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Original article

Effect of delayed mating on spermathecal activation in *Melipona quadrifasciata anthidioides* (Hymenoptera, Apidae) queens*

Edmilson Amaral SOUZA[1], Lúcio Antônio Oliviera CAMPOS[1], Clóvis Andrade NEVES[1], José Cola ZANUNCIO[2], José Eduardo SERRÃO[1]

[1] Departamento de Biologia Geral, Universidade Federal de Viçosa, 36570-000 Viçosa, Minas Gerais, Brazil
[2] Departamento de Biologia Animal, Universidade Federal de Viçosa, 36570-000 Viçosa, Minas Gerais, Brazil

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Abstract – In this work, we examined the effect of delayed mating on spermathecal development in *Melipona quadrifasciata anthidioides* queens. The histology and histochemistry of the spermatheca of virgin queens zero, 15, 20, and 30 days old and physogastric queens were compared. There were no significant differences in the thickness of the epithelium and the volume of the spermathecal reservoir among virgin queens of different ages. However, physogastric queens had more voluminous spermathecae and thinner epithelia than virgin queens. The structure of the spermathecal glands was also influenced by mating. These findings suggest that mating leads to activation of the spermatheca and its glands.

mating / spermatheca / stingless bees / virgin queen

1. INTRODUCTION

The reproductive process in social bees spans a long period throughout which the spermatozoa must remain within the female. The maintenance of viable spermatozoa is fundamental for successful reproduction (Hitchcock, 1956), and this viability is ensured by the spermatheca, a specialized organ present in the female insect reproductive tract (Snodgrass, 1933, 1935). The storage of spermatozoa in spermathecae eliminates the need for females to mate before each oviposition, allows stored spermatozoa to be used as required, and reduces the risk of being preyed upon during mating flights (Thornhill and Alcock, 1983).

The spermatheca of bees is a round sac that communicates with the outside via a short duct located in the transition region from the common oviduct to the vagina (Camargo and Mello, 1970; Dallai, 1975; Pabalan et al., 1996; Martins and Serrão, 2002). The spermathecal wall consists of a single layer of columnar cells with a cuticular lining on the luminal surface, a basal lamina on the outer surface and a well-developed tracheal network. The spermatheca controls the pH and gas exchange of the spermatozoan milieu and the release of spermatozoa (Snodgrass, 1956; Lensky and Schindler, 1967; Poole, 1970). In newly emerged queen bees, the spermathecal epithelium, which ensures the viability of spermatozoa (Taber and Blum, 1960), is incompletely developed and may require maturation before mating (Poole, 1970).

A pair of glands is associated the spermatheca. In the honey bee *Apis mellifera* L., these glands consist of two tubules that join to form a duct that links the spermatheca to the vagina (Dallai, 1975). In the stingless bee *Melipona bicolor* Lepeletier, 1836, the spermathecal glands are smaller and the gland ducts open directly into the spermatheca (Cruz-Landim and Serrão, 2002). In mature *A. mellifera* queens,

Corresponding author: J.E. Serrão
jeserrao@ufv.br
* Manuscript editor: Klaus Hartfelder

proteins released by the spermathecal gland into the spermathecal reservoir may play a role in maintaining spermatozoan viability (Klenk et al., 2004).

In view of the importance of spermathecae in storing spermatozoa and ensuring their viability and the fact that the spermathecae of newly emerged queens are not fully developed, the aim of this study was to evaluate whether a delay in mating influenced spermathecal development and the activity of spermathecal glands in queens of the stingless bee *Melipona quadrifasciata anthidioides* Lepeletier, 1836.

2. MATERIALS AND METHODS

Queens and worker bees of *M. quadrifasciata anthidioides* were obtained directly from colonies maintained by the Central Apiary of the Federal University of Viçosa in the State of Minas Gerais, Brazil. Brood combs from the colonies were maintained at 29 ± 1 °C and examined daily for newly emerged queens. The new queens were separated in Petri dishes (12 cm in diameter) containing 10 worker bees each and received pollen and honey ad libitum in the first five days of adult life. Five days after emergence, the queens were transferred to glass-covered wooden boxes (15 cm × 7.5 cm × 15 cm) and were fed on pollen and honey ad libitum and received cerumen. New worker bees were added to the boxes each day.

