Insectes Sociaux (2023) 70:17–27
https://doi.org/10.1007/s00040-022-00887-z

Insectes Sociaux

RESEARCH ARTICLE

Virgin queen behaviour and controlled mating in the stingless bee *Tetragonula carbonaria* (Meliponini)

F. G. B. Bueno[1] · **R. Hajjar**[2] · **T. Colin**[2] · **G. Buchmann**[1] · **T. Latty**[2] · **R. Gloag**[1]

Received: 25 July 2022 / Revised: 20 October 2022 / Accepted: 22 October 2022 / Published online: 14 November 2022
© The Author(s) 2022

Abstract

Improving knowledge of virgin queen behaviour and mating biology in stingless bees (Meliponini) is an important step towards artificial selection programmes for managed colonies. Here we study the Australian stingless bee *Tetragonula carbonaria* to (1) describe the behaviour of queens from eclosion until egg-laying, and (2) assess whether young queens can be reared and mated under constrained conditions. We extracted mature virgin queen cells of *T. carbonaria* from large colonies and reared them in queen maturation boxes containing a limited number of workers ("micro-colonies"). Queen behaviour followed a typical pattern with three phases: a period of high activity and wing-flapping on top of the brood (5 ± 2 days of age), attempts to leave the colony for the nuptial flight (12 ± 3 days of age), and oviposition (20 ± 6 days of age). In total, 71% (68 of 95) of queen cells hatched in our maturation boxes and 57% (39 of 68) survived to mating age, with some observed to be actively killed by workers within the first week of life (8 of 68; 11%). For a subset of our queens, we performed controlled matings (either with or without CO_2 narcosis) by presenting constrained females to males in mating aggregations, allowing one male to mate (deposit mating plug) and then returning queens to their box. In the absence of CO_2 narcosis, 30% of queens mated this way were subsequently found to have sperm in their spermathecae, indicative of a successful mating (5 of 17). The remaining 70% however contained no sperm, despite the presence of the male mating plug. Amongst queens that were CO_2 narcotized before and during the controlled mating, none had sperm in their spermathecae (0 of 12), though most went on to activate their ovaries following the mating and lay male (haploid) eggs. Together, these trials indicate that controlled matings with *T. carbonaria* are possible, but suggest that queens may need to actively accept mating for successful sperm transfer.

Keywords Reproductive behaviour · CO_2 narcosis · Ovary activation · Oviposition

Introduction

Stingless bees (Meliponini) are a group of eusocial insects found in the tropics and subtropics. They are important pollinators of wild plants and some crops, with many stingless bee species propagated for crop pollination, honey production and recreational beekeeping (Grüter, 2020). Although stingless bees have been kept and propagated for centuries in many parts of their range, recent interest in the commercial uses of stingless bees has led to calls for the large-scale breeding of some stingless bee species (Menezes, Vollet-Neto, & Imperatriz-Fonseca, 2013).

The ability to rear and mate stingless bee queens under controlled conditions would help to accelerate the propagation of managed colonies (Cortopassi-Laurino et al., 2006; Jaffé et al., 2015; Menezes et al., 2013). This is due to the low reproductive rate of colonies, both in the wild and in meliponaries because queen availability can be a limiting factor during colony propagation (Eleutério, Rocha, & Freitas, 2022; Imperatriz-Fonseca & Zucchi, 1995; Jaffé et al., 2015; Menezes et al., 2013). Stingless bee queens are produced throughout the year in most species but, with the exception of *Melipona*, new queens are a very small proportion of total brood (1–2%) (de Souza Santos-Filho, de Araujo Alves, Eterovic, Imperatriz-Fonseca, & Kleinert,

---

✉ F. G. B. Bueno
fgarciabulle@gmail.com

[1] Behaviour Ecology and Evolution Laboratory, School of Life and Environmental Sciences A12, University of Sydney, Sydney, NSW 2006, Australia

[2] Insect Behaviour and Ecology Laboratory, School of Life and Environmental Sciences A12, University of Sydney, Sydney, NSW 2006, Australia

2006). Furthermore, the ability to perform controlled matings in stingless bees would open the door to selection programmes that favour high productivity colonies, similar to programmes used for honey bees (e.g. (Oxley, Hinhumpatch, Gloag, & Oldroyd, 2010; Plate, Bernstein, Hoppe, & Bienefeld, 2019). The reproductive biology of queens for most of the world's 600 or so stingless bee species, however, remains poorly known (Vollet-Neto et al., 2018). Even for those species that are widely propagated, there are often key gaps in our knowledge of queen behaviour. Thus, a first step towards controlled queen rearing and mating in stingless bees is a better understanding of young queen behaviour.

Like many tropical regions around the world, Australia has a thriving stingless bee keeping industry. The most popular and commonly kept species is *Tetragonula carbonaria*, which is kept in wooden box hives throughout its range in tropical and subtropical East Coast Australia. This species can pollinate a range of Australian fruit crops, including lychee, coconut, carambola, macadamia, blueberry and mangoes (Heard, 2016; Kendall, Stavert, Gagic, Hall, & Rader, 2022) and is currently under investigation as a managed pollinator of many more crops, including those grown in greenhouses and poly-tunnels (Greco, Spooner-Hart, Beattie, Barchia, & Holford, 2011; Hall, Jones, Rocchetti, Wright, & Rader, 2020).

