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SHORT COMMUNICATION

# Evidence for male genitalia detachment and female mate choice in the Australian stingless bee *Tetragonula carbonaria*

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## Abstract

Mating systems are poorly understood for the majority of the world's estimated 600 stingless bee species (Meliponini). It is assumed that in most species, a virgin queen takes a single nuptial flight, mates with a single drone and returns to the nest, using the stored sperm from this one event for the remainder of her life. Multiple genetic studies focused on stingless bee species from around the world have supported the hypothesis of single mating—monandry—by demonstrating shared paternity of workers within colonies. One possible mechanism for monandry is through male genitalia detachment during mating, which forms a residual mating plug inside the queen. Despite being thought to be the norm for stingless bees, male genitalia detachment has only been observed in a small number of species from the neotropics. Here I report on detailed observations of mating interactions for four queens of the Australian stingless bee *Tetragonula carbonaria*. Male genitalia detachment was observed in each case of copulation, providing the first evidence of male genitalia detachment in any Asian–Australian stingless bee lineage. Observations of the behaviour of queens also provide possible evidence for female mate choice in *T. carbonaria*. To more fully understand the mating system of *T. carbonaria*, further observations must be made of wild, natural copulation events, and the behaviour of the queen and the workers within the nest upon her return from the nuptial flight.

**Keywords** Reproduction · Drone · Virgin queen · Queen crowding · Meliponary · Apidae

## Introduction

Mating systems in stingless bees (Meliponini) are poorly understood for the vast majority of the estimated 600 species that occur globally. For most species, it is thought that young, virgin queens leave the nest on a single nuptial flight, during which she mates with a single male whilst flying (Strassmann 2001; Vollet-Neto et al. 2018). The mated queen then returns to the nest and stores this sperm alive in her spermatheca for the remainder of her life. Evidence for single mating—monandry—comes from a small number of observations of neotropical stingless bee species (Kerr et al. 1962; Silva et al. 1972) and from genetic studies of worker paternity within colonies of stingless bee species around the world (Machado et al. 1984; Peters et al. 1999; Drumond et al. 2000; Green and Oldroyd 2002; Palmer et al. 2002; Tóth et al. 2002). While there does exist limited evidence for polygamy in some stingless bee species (Wille 1983; Imperatriz-Fonseca et al. 1998; Lopes et al. 2003; Vollet-Neto et al. 2019), monandry is thought to be the typical mating system (Strassmann 2001; Hughes et al. 2008; Vollet-Neto et al. 2018).

Detachment of male genitalia inside the female during copulation is a mechanical mechanism thought to contribute to monandry in stingless bees (Strassmann 2001; Colonello and Hartfelder 2005; Vollet-Neto et al. 2018). The detached genitalia may form a residual mating plug inside the female, blocking further males from successfully inseminating her, and thus ensuring the male who successfully mates becomes the single father to the colony's female offspring (Colonello and Hartfelder 2005). The presence of the residual mating plug may also have a role in ovarian activation of the queen (Vollet-Neto et al. 2018). Despite being thought to be the norm for stingless bees (Melo et al. 2001), male genitalia

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detachment and plugging have been reported in only a small number of stingless bee species in the neotropics (Kerr et al. 1962; Camargo 1972; Silva et al. 1972). In these cases, the queen flies back to the nest after her mating flight with the residual mating plug (detached male genitalia) inside her. In two species, queens have been observed removing the single genitalia plug themselves within the nest (Camargo 1972; Silva et al. 1972). It has been suggested that in some other species, workers in the nest may assist the queen in removing the genitalia plug (Colonello and Hartfelder 2005).

