



The queens of the stingless bees: from egg to adult

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Abstract

Queens play an essential role in the colonies of stingless bees. Typically, only one queen occurs in a colony at any time, and she dominates the egg laying. Their presence maintains colony cohesion and ensures the development and survivorship of these matriarchal societies. Yet there remain significant gaps in our knowledge of queen life cycles as compared to their daughters, the workers. In this review, we follow chronologically the life of queens from pre-emergence inside brood cells (caste determination), to their interaction with workers (queen selection) and males (sexual selection), and up to adulthood. Stingless bee queens can be determined either trophically or genetically. After emergence, the virgin queens undergo a selection process whereby many are executed by workers. The body size, pheromones, age and behaviour of virgin queens may play a role in queen selection. Queens then leave the nest on a nuptial flight during which they mate just once. After mating, queens are still susceptible to workers' harassment. For example, if they produce diploid males they are killed by workers. Previous studies have successfully in vitro reared and mated virgin queens under laboratory conditions, which have revealed new insights of queen development time, the threshold of minimum and maximum provided food to larvae developing into queens, and lethal and sublethal effects of agrochemical substances. These new techniques have also provided new information about queen physiology. However, the daily routine of queens inside colonies demands further well-designed experiments to capture other patterns of behaviour which remain poorly understood, including their interactions with other queens, workers and even males.

Keywords Caste determination · In vitro rearing · Gynes · Mating · Pesticides · Sexual pheromones · Queen selection

Stingless bees

The stingless bees (Hymenoptera: Apoidea: Meliponini) are a group of highly eusocial bees native to the world's tropical and subtropical regions (Michener 1974). They are the most diverse group of social bees, with an estimated 550 species belonging to ~ 58 genera (Grüter 2020). These are divided between three monophyletic clades—Neotropical, African and Indo-Malayan-Australasian—that diverged from each other 50–70 million years ago (Rasmussen and Cameron 2010), with the Neotropics harbouring most of the species diversity (> 400 species). In all stingless bees, the queen is the sole reproductive female in the colony capable of laying female (diploid) eggs, whereas the female workers forage for food, protect the colony against predators and care for the queen's brood (Michener 1974).

Stingless bees are common flower visitors in tropical ecosystems due to the high number of species, the fact that each colony contains thousands to tens of thousands of

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workers, differences in morphology and foraging strategies (Michener 1974; Grüter 2020). Many stingless bee species are already propagated in hives for use in crop pollination, honey production and by hobby, which is called meliponiculture (Heard 1999; Cortopassi-Laurino et al. 2006; Slaa et al. 2006; Jaffé et al. 2015). Hence, the ability to rear and mate stingless bee queens underpins the efforts to accelerate the propagation of managed colonies and be used in conservation (Menezes et al. 2013; Jaffé et al. 2015). 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 (Imperatriz-Fonseca and Zucchi 1995; Menezes et al. 2013; Jaffé et al. 2015). The stingless bee queens' basic biology remains poorly understood due to their habits. When unmated, many species hide inside the nest avoiding execution from the workers, once accepted they only leave the nest to mate and once mated they are constantly guarded by workers (Imperatriz-Fonseca and Zucchi 1995).

In this review, we synthesise what is currently understood about the life history of stingless bee queens, including larval development, sexual maturation, social interactions, mating and pheromone production. We highlight recent advances but also point to key gaps that remain in our knowledge of queen biology.

To identify relevant studies for our review, we first revised all articles citing Imperatriz-Fonseca and Zucchi (1995), which summarized many aspects of the stingless bee queens. After that, we looked for more recent data so far by searching keywords as “stingless bees AND queens”, “Stingless bees AND reproduction” and “stingless bee gynes” in databases including ISI Web of Science (<https://www.webofknowledge.com>), Google Scholar (<https://scholar.google.com/>), and Scielo (<https://scielo.org>). The assessed studies were used to build up this review and additionally, all authors contributed with information based on their knowledge of the stingless bee literature.

Virgin queen (gynes)

Queen production

In stingless bees, new queens may be determined genetically, as in the *Melipona* genus (although there is a slightly plastic ratio, which is affected by nutritional conditions) (Kerr 1950; Hartfelder et al. 2006; Brito et al. 2015), or trophically, whereby any female larvae that are reared in larger brood cells and, consequently, receive more larval food than the larvae in regular brood cells, will develop as queens (Fig. 1a–e). Additionally, in a few stingless bee species (e.g. *Frieseomelitta varia*), larvae reared in workers' cells may perforate auxiliary cells and eat extra food there,

also then developing as queens (Terada 1974; Faustino et al. 2002; Nunes et al. 2015; Luz et al. 2017) (Fig. 1F). Commonly, queen and worker castes in stingless bees are well-defined physiologically, morphologically and behaviourally. However, it has been observed that adult female bees can sometimes possess both corbiculae and spermatheca, making them intercastes (Hartfelder and Engels 1992; dos Santos et al. 2016a). The process that triggers the intercaste condition is unclear.

In some species, as *Nannotrigona testaceicornis*, *Plebeia remota* and *Schwarziana quadripunctata*, alternative queen phenotypes, known as dwarf or miniature queens, may also occur and head new colonies (Camargo 1974; Imperatriz-Fonseca et al. 1997; Wenseleers et al. 2005; Ribeiro et al. 2006; Nogueira-Ferreira et al. 2009). Miniature queens are consistent with the theory that under some conditions, female larvae in social insects should self-determine their own developmental fate (Nonacs and Tobin 1992; Bourke and Ratnieks 1999; Ratnieks 2001; Wenseleers et al. 2003). It means that larvae reared in brood cells destined to become workers might adopt a selfish strategy by evading the worker fate and developing into miniature queens (Wenseleers et al. 2005; Ribeiro et al. 2006).