T. carbonaria colonies contain an estimated average of 10,000 workers and a single egg-laying queen (Heard, 2016). Queens in this species lay all the eggs, including all males, as workers are entirely sterile and never lay reproductive eggs (Garcia Bulle Bueno, Gloag, Latty, & Ronai, 2020; Gloag, Beekman, Heard, & Oldroyd, 2007). Queens are determined nutritionally (Heard, 2016), since they are reared in special, larger royal cells (Fig. 1). Thus, a larva in a royal cell can feed much longer, being larger than workers. Moreover, *T. carbonaria* is capable of creating emergency queen cells by the fusion of two worker cells whenever they are queenless (Nunes, Heard, Venturieri, & Oldroyd, 2015). The fate of virgin *T. carbonaria* queens when they emerge is unknown, but queenright colonies seemingly retain multiple virgin queens at all times because virgin queens are quick to appear once a resident queen has been removed; sometimes within 30 min (FGBB, pers. obs). These excess virgin queens presumably act as an insurance against the death of the mated queen, ensuring rapid queen replacement (Imperatriz-Fonseca & Zucchi, 1995). They may also facilitate active queen replacement, such as regicide by workers of queens laying diploid males (Conceiçao Aparecida de Camargo, 1979; Vollet-Neto, Imperatriz-Fonseca, & Ratnieks, 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 *T. carbonaria* virgin queens are ready to mate, they fly from their colony to a male aerial aggregation composed of several hundreds to thousands of males. These aggregations typically form within a few metres away from the virgin queen's colony (Garcia Bulle Bueno et al., 2022). Queens then mate with a single male before returning to the colony (Green & Oldroyd, 2002). During the mating, the male loses his genitalia, leaving them attached to the queen's genital chamber as a mating plug (Smith, 2020). Presumably this mating plug reduces the chance of further matings, as shown to be the case in other stingless bees (Da Silva, Zucchi, & Kerr, 1972; Conceição Aparecida de Camargo, 1972; Green & Oldroyd, 2002; Imperatriz-Fonseca, Matos, Ferreira, & Velthuis, 1998; Kerr, Zucchi, Nakadaira, & Butolo, 1962). Once

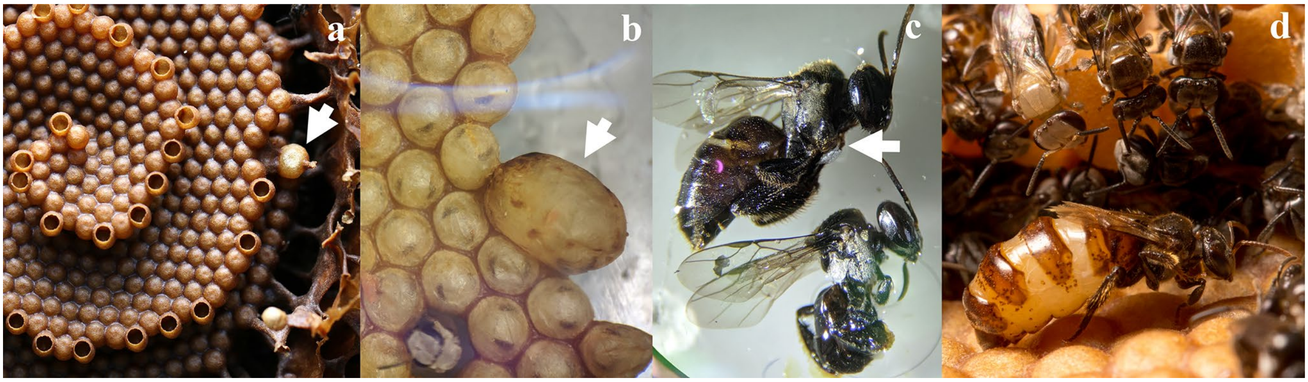

Fig. 1 Life stages of a *T. carbonaria* queen. **a** A queen cell (indicated by arrow) on the edge of a *T. carbonaria* brood sprial. Queen cells are continuously produced throughout the year and always built at the edge of the comb combs (Photo by Glenbo Craig). **b** Queen cells (indicated by arrow) 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 separate, giving a typical striped appearance (photo by James B. Dorey)

egg-laying, queens never again leave the colony, storing enough semen in the spermatheca to fertilise eggs throughout their lifetime (Kerr et al., 1962).

Previous work suggests that the controlled mating of *T. carbonaria* queens may be possible; that is, matings which allow bee breeders to choose the queens and males that mate. Natural male aggregations are easy to attract, and mature males can also be sampled directly on their exit from their colonies (Garcia Bulle Bueno et al., 2022). Furthermore, males from mating aggregations have been shown to mate with tethered, recently deceased queens (Smith et al. 2019).

In this study, we observed *T. carbonaria* virgin queens in small observation boxes. We had two aims: (i) to describe the progression of queen behaviour from emergence until egg-laying, and (ii) to hatch and rear queens in closed micro-colonies until sexual maturity, and then to mate them under controlled conditions. We devised a mating technique in which a constrained queen was presented to males in a natural male aggregation. After mating, the male that mated was collected. Because artificial mating of honey bees regularly narcotizes queens with carbon dioxide (CO_2) before and during insemination (Gillard & Oldroyd, 2020), we attempted mating of *T. carbonaria* queens both with and without CO_2 narcosis. Together, our efforts are a step towards new propagation techniques for *T. carbonaria*, and also provide insights into the reproductive biology of *Tetragonula* stingless bees.

Methods

Biological material

We carried out this work at The University of Sydney, Australia, between October 2019 and January 2020 ("batch 1 queens") and October 2020 and December 2020 ("batch 2 queens") 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 & 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 brood combs (Fig. 1). We extracted 95 mature queen cells (60 for batch 1 and 35 for batch 2) from 25 colonies (1–5 per colony). Mature 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 comb of mature pupal brood, from a different set of 20 colonies, and used a further six colonies to supply adult workers.

Incubating boxes

Prior to emergence, the 95 queen cells were placed into an incubating box. Incubating boxes were industry-standard wooden boxes (23 × 15 × 11 cm, volume 3.8 L) covered with transparent acetate sheets, allowing us to observe the bees' behaviour and the emergence of virgin queens. These boxes contained no adult workers, but each was provided with one comb of brood (approximately 1000 brood cells: comprising workers and a few males), 3–4 pots of pollen (with approximately 2–3 gr each), 1 mL of honey, and 2 mL of water on a piece of cotton placed in a plastic lid (5 cm in diameter). Queen, worker and drone cells emerged freely within the incubating boxes and the callows started working on the brood as soon as they emerged. We also included a 5 cm diameter block of propolis (building material made of wax and plant resins (Heard, 2016) fix double brackets. Pollen, honey and propolis were sourced from *T. carbonaria* colonies. The pollen was stored at −20 °C whilst 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 honey and the pollen were refilled ad libitum. All the boxes were kept in darkness and at constant temperature of 28 °C.

