CHAPTER V

INSTRUMENTAL INSEMINATION OF *Apis mellifera* L. QUEENS WITH HETERO-AND CONSPECIFIC SPERMATOZOA RESULTS IN DIFFERENT SPERM SURVIVAL

ABSTRACT

Sixty three queens of *A. mellifera* were inseminated each with about 8 million spermatozoa from either 1 *A. mellifera*, 8 *A. cerana*, 5 *A. dorsata* or 20 *A. florea* drones. Spermatozoa were collected from seminal vesicles, diluted in buffer and reconcentrated at 1,000 g for 10 min. Between 1.4% and 2.8% of the spermatozoa reached the spermatheca. Motility of spermatozoa of *A. mellifera* and *A. cerana* did not change within 4 weeks, it was nearly 100%. The motility of *A. florea* spermatozoa decreased to 83.4% after 3 days and to 33.9% after 4 weeks, and of *A. dorsata* spermatozoa it decreased to 61.2% after 3 days and to 26% after 4 weeks. Fertilization of *A. mellifera* eggs was 57% by *A. mellifera* spermatozoa. Calculation based on non hatching eggs showed that about 40% were fertilized by *A. cerana* and *A. florea* and less than 20% by *A. dorsata* spermatozoa. The composition of spermathecal fluid seems to be different within the species and its significance for long term sperm storage is discussed.

key words: sperm survival / spermathecal fluid / hetero specific insemination / *Apis mellifera* queens / *Apis* species

5.1. INTRODUCTION

The storage of spermatozoa after mating in a special organ of the female, the spermatheca, is a widespread phenomenon in the animal kingdom. It is known in many genera as diverse as crustacea, insects, spiders and mammals (Eberhard, 1996). The storage duration differs from a few days up to several years. In somes ants spermatozoa are able to fertilize eggs up to 30 years (Buschinger, unpublished data). In the honey bee, *A. mellifera*, spermatozoa can be stored up to 6 years (Butler, 1954).

Spermathecal organs are ectodermal in origin and lined with cuticle. They vary considerably in their overall structure and their number vary from one to three. The contents of the spermatheca often derive from glands or glandular epithelium and are known to contain proteins, but the functions of the spermathecal fluids are not known for certain (Chapman, 1998; Resh and Cardé, 2003). In some insects, such as *Drosophila melanogaster*, male derived proteins play a major role for the physiology of the spermatozoa (Chapman, 2001). For the long term storage of 3 up to 5 years in *A. mellifera*, secretions of the queen into the spermatheca seem to be of significance (Klenk *et al.*, 2004, in press). Further the the spermathecal fluid contains several sugars (Alumot et al., 1969) and its pH value is 8.6 (Gessner and Gessner, 1976).

All honeybee species have one spermatheca connected with the oviduct by the spermaduct. In *A. mellifera* the spermatheca is a globular sac with a diameter of about 1.1mm. It consists of a chitinous membrane with a one layer epithelium, surrounded by a dense tracheal net (Bishop, 1920; Snodgrass, 1956; Ruttner *et al.*, 1971). A pair of tubular glands is connected to the lumen which, in virgin queens, is filled with a transparent fluid. 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 amount of spermatozoa of mated queens varies among the species of the genus *Apis* but the concentration per µl seems to be similar as in all *Apis* species. Spermatozoa are densely packed in the lumen of mated

queens (*A. florea* Koeniger *et al.* 1989; *A. cerana* Punchihewa 1992; Woyke 1975; *A. dorsata* Tan *et al.*,1999; *A. koschevnikovi* Koeniger *et al.*, 1994).

A. mellifera queens were instrumentally inseminated with spermatozoa of *A. mellifera, A. cerana, A. dorsata* and *A. florea*. As *A. mellifera* is naturally allopatric to the other *Apis* species, mating barriers between *A. mellifera* and the Asian honeybee species do not have any adaptive significance. Therefore, the passage of spermatozoa from the oviduct into the spermatheca and the survival of heterospecific spermatozoa in the spermatheca of an *A. mellifera* queen is expected to reflect a genetic incompatibility independently of any recent selective and adaptive pressures.

