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Evolução dos sistemas de acasalamento em abelhas sem ferrão (Apidae, Meliponini)

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"A resposta certa, não importa nada: o essencial é que as perguntas estejam certas"
Mario Quintana

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RESUMO

O sistema de acasalamentos das abelhas sociais é intrigante. As rainhas acasalam durante uma curta janela de tempo nas fases inicias de suas vidas, armazenando o esperma pelo resto de suas vidas. Enquanto as rainhas das abelhas sem ferrão (Meliponini) e de mamangavas (Bombini) se acasalam com um ou poucos machos (monândricas), as rainhas de espécies representantes do gênero *Apis* (Apini) acasalam-se com vários machos (poliândricas). Diversas hipóteses têm sido propostas para compreender os benefícios do comportamento extremamente promíscuo das rainhas do gênero *Apis*. Porém, pouco foi feito para entender as igualmente intrigantes pressões seletivas que mantém a monandria em um grupo tão diverso quanto o das abelhas sem ferrão. No presente estudo, investigamos como as forças seletivas causadas pelas produção de machos diploides (uma consequência natural do sistema de determinação sexual em Hymenoptera) podem afetar o sistema de acasalamento da abelha sem ferrão brasileira, *Scaptotrigona depilis*. Em particular, rainhas que realizam um acasalamento pareado para o *locus* sexual (i.e., acasalam com um macho que possui o mesmo alelo sexual) terão o seu fitness reduzido porque elas serão executadas em colônias com 50% de machos diploides entre sua cria diploide. Acasalar-se com mais de um macho aumentam as chances de um acasalamento pareado, mas reduz a proporção de machos diploides na cria. Por meio da manipulação dos favos de cria de colônias experimentais, nós testamos se a mortalidade de rainhas com proporções menores de machos diploides na cria terão taxas de mortalidade similares à de rainhas em colônias com 0% ou 50% de machos diploides na cria. Para isso, obtivemos rainhas produzindo machos diploides nesta espécie e estudamos alguns detalhes de sua biologia, em particular, a viabilidade e morfologia de seus espermatozoides e comportamento fora de suas colônias (Capítulo 1). Nós verificamos se a rainha morre na presença de machos diploides, como este comportamento é predominante na população, e quais os possíveis mecanismos que desencadeiam este comportamento (Capítulo 2). Finalmente, verificamos se a mortalidade de rainhas em colônias com proporções menores de machos diploides na cria (simulando acasalamentos múltiplos) é mais próxima da mortalidade de rainhas em colônias com 0 ou 50% de machos diploides (Capítulo 3). Verificamos que os machos diploides de *S. depilis* são viáveis e juntam-se a agregados reprodutivos. Seus espermatozoides possuem a mesma viabilidade que os dos machos haploides, porém possuem a cabeça e a cauda maiores. As rainhas são mortas na presença de cerca de 50% de machos diploides na cria e esse comportamento é muito difundido na população estudada, com 100% de morte das rainhas (n=20). O perfil químico de hidrocarbonetos cuticulares dos machos diploides é quantitativamente diferente dos machos haploides, sugerindo que pode conter informações para que a rainha seja executada. No entanto, por meio da contagem de espermatozoides na espermateca de rainhas recém acasaladas e com um ano de idade foi possível observar que o esgotamento de espermatozoides pode afetar a expectativa de vida das rainhas. Isso sugere que o sinal para execução da rainha também pode estar associado a uma quantidade grande de machos emergindo, como um sinal de esgotamento de espermatozoides da rainha, ao invés da ploidia dos machos. Rainhas em colônias com cerca de 25% de machos diploides emergindo foram mortas em proporções semelhantes à de rainhas em colônias com 50% de machos diploides. Isso indica que esse comportamento causa uma pressão seletiva contra os acasalamentos duplos nessa espécie, pois ao acasalar duas vezes a rainha dobra as chances de realizar um acasalamento pareado e a mortalidade é igual à de rainhas que se acasalam uma única vez.

Palavras-chave: machos diploides; execução da rainha; monandria; *Scaptotrigona depilis*;

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ABSTRACT

The mating systems of social bees is intriguing. Queens mate within a narrow time window very early in their lives, storing male sperm for their entire lifespan. In bumblebees (Bombini) and stingless bees (Meliponini), queens usually mate with a single male (monandry), while the queens of *Apis* species (Apini) mate with several males (polyandry). Several hypothesis have been proposed to understand the benefits of the extreme polyandry of honey bees. However, very little have been done in order to understand the equally intriguing selective forces that make such a diverse group as stingless bees as monandrous. In the present study we investigate how the selective force imposed by the chances of producing diploid males (a natural consequence of sex determination system of Hymenoptera) can affect the mating system of the Brazilian stingless bee *Scaptotrigona depilis*. In particular, queens that make a matched mating for the sex locus (i.e., mating with a male with the same sex allele) will have reduced fitness because queens are executed when producing 50% of diploid males amongst her diploid offspring. Mating with more males increase the chances of a matched mating, but reduces the proportion of diploid males on their brood. By manipulating the brood combs of experimental colonies, we tested whether queens with smaller proportions of diploid males (25%) will have mortality rates similar to queens in colonies with 0% or 50% diploid males. For that, we obtained queens producing diploid males and studied details of diploid males biology, in particular their sperm viability and morphology, and their behaviour out of their mother colonies (Chapter 1). We verified how prevalent queen execution behaviour under diploid male production is on this species, and the possible proximate mechanisms that trigger this behaviour (Chapter 2). Finally, we verified whether queen mortality in colonies with lower proportion of diploid males (simulating multiple mating) is closer to colonies with 50% or 0% of diploid males (Chapter 3). We verified that diploid males of *S. depilis* are viable and can join reproductive aggregations. Their sperm cells have the same viability of haploid males, but have longer sperm head and tail cells. Queens are executed in the presence of 50% of diploid males, and this behaviour is very prevalent on the studied population, with 100% of queen death (n=20). The cuticular hydrocarbon chemical profile of diploid males is quantitatively different from haploid males, suggesting that workers could use it as a signal for queen execution. However, sperm count of newly mated queens and one-year-old queens showed that sperm depletion might affect queen lifespan. This suggests that the signal for queen execution could also be related to a high number of males emergence, as a signal of queen sperm depletion, instead of male ploidy. Queens mortality in colonies with 25% of diploid males was similar to queens in colonies with 50%. This indicates that double mating is selected against regarding this selective pressure, since mating twice will increase the chances of a matched mating and queen mortality will be the same as single mated queens.

Keywords: diploid males; queen execution; monandry; *Scaptotrigona depilis*;

SUMÁRIO

GENERAL INTRODUCTION

Mating systems

Mating systems describes the way in which a sexually reproducing animal group is structured in relation to mating behaviour (Davies & Krebs 2012). In other words, it refers to the number of mating partners that males and females look for. There are basically four types of recognised mating systems, namely: monogamy, polygyny, polyandry and promiscuity (Table 1), that basically refers to the number of mating partners each sex type has. Such difference of strategies that each sex makes to reproduce, evolutionarily arises on the different investment that males and females makes to reproduce.

When studying sexual selection in Drosophila, Bateman (1948) concluded that due to the differential investments made by males and females on reproduction, in terms of time and energy spent, usually there are different interests for each gender in relation to mating behaviour. Such differences are originated on the differences between sexual gametes: females usually invest more energy on their gametes. Hence, the number of offspring a female is able to have is not dependent on the number of copula they perform, since one ejaculates is quite enough to fertilize all her eggs. On the other hand, male number of offspring is directly linked to the number of mates a male had. Therefore, considering only the gamete size, the male fitness is maximized the more females they mate with, while the female fitness usually do not increase with more than one mating.

However, the scenario is not so simple for majority of animal species. Many other behavioural and ecological traits can act as a selective pressure on the mating system. Emlen & Oring (1977) defined some ecological parameters to understand the evolution of mating systems. They identified that the pattern of spatial distribution of key resources and the temporal availability of mating partners greatly influence mating systems. Examples of key

resources are food, refuges, and even the potential mating partners. They suggest that the bigger the potential to monopolize the resources and/or mating partners, the higher is sexual selection and consequently the higher is the potential to polygamous systems, instead of monogamous. They also suggest that since those ecological variables can change across time, there is a great plasticity and variation on the mating systems from time to time, and even amongst different intraspecific individuals.

Table 1: Brief description of the mating systems found in animal species

Mating system		Description
Monogamy		Male and female have a exclusive mating relationship for a big period of their lifes, or even during a mating season
Polygamy	Polygyny	A male mate with two or more females
	Polyandry	A female mate with two or more males
Promiscuity		Males and females mate with multiple partners during a mating season

Mating systems in social Hymenoptera

The mating system of social hymenopterans received special attention because presents peculiarities and puzzles that challenge our understanding. Social insects share a fundamental feature of division of reproductive labour, in which usually a single queen reproduce, while the workers are usually sterile. The queen is usually the only colony member that mate. In majority of hymenopteran species, queens mates with a single male (Boomsma e Ratnieks 1996; Strassman 2001), that is likely the ancestral condition of social species (Hughes et al. 2008). Copulation occurs in the air, during a narrow time window early in queen life. Male sperm is stored in a specialized organ, the spermatheca, where are kept alive throughout the queen life and gradually used to fertilize her eggs (Michener 2000).

The number of males that effectively mate with a queen was only revealed when molecular techniques, such as microsatellite analysis, were used. Most of the social species have shown monandrous queens (Boosma & Ratnieks 1996; Strassmann 2001). On the other hand, a small number of species showed an extreme effective paternity frequency amongst a single queen brood. Curiously, they are representative of groups of great ecological success, such as the *Apis* bees, *Atta* ants and *Vespula* wasps (Boomsma & Ratnieks 1996).

Those extreme promiscuous queens are an evolutionary puzzle. The costs of mating multiple times are evident: the mating flight is the most critical period in a life of a queen, where she can be preyed, males can transmit diseases and harm the queen during attempts of copula. Moreover, a high effective paternity can drastically decrease the relatedness coefficients amongst colony members potentially affecting the conflicts occurring between them (Ratnieks & Boomsma 1995). That could happen because the theory of inclusive fitness predicts that a high relatedness coefficient is necessary for evolution of an altruistic social behaviour (Hamilton 1964). According this theory, altruistic behaviours would be favoured because they would increase the chances of gene transmission to the next generation through an increase in reproduction of relatives, to the costs of their own reproduction. Low levels of relatedness cause little own gene transmission to the next generation. In fact, the great number of independent origins of social behaviour in Hymenoptera is partially attributed to the high levels of relatedness that haploid males determine, since their sperm cells are genetically identical, giving rise to diploid individuals sisters (hymenopteran diploid individuals are females – see details below) sharing most of their genetics. A higher number of patrilineages greatly reduces the relatedness coefficient between half-sisters (sisters of different patrilines). Therefore, the inclusive fitness of workers that care for low-related individuals is much lower, affecting the maintenance of altruistic behaviours (Boomsma e Ratnieks 1996; Boomsma e Ratnieks 1996).

On the other hand, understanding the benefits of this extreme polyandry it is not so obvious. Several hypothesis were proposed to explain the evolution of polyandry in social Hymenoptera (Crozier & Page 1985 Boomsma & Ratnieks 1996, Crozier & Fjerdingstad 2001). However, few of them have been properly investigated. The hypothesis that have mostly draw the attention of scientific community is the "genetic diversity" hypothesis (Table 2). This hypothesis suggests that multiple matings cause a high genetic diversity, and consequently phenotypic, among colony members. Such diversisty confers a better colonial performance on general tasks, such as foraging (Mattila & Seeley 2007; Mattila et al. 2008) nest thermoregulation (Jones *et al.* 2004), and increase overall disease resistance (Tarpy & Seeley 2006, Seeley & Tarpy 2007; Baer & Schmid-Hempel 1999, 2003; Hughes & Boomsma 2004). A related hypothesis, regarding the sex determination system of Hymenoptera, specifically on how the diploid male fitness load could be a selective force acting on mating systems of social Hymenoptera have also been considered (Page 1980), but lack empirical support. Herein, we investigated aspects of this hypothesis, focusing on its selective pressure in mating systems of stingless bees. Hence, hereafter we will present this hypothesis and how it may affect the group of the social stingless bees.

Table 2: Hypothesis to explain the evolution of polyandry and its relevance to the group of highly eusocial bees. Modified from Boosma & Ratnieks (1996)

Hypothesis	Hypothesis description	Relavant to highly eusocial bees? Why?	References
Costs of avoiding male copulation attempts	Avoiding conflicts with males is costly to queens than mating several times	No; honey bee queens can perform multiple mating flights	Tan et al. 1999
Sperm limitation	Need/capacity of sperm storage of queen spermatheca is bigger than the quantity of sperm a single male can produce	No; the amount of sperm produced by a single male is equal or bigger than spermatheca capacity	Cole (1983), Boomsma & Ratnieks (1996), Fjerdingstad & Boomsma (1998)
Nuptial gifts/nutritive sperm	Females gain mating with several males due to direct nutritional gains associated with the copula	No; gains would be very small, specially considering that highly eusocial queens have the support of workers for nutrition	Lamunyon & Eisner (1994)
Good genes	Increase the probability of mating with a male genetically superior	Yes; however there is no evidences of partner choosen by the queens (Koeninger 1990)	Zeh & Zeh (1997)
Spermatic competition	Passive way off emale choosen that allows the best male to have a bigger fertility	Yes; however it was tested and not supported	Parker (1984); Keller & Reeve (1995)
Effective population size	Increase the effective population size (Ne)	No; polygyny is more effective to increase Ne than polyandry (Crozier e Page 1985)	Wilson (1963)
Genetic Variability i. Better colonial performance	Increase on colony fitness due to performing better on tasks that involves division of labour: more effective foraging exploitation; better at colonial thermoregulation	Yes;	Oster & Wilson (1978), Robinson & Page (1989), Oldroyd et al. (1991), Oldroyd et al. (1992), Oldroyd et al. (1993), Fuchs & Moritz (1999), Calderone et al. (1989), Page et al. (1995)
ii. Parasite tolerance	Higher fenotipic variety would decrease chances of disease transmission	Yes;	Hamilton (1987), Sherman et al. (1988), Schmid-Hempel (1998);
iii. Diploid male load	Reduce the variance of diploid male production between colonies	Yes;	Page (1980), Page & Metcalf (1982), Crozier & Page (1985), Ratnieks (1990), Pamilo et al. (1994), Crozier & Pamilo (1996)

Mating systems in eusocial bees with focus on stingless bees

The pattern of mating system of social bees it is not too different of social Hymenoptera in general. There is a great diversity of monandrous species, or with very low levels of polyandry, while a small number of species have extreme polyandrous queens (Table 3). There is a high phylogenetic signal on this trait: Apini bees, comprising 11 species eusocial of *Apis* genus, are a polyandrous group. The bumblebees (Bombini), a primitively social species of *Bombus* genus (Michener 2000), and the stingless bees (Meliponini) with ca. of 500 eusocial species distributed over more than 60 taxonomic groups (Ramussen and Cameron 2010), have monandrous queen or with low polyandry levels (Table 3).

The number of males that successfully copulated with a stingless bee queen was a mystery before molecular markers came into use. The very few earlier observations indicated that one (Kerr & Krause 1950) or two males (*Tetragonisca angustula* - Imperatriz-Fonseca et al. 1998) were copulating with a single queen. Conclusions on *T. angustula* were, however, speculative, since the inference on the number of copulation events was based on the number of males that lost their genitalia apparatus, whereas fecundation could not be assured (Imperatriz-Fonseca et al. 1998). Indirect evidence, such as sperm counts in the spermatheca of newly mated queens, compared to the seminal vesicle of mature males, indicated that single mating occurred in *M. quadrifasciata* (Kerr 1962, Camargo 1972).

Another indirect evidence for mating is the presence of a mating plug, a mechanism that, apparently, allows the male forced control over the queen's monogamy. The mating plug is a sclerotized spine shaped structure of the male genital system, which stays attached to the female genital chamber after copulation (Kerr & Krause 1950). It is suggested that this structure could prevent further copulations (Kerr & Krause 1950; Kerr 1962). Yet, very little is known about the role and effects of the mating plug in the mating system of stingless bees. There is weak evidence that the duration of attachment of the mating plug in the newly mated

queen is correlated to her ovary development, as indicated by observations on *M. quadrifasciata* queens mated under controlled conditions. The results suggested that the mating plug needs to be attached to the female genital chamber for up to three day to trigger ovary development (Melo et al. 2001). However, naturally mated queens of *M. quadrifasciata* and *M. quinquefasciata* took only 21 min and 1 h 17 min to get rid of the mating plug by themselves, (da Silva et al. 1972), and it took about one hour in *M. beecheii* (Van Veen & Sommeijer 2000). The queens apparently needed a substrate for rubbing their abdomen to remove the male genitalia.

Hence, it is possible that queens can get rid of the mating plug soon after returning to the nest and perform another mating flight, just as honey bee queens do when not enough matings had been performed in a single mating flight (Schlüns et al. 2005). In fact, subsequent mating flights were already reported for *T. angustula* virgin queens (Van Veen & Sommeijer 2000). Also, egg-laying physogastric queens of *M. quadrifasciata* could be mated with a second male in controlled conditions (Campos & Melo 1980). The sperm is successfully transferred to the spermatheca and later used for egg fertilization (Lopes et al. 2003), showing that physogastric queens are physiologically able to receive sperm from extra copulations. Sakagami & Laroca (1963) observed a male copulating with the physogastric queen inside the nest in *Lestrimelitta ehrhardti*, during a colony manipulation event. They suggest that this could be an unusual event due to the colony disturbance. Physogastric queen mating was never observed in natural environments or observation hives. Physogastric queens are unable to fly.

The use of microsatellite genotyping to assess the number of patrilines in colonies of stingless bee species largely confirmed what the indirect lines of evidence had already indicated: single or very low numbers of patrilines (Table 3). The first studies showed low levels of polyandry (Falcão & Contel 1991; Paxton *et al* 1999b; Peters *et al* 1999; Paxton

2000), but these were not totally conclusive, mainly due to many other possibilities than polyandry to explain the presence of anomalous genotypes amongst workers of a colony. Two main factors were considered as contributing to these anomaly results: a) genotyped individuals were old foragers, allowing the possibility of genotyping drifting workers; and b) the mother queens were not marked or followed, allowing sampling of two different matrilines due to queen supersedure (Paxton 1999).

Later studies considered some of these issues, and results on microsatellite analysis on several stingless bee species were consistent with a monandrous queen (Palmer et al. 2002, Peters et al. 1999, Wenseleers et al. 2011). The main caution in these studies was sampling individuals at the pupal stage or callow workers, thus eliminating the possibility of worker drifiting. However, up to date only Wenseleers et al. (2011) controlled the mother queens by sampling the wing tips of *Melipona scutellaris* queens and genotyping them, as well as the worker and male pupae of the studied colonies. Therefore, they could track the mother queen genes in the brood, confirming monandry and discarding the possibility of queen supersedure. An alternative method to ensure the matriline is to paint-mark a queen before sampling her brood for microsatellite analysis.

Even with such precautions there are alternative hypothesis that should be considered to explain the presence of anomalous genotypes that at first sight seem to indicate low levels of polyandry. A small number of anomalous genotypes could arise from null alleles, the accidental inclusion of males among the analyzed workers, or from polygyny, rather than polyandry (Peters *et al* . 1999; Paxton *et al* 2001b, Palmer 2002). Even though being a rare event, a microsatellite mutation in the fathering male's germline could also explain apparent polyandry (Gardner et al. 2000) and, hence, should be taken into consideration while interpreting results. It is suggested to increase the sample size if the issue of polyandry came up in a first analysis on Meliponini colonies.

Recently, Vianna et al. (2015) reported that seven out of nine queens of *Melipona mondury* presented some degree of polyandry (2 – 7 patrilines amongst 10 pupae workers genotyped). This is the only study reporting a relatively high degree of polyandry in a stingless bee species, so far. However, it is important to interpret the results carefully, since the authors did not take many of the previous mentioned precautions. For instance, queen replacement or polygyny were not considered as a possible cause of the anomalous genotypes. Also, the number of useful molecular markers were relatively low for some colonies. There was also great variation in the number of molecular markers that worked for each colony. This suggests that the primers could not have worked as predicted, and the results are overestimating the number of different alleles. These points should have been considered while taking the conclusions on patriline numbers in the referred study.

Therefore, with very few exceptions, the current molecular tools support the view that stingless bees are a monandrous group of eusocial bees. The anomalous genotypes indicating low polyandry levels must be carefully interpreted, but there is the possibility that queens of some species occasionally mate with two or more males.

Table 3: Paternity frequency of 58 eusocial bee species. Only studies using genetic markers were used. Paternity frequency is divided as observed partenity (K_{obs}) and effective paternity (m_e). the number of analysed colonies (n) and the colony size (mean number of workers) are also shown. Data extracted and modified from Jaffé et al. (2014).

Tribo	Espécie	K_{obs}	m_e	n	Tamanho colonial	Referências
Apini	*Apis andreniformis*	13.50	10.50	60	4,9	Oldroyd et al. 1997; Koeniger et al. 2011
Apini	*Apis cerana*	18.80	14.10	74	6,884	Oldroyd et al. 1998; Hammond & Keller 2004
Apini	*Apis dorsata*	54.90	44.20	140	36,63	Hammond & Keller 2004; Wattanaachaiyingcharoen et al. 2003
Apini	*Apis florea*	11.60	7.90	81	6,271	Hammond & Keller 2004; Palmer & Oldroyd 2001
Apini	*Apis koschevnikovi*	16.20	13.30	74	7	Rinderer et al. 1998; Koeniger et al. 2011
Apini	*Apis laboriosa*	34.40	18.28	135	36,63	Paar et al. 2004; B. Oldroyd pers. comm.
Apini	*Apis mellifera*	12.00	11.60	61	19,524	Estoup et al. 1994; Michener 1974; Schneider & Blyther 1988
Apini	*Apis nigrocincta*	54.00	40.30	159	6,884	Palmer et al. 2001; B. Oldroyd pers. comm.
Augochlorini	*Augochlorella striata*	1.00	1.00	24	7	Mueller et al. 1994
Bombini	*Bombus affinis*	1.00	1.00	1	176	Payne et al. 2003; Michener 1974
Bombini	*Bombus ardens*	1.00	1.00	5	26	Kokuvo et al. 2009; Katayma 1997
Bombini	*Bombus auricomus*	1.00	1.00	1	35	Payne et al. 2003; Katayma 1997
Bombini	*Bombus bimaculatus*	1.25	1.05	8	60	Payne et al. 2003; Michener 1974
Bombini	*Bombus citrinus*	2.50	1.76	10	50	Payne et al. 2003; B. Baer pers. comm.
Bombini	*Bombus fervidus*	1.00	1.00	1	88	Payne et al. 2003; Michener 1974
Bombini	*Bombus griseocollis*	1.00	1.00	1	46	Payne et al. 2003; Cameron 1989
Bombini	*Bombus honshuensis*	1.00	1.00	1	200	Kokuvo et al. 2009; Ochiai & Katayama 1982
Bombini	*Bombus hortorum*	1.00	1.00	5	100	Schmid-Hempel & Schmid-Hempel 2000; Michener 1974
Bombini	*Bombus hypnorum*	1.87	1.18	23	29	Paxton et al. 2001; Schmid-Hempel & Schmid-Hempel 2000; Hammond & Keller 2004
Bombini	*Bombus impatiens*	1.55	1.04	10	450	Payne et al. 2003; Cnaani et al. 2002; Michener 1974
Bombini	*Bombus lapidarius*	1.00	1.00	11	200	Schmid-Hempel & Schmid-Hempel 2000; Westphal et al. 2006
Bombini	*Bombus lucorum*	1.00	1.00	12	200	Schmid-Hempel & Schmid-Hempel 2000; Michener 1974
Bombini	*Bombus mixtus*	4.00	3.57	5	50	Payne et al. 2003; B. Baer pers. comm.
Bombini	*Bombus occidentalis*	1.00	1.00	23	55	Owen & Whidden 2013; Whittington & Winston 2003
Bombini	*Bombus pascuorum*	1.00	1.00	6	120	Schmid-Hempel & Schmid-Hempel 2000; Westphal et al. 2006
Bombini	*Bombus pratorum*	1.00	1.00	5	100	Schmid-Hempel & Schmid-Hempel 2000; Westphal et al. 2006
Bombini	*Bombus ternarius*	2.00	2.04	12	100	Payne et al. 2003; Michener 1974
Bombini	*Bombus terrestris*	1.00	1.00	17	350	Schmid-Hempel & Schmid-Hempel 2000; Westphal et al. 2006
Bombini	*Bombus vagans*	1.00	1.00	4	70	Payne et al. 2003; Michener 1974
Halictini	*Lasioglossum malachurum*	1.36	1.15	30	33	Paxton et al. 2002; Richards et al. 1995; Soro et al. 2009
Halictini	*Lasioglossum zephyrum*	1.00	1.00	8	20	Barrows 1975; Crozier et al. 1987
Meliponini	*Austroplebeia australis*	1.00	1.00	2	2	Drumond et al. 2000; Hammond & Keller 2004

Meliponini	*Austroplebeia symei*	1.00	1.00	4	2	Palmer et al. 2002; Hammond & Keller 2004
Meliponini	*Heterotrigona hockingsi* [a]	1.00	1.00	4	7	Palmer et al. 2002; Tóth et al. 2004
Meliponini	*Heterotrigona mellipes* [b]	1.00	1.00	4	2	Palmer et al. 2002
Meliponini	*Lestrimellita limao*		1.23	2	900	Peters et al. 1999; Roubik 1983
Meliponini	*Melipona beecheii*	2.20	1.13	10	1,192	Paxton et al. 1999; Tóth et al. 2004; R. Paxton unpubl. data
Meliponini	*Melipona marginata*	1.00	1.00	5	202	Tóth et al. 2002b; Tóth et al. 2004
Meliponini	*Melipona panamica*	1.00	1.00	9	550	Peters et al. 1999; Tóth et al. 2004
/Meliponini	*Melipona quadrifasciata*	1.00	1.00	7	350	Peters et al. 1999; Tóth et al. 2002b; Tóth et al. 2004
Meliponini	*Melipona scutellaris*	1.00	1.00	54	992	Tóth et al. 2002b; Alves et al. 2009; Tóth et al. 2004; Wenseleers et al. 2011
Meliponini	*Nannotrigona perilampoides*		1.19	7	1,125	Peters et al. 1999
Meliponini	*Paratrigona subnuda*		1.16	11	3,75	Peters et al. 1999; Tóth et al. 2002a
Meliponini	*Partamona* aff. *cupira*	1.00	0.91	12	3,39	Peters et al. 1999; Slaa et al. 2003
Meliponini	*Plebeia* aff. *minima*		1.42	5	125	Peters et al. 1999; Roubik 1983
Meliponini	*Plebeia droryana*	1.00	1.00	2	2,96	Tóth et al. 2002b; Tóth et al. 2004
Meliponini	*Plebeia remota*	1.00	1.00	7	3,5	Tóth et al. 2002b; Tóth et al. 2004
Meliponini	*Plebeia saiqui*	1.00	1.00	4	1,5	Tóth et al. 2002b; Hammond & Keller 2004
Meliponini	*Scaptotrigona depilis* [c]	1.20	1.02	19	10,375	Paxton et al. 2003; Tóth et al. 2004
Meliponini	*Scaptotrigona barrocoloradensis*	1.00	0.85	4	5	Peters et al. 1999; Tóth et al. 2004
Meliponini	*Scaptotrigona mexicana*	1.00	1.00	5	2	Palmer et al. 2002;
Meliponini	*Scaptotrigona pectoralis*	1.00	1.00	7	4,6	Palmer et al. 2002; Slaa et al. 2003
Meliponini	*Schwarziana quadripunctata*	1.00	1.00	22	1,65	Peters et al. 1999; Tóth et al. 2003; Tóth et al. 2004
Meliponini	*Tetragona clavipes*	1.00	1.00	17	7	Peters et al. 1999; Tóth et al. 2002b; Tóth et al. 2004
Meliponini	*Tetragonula carbonaria* [d]	1.00	1.00	5	2,75	Green & Oldroyd 2002
Meliponini	*Tetragonula clypearis* [e]	1.00	1.00	4	500	Palmer et al. 2002; Hammond & Keller 2004
Meliponini	*Trigona fulviventris*		1.15	7	8,5	Peters et al. 1999; Slaa et al. 2003
Meliponini	*Trigona spinipes* [f]	1.00	1.00	44	92,5	Jaffé et al. 2014; Lindauer & Kerr 1960

[a] previously *Trigona hockingsi*, [b] previously *Trigona mellipes*, [c] previously *Scaptotrigona postica*, [d] previously *Trigona carbonaria*, [e] previously *Trigona clypeari*, [f] previously *Trigona ruficrus*

Evolution of the mating systems in eusocial bees

Most studies have focused on understanding the evolution of the high polyandry levels in *Apis* bees. New queens are produced in honey bee colonies for swarming (reproduction) or queen replacement, in case of mother queen death. The queens make the mating flight early in their life, within a narrow time window. They usually flight into a male congregation site that is

located in the air. During her flight, hundreds of males follow her, while very little are able to grab the queen in the air and mate. After mating, males evert their genitalia and dies (Koeninger et al. 1979). They leave a mating signal (or mating plug) in the queen genitalia that do not prevent further copulations, but could function to prevent the sperm to leak out from the genital chamber (Koeninger 1990).

