Technical, sanitary and environmental sequences to improve artificial insemination of honey bee, *Apis mellifera*. Part I. Experimental results

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Abstract. By this paper we wanted to investigate the importance of drones and queens dejection before the insemination procedure, because it is a serious contamination risk of the seminal material, of the tools and implicitly of the queens with fecal matters. The results of our research show 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.
Key Words: insemination instrumental, artificial reproduction, honey bee, defecation.

Introduction. In Romania, due to the pedo-climatic and environmental conditions, the selection, improvement and maintenance in pure state of the selected genetic material may be conducted in a 100% controlled manner by artificial insemination.

In this introductory chapter of the paper we will mention some of the advantages of artificial insemination as compared to natural mating, but also some disadvantages that may occur after several years of repeated artificial insemination.

As shown above, this insemination technique helps the beekeeper in the beekeeping activity by the fact that it provides the opportunity to patiently make a safe and thorough selection of the genetic material, to work with pure genetic material, to obtain high quality genitors that can ensure the highest output in terms of productivity in a shorter term than natural mating (Cobey 2007). It is the technique which is best suited in the areas where natural mating control cannot be ensured.

Another feature (and advantage) of artificial insemination is that it can create specific retrocrossings by extracting the seminal material from the spermatheca of a queen and insemination of another queen using this material (Harbo 1986).

Beside the success of artificial selection, controlled reproduction (inbreedings, retrocrossings, fixation of desired features, hybridizations etc), which is the main advantage, the other advantages of artificial insemination if this is successfully carried out, are most of the time related to avoiding some diseases transmitted by natural mating (Amiri et al 2016). As we know, the queen mates with many drones during its flight (Woyke 1962; Cobey 2007) and the probabilistic risk to get diseases is higher (Odagiu & Oroian 2010).

Moreover, it appears that a larger volume of sperm inoculated to the queen enables it to fecundate several eggs and for a longer period, by physiological regulation proven by Baer et al (2016). Artificial insemination may ensure the adequate volume of seminal material for the queen.

Artificial insemination may have adverse effects, such as the reduction of genetic variability (Pieplow et al 2017) and of the behavioral variability at hive level (Tarpy &

Seeley 2006; Mattila & Seeley 2007; Lattorff & Moritz 2013) due to using brother drones coming from joint "father hives". Avoiding these phenomena may be achieved by inseminating the queens with sperm from multiple non-related drones (Pieplow et al 2017; Brauße & van Praagh 2010). However, it is important to note that the excessive inbreeding risk occurs only after several years of unidirectional selection.

Investigating the published literature we find that the most notable paper which is closest in title and objectives with our research is entitled "Research on improving the artificial insemination technology in honey bees" and it was written by engineer Eliza Căuia (Căuia 2005).

The paper mentioned above intended to: improve the artificial insemination device; optimize the achievement, autoselection and storage of sexually mature drones; develop a technology for breeding queens and drones in the apitron under extra season conditions; correlate the queens' insemination with their functional quality; correlate proteic feeding with certain physical, anatomo-morphologic and physiological parameters of the genitors; improve the technique for storing the queens in orphan hive in a "queen bank" system and optimize the blending and homogenization technique of the honeybee sperm by using some artificial thinners, improved with seminal liquid.

Another paper relevant for our research is the book written by Pătruică & Bura (2017). This book is one of the most exhaustive papers that address instrumental insemination of the bees.

Objectives. By this paper we wanted to investigate the importance of drones and queens dejection before the insemination procedure, because it is a serious contamination risk of the seminal material, of the tools and implicitly of the queens with fecal matters. We presumed that by neglecting these aspects the result of insemination may be negatively affected.

During sperm collection, the drones often eliminate faeces. During insemination, the queens also eliminate faeces. The published literature mentions that in order to reduce the risk of sperm and queen contamination with faeces it is recommended that the drones eliminate the fecal matters before collecting sperm from them (Ruttner 1976). However, it is not specified how, when and in what conditions this is performed. Regarding the queens, the published literature does not mention whether they should fly or not before the artificial insemination procedure.

More precisely, by this thorough study we intended to optimize the artificial insemination procedure, adding to the common technique some sequences meant to hygienically improve the implementation conditions and the final results. The answers that we expect from our experiments should answer the following questions:

1) Is the quality of artificial insemination results influenced or not by inducing the previous dejection by the drones?

2) Is the quality of artificial insemination results influenced or not by the queen flying before the procedure?

3) If these actions influence the quality of the insemination results, how can they be included in a program for optimizing the artificial insemination technique?

Also, we propose as a final objective to edit an improved operating protocol, which will include the essence of the original part of our studies in this paper. This last idea will be the subject of a future paper.

Material and Method

Location of the research and geography of the area. Our research was conducted in Ciucurova commune, Tulcea county, Romania (Figure 1).

Ciucurova is located in the central southern part of the Tulcea county, with the following surroundings: Tulcea municipality (45 km) and the Danube Delta (60 km) to the north, Babadag forest and Babadag town (35 km), Razem Lake and the Black Sea (100 km) to the east, a large forest area to the south and the Măcin Mountains and Măcin forest (60 km) to the west.

The coordinates of the Ciucurova commune (latitude, longitude) are: 44.9333, 28.4833.

The population of Ciucurova is 1,941 people.

The climate is continental, with hot summers and little rain, cold winters with strong winds, average annual temperature +10.8°C; average annual amount of rainfall 469.4 mm/m^2. However, the atmospheric influence caused by the Black Sea and the Danube Delta is felt and an atypical climate is often felt, with intense and short-term rainfall.

The area is slightly hilly, with altitudes up to 400 m, with very productive agricultural lands but also large areas of forests.

In the area of Ciucurova there is the largest honeybee basin in the form of tile forest in the South-Eastern Europe. Beside the tile, the flora of the area is diverse and abundant, with species like: corn poppy (*Papaver rhoeas*), rape (*Brassica rapa*), acacia (*Robinia pseudacacia*), sunflower *(Helianthus annuus*), snowdrops (*Galanthus nivalis*), hazel trees (*Corylus avellana*), European cornel (*Cornus mas*), coriander (*Coriandrum sativum*), charlock mustard (*Sinapis arvensis*), lavender (*Lavandula angustifolia),* brotherwort (*Thymus serpyllum*), lemon balm (*Melissa officinalis* L.), facelia (*Phacelia tanacetifolia*), wild garlic (*Allium ursinum*) etc. The area is very favorable both for monofloral honey (Figure 2), and for the polyfloral honey production (Figure 3).

Figure 1. The geographical coordinates of Ciucurova locality.