5.2. MATERIALS AND METHODS

5.2.1. Producing and keeping queens

A. mellifera queens were reared in queenless colonies according to the method of Ruttner (1983). They were kept in small colonies (nucleus boxes) with about 1,000 worker bees and top bars for frames.

5.2.2. Buffer solution for collection of spermatozoa and instrumental insemination

As diluent for the collection of spermatozoa Tris buffer was used: (Trizma HCL and Trizma Base combined to pH: 8.5). In addition, glucose D 1.0 g, sodium chloride 11.0 g, lysine 0.1 g and arginine 0.1 g were added and solved in 1,000 ml distilled water. About 0.01 g penicillin G (K-salt) and 0.01 g streptomycin sulfate were added to prevent infections.

5.2.3. Semen collection from seminal vesicles

To overcome the problem that the number of spermatozoa of drones differ between 0.4 $x10^6$ and 8 to 10 $x10^6$ (Koeniger and Koeniger, 2000), the number of spermatozoa for each insemination was adjusted to one drone of *A. mellifera* (to about 8

million). Further we combined the technique of collecting spermatozoa from the seminal vesicles (Mackensen and Ruttner, 1976) and sperm collection in buffer solution (Kaftanoglu and Peng, 1980), which both proved to be successful.

Drones were collected during the mating flight times of each species (Koeniger and Koeniger, 2000) narcotized with CO_2 to prevent semen ejaculation. For collection of spermatozoa the last tergite and sternite the abdomen was cut off under a stereomicroscope, the endophallus was grasped with the forceps and the reproductive tract was pulled out with the attached mucus glands and seminal vesicles. Seminal vesicles were separated and transferred into a small black vessel (to increase contrast of tissue against container) that contained the described diluent. The seminal vesicles were torn and pressed with a fine pair of needles to release semen. The time needed for collection of spermatozoa was less than 1 min per drone. Thus the total time for sperm collection was less than 30 min for *A. florea*, less than 10 min for *A. dorsata* and *A. cerana*. Spermatozoa were transferred into a micro-centrifuge tube to centrifuge at 1,000 g for 10 min. Then, spermatozoa were collected. For instrumental insemination they were sucked into an insemination syringe and immediately inseminated.

5.2.4. Instrumental insemination of queens

A. mellifera queens were instrumentally inseminated at the age of 6 days with about 8.0 mio spermatozoa per queen using the standard insemination apparatus of Schley. Twenty four hours before insemination, queens were anaesthetized for 10 min with CO_2 to stimulate egg laying. After insemination, the right wing of the queens were clipped and were marked with a number on the thorax. Each queen was maintained in a mating nucleus that had a queen excluder attached to the entrance.

5.2.5. Counting spermatozoa from seminal vesicles and from spermathecae and testing motility.

Spermatozoa were collected from the seminal vesicles as described above. They were thoroughly dispersed and further diluted with distilled water to a volume of 20 ml. The spermatozoa were counted with a Fuchs - Rosenthal haemocytometer. The spermathecae of inseminated queens were dissected and transferred into a drop of buffer. The tracheal net around the spermatheca was removed.

In case of transparent appearance, the spermatheca was placed directly on counting squares of a haemocytometer. It was squashed with a cover glass until the membrane was broken and the fluid dispersed. With this method, even single spermatozoa could be recognized and counted. The motility was recorded during a period of 1 hour in which 100 spermatozoa were observed twice and each time the number of moving spermatozoa were noted.

In the case of opalescent spermathecae, they were squashed on an object slide and the sperm motility was recorded. For counting, spermatozoa were transferred carefully with a fine Pasteur pipette and after several rinsing with some buffer solution into a small black vessel. After dispersion the spermatozoa were further diluted to exactly 1 ml with distilled water and counted in the hemocytometer.

