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Breakthrough research on reinsemination of bee queens with imaging of reproductive system elements

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The sperm reservoir in the spermatheca of bee queens with desirable genotypes may be depleted. In order to recreate a breeding line, instrumental reinsemination can be performed although beekeepers are generally convinced that ovipositing queens cannot be reinseminated due to the hardening of their valve fold. The beekeeping literature does not provide macroscopic-scale anatomical images of the valve fold. In this two-year study, in total 28 bee queens laying fertilized and unfertilized eggs were subjected to reinsemination with drone semen and ten-fold diluted hematoxylin. The reproductive system was dissected from 24 queens, and the valve fold was subjected to anatomical and histological analysis. The reinseminated queens were aged three months and three years. This study has confirmed that it is possible to carry out instrumental reinsemination in bee queens laying only unfertilized eggs. Afterwards, such queens begin to lay fertilized eggs from which fully developed workers hatch. The mean number of fertilized eggs laid by such queens was 514. The reinsemination of the queens with 2 µl of ten-fold diluted hematoxylin and the histological analyses performed with the use of a stereomicroscope in the macro scale and a light microscope have revealed the actual structure of the stained valve fold, whose shape resembles a "rose flower". The valve fold consists of one main structure supported by two smaller ones on the sides. In terms of histology, the structure of the valve fold in three-month-old queens is composed of a thick cuticle covering the secretion of epithelial secretory cells and muscles. This structure is responsible for the flexibility of the valve fold. In turn, the valve fold in three-year-old bee queens has an altered histological structure, as its cuticle is lost, leaving only the secretory cells of the epithelium.

Keywords Reproductive tract, Valve fold, Reinsemination, *Apis mellifera* queen

Bee breeding differs from the breeding of other farm animals¹, because queens are involved in mid-flight mating with many drones²⁻⁴. Therefore, it is difficult to control the paternal origin in bee breeding. Partial control of the origin of drones is possible through natural geographical isolation of breeding apiaries/queen-mating stations, which prevents the presence of drones of unknown origin. Queens and drones are able to mate within a distance of 10–15 km from the apiary⁵⁻⁷; hence, a very large area must be free from bee colonies of unknown origin. Breeding apiaries/queen-mating stations are most often established in forests, mountains, or on islands where there are no bee colonies of unknown origin^{8,9}. However, the success of queen insemination on islands is often low, which is attributed to poor weather conditions that do not encourage mating flights¹⁰⁻¹³, hence, the various estimated sizes of losses during natural queen insemination from as low as 3–30%¹⁴⁻¹⁸ to even 50–99%^{19,20}. This finding was the basis for the development and improvement of the instrumental insemination technique, which ensured full control of the paternal side in the mating of bee queens and helped to design bee breeding programs^{21,22}.

In the world, an estimated 6000 to 10,000 queens are inseminated instrumentally²³. In 2000, approximately 4000 instrumentally inseminated queens were used in Europe, excluding Poland²⁴. In Poland, approximately

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23,000 bee queens undergo instrumental insemination in 72 registered apiaries, which maintain 79 breeding lines in breed purity programs^{25,26}.

As reported by Cobey et al.²⁷, the apiculture based on instrumental insemination of bee queens in Poland is unique, as the procedure is also applied in colonies intended for pollination and production of honey. It increases the total number of instrumentally inseminated bee queens in Poland in the range from 50,000 to 90,000, depending on the year and the demand for queens. While learning to inseminate queen bees instrumentally, young inseminators are instructed that the valve fold should be pushed aside, which depends on the inseminator's skills^{28,29}. However, most textbooks and publications provide only an imprecise image of the structure^{30,31}. Therefore, the knowledge of the valve fold structure will facilitate and speed up the process of learning the insemination technique. It will show that the delicate surface of the valve fold may be involved in insemination capillary clogging or in damage to the valve fold during the instrumental insemination procedure. Instrumentally inseminated queens are often not accepted or replaced by bees in the colony. The knowledge of the anatomy of bee queens will help to elucidate the function of the valve fold and may contribute to further improvements in the instrumental insemination technique in the future. It will also provide more information about the anatomy and biology of honey bees. To date, only three authors, i.e. Fyg³², Stell³³, and Kozii et al.³⁴, have published histological sections of the valve fold, but the valve fold structure viewed in macro magnification under a light microscope showing the anatomy of the bee queen has not yet been reported. The publication by Fyg³² is difficult to access, and the images shown therein are black and white. Stell³³ showed a rudimentary structure of the valve fold. In turn, Kozii et al.³⁴ described a valve fold collected from queens that were naturally mated during early summer 2015 and collected in 10% formalin at the end of summer. Kozii et al.³⁴ presented their research conducted in the 2015 season on queens inseminated at the beginning of the season and performed the dissection during the summer; therefore, it can be concluded that the queens were approximately half a year old.

