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Research article

Sperm utilisation by *Melipona quadrifasciata* Lepeletier (Hymenoptera, Apidae) queens subjected to multiple mating

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Summary. In most Hymenoptera species the queen mates once but in a small number of species, multiple matings can occur normally. So, in this study, physogastric *M. quadrifasciata* queens were mated with a second male to investigate how these queens, naturally inseminated and laying eggs, use spermatozoa stored in their spermatheca, when they are mated with a second male. Results demonstrate that spermatozoa of different males mix in the spermatheca of *M. quadrifasciata* queens and that there is a gradual increase in the utilisation of spermatozoa of the second male, which could be explained by a competition among spermatozoa of different drones over the way in which spermatozoa are stored in the spermatheca.

Key words: Hymenoptera, *Melipona*, stingless bees, sperm utilisation, molecular markers.

Introduction

In most social Hymenoptera species the queen mates once (monandry) but in a small number of species, multiple matings can occur normally (polyandry). In polyandrous species, the sperm distribution inside the spermatheca, intraspermathecal sperm competition and the manner in which queens utilise the spermatozoa stored in their spermatheca may alter the patterns of genetic relatedness inside the colony.

Polyandrous females of different Hymenoptera species, however, can utilise the spermatozoa stored in their spermatheca in different ways. In *Dahlbominus fuscipennis* Zetterstedt for example, Wilkes (1966) demonstrated that there was sperm competition when two successive copulations occurred in a period of less than 24 h. However, when this period is longer, spermatozoa of the first male are preferentially used. Precedence of the first male spermatozoa has also been documented in other Hymenopteran species (Parker, 1970; Holmes, 1974; Metcalf and Witt, 1977; El Agoze et al., 1995).

Page and Metcalf (1982), in contrast, used enzymatic data to show that multiple inseminated queens of *Apis mellifera* Linnaeus utilise the sperm of at least three males at any given time and that the sperm are, to some extent, mixed in the queen's spermatheca. Sasaki et al. (1995) using DNA fingerprinting, also demonstrated that, in *A. mellifera*, sperm from different males are used non-preferentially.

Manipulation of *Melipona quadrifasciata* Lepeletier colonies, a monandrous stingless bee species, induces males to mate with the physogastric queen of the colonies, which suggests that these queens maintain their attractiveness and, possibly the production of substances (pheromones) that attract males, permitting copulation. So, in this study, physogastric *M. quadrifasciata* queens were mated with a second male to investigate how these queens, naturally inseminated and laying eggs, use spermatozoa stored in their spermatheca, when they are mated with a second male.

Materials and methods

Four physogastric *M. quadrifasciata* queens (colonies 838, 839, 843 and 845) maintained in the Central Apiary of the Federal University of Viçosa (MG, Brasil) were allowed to mate with a second drone collected from the field In these crosses the physogastric queens were placed in a small wooden box along with, one, wild-caught, drone.

Progeny combs were withdrawn from these colonies before the mating with the second male and progeny were allowed to emerge in an incubator at 28 °C. Five workers of these combs were analysed by PCR-RAPD to determine the band pattern of workers daughters of the first male that mated with the queen. Fifty days after the mating with the second male, new progeny combs were withdrawn from these colonies. Progeny combs were then collected for a period of two to three months, depending on the colony. Ten workers from each comb were used for analysis.

Following identification of RAPD markers that were present in the drone used in the second mating and absent in workers eclosing from combs collected before this second mating, colony 843 was chosen to verify the frequency of worker daughters of the first and second drone. This colony was chosen because it presented a clearer band pattern than the ones found in other colonies. Three samples of twenty individuals each were collected from this colony with an interval of twenty days between each sample (50, 70 and 90 days after the second mating). Ten months after the second mating, a new sample of 20 workers was collected and analysed.

DNA extraction and PCR reactions

The genomic DNA was extracted as described by Waldschmidt et al. (1997). The amplification reaction mixture (25 µl) contained 3.5 mM $MgCl_2$, 10 mM/50 mM Tris-KCl (pH 8.3), 0.1 mM of each dNTP (dATP, dTTP, dGTP, dCTP), 0.4 µM of a decamer primer (Operon Technologies, Alameda, CA, USA), one unit of Taq DNA polymerase and 25 ng of genomic DNA. The mixture was placed in a thermocycler model PTC-100 (MJ Research) programmed for 40 cycles. Amplification products were resolved in 1.2% agarose gels containing 0,2 µg/ml ethidium bromide immersed in TBE buffer.

Results

Seventy two RAPD primers were used to compare the electrophoretic pattern of genomic DNA from the males that mated with the physogastric queens and the genomic DNA of five workers eclosing before the second mating in the four selected colonies. Of these, four primers (OPI-13, OPJ-13, OPM-08 and OPR-17) generated useful markers that were present in the drone that mated with the queen of colony 843 and absent in the workers eclosing before this second mate, while four other primers (OPO-01, OPO-06, OPR-17 and OPS-04) generated markers for the colony 839. Colonies 838 and 845 did not have markers with the required characteristics and therefore they were withdrawn from further analysis.