A substance consumed by female larvae has been known to be a factor affecting caste determination of *Melipona* genus, in which queens are genetically determined (Kerr 1950; Brito et al. 2015). It was suggested that a cohort of adult bees (nurse workers) could apparently coerce female larva to become queens by adding geraniol to larval food (Jarau et al. 2010). Geraniol can also be found in pollen from different plant species, and it has been hypothesized by van Veen (2018) that the availability of fresh pollen, which in *Melipona beecheii* is collected in short periods in relatively large quantities, coinciding with gyne production in the colonies, may function as a natural regulatory mechanism which limits the excess production of virgin queens to periods of swarming and supersedure. Contrary, recent research re-examined these hypotheses by artificially increasing the levels of geraniol on female larvae food and revealed no effect of the substance on the rearing of new queens (Oliveira et al. 2022).

In the majority of stingless bee species in which the amount of food is important for caste determination (non-*Melipona* genus) (Hartfelder et al. 2006; Grüter 2020). However, could female larvae evade their caste fate? The development of protocols to rear stingless bee queens in vitro has helped to answer this question (Prato 2010; Baptistella et al. 2012; Menezes et al. 2013; dos Santos et al. 2016a). These studies have revealed that there is a threshold for the amount of larval food offered to female larvae, below which the larva becomes a worker. Conversely, volumes of the same larval food above this threshold, and within a larger brood cell, the royal cell, lead to all female larvae developing into queens

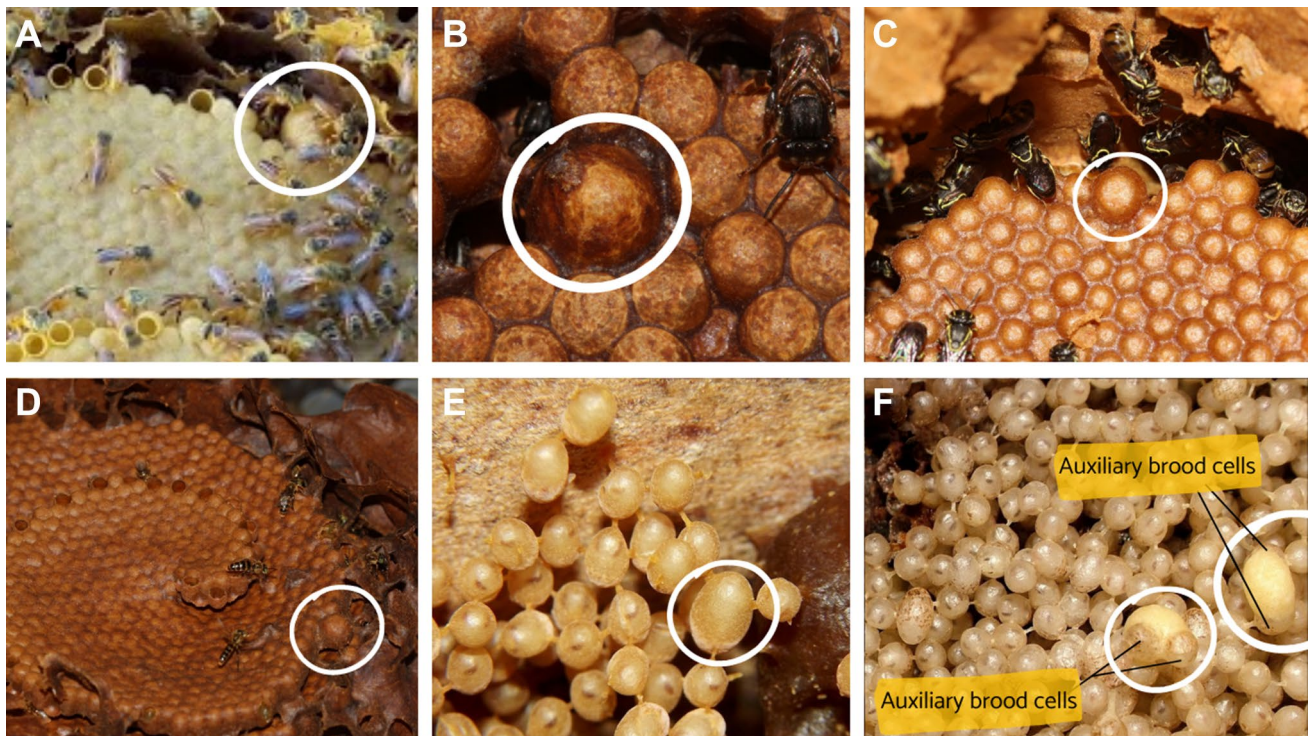


Fig. 1 Royal brood cells (white ellipses) of some stingless bee species on: horizontal (disc-like) brood combs of **A** *Tetragonisca angustula*, **B** *Scaptotrigona* aff. *depilis* and **C** *Paratrigona subnuda*; Spiral brood comb of **D** *Tetragona clavipes* and clustered brood cells of **E** *Plebeia minima* and **F** *Leurotrigona muelleri*. Note: commonly the royal brood cells are built in the edge of combs as in A, C, D, except

B. However, some species that build clustered brood cells may construct the royal cells randomly distributed, depending on whether the larvae are consuming extra larval food adjacent to auxiliary cells as in F. Image source: A—Charles F. dos Santos; B-F retrieved from Fototeca Cristiano Menezes <http://splink.cria.org.br/manager/detail?setlang=pt&resource=FCM>

(Prato 2010; Baptistella et al. 2012; Menezes et al. 2013; dos Santos et al. 2016a). In general, the quantity of larval food provided to potential queens is, at least, twice as much as that given to potential workers (Table 1). This appears to guarantee the emergence only of queens, since to date no “giant worker” has been observed emerging from royal cells, except giant males (references in Table 1).

In some stingless bee species with trophically determined castes, there is an alternative pathway by which eggs laid into worker cells can develop as standard-sized queens.

