Original article

Flow cytometry evidence about sperm competition in honey bee (*Apis mellifera*)

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Abstract – We tested whether flow cytometry can be used for assessment of viability of honey bee (*Apis mellifera*) sperm. The method was used to detect possible competition between the sperm of different drones. The flow cytometry analysis of semen stained with SYBR-14/propidium iodide revealed significant differences between fresh and freeze-thawed samples. The identification of populations corresponding to viable and nonviable sperm allowed us to assess the sperm viability. The comparison of single-drone semen with mixed semen of two unrelated drones showed that sperm viability was not affected by mixing, but there were differences between mixed and unmixed semen in side scatter, which correlates with shape and optical homogeneity of particles. The proportion of particles in different populations also was affected by mixing of the semen. The results suggest that there are interactions between ejaculates of different drones, possibly related to sperm competition.

honey bee / *Apis mellifera* / spermatozoa / sperm competition

1. INTRODUCTION

During a single mating flight, the honey bee (*Apis mellifera*) queen mates with multiple drones (Woyke 1960; Schlüns et al. 2005). Their semen is transferred to the oviducts (Woyke 1956) where it is mixed by contraction of the oviduct muscles (Page 1986). Only a small fraction of spermatozoa reaches the spermatheca and can be used later for fertilization of eggs, and the rest is expelled through the sting chamber (Laidlaw 1944). These circumstances favor intraoviductal sperm competition (Woyciechowski and Król 1996).

One mechanism of sperm competition is sperm incapacitation, by which substances present in seminal fluid kill the sperm of other males (Harshman and Prout 1994). If this mechanism operates, then mixing the semen of two or more drones should result in higher mortality of spermatozoa. Another possible mechanism of sperm competition is related to sperm polymorphism. In some species, there are two distinct types of sperm. It has been suggested that only one of those types is suitable for fertilization and that the other can block or eliminate the spermatozoa of other males (Swallow and Wilkinson 2002). At the moment, there is no data about the sperm polymorphism in honey bees.

Most of the earlier attempts to confirm sperm competition in honey bees have been unsuc-

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cessful (Harbo 1990; Woyciechowski and Król 1996; Moritz 1986; for review, see Shafir et al. 2009). Analyses of patrilines in honey bee colonies showed that some drones, which mated with the queen, were underrepresented in her offspring (Franck et al. 1999; Haberl and Tautz 1998), but it was not clear if this was the result of sperm competition. In one experiment, the viability of sperm in the presence of seminal fluids of other males was found to be lower than in the presence of only its own seminal fluids (den Boer et al. 2010).

Here we used flow cytometry to verify whether the spermatozoa of two drones show higher mortality after mixing. This method allows quantitative analysis of semen, permits sperm viability to be assessed, and can also detect sperm polymorphism.

2. MATERIAL AND METHODS

The drones used in this study came from three unrelated colonies of honey bee (*A. m. carnica*). In each of the colonies, the queen was caged on the drone comb for 24 h in order to obtain drones of known age. The experiment was done when the drones were at 20 days of age. Semen was collected in a calibrated capillary tube by standard technique (Laidlaw 1989; Collins and Donoghue 1999). Semen volume was measured by the height of the liquid column in the capillary. The semen was diluted 1:1,000 *v:v* in Kiev buffer (0.3 g glucose, 0.41 g potassium chloride, 0.21 g sodium bicarbonate, 2.43 g sodium citrate per 100 mL deionized water; Collins and Donoghue 1999). The volume of collected semen was always greater than 0.8 μL, yielding at least 0.8 mL diluted sperm. From each sample, 0.5 mL suspension was mixed with the same volume of a sample collected from an unrelated drone. The remaining volume (at least 0.3 mL) of each sample was used for the analysis of unmixed semen (Figure 1).

The order in which the semen was collected and analyzed was controlled to eliminate the effect of the time between collection of the sperm and its analysis. For example, the order of collection was A1, B1, B2, A2, … and the order of analysis was A1, B1, A1+B1, A2+B2, B2, A2, …, where the different capital letters

indicate different unrelated colonies and the different numbers indicate different drones. The semen of 60 drones was collected and 90 samples were analyzed. There were 60 samples of unmixed semen of single drones and 30 samples of mixed semen of two drones.