Twelve queens were used to study the morphological alterations in the spermathecae and spermathecal glands at zero (newly emerged), 15, 20 and 30 days of age (three queens/interval). Physogastric queens were used to assess whether mating influenced spermathecal morphology. At each interval, the queens were killed and the reproductive tract was dissected in insect saline solution (0.1 M NaCl, 0.1 M Na_2HPO_4 and 0.1 M KH_2PO_4), transferred to Zamboni solution (Stefanini et al., 1967), and then dehydrated in a graded ethanol series and embedded in JB4 historesin. Serial sections 5 μm thick were stained with hematoxylin and eosin. Some sections were also stained with periodic acid-Schiff (PAS) reagent to detect polysaccharides and glycoconjugates followed by counterstaining with hematoxylin. Proteins were detected by staining with mercury-bromophenol at pH 2.5 (Pearse, 1985).

The serial sections were used to determine the epithelial thickness and spermathecal reservoir volume by the method of Cavalieri (Coggeshall, 1992). The images were processed using the software package Image Pro Plus^{TM}, version 4.0 for Windows (Media Cybernetics) and the numerical data were analyzed by analysis of variance (ANOVA) to determine whether spermathecal development in virgin queens was age-dependent. A similar analysis was used to assess whether there was any difference in spermathecal development between virgin and mated queens.

3. RESULTS

The spermatheca of *M. quadrifasciata anthidioides* queens was a round sac connected to the common oviduct by the spermathecal duct (Fig. 1). The wall lining the spermathecal reservoir consisted of a single layer of epithelium with a cuticle on the luminal surface and a basal lamina on the outer surface (Fig. 2). In virgin and physogastric queens, the spermathecal wall contained columnar cells with central nuclei. These cells had a predominantly basophilic cytoplasm with scattered filamentous material at their base and a homogenous organization at their apex in virgin queens. In contrast, the cytoplasm was homogenous in physogastric queens (Figs. 3, 4).

In virgin queens, the spermathecal basal lamina and cuticle were PAS-positive, whereas in physogastric queens there was additional weak staining in the cytoplasm (Fig. 5). The apical region of the spermathecal epithelium was positive for proteins in all of the queens studied, whereas the basal region reacted very little with the mercury-bromophenol stain. In the subcuticular space, the cells stained strongly for proteins (Fig. 6) and large nuclei with evident nucleoli were present in the spermathecal epithelium (Fig. 7). There were no signs of death or cell division in the spermathecal epithelial cells or spermathecal glands.

The spermathecal duct epithelium consisted of a single layer of flattened cells lined with cuticle, with a well-developed muscle layer occurring at the junction with the spermathecal reservoir. Delayed mating did not significantly alter the spermathecal volume ($F = 2.34$; $P > 0.05$) or epithelial thickness ($F = 2.42$; $P > 0.05$) in virgin queens. The reservoirs of