The mating flight is one of the most critical moments in the life of a queen. Predation risks coupled with the male competition for accessing her are factors that makes the mating flight a risky behaviour. In fact, the risk of not returning from a mating flight was estimated, and reach levels of 4.85% (Ratnieks 1990) and 6.25% (Tarpy e Page 2000). Therefore, multiple mating might bring benefits to queens that overcome such costs.

Several hypothesis were proposed to explain the benefits of multiple mating by honey bee queens (Cook & Crozier 1995). The most obvious idea is that queens are not able to avoid males. In other words, males enforcement for mating is the reason why queens mates so many times. However, it is known that queens mates deliberately with more than one male, since they can perform more than one mating flight (Tan et al. 1999; Schünls et al. 2004). Another obvious hypothesis is that queens needs more sperm than a single male would be able to offer, because honey bee queens are highly fertile (high rate of egg laying). However, there is evidence that the sperm of a single male would be enough to fill their spermatheca (Moritz & Southwick 1992).

Up to date, the most investigated and well supported hypothesis is the genetic variability hypothesis (Fuchs & Moritz 1998). Under polyandry, colonies will present workers that have different patrilines. Such difference potentially affects the division of labour within a colony, since results in different thresholds for starting different tasks. Empirical evidence have supported this hypothesis. Colonies with high genetic variability are more efficient at thermoregulation (Jones *et al.* 2004), foraging (Mattila & Seeley 2007; Mattila et al. 2008)

and disease resistance (Tarpy & Seeley 2006, Seeley & Tarpy 2007; Baer & Schmid-Hempel 1999, 2003; Hughes & Boomsma 2004) than colonies with a single patrilinear. The experimental colonies are artificially inseminated with sperm from a single male with colonies inseminated with sperm from several males. The sperm use for egg fertilization is random (Haberl & Tautz 1998), creating a mixed colony in terms of patrilines. This hypothesis was mostly investigated in polyandrous species, experimentally simulating a monandrous condition. Therefore, conclusions about the importance of genetic diversity on the evolution of multiple mating are not so strong, since it is possible that derived adaptations could bias the results. In other words, polyandry could have evolved under another selective pressures and later adaptations regarding division of labour efficiency could have evolved in honey bees.

A complementary hypothesis that is very likely important to the evolution of polyandry but is less explored mainly due to methodological constraints is the diploid male load hypothesis. It is based on the complementary sex determination system that control sex development in honey bees and most Hymenoptera. Under this system, a single multiallelic locus controls sex determination of an individual. Heterozygous individuals for this locus develop into diploid females, while hemizygous (individuals carrying a single copy of an allele) turn into haploid males (Fig. 1). Homozygous individuals for this locus turn into diploid males (Whiting 1943) (Fig. 1), that are usually infertile (Heimpel & Boer 2008). In addition, diploid males arises of fertilized egg, replacing a supposed worker, since queens would lay unfertilized eggs to produce males. Therefore, a queen that make a matched mating will have a decreased fitness, since competition at colonial level will be reduced by a small workforce, which is replaced by unfertile males.

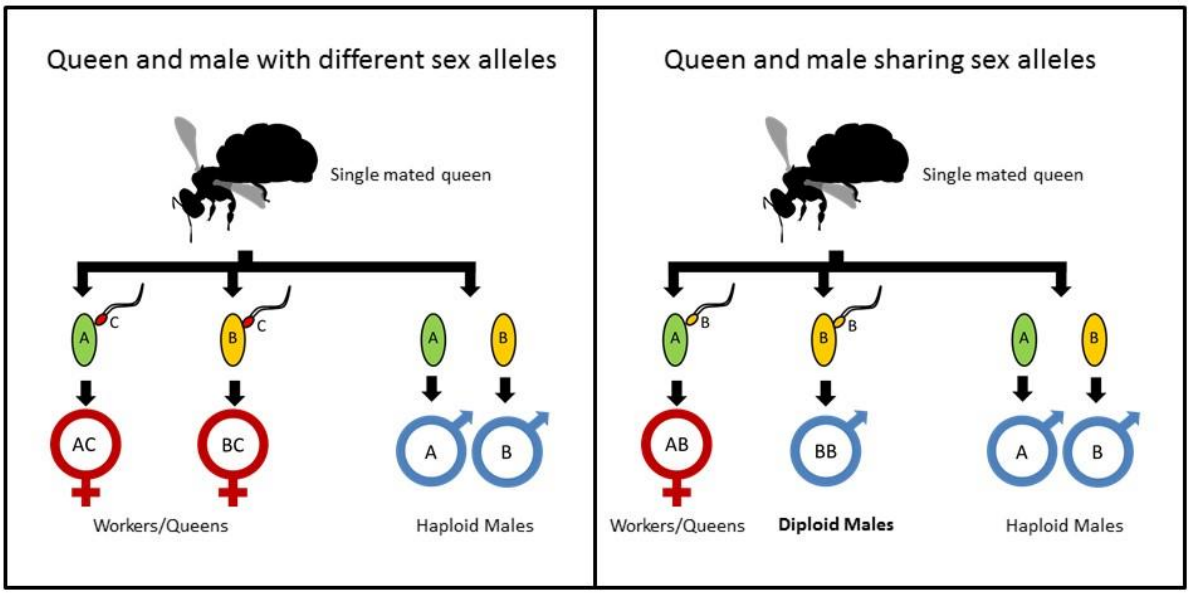

Figure 1. Possible scenarios of sex alleles on the offspring of a stingless bee' queen. Mating with a male with a different sex allele would give rise only to females amongst her diploid offspring (left). When the queen and the male share the same sex allele (right), half of their diploid offspring develop into infertile diploid males.

The production of diploid males occurs when a queen mate with a male sharing one of her sex alleles (she have two different sex alleles, since she is a diploid female), namely a "matched mating". A queen that mates with a single male have 2/n chances of making a matched mating, where "n" is the number of sex alleles in a given population, that results from a chance of 1/n of sharing one of her sex alleles, added to 1/n chances of sharing the second one. The more males a queen mates with, the higher are the chances of making a matched mating. However, at the same time the proportion of diploid males amongst her brood tend to be lower compared to the extreme situation of producing 50% of diploid males when mating with a single male that share one of her sex alleles. Considering a hypothetical ancestral state of monandrous queens, likely the next evolutionary step would be mating with two males occasionally, resulting in twice more queens producing ca. of 25% of diploid males amongst their brood. If the fitness of such queens would be closer to the fitness of queens producing 0% of diploid males than to the fitness of colonies producing 50% of diploid males, then the selection force would act in favour of multiple matings (Page & Matcalf 1982). In

honey bees, diploid males are recognized and eliminated by the workers during their early stages of development (Woyke 1963). Ratnieks (1990) analysed the effects of the behaviour of diploid males elimination in honey bees on their mating systems evolution. He concluded that this behaviour could attenuate the diploid male load on the fitness of queens that mates with several males, approximating the fitness of multiple mated queens to the fitness of colonies producing 0% of diploid males compared to colonies that produces 50% of diploid males. This situation would result in a selection force in favour of multiple mating in honey bees.

The evolution of mating systems in stingless bees

To understand the selective pressure of diploid male load on the evolution of stingless bee mating system it is necessary to know the effects of diploid males on their queen fitness. Up to date, diploid males are reported in ten stingless bee species (Table 4). They were first detected in *Melipona quadrifasciata* with techniques that induced inbreeding by mating queens with their brothers under controlled conditions (Camargo, 1979). Using the proportion of males amongst the offspring of a queen (approximately 50% males) as a first sign of diploid male production, cytogenetic techniques were used to certify their ploidy (Camargo, 1979; Kerr 1987; Carvalho et al. 1995). Results of crossing experiments also indicated that there should be a single locus controlling sex determination in *M. quadrifasciata* (Camargo, 1979), as is the case in the majority of the Hymenoptera analyzed up to date (Heimpel & Boer 2008).

Table 4: Stingless bees' species where diploid male occurrence is confirmed.

Species	Diploid male detection method	Estimated number of sex alleles	Reference
Lestrimelitta limao	Cytogenetics	-	Tavares et al. 2010
Melipona bicolor	Molecular	10	Alves et al. 2011
Melipona interrupta	Cytogenetics	22	Francini et al. 2012
Melipona compressipes	Cytogenetics	20	Kerr 1987
Melipona quadrifasciata	Cytogenetics	-	Camargo 1979
Melipona seminigra	Cytogenetics	16	Francini et al. 2012
Melipona scutellaris	Molecular	3.8[a] - 25.9[b]	Alves et al. 2011
Scaptotrigona postica[c] *(S. depilis*)*	Molecular	10[c]	Paxton, 2003; Vollet-Neto et al. 2014*
Tetragonisca angustula	Molecular	-	Santos et al. 2013
Tetragonula carbonaria	Molecular	6	Green & Oldroyd 2002

a – small managed population, without geographic contact with natural population; b – big managed population in geographic contact with natural population; c – later known as *S. depilis*;

Stingless bees mother queens die, probably executed by the workers, in response to diploid male emergence. This behaviour was first described in *M. quadrifasciata* (Camargo 1976), and was later verified in *M. scutellaris* with the help of molecular markers (Alves et al. 2011). It is suggested that this behaviour is an adaptation at the colony level to the diploid male load, leading to a last round of queen production followed by queen replacement (Alves et al. 2011), however, this was not yet properly tested.

Queen death due to a matched mating and diploid male production can potentially have evolutionary consequences on the mating system of stingless bees. This is still held as one of the major hypotheses to explain differences between the honey bee and stingless bee mating systems (Page 1990; Ratnieks 1990; Cook & Crozier 1995). However, most studies are still focused on honey bees only, and hardly any recent advances were made regarding monandry in stingless bees. If a stingless bee queen mates with two males she will double her chances of having a matched mating. On the other hand, assuming equal sperm use, mating with more males would reduce the proportion of diploid males amongst her diploid offspring.

If the fitness costs for queens producing ca. of 25% of diploid males amongst their diploid brood (in the case of a double mating) are closer to the fitness of the queens producing 50% of diploid males amongst their diploid offspring (single mating situation), then there should be selection against double mating. In contrast, if the fitness of queens producing 25% of diploid males is closer to the fitness of queens having 0% of diploid males within their brood, the selection pressure should act in favour of double mating (Page 1990; Ratnieks 1990; Cook & Crozier 1995). Measuring the fitness of colonies (and consequently of queens) is quite complex and difficult. Ratnieks (1990) proposed to measure it as queen survival, since a premature death would represent almost a null fitness.

In this way, in the present study we investigated this hypothesis by experimentally manipulating the amount of diploid males amongst the emerging brood of the stingless bee *Scaptotrigona depilis* and checking the effects on queen survival. For that, we first established a method for having matched mated queens of *S. depilis* (Chapter 1). We also investigated the sperm morphology and viability of diploid males in this species (Chapter 1). Next, we determined whether the queen execution behaviour also occurs in *S. depilis*, determining its prevalence on the studied population and also investigating possible mechanisms related to chemical signalling that could trigger such behaviour (Chapter 2) We also investigated a possible alternative hypothesis to explain queen death under diploid male production, which is queen sperm depletion (Chapter 2). Finally, we tested how the proportion of diploid males emergence amongst the offspring (0, 10, 25 e 50%) affect queen mortality, defining how this selective force might have been acting on the evolution of the stingless bees mating systems (Chapter 3).

Model species and study sites

At first, this study intended to use *Melipona quadrifasciata* as a model species, since the behaviours necessary to test the proposed hypothesis were already know to occur in this species. However, we previously knew that experiments would require a great quantity of biological material and combined with the difficulty to find *M. quadrifasciata* colonies and their low resistance to manipulation, we decide to try if *S. depilis* would present the same behaviour necessary for the hypothesis testing. This species is highly resistant to manipulation and we already had ca. of 80 colonies available to be used. Moreover, while investigating whether this species could fit on the hypothesis testing, we generated new information on another species not confined to *Melipona* genus. Most experiments were carried out at the experimental apiary of the Biology Department of the University of São Paulo at Ribeirão Preto (USP RP). A fraction of the experiments were carried out at the apiary of PROMIP near the town of Engenheiro Coelho, São Paulo State, Brazil, where we had available colonies of *S. depilis* to repeat some experiments. The two locations are 150 km apart and *S. depilis* naturally occurs in this region (Camargo & Pedro 2013). We have collaborated with several other researchers during the development of this project. In the beginning of each Chapter we inform the names and affiliation of involved people. Every Chapter have its own Figure and Table numbering. The experiments related to the central question of this thesis (Chapter 3) were done in collaboration with Professor Francis Ratnieks, from University of Sussex, United Kingdom. He came up with the hypothesis and collaborated on experimental design, data analysis and writing of the manuscript.

S. depilis is monandrous and diploid males were already reported on this species (Paxton et al. 2003), two conditions necessary for the hypothesis testing. This species was previously referred as *S. postica*, however, nowadays it is being designated as *Scaptotrigona depilis*. The taxonomic classification of this species is controversial and still needs revision.

According to Silvia Regina de Meneses Pedro it is very likely that this species it is very similar, but not the same as *S. depilis*. She, therefore, suggest that the correct designation is *Scaptotrigona* sp. aff. *depilis*, due to the lack of a refined taxonomic study on this species. That said, we state that here, for practical purposes, we will use only *Scaptotrigona depilis* throughout this manuscript.

CHAPTER 1: Diploid males in *Scaptotrigona depilis*

On this Chapter, we present the methods used to obtain the matched mated queens and the diploid males that were used in the studies presented on the next Chapters (1.1). We also made a descriptive analysis of the viability and morphology of sperm cells of haploid and diploid males of *S. depilis* (1.2). Finally, we present a study on the capacity of diploid males joining reproductive aggregations (1.3).

1.1. The diploid males

Diploid males are produced by queens that have mated with a male sharing one of her sex alleles, i.e. a *matched mating* (see *Introduction* for more details). In order to obtain queens that had made a matched mating, we allowed several virgin queens to mate with males randomly. Therefore, queens would make matched males by chance. During the whole duration of the present PhD we removed the laying queen from 99 colonies of *S. depilis*. This resulted in a new, daughter, queen taking over. Approximately 30 days after the daughter queen starts laying eggs, her oldest offspring are pupae with black eyes (Fig. 1). At this time, we inspected samples of pupae taken from brood cells in order to identify colonies in which approximately 50% of the pupae had the characteristic male morphology of smaller heads and bigger eyes (Fig. 2).

Figure 1. Uncapped brood comb showing the pupae offspring of a queen which performed a matched mating. The male:female sex ratio becomes close to 1:1, whereas the usual condition is a female biased ratio. Males (identified by larger eyes and smaller heads) are highlighted in light blue. Most of these males are diploid.

Figure 2: A male (left), a queen (center) and a worker (right) pupae of *S.* aff *depilis*. Males have smaller heads and larger eyes compared to workers.

Eight queens producing c. 50% male offspring were obtained and were marked by clipping their wings (Fig. 3). In these colonies, 10 males were collected in absolute ethanol and stored at -20°C. They were checked using DNA microsatellites at three *loci* to confirm that they were diploid (see details below) in different periods.

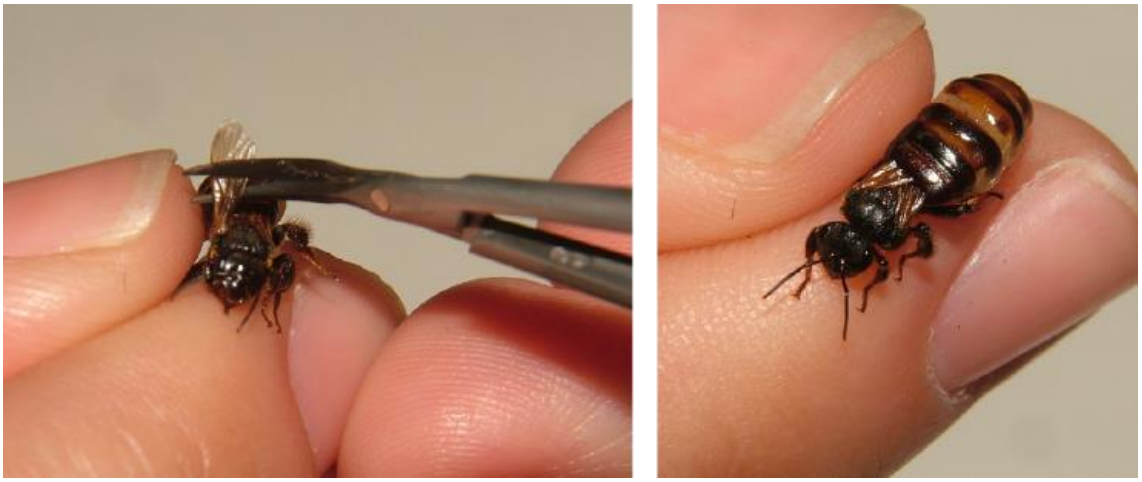

Figure 3. Queen marking. To detect the queen execution, we observed signals of queenless conditions in the hive or a new queen with the full wings.

We prevented the execution of queens that had made a matched mating by removing them from their own colonies and placing them in colonies without diploid males, from which we had removed the queen (see Chapter 2).

1.2 Sperm viability and morphology of haploid and diploid males of the stingless bee *Scaptotrigona depilis*

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Abstract

In stingless bees, as well as in most Hymenoptera, sex is determined by the combination of alleles at the complementary sex determination (*csd*) *locus*. Normally, hymenopteran males in *csd* species develop from haploid, unfertilized, eggs. However, diploid males can occur when a fertilized egg is homozygous at the *csd* locus. This occurs when a female (females are heterozygous at the *csd* locus) makes a "matched mating" with a male sharing a *csd* allele with her (e.g., AB x A), which results in half of their diploid offspring being males. Diploid males are usually unviable or sterile, imposing a great fitness cost to their parents. Here, we investigated the sperm viability and morphology of diploid males of the stingless bee *Scaptotrigona depilis*, by using a Sperm Viability Kit LIVE/DEAD Invitrogen L-7011 and a fluorescence microscope coupled with a camera for morphology measurements. We verified that there is no significant difference in sperm viability of haploid and diploid males. Sperm cells of diploid males are larger than haploid males. The head of the sperm cell is 154% longer in average in diploid males and could be used as a male ploidy indicator.

Introduction

Males of Hymenoptera species are normally haploid, since they develop from unfertilized eggs due to their haplodiploid system of sex determination, while females are diploid and originate from fertilized eggs (Heimpel & Boer 2008). However, occasionally fertilized eggs give rise to diploid males, due to the complementary sex determination (*csd*) system, a genetic mechanism that control sex determination in most species. On this system, the combination of sex alleles on a single or multi-locus determine the sex: heterozygous are diploid females; hemizygous (haploid individuals) and homozygous are haploid and diploid males,

respectively (Whiting 1943). Females that make a matched mating (mating with a male sharing one of her two sex alleles, e.g., AB x A) will sire approximately 50% of diploid males amongst her diploid brood.

Diploid males are, with very few exceptions, unviable, sterile or sire triploid offspring due to diploid sperm (Heimpel & Boer 2008). Additionally, they arise from a fertilized egg that was supposed to be a female, decreasing the workforce of a colony in social species (Wilgenburg et al. 2006). Therefore, diploid males impose high fitness costs on the individual level of females that performed a matched mating. In small populations, the increased frequency of matched mating due to reduced allelic diversity at the *csd* locus may increase extinction risk (Zayed & Packer 2005). Understanding the reproductive potential of diploid males is also important for conservation purposes, since reproductive active diploid males imposes an additional cost at the population level, causing a load to the females they potentially mate with (Zayed & Parker 2005; Harpur et al. 2013). This is expected because female mortality would increase over two generations, first in the diploid offspring that was supposed to be female but ends as an infertile diploid male, and secondly because the females that mate with diploid male would produce triploid daughters or only haploid males (Zayed & Packer 2005; Harpur et al. 2013). Indeed it has been shown that in several hymenopteran species the diploid males are able to mate (reviewed in Heimpel & Boer 2008).

The stingless bees comprise several hundred eusocial species living worldwide in the tropics and subtropics (Schwarz, 1948; Michener, 2007;Camargo & Pedro 2013). Their populations can be highly affected by diploid male load when submitted to a genetic bottleneck (Alves et al. 2011). It is still not known whether stingless bee's males are sterile or sire triploid offspring. In all species investigated so far diploid males are viable, i.e., reach adulthood and emerge inside the colony, and are able to join reproductive aggregations (Santos et al. 2013; Vollet-Neto et al. 2015). Understanding the reproductive capacities of diploid males is, therefore, important for conservation purposes. Moreover, distinguishing the ploidy of males by morphology can be useful for research studies and breeding programs. On this context, here we investigated the sperm morphology and viability of diploid males of *Scaptotrigona depilis*.

Methods

Obtaining matched mated queens

In order to obtain queens that had made a matched mating, we first removed the laying queen from 26 colonies of *S. depilis*. This resulted in a new, daughter, queen taking over. Approximately 30 days after the new queen starts laying eggs, her oldest offspring are pupae with black eyes. At this time, we inspected samples of pupae taken from brood cells in order to identify colonies in which approximately 50% of the pupae had the characteristic male morphology of smaller heads and bigger eyes. Two queens producing c. 50% male offspring were obtained and were marked by clipping their wings. Supposed diploid males were collected randomly from such colonies, analyzed regarding their sperm viability and morphology (see below) and had their ploidy checked using DNA microsatellites at six *loci* to confirm that they were diploid (see below).

Male samples and sperm extraction

We collected 36 males from three different source colonies that had matched mated queens and 26 haploid males from three different source colonies with non-matched mated queen, and other 13 from reproductive aggregations outside the colonies. Sampled males from colonies were collected from brood combs with age "zero", and were placed in Petri dishes with other males and workers to age until 11-14 days to complete maturation. They were supplied with sugar syrup (1:1), water and pollen *ad libitum*. Males from aggregation were collected and had their sperm extracted after collection. We followed Meneses et al. (2014) protocol for semen collection.

Viability analysis

In order to assess sperm viability we followed the protocol designed by Meneses et al. (2014). We used the Sperm Viability Kit LIVE/DEAD Invitrogen L-7011. Employing a fluorescence microscope Axiovert 40 CFL Zeiss with filter Cy3 and a cell counter, we counted the first 400 cells found from the centre of the cover slip. Sperm cells were classified as green (live), red (dead) and green / red (dying).

Morphology analysis

To analyse the measurements on the head and tail we used sperm from 52 individuals out of the 75 used for viability. The sperm used was collected from the stock material prepared to

viability, however, we stained sperm cells using DAPI at a concentration of 4 ng /µl. DAPI (dihydrochloride 4 ', 6-diamidino-2-phenylindole) is a fluorescent blue dye that marks preferably double stranded DNA, with a peak excitation/emission of 358/461nm, respectively. We followed the protocol of morphology analysis established by Meneses et al. (2014). We took pictures of 10 sperm cells for each individual. In order to perform the measurements, we used the software ImageJ 1.48. We measured the tail and head length.