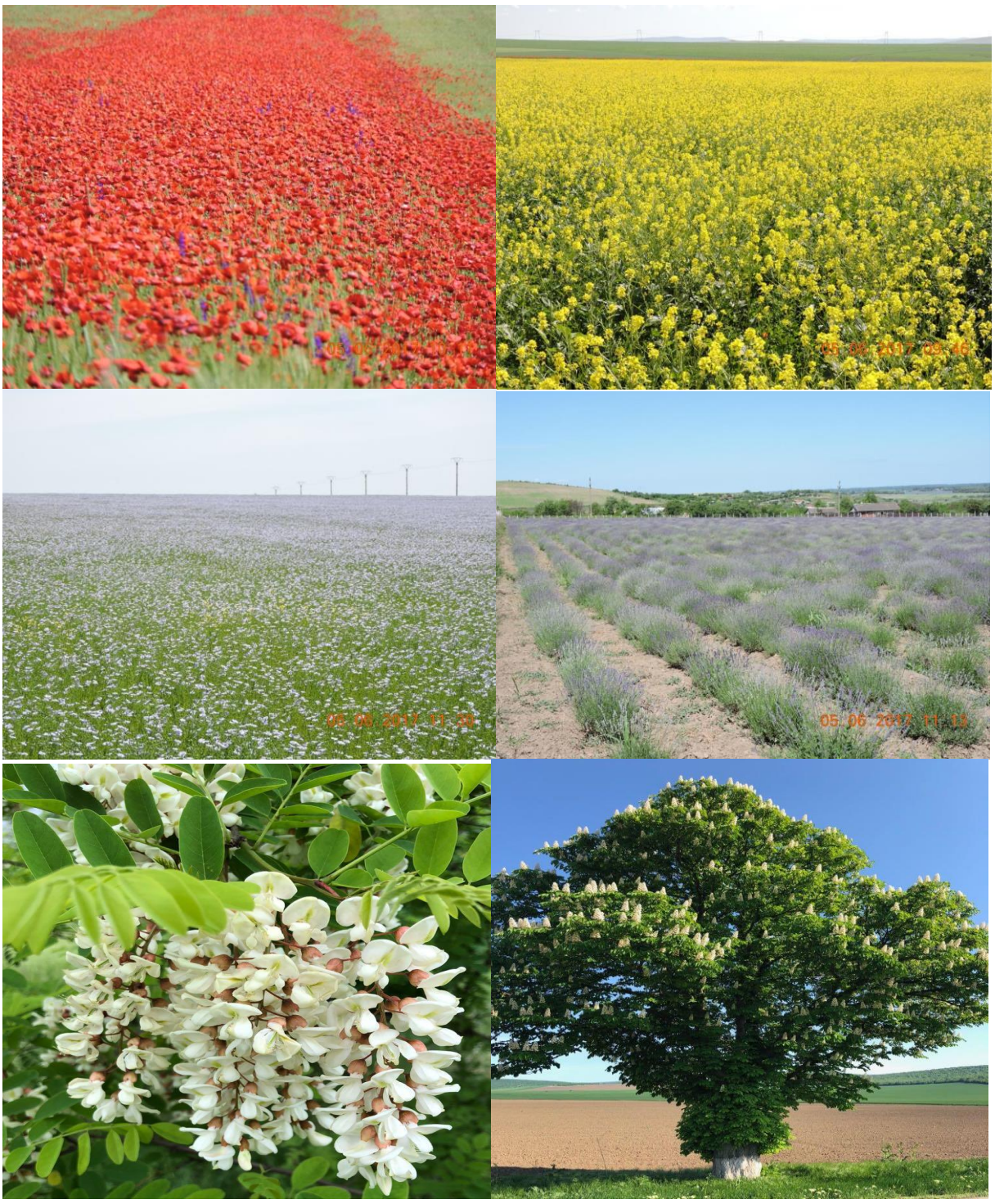

Figure 2. The monofloral honey production potential of Ciucurova area (original).

Figure 3. The polyfloral honey production potential of Ciucurova area (original).

Calendar of the research plan. The research was conducted in 2015 (May-June) and it was repeated in 2016 (May-June) and in 2017 (May-June). Our research is based on the results of a study with three repeated analyses conducted in relatively identical conditions

from all points of view: methodology, biology, ecology, season, geography, soil and climate.

Procedure, biologic material and instruments used. We should mention from the very beginning the fact that there are no multiple standard techniques in terms of artificial/instrumental insemination, the technique is approximately the same each time. The only aspect that is different is the insemination device that is used, the timing and planing of work, as well as the correct implementation of the cleanness principle and work discipline. Nowadays, the most commonly used insemination devices are: Swienty (Figure 4), Schley (Figure 5) and Laidlaw. The most appreciated insemination devices are those which are the easiest to handle, which help the beekeeper to work as precisely and as comfortably as possible.

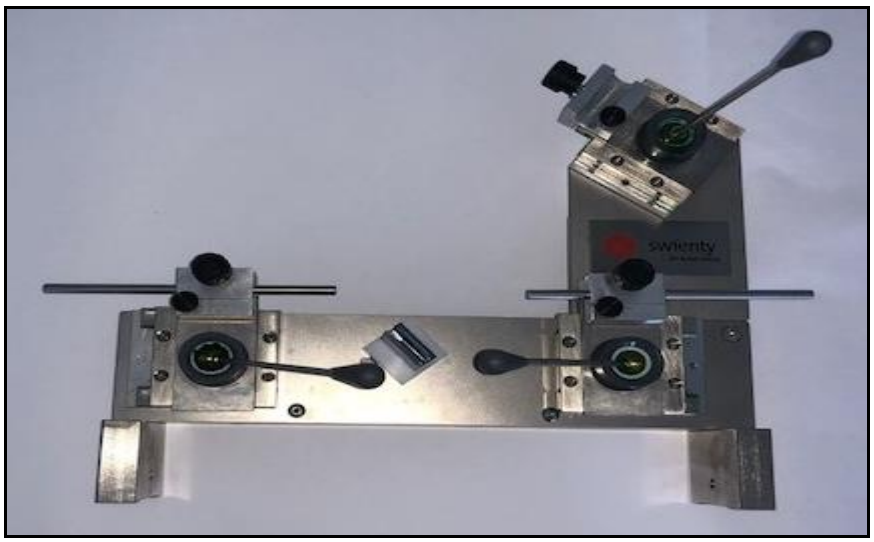

Figure 4. Swienty device used in our research (original).

Figure 5. Schley device used in our research (original).

As the sperm collection from the drones is carried out in the laboratory, we have built a special space so that the drones fly before their sperm is collected. Inside the laboratory we have built a wall separating this space from the laboratory room itself. 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.

Instruments used for semen collection and insemination. In this experiment we have used the following beekeeping and laboratory objects: 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 (all these were presented in Stoian et al 2018) (Figure 6). 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 ethyl alcohol.

Figure 6. Overview photo of the insemination equipment: macroscope, Schley insemination device, gas bottle, salt solution, cold light lamp, video camera (original photo). General view of the insemination equipment: the macroscope, Schley device, gas bottle, Schley syringe, saline solution, cold light lamp, video camera.

Before starting the experiment, I made 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 of wood, were carefully disinfected and sterilized.

Other aseptic working conditions and illness prevention measures (outside the insemination laboratory), that we implemented in practice were:

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.

Biologic material; genitors selection. I own an apiary of 225 production hives and a selection apiary (elite) of approx. 60-100 hives (this number differs from one year to another). I produce and sort the queens for selection (fig. 7-8). I work constantly and exclusively with the *Apis mellifera species*.

In order to be sure of the quality of hives used in the experiment, I used 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.

In the apiary I own, the hives and individuals intended for the experiment have been chosen by the criteria presented below.

Figure 7. Our apiary, a view of stationary beekeeping; micronuclei with bees and queen bees prepared to be artificially inseminated (original).

Figure 8. Our apiary, a more dynamic view of the beekeeping practice, showing bee care activities (original).

Selection of drones for sperm collection. The drones intended for sperm collection for our experiment have been selected according to the following morpho-productive criteria: healthy, corpulent animals, born in prolific hives (elite hives). The sample we used consisted in 5 bee "father" hives, each queen being inseminated with semen from drones coming from different father hives.

In their paper, Cobey et al (2013) say that, generally, 8-12 drones are used for inseminating each queen. To ensure the numerical and quantitative success (Richard et al 2011), we have assigned approx. 17 drones to each queen for insemination.

According to recent studies performed by Richard et al (2011), the activities of the worker bees in the hive are stimulated by two factors: the inoculated sperm volume and the multiple origin of the inoculated sperm; that is why the drones have been brought for an intercalated harvesting.

Selection of queens for insemination. In order to obtain the larvae transplants in the artificial queen cells (see chapter "Larvae transplant" presented in Stoian et al 2018) which subsequently have been moved in the starter hives to be bred (Figures 9-10).

The larvae in the artificial queen cells, after being given for breeding in the starter hives and after they are capped, on X day they will be moved in micronuclei hives populated with worker bees (Figure 11), where they will hatch and will stay until they reach the age of 8 days, and then they will be artificially inseminated.

The queens necessary for our research have been chosen by the following morpho-productive criteria: healthy, corpulent, with well developed abdomen, no genetic or acquired handicap, from elite hives.

In this regard, we have selected 12 bee hives 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 were the source of queens for the insemination itself.

Figure 9. Starter for queen growth (left) and portable incubator for queens (right) (original).

Figure 10. The starter, full of bee brood (original).

Figure 11. Pallet with eight micronuclei, each micronucleus with its own queen; miniature hives with virgin queens (original).

Preparation of drones for sperm collection. The drones proposed for the achievement of our research objectives (4777 drones) were split randomly in two approximately equal batches (2397 and 2380 individuals). Approximately half of the drones will be used for collection as such (2397 drones, control batch), and the other half (2380 drones, experimental batch) will be induced dejection by repeated previous flight, and then they will also be used for sperm collection. We have used approximately 17 drones per inseminated queen (281 queens x 17 drones = 4777 drones used annually). The two drones batches will then be divided once again and will become four batches, according to the four experimental variants (V1, V2, V3 and V4).

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

The isolation cage (Figure 12) is the box where the drones were grown in the father-hives. This isolation cage 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 achieving 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 (Figure 14) and inserted either in the isolation cage again or in the box for keeping the drones (Figure 13) to be used for sperm collection.

Figure 12. Isolation cage for holding the drones before collecting the sperm (original).