The sperm reservoir in the spermatheca of breeding queens (three years old and older) with desirable genotypes may be depleted. Such queens become useless for further breeding, as they lay only unfertilized eggs, from which only drones will hatch. Therefore, in order to recreate a breeding line, instrumental reinsemination can be applied. However, there are only sparse studies of this issue in the available literature. Beekeepers and breeders of bee queens commonly believe that, one month after queens are born, the valve fold hardens and the queens will not engage in the mating flight or cannot be subjected to instrumental insemination, as it will be impossible for the inseminator to push aside the valve fold³⁵. As confirmed by Fyg³², the valve fold muscles harden and lose their mobility in the first year of queen's life; hence, it is impossible to perform instrumental reinsemination in queens that lay only unfertilized eggs. As indicated by Adam³⁶, the valve fold muscles press oocytes towards the aperture of the spermathecal duct to facilitate fertilization of oocytes present in the median oviduct. Aging queens lay unfertilized eggs because the valve fold stops functioning.

Therefore, the aim of the study was to check whether it is possible to apply instrumental reinsemination in bee queens laying eggs. An additional goal was to expand the knowledge of the anatomical structure of the valve fold.

Results

The instrumental reinsemination of the three-year-old queens with drone sperm was successful. The reinsemination with hematoxylin allowed visualization of a sperm-filled lateral oviduct and active ovaries with nurse cells of a three-year-old queen laying only unfertilized eggs (Fig. 1a.). The reinsemination with hematoxylin allowed visualization of oocytes awaiting fertilization in the lateral oviducts (Fig. 1a).

The empty lateral oviducts of the bee queens are strongly folded (Fig. 1b); they stretch only after they have been filled with sperm (Fig. 1a) or eggs released from the ovaries (Fig. 1c). An oocyte passing through the median/common oviduct and through the valve fold is shown in Fig. 1d. Instrumental insemination with the hematoxylin injection allowed better visualization of the lateral oviducts and the valve fold, as shown in Fig. 1e, f, g, h. A detailed stained valve fold is presented in Fig. 1f and h in transverse view of the median/common oviduct with a characteristic bend at the valve fold.

Figure 2a, b, c, and d confirm the complex structure of the valve fold, which is composed of one main structure and two lateral structures supporting the main structure, as shown in Figs. 1e and 2c.

The histological micrographs of the reproductive tracts of the three-month-old queens show the main valve fold structure (Fig. 2a, b) and one of the lateral valve fold structures (Fig. 2c). The valve fold of the young queens (12 valve folds subjected to histological analysis) is covered by a thick cuticle, under which a substance secreted by the secretory epithelium and muscles can be observed (Fig. 2b). The valve fold of the three-year-old brooding queens (12 valve folds subjected to histological analysis) has no outer cuticle but only secretory epithelium cells (Fig. 2d). No deviations in the structure of the secretory epithelium and muscles described above were found in all the analyzed valve folds of the three-month-old and the three-year-old queens.

In the first year of the experiment (2023), the queens were not prevented from laying eggs. Therefore, after the instrumental insemination and return to the bee colonies, bees eliminated such queens from the colony after approximately three days. The cause of the elimination of the queens by the bees may have been the end of the nectar season, as the bees began to prepare for winter after the lime trees had stopped flowering. Additionally, three-year-old queens secrete pheromones less abundantly, and the instrumental insemination may also have disrupted the natural process of oviposition by the queens. All this contributed to the elimination of queens that would not be able to prepare the colony for wintering. In the second year of the experiment (2024), three-year-old queens that laid only unfertilized eggs were kept in transport cages with a queen excluder so that they discontinued laying eggs, and bees had access to the queens. After 12 days of isolation, the queens were subjected to instrumental insemination and returned to the bee colonies. All these queens began brooding after approximately 7 days. The inseminated queens laid from 460 to 536 (mean 514) fertilized eggs. The brooding period lasted 3 days. The bees fed the larvae and sealed the brood (Fig. 3), from which mature workers emerged

