

Article

Queens remate despite traumatic mating in stingless bees

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Abstract

Males can control female reproduction using genital plugs to impede access by rivals. In social bees, ants, and wasps, plugging may involve traumatic mating, with females being harmed. In stingless bees, chances are that plugs may promote ovarian activation, and are thought to ensure single mating—a general tendency among the social Hymenoptera. However, understanding on relationships between mating plugs, traumatic mating, and mating systems in stingless bees remains limited. To address this, we (1) compared mated queens of 7 Neotropical species to understand the patterns of copulatory marks in females and (2) compared pre- and post-mating genitalia of males and females in *Melipona fasciculata* to depict plug functional morphology. Data revealed an unprecedented consequence of mating in stingless bees: the characteristic marks left by mating plugs on female abdomens and the inferences that can be made from them. To our surprise, in 1 species *M. fasciculata* we found that queens retain the plug long after mating, and may carry it for the rest of their lives. All the other 6 species retained the plug for only a short period. Remated queens were only found in *M. seminigra*, whose multiple copulatory marks match previous findings of polyandry in this species. Our study shows that queens can remate, and suggests that male genital morphology may determine in part the time persistence of plugs. We conclude that traumatic mating plugs do not fully prevent remating in stingless bees and that mating systems are not uniform in this group. Nonetheless, exceptional cases of facultative polyandry in social insects—for example, when mating plugs fail—may confirm a general tendency for single mating in close link with efficient mating plugs.

Key words: copulatory marks, facultative polyandry, genital morphology, male harm, sexual conflict

Sexual conflict, a potent force shaping the coevolution of sexes, emerges from male and female antagonistic sexual interests (Chapman et al. 2003; Parker 2006; Arnqvist and Rowe 2013). The

mating-guard hypothesis predicts that males benefit from monopolizing females after insemination (i.e., post-insemination associations) because it may reduce the chance that a mate will copulate

again during this period (Eberhard 1985; Alcock 1994). Lengthy copulations, lasting days or even weeks, prolonged guarding through non-genital contact, and suicidal donation of mating plugs are classical examples of this strategy (Alcock 1994), all serving to restrict female copulation with rival males (Eberhard 1985; Alcock 1994). Mating plugs may function by transfer of chemical secretions, that block access to the female's genital tract, like in some butterflies, moths, and fruit-flies (Alcock 1994; Polak et al. 1998; Wedell 2005), although others involve the sacrifice of part of the male abdomen, which remains attached to the genital opening of his partner—the mechanical plugs—as frequently observed in ants, wasps, and bees (Roig-Alsina 1993; Alcock 1994). Even though these strategies may carry high costs to males, for instance reducing or suppressing their future mating opportunities, they have been proposed as advantageous by preventing female remating (Eberhard 1985; Alcock 1994; Boomsma et al. 2009).

In social Hymenoptera (e.g., bees, ants, and wasps), mating plugs have been invoked as part of the explanation for the maintenance of single mating queens (Boomsma et al. 2009). A growing body of evidence has reinforced the correlation between mating plugs and mating systems (Koeniger 1990; Roig-Alsina 1993; Colonello and Hartfelder 2005; Baer and Boomsma 2006; Boomsma et al. 2009), suggesting that mating plugs are an efficient means to prevent female remating. In fact, single mating is the ancestral condition in this group, and thus the general tendency (Hughes et al. 2008a; Boomsma et al. 2009). However, in cases where polyandry occurs, mating plugs seem to fail, either because plugs are short-term, like the chemical plugs found in *Atta* ants and bumble bees (*Bombus hypnorum* Linnaeus 1758; Brown et al. 2002), or because they can be displaced by other males during mating, as observed in polyandrous honeybees (*Apis* Linnaeus, 1758) (Koeniger 1990; Palmer and Oldroyd 2000). Only recently studies have pointed out that in both monogamous and polyandrous systems of social Hymenoptera, mating plugs may be accompanied by traumatic mating, which means that grasping structures of male genitals damage females, that is, causing a trauma (Lange et al. 2013). Male-induced harm has been evidenced by signs of melanization in females, typically found after wound healing in insects (Baer and Boomsma 2006; Kamimura 2008). This has been indirectly evidenced by post-mating-induced immune responses detected in bumblebees (*B. terrestris* Linnaeus, 1758, Barribeau and Schmid-Hempel 2016) and, directly, by copulatory marks, as observed in ants of the genera *Atta* Fabricius, 1804 (Baer and Boomsma 2006) and *Formica* Motschoulsky, 1866 (Kamimura 2008). In this sense, male genital morphology and traumatic mating consequences for females may be used to infer queens' mating status (e.g., unmated, once mated, or remated) in some social hymenopterans, and to better understand their mating systems.

In stingless bees (tribe Meliponini), observations on mating plugs are sparse and often anecdotal. The whole male genital capsule is known to turn into a mating plug by the activation of a pair of sclerotized claw-like spines—the penis valves—when attached to the female's genital chamber (Camargo et al. 1967; Michener 1990, 2007). There are indications that plugs may be involved in the activation of queens' ovaries in 1 species (*Melipona quadrifasciata*, Melo et al. 2001), and are believed to be removed by the queens themselves, or with the help of workers, as soon as they return to the nest from the nuptial flight (Da Silva et al. 1972; Van Veen and Sommeijer 2000). Although it is suggested that this mechanical plug may prevent queen remating (Kerr et al. 1962; Eberhard 1985; Alcock 1994), multiple matings have been detected in the stingless bee *M. seminigra*, as suggested by molecular evidences of multiple

patrilines among brood in this species (Francini 2013). Indeed, mating plugs seem to fail sometimes, as observed under experimental conditions: males may lose precedence for rivals, with more than 1 male plugging a queen simultaneously in the same mating event (Smith 2020), or males may simply lose their genital capsules without successfully attaching it to a queen (Veiga et al. 2017, 2018). Taken together, these reports suggest that mating plugs in stingless bees may be (1) sources of copulatory lesions in females due to the barbed structures in male genital capsules, (2) key to stimulate female fecundity, and that (3) plugs may be linked to single mating in the Meliponini (Strassmann 2001; Vollet-Neto et al. 2018; Grüter 2020)—a general tendency expected for most social Hymenoptera (Boomsma et al. 2009)—by preventing queen remating (Alcock 1994). However, studies on mating plugs are rare, including aspects of functional morphology, thus the understanding of relationships between mating plugs, traumatic mating, and mating systems in stingless bees remains limited.

