Technical, sanitary and environmental sequences to improve artificial insemination of honey bee, *Apis mellifera*. Part II. Improved procedure

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Abstract. Previous studies developed by us have shown that the improvement of the hygiene state during the artificial insemination induced by preinsemination flight for dejection and emptying the distal portion of the male and female individuals' intestines has a positive effect on the success of the artificial insemination. This paper was intended by the authors as an improved protocol for the artificial insemination of the honey bee, a protocol which included the preinsemination flight sequences. The documentation and experimental research that set the basis of this protocol have already been described in details by Stoian et al (2018a,b).
Key Words: improvement, hygene state, preinsemination, male, female.

Introduction. Previous studies (Stoian et al 2018a,b) have shown that the improvement of the hygiene state during the artificial insemination induced by preinsemination flight for dejection and emptying the distal portion of the male and female individuals' intestines has a positive effect on the success of the artificial insemination.

This paper was intended by the authors as an improved protocol for the artificial insemination of the honey bee (*Apis melifera*), a protocol which included the preinsemination flight sequences. The documentation and experimental research that set the basis of this protocol have already been described in details by Stoian et al (2018a,b).

Artificial insemination improved protocol. As the sperm collection from the drones is carried out in the laboratory, a special space is created so that the drones fly before their sperm is collected. The specially designed space is dark and provided at the end with a window so that the drones are excited by sunlight and fly towards it. The space is approx. 3 m long, 1.5 m wide and ceiling (2.20 m) high. The drones will defecate during the flight and will be collected easily at the window pane.

The following beekeeping and laboratory objects are necessary for insemination: Brunel IMXZ macroscope, with X10 lens, CO_2 bottle with pressure regulator, Brunel cold light lamp, tweezers, Schley insemination device, tube for queen fixation, Harbo insemination syringe, ventral hook, dorsal hook and bumper. Other accessories used are: salt solution for pumping the semen through the syringe, white vaseline for sealing the sperm tubes, sterile wet napkins, Sharp scissors, cages for queens and drones, distiled water, holder, 96% concentration ethanol.

Before starting the work, make sure that all the materials, starting from those strictly related to the laboratory and ending with those that are found in nature, both made of plastic or metal, as well as those made of wood, were carefully disinfected and sterilized.

Other aseptic working conditions and illness prevention measures (fragment taken from Ruttner 1976) (outside the insemination laboratory): i) The disinfection of the frames and of the mating nucleus by submersing the objects in hot leach; ii) The forming of nuclei for mating with young bees in healthy and strong colonies; iii) The feeding of bees with Fumidil B sorbet.

When the disease has been confirmed, all tools that come in contact with the queen, must be sterilized (Ogradă 1986; Wolfgang 2000; Bura 1996).

Hygiene should be the first rule in the artificial insemination laboratory, especially not to leave dead or lost bees in the room. The surface of the working table should be plated with smooth material, easily and repeatedly washable with detergents. The hands must be washed with disinfectant. The fingers that came in contact with drone faeces are considered very dangerous contamination sources.

If after insemination one notices that in the needle tip channel there is sperm and mucus, the tip is immediately washed with disinfectant solutions, as the fresh deposits come off easily. After they dry, it is more complicated to clean them. It is recommended to repeat this operation at least after the 3rd or 4th insemination. The mucus flocculations on the outside of the needle tip are cleaned with a clean cotton pad, dampen with supracillin or a similar efficient product.

No tool used for insemination is used within the insemination station for purposes other than those for which they have been intended. The probes, the tweezers and the pipettes shall be immersed in ethanol and then rinsed with sterile water before each use.

The sperm that came in contact with: the drone body, its hemolymph, its faeces or the beekeeper's fingers, is stored in a biohazard container for a short time and then they are compulsorily disposed of. In many cases it happens that when inserting the hooks in the queen sting chamber, it defecates. In such situations, as the pathogen agents may penetrate the oviducts very easily, the insemination is immediately interrupted and the queen is rescheduled for the next day. The hooks are carefully cleaned, as explained above.

It is very important to carefully clean all the tools, especially the tip, at the end of the day. Approximately 30 minutes or more, as needed, will be allocated for this operation. Any mucus residue left on the tip is a favorable growth medium for microbial proliferation. Sometimes it is enough to rinse the tip with water and then to insert it in the "tego". The mucus residues that do not come off easily are removed by means of a fine copper wire. The plastic tip and the base can be left over night in the disinfectant solution. For a longer period of time they should be kept dry, at all times.