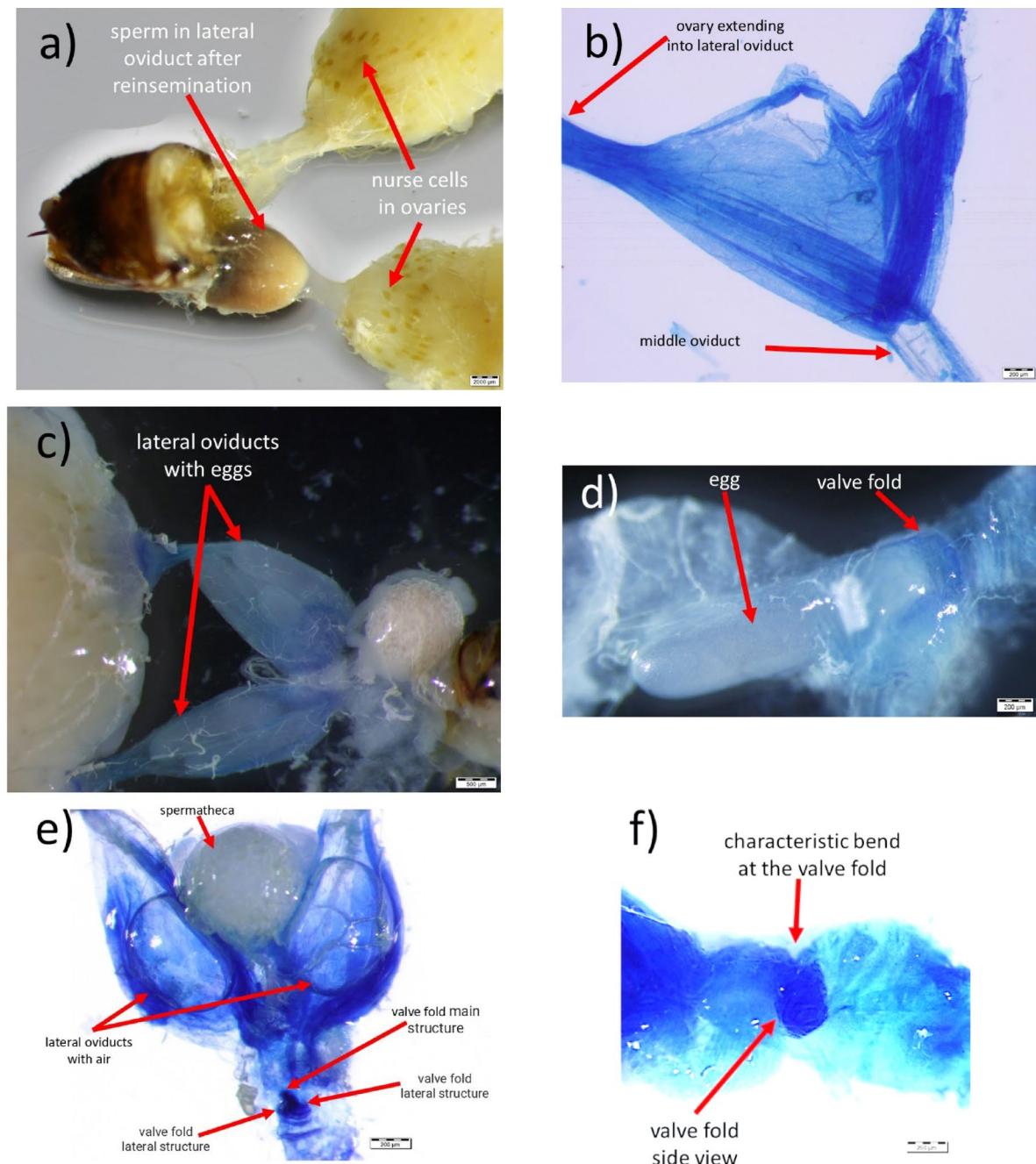


Fig. 1. Microscopy image of the reproductive system: (a) sperm-filled lateral oviduct after reinsemination in three-year-old queen, (b) stained empty lateral oviduct, (c) lateral oviducts with oocytes in three-month-old queens after reinsemination, (d) oocyte passing through the valve fold of three-month-old queens after reinsemination, (e) lateral oviducts of three-month-old queens after reinsemination; visible valve fold as the main structure supported on the sides by two smaller ones, (f) valve fold viewed from the side of the median oviduct, (g) valve fold viewed from the top of the median oviduct, (h) smaller and larger valve fold structure viewed from the side of the median oviduct.

