CHAPTER III

GENERAL MATERIALS AND METHODS

3.1. Collecting and maintaining drones

Mature drones of *Apis mellifera*, *A. cerana*, *A. dorsata* and *A. florea* were sampled for insemination with *A. mellifera* queens. There were different methods to collect mature drones of *A. mellifera*, *A. cerana*, *A. dorsata* and *A. florea* in this experiment as follow:

3.1.1. Collecting drones of *A. mellifera*

The drone flight period of European honeybees *A. mellifera* was between 12.15 - 17.00 (Rutter, 1966; Tribe,1982; Koeniger,1989). Mature drones of *A. mellifera* were caught at the entrance of colonies during mating flight time at Chulalongkorn University (Bangkok, Thailand) and Institut für Bienenkunde, (Oberursel, Germany).

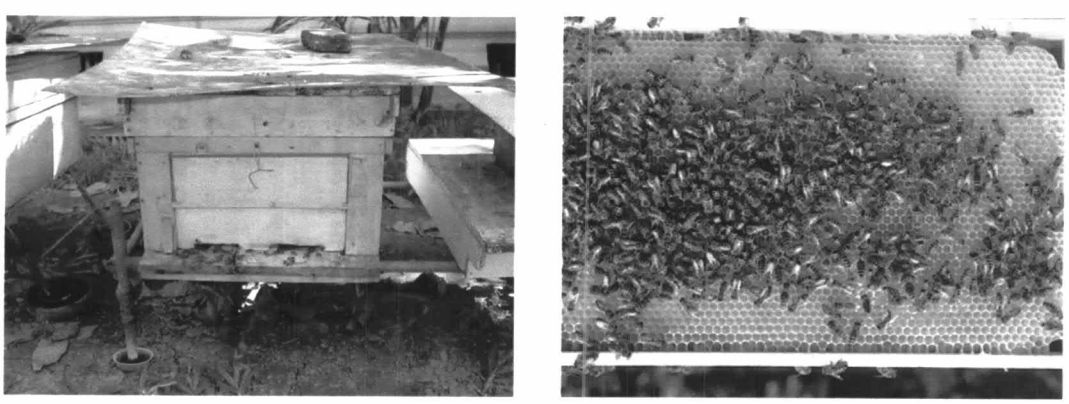


Figure 3.1. *A. mellifera* colonies were introduced to establish at Chulalongkorn University.

Figure 3.2. *A. mellifera* drones at the entrance of colony during mating flight time.

3.1.2. Collecting drones of *A. cerana*

In Eastern honeybees *A. cerana*, the period of drone flight in Thailand was 15.15 - 17.30 (Rinderer *et al.*, 1993). Mature drones of *A. cerana* were caught at the entrance of colonies during mating flight time at Chulalongkorn University (Bangkok, Thailand).

Figure 3.3. *A. cerana* colonies were introduced to establish at Chulalongkorn University.

Figure 3.4. *A. cerana* drone at the entrance of colony during mating flight time.

3.1.3. Collecting drone of *A. florea*

The colonies of *A. florea* with plenty of drones were taken from Samut Songkram province afterwards the colonies were introduced to hung under branches of small trees at the balcony on the second floors in front of Bee Biology Research Unit in Chulalongkorn University. The drone flight period of *A. florea* in Bangkok occurred between 13.45 and 15.30 (Koeniger *et al.*, 1989). From observation, mature drones climbed up to the top of the colony before flying from the colony for mating flight. After coming back from mating flight, they landed on the top of the colony. Thus, mature drones could be sampled, caught on the top of the colony during mating flight time by using forceps, and put into a cage. When all drones were sampled from the colony, new colonies were taken from Samut Songkarm province. Alternatively, sealed drone broods were brought from Jatujark market in Bangkok.

There were two methods for management of sealed drone broods comb.

- First; sealed drone broods were hung directly by connecting them at the bottom of the colony. The worker bees in the colony would take care of sealed drone broods comb until drone emergence.
- Second; sealed drone broods comb were kept in an incubator at 34 °C, 60-70% RH until they emerged. Newly emergent drones were transferred directly onto the top of colony. In other choice, took a sealed brood comb containing

emergent drones under the colony and touch together slightly in order that drones climbed to the colony.

Figure 3.5. *A. florea* colonies were introduced to hung under small branches of trees.

Figure 3.6. *A. florea* drones were located on the top of the colony. (Photo from Institut für Bienenkunde).

3.1.4. Collecting drones of *A. dorsata*

Drones of *A. dorsata* (Giant honeybee) fly at the dusk from 18.15 until 19.05 (Koeniger,1988,1994). Mature drones of *A. dorsata* were caught at a drone congregation area (DCA) under the canopy of bee tree at the agricultural research station near Tenom (Sabah, Malaysia). For catching drones at DCA, we used a big trap and a dummy (a small piece of a black pencil; 3.4 cm length, 8 mm diameter) containing 1 mg 9-oxo-2-trans decenoic acid (9-ODA) to attract drones to the dummy

(Koeniger *et al.*, 1979). The big trap (6 feet Ø) was made from a mosquito net. The dummy was tied with thread and fixed at the trap about 5 m below the opening. Also, big trap was fixed about 5 m below the canopy of a tree. When a large number of *A. dorsata* drones were attracted by the dummy, the line of trap was loosen. The trap rapidly fall down crashing on the ground. The drones inside the trap were caught carefully and collected into cages.

Figure 3.7. (A) A drone congregation area of *A. dorsata*; (B) A hanging trap under a tree for catching drones; (C) Drones collecting inside the trap. (Photo from Institut für Bienenkunde).

Figure 3.8. *A. dorsata* colony hanging under a branch of a big tree. (Photo from Institut für Bienenkunde).

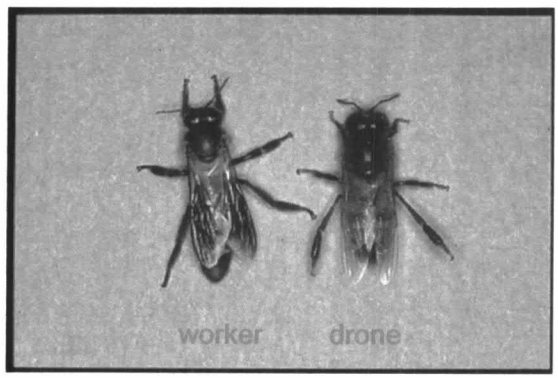


Figure 3.9. Drone and worker of *A. dorsata*. (Photo from Institut für Bienenkunde).

3.2. Storage drones

Captured drones were put in a cage at least 1 hr before obtaining semen. In case of *A. dorsata* and *A. cerana*, whose drones could not be used on the same day of collection, drones would be kept in a cage containing workers and small pieces of candy to feed drones. The ratio of drone and worker in the cage was 1:1. Afterwards, they were kept in a dark place at room temperature (25°C). In *A. mellifera*, drones were kept in a drone cage within the colony (figure 3.10.). A cage contain a wood frame (width: 10 cm, length: 11.5 cm and height: 2.5 cm), both sides consist of queen excluders that worker can pass into the cage to feed drones but drones can not leave the cages.