Female larvae may access either auxiliary cells (lacking larva) or neighbouring brood cells (containing eggs, or young larvae) and ingest the additional food stored there, consequently developing as new queens (*Frieseomelitta varia*, *Leurotrigona muelleri*, *Plebeia lucii* and *Tetragonula carbonaria*) (Terada 1974; Faustino et al. 2002; Nunes et al. 2015; Luz et al. 2017). When this occurs in colonies that have been orphaned, such a process can be considered a mechanism for emergency queen production. For example, queenright colonies of *Tetragonula carbonaria* build royal

Table 1 Larval food (in μL) ratio between queens and workers in stingless bee queens

Species	Volume (queen: worker)	Ratio	References
<i>Frieseomelitta varia</i>	61.7: 26.7	2.3:1	Baptistella et al. 2012
<i>Nannotrigona testaceicornis</i>	45: 20	2.2:1	Prato 2010
<i>Plebeia droryana</i>	60: 8.4	7.1:1	Santos et al. 2015
<i>Scaptotrigona</i> aff. <i>depilis</i>	130: 31	4.2:1	Menezes et al. 2013
<i>Scaptotrigona postica</i>	140: 35	4.0:1	Hartfelder and Engels 1992
<i>Tetragonisca angustula</i>	51.5: 7.85	6.5:1	Prato 2010
<i>Partamona helleri</i>	80: 40	2.0:1	Araujo et al. 2019
<i>Trigona spinipes</i>	360: 36	10.0:1	Lisboa et al. 2005

cells in which to produce their new queens, and build auxiliary cells for queen production only when queenless (Nunes et al. 2015). On the other hand, in *Leurotrigona muelleri*, larvae can themselves perforate neighbouring brood cells (containing eggs, or young larvae) and consume their food content, turning into a new queen (Terada 1974).

This special case of queen emergence has two features in common to all species where it has to date been observed: (i) fusion of two neighbouring cells and (ii) no occurrence of males emerging from those fused cells (Terada 1974; Faustino et al. 2002; Nunes et al. 2015; Luz et al. 2017). When workers build auxiliary cells to raise emergency queens, they may recognise brood cells containing female larvae and ignore those with male larvae. However, to date there is no information on whether particular larvae are chosen at the expense of another or even if such larvae emit any type of signal to be chosen (Grüter 2020). Likewise, when larvae perforate adjacent cells themselves, male larvae might refrain from doing so because presumably there is no fitness advantage to being a “giant male”. Those oversized males do occur in several species, as rare events in some (*Tetragonula carbonaria*; Gloag et al. 2007) and common in others (*Friesella schrottkyi*, *Paratrigona subnuda*, *Plebeia droryana*, *P. emerina*, *P. remota*, *Scaptotrigona postica*, *Schwarziana quadripunctata*) (Camillo 1971; Bego and Camargo 1984; Imperatriz-Fonseca 1976; Santos-Filho et al. 2006; Alves et al. 2009). These males take longer to develop and produce more sperm cells (Camillo 1971), though it is yet to be determined if they are capable of fertilizing queens (Grüter 2020).

Queen selection by workers

Since stingless bee colonies are usually monogynic and monandric (i.e. contain only a single mated queen), any excess of virgin queens emerging in colonies are commonly killed by the workers or expelled from the colony shortly after emergence (e.g. *Melipona* genus) (Silva et al. 1972; Imperatriz-Fonseca and Zucchi 1995; Sommeijer et al. 2003; Jarau et al. 2009). Koedam et al. (1995) supposed that the competition between mated and unmated queens could be a mechanism by which the quality of the egg-laying queen is continuously tested by workers. Under queenless conditions, the enlargement (inflation) of the abdomen of gynes was found to be crucial for acceptance (van Veen et al. 1999; Kleinert 2005; Kärcher et al. 2013). *Melipona beecheii* gynes that are not able to escape from worker aggression through hiding inside empty food pots or “feigning death” are killed by workers shortly after emergence (Koedam et al. 1995; van Veen et al. 1999; Kleinert 2005; Kärcher et al. 2013). Only 19.6% of gynes survived the first 12 h after emergence, all survivors had enlarged abdomens, indicating that this condition in

queens might be more accepted or tolerated by the workers (van Veen et al. 1999). Similarly, gynes of *Melipona favosa* with an enlarged abdomen have an increased number of workers in the court around them (Koedam et al. 1995). Abdominal inflation is likely associated with the activation of the abdominal gland, indicating pheromone production and therefore acceptance by the workers (van Veen et al. 1999). Thus, the success of gynes with inflated abdomens suggests that queen pheromones play an important role in the acceptance of virgin queens by workers.

In honeybees, queens have a functional stinger that can be used multiple times, and when new queens emerge in queenless colonies they will typically kill all the rival queens (Butz and Dietz 1994). In contrast, the constant supply of new queens in stingless bees may give more power over queen selection to workers. Workers rear the brood and so control queen production. Workers outnumber the virgin queens and so can select the best one by killing the others (Koedam et al. 1995; Wenseleers et al. 2004; Jarau et al. 2009; Kärcher et al. 2013). This strategy comes with the obvious risk that if all new queens fail, the colony will be left without an egg-laying female and unable to produce new worker brood, leading the colony to die (Beekman and Ratnieks 2003).

Most stingless bee species are believed to retain multiple virgin queens at all times as a way of insurance against death of the mated queen (Kerr et al. 1962; Nogueira-Ferreira et al. 2009; Inoue et al. 1984; Engels and Imperatriz-Fonseca 1990; Bueno et al. 2020). Up to 44 “spare queens” have been observed in *Schwarziana quadripunctata* (Nogueira-Ferreira et al. 2009). This strategy enables a rapid and active queen replacement in the event that a queen is producing diploid male offspring (Vollet-Neto et al. 2019). For example, stingless bee queens producing diploid males tend to be killed by their workers (Camargo 1979; Alves et al. 2011; Vollet-Neto et al. 2017, 2019).

In *Melipona scutellaris*, the life expectancy of queens that produced diploid males was half of those producing haploid males (Alves et al. 2011). Similarly, in *Melipona quadrifasciata* and *Scaptotrigona depilis*, the emergence of diploid males triggers queen execution, usually within 6–30 days after the first diploid males begin to emerge (Camargo 1979; Vollet-Neto et al. 2017). This timing is related to the moment when the cuticular hydrocarbons of haploid and diploid males are different, suggesting that the queen’s execution might be triggered by these chemical differences (Vollet-Neto et al. 2017). Workers may decide to execute queens for other motives too, though which other cues might trigger regicide in stingless bees remains understudied.

Under queenright situations, most virgin queens are killed by workers soon after emergence or whenever they become sexually attractive (Imperatriz-Fonseca and Zucchi 1995;

Wenseleers et al. 2004; Jarau et al. 2009; Kärcher et al. 2013; de Souza et al. 2017).