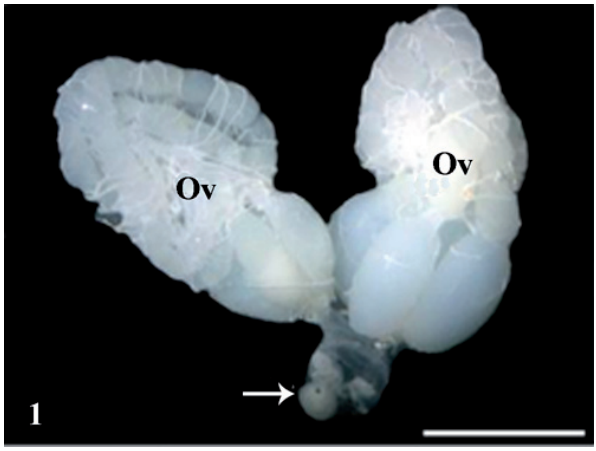
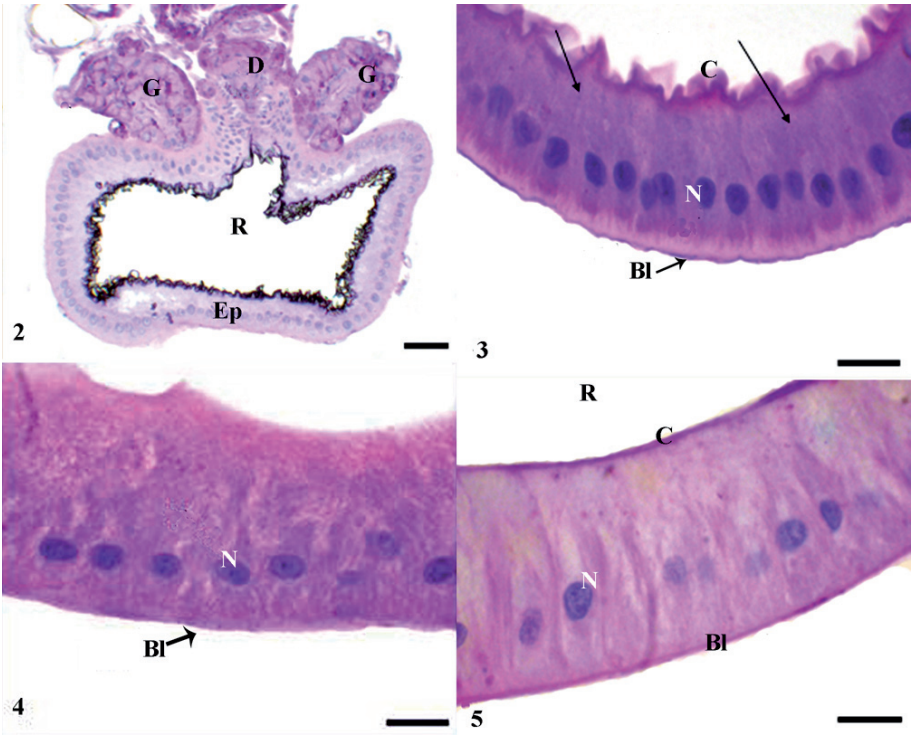
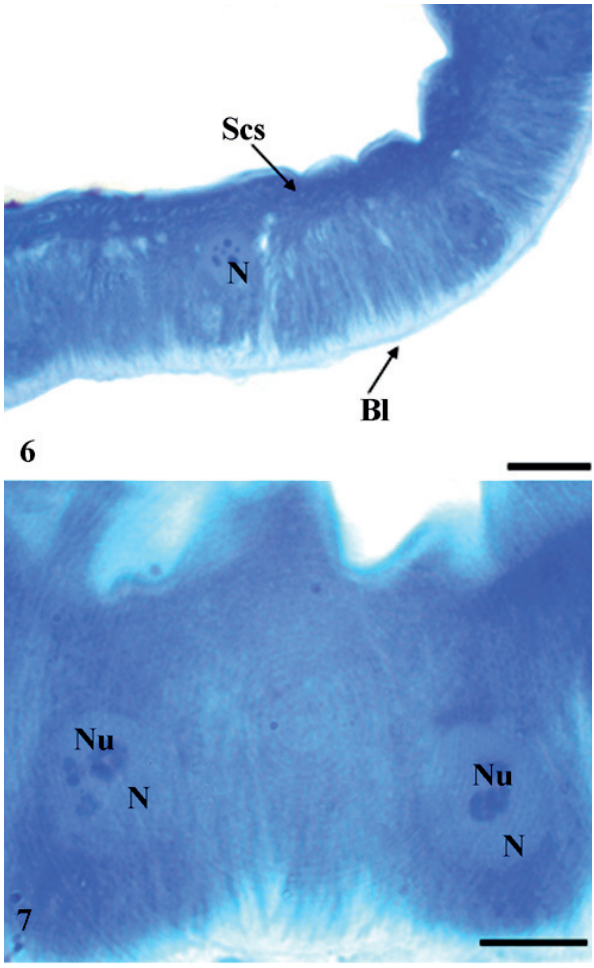


Figure 1. Anatomy of the female reproductive system of *Melipona quadrifasciata anthidioides* queens. The ovaries (Ov) consist of follicles connected to the lateral oviducts. The arrow indicates the spermatheca linked to the common oviduct. Bar: 2 mm.