Genetic analysis to confirm ploidy

After above mentioned analysis, the males were preserved in absolute ethanol for genetic analysis; The DNA was extracted using the Chelex method and males were genotyped at six microsatellite *loci*, T3, T4, T7 and T8 (Paxton et al. 1999), and Sxant06 and Sxant18 (Duarte et al. 2011). Microsatellite amplification and visualization were done as described previously by Francisco et al. (2011). We categorized males as diploid if they were heterozygous at one or more *loci*.

Statistical analysis

All data was analysed in R 2.9.2 (R Development Core Team 2009). To compare the viability and sperm size of haploid and diploid males, we ran a linear mixed effect models (LMEs) of the viability/size in function of ploidy. Colony origin was always included as random effect to control for non-independence of data.

Results

Ploidy analysis

The primer T4 presented anomalous readings and was, therefore, discarded from our analysis. Two supposedly diploid males presented a single allele for all *loci* analysed. These males were discarded from our analysis, totalizing 34 diploids and 39 haploids males (see table 1). The other diploid males showed two different alleles at least for one of the *loci*. All supposed haploid males genotyped presented only one allele for each *loci*.

Table 1: Microsatellite analysis of the *S. depilis* males used for viability and morphology analysis for ploidy confirmation. Not all the haploid males used on both analyses were genotyped. Males produced in colonies with matched mated queen that did not presented any heterozygous *loci* were discarded from our analysis.

Male	Colony	Expected ploidy	Primers										Num. of heterozygous *loci*	Observed ploidy
			T3		T7		T8		Sxant06		Sxant18			
2	A4	2n	128	132	-	-	147	-	179	181	174	178	3	2n
3	A4	2n	132		98	-	147	-	175		194	-	0	n
4	A4	2n	128	132	-	-	147	-	179	181	-	-	2	2n
5	A4	2n	128	132	-	-	147	-	175	181	-	-	2	2n
6	A4	2n	128	132	-	-	147	-	175	181	-	-	2	2n
8	A4	2n	128	132	100	-	147	-	175	181	-	-	2	2n
11	A4	2n	128	132	100	-	147	-	179	181	174	178	3	2n
13	A4	2n	128	132	100	-	147	-	179	181	168	178	3	2n
14	A4	2n	128	132	100	-	147	-	175	181	174	178	3	2n
15	A4	2n	128	132	100	-	147	-	179	181	168	178	3	2n
16	A4	2n	128	132	-	-	147	-	179	181	-	-	2	2n
18	A4	2n	128	132	-	-	147	-	175	181	-	-	2	2n
19	A4	2n	128	132	100	-	147	-	179	181	168	178	3	2n
21	A4	2n	128	132	98	100	147	-	179	181	174	178	4	2n
22	A4	2n	128	132	100	-	147	-	179	181	174	178	3	2n
23	A4	2n	128	132	98	100	147	-	179	181	174	178	4	2n
24	A4	2n	128	132	100	-	147	-	175	181	168	178	3	2n
25	A4	2n	128	132	100	-	147	-	179	181	168	178	3	2n
27	A4	2n	128	132	-	-	147	-	179	181	-	-	2	2n
28	A4	2n	128	132	-	-	147	-	-	-	-	-	1	2n
29	ra34	2n	128	134	-	-	153	155	-	-	-	-	2	2n
30	ra34	2n	128	134	104	-	153	155	175	181	172	194	4	2n
31	ra34	2n	128	134	98	104	153	155	175	179	194	-	4	2n
32	ra34	2n	128	-	-	-	155	-	179		168	-	0	n
33	ra34	2n	128	-	98	104	147	153	175	181	194	-	3	2n
34	ra34	2n	128	134	104	-	153	155	175	179	194	-	3	2n
35	ra34	2n	128	134	98	104	153	155	175	181	194	-	4	2n
A9	aggreg	n	130	-	100	-	147	-	-	-	176	-	0	n
A10	aggreg	n	128	-	98	-	149	-	-	-	178	-	0	n
A11	aggreg	n	134	-	100	-	165	-	-	-	192	-	0	n
A12	aggreg	n	134	-	102	-	165	-	-	-	182	-	0	n
A13	aggreg	n	128	-	98	-	149	-	175	-	178	-	0	n
A14	aggreg	n	128	-	104	-	147	-	183	-	174	-	0	n
A15	aggreg	n	130	-	100	-	147	-	175	-	170	-	0	n
A16	aggreg	n	128	-	98	-	149		173	-	196	-	0	n
5-2n	Rb24	2n	128	130	98	104	147	153	-	-	168	172	4	2n
6-2n	Rb24	2n	126	128	98	-	151	153	-	-	172	-	2	2n
8-2n	Rb24	2n	126	128	98	104	151	153	-	-	172	-	3	2n
9-2n	Rb24	2n	128	130	98	-	151	153	181	-	172	-	2	2n
16-2n	Rb24	2n	126	128	98	104	147	153	181	-	168	172	4	2n
17-2n	Rb24	2n	126	128	98	-	151	153	181	-	168	172	3	2n
18-2n	Rb24	2n	126	148	98	104	151	153	181	-	172	-	3	2n
21-2n	Rb24	2n	128	130	98	104	151	153	181	-	172	-	3	2n
22-2n	Rb24	2n	128	130	98	-	151	153	181	-	168	172	3	2n

Viability and morphology analysis

The sperm viability of haploid and diploid males is not significantly different (LME, N=73, p = 0.94). The mean viability of haploid males (52% ± 14) is similar to mean viability of diploid males (54% ± 11). (Fig. 1). On the other hand, sperm morphology of diploid and haploid males is significantly different. Diploid male sperm have significantly longer tail (LME, N=500, p = 0.003) and have head (LME, N=500, p < 0.001) (Fig. 2).

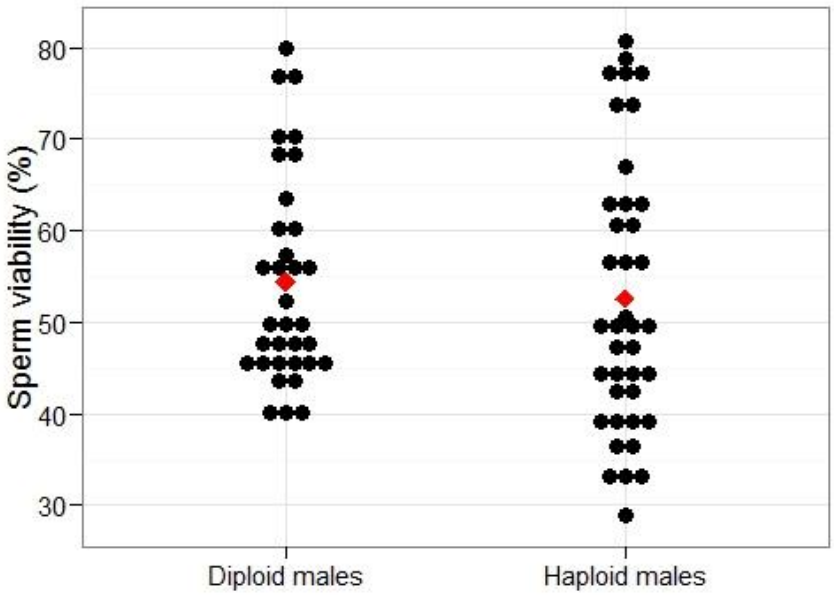

Figure 1: Sperm viability of diploid and haploid males of *S. depilis*. The red dot represents the average. There is no statistical difference between the two groups (LME, N = 73, p = 0.94)

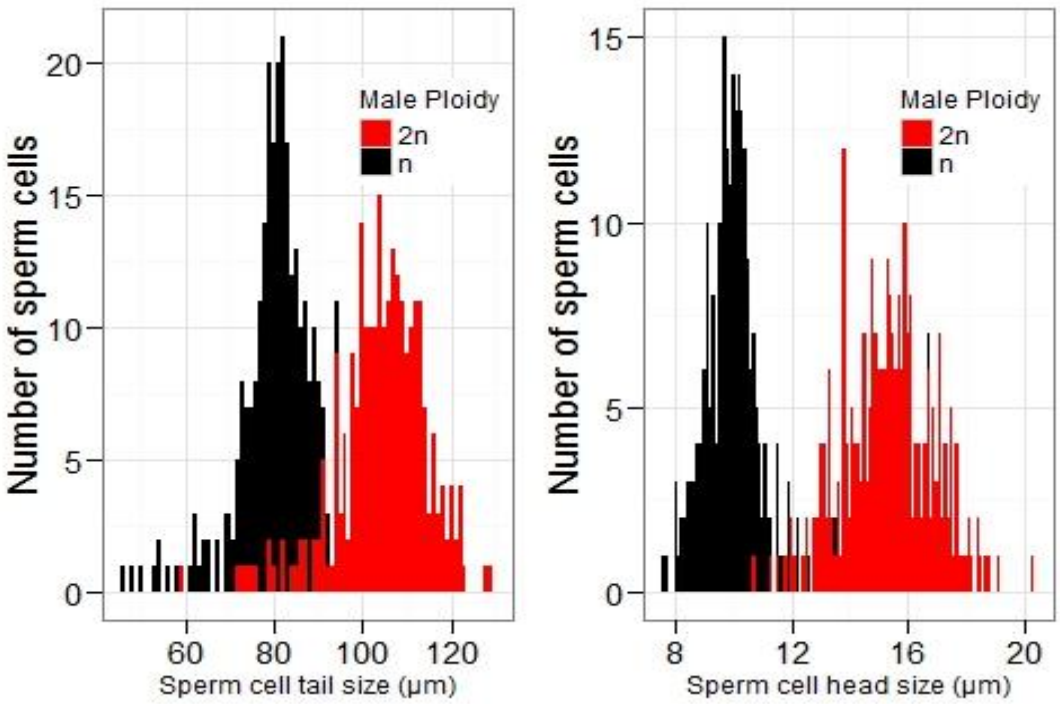

Figure 2: Sperm cell length of haploid (black) and diploid (red) males of *S. depilis*. There is statistical significant difference between head (LME, N=500, $p < 0.001$) and tail (LME, N=500, $p = 0.003$) sperm cell length.

Discussion

Our results showed that sperm viability of diploid males is similar to haploid males aged within 11-15 days old (Fig. 1). The sperm viability of haploid males ranged from 29 to 80%, matching well with sperm viability found in another study using similar methods and the same species (range 52% - 87%) (Meneses et al. 2014). This is the first measurement of sperm ploidy of a stingless bee species. Sperm viability of honeybee diploid males was never measured, mainly because of the very little amount of sperm that a honeybee diploid male produces (Woyke, 1983). We did not count the number of sperm cells in the diploid males of *S. depilis*. However, sperm density was not a problem while measuring sperm viability, indicating that if there are differences between the amount of sperm produced by diploid and haploid males, they are not too big. In honeybees, the very few sperm cells are viable, because triploid brood was produced when queens were artificially inseminated with diploid drone's sperm (Chaud-Netto 1972).

Bumblebees diploid males are able to naturally mate and produce triploid females (Darvill et al. 2012 – *B. muscorum and B. jonellus*) and in laboratory conditions

(Duchateau & Mariën, 1995 – *Bombus terrestris*). In stingless bees, triploid workers were never reported, as well as diploid males mating. Diploid males of *S. depilis* are able to join reproductive aggregations, therefore, exhibiting "normal" male behaviour (Vollet-Neto et al. 2015). Confirming the ability of diploid males mating is of great importance to conservation purposes, as this could increase the population extinction risks, due to invalidation of newly mated queens (Zayed & Parker, 2005).

Our data clearly showed that diploid male sperm are longer than haploid male sperm. The sperm head length is proportionally longer (154% in average) in diploid compared to haploid males, while sperm tail length is less big (131% in average) in diploid male sperm. This result is very similar to *Apis mellifera* sperm length, where the head is also proportionally bigger in diploid male sperm (head length 154% of haploid male sperm), compared to the total length (129% of the haploid male sperm). The same pattern of bigger sperm cells is also found in other Hymenoptera species that produces diploid males (Duchateau & Mariën, 1995 - head length of *Bombus terrestris* 134% longer; Armitage et al. 2010 - total sperm length of *Atta sexdens* 150% longer).

In honey bees, diploid males spermatogenesis do not reduce the number of chromosomes, as it happens with normal haploid males (Woyke & Skowronek 1974). Therefore, the DNA content in the head of a diploid male sperm cell is twice as much as there is in haploid males' sperm cell. This is probably the main reason of why the sperm cell head is bigger in diploid males. Even though we did not measure the DNA content of sperm cells, it is very likely that diploid male sperm cells of *S. depilis* are also diploid, given their bigger size and the similar situation in close phylogenetic group of honeybees. This suggest that diploid males of *S. depilis* would sire triploid offspring if they would be able to mate. This would increase the extinction risks if they would present an active mating behaviour (Zayed & Paker, 2005). However, further studies are necessary to confirm this assumption. The sperm size difference could be used as a method for male ploidy determination in *S. depilis*, since it is faster, cheaper and easier to be done, compared to cytogenetic, flow cytometry and microsatellite analysis.

1.3. Diploid males of *Scaptotrigona depilis* are able to join reproductive aggregations (Apidae, Meliponini)

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Abstract

The sex determination system in the eusocial stingless bees (Apidae, Meliponini) is based on the combination of alleles at the complementary sex determination (CSD) locus. In this system, males are haploid and females are diploid. However, diploid males can develop from fertilized eggs when they are homozygous at single or multiple sex loci. The production of such males can negatively affect population viability, since they are usually infertile or inviable. Moreover, when they are viable but infertile, or siring sterile triploid offspring, this could cause another load on the population, leading the fertilized offspring of other females to be only haploid males or triploid sterile daughters. In this context, our aim was to verify whether diploid males of the stingless bee *Scaptotrigona depilis* join reproductive aggregations. We showed that out of 360 marked males from two different colonies, five were participating in a reproductive aggregation ca. 20 meters from their natal colonies. Using microsatellites markers, it was confirmed that three males were diploid. They were captured in the mating aggregations when they were 15 to 20 days old. Further research is necessary to determine the mating success of stingless bee diploid males under natural conditions and to determine their impact on stingless bee population extinction risks.

Keywords: inbreeding; diploid male load; population viability; stingless bees.

The system of sex determination in the eusocial stingless bees (Apidae, Meliponini) has been shown to be based on the combination of alleles at the complementary sex determination (CSD) locus (Camargo 1979), like many other Hymenoptera (Whiting 1943). Under this system, individuals that are heterozygotes at the CSD locus are females, while the hemizygotes (haploid individuals) and the homozygotes are males (Whiting 1943; Cook & Crozier 1995).

With few exceptions (El Agoze et al. 1994; Cowan & Stahlhut 2004; Kureck et al. 2013), diploid males are highly disadvantageous for the Hymenoptera species they occur in, imposing fitness costs on their relatives. Most of the time they are unviable, infertile or lead to the production of infertile triploid broods (Heimpel & Boer 2008). They also lead to the decrease of females in the population, since the diploid individuals were supposed to develop into females, increasing the extinction risks of the population (Zayed & Packer 2005).

Simulation models have shown that extinction risks increase when diploid males are viable and can mate (Zayed & Packer 2005). This is expected because females mortality would increase over two generations, first in the diploid offspring that was supposed to be female but ends as an infertile diploid male, and secondly because the females that mate with diploid male would produce triploid daughters or only haploid males (Harpur et al. 2013). Indeed it has been shown that in several hymenopteran species the diploid males are able to mate (reviewed in Heimpel & Boer 2008).

The extinction risks are even more extreme for stingless bees, since their nests are sparse and, together with habitat fragmentation, there are low effective population sizes (Packer & Owen 2001; Zayed 2009). It is well known that the queens mate only once in this group (Peters et al. 1999), causing half of their diploid brood to develop into diploid males when there is a matched mating (queen mating with a male with the same sex allele), greatly reducing their fitness (Cook & Crozier 1995) and increasing population extinction risks when there is low population size or low sex alleles variability (Alves et al. 2011). Therefore, to assess the effects of the diploid males on stingless bee conservation, it is important to know the viability of diploid males and their participation on the reproductive events. Despite some previous observations of aggressive behaviour of workers towards the diploid males in *Melipona interrupta*, suggesting that though the diploid males are viable they would be killed before leaving the nest (Francini et al. 2012), diploid males of *Tetragonisca angustula* were found in a

reproductive aggregation close to the entrance of a queenless colony (Santos et al. 2013).

Here we investigate the fate of diploid males of *Scaptotrigona depilis* and aimed to answer the question: Do diploid males leave their colonies to join mating aggregations? In order to answer this question, two free-foraging colonies kept in wooden boxes in the Biology Department at São Paulo University (Ribeirão Preto, Brazil) were utilised. These colonies were chosen because their queens were producing diploid males, as previously confirmed through genotyping ten male pupae from each colony following the methods described below. We collected brood combs containing mature pupae and let the bees emerge in an incubator. Thirty newly emerged males of each colony were marked with nontoxic paint (Revell GmbH & Co. KG, Germany) on their thorax for six days, using a different colour for each day (total 360 marked males) and all the males were reintroduced into their natal nest on the same day of marking. During the time of the study, the mating aggregations were observed twice a day, and any marked males present were collected. They were preserved in absolute ethanol for genetic analysis. The DNA was extracted using the Chelex method and males were genotyped at six microsatellite *loci*, T1, T3, T4 and T8 (Paxton et al. 1999), and Sxant06 and Sxant18 (Duarte et al. 2011). Microsatellite amplification and visualization were done as described previously by Francisco et al. (2011). Males were categorized as diploid if they were heterozygous at one or more loci (Alves et al. 2011).

Five marked males were found at two mating aggregations which were approximately 20 meters from their natal colonies. Three of them were diploid, since almost all loci showed two different alleles (Table 1), whereas the other two were likely haploid. The age of diploid males within the aggregation ranged from 15 to 20 days old (Table 1), while the haploid were from 16 and 20 days old.

These findings suggest that the presence of stingless bee diploid males in mating aggregations may be more common than previously suspected. Our data are in accordance with those obtained by Santos et al. (2013), who also found diploid males in a mating aggregation of *Tetragonisca angustula*. Although the workers of stingless bees are supposed to recognize and kill diploid males inside the nests (Francini et al. 2012), it is therefore clear that some of them escape worker policing, depart from their colonies, and become a member of a reproductive aggregation (Santos et al. 2013; this study). It is important to note that removing the brood combs and allowing the pupae to emerge out of the colony context might have enhanced their survival. However, we never

observed any aggressive behaviour towards males in colonies producing diploid males during their emergence (personal observations). Camargo (1982) verified that the average lifespan of diploid males of *Melipona quadrifasciata* is only three days, while haploid males live 15 days on average, both in artificial conditions. The average lifespans of haploid and diploid males of *S. depilis* were not compared, but if there are any physiological constraints that limit the diploid males life span, at least some of them must live long enough to be able to join mating aggregations.

Some new information about the mating strategies of *Scaptotrigona depilis* males was discovered. Despite the fact that stingless bee males disperse away from their natal nests to avoid inbreeding (Paxton 2000; Cameron et al. 2004; Kraus et al. 2008), some of them (haploid or diploid) join aggregations very close to their original colonies. It is important to note that this is a managed population kept in a stingless bee apiary maintaining up to 100 nests in a small area. Further research on this topic is needed to draw stronger conclusions.

These results add more precision as to the age of sexual maturity age of male stingless bees, since previous studies considered only the age that males of leave the nest (an average of 18.6 days old in *Melipona favosa*; van Veen et al. 1997). It is showed here that both haploid and diploid males join mating aggregations from 15 to 20 days old. It was not possible to ascertain that this was their first experience in a mating aggregation since they could have already visited one previously. However, considering that Pech-May et al. (2012) found that *M. beecheii* males are sexually mature at ca. 14 days old, it is possible that males of *Scaptotrigona depilis* also mature later in life, and would join reproductive aggregations only when mature.

Despite the few diploid males found in mating aggregation compared to the marking effort and presence of approximately thousand individuals therein, there is a great chance that a much higher fraction of diploid males produced by *Scaptotrigona depilis* leave the nest to search for an aggregation site, since there is good evidence that males disperse from their natal nests (Cameron et al. 2004; Kraus et al. 2008). This could also explain why only ca. 1.4% of the marked males were recaptured. Probably, the majority of males had dispersed to distant aggregations that we did not sample. However, some did not disperse and thus increased the risks of inbreeding. It is important to note that our manipulation could may have allowed the males to live normally by minimizing worker policing. Additionally, diploid male production in social insects under monandry is predicted to be very high, since half of the diploid

brood are diploid males when a matched mating is performed. As this species produces about 200 or more new brood cells per day (personal observation), even if just a small fraction of the diploid males succeeded in leaving the nest, the total amount could be high.

All these factors together (high rate production of diploid males, survivorship, and visit to mating sites) strongly suggest that diploid males could be dangerous to stingless bee populations from the females' point of view (Harpur et al. 2013). Further research is needed to verify the potential mating success of *Scaptotrigona depilis* diploid males, since we had no success when testing this under controlled conditions, following the protocol established by Engles (1984). If it is established that diploid males of *S. depilis* can successfully mate, this would suggest that this species is under a higher risk of extinction due to the decreased fitness of queens that mate with them (Zayed 2009; Harpur et al. 2013).

Table 1. Age at collection and genotypes of diploid males of *Scaptotrigona depilis* in a reproductive aggregation

Colony and male	Age (days)	Males' genotypes at locus					
		T1	T3	T4	T8	Sxant 06	Sxant 18
1-a	18	140/142	125/129	132/140	147/153	175/177	172/192
1-b	15	140/142	125/129	132/138	153/153	177/181	170/172
2-c	19	138/142	125/129	132/140	147/153	177/179	172/174

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CHAPTER 2: Queen death under diploid male emergence: possible proximate mechanisms

On this chapter we determined the behavior of queen death under diploid male offspring emergence in *S. depilis* (2.1). We also investigated the proximate mechanisms that might trigger this behavior. In particular, we verified the differences on cuticular hydrocarbon profile of diploid and haploid males that could be used to detect a matched mated queen (2.1). We also investigated queen sperm use to estimate how it may affect queen lifespan (2.2). Finally, we presents the attempts of having queens producing only haploid males amongst their offspring, in order to have biological material to test the alternative hypothesis on the mechanisms that triggers queen death under diploid male emergence (2.3).

2.1. Diploid male production results in queen replacement in the stingless bee *Scaptotrigona depilis*

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Abstract

As in most Hymenoptera, stingless bees (Meliponini) have a complementary sex determination (CSD) system. When a queen makes a "matched mating" with a male that shares a CSD allele with her, half of her diploid offspring are sterile, diploid, males rather than females. Previous research suggests that worker *Melipona* bees execute queens that have made a matched mating, thereby allowing a new queen to head the colony. Here we provide clear evidence that in the stingless bee *Scaptotrigona depilis*, the emergence of diploid males induces the queen death. The queen is likely killed by the workers inside the colony, mainly within 10-20 days of the emergence of diploid male offspring from their pupae. Queens who have not made a matched mating are killed if introduced into a colony in which diploid males are emerging. This shows that the diploid males, not the queen who has made a matched mating, are what causes the workers to execute the queen. Analysis of the cuticular hydrocarbon profiles of haploid and diploid males shows twelve compounds with significant differences. This suggests that mother queens that have made a matched mating are detected based on chemical differences in the cuticular hydrocarbon profiles of diploid male offspring.

Keywords: sex determination; diploid male load; queen execution; chemical recognition

Introduction

Male Hymenoptera are normally haploid and develop from non-fertilized eggs while females are always diploid and develop from fertilized eggs. Nevertheless, in some species, diploid individuals can develop into males as a result of the complementary sex determination (CSD) system that is characteristic of many Hymenoptera. Under this system, individuals that are heterozygous at the CSD locus develop as females, whereas haploid (hemizigous) individuals or individuals that are homozygous at the CSD sex locus develop into males (Whiting 1943). Since diploid males are non-viable or sterile, females that make a matched mating and mate with a male sharing one of her two sex alleles will end up having reduced fitness. In small populations, the increased frequency of matched mating due to reduced allelic diversity at the CSD locus may increase extinction risk (Zayed & Packer 2005). In eusocial species, this effect is further exacerbated by the fact that males do not work and that the replacement of workers with diploid males will therefore strongly reduce colony performance (Heimpel & Boer 2008).

Mechanisms to avoid the production of diploid males are relatively common amongst Hymenoptera species, and are mainly related to inbreedin avoidance, i.e., reducing the chances of having a matched mating (van Wilgenburg et al. 2006). Some eusocial species have mechanisms to reduce the cost of matched matings and diploid male production (van Wilgenburg et al. 2006). In the honeybee, *Apis mellifera*, there are two such mechanisms. First, because honeybee queens are highly polyandrous, the variance in diploid male production among queens is reduced. Although this results in a higher proportion of queens making a matched mating, it reduces the proportion of diploid male offspring derived from each matched-mated queen (Page & Metcalf 1982). Second, honeybee workers detect diploid male larvae and eliminate them within 36 hours after hatching (Woyke 1962, 1963). This avoids wasting resources in rearing them and allows the cell to be reused without great delay (Ratnieks 1990). By contrast, stingless bees (Meliponini) rear brood in sealed cells and have no option to detect and eliminate diploid male larvae in an early stage (Camargo 1979). However, it has been suggested that stingless bees also possess an effective mechanism to protect themselves against diploid male load, namely by executing queens that have made a matched mating. This was shown in *Melipona* species, *M. quadrifasciata* and *M. scutellaris*, where matched mated queens died 25-30 and 11-67 days, respectively, after diploid

male brood first began to emerge (Camargo 1976; Alves et al. 2011). Alves et al. (2011) suggested that queen execution could greatly reduce the negative fitness effects of diploid male production, as it would result in the genetically defective queen being replaced by a healthy daughter. For stingless bees, this is particularly important, as queens are monandrous (Peters et al. 1999), and if uncountered thereby leading colonies to waste considerable resources on rearing ca. 50% of the diploid brood into sterile or unviable diploid males.