Although a clearer comprehension of how mating plugs work in the stingless bee mating biology can provide a basis for a better understanding of their mating systems and underlying sexual conflict, the role of mating plugs in the Meliponini remains poorly understood. In line with this knowledge gap, and considering that traumatic mating plugs may harm queens, but prevent their remating, our aim was to investigate if mating plugs are sources of trauma, and how trauma levels and plug persistence vary in 7 Neotropical stingless bees species. To address it, we (1) detected and described the copulatory marks in females, (2) used this knowledge to build a diagnostic tool of traumatic mating based on plug persistence and trauma levels, and (3) depicted the functional morphology of mating plugs. Our study shows an unprecedented consequence of mating in stingless bees: the characteristic marks left by mating plugs on female abdomens—the copulatory marks evidenced by melanized patches—and the possible inferences that can be made from those marks. Importantly, our study reveals a traumatic mating system in this group, in which some queens may be enforced into monogamy through lifelong mating plugs, although others may remate despite traumatic mating.

Material and Methods

Specimen collections took place in 2 regions of the Brazilian Amazon (Supplementary Table S1): Belém, Pará State (1°26'11.8"S 48°26'37.4"W) and Manaus, Amazonas State (3°05'45.4"S 59°59'22.0"W). Between May and October 2017, we collected males and queens of 7 Neotropical stingless bee species: *Melipona fasciculata* Smith, 1854, *M. flavolineata* Friese, 1900, *M. melanoventer* Schwarz, 1932, *M. seminigra*, *Frieseomelitta longipes* Smith, 1854, *Scaptotrigona* aff. *postica* Latreille, 1807, and *Plebeia minima* Gribodo, 1893. All queens, virgins, or mated were sampled from living colonies housed in wooden artificial beehives.

We chose *M. fasciculata* as our reference species for illustrations and mechanism descriptions, because we had a complete set of specimens: unmated females (i.e., virgin queens), newly mated non-physogastric queens (i.e., recently mated, but not showing abdominal extension), mated physogastric queens (i.e., enlarged abdomen due to ovary development), newly emerged males, and sterile males (i.e., males in reproductive aggregations that have lost their genital capsules during previous mating attempts, Veiga et al. 2018). Even though behavioral differences among species are expected (Michener 2007; Grüter 2020), we believe our general descriptions

of functional morphology may be extended to the other genera studied here, and others within the Meliponini tribe.

Traumatic mating: detecting and comparing copulatory marks in queens

We compared virgin and mated queens to describe patterns of traumatic mating. Then we compared these patterns among 7 Neotropical species (*M. fasciculata*, *M. flavolineata*, *M. melanoventer*, *M. seminigra*, *F. longipes*, *S. aff. Postica*, and *P. minima*) to understand how traumatic mating varied among species. To do so, we inspected queens' genital chambers to (1) identify copulatory marks that could indicate previous mating events and, consequently, (2) determine their mating status (e.g., unmated, single mated, and remated). We also inspected virgin queens, considered as a control group for genital morphology and tissue integrity. All inspected queens were individually inserted in plastic tubes (Supplementary Figure S1), having their heads positioned toward the tube tip and leaving the terminal part of their abdomens toward the tube entrance. We inspected each queen under a stereomicroscope, with the help of entomological tweezers used to hold the tergum T-VI and the sternum S-VI apart, and thus access the genital chamber (Supplementary Figure S1). During these inspections, we looked for melanized patches as recommended by previous protocols of copulatory marks in ants and flies (Kamimura 2008, 2016). We then compared the membranous region, below the gonopore (henceforth membranous pouches), between unmated and mated queens of all species. Based on this comparison, we collected data on the (1) occurrence, (2) number, (3) location, and (4) position of melanized patches in the genital chamber—all of which were used to determine mating status.

We analyzed a total of 320 queens (e.g., 160 mated physogastric and 160 virgin queens) from 7 stingless bee species ($N=15\text{--}35$ physogastric queens per species), including 4 *Melipona* species (*M. fasciculata*, *M. flavolineata*, *M. melanoventer*, and *M. seminigra*) and 3 non-*Melipona* species (*F. longipes*, *S. aff. Postica*, and *P. minima*) (Table 1). Virgin queens were sampled directly from brood combs or within nests, then immediately frozen to preserve shape, structure, and color of internal tissues (Quicke et al. 1999). To ensure the mated physogastric queens were active, specimens were sampled after we observed them laying eggs. All mated queens were temporarily accommodated in Petri dishes and taken to the

laboratory to be immediately inspected. We then returned them to their original nests, except for *P. minima* whose queens had been sacrificed to allow inspections due to their small body size.

To estimate the age of sampled queens, we classified all individuals by cumulative wing wear marks (Mueller and Wolf-Mueller 1993; Coelho 2002). Queens were grouped into the following categories: (1) “newly mated,” intact wings (0–2 wear marks); (2) “young,” slightly worn wings (3–5 wear marks); (3) “middle aged,” worn wings (6–12 wear marks); and (4) “old,” very worn wings (>12 wear marks). For some samples, data on wing wear were not collected thus being labeled as “no info.”

To determine mating status of queens we considered 4 aspects: (1) the presence of melanized patches in the female genital chamber—if present, queen was considered mated, if not, was considered unmated; (2) number of paired melanized patches (e.g., single pair or multiple pairs)—if there was a single pair, the queen was considered singly mated, if there were multiple pairs, the queen was considered remated; (3) persistence of mating plug attachment based on the combined information of the presence of melanized patches, the presence of a plug inside the queen, and inferred age—if melanized patches were present, but plug was absent, irrespective of queen age, the plug was considered short term, but if the plug was present, irrespective of queen age, then it was considered a long-term plug; (4) newly mated queens (i.e., recently mated queens returning from a nuptial flight; Kerr et al. 1962; Da Silva et al. 1972), were those individuals with an attached mating plug, but not yet physogastric. We did not find any physogastric queens with melanized patches and attached mating plugs (see the “Results” section).