Figures 2–5. Transversal section of the spermatheca of *Melipona quadrifasciata anthidioides.* 2. Spermatheca of queens with a single cell layer (Ep), a pair of glands (G) and a duct (D). R, reservoir. Bar: 50 μm. 3. The columnar epithelium of the spermatheca with cells with basophile cytoplasm (arrows) of a virgin queen. Bar: 20 μm. 4. The columnar spermathecal epithelium of a physogastric queen with basophile cytoplasm. Bar: 20 μm. 5. The epithelium of a physogastric queen with cytoplasm, basal lamina and cuticle PAS-positive. Bar: 20 μm. Bl, basal lamina; C, cuticle; N, nucleus.



Figures 6–7. Histological section of the spermatheca of *Melipona quadrifasciata anthidioides.* 6. The epithelium of a physogastric queen in the mercury–bromophenol test with positive cytoplasm cells, especially in the apex. Bl, basal lamina; N, nucleus; Scs, subcuticular space. Bar: 20 μm. 7. Detail of the spermathecal epithelium showing the nucleus (N) and nucleolus (Nu). Bar: 10 μm.

physogastric queens ($(64.4 \pm 42) \times 10^6$ μm^3) were significantly larger than in virgin queens ($(13.7 \pm 6) \times 10^6$ μm^3 ; F = 19.32; $P < 0.05$; Fig. 8), and the spermathecal epithelium of physogastric queens (50.3 ± 4.5 μm) was taller than that of virgin queens (34.3 ± 6.1 μm; $P < 0.05$; Fig. 9).

The spermathecal glands consisted of an inner, non-secretory layer covered with cuticle on the luminal surface and an outer secretory layer (Fig. 10). The inner layer of cells formed the intima and gave rise to the gland duct. The secretory cells were round and had a collecting canal that linked them to a pore in the cuticle. This pore released the secretion into the gland duct, which in turn open into the spermathecal reservoir.

The spermathecal glands of virgin queens stained positive for PAS. With advancing age, the intensity of this positive reaction declined (Figs. 11, 12). PAS-positive muscle cells were seen in the spermathecal duct of virgin queens up to 15 days old. The spermathecal glands and spermathecal ducts of

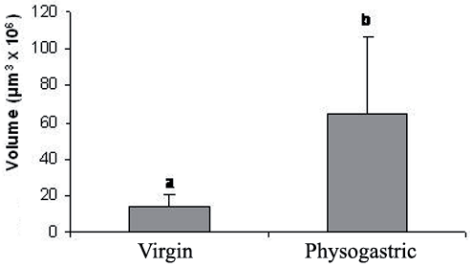


Figure 8. Morphometrical data of the spermatheca of *Melipona quadrifasciata anthidioides.* Reservoir volume of the spermatheca in virgin and physogastric queens.

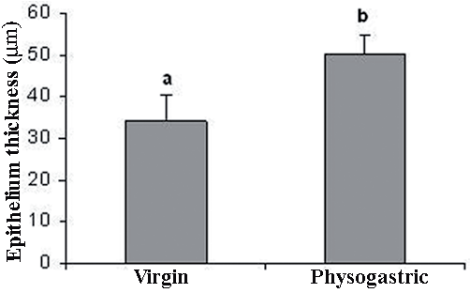


Figure 9. Morphometrical data of the spermatheca of *Melipona quadrifasciata anthidioides.* Thickness of the spermathecal epithelium of virgin and mated queens. Different letters above the bars indicate differences in the ANOVA test ($P < 0.05$).

virgin queens stained positive for protein. Similarly, the gland cells and spermathecal duct of physogastric queens stained positive for PAS (Fig. 13) and weakly positive for proteins (Fig. 14).