5.2.6. Oviposition

All nucleus colonies were checked every 2 days for eggs and larvae. The hatching rates were determined by putting a transparent sheet over the comb and marking cells with an egg with red color on the sheet. Two or four days later the emerged egg was noted by a blue color. Capped brood was kept in an incubator (34.5°C and 60 – 70% RH) and the emerged imagos were sexed and counted.

5.2.7. Statistics

Differences in number and motility of spermatozoa for the *Apis* species were analyzed by Mann Whitney U-tests.

5.3. RESULTS

5.3.1. Number of drones used for each insemination of a *A. mellifera* queen

The number of spermatozoa per drone was determined for each species (table 5.1). The number of spermatozoa in seminal vesicles were different between species. The average number of spermatozoa in seminal vesicles of *A. mellifera*, *A. cerana*, *A. dorsata* and *A. florea* were 7.59± 1.47 (n = 10), 1.00 ± 0.11(n= 5), 1.80 ± 0.18 (n=5) and 0.38 ± 0.03 x 10^6 (n= 5) spermatozoa respectively.

According to these data spermatozoa of 1 *A. mellifera* drone, 5 *A. dorsata* drone, 8 *A. cerana* drones and 20 *A. florea* drones were collected as one batch. The result are presented in table 4.6. The seminal vesicles of one drone in *A. mellifera* contained 7.59 ±1.47 x 10^6sptz (n=10). In 8 drones of *A. cerana*, the number of spermatozoa was 7.96 ±1.19 x 10^6 (n=10). In 5 drones of *A. dorsata*, the average number of spermatozoa was 7.81 ± 0.28x 10^6 (n=5). In 20 drones of *A. florea*, the average number of spermatozoa was 7.13 ± 1.31 x 10^6 (n=8).

There were no significantly difference for the amount of spermatozoa in seminal vesicles between one drone of *A .mellifera,* 8 drones of *A. cerana*, 5 drones of *A. dorsata* and 20 drones of *A. florea.* All queens were thus inseminated with similar number of spermatozoa of all species.

Table 5.1. Number of spermatozoa in the seminal vesicles of 4 *Apis* species.

Origin of spermatozoa	numbers of spermatozoa per drone (x 10^6)	numbers of spermatozoa adjusted to *Apis mellifera* drone (x 10^6)
A. mellifera	7.6 ± 1.47 (n = 10)	1 drone: 7.6 ± 1.5 (n = 10)
A. cerana	1.0 ± 0.11 (n =5)	8 drones: 8.0 ± 1.0 (n = 10)
A. dorsata	1.8 ±0.18 (n = 5)	5 drones: 7.8 ± 0.3 (n = 5)
A. florea	0.38 ±0.03 (n = 5)	20 drones: 7.1 ± 1.3 (n = 8)