Keeping all the glass or metal objects in a box under an UV lamp was proven to be very efficient in practice. The lamp will be turned on daily for half an hour. The plastic material (such as the tip, for example) cannot support this treatment, as it is damaging.

The capsules (except the one with alcohol and the one with "tego") are emptied and cleaned hygienically every day. The necessary amount of distilled water is boiled every day, so that it is fresh. The supracillin dilution solution may be kept in the fridge for three days. After that it also needs to be renewed.

In order to be sure of the quality of hives used in the experiment, I will use bee hives with two year old queens which in the first two years have gone through the selection program in order to be classified as elite.

The drones intended for sperm collection must be chosen according to the following morpho-productive criteria: healthy, corpulent animals, born in prolific hives (elite hives), coming from different "father-hives".

To ensure the numerical and quantitative success, approx. 8-17 drones will be assigned to each queen for insemination and the drones will be brought for an intercalated harvesting.

To obtain queens, the larvae transplants in the artificial queen cells method will be used. Subsequently, these will be moved in the starter hives to be bred.

The larvae in the artificial queen cells, after being given for breeding in the starter hives and after they are capped, on the tenth day they will be moved in micronuclei hives populated with worker bees, where they will hatch and will stay until they reach the age of 8 days, and then they will be artificially inseminated.

The queens will be chosen by the following morpho-productive criteria: healthy, corpulent, with well developed abdomen, no genetic or acquired handicap, from elite hives.

In this regard, bee hives will be selected which were not related to the father hives, which have been carefully monitored over the last two years, which have obtained the highest score in terms of honey production, disease resistance (varroosis), aggressiveness, decreased tendency towards swarming, and wintering resistance. These hives will be the source of queens for the insemination itself.

The drones are kept in the father-hive, in the isolation cage, with a special box (47cm x 8cm x 30cm), where they are provided with the food and heat necessary for the development until they reach the optimum age for mating.

The isolation box is the box where the drones were grown in the father-hives. This isolation box remains permanently in the bee hive when we breed drones. Inside it there is a frame with plenty of honey, bee bread and pollen and 1-2 drone brood frames (in the case of the drone brood we know exactly the date when they will hatch) the drones will come out from. These drones will be used for sperm collection. They are kept in the isolation cage to know their exact age, i.e. to know exactly when they can be used for mating. We are interested in this aspect so that we do not collect immature or old drones and in order to synchronize with the stage of achieving unprolific queens that will be inseminated with these drones' sperm.

Subsequently, after reaching the appropriate age, when they will be able to be used for sperm collection, the drones are released from the isolation cage in the specially designed space in the laboratory so that they can fly. After flight, these will be collected one by one using the small box and inserted either in the isolation cage again or in the box for keeping the drones to be used for sperm collection.

We will further present the preparation for sperm collection from the drones. As we mentioned, a specially designed space is created, separately from the queens' insemination room. This space has the following dimensions: L = 3.5 m, l = 2 m, h = 2 m. The drones are released from the isolation cage and excited by the sun light they fly towards the window. After approx. five minutes of flight, they will be collected in a small box (12 cm long x 5 cm wide x 10 cm high) provided with a transparent window and an orifice for drone insertion. This operation is repeated the next day. The purpose of this repeated flight is that drones eliminate the faeces from the intestine as they did not have the possibility to fly as long as they were kept locked in the isolation cage. This way, we reduce the risk of contaminating the laboratory equipment, the sperm and the queen.

Also, the flight has a stimulating/excitation effect on the drone's body. It is therefore useful for making the sperm collection operation easier. The migration of spermatozoa is achieved by flying. The temperature in the working room and the special room will be maintained at values between 26 and 28^0C, in order to imitate as best as possible the natural atmospheric conditions.

After the 5-7 minutes of flight, the drones are collected from the window in a small box, after which they are inserted back in the isolation cage and subsequently in the bee hive.

The preinsemination flight operation described above for the drones is also applied for the receptive unprolific queens. However, at this point we have to make a remark: the queens are allowed to fly to eliminate faeces, one by one, only on the insemination day because the accumulated faeces are less than in case of drones, and the insemination, as mentioned above, is carried out in the eighth day from hatching.

The drones differ very much from one another in terms of the easiness they ejaculate, but also by the sperm volume that can be collected from one individual, especially when consanguine genetic material is used. Some drones do not have sperm; in others the turning of the endophallus and the ejaculation do not occur naturally when artificially stimulated; while in others the turning is so violent that the sperm is projected and lost or the endophallus explodes (Mackensen & Ruttner 1976).

