CHAPTER II

LITERATURE REVIEW

2.1. Classification of honeybee

The honeybee is a highly eusocial insect. Cooperative brood care, overlap of generations, reproductive division of labor and the presence of non-reproductive helpers of a later generation to the reproductive are the prime traits defining eusociality (Wilson, 1971).

Honeybee is classified in;

Kingdom Animalia

Phylum Arthropoda

Class Insecta

Order Hymenoptera

Family Apidae

Genus *Apis*

Apis spp.

Nowadays there are nine *Apis* species which are recognized. The newly recognized species were intergraded into three groups (Ruttner, 1988; O'Toole and Raw, 1999).

1. The small free open-nesting type with a single comb around a single branch of a small tree,shrub or bush

- *A. florea* Fabricius, 1787
- *A. andreniformis* Smith, 1858

2. The open-nesting giant species with a single comb under a horizontal support like branches of the tree or cliffs of rocks.

- *A. dorsata* Fabricius, 1793
- *A. laboriosa* F. Smith, 1871

3. The cavity- nesting type with combs

- *A. mellifera* Linneaus, 1758
- *A. cerana* Fabricius, 1798
- *A. nigrocincta* Smith,1861
- *A. koschevnikovi* Buttel-Reepen, 1906
- *A. nuluensis* Tingek, Koeniger and Koeniger, 1996

2.2. Honeybees in Thailand and distribution

Four from nine species of honeybees; *A. dorsata*, *A. cerana*, *A. andreniformis* and *A. florea* are native species in Thailand. While *A. mellifera* is an introduced species in Thailand (Wongsiri *et al.*, 2000).

2.2.1. *A. florea* Fabricius, 1787 (the dwarf honeybee) and *A. andreniformis* Smith, 1858 (the small dwarf honeybee)

A. florea and *A. andreniformis* are the open-nesting species. Both species normally build single comb around the small branch of tree or other support in open nesting sites without protective cover. They produce a band of sticky resin like substance around the branch of their nesting to prevent the ant or other enemies to invade the colony (for *A. florea*: Lindauer, 1956, 1957, Ruttner, 1982, for *A. andreniformis*: Wongsiri *et al.*, 2000). The difference of distinguishing morphological characteristic between species are the color of the body. The overall body color of *A. florea* is yellow-red whereas *A. andreniformis* is black and white (Rinderer *et al.*, 1995). The body size of *A. florea* is a little larger than *A. andreniformis* both are called the dwarf honeybee. Both species are distributed in Southeast Asia. *A. florea* is found in Southern

China, East India, Myanmar, Thailand, Lao, Combodia and Vietnam, but never found in the Malaysian peninsular or the surrounding island. For *A. andreniformis* is found in West Bangladesh, Southern and North China, East Vietnam and Malaysia and the island of Sumartra, Borneo and Phillipines. In Thailand, *A. florea* is found almost everywhere, even in big city Bangkok and Chiang Mai but there are very few in the South of Thailand whereas *A. andreniform* is found in South Thailand, but have been never found in central of Thailand. *A. andreniformis* is more strict in its nest sites, they are only found in dense bush and in limited areas (Wongsiri *et al.*, 1990, 1996).

2.2.2. *A. dorsata* Fabricius, 1793 (the giant honeybee)

A. dorsata is an open-nesting species and has the large - sized body and colony. *A. dorsata* build a large comb under tree branches or rocky cliffs or man-made structure such as water tower or tall building. They build their nests higher compared to *A. florea* and *A. andreniformis*. *Apis dorsata* is found throughout India and Southeast Asia including Palawan, Borneo, and the island of Indonesia from Sumartra to Timor. In Thailand, *A. dorsata* is found almost everywhere even in big cities like Bangkok and Chiang- Mai. *A. dorsata* is a seasonal migratory species. It nests in northern Thailand during November-April when flowers are blooming. Afterwards they migrate to the lowland and more than 100 colonies build their nests on a special tree, *Kompassia alaccensis* (Wongsiri *et al.*, 1996).

2.2.3. *A. cerana* Fabricius, 1793 (the Eastern honeybee) and *A. mellifera* Linnaeus, 1758 (the European or Western honeybees)

A. cerana and *A. mellifera* are cavity-nesting species, they build several parallel vertical combs under the ceiling of a cavity. *A. cerana* is spread widely throughout Southeast Asia, extending from Sri Lanka and India to Japan and Southeast to the

Moluccas (Michener,1974). *A. cerana* is found throughout Thailand. *A. cerana* is a honeybee species used for beekeeping especially in the South of Thailand.

A. mellifera is introduced for commercial beekeeping replace *A. cerana* especially in Northern Thailand.

2.3. Castes and tasks in honeybee

In honeybee, there are two castes; queen and worker. Queen and worker are female developed from fertilized egg, heterozygous (diploid, 2n=32). Fertilized eggs develop to queen or worker depends on quality and quantity of food fed to female larvae. Queen development is due to feeding on royal jelly. Queen larva is reared on a food containing hypopharyngeal and mandibular gland secretions of attendant workers. This royal jelly differs from food fed to worker. The developing queen larva receives ten times more biopterin and pantothenic acid and three times as much sugar in her food as does a worker larva (Hanser and Rembold, 1960), and she gets a larger amount and more frequently. Queen's task is to lay eggs and to control certain colony processes by pheromone for influencing worker behavior. Workers have division of labor and have multiple tasks based on age; (1) cell cleaning and capping, (2) brood and queen tending, (3) comb building, cleaning and food handling and (4) outside tasks, including ventilating, guarding and foraging. The workers change jobs when their age changes. A form of behavioral development is called "age polyethism" (Rösch, 1927; Lindauer 1952; Seeley, 1982; Huang, 1991). Drone is a male that develops from unfertilized egg, with one type of sex allele. If the sex alleles are homozygous diploid drones would develop but in colony these larvae are removed by the workers (Woyke, 1963). Drones do not contribute to the labor but have one task only to mate queens. After mating he dies.