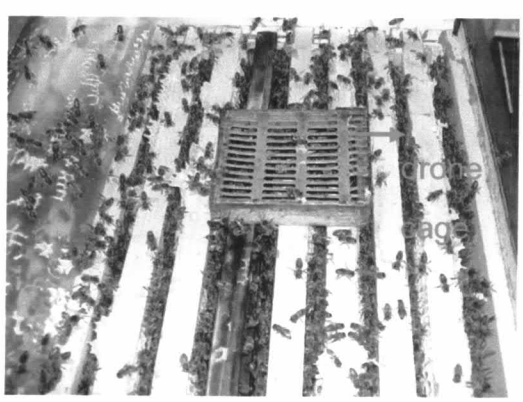


Figure 3.10. *A. mellifera* drones were kept in a drone's cage on the top of a colony.

3.3. Preparation of drones for collection of spermatozoa

Drones were transferred from the cage to a small box (width: 23 cm, length: 17 cm and height: 23 cm). The small box should be big enough for drone flight and for a researcher's hand to insert inside to catch drones for semen collection. One side of the small box should be easily open and made from transparent plastic. In the box, there were workers and candy in a small rectangular cage to feed drones. About a rectangular cage (width: 15 cm, length: 11 cm and height : 3 cm), one side was made of clearly plastic which was easily open while the other side was made of wire that had small hold in order not to let workers pass.

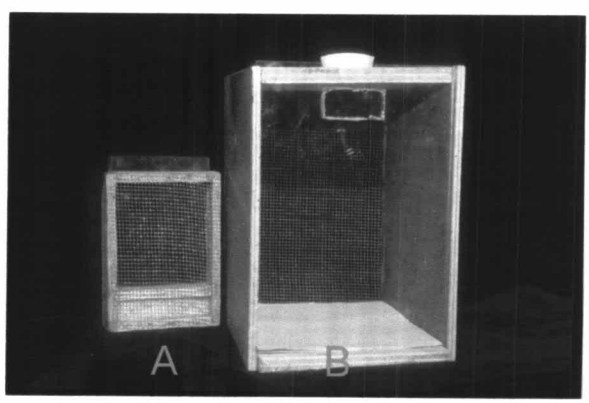


Figure 3.11. (A) A rectangular cage; (B) A small box
(Photo from Institut für Bienenkunde).

3.4. Obtaining spermatozoa from drones

3.4.1. Obtaining semen from eversion of drone's endophallus

Eversion of endophallus was accompanied by ejaculation of the seminal fluid. A drone was easily stimulated by hand in a two step process; the partial and the full eversion.

3.4.1.1. The partial eversion

A partial eversion was obtained by holding a drone by a head and thorax and teasing abdomen. Further stimulation was necessary, the drone was crush at the head and thorax. It was also necessary to apply gentle pressure to the tip of the abdomen to stimulate the eversion. During partial eversion, the abdomen contracted and exposed the cornua (horns) of the endophallus.

3.4.1.2. The full eversion

To obtain a full eversion, a drone was pressed along the sides of the anterior abdomen with a thumb and forefinger. The abdomen was squeezed laterally, starting at the anterior base of the abdomen and working toward the tip by rolling the fingers together in strong and steady motion until the abdominal muscle contracted and endophallus inside out to expose the semen (Woyke and Ruttner, 1958).

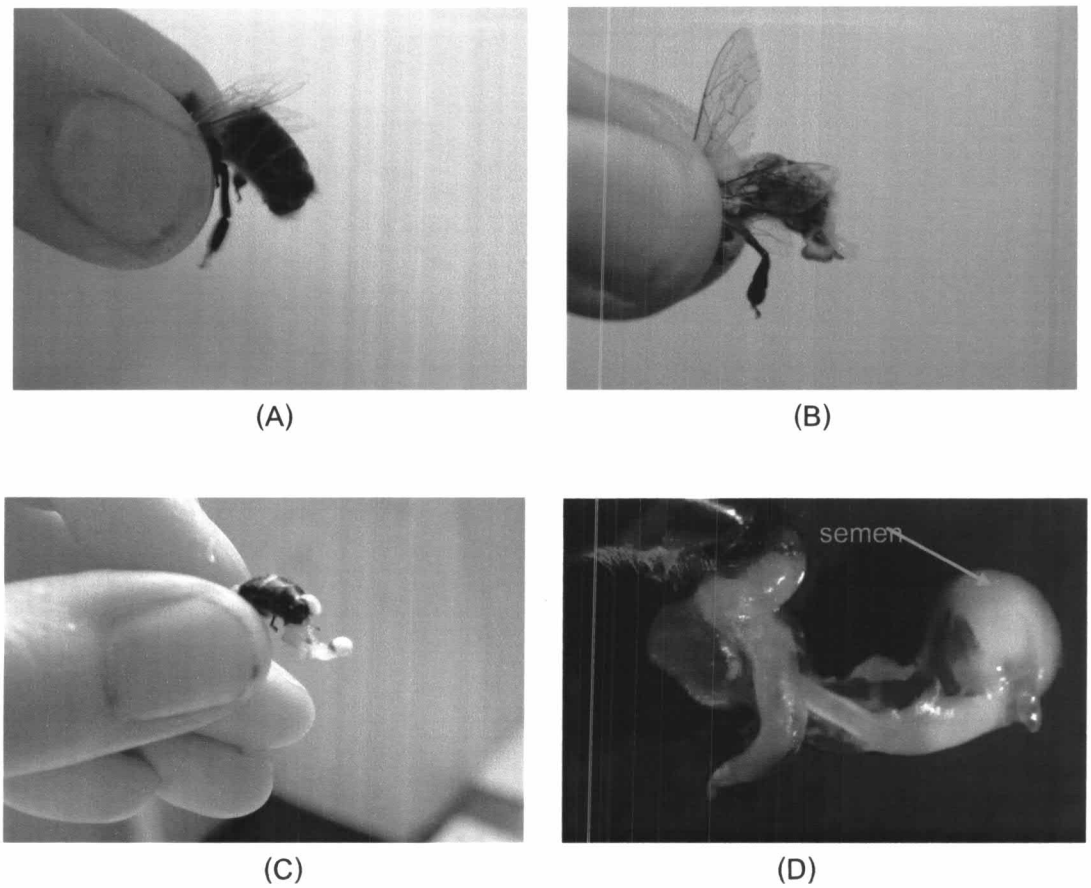


Figure 3.12. Obtaining semen from a mature drone of *A. mellifera* by eversion of the endophallus. (A) Squeezing the head and thorax of drone; (B) Abdomen contracted and exposed the cornua (horns) of the endophallus; (C) and (D) Lateral squeezing of the tergid abdomen, semen and mucus were discharged at the end of everted endophallus.

3.4.2. Obtaining spermatozoa from seminal vesicles

Drones were narcotized with CO_2 to prevent semen ejaculation during dissecting under stereo-microscope (approximately 10x, 20x or 40x magnification which depends on drone species). Drones were grasped at the thorax (head down) between a thumb and an index finger on the left hand. The first tergite of the tip of the abdomen was cut, afterwards the base of endophallus was grasped with fine forceps and the whole reproductive system was pulled out carefully from the abdomen (figure 3.13).