Few reports of natural stingless bee copulation exist in the literature because it occurs rarely, and the bees are small and fast flying. In Australia, published accounts of copulation do not exist for any of the 11 described stingless bee species, despite one species, *Tetragonula carbonaria*, being one of the most well-studied bee species in the world. *T. carbonaria* live in colonies typically of up to 10,000 and have a single mated queen (Heard 2016). Despite a lack of observational evidence, direct evidence for *T. carbonaria* queens being monandrous has existed since 2002, when Green and Oldroyd (2002) demonstrated consistent single paternity among female workers within colonies using microsatellite analysis. A small number of videos do exist showing Australian stingless bee queens and drones interacting outside of nests, usually in frenzies of queen-crowding activity (e.g. Smith 2019), but none show enough detail to be confident of actual copulation or see details of the physical condition of the queen post-crowding (Author, pers. obs.).

Here I report observations of mating behaviour for four separate *T. carbonaria* young, non-gravid queens and provide the first direct evidence of male genitalia detachment and possible plugging during copulation in any Asian–Australian stingless bee lineage. These observations also provide evidence that supports the hypothesis that stingless bee queens make the choice whether to copulate with a crowding drone or not.

## Methods

The following experiments and observations all took place at a meliponary at Currumbin Waters (postcode 4223), Queensland, Australia. Four young, non-gravid *T. carbonaria* queens were collected and experimentally presented to drone congregations, followed by detailed inspections of their bodies under a dissecting microscope. The four queens were in various states of health. One queen was dead ('dead queen') when presented to drones, one was alive but close to death ('dying queen') and two queens were healthy and vigorous ('vigorous queen 1' and 'vigorous queen 2'). 'Dead queen' was collected alive at the experimental meliponary in Currumbin Waters (on the 17th of August, 2019), whereas 'dying queen' and the two 'vigorous' queens were collected alive during a transfer of a queen-right colony from a rotting hive box at a second meliponary nearby (between 9 a.m. and 10 a.m., 4th of September, 2019), and brought to the Currumbin Waters meliponary for experimentation on the same day (between 12 p.m. and 2 p.m.).

Observation of the 'dead queen' was made in the presence of a congregation of approximately 100–200 drones. Observations of the remaining three queens were made in the presence of a congregation of drones estimated at 1000 +. Three of the queens ('dying queen' and the two 'vigorous' queens) were videoed as part of observations. In these cases, queens were mounted to a wire and presented to the drone congregation, fixed in front of a Nikon D7000 camera with 60 mm macro lens and artificial lighting sources. Queens were mounted with fast drying glue attached to the wings.

Post-experimentation, the bodies of all queens and a single drone (detailed below) were individually stored in 70% ethanol and deposited in the Mayfield Lab bee collection, School of Biological Sciences, University of Queensland.

## Results

### Dead queen

This queen was found alive at approximately 4:30 p.m., on the ground, being crowded by approximately 5–10 drones. Other drones were flying directly above her. Approximately 30 s after first being spotted, and still on the ground being crowded, the queen was collected in a 5 ml plastic specimen jar. She was inspected alive under a microscope and no visible evidence of any protrusion from the end of her metasoma was seen. She was kept overnight in the specimen jar.

The following morning (~ 7:30 a.m.) the queen was found dead. The queen's abdominal opening, and the inside of the specimen jar, was inspected under a microscope and no male genitalia was present. At 12:30 p.m. that day the jar was taken outside to the meliponary where drones were still loosely congregating in the air. The young queen's dead body was placed on the ground. Within 1 min, drones landed near her and quickly crowded her body, climbing over her in a mass. After approximately 15 s of crowding by up to ten males, and what appeared to be actual copulation by one male, her body was again collected. With the naked eye, a small white mass was visible at the apex of her metasoma. She was immediately observed under a microscope. Only the white mass was visible externally, and the apex of her metasoma appeared closed (Fig. 1a). Using forceps, the queen's metasoma was opened apically and a whole male genitalia was removed from within her, intact (Fig. 1b). A small amount of milky white liquid was observed as the male genitalia was extracted. The genitalia was wholly inside the queen prior to being extracted. After removing the genitalia,