Genital functional morphology, hypothesized plugging steps, and mating positions

We compared the male genital capsule of *M. fasciculata* in its resting position (inside the male's body) and in its triggered state as a mating plug (inside the female's genital chamber) to understand its functional morphology. We described and illustrated the functional morphology based on the differences of initial and final positions of the following male genital structures: the penis valves, the gonocoxites, and the gonobase. After being able to characterize the resting state and the triggered state of the mating plug, we proposed intermediate states for the structures of the genital capsule to represent their changes during the plug activation. Additionally, we revised

Table 1. Determination of mating status in mated queens of 7 Neotropical species of the Meliponini tribe

Groups	Species	Number of mated queens	Diagnose				Inferred queen mating status
			Mating evidence	Number of patches	Patch pattern	Plug persistence	
<i>Melipona</i> species	<i>M. fasciculata</i>	35	Mating plug	0	No patches	Long-term	Singly mated
	<i>Melipona flavolineata</i>	30	Melanized patches	2	Single paired patches	Short-term	Singly mated
	<i>Melipona melanoventer</i>	10	Melanized patches	2	Single paired patches	Short-term	Singly mated
	<i>Melipona seminigra</i>	33	Melanized patches	2–7	Single paired patches and multiple paired patches	Short-term	Singly mated or remated
Non- <i>Melipona</i> species	<i>Scaptotrigona aff. postica</i>	15	Melanized patches	2	Single paired patches	Short-term	Singly mated
	<i>F. longipes</i>	18	Melanized patches	2	Single paired patches	Short-term	Singly mated
	<i>P. minima</i>	19	Melanized patches	2	Single paired patches	Short-term	Singly mated
Total	7	160 ^a					

Predicted mating systems are given based on the 4 steps proposed within our diagnostic approach (e.g., mating evidence, number of patches, patch pattern, and plug persistence). ^a All these specimens were compared with an equal number of virgin queens, therefore, totalizing 320 queens analyzed.

the literature to collect data on male genital morphology of other genera and visually compare data from previous studies to our sampled species. Our cross-species comparison consisted of a categorized classification of the penis valves aspect (robust or slim), curvature (strong, intermediary, or weak), and spatha's development (developed, reduced, or absent) (Supplementary Table S2).

We compared the genital morphology between pre- and post-mating, of both males and queens of *M. fasciculata*, to understand the stages of the plugging mechanism. We chose the species *M. fasciculata* to reconstruct the mechanism of mating plug because all mated queens in this species retained mating plugs regardless of their age (see the "Results" section). For this reason, we believe this species allowed us to assess females presenting the mating plug during its most likely activated position. We compared a set of 3–5 specimens belonging to different categories—(1) newly emerged adult males from brood combs; (2) sterile penisless males; (3) physogastric queens with mating plug; and (4) virgin queens. We then described and illustrated the functional morphology between the initial and final stages of mating. To do so, we accessed the genital region of females and males and dissected each specimens' metasoma. We removed the final part of the abdomens of 1 individual per sex per species (from tergum T-IV and sternum S-IV in females; and from tergum T-VI and sternum S-VI in males; Supplementary Figure S1). After removing it, we manually exposed the marks by using tweezers to holding apart the terminal sternum and tergum. For males, we exposed their genital capsule by fully removing it from the dissected part. After structures were dissected and exposed, we represented them by drawings. Based on the illustrations of a complete set of mating plug states, we speculate about male and female genital movements to describe the hypothetical function and action of plugs in stingless bee mating.

To support our assessment of queen mating status and descriptions of functional morphology, we described male and female interactions to understand their positions during mating. To do so, we used fresh specimens (preserved in 70% ethanol)—1 newly emerged male and 1 virgin queen of *M. fasciculata*—and manually arranged both male and female specimens under a stereo-microscope, following previously sketched mating positions. We prepared the sketches based on a video of a mating pair of *M. flavolineata* (Supplementary Video S1), a close related species. After this positioning session, we proceeded to the subsequent descriptions and correspondent illustrations of the mating positions.

Specimens dissection, illustrations, and multifocus high-resolution photographs

To photograph copulatory marks and the male genital morphology of each species, we accessed female and male genitalia through dissections. For this purpose, we sampled extra queens (both virgin and physogastric mated ones) and extra newly emerged males of each species, following the same sampling criteria previously described (see the previous sections). We then dissected each specimens' metasoma to access the genital region of females and males. After dissection, we immediately photographed these structures to ensure the integrity of membranous tissues, especially in females. Dissections followed the procedure previously described in Genital functional morphology, hypothesized plugging steps and mating positions. Finally, we used a Leica DM 1000 stereomicroscope, coupled with a drawing tube to prepare the illustrations of the mating pair ($\times 20$ magnification, Genital functional morphology, hypothesized plugging steps and mating positions) and the genital functional morphology ($\times 80$ magnification, Genital functional morphology, hypothesized plugging steps and mating positions). We vectorized

and treated these illustrations using an image editor software. We also used a Leica DFC420 digital camera attached to a Leica M205A stereomicroscope to produce high-resolution images of females and males genitalia ($\times 1.0$ lens and $\times 50$ magnification, Section 2.4). First, we took multifocus color photographs, then we stacked them in a single image using the Leica application suite version 3.4.1. We repeated this procedure for each genitalia (female and male) from all sampled stingless bee species, but one, *M. melanoventer*, due to sampling limitations.

Results

Traumatic mating in stingless bees

No melanized patches were detected in inspected unmated virgin queens. In all species, physogastric queens showed melanized patches on their membranous pouches (Table 1), except for *M. fasciculata* (Figure 1). The membranous pouches were the only region with copulatory marks. In each mated queen, patches were usually paired, matching the inferred position of the pair of penis valves in the male genital capsule. Patches were located on each pouch, and symmetrically positioned (Figure 1). Additionally, melanized patches appeared to be permanent, because we observed them in mated queens of different ages, according to our wing wear classification (Supplementary Figure S3).

In *M. fasciculata*, instead of melanized patches, we always found a single mating plug attached to the membranous pouches in mated queens (Figure 1), irrespective of their age (Supplementary Figure S3). Inside queens, the mating plug was ventrally oriented in relation to the female's body, lacking both gonostyles, and not showing any visible signs of a melanization process (even below the attachment areas). In this species, we tested if the melanization process would happen by removing the plugs of 3 fully physogastric mated queens, and then returning them, marked with a painting in their thorax, to the nests. After 7 days, we recollected them and found single paired melanized patches where mating plugs were attached before.

In *M. seminigra* queens, we observed both single pair and multiple pairs of melanized patches (Figure 1 and Supplementary Figure S4), and we never found permanent mating plugs in this species. The distribution of multiple patches varied between 3 and 7 individualized areas, with different types of arrangements. From the 33 *M. seminigra* specimens analyzed, 15 presented single paired patches pattern, 12 presented a single paired patches in combination with a central patch, and 6 specimens presented a multiple patch pattern (Supplementary Figure S4). In the latter case, we thus could distinguish both central patches and >1 patch on each side of the membranous tissue (Figure 1 and Table 1). In these arrangements, the central melanized patches matched the inferred position of the spatha—the central structure in the mating plug (Figure 2E,I). It is worth mentioning that we captured an extra newly mated *M. seminigra* queen, which was carrying 2 mating plugs: 1 was attached inside her genital chamber, although the other was onto the external surface of her abdomen, between sterna IV and V (Supplementary Figure S4A). This newly mated was not fully physogastric, and thus was not included in our main dataset.