Quantitative differences in the chemical profile of diploid and haploid first instar male larvae were shown to be what allows workers to detect diploid males in the honey bee (Santomauro et al. 2004). However, no differences were observed in the chemical profiles of diploid and haploid *M. quadifasciata* males (Borges et al. 2012). However, in this study only newly-emerged individuals were analysed, who may not have a fully mature cuticular profile yet and who may also have lower amounts of cuticular hydrocarbons than older individuals (LeConte & Hefetz 2008; Falcón et al. 2014), thereby explaining the lack of differences in their chemical profiles. Alternatively, there are two other possible sources of signal triggering queen death: (i) the matched mated queen could release a signal herself resulting from mating with a male with the same sex allele; or (ii) the odour of too many males emerging at the same time, independent of their ploidy.

The aim of this study was twofold. First, we investigated if in *Scaptotrigona depilis*, the emergence of diploid male offspring triggers the mother queen to be executed and replaced. Second, we investigated whether the chemical profiles of diploid males differed from that of haploid ones, and hence could be used as a cue to infer whether or not a matched mating had occurred. Finally, we estimated the proportion of queens that made matched matings and estimate the effective number of alleles at the CSD locus present in our study population.

Material and methods

Study species and site

The study was carried out using colonies of *Scaptotrigona depilis* kept in hives at the experimental meliponary of the Biology Department at the University of São Paulo, Ribeirão Preto, Sao Paulo State, Brazil. Colonies are perennial, generally contain 5,000-10,000 workers and are headed by a single-mated queen (Paxton et al. 2003).

Obtaining matched mated queens

In order to obtain queens that had made a matched mating, we first removed the laying queen from 68 colonies of *S. depilis*. This resulted in a new, daughter, queen taking over. Approximately 30 days after the daughter queen starts laying eggs, her oldest offspring are pupae with black eyes. At this time, we inspected samples of pupae taken from brood cells in order to identify colonies in which approximately 50% of the pupae had the characteristic male morphology of smaller heads and bigger eyes.

Five queens producing c. 50% male offspring were obtained and were marked by clipping their wings. In these colonies, 10 males were collected and checked using DNA microsatellites at three *loci* to confirm that they were diploid (see below). We prevented the execution of queens that had made a matched mating by removing them from their own colonies and placing them in colonies without diploid males, from which we had removed the queen.

Time course of queen replacement

In order to determine the time for natural queen replacement we studied 43 replacement queens in detail. We opened the colony every 10 days after removing the original queen in order to verify the presence of a new physogastric queen, or signs of her presence (organized brood cell arrangement).

Effective number of sex alleles in the population

The effective number of sex alleles present on this population was estimated using the following equation,

$$n = \frac{2}{m} \ ,$$

where *m* is the observed proportion of matched matings, and *n* is the unknown effective number of sex alleles (Cook & Crozier 1995). The 95% confidence intervals were also estimated.

Queen execution bioassays

To determine whether a queen dies after the emergence of diploid males from brood cells following a matched mating, we removed the matched mated queen from her colony a few days before diploid male emergence and replaced her with a non-matched mated marked queen (see Fig. 1 and 2 for more details). We then inspected the colony

every 10 days for 50 days. We determined that a queen had died by observing that the colony was queenless, when either there was no production of new brood cells or by observing the presence of a new, non-marked, physogastric queen.

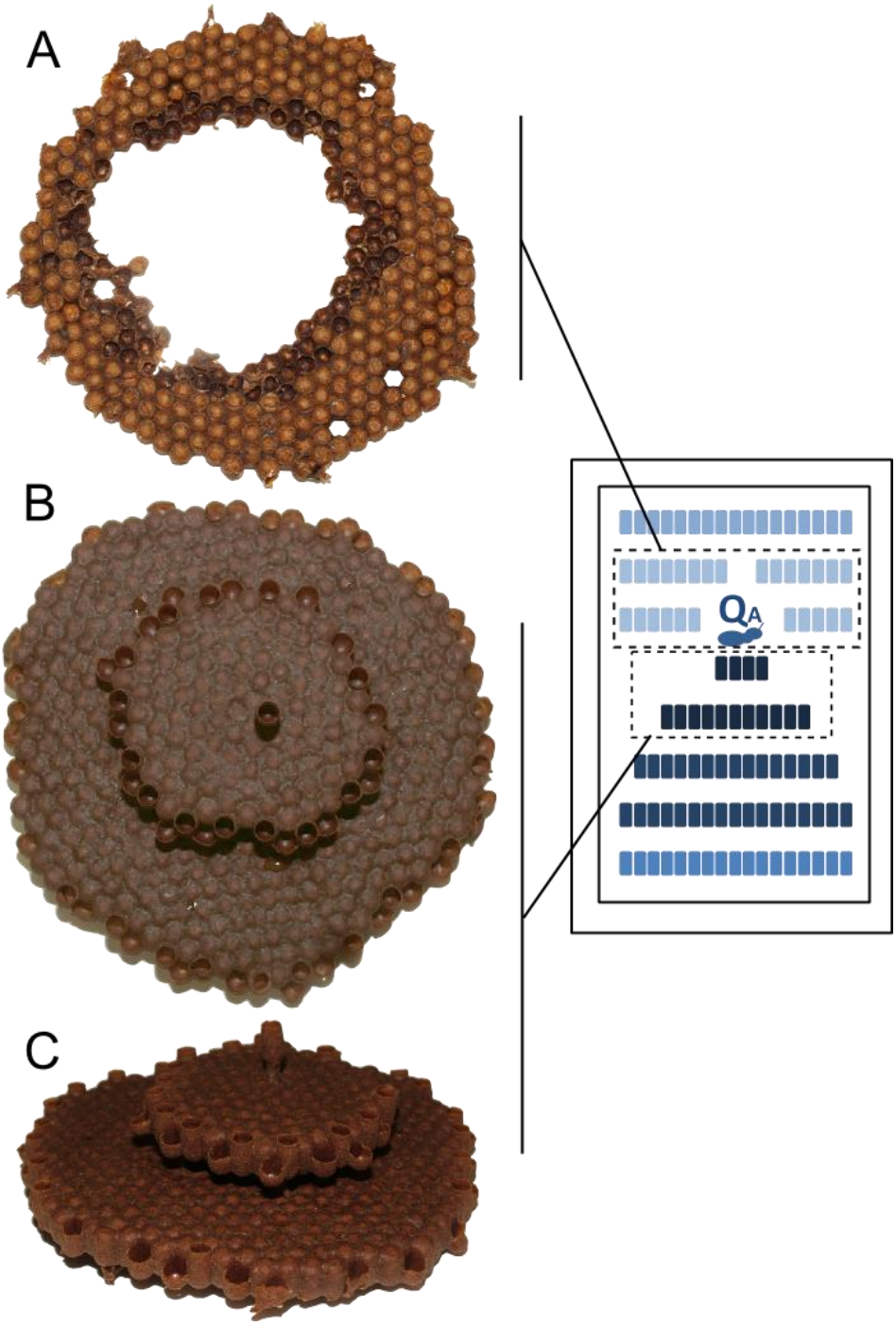

Figure 1. Layout representing the brood dynamics inside a beehive (represented by the box in a side view). The adults emerge first in the centre of the brood comb (C) and are represented by lighter colours in the scheme. The old cells are destroyed after adult emergence. New cells are constantly added on the borders of the new brood comb (B and C) containing eggs, and are represented by the darker colours. The egg to adult time of development takes ca. of 35 days to complete

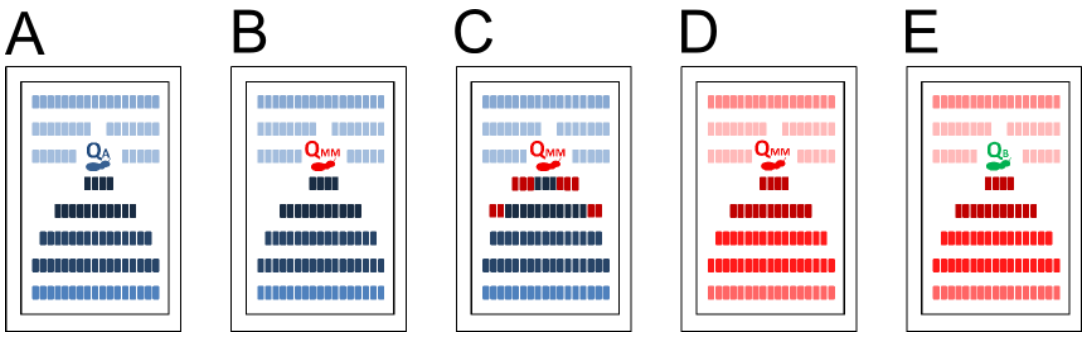

Figure 2. Layout representing the chronological steps of the "treatment" bioassay: we select a colony with a normal queen (Q_A) which have her own offspring from eggs to adults in the brood combs (A) and replace her by a *matched mated queen* (Q_{MM}) (B). This new queen will quickly start her own egg laying (C) and after ca. of 35 days there will be only her offspring on the brood combs (D). Then, we replace her by a marked normal queen (Q_B) (E). The execution of Q_B is followed every 10 days for 50 days

This process was repeated four times for each of the five matched mated queens, to give 20 trials. As a control for the use of non-nestmate introduced queens, we also removed queens that had not made a matched mating and replaced these with another queen that had not made a matched mating. We carried out 20 trials, this time using 20 different removed queens and 20 different replaced queens.

It was not necessary to record data blind because our study involved a binary observation (queen death) that it is not subject to any bias.

Chemical and genetic analyses

Combs from three different colonies classified as producing or not producing diploid males were collected and placed in petri dishes in an incubator at 28º C until the adult males emerged. Subsequently, males were placed in petri dishes in groups of 8, and aged until 5 and 10 days. Bees were fed with sugar syrup and pollen *ad libitum*.

The males were then freeze killed at -20º C and extracted in 1ml of pentane for 10 minutes. The solution was evaporated at room temperature and resuspended in 150µl pentane. Samples were analyzed on a Shimadzu QP 2010 Ultra couple gas chromatograph/mass spectrometer by injecting 2µl of this solution. We used an initial column temperature of 70°C held for 1 minute, increasing to 150°C at a rate of 20° C min^{-1}, and then to 320°C at 3°C min^{-1}. The final temperature of 320°C was held for 15 minutes with final pressure of 75kPa. Helium was used as carrier gas at a flow rate of 1mL min^{-1}. Samples were ran with splitless injection and inlet temperature of 280°C. The electron ionization voltage was auto-tuned to enhance the acquisition performance

according to the molecular weight of the compounds, and the ion source temperature was set to 300°C. Peaks in the chromatogram were integrated using GCMS Solutions software, and compounds were identified based on their retention times and diagnostic ions in the mass spectra as well as through comparison with known standards and library searches, using the NIST 11 mass spectral database. Double bonds positions of alkenes were tentatively identified through both their elution order and retention indices.

After cuticular hydrocarbon extraction, individuals were kept in absolute ethanol for genetic analysis to confirm their ploidy. DNA was extracted using the Chelex method and males were genotyped at three microsatellite *loci*, T3 (Paxton et al. 1999), and Sxant06 and Sxant18 (Duarte et al. 2011). Microsatellite amplification and visualization were done as described previously by Francisco et al. (2011). We categorized males as diploid if they were heterozygous at one or more *loci* (Alves et al. 2011).

Statistical analyses

All statistical analyses were performed using R 3.2.2 (R core team 2015). The survival probability of matched mated queens versus unmatched mated queens was assessed using generalized linear model (GLM) with a binomial error distribution. Queen survival was the response variable with an interaction effect factor between group (matched or unmatched mating) and day of observation (0, 10, 20, 30, 40 and 50) were considered explanatory variables. Wald Z tests were used to assess significance.

For the chemical analyses, absolute abundances of the chemical compounds were transformed according to Aitchison (1986) to decrease the dependence of compositional data. In order to investigate whether particular cuticular hydrocarbons could be used as a cue for workers to discriminate between haploid and diploid males we performed linear models in which the abundance of a given chemical compound was the dependent variable, age and ploidy groups independent variables, and colony a random factor (package *lme4* 1.1.7). The models were *posthoc* corrected for multiple comparisons using general linear hypotheses test in the *multicomp* package (ver. 1.4-0). Subsequently, the overall differences in chemical profiles among age and ploidy groups were determined using Permanova with 1000 permutations (package *vegan* 2.3-0).

Results

Time course of queen replacement

Following removal of the egg-laying queen from 43 colonies, 35 (81%) had a new physogastric queen when the colonies were inspected for the first time 10 days later, and the other 8 at the 20 day inspection.

Effective number of sex alleles in the population

We observed 5 matched matings out of 68 matings. Assuming that queens mate to a single male, this gives an estimate of 27.2 sex alleles in the population. The 95% confidence interval is 14.7 to 173.9 alleles.

Queen execution bioassays

All 20 queens (who had not themselves made a matched mating) that were transferred into colonies with emerging diploid males, died, presumably by execution. Three (3/20, n = 15%) had died by the 10 day inspection, 14 (70%) by the 20 day inspection, and the last 3 (15%) by the 30 day inspection (Fig. 3). In all the cases, queen death was followed by queen replacement. By contrast, only one out of 20 control replacement queens died, within 10 days of replacement (Fig. 3). The survival probability of queens in a colony with emerging diploid males was significantly lower than of queens in a colony without emerging diploid males (binomial GLM, $Z = -4.124$, $P < 0.001$). We were unable to observe queen execution directly. However, given the evidence for virgin queen execution by workers in many stingless bees species (Imperatriz-Fonseca & Zucchi 1995), the most probable reason for queen death is execution by the workers. Therefore, we will frequently refer to queen death as queen execution. However, further investigations are necessary to determine exactly how the mother queens dies.

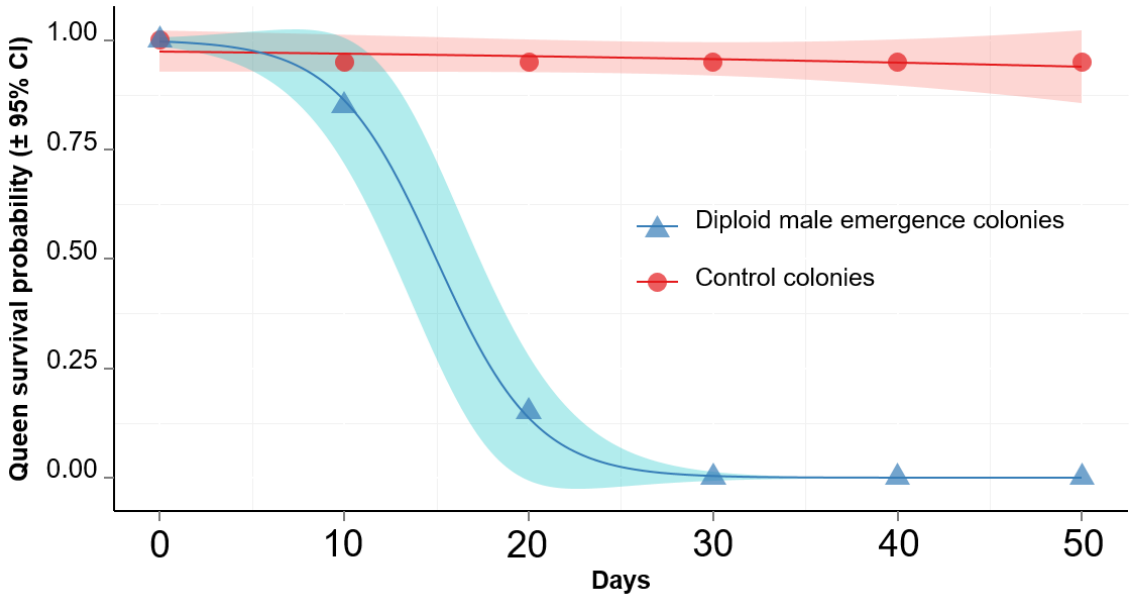

Figure 3. Survival curve of queens that replaced matched mated queens versus non-matched mated queens (control). The probability of queen survival with the 95% confidence intervals (shaded areas) per group are indicated at each time point

Genetic analyses

Genetic analyses of male offspring from the five queens producing large numbers of male offspring confirmed that they had made matched matings as 95% of the 50 males analysed were diploid. Two males used for the chemical analysis were found to be haploid (Table 1) and excluded from our chemical analysis.

Chemical analyses

Thirty-one hydrocarbons were identified on the cuticles of *S. depilis* males, and including saturated and unsaturated alkanes and alkenes (Table 2). Statistical analyses showed that the cuticular chemical profiles of diploid and haploid males show little difference five days after emerging from their cells, with only heneicosane ($n - C_{21}$) being relatively more produced in haploid males. After 10 days, however, eleven compounds show significant differences in their relative abundance between haploid and diploid males. Some were relatively more abundant in haploid males: heptacosane ($n - C_{27}$), a mixture of branched heptacosanes (13-, 11-, 9-, 7- MeC_{27}), two nonacosenes (11-$C_{29:1}$ and 5-$C_{29:1}$) and a branched nonacosane (15-MeC_{29}), whereas some others were more abundant on the cuticle of diploid males: tetracosane ($n - C_{24}$), 9-pentacosene ($C_{25:1}$), 11-hexacosene ($C_{26:1}$), 7-octacosene ($C_{28:1}$), 13-methylnonacosane

(13-MeC$_{29}$) 3,15-dimethylheptacosane (3,15-diMeC27). Both age and ploidy significantly affected the overall cuticular chemical profile (age: Permanova, $F = 10.25$, $p < 0.0001$; ploidy: Permanova, $F = 3.85$, $p = 0.003$; age and ploidy interaction: Permanova, $F = 3.44$, $p < 0.0001$). Aging affects the cuticular chemical composition somewhat similarly for haploid and diploid males with the differences between the two groups being more pronounced only at the age of 10 days, indicating that only when the cuticle is fully mature workers would be able to assess the males ploidy (Fig. 2). Therefore, our data suggest that chemical cues could be used by workers to detect the occurrence of diploid males in their colony (Fig. 2, Table 2).

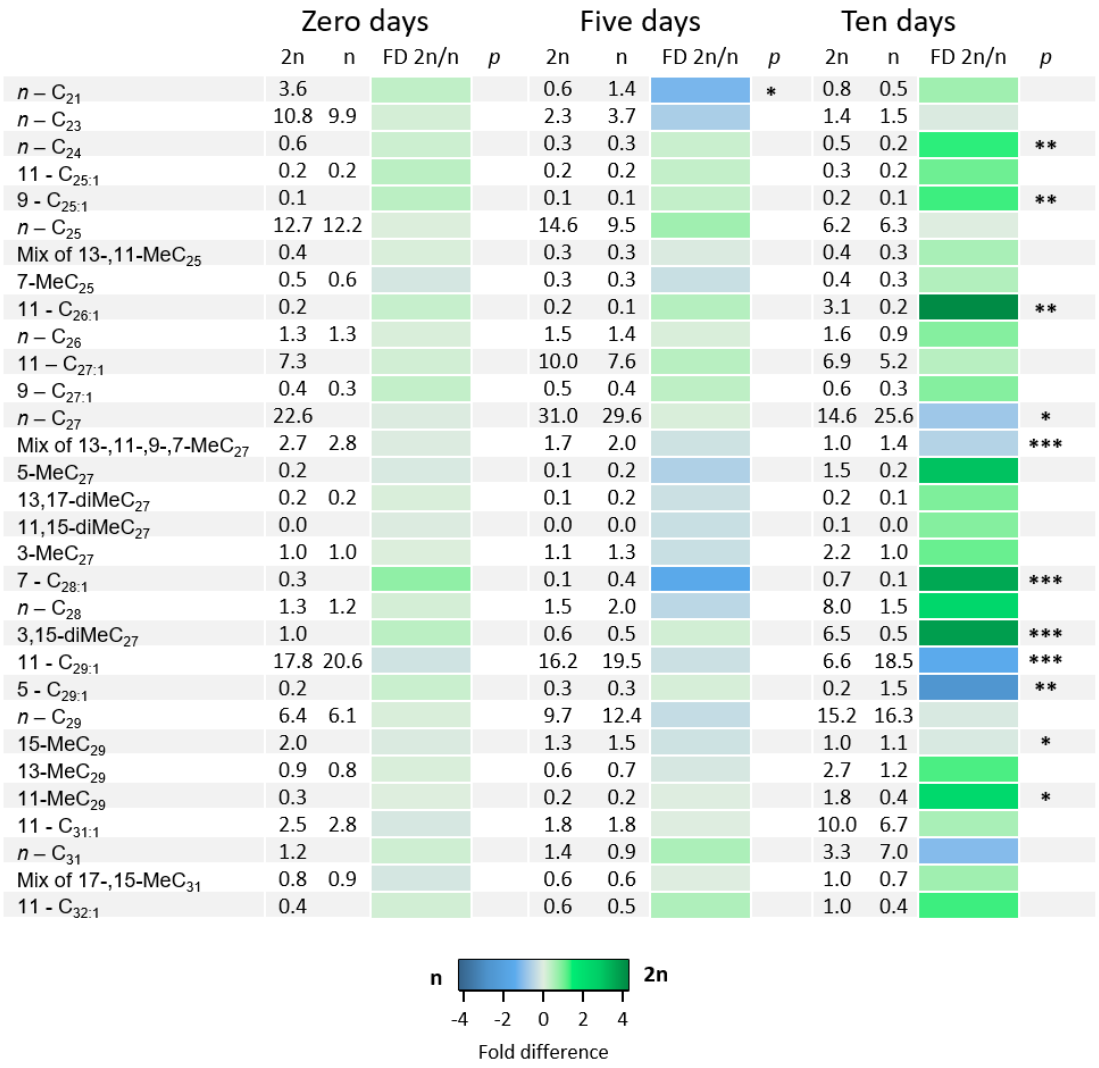

Fig. 2 Relative abundance in percentage of cuticular hydrocarbons *S. depilis* haploid (n) and diploid (2n) males in three different ages (zero, five and ten days old). The heatmap shows the fold difference between the average relative abundance of different age groups represented by subtracted $\log_2$ transformed relative areas (FD 2n/n). Chemical compounds with colors toward blue pallet were overrepresented in haploid groups whereas substances with colors towards green pallet were overrepresented in diploid males. No significant differences were observed at age of zero days. *P* value significance levels: * P < 0.05; ** P < 0.01; *** P < 0.001. A complete table with chemical compound areas together with effect sizes and significance levels is present in the Table 2.