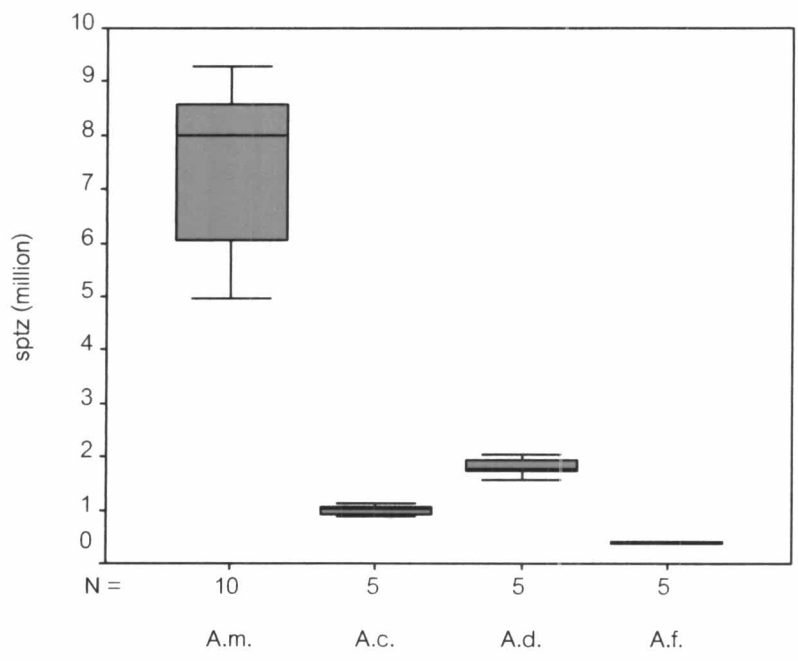

Figure 5.1. Number of spermatozoa per drone in seminal vesicles (x 10^6) of *A. mellifera*, *A. cerana*, *A. dorsata* and *A. florea*.

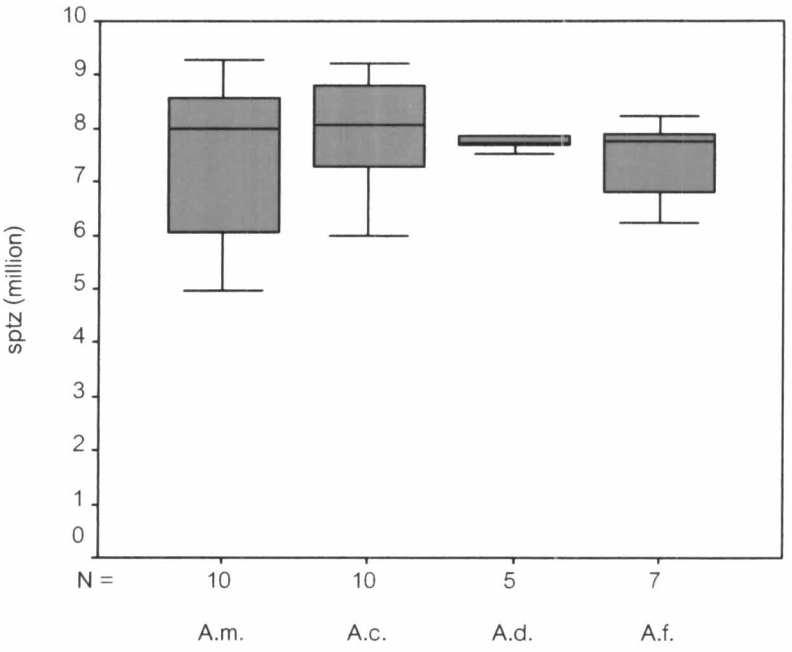

Figure 5.2. Number of spermatozoa in seminal vesicles ($x10^6$) in one drone of *A mellifera*, 8 drones of *A. cerana*, 5 drones of *A. dorsata* and 20 drones of *A. florea*.

5.3.2. Concentration of spermatozoa for insemination

The concentration of spermatozoa used for insemination was only measured for *A. mellifera*. After reconcentration by centrifugation for 10 min with 1,000 g, it was 2.1 x $10^6 \pm 0.3$ pro µl (n = 10).

5.3.3. Number of spermatozoa reaching the spermatheca

The number of spermatozoa reaching the spermatheca showed no difference for *A. mellifera* and *A. florea*. From *A. cerana* a significantly higher number and from *A. dorsata* significantly lower number of spermatozoa entered the spermatheca (table 5.2. and figure 5.3.). Based on the number of inseminated spermatozoa it was 1.4% for *A. dorsata*, 1.9 for *A. florea*, 2% for *A. mellifera* and 2.8% for *A. cerana*.