In each sample, the proportions of live and dead spermatozoa were determined by SYBR-14/propidium iodide (PI) fluorescent staining with the LIVE/DEAD Sperm Viability Kit (Molecular Probes L-7011). From each sample, 300 μL of diluted semen was collected; 5 μL SYBR 14 (stock solution, deionized, sterile water 1:50) was added and then incubated for 5 min at 36°C. Then 4 μL PI was added and stirred. SYBR-14 stains live cells green and PI stains dead cells red. The counts were obtained by flow cytometry (Becton Dickinson FACSCalibur, USA) with a 488-nm argon laser. Green fluorescence was measured in the LFL1 channel and red fluorescence in the LFL3 channel, using the manufacturer's fluorescence compensation filters. Forward scatter and side scatter were also measured. The forward scatter tends to be more sensitive to size of particles and the side scatter tends to be more sensitive to shape and optical homogeneity of particles. Twenty thousand particles were analyzed in each sample. The groups of particles with similar properties are called populations. The cytometry data were analyzed with WinMDI 2.8 software. The red fluorescence, green fluorescence, forward scatter, and side scatter are reported as arbitrary units. Measurements in those units can be compared only within this study.

In order to verify if flow cytometry can be used for assessment of honey bee sperm viability, we conducted freeze-thawing experiment. The experiment allowed us to identify populations with viable and nonviable sperm. Samples of semen from four drones were frozen at −17°C for 2 h in order to kill the sperm. The samples were analyzed after thawing. Earlier work shows that honey bee sperm does not survive freezing without cryoprotectant (Peng et al. 1992). In the freeze-thawing experiment, each of the four samples consisted of unmixed semen. It was assumed that live spermatozoa are in populations which disappeared after freeze-thawing and dead spermatozoa are in populations which appeared after freeze-thawing.

Figure 1. Preparation of honey bee semen for flow cytometry. Each of the samples *A* and *B* contained semen of single drone and sample *A+B* contained semen of two drones mixed together.
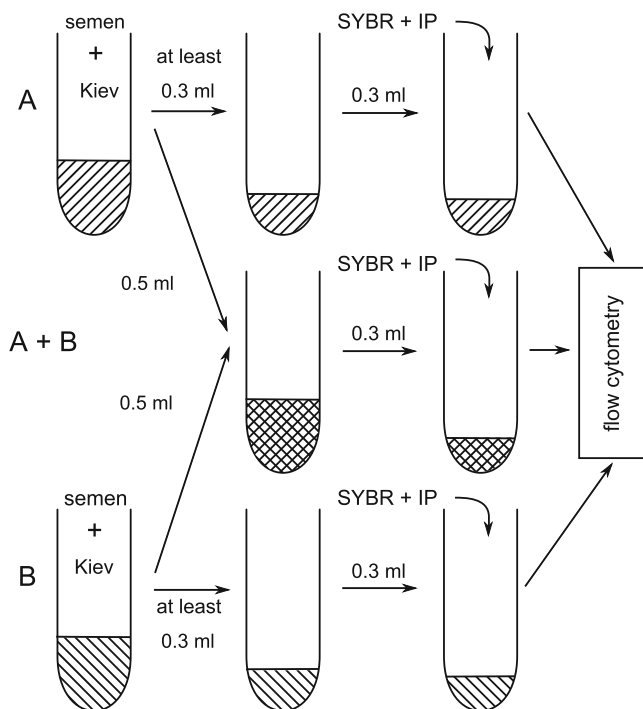
Coming from a total population of semen, the proportions of particles represented in the different flow cytometry populations of a single sample are, of course, not independent from each other, such that when the number of particles increases in any one of these populations, there will be corresponding changes in one or several of the others. Thus, in order to detect changes in particle numbers in any one of the four populations, six ratios were calculated: $n1/(n1+n2)$, $n1/(n1+n3)$, $n1/(n1+n4)$, $n2/(n2+n3)$, $n2/(n2+n4)$, and $n3/(n3+n4)$ where n1, n2, n3, and n4 correspond to the number of particles in populations P1, P2, P3, and P4, respectively. Some of the ratios are independent of each other. For example, if mixing of semen affected number of particles only in population P1, the ratios $n1/(n1+n2)$, $n1/(n1+n3)$, and $n1/(n1+n4)$ should be affected and ratios $n2/(n2+n3)$, $n2/(n2+n4)$, and $n3/(n3+n4)$ should not be affected.