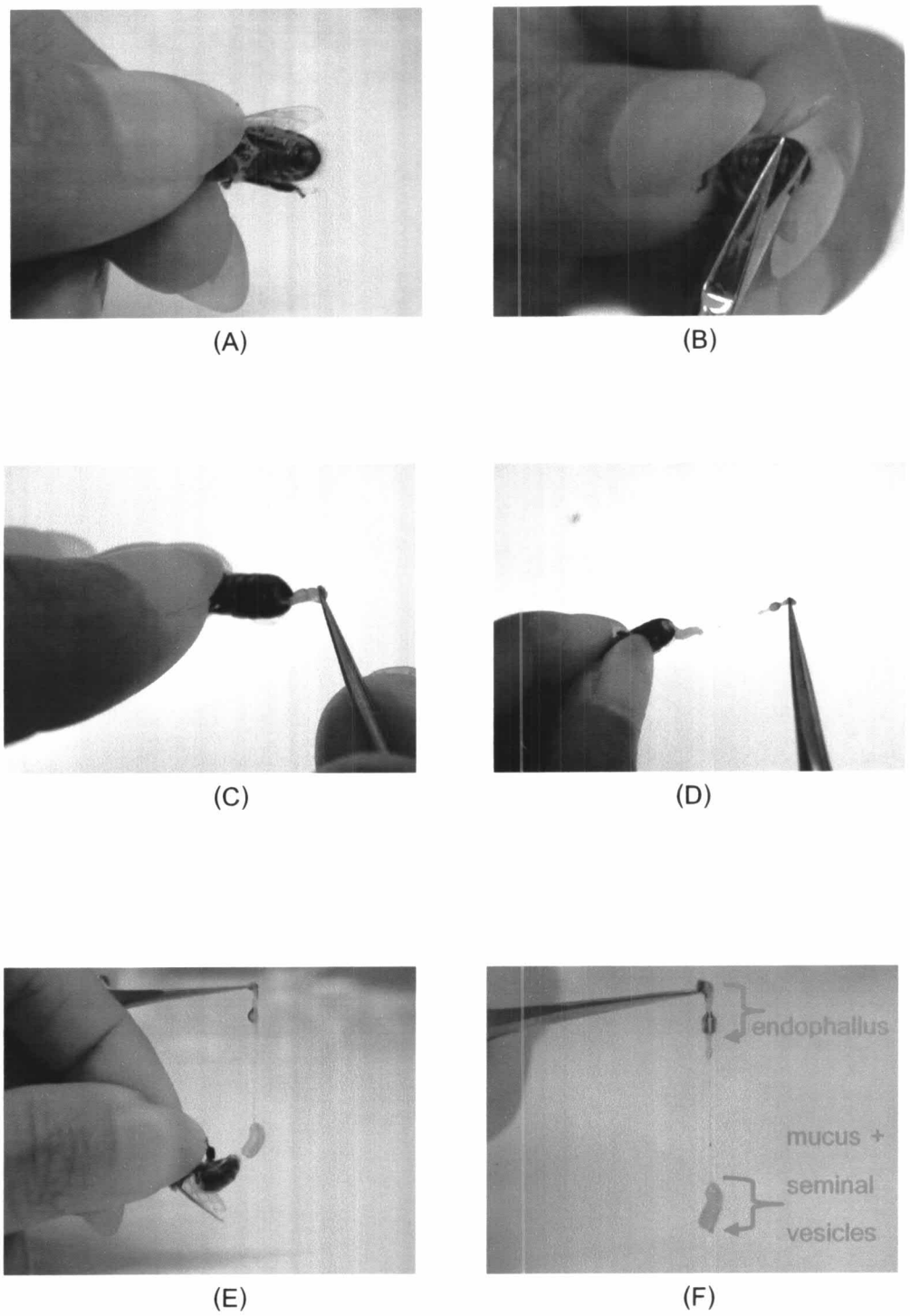


Figure 3.13. Dissection of drone to remove reproductive organs; (A) A drone was grasped at the thorax; (B) The first tergite of abdomen was cut; (C)- (F) Reproductive organs were removed with fine forceps.

The reproductive organs were transferred into a small black vessel (to increase contrast of tissue against a container) containing Tris buffer, Hyes solution or 0.9% NaCl. The mucus glands and seminal vesicles were separated from the endophallus with fine forceps at ejaculatory duct (figure 3.14 (A)). The mucus glands and the seminal vesicles were washed with Tris buffer, Hyes solution or 0.9% NaCl ,3 times (approximately 2 ml per wash) to prevent contamination from the intestine. The seminal vesicles were separated from mucus glands at their basal connective (figure 3.14 (B)) with fine forceps and transferred into a new black vessel containing 0.5 ml Tris buffer, Hyes solution or 0.9% NaCl . Seminal vesicles were grasped and torn at the tip with fine forceps. Afterwards, they were pressed continuously from the basal connective with fine forceps to the cut end to release semen from seminal vesicles. Semen were moved out and dispersed in buffer (figure 3.14 (C)).

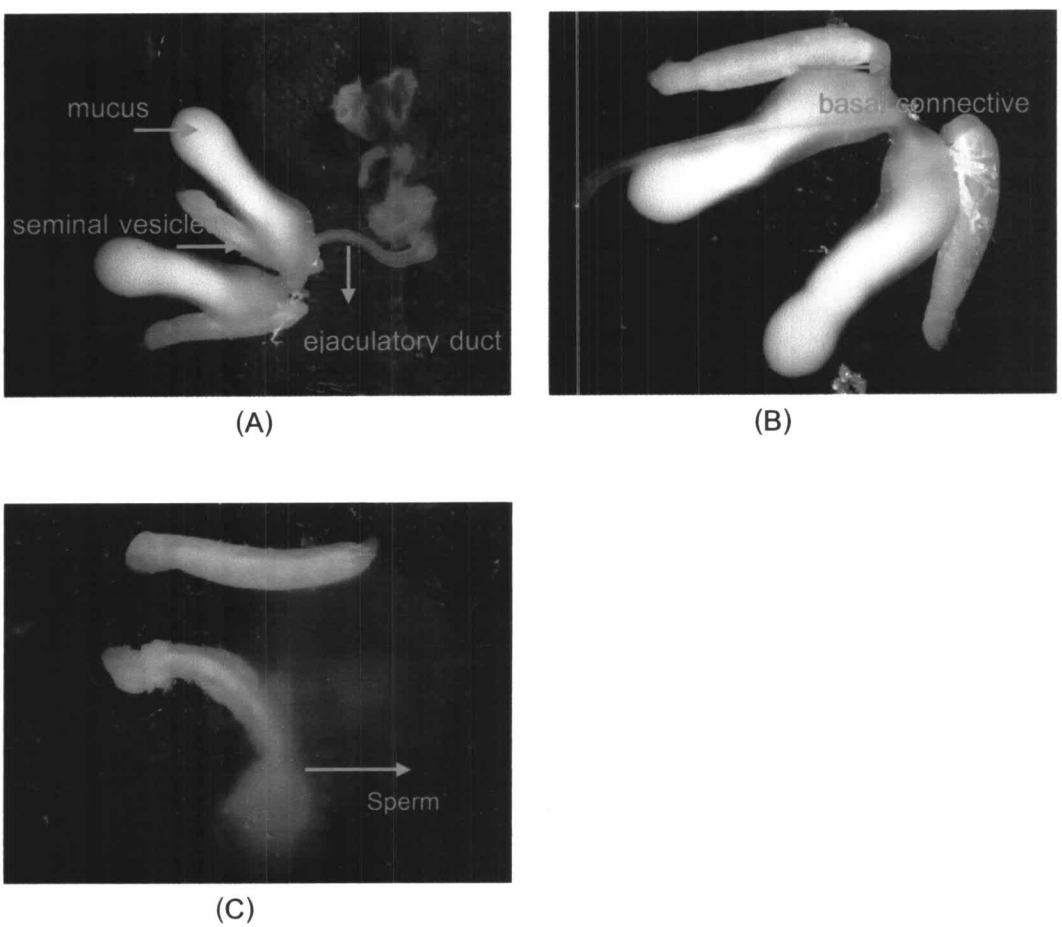


Figure 3.14. Obtaining semen from seminal vesicles of *A. mellifera* drone.