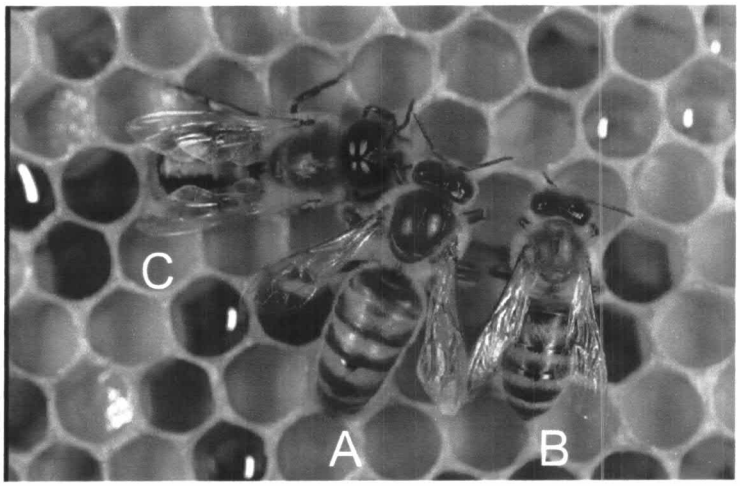


Figure 2.1. (A) Queen; (B) worker; (C) and drone of *A. mellifera*.

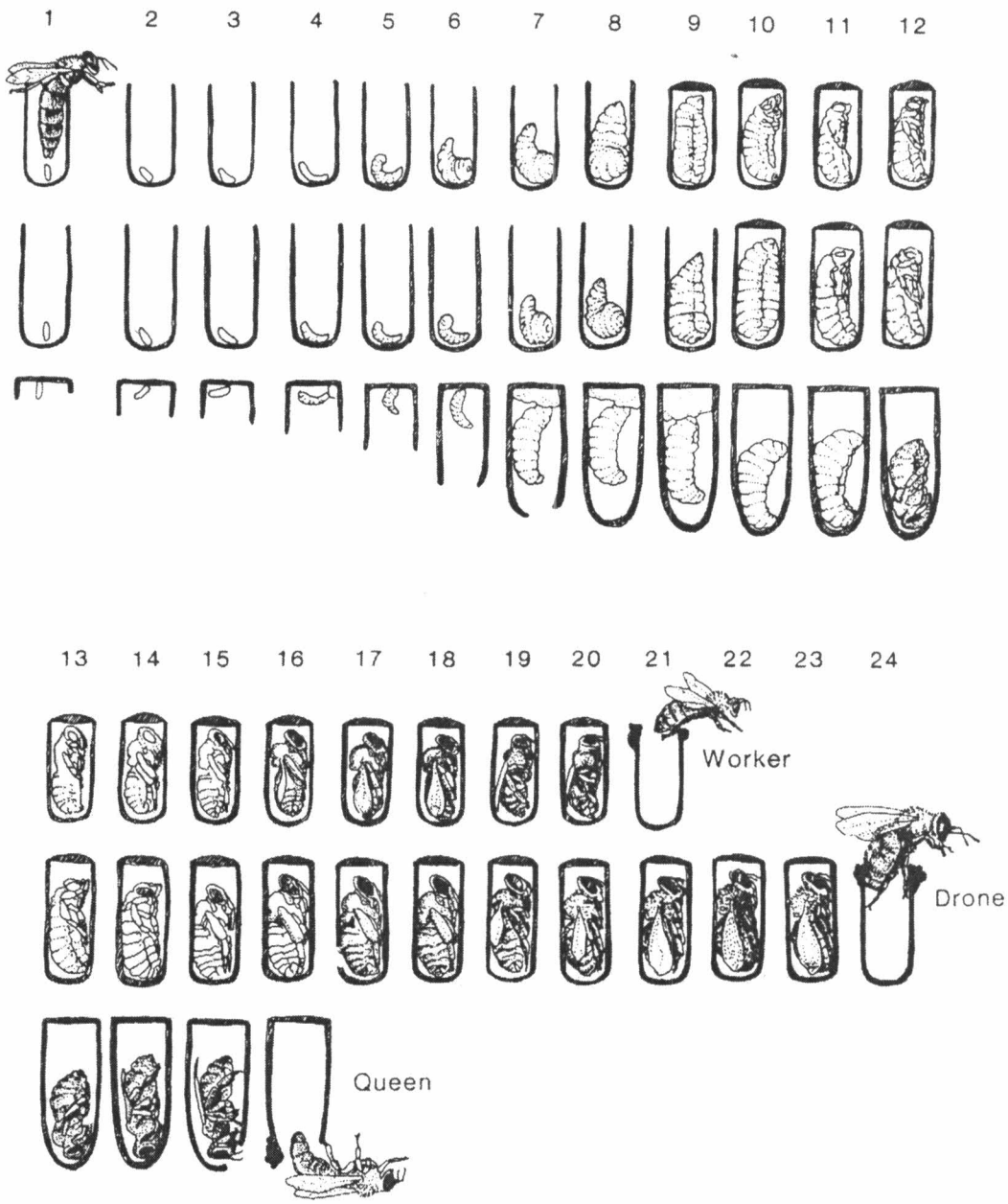
2.4. Development of honeybee

Honeybees have a complete metamorphosis have 4 stage; egg, larva, pupa and adult. From egg to adult develop with in the cells of the wax comb. The eggs laid by honeybee queen. The egg are elongate and gentle curved whitish with a soft membranous chorion. After 3 days, the larvae emerge from the egg (Nelson, 1915). Larvae are soft, whitish, legless grub. There are five larval instar. During the first few days, the larvae are fed with royal jelly later on with honey and pollen. All brood are given royal jelly until middle of larva 4 and whose are feed with royal jelly after middle of L4 will develop to queen (Dietz and Lambremont 1970; Hartfelder, 1990). In the L5, Larvae feed for a time follow by a spinning phase. In the prepupal stage are sealed in their cells with wax capping by adult workers. After capping, few days of larval life are spent constructing a cocoon within the cell. The larvae stretch out fully in the cells with their heads toward the capped end (Jay, 963). Pupae are the last period before molting to adult. Pupae are relatively delicate and have rapidly development process. To emerge, they perforate the wax capping with small holes by using mandible, antennae protrude through the hole. The adult workers help to take them out from capping cell.

The development times from egg until adult emerge are 16 days for queen, 21 days for worker and 24 days for drone. However, the period of development from egg until adult emergence are varied on environment, nutrition or temperature.

Figure 2.2. Development times and stage of honeybee

(Modified from Winston (1987))



2.5. Reproductive organs of honeybee

2.5.1. Reproductive organs of queen

The reproductive organs of a queen consist of the ovaries, the paired lateral oviduct, a median common oviduct and the genital chamber. The genital chamber is differentiated into a bursa copulatrix opening at the base of the sting and a vagina that receives the common oviduct. A sperm receptacle or spermatheca opens by a short duct from the dorsal wall of the median oviduct.