(Fig. 4). The histological analysis of the spermathecae performed after the end of the brooding period revealed the absence of sperm therein (Fig. 5).

Discussion

The present study has evidenced that it is possible to reinseminate three-year-old queens laying only unfertilized eggs and three-month-old queens laying fertilized and unfertilized eggs. This is confirmed in Fig. 1a, which shows a sperm-filled lateral oviduct and the presence of nurse cells, proving the functioning of the ovaries. This finding is consistent with the results reported by Biliński and Jaglarz³⁷ and Dade³⁸. However, the brooding period

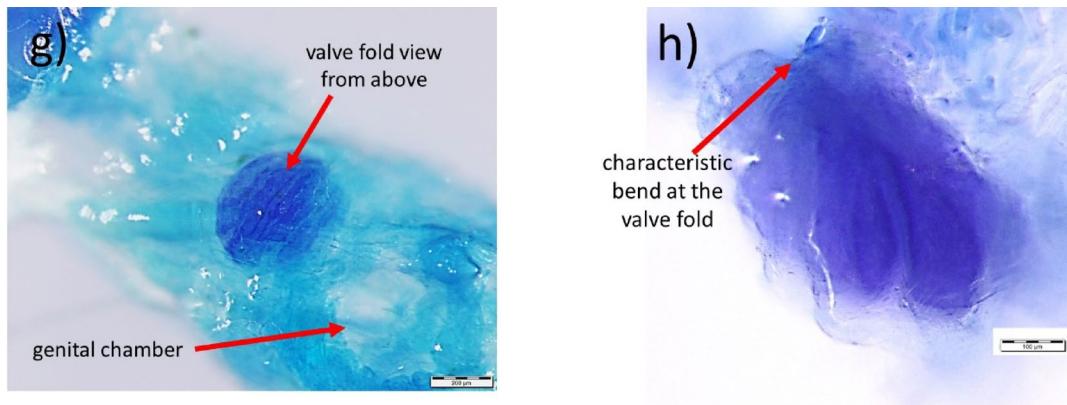


Fig. 1. (continued)