Genital functional morphology, hypothesized plugging steps, and mating positions

Functional morphology of the male genital capsule

The male genitalia of *M. fasciculata* has 3 components: 2 overlapping metasomal sterna (S-VIII and S-IX) and a genital capsule

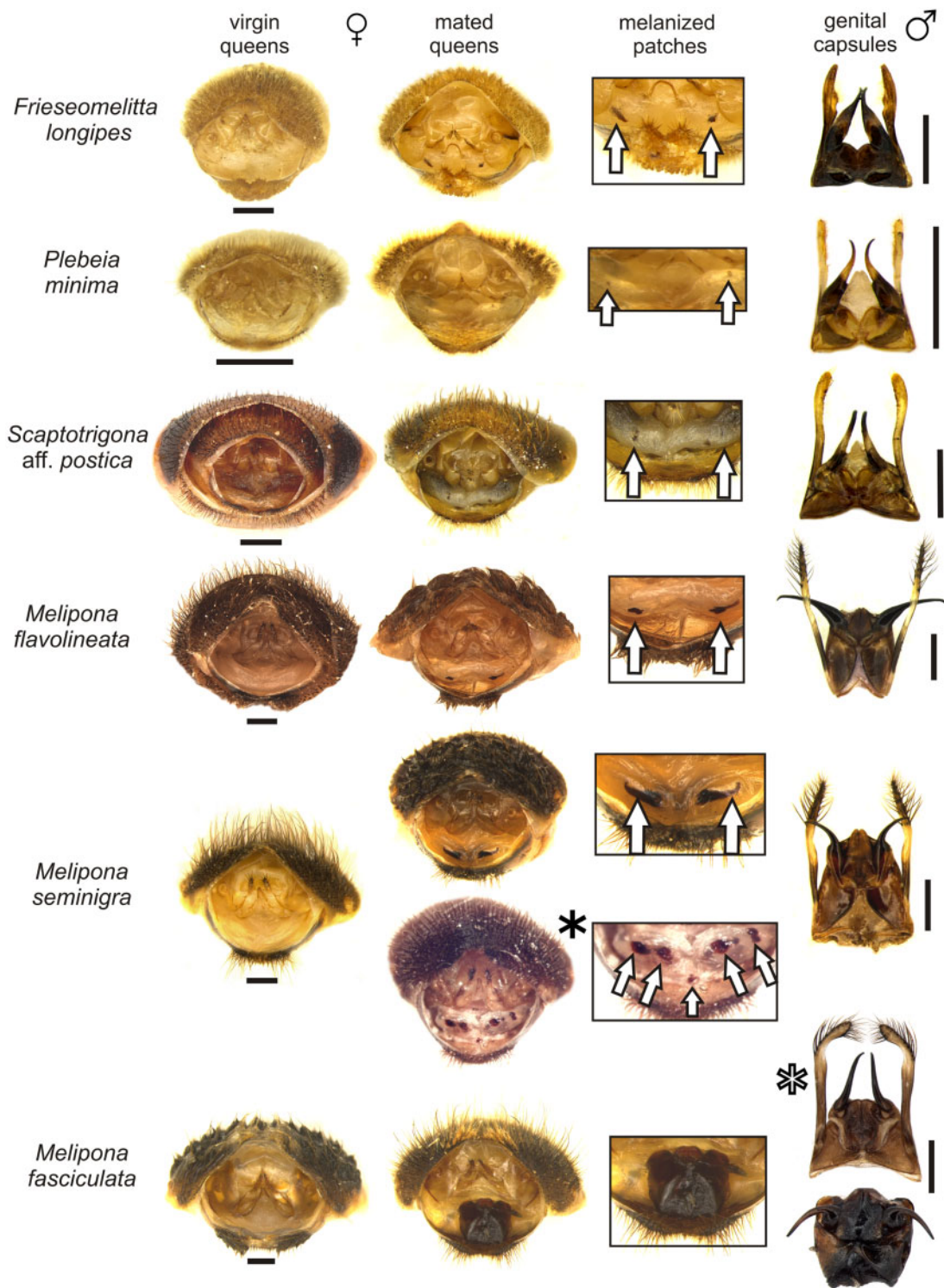


Figure 1. Female genital chamber, pattern of copulatory marks, and ventral view of male genital capsules of *F. longipes*, *P. minima*, *Scaptotrigona* aff. *postica*, *Melipona flavolineata*, *M. seminigra*, and *M. fasciculata*. From left to right, images show the genital chamber of virgin queens, the genital chamber of mated queens, and the melanized patches found on queens, which are indicated by white arrows in the third column of images. Singly mated queens show a single pair of melanized patches, shown for *F. longipes*, *P. minima*, *S. aff. postica*, *M. flavolineata*, and *M. seminigra*; or a long-term attached plug, only found in *M. fasciculata*. Remated *M. seminigra* queens, indicated by the black asterisk, show multiple pairs of melanized patches and central patches. In the right hand set of images, male capsules are presented as activated mating plugs only for *Melipona* species, showing rotated penis valves. We also show an inactivated mating plug for *M. fasciculata*, as indicated by the white asterisk. Black bars represent a scale of 0.5 mm. This figure includes images of 6 out of our 7 studied species (see Material and Methods).