Figure 13. Storage box for drones keeping before collecting the sperm (original).

Half of the sperm collected from the drones that flew and probably defecated is intended for half of the number of queens in the batch that flew and probably defecated, and the other half is intended for insemination of queens that did not fly. Half of the sperm collected from the drones that were used directly, without previously flying will be used for the insemination of half of the number of queens that did not fly and probably did not defecate before, and the other half of the sperm volume is intended for insemination of remaining half of queens that did fly.

Essentially, there will be four experimental variants, V1, V2, V3 and V4, according to the following criteria:

- V1) batches whose minimum hygiene rules are met before the insemination (queens that did not fly, inseminated with sperm from drones that did not fly);
- V2) batches whose genitor drones flew, but the drones did not fly before the insemination;
- V3) batches whose genitor drones did not fly and queens that did fly before the insemination;
- V4) batches whose hygiene rules before insemination are improved by flying and implicitly by emptying the distal portion of the intestine (queens that flew, inseminated with sperm from drones that flew) (see Table 1).

Table 1

The experimental variants, the number of genitors that participated in artificial insemination and the theoretical number of resulting descendant bee hives

*Experimental variants 2015**	*V1) Drones that did not fly; queens that did not fly*	*V2) Drones that flew; queens that did not fly*	*V1) Drones that did not fly; queens that flew*	*V2) Drones that flew; queens that flew*
Number of genitors (approximately)	♂ 1199; ♀ 71	♂ 1190; ♀ 70	♂ 1198; ♀ 70	♂ 1190; ♀ 70
Number of hives/ egg sets resulted theoretically	71	70	70	70

*The experiment was repeated twice more following the same experimental plan (in the years 2016 and 2017).

We will further present the preparation for sperm collection from the drones intended for batches V2 and V4.

As we mentioned, a specially designed space was 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 were released from the isolation cage and excited by the sunlight they flew towards the window. After approx. 5-7 minutes of flight, they were 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 was repeated the next day. The purpose of this repeated flight was 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, at least theoretically, we reduce the risk of contaminating the laboratory equipment, the sperm and the queen.

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

After the 5-7 minutes of flight, the drones were collected from the window in a small box, after which they were inserted back in the isolation cage and subsequently in the bee hive (Figure 13).

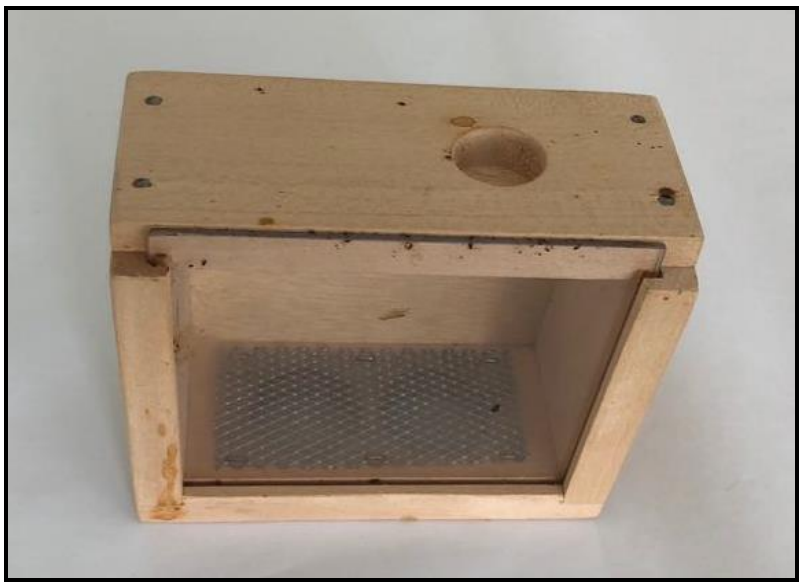

Figure 14. A small box (original design) fitted with glass, used to collect the drones after 5-7 minutes of flight. The box can accommodate about 20-30 drones (original).

Preparation of queens for insemination. The preinsemination flight operation described above for the drones was also applied for the receptive unprolific queens. However, at this point we have to make a remark: the queens in the experimental batch (140 individuals, corresponding to the experimental variants V3 and V4) were allowed to fly to eliminate faeces, one by one, only on the insemination day. We did so because we presumed that the accumulated faeces are less than in case of drones, and the insemination is carried out in the eighth day from hatching. The control queens (141 individuals, corresponding to the experimental variants V1 and V2) did not perform this flight, but were inseminated directly.

Sperm collection. The sample consisted in 5 "father" hives of which we chose, according to the above procedure, the drones whose sperm was collected as presented in the paper Mackensen & Ruttner (1976) (see also Stoian et al 2018).

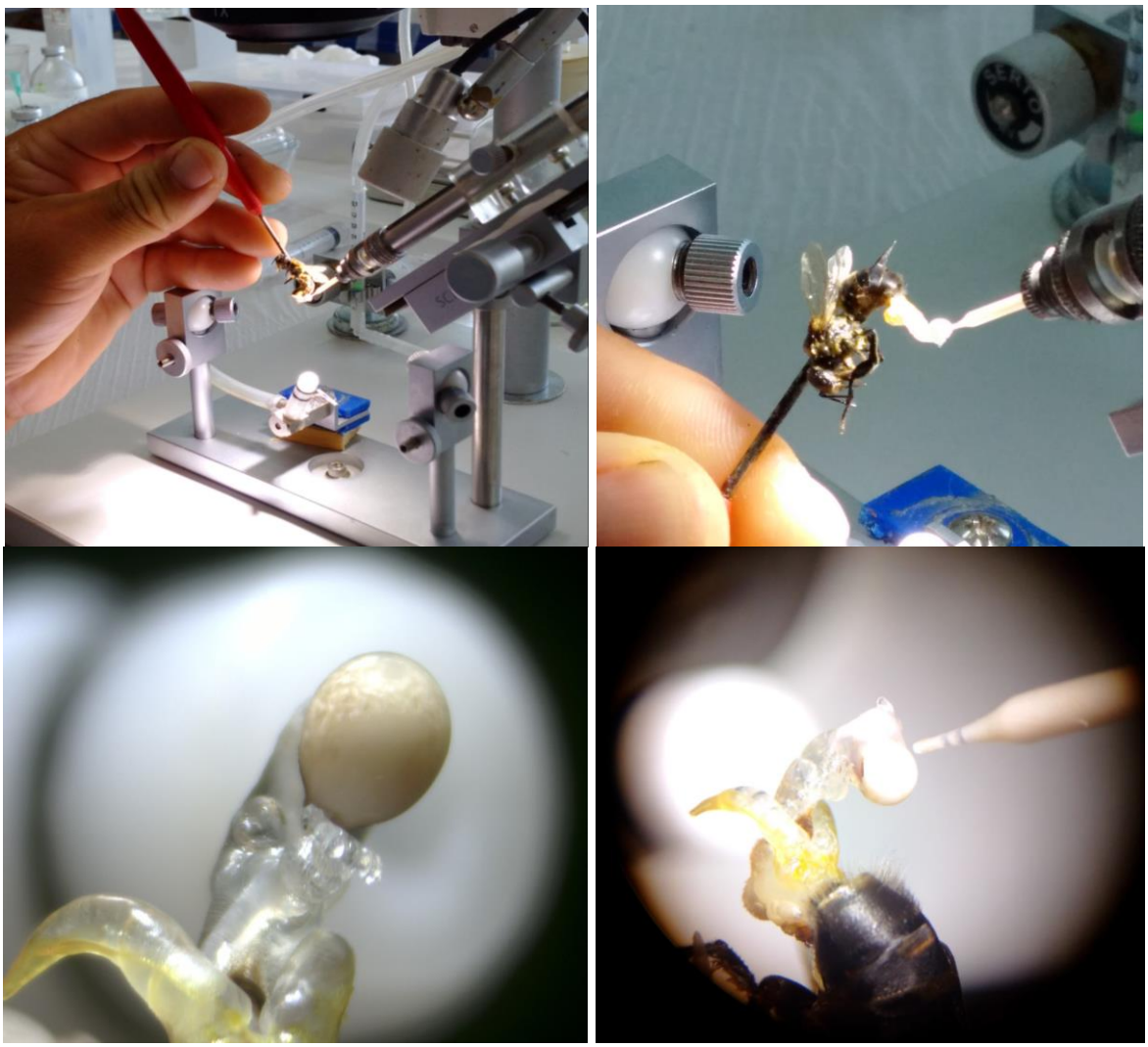

Figure 15. Sperm sampling from drones (original).

