CHAPTER IV

MOTILITY OF SPERMATOZOA (*Apis mellifera*) IN DIFFERENT DILUENTS AND METHOD OF COLLECTIONS

ABSTRACT

Spermatozoa of honebee *Apis mellifera* which collected from ejaculation by everted endophallus or from seminal vesicles by dissection were stored in different diluents, kept in capillary tubes at 34.5^{0}C for 12 days. The method of collection and diluent for spermatozoa were assessed by motility of spermatozoa. No significant difference was found in the motility of spermatozoa between spermatozoa collected both from ejaculation and from seminal vesicles. Significant differences of spermatozoa motility were found in different diluents for spermatozoa storage. A higher percentage of spermatozoa were motile in Tris buffer diluent than in Hyes diluent and 0.9%NaCl. Tris buffer diluents (pH: 8.5) containing mainly Glucose and NaCl as well as some arginine and lysine was an appropriated diluent for storing honeybee spermatozoa. The technique of centrifugation for spermatozoa reconcentration affected on spermatozoa motility and the number of spermatozoa reaching the spermatheca after insemination of queens. Using high speed and long duration of centrifugation (10,000 g for 10 min and 30 min) caused a higher reduction of motility than using low speed and short duration (1,000 g for 10 min).

The combination of these 3 techniques, collecting spermatozoa directly from seminal vesicles, washing in Tris buffer diluent and reconcentrating spermatozoa by centrifugation at 1,000 g for 10 min was used successfully for instrumental insemination

key words: spermatozoa motility/ seminal vesicles/ centrifugation/ *Apis mellifera*

4.1. INTRODUCTION

In honey bee the technique of instrumental insemination has been developed and improved since the 20th century. Its usefulness is in the field of research of genetics and improvement of honeybees breeding stock for commercial beekeeping. The success of instrumental insemination depends on the diluents and periods of sperm storage (Jaycox, 1960; Lensky and Schindler, 1967; Poole and Taber, 1969, 1970; Camargo,1975; Verma, 1978; Moritz,1984). Many researches have been done on developing sperm storage and diluents for storing honeybee spermatozoa in vitro. Jaycox (1960) tested motility of sperm diluted in several saline solution after storage in capillaries. He found moderate motility after 10 days when sperm was diluted in a saline solution containing glycine. Taber and Blum (1960) kept undiluted spermatozoa of honeybee alive for 68 days at room temperature. Poole and Taber (1969, 1970) kept semen alive for 12-13 weeks at 13-15°C in capillaries. Mackenson (1969) inseminated queen with semen diluted in saline (pH: 6.9) which containing 0.1% glucose, fructose or trehalose (dilution 1:1). He found the number of spermatozoa entering the spermatheca did not differ from control. Camargo (1975) reported that Coconut water (pH: 7.0) was a good diluent for preservative *A. mellifera* semen. Verma (1978) stored honeybee semen in Tris buffer with different diluted components and also different pH, Glycine buffer, Coconut water *etc.* and used motility of spermatozoa for assessing sperm quality. He reported that Tris buffer diluent (pH: 7.9) containing K^+ and Na^+ as found in the spermatheca of the queen acted as a reversible inhibitor of sperm motility, even after 9 months of storage.

Kaftanoglu and Peng (1980) initiated a washing technique that separated the sperm from mucus by rinsing in diluents and reconcentrated spermatozoa by centrifugation at 2,500 rpm and used it to successfully inseminate queen. Moritz (1983) and Harbo (1990) pooled semen of more than 100 drones by mixing them in a diluent and reconcentrated it by centrifugation at 10,000 g for 10 min for instrumental insemination. Moritz (1984) studied the effects of semen diluents on the onset of oviposition by inseminating queens with semen diluted in Tris buffer diluent, Hyes diluent

and Kiev diluent and centrifuged at 10,000 g for 10 min. He found that the onset of queen was much earlier in Tris buffer diluent than Hyes diluent and Kiev diluent. Koeniger *et.al.* (1996, 1998) took sperm directly from seminal vesicles which was diluted during dissection in Hyes diluent and re-concentrated by centrifugation (8,000 rpm for 15 min) for interspecific instrumental insemination. There are various assays used for assessing sperm quality. Sawada and Chang (1964) and Verma (1978) used motility of spermatozoa for assessing sperm quality. Bolton (1982), Harbo (1979, 1983, 1987), and Kaftanoglu and Peng (1980) counted the number of spermatozoa in spermatheca of an instrumentally inseminated queen. Collins and Donoghue (1999) used the technique of live: dead dual fluorescent staining to determine sperm viability in honeybees. Collins (2003) evaluated the viability of spermatozoa after centrifugation by live: dead dual fluorescent staining.

The investigation of the present research was to find an appropriated diluent for honeybee spermatozoa and to develop the method of collecting spermatozoa from seminal vesicles. After dissection of seminal vesicles in a diluent spermatozoa have to be reconcentrated by centrifugation for instrumental insemination. Therefore, motility of spermatozoa after storage in different diluents and centrifugation were studied. In addition, the number of spermatozoa entering the spermatheca after different treatments was determined.

4.2. MATERIALS AND METHODS

4.2.1. Drone collection

The experiments were conducted in Oberursel, Germany. Mature drones of *A. mellifera* were caught at the entrance of colonies when they returned from mating flight in the afternoon from 14.00 to 17.00 h. Captured drones were put in a drone cage for at least 1 hr before obtaining semen. In case that drones could not be used on the same day of collection, the drones in the cage were kept in a nursery colony overnight until the next day.