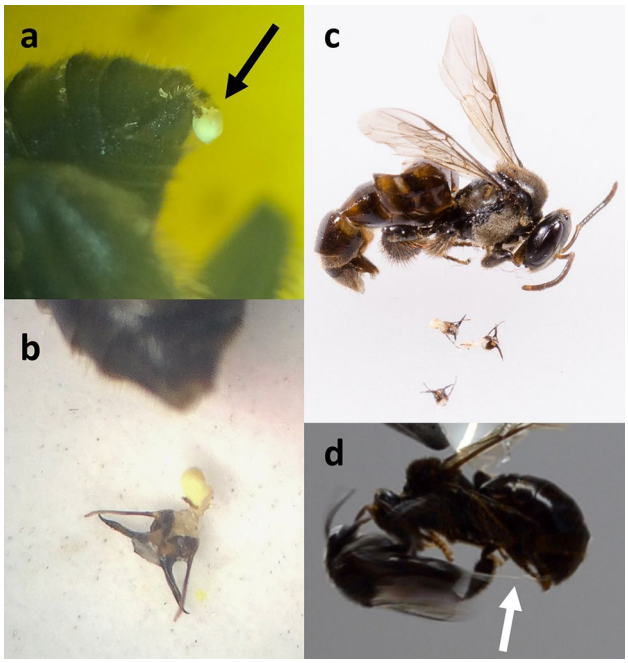


**Fig. 1** **a** Apex of the 'dead queen's' metasoma showing a white mass protruding (highlighted by arrow), **b** first male genitalia extracted from the 'dead queen's' metasomal opening, **c** the 'dead queen's' body next to the three genitalia extracted from her metasomal opening over repeated experiments, **d** still image taken from video of the 'dying queen' being copulated by a male, at the moment he detached from her. A thread of body tissue (indicated by arrow) extends between the apex of his abdomen and the queen's abdominal opening and broke away from him immediately after this moment. Male genitalia was subsequently removed from the queen's abdominal opening

the queen's body was again taken outside and placed on the ground near the drone congregation. Again she was crowded for approximately 15 s, and subsequently collected and taken for microscope inspection along with a male that was engaged in copulation with her (he detached in the collection container, and was kept alive). Upon inspection, another male genitalia was present and was again removed from the queen's metasomal opening. This process was repeated a third time outside with the drone congregation, and again upon inspection a third male genitalia was removed from her metasomal opening. Following this third extraction, her metasoma was distorted and damaged, and no further repeat was made (Fig. 1c). The male who was captured during the second event survived, without genitalia, in a specimen jar for 8 h until he was killed for identification.

## Dying queen

'Dying queen' was found floundering in spilled honey. She was washed gently with water, dried and placed in a 5 ml specimen jar. In the jar she remained alive, but was limp and moving very little, seemingly almost dead. External observation showed her abdominal opening closed, with no obvious signs of past mating. At 12:15 p.m. she was presented, on the ground, to males for a period of 5 min, during which she was crowded by 50 or more males. Still alive, she was subsequently collected and inspected under a microscope. Within her abdominal opening were found three separate male genitalia. These were partially visible from the outside. The three genitalia were tangled with each other and with dust and fibres from the environment. This queen, with all male genitalia extracted from her and still alive, was then mounted to a wire and presented to drones outside while being videoed. She was filmed until copulation appeared to have occurred (1.5 min), and again inspected under a microscope. A further male genitalia was extracted from her abdominal opening. Reviewing the video also revealed copulation and the moment of male genitalia detachment (Fig. 1d, Supplementary Material 1), after the male struggled for several seconds to detach from the queen.

## Vigorous queen 1

'Vigorous' queen 1 was found and stored in a 5 ml specimen jar. At 1:10 p.m. she was mounted to a wire and presented to the drone congregation and videoed. For a period of nine and a half minutes, she was almost continuously crowded by drones, after which she was again collected. The queen was kept alive in the jar for 9 h, after which her abdominal opening, and whole body, was inspected under a microscope. The jar and lid were also inspected under a microscope. No male genitalia was found within her abdominal opening, on her body or in the jar. On reviewing the video of this queen, one male appeared to ejaculate outside of the queen's body, near the side of her abdominal opening, but no clear copulation event occurred despite being crowded by hundreds of males. The queen appeared to use her legs to continuously attempt to dislodge males who were holding on to her.