In *Melipona* species, it is common to find several virgin queens fallen in front of their colonies. However, there is no consensus if such individuals are trying to escape from workers' harassments or whether they are expelled by them (Sommeijer et al. 2003; Wenseleers et al. 2011).

Once the resident queen is removed or dies, the virgin queen(s) are quick to appear on the brood comb; sometimes even within 30 min (*Tetragonula carbonaria*) (Bueno 2021). From the body posture of the virgin queens, and the trophallaxis that occurs, it can be assumed that there is a hierarchy among them (Silva 1972; Imperatriz-Fonseca and Zucchi 1995). The selection and acceptance of a new queen may depend on the interaction of several factors.

a) Body size

Body size and mass seem to play a role in queen selection and attractiveness in some stingless bee species. For example, in *Schwarziana quadripunctata*, a species that sometimes produces miniature queens, workers prefer large queens (Nogueira-Ferreira et al. 2000; Ribeiro and Alves 2001; Wenseleers et al. 2005). Indeed, workers in this species kill most miniature queens and cage many large queens in imprisonment chambers (or royal chambers) to keep them as insurance against queen loss (Engels and Imperatriz-Fonseca 1990; Ribeiro et al. 2006). Workers might respond to fertility indicators shown by morphological, pheromonal or behavioural patterns in the miniature queens (Ribeiro et al. 2006). However, in *Melipona quadrifasciata*, body mass does not seem to determine the acceptance of the queens (Kärcher et al. 2013).

b) Chemical profile

Stingless bee workers recognise virgin queens by the chemical profile of hydrocarbons on their cuticle, which can indicate age, caste and sex (Leonhardt 2017). This cuticular hydrocarbon profile differs between castes (Abdalla et al. 2003; Kerr et al. 2004; Grajales-Conesa et al. 2007; Ferreira-Caliman et al. 2013) and they confer information about the reproductive status and fertility of the queens (Jarau et al. 2009; Veiga et al. 2017), as well as signalling their presence and suppressing ovary activation in workers (Nunes et al. 2014).

At the peak of attractiveness, the virgin queens from several stingless bee species inflate their abdomens constantly, move very quickly around the nest requesting food from the workers and exposing their tergite pockets as they walk around, presumably to release a pheromone

(Imperatriz-Fonseca and Zucchi 1995; Jarau et al. 2009; Kärcher et al. 2013). These abdominal inflations seem crucial during the interactions with workers indicating a complex interaction between pheromones emitted by different parts of the body from the queens (Alves et al., unpublished data). This behaviour stimulates the worker's courtship behaviour towards her (Kärcher et al. 2013), but it may also trigger persecution and execution (Jarau et al. 2009).

c) Sexual maturation

Age might be related to the secretion of specific chemical compounds that dictate the workers' preference for some virgin queens over the other ones (Silva et al. 1972). In an experimental set-up with the Australian species *Tetragonula carbonaria*, when three queens were introduced to small queenless colonies, the workers preferred the first one to be introduced (also the oldest one). The subsequent queens (introduced into the nest box one day later and also one day younger than the queen introduced one day before) remained secluded in the trash deposit of the nest box. These younger queens were ignored by the workers and were finally killed (Bueno 2021; Bueno et al. 2022b). Similarly, another study found that newly emerged virgin queens have higher chances of becoming the new resident queen if they emerge shortly after the removal of the physogastric queen (24–48 h) and if they are successful in hiding and avoiding worker attacks until queen choice takes place (Kärcher et al. 2013). The age of workers interacting with new queens is also important, since virgin queens introduced into small colonies containing both callow (younger) and mature (older) workers are often rejected, while those introduced into colonies with only callow workers presented a rate of acceptance above 75% (dos Santos et al. 2015).

d) Behaviour

It has been assumed that queen selection is related to a virgin queen's dominance over the workers, as well as other virgin queens. This dominance is established by the queen's aggression towards the workers. Some studies have observed unmated queens of stingless bees grouped in areas like the involucre (Kleinert and Imperatriz-Fonseca 1994) and establishing dominance hierarchies (Engels and Imperatriz-Fonseca 1990) without aggressive interaction among them (Wenseleers et al. 2004; Kärcher et al. 2013). In *Melipona quadrifasciata*, aggression among virgin queens has rarely been observed, but fights may occur between the resident queen and one or more virgin queens (Kärcher et al. 2013). This lack of aggression between gynes is interesting given the large number of young queens attempting to become the

resident queen in some stingless bee species (i.e. *Melipona*). Given their lack of a stinger, stingless bee virgin queens could kill another queen only through biting, which might endanger both virgin queens and risk leaving no unharmed winner, jeopardizing the survival of the entire colony. Presumably for the same reason, virgin queens are no threat to a physogastric queen and instead are continuously executed by workers (Peters et al. 1999). Nevertheless, queen aggression may occur in some species. Two or more *Tetragonula carbonaria* virgin queens in a queenless colony were seen chasing one another, exchanging brief aggressive interactions (Bueno et al. 2022a), and *Plebeia remota* virgin queens may also attack one another (Ribeiro et al. 2003).

Some behaviours possibly linked to the queen's survival are resting motionless, running and performing rapid turns to avoid being attacked by workers, and hiding around inside empty food storage pots (Engels and Imperatriz-Fonseca 1990; van Veen et al. 1999; Kärcher et al. 2013). In *Melipona quadrifasciata* however, behaviours such as running, walking, antennating or trophallaxis played little or no role, as did the factors body mass or nestmate (Kärcher et al. 2013). These behaviours may vary between species, and further studies are needed to understand queen succession, and the role of such behaviours in queen selection and survival.

e) Relatedness

Current evidence suggests that genetic relatedness does not play a strong role in queen selection, since foreign virgin queens are accepted by colonies at similar rates than their nestmates. In *Melipona quadrifasciata*, queen acceptance rates were not affected by whether a virgin queen was a nestmate or was introduced from another colony (Kärcher et al. 2013). Beekeeping practices in many species include transplanting brood between colonies, whereby queens that hatch from the donor brood cells are readily accepted (Nogueira-Neto 1997).