Table 1. Molecular analysis of ploidy levels in all expected diploid males and some haploid males. The expected diploid males that were actually haploid according to our analysis are highlighted

Sample	Colony	Age	Expected Ploidy	Molecular Marker										Number of Heterozigous Loci	Observed Ploidy
				S18		S6		T1		T4		T8			
1	Rb24	0	2n	153	153	165	165	123	125	119	119	134	136	2	2n
2	Rb24	0	2n	153	153	165	165	123	129	119	125	129	136	3	2n
3	Rb24	0	2n	157	157	160	160	125	125	121	129	127	129	2	2n
4	Rb16	0	2n	153	155	160	160	123	125	121	129	127	136	4	2n
5	Rb16	0	2n	151	155	155	167	123	125	119	119	129	136	4	2n
6	Rb16	0	2n	157	157	161	161	123	125	127	136	121	129	3	2n
7	Rb16	0	2n	157	157	161	161	123	125	127	129	110	129	3	2n
8	Rca08	0	2n	153	155	155	161	123	123	129	142	108	121	4	2n
9	Rca08	0	2n	153	153	151	151	123	123	116	120	128	141	2	2n
10	Rca08	0	2n	153	160	153	159	123	123	116	120	128	141	4	2n
11	Rca08	0	2n	152	152	159	163	123	123	106	120	128	141	3	2n
12	Rb24	0	2n	151	155	165	165	123	125	118	118	132	134	3	2n
13	C22	0	n	158	158	161	161	125	125	120	120	128	128	0	n
14	2013-12	0	n	156	156	161	161	123	123	112	112	128	128	0	n
15	2013-12	0	n	156	156	166	166	125	125	116	116	134	134	0	n
16	C22	0	n	153	153	152	152	125	125	118	118	128	128	0	n
17	RP41	0	n	-	-	-	-	-	-	-	-	-	-	-	-
18	C22	0	n	-	-	-	-	-	-	-	-	-	-	-	-
19	C22	0	n	-	-	-	-	-	-	-	-	-	-	-	-
20	RP41	0	n	-	-	-	-	-	-	-	-	-	-	-	-
21	C22	0	n	-	-	-	-	-	-	-	-	-	-	-	-
22	C22	0	n	-	-	-	-	-	-	-	-	-	-	-	-
23	CM05	0	n	-	-	-	-	-	-	-	-	-	-	-	-
24	CM05	0	n	-	-	-	-	-	-	-	-	-	-	-	-
25	Rb16	5	2n	157	157	161	161	123	125	108	129	126	134	3	2n
26	Rb24	5	2n	155	155	165	165	123	129	118	124	?	?	2	2n
27	Rb24	5	2n	155	155	165	165	123	125	118	118	128	134	2	2n
28	Rb16	5	2n	154	157	159	159	125	125	120	128	?	?	2	2n

29	Rb24	5	2n	149	153	165	165	123	129	118	118	128	134	3	2n
30	Rb24	5	2n	151	155	165	165	123	129	118	118	132	134	3	2n
31	Rca08	5	2n	155	162	155	159	123	123	116	120	?	?	3	2n
32	Rb16	5	2n	155	157	159	159	125	125	108	129	126	134	3	2n
33	Rca08	5	2n	153	161	159	163	123	123	116	120	128	141	4	2n
34	Rb16	5	2n	157	157	159	159	125	125	109	128	126	128	2	2n
35	Rca08	5	2n	153	155	155	159	123	123	106	120	?	?	3	2n
36	Rca08	5	2n	153	155	155	159	123	123	106	120	128	128	3	2n
37	C22	5	n	-	-	-	-	-	-	-	-	-	-	-	-
38	C22	5	n	-	-	-	-	-	-	-	-	-	-	-	-
39	C22	5	n	-	-	-	-	-	-	-	-	-	-	-	-
40	C22	5	n	-	-	-	-	-	-	-	-	-	-	-	-
41	C1	5	n	-	-	-	-	-	-	-	-	-	-	-	-
42	C1	5	n	-	-	-	-	-	-	-	-	-	-	-	-
43	C1	5	n	-	-	-	-	-	-	-	-	-	-	-	-
44	C2	5	n	-	-	-	-	-	-	-	-	-	-	-	-
45	C4	5	n	-	-	-	-	-	-	-	-	-	-	-	-
46	C4	5	n	-	-	-	-	-	-	-	-	-	-	-	-
47	C4	5	n	-	-	-	-	-	-	-	-	-	-	-	-
48	C22	5	n	-	-	-	-	-	-	-	-	-	-	-	-
49	Rb24	10	2n	149	153	165	165	123	125	118	124	128	134	4	2n
50	Rb24	10	2n	151	155	165	165	123	125	118	124	132	134	4	2n
51	Rb24	10	2n	150	154	165	165	123	125	118	118	132	134	3	2n
52	Rb24	10	2n	150	150	163	163	127	127	110	110	128	128	0	n
53	C45	10	n	-	-	-	-	-	-	-	-	-	-	-	-
54	C45	10	n	-	-	-	-	-	-	-	-	-	-	-	-
55	C45	10	n	-	-	-	-	-	-	-	-	-	-	-	-
56	C45	10	n	-	-	-	-	-	-	-	-	-	-	-	-
57	C45	10	n	160	160	159	159	125	125	114	114	128	128	0	n
58	C45	10	n	183	183	161	161	125	125	120	120	?	?	0	n
59	Rca08	10	2n	155	161	155	159	123	123	116	120	?	?	3	2n
60	Rca08	10	2n	154	161	159	163	123	123	106	120	?	?	3	2n
61	Rca08	10	2n	154	161	159	163	123	123	106	120	?	?	4	2n

62	Rca08	10	2n	154	161	159	163	123	123	116	120	?	?	3	2n
63	Rca08	10	2n	151	153	159	163	123	123	116	120	128	128	3	2n
64	Rca08	10	2n	153	155	159	163	123	123	116	120	128	141	4	2n
65	C06	10	n	159	159	163	163	125	125	129	129	134	134	0	n
66	C06	10	n	155	155	161	161	125	125	108	108	134	134	0	n
67	C06	10	n	157	157	159	159	123	123	108	108	128	128	0	n
68	C06	10	n	159	159	163	163	123	123	112	112	128	128	0	n
69	Rb16	10	2n	147	150	167	167	123	123	122	122	130	130	1	?
70	Rb16	10	2n	151	155	165	165	123	125	118	118	?	?	2	2n
71	Rb16	10	2n	153	155	161	161	123	125	120	129	126	134	4	2n
72	Rb16	10	2n	153	155	165	165	123	125	118	118	132	134	3	2n
73	C37	10	n	-	-	-	-	-	-	-	-	-	-	-	-
74	C37	10	n	-	-	-	-	-	-	-	-	-	-	-	-
75	C37	10	n	-	-	-	-	-	-	-	-	-	-	-	-
76	C37	10	n	-	-	-	-	-	-	-	-	-	-	-	-
77	C37	10	n	-	-	-	-	-	-	-	-	-	-	-	-
78	C37	10	n	-	-	-	-	-	-	-	-	-	-	-	-
79	C42	10	n	-	-	-	-	-	-	-	-	-	-	-	-
80	C42	10	n	-	-	-	-	-	-	-	-	-	-	-	-
81	C42	10	n	-	-	-	-	-	-	-	-	-	-	-	-
82	C42	10	n	-	-	-	-	-	-	-	-	-	-	-	-

Table 2. Averages and standard deviations of the relative abundance of cuticular chemical compounds of *S. depilis* males per age and ploidy groups with the coefficients and significance levels of the general linear hypothesis *posthoc* comparison test per age and ploidy group. Significance differences are show in bold

	0 days						5 days						10 days						General Linear Hypotheses Binomial Generalized Linear Model					
Compound	**Mean n**		**SD n**	**Mean 2n**		**SD 2n**	**Mean n**		**SD n**	**Mean 2n**		**SD 2n**	**Mean n**		**SD n**	**Mean 2n**		**SD 2n**	**0d2n - 0dn**	***P value***	**5d2n - 5dn**	***P value***	**10d2n - 10dn**	***P value***
n-C21	2.10	±	1.80	2.60	±	1.80	1.10	±	0.30	0.20	±	0.50	0.40	±	0.10	0.30	±	0.20	0.13	0.59	**-0.31**	**0.02**	0.03	0.99
n-C23	7.20	±	2.70	7.60	±	1.80	2.80	±	0.60	0.10	±	1.60	1.20	±	0.70	0.60	±	0.40	0.02	0.99	-0.15	0.26	-0.17	0.19
n-C24	0.40	±	0.10	0.40	±	0.10	0.20	±	0.03	0.10	±	0.20	0.20	±	0.10	0.20	±	0.30	0.03	0.93	0.08	0.43	**0.17**	**0.01**
11-C25:1	0.10	±	0.10	0.20	±	0.10	0.10	±	0.10	0.10	±	0.20	0.10	±	0.10	0.10	±	0.10	0.10	0.63	0.14	0.31	0.20	0.06
9-C25:1	0.05	±	0.02	0.10	±	0.02	0.10	±	0.01	0.02	±	0.10	0.05	±	0.03	0.10	±	0.03	0.08	0.60	0.11	0.33	**0.22**	**0.00**
n-C25	8.80	±	0.80	8.80	±	0.80	7.30	±	1.00	3.60	±	10.10	4.90	±	3.90	3.40	±	4.60	-0.02	1.00	0.19	0.83	-0.49	0.15
Mix of 13-,11-MeC25	0.30	±	0.10	0.30	±	0.10	0.20	±	0.10	0.05	±	0.20	0.20	±	0.10	0.20	±	0.10	-0.03	0.96	0.03	0.94	-0.01	0.99
7-MeC25	0.40	±	0.20	0.40	±	0.10	0.20	±	0.10	0.10	±	0.20	0.20	±	0.10	0.20	±	0.10	-0.07	0.57	-0.05	0.79	-0.02	0.98
11-C26:1	0.10	±	0.04	0.10	±	0.10	0.10	±	0.04	0.10	±	0.10	0.10	±	0.10	1.80	±	5.50	0.02	1.00	0.16	0.56	**0.38**	**0.01**
n-C26	0.90	±	0.10	0.90	±	0.10	1.10	±	0.10	0.10	±	1.00	0.70	±	0.40	0.90	±	1.50	-0.01	1.00	0.04	0.99	-0.11	0.81
11-C27:1	4.80	±	1.70	5.20	±	1.90	5.90	±	1.80	2.60	±	7.10	4.00	±	2.50	3.90	±	4.10	0.01	1.00	0.15	0.86	-0.11	0.94
9-C27:1	0.20	±	0.10	0.30	±	0.10	0.30	±	0.10	0.20	±	0.40	0.20	±	0.20	0.30	±	0.30	0.05	0.90	0.12	0.42	0.15	0.19
n-C27	16.60	±	2.40	15.60	±	1.90	22.90	±	2.10	21.40	±	2.70	20.00	±	11.60	8.10	±	9.50	-0.04	1.00	0.03	1.00	**-0.59**	**0.02**
Mix of 13-,11-,9-,7-MeC27	2.00	±	0.50	1.90	±	0.50	1.60	±	0.40	0.20	±	1.20	1.10	±	0.40	0.50	±	0.40	-0.02	0.98	-0.03	0.98	**-0.33**	**0.00**
5-MeC27	0.20	±	0.02	0.20	±	0.04	0.20	±	0.05	0.02	±	0.10	0.10	±	0.03	0.30	±	0.40	-0.07	0.91	-0.15	0.44	0.18	0.25
13,17-diMeC27	0.20	±	0.05	0.20	±	0.10	0.10	±	0.10	0.02	±	0.10	0.10	±	0.03	0.10	±	0.10	-0.02	0.99	-0.05	0.86	0.05	0.82
11,15-diMeC27	0.04	±	0.01	0.03	±	0.01	0.02	±	0.01	0.00	±	0.01	0.03	±	0.02	0.03	±	0.02	-0.03	0.99	-0.02	1.00	0.07	0.89
3-MeC27	0.70	±	0.10	0.70	±	0.10	1.00	±	0.20	0.20	±	0.70	0.80	±	0.10	1.20	±	1.90	-0.02	1.00	-0.07	0.86	-0.13	0.41
7-C28:1	0.10	±	0.10	0.20	±	0.20	0.30	±	0.40	0.10	±	0.10	0.04	±	0.10	0.30	±	0.30	0.09	0.95	-0.32	0.30	**1.14**	**0.00**
n-C28	0.90	±	0.20	0.90	±	0.20	1.60	±	0.30	0.40	±	1.00	1.20	±	0.50	4.40	±	8.50	0.01	1.00	-0.12	0.72	0.05	0.97

3,15-diMeC27	0.50	±	0.10	0.70	±	0.10	0.40	±	0.10	0.10	±	0.40	0.30	±	0.20	1.50	±	1.80	0.09	0.87	0.07	0.94	**0.52**	**0.00**
11-C29:1	14.70	±	1.60	12.30	±	2.00	15.10	±	1.30	3.00	±	11.40	13.10	±	4.70	3.70	±	3.30	-0.10	0.80	-0.06	0.94	**-0.69**	**0.00**
5-C29:1	0.10	±	0.02	0.20	±	0.05	0.20	±	0.03	0.05	±	0.20	1.00	±	3.20	0.10	±	0.20	0.03	0.99	0.06	0.95	**-0.40**	**0.00**
n-C29	4.30	±	0.90	4.40	±	0.90	9.60	±	1.90	4.20	±	7.10	12.60	±	8.10	8.30	±	9.90	0.00	1.00	-0.13	0.73	-0.23	0.23
15-MeC29	1.50	±	0.30	1.40	±	0.30	1.20	±	0.20	0.20	±	0.90	0.80	±	0.40	0.50	±	0.30	-0.04	0.92	-0.05	0.84	**-0.18**	**0.03**
13-MeC29	0.60	±	0.10	0.60	±	0.10	0.50	±	0.10	0.10	±	0.40	0.80	±	0.60	1.20	±	2.20	-0.02	1.00	-0.03	0.99	0.10	0.83
11-MeC29	0.20	±	0.03	0.20	±	0.03	0.20	±	0.03	0.03	±	0.20	0.20	±	0.20	0.80	±	1.20	-0.04	0.99	0.01	1.00	**0.39**	**0.03**
11-C31:1	2.00	±	0.20	1.80	±	0.50	1.40	±	0.40	0.20	±	1.20	4.20	±	5.90	2.30	±	2.70	-0.07	0.98	0.02	1.00	-0.12	0.91
n-C31	0.80	±	0.10	0.80	±	0.30	0.70	±	0.10	0.30	±	1.00	4.30	±	5.70	1.70	±	2.10	0.01	1.00	0.16	0.74	-0.21	0.55
Mix of 17-,15-MeC31	0.60	±	0.10	0.50	±	0.10	0.50	±	0.10	0.10	±	0.40	0.40	±	0.30	0.50	±	0.60	-0.08	0.88	0.00	1.00	-0.05	0.97
11-C32:1	0.30	±	0.10	0.30	±	0.10	0.40	±	0.10	0.10	±	0.40	0.30	±	0.20	0.40	±	0.30	0.00	1.00	0.17	0.13	0.13	0.25

Discussion

Our data show that *S. depilis* queens transferred into a colony producing adult diploid males are killed, presumably executed by the workers, within 10 to 30 days of the start of diploid male emergence. This is very similar to the situation in *Melipona* (Camargo 1976; Alves et al. 2011), and suggests that the execution of queens that have made a matched mating at the CSD locus is not confined to *Melipona* in the stingless bees. Since the queens that were killed did not actually make a matched mating but were transferred into colonies producing diploid males, our results also show that the cues triggering execution are not present on the matched-mated queen but arise from the diploid males themselves. Our results strengthened how prevalent this behaviour is in a stingless bee population, since previous conclusions on *Melipona* species were based in low sample size data and non-systematized bioassays (Camargo 1976; Alves et al. 2011).

The cuticular hydrocarbons of diploid and haploid males were significantly different in males 5 and 10 days after emergence from their sealed cells, but not immediately after emergence. This suggests that the cue, or possibly signal, triggering queen execution is the presence in the colony of males of a distinct diploid-male odour. Alternatively, it is possible that the workers might detect a matched mating by the presence of unusually large numbers of males in the colony. However, because queen execution normally took place within 10 to 20 days of the start of the emergence of diploid males, this would be too soon for the number of individuals in the colony to become more than half male (i.e., the haploid males + 50% of the diploid adults), unless the workers could simply assess the presence of "too many young males" rather than "too many males in total". Haploid males can be produced in large numbers in *S. depilis* colonies, reaching peaks of ca. of 30% during times of pronounced male production (Bego 1982). Further research is needed to test among these competing hypotheses. However, there is a precedent in the use of odours to detect diploid males, as this is what takes place in the honey bees *A. mellifera* and *A. cerana* (Woyke 1962, 1963, 1979). Here, however, the individual diploid males are recognized and killed.

We showed that several compounds are present on the cuticle of diploid males in different relative amounts when compared with haploid males. Although no qualitative differences were found, workers are definitely able to detected quantitative differences

in cuticular chemical profiles as is the case for nestmate recognition, policing and queen pheromones, for example (e.g. Smith et al. 2009; van Zweden & D'Ettorre 2010; Van Oystaeyen et al. 2014). In the honeybee, the diploid male larvae cannibalism is triggered by an odd pattern of five different chemical compounds, mostly alkenes, present in the cuticle (tricosane, pentacosene, heptacosene, nonacosene and squalene) (Santomauro et al. 2004). In our study several alkenes were indeed also differently expressed on the cuticle of diploid males including some of the same compounds found in the honeybees, namely pentacosene, hexacosene, octacosene and nonacosene. Although the position of the unsaturation was not determined, it is possible that there is conservation of the chemical signaling, and the same compounds are responsible for the diploid male detection by workers in both groups of bees.

Our estimate on the proportion of matched matings is not significant different from Paxton et al. (2003) estimate, with the same species (*S. postica* at the time) and location, in which 2 out of 10 colonies were producing diploid males, resulting in 10 sex alleles (Fischer's exact test, $p = 0.2188$). Our results also showed no significant differences with other estimates from other studies using different stingless bees' species: 4 matched mated out of 49 non-matched mated queens in *M. compressipes fasciculata* (Kerr 1987; Fischer's exact test, $p = 1$); 1 out of 5 in *Tetragonula carbonaria* (Green and Oldroyd 2002; Fischer's exact test, $p = 0.4091$); 2 out of 38 in *M. scutellaris* (Alves et al. 2011; Fischer's exact test, $p = 1$); 3 out of 28 in *M. seminigra merrilae* (Francini et al. 2012; Fischer's exact test, $p = 0.7027$); and 2 out of 30 in *M. interrupta manaosensis* (Francini et al. 2012; Fischer's exact test, $p = 1$);

The diploid male load, due to the *complementary sex determination*, is an important selective pressure acting in both social and solitary Hymenoptera. Meliponini bees are specially affected due to their monandrous queens, which have a heavy load on their fitness when performing a matched mating. Even in populations with relatively high number of sex alleles like the one studied here, the probability of a queen mating with a male carrying the same sex allele is somewhat high. As previously suggested, this behaviour of replacement genetically deficient queens would greatly help populations to survive genetic bottlenecks and is of great importance in a genetic conservation context (Alves et al. 2011). Our results strength the hypothesis that the queen of stingless bees are executed in response to a matched mating, leading to a chance of a last reproductive event for a colony. It is possible that the diploid male

specific odour trigger queen death. This would mean another specific adaptation to diploid male load among social Hymenoptera.

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2.2. Stored sperm depletion with aging in queens of the stingless bee *Scaptotrigona depilis*

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Abstract

Eusocial bee queens mate early in their lives and do not remate again, storing sperm on their spermathecal for their entire lifespan. Lack of sperm will eventually cause colony death, so it is expected that queens with sperm depletion are supersedure. Here we investigate the sperm use on the stingless bee *Scaptotrigona depilis*. We counted the number of sperm and verified the sperm viability of the spermatheca of newly mated queens (one month age) and one-year-old queens (13 months age). We also determined the proportion of male offspring of each of the queens. Additionally, we estimated the egg-laying rate of a group of queens with uncontrolled age. We found that newly mated queens had in average 880 thousand sperm cells on their spermathecal, and the viability was 63%. One-year-old queens had the sperm quantity reduced by a factor of 30%, to an average of 611 thousand cells, and the viability was 49%. Male production was not affected by sperm cell reduction. We estimated that queens laid approximately 50 thousand egg per year. By combining these results and assuming that fertilization mechanism would be similar to honey bees queens, we modelled sperm use per egg in function of queen age. Our estimates resulted in 3 viable sperm cells per egg during the first year of the queen. By the age of 2.7 years old, queens start using less than one sperm cells per egg, what might result in result in lack of sperm cells for fertilization and consequently unintentional male production. Our results indicated that *S. depilis* queens' life expectancy could be influenced by sperm depletion, however, further studies are necessary to strength our assumptions.

Keywords: sperm use; queen fertility; queen longevity;

Introduction

Queens of social Hymenoptera species mate early in their lives within a narrow window of time, to acquire and store a lifetime supply of sperm (Wilson 1971). They store the sperm in their spermatheca, a specialized organ that keep sperm alive and viable even for several decades in some species (Boomsma et al. 2005). The queen gradually uses the sperm for fertilizing her eggs. If a Hymenoptera queen runs out of sperm she will lay unfertilised eggs. Due to the haplodiploid sex determination system of Hymenoptera, unfertilized eggs will turn into males, while fertilized eggs will originate diploid females (Cook and Crozier 1995). Producing only male offspring will eventually lead to colony death, since males do not perform nest internal tasks for colony maintenance (Baer 2016).

In species where the queen can be replaced by one of her daughters virgin queens, it is expected the mother queen is superseded under sperm depletion (Butler 1957; Baer et al. 2016). The replacement of the queen should be initiated as soon as a queen's fertility declines and workers can, in principle, assess queen fertility by monitoring her egg laying frequency and/or the success rate of egg fertilization (Baer et al. 2016). Independently of the causes of the queen death (colony death or by being replaced), sperm depletion might potentially influence queen lifespan. The sperm quantity stored in the spermatheca decreases as sperm is used for egg fertilization, since no remating occurs (Boomsma et al. 2005). In addition, viability of sperm cells decreases, since the sperm cells can die inside the spermatheca (Tarpy & Olivarez 2014), which may also increase the number of sperm used per egg (den Boer et al. 2009). Sperm use will, therefore, depend on the rate of egg laying (queen fertility), the capacity of sperm viability maintenance and the efficiency of sperm use.

Honey bees and stingless bees form the group of eusocial bees, which have perennial colonies that are headed by a single queen (Engels & Imperatriz-Fonseca 1990). Both groups have the capacity of replacing their mother queens in case of death. Honey bee queens mean life expectancy of is ca. of one year (Seeley 1978; Gordon et al. 1995; Guzmán-Novoa et al. 1998), however there are reports that they can live up to 8 years (Bozina 1961). Queens are extremely fertile, laying up to 1.7 million eggs during their lifespan. Aging caused a reduction on sperm cells used per egg fertilisation in honey bee queens (Baer et al. 2016). Since their spermatheca volume does not

reduce, it is suggested that a constant volume of spermathecal fluid containing the sperm cells is sampled from the spermatheca and is transferred onto the eggs (Baer et al. 2016). Baer et al. (2016) showed that honey bee queens are very economic in sperm use, with estimates of a median use of two sperm cells per egg fertilisation and even though their model suggested that sperm depletion might affect queen longevity. Stingless bees queen fertility greatly varies within the several hundred species comprising this group. With exception of some few species with large worker populations, most species have clearly lower egg laying rates compared to honey bees (Sakagami 1982). Low fertility is frequently related to higher lifespan due to intrinsic factors in animal kingdom (Williams 1957; Kirkwood 1977).Therefore, it is expected that stingless bee queens have higher lifespan than honey bee queens, resulting in greater importance of sperm depletion for stingless bee queens. Although few studies have recorded queen lifespan, anecdotal reports of stingless bees queen lifespan reach up to 7 years in *Melipona* species (Carvalho-Zilse & Kerr 2004).

Queen sperm use could be a factor contributing to queen lifespan in stingless bees. Here we investigated queen sperm use in the Brazilian stingless bee *Scaptotrigona depilis*. We conducted an analysis on the sperm quantity and viability of newly mated and one-year-old queens. Additionally, we estimated the proportion of males produced in their colonies. We also estimate queen egg laying rate during summer and winter. By combining those results we developed a model to assess queen sperm use and whether the sperm depletion could possibly have an effect on queen lifespan.

Methods

Study species and site

The study was carried out using queens of *Scaptotrigona depilis* collected from colonies placed in wooden hives at the experimental apiary of the Biology Department of the University of São Paulo *campus* Ribeirão Preto, São Paulo State, Brazil. Queen egg-laying data was collected using nine established colonies kept in wooden hives (internal measures of 20 x 20 x 40 cm). The nine colonies used for egg laying were placed side by side into plastic shelters outside of the laboratory. A roof tile avoided direct exposition to sun and rain. Colonies used for queen egg-laying ratio were not the same as the one used for queen aging. Sperm count and viability analysis were performed at

the Department of Ecology of University of São Paulo *campus* São Paulo, São Paulo State, Brazil. *S. depilis* colonies are perennial and have a singly mated queen (Paxton et al. 2003). Mature colonies have approximately 5-10 thousand workers.

Queen aging and spermatheca removal

In order to control queen age we removed 8 mother queens from colonies and allowed a replacement daughter queen to take over the colony. This new queen was marked by clipping her wings and aged for one year and one month. Their colonies were not disturbed during this period. One of the queens died in the meantime, resulting in a total of seven queens with controlled age. Seven newly mated queens were obtained in the same way, however were collected one month after the mating flight. Colonies were visually classified by their "strength" in a rank of 1-4, which reflected their population size. We collected the queens and kept them inside a small wooden box (internal measures of 12 x 12 x 6 cm) with ca. of one hundred workers and pollen and honey *ad libitum*. Queens were transported from Ribeirão Preto to São Paulo (~350km) on the day before spermatheca's extraction. For the spermatheca's extraction, we killed the queens by cutting their heads and dissected their abdomen under a stereomicroscope. By removing the tergites, we exposed the espermathecae, which was entirely removed using a tweezer. . The spermathecae was placed them into a lid of an Eppendorf tube containing 20uL of Hayes solution (9g NaCl, 2g $Ca(Cl)_2$, 0.2g KCl, and 0.1g $NaHC(O)_3$ in 1000ml of deionized water, pH adjusted to 8.7), and was ruptured using tweezers. Outflowing sperm was gently mixed and this sperm solution was homogenized with 100uL of Hayes solution inside an eppendof tube.

Sperm viability

In order to assess sperm viability we followed the protocol established by Meneses et al. (2014). We used the Sperm Viability Kit LIVE/DEAD Invitrogen L-7011. After homogenizing, a sample of 5ul of the sperm solution was stained with 5ul of SYBR14, and 3ul of propidium iodide, and incubated in a dark and humid chamber. Employing a fluorescence microscope Axiovert 40 CFL Zeiss with filter Cy3 and a cell counter, we counted the first 400 cells found in the center of the cover slip. Sperm cells were classified as live (green stained), dead (red stained) and dying (dual stained). Dual stained cells comprised less than 5% and were considered as live sperm cells

Sperm count

Sperm count was performed adapting the existing protocol for counting sperm in males of *Scaptotrigona* (Meneses et al. 2014). We dilluted12 uL of the sperm solution in 488 ul of Hayes solution. Five droplets of 1 ul each of this diluted solution were placed on a microscope slide and air-dried. Each sample was stained using 5 ul of DAPI (dihydrochloride 4 ', 6-diamidino-2-phenylindole) solution (4 ng/ul), covered with a cover slip, and inspected using a fluorescence microscope. To obtain the number of sperm in the spermatheca, we multiplied the number of sperm counted in each 1ul droplet by the dilution factor (5.000 times). For statistical analyses, we used the mean number of sperm counted for the five droplets.

Proportion of male offspring

One large brood comb containing red-eyed pupae was collect from the colony of the newly mated and one-year-old queens in the moment of their collection. As newly mated queens were sampled after one month from their mating flight, we could sample their offspring on these combs, since the time taken from egg to adult is ca. of 35 days. We uncapped 500 brood cells of each comb and determined the sex of individuals based on head morphology (males have smaller heads and larger eyes). To avoid sampling a brood comb region that would have higher male concentration, we uncapped cells by opening the first row comprising the diameter of the brood comb. Then we progressively opened new rows in each of the sides of the first row.

Queen egg-laying rate

In stingless bees, the brood cells are not reused as like in honey bees, so workers always build a new brood cell made of wax and fill it with larval food, where the queen will lay her egg. Therefore, counting the number of new brood cells is a good method to estimate queens' egg-laying rate. In order to do that, we compared consecutives daily pictures of the new brood cell area (Fig. 1). All the pictures were taken at the same time interval of the day, around noon. We quickly opened the colonies at the region of new brood cells construction and took pictures in the same position. Photos were analysed into image editing programs in order to mapping the new brood cells built (Fig. 1). We counted the number of new brood cells in two different periods of the year: summer

(January, mean temperature of 26.5ºC) and winter (July, mean temperatures of 18.2 ºC). We counted the number of new brood cells produced by each of the colonies for 9 days in a row during summer. During winter we used the same colonies observed in summer, with exception of 2 colonies, and counted the number of new brood cells for 18 days in a row (Table 2).

Figure 1: New brood cell area of a single colony on three consecutive days (a, b and c). The number of new cells built per day was estimated by comparing pictures of two consecutive days. New brood cells are added on the margins of the first two superior combs.

Statistical analysis

All data was analysed in R 2.9.2 (R Development Core Team 2009). To compare the viability and sperm counts of newly-mated with one-year-old queens we ran linear models (LMs) of the viability/count/male production in function of age. We included colony strength as a factor to test for the interaction with this variable. Queen egg-laying rate was also compared using a linear model in function of season of the year, and the results were incorporated into a model (see below) to estimate queen sperm use.