Insemination of the queens. The insemination of the queens was made according to the method presented by Ruttner (1976), as detailed in Stoian et al (2018) (see Figure 16).

In carrying out this activity we were helped by a collaborator. Thus, we managed to inseminate these 281 queens in only 4 days. The activity was organized as follows: i) first the sperm was collected from all the drones. The sperm was stored in special tubes called capillary tubes. These tubes (made of borosilicate glass with a capacity to inseminate 10 queens, 10 µL/queen, see Figure 17) were sealed at the ends and stored

in a dark place, at a constant temperature of 17.2-17.5^{0}C; ii) after completing this activity, the following one lasted for 4 days and implied the actual insemination of receptive unprolific queens.

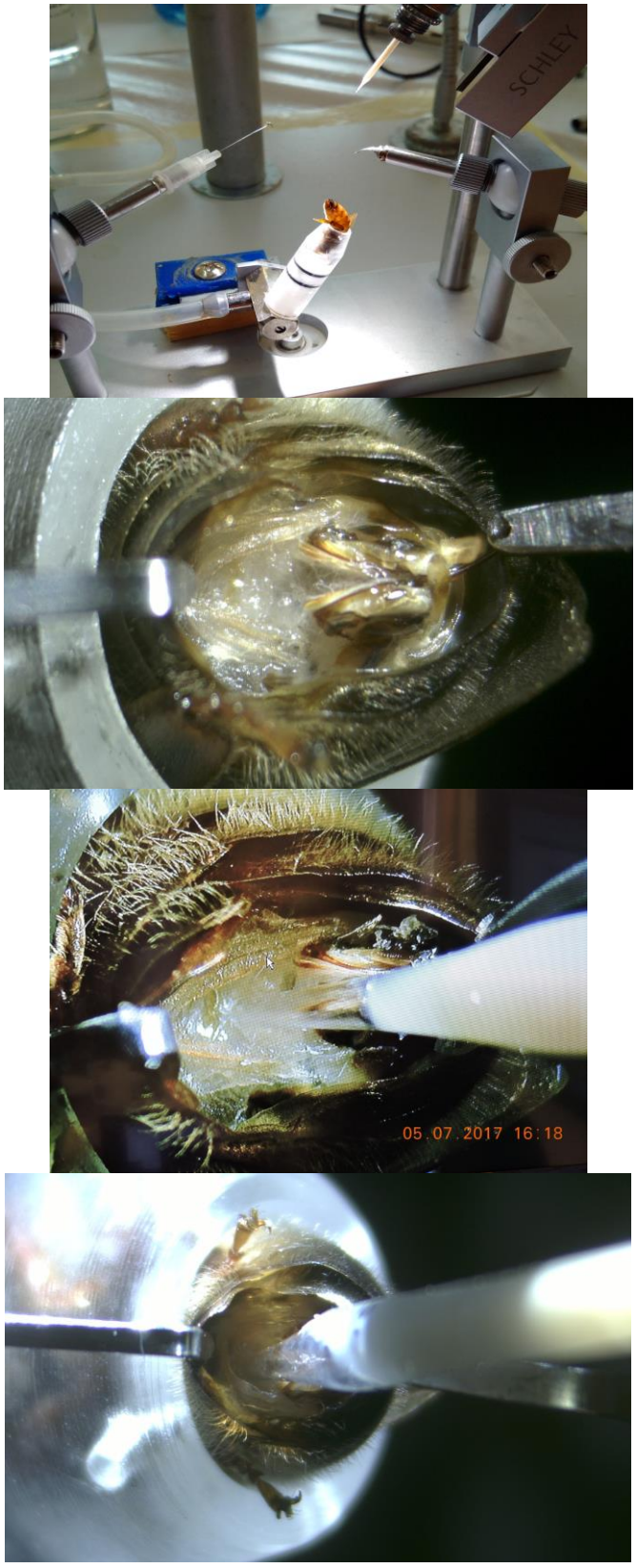

Figure 16. Artificial insemination of bee queens (original).

Figure 17. Borosilicate glass capillary tubes; they are stored in a protective metal packaging for the short-term preservation of drone sperm.

Within the sperm collection activity, the work was 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 could only be transferred as they were used. The time between the transfer and the use of the drone is maximum 30 minutes; otherwise, the physical cooling of the drone made the operation impossible. The transfer of the drones was accomplished in small number, and after their use the operation was repeated. Another task was that after their transfer in the special keeping box, the drones were 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 was working with the insemination device. In this case we used the Harbo syringe to collect the sperm of the drones. This person did not get in contact at all with other devices or with the drones; the only task was to take the drones on the spike and to collect the sperm.

Data collection and statistics. The research results data were recorded as notes in a notebook (Figure 18) for the three consecutive experimental years (2015, 2016 and 2017) and they were included in statistic programs. The study of correlations was conducted by means of the Statistica software, version 10.

Taking into consideration that the repetitions no. 2 (2016) and no. 3 (2017) should have been the identical copy of the first year experiment, we have striven to keep the numbers (individuals), the schedule (days of the year, month, season, temperatures) and the protocol (the experimental technique) identical or as similar as possible with those in the first year of research.

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 are alive in those colonies. After other approximately 4-6 days they are about to lay the egg sets in the honeycomb.

During these days we monitored the presence or the absence of the queen and whether the queens had a normal behavior or, on the contrary, an aberrant behavior.

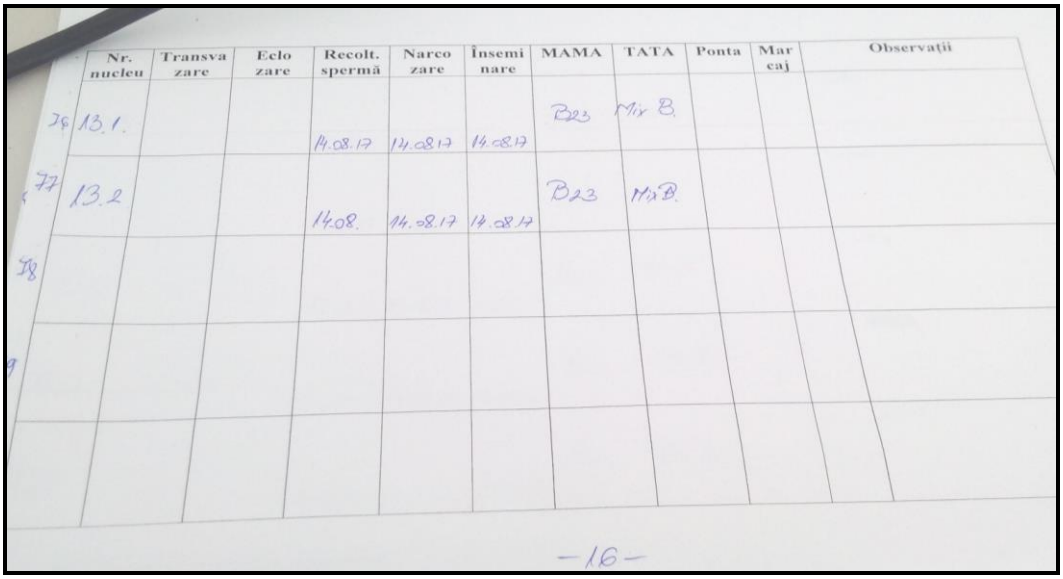

Figure 18. Retrieving raw data as notes on notebooks, registers, data sheets, etc.

Results and Discussion. The results of our research show 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 (Tables 2, 3 & 4).

Thus, the experimental variant V4 (drone that flew and queens that flew) has given the best results in terms of insemination success (Tables 2-4, Figure 19), survival of the queens from the initial number up to 5 days after insemination (Table 2-4, Figure 20), the eggs successfully laid from the initial number of queens (Tables 2-4, Figure 21) and the number/percentage of healthy descendant hives (as a result of the insemination performed) against the theoretical number (Table 2-4, Figure 22), while the experimental variant V1 (where neither the drones, nor the queens flew) has resulted in the weakest outcomes for the same health and productivity features.