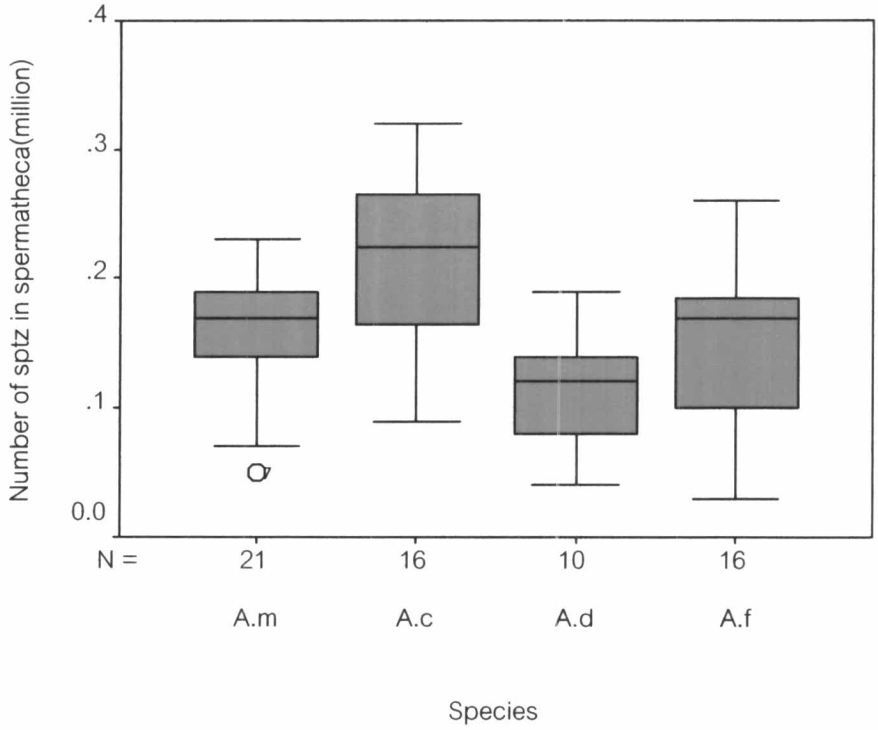

Figure 5.3. Number of spermatozoa of 4 species entered spermatheca of *A. mellifera*

5.3.4. Motility of spermatozoa 3 days and 4 weeks after insemination

Motility of spermatozoa of *A. mellifera* and *A. cerana* drones in the spermatheca did not change within the period of 4 weeks, it was nearly 100%. The motility of *A. florea* spermatozoa decreased significantly after 3 days to 83.4 ± 22.5% and after 4 weeks to 33.9 ± 38.9% (table 5.2., fig. 5.4), while in *A. dorsata* after 3 days only 61.2 ± 37.8 % were motile and even less after 4 weeks (26.0 ± 37.2%; table 5.2., figure 5.4.).

Table 5.2. Number and motility of spermatozoa of 4 species stored in the spermatheca of an *A. mellifera* queen.

origin of spermatozoa	number of spermatozoa in spermatheca (x 10^6)	motility of spermatozoa 3 days after insemination (%)	motility of spermatozoa 4 weeks after insemination (%)
A. mellifera	0.16 ± 0.05 (n = 21)	98.46 ± 3.15 (n = 13)	96.88 ± 4.58 (n = 8)
A. cerana	0.22 ± 0.07 (n = 16)	97.50 ± 5.35 (n = 8)	93.75 ± 10.26 (n = 8)
A. dorsata	0.11 ± 0.04 (n = 10)	61.20 ± 37.78 (n = 5)	26.00 ± 37.15 (n = 5)
A. florea	0.15 ± 0.06 (n = 16)	83.38 ± 22.53 (n = 8)	33.86 ± 38.91(n = 8)