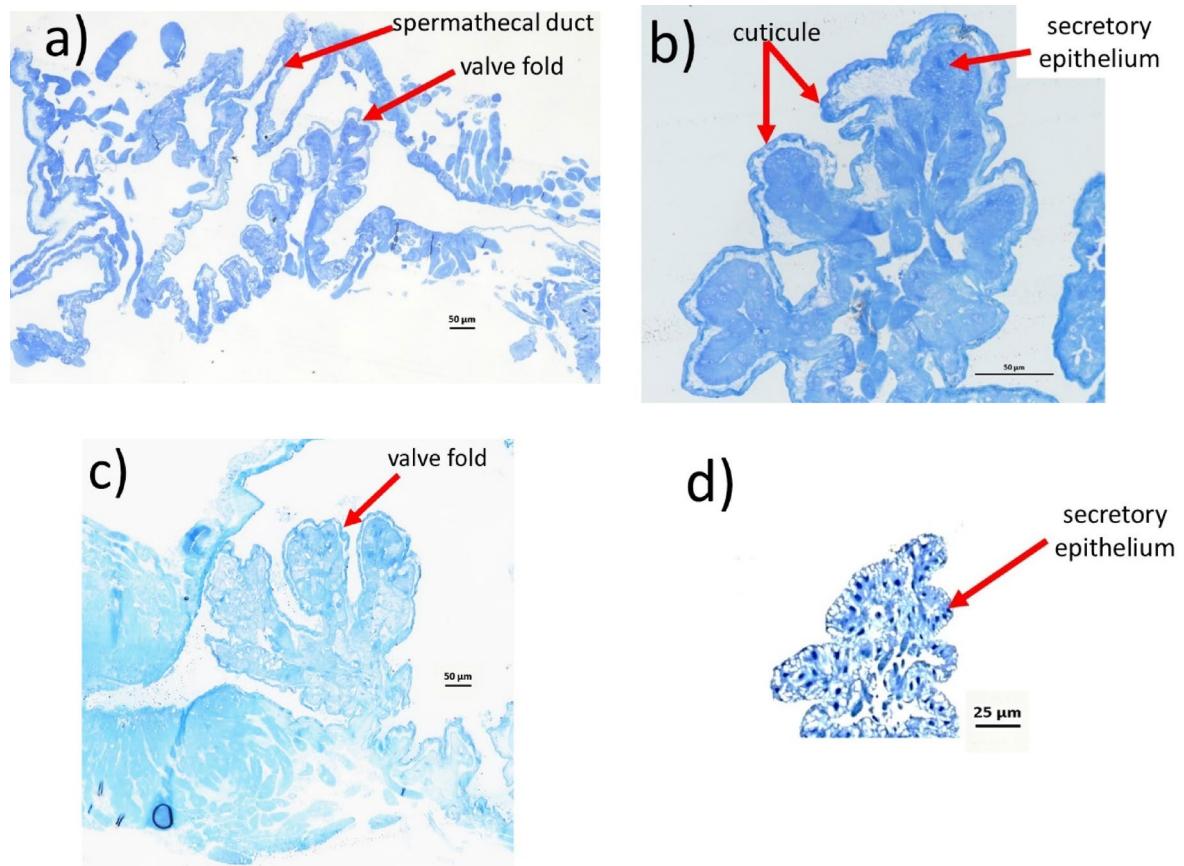


Fig. 2. Histological micrographs of the reproductive system: (a, b) main structure of the valve fold of three-month-old queens, (c) minor supportive structure of the valve fold of three-month-old queens, (d) valve fold of three-year-old queens.

was short (3 days), and the queens produced on average 514 fertilized eggs from which worker bees emerged (Fig. 4). After this time, the queens discontinued oviposition. The histological analysis of the spermathecae revealed the absence of sperm therein (Fig. 5). This confirms that the valve fold in three-year-old queens becomes flaccid³²; hence, only some spermatozoa entered the spermatheca and some were expelled. However, this process has not been sufficiently studied and opens the way for further research. This could explain why the queens continued brooding for a short time and laid such a small number of fertilized eggs. The changes in the anatomical structure of the valve fold, which is then unable to retain sperm, are probably responsible for the oviposition of only unfertilized eggs by three-year-old queens.

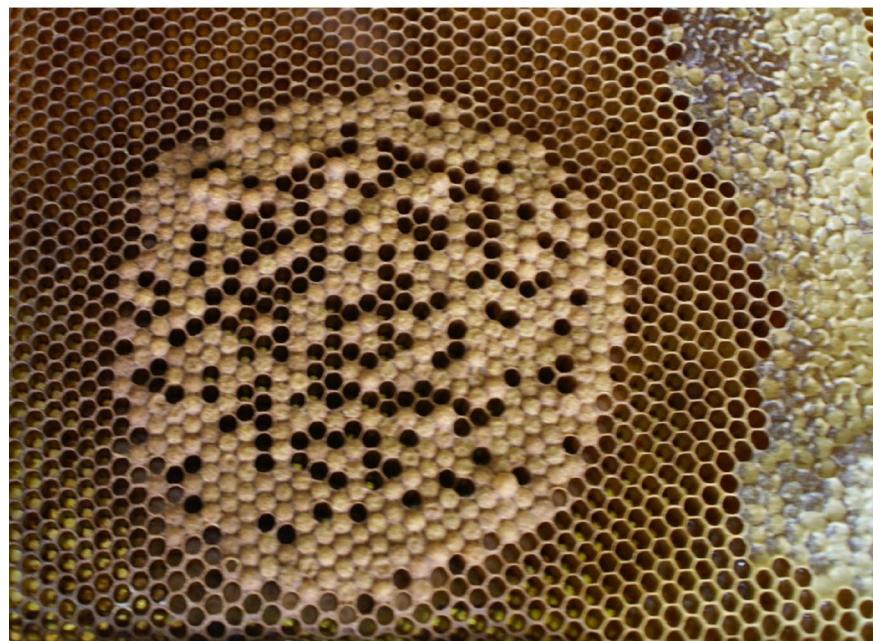


Fig. 3. Sealed brood from three-year-old queens after instrumental reinsemination.