Table 2

Results of experimental artificial insemination from 2015 (May to June)

Experimental variant	*V1*	*V2*	*V3*	*V4*
Number of queens available at the beginning of the experiment	71 queens (100%)	70 queens (100%)	70 queens (100%)	70 queens (100%)
Successful insemination	66 queens (92.96%)	67 queens (95.71%)	66 queens (94.29%)	68 queens (97.14%)
Survival of the queens from the initial number (up to 5 days after insemination)	21 queens (29.58%)	47 queens (67.14%)	30 queens (42.86%)	68 queens (97.14%)
Egg sets successfully laid (from the initial number of queens)	20 egg sets (28.17%)	43 egg sets (61.43%)	28 egg sets (40.00%)	67 egg sets (95.71%)
Healthy offspring/hives (as a result of instrumental insemination) against the theoretical number	18 hives (25.35%)	40 hives (57.14%)	25 hives (35.71%)	67 hives (95.71%)

Table 3

Results of experimental artificial insemination from 2016 (May to June)

Experimental variant	*V1*	*V2*	*V3*	*V4*
Number of queens available at the beginning of the experiment	71 queens (100%)	70 queens (100%)	70 queens (100%)	70 queens (100%)
Successful insemination	65 queens (91.55%)	66 queens (94.29%)	66 queens (94.29%)	68 queens (97.14%)
Survival of the queens from the initial number (up to 5 days after insemination)	22 queens (30.99%)	46 queens (65.71%)	31 queens (44.29%)	67 queens (95.71%)
Egg sets successfully laid (from the initial number of queens)	21 egg sets (29.58%)	42 egg sets (60.00%)	29 egg sets (41.43%)	67 egg sets (95.71%)
Healthy offspring/hives (as a result of instrumental insemination) against the theoretical number	18 hives (25.35%)	35 hives (50.00%)	28 hives (40.00%)	67 hives (95.71%)

The experimental variants V2 (where only the drones flew) and V3 (where only the queens flew) have indicated intermediate values between V1 and V4. Although, apparently, the hygiene of the genitors of both genders before the insemination influences the health and productivity features after insemination, one can see that the experimental variant V2 has given better results than variant V3. This is translated in the slightly bigger importance of drone hygiene than of the queen (Figures 19-22). In all cases, the statistical connection is significant and in most of the cases $p<0.001$, the statistical connection is highly significant (Tables 5-8).

Table 4

Results of experimental artificial insemination from 2017 (May to June)

Experimental variant	*V1*	*V2*	*V3*	*V4*
Number of queens available at the beginning of the experiment	71 queens (100%)	70 queens (100%)	70 queens (100%)	70 queens (100%)
Successful insemination	67 queens (94.37%)	68 queens (97.14%)	67 queens (95.71%)	68 queens (97.14%)
Survival of the queens against the initial number (up to 5 days after insemination)	23 queens (32.39%)	41 queens (58.57%)	38 queens (54.29%)	68 queens (97.14%)
Egg sets successfully laid (against the initial number of queens)	19 egg sets (26.76%)	39 egg sets (55.71%)	30 egg sets (42.86%)	68 egg sets (97.14%)
Healthy offspring/hives (as a result of instrumental insemination) against the theoretical number	17 hives (23.94%)	38 hives (54.29%)	26 hives (37.14%)	68 hives (97.14%)

Beside those presented above we calculated the correlation degree between the four experimental factors related to health and productivity, more exactly: F1- successful insemination; F2- survival of the queens against the initial number (up to 5 days after insemination); F3 - egg sets successfully laid (against the initial number of queens); F4 - healthy offspring/hives (as a result of instrumental insemination) against the theoretical number, for each of the four experimental variants (V1-V4). In most cases, although each factor visibly influences the other, the correlation degree between them is not significant from the statistical point of view. However, we found a significant direct correlation in the case of experimental variant V1, between the factors F1 (successful insemination) and F3 (egg sets successfully laid), as well as in the case of the experimental variant V2, between the factors F2 (survival of the queens against the initial number) and F3 (egg sets successfully laid) (Tables 9-12).

Table 5

Influence of the genitors preinsemination flight on the success of the insemination

Experimental variant	*Average*	*Std.Dv.*	*N*	*Std.Err.*	*Reference - Constant*	*T-value*	*Df*	*P*
V1	92.957	1.405	3.000	0.811	0.000	114.595	2.000	0.000
V2	95.707	1.425	3.000	0.823	0.000	116.329	2.000	0.000
V3	95.237	0.820	3.000	0.473	0.000	201.204	2.000	0.000
V4	97.140	-	3.000	-	0.000	-	2.000	-

Note 1: V1 - drones that did not fly; queens that did not fly; V2 - drones that flew; queens that did not fly; V3 - drones that did not fly; queens that flew; V4 - drones that flew; queens that flew.
Note 2: p <0.05 - the statistical correlation is significant (S, 95% confidence); p <0.01, the statistical correlation is significant (S, 99% confidence). p <0.001, the statistical correlation is highly significant (HS, 99.9% confidence); p> 0.05, the statistical correlation is insignificant (NS).

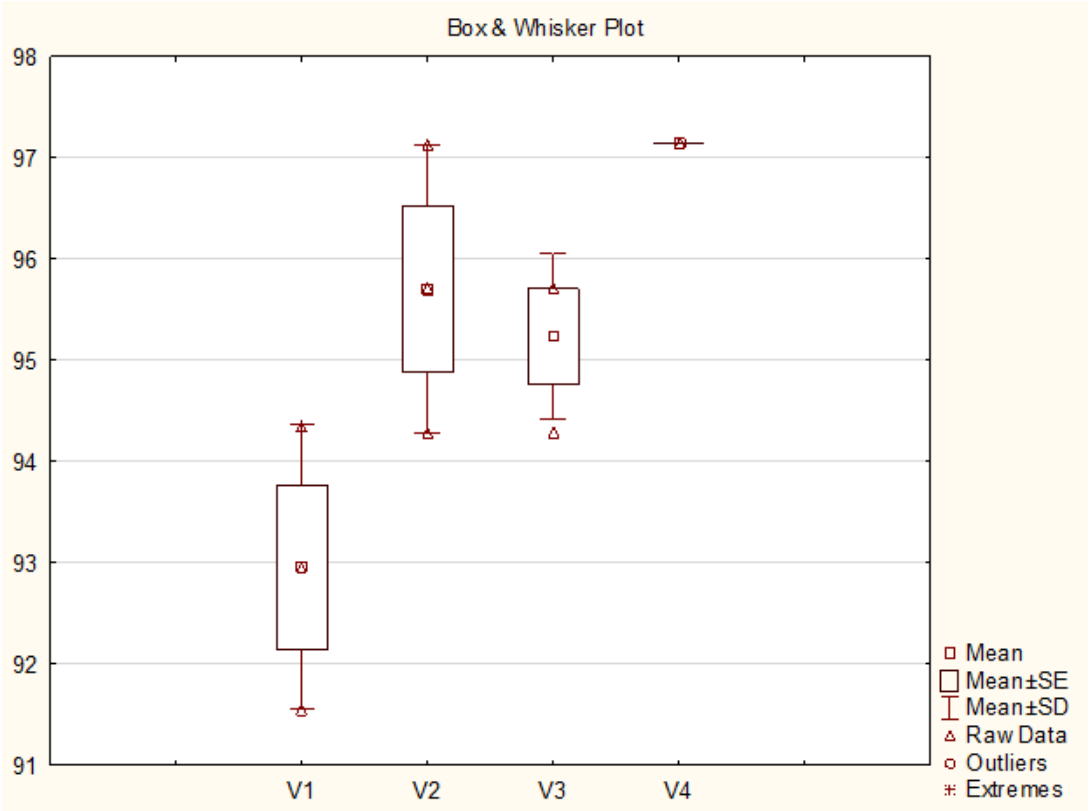

Figure 19. Influence of the genitors preinsemination flight on the success of the insemination. Note: V1 - drones that did not fly; queens that did not fly; V2 - drones that flew; queens that did not fly; V3 - drones that did not fly; queens that flew; V4 - drones that flew; queens that flew.

Table 6

Influence of the genitors preinsemination flight on queen survival (against the initial number of queens; up to 5 days after insemination)

Experimental variant	*Average*	*Std.Dv.*	*N*	*Std.Err.*	*Reference*	*T-value*	*Df*	*P*
V1	30.983	1.405	3	0.811	0.000	38.195	2	0.001
V2	63.803	4.597	3	2.654	0.000	24.041	2	0.002
V3	47.140	6.224	3	3.593	0.000	13.118	2	0.006
V4	96.663	0.826	3	0.477	0.000	202.790	2	0.000

Note 1: V1 - drones that did not fly; queens that did not fly; V2 - drones that flew; queens that did not fly; V3 - drones that did not fly; queens that flew; V4 - drones that flew; queens that flew.
Note 2: p <0.05 - the statistical correlation is significant (S, 95% confidence); p <0.01, the statistical correlation is significant (S, 99% confidence). p <0.001, the statistical correlation is highly significant (HS, 99.9% confidence); p> 0.05, the statistical correlation is insignificant (NS).