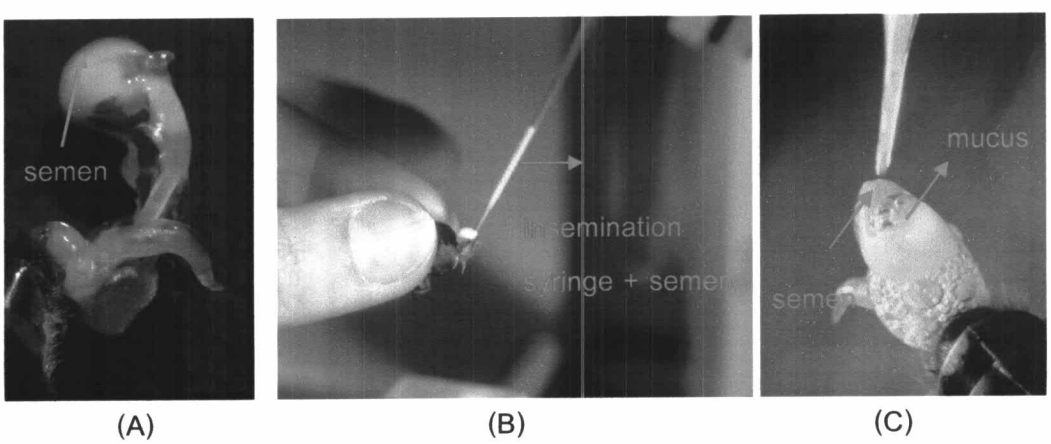
3.4.3. Obtaining spermatozoa from seminal vesicles and reconcentrated spermatozoa by centrifugation

Spermatozoa collected from seminal vesicles (according to 3.4.2) were diluted and dispersed in 300 µl Tris buffer. The spermatozoa were transferred to 0.5 ml sterile micro-centrifuge tube, and were spun to reconcentrate spermatozoa by centrifugation at 1,000 g for 10 min, 10,000 g for 10 min and for 30 min.

3.5. Collecting semen into an insemination syringe

3.5.1. Collecting semen from eversion's endophallus into a insemination syringe

A glass tip and insemination syringe had to be sterilized and filled with Tris buffer before semen collection. Before taking semen into tip of insemination syringe, the Tris buffer had to be moved back from the tip about 3 µl. It was essential to have air space from the tip of insemination syringe to separate buffer and semen to prevent dilution of the semen. Focusing a microscope at the end of syringe tip and ejaculated semen, the syringe tip touched with the semen and then pulled the semen slightly to avoid contamination from mucus. Semen was drawn into the tip. After the syringe was filled with semen, about 1 µl of Tris buffer was taken into the tip to prevent drying of semen.



(A) (B) (C)

Figure 3.15. (A), (B) and (C) Collecting ejaculated semen from eversion's endophallus into a insemination syringe.

3.5.2. Collecting spermatozoa from seminal vesicles into an insemination syringe

Spermatozoa collected from seminal vesicles and diluted with Tris buffer (according to 3.4.2) were sucked directly in a glass tip of insemination syringe.

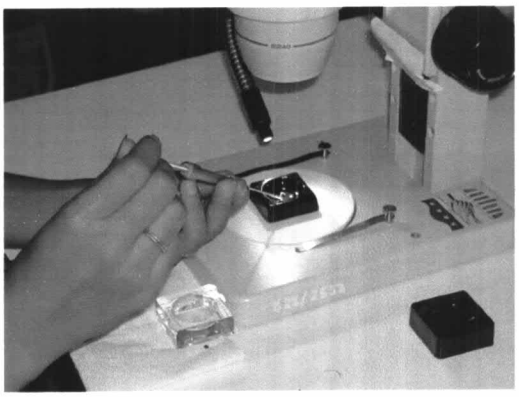


Figure 3.16. Collecting semen from seminal vesicle into insemination syringe.

3.5.3. Collecting spermatozoa from seminal vesicles into an insemination syringe tip after reconcentration by centrifugation

Spermatozoa were collected from seminal vesicles and reconcentrated by centrifugation (according to 3.4.3). After centrifugation, there were 2 fractions in micro-centrifuge tube; pellet and supernatant. The pellet was the spermatozoa and the supernatant was Tris buffer. Tris buffer was removed carefully to avoid the mixture of spermatozoa and buffer. To collect spermatozoa, use a sharp knife to cut the tube above the pellet. Focusing a microscope at the tip of insemination syringe and the part of the cutting tube containing spermatozoa. Touch the tip of insemination syringe at the surface of spermatozoa and suck the sample.

3.6. Rearing queens

3.6.1. Preparation of a queen rearing colony

A strong colony was used for a queenless colony to rear queens. Before the queenless cell builders were made up, the bees were fed with sugar syrup and pollen for at least a day or two days. The queenless cell builders consisted of 2 frames of honey (close the hive walls), 2 frames of pollen (next to honey frames) and several frames of capped worker broods (in the middle of the hive); 1 frame for each side and a lot of young worker bees. Twenty four hrs before grafting, the queen was removed from the colony. Before a second queen rearing, a capped brood frame was inserted to ensure sufficiency of young worker bees for good nursing capacity.

3.6.2. Preparation for grafting

3.6.2.1. Queen cell cups

Artificial queen cell cups were produced by dipping wooden stick in liquid wax. The forming stick for dipping was made from hardwood and about 3 inches long. It had diameter of 9 mm. The end of stick was round and smooth to give the bottom of a wax cell cup smooth and concave form. The beeswax for dipping was clean and melt on a hotplate. The stick was dipped into water until soaked. To remove surplus water, the stick was touched with back of the hand before dipping it in the wax. The stick was dipped in the wax at the approximately 1 cm depth and removed quickly in the air until the wax became solid. This process was repeated three or four times until the wall of wax cup was thick enough. The wax cup was removed from the stick by a slight twist. The next wax cup was formed by the same process. The wax cups were attached to the cell bars with melt beeswax (12 cells/bar Langstroth). There were 2 cell bars (24 wax cups) on a standard frame for grafting.

3.6.2.2. Grafting and transferring larvae to queen cell cups

Grafting is a process of transferring larvae from a worker cell of the breeder's colony to an artificial queen cell. A comb of larvae was taken from a breeder colony and put on a table for grafting under a magnifying lamp. A Chinese grafting tool was used to slip under a 24 h old larvae. Afterwards, it was lifted and placed on the top of a small drop of royal jelly in the bottom of the wax queen cups. Before placing a grafted frame in a cell builder, it was inspected for natural queen cups, which would be destroyed if present. The grafted frame was placed in the center between sealed brood frames of the cell builder. Twenty- four hours after grafting, queen cell acceptance was checked. In case of low acceptance of queen cell, grafting was repeated (double grafting). The larvae from accepted cells were removed from the wax cups in order to replace by new larvae in the same queen wax cups (follow the same process of grafting).