Queen maturation boxes

A total of 68 queens emerged (48 of 60 in batch 1 and 20 of 35 in batch 2) with the remainder failing to hatch. Upon emergence in the incubating boxes, queens were marked for individual identification with a coloured spot on the thorax using POSCA pens and they were directly placed into its own 'maturation box' (another wooden box with closed entrance) with the same provisions as the incubating boxes, a new brood comb with 200–300 workers' pupae, and fully pigmented adult workers. Workers were introduced to boxes 24–48 h before the newly eclosed queen was introduced. Maturation boxes were intended to keep queens alive in sealed conditions until mating age, that is, to rear them until an age suitable for controlled mating with the certainty that they had not left the colony and mated naturally. These boxes also allowed us to easily observe the behaviour of young queens, and they were inspected every day for 30 days or until the queen died. Excreta and dead bees were manually removed every day, via forceps or an aspirator.

The number and age of workers in maturation boxes could affect the survivorship of young queens. In an initial trial, five queens were left in boxes with 50–100 callow workers only and all these queens died within a few days of hatching, suggesting that some mature workers must be present to care for callow queens. For our initial batch of queens therefore (batch 1), we allocated the young queens to either "large micro-colonies" (starting population of 800–1000 mature workers; n = 20) or "small micro-colonies" (starting population of 50–100 mature workers; n = 10). To determine whether keeping the colonies closed affected queen survival, we also allocated some batch 1 queens to maturation boxes with 800–1000 workers but open entrances (n = 7) such that both queens and workers were allowed to fly freely. We then compared the proportion of queens in batch 1 that reached mating age in different types of maturation boxes (large vs. small, and large open vs large closed) using Fisher's Exact tests for equality of proportions in R v4.0.0 (R Core Team, 2013). The remaining nine batch 1 queens that hatched were allocated to three "multi-queen" boxes, each of which received three queens one day apart (and 50–100 workers). Under natural conditions, *T. carbonaria* colonies may have more than one virgin queen at a time, and the purpose of these boxes was to observe interactions between virgin queens. Finally, for batch 2 queens, which were reared the following spring/summer, all queens were reared in "large microcolony" conditions (800–1000 adult workers; n = 17 closed, n = 3 open).

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 per box. When queens were present and not hiding under the brood nest, we noted their behaviour. Any behaviour that was considered interesting was filmed (IPhone X and Google Pixel 4). We categorised queen behaviour into ten categories based on virgin queen behaviour reported for other stingless bees (Kleinert, 2005); (1) *motionless or low activity*; (2) *trophallaxis*, between virgin queen and workers; (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 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) *readiness to mate,* 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 "large micro-colonies") outside and allowed queens to leave; in all cases, presence in the nest tube corresponded to actually leaving the colony; (10) *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

Batch 1: without CO_2 narcosis

In our first attempt at controlled matings of *T. carbonaria*, we performed controlled matings of 17 virgin queens that were constrained from flying. We extracted queens from their boxes once they were observed to enter the plastic entrance tube, indicating readiness for a mating flight. We 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 could not escape. This left their abdomen free in the open air. 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 collected the male and stored him at -20 °C freezer for genetic analyses. We then checked 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 h, and daily thereafter.

Most queens died within three weeks of mating (either from unknown causes or worker attack) at which point we dissected them under 10× magnification to determine if the spermatheca was filled with sperm, as evidence of a successful mating. Any queens still alive at 120 days post mating were sacrificed and dissected. A full spermathecae can be readily distinguished from an empty one based on its colour (Garcia Bulle Bueno et al., 2020; Gerula, Panasiuk, Węgrzynowicz, & Bieńkowska, 2012). Most queens did not lay eggs in this post-mating period, but in many boxes, this may have been because the relatively small number of workers present had not built brood cells into which a queen could lay. For those queens that did begin egg-laying, we collected brood to check whether it was female (produced from fertilised eggs) or male (produced parthenogenically from unfertilized eggs). If possible, brood sex was determined from visual inspection of pupa. When no pupae were available, however, we instead deemed brood to be male if they were haploid at all seven micro-satellite loci. We extracted the DNA from larva by grinding whole abdomens in 5% Chelex (Walsh, Metzger, & Higuchi, 1991). We genotyped each bee at seven micro-satellite loci (Tc3. 155, Tc4. 63, Tc3. 302, Tc7. 13, Tc4. 287, Tang60 and Tang70) (Brito et al., 2009; Green, Franck, & Oldroyd, 2001) and the resulting products were analysed using a 3130xl Genetic Analyser and Genemapper (Applied Biosystems, USA).

Batch 2: with CO_2 narcosis

During controlled matings with batch 1 queens, we noted that queens often struggled to free themselves from the constraint. Evidence from honey bees suggests that the success of controlled mating protocols are improved by reducing the stress of queens during mating. A common practice used during artificial insemination of *Apis* species is carbon dioxide narcosis of queens (Gillard & Oldroyd, 2020). In our second attempt at controlled matings, we therefore CO_2-narcotised *T. carbonaria* virgin queens (n = 12). Queens were exposed to CO_2 during two sessions of 3 min each; the first time 24 h before the constrained mating (after which they were returned to their colony), and the second time during the mating itself. This schedule of narcosis is one commonly used for *Apis mellifera* (Gillard & Oldroyd, 2020). Queens were gassed by sealing them in a screw-cap tube (20 × 60 mm diameter, 5-mL) attached to a tank of CO_2. These batch 2 immobilised queens were mated at natural male aggregations in the same manner as that described above for batch 1 queens. As for batch 1, the brood of any queens that began laying was sexed based on either morphology or micro-satellite genotype.