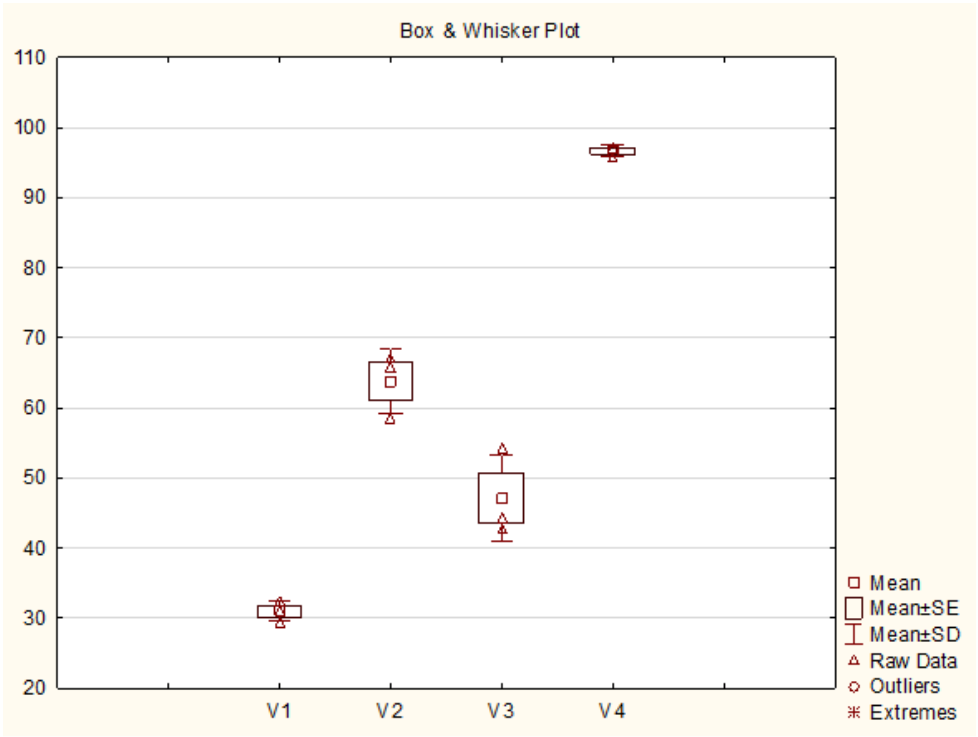

Figure 20. Influence of the genitors preinsemination flight on queen survival (against the initial number of queens; up to 5 days after insemination). Note: V1 - drones that did not fly; queens that did not fly; V2 - drones that flew; queens that did not fly; V3 - drones that did not fly; queens that flew; V4 - drones that flew; queens that flew.

Table 7

Influence of the genitors preinsemination flight on successful egg laying (against the initial number of queens)

Experimental variant	*Average*	*Std.Dv.*	*N*	*Std.Err.*	*Reference*	*t-value*	*df*	*p*
V1	28.167	1.405	3	0.811	0.000	34.723	2	0.001
V2	59.047	2.977	3	1.719	0.000	34.357	2	0.001
V3	41.423	1.425	3	0.823	0.000	50.349	2	0.000
V4	96.183	0.820	3	0.473	0.000	203.204	2	0.000

Note 1: V1 - drones that did not fly; queens that did not fly; V2 - drones that flew; queens that did not fly; V3 - drones that did not fly; queens that flew; V4 - drones that flew; queens that flew.
Note 2: p <0.05 - the statistical correlation is significant (S, 95% confidence); p <0.01, the statistical correlation is significant (S, 99% confidence). p <0.001, the statistical correlation is highly significant (HS, 99.9% confidence); p> 0.05, the statistical correlation is insignificant (NS).

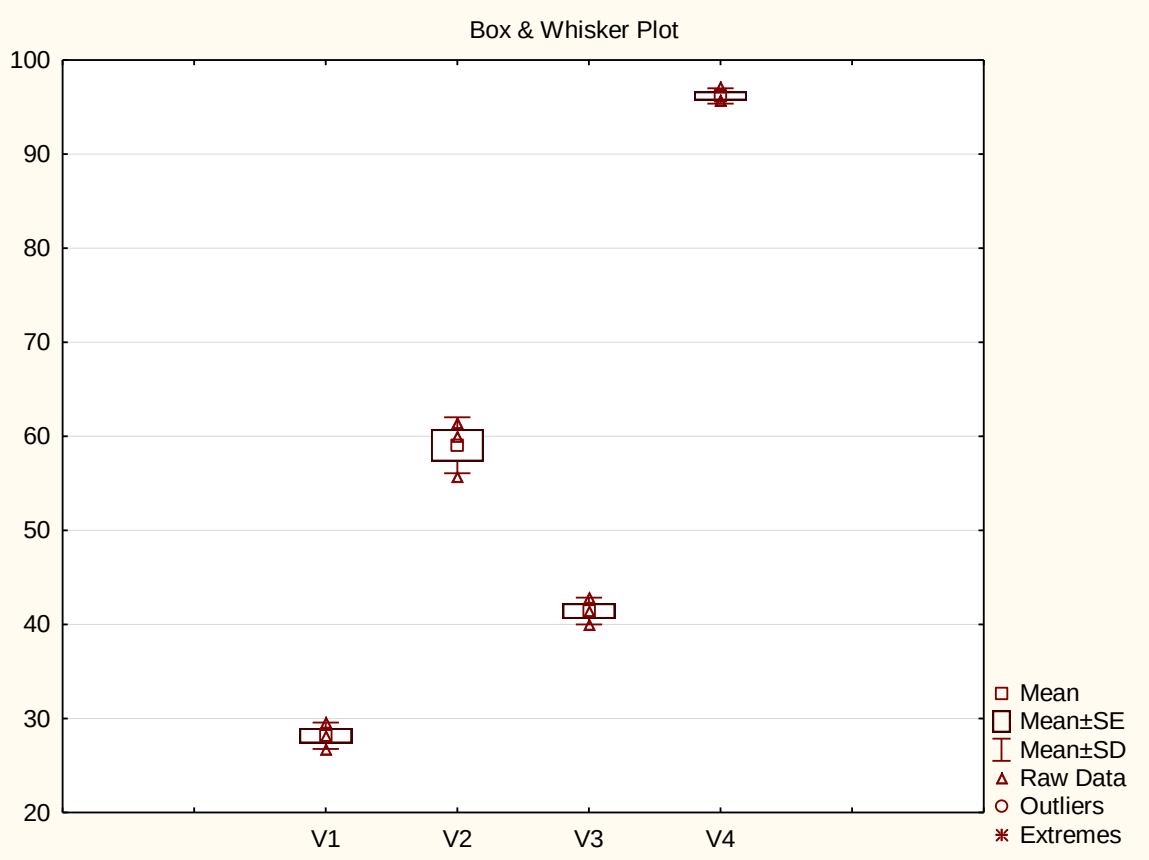

Figure 21. Influence of the genitors preinsemination flight on successful egg laying (against the initial number of queens). Note: V1 - drones that did not fly; queens that did not fly; V2 - drones that flew; queens that did not fly; V3 - drones that did not fly; queens that flew; V4 - drones that flew; queens that flew.

Table 8

Influence of the genitors preinsemination flight on healthy offspring/hives (as a result of instrumental insemination) against the theoretical number

Experimental variant	*Average*	*Std.Dv.*	*N*	*Std.Err.*	*Reference*	*t-value*	*df*	*p*
V1	24.880	0.814	3	0.470	0.00	52.936	2	0.000
V2	53.803	3.599	3	2.078	0.00	25.895	2	0.001
V3	37.610	2.180	3	1.259	0.00	29.882	2	0.001
V4	96.183	0.820	3	0.473	0.00	203.204	2	0.000

Note 1: V1 - drones that did not fly; queens that did not fly; V2 - drones that flew; queens that did not fly; V3 - drones that did not fly; queens that flew; V4 - drones that flew; queens that flew.
Note 2: $p < 0.05$ - the statistical correlation is significant (S, 95% confidence); $p < 0.01$, the statistical correlation is significant (S, 99% confidence). $p < 0.001$, the statistical correlation is highly significant (HS, 99.9% confidence); $p > 0.05$, the statistical correlation is insignificant (NS).