3.6.3. Handling of queen cells

Five days after grafting, queen cells were sealed. They were removed by twisting carefully from the bars and each queen cell was transferred into a cage (15 mm diameter). They were incubated at 34 -35°C in an incubator with 60 – 70% RH. The RH was provided by placing a tray filled with H_2O in an incubator. Six or seven days after transferring, the day before queen emergence, a small piece of candy was attached on the cage to feed queens after emerged.

Figure 3.17. Sealed queen cells at the grafted frame.
(Photo from Institut für Bienenkunde).

Figure 3.18. Each queen cell was placed in a cage and kept in incubator.

3.7. Maintaining queens after emergence

After queens emerged, queen cells were removed from cages to avoid queen death by sticking to a queen cell. Each emerged queen was provided with 4 worker bees and a small piece of candy for feeding. Queens were kept in this way before transferring them to a nucleus box.

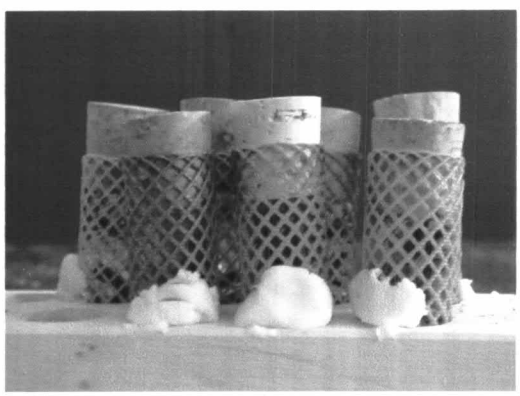


Figure 3.19. Emerged queen and worker bees in each cage.

3.7.1. Preparation of nucleus box

Nucleus box was copied from a Kirchainer Styrofoam mating box. The nucleus box was made from hardwood (18 cm width, 24 cm length, and 13 cm height) and contained four small foundations and a large chamber for candy. A plastic sheet was placed above the combs. A queen excluder was placed above the entrance on the bottom of the nucleus to prevent queen to leave the colony for mating and escape.

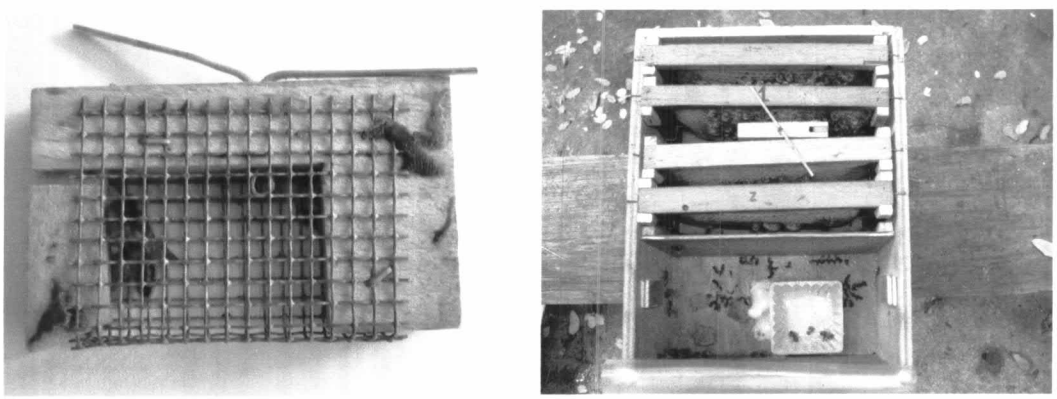
(A), (B),

Figure 3.20. (A) and (B) Nucleus boxes are managed at Chulalongkorn University.

3.7.2. Fill worker bees in a nucleus box

An entrance of nucleus was closed. Four small foundations (12 cm width, and 17.5 cm length) and candy were put into the nucleus box. A queen was introduced into a queen cage (a wire mesh push-in cage), which entrance was blocked with candy. \A queen was self-released from a cage sealed with candy. The cage was hung between the first comb and the second comb. The young worker bees from many combs were sprayed with H_2O and brushed into a swarm box. They were transferred from the swarm box into the nucleus box by ladle which can hold approximately 120 g of bees in each nucleus box. Afterwards, the lid of the nucleus was closed and the nucleus boxes were

stored in cold room (20°C). Three days later, they were moved to the bee yard. The entrance of the nucleus box was opened in the evening. One day later, the survival of queens was controlled. In case that, the queen died, it was replaced by a new queen which was in a queen cage. Two- three days after introduction, caged queen was released inside by still sealing the entrance of the cage with candy.



(A) (B)

Figure 3.21. (A) An emerged queen is kept in queen cage; (B) A queen in cage is placed in a nucleus box.

3.8. Maintaining queens before insemination

Each queen was free in a nucleus box. At least 24 hr before insemination, the queens were anaesthetized with CO_2 for 10 min to induce egg laying. When they were moving again, they were pushed in a cage, covered with candy and put them back in the same nucleus box. Virgin queens should be 6-12 days old at the time of insemination. A queen younger than 5 days old is risk for mortality. On the other hand, a queen older than 12 days stores less semen in spermatheca.

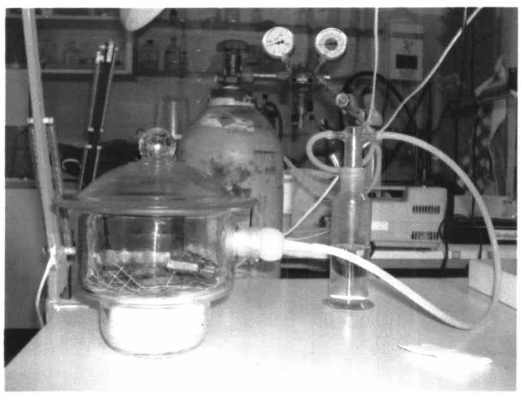
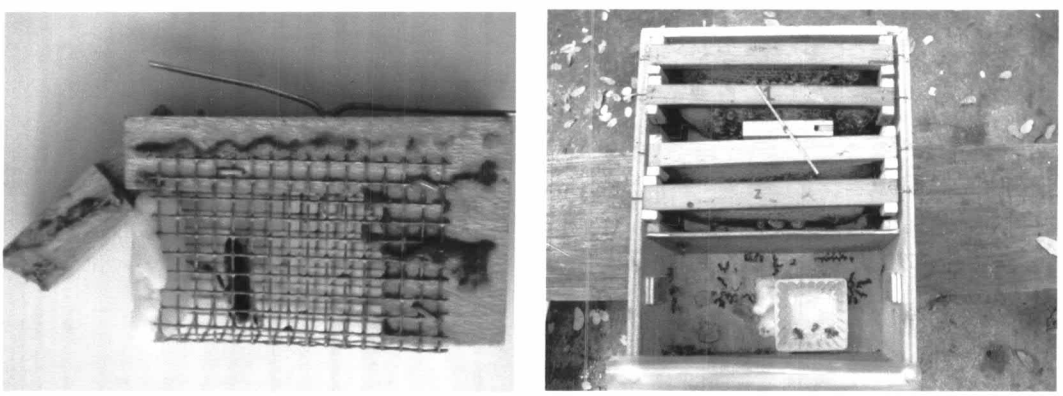


Figure 3.22. Queens were anaesthetized with CO_2 for 10 min.

