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The Effect of Semen Dilution on the Number of Spermatozoa Entering the Spermatheca of the Queen Honey Bee (*Apis mellifera* L.)[#]

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ABSTRACT

This study aimed to determine whether the addition of diluent to fresh semen before injection affects the number of spermatozoa entering the spermatheca of the instrumentally inseminated queens. The queens reared by the grafting method were introduced into mating nucs one day before adult emergence. When 7-day old, the queens were allocated into four groups for the following instrumental insemination treatments. The queens in group 1 and group 3 were inseminated with 4 µl and 8 µl fresh semen, respectively (FS - 4 µl and FS - 8 µl). In group 2, the queens were inseminated with 4 µl fresh semen diluted 1-fold with NaCl solution (DS - 8 µl). In group 4, the queens were inseminated with 8 µl fresh semen diluted 1-fold with NaCl solution (DS - 16 µl). Each queen was dissected after the onset of oviposition for counting the number of spermatozoa in the spermatheca. There was not a significant difference in spermatozoa number between queens inseminated with 4 µl FS (2.57 million) and 8 µl DS (2.68 million). The queens inseminated with 16 µl DS (3.51 million) had fewer spermatozoa than the queens inseminated with 8 µl FS (4.46 million). The queens inseminated with 16 µl DS had more spermatozoa (3.51 million) than the queens inseminated with 4 µl FS (2.57 million, and 8 µl DS (2.68 million). The results revealed that the addition of 1-fold diluent to 4 µl fresh semen did not alter the number of spermatozoa. In contrast, adding 1-fold diluent to 8 µl fresh semen adversely affected the number of spermatozoa entering the spermatheca.

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Introduction

The honey bee queen (*Apis mellifera*) is polyandrous and mates with 7-17 or more drones in the drone congregation area, away from the hive. The semen acquired from several drones via subsequent copulations was accumulated in the lateral oviducts. The lateral oviducts of the queen returning from the mating flight are filled less or more with semen (0.6 to 28 µl), depending on the number of successfully mated drones (Woyke, 1962). The lateral oviducts are the temporary storerooms of the queen for the accumulated semen containing about 100 million spermatozoa (Koeniger and Koeniger, 1991). Immediately after returning from the mating flight, temporarily stored spermatozoa in the lateral oviducts migrate to the spermatheca within 24 hours (Woyke, 1962, 1983).

The natural mating behaviour in honey bees does not permit human interference aiming to provide matings of individually selected queens and drones. Yet, the controlled mating of parents is the first prerequisite of selective breeding. Instrumental insemination is the only effective way to pair the individually selected parents in honey bee breeding.

The performance of the queen is closely related to the number of spermatozoa in the spermatheca. The number of spermatozoa in spermatheca is crucial for the longevity and reproductive success of the queen. The number of spermatozoa in the spermatheca is one of the most common quality indicators of the queen (Kahya et al., 2008; Hatjina et al., 2014; Delaney et al., 2010; Arslan et al., 2021). The naturally mated queens have an average of 4-6 million spermatozoa in their spermatheca (Woyke, 1962, 1983). The queens having less than 3 million spermatozoa in the spermatheca are considered poor-quality (Woyke, 1962). The spermathecae of queens naturally mated or instrumentally inseminated contain about 98% live spermatozoa, regardless of the number of spermatozoa entering spermatheca (Gençer and Kahya, 2011a).

There is abundant literature concerning the various environmental factors affecting the success of instrumental insemination of queens. Nevertheless, the studies on semen dilution are limited (Mackensen, 1969; Harbo, 1977, 1983; Verma, 1978; Moritz, 1984; Skowronek et al., 1995; Lodesani et al., 2004). In this study, we instrumentally inseminated the queens with diluted and undiluted semen to compare the number of spermatozoa entering the spermatheca.