Results

Sperm count, viability and proportion of males on queens offspring

As expected, one year-old-queens showed lower sperm counts than newly mated queens, with 30% less sperm cells in average on their spermatheca (LM, $F = 4.615$, $p = 0.03504$) (Fig. 2; Table 1). The number of sperm cells in queens' spermatheca was not affected by colony strengh (LM, $F = 4.615$, $p = 0.898$). The proportion of males in queens' offspring was not different between the two age groups, which was also not affect by the number of sperm on queen spermatheca (LM, $F = 0.1889$, $p = 0.8305$) (Fig. 2).

Table 1: Mean number (±std. dev.) of sperm cells and sperm viability on 1 and 13 months old queens' spermatheca. The mean proportion of males amongst 500 individuals offspring of queens in each group is also shown. The reduction or increase of each parameter is also indicated.

	Queen age (months)		Reduction (%)
	1	13	
Mean num. sperm cells (± std. dev.)	881428 (±172953)	611000 (±117506)	- 30.5
Mean sperm viability (± std. dev.)	63.2 (±9.1)	49.2 (±17.2)	- 22
Mean proportion of male offspring (± std. dev.)	0.071 (±0.08)	0.0814 (±0.114)	+14

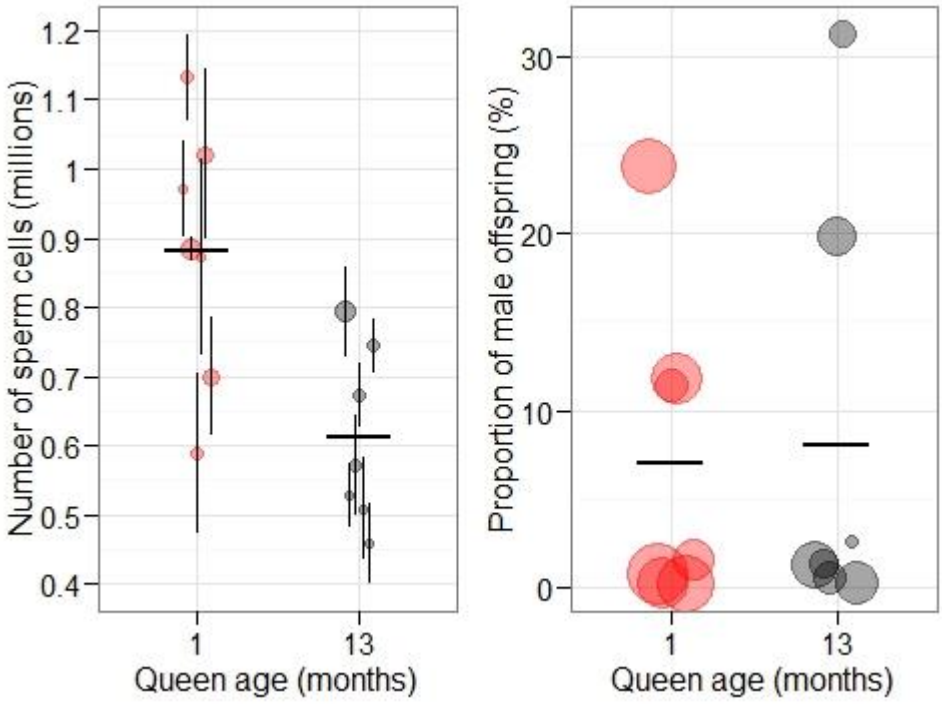

Figure 2: Sperm counts and proportion of male in the offspring of newly mated (1 month old) and one-year-old (13 months old) queens. For the sperm count graph, the size of each dot represents queen' colony strength. For the proportion of male offspring graph, the size of each dot represents queen' sperm number. The lines are showing the means.

Sperm viability was on average 63.2% (± 9.1) for newly mated queens, and 49.2 % (±17.2) for one year-old queens (Fig. 3; Table 1). Despite the big effect size of a reduction by a factor of 22%, it is not significant different (LM, $F = 2.282$, $p = 0.0713$), and was not affected by colony strength (LM, $F = 2.282$, $p = 0.2699$).

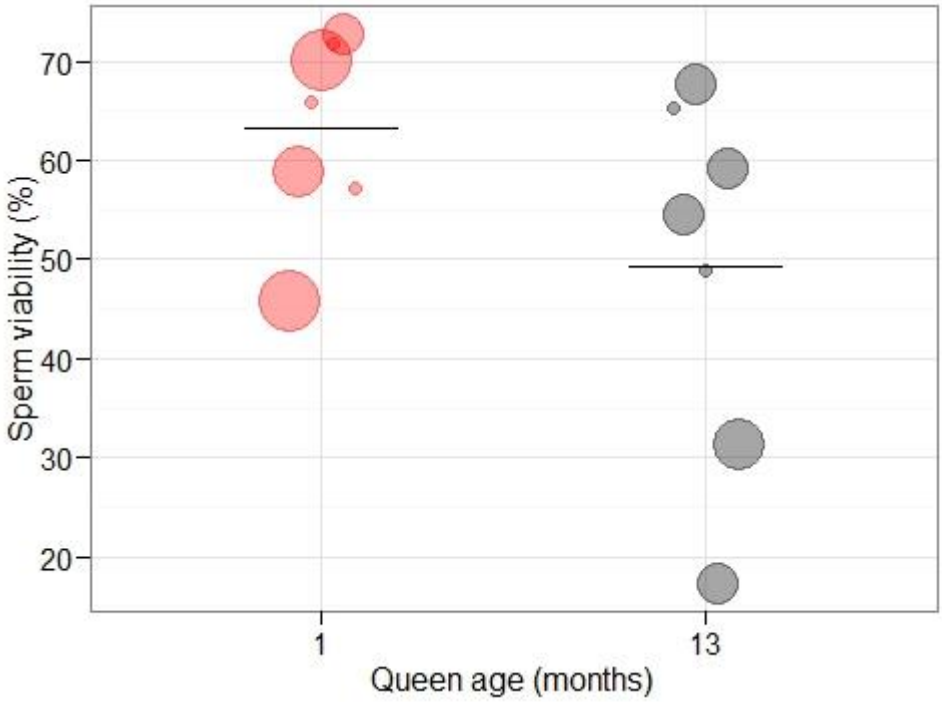

Figure 3: Sperm viability of spermatheca of newly mated (1 month old) and one-year-old (13 months old) queens. The size of each dot represents queen' colony strength (rank of 1 to 4). The lines are showing the means.

Queen egg-laying rate

Colonies produced more than twice the amount of new brood cells during the summer days, in comparison with the winter (LM, $F = 18.19$, $p = 0.0007845$) (Fig. 4; Table 2). In order to estimate the number of fertilized eggs a queen will lay throughout a year, we considered 8 months of summer egg-laying rate and the other 4 with winter rate. Not all the eggs laid by a queen will be fertilized, since it is very likely that that queens can control egg fertilisation in order to produce male offspring, as honey bee queens do (Ratnieks & Keller 1998). Based on male production rates (Bego 1990) and proportion of queen maternity of males (Paxton et al. 2003), we assumed that 10% of all eggs were not fertilized. That results in an estimate of 50441 fertilized eggs laid by one queen per year.

Table 2: Average number of new brood cells produced per day, in two distinct temperature and food availability regimes: summer and winter. The number of colonies and the number of days in which brood cells production was measured are shown.

	Mean number of new brood cells produced (±std. dev.)	Number of colonies	Number of serial days of measurement	Mean temperature (°C)
Summer	193 (±56)	9	9	26.5
Winter	81 (±37.3)	7	18	18.2

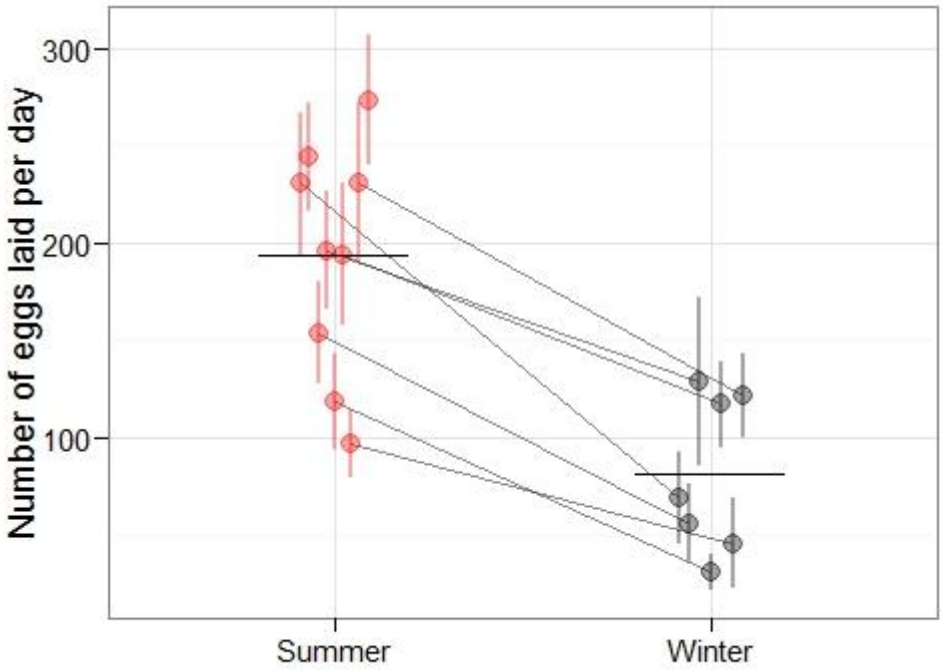

Figure 4: Queen egg-laying rate of queens during summer (January) and winter (July), based on the number of new brood cells produced per day. Lines are connecting two data points of the same colony.

A model of queen sperm use

In order to assess queen sperm use throughout her life we developed a model in which queens will use a constant volume of spermathecal content when fertilizing an egg and that spermatheca volume will not decrease with time, similar to what is found in honey bees (Baer et al. 2016). Under this model, the number of sperm stored in a queen spermatheca at a given time (X_t) depends on the initial amount of sperm cells inside a queen spermatheca (X_i), the proportion of sperm cells she will use per year (U) and her age (t):

$$X_t = X_i\,(1-U)^t \qquad (1)$$

Considering the average initial sperm cells quantity stored in newly-mated queens of X_i = 881429, and the proportion of sperm used U = 30.6% calculated as the percentage of sperm reduction from newly-mated queens to one-year-old queens, we have the curve expressed in Figure 5 (total). Since only live sperm cells will be able to fertilize eggs, sperm viability might have an effect on the effective number of sperm cells. Considering that sperm viability will change from year to year with the same rate, then sperm viability will be related to queen age. The sperm viability at a given time (V_t) will be in function of initial viability (V_i) and the rate of sperm viability reduction (r):

$$V_t = V_i(1-r)^t \quad (2)$$

Then the viable number of stored sperm cells (Xvt) will be:

$$X_{vt} = X_t V_t \quad (3)$$

With the rate of sperm viability reduction of 0.22 calculated as the percentage of sperm viability of one-year-old queens in relation to newly mated queens, we have a lower number of viable sperm cells stored in queen spermathecal in function of time (Fig. d – "Alive"). This model offered us the possibility to estimate the number of sperm cells used per eggs, by combining it with the estimated value of fertilised eggs laid by a queen during one year. The estimated number of sperm cells used per egg at a given queen age (S_t) will depend on the number of eggs laid per year (E) and the amount of sperm used at a given queen age (A_t). Since,

$$A_t = X_i - X_t \quad (4)$$

Then we have:

$$S_t = \frac{A_t}{E_t} \quad (5)$$

This results in 5.4 sperm cells used per egg during the first year, decreasing by a factor of 30% per year. Using the viable number of sperm cells for the amount of sperm cells used at a given time,

$$A_{vt} = X_{vi} - X_{vt} \quad (6)$$

$$S_{vt} = \frac{A_{vt}}{E_t} \quad (7)$$

We have the estimate of 3 viable sperm used per egg, decreasing by a factor of 46% per year. The model predicts that by the age of 2.7 years queens will start using less than one sperm cell per egg, which would result in lack of sperm cells for fertilization and consequently unintentional male production.

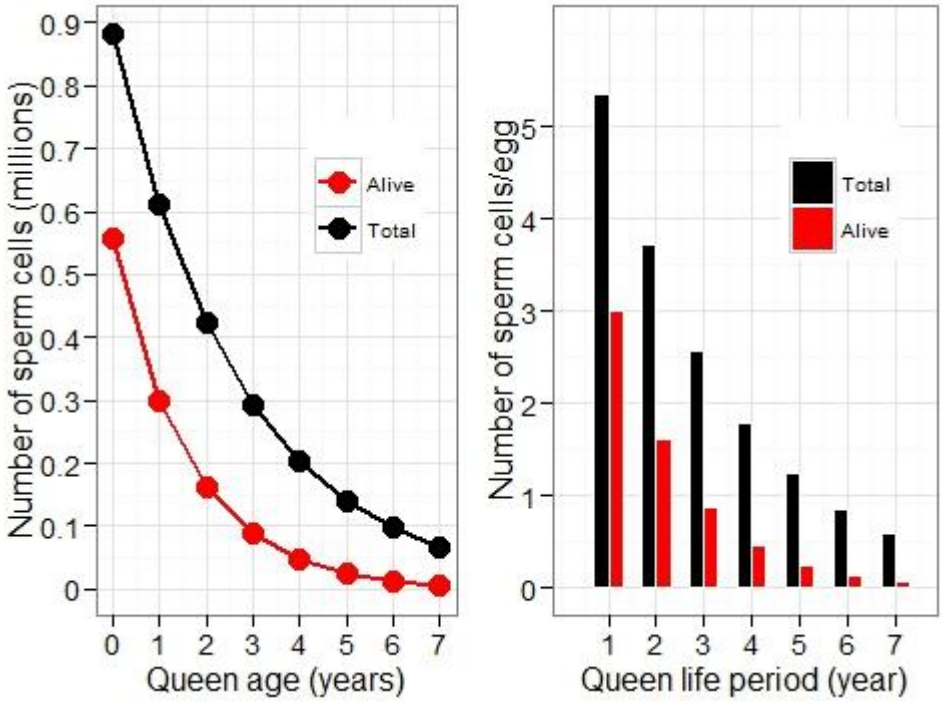

Figure 5: estimated number of sperm cells on queen spermatheca (left) and number of sperm cells used per egg fertilisation (right) in function of queen lifetime. Black bars and dots are showing the total amount of sperm cells, while the red dots and bars are showing only the viable sperm cells. Estimations were based on the model described on the text.

Discussion

Newly mated queens showed an average sperm count of 880,000 (±172,953). We can estimate the total amount of sperm in *S. depilis* queens' spermatheca just after mating. The newly mated queens were one month old at the time of their dissection and analysis, so we estimate that her sperm use was 1/12 of what we found for one year. This gives us an estimate of 903,964 sperm cells. This amount is ca. of 60% of the estimate for mature males sperm seminal vesicles of 1,487,778 (Meneses et al. 2014). The sperm viability of mature males ranged from 52 to 87% (Meneses et al. 2014), resulting in a range of ca. 770,000 to 1.3 million viable sperm cell. It is not know how *S. depilis* sperm migrate to spermatheca, but it is very likely that sperm viability and motility is linked to sperm migration in stingless bees (Kerr 1962). In honey bees, there is evidence that sperm cells have to be viable to actively migrate to queen spermatheca, however during this process a proportion of dead sperm is carried along with the live sperm (Collins 2000). Considering that only live sperm cells migrate from queen vaginal chamber to spermatheca, our results matches well with previous estimates of

sperm quantity on males (Meneses et al. 2014). However, further studies are necessary to determine the proportion of dead sperm cells that might be carried along with the live sperm cells to queen spermatheca.

Sperm viability on spermatheca decreased from initial proportion of 63.2 % of live sperm cells in newly mated queens to 49.2% in one-year-old queens. Our estimates on sperm viability inside queen spermatheca matched well with previous studies on sperm viability of seminal vesicles of mature males of *S. depilis* (menses 2014). Despite the possibility of this result represents reality because sperm viability indeed might have the tendency to reduce with time, our results should be carefully interpreted. Methodological constraints can affect sperm viability, since sperm cells are considerably fragile and might die during experimental procedures (Collins & Donoghue1999; Gençer & Kahya 2011). However, the relative sperm viability of both age groups was controlled, since both queens were analysed under the same conditions. Such reduction on sperm viability can be associated with the challenge of queens keeping sperm cells alive inside their spermatheca. The sperm viability stored in honey bee queen's spermatheca also reduces with time. However, the reduction was not so pronounced as we observed for stingless bees at the first year: two-months-old queens showed 79.5% of live cells, very similar to 78.5% in one-year-old. Two-years-old queens showed a considerable reduction to 66.5% of live cells (Lodesani et al. 2004). Tarpy & Ollivarez (2014) also observed decreased sperm viability in honey bee queens spermatheca declined from 90.3 to 81.3 % after the first 5 months of queens life.

Our results showed that the sperm quantity stored on the spermatheca of queens of *S. depilis* decreased by a factor of 30% in average, and by 22% in viability after a period of one year. Such reduction on viable sperm availability after one year of egg-laying did not affect queen capacity of egg fertilisation, since queen male offspring is not higher nor correlated to sperm quantity in one-year-old queens (Fig. 2). We combined these results with the egg-laying rate data of ca. of 50 thousand eggs laid throughout one year, to model the sperm use in queens of *S. depilis*. We considered that queens use a fixed amount of volume from spermatheca, which is continuously replaced by spermathecal fluids, leading to no reduction on spermathecal volume, just as observed for honey bees (Baer et al. 2016). This leads to a reduction on the concentration of sperm cells on the queen spermathecal with time, and consequently a reduction on the number of sperm cells used to fertilise an egg. Our model estimated

that *S. depilis* queens use an average of 5 sperm cells per egg during the first year of her life. If our model is correct, sperm use per egg is higher in *S. depilis* when compared to honey bees, which uses a median use of two sperm cells per egg fertilisation (Baer et al. 2016). Since the model assumes that the proportion of sperm cells will decrease inside spermathecal with time, it estimated that eggs will be fertilized with more than one viable sperm cell per egg until queen age of 2.7 years old (Fig 5). After this threshold, eggs might start to be randomly unfertilised, giving rise to unintentional male production.

High male production might be detrimental in a colony level, since males are not able to work on nest maintenance and decrease colony performance. Eventually, completely lack of sperm will result in the death of the colony because workers and virgin queens can only be reared from fertilized eggs. It is suggested that sperm depletion might provide an honest signal of queen fertility in honey bees, as it is her interest to get replaced by one of her daughter queens before she becomes completely infertile (Butler 1957; Baer et al. 2016). Therefore, the appearance of haploid eggs in diploid comb could be a key factor initiating queen supersedure (Butler 1957). A condition for such adaptation is that the queen is executed before her stock of sperm is completely depleted.

Our results indicate that sperm depletion might have an effect on *S. depilis* queen's lifespan. Stingless bee queens are expected to have longer lifespan due to their lower fertility levels compared to honey bees. Honey bee queens are extremely fertile, laying ca. of 2000 eggs per day (Snodgrass 1984), producing up to 1.7 million fertilised eggs over their lifespan. The average lifespan of managed honey bee queens is relatively low, mostly living one year or less (Seeley 1978; Gordon et al. 1995; Guzmán-Novoa et al. 1998), showing that queens might being replaced. Our data have shown that *S. depilis* queens egg-laying rate is ca. of 10% of honey bee queens (approximately 150 eggs per day; Fig 4). Fertility is highly associated with lifespan in animal kingdom (Williams 1957; Kirkwood 1977) and in social insects (Keller & Jemielity 2006, Remolina & Hughes 2008). Therefore, it is likely that queen mortality on stingless bees queens might be also related to extrinsic factors, such as sperm depletion, than only due to intrinsic factors. Very little is known about stingless bee mother queens mortality or replacement. In general, stingless bees colonies are always producing new virgin queens, which can head new colonies by swarming, replace their mother queens

(Sakagami 1982, Imperatriz-Fonseca & Zucchi 1995) or even enter in foreign colonies as social parasites (Wenseleers et al. 2011). As it is suggested for honey bees, it is expected that stingless bees mother queens with low fertility should be detected and replaced.

Queen replacement occurs in stingless bees species when the queen mates with a male sharing of her sex alleles (a matched mating), resulting in the production of 50% of diploid males amongst her diploid brood (Camargo 1976; Alves et al. 2011; Vollet-Neto et al. submitted – Chapter 2). It is suggested that this is an adaptation for attenuating the negative effects of diploid male production. It is suggested that the signal for triggering queen execution are the diploid males chemical profile (Vollet-Neto et al. submitted; Chapter 2). However, an alternative hypothesis is that the signal could come from the presence of "too many males" inside the colony. In particular, the male ploidy could have no effect on this behaviour, but the quantity of males. On this case, executing the queen under diploid male production could be adaptive or not. Queen execution would occur as a consequence of workers ability to recognize overproduction of males, since this is adaptive considering a queen with sperm depletion.

We can conclude that queens of *S. depilis* still have enough viable sperm stored on their spermatheca to fertilize their eggs after one year of life. They showed a smaller rate of egg laying compared to honey bee queens. We extended our findings of one year of sperm use, reduction on viability and egg laying by modelling the number of sperm cells used per egg based on the way honey bee queens fertilise their eggs. Our results indicated that *S. depilis* queens' life expectancy could be influenced by sperm depletion, however, further studies are necessary to refine the data and check how sperm is used later than one year in older queens.

2.3. Attempts to have queens producing only haploid males

As discussed in the previous section, there is an additional hypothesis to explain the proximate mechanism that triggers queen death under diploid male emergence. Alternatively to the hypothesis that diploid males odour bouquet could trigger queen death, the presence of "too many males" inside the colony could trigger queen death. To test this hypothesis it is necessary to have a great quantity of haploid males in pupal stage to insert in the proportion of 50% in colonies to check queen execution. Here we describe the trials we did to have queens producing only males (or a huge quantity). Unfortunately, we did not achieve this situation and we were not able to test this hypothesis. However, we believe it worth including the fail attempts in order to provide insights for further experiments.

Queens producing only males were already obtained in *Apis mellifera* through stimulating queens that did not mated to initiate egg laying. Since they do no have sperm in their spermatheca, her eggs are not fertilized and end up developing into haploid males. Submitting virgin queens of *A. mellifera* to two subsequent carbonic gas (CO_2) narcosis stimulated ovarian development. By not allowing them to perform the mating flight, they start egg laying without copulating (Mackensen 1947). We performed preliminary tests with CO_2 narcosis on virgin queens of *S. depilis*. Virgin queens were kept confined inside small colonies with hundreds of workers, pollen and honey. They did not developed physiogastry and end up dying (probably executed by workers). We also clipped their wings to avoid that they could perform mating flight and inserted them into free foraging colonies, but they also died in such conditions. Since we did not developed a systematic study on these bioassays, we cannot conclude that CO_2 narcosis have the same effect in stingless bees compared to honey bees. Therefore, we would like to encourage further research on this topic, especially varying the CO_2 concentration and period by which queens are submitted to narcosis.

Hence, we tried three alternative methods to have queens laying big proportions of male (unfertilized) eggs: (i) based on gonadotropic effects of Juvenile Hormone (JH) already known in several insect groups (Robinson & Vargo 1997), we tested it in virgin queens; (ii) we performed a surgical intervention into physogastric queens aiming to remove the whole spermatheca, expecting that queens would be unable to fertilize their eggs; and (iii) we submitted the posterior region of abdomen of physogastric queens

(where spermatheca is located) to different levels of X-ray radiation, aiming to kill the sperm cells stored inside the spermatheca;

Methods

Effects of topical application of Juvenile Hormone III on virgin queens of S. depilis

In order to have a big sample size we performed the following tests with in vitro reared queens (see detailed proceedings in Menezes et al. 2013). Such queens have already showed normal fecundity (Menezes et al. 2013). Juvenile Hormone III was dissolved in acetone in different specific concentrations (see details below). We applied it topically into the ventral portion of the abdomen of virgin queens. Two different approaches were used to verify the effects of JH-III into fecundity: (i) different aged virgin queens received the treatment (JH-III in different concentrations or control with acetone) and were placed in petri dishes (10 cm diameter x 1 height) with food ad libtum. After six days queens were dissected using a stereomicroscope and had their ovary development assessed in a rank of 1 to 3, where 1 is undeveloped and 5 totally developed; and (ii) queens received the specific treatment with JH-III and were placed into wooden boxes (12x12x4 cm) containing ca. of 200 workers, and food (honey and pollen) *ad libtum*. There was no control treatment on this case. Every 5 days queens were observed in relation to their survival and physogastry condition.

Surgical intervention to remove queen' spermatheca

These interventions were done from May 18 to 23, 2015, at EMBRAPA Meio Norte, Belém, PA. We performed interventions on several virgin queens of *M. flavolineata* and *S. depilis* for practicing and 10 physogastric queens of *S. depilis*. Such proceedings were done in Belém due to the availability of an artificial inseminator of honey bee queens, and also to the presence of Dr. Cristiano Menezes and his Master student, Jamille Veiga, whose knowledge in anatomy and management of the equipment collaborated to the development of this project.

Five virgin queens of *S. postica* and other five of *M. flavolineata* were collected from bee hives at Belém/PA. They were used to test the equipment adequation to dissection, once the first finality of this equipment is to artificial insemination, besides to know the anatomy of the posterior portion of stingless bee queens abdomen. The

physogastric queens of *S. depilis* were collected in Ribeirão Preto/SP and transported into small wooden boxes (12x12x4 cm) containing several newly emerged workers and food *ad libtum* (honey, water and pollen). Based on analysis of virgin queens, three of these physogastric queens were used to test different methods of interventions aiming to determine the best way to access the spermathecal: (i) ventral incision in the final sternite; (ii) ventral incision in the pleura between the final and penult sternite; and (iii) lateral incision in the pleura between the final and penult sternite (Fig. x); A "U" shaped tool was designed to assess the spermatheca and remove it. It is basically a entomological pin bended 180° (Fig. x). The other seven queens were used for spermathecal removal using the method that seemed less invasive (ii). The posteriors proceedings to incision are described on the Results section.