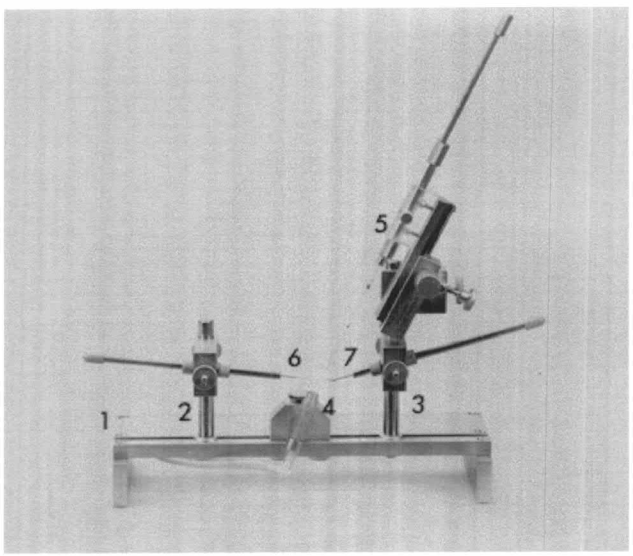


(A) (B)

Figure 3.23. (A) A queen was placed in a cage covered with candy; (B) Later, it was transferred in a nucleus box.

3.9. Preparing the insemination instrument

It consists of insemination instrument (Schley, model: Standard), a cold-light-source, a stereo-microscope, syringe (50 ml volume) with a bacteria filter (0.20 µm pore size), Co_2 and Co_2 container. Before insemination, an insemination syringe and the glass tip of insemination syringe were sterilized by autoclaving to prevent contamination.



1- base
2- left column with ball bearing-block
3- right column adding with a mechanic for insemination syringe
4 - queen-holder with a holding tube
5 – insemination syringe with a tip
6- left hook (ventral-hook)
7- right hook (dorsal-hook

Figure 3.24. Standard insemination instrument (Modified from Schley, http://www.besamungsgeraet.de).

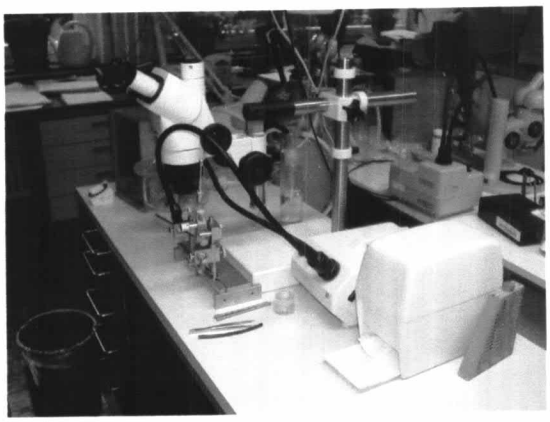
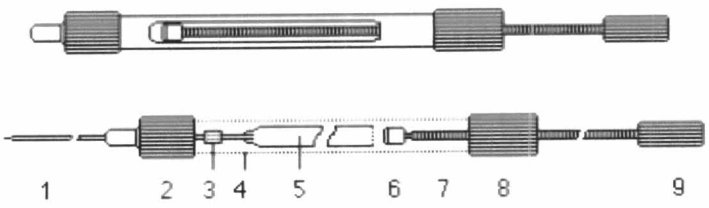


Figure 3.25. Equipment for insemination.

3.9.1. Filing Tris buffer in insemination syringe

The 50 ml syringe was filled with Tris buffer, capped with a bacteria filter and were attached to a needle. Before filling Tris buffer in an insemination syringe, the end of a glass tip of insemination syringe was sealed with tubing silicone sealer (approximately 4 mm length) and plunged in a sleeve. The insemination syringe cylinder was placed in syringe casing and screwed a control knob up to provide space in cylinder of insemination syringe in order to fill Tris buffer by applying from the 50 ml syringe. Afterwards, a sleeve was placed and screwed in the syringe casing. The control knob of insemination syringe was screwed a little downward to expel small buffer and air bubble away from the glass tip. Afterwards, screwed a little downward of control knob to have small air space at the glass tip before sucking semen to prevent dilution of semen. The insemination syringe was put in a syringe holder of insemination instrument.



1 – glass tip	4 – syringe casing	7 – winding spindle
2 - sleeve	5 – syringe cylinder	8 – screw cap
3 – squeeze sealing	6 - flask	9 – control knob

Figure 3.26. Details of insemination syringe with exchangeable flasks and cylinders. (Modified from Schley, http://www.besamungsgeraet.de).

3.10. Collecting spermatozoa for insemination

Spermatozoa from seminal vesicles of 1 *A. mellifera*, 8 *A. cerana*, 5 *A. dorsata* and 20 *A. florea* drones were collected (according to 3.4.2). Spermatozoa were spun to reconcentrate by centrifugation at 1,000 g for 10 min (according to 3.4.3). Then, spermatozoa were collected into the tip of insemination syringe for insemination (according to 3.5.3).

3.11. Insemination of queen

3.11.1. Placing a queen on a queen holder

A queen was taken from nucleus box for insemination. A thorax of queen was held with two fingers and placed head downwards into the cylindrical backup tube and closely connected to a holding tube. The queen was moved backward into the holding tube. Take this tube with queen to fit on the queen holder of the insemination instrument

(figure 3.27). The queen holder was connected to CO_2 line. CO_2 was adjusted to flow slowly to anaesthetize queen while she was inseminated. The tube must be turned to set the back of queen to the right side of the instrument for sting hook.

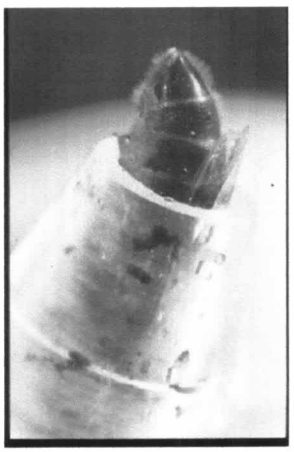


Figure 3.27. A queen backed up in a queen holder which is in a holding tube. (Photo from Institut für Bienenkunde).

3.11.2. Opening a sting chamber

The tip of queen abdomen protruded outside the holding tube. The queen abdomen must be focused under a stereo-microscope. After queen was anesthetized to be motionless, both hooks, a ventral hook and a sting hook, were positioned near the opening of the sting chamber. A ventral hook was held on the tip of the queen's abdomen, later on a sting hook was inserted, lifted the sting up and pulled dorsally to the right (figure 3.28). Tissue would be extended to form a large triangle. Within the triangle, there was a smaller "V" of wrinkled tissue defining vaginal orifice and location of the valve fold.