Material and Methods

The study was carried out at Ankara University, Turkey (39°57'47.3"N, 32°52'00.1"E) in the summer season of 2011. The sister queens were reared from a Caucasian (*A. m. caucasica)* colony by standard grafting method (Laidlaw and Page, 1997). Each queen cell was introduced into a trapezoid styrofoam mating nuc (Kirchain) containing four combs covered about 1000 worker bees one day before adult emergence. The flight entrances of mating nucs were closed with a queen excluder to prevent the virgin queens from natural mating. When 7-day old, the virgin queens (n = 63) were randomly allocated into four groups for the following instrumental insemination treatments. The queens in group 1 (FS - 4 µl) and group 3 (FS - 8 µl) were inseminated with 4 µl and 8 µl fresh semen, respectively. In group 2 (DS - 8 µl), the queens were inseminated with 4 µl fresh semen diluted 1-fold with NaCl (0.09%) solution. In group 4 (DS - 16 µl), the queens were inseminated with 8 µl fresh semen diluted 1-fold with NaCl (0.09%) solution.

The drones going out for mating flight were captured in front of the hives and transported to the laboratory for the semen collection procedure. The mature drones were manually provoked to ejaculate by pressing on the thorax. The semen on the fully everted endophallus of each drone was collected into the glass tip of the Harbo syringe connected to the Schley insemination instrument (Peter Schley Equipment, Lich, Germany). The volume of semen was measured by the Gilmont micrometer (Gilmont Instruments; Barrington, IL, USA) of the Harbo syringe. The required amount of semen (4 µl or 8 µl) was separately collected for each queen. The semen (4 µl or 8 µl) collected in the glass tip of the syringe was discharged into a microcentrifuge tube (volume: 0.2 ml) containing 4 µl or 8 µl NaCl (0.09%) solution. Thus, an equal proportion of semen-diluent mixture (1: 1) was prepared for each virgin queen. The semen-diluent mixture in the microcentrifuge tube was homogenised by gently pipetting. Then the diluted semen was redrawn into the glass tip of the syringe to inseminate the virgin queen. Immediately after inseminating with the respective composition or volume of semen, the queen was introduced into her mating nuc. Each queen was subjected to CO_2 anaesthesia during the insemination process. The queens were not treated with additional CO_2 before or after instrumental insemination. The inseminated queens in mating nucs were detected daily to determine the onset of oviposition.

When the queens commenced laying eggs, they were taken from their nucs to determine the number of spermatozoa in the spermatheca. The spectrophotometric method was used count the number of spermatozoa in the spermatheca (Gençer and Kahya, 2011b). Each queen was dissected under a macroscope (Leica, Z16 Apo) to take out the spermatheca. After removing the tracheal net, the spermatheca was placed in a quartz cuvette (Hellma, 1.4 ml) containing 1000 µl modified Kiev solution (MKS, Moritz, 1984). The semen in the spermatheca was then discharged by compressing the spermatheca with fine-pointed forceps. The emptied spermatheca was taken from the cuvette. Then, the released semen was homogeneously dispersed in the solution with a micropipette. The quartz cuvette was placed into the spectrophotometer (Shimadzu UV1800). The absorbance values of semen-MKS solution were obtained by kinetic measurements at 260 nm wavelength for 3 minutes for each spermathecal content. Then, the average absorbance values were converted into spermatozoa numbers by using the calibration curve estimated by the computer-based software (Shimadzu-UV Probe, Version 2.33; Gençer and Kahya, 2011b).

All statistical analyses and graphical representations of the data were performed by RStudio (Version 1.3.1073) and its libraries (*rstatix, dplyr, tidyverse, ggpubr and agricolae)*. After testing for normality and homogeneity by Shapiro–Wilk's test and Levene's test, respectively, the data on the data on spermatozoa counts in the spermatheca were analyzed with ANOVA followed by Duncan test for multiple comparisons. The non-parametric data on the interval (day) from to onset of oviposition were analysed with Kruskal-test.

Results and Discussion

In total, 63 instrumentally inseminated queens were dissected; 13 queens in FS - 4 µl, 16 queens in DS - 8 µl, 17 queens in FS - 8 µl, 17 queens in DS - 16 µl, respectively. The number of spermatozoa entering the spermatheca varied from 1.49 million to 6.35 million. The mean number of spermatozoa in FS - 4 µl, DS - 8 µl, FS - 8 µl and DS - 16 µl were 2.57, 2.68, 4.55, and 3.51 million, respectively (Table 1). There were significant ($P<0.0001$) differences in the number of spermatozoa between insemination groups (Figure 1). The highest mean value was obtained from the queens inseminated with 8 µl FS. The lowest mean value was from the queens inseminated with 4 µl FS. There was not a significant difference ($P>0.05$) in spermatozoa count between queens inseminated with 4 µl FS (2.57 million) and 8 µl DS (2.68 million).