These primers were used in the amplification of genomic DNA of workers eclosing after the second mating of queens from colonies 843 and 839. Results demonstrated that some workers eclosing after the second mating possessed the genetic markers present only in the drone utilised in the second mating, while others did not (Fig. 1). This is consistent with the fact that some workers produced after the second mating were daughters of the second drone. Analysis of these workers with different primers confirmed that they were daughters of the second drone mated with the queen, because the different markers found in the drone could always be found in the same workers.

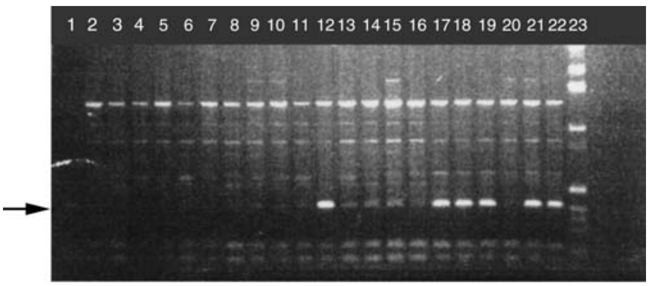

Figure 1. RAPD products generated with primer OPR-17 on the DNA extracted from workers eclosing before the second mating (lanes 2–11), the drone used in this mating (lane 12) and workers eclosing after the refereed mating (lanes 13–22). Note the presence of a RAPD marker present in the parental drone (very brightly band) and absent in some workers eclosing after the second mating (arrow). Lane 1: negative control; lane 23: 1-kb ladder used as size marker

Primer OPR-17 and primer OPJ-13 were used to estimate the frequency of spermatozoa's use of each male in colony 843. In the first sample (workers collected 50 days after the second mating) only two workers had the marker present in the second drone. This represents 90% utilisation of spermatozoa from the first male. In the second and third samples, respectively (workers eclosing 70 and 90 days after the second mating), four and fourteen workers possessed the marker identical to that of the second drone. The utilization of the first male's spermatozoa decreased from 90 to 30% during this interval. This marker was also present in all 20 workers collected ten months after the second mating, which demonstrates that the queen was utilising only spermatozoa from the second male at this time.

Discussion

The results of the present study clearly demonstrate that physogastric *M. quadrifasciata* queens, when mated with a second male, use at the same time spermatozoa of both drones that mated with her to fertilise her eggs. However, the use of the spermatozoa of the second male increased with time corroborating data by Campos and Melo (1990). These authors analysed the sperm utilisation in physogastric *M. quadrifasciata quadrifasciata* queens mated with a second male of the subspecies *M. q. anthidioides* over one year. These two subspecies and their hybrids are distinguishable by the pattern of tergal bands presented in the abdomen of workers and drones. The authors verified that the spermatozoa of the second male mixes with those already present in the spermatheca and that there was a gradual increase in the proportion of hybrid phenotype workers. This could be explained by a slow mixing of the spermatozoa within the spermatheca during the sampling period. However, the variation observed by Campos and Melo (1990) was much lower (30%) than that verified in this study (100%, 10 months after the second mating). This difference may be related with the fact that Campos and Melo (1990) analysed different subspecies. In this case, spermatozoa can present physiological and structural differences that could favour the use of one subspecies' spermatozoa to the detriment of the other.

Irsigler (2002) also worked with *M. quadrifasciata* queens submitted to experimental multiple mating. His experiments suggested that the queen has a cyclic pattern of spermatozoa use, with periods where there is predominance of one drone over the other. This cyclic pattern was not observed in the present work. Campos and Melo (1990) whose samples were taken in a greater period (15 months) did not found this cyclic pattern either.

Another factor that could influence sperm utilisation is the age of the queen. Woyke (1971) demonstrated that the number of spermatozoa stored in an *A. mellifera* spermatheca depends on the age at which the queen mated and on their size. Therefore, an older queen could store a greater quantity of sperm from a second drone than a young one, because the amount of spermatozoa in her spermatheca decreases with time.

In summary, we can conclude that in cases of multiple matings, spermatozoa of different males mix in the spermatheca of *M. quadrifasciata* queens, because we verified representative genotypes of the two matings in the analysed samples. This mixture, however, can not be totally homogeneous nor is the use of these spermatozoa completely random, because we verified a gradual increase in the utilisation of spermatozoa of the second male over time. So, further analyses, over a long period of time, should be done to carefully document the pattern of spermatozoa use by *M. quadrifasciata* queens undergoing multiple mating.

The use of spermatozoa from different males will certainly alter the average relatedness of the workers inside the colony, generating different proportions of half-sister and full-sister workers at different times. This probably will also alter their behaviour in the colony and so represents another issue that should be addressed in future analysis.

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