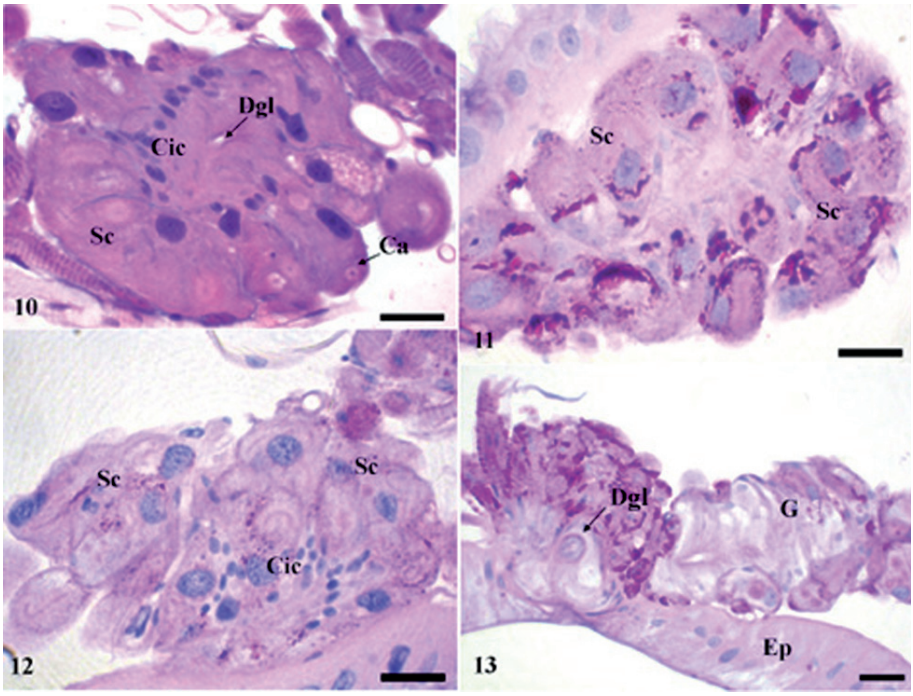
4. DISCUSSION

The spermathecal structure in *M. quadrifasciata anthidioides* is similar to that of other bee species (Camargo and Mello, 1970; Dallai, 1975; Pabalan et al., 1996; Martins and Serrão, 2002). In *A. mellifera*, the thickness of the spermathecal wall epithelium is age-dependent. The epithelial cells are cubic in virgin queens and columnar in physogastric queen since the spermathecae of newly emerged queens are not completely developed and may require differentiation and maturation before mating. In this species, spermathecal development depends more on age than on mating (Poole, 1970). In contrast, as shown here, there were no alterations in the epithelial thickness or spermathecal volume in virgin *M. quadrifasciata anthidioides* queens of different ages. However, the epithelial thickness and spermathecal volume were greater in physogastric queens than in virgin queens, indicating that development of the spermathecal epithelium in *M. quadrifasciata anthidioides* is influenced by age and mating. The increase in volume probably reflected the quantity of spermatozoa stored in the spermatheca after mating.

No cell death or cell division was seen in the spermathecal epithelium of virgin or physogastric queens. Poole (1970) also reported that there was no cell division in the spermathecal epithelium of *A. mellifera*, and we concluded that the increase in spermathecal volume was attributable to an increase in the size rather than number of epithelial cells. This conclusion is corroborated by the thicker spermathecal epithelium seen here in physogastric queens of *M. quadrifasciata anthidioides*.

The cuticle and basal lamina of *M. quadrifasciata anthidioides* contain PAS-positive materials probably glycoconjugates, as also reported for *A. mellifera* (Poole, 1970). The primary components of the basal lamina are fibrous proteins, collagen, glycoprotein and glycosaminoglycans, with the latter two accounting for the positive PAS staining. Chitin, a characteristic constituent of the insect cuticle, consists of N-acetylglucosamine (NAG) residues and other components (Chapman, 1998), with NAG probably being the main contributor to positive PAS staining (Pearse, 1985). The spermathecal epithelium of virgin and physogastric queens of *M. quadrifasciata anthidioides* contained proteins, a finding that agrees with the detection of protein from the pupal to the adult stage in *A. mellifera* (Camargo and Mello, 1970).