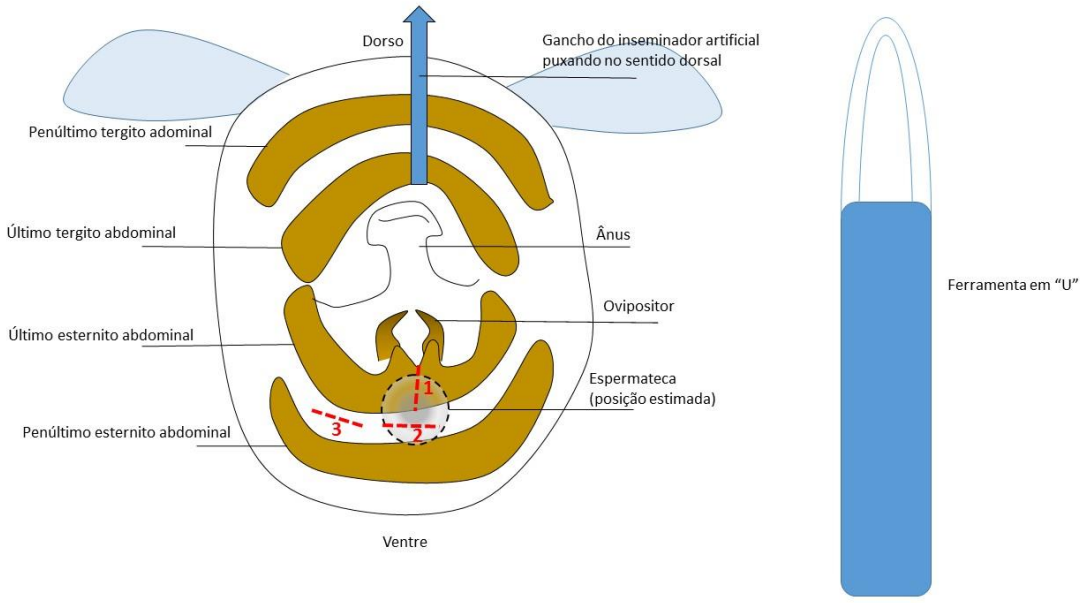

Figure 1. Scheme representing the positioning and view of the queen placed into the artificial inseminator. The posterior region is faceup. A hook of the own equipment is fixed into the last abdominal tergite, pulling the genital chamber dorsally. The queen is constantly anesthesized with CO_2. The anus is expelled out during this process.

X-rays exposure

Nine physogastric queens were submitted to different doses of X-ray. Each one of them received a different dose (1, 2, 4, 6, 8, 10, 12, 14 and 16 Grays). For that, queens were placed inside eppendorfe tubes that had the end bottom cut, exposing only the far end of their abdomen. In this way, queens were covered with a lead plate exposing the posterior half of their abdomen. Queens were then placed inside the X-ray emission

equipment and had the doses determined by the exposure time. After the treatment, the queens were marked and reinserted into their colonies and had their survival monitored. After 60 days one brood comb containing pupae about to emerge was collected from each colony and the male proportion was assessed by determining the sex of 50 individuals.

Results

Effects of topical application of Juvenile Hormone III on virgin queens of S. depilis

Juvenile Hormone did not show any effect on the ovary size and physiograstry development of *S. depilis* queens with the doses used (Table 1). Therefore, there are apparently three possibilities to explain the results: (i) the JH-III hormone have no effect on the ovary development metabolic pathways in *S. depilis* queens; (ii) there are other stimuli necessary to activate ovaries that need to act combined with JH-III; or (iii) the method used, regarding concentration and application method, were not enough to trigger ovary development;

In honey bees, Juvenile hormone likely switched its function over the course of evolution and is associated with behavioural control of division of labour (Giray et al. 2005). The same scenario could be true for *S. depilis*, however further investigation is necessary. If the gonadotropic hormone regulation in *S. depilis* is similar to *A. mellifera*, then we could expected that ecdysteroid hormones can mediate ovary development (Robinson 1991).

Table 1: Effect of topical application of Juvenile Hormone-III in virgin queens of *S. depilis*. Queens that were kept in wooden boxes with ther bees and food were followed till 30 days old. Queens kept in petri dishes were dissected within 6 days. (-) represent no data to be shown

Treatment	Number of treated individuals	Age at treatment (days)	Treatment (total volume applied)	JH-III concentration	Date of treatment	Survival (number of queens alive till the end of the experimente/number of treated queens)	Level of ovary development	Physiogatry development
Queens kept in wooden boxes with a social context	1	3	JH-III (5µl)	40µg/µl	05/02/2015	1/1	-	No
	1	3	JH-III (5µl)	40µg/µl	06/02/2015	1/1	-	No
	1	3	JH-III (3µl)	40µg/µl	07/02/2015	1/1	-	No
	1	3	JH-III (3µl)	40µg/µl	08/02/2015	1/1	-	No
	1	5	JH-III (5µl)	40µg/µl	09/02/2015	0/1	-	No
	1	5	JH-III (5µl)	40µg/µl	10/02/2015	1/1	-	No
	1	5	JH-III (3µl)	40µg/µl	11/02/2015	0/1	-	No
	1	5	JH-III (3µl)	40µg/µl	12/02/2015	1/1	-	No
Queens kept in petri dishes with other queens	2	9	Acetone (2µl)	20µg/µl	30/04/2015	0/1	-	-
	2	9	Acetone (3µl)	20µg/µl	30/04/2015	0/1	-	-
	2	9	JH-III (2µl)	20µg/µl	30/04/2015	0/1	-	-
	2	9	JH-III (3µl)	20µg/µl	30/04/2015	0/1	-	-
	2	9	Negative Control (CO_2)	20µg/µl	30/04/2015	0/1	-	-
	2	9	Negative Control (CO_2)	20µg/µl	30/04/2015	2/2	0	-
	2	3	Acetone (2µl)	20µg/µl	30/04/2015	2/2	0	-
	2	3	Acetone (3µl)	20µg/µl	30/04/2015	2/2	0	-
	2	3	JH-III (2µl)	20µg/µl	30/04/2015	2/2	0	-
	2	3	JH-III (3µl)	20µg/µl	30/04/2015	2/2	0	-
	2	3	Negative Control (CO_2)	20µg/µl	30/04/2015	2/2	0	-
	2	3	Negative Control (CO_2)	20µg/µl	30/04/2015	2/2	0	-

Surgical intervention to remove queen' spermatheca

The preliminary tests showed that virgin queen spermathecal is located on the final end of the abdomen, closest to the ventral face. The intestinal hook is dorsally place in relation to the spermathecal, as well as the Dufour' gland. In this way, accessing the spermathecal dorsally would have to go through these two organs. Then, accessing ventrally showed to be more adequate.

The trials to determine the type of incision were not conclusive in terms of effects on the queens, since the sample size was low, making the choice between the three types of incision on low grounded results. However, since the number of queens was restrict, it was necessary to choose between one of the methods to have a significant sample size. Therefore, we would like to make it clear that the other two methods that were not used here could show good results, not being discarded regarding future investigations.

Proceeding 1 quickly exposed the spermatheca through an incision on the last esternite made with an entomological scissor, cutting it entirely (Fig. 1). However, the incision made on the sclerotized tissue of the exoskeleton disrupted the anatomical configuration of the final end of the abdomen. To fix it, both esternites pieces should be stuck together, maybe using normal glue or even resin. Since this process is detailed and material that would be used to stick both pieces should also be tested, we discarded this proceeding.

The second proceeding also showed viable to remove the spermathecal. The incision was made in the pleura, the soft tissue of the abdomen, between the esclerotized plates. The tissue was perforated with a very fine entomological pin. An entomological scissor was used to open the incision in case of necessity. After the perforation, a drop of haemolymph is released. We inserted the "U"-shaped tool into this incision and randomly and slowly moved it inside the abdomen of the queen, aiming to noose the spermatheca. When the "U"-shaped tool is pulled from the incision, the spermathecal duct is broken and the spermathecal is removed from the abdomen. This tool allows noosing the spermatheca because of its round shape, while tubular organs such as ovarian and intestine are not catch. The round tip also do not perforate the internal organs.

After this proceeding the incision is dried with a piece of cotton and the CO_2 narcosis is turned off. The queen was placed alone in a *petri* dish for 3 hours and later on the wooden box she was before. The incision is naturally closed and a dark sign appears on the scar, suggesting a cicatrisation process. This proceeding have the disadvantage that the nerve cord is in between the spermathecal and the ventral surface. The queen submitted to this

proceeding died, suggesting that the nerve cord could have been harmed, leading us to make the incision on the lateral pleura. The third proceeding (iii) is similar to the second (ii) in every step, except for the position of the incision. On this case, the ovary seem to be in between the spermatheca and the incision, requiring care when using the "U"-shaped tool.

Seven queens were submitted to the third (iii) proceeding. They were kept in the wooden boxes with ca. of 100 workers and food *ad libtum* (honey, water and pollen). Two of them died after one day, and another two after five days. After six days from their surgery the three survivors queens were marked and placed into normal queenless colonies with different population size: small, medium and big. After two days the brood combs showed signs that the oviposition was no occurring (opened brood cells and an irregular brood comb). The colonies were observed subsequently and the replacement of those queens was observed.

X-rays exposure

Only one queen died after the X-ray exposure, treated with an intermediate doses of 10 Gy. All the other queens survived at least for 50 days after the treatments. The proportion of males was not affected in any treatment. Doses higher than 10Gy apparently affected egg viability, since their brood combs presented considerably mortality (concluded from empty cells on the brood comb). The percentage of mortality was not measured.

Conclusion

We did not successfully obtained any queen of *S. depilis* producing a large proportion of haploid males. Exposure to X-rays was not enough to kill the sperm cells inside the spermatheca. X-ray doses could be increased to have better results, however, our data suggest that high doses can affect the egg viability, likely because oogenesis is affected by the radiation. However, on that case, males were submitted to radiation exposure, and the spermatogenesis might have been affected, since cells under division process are more affected by radiation.

Topical application of JH-III also did not have any effect on ovary development in virgin queens (Table 1). The concentration of JH-III used were based on previous studies with social Hymenoptera. Therefore, higher concentrations should be tested in order to confirm that JH-III have no gonadotropic effect in *S. depilis*. The age that virgin queens received the doses could also affect ovary development and should also be considered in future trials.

Despite the methodological constraints, there is no evidence that JH-III could act on ovary development of *S. depilis* virgin queens, suggesting that this hormone could affect other physiological aspects of queens. Studies on behavioural effects should be considered, since it was observed that this hormone participate on the control of division of labour in honey bees (Robinson & Vargo 1997).

Surgical interventions aiming to remove the spermatheca were promising, since queens survived the surgery. However, further investigations are necessary to determine the reasons why queens were not able to oviposit. Longer healing process, without a complex social context in terms of population size and demand for egg laying, should be necessary. However, it is likely that internal organs were too harmed that egg laying was not possible anymore. New tools and procedures should be tested in order to achieve better results.

CHAPTER 3: Effects of diploid males and queen execution on the evolution of the queen mating frequency in the Brazilian stingless bee *Scaptotrigona depilis*

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Abstract

Queen mating frequency greatly varies in eusocial Hymenoptera. Determining the causes of this variation is an active area of research. Here, we tested the hypothesis that single mating in stingless bees, Meliponini, is due to the execution of queens that have made a matched mating at the CSD (complementary sex determination) hypothesis and have diploid male offspring. In particular, double mating increases the probability of making a matched mating, although the proportion of diploid males is reduced from 50% of all diploid offspring to 25%. We studied the common Brazilian species *Scaptotrigona depilis*. We allowed 99 queens to mate to estimate the proportion of matched matings (8/99, 8.1%) and to provide sources of diploid male and normal brood. We transferred brood from two source colonies into 70 test colonies so that 50, 25, 12.5, 0% of the emerging brood were diploid males. Execution of the queens in the test colonies following the emergence of diploid males was equal and high in colonies producing 50 (n = 13, 77% executed) and 25% (n = 32, 75%) diploid males. Queen execution was equal and low in colonies producing 12.5% (n = 14, 93%) and 0% (n = 11, 0%) diploid males. In combination with a mathematical model to determine the total probability of queen execution, our results show: 1) double mating with equal paternity results in higher total probability of queen execution than single mating, showing that double mating is selected against in *S. depilis*; 2) double mating with highly unequal paternity is selectively neutral compared to single mating; 3) mating with 4 males with equal paternity would result in decreased queen execution when the probability of making a matched mating is less than c. 0.17. Overall, our results show that queen execution causes a fitness landscape with two adaptive peaks at single and multiple mating. Even though the multiple mating adaptive peak

may result in higher fitness than single mating, it seems that it cannot be accessed through a gradual process of evolution as double mating with equal paternity results in lower fitness.

Introduction

The number of males that females mate with varies greatly in animals ranging from monandry to promiscuity (Pizarri & Wedell 2013; Taylor et al. 2014). In different species of eusocial Hymenoptera, queens mate with 1 to 20 or more males at the start of their adult lives and do not remate (Boosma & Ratnieks 1996; Strassmann 2001). The stingless bees, Meliponini, comprise several hundred eusocial species living worldwide in the tropics and southern subtropics (Camargo & Pedro 2013). As in the honey bees, *Apis*, there is strong queen-worker reproductive division of labour and morphology, with long-lived colonies and the ability to replace a failing or dead queen (Michener 1974). In contrast to the honey bees, in which queens of all species are highly polyandrous (Oldroyd et al. 1997), stingless bee queens are typically mated to a single male (Peters et al. 1999; Palmer et al. 2002; Paxton et al. 2003).

There are many potential adaptive reasons for variation in queen mating frequency (Crozier & Page 1985 Boomsma & Ratnieks 1996, Crozier & Fjerdingstad 2001), including colony-level benefits of polyandry in the honey bee *Apis mellifera* (Tarpy & Page 2001; Mattila & Seeley 2007) and constraints on multiple mating in the bumble bee *Bombus terrestris* (Baer and Schmidt-Hempel 2001). In the stingless bees, one hypothesized reason for monandry involves the production of diploid males (Ratnieks 1990). In many Hymenoptera, sex is determined by a genetic system known as complementary sex determination (CSD) (Whiting 1943, Heimpel & Boer 2008). Individuals that are heterozygous at the CSD locus are female. Homozygotes and hemizygotes (haploids) are male. When a queen makes a matched mating, mating with a male sharing one of her two (as she is a heterozygote) alleles at the CSD locus, then 50% of their diploid offspring will be males (e.g., AB (queen) x A (male) → 50% AB (diploid females) + 50% AA (diploid males)). By contrast, when a queen makes an unmatched mating then all her diploid offspring are female (e.g., AB (queen) x C (male) → 50% AB (diploid females) + 50% AC (diploid females) (Camargo 1979). Matched matings are rare, only 5-10% (Kerr 1987; Alves et al. 2011, Francini et al. 2012), because frequency-dependent selection favours rare alleles at the CSD locus (Cook & Crozier 1995) and so generates high allelic diversity (Adams et al. 1977; Duchateau et al. 1994; Harpur et al. 2013).

Stingless bee queens that have made a matched mating die, presumably executed by the workers in the colony, soon after their first offspring, including adult diploid males, emerge from their cells (Camargo 1979; Ratnieks 1990; Alves et al 2011; Chapter 2). This is presumably because a colony cannot function when half of the offspring that would normally become workers instead become diploid males, who do no work. As a result, the workers (and also the executed queen) would benefit from killing the queen that has made the matched mating thereby allowing the colony to be requeened by one of her daughters, even though this will result in a relatedness reduction that will reduce the inclusive fitness of both the executed queen and the executing workers. One hypothesized consequence of queen execution in stingless bees is that it selects for monandry by queens (Ratnieks 1990). The underlying logic depends on the relationship between a queen's execution probability and the proportion of diploid males in her offspring. In particular, double mating increases the probability of making a matched mating, which is approximately doubled (see Box 1), but will reduce the proportion of diploid males in the brood from 50% to 25% assuming equal paternity. If queen execution is triggered by 25% diploid males in a queen's offspring, then double mating will be selected against as the overall execution probability will increase. However, if 25% diploid males does not trigger execution, or causes only a low probability of execution, then double mating will be selected for, as the overall probability of execution will be reduced.

We tested the increased probability of execution hypothesis in the Brazilian stingless bee *Scaptotrigona depilis*, a species known to execute queens that make a matched mating (Chapter 2) and to have paternity frequency very close to 1 (Paxton et al. 2003). We transferred brood combs from colonies of queens producing 0% and 50% diploid males to create test colonies in which approximately 25% of the diploid offspring were diploid males, thereby simulating the outcome of mating with two males with equal paternity shares, one being a matched mating the other an unmatched mating. The execution of test queens in these colonies was equal to that of colonies producing 50% diploid males. This shows that queen execution selects against double mating. In addition, queen execution was also determined in colonies producing 12.5% diploid males, equivalent to mating with 4 males with equal paternity with one of being a matched mating. Here, rates of execution were low and similar to colonies producing 0% diploid males. Overall, the results indicates that there is a fitness landscape with two fitness peaks (single mating, polyandry) with reduced fitness at double mating unless paternity shares are unequal, in which case double mating with high and low paternity shares is selectively neutral.

Methods

Study species and site

The study was carried out using colonies of *Scaptotrigona depilis* kept in outdoor wooden hives (internal cavity measurements 42 x 28 x18 (high) cm) at the experimental apiary of the Biology Department of the University of São Paulo at Ribeirão Preto (USP RP) and at the apiary of PROMIP near the town of Engenheiro Coelho, São Paulo State, Brazil. The two locations are 150 km apart. *S. depilis* naturally occurs in this region (Camargo & Pedro 2013). Colonies have a single egg-laying queen. Mature colonies have approximately 5-10 thousand workers.

Obtaining matched mated queens

S. depilis is common on the USP RP campus. A recent census over nine years (http://www.apacame.org.br/mensagemdoce/100/artigo3.htm, accessed on 26/05/2016) found 109 colonies nesting in hollow trees in the 5.8 km^2 campus. Almost certainly there were many more colonies undetected. To obtain queens that had made a matched mating we made a total of 99 removals of the mother queen, using the USP RP hives. We then allowed a replacement daughter queen to take over and start egg laying which was detected by the presence of newly-sealed brood cells. (Stingless bees mass provision brood cells, with cell sealing taking place immediately after the queen has laid an egg in the cell [Sakagami 1982; Michener 1974].)

We had previously determined that the brood period, from egg to adult, in *S. depilis* is approximately 36 days. Therefore, approximately 30 days after a new queen began egg laying we inspected pupae taken from brood combs in order to identify colonies in which approximately 50% were male, based on their characteristic morphology (i.e., smaller heads, larger eyes).

From each of these colonies 10 males were collected and checked using DNA microsatellites at three loci to confirm that they were diploid. Males were kept in absolute ethanol until DNA was extracted using the Chelex method. They were then genotyped at three microsatellite loci, T3 (Paxton et al. 1999), and Sxant06 and Sxant18 (Duarte et al. 2011). Microsatellite amplification and visualization were done as described by Francisco et al. (2011). We confirmed males as diploid if they were heterozygous at one or more loci (Alves et al. 2011).

In total, 8 queens that had made a matched mating were obtained. As *S. depilis* queens have single or close to single paternity (Paxton et al. 2003) this also gave us an estimate of the probability of making a matched mating (= 8/99 = 0.0808). These matched-mated queens were marked by clipping their wings and were introduced into queenless colonies with brood, workers and food stores. Egg-laying *S. depilis* queens are easily accepted in queenless colonies (see Chapter 2). We prevented the execution of our matched-mated queens by repeatedly putting them into new queenless colonies without diploid males before the emergence of their own diploid male adults caused them to be executed.

Producing experimental colonies with different proportions of diploid males

In order to simulate colonies in which the queen has mated with two males with equal paternity, one of whom has made a matched mating (i.e., 25% diploid males), we set up test colonies in which half the brood came from a source colony headed by a matched-mated queen (50% diploid males) and half from a non matched-mated queen (0% diploid males) (Figure 1). By choosing brood source colonies of equal strength, we ensured that there were similar numbers of emerging adult bees from each. In addition, to simulate one matched mating out of four matings, or double mating with unequal paternity (25% v 75%), we set up test colonies in which approximately 12.5% of the emerging bees were diploid males. We did this in a similar way, but with only one quarter of the brood from the matched-mated queen versus three quarters from the non-matched mated queen. To control for the use of test colonies with brood from two source colonies, test colonies producing 50% and 0% diploid males were also given brood from two source colonies and received all the same manipulations.

For each test colony, combs containing pupae were introduced 4 times at intervals of approximately 18 days. At every comb introduction we removed approximately the same amount of older brood combs containing pupae from the test colony that were the progeny of the test colony's queen. Any adult bees on these combs were gently brushed from the combs back into their hive. During the test period, the only brood that gave rise to adult bees was the brood that had been transferred into the colony from the source colonies. This resulted in a period of approximately 70-80 days in which the test colonies had bees emerging from their cells in the designated experimental proportions (e.g., 0, 12.5, 25, 50% diploid males). This was a sufficient test duration as queen execution generally occurs within c. 40 days (see results).

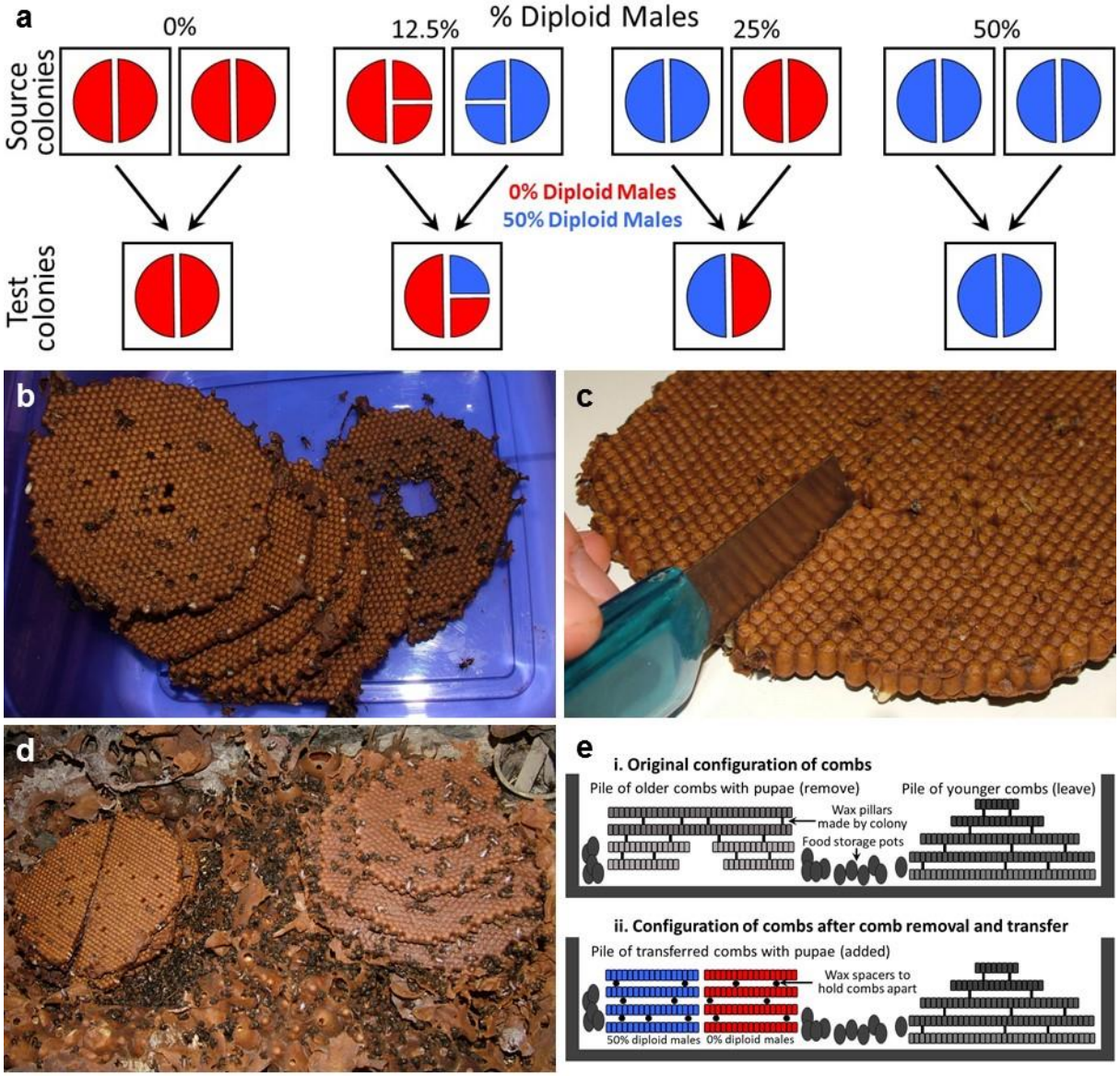

Figure 1. *Set up of test colonies with different proportions of emerging diploid males*. (a) As shown, all test colonies received brood from 2 source colonies. Source colonies were matched for their amounts of brood to facilitate providing equal amounts of brood to test colonies with 0, 12.5, 25, 50% diploid males. Only older combs comprising only pupae, in their silk cocoons, were transferred. Combs containing larvae are too fragile for handling. As the brood period in *S. depilis* is c. 36 days, we transferred brood every 18 days (i.e., half the brood period). Source colonies generally provided 4-6 combs per transfer. (b) six combs of pupae taken from a single source colony for transfer to test colonies; the lowest comb is the oldest and the pupae in the centre, from the oldest cells, have already emerged to leave a large central hole; (c) cutting a brood comb in half; the pupae are in cocoons with the wax already removed by the worker bees. (d) test hive showing 2 brood piles, as occurs normally in *S. depilis* colonies; the pile on the left is composed of brood transferred in from the source colonies; the pile on the right is young brood produced in the colony; many food storage pots and bees can also be seen. (e) Schematic (not to scale) of photograph d from the side showing how the left hand pile of older brood combs had been removed, i, and was replaced with a brood pile composed of comb halves taken from two source colonies, ii; these were introduced as a group and were separated vertically using small wax balls, similar to the wax pillars built naturally by each colony for this purpose.