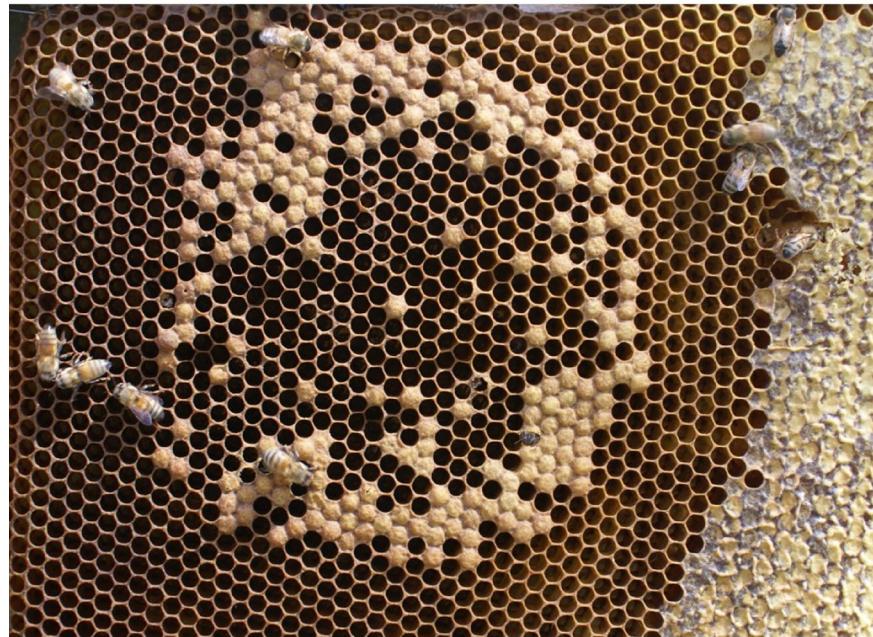


Fig. 4. Partially hatched brood produced by three-year-old queens after instrumental insemination, alongside recently emerged worker bees.

Bee queens have a phenomenal reproductive system, which is quite surprising to young inseminators, especially during their first attempts at instrumental insemination. Empty lateral oviducts, which are very strongly folded (Fig. 1b), can stretch to hold 8–10 μ l of sperm^{39–41}. Lateral oviducts can also be stretched by oocytes released from the ovaries (Fig. 1c). If queens lay 2000 eggs a day⁴² or more, the number of mature oocytes released from the ovaries is so large that some oocytes remain in the lateral oviducts and then are individually transferred to the median/common oviduct for fertilization and subsequent oviposition.

The reinsemination of the three-month-old queens with 2 μ l of ten-fold diluted hematoxylin, applied for the first time in bee research, made it possible to visualize oocytes in the lateral oviducts and an oocyte retained by the valve fold (Fig. 1d) and probably awaiting fertilization, as shown by Camargo and Mello⁴³. The valve fold has

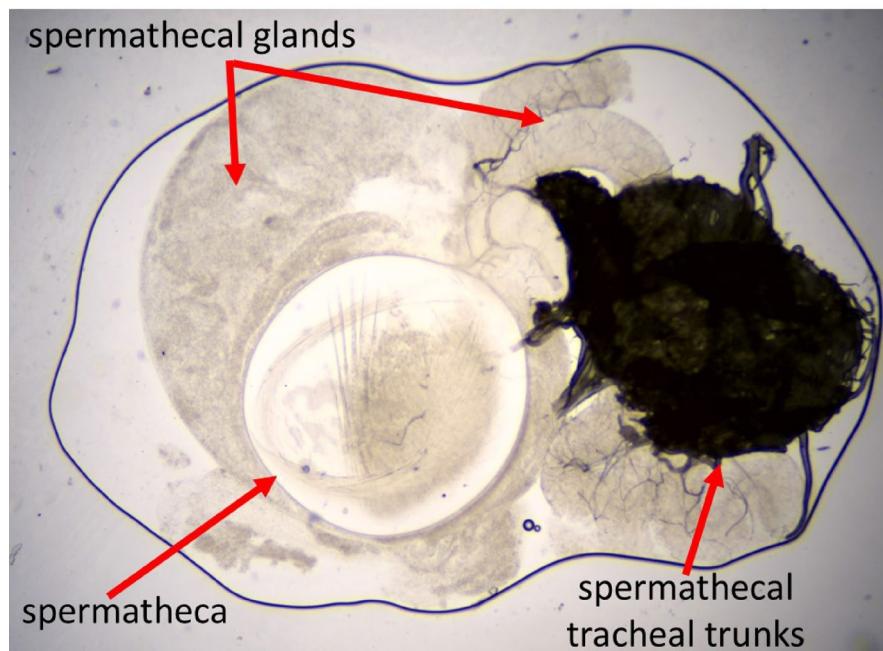


Fig. 5. Empty spermatheca dissected from three-year-old queens after instrumental reinsemination.

a role in delaying the transit of an unfertilized oocyte, while spermatozoa are released from the spermathecal duct to fertilize the egg^{33,36}. Kozii et al.³⁴ provided similar images; however, they used a 10% formalin solution in the preparation procedure, which macerated the delicate tissues of the queen reproductive system and the oocytes, hence the lower visibility of the images obtained by these authors in comparison with those presented in this study.