Spermathecal epithelial cells release material into the reservoir and may be involved in gas exchange between the spermatheca and trachea and in the transport of substances from the hemolymph to the spermatheca



Figures 10–13. Histological section of the spermathecal gland of *Melipona quadrifasciata anthidioides*. 10. Two cell layers, with the outer of secretory cells (Sc) and an inner, non-secretory with two cell types: the cells of the cuticular intima (Cic) and the cells that form the collecting canal (Ca). Bar: 20 μm. 11. Newly emerged queen showing PAS-positive secretory cells (Sc). Bar: 20 μm. 12. A virgin queen 30 day-old with spermathecal gland PAS-negative. Bar: 20 μm. 13. Physogastric queen showing the PAS-positive spermathecal duct and spermathecal gland with some PAS-positive regions. Bar: 30 μm. Dgl, duct of the spermathecal gland; Ep, spermathecal epithelium; G, spermathecal gland.

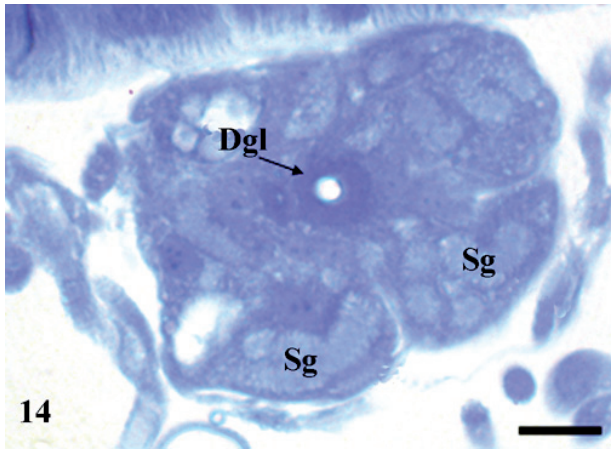


Figure 14. Histological section of the spermathecal gland of physogastric queens of *Melipona quadrifasciata anthidioides* with secretory granules (Sg) positive for protein. Bar: 15 μm. Dgl, duct of the spermathecal gland.

(Laidlaw, 1944). Camargo and Mello (1970), Dallai (1975) and Kressin et al. (1996) suggested that this epithelium was not involved in the production of substances in *A. mellifera* queens. This conclusion was based on the observation that spermathecal epithelial cells contain little rough endoplasmic reticulum and Golgi complexes, but have mitochondria and invaginations of the basal plasma membrane suggestive of involvement in the transportation of substances between the hemolymph and spermathecal lumen (Dallai, 1975; Kressin et al., 1996). Similar observations were made for the spermathecae of *M. bicolor*, and suggested that spermatozoa are nurtured by substances captured from the hemolymph in addition to the secretion produced by the spermathecal gland (Cruz-Landim et al., 2003). As shown here, the spermathecal epithelium contained centrally located nuclei with descondensed chromatin and an evident nucleolus in virgin and physogastric queens, an arrangement characteristic of cells with high biosynthetic activity. The apex of spermathecal epithelial cells in both types of queens stained strongly for protein, indicating that these cells can release proteins and/or cuticular components into the spermathecal reservoir.

The morphology of the spermathecal glands of *M. quadrifasciata anthidioides* was similar to that of *A. mellifera* and *M. bicolor* (Dallai, 1972; Cruz-Landim and Serrão, 2002). Noirot and Quennedey (1991) classified the gland cells of insects that had a similar structure to that of the spermathecal glands of bees as being type III gland cells in which the cells that form the intima layer correspond to the epidermis.

Histochemical analyses have shown that the secretion that coats the spermathecal lumen in virgin females is probably a glandular secretion containing glycoproteins or glycosaminoglycans (Davey and Webster, 1967; Clements and Potter, 1967; Bhatnagar and Musgrave, 1971). In *A. mellifera* queens, the spermathecal gland is PAS-positive (Camargo and Mello, 1970; Dallai, 1972). A similar arrangement was seen here in the spermathecal glands of *M. quadrifasciata anthidioides* queens. The decrease in PAS positivity with age may reflect the fact that in younger queens this gland provides the energy reserves for development and the onset of activity (Cruz-Landim and Serrão, 2002).