The willingness of stingless bees eventually to accept unrelated queens helps explain why some species are susceptible to infiltration by unrelated, foreign queens (Reis et al. 2011; Wenseleers et al. 2011; van Oystaeyen et al. 2013; Jaffé et al. 2014), and even the queens of other stingless bee species (Cunningham et al. 2014). However, in this latter situation, it differs from queen parasitism in *Melipona*, since colonies are taken by force and many adult workers are killed and only callow workers and brood are enslaved and accept the incoming queen (Cunningham et al. 2014). In *Melipona scutellaris* the invading queen's strategy to successfully infiltrate an unrelated and queenless colony is associated with her time of arrival at the nest, with success more likely at late hours of the day (after 6:00 p.m.) when guards have lower levels of activity and are less efficient

(van Oystaeyen et al. 2013). It remains a question for further studies to understand more about the recognition abilities of non-nestmate virgin queens within a colony (Monteiro and Kerr 1990); specifically, how they can outmanoeuvre the guards, given that guards are very good at recognising non-nestmates (Breed and Page Jr 1991; Grüter 2020).

Acceptance inside the nest

Ultimately, the “accepted” virgin queens are those that are active in the area where the new brood cells are built (Silva et al. 1972), indicating a strong relationship between dominance and territory (Imperatriz-Fonseca 1977). Accepted queens (mated or not yet mated) are fed by workers via trophallaxis (Silva et al. 1972; Engels and Imperatriz-Fonseca 1990) and may perform “dominance dances” in which the queen shakes her inflated abdomen, exposed their pheromone glands and touches workers with it (Engels and Imperatriz-Fonseca 1990; Imperatriz-Fonseca and Zucchi 1995).

Queen-male interaction, mating and fertilization

Queen mating under natural conditions

In stingless bees, males engage in neither courtship nor territory defense (Engels et al. 1990; Roubik 1990). Instead, mate selection occurs via indirect male-male competition. Males aggregate in large numbers near colonies where there are new virgin queens to be fertilized (Cortopassi-Laurino 2007; dos Santos et al. 2014). Similarly, these males can aggregate near new nests that have been founded by workers (van Veen and Sommeijer 2000a; dos Santos et al. 2016b). In both cases, the males typically wait several days for the nuptial flight of virgin queens (Van Veen and Sommeijer 2000b; Cortopassi-Laurino 2007; dos Santos et al. 2014). Even though males commonly aggregate in front or near the nests, virgin queens may fly far from their nests, moment in which they are pursued by several males waiting for her flight (Kerr et al. 1962; Silva et al. 1972; Roubik 1990). The exact distance of the queen's nuptial flight is unknown, but from the intertegular distance, it was estimated to be between 420 to 966 m, for *Plebeia droryana* and *Scaptotrigona depilis*, respectively (Table 2). The reproductive system, the chemical profile and the size of the glands are all less complex and activated in virgin than in physogastric queens (Cruz-Landim et al. 2005; Abdalla 2006) (Fig. 2A-D). However, after copula, the newly mated queen will store the semen of the successful male in her spermathecae (Fig. 2E) and the abdomen will enlarge considerably due to ovary activation and the intensification of oogenesis in the

Table 2 Estimated flight range of stingless bee queens based on their intertegular distance

Species	Rearing conditions	Intertegular span (mm)	Estimated flight range (m)	References
<i>Plebeia droryana</i>	Natural	1.484	433	dos Santos et al. 2015
<i>Plebeia droryana</i>	In vitro	1.465	420	
<i>Scaptotrigona</i> aff. <i>depilis</i>	Natural	2.050	914	Menezes et al. 2013
<i>Scaptotrigona</i> aff. <i>depilis</i>	In vitro	2.100	966	

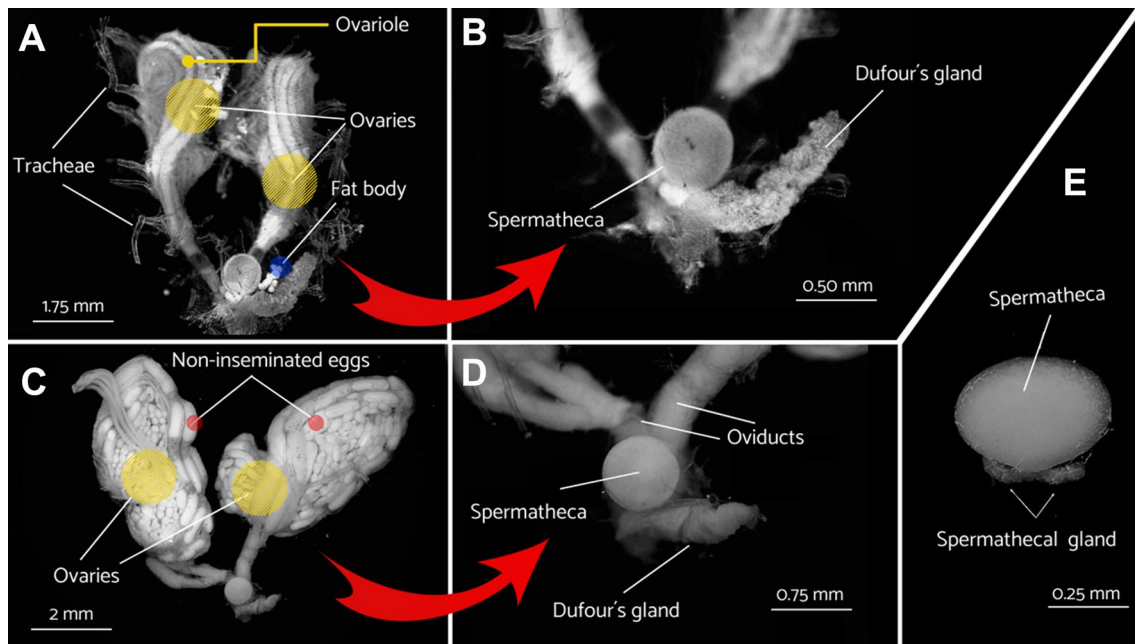


Fig. 2 Reproductive systems of virgin (A–B) and mated queen (C–D) of *Tetragnisca angustula*. A—The entire reproductive apparatus of a virgin queen; B—Details of the spermatheca and Dufour's gland from A; C—The entire reproductive apparatus of a mated, physogastric queen; D—Details of the spermatheca and Dufour's gland from C; E—Isolated spermatheca of mated, egg-laying queen of *Scaptotrigona* aff. *depilis*. Notes: The ovariole is the basic unit of egg pro-