The ovaries consist of two huge, pear-shaped masses of egg tubules, the ovarioles. Each ovary contain 160 or 180 tubules. The ovarioles arise from the anterior ends of the lateral oviduct and taper to slender threads, which finally in each ovary unite in a single suspensory strand attached beneath the ventral wall of the heart. In fertile queen, the ovarioles increase in diameter behind their tips; and the ovaries are very large. Oogenesis occurs in an ovariole. At the posterior ends the ovarioles open into the lateral oviducts. The paired lateral oviducts join a short, common median oviduct. The oviduct opens into vagina. The opening of the posterior end of the vagina is in the form of horizontal slit, beyond which lies the bursa copulatrix, a wide membranous pouch at the anterior end of the sting chamber. At each side of the bursa is a lateral pouch. Eggs pass from the ovarioles into the oviduct beneath the opening of the spermathecal duct. The spermatheca is attached to the common oviduct by a single spermathecal duct. Spermatheca is the organ that store spermatozoa after mating (Snodgrass,1956; Dade, 1977).

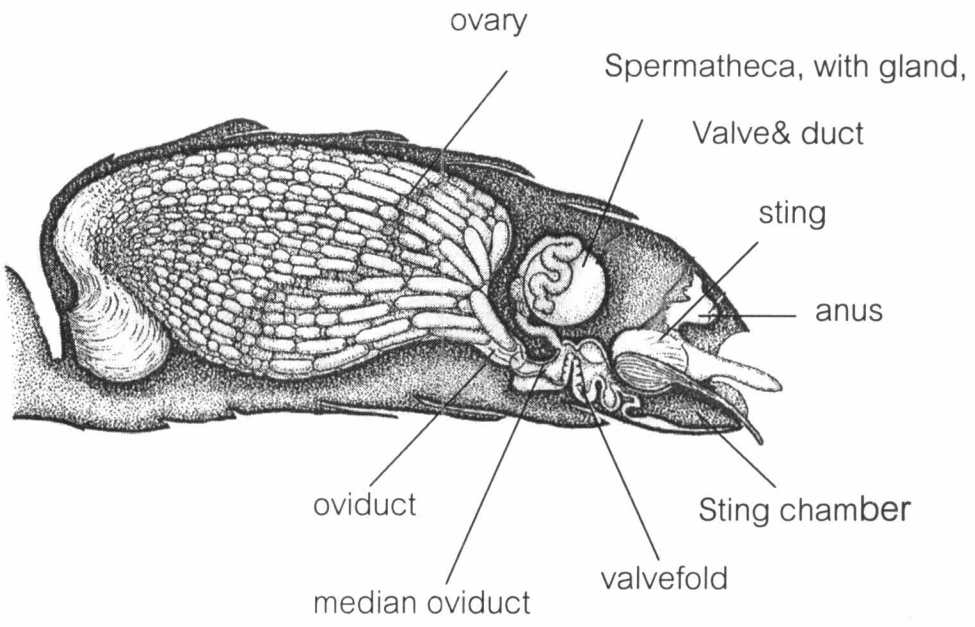


Figure 2.3. Reproductive organs of *A. mellifera* queen (Modified from Dade (1977)).

2.5.2. Reproductive organs of drone

The reproductive organs of drone consists of a pair of testes, their outlet ducts or vasa deferentia, which are partly enlarged as seminal vesicles, a pair of huge mucus glands united at their posterior ends, the single ejaculatory duct, and a large intromittent organ or the endophallus. The testes are composed of bundles of tubules in which the spermatozoa are produced and matured. In drones which have just emerged from their cells the testes are enormous, white and bean shaped bodies. When the drone is mature, they are reduced to small, greenish-yellow scraps of tissue, all their contents having passed on through the coiled tubes of the vasa deferentia into the seminal vesicles. The vasa deferentia is differentiated into three parts. The ducts leaves the testis as a short, tightly coiled tube, followed by a long tube, and sausage-shaped enlargement which is the seminal vesicles. The spermatozoa leaving from testes are retained and packed in seminal vesicles. The mucus glands are very large club-shaped sacs filled with mucus, which join at their bases to make a large U, where the seminal

vesicles are connected by short narrow tubes. The ejaculatory duct, through which the spermatozoa pass at the moment of copulation, springs from the middle of the U near to the openings of the seminal vesicles. These openings are brought close to the opening of the duct when the seminal vesicles are emptied, and the semen passes into the duct, followed by the contents of the mucus glands. The endophallus is differentiated into three parts; 1. the bulbus; 2. the cervix with hairy patches and a special lobe 'fimbriate lobe' ; and 3. the vestibulum with cornua and hairy patches. The bulbus opens to the ejaculatory duct. it is large ovoid body and has chitinous plate. The cervix is a thin tube and has hairy patches on the ventral and dorsal side of the cervix. On its dorsal surface it bearsa large, doubly pinnate fimbriated lobe. The vestibulum is a chamber and a final part of the tube that ends at the genital opening. From the side of vestibulum is a pair of cornua. The cornua is wrinkled and folded. In newly emerged drones the cornua have no pigment while in sexually mature drones have an orange-colored secretion inside the cornua (Snodgrass,1956; Dade, 1977; Koeniger, 1991; Koeniger *et al.*, 1990).

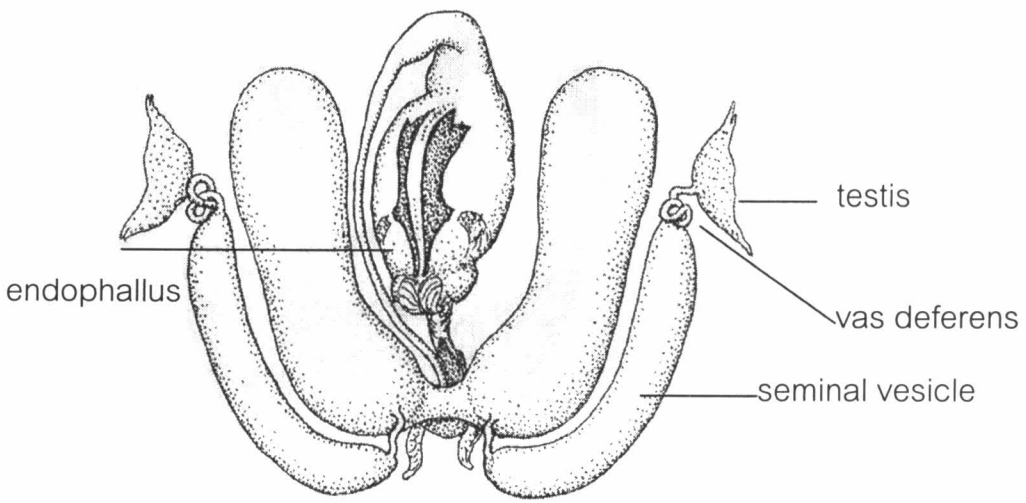


Figure 2.4. Reproductive organs of *A. mellifera* drone (Modified from Dade (1977)).