4.2.2. Semen collection for test motility

Semen was collected from mature drones by three different methods. First, semen was collected from ejaculation by everted endophallus. Second, it was collected from seminal vesicles by dissection. Last, it was collected from seminal vesicles, but spermatozoa were dispersed in different diluents and reconcentrated by centrifugation at different speed and durations (g/time treatment: 1,000 g for 10 min and 10,000 g for 10 min, 30 min).

Three different diluents were tested for motility of spermatozoa;

1. Tris buffer diluent (pH: 8.5): 2.21 g Trizma hydrochloride; 4.36 g Trizma base; 1.00 g α- D (+) Glucose; 11.00 g NaCl; 0.10 g L- Lysinmonohydrochloride; 0.10 g L- Argininmonohydrochloride; 1,000 ml distilled H_2O (Verma, 1973, 1978)
2. Hyes diluent (pH: 8.5): 9.00 g NaCl; 0.20 g $CaCl_2$; 0.20 g KCl; 0.10 $NaHCO_3$; 1,000 ml distilled H_2O; and adjusted pH with NaOH (Ruttner, 1975)
3. NaCl 0.9% (w/v): 9.00 g NaCl; 1,000 ml distilled water

Penicillin G potassium and Streptomycin sulphate were added to each of solution as antibiotics.

To find the most suitable diluent, the collected spermatozoa from each method were diluted with 100 µl of each diluent. Three µl of each sample were stored in a 5 µl micropipette tube. After filling spermatozoa, the end of micropipette tubes were sealed with parafilm. This resulted in 9 samples per drone per treatment. All the tubes containing the diluted spermatozoa were kept in an incubator at 34.5°C with 60-70 % RH. The percentage of motility was determined.

4.2.3. Evaluation of spermatozoa motility

Spermatozoa were checked for the motility in the period of 12 days. All spermatozoa were transferred by blowing a collecting tube on 76x26 mm slide glass

covered with 22x22 mm cover glass. The motility of spermatozoa was checked under a phase contrast microscope (Leica DMLB) at magnification of 200x and 400x. From every sample 100 spermatozoa were observed.

For classification of motility, 5 grades of motility of spermatozoa was estimated from the percentage of motile spermatozoa

0 = all spermatozoa were immotile

1 = Up to 10 % of spermatozoa were moving

2 =10 - 50 % of spermatozoa were moving

3 = 50 -90 % of spermatozoa were moving

4 = 90 - 100% of spermatozoa were moving

4.2.4. Concentration of spermatozoa from different spermatozoa collection

Spermatozoa were collected from 5 *A. mellifera* drones as ejaculate or from seminal vesicles in diluent. Then, they were reconcentrated by centrifugation at 1,000 g and 10,000 g for 10min. Spermatozoa of each sample were collected in an insemination syringe and the volume was measured. Then they were transferred to a small black container, diluted with 1ml Tris buffer diluent, and dispersed by blowing air with Pasteur pipette. Distilled H_2O (19 ml) was added in order to kill spermatozoa. The changing of osmotic pressure killed the spermatozoa and twisted it in a circular form. They were counted in a Fuchs– Rosenthal haemocytometer under a phase contrast microscope at magnification of 100 x and 200 x.

4.2.5. Instrumental insemination

A queen was inseminated by spermatozoa collected from 3 methods as described previously. Ten queens were used for each treatment group. Three days after insemination, queens were killed in order to count the number of spermatozoa which had entered the spermatheca. All inseminated queens were dissected. Spermathecae were transferred into a small black vessel. The tracheal net around a spermatheca was removed. Each spermatheca was punctured with a fine needle or forceps. Spermatozoa

were released and diluted with 1 ml Tris buffer diluent and dispersed by blowing. Afterwards, 19 ml distilled H_2O were added and the number of spermatozoa was counted in a Fuchs– Rosenthal haemocytometer.

4.2.6. Statistical analyses

Kruskal-Wallis and Mann-Whitney U procedures were used to test for significant differences in motility of spermatozoa in various buffers and methods of semen collection and differences in concentration of spermatozoa and number of spermatozoa reaching the spermatheca from different methods of semen collection.

4.3. RESULT

4.3.1. Motility of spermatozoa collected from ejaculate and seminal vesicles in Tris buffer diluent

The motility of spermatozoa was observed in Tris buffer diluent obtained as ejaculate and from seminal vesicles after different storage duration (table 4.1.). The motility decreased depending on the time of storage. The highest motility of spermatozoa was on day 2 and the lowest was on day 12. Two days after storage, the movement was as strong as in fresh spermatozoa. The movement of spermatozoa was gradually slowing down during long term storage. The motility was not significantly different in both collection methods. .

Table 4.1. Motility of spermatozoa collected from ejaculate or from seminal vesicles in Tris buffer diluent.