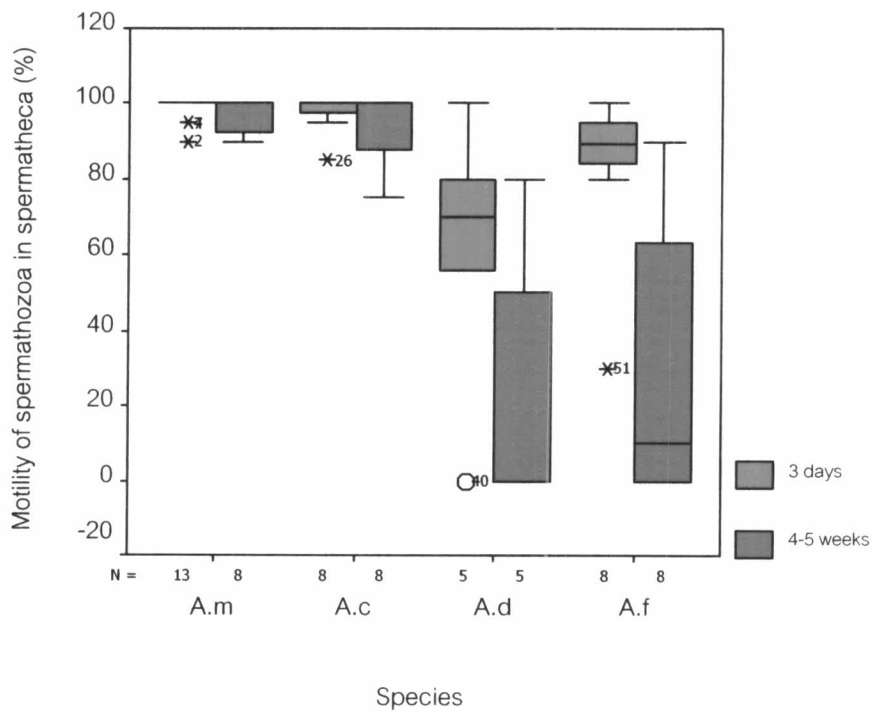

Figure 5.4. Motility of spermatozoa stored in spermatheca 3 days and 4 weeks after insemination.

5.3.5. Oviposition and egg hatching

All queens started to lay eggs between 6 and 13 days after instrumental insemination. Only *A. mellifera* queens inseminated with *A. mellifera* spermatozoa produced worker brood, in average 57%. Egg hatching did not differ after insemination with *A. mellifera* and *A. dorsata* spermatozoa, but it was significantly lower after insemination with *A. cerana* and *A. florea* spermatozoa (table 5.3.).

Table 5.3. Development of the eggs laid by the *A. mellifera* queens 6 up to 13 days after insemination.

Origin of spermatozoa	egg hatching index	% worker offspring	% fertilized eggs
A. mellifera (8 nuclei)	100 (a)	57.3% n = 1,119	57.3%
A. cerana (8 nuclei)	0.56 ± 0.32 (b)	0 n = 1,348	44%
A. dorsata (5 nuclei)	0.82 ± 0.23 (a)	0 n = 1,191	18%
A. florea (8 nuclei)	0.59 ± 0.24 (b)	0 n = 598	41%

5.4. DISCUSSION

Instrumental insemination with sperm of a single drone and insemination of centrifuged sperm has been used in several studies (Kaftanoglu and Peng, 1980; Harbo, 1986). Also insemination of spermatozoa collected from seminal vesicles proved to be successful (Mackensen and Ruttner, 1976). Previously, the combination of these 3 techniques and subsequent instrumental insemination was used successfully for *A. koschevnikovi* spermatozoa inseminated into *A. cerana* queens. Those queens produced fertilized eggs from which adult gynandromorph hybrids emerged (Koeniger and Koeniger, 2000).

In all experiments about 8 million spermatozoa of each species were inseminated and in all cases spermatozoa reached the spermatheca. This is in accordance with earlier experiments. *A. mellifera* spermatozoa entered the spermatheca of *A. florea* queens (Woyke, 1993) and those of *A. koschevnikovi* and *A. cerana* spermatozoa entered the spermatheca of *A. koschevnikovi* and vice versa (Koeniger and Koeniger, 2000). Further *A. dorsata* spermatozoa were supposed to enter those of *A. mellifera* (Woyke *et al.*, 2001), a low percentage (about 3.5%) of the inseminated spermatozoa entered the spermatheca of *A. koschevnikovi* (Koeniger and Koeniger 2000).