The morphology of endophallus is different in each species. There are three main types of endophalli (Koeniger *et al.*, 1991);

1. The bulbus is thick and bent dorsally; the ventral cornua is bent ventrally; production of mucus and lobe with fimbria are observed. This type is found in *A. mellifera*, *A. cerana* and *A. koschevnkovi.*

2. The bulbus is thin and elongated; the cervix short, the ventral cornua bent dorsally; there is little or no mucus; the lobe is without fimbria. This type is found in *A. florea* and *A. andreniformis.*

3. The bulbus is small and bent ventrally; the cervix is extremely long; there are ventral cornua with 2 long tubes; the lobe is divided into 4 parts and without fimbria. This type is found in *A. dorsata.*

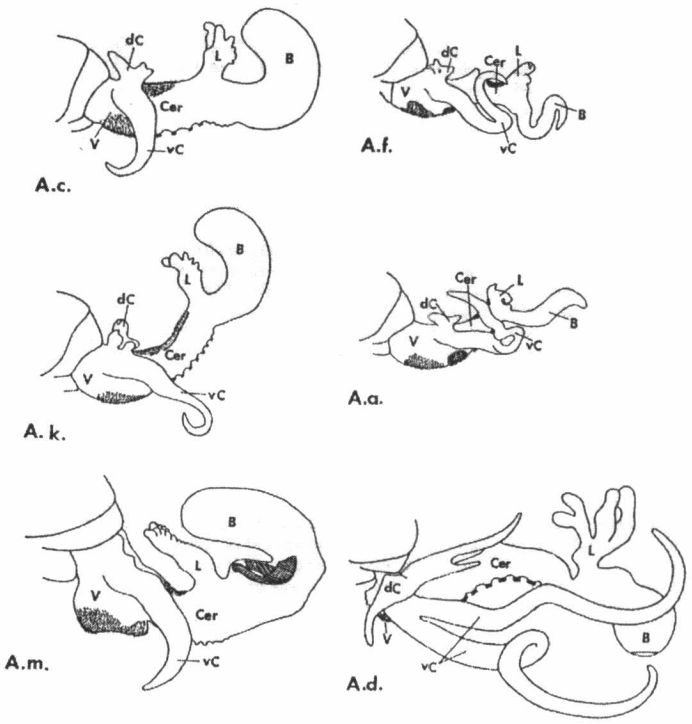


Figure 2.5. Lateral view of everted endophalli of *A. mellifera*, *A. koschevnkovi*, *A. cerana*, *A. dorsata*, *A. florea*, and *A. andreniformis* (Modified from Koeniger *et al* (1991)).

2.5.2.1. Semen and compounds of seminal fluid

In *A. mellifera*, semen is a cream colored fluid which consist of sperm and seminal plasma. The pH of ejaculated semen is 6.8-7.0 (Taber,1977). In the seminal plasma several components could be identified: three ypes of sugar (fructose, glucose and trehalose); many ions (magnesium, calcium, sodium, copper, iron and manganese); amino acid (tyrosine, methionine, leucine, cystine, isoleucine, tryptophan, lysine, phenylalanine, arginnie, glutamic acid, alanine, aspartic acid, serine and threonine); group of dehydrogenase enzyme (lactate, glucose6-phosphate, glutamate, malate, succinate, malate, β-hydroxybutyrate, NADH2 and NADPH2 and α-glycerophosphate); and free fatty acid (phospholipids, tryglycerides and sterols) (Blum *et al.*, 1962, 1967; Blum and Taber, 1965; Novak *et al.*,1960). The compound of seminal plasma of another species have not yet been studied.

2.5.2.2. Spermatozoa structure

Spermatozoa of *A. mellifera* are thin elongated cells very slender threads, about 250 μm long and 0.7 μm wide. The spermatozoa consist of two parts; acrosomal complex and tail. The sickle-shaped acrosomal complex is 5 μm long and from 0.4 to 0.5μm wide and consists of acrosome and nucleus. The tip of acrosome complex or the perforatorium is pointed, flat, and slightly bent at the end. The perforatorium widens posteriorly and forms the galea. The acrosomal rod accompanied by tubular-like structures in the galea region. The acrosomal rod is attached to the nucleus by a peg-like asymmetric insertion. Nucleus is oval-shaped (length; 5 μm, width; 0.5 μm and thick; 0.3 μm). The anterior tip of nucleus is eccentric and shaped like a truncated cone. The posterior is cone-shaped. The peg-like end of the acrosomal rod passed through the truncated cone and penetrates into the nucleus. The tail consists of axoneme(flagellum)

and mitochondria. The axoneme is accompanied by two triangular rods, each facing one of the corresponding mitochondrial derivative. The tail compose of two long mitochondrial derivatives parallel to the flagellum. The two mitochondrial derivatives become slender towards the end (Lensky *et al.*, 1979).

2.6. Copulation

In genus *Apis*, drones are monogamous because they die after mating. In contrast queens are polyandrous, mate with multiple drones during mating flight period. Drones and queens mate in the air. In *A. mellifera*, the drone mounts the queen from behind. After making contact with his front and middle legs on the female's abdominal tergites, drone uses the metatarsi at hind legs cling to the ventral side of the queens abdomen. When the queen opens the sting chamber, drone inserts the endophallus. With the eversion of the endophallus the wings of the drone stops moving and is paralyzed. He swings back. After full eversion of the endophallus he separates from the queen and falls to the ground and dies (Koeniger, 1981,1988). In *A. mellifera*, it is mainly the large membranous endophallus which connects the drone to queen. Filled under high muscular pressure with leucolymph and mucus it seems to guarantee a sufficiently strong connection between the flying queen and the motionless drone until the sperm transfer is completed.