Duration of spermatozoa storage (days)	Spermatozoa motility	
	ejaculate	seminal vesicles
2 (n=18)	3.78 ± 0.43	3.72 ± 0.46
3 (n=6)	3.67 ± 0.52	3.50 ± 0.55
4 (n=18)	3.65 ± 0.49	3.44 ± 0.62
5 (n=6)	3.50 ± 0.55	3.17 ± 0.75
6 (n=18)	3.56 ± 0.62	3.06 ± 1.00
7 (n=6)	3.33 ± 0.52	2.67 ± 1.03
8 (n=12)	3.42 ± 0.67	2.42 ± 1.56
10 (n=12)	2.67 ± 1.07	1.83 ± 1.19
12 (n=12)	2.08 ± 1.08	1.53 ± 1.16

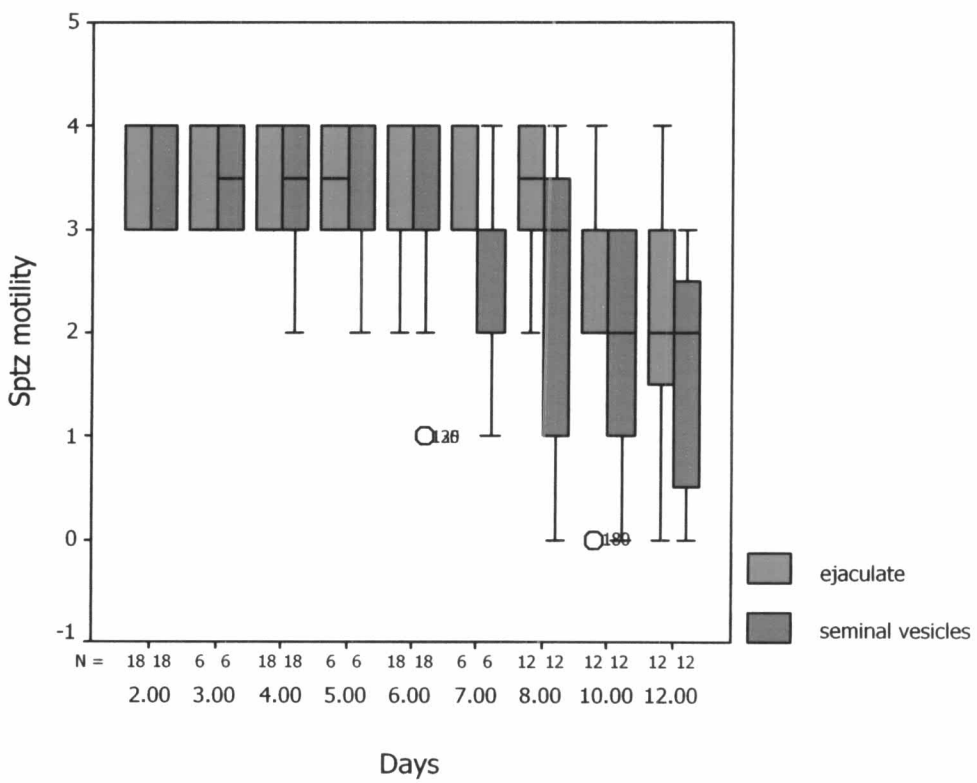

Figure 4.1. Motility of spermatozoa collected from ejaculate or from seminal vesicles in Tris buffer diluent.

4.3.2. Motility of spermatozoa from vesiculae seminales in Tris buffer diluent, Hyes diluent, and 0.9%NaCl

Spermatozoa were motile in all buffers (table 4.2.). The motility of spermatozoa decreased in all buffers after some days of storage, but at different grades. There were significant differences among Tris buffer diluent, Hyes diluent and 0.9%NaCl at day 4, 6, 7, and 12 (Kruskal-Wallis Test, at day 4, $P = 0.004$; at day 6, $P = 0.004$; at day 7, $P = 0.038$; and at day 12, $P = 0.028$). To determine the best diluent for storing spermatozoa, Mann-Whitney Test was used to analyse differences between single pairs of diluents: Tris buffer diluent and Hyes diluent; Hyes diluent and 0.9% NaCl; and Tris buffer diluent and 0.9% NaCl. The motility of spermatozoa was not significantly different between Tris buffer diluent and Hyes diluent; and between Hyes diluent and 0.9%NaCl. Comparing Tris buffer diluent and 0.9% NaCl, the motility of spermatozoa was significantly different at day 4, 5, 6, 7, 8, and 12 (Mann-Whitney Test, at day 4, $P = 0.001$; at day 5, $P = 0.015$, at day 6, $P = 0.001$; at day 7, $P = 0.015$, at day 8, $P = 0.039$; and at day 12, $P = 0.028$). The motility in Tris buffer diluent was stronger than in Hyes diluent. Also, the motiity in Hyes diluent was stronger than in 0.9% NaCl. This indicated that the highest percentage of motility of spermatozoa was in Tris buffer diluent.

Table 4.2. The motility of spermatozoa from seminal vesicles in Tris buffer diluent, Hyes diluent, and 0.9% NaCl.

Duration of spermatozoa storage (days)	Spermatozoa motility		
	Tris buffer diluent	Hyes diluent	0.9% Nacl
2 (n=18)	3.72 ± 0.46	3.39 ± 0.78	3.28 ± 0.83
3 (n=6)	3.50 ± 0.55	3.00 ± 0.89	2.83 ± 0.75
4 (n=18)	3.44 ± 0.62	3.00 ± 1.08	2.44 ± 0.92
5 (n=6)	3.17 ± 0.75	1.83 ± 1.47	1.67 ± 0.82
6 (n=18)	3.06 ± 1.00	2.44 ± 1.25	1.83 ± 0.99
7 (n=6)	2.67 ± 1.03	1.33 ± 1.21	0.83 ± 0.98
8 (n=12)	2.42 ± 1.56	2.17 ± 1.53	1.77 ± 0.94
10 (n=12)	1.83 ± 1.19	1.25 ± 1.36	0.83 ± 1.03
12 (n=12)	1.53 ± 1.16	0.92 ± 1.16	0.33 ± 0.49

