN, H. & RODRIGUEZ-SAONA, C. 2011. Identification and field evaluation of attractants for the cranberry weevil, *Anthonomus musculus* Say. *Journal of chemical ecology,* 37**,** 387-397.

TAVARES, M. G., ALMEIDA, B. S., PASSAMANI, P. Z., PAIVA, S. R., RESENDE, H. C., CAMPOS, L. A. D. O., ALVES, R. M. D. O. & WALDSCHMIDT, A. M. 2013. Genetic variability and population structure in *Melipona scutellaris* (Hymenoptera: Apidae) from Bahia, Brazil, based on molecular markers. *Apidologie,* 44**,** 720-728.

TAYLOR JR, O. R. 1984. An aerial trap for collecting drone honeybees in congregation areas. *Journal of Apicultural Research,* 23**,** 18-20.

THANGADURAI, D., ANITHA, S., PULLAIAH, T., REDDY, P. N. & RAMACHANDRAIAH, O. S. 2002. Essential oil constituents and in vitro antimicrobial activity of *Decalepis hamiltonii* roots against foodborne pathogens. *Journal of Agricultural and Food Chemistry,* 50**,** 3147-3149.

THE PLANT LIST. 2010. *Version 1. Published on the Internet;* http://www.theplantlist.org/ [Online]. [Accessed 3 Dec 2020].

THORNHILL, N. W. 1993. *The natural history of inbreeding and outbreeding: theoretical and empirical perspectives*, University of Chicago Press.

TORRES, C. & GALETTO, L. 2002. Are nectar sugar composition and corolla tube length related to the diversity of insects that visit Asteraceae flowers? *Plant biology,* 4**,** 360-366.

TORTO, B., SUAZO, A., ALBORN, H., TUMLINSON, J. H. & TEAL, P. E. 2005. Response of the small hive beetle (*Aethina tumida*) to a blend of chemicals identified from honeybee (*Apis mellifera*) volatiles. *Apidologie,* 36**,** 523-532.

TÓTH, E., QUELLER, D. C., DOLLIN, A. & STRASSMANN, J. E. 2004. Conflict over male parentage in stingless bees. *Insectes Sociaux,* 51**,** 1-11.

TÓTH, E., STRASSMANN, J. E., NOGUEIRA-NETO, P., IMPERATRIZ-FONSECA, V. L. & QUELLER, D. C. 2002. Male production in stingless bees: variable outcomes of queen–worker conflict. *Molecular Ecology,* 11**,** 2661-2667.

TRIVERS, R. L. & HARE, H. 1976. Haploidploidy and the evolution of the social insect. *Science,* 191**,** 249-263.

UCHIYAMA, M., ABE, H., SATO, R., SHIMURA, M. & WATANABE, T. 1973. Fate of 3-allyloxy-1, 2-benzisothiazole 1, 1-dioxide (oryzemate®) in rice plants. *Agricultural and Biological Chemistry,* 37**,** 737-745.

UTAIPANON, P., HOLMES, M. J., CHAPMAN, N. C. & OLDROYD, B. P. 2019a. Estimating the density of honey bee (*Apis mellifera*) colonies using trapped drones: area sampled and drone mating flight distance. *Apidologie,* 50**,** 578-592.

UTAIPANON, P., SCHAERF, T. M. & OLDROYD, B. P. 2019b. Assessing the density of honey bee colonies at ecosystem scales. *Ecological Entomology,* 44**,** 291-304.

VALDEZ-HERNÁNDEZ, M., SÁNCHEZ, O., ISLEBE, G. A., SNOOK, L. K. & NEGREROS-CASTILLO, P. 2014. Recovery and early succession after experimental disturbance in a seasonally dry tropical forest in Mexico. *Forest Ecology and Management,* 334**,** 331-343.

VAN DEN DOOL, H. & KRATZ, P. D. 1963. A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. *Journal of Chromatography,* 11, 463-471.

VAN EECKHOVEN, J. & DUNCAN, E. J. 2020. Mating status and the evolution of eusociality: Oogenesis is independent of mating status in the solitary bee *Osmia bicornis*. *Journal of Insect Physiology,* 121**,** 104003.

VAN NIEUWSTADT, M. & IRAHETA, C. R. 1996. Relation between size and foraging range in stingless bees (Apidae, Meliponinae). *Apidologie,* 27**,** 219-228.