By the previous contraction of the abdominal muscles the drone had pressed mucus into membranous endophallus which very firmly fills the sting chamber. The cornua with their stick secretion increase the strength of the attachment. Thus, the endophallus is anchored in the queen by its like "a cork in bottle". In this stage the queen continues to fly and for a moment carries the motionless drone. The queen contracts the bursa and presses the endophallus which results in sperm transfer into the

median oviduct. The drone falls down, leaving mucus and the corneal secretions as mating sign in the sting chamber of queen. The mating sign at the tip of the flying queen's abdomen enhances the attraction of further drones and facilitates subsequent copulations (Koeniger, 1986, 1990). In *A. florea* and *A. andreniformis* drones have a forceps like appendix at the metatarsus of the hind leg. In *A. andreniformis* has the metatarus of the drone's legs has an appendix a little shorter than *A. florea*. With this "thumbs" the drone locks himself to the hind legs of the queen (Ruttner, 1988), supported again by the sticky cornua pressed into sting chamber. Thus the pair stays connected until the queen turns her leg in a way that the drone is released. Because of this mechanism, mucus is not necessary for the mating process and mucus gland are tiny in these species. *A. florea* and *A. andreniformis* drone have rudimentary mucus glands and no mucus for a mating sign is produced. No mating sign is left in the queen's sting chamber after mating and sperm is transferred directly to the spermatheca. The shape of the endophallus especially the tip at the distal end, seems to fit the hypothesis of direct sperm transfer (Koeniger,1991). In *A. dorsata*, drone has a more elongated endophallus with four long curled cornua. The elongation is caused by the extend cervix. The mucus glands are relative small. The drone has broadened metatarsi at the hind legs which show conspicuous feathered hairs that on the drone's metatarsus is believed to reinforce the attachment to the queen during copulation (Ruttner, 1975, 1988). The mating behaviour of this species is not study yet.

2.7. Sperm transfer

The transfer of spermatozoa into spermatheca is complex process, involving the muscle of the queen, the fluid of the spermatheca and its gland and the movement of spermatozoa. Spermatozoa transfer to the wall of oviducts by contraction of the oviduct and abdominal muscles. They are able to migrate through the spermaduct from their

orifice. In *A. mellifera* and *A. cerana*, after copulation drone inject spermatozoa into the median and lateral oviduct of queen. Up to 24 hr spermatozoa reach in spermatheca of queen (Woyke,1983). In *A. florea* and *A. andreniformis*, after mating spermatozoa transfer directly into spermaduct of queen (Koeniger *et al.*, 1989). The process of sperm transfer in *A. dorsata* is still not clear. Three taxonomic groups (dwarf, giant and cavity-dwelling honeybee), sperm transfer very likely is prevented by the morphology of the endophalli.

Drone of *A. mellifera* produce spermatozoa 10 -12 X 10^6 spermatozoa in the seminal vesicle (Woyke, 1975; Rinderer *et al.*, 1985), 1.2 X 10^6 spermatozoa in *A. cerana*; 0.43 X 10^6 spermatozoa in *A. florea* (Koeniger *et al.*, 1989), 0.13 X 10^6 spermatozoa in *A. andreniformis*, and 2.46 X 10^6 spermatozoa in *A. dorsata* (Koeniger *et al.*, 1990). After mating, a small percentage of spermatozoa transfer in a spermatheca of the queen where it is kept for several year. The percentage of spermatozoa per drone stored in the spermatheca is differenct in each species. Spermatozoa reaching the spermatheca was 66% in *A. andreniformis*, 44% in *A. florea*, 7% in *A. dorsata*, 10% in *A. cerana*, and 3% in *A. mellifera* (Koeniger *et al.*, 1990). While Palmer and Oldroyd (2000) reported, the spermatozoa retained in spermatheca was 74.1% in *A. andreniformis*, 28.1% in *A. florea* , 5.5% in *A. dorsata* , 7.2% in *A. cerana*, and 4.5% in *A. mellifera*. In *A. mellifera*, drone produce large number of spermatozoa whereas queen expel extreme excess semen (only 3-4.5% of spermatozoa in spermatheca). For *A. cerana* and *A. dorsata* which produce fewer spermatozoa than *A. mellifera*, queens store more spermatozoa in spermatheca than *A. mellifera*. Whereas in *A. florea* and *A. andreniformis* produce less amount of spermatozoa but a high percentage of spermatozoa per drone is stored in the spermatheca. It seems that in cavity-nesting species (*A. mellifera* and *A. cerana*), drone inject sperm into oviduct, less than 10% of

spermatozoa in spermatheca, queen expel spermatozoa more than 90%. Whereas in open-nesting species (*A. florea* and *A. andreniformis*), drone inject spermatozoa in spermaduct, about 50% of spermatozoa are rejected. The storage of spermatozoa in spermatheca support the idea of sperm transfer influences the filling process of the spermatheca. Beside multiple mating is support about sperm transfer in spermatheca. It seem to be in *A. florea* and *A. andreniformis* that sperm transfer directly to the spermatheca. There is less waste (about 50%) of semen like in *A. mellifera* and *A. cerana* where the sperm is deposited in lateral oviducts and only 10%migrated in spermatheca. Then partinity frequency of *A. florea* (8 and 10) and *A. andreniformis* (13.5) are fewer than *A. mellifera* (13.8) and *A. cerana* (26.7) (Palmer and Oldroyd, 2000, 2001).