The correct immobilization of the drone when collecting the sperm is extremely important. In order to cause eversion and correct ejaculation, the head and thorax of the drone are grabbed between the thumb and the index finger of the left hand, holding the

drone with the ventral part up. Then, while the head and the front part of the thorax are gripped in the left hand, the dorsal part of the abdomen is slightly and repeatedly squeezed between the thumb and the index finger of the right hand. This action usually causes the contraction of the abdominal muscles and a more or less complete eversion of the penis and ejaculation. If the penis is only partially turned and the sperm does not occur, the abdomen is progressively squeezed from the dorso-front part towards the ventro-posterior part in order to continue the forced turning up of the penis until the sperm is eliminated. The sperm is rarely obtained without abdominal contraction, but when the abdomen is contracting without the partial turning up of the penis, this can be fully turned by pushing it, thus achieving a larger amount of sperm (Mackensen & Ruttner 1976).

The amount of sperm and mucus eliminated are variable in the drones. As the penis is turned up, the cream-colored sperm appears first, followed by the white, thicker mucus. Sometimes only sperm is released, but usually, after sperm, a small amount of mucus comes out, both spreading on the upper part of the penis, in varying proportions. The spermatozoa movements cause the sperm to spread in a thin layer over the mucus layer, which often renders its collection with the syringe very difficult. For this reason, it is important that any delay is avoided when collecting the sperm (Mackensen & Ruttner 1976).

The drone that has just ejaculated is brought to the top of the syringe with the left hand. The piston is withdrawn to create an air bubble between the sperm and the salt solution and to estimate the collected amount of sperm. The sperm surface is then brought in contact with the tip of the syringe under an angle of approximately 45°. If the drone is slowly withdrawn from the tip of the syringe, without interrupting their contact, the sperm will continue to adhere to it and will flow as the piston is withdrawn (unfastened). This procedure helps the operator avoid the sampling of the mucus – as it is viscous it does not flow. The mucus is too consistent to go into the tip of the syringe and would stop the passing of the sperm. If this accident occurs, the piston must be unfastened (advanced) until the lumen is clean; then the sperm collection operation is resumed. The drone sperm is collected until the syringe is filled to the desired level. The amount of sperm that can be collected from one drone is approx. 0.8-1 µL. The beginner operator must sample the sperm very slowly; with experience, he will be able to work faster (Mackensen & Ruttner 1976).

When the insemination device has been changed so that the fixing of the queen happens very fast, many times the insemination works conversely, i.e. The sampling of the sperm in the syringe – fixing and anesthetization of the queen – insemination with seminal material. If mature drones are available, the time needed for the procedure is as follows: filling of the syringe - 8 minutes, insemination - 2 minutes. If all is well prepared, approx. 15 minutes are calculated on average for one insemination, including changing the queens and getting the drones (Mackensen & Ruttner 1976).

The queens are inseminated between 6 and 12 days after their hatching from queen cells, when they become mature. According to some researchers, it is ideal in the eighth day (Ruttner 1976). The procedure is carried out in several stages. According to the state of the art presented in the published literature (Cobey et al 2013), with minor changes from one practitioner to another, these stages are:

1) Two carbon dioxide (CO_2) treatments. The first treatment implies an exposure of 5 minutes the most and it is achieved one or two days before the artificial insemination procedure. The second treatment is applied during the procedure itself. It is important to mention here that, if this second anesthetization is performed correctly, it stimulates the oviduction of the queen.

2) The syringe and the queen holder are aligned on the tool holder at an angle of 30°, 45° or 60° (depending on the tool that is used) to facilitate the penetration of the syringe needle into the vaginal orifice.

3) Then, the queen is inserted inside the retention tube of the insemination device for immobilization and fixation, head down, towards the carbon dioxide source. A continuous, slow flow of carbon dioxide is administered to put the queen to sleep.

4) The visible folds of the abdomen are separated, more precisely the sternite, the tergite, and the chamber of the sting to expose the vaginal orifice, using a pair of hooks or tweezers.

5) The syringe needle tip is placed at the rear part, above the "V spot", defining the vaginal orifice. The tip of the needle is inserted in the vaginal orifice, at a depth ranging between 0.5 and 1.0 mm, slightly before the tip of the "V spot".

6) The tip is further inserted in depth, for another 0.5 to 1.0 mm, while making a slight zig-zag movement to bypass the entrance valve in the vaginal orifice.