The number of spermatozoa reaching the spermatheca was low: it was between 0.11×10^6 (1.4%) (*A. dorsata* spermatozoa) and 0.22×10^6 (2.8%) (*A. cerana* spermatozoa). The concentration of the inseminated spermatozoa after centrifugation amounted to 2.1×10^6 per 1 µl. In another report a dilution of spermatozoa from ejaculate of 1:1 the concentration was 3.1×10^6 per 1 µl and insemination of 1.4 µl resulted in 1.2 million in the spermatheca (Bolten and Harbo, 1982). Thus sperm concentration (viscosity) seems to be an important factor for the ratio of spermatozoa reaching the spermatheca. After natural mating only about 3% of spermatozoa are found in the spermatheca (Koeniger and Koeniger, 2000; Palmer and Oldroyd, 2000) though the concentration in ejaculate is about 7×10^6 per 1µl (Woyke, 1960). Even after

mating with 1 or 2 drones,it is less than after instrumental insemination (Koeniger, 1991; Schluens *et al.*, **unpublished data**).

The fact that there was no difference in the number of spermatozoa reaching the spermatheca between *A. mellifera* and *A. florea* was unexpected. During mating of *A. florea* spermatozoa are injected into the spermaduct from where they have to overcome only a short distance to reach the spermatheca while in the other species sperm is deposited in the oviduct. Further after natural mating in *A. florea* queens a percentage from 30 to 40% per drone reaches the spermatheca (Palmer and Oldroyd, 2000; Koeniger and Koeniger, 2000). The similar range in percentage of spermatozoa of all species reaching the spermatheca can be interpreted that queens active support the filling process of the spermatheca. This is in accordance with the earlier results for *A. mellifera* queens (Ruttner and Koeniger, 1971; Gessner and Ruttner, 1977). But spermathecal fluid or gland secrete also may have similar attractants for spermatozoa in all species.

There was no difference in the duration between the insemination and the onset of egg laying. So we can exclude any effects of heterospecific spermatozoa on the queen's physiological changes which was induced in the experiment by 2 CO_2 narcosis and lead to oviposition.

Significant differences occured in the motility of spermatozoa in the spermatheca after a storage of only 3 days. The range of motility is in accordance with the range of relatedness of the species suggested from various studies on DNA, morphology and other characters (Alexander, 1991a, 1991b). *A. mellifera* is positioned between *A. cerana* on one side and *A. dorsata* and *A. florea* at the other. Already after 3 days the motility of spermatozoa from the free nesting species decreased significantly, in *A. florea* (17%) it was significantly less than in *A. dorsata* (39%). After 4 weeks less that 35% spermatozoa were motile in both free nesting species. There were no significant differences in motility within the two cavity- nesting species and within the two free-

nesting species. Probably the composition of the spermathecal fluid is different which has some influence on sperm survival.

There were significant differences in the egg hatching rate between conspecific and heterospecific insemination. While after insemination with *A. mellifera* and *A. dorsata* spermatozoa no significant difference occurred, a significantly lower percentage of hatching eggs was found after insemination with *A. cerana* and *A. florea* spermatozoa. Experiments of Ruttner and Maul (1983) showed that after cross insemination of *A. mellifera* and *A. cerana* eggs were fertilized but embrydied in the blastula stage. Consequently only 8% of the eggs hatched, which developed into drones.

Because of the low numbers of *A. mellifera* spermatozoa only about 57% of emerging bees were worker bees, thus we calculate that the same percentage of eggs were fertilized from *A. mellifera* spermatozoa. Considering the low hatching index of 0.56 after insemination with *A. cerana* spermatozoa, we assume that 44% of the eggs were fertilized and these did not develop. Similarly 41% of *A. mellifera* eggs were fertilized by *A. florea* spermatozoa. Only after insemination of *A. dorsata* spermatozoa the number of hatching eggs was not significantly different to the conspecific insemination, so no or only few eggs were fertilized. This is in accordance with the high loss of motility of spermatozoa after 3 days, as the first eggs were laid only after 6 to 13 days.