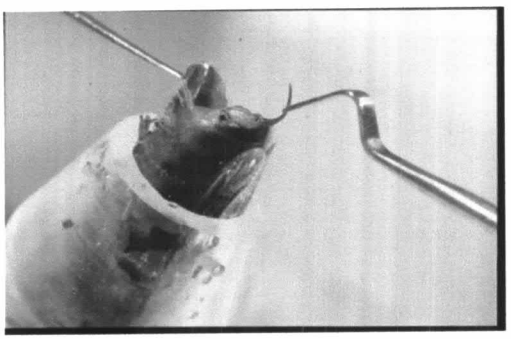
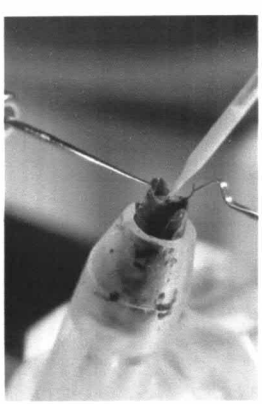


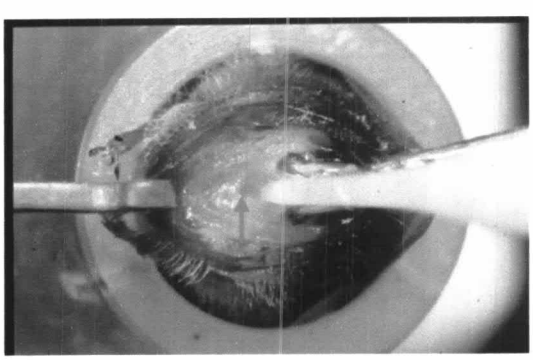
Figure 3.28. Opening the sting chamber; a ventral hook was held on the tip of the queen's abdomen on a left side, a sting hook was lift up the queen's sting on a right side. (Photo from Institut für Bienenkunde).

3.11.3. Injection of semen

An insemination syringe tip was lowered into a sting chamber. The tip was positioned above and slightly to the right of the "V" area. The tip was inserted about 0.5 mm depth and moved slightly to the left. Later, the tip was inserted about 0.5 - 1 mm depth into a median oviduct. Spermatozoa were injected and delivered to a median oviduct. The syringe tip must be easily slipped into the median oviduct in order that semen will not be leak during injection. Semen injection is done, the syringe was raised carefully from the vaginal and abdomen of queen.



(A)

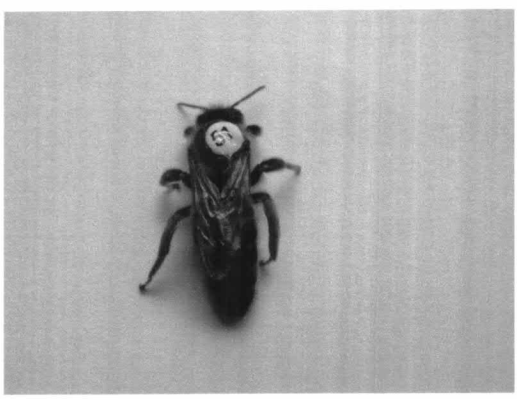


(B)

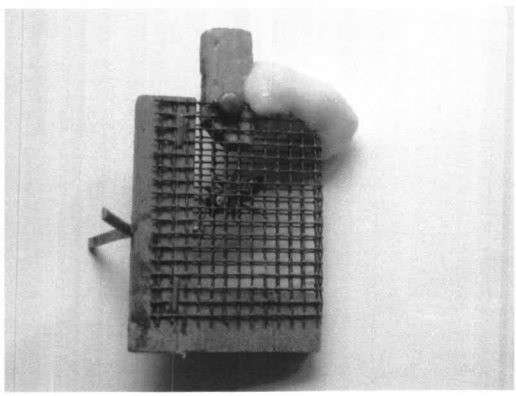
Figure 3.29. (A) A queen was inseminated. (B) The position of "V" area that inserted an insemination tip. (Photo from Institut für Bienenkunde).

3.12. Maintaining an inseminated queen

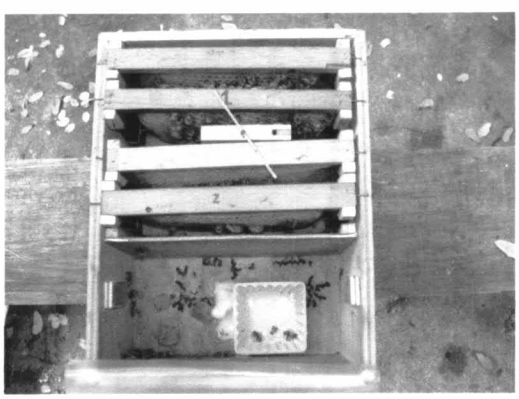
After insemination, a queen was blew out of the holding tube. The queen was marked on the thorax by number color tag, and its right wing was clipped. Then, the queen was placed into a cage which its entrance was covered with candy. When the queen was recovered from CO_2 and walked in the cage, the queen was introduced to the same nucleus box. Worker bees would remove candy to let the queen free in the nucleus box. Twenty Four hours after insemination, the queen was checked for the survival.



(A)



(B)

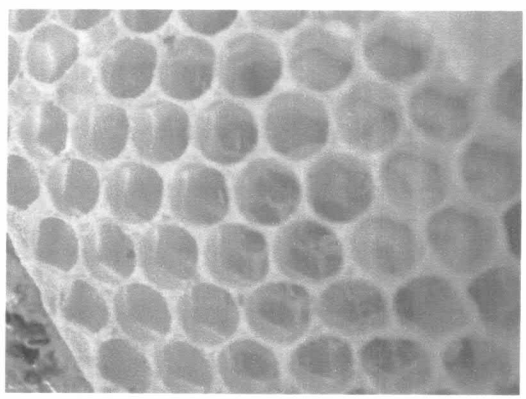


(C)

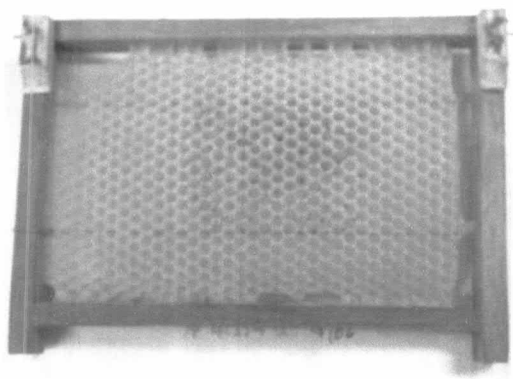
Figure 3.30. (A) An inseminated queen was marked with a number plate; (B) The queen was placed in a cage; (C) The queen was put in the nucleus box.

3.13. Evaluation of egg hatching and offspring

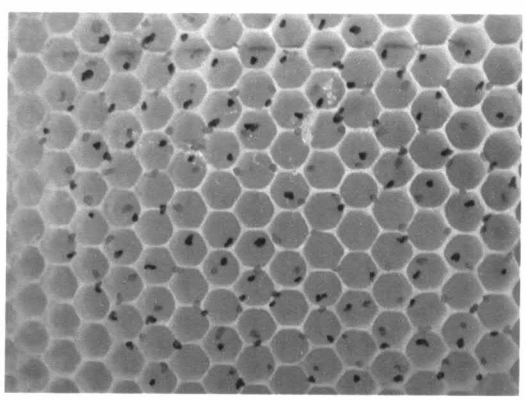
Queens were checked for starting oviposition every 2 days and the duration time after insemination until a start of egg laying were noted. The hatching rates were checked by mapping eggs randomly and by using a transparent sheet. The transparent sheet was put on the comb and marked the position of egg with red color. Hatching eggs were checked continuously for 3 days and marked with blue color at the same position of eggs.