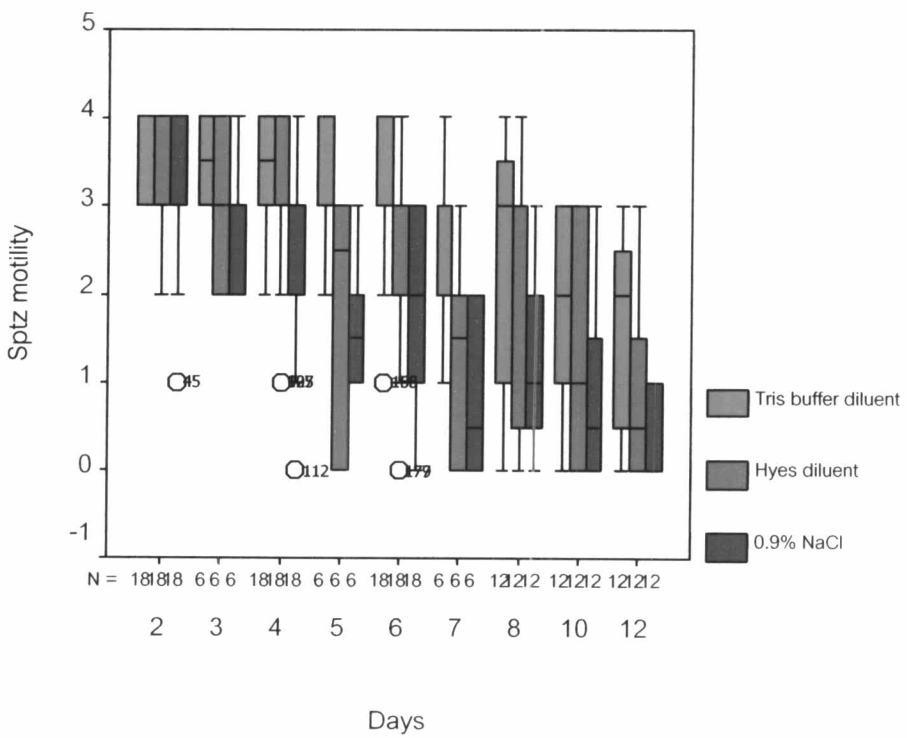

Figure 4.2. The motility of spermatozoa from seminal vesicles in Tris buffer diluent, Hyes diluent, and 0.9% NaCl.

4.3.3. Motility of spermatozoa from seminal vesicles after re-concentration by centrifugation at different speed and time (g/time) in Tris buffer diluent.

The result of spermatozoa motility is shown in table 4.3. The motility was observed in treatments without centrifugation, after re-concentration by centrifugation at 1,000 g for 10 min, and 10,000 g for 10 min and 30 min after storage in Tris buffer diluent. Motility was checked from day 2 until day 12. The motility of spermatozoa decreased the longer they were stored in Tris buffer diluent. The motility of spermatozoa reconcentrated at 10,000 g for 30 min was very low already after 2 days. After 3 days there were less than 10% of moving spermatozoa. All spermatozoa were dead at day 12 after storage. The motility of spermatozoa was significantly different at day 2,3,4, 5, 6, 7, 8, 10, and 12 (Kruskal-Wallis Test, at day 2, $P = 0.000$; at day 3, $P = 0.002$; at day 4, $P = 0.000$; at day 5, $P = 0.004$; at day 6, $P = 0.000$; at day 7, $P = 0.009$; at day 8, $P = 0.003$; at day 10; $P = 0.001$; and at day 12, $P = 0.001$). The Mann-Whitney Test was used to compare the spermatozoa motility among samples without centrifugation, with centrifugation at 1,000 g, and at 10,000 g for 10min. Between dilution without centrifugation and dilution + re-concentration at 1,000 g for 10 min, the motility was significantly different at day 4, 6, and 12 (Mann-Whitney Test, at day 4, $P = 0.029$; at day 6, $P = 0.008$; and at day 12, $P = 0.039$). Between dilution without centrifugation and dilution + re-concentration at 10,000g for 10 min, the motility was significantly different at day 2, 4, 6, and 10 (Mann-Whitney Test, at day 2, $P = 0.031$; at day 4, $P = 0.012$; at day 6, $P = 0.001$; and at day 10, $P = 0.024$). The motility of spermatozoa was not significantly different between samples reconcentrated at 1,000 g and at 10,000 g for 10 min. The motility of spermatozoa in the group after reconcentration at 1,000 g was stronger than in that after re-concentration at 10,000 g because the motility of spermatozoa at day 2 was significantly different between treatment without centrifugation and treatment reconcentrated at 10,000 g for 10 min. In contrast, it is not significantly different between treatment without centrifugation and treatment reconcentrated at 1,000 g.

The motility of spermatozoa in control without centrifugation was the highest while the lowest was after reconcentration at 10,000 g for 30 min. The motility of spermatozoa after re-concentration at 1,000 g for 10 min was better than that after re-concentration at 10,000 g for 10 min.

Table 4.3. Motility of spermatozoa from seminal vesicles after reconcentration at different g/time in Tris buffer diluent.