Results

Queen survival in maturation boxes until mating age

Of 68 virgin queens that were reared from emergence in maturation boxes, 39 survived until mating age (57%). The proportion of queens that reached mating age was lower in large maturation boxes than small ones (batch 1: n = 10 of 20 and 10 of 10, respectively; Fisher's Exact Test, df = 1, p = 0.01), but did not differ between maturation boxes that were open or closed (i.e. between queens allowed to leave freely and those confined to the box (6 of 7 and 32 of 47 respectively; Fisher's Exact Test, df = 1, p = 0.4).

Behavioural observations of young queens

Upon emergence, all virgin queens followed a similar trajectory of behaviours during the first two weeks after emergence. 1) Queens emerge from a period of hiding or low activity to become highly and consistently active on top of the comb (age: 5 ± 2 days old, n = 41). 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, and are frequently on top of the brood comb. 2) Activity in the entrance tube, indicating a need to go outside and mate (age: 12 ± 3 days old, n = 33). This usually occurred each day at early or mid-afternoon. 3) Onset of oviposition (age: 20 ± 6 days old, n = 17); the queen was not visually physogastric at the time egg-laying was initiated but became enlarged over a period of 10–14 additional days (Figs. 2, 3).

We additionally observed three other behaviours of note amongst workers in response to queens. First, workers often formed a court around the recently mated queen, engaged in trophallaxis and constant antennal contact, and did not allow the queen to move freely (n = 19) (Fig. 3, Sup Mat. Fig. 1). Workers in these courts sometimes engaged in aggression, walking over the queen and biting her. Second, workers were observed to remove the mating plug from the queen in two cases, by pulling it with their mandibles. Mating plug removal in both cases occurred within 20 min of mating. Third, workers were observed to actively kill eight queens. All these killings occurred in the first nine days after emergence, and most before the queens were mated (unmated queens, n = 7; mated, n = 1). Workers bit the queen's antennae and legs, and/or attached resin on the queen's body until she was fully immobilised and dead. A further nine queens were possibly killed by workers via starvation (no

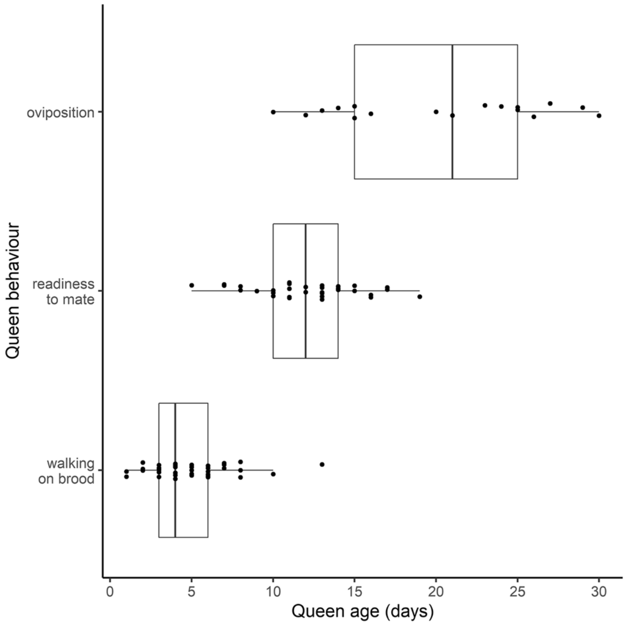

Fig. 2 Age at which *T. carbonaria* queens in micro-colonies were first observed (1) walking on the brood (39 queens), (2) showing readiness to mate by waiting at hive entrances (33 queens) and (3) ovipositing once mated (18 queens)

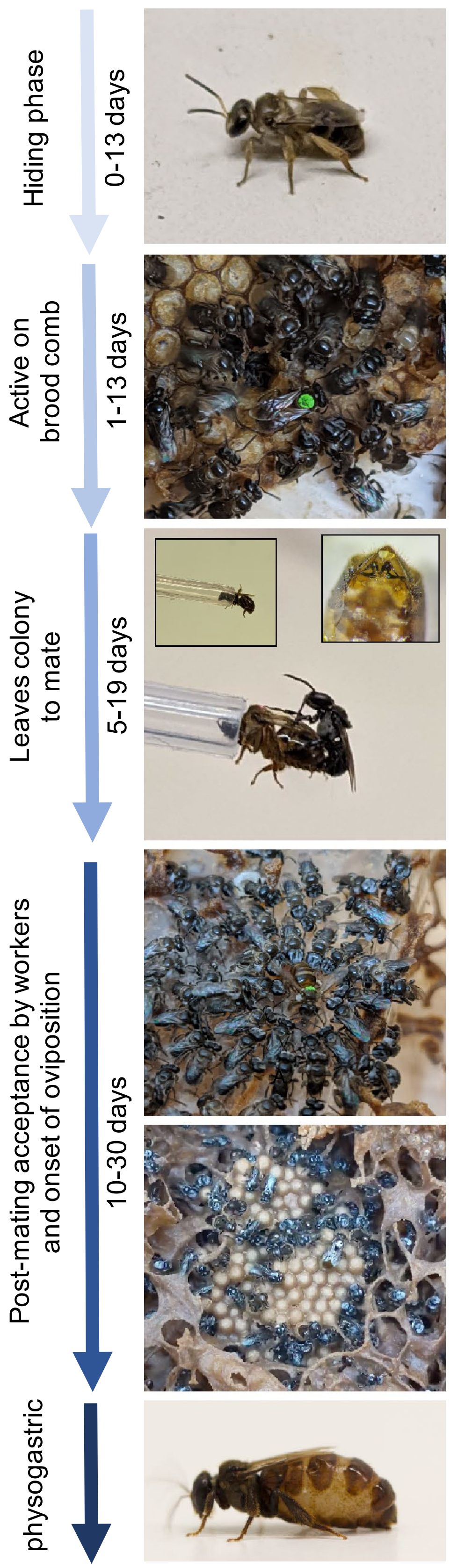

Fig. 3 Life of a young *T. carbonaria* queen in a queen-less colony. In the days after eclosion, the young queen hides under the brood. At 1–13 days days old, the queen becomes very active, walking quickly across the brood, rubbing her abdomen with her hind legs and fanning her wings. She shows a readiness to leave the colony to mate at 5–19 days of age (inset: mating plug attached to queen's abdomen). On her return to the nest post mating, she interacts with workers. If accepted by workers, they form a retinue around the queen making antennal contact; if not accepted, worker interactions may be aggressive. Onset of oviposition is 10–30 days. Finally, queens become visibly physogastric 10–14 days after egg-laying begins

trophallaxis observed between workers and the queens at any observation session in the days prior to queen death), although queen-killing could not be confirmed in these cases.