duction, i.e. the functional unit of the ovary (hatched yellow circles); the spermatheca is a sperm reservoir that stocks sperm from the male; a large number of mature eggs (hatched red circles in C) and other developing eggs can be seen in the activated ovaries of mated queens; the hatched blue circle (A) shows a fragmented fat body (adipocytes) that is a tissue with the functions as storing and releasing of nutrients for growth and reproduction of insects

ovaries (Melo et al. 2001; de Souza et al. 2007; Tanaka et al. 2009; Bueno et al. 2020).

How do males locate colonies with virgin queens ready to mate? 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 (Bueno et al. 2022a). Three hypotheses have been suggested to explain male attraction to nests that are requeening: (i) 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)), (ii) workers that leave the nest (e.g. foragers) spread a pheromone outside of the nest (Engels et al. 1990; Roubik 1990) or (iii) virgin queens inside colonies produce a pheromone that wafts out from nest entrances (Engels 1987; Fierro et al. 2011; Verdugo-Dardon et al. 2011). To date, this third

scenario has been most studied, via efforts to identify unique chemical compounds produced by virgin queens.

Virgin queens do release highly volatile chemical compounds that can attract males (Engels et al. 1997; Grajales-Conesa et al. 2007; Fierro et al. 2011; Verdugo-Dardon et al. 2011; Araújo et al. 2017). Such volatile compounds may be present in the cephalic glands of *Scaptotrigona* species (Engels et al. 1997; Grajales-Conesa et al. 2007; Verdugo-Dardon et al. 2011) or in abdominal glands such as Dufour's (Fig. 2D, E) and tergal glands of *Tetragnisca angustula* (Fierro et al. 2011; Araújo et al. 2017).

In *Tetragnisca angustula*, the candidates for sexual pheromones are the isopropyl hexanoate, butyl hexanoate and hexyl hexanoate present in abdominal glands (Fierro et al. 2011; Araújo et al. 2017). In contrast, in *Scaptotrigona* species it is the 2-alcohols present in the cephalic glands of

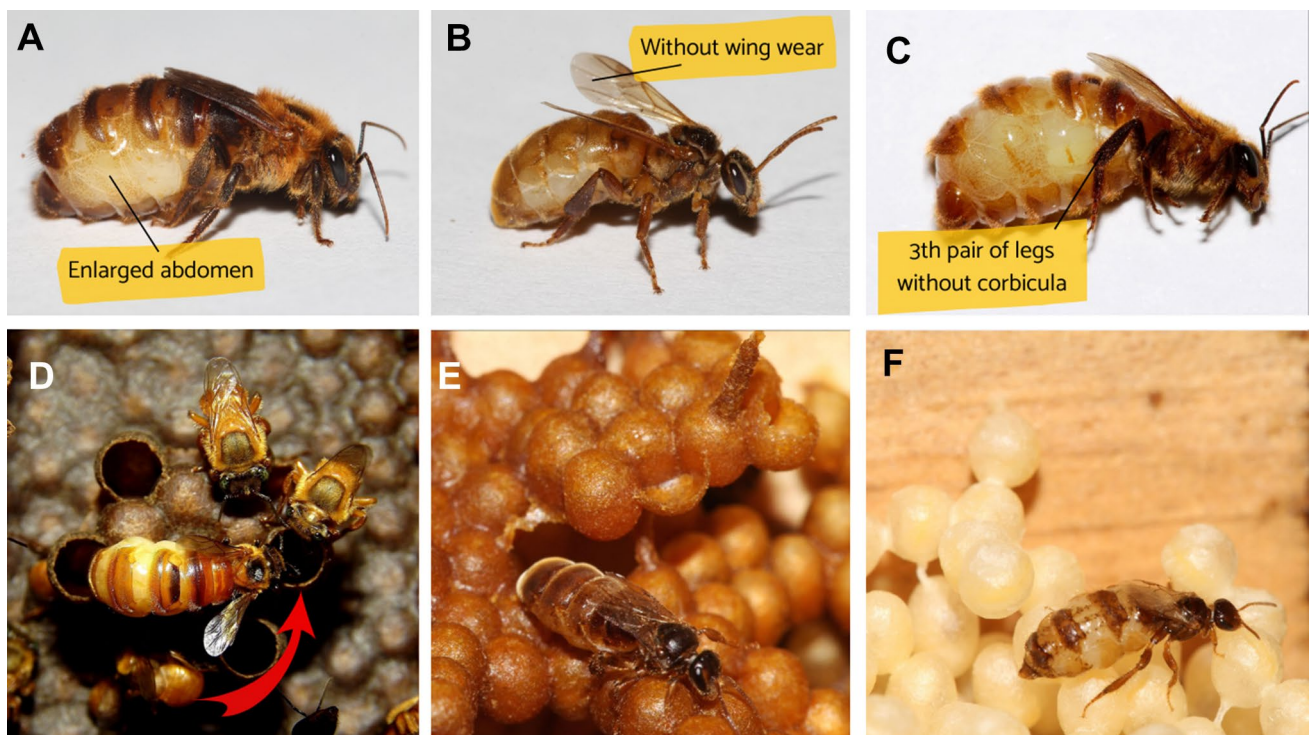


Fig. 3 Physogastric queens of some stingless bee species: **A** *Melipona melanoventer*, **B** *Frieseomelitta flavicornis* **C**, **D** *Melipona flavolineata*, **E** *Friesella schrottkyi* and **F** *Leurotrigona muelleri*. Note: **A** Distended abdomen of physogastric queen as a result of over production of eggs, as shown in Fig. 3C. **B** Young physogastric

queen with their hindwings still worn. **C** The stingless bee queens do not have corbiculae. **D** The red arrow points towards a brood cell being inspected by a queen before oviposition. Images retrieved from Fototeca Cristiano Menezes <http://smlink.cria.org.br/manager/detail?setlang=pt&resource=FCM>

virgin queens that appear to attract males to copulate (Engels 1987; Engels et al. 1997; Verdugo-Dardon et al. 2011).