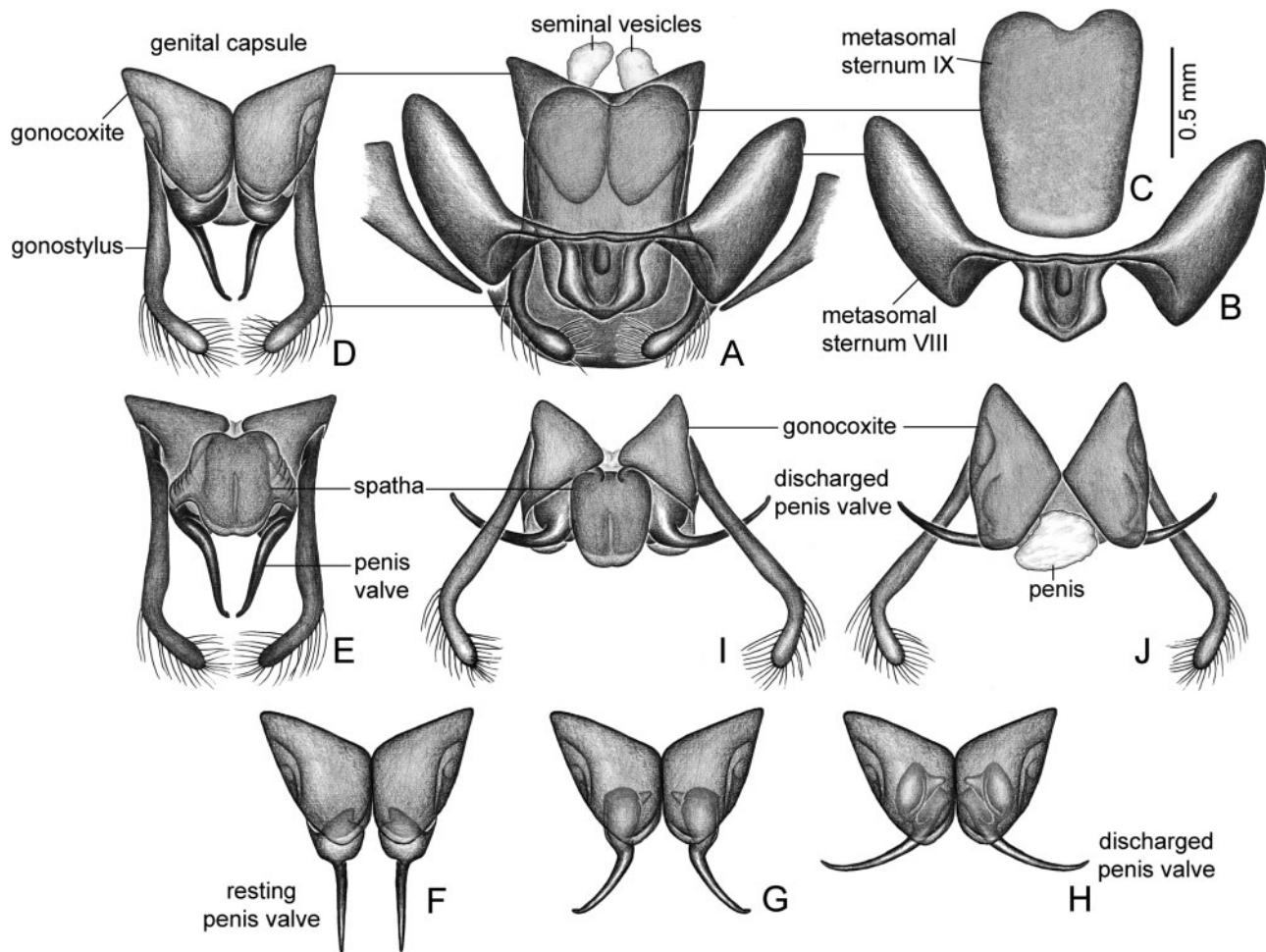


Figure 2. Male genital capsule and genital sternum of *M. fasciculata*. Ventral view of genital capsule, metasomal sternum S-VIII, and metasomal sternum S-IX, as found inside virgin males (A). Ventral view of metasomal sternum S-VIII (B) and metasomal sternum S-IX (C). Genital capsule in ventral view, showing the gonocoxites and a pair of gonostyli (D). Genital capsule in dorsal view, presenting the spatha and a pair of penis valves (E). Genital capsule in ventral view, showing in sequence, the rotation of the penis valves, before gonocoxites lateral compression (F–H). Genital capsule with triggered penis valves and gonocoxites compressed laterally, in dorsal (I) and ventral view (J).

(Figure 2). The genital capsule has a pair of nearly triangular gonocoxites, in which a pair of long gonostyli and the pair of piercing penis valves are articulated; dorsally there is a narrow gonobase and a median spatha (Figure 2E,I). These sclerites are strongly held together by a series of membranes that allow their free articulation. All components are strongly sclerotized, except for the putatively sensitive setae on the distal third of gonostyli; and are positioned ventrally in the male body.

The female genitalia of *M. fasciculata* is located inside a chamber formed by the tergum and sternum of the 6th abdominal segment (Figure 3). The final portion of the female body is membranous, poorly sclerotized, held by pairs of lateral hemitergites (T-VIII and T-IX). Remarkably arising from the membranous rear end, we see a pair of short setose gonostyli, a central bulb (the gonopore area), and a pair of thin, lateral rami. Between these structures and the sternum S-VI beneath, just below the gonopore area, there are large membranous pouches—tissue pockets with reduced sclerotization—onto which the male mating plug is attached during the mating (Figure 3A,J) and copulatory marks are found (Supplementary Figure S5). Membranous pouches are represented by a white space below the gonopore in Figure 3. These membranous structures are

shown in Figure 1 for all species, in both virgin and mated queens, although their relative size and orientation can be viewed in detail in Supplementary Figure S5.

In the non-*Melipona* species (*F. longipes*, *S. aff. Postica*, and *P. minima*), we observed robust penis valves with intermediary curvature and a reduced spatha (Figure 1). Comparing the general morphology of penis valves and spatha of *M. fasciculata* to the other studied species, we found that both *M. flavolineata* and *M. seminigra* have slim penis valves with low curvature. However, *M. seminigra* has shorter valves than *M. flavolineata*, and a more developed spatha when compared with the other *Melipona* (Figure 1).

Description of hypothesized plugging steps

The final position of mating plugs inside mated females matched the position of the male's abdomen during copulation due to “J” movement and positioning under female's abdomen (Figure 4C). In all physogastric queens of *M. fasciculata*, we observed the mating plug attached to the membranous genital pouches by the penis valves, with the spatha surface oriented toward the female ventral portion, placed under the gonopore (Figure 3J). We propose that the mating