Determining survival of queens in colonies with different proportions of diploid males

Every test colony had an egg-laying queen, marked by clipping her wings, who we knew had not made a matched mating as her offspring pupae contained a low proportion of males. (Some haploid males are also reared by colonies.) Previous research has shown that a queen who has made a matched mating is not detected by her mating status but by the presence of adult diploid males in the colony (Chapter 2). Thus, a matched-mated queen whose brood is prevented from emerging is not killed (Camargo 1979; Chapter 2; this study), and a non-matched mated queen in a colony with 50% diploid males is usually killed (Chapter 2, this study).

The survival of the queen in each test colony was followed until she was killed or for a maximum of 90 days. We determined that the marked queen was dead when we observed either a new non-marked egg-laying queen or a break in the production of new brood cells, which indicates a colony without an egg-laying queen.

Results

Figure 2 shows the survival of the 70 queens in the 4 groups of test hives. As expected, queen mortality was high in colonies producing 50% (77%, 10/13) diploid males and low in colonies producing 0% diploid males (0%, 0/11) ($P = 0.0002$, Fisher's Exact Test, 2-tailed). Queen mortality in colonies producing 25% diploid males was also high (75%, 24/32), very similar to that in the colonies producing 50% diploid males ($P = 1$, Fisher's Exact Test, 2-tailed). Queen mortality in colonies producing 12.5% diploid males was low (7%, 1/14), very similar to that in the colonies producing 0% diploid males ($P = 1$, Fisher's Exact Test, 2-tailed) Queen mortality in colonies producing 12.5% diploid males was significantly lower than in colonies producing 25% diploid males ($P < 0.0001$, Fisher's Exact Test, 2-tailed).

Our data are insufficient to precisely determine the exact shape of the sigmoid relationship between queen mortality and the proportion of diploid males. However, the data clearly show two important results based on the general sigmoid shape of this relationship. First, below a certain threshold proportion of diploid males, approximately 12.5%, queen mortality is low, similar to 0% diploid males. Second, above a certain threshold, approximately 25%, queen mortality reaches a maximum similar to 50% diploid males. These results are sufficient for testing the queen execution hypothesis.

Although the mortality of our queens in colonies with 50% diploid males is slightly lower than in a previous study, 10/13 v 20/20 (Chapter 2), this difference is not significant (P = 0.0524, Fisher's Exact Test, 2-tailed). The two studies have slightly different methodologies as they addressed different questions. In Vollet-Neto et al (Chapter 2), queens that had not made a matched mating were introduced into colonies with 50% diploid male production from which the matched-mated queen had been removed. In the current study brood combs were introduced into, and removed from, colonies with a queen that had not made a matched mating and was not removed. In the current study, we used a design (Figure 1) that resulted in equal experimental manipulations across all test colonies and so could not have caused the variation in queen mortality seen among the 4 test groups (0, 12.5, 25, 50% diploid males).

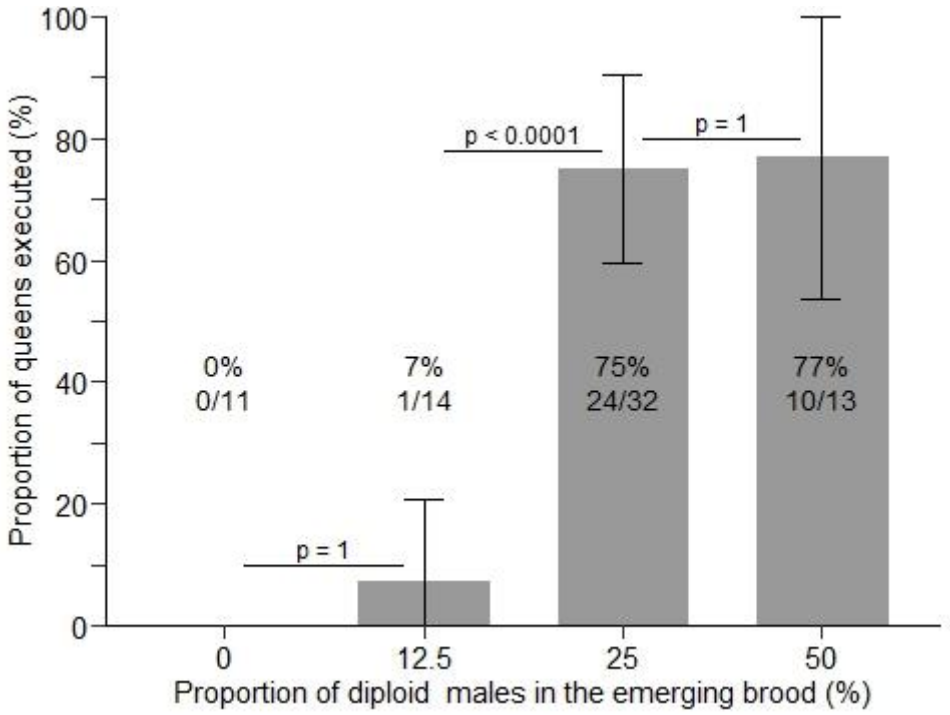

Figure 2. Proportion of queens executed in the test colonies (n = 70) with different experimental proportions of diploid males. P values are from 2x2 contingency tests using Fisher's Exact Theorem, 2-tailed. Numerators show number of queens executed, and denominators show number of queens tested.

Discussion

Our results support the increased probability of execution hypothesis for single mating in stingless bees. The results show that *S. depilis* queens that mate with 2 males and use the sperm equally will have higher total mortality than queens that mate with only one male. In particular, the results show that queen execution probability is almost identical for queens

heading colonies with 25% (1 matched mating of 2 matings with equal sperm use) versus 50% (1 matched mating of 1 mating) diploid males (Figure 3). However, when a queen mates with 2 males, this approximately doubles her chance of making a matched mating (see Box 1). Overall, therefore, double mating will cause increased queen mortality and select for single mating (Box 1).

Our second set of results shows that the mortality of queens heading colonies with 12.5% diploid males is almost identical to queens heading colonies with 0% diploid males (7% v. 0%, Figure 3). This has two important additional implications for the possible evolution of the mating system away from monandry.

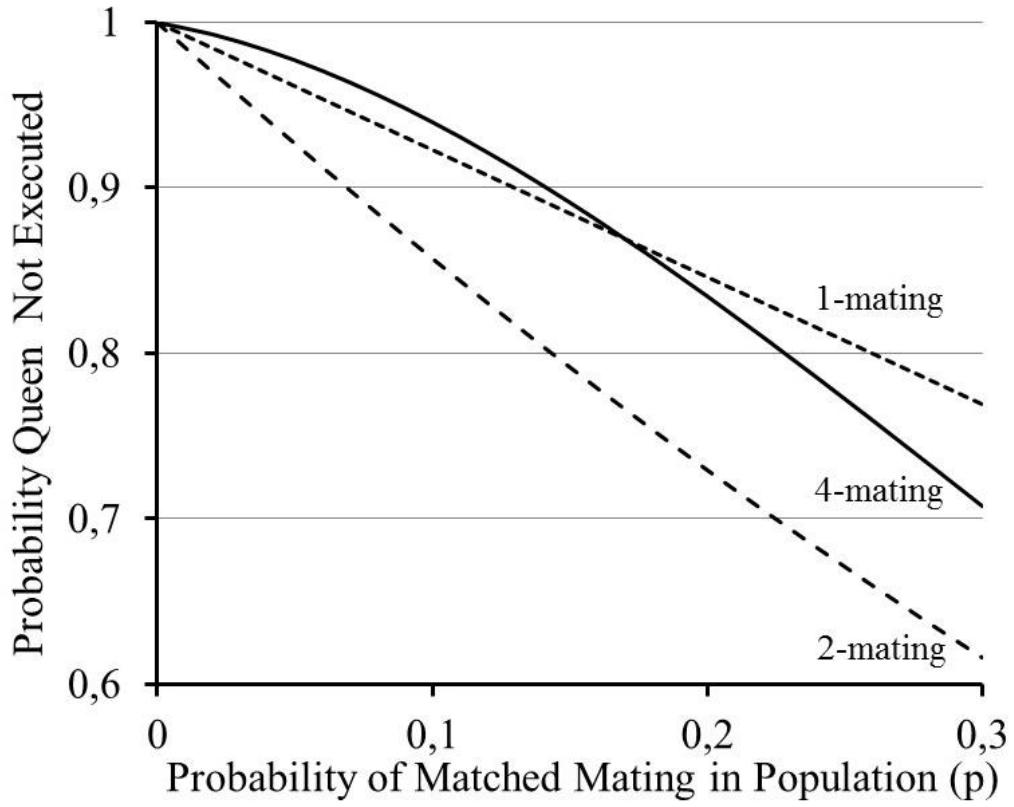

Figure 3. Probability that a queen who has mated with 1, 2, or 4 males with equal paternity will not be executed due to diploid male production in her colony as a function of the probability or making a matched mating in the population.

Although our data indicate that double mating with equal sperm use will be selected against, these additional queen mortality data show that double mating with unequal sperm use will only be weakly selected against and may well be selectively neutral. In particular, the results show that if the minority male contributes a small proportion of the paternity, less than approximately one quarter, then this will not result in increased total queen execution

probability. When a queen mates with two males, one of whom is a matched mating, then with a probability of one half the matched male will be the minority male and so will not trigger execution (Box 1). Our data indicate that this non-execution threshold is close to 12.5% diploid males, equivalent to 25:75% paternity shares of two males.

Interestingly, analysis of progeny genotypes with DNA microsatellite markers indicates that some *S. depilis* queens mate with two males but have highly unequal paternity (Paxton et al. 2003). A second patriline was detected in the progeny of 2 out of 10 queens studied, with the minority male contributing 1 of 20 (5%) and 1 of 16 worker progeny analysed (6%). Our results indicate that this situation, in which the effective paternity frequency of double-mated queens is close to 1, will be selectively neutral with respect to queen execution. Paternity skew within social polyandrous Hymenoptera in general is, in fact, higher in species with low paternity frequency (Jaffé et al. 2012). Double mated queen of four species of the yellow jacket wasp *Dolichovespula* showed high unequeal sperm use, resulting in biased paternity (Foster et al. 2001), while the facultative polyandrous queens of *Vespula vulgaris* presents more equal paternity (Foster and Ratnieks 2001).

The queen mortality data from colonies with 12.5% diploid males also indicate that polyandry will be selected for if paternities are equal (Box 1b) If a queen mates with 4 males and uses sperm equally, then a single matched mating will result in 12.5% diploid males and low or zero queen execution. In some cases a polyandrous queen will make more than one matched mating. However, the probability of making 2 or more matched matings out of 4 is low when the probability of making a matched mating is low. The chances of making more than one matched mating increases with the chances of making a matched mating. In particular, this results in a concave relationship for multiple matings in function of the probability of making a matched mating, while single and double mating have a linear relationship (Box 1; Fig 3). This means that at lower chances of making matched matings, the probability of mortality of four mated queens is lower that single mated queens. However, the mortality probability of four mated queens increases faster than singly mated queens and will be higher at some point. Therefore, for queens mating multiple times (>2), the direction of selection will also depend on the probability of making a matched mating (i.e. on the number and frequency of sex alleles present on the population).

However, it is very likely that the probability of matched matings are concentrated at the range where the mortality of multiple mated queens is lower than single mated queens (i.e., low p levels). That is because frequency-dependent selection favours rare alleles at the

CSD locus and so generates high allelic diversity (Cook & Crozier 1995), as observed for several populations of eusocial bees (Kerr 1987; Adams et al. 1977; Alves et al. 2011, Francini et al. 2012). For our data (p = 8%), the probability of making 2 or more matched matings, which will result in 25% or more diploid males and a high probability of queen execution, is only 3.5%. By contrast, with double mating, the chance of making 1 matched mating, and so having 25% diploid males is 15%, and 8% for single mated queens. For our data on probability of queen mortality, selection will then be neutral at a value of p = 0.17 for single and 4 matings (Box 1; Fig 3).

Therefore, if polyandry occurs then the natural selection consequences will depend on how unequal the paternity shares are and on the probability of making a matched mating. In the case of mating with 4 males, and considering low levels of "p", the outcomes are:

1) Very low paternity in all but one male. Selectively neutral (equivalent to single mating).
If all but one male has a low share of paternity (e.g., 85, 5, 5, 5%) then this will be similar to single mating. This is because a matched mating will only cause queen mortality if it is with the majority male. Matched matings in the minority males will have no effect, even if all of them have made a matched mating, as this will only result in a small proportion of diploid males in the colony (2.5-7.5% in the example), and be below the execution threshold.

2) Low paternity in all but one male. Weakly selected against (nearly equivalent to single mating). All but one male has a low share of paternity (e.g., 50, 16.7, 16.7, 16.7%). The majority male has enough paternity to cause queen execution if he is a matched mating. The minority males have insufficient paternity individually to cause queen execution if they are matched matings, but if several or all of them are matched matings this will exceed the execution threshold. Selection is very weak as the probability that all the minority males will make a matched mating is extremely low.

3) High paternity in 2 males. Selection against (equivalent to double mating with equal sperm use). If two of the males each have sufficiently high paternity to individually cause queen execution, if one of them is a matched mating (e.g., 48, 48, 2, 2%) then this will be similar to double mating and will cause greater total queen mortality.

4) Equal paternity in all males. Selection for polyandry.

If a queen mates with multiple males that have equal or near equal paternity (e.g, 25, 25, 25, 25%), then a single matched mating will not exceed the execution threshold and the total probability of queen execution will reduce in comparison to monandry. Although two or more of a queen's mates may make matched matings, resulting in queen execution, the chance of this occurring is low when the probability of making a matched mating is low. For example, the probabilities of making 0, 1, 2, 3, 4 matched matings, for a queen that mates with 4 males, are: p^4, $4p^3(1-p)$, $6p^2(1-p)^2$, $4p(1-p)^3$, $(1-p)^4$. For $p = 0.08$, these values are 0.7139, 0.2510, 0.0331, 0.0019, <0.0001, respectively. That is, most matched matings do not trigger queen execution as they occur in only one of a queen's mates versus in 2 or more.

Overall, our results suggest a fitness landscape with two adaptive peaks: 1) monandry or multiple mating with unequal sperm use such that the effective paternity is close to 1; 2) polyandry in which queens mate with 4 or more males and use sperm equally. Intriguingly, our analysis indicates that it is not possible to evolve gradually from the monandry peak to the polyandry peak. If sperm use is equal, then double mating is selected against. Double mating is neutral, with respect to queen execution, when sperm use is unequal. However, if sperm use under double mating evolves to be more equal than approximately 25:75% it will be selected against.

Our analysis so far has focused on the costs and benefits of different paternity frequencies with respect to the effects of the proportion of diploid males on queen execution. Here we consider two additional costs of making a matched mating and producing diploid males on the fitness of different mating strategies. 1) the relatedness cost that will result from a queen being executed and replaced by her daughter; 2) the effect of diploid males on colony-level performance.

The relatedness cost only occurs when the queen is executed. An executed queen will be replaced with her daughter as at the time of execution any young queens being reared in the colony will be her daughters not her sisters. This will reduce the relatedness of the executed queen to the reproductives produced in the colony, haploid males and young queens, by exactly 50%. This is because a queen is twice as related to her offspring as to her grand-offspring, and in the case of workers' sons (which are produced in some stingless bees even in colonies with a queen), twice as related to grandsons (the sons of daughter workers) as great-grandsons (the sons of grand-daughter workers). This cost will tend to increase the fitness of

queens that mate with 4 males and use sperm equally, as they will rarely be executed if they make a matched mating, versus queens that mate with a single male, which will usually be executed if they make a matched mating. Further investigations are necessary to determine whether an executed matched-mated queen will be replaced by one of her daughter over natural conditions, and to determine the success of the colony.

Since each diploid male that is reared replaces a worker that could have been reared in the same cell on the same food provision mass, this will reduce the colony's work force giving rise to colony-level costs. When a matched-mated queen is executed the efficiency cost will last only c. 2-3 months, the duration of the period in which the queen that has made a matched mating is laying eggs plus the time taken to be executed and then replaced. The total efficiency cost is not known, but could possibly be estimated in further research aimed at this question. However, it is likely to be low as it is of relatively short duration given that stingless bee colonies are perennial not annual societies. The magnitude of this short term efficiency cost will be greater for monandry than for double mating as during the diploid male rearing period, 50% of the diploid brood will be diploid males for monandry versus 25% for double mating with a single matched mating and equal sperm use. Overall, this will tend to reduce selection against double mating versus single mating.

With polyandry and equal paternity, a queen who has made a single matched mating will not be executed. Therefore, the colony efficiency cost arising from the production of, for example, 12.5% (4 matings) or 10% (5 matings) diploid males is borne throughout her whole life. In addition, if she mates with 4 or 5 males, then there is 4 or 5 times the chance of making a single matched mating compared to a queen who mates with a single male. The key question, therefore, is whether the relatedness cost of being executed (which is a fitness benefit to the queen of polyandry versus monandry) is greater or less than the long term colony efficiency cost (which is an additional cost to the queen of polyandry versus monandry). This also cannot be fully determined without empirical data. However, it is likely that these costs are similar. If the efficiency cost of producing diploid males is linear (i.e., 12.5% fewer workers gives a colony level cost of 12.5%), then the total cost is 50% (i.e., 12.5% x 4). This is the same as the 50% relatedness cost. In addition, both costs are of equal duration: the lifetime of one queen.

There are many other costs and benefits not related to the diploid male load that also effect the evolution of queen mating frequency in eusocial Hymenoptera, and which may also be relevant to stingless bees (Ratnieks 1990; Tarpy and Page 2000 Pamillo 1991). Our results

and analysis show that the existence of diploid males and matched matings can influence the evolution of the mating system in a stingless bee. We hypothesize that the apparent ubiquity of monandry in stingless bees (Jaffé et al. 2014) could have the same cause. This is because all stingless bees rear brood in sealed cells on a provision mass (Michener 1974) which result in diploid males causing a high cost at the colony level, since each diploid male is reared to adulthood. Queen execution is known to occur in two other stingless bee, both *Melipona* (Camargo 1976; Alves et al. 2011). Diploid males also occur in other eusocial Hymenoptera. In contrast to the stingless bees, in the honey bee, *Apis mellifera*, there is evidence that the colony-level costs of diploid male production favour multiple mating (Page 1980, Ratnieks 1990). The reason for this difference is that honey bees rear brood in open cells, and remove diploid males as young larvae before much time or energy has been spent on rearing them (Ratnieks 1990). There are many factors that may cause queens to mate multiply, and there is evidence that at the multiple origins of eusociality in the Hymenoptera paternity frequencies were low (Hughes et al. 2008). However, the question is not just "what causes polyandry to evolve from monanadry" but "what causes the observed variation in queen mating frequency", which ranges from monandry to extreme polyandry, and also includes intraspecies variation.

Box 1. Effect of queen execution on the fitness of queens mated to different numbers of males

1. Single mating versus double mating

This analysis considers the effects of queen execution resulting from diploid male production on the fitness of single versus double mating strategies by queens.

Parameters

M_i	Total execution probability of a queen of mating type i
p	Probability of a matched mating in the population
m_x	Execution probability of a queen in a colony producing proportion x diploid males

Total Execution Probabilities

*Single matin*g: Scenario. Random mating. If there is a matched mating then the proportion of diploid males in the colony is 50%. Note that $m_0 = 0$.

$$M_1 = m_0(1-p) + m_{0.5}p = m_{0.5}p \quad [\text{As } m_0 = 0] \qquad (1)$$

Double mating: Scenario: Random mating. The paternities of the two males are x and (1-x). If one of the two males is a matched mating, then the proportion of diploid males is either (x/2) or (1-x)/2. If both males are matched matings then the proportion of diploid males is 0.5.

$$M_2 = m_0(1-p)^2 + m_{x/2}[p(1-p)] + m_{(1-x)/2}[p(1-p)] + m_{0.5}p^2 \qquad (2)$$

Queen Fitness

For single mating to be favoured over double mating, then M_1 must be less than M_2. From equations 1 and 2 above this is:

$$m_{0.5}p < m_{x/2}[p(1-p)] + m_{(1-x)/2}[p(1-p)] + m_{0.5}p^2 \qquad (3)$$

$$m_{0.5}[p(1-p)] < m_{x/2}[p(1-p)] + m_{(1-x)/2}[p(1-p)]$$

$$m_{0.5} < m_{x/2} + m_{(1-x)/2} \qquad (4)$$

Inequality (4) shows that the probability of making a matched mating does not affect the result as parameter p cancels out. Single mating is favoured when the queen execution probability in a colony producing 50% diploid males is less than the sum of the two execution probabilities for a colony with double mating, corresponding to the paternities of the two males (e.g., the majority male and the minority male). When males contribute equal paternity (i.e., x = (1-x) = 0.5) then the proportions of diploid males are both 25% (i.e., x/2 = (1 –x)/2 = 0.25 = 25%). When this occurs our data show that inequality (4) is true as $m_{50} = 0.77$ and $m_{0.25} = 0.75$ (i.e., 0.77 < 0.75 + 0.75). When paternity is unequal, then in the limiting case of highly unequal paternities (i.e., x/2 approaches 0.5 and (1-x)/2 approaches 0) inequality (3) will become the equality $m_{50} = m_{50}$, showing that double mating is neutral. Our data show that inequality (3) is approximately an equality for x = 0.75, as $m_{0.125}$ is low, 0.07. Although we do not have empirical data for $m_{0.375}$, we do have data for $m_{0.5}$ and $m_{0.25}$. These are almost the same numerically, 0.77 and 0.75, and not statistically different (P = 1.0), showing that double mating with paternities of 75:25% is approaching neutrality. Inequality (4) also shows that double mating would be selectively favoured for a wide range of paired values of $m_{x/2}$ and $m_{(1-x)/2}$.

2. Single mating versus polyandry

This analysis considers the effects of queen execution resulting from diploid male production on the fitness of queens mated to a single male versus to 4 males with equal paternity.

Total Execution Probabilities

Single mating: as above (1)

Quadruple mating: Scenario Random mating. The paternities of the 4 males are equal, 25%. The proportion of diploid males is 0%, 12.5%, 25%, 37.5%, 50% for queens who have made 0, 1, 2, 3, 4 matched matings. The probabilities of these events are given by the terms of the binomial theorem: p^4, $4p^3(1-p)$, $6p^2(1-p)^2$, $4p(1-p)^3$, $(1-p)^4$. Paralleling equation (2), the total mortality of queens mated to 4 males is:

$$M_{4equal} = m_0p^4 + m_{0.125}[4p^3(1-p)] + m_{0.25}[6p^2(1-p)^2] + m_{0.375}[4p(1-p)^3] + m_{0.5}[(1-p)^4] \quad (5)$$

Mating with 4 males is favoured if

$$m_{0.5}p > m_0p^4 + m_{0.125}[4p^3(1-p)] + m_{0.25}[6p^2(1-p)^2] + m_{0.375}[4p(1-p)^3] + m_{0.5}[(1-p)^4] \quad (6)$$

Where $m_0 = 0$, $m_{0.125} = 0.07$, $m_{0.25} = 0.75$, $m_{0.375} = 0.76$, $m_{0.5} = 0.77$ (m_x values taken from from Fig. 2, except $m_{0.375}$ which is interpolated between $m_{0.25}$ and $m_{0.5}$). When (6) is evaluated it is found to be true ($0.062 > 0.044$), showing that multiple mating is favoured as it results in a lower total probability that a queen will be executed. However, unlike single versus double mating this result also depends on p, the probability of a matched mating. As p increases the total mortality of queens mated to 4 males increases more rapidly than queens mated to a single male and at a value of $p = 0.17$ (approximately) the total survival is equal (Fig. 3). The reason is that when p increases the magnitudes of the higher order terms in p, especially p^2, which generate enough diploid males to result in queen execution, are greater.

In general terms, a sigmoid function could be fitted to the queen execution data in Figure 2 and used to calculate the total survival of queens of any mating frequency and any pattern of paternity using the binomial theorem to determine the relevant frequencies of the different outcomes in terms of the proportion of diploid males. In practice, it is premature to do this as additional data points would be needed to fit the function in the middle part, between 12.5% and 25% diploid males, where the execution probability changes greatly. The text provides a discussion of special cases.

GENERAL DISCUSSION

Recent advances on molecular biology have shown that polyandry is quite widespread in nature (Taylor et al. 2014), contradicting earlier predictions on animal mating systems (Bateman 1948, Emlen & Oring 1977). This also revealed a few species of social Hymenoptera that show a strategy of extreme multiple paternities (Boomsma 2013). There is evidence that at the multiple origins of eusociality in the Hymenoptera paternity frequencies were low (Hughes et al. 2008), which makes the question of "what causes polyandry to evolve from monandry" being widely investigated by scientific community. There are many factors that may cause Hymenoptera queens to mate multiply. Given that there are clear benefits for queens having multiple matings, and that there is usually a variation on the paternity number within species, the most impostant question is "what causes the observed variation in queen mating frequency". In particular, the question of what forces are maintaining queens as monandrous has received very little attention, especially considering that the vast majority of social Hymenoptera species have queens with low paternity frequency.

Here, we addressed the question of a particular selective force that might be selecting against multiple mating in a stingless bee species, *S. depilis* (Chapter 3). Combined with all the others selective forces acting on stingless bees mating system, natural selection seems to drive queens mating frequency towards monandry, since many stingless bee species with a great variety of natural history strategies have shown a low paternity frequency (Jaffé et al. 2014). Since 1976, the behaviour of *Melipona quadrifasciata* queen execution under diploid male emergence has not been studied in details (Camargo, 1976). Alves et al. (2011) showed indirect evidence that this behaviour also occurs in *M. scutellaris*. Here, besides showing that this behaviour could select against multiple mating in *S. depilis*, we provided a great finding regarding the possible occurrence of queen execution behaviour under diploid male production in the group of stingless bees (Chapter 2). Since *S. depilis* is not phylogenetically related to *Melipona* (Rasmussen et al. 2010), there is a chance this is a ubiquitous behaviour on stingless bee species. If confirmed by future studies, it might be possible that this is one of the main causes of selection for monandry in stingless bees. We also provided information on the biology of diploid males of *S. depilis* (Chapter 1), which may give support for conservation studies and policies.

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