The differences between populations of particles in forward scatter and side scatter were analyzed using ANOVA. The green and red fluorescence were not included in the comparisons because the populations were determined using those variables. In the statistical analysis of forward scatter and side scatter, sample means were used. In comparing the mixed and unmixed semen, paired Student's *t* test was used. The pairs consisted of mean values of two unmixed samples and value of sample made by mixing the two samples. The data are presented as means±SD. Proportions were arcsin transformed before the statistical analysis.

3. RESULTS

In fresh samples of the semen of honey bee drones stained with SYBR-14/PI, there were three main populations of particles (P1, P2, P3), which differed mainly in green fluorescence (Figure 2a, b). After freeze-thawing, there was one new population (P4) of particles showing higher red fluorescence, P1 was still visible, and P2 and P3 disappeared almost completely (Figure 2d). The boundaries of the populations were determined using minima of the red and green fluorescence histograms (Figure 3). The populations differed significantly in forward scatter and side scatter (ANOVA: forward scatter of fresh unmixed samples, $F_{(3,\ 236)}=13.4$, $P<0.001$; side scatter of fresh unmixed samples, $F_{(3,\ 236)}=61.9$; $P<0.001$; forward scatter of fresh mixed samples, $F_{(3,\ 116)}=13.6$, $P<0.001$; side scatter of fresh
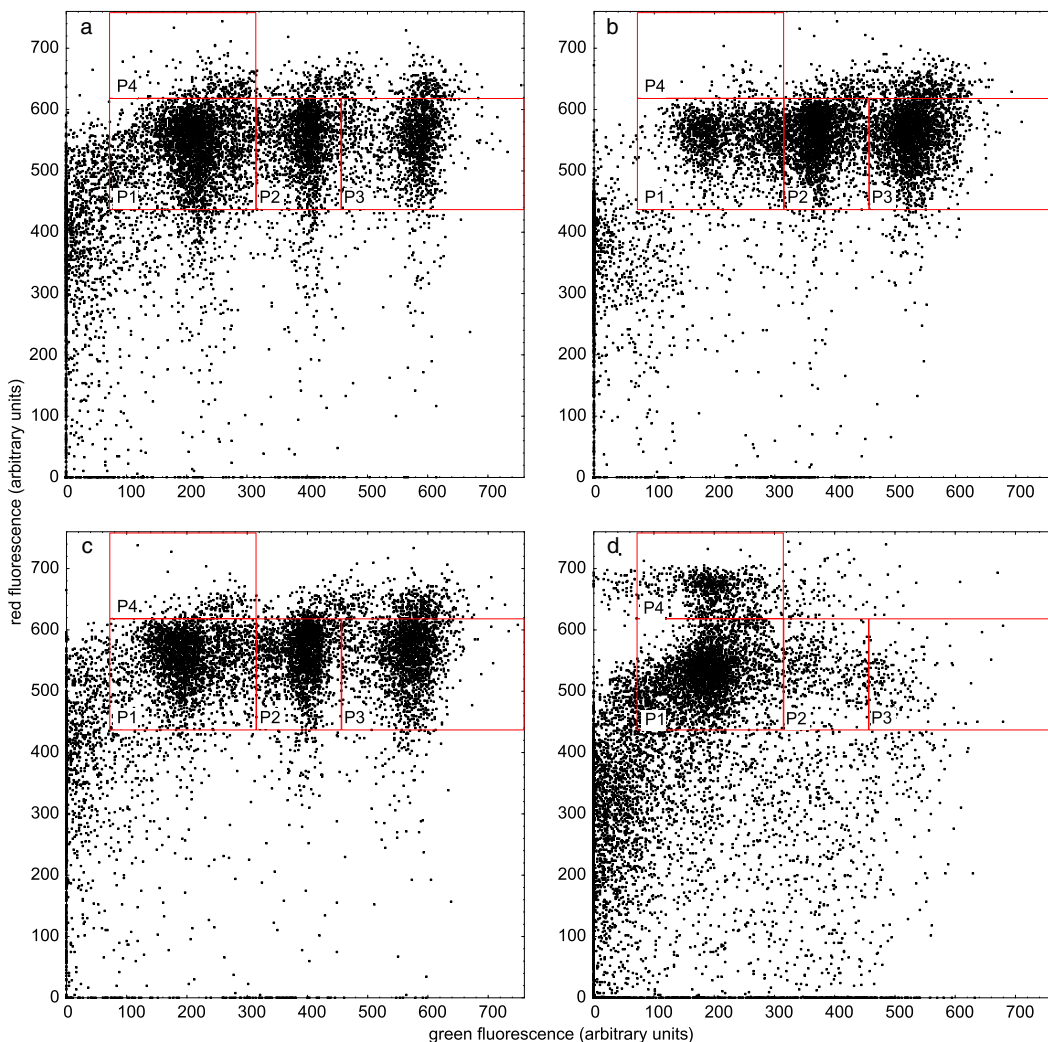
Figure 2. Green and red fluorescence of particles in honey bee semen immediately after collection (**a**, **b**, **c**) and after freeze-thawing (**d**); **a**, **b**, **d** - semen of single drone; **c** - mixed semen of the two drones presented at **a** and **b**. *Rectangles* indicate four populations of particles (P1, P2, P3, P4). Each *point* represents one particle.