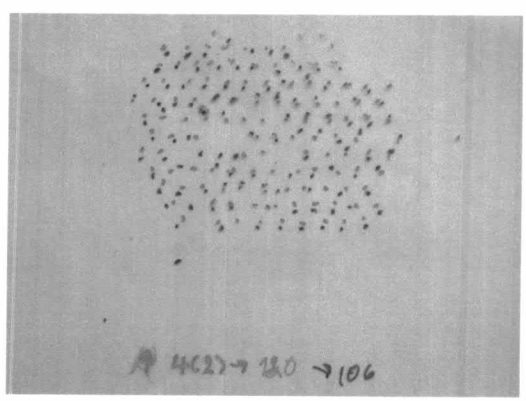
(A)



(B)



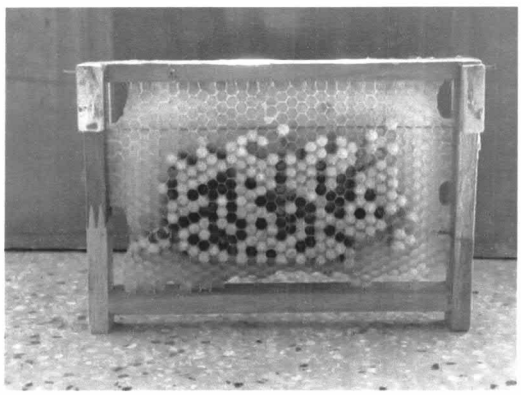
(C)



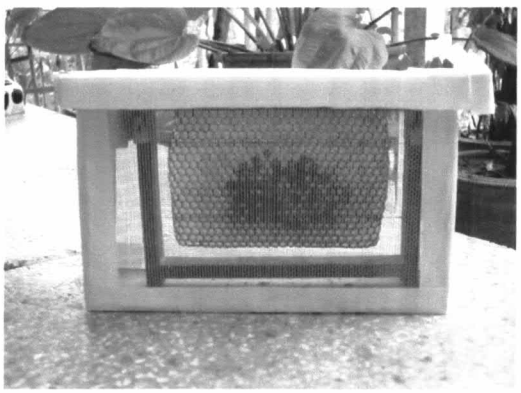
(D)

Figure 3.31. (A), (B), (C) and (D) The step of mapping eggs.

A comb of sealed brood cells was removed after eggs had been laid for 21 days. Each sealed brood comb was placed in a separate cage. Both sides of cages are made of wire (5 cm width, 20 cm length, and 15 cm height) so that emerged progeny can not pass outside the cage. They were kept in an incubator at 34.5°C and 60-70% RH and offspring emergence was checked. The newly emerged progeny were checked everyday for morphological characters, sampling offspring are preserved in 70% ethanol. The number of offspring emergence were noted.



(A)



(B)

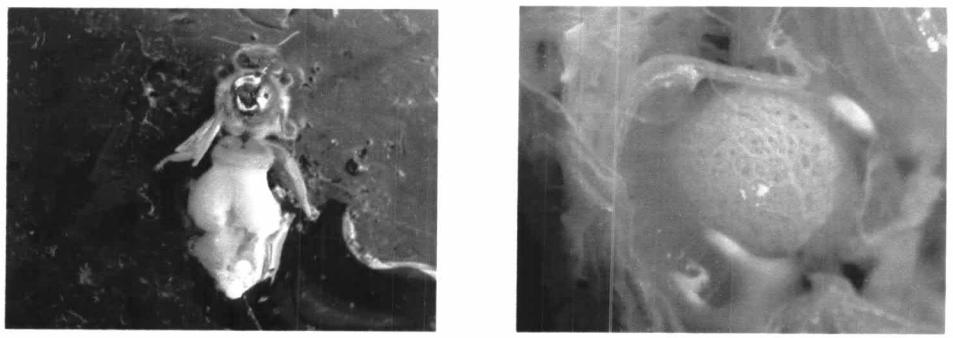
(C)

Figure 3.32. (A) Sealed broods; (B) A sealed brood comb was placed in a cage; (C) Sealed brood combs were kept in an incubator.

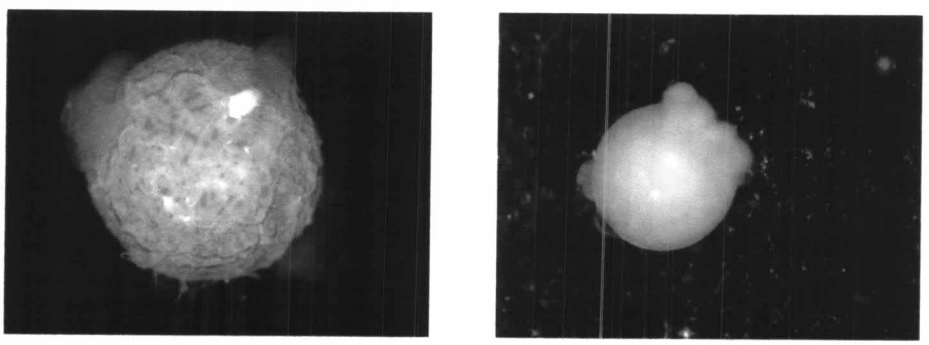
3.14. Counting number of spermatozoa and testing motility of spermatozoa in spermatheca

All inseminated queens were dissected and the spermathecae were transferred into a small black vessel (figure 3.33 (A)-(C)). The tracheal net around the spermatheca was removed (figure 3.33 (D)). The spermatheca was placed on counting squares of a haemocytometer. It was squashed with a cover glass until the membrane was broken and the fluid dispersed (figure 3.34). With this method, even single spermatozoa could be recognized and counted. The spermatozoa motility was recorded during a period of 1 hr. Two times, 100 spermatozoa were observed and each time the number of moving spermatozoa were noted.

For counting, spermatozoa were rinsed carefully with 0.2 ml of Tris buffer and 0.8 ml of distilled H_2O in order to remove them from the counting squares of the haemocytometer and cover glass into a small black vessel. The spermatozoa were counted with a Fuchs – Rosenthal haemocytometer under a phase contrast microscope at magnification of 100x and 200 x.

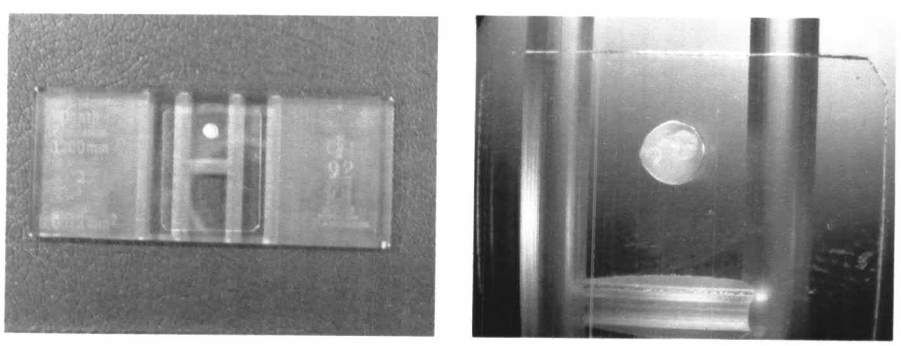


(A) (B)



(C) (D)

Figure 3.33. (A) and (B) A queen was dissected to remove spermatheca; (C) Spermatheca with tracheal net; (D) Spermatheca without tracheal net..



(A) (B)

Figure 3.34. (A) and (B) Spermatheca was squashed by a cover glass for checking the spermatozoa motility.