Table 1. The spermatozoa counts (million) in the spermathecae of instrumentally inseminated queens.

Insemination groups		n	Mean±SD*	Minimum	Maximum
FS - 4 µ	4 µl fresh semen	15	2.57 ± 0.621^{a}	1.49	3.66
DS - 8 µl	4 µl fresh semen + 4 µl diluent	16	2.68 ± 0.985^{a}	1.34	4.89
FS - 8 µl	8 µl fresh semen	17	4.55 ± 0.926^{c}	2.75	6.35
DS - 16 µl	8 µl fresh semen + 8 µl diluent	17	3.51 ± 1.040^{b}	1.69	4.97

*Different letters denote significant differences between means.

Table 2. The variation in the interval from insemination to the onset of oviposition in instrumentally inseminated queens

Insemination groups		n	Mean±SD*	Minimum	Maximum
FS-4 µl	4 µl fresh semen	15	22.3 ± 8.56[a]	9	33
FS:D-8 µl	4 µl fresh semen + 4 µl diluent	16	18.2 ± 7.95[a]	7	31
FS-8 µl	8 µl fresh semen 4 µl fresh semen + 4 µl diluent	17	22.7 ± 7.55[a]	8	32
FS:D-16 µl	8 µl fresh semen + 8 µl diluent	17	21.4 ± 71.7[a]	5	31

*Different letters denote significant differences between means.

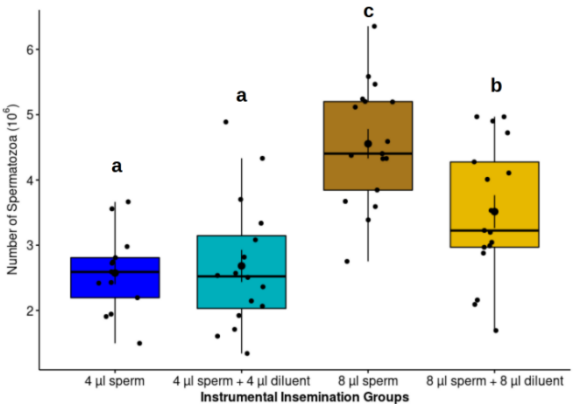

Figure 1. The spermatozoa counts (million) in the spermathecae of queens in instrumental insemination groups.
Significant differences (P<0.05) between mean values are indicated with different letters

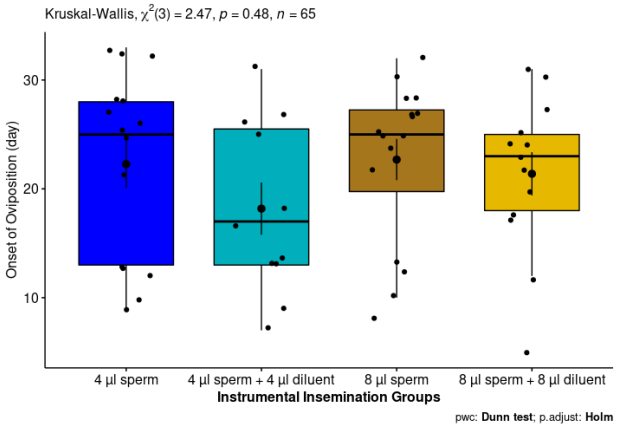

Figure 2. The variation in the interval from insemination to onset of oviposition (day) in instrumentally inseminated queens.

The dilution of 4 µl semen one fold with NaCl solution resulted in only 0.11 million increase in spermatozoa count. The queens inseminated with 16 µl DS (3.51 million) had significantly fewer spermatozoa than the queens inseminated with 8 µl FS (4.55 million, P<0.05). The dilution of 8 µl FS adversely affected the spermatozoa count in the spermatheca, causing 1.04 million fewer spermatozoa. The queens inseminated with 16 µl DS had more spermatozoa (3.51 million) than the queens inseminated with 4 µl FS (2.57 million, P<0.05), and 8 µl DS (2.68 million, P<0.05).