2.8. Sperm storage (only *Apis mellifera*)

Apis mellifera queens are known to store spermatozoa for all their life of 3-5 years in the spermatheca. Sometimes they even live longer (Butler, 1954). The lumen is filled with a transparent fluid and connected to a pair of tubular glands. About 2 days after last mating densely packed spermatozoa lay in bundles in the lumen giving the spermatheca a whitish marbled pattern. The spermatheca is separated from the lumen of the oviducts by a muscular system which keeps the spermathecal duct closed thus forming a separate spermathecal compartment. The muscles function as a pump for sperm transport (Bresslau's sperm pump, Bresslau, 1905). The pH value of the spermathecal fluid is high (8.6). While the Na^+concentrations in the hemolymph show the usual value for insect body fluids, the K^+ concentration in the spermathecal fluid is higher by about eightfold (Gessner and Gessner, 1976). The stored spermatozoa have a reduced metabolism (Verma, 1973) which is thought to depend on the high pH. Spermatozoa became immotile within 3 weeks after removal of only part of the dense tracheal net. After removal of the spermathecal gland the queen laid unfertilized eggs,

even though a high percentage of the spermatozoa were motile for more than 90 days (Koeniger, 1970). Poole (1972) found after removal of some trachea that the tall columnar cells of the spermathecal epithelium shortened at the tracheectomized areas. Thus it is not clear if the infertility of these queens is caused directly by impairment of the oxygen supply to the spermatozoa or if it is caused by disturbing the isolating structure of the spermathecal complex. The spermathecal complex contains several sugars (Alumot *et al.*, 1969). Comparisons of proteins from hemolymph and spermathecal complex by disk electrophoresis and immunological tests revealed that both are separate fluid systems (Lensky and Alumot, 1969). There is only little information on the amount and function of proteins in the spermathecal complex that may contribute to the survival of sperm during storage. Recently, antioxidases (CAT, SOD and GST) were found in the spermathecae of mated queens which may be involved with long-term storage by protecting the spermatozoa from oxidative stress (Weirich *et al.*, 2002). Klenk *et al* (2004) showed that a high concentration of protein is found in the spermathecal fluid in *A. mellifera* (from 8.5 mg/ml to 15.3 mg/ml). The secretion of the spermathecal gland has about half the concentration. Mated, egg laying queens had a concentration of 6.4 in the gland secretion and 11.3 in the spermathecal fluid. They hypothesize that in *A. mellifera*, in addition to the high pH, many proteins are produced by the queen which have a function in long sperm storage. For functional studies the 29 kDa and other proteins have to be characterized.

2.9. Reproductive isolation

The reproductive isolation is the decisive step in speciation. Traditionally reproductive isolation mechanisms are categorized according to their temporal relation as prezygotic or postzygotic barriers (Mayr, 1963). The prezygotic phase is divided into 3 subgroups of isolation factors; 1. behavioural barriers operate early that prevent the physical contact between queen and drone; 2. copulatory barriers operate during the process of copulatipon, prevent sperm transfer to a queen; and 3. physiological barriers

operate after copulation. This barriers may block sperm transfer and storage in spermatheca and egg fertilization. The postzygotic barrier have effect of fertilization, normal development, resulting in the death or infertility of hybrids (Koeniger and Koeniger, 2000).

Behavioral mating barrier is discussed here because it is the main reproductive isolation among sympatric species of the genus *Apis* that operate early to prevent physiological contact.

2.10. Behavioral mating barriers among sympatric species of the genus *Apis*

2.10.1. Reproductive isolation by seasonally different mating periods

Honeybee is monogeny. This links the rearing of new queens to the process of colony multiplication (swarming). For survival, a new swarm need more nectar and pollen for comb building and brood rearing. Otherwise the natural mortality of workers can not be compensated and later swarm colony is reduced to beyond the critical threshold (Seeley, 1985). Then, mating season in honeybee populations depend on seasonal blooming cycles. This holds true for populations of sympatric species. Accordingly, in Thailand, Sri Lanka and Borneo, all sympatric *Apis* species produce drones simultaneously (Rinderer *et al.*, 1993; Koeniger and Wijayagunesekera, 1976; Koeniger *et al.*, 1996).

2.10.2. Reproductive isolation by different mating places

Mating of honeybee queens and drones takes place during flight at some distance from the colony. The mating place call drone congregation area (DCA). The first

DCAs of *A. mellifera* in Europe and USA were discovered by hearing the hum of flying drones (Jean-Prost, 1957; Zmarlicki and Morse, 1963; Ruttner and Ruttner,1965) The DCAs of *A. mellifera* seems to vary greatly. *A. mellifera* drones which congregate in the open air. A congregation areas have a diameter of 30-200 m. The actual area of drone distribution measured by radar as 16000 m^2 (Loper *et al.*, 1987). Drone fly above the ground varies from 5-40 m. A congregation area has a limited spatial extension, *A. mellifera* drone are not attracted by a queen flying outside the area (Ruttner and Ruttner, 1968).

The DCAs of *A. koschevnikovi* in Borneo occurred under thick cover of vegetation and the height above that ground of different DCAs varied between 1.5-12 m (Koeniger *et al.*, 1998)

A. cerana indica drone in Borneo and in Sri Lanka congregate in an open space within or near the canopies of the tree. They do not follow the queen far into the open air (Punchihewa *et al.*, 1990). In Japan, drone of *A. cerana japonica* congregate in open area above high tree (Fujiwara *et al.*, 1994). In Borneo, drone of *A. cerana* indica congregate outside the canopy of the tree and larger shrubs and flight 10-12 m above the ground (Koeniger and Koeniger, 2000).

The DCAs of *A. dorsata* differ from *A. mellifera* and *A. cerana*. In Borneo, *A. dorsata* drones congregate under the canopy at a height 20-25 m from the ground and do not follow the queen to the open air. Drone of *A. dorsata* fly nearly everyday not depend on the weather (Koeniger *et al.*, 1994) whereas drone of *A. mellifera* and *A. cerana* are reported to fly under blue sky (Jean-Prost, 1957; Zmarlicki and Morse, 1963; Ruttner and Ruttner,1965). The DCAs of *A. andreniformis* and *A. florea* have not yet been found.

Research on drone congregation areas of sympatric species (*A. dorsata*, *A. cerana* and *A. koschevnikovi*) have been carried out in Borneo by using standard dummy which was impregnated with 1 mg of 9-ODA (queen pheromone) (Koeniger *et al.*,1998). Drone of *A. cerana* had their maximal flight frequency measured by attraction to standard dummy slightly ouside the canopy of the tree and larger shrubs and flight 10-12 m above the ground. Drones of *A. koschevnikovi* remained under the dense cover of the canopy and flew in a space 6-8 m above the ground. While *A. dorsata* drones flew under the first layer of branches in a height of 20-25 m about 5 m below the canopy. So, the distribution of drones resulted in a clear spatial separation without any overlap between these 3 species. The mating place can be factor of reproductive isolation between *Apis* species.