The air bubbles introduced with the air buffer after the reinsemination were located evenly in the two lateral oviducts. This indicates that the inseminator managed to push the valve fold aside using the insemination capillary and introduced hematoxylin with the air buffer present in the capillary during the instrumental insemination (Fig. 1e). This procedure facilitated staining and visualizing the valve fold. It was shown to have a structure resembling a “rose flower”, which was only visible after the hematoxylin staining (Fig. 1f, g, h). Noteworthy, the histological images are flat and show the cross-section fragment of a specimen. In turn, the image from the stereoscopic microscope and the hematoxylin staining revealed the “rose flower” structure of the valve fold. The valve fold was observed to be composed of one main structure (Fig. 2a, b, d) and two lateral structures (Figs. 1e and 2c). The histological study showed that it was covered by a thick cuticle, under which there was a substance produced by epithelial secretory cells (Fig. 2b) and muscles. This structure is responsible for the flexibility of the valve fold, which can thus be pushed aside by the capillary during the instrumental insemination of bee queens. The valve fold is a stretchy flap of tissue covering the median oviduct²⁷. As bee queens age, the valve fold loses the cuticle on its outer surface, leaving only the secretory cells of the epithelium (Fig. 2d). Probably, it also loses elasticity, as confirmed by Fyg³², and the insemination capillary penetrates into only one lateral oviduct; hence, the sperm was present in only one lateral oviduct in the three-year-old reinseminated queens (Fig. 1a).

The structure of the valve fold, resembling a “rose flower”, relatively often blocks the end of the capillary during the procedure of instrumental insemination of bee queens and impedes the release of the sperm from the capillary into the median/common oviduct of the bee queen.

As suggested by Fyg³², the valve fold muscles lose their original flexibility very quickly. Considerable degeneration is noticeable in two- or three-year-old queens⁴³. This was confirmed by our histological micrographs of the reproductive system. The analysis of the histological micrographs presented by Kozii et al.³⁴ allowed a conclusion that their images showed valve folds of half-year-old queens, which were replaced by beekeepers in bee colonies by young ones; hence the consistency of the histological image of the valve fold reported by Kozii et al.³⁴ with the valve fold of the three-month-old queens presented in our study (Fig. 2a, b).

Our study challenges the stereotypes that laying queens cannot be reinseminated due to valve fold stiffening. Our observations indicate that it is possible to push the valve fold aside during the reinsemination procedure. The present study complements the available knowledge/literature that has mainly provided schemes of structures or images of histological sections with no macro-scale images of the structures of the queen reproductive system. The images presented in this study show the valve fold, and they will hopefully be helpful in training young inseminators and beekeepers. Upon the analysis of the histological structure of the valve fold, we recommend that inseminators release a drop of semen at the end of the capillary before inserting the capillary into the queen reproductive tract. The drop of semen will be the first to lubricate the capillary in the reproductive tract of the queen, and the semen present at the capillary tip will be the first to come into contact with the valve fold and protect its structures. The present study expands the knowledge of the instrumental insemination of bee queens.

Years of research	Status of queens laying eggs			
	fertilized and unfertilized		only unfertilized	
	Number of queens	Age of queens	Number of queens	Age of queens
2023	12	3 months	4	3 years
2024	–	–	12	3 years

Table 1. Number, age, and status of queens used in the study.

and provides a new opportunity to obtain genetic material from apiculturally valuable queens that have already started to lay only unfertilized eggs.

Materials and methods

Queens

All the queens were reared in our apiary and kept in Dadant nuc hives in the apiary of the University of Life Sciences in Lublin, Poland ($51^{\circ} 13' N$, $22^{\circ} 38' E$). Four queens that laid only unfertilized eggs and emerged in 2021, i.e. they were three years old, were used in this study in 2023 (the first experimental year) (Table 1). The queens were subjected to instrumental reinsemination with 4 μ l of semen immediately after it was confirmed that they laid unfertilized eggs.