Woyke *et al.* (2001) observed a hatching rate of eggs of only 3% after insemination of 3 *A. mellifera* queens with spermatozoa from ejaculate of *A. dorsata*. They concluded that in this case all eggs were fertilized but died during development. But unfortunately the queens died already after 35, 100 and 130 days. The authors did neither check for number of spermatozoa in the spermatheca nor for their motility. It is possible that the spermatozoa were motile for a longer period after collection from ejaculate. But the early death of all queens may also point to some unusual infection which might be a result of the death of spermatozoa.

In all *Apis* species the spermatheca has a spermathecal gland and a dense tracheal net. Up to now physiological conditions were only studied in *A. mellifera*. The pH value of the spermathecal fluid is 8.6 (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 gland the queen laid unfertilized eggs, even though a high percentage of the spermatozoa were motile for more than 90 days (Koeniger, 1970). Further the spermathecal compartment contains several sugars (Alumot *et al.*, 1969) and soluble proteins (Lensky and Alumot, 1969). The concentration of proteins varies from 5 to 15.3 mg / ml according to age and season (Klenk *et al.*, 2004). A 29kDa protein only occurred in the spermathecal fluid of sexually mature queens. It may have a function for long-term sperm storage, but further studies are required (Klenk *et al.*, 2004). Antioxidases (CAT, SOD and GST) found in the spermathecae of mated queens may be also involved in the storage by protecting the spermatozoa from oxidative stress (Weirich *et al.*, 2002).

The present experiments mainly investigated the physiological conditions in the spermatheca, as spermatozoa were inseminated without seminal plasma to reduce or largely eliminate male-derived accessory components. The results can be interpreted as differences in the physiological conditions between the two multiple-comb cavity and the two single-comb open-air nesting *Apis* species. This supports the idea of the significance of spermathecal fluid for sperm storage, as suggested by Klenk *et al.* (2004). Spermathecal fluid and spermathecal gland secretion should be compared in the species.

It is generally accepted that the genus *Apis* consists of 3 taxonomic units, the cavity nesting species including *A. mellifera* and *A. cerana* (Koeniger, 1991), the dwarf honeybees (*A. florea* and *A. andreniformis*) and the giant honeybees (*A. dorsata* group). The similarity in sperm survival between of *A. cerana* spermatozoa and conspecific *A. mellifera* sperm is a further confirmation of the above concept, while it is different for the other 2 species. In a cladistic analysis on phylogenetic relationship between the *Apis*

species based on 20 characters (Alexander, 1991a,b) the dwarf honeybees (*A. florea* and *A. andreniformis*) branched away at the basis and the giant bees (*A. dorsata* group) plus the cavity nesting bees (with *A. mellifera* and *A. cerana etc)* form a monophyletic sister group. Similar results were reported by Tanaka *et al* (2001) in a phylogram of *Apis* 16S (477 characters of aligned sequences) and of *Apis* CO1 (based on 1041 base-pair length of sequences). Our results would support a different cladogram. Though the difference in sperm motility between *A. florea* (83%) and *A. dorsata* (61%) was not significant the difference in the rate of non hatching eggs (e.g. fertilized eggs) was significant. This would support *A. dorsata* 's position as the basic group. Accordingly, the other species complex (the dwarf bees and cavity nesting species) branched away later as monophylletic unit.

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To whom it may concern

I hereby confirm that the manuscript

"Instrumental insemination of Apis mellifera L. queens with hetero- and conspecific spermatozoa results in different sperm survival"

submitted by Mananya Phiancharoena, Siriwat Wongsiria, Nikolaus Koeniger and Gudrun Koeniger has been accepted for publication in APIDOLOGIE

With best regards

S. Fuchs S. Fuchs