In our "multi-queen" boxes in which three newly emerged queens were introduced one per day for three days, 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 before dying 2–3 days after their introduction. We did not observe aggression between virgin queens in the same box.

Natural mating flights

Of four queens that were allowed to embark on natural mating flights, one left the colony at 1:15 PM, reappeared near the nest entrance 10 min later 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

Without CO_2 narcosis (batch 1)

Despite the presence of a mating plug in all 17 queens mated under constraint, only five queens (30%) were found to have full spermathecae following dissection, indicating the successful transfer of sperm. The remaining 12 queens had empty spermathecae. This included two queens that began egg-laying post mating. 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 spermatheca (Sup mat. Fig. 2). Workers showed no aggression towards these two queens, despite them laying only un-fertilized eggs.

With CO_2 narcosis (batch 2)

Of 12 queens narcotized with CO_2 and mated under constrained conditions, eleven were found to have empty spermatheca following dissection, with the spermatheca of one queen damaged during dissection and thus inconclusive. Nine of these queens nevertheless laid eggs and became physogastric. We were able to confirm only-male eggs for seven queens via viewing pupal brood (4 queens) and/or genotyping larva (3 queens; 4–8 larvae genotyped per queen).

Discussion

In this study, we harvested pupal-stage queen cells of *T. carbonaria*, allowed them to eclose in closed micro-colonies of < 1000 workers where we could observe their behaviour and prevent natural mating, and then mated them under constraint. These efforts reveal that queen behaviour from emergence until mating age follows a predictable timeline, with queens showing readiness to mate 10–14 days old and egg-laying around 20 days post eclosion. They also reveal that constrained matings are possible, with a subset of our queens having filled spermatheacea following matings, though further efforts will be needed to improve queen insemination rates and maintain mated queens through to egg-laying. Finally, we find that CO_2 narcosis did not improve rates of sperm transfer for constrained matings, though CO_2 exposure before and during mating did reliably stimulate the oviposition of un-fertilized (male) eggs. We expand on each of these points below, in the context of the broader aim of developing controlled queen rearing and mating in *T. carbonaria*.

Virgin queen behaviour

The behaviour of young *T. carbonaria* queens in the absence of a resident queen is similar to that described for other stingless bee species investigated to date. Upon emergence, *T. carbonaria* callow queens have less activity and attract little attention from workers until close to one week of age. They are born unattractive to workers, with little pigmentation, and remain stationary below the comb, similar to the queens of Neotropical species, such as *Scaptotrigon*a, *Paratrigona*, *Schwarziana* and *Nannotrigona*) (Imperatriz-Fonseca & Zucchi, 1995; Nogueira-Ferreira, Silva-Matos, & Zucchi, 2009). A few days after emergence (5–9 days old), *T. carbonaria* queens start to be noticed by the workers, become active on the brood comb, running excitedly, rubbing their abdomen with their legs and vibrating their wings intensively in search of trophallaxis with the workers (de Souza, Trigo, Santos, Vieira, & Serrão, 2017; Van Veen, Sommeijer, & Monge, 1999). At this point, it is likely that stingless bee queens begin to produce volatiles which signal their queen status to both nest-mate workers and males (potential mates) outside the colony (Engels, Engels, Lübke, Schröder, & Francke, 1993; Fierro, Cruz-López, Sánchez, Villanueva-Gutiérrez, & Vandame, 2011; Imperatriz-Fonseca & Zucchi, 1995; Verdugo-Dardon, Cruz-Lopez, Malo, Rojas, & Guzman-Diaz, 2011) and these pheromones may play an critical role in the acceptance of the virgin queen by workers (Imperatriz-Fonseca & Zucchi, 1995; Van Veen et al., 1999). Indeed, in queen-less 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 (Garcia Bulle Bueno et al., 2022). Under queenright conditions, however, virgin queens presumably continue hiding until the egg-laying queen dies, the colony reproduces, or until they are killed or evicted by workers (Imperatriz-Fonseca & 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 aggregations typically peak in size (Garcia Bulle Bueno et al., 2022). This timing is also consistent with two personal observations of FGBB: a queen 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). Whether *T. carbonaria* queens sometimes make orientation flights on the day or days prior to mating flights is unknown but the one *T. carbonaria* queen we observed to make a natural mating flight did so on her first flight outside the nest. In any case, both types of queen flights indicate preparation to mate, as virgin queens are mature and capable of mating at the time orientation flights begin (Van Veen & Sommeijer, 2000).

Three of the four queens in our observations failed to return to the nest. 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 & Koeniger, 2007). Whether return rates were affected by the small size of our experimental colonies however is unclear. An alternative reason for the low return rate may be that queens instead try to infiltrate other colonies as reproductive parasites. In at least one South American stingless bee (*Melipona scutellaris*), newly mated queens actively seek out and parasitize other colonies (Kärcher et al., 2013; Van Oystaeyen et al., 2013; Wenseleers, Alves, Francoy, Billen, & Imperatriz-Fonseca, 2011). Such behaviour in *T. carbonaria* queens remains unconfirmed, but dead virgin queens have been collected outside hives to which they are not related, which could be consistent with attempted parasitism of this kind (Bueno, 2021, pp. 134; Lau, Hereward, Smith, Heard, & Walter, 2022).