As in other insects, the spermathecal glands of physogastric *M. quadrifasciata anthidioides* queens were PAS-positive, suggesting that the lack of PAS-positivity in 30-day-old virgin queens may reflect a lack of mating that would normally stimulate the production of gland secretion. Since the spermathecal gland opens directly into the spermathecal reservoir, we suggest that this gland contributes to spermatozoan nutrition.

The spermathecal duct cells of some bumble bee queens release polysaccharides that nourish the spermatozoa before fecundation (Schoeters and Billen, 2000). As shown here, the spermathecal duct was PAS-positive in virgin queens up to 15-day-old, whereas in physogastric queens glycoconjugates were found in muscle cells but not in the cells that form the duct. This finding indicates that the spermathecal duct is not involved in nourishing the spermatozoa in this species of stingless bee.

In conclusion, the results of this study indicate that mating positively influences spermathecal development and the activity of spermathecal glands, and that delayed mating has no deleterious effect on the spermathecal morphology of virgin queens.

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Un retard dans l'accouplement agit sur l'activation de la spermathèque chez les reines de l'abeille *Melipona quadrifasciata anthidioides* (Hymenoptera, Apidae).

abeille sans aiguillon / *Melipona* / reine vierge / accouplement / spermathèque / histologie / histochimie

Zusammenfassung – Die Auswirkung einer verzögerten Paarung auf die Aktivierung der Spermatheka bei Königinnen der Stachellosen Biene

***Melipona quadrifasciata anthidioides* (Hymenoptera, Apidae).** Die Erhaltung der Lebensfähigkeit der Spermatozoen ist ein kritischer Aspekt einer erfolgreichen Reproduktion und insbesondere bei sozialen Bienen ist dies über einen langen Zeitraum hin erforderlich. Die Spermatheka ist eine spezifische Struktur im weiblichen Reproduktionstrakt, die genau diese Funktion erfüllt. Da bei frischgeschlüpften Königinnen die Spermatheka noch nicht voll entwickelt ist, war es das Ziel dieser Studie die Auswirkung einer Verzögerung in der Paarung auf die Entwicklung der Spermatheka und ihrer Drüsen bei der Stachellosen Biene *Melipona quadrifasciata anthidioides* zu untersuchen. Dazu verglichen wir die Spermatheken von nicht verpaarten 0, 15, 20 und 30 Tage alten Jungköniginnen mit denen von physogastrischen Königinnen hinsichtlich ihrer Histologie und histochemischer Parameter. Bei den unverpaarten Jungköniginnen verschiedenen Alters fanden wir keine signifikanten Unterschiede in der Epitheldicke und dem Innenvolumen der Spermatheka. Physogastrische Königinnen hingegen hatten wesentlich voluminösere Spermatheken mit dickeren Epithelien. Auch auf die Spermathekaldrüsen hatte die Paarung offensichtlich eine deutliche Wirkung. Bei frischgeschlüpften Jungköniginnen war der Polysaccharidgehalt zunächst noch relativ hoch und während bei 30 Tage alten Jungköniginnen nur noch ein geringer Polysaccharidgehalt zu verzeichnen war, zeigten physogastrische Königinnen bereits wieder einen deutlich erhöhten Polysaccharidgehalt in ihren Spermathekaldrüsen. Das Spermathekenepithel sowohl von Jungköniginnen als auch von physogastrischen Königinnen zeigte positive Reaktionen in Proteintests. Ausserdem fanden wir Proteingranula in den Drüsen von physogastrischen Königinnen. Diese Ergebnisse lassen den Schluss zu, dass der Paarungsprozess die Spermathekenentwicklung und die Aktivität der Spermathekaldrüsen in positiver Weise beeinflusst. Eine Verzögerung in der Paarung zeigte hingegen keine negativen Auswirkungen auf die Spermatheka von Jungköniginnen.

Bienen / Paarung / Spermatheka / Stachellose Bienen / Jungköniginnen

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