Male attraction to mated queens

It is well established that virgin queens in stingless bees copulate only once and with a single male (Kerr et al. 1962; Peters et al. 1999; Palmer et al. 2002; Vollet-Neto et al. 2018). Therefore, we could expect that mated and physogastric queens would lose their sexual attractiveness after mating. However, at least for Neotropical species, it has long been known that if colonies are artificially disturbed, the egg-laying queens may be harassed by males attempting copulation inside nests (Nogueira-Neto 1957; Sakagami and Laroca 1963; Imperatriz-Fonseca 1973; Cortopassi-Laurino 1979). Interestingly, some of the same volatile compounds produced by virgin queens are also produced by physogastric queens as observed for *Tetragonisca angustula* (Fierro et al. 2011; Araújo et al. 2017). Recently, it has been also observed that physogastric queens outside nests are as sexually attractive to aggregated males as virgin queens (Fierro et al. 2011; Araújo et al. 2017).

To date, there is no work experimentally demonstrating whether those molecules released by physogastric queens

outside nests, as aforementioned, are the same released when these queens are inside nests. However, it demonstrates that the power to sexually attract males to copula remains latent in egg-laying queens from at least seven species of stingless bees. For example, it was recorded and depicted during the opening of a natural nest of *Lestrimelitta ehrhardti* (Sakagami and Laroca 1963). Later, when a physogastric queen of *Schwarziana quadripunctata* egg-laid inside a brood cell, some nearby males attempted to copulate with her (Imperatriz-Fonseca 1973). After that, other cases have been rarely but continuously observed inside and outside nests or in confinement conditions as for *Paratrigona subnuda* (Imperatriz-Fonseca 1977), *Melipona quadrifasciata* (Campos and Melo 1990), *Scaptotrigona postica* (Engels and Engels 1988), *S. depilis* (Menezes, unpublished data) and *Tetragonisca angustula* (Fierro et al. 2011; Araújo et al. 2017). Outside the nest, physogastric queens perform a persistent and erratic behaviour in which they vibrate their wings, swell their abdomens and visually expose their abdominal glands (Araújo et al. 2017). As suggested by Araújo et al. (2017), even though *T. angustula* physogastric queens release chemical compounds sexually attractive to males outside nests, inside the nests such molecules may have other biological meaning to workers.

The male attempts at copulating (non-natural conditions) with physogastric queens in stingless bees is most likely a non-adaptive behaviour and costly to both physogastric queens and males. The evidence for this: (i) physogastric queens that are sexually harassed by males try to deter them with vigorous abdominal movements (Imperatriz-Fonseca 1973; Cortopassi-Laurino 1979), (ii) if mating occurs, then the physogastric queen may be seriously injured and die shortly afterwards (Campos and Melo 1990), (iii) workers assault the males involved, injuring their antennae and legs (Imperatriz-Fonseca 1977; Cortopassi-Laurino 1979), (iv) physogastric queens are able to use the spermatozoa from a second male (under laboratory conditions) even mated for a long time (Campos and Melo 1990; Lopes et al. 2003) (Campos and Melo 1990; Lopes et al. 2003). Moreover, such behaviour has not been selected through evolutionary history of stingless bee queens. The mating (v) with a highly related partner (matched mating) may make that queens producing diploid (infertile) males and they are killed by their workers (Camargo 1979; Vollet-Neto et al. 2017, 2019). If hypothetically successful in an inbreeding mating (vi) such males would waste the opportunity to fertilize unrelated queens, because after mating they lose their genitalia and die (Kerr and Krause 1950; Kerr et al. 1962).

The points raised above suggest that, when performing any management in queenright hives, the physogastric queens should be immediately removed or isolated from colonies to avoid any additional and unnecessary attempts of copulation by nearby males.

Queen mating under laboratory conditions

Queen-rearing under laboratory conditions is used to produce stingless bee queens on a large scale (Hartfelder and Engels 1992; Baptistella et al. 2012; Menezes et al. 2013; dos Santos et al. 2015; Bernardes et al. 2018). The controlled copulation or instrumental insemination of these queens after their emergence remains a challenge. Nuptial flights of lab reared queens are rare or risky (Menezes et al. 2013)—“the gyne waste”. Yet under laboratory conditions, the males may ignore the gynes or they may evert their genitalia without copulating with them (Veiga et al. 2017)—“the male waste”.

Even though mating of stingless bees is believed to usually occur naturally in flight and outside nests, some experiments have shown that sexually attractive virgin queens confined with a small number of males inside boxes (*Melipona quadrifasciata*, *M. flavolineata*, *Scaptotrigona postica*) may copulate under laboratory conditions (Camargo 1972; Engels and Engels 1988; Veiga et al. 2017). The age and glandular development of the queens and the social context (e.g. number and age of workers where virgin queens are sheltered) seem to be important factors determining their

sexual attractiveness to males (Engels and Engels 1988; Araújo et al. 2017; Veiga et al. 2017). Controlled mating procedures require further investigation.

Although using mating boxes allows some control over which individuals (virgin queens and males) will mate, this procedure still depends on male and virgin queen behaviour. As such, artificial insemination could give a greater control over the semen donor to a particular queen. Protocols to assess the sperm collection and quality already exist for *Scaptotrigona* aff. *depilis* (Menezes et al. 2014; Koffler et al. 2021) and could be adapted to other stingless bee species. However, its use to manually inseminate virgin queens under laboratory conditions remains to be tested. The artificial insemination technique was attempted 50 years ago with *Melipona bicolor* queens; however, they were inseminated with *Apis mellifera* sperm (Silva et al. 1972) and to date there is still no procedure available for any stingless bee species.