Duration of spermatozoa storage (days)	Spermatozoa motility			
	Without centrifugation	Centrifugation at 1,000 g, 10 min	Centrifugation at 10,000 g,10 min	Centrifugation at 10,000 g, 30 min
2 (n=18)	3.72 ± 0.46	3.39 ± 0.50	3.17 ± 0.79	1.61 ± 0.98
3 (n=6)	3.50 ± 0.55	3.17 ± 0.75	3.33 ± 0.52	0.83 ± 0.75
4 (n=18)	3.44 ± 0.62	2.89 ± 0.68	2.67 ± 0.91	1.06 ± 1.00
5 (n=6)	3.17 ± 0.75	2.33 ± 0.52	2.50 ± 1.22	0.67 ± 0.82
6 (n=18)	3.06 ± 1.00	2.22 ± 0.73	1.89 ± 0.96	0.56 ± 0.86
7 (n=6)	2.67 ± 1.03	1.67 ± 1.03	2.00 ± 1.26	0.17 ± 0.41
8 (n=12)	2.42 ± 1.56	1.67 ± 0.78	1.25 ± 0.87	0.5 ± 0.80
10 (n=12)	1.83 ± 1.19	1.00 ± 0.85	0.75 ± 0.75	0.08 ± 0.29
12 (n=12)	1.53 ± 1.16	0.58 ± 0.67	0.67 ± 0.78	0

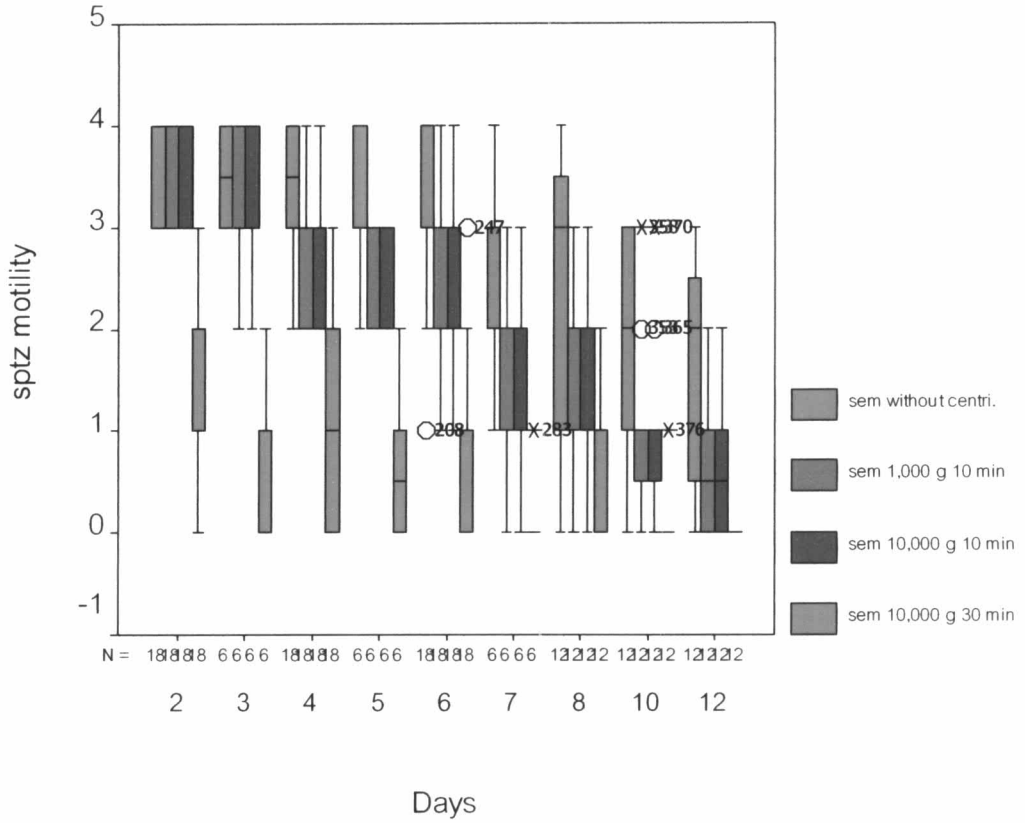

Figure 4.3. Motility of spermatozoa from seminal vesicles after reconcentration at differences g/time in Tris buffer diluent.

4.3.4. Concentration of spermatozoa for insemination

According to table 4.4, the concentration of undiluted spermatozoa collected from ejaculate was 3.84 ± 0.79 spermatozoa $x10^6$/µl (n=10), from seminal vesicles, diluted and reconcentrated by centrifugation at 1,000 g for 10 min it was 2.06 ± 0.30 spermatozoa $x10^6$/µl (n=10); and from seminal vesicles diluted and reconcentrated by centrifugation at 10,000 g for 10 min was 3.05 ± 0.53 spermatozoa $x10^6$/µl (n=10). The concentrations of spermatozoa were significantly different between groups (NPar Tests; Kruskal-Wallis, $P = 0.000$).

Table 4.4. Concentration of spermatozoa for insemination (spermatozoa $x10^6/\mu l$) from different methods of collection

ejaculate	seminal vesicles 1,000 g, 10 min	seminal vesicles 10,000 g, 10 min
4.63	2.83	3.73
4.55	1.78	2.78
4.91	2.05	3.12
4.38	1.89	3.39
3.4	2.12	3.27
3.23	1.81	3.91
3..98	1.89	2.52
3.28	2.15	2.66
3.64	2.11	2.54
2.40	1.69	2.50
$\bar{x}$ = 3.84 ± 0.79	$\bar{x}$ =2.06 ± 0.30	$\bar{x}$ =3.05 ± 0.53