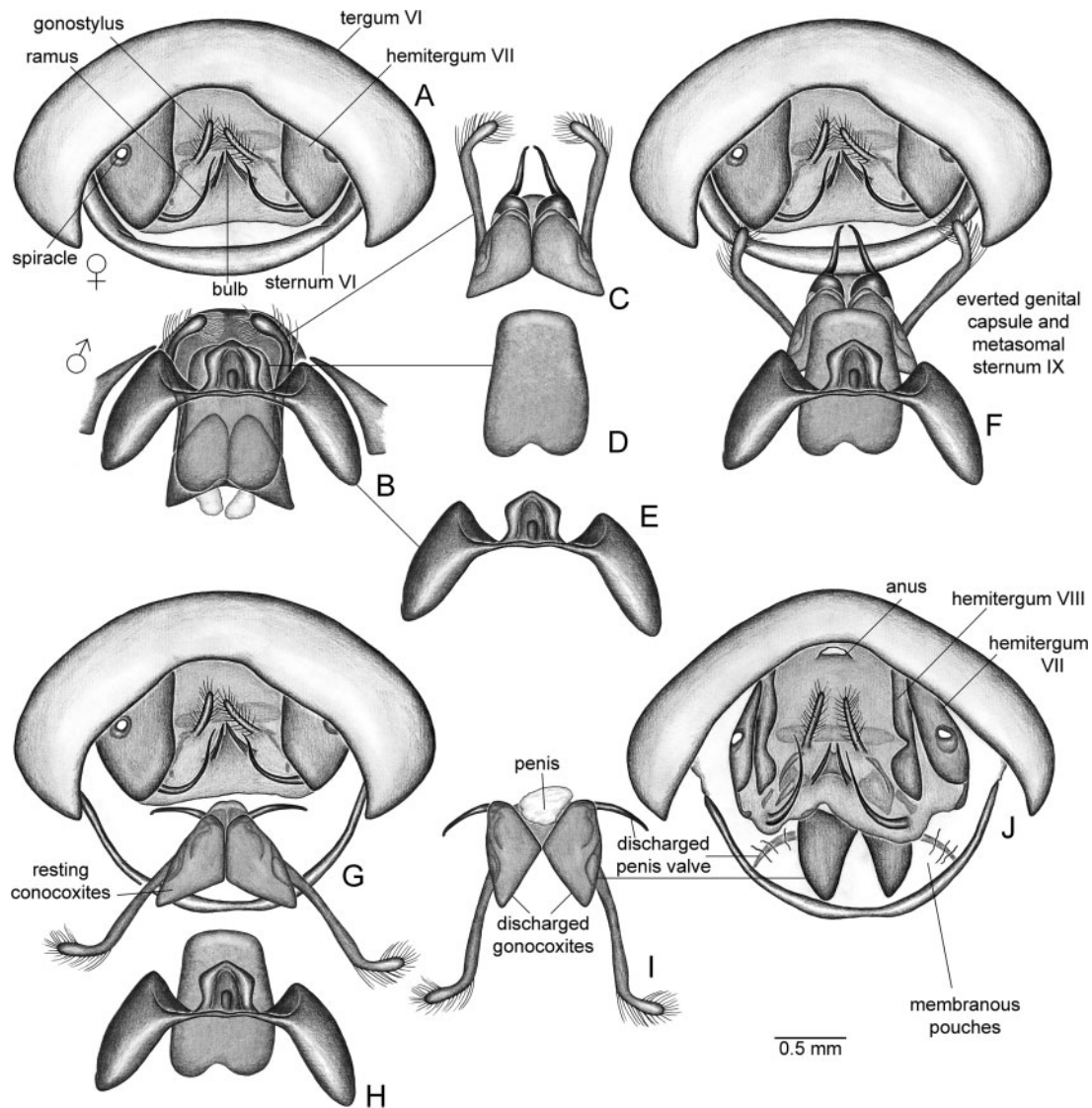


Figure 3. Female genitalia of *M. fasciculata* and steps for mating plug coupling. (A) Virgin female genitalia in frontal view. (B) Male genitalia of pre-copula male, approaching the female genitalia. (C–E) Genital capsule, metasomal sterna S-VIII and S-IX. (F) Male genitalia starting the coupling of the plug: ventral view of genital capsule and metasomal sternum S-IX projecting out of the body of the male by a telescopic movement. (G) Male genital capsule detached from male's body, with penis valves triggered, gonostyli facing the opposite position in relation to the initial. (H) Metasomal sterna S-VIII and S-IX, where sternum S-IX assumes its original position inside the male's abdomen. (I) Mating plug in detail, showing the lateral compression of gonocoxites. (J) The genital chamber of a plugged female: the membranous region containing the mating plug attached.

plug mechanism is triggered when the male genital capsule is irreversibly protruded from male's body, which results in modified positions of the gonostyli, penis valves, and gonocoxites.

Based on the comparison between resting state of genital capsules and triggered state of mating plugs, its functional morphology, and in video records on mating behavior of a closely related species (Supplementary Video S1), we suggest that triggering of mating plug occurs in 5 hypothetical steps (Figures 3 and 4):

- i. After immobilizing the female body, the male inserts his metasomal sternum S-VIII under the female tergum T-VI, lifting it (Figure 4C).
- ii. Once the female genital chamber is opened (Figure 3A), the male gonostyli touches the female's genital region. After positioning the sternum S-VIII, the male metasomal sternum S-IX protrudes out of the male's body toward the female's genital

chamber along with the genital capsule, by a telescopic movement (Figure 3F). The insertion of male genital capsule into the female genital chamber is not followed by the gonostyli, which move backward during the process, remaining outside the female genital chamber (Figure 3G).

- iii. In this moment, the penis valves' rotational movements start (Figure 2F–H), resulting in a position where the tapering and terminal portion of the valves, previously turned up (Figure 3F), become turned laterally, to the left and right (Figure 3G), against the female genital membrane, without rupturing it in a first moment.
- iv. Then the gonocoxites are laterally compressed, and the gonobase is retracted (Figure 3I), increasing the angulation between the pair of penis valves and resulting in the perforation into the membranous pouches, below the gonopore, and subsequent attachment (Figure 3J).

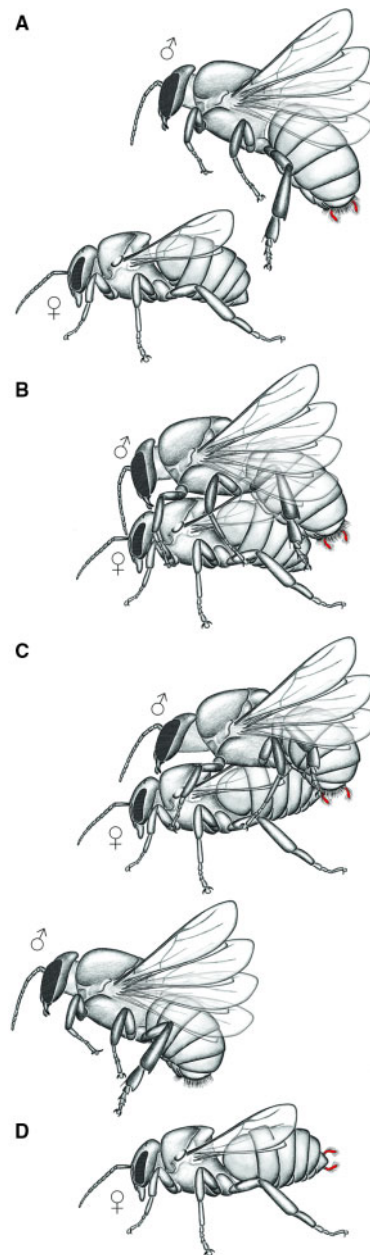


Figure 4. The mating pair of *M. fasciculata*, based on a video record of mating of a related species. From top to bottom: (A) male in flight, seeking to position itself over the female; (B) positioned male, showing pairs of legs fitted along the female's body; (C) male raising the abdomen of the female; (D) male leaving the female after successful mating, that is, the mating plug attachment. The gonostyli are highlighted in red, and can be explicitly observed in newly mated queens. In all parts, male wings are illustrated to represent their movement during mating, based on [Supplementary Video S1](#).