Workers play an important role in the survival of young queens amongst the stingless bees (de Souza et al., 2017; Jarau, Van Veen, Aguilar, & Ayasse, 2009; Kärcher et al., 2013; Sommeijer, Koedam, & Monge, 1994; Van Veen et al., 1999; Veiga, Menezes, & Contrera, 2017; Wenseleers, Hart, Ratnieks, & Quezada-Euán, 2004). We report here for the first time that *T. carbonaria* workers do actively kill virgin queens, a common practice amongst 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. Death by neglect is difficult to confirm without constant observation, but we noted that virgin queens that were accepted by workers enjoyed almost constant trophallaxis with workers. We also observed workers remove the mating plug from queens in some cases, but the importance of this for queen survival is unclear (Melo, Buschini, & Campos, 2001) In some other stingless bee species, the queen removes the male genitalia herself (Da Silva et al., 1972; Conceição Aparecida de Camargo, 1972; Engels et al., 1993), or the male genital capsule remains attached to the queen for several days and detaches on its own (Melo et al., 2001).

The extent to which our micro-colonies reflect typical worker–queen interactions, however, is unclear. Natural colonies are far larger, workers and queens are free to fly, and they are typically full sisters (whilst queens and workers in our study were not kin). Furthermore, under natural conditions, colonies of *T. carbonaria* and most other stingless bees will usually have more than one virgin queen (Imperatriz-Fonseca & Zucchi, 1995; Silva, 1972). Not much is known about the interactions between virgin queens in full size *Tetragonula* colonies. However, we have observed two virgin queens in a full-sized queen-less 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. Likewise, in the three micro-colonies in this study that received multiple virgin queens, the queens did not interact directly.

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 nest (Butz & Dietz, 1994; Gilley, 2001). Contrastingly, in stingless bees, the constant supply of new queens may give more power to the workers. They rear the brood, having control over queen production and choosing the best one by killing the rest (Jarau et al., 2009; Sommeijer et al., 1994; Wenseleers et al., 2004). This strategy comes with the obvious risk that all queens fail, leaving the colony without a queen to produce new brood (Beekman & Ratnieks, 2003). Further work is needed to understand the role of *T. carbonaria* workers in choosing new queens for the colony.

Controlled mating techniques for *T. carbonaria*

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 food. We were able to rear 61% of emerged queens to mating age in this way. Some pre-mating queens were confirmed as killed by workers, but most died soon after emergence from unknown causes. In contrast, four queens reared in open micro-colonies all survived (i.e. colonies in which workers were free to forage). One possible explanation for this difference in queen mortality is that the provisions available to our closed colonies did not fully replicate those provided by foraging workers in open colonies, reducing young queen survival. Future efforts might therefore consider alternative ways to prevent queens mating naturally whilst still keeping colonies free to forage, such as leaving the colony open whilst queens are young and closing it only once queens approach mating age. Mesh queen excluders at the hive entrance, like those used in honey bees, may also be possible. Notably though, low survivorship of queens is reported for other stingless bee species, and might simply reflect a high natural mortality rate for young queens (Da Silva et al., 1972; Kerr et al., 1962; Sommeijer et al., 1994).

We demonstrated that constrained live virgin queens will attract *T. carbonaria* males, and that these males can mate and fill the spermatheca. This represents an exciting step towards controlled mating techniques for this species, which would benefit both bee breeding programmes and research. Importantly, however, neither the presence of the mating plug nor egg-laying after mating were reliable evidence of a successful mating via our technique. Only one third of our queens mated under constraint (without CO_2) carried sperm in their spermathecae, and none of those narcotized with CO_2 during mating did so, despite the presence in all cases of a mating plug.

T. carbonaria queens may need to actively accept mating for sperm transfer to be successful. Smith et al. (2019) proposed that *T. carbonaria* queens exercise control over mating based on observations that males left mating plugs in two dead or dying young queens exposed to male swarms, whilst no mating plugs were found in two healthy tethered queens similarly exposed to males. Indeed, the live queens appeared to repel males from their abdomens using their legs (Smith, 2020). In our study, constrained queens never prevented a male depositing a mating plug, but they did regularly wriggle their abdomens and legs in ways that might have made mating difficult for males. Most tellingly, CO_2-narcotised queens never had filled spermathecae, suggesting that some action by queens is needed, and thus that queens could exercise choice when mating. An alternative explanation, however, is that stationary constrained queens do not sufficiently mimic the body position of queens during natural mid-flight matings; that is, males may have had low insemination success for reasons unrelated to female choice. Whichever the case, other protocols for controlled mating in this species are worth exploring. For example, attempts to mate constrained queens might have more success if queens' antennae are exposed, allowing her to better sense her environment (in our experiments, the entire head of the queen was covered). Alternatively, virgin queens could be released into flight cages or glasshouses containing males to allow on-the-wing mating. Artificial insemination like that performed in honey bees (in which sperm is harvested and inserted directly into the oviduct of a restrained queen (Gillard & Oldroyd 2020), is another possible route to selective breeding programmes in *Tetragonula*, though the small size of the bees present significant challenges for that approach.

Aside from advancing the goal of controlled mating, the methods we describe for establishing *T. carbonaria* colonies from harvested queen cells represent a step towards protocols for producing new colonies from the excess virgin queens produced by mother colonies. Such protocols have the potential to produce a large number of new colonies descending from virgin queens that are normally eliminated, with modest resources and in a short period of time, as recently proposed for some *Melipona* (Eleutério et al., 2022; Veiga et al., 2017).

Triggers of ovary activation in queens

Following controlled matings, the *T. carbonaria* queens from our experiment, were immediately surrounded by a court of workers and became greatly attractive, inflated their abdomens, walked excitedly through the hive, vibrating their wings, and had more trophallactic and antennal contact with courting workers. Days later, some queens in our study became physogatric and laid eggs but were later found to have an empty spermatheca and be laying only haploid eggs. This included two queens not treated with CO_2 and all twelve queens that were CO_2-narcotized during mating. Thus, ovary activation and the onset of oviposition in *T. carbonaria* is not directly linked to successful mating. Rather, the key trigger may be the mechanical stimulation of mating and the mating plug, as has been proposed for *Melipona quadrifasciata* (Melo et al., 2001).