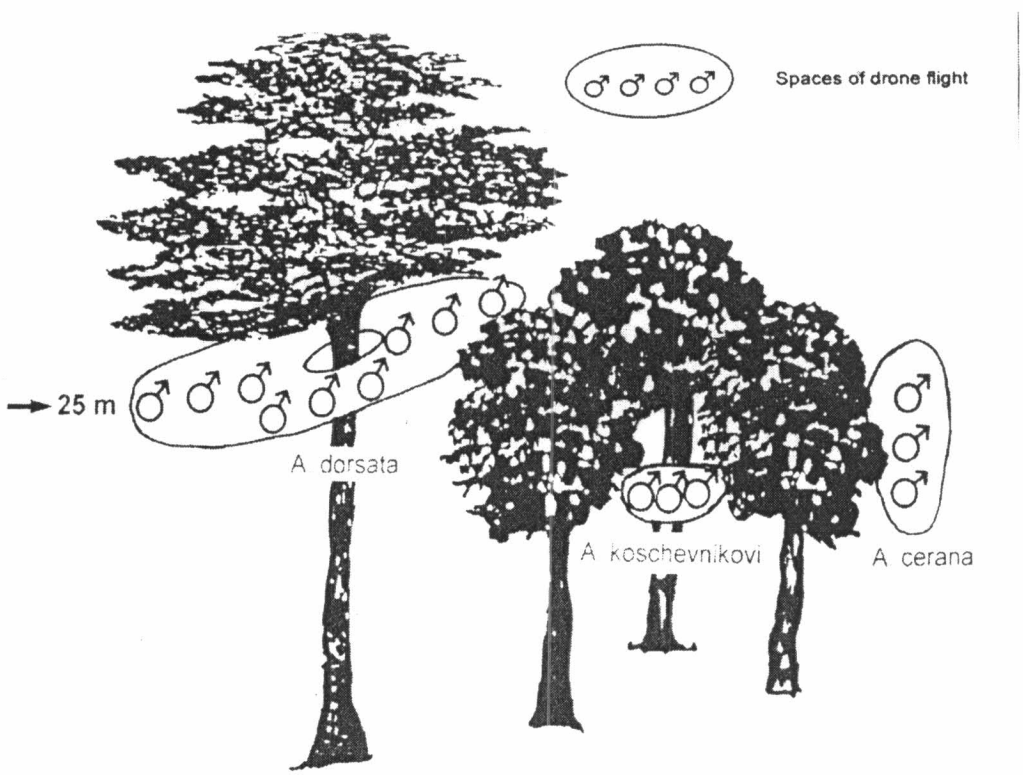


Figure 2.6. Drone congregation areas of *A. dorsata*, *A. cerana* and *A. koschevnikovi* in Borneo (Modified from Koeniger *et al* (1994)).

2.10.3. Different sexual signals as means of reproductive isolation

The first reaction of drones to visual stimuli seems to depend on "unspecific" movements. At DCAs, *A. mellifera* drones react to various moving objects by a fand short turning reaction. Flying bird, butterflies and even stones thrown into the air area will momentarily attract some drones (Jean-Post, 1957).

(E)-9-oxox-2-decenoic acid (9-ODA) is a major active component of *A. mellifera* mandibular gland (Callow and Johnston, 1960) which found to be the main component of the *A. mellifera* queen's sex attractant (Gary, 1962; Pain and Rutter, 1963). Later, it was demonstrated that extracts of queens of 3 other *Apis* species (*A. dorsata*, *A. florea* and *A. cerana*) attract *A. mellifera* drones, and that these extracts contained 9-ODA. *A. dorsata* and *A. cerana* queens had a quantity of 9-ODA similar *A.mellifera* queen (Botler *et al.*, 1967; Shearer *et al.*, 1970). There are specific differences spectrum in the mandibular gland signals between queen of *A.mellifera*, *A.dorsata*, *A. cerana* and *A. andreniformis* (Plettner *et al.*, 1997).

Direct observations and tests of interspecific reactions were carried out with *A. mellifera* and imported *A. cerana* in Germany by Rutter and Kaissling (1968). At DCAs in Germany, *A. mellifera* drones attracted *A.mellifra* queen (in caged) more than A. cerana queen (in caged). This result showed that the species-specific differences in queen pheromones between *A. mellifera* and *A. cerana*. However, the preference for conspecific queens does not prevent the mating of heterospecific queen. Rutter and Maul (1983) reported younger *A. cerana* queen which had her bursa cpulatrix blocked by a mating sign of *A. mellifera* drone. The main olfactory signal and essential sex pheromone seems to be 9-ODA in all species. However, the differences in sexual signals do not play a major role as behavioral barrier between sympatric honeybee species.

2.10.4. Reproductive isolation by different daily mating periods

In *Apis* species, drone and queen leave the colony for mating during mating flight time. Drone and queen in each species leave the colony at approximately the same time of day for mating. Mating flight times are not species specific depend on place, environment and geographic location.

In *A. mellifera*, the drone flight times start shortly after noon and covers a period of 4 to 5 h. Ruttner (1966) reported drone start flying after the sun pass zenith (12.15), and stop in the late afternoon (17.00). In Africa near Pretoria, drone of *A. mellifera* scutella fly 12.45-16.45 (Tribe, 1982). In Malawi, the drone flight period occurs from 11.20-16.00 (Lahner, 1998). The mating flight of *A. mellifera* queen occurs during the peak period of drone flight between 14.20 and 16.10 in Austria (Koeniger *et al.*, 1989), 13.00 and 15.30 in Africa (Lahner, 1998).

The following table 2.2 focusses on observations and research which present data on mating flight of sympatric species at one location (In Thailand, Sri lanka and Borneo).

In *A. andreniformis*, the drone has short flight period about 1.5 hr. In Thailand, drone fly at 12.15-13.45 (Rinderer *et al.*, 1993) and nearly the same time drones fly in Borneo at 12.00-13.45 (Koeniger *et al.*, 1996). The successful mating flights of *A. andreniformis* queen in Borneo was between 12.33 and 12.50 (Koeniger *et al.*, 1996).

The drone flight period of *A. florea* is 12.00-14.30 in Sri Lanka (Koeniger and Wijayagunesekera, 1976) whereas in Southeastern Thailand it is 14.00-16.45 (Rinderer *et al.*, 1993). In Thailand, drone flight period also differed in Southeastern Thailand

(Chantaburi) and Central Thailand (Bangkok), drones fly earlier at 13.45-15.30 in Central Thailand and the period of queen flight observed between 14.04 and 14.25 (Koeniger *et al.*, 1989)

Drones of *A. cerana indica* fly from14.00-16.15 in Borneo (Koeniger *et al.*, 1996), from 15.15-17.30 in Southeastern Thailand (Rinderer *et al.*, 1993), from 16.15 to 17.15 in Sri Lanka (Koeniger and Wijayagunesekera, 1976). The successful mating flight of *A. cerana* queen, in Sri Lanka is 16.15 and 16.55 (Punchihewa *et al.*, 1990). The, drone flight period of *A. cerana* are more variability than among Asian honeybee species.