- v. Once attached by the pair of penis valves, the genital capsule is irreversibly decoupled from male's body, lodged within the female genital chamber, and leaving only the gonostyli, visible outside the female body ([Figure 4](#) and [Supplementary Figure S2](#)). The mating plug is finally attached, the male permanently loses his genital capsule, keeping intact in his body the sterna S-VIII and S-IX. Then the female is finally "plugged" ([Figures 3J](#) and [4D](#)).

In newly mated queens, gonostyli are still present and visible outside their body and the gonocoxites harbor a large part of the seminal vesicles ([Supplementary Figure S2](#)). After a while, gonostyli are

lost, like observed in older *M. fasciculata* queens ([Figure 1](#)), with mating plugs being visible inside females only under inspection of the genital chamber ([Supplementary Figure S1](#)).

Reconstruction of mating pair interactions

When a male in flight finds a female in the field ([Figure 4A](#)), he mounts the female's body and starts moving his wings uninterruptedly as the copulation begins ([Figure 4B](#)). By positioning himself entirely over the female, the male firmly adjusts each pair of legs at specific locations of the queen's body: (1) his forelegs anchors under the femurs of the forelegs of the female; (2) his midlegs, under the

trochanter of the hindlegs of the female; and (3) his hindlegs anchor under the tip of female's abdomen, thereby lifting it (Figure 4C). The male places the tip of his abdomen down the female's abdomen, forming a "J," moving it backward and upward (Figure 4C). After genital protrusion, and subsequent mating plug triggering, a successfully mated male leaves his genital capsule inside the female's genital chamber, which is indicated by the presence of an apparent pair of gonostyli (Figure 4D and Supplementary Figure S2).

Discussion

Our study shows that mating in 7 Neotropical stingless bees is a harmful male–female interaction, a traumatic mating system. Based on (1) observations of mated and unmated female genital morphology and tissue integrity, (2) the male genital mechanism description, and (3) the behavior of the mating pair, we identified that mating plugs leave characteristic marks on mated queens, that the pattern and position of these marks match the mating plug morphology, and the number of marks corresponds to the likely number of queen mating partners detected in different species evidenced by molecular data on the literature. Specifically, our study indicates that the molecular evidence of multiple patriline among brood of *M. seminigra* (Francini 2013) is now supported by evidence on the number of mating pairs represented by the number of copulatory marks found in mated queens. Our results suggest that traumatic mating plugs may be pervasive in stingless bees, but that such a mechanism may not fully prevent queen remating in some species of this group. A key application of our research is that copulatory lesion patterns may be a reliable and low-cost diagnostic tool to identify mating conditions (e.g., unmated, once mated, or remated) of Meliponini queens.

In all 7 studied species, mated queens showed melanized patches, although virgins did not (Figure 1). This is the first evidence of traumatic mating in this group, suggesting male–female harmful interactions during mating. Copulatory lesions caused by penile spines in the female genital tract were previously detected in ants (*Atta* sp. in Baer and Boomsma 2006; *Formica japonica* in Kamimura 2008). The pattern was described as traumatic penetration, thus meaning harmful penetration, but harmless insemination (Lange et al. 2013), which also applies to the stingless bees studied here. This suggests that harm to females may simply result from a strong physical anchorage of male genital structures to ensure copulation. In stingless bees, dense mating swarms are common (Imperatriz-Fonseca et al. 1998; Van Veen and Sommeijer 2000; Sommeijer et al. 2003; Koffler et al. 2016; Veiga et al. 2018), where sex ratio is highly skewed to males, in particular in the non-*Melipona* species (Boomsma et al. 2005; Koffler et al. 2016), thus implying that rival males could dislodge the mating pair. In this scenario of intense male–male competition, both male and female may benefit from piercing structures, thus traumatic mating may also have an adaptive value, such as increased paternity or fecundity stimulation (Lange et al. 2013). Indeed, it has been demonstrated that the mechanical stimulation of mating plugs triggers ovarian activation in at least 1 species of stingless bee (*M. quadrifasciata*; Melo et al. 2001). Nonetheless, whether neutral or adaptive, effects of copulatory lesions caused by traumatic mating plugs on female reproductive fitness deserve further investigation.

We also identified that the mating plug leaves a characteristic mark on the abdominal tissues of mated queens, the melanized patches, and that the number of marks likely corresponds to the number of potential mates reported in the literature, thus providing

robust evidence of remating in this group. Both evidence of single and multiple paired marks find support in the literature of stingless bees: although most of our studied species showed a single pair of copulatory marks (Figure 1 and Table 1), potentially corresponding to a general tendency for monandry in the group (Strassmann 2001; Vollet-Neto et al. 2018; Grüter 2020), we found multiple patches in *M. seminigra* (Figure 1 and Supplementary Figure S4), a species that has already been suggested to show levels of polyandry (Francini 2013). Colonies of *M. seminigra* headed by 1 queen were found to show multiple patriline among brood, as revealed by molecular data, suggesting that queens mated with multiple males (Francini 2013). Now our study indicates that the molecular evidence of polyandry in *M. seminigra* (Francini 2013) is supported by the number of copulatory marks we found in queens (Figure 1 and Table 1). Additionally, data on the genital functional morphology and mating interactions (Figures 2 and 3 and Supplementary Video S1) suggest mating plug activation is unstoppable after triggered. Also, they indicate that males are not capable of protruding plugs partially or completely without losing it, thus being unlikely that 1 male can leave multiple marks on a queen. In this sense, genital functional morphology and the hypothesized mating interactions jointly support inference of remating in *M. seminigra*. Taken together, our results and the literature support the use of copulatory marks to infer the likely number of queen's mating partners in stingless bees, or at least the queen mating condition (e.g., unmated, once mated, or remated).

Our evidence suggests that short-term plugs are the rule, although long-term plugs are the exception, and that their persistence may be determined by morphological traits making them either easier or harder for females to detach. Interspecific variation in the morphology and movement of male genital capsule may be responsible for the distinct reproductive behavior found in stingless bees, that is, short-term plug versus long-term, and single mating versus remating. Although we did not quantify mating plug size and shape in our sampled species, we were able to visually compare mating plug morphological traits among them (Figure 1). We found short-term mating plugs in all species, except for *M. fasciculata* whose queens consistently retained the plug irrespective of their age (Figure 1 and Supplementary Figure S3). Even though *M. fasciculata* and *M. seminigra* show similar penis valve traits, both being robust and strongly curved (Figure 1), *M. fasciculata* mating plugs differ in their wide rotation capacity of $>90^\circ$ of opening, although *M. seminigra* plugs differ in their shorter penis valves, with weaker rotation capacity (Figure 1). The specific combination of penis valves length and rotation in each species possibly makes plugs harder to detach in the first, but easier to remove in the second, suggesting that male genital morphology may be related to time persistence of plugs and, as a consequence, to the probability of remating. Further investigation should quantify intrapopulation variability in mating plug size and shape of *M. seminigra*, or similar species in terms of mating system, to understand how morphology determines functional traits as time persistence, and how it may impact the probability of remating.