VAN OYSTAEYEN, A., OLIVEIRA, R. C., HOLMAN, L., VAN ZWEDEN, J. S., ROMERO, C., OI, C. A., D'ETTORRE, P., KHALESI, M., BILLEN, J. & WÄCKERS, F. 2014. Conserved class of queen pheromones stops social insect workers from reproducing. *Science,* 343**,** 287-290.

VAN VALEN, L. 1971. Group selection and the evolution of dispersal. *Evolution***,** 25, 591-598.

VAN VEEN, J., ARCE, H. & SOMMEIJER, M. J. Production of males in colonies of *Melipona beecheii*, Costa Rica. Actes Coll. Ins. Soc., 6, 57-62.

VAN VEEN, J. & SOMMEIJER, M. 2000a. Colony reproduction in *Tetragonisca angustula* (Apidae, Meliponini). *Insectes sociaux,* 47**,** 70-75.

VAN VEEN, J. & SOMMEIJER, M. 2000b. Observations on gynes and drones around nuptial flights in the stingless bees *Tetragonisca angustula* and *Melipona beecheii* (Hymenoptera, Apidae, Meliponinae). *Apidologie,* 31**,** 47-54.

VAN VEEN, J., SOMMEIJER, M. J. & MEEUWSEN, F. 1997. Behaviour of drones in *Melipona* (Apidae, Meliponinae). *Insectes Sociaux,* 44**,** 435-447.

VANBERGEN, A. J. & INITIATIVE, T. I. P. 2013. Threats to an ecosystem service: pressures on pollinators. *Frontiers in Ecology and the Environment,* 11**,** 251-259.

VANENGELSDORP, D., TRAYNOR, K. S., ANDREE, M., LICHTENBERG, E. M., CHEN, Y., SAEGERMAN, C. & COX-FOSTER, D. L. 2017. Colony Collapse Disorder (CCD) and bee age impact honey bee pathophysiology. *PLoS One,* 12**,** e0179535.

VEIGA, J. C., MENEZES, C. & CONTRERA, F. A. L. 2017. Insights into the role of age and social interactions on the sexual attractiveness of queens in an eusocial bee, *Melipona flavolineata* (Apidae, Meliponini). *The Science of Nature,* 104**,** 31.

VELÁZQUEZ-BECERRA, C., MACÍAS-RODRÍGUEZ, L. I., LÓPEZ-BUCIO, J., ALTAMIRANO-HERNÁNDEZ, J., FLORES-CORTEZ, I. & VALENCIA-CANTERO, E. 2011. A volatile organic compound analysis from A*rthrobacter agilis* identifies dimethylhexadecylamine, an amino-containing lipid modulating bacterial growth and *Medicago sativa* morphogenesis in vitro. *Plant and soil,* 339**,** 329-340.

VELTHUIS, H. H., DE VRIES, H. & IMPERATRIZ-FONSECA, V. L. 2006. The polygyny of *Melipona bicolor*: scramble competition among queens. Apidologie 37:222-239.

VELTHUIS, H. H., KOEDAM, D. & IMPERATRIZ-FONSECA, V. L. 2005. The males of *Melipona* and other stingless bees, and their mothers. *Apidologie,* 36**,** 169-185.

VERDUGO-DARDON, M., CRUZ-LOPEZ, L., MALO, E. A., ROJAS, J. C. & GUZMAN-DIAZ, M. 2011. Olfactory attraction of *Scaptotrigona mexicana* drones to their virgin queen volatiles. *Apidologie,* 42**,** 543-550.

VERGARA, C., DIETZ, A. & PEREZ DE LEON, A. 1993. Female parasitism of European honey bees by Africanized honey bee swarms in Mexico. *Journal of Apicultural Research,* 32**,** 34-40.

VERMA, R. S., PADALIA, R. C., SINGH, V. R., GOSWAMI, P., CHAUHAN, A. & BHUKYA, B. 2017. Natural benzaldehyde from *Prunus persica* (L.) Batsch. *International journal of food properties,* 20**,** 1259-1263.

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.

VIT, P., PEDRO, S. R. & ROUBIK, D. W. 2018. *Pot-pollen in Stingless Bee Melittology*. Springer.

VIUDA-MARTOS, M., RUIZ-NAVAJAS, Y., FERNÁNDEZ-LÓPEZ, J. & PÉREZ-ÁLVAREZ, J. 2008. Functional properties of honey, propolis, and royal jelly. *Journal of food science,* 73**,** R117-R124.

VOLLET-NETO, A., IMPERATRIZ-FONSECA, V. L. & RATNIEKS, F. L. 2019. Queen execution, diploid males, and selection for and against polyandry in the Brazilian stingless bee *Scaptotrigona depilis*. *The American Naturalist,* 194**,** 725-735.