A. koschevinkovi drones fly during a long period nearly 2 hrs from 16.45-18.30. Queen flew between 17.00-18.15 (Koeniger *et al.*, 1994).

A. dorsata fly consistently at sunset and occur for a short period. Drone fly from 18.15-18.45 in Southeastern Thailand (Rinderer *et al.*, 1993), from 18.00-18.45 in Sri Lanka (Koeniger and Wijayagunesekera, 1976), and from 18.15-19.05 in Borneo (Koeniger *et al.*, 1996).

Mating flight period is correlated with the size; it start with the smallest *A. andreniformis* and *A. florea*; the next with cavity-dwelling species *A. mellifera* and *A. cerana*; and ends with the largest *A. dorsata*. The difference in the mating flight time in *Apis* species is thought to be a key factor of interspecific reproductive isolation.

Table 2.1. Drone flight periods of sympatric Asian honeybee species (Modified from Koeniger and Koeniger (2000)).

Apis species	Koeniger and Wijaygunesekera (1976) **Sri Lanka**	Rinderer et al (1993) **Thailand**	Koeniger et al (1996) **Sabah, Borneo**
A. andreniformis	-	12.15-13.45	12.00-13.45
A. florea	12.00-14.30	14.00-16.45	-
A. cerana	16.15-17.15	15.15-17.30	14.00-16.15
A. koschevnikovi	-	-	16.45-18.30
A. dorsata	18.00-18.45	18.15-18.45	18.15-19.05

2.11. Instrumental insemination

Honeybees are economically important species, especially *Apis mellifera* is a commercial species for beekeeping which have many subspecies. It is important for improvement of bee subspecies, strains, and breeding stocks for commercial. Then, it is necessary to control mating. However, it is difficult in controlled mating because mating of honeybee occurs in the air. For at least two centuries beekeepers have been trying to control breeding, first by attempting to get queens and drones to mate in enclosed space, this method is failed.

Instrumental insemination is a technique to transfer instrumentally sperm from the male into the female's reproductive organs. Instrumental insemination is an alternative for honeybee breeding stock for commercial and also enable to make mating that are not possible for natural mating. The major use of instrumental insemination has been in research. It has been used to develop inbred lines, maintain mutant markers, and make specific mating for generic research such as backcross and single drone insemination. Therefore, the improvement of instrumental insemination technique has become important.

The principle of instrumental insemination was development between 1926 and 1947. Watson (1927) initiated modern development of instrumental insemination, and he collected honeybee semen in microsyringe. The success of Watson's technique is comfirmed by Nolan (1929), he also constructed a new type of syringe, and also designed an insemination apparatus in 1937. Laidlaw (1944) improved the success rate of instrumental insemination tip pass the valvefold that cover the entrance of the median oviduct. He depressed the valvefold and injected the semen directly into median oviduct. Mackenson (1947) used Co_2 to immobilize during insemination. This made it easier to insert the insemination syringe tip into the median oviduct. Moreover, he found Co_2 narcosis caused queen to begin laying eggs sooner after insemination. Mackenson (1955) later designed a diaphragm type syringe for semen collection and insemination of queens, which is universally used. Semen must be taken into syringe before each queen is injected. It seemed desirable to take enough semen into a syringe to inseminate several queens in succession.

The success of instrumental insemination is measured by the ability of queens to produce sufficient quantities of normal female (workers, or more importantly, daughter queens). One factor of successful in insemination is diluent and period of sperm

storage. Much research has been done on developing sperm storage and diluents for storing honeybee spermatozoa in vitro. (Jaycox, 1960; Taber and Blum,1960; 1969, 1970; Mackenson,1969; Camargo, 1975; Verma ,1978).

2.12. Cross insemination

The first pioneer research on reproductive isolation among honeybees was initiated by Ruttner. It is not possible for natural mating between species because of reproductive isolation in phase of behavioral barriers that prevent physiological contact between queen and drone. Then, the technique of instrumental insemination can be overcome this problem to study reproductive imcompatibility among species. Both *A. mellifera* and *A. cerana* were inseminated with heterospecific semen that collected from everted drone endophalli. Spermatozoa reached the spermatheca. Egg fertilized but died in blastular stage (Ruttner and Maul, 1983). Woyke (1993) inseminated *A. florea* queen with *A. mellifera* semen that collect from everted endophalli of drone. He reported only some spermatozoa of *A. mellifera* reaches the spermatheca of *A. florea* queen. Koeniger *et al* (1996) examined whether spermatozoa are stored in heterospecific spermathecae of sympatric species *A. cerana*, *A. koschevnikovi* and *A. dorsata*. Spermatozoa of these species were collected drom seminal vesicles by dissection and stored in hyes solution. Afterwards, it was concentrated by centrifugation (8,000 rpm for 15 min). For a control, *A. cerana* queens were inseminated each with 7.3 million spermatozoa of *A. cerana* drones. The spermatheca contained 0.8 million spermatozoa. Also, *A. cerana* queens were inseminated each with 8.0 mio sperm of *A. koschevnikovi*. After insemination, spermatheca contained 0.6 million spermatozoa. In *A. koschevnikovi*, queens were inseminated with 11.7 million spermatozoa of their own species. The spermatheca contained 0.8 million spermatozoa. When *A. koschevnikovi* queens were inseminated with 6.4 million spermatozoa of *A. cerana*, the spermatheca

contained 0.6 millio spermatozoa. Moreover, *A. koschevnikovi* queens were inseminated with spermatozoa of *A. dorsata*, and also spermatozoa reached the spermatheca of *A. koschevnikovi*. In all cases spermatozoa were active in te spermatheca when dissected 3 days later. Koeniger *et al* (1998) inseminated two queens of *A. cerana* with *A. koschevnikovi* sperm. They found hybrid with gynandromoph characters. Woyke (2001) observed a hatching rate of eggs of only 3% after insemination of 3 *A. mellifera* queens with spermatozoa spermatozoa of *A. dorsata* that collected from ejaculate by everted endophallus.