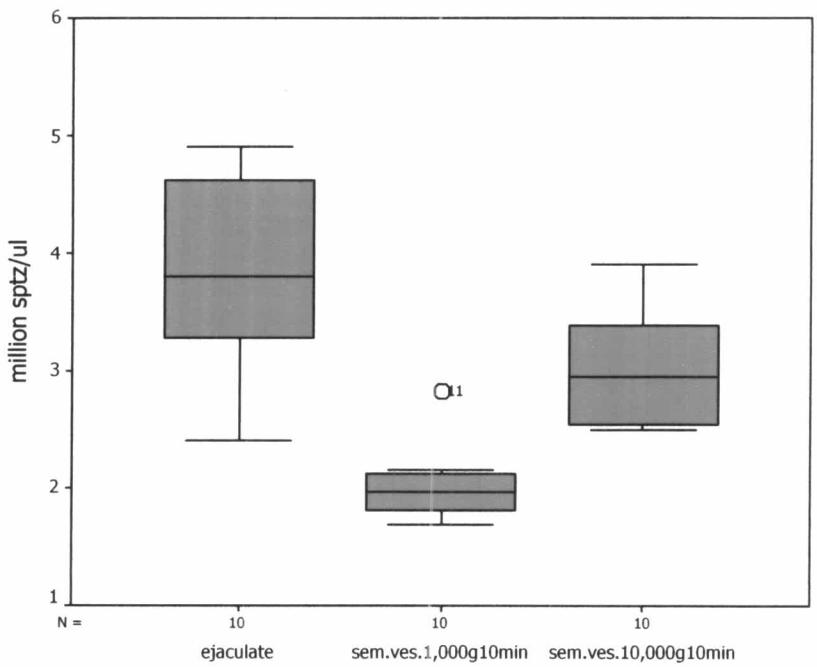

Figure 4.4. Concentration of spermatozoa for insemination (sptz $x10^6/\mu l$) from different methods of collection.

4.3.5. Instrumental insemination

The result of the number of spermatozoa in spermatheca is presented in table 4.5. The average number of spermatozoa in spermathecae of queens inseminated with spermatozoa collected from ejaculate was 2.35 ± 0.78 x 10^6 spermatozoa (n = 10). When queens were inseminated with spermatozoa collected from seminal vesicles and reconcentrated by centrifugation at 1,000 g for 10 min, there were 1.14 ± 0.24 x 10^6spermatozoa (n = 10) in the spermathecae. Further, there were 1.13 ± 0.32 x 10^6 spermatozoa (n = 10) in the spermathecae when queens were inseminated with spermatozoa collected from seminal vesicles and reconcentrated by centrifugation at 10,000 g for 10 min.

Significant differences of number of spermatozoa in spermathecae were found among collections from ejaculate and from seminal vesicles and reconcentrated by centrifugation (NPar Tests; Kruskal-Wallis Test, $P = 0.001$).

Comparison the number of spermatozoa in spermathecae collected from seminal vesicles, there is no difference among collections from seminal vesicles and reconcentrated by centrifugation between at 1,000 g and 10,000 g for 10 min.

Table 4.5. Number of spermatozoa ($x10^6$) in spermatheca after insemination with spermatozoa from different collection methods

ejaculate	seminal vesicles 1,000 g 10 min	seminal vesicles 10,000 g 10min
3.10	1.43	0.89
3.18	1.10	1.61
3.36	1.20	1.02
1.26	0.77	0.73
1.29	1.02	1.26
1.82	1.04	1.32
1.76	1.62	1.38
2.32	0.99	1.45
2.54	0.98	0.86
2.83	1.22	0.73
$\bar{x}$ = 2.35 ± 0.78	$\bar{x}$ = 1.14 ± 0.24	$\bar{x}$ = 1.13 ± 0.32

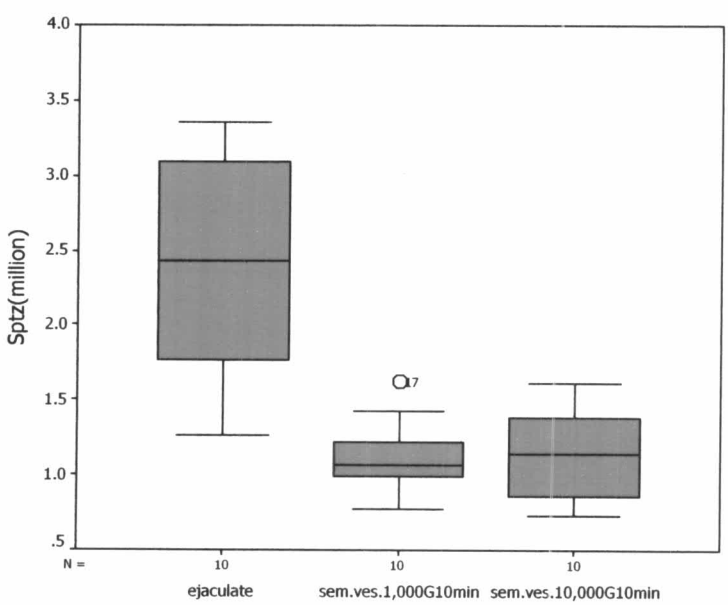

Figure 4.5. Number of spermatozoa in spermatheca after insemination with spermatozoa from different collection methods.

4.4. DISCUSSION

The motility of spermatozoa was not different between semen collected from ejaculate and from seminal vesicles when stored in Tris buffer diluent. Furthermore, there was no significant decrease in spermatozoa motility in both method of sperm collection. Thus, there was no effect of spermatozoa motility after obtaining directly semen from seminal vesicles in a diluent. Apparently most of the male deriving substances of the seminal plasma in the ejaculate does not play a major role. The substances glucose, arginine and lysine present in the Tris buffer diluent be important for the spermatozoa.