VOLLET-NETO, A., KOFFLER, S., DOS SANTOS, C., MENEZES, C., NUNES, F., HARTFELDER, K., IMPERATRIZ-FONSECA, V. & ALVES, D. 2018. Recent advances in reproductive biology of stingless bees. *Insectes Sociaux***,** 65, 1-12.

VON ZUBEN, L. G. 2017. *The mating communication of stingless bees (Hymenoptera: Apidae, Meliponini).* Ph.D. Thesis, University of São Paulo, Ribeirão Preto, Brazil.

VOSSLER, F. G. 2013. *Estudio palinológico de las reservas alimentarias (miel y masas de polen) de abejas nativas sin aguijón (Hymenoptera, Apidae, Meliponini): un aporte al conocimiento de la interacción abeja-planta en el Chaco seco de Argentina.).* Ph.D. Thesis National University of La Plata, Buenos Aires, Argentina.

WAGER, B. R. & BREED, M. D. 2000. Does honey bee sting alarm pheromone give orientation information to defensive bees? *Annals of the Entomological Society of America,* 93**,** 1329-1332.

WAGNER, A. & DOLLIN, L. 1982. Swarming in Australian native bees: help solve the mystery. *Australasian Beekeeper,* 84**,** 15-18.

WALLACE, H. M. & TRUEMAN, S. J. 1995. Dispersal of *Eucalyptus torelliana* seeds by the resin-collecting stingless bee, *Trigona carbonaria*. *Oecologia,* 104**,** 12-16.

WALSH, P. S., METZGER, D. A. & HIGUCHI, R. 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques,* 10**,** 506-513.

WANG, H., ZHANG, S.-W., ZENG, Z.-J. & YAN, W.-Y. 2014. Nutrition affects longevity and gene expression in honey bee (*Apis mellifera*) workers. *Apidologie,* 45**,** 618-625.
WANG, J. 2004. Sibship reconstruction from genetic data with typing errors. *Genetics,* 166**,** 1963-1979.
WASER, P. M. & STROBECK, C. 1998. Genetic signatures of interpopulation dispersal. *Trends in Ecology & Evolution,* 13**,** 43-44.
WATSON, L. R. 1928. Controlled mating in honeybees. *The Quarterly Review of Biology,* 3**,** 377-390.
WEI, Q. & YIN, C. W. 2019. Chemical Composition of Essential Oils from the Stems of *Taxus chinensis* var. *mairei*. *Journal of Essential Oil Bearing Plants,* 22**,** 1144-1149.
WENIG, P. & ODERMATT, J. 2010. OpenChrom: a cross-platform open source software for the mass spectrometric analysis of chromatographic data. *BMC bioinformatics,* 11**,** 405.
WENSELEERS, T., ALVES, D. A., FRANCOY, T. M., BILLEN, J. & IMPERATRIZ-FONSECA, V. L. 2011. Intraspecific queen parasitism in a highly eusocial bee. *Biology Letters,* 7**,** 173-176.
WENSELEERS, T., HART, A. G., RATNIEKS, F. L. & QUEZADA-EUÁN, J. J. 2004a. Queen execution and caste conflict in the stingless bee *Melipona beecheii*. *Ethology,* 110**,** 725-736.
WENSELEERS, T., HELANTERÄ, H., HART, A. & RATNIEKS, F. L. 2004b. Worker reproduction and policing in insect societies: an ESS analysis. *Journal of evolutionary biology,* 17**,** 1035-1047.
WILLE, A. 1983. Biology of the stingless bees. *Annual review of entomology,* 28**,** 41-64.
WILLIAMS, J. L. 1987. Wind-directed pheromone trap for drone honey bees (Hymenoptera: Apidae). *Journal of economic entomology,* 80**,** 532-536.
WILLIAMS, R. A. 2015. Mitigating biodiversity concerns in *Eucalyptus* plantations located in South China. *Journal of Biosciences and Medicines,* 3**,** 1.
WILLMER, P. & STONE, G. 2004. Behavioral, ecological, and physiological determinants of the activity patterns of bees. *Advances in the Study of Behavior,* 34**,** 347-466.
WILMS, W. & WIECHERS, B. 1997. Floral resource partitioning between native *Melipona* bees and the introduced Africanized honey bee in the Brazilian Atlantic rain forest. *Apidologie,* 28**,** 339-355.
WILSON, E. O. 1971. *The insect societies,* Harvard University Press.
WILSON, R. S., KELLER, A., SHAPCOTT, A., LEONHARDT, S. D., SICKEL, W., HARDWICK, J. L., HEARD, T. A., KALUZA, B. F. & WALLACE, H. M. 2021. Many small rather than few large sources identified in long-term bee pollen diets in agroecosystems. *Agriculture, Ecosystems & Environment,* 310**,** 107296.
WINFREE, R., WILLIAMS, N. M., DUSHOFF, J. & KREMEN, C. 2007. Native bees provide insurance against ongoing honey bee losses. *Ecology letters,* 10**,** 1105-1113.
WOSSLER, T. & CREWE, R. 1999a. The releaser effects of the tergal gland secretion of queen honeybees (*Apis mellifera*). *Journal of insect behavior,* 12**,** 343-351.
WOSSLER, T. C. & CREWE, R. M. 1999b. Honeybee queen tergal gland secretion affects ovarian development in caged workers. *Apidologie,* 30**,** 311-320.
XIN, X.-X., CHEN, Y., CHEN, D., XIAO, F., PARNELL, L. D., ZHAO, J., LIU, L., ORDOVAS, J. M., LAI, C.-Q. & SHEN, L.-R. 2016. Supplementation with major royal-jelly proteins increases lifespan, feeding, and fecundity in *Drosophila*. *Journal of agricultural and food chemistry,* 64**,** 5803-5812.
YAMANE, S. & SAKAGAMI, S. F. 1995. Oviposition Behavior of the Stingless Bees (Apidae, Meliponinae) XVI. *Trigona* (*Tetragonula*) *carbonaria* Endemic to Australia, wiht a Highly Integrated Oviposition Process. *昆蟲,* 63**,** 275-296.