Exposure to CO_2 triggers the onset of oviposition in honey bees (Mackensen, 2014), where it is used to stimulate egg-laying following artificial insemination, and bumble bees (Röseler, 1985), where it is used to bypass seasonal queen diapause. Indeed, honey bee queens artificially injected with semen will typically fail to activate ovaries and begin egg-laying in the absence of CO_2 treatment (Gillard & Oldroyd 2020). Furthermore, CO_2 will trigger oviposition in unmated virgin queens of both honey bees (Engels et al., 1976) and bumble bees (Amsalem & Grozinger, 2017). In stingless bees, CO_2 effects on ovaries remain largely untested, although CO_2 reportedly failed to trigger oviposition in *Melipona quadrifasciata* queens (unpublished data cited in Melo et al., 2001). Our results show that CO_2 exposure consistently leads to ovary activation in *T. carbonaria* queens when combined with mating. Further work on *T. carbonaria* is needed to determine the relative importance of CO_2 and mating as triggers of oviposition.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00040-022-00887-z.

Acknowledgements We thank Kuringai Council Sydney for supplying queen cells, Julianne Lim for assistance with molecular lab work and Benjamin P. Oldroyd for advice and discussion at various stages of the work.

Funding Open Access funding enabled and organized by CAUL and its Member Institutions.

Data availability Any raw data additional to that provided inSupplementary material can be provided on request of the authors.

Declarations

Conflict of interest The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report. We certify that the submission is original work and is not under review at any other publication.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

References

Amsalem E, Grozinger CM (2017) Evaluating the molecular, physiological and behavioral impacts of CO_2 narcosis in bumble bees (Bombus impatiens). J Insect Physiol 101:57–65

Beekman M, Ratnieks FL (2003) Power over reproduction in social Hymenoptera. Philos Trans R Soc Lond Ser B Biol Sci 358(1438):1741–1753

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). Conserv Genet Resour 1(1):183

Butz VM, Dietz A (1994) The mechanism of queen elimination in two-queen honey bee (*Apis mellifera* L.) colonies. J Apicult Res 33(2):87–94

Cortopassi-Laurino M, Imperatriz-Fonseca VL, Roubik DW, Dollin A, Heard T, Aguilar I, Venturieri GC, Eardley C, Nogueira-Neto P (2006) Global meliponiculture: challenges and opportunities. Apidologie 37:275–292

Da Silva DLN, Zucchi R, Kerr WE (1972) Biological and behavioural aspects of the reproduction in some species of *Melipona* (Hymenoptera, Apidae, Meliponinae). Anim Behav 20(1):123–132

de Camargo CA (1972) Mating of the social bee *Melipona quadrifasciata* under controlled conditions (Hymenoptera, Apidae). J Kansas Entomol Soc 1:520–523 de Camargo CA (1979) Sex determination in bees. XI Production of diploid males and sex determination in *Melipona quadrifasciata*. J Apicult Res 18(2):77–84

de Souza EA, Trigo JR, Santos DE, Vieira CU, Serrão JE (2017) The relationship between queen execution and cuticular hydrocarbons in stingless bee *Melipona scutellaris* (Hymenoptera: Meliponini). Chemoecology 27(1):25–32

de Souza Santos-Filho P, de Araujo Alves D, Eterovic A, Imperatriz-Fonseca VL, Kleinert A (2006) Numerical investment in sex and caste by stingless bees (Apidae: Meliponini): a comparative analysis. Apidologie 37(2):207–221

Eleutério P, Rocha EE, Freitas BM (2022) Production of new colonies of *Melipona subnitida* Ducke (Hymenoptera: Apidae) by reclamation of excess virgin queens. J Apicult Res 61:1–11

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(6):539–548

Fierro MM, Cruz-López L, Sánchez D, Villanueva-Gutiérrez R, Vandame R (2011) Queen volatiles as a modulator of *Tetragonisca angustula* drone behavior. J Chem Ecol 37(11):1255–1262

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*. J Exp Biol 223(18):230599. https://doi.org/10.1242/jeb.230599

Garcia Bulle Bueno F, Garcia Bulle Bueno B, Buchmann G, Heard T, Latty T, Oldroyd BP, Hosoi AE, Gloag R (2022) Males are capable of long-distance dispersal in a social bee. Front Ecol Evolut 10:149

Garcia Bulle Bueno F (2021) Native bees as alternative crop pollinators: reproductive behaviour of *Tetragonula carbonaria*

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. J Apicult Sci 56(1):159–167

Gillard TL, Oldroyd BP (2020) Controlled reproduction in the honey bee (Apis mellifera) via artificial insemination. Adv Insect Physiol (Elsevier) 59:1–42

Gilley DC (2001) The behavior of honey bees (*Apis mellifera ligustica*) during queen duels. Ethology 107(7):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 Soc 54(4):412–417

Greco MK, Spooner-Hart RN, Beattie AG, Barchia I, Holford P (2011) Australian stingless bees improve greenhouse *Capsicum* production. J Apic Res 50(2):102–115

Green CL, Oldroyd BP (2002) Queen mating frequency and maternity of males in the stingless bee *Trigona carbonaria* Smith. Insectes Soc 49(3):196–202

Green CL, Franck P, Oldroyd BP (2001) Characterization of microsatellite loci for *Trigona carbonaria*, a stingless bee endemic to Australia. Mol Ecol Resour 1(1–2):89–92

Grüter C (ed) (2020) Stingless bees: an overview. In: Stingless bees. Springer, Berlin

Hall MA, Jones J, Rocchetti M, Wright D, Rader R (2020) Bee visitation and fruit quality in berries under protected cropping vary along the length of polytunnels. J Econ Entomol 113(3):1337–1346

Heard TA (2016) The Australian native bee book: keeping stingless bee hives for pets, pollination and sugarbag honey. Sugarbag Bees, Brisbane

Imperatriz-Fonseca V, Zucchi R (1995) Virgin queens in stingless bee (Apidae, Meliponinae) colonies: a review. Apidologie 26(3):231–244