The result of the test of spermatozoa motility after collection of semen from seminal vesicles in various diluents (Tris buffer diluent, Hyes diluent and 0.9% NaCl) showed, that the motility of spermatozoa was the strongest in Tris buffer diluent pH 8.5. Based on motility, Tris buffer diluent is a good solution for storing spermatozoa is. This diluent contains in addtion to the ions Na+ and Cl- the amino acids arginine and lysine which were found in high concentration in seminal plasma of drone honeybee (Novak *et al.*, 1960). As sugar glucose was present in seminal plasma of honeybee (Blum *et al.*, 1962; Alumont *et al.*, 1969). Moreover, Na^+ is present in the honeybee spermatheca and is one of the important factor for the reversible suppression of sperm motility and longevity both in *vivo* and in *vitro* (Verma, 1973). The presented result is agreement with the experiment of Verma (1978) and Moritz (1984) that Tris buffer diluent containing arginine, lysine and glucose was a good diluent for obtaining spermatozoa. The adjustment of pH 8.5 of diluent may also have an important effect on motility of spermatozoa and prolongation survival of spermatozoa in a period of sperm storage because the pH value of spermathecal fluid is 8.6 (Gessner and Gessner, 1976)

Using centrifugation technique in order to reconcentrate semen that was collected in a diluent from seminal vesicles speed / time of centrifugation had an effect on motility of spermatozoa. There was a correlation between the motility of spermatozoa and the speed and time of centrifugation. There seems to be a negative relation of spermatozoa motility with speed and longer time of centrifugation. Centrifugation of honeybee

spermatozoa cause damage and kill honeybee spermatozoa, especially when using high speed of centrifugation (Collins, 2003). Centrifugation at speed 82 g, 30 min, 180 g, 5 min, 510 g, 10 min, 8,160 g, 1 min, 10 min had a percentage of live spermatozoa of 83.4, 84.0, 81.3, 80.1, 77.4 and 70.7 respectively. In these experiments live: dead dual fluorescent staining was used to determine sperm viability in honeybees (Collins, 2003). The percent of live spermatozoa at centrifugation 1,000 g for 10min was estimated between 70.7-80.1% according to Collins (2003). Although, centrifugation had effect on motility and mortality of honeybee spermatozoa, even queen inseminated with only 50% live spermatozoa produced normal worker (Collin, 2000). Accordingly, centrifugation at 1,000 g for 10 min as used in this experiment should not have effect on quality of worker brood. Taber (1961), Moritz (1983), Harbo (1990) also used the technique of centrifugation (at 8,160 g - 10,000 g for 1 and 10 min) to reconcentrate semen for insemination of a queen, they observe that queen produced worker brood as naturally mated queen. Moreover, Koeniger *et al* (1996, 1998) also used spermatozoa collected from seminal vesicles and use centrifugation (8,000 rpm for 15 min) for successfully cross insemination between *A. koschevnikovi* and *A. cerana* that got gynandromoph hybrid.

The amount of number of spermatozoa reaching spermatheca in inseminated queen with sperm collection from ejaculate (2.35 ± 0.78 spermatozoa x 10^6) was higher than from seminal vesicle that was reconcentrated semen by centrifugation at 1,000 g and 10,000 g for 10 min (1.14 ± 0.24 and 1.13 ± 0.32 sptz x 10^6). This result is supported by Mackenson (1955), he found that more spermatozoa reached spermatheca in queens inseminated with semen collected from ejaculate than from seminal vesicles. During washing and centrifugation, the water-soluble portion of seminal fluid was dissolved in diluents and most of it was removed after centrifugation. Thus seminal fluid was substituted by the diluent which may have had an effect on the viscosity. Further, following concentration of spermatozoa did reach the same level as it is in ejaculate. Concentration of spermatozoa seems to be an important factor for the ratio of spermatozoa reaching the spermatheca

Although the concentration of spermatozoa at 10,000 g for 10 min (3.05 ± 0.53 sptz x 10^6/µl) was higher than at 1,000 g for 10 min (2.06 ± 0.30 sptz x 10^6/µl), the amount of spermatozoa reaching spermatheca was similar (in 1,000 g was1.14 ± 0.24 x 10^6 ,and in 10,000 g was 1.13 ± 0.32 sptz x 10^6). Probably the use of high speed of centrifugation had a higher negative effect on viability of spermatozoa than using low speed of centrifugation (Collins, 2003).

Therefore, the method of collecting spermatozoa from seminal vesicles, dispersed in Tris buffer diluent and reconcentrated spermatozoa by centrifugation at 1,000 g for 10 min was used to success for instrumental insemination between species of honeybee. In the future, this maybe important for breeding programs in *A. cerana*. The transfer of the technique of instrumental insemination from the Western honeybee proved to be very difficult because of the low number of spermatozoa in drones and also the low number of mature drones per colony of *A. cerana*

ACKNOWLEDGMENTS

I would like to thank the staff of Insitut für Bienenkunde, Oberursel, Germany for their helpfulness, friendship, and field assistance. This research was supported by grants from the Royal Golden Jubilee Project, Thailand Research Fund.

REFERENCES

Alumot, E., Lensky, Y. and Holstein, P. 1969. Sugars and trehalase in the reproductive organs and hemolymph of the queen and drone honey bees (*Apis mellifera* L. Var. Ligustica Spi.). *Comp. Biochem.Physiol.* 28: 1419-1425.