YANG, W., TIAN, Y., HAN, M. & MIAO, X. 2017. Longevity extension of worker honey bees (*Apis mellifera*) by royal jelly: optimal dose and active ingredient. *PeerJ,* 5**,** e3118.

YOU, L. X. & WANG, S. J. Chemical Composition and Allelopathic Potential of the Essential Oil from *Datura Stramonium* L. Advanced Materials Research, 2011. Trans Tech Publ, 2472-2475.

YUE, X.-F., SHANG, X., ZHANG, Z.-J. & ZHANG, Y.-N. 2017. Phytochemical composition and antibacterial activity of the essential oils from different parts of sea buckthorn (*Hippophae rhamnoides* L.). *journal of food and drug analysis,* 25**,** 327-332.

YUND, P. 1995. Gene flow via the dispersal of fertilizing sperm in a colonial ascidian (*Botryllus schlosseri*): the effect of male density. *Marine Biology,* 122**,** 649-654.

ZAVODNA, M., ARENS, P., VAN DIJK, P. J., PARTOMIHARDJO, T., VOSMAN, B. & VAN DAMME, J. M. 2005. Pollinating fig wasps: genetic consequences of island recolonization. *Journal of Evolutionary Biology,* 18**,** 1234-1243.

ZAYED, A. 2009. Bee genetics and conservation. *Apidologie,* 40**,** 237-262.

ZAYED, A., ROUBIK, D. W. & PACKER, L. 2004. Use of diploid male frequency data as an indicator of pollinator decline. *Proceedings of the Royal Society of London. Series B: Biological Sciences,* 271**,** S9-S12.

ZHANG, J., LIU, J., YANG, Y. & YUAN, K. 2010a. Analysis the chemical constituents of volatile oils in *Medinilla arboricola* by SPME-GC-MS. *Zhong yao cai= Zhongyaocai= Journal of Chinese medicinal materials,* 33**,** 225-229.

ZHANG, Y.-T., WANG, G.-X., JING, D., ZHONG, C.-F., JIN, K., LI, T.-Z. & HAN, Z.-H. 2009. Analysis of volatile components in strawberry cultivars Xingdu 1 and Xingdu 2 and their parents. *Agricultural Sciences in China,* 8**,** 441-446.

ZHANG, Z. F., ZHOU, X. Y., WU, F. J. & MA, Q. Z. GC/MS Analysis on Biomedical Resources of Extractives of *Eucalyptus* Leaves for Biomedical Engineering. Advanced Materials Research, 2010b. Trans Tech Publ, 719-723.

ZHOU, Y., WEI, Z., ZHONG, Z. & LI, Z. 2009. Analysis of essential oil from the leaves and fruits of *Eucalyptus tereticornis* in Guangxi Province by GC-MS. *Zhong yao cai= Zhongyaocai= Journal of Chinese medicinal materials,* 32**,** 216-219.