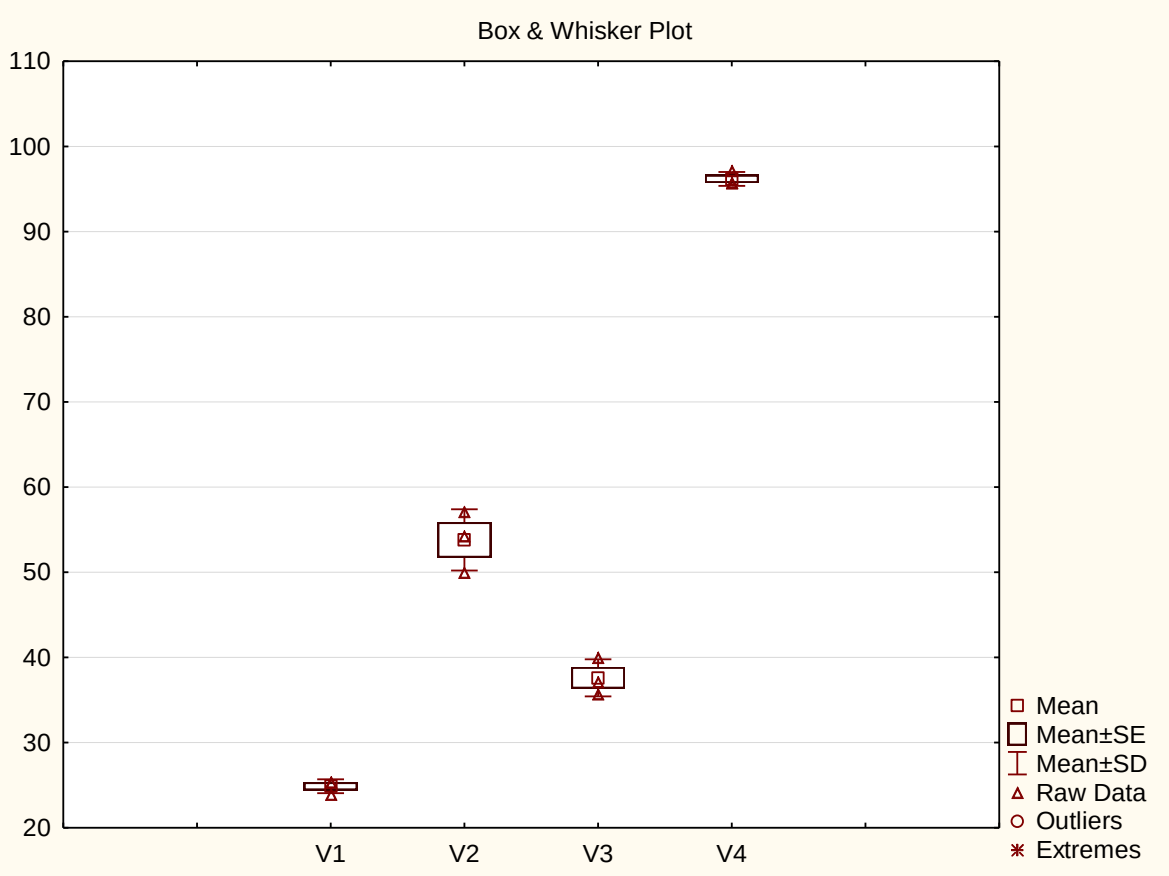

Figure 22. Influence of the genitors preinsemination flight on healthy offspring/hives (as a result of instrumental insemination) against the theoretical number. Note: V1 - drones that did not fly; queens that did not fly; V2 - drones that flew; queens that did not fly; V3 - drones that did not fly; queens that flew; V4 - drones that flew; queens that flew.

Table 9

Correlations between experimental factors for V1- drones that did not fly; queens that did not fly

	F1	*F2*	*F3*	*F4*
F1	-	0.667	0.003	0.335
F2	0.667	-	0.664	0.332
F3	0.003	0.664	-	0.332
F4	0.335	0.332	0.332	-

Note 1: F1 - Successful insemination; F2- Survival of the queens from the initial number (up to 5 days after insemination); F3 – Egg sets successfully laid (against the initial number of queens); F4 - Healthy hives (as a result of instrumental insemination) against the theoretical number.

Note 2: p <0.05 - the statistical correlation is significant (S, 95% confidence); p <0.01, the statistical correlation is significant (S, 99% confidence). p <0.001, the statistical correlation is highly significant (HS, 99.9% confidence); p> 0.05, the statistical correlation is insignificant (NS).

Table 10

Correlations between experimental factors for V2- drones that flew; queens that did not fly

	F1	*F2*	*F3*	*F4*
F1	-	0.434	0.489	0.592
F2	0.434	-	0.055	0.974
F3	0.489	0.055	-	0.919
F4	0.592	0.974	0.919	-

Note 1: F1 - Successful insemination; F2- Survival of the queens from the initial number (up to 5 days after insemination); F3 – Egg sets successfully laid (against the initial number of queens); F4 - Healthy hives (as a result of instrumental insemination) against the theoretical number.
Note 2: p <0.05 - the statistical correlation is significant (S, 95% confidence); p <0.01, the statistical correlation is significant (S, 99% confidence). p <0.001, the statistical correlation is highly significant (HS, 99.9% confidence); p> 0.05, the statistical correlation is insignificant (NS).

Table 11

Correlations between experimental factors for V3 - drones that did not fly; queens that flew

	F1	*F2*	*F3*	*F4*
F1	-	0.594	0.335	0.455
F2	0.594	-	0.259	0.951
F3	0.335	0.259	-	0.790
F4	0.455	0.951	0.790	-

Note 1: F1 - Successful insemination; F2- Survival of the queens against the initial number (up to 5 days after insemination); F3 – Egg sets successfully laid (against the initial number of queens); F4 - Healthy hives (as a result of instrumental insemination) against the theoretical number.
Note 2: p <0.05 - the statistical correlation is significant (S, 95% confidence); p <0.01, the statistical correlation is significant (S, 99% confidence). p <0.001, the statistical correlation is highly significant (HS, 99.9% confidence); p> 0.05, the statistical correlation is insignificant (NS).

Table 12

Correlations between experimental factors V4 - drones that flew; queens that flew

	F1	*F2*	*F3*	*F4*
F1	-	-	-	
F2	-	-	0.667	0.667
F3	-	0.667	-	-
F4	-	0.667	-	-

Note 1: F1 - Successful insemination; F2- Survival of the queens against the initial number (up to 5 days after insemination); F3 – Egg sets successfully laid (against the initial number of queens); F4 - Healthy hives (as a result of instrumental insemination) against the theoretical number.
Note 2: p <0.05 - the statistical correlation is significant (S, 95% confidence); p <0.01, the statistical correlation is significant (S, 99% confidence). p <0.001, the statistical correlation is highly significant (HS, 99.9% confidence); p> 0.05, the statistical correlation is insignificant (NS).

Further on we will comparatively address and discuss the results obtained in the experimental variants V1 and V4, variants that significantly differ from one another in terms of the values of all the four factors related to health and productivity that were monitored (F1-F4).

While in case of the experimental variant V4 (97.14% successful inseminations) the failure difference up to 100% is due to some occupational accidents, to incorrect handling of the queens, to negligence or some natural defects of the queens, in the variant V1 (92.96% successful inseminations) the frequency of unsuccessful inseminations is amplified by the difficulty of using some genitors with the digestive tube full of faeces, which makes the artificial insemination work much more difficult (tab.5).

Also, the mortality of queens over the following 5 days after the artificial insemination differs significantly between V1 (30.98% mortality against the initial number) and V4 (96.66% mortality against the initial number) (tab. 6) due to some

diseases occurred in the case of V1 and mainly diagnosed as septicemia (melanosis B/ bacterial melanosis, Figure 23) and paralysis.

In some of the colonies of the experimental variant V1 (but also in case of some colonies in V2 and V3) we noticed the absence of the queens (it is likely they died and they were removed from the colony). In case of other colonies (most of them) of the same experimental variants we noticed that the queens had swollen abdomen and they hardly moved on the honeycomb, they were walking slowly, behavior that characterized some diseases. In some colonies of the experimental variants V1, V2 and V3, the queens were found dead in the colonies.

Other queens of the experimental variants V1, V2 and V3 have laid the eggs sets, but shortly they were found dead on the bottom of the micronucleus after about 5-6 days. For V4 the egg sets were laid successfully in about 96.18% of the cases against the initial number of queens and theoretically expected egg sets. For the V1 variant only 28.17% of the egg sets were laid successfully (Table 7).