Blum, M.S., Glowska, Z. and Taber, S.III. 1962. Chemistry of the drone honey bee reproductive system.II. Carbohydrates in the reproductive organ and semen. *Ann. Ento. Mol. Soc. Am.* 55: 135-139.

Bolten, A.B. and Harbo, J. 1982. Numbers of spermatozoa in the spermatheca of the queen honey bee after multiple insemination with small volumes of semen. *J. Apic. Res.* 21: 7-10.

Camergo, C.A.de. 1975. Biology of the spermatozoon of *Apis mellifera*. I. Influence of diluents and pH*. *J. Apic. Res.* 14:113-118.

Collins, A.M. 2000. Relationship between semen qualitu and performance of instrumentally inseminated honey bee queens. *Apidologie* 31: 421-429.

Collins, A. M. 2003. A scientific note on the effect of centrifugation on pooled honey bee semen. *Apidologie* 34: 469-470.

Collins, A. M. and Donoghue, A.M. 1999. Viability assessment of honey bee, *Apis mellifera*, sperm using dual fluorescent staining. *Theriogenology* 51: 1513-1523.

Gessner, B. and Gessner, H. 1976. Inorganic ions in spermathecal fluid and their transport across the spermathecal membrane of the queen bee, *Apis mellifera*. *J. insect. Physiol.* 22: 1469-1474.

Harbo, J.R. 1979. The rate of depletion of spermatozoa in queen honeybee spermatheca. *J. Apis. Res.* 18: 204-207.

Harbo, J.R. 1983. Survival of honey bee (Hymenoptera: Apidae) spermatozoa after two years in liquid nitrogen (-196°C). *Ann. Entomol. Soc. America.* 76: 890-891.

Harbo, J.R. 1990. Artificial mixing of spermatozoa from honeybees and evidence for sperm competition. *J. Apis. Res.* 29: 151-158.

Harbo, J.R. and Williams, J.L. 1987. Effect of above-freezing temperatures on temporary storage of honeybee spermatozoa. *J. Apic. Res.* 26: 53-55.

Jaycox, E.R. 1960. The effect of drying and various diluents on spermatozoa of the honey bee (*Apis mellifera* L.). *J. Econ. Ent.* 53(2): 266-269.

Kaftanoglu, O. and Peng, Y.S. 1980. A washing technique for collection of honeybee semen. *J. Apic. Res.*19: 205-211.

Koeniger, N., Koeniger, G. and Tingek, S. 1996. Interspecific instrumental insemination of *Apis cerana* Fabricius, 1793, *Apis koschevnikovi* Buttel-Reepen,1906 and *Apis dorsata* Fabricius, 1793. *Apidologie* 27: 303-304.

Koeniger, N., Koeniger, G., Tingek, S. and Kelitu, A. 1998. Preliminary report on hybrids between *Apis cerana* Fabricius, 1793 and *Apis koschevnikovi* v.Buttel-Reepen, 1906 produced by instrumental insemination. *Apidologie* 29: 462-463.

Lensky, Y. and Schindler, H. 1967. Motility and reversible inactivation of honey bee spermatozoa *in vivo* and *in vitro*. *Annls Abeille* 10(1): 5-16.

Mackensen, O. 1955. Experiments in the technique of artificial insemination of queen bees. *J. Econ. Ent.* 48(4): 418-421.

Mackensen, O. 1969. Effect of diluents and temoerature on instrumental insemination of queen honey bees. *J. Econ. Ent.* 62(6): 1370-1372.

Moritz, R.F.A. 1983. Homogeneous mixing of honeybee semen by centrifugation. *J. Apis Res.* 22: 249-255.

Moritz, R.F.A. 1984. The effect of different diluents on insemination success in the honeybee using mixed semen. *J. Apic. Res.* 23: 164-167.

Novak, A. F., Blum, M.S., Taber S.III. and Liuzzo, J. A. 1960. Separatation and determination of seminal plasma and sperm amino acids of the honeybee, *Apis mellifera*. *Ann. Entomol. Soc. Am.* 53: 842-843.

Poole, H.K. 1970. The wall Structure of the honey Bee spermatheca with comment about its function. *Ann. Ent. Soc. Ame.* 63:1625-1628.

Poole, H.K. and Taber, S. 1969. A method of in vitro storage of honey-bee semen. *American Bee Journal.* 109: 420-421.

Poole, H.K., and Taber, S. 1970. In vitro preservation of honey bee semen enhanced by storage at 13-15°C. *Annals of the Entomological Society of America.* 63: 1673-1674.

Ruttner, F. Te. 1975. *Instrumental insemination of the queen bee.* Romania: Apimondia Bukarest.

Sawada, Y. and Chang, M.C. 1964. Tolerance of honey bee sperm to deep freezing. *J. Eco. Ent.* 57(7): 891-892.

Taber, S.III. 1961. Successful shipments of honeybee semen. *Bee World* 42: 173-176.

Taber, S.III. and Blum, M.S. 1960. Preservation of honey bee sperm. *Science* 131: 1734-1735.

Verma, L.R. 1973. An ionic basis for apossiblemechanism of sperm survival in the spermatheca of the queen honey bee (*Apis mellifea* L.). *Comp. Biochem.Physiol.* 44: 1325-1331.

Verma, L.R. 1978. Biology of honeybee (*Apis mellifera* L.) spermatozoa 1. effect of different diluents on motility and survival. *Apidologie* 9: 167-174.