Little is known about how mating plugs could be removed and the time taken for removal, but there are indications that in *Melipona* species it could take from less than 25 min to about 3 days (Vollet-Neto et al. 2018). If queens are able to remove plugs soon after they return from mating flight, quickly enough before gaining weight through ovarian activation, then a second mating flight could take place, as already observed in honeybees (Woyke 1964; Schlüns et al. 2003). Subsequent flights on consecutive days have been reported in stingless bees, although at a very low frequency (2 virgin

queens of *T. angustula*, Van Veen and Sommeijer 2000), which could explain what is happening with *M. seminigra* queens. Another possibility is that queens remate in the field. Reports suggest mating plugs are removed by the queens themselves in the nest, or with the help of workers (Kerr et al. 1962; Da Silva et al. 1972), but it has never been reported that males could do so, either at the nest, or in the field, as in honeybees (Koeniger 1990; Koeniger and Koeniger 1991). So, although we have some evidence of the persistence of mating plugs being related to their morphological traits, further research in stingless bee mating behavior is needed to investigate how the traumatic mating plugs are removed, where, and the time taken for removal.

We have shown that traumatic mating in stingless bees does not always prevent remating, but equally we do not have unequivocal evidence that mating plugs prevent it, as predicted by the mating-guard hypothesis (Alcock 1994), in any of the studied species. Although we found only clear evidence for remating in *M. seminigra*, it is important to state that queens of other species may still be able to remate following plug removal, but do not do so for other reasons. Even in *M. fasciculata* it is not possible to say it without confirmation from complementary studies. In this sense, molecular data could inform us the number of paternal lineages among brood (Strassmann 2001; Vollet-Neto et al. 2018), although behavioral studies could reveal the role of queens' choice or resistance to mate (Eberhard 1985, 1996) in determining their number of mating partners. Additionally, our study shows that traumatic mating plugs are more than just a barrier to rival males, as they harm females. The lesions may affect female receptivity after mating, with behavioral and physiological changes potentially linked to remating avoidance, like investment in healing damaged genital tissues (e.g., ants, Baer and Boomsma 2006; Kamimura 2008) and in ovary activation (Melo et al. 2001), which could indirectly enhance male control over queen remating chances.

Finally, by demonstrating the existence of queen remating in stingless bees, our study raises an interesting question: how does the traumatic mating plug correlate to mating systems in stingless bees? It has been argued that mating plugs are a common strategy in monogamous systems (Boomsma et al. 2009), and have been considered as a primary hypothesis to explain the maintenance of single mating queens in the majority of eusocial Hymenoptera (Strassmann 2001; Boomsma et al. 2009). In stingless bees, monogamy has been considered a paradox because it challenges a very strong hypothesis in social insects: high genetic diversity is important to maintain large insect societies (Hughes et al. 2008b; Boomsma et al. 2009; Jaffé et al. 2012, 2014). In most social insects with large societies, queens are highly polyandrous (e.g., honeybees, yellowjacket wasps, and *Acromyrmex* leaf-cutter ants, Ross and Carpenter 1991; Boomsma et al. 1999, 2009), suggesting that males failed to control remating. However, stingless bee males seem to have succeeded in most cases. An efficient plug that prevents queens from remating is a plausible proximate/mechanistic hypothesis to explain the monogamy paradox in stingless bees. In this sense, facultative remating in *M. seminigra* may be an example of an exception that confirms a general tendency of single mating being linked to the efficiency of mating plugs in this group. Indeed, in the social Hymenoptera as a whole, there are indications that when mating plugs fail, remating is more likely to take place (Boomsma et al. 2009). Moreover, there may be ultimate/evolutionary reasons for queens mating with a single male. The few studies on trying to understand this condition in stingless bees argue that single mating may also be linked to the fitness costs associated with inbred queens producing unviable or unfertile diploid males, rather than viable workers and fertile queens (Cook and Crozier 1995). Ratnieks (1990) have suggested that biological features in

stingless bees, such as the impossibility of identifying the ploidy of brood before it emerges, may favor selection to single mating due to reduced diploid male load to colony fitness, in opposition to the honey bees, in which the early removal would favor selection to multiple mating. Additionally, Vollet-Neto et al. (2019) have suggested that the queen execution behavior under the presence of diploid males may also create conditions under which selection would favor single mating. Undoubtedly, mating plugs are only part of the explanation for the widespread monogamy in the Meliponini and further investigation on the interplay between mechanistic and evolutionary reasons is necessary to draw stronger conclusions.

In summary, traumatic mating plugs do not fully control female remating in stingless bees and mating systems are not uniform in this group. Nonetheless, it is the exceptional cases of facultative polyandry in social insects, when mating plugs fail to prevent remating, that may confirm a general tendency of single mating in social insects being in close link with efficient mating plugs. Furthermore, morphological traits may determine in part the time persistence of mating plugs (whether short-term or long-term) and, consequently, impact the remating chances of a queen. The traumatic mating phenomenon may be a widespread strategy among Meliponini bees due to shared male genital traits by various species. However, the observed differences among our studied species highlight that we need to be cautious when generalizing mating systems in stingless bees. Finally, our study demonstrates that the traumatic mating system in this group provides a reliable and low-cost diagnostic tool to identify mating conditions (e.g., unmated, once mated, or remated) of Meliponini queens. We hope this approach can be useful for future research aiming to infer the exact number of mating partners of stingless bee queens, a key knowledge to understand colonies' genetic diversity and social conflicts. Further studies should combine morphological, ecological, and molecular data to investigate remating in stingless bee species and understand the conditions under which such phenomenon is more likely to occur, including its costs and benefits to queens.

Author's Contributions

J.C.V., F.A.C.L., and G.R.S.R. conceived the sampling design. J.C.V. collected and analyzed the data. G.R.S.R. prepared the illustrations. J.C.V. wrote the initial draft of manuscript and all authors contributed to subsequent revisions.

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Supplementary Material

Supplementary material can be found at <https://academic.oup.com/cz>.

Conflict of Interest Statement

The authors declare that they have no competing interests.

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