## Vigorous queen 2

'Vigorous' queen 2 was found and stored in a 5 ml specimen jar. At 1:30 p.m. she was mounted to a wire and presented to the drone congregation and videoed. While mounted, she was exposed to almost continuous crowding by drones and appeared to use her legs to try to dislodge males that were holding on to her. After 7 min of exposure, she was collected and stored in a 5 ml jar. Two hours later she was alive in the jar, but barely moving and seemingly almost dead. Under a microscope, her abdomen was dissected and her whole body and the jar inspected. No male genitalia was found.

## Discussion

These observations provide the first evidence of male genitalia detachment and lodgement in the female during copulation in *Tetragonula*, and indeed in any Asian–Australian stingless bee lineage. This report also provides the first detailed observations of any Asian or Australian stingless bee queen's body immediately after copulation has occurred. The male genitalia removed from the two queens in these instances match images and drawings of *T. carbonaria* male genitalia previously reported in the literature from male dissections (Dollin et al. 1997; Heard 2016).

The fact that the two queens that were copulated successfully were either dead or dying obviously narrows the inferences that can be made for natural, live matings, although it is reasonable to believe that natural copulation events would be similar in regard to genitalia detachment and lodgement in the queen. Genitalia detachment from live drones has been demonstrated here and provides evidence for a morphological mechanism for monandry in *T. carbonaria*. Complicating the hypothesis that the male genitalia plugs the queen, ensuring monandry, is the observation of three male genitalia simultaneously within the 'dying queen'. Evidently, in some circumstances, the presence of male genitalia within the queen does not physically exclude further males from inserting their genitalia within the queen. However, given that the 'dying queen' was close to death, unmoving and intensely exposed to males over 5 min, it is likely that males two and three in this instance had opportunities that would not exist with a heathy, vigorous queen. It is possible that the queen's metasoma in this case was damaged and opened in a way it would not be in a healthy, vigorous queen. It is interesting that the two vigorous queens observed here were not successfully mated with by any males, despite many attempts by many males.

Coupled with the observations of the matings of dead and dying queens, and past genetic evidence of single paternity in *T. carbonaria* colonies (Green and Oldroyd 2002), the fact that the vigorous queens here did not copulate may suggest some degree of active female mate choice. Maybe it is, in part, a queen's ability to resist mating attempts by multiple males that ensures monandry, rather than simply by the presence of a residual mating plug. Evidence for female mate choice in various forms exists for many different bee groups around the world (Danforth et al. 2019), and by many other insects. So it is unsurprising that it would also occur in stingless bees. It is possible that queens have the ability to withhold, or accept, the insertion of male genitalia into their abdominal opening. If the hypothesis of active choice by the queen is correct, how she chooses which single male to mate with whilst flying and presumably being perused by hundreds or thousands of frantic males, is unclear. Alternatively, it could be that the two vigorous queens observed here were so distressed during the experiments that they were somehow simply unable to mate? It has been demonstrated in one neotropical stingless bee species that young queens are continuously attractive to males regardless of age (Veiga et al. 2017), although it is possible that in other species queens go through a period of attractiveness to males depending on age. In the present study, the age of the queens was unknown, but all four queens appeared to be equally attractive to males given the crowding that occurred for each.

An obvious next step in understanding queen mating in *T. carbonaria* is to observe wild copulation of live, untethered *T. carbonaria* queens, and to observe the behaviour of queens and workers within the nest immediately after the return of queens from nuptial flights. Observing the former will require a great deal of luck and patience, but observing the latter may be relatively easy in purpose-built artificial hives boxes fitted with observation windows. Further experiments with tethered healthy and vigorous queens may reveal whether or not detached genitalia from a single male acts as a mechanical plug to exclude further copulation during the nuptial flight.

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