7) A precise amount of semen is inserted directly into the median oviduct. The standard injected dose is 8-12 µL for each queen. After administering the sperm, the queen is inserted directly into the colony of its mating nucleus to promote the migration of the sperm or to be able to administer two doses of 6 µl of spermatic material in a 48 hours interval. Otherwise, the maximum volume of spermatic material cannot fit the median and collateral oviducts.

8) At the end of the insemination, the tip of the syringe collects a small air space with a small drop of salt solution (approximately 0.5 µL) for the next insemination, only to prevent the drying of the sperm.

In conducting this artificial insemination activity, the help of a collaborator is very useful. This way, 200-300 queens can be inseminated in only 3-4 days. The activity is organized as follows: first, the sperm is collected from all the drones. The sperm is stored in special tubes called capillary tubes.

These tubes (made of borosilicate glass with a capacity to inseminate 10 queens, 10 µL/queen) sealed at the ends and stored in a dark place, at a constant temperature of 17.2-17.5^{0}C. After completing this activity, the following one lasts for 3-4 days and it implies the actual insemination of receptive unprolific queens.

Within the sperm collection activity, the work is divided between the two persons as follows:

- The first person has the following tasks: collection of the drones (from the isolation cage) and their transfer to the laboratory where they are placed in the special keeping box. One should mention that the drones can only be transferred once they are used. The time between the transfer and the use of the drone is maximum 30 minutes; otherwise, the physical cooling of the drone makes the operation impossible. The transfer of the drones is accomplished in small number, and after their use the operation is repeated. Another task was that after their transfer in the special keeping box, the drones are taken one by one and prepared for sperm collection by pushing on their abdomen until the eversion of the endophallus and of the reproductive organs with sperm. The following step is killing them by piercing them very fast with a sharp metal spike and then placing them in a glass-type recipient (just as the pencils are placed in a cup).
- The second person works with the insemination device, in this case with the Harbo syringe, to collect the sperm of the drones. This person does not get in contact at all with other devices or with the drones; the only task is to take the drones on the spike and collect the sperm.

After insemination, each queen is returned to the bee micronucleus it comes from. After approximately two days since the insemination we checked whether the queens were accepted and whether they were alive in those colonies. After other approximately 4-6 days, they are about to lay the egg sets in the honeycomb (Căuia 2005; Pătruică & Bura 2017).

During this period, the presence or the absence of the queen is monitored and whether the queens have a normal behavior or, on the contrary, an aberrant behavior.

Conclusions. One should take into account the fact that the bees, regardless of their gender, do not defecate in the hive. This behavior is one achieved along the history of the species by natural selection and it is very important for the hygiene of the bee colony. The faeces are eliminated during the flight, away from the hive. That is why including a preinsemination flight sequence is beneficial for the hygiene of the inseminated queens and for the success of the insemination work.

References

Bura M., 1996 Creşterea intensivă a albinelor. Editura Helicon.

Căuia E., 2005 Cercetări privind îmbunătăţirea tehnologiei însămânţărilor artificiale la albine. Teză de doctorat. Facultatea de Zootehnie. Universitatea de Ştiinţe Agronomice şi Medicină Veterinară, Bucureşti.

Cobey S. W., Tarpy D. R., Woyke J., 2013 Standard methods for instrumental insemination of *Apis mellifera* queens. In: Coloss Bee Bool. Vol.I., Standard Methods for *Apis mellifera* Research. International Bee Research Association, Department of Entomology, Washington State University, USA.

Mackensen O., Ruttner F., 1976 Însămânțarea artificială a mătcii. Ediţia a 2-a. Cap. V. Tehnica însămânţării. Editura Apimondia, Bucureşti, pp. 69-86.

Ogradă I., 1986 Bolile şi dăunătorii albinelor. Ediţia a III a, ACA, Bucureşti.

Pătruică S., Bura M., 2017 Insamantarea artificiala ca metoda de ameliorare a albinelor. Editura Eurobit, Timisoara.

Ruttner F., 1976 Însămînţarea artificială a mătcii. Ediţia a 2-a. Apimondia, Bucureşti.

Stoian R. O., Botha M., Petrescu-Mag I. V., 2018 Beekeeping in Romania and artificial insemination of honey bee, Apis mellifera. State of the art. ABAH Bioflux 10(2):93-121.

Stoian R. O., Mălinaş C., Botha M., Petrescu-Mag I. V., 2018 Technical, sanitary and environmental sequences to improve artificial insemination to honey bee, Apis mellifera. Part I. Experimental results. ABAH Bioflux 10(2):122-149.

Wolfgang R., 2000 Bolile albinelor. Editura Mast, Bucureşti.

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