Additionally, twelve naturally mated queens that laid fertilized and unfertilized eggs and emerged in 2023 (three months old) were included in the study (Table 1).

In 2023, twelve three-month-old queens were subjected to a single instrumental reinsemination with 2 μ l of ten-fold diluted hematoxylin (Kolchem). Immediately after the reinsemination, the queens were euthanized by freezing at $-80^{\circ} C$ for 20 min. Next, the reproductive tract was dissected in a Petri dish in the presence of 0.6% NaCl and viewed under an Olympus SZX16 microscope.

In 2024 (the second year of the experiment), twelve queens that emerged in 2022 (three years old) were naturally fertilized and started laying only unfertilized eggs (Tab. 1). These queens were kept in transport cages with a queen excluder so that they stopped laying eggs, and bees had access to the bee queens. After 12 days of isolation, the queens were subjected to instrumental reinsemination and returned to the bee colonies. All the three-year-old queens underwent a single instrumental reinsemination with 4 μ l of semen.

Drones

Semen was collected from 14-day-old drones from one colony. In each year of the study, the drone comb together with the queen was placed in a single-compartment isolator to ensure that the emerged drones were of similar age. For 4 days, the emerging drones were marked with a water marker, which allowed the determination of their exact age. After the emergence, a cage covered with a queen excluder was placed on the entrance so that the drones could fly around and defecate outside the colony. This also prevented the spread of the drones to all the other colonies in the apiary.

Process of insemination of queens

Queens taken from the Dadant nuc hives were placed in an immobilizing sleeve with a flow of water-expanded CO_2 to anesthetize the queens. Concurrently, semen was collected from the drones using a syringe with a capillary filled with physiological saline. A buffer was made at the tip of the insemination capillary, i.e. sperm was mixed with physiological saline. After an air space had formed, sperm was collected from the drones for insemination. Upon the collection of sperm from 4–5 drones (4 μ l) and complete cessation of vital signs (after approx. 2 min), the queens were placed in the insemination instrument. The ventral hook was used to separate the abdominal plates of the sting chamber. Next, the vaginal cavity was exposed using the sting hook mounted on the sting. A drop of semen was released on the insemination capillary tip and the syringe together with the capillary was gently inserted into the vagina. After insertion to a depth of approx. 1.0 mm, the semen was released from the capillary until the air space was visible. The syringe with the capillary and the hooks were removed, and the anesthetized queens were returned directly to the Dadant nuc hives. The entire insemination process was performed under a stereoscopic microscope with six-fold magnification.

Histological analyses

The dissected parts of the reproductive system of the bee queens were fixed in a mixture of 2% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer (PBS), pH = 7.2, at room temperature for 10 h. To enable the fixative to penetrate, the material was deaerated in a desiccator connected to a PL 2 vacuum pump (5×15 min at 0.7 Atm.). The fixed material was washed with 0.1 M phosphate buffer (3×15 min) and then with distilled water (3×15 min). Next, the material was dehydrated in an ethyl alcohol series: 30%, 50%, 70%, 90%, 96% ($\times 15$ min), and 99.8% (2×30 min). The dehydrated material was saturated in mixtures of LR-White resin and acetone in the following proportions: 1:3 (3 h), 1:1 (3 h), and 3:1 (3 h) and in pure LR White (12 h).

The saturated material was placed in glycerin capsules, embedded in LR-White resin, and placed in a Memmert incubator (temp. $55^{\circ} C$) for 24 h. The embedded material was cut into 1.5 μ m semithin sections using glass knives in a Leica EM UC7 ultramicrotome. The semithin sections were stained with 0.5% Toluidine blue in 0.5% borax for 1 min on an HP 100 hotplate at $45^{\circ} C$. After rinsing off the excess dye with distilled water, the preparations were dried on the hotplate at $50^{\circ} C$.

In total, reproductive systems from 24 queens were dissected during the two-year-study and subjected to histological analysis. The sections were observed using a Nikon Eclipse Ni microscope equipped with a Nikon DS Ri2 camera.

Data availability

All data is provided within the manuscript.

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Author contributions

PD and GB conceived this research and designed the experiments; GB, PD, MD, RPŁ and KO performed the experiments and analysis; All authors have read and approved the final manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Ethical approval

No approval of the Research Ethics Committee was required to achieve the goals of this study, as the experimental work involved unregulated invertebrate species (*Apis mellifera*).

Additional information

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