Exposure to agrochemicals

It is well known that (bio)pesticides can alter physiological aspects of non-target insects, such as stingless bees, consequently causing changes in their development, behaviour, and health (Barbosa et al. 2015; Rosa et al. 2016; Araujo et al. 2019; Dorneles et al. 2021; Almeida et al. 2022). In most social insects like stingless bees, the queens are required to fulfil some behaviours early in life as a prerequisite to be selected by workers as the new reproductive queen of the colony (Silva et al. 1972; Jarau et al. 2009; Veiga et al. 2017). If the queens do not have the optimal characteristics needed within this short and risky period, they are more likely to be killed by the workers than selected by the colony (Silva et al. 1972; Jarau et al. 2009; Veiga et al. 2017).

Development

Some studies have shown that larvae can have their caste fate changed by the action of pesticides. Furthermore, larval queens poisoned with residual doses of insecticides have shown delayed development, while emerged virgin queens presented altered behaviour and malformations (dos Santos et al. 2016a; Bernardes et al. 2018; Otesbelgue et al. 2018). For example, an organophosphorous insecticide changed larvae of *P. droryana* that were destined to become queens into workers (dos Santos et al. 2016a). Even the low-risk insecticide azadirachtin caused alterations in the development time, malformations and reduced reproductive organs in *Partamona helleri* queens (Bernardes et al. 2018). A reduction of 36% in the area of the reproductive system was found in exposed queens of *Partamona helleri* (Bernardes

et al. 2018). Additionally, the development larval period of queens exposed to agrochemicals increased from 4.5% to 20.5% depending on species (Table 3).

In other cases, exogenous substances may induce an elevated queen production in stingless bees. The insecticides that mimic juvenile hormones (JH) in young insects (i.e. insect growth regulators) applied during the cocoon-spinning phase (predefaecating larvae) in some stingless bee species can induce most female larvae to develop into queens (Campos and Coelho 1993; Bonetti et al. 1994; Pinto et al. 2002). For example, the insecticide pyriproxyfen, an analogue of juvenile hormone, disturbed caste differentiation in *Melipona quadrifasciata* when more than 80% of the female larvae exposed to this substance became queens compared to 0% in the control (Pinto et al. 2002). Under natural conditions, many queens may compromise the maintenance and survival of colonies in the long-term since they usually do no work and are killed.

Behaviour

Queens exposed to the neonicotinoid imidacloprid when larvae, presented less wing vibrating (78%) and made less trophallaxis (83%) (Otesbelgue et al. 2018), which are important behaviours for their acceptance by workers (Silva et al. 1972; Imperatriz-Fonseca and Zucchi 1995; Araújo et al. 2017). Additionally, imidacloprid-treated queens suffered more harassment (increased by 219%) by workers than non-exposed queens and, over a period of days, such exposed queens reduced (79%) her defensive responses against worker attacks compared to non-exposed virgin queens (Otesbelgue et al. 2018). These variations in behavioural repertoire caused by residual substances can raise the number of queen executions, contributing to a lower number

of physogastric queens and new nests, which are harmful for reproduction and colony swarming.

Stingless bees can contaminate each other (and even the queen) by horizontal poisoning (Boff et al. 2018). It means that if a queen is poisoned, probably the whole colony is affected, given that the workers are more exposed to agrochemicals when foraging and are thus the first ones to be in contact with the contaminated food, bringing it back into the colony. When exposed to pesticides, it is known that workers can change not only individual behaviours, but also their collective responses (Tomé et al. 2015; Boff et al. 2018), which surely affect the egg-laying queens; e.g. workers of *Melipona quadrifasciata*, when exposed to a pesticide composed by acetamiprid and alpha-cypermethrin, presented less trophallaxis and antennation (Boff et al. 2018). Even though the effects in the interactions with the queen were not studied, it is possible that the feeding and communication of the queen would be negatively affected, as it is directly dependent on the workers' behaviours that presented changes caused by agrochemicals in previous studies. Thus, we can predict that there are several ways, yet unstudied, in which pesticides may indirectly interfere in the social interactions of stingless bee queens. Besides that, possible effects of pesticides in other aspects, such as queens' oviposition rate, longevity and eggs sizes need to be further studied in stingless bees.

Concluding remarks

The queens of stingless bees are essential for the growth, maintenance and survivorship of their colonies, but they face many challenges from larval development to their adulthood as an egg-laying queen, in which, little seems to be under their control. Although female larvae are postulated to be able to sometimes evade their fates as a future worker, in many cases, it is the workers that control when and how many new queens will emerge. Similarly, when they are just young, unmated queens, they need to display a range of behavioural repertoire to be accepted by workers. Likewise, their mates appear to be selected via indirect competition between males in an aggregation, since we do not have evidence for a direct mate choice by the virgin queens during the nuptial flight. Finally, physogastric queens may be exposed to multiple challenges to sustaining a high rate of oviposition. Agrochemicals and their sublethal effects may put still more pressure on the viability and survival of the queen caste.

Investigation of the life of stingless bee queens is challenging since colonies are dark and stingless bee nests are complex structures obstructing the observation of their behaviour inside of the brood area. Only well-designed experiments can capture their natural routine and their patterns of behaviour towards other queens, workers and even

Table 3 In vitro rearing and larval development of stingless bee queens exposed to agrochemicals

Species	Status	Development period (days)	References
<i>Partamona helleri</i>	Control	37.65 (mean)*	Bernardes et al. 2018
	Exposed ¹	39 (4.5%↑)§	
<i>Plebeia droryana</i>	Control	40 (median)	Otesbelgue et al. 2018
	Exposed ²	44 (10%↑)	
<i>Plebeia droryana</i>	Control	34 (mean)	dos Santos et al. 2016a, b
	Exposed ³	41 (20.5%↑)	

*Mean day according to provided regression formula. §Higher dose (0.48 µg a.i. * bee⁻¹);

¹azadirachtin (botanical biopesticide obtained from neem), ²chlorpyrifos (organophosphate), ³imidacloprid (neonicotinoid)

males. Therefore, even though recent work has brought new insights into the lives of queens of stingless bees, further studies on their physiology, genetics, chemical ecology and behaviour are needed. Such studies would ideally be conducted across a diverse range of stingless bee species.

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Data availability statement There is no dataset.

Declarations

Conflict of interest The authors have declared no conflict of interest.

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