The survival of the queen and the successful laying of the egg sets facilitated a good output of those colonies, measured both in terms of number of colonies (V1 - 24.88, V4 - 96.18; Table 8), but especially as the density of worker bees and productivity within these colonies (Figures 24 & 25).

Figure 23. Queen killed by melanosis (source: http://windowbee.com/melanosis_en/).

Figure 24. Honeycomb with larvae in different stages of development, a positive result of the artificial insemination (original).

Figure 25. Healthy and vigorous hives obtained by insemination, with "plenty" of biomass and harvesting products (original).

Regarding the two diseases, the septicemia and the paralysis, none of them is considered an extremely dangerous disease in common beekeeping management. However, in the artificial insemination procedure, they can cause very big losses if not kept under control.

Ruttner (1976) said that a queen that has recently gotten septicemia (melanosis B) during the insemination dies after about 1-2 days, which is also proven by our experiments. In the case of septicemia, one can notice typical organic changes as black spots on various internal organs: venom glands, ovaries, Malpighi tubes, etc, an aspect that Fyg described in 1934 as "bacterial melanosis" (Fyg 1964). After investigating the published literature it appears that several bacterial species are involved in this bee disease.

In the European continent, the septicemia presents symptoms similar to the bee paralysis, i.e. after insemination the queen does not start to lay eggs or simply pauses shortly after initiation. The movements of the queen become soft and insecure, the abdomen is visibly swelling. After this episode, the queen falls from the honeycomb on the bottom of the hive and remains there for a while before it dies (Ruttner 1976).

In 1969 Mackensen (cited by Ruttner 1976) has noticed and described an acute form of the septicemia in the United States. In this case the queens begin to die already one or two days after insemination, as their body comes apart in a very specific manner.

In a personal communication, Maul was writing to Ruttner (1976) about his observations at the Kirchhain insemination station. He said that this illness occurs already before the insemination also in the queens inseminated naturally. Therefore, the researcher concluded that contamination may take place without insemination, in the mating nuclei.

The other disease we identified as a cause of mortality within the experiment, the paralysis, is caused by a virus. The queens contaminated with this virus, reinserted in their nucleus after the artificial insemination live a few days and then they disappear or gradually become apathetic and swollen with liquid. Under such illness conditions, they can live a few more days on the honeycombs or fallen on the bottom of the hive, and some lay some eggs before they show the disease symptoms (but the egg laying fails in most of the cases).

Paralysis can be avoided to a significant extent by using marked drones that fly freely or drones kept closed and that are not three weeks old yet (Ruttner 1976).

Thus, inspired by Ruttner, we conducted numerous preliminary studies on the influence of the drones and queens preinsemination flight on the success of artificial reproduction. Such preliminary studies were based on pure observations and they were initiated before the present studies, and the latter have numerically and statistically confirmed the first hypotheses that we have issued.

The contamination of the sperm may be a major cause for the diseases that occurred and hence for queens mortality. One of the main requirements is to pay

permanent attention to the hygiene conditions. When the disease exists in the apiary, all tools that come in contact with the queen, must be sterilized. According to the published literature (compiled by Ruttner, 1976) and based on our experience, the incidence of queen mortality as a result of the artificial insemination procedure is rather high and it is due mostly to the melanosis (septicemia).

At the same time, we also identified the moment when the queens fell ill because of the procedure. During the sperm collection activity and queen artificial insemination, both the drones and the queens defecate. This is the sequence where the contamination of the queens occurs. Septicemia and the paralysis spread removal hooks, and the valve probe.

As the drones are kept captive (about 15 days after hatching) in the isolation cage in the hive from their "birth" to the moment of sperm collection, they accumulate faeces in the digestive tube. When the endophallus eversion operation for sperm collection is complete, due to the pressure exerted on the thorax and the abdomen with two fingers, the drones have the tendency to eliminate faeces together with the sperm. This results in the contamination of sperm, hands and instruments used for insemination.

Although the published literature recommends that drones should be allowed to fly before collecting sperm from them (Ruttner, 1976), it does not mention how and when this should be performed, nor how important this aspect is. As for the queens, there is no mentioning in this regard.

Our study shows that by including some preinsemination flight sequences has significant advantages on the success of the insemination (4.18% better), survival of the queens in the initial number (up to 5 days after insemination) (65.68% higher), the successfully laid egg sets (against the initial number of queens) (68.02 more frequent) and the frequency of healthy descendant hives (as a result of instrumental insemination) against the expected theoretical number (71.30% more numerous).

It is important to note here that our calculation procedure is additive to the findings related to the monitored factors, and the final values were related to the initial number of individuals in the experimental batch. Therefore, the frequency of the healthy descendant hives is the additive result of the other three monitored factors, i.e. the successful insemination, the survival of the queens against the initial number and the egg sets successfully laid. This way, the differences are much easier to comparatively analyze and if the preinsemination flight has indeed an influence on the reproduction by artificial insemination, then these are more perceptible.

Conclusions and Recommendations. The results of our research show 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.

Therefore, the experimental variant V4 (drones that flew and queens that flew) has given the best results in terms of the successful insemination, the survival of the queens against the initial number up to 5 days after insemination, the successfully laid egg sets against the initial number of queens and the number/percentage of healthy offspring/hives (as a result of instrumental insemination) against the theoretical number, while the experimental variant V1 (where neither the drones, nor the queens flew) has resulted in the weakest outcomes for the same health and productivity features.

The experimental variants V2 (where only the drones flew) and V3 (where only the queens flew) have indicated intermediate values between V1 and V4. Although, apparently, preinsemination hygiene of both genders of genitors influences the health and productivity features after insemination, one can see that the experimental variant V2 has given better results than variant V3. This is translated in the slightly bigger importance of drone hygiene than of the queen. In all cases, the statistical connection is significant and in most of the cases $p<0.001$, the statistical connection being highly significant.

While in case of the experimental variant V4 (97.14% successful inseminations) the failure difference up to 100% is due to some occupational accidents, to incorrect

handling of the queens, to negligence or some natural defects of the queens, in the variant V1 (92.96% successful inseminations) the frequency of unsuccessful inseminations is amplified by the difficulty of using some genitors with the digestive tube full of faeces, which makes the artificial insemination work much more difficult.

Also, the mortality of queens over the following 5 days after the artificial insemination differs significantly between V1 (30.98% mortality against the initial number) and V4 (96.66% mortality against the initial number) due to some diseases occurred in the case of V1 and mainly diagnosed as septicemia (melanosis B/ bacterial melanosis) and paralysis.

In some of the colonies of the experimental variant V1 (but also in case of some colonies in V2 and V3) we noticed the absence of the queens (it is likely they died and they were removed from the colony). In the case of other colonies (most of them) of the same experimental variants we noticed that the queens had swollen abdomen and they hardly stayed on the honeycomb, they were walking slowly, behavior that characterized some diseases. In some colonies of the experimental variants V1, V2 and V3, the queens were found dead in the colonies.

Other queens of the experimental variants V1, V2 and V3 have laid the eggs sets, but shortly they were found dead on the bottom of the micronucleus (about 5-6 days). For V4 the egg sets were laid successfully in about 96.18% of the cases against the initial number of queens and theoretically expected egg sets. For the V1 variant only 28.17% of the egg sets were laid successfully.

The survival of the queen and the successful laying of the egg sets facilitated a good output of those colonies, measured both in terms of number of colonies (V1 – 24.88, V4 – 96.18), but especially as the density of worker bees and productivity within these colonies.

In terms of inducing the dejection of queens and drones by preinsemination flight to be sure that the faeces have been eliminated, we determined that the drone flight in the specially designed space in the laboratory should be performed twice (on the day before the insemination and once more on the following day, about 15 minutes before collecting the sperm), and for the queens we concluded that these should only fly on the insemination day.

As for the recommendations during the insemination procedure itself, in order to reduce the risk of queen contamination it is ideal to perform this operation by carefully planning this work. This can be carried out by two persons on different days or in distinct stages. During the first stage, the sperm collection activity is carried out. The first person prepares the drones for sperm collection by stimulating them and by endophallus eversion. The second person comes in contact only with the sperm collection device and the syringe. Thus, the risk of sperm and tools contamination with pathogen agents is greatly reduced. During the second stage the insemination itself will be carried out. Again, the activity needs to be carefully planned: the first person prepares the biological material (receptive unprolific queen) consisting in the activities listed above and the second person performs the insemination.

By the strict division of the activities between the two persons, the gain will be twofold: on the one hand, the contamination risk decreases considerably and on the other hand the number of artificially inseminated queens increases per time unit.

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