Imperatriz-Fonseca V, Matos E, Ferreira F, Velthuis H (1998) A case of multiple mating in stingless bees (Meliponinae). Insectes Soc 45(2):231–233

Jaffé R, Pope N, Carvalho AT, Maia UM, Blochtein B, de Carvalho CAL, de Fátima Ribeiro M (2015) Bees for development: Brazilian survey reveals how to optimize stingless beekeeping. PLoS ONE 10(3):121157

Jarau S, Van Veen JW, Aguilar I, Ayasse M (2009) Virgin queen execution in the stingless bee *Melipona beecheii*: the sign stimulus for worker attacks. Apidologie 40(4):496–507

Kärcher MH, Menezes C, Alves DA, Beveridge OS, Imperatriz-Fonseca V-L, Ratnieks FL (2013) Factors influencing survival duration and choice of virgin queens in the stingless bee *Melipona quadrifasciata*. Naturwissenschaften 100(6):571–580

Kendall LK, Stavert JR, Gagic V, Hall M, Rader R (2022) Initial floral visitor identity and foraging time strongly influence blueberry reproductive success. Basic Appl Ecol 60:114–122

Kerr WE, Zucchi R, Nakadaira JT, Butolo JE (1962) Reproduction in the social bees (Hymenoptera: Apidae). J N Y Entomol Soc 1:265–276

Kleinert A (2005) Colony strength and queen replacement in *Melipona marginata* (Apidae: Meliponini). Braz J Biol 65(3):469–476

Koeniger N, Koeniger G (2007) Mating flight duration of *Apis mellifera* queens: as short as possible, as long as necessary. Apidologie 38(6):606–611

Lau IH, Hereward JP, Smith TJ, Heard TA, Walter GH (2022) Inter-colony fights in Tetragonula stingless bees result in temporary mixed-species worker cohorts. Apidologie 53(4):1–13

Mackensen O (2014) Effect of carbon dioxide on initial oviposition of artificially inseminated and virgin queen bees. J Econ Entomol 40(3):344–349

Melo GA, Buschini MLT, Campos LA (2001) Ovarian activation in *Melipona quadrifasciata* queens triggered by mating plug stimulation (Hymenoptera, Apidae). Apidologie 32(4):355–361

Menezes C, Vollet-Neto A, Imperatriz-Fonseca VL (2013) An advance in the in vitro rearing of stingless bee queens. Apidologie 44(5):491–500

Nogueira-Ferreira F, Silva-Matos E, Zucchi R (2009) Interaction and behavior of virgin and physogastric queens in three Meliponini species (Hymenoptera, Apidae). Genet Mol Res 8(2):703–708

Nunes TM, Heard TA, Venturieri GC, Oldroyd BP (2015) Emergency queens in *Tetragonula carbonaria* (Smith, 1854) (Hymenoptera: Apidae: Meliponini). Austral Entomol 54(2):154–158

Oxley PR, Hinhumpatch P, Gloag R, Oldroyd BP (2010) Genetic evaluation of a novel system for controlled mating of the honeybee, *Apis mellifera*. J Hered 101(3):334–338

Plate M, Bernstein R, Hoppe A, Bienefeld K (2019) The importance of controlled mating in honeybee breeding. Genet Sel Evol 51(1):74

R Core Team (2013) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna

Röseler P-F (1985) A technique for year-round rearing of *Bombus terrestris* (Apidae, Bombini) colonies in captivity. Apidologie 16(2):165–170

Silva DD (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

Smith T (2020) Evidence for male genitalia detachment and female mate choice in the Australian stingless bee *Tetragonula carbonaria*. Insectes Soc 67(1):189–193

Sommeijer M, Koedam D, Monge IA (1994) Social interactions of gynes and their longevity in queenright colonies of *Melipona favosa* (Apidae: Meliponinae). Netherlands J Zool 45(3–4):480–494

Van Oystaeyen A, Alves DA, Oliveira RC, do Nascimento DL, do Nascimento FS, Billen J, Wenseleers T (2013) Sneaky queens in Melipona bees selectively detect and infiltrate queenless colonies. Anim Behav 86(3):603–609

Van Veen JW, Sommeijer MJ (2000) Observations on gynes and drones around nuptial flights in the stingless bees *Tetragonisca angustula* and *Melipona beecheii* (Hymenoptera, Apidae, Meliponinae). Apidologie 31(1):47–54

Van Veen JW, Sommeijer MJ, Monge IA (1999) Behavioural development and abdomen inflation of gynes and newly mated queens of *Melipona beecheii* (Apidae, Meliponinae). Insect Soc 46(4):361–365

Veiga JC, Menezes C, Contrera FAL (2017) Insights into the role of age and social interactions on the sexual attractiveness of queens in an eusocial bee, *Melipona flavolineata* (Apidae, Meliponini). Sci Nat 104(3–4):31

Verdugo-Dardon M, Cruz-Lopez L, Malo EA, Rojas JC, Guzman-Diaz M (2011) Olfactory attraction of *Scaptotrigona mexicana* drones to their virgin queen volatiles. Apidologie 42(4):543–550

Vollet-Neto A, Koffler S, dos Santos C, Menezes C, Nunes F, Hartfelder K, Alves D (2018) Recent advances in reproductive biology of stingless bees. Insectes Soc 65(2):201–212

Vollet-Neto A, Imperatriz-Fonseca VL, Ratnieks FL (2019) Queen execution, diploid males, and selection for and against polyandry in the Brazilian stingless bee *Scaptotrigona depilis*. Am Nat 194(5):725–735

Walsh PS, Metzger DA, Higuchi R (1991) Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques 10(4):506–513

Wenseleers T, Hart AG, Ratnieks FL, Quezada-Euán JJ (2004) Queen execution and caste conflict in the stingless bee *Melipona beecheii*. Ethology 110(9):725–736

Wenseleers T, Alves DA, Francoy TM, Billen J, Imperatriz-Fonseca VL (2011) Intraspecific queen parasitism in a highly eusocial bee. Biol Let 7(2):173–176

Yamane S, Sakagami SF (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(2):275–296