The interval (day) between insemination and the onset of oviposition varied from 5 to. The mean interval from insemination to the onset of oviposition in FS - 4 µl, DS - 8 µl, FS - 8 µl and DS - 16 µl insemination groups were 22.3, 18.2, 22.7, and 21.4 days, respectively (Table 2). The duration of the interval (Figure 2) was not affected (P>0.05) by insemination groups (overall mean = 21.4 days). The duration between insemination and onset of oviposition is generally considered to be somewhat longer and more variable in instrumentally inseminated queens than in naturally mated queens (Cobey, 2007). Kahya et al. (2008) reported that the duration between last mating flight and onset of oviposition was 3.05 days in naturally mated queens. Instrumentally inseminated queens are stimulated to start egg laying by treating CO_2 before or after insemination besides CO_2 anesthetisation during the insemination process (Mackensen, 1947). We did not apply additional CO_2 treatment to the queens in our study. Therefore, the mean duration between insemination and onset of oviposition was somewhat longer. It could be claimed that if we had applied additional CO_2 treatment to the queens, the interval from insemination to the onset of oviposition would have been shorter than 21.4 days.

The optimum volume of semen injected into the queens was determined as 8 µl in previous studies (Woyke, 1962; Mackensen, 1964). We found the highest spermatozoa count in queens inseminated with 8 µl FS. On the other hand, the mean spermatozoa number in queens inseminated with 8 µl FS (4.55 million) is consistent with the previous reports (Mackensen and Roberts, 1948; Gençer and Firatli, 2005). Our result demonstrated that the queen inseminated with 8 µl FS had as many spermatozoa as naturally mated queens. We found that the dilution of 8 µl FS with an equal volume of diluent caused a decrease of one million spermatozoa. However, the one fold-dilution of 4 µl FS did not cause any significant increase or decrease in spermatozoa count.

Limited data are available on the effect of semen dilution on instrumentally inseminated queens. The first study on the insemination of the queens with diluted semen was conducted by Mackensen (1969). Mackensen (1969) inseminated the queens with small volumes of fresh semen undiluted (1.25 µl) and diluted (2.5 µl). Similar to our finding, Mackensen (1969) found no difference in the spermatozoa count between the queens inseminated with 1.25 µl FS (1.62 million) and 2.5 µl DS (1.44 milion). Skowronek et al. (1995) obtained data on the dilution of semen comparable with our study. Skowronek et al. (1995) did not find a difference in the spermatozoa count when the queens were inseminated with 8 µl FS (3.48 million) and 8 µl FS diluted 1-fold with Hyes solution (3.13 million). We, however, found that the dilution of 8 µl FS 1-fold with saline solution caused about 1 million fewer spermatozoa in the spermatheca. This inconsistency may be attributed to the use of different solutions. Skowronek et al. (1995) used Hyes solution as the diluent, whereas we used saline solution (NaCl, 0.09%). In another similar study, Gąbka

and Cobey (2018) inseminated the queens with 8 µl FS and 8 µl FS diluted with 2 µl saline solution. They also did not determine a significant difference in the spermatozoa counts between the queens inseminated with 8 µl FS (3.05 million) and 10 µl DS (2.83 million).

The queens in the DS - 16 µl insemination group had the highest volume of semen in their lateral oviducts after injection. However, the queens inseminated with had fewer spermatozoa than those inseminated with 8 µl FS. The reduction of the concentration the concentration of 8 µl semen by 1-fold dilution adversely affected the spermatozoa migration. In contrast, one fold dilution of 4 µl FS did not cause any considerable increase or decrease in spermatozoa count. The queens inseminated with 4 µl FS and 8 µl DS had similar spermatozoa counts. The number of spermatozoa entering the spermatheca decreased when a large volume of DS (16 µl) was injected into the queen. We, therefore, suggested that the volume increase probably caused more pressure on DS in the extended lateral oviducts. Besides, the semen became less dense due to 1-fold dilution, facilitating the flowing out of semen from the reproductive tract. The higher pressure in lateral oviducts resulted in less viscous semen being quickly expelled through the reproductive tract. Eventually, fewer spermatozoa migrated into the spermatheca. The sperm migration dynamics in queens should be further investigated by conducting different experimental setups using the instrumental insemination technique.

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