mixed samples, $F_{(3, 116)}=50.4$; $P<0.001$). Particles in P1 differed markedly from particles in all other populations, whereas particles in P2 and P3 were similar to each other (Table I).

3.1 Differences between fresh unmixed and freeze-thawed samples

Freeze-thawed samples differed significantly from fresh unmixed samples in all six proportions

calculated from number of particles in the four populations (Table II). After freeze-thawing, the number of particles in P4 increased in comparison to all other populations. Moreover, the number of particles in P2 and P3 decreased in comparison to P1. Those results (Table II) confirm the above mentioned marked differences between freeze-thawed and fresh samples. From the results, it can be concluded that P1 did not consist of live cells, P2 and P3 were viable
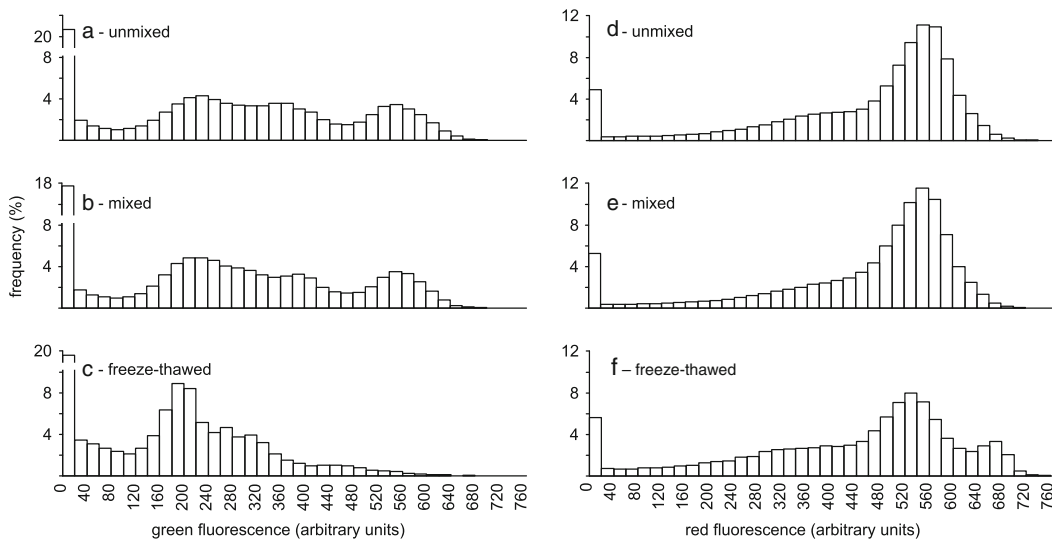
Figure 3. Histograms of green (**a**, **b**, **c**) and red (**d**, **e**, **f**) fluorescence of particles in honey bee semen stained with SYBR14/PI. The graphs correspond to: samples of fresh semen of one drone (**a**, **d**), samples of fresh semen of two drones mixed together (**b**, **e**), and samples of semen of one drone after freeze-thawing (**c**, **f**). The graphs show combined data from all samples in each group.

sperm, and P4 was nonviable sperm. The proportion of live spermatozoa was calculated as $(n2+n3)/(n2+n3+n4)$. It was significantly lower in freeze-thawed samples (0.250 ± 0.152) than in fresh unmixed samples (0.952 ± 0.048; Student's t test: $t_{(62)}=-23.5$, $P<0.001$).

3.2 Differences between fresh mixed and fresh unmixed samples

After mixing, there were more particles in P1 in relation to all other populations (Figure 2c).

This can be concluded because the proportions $n1/(n1+n2)$, $n1/(n1+n3)$ and $n1/(n1+n4)$ increased (Table II). In the first two cases, the increase was highly significant (Table II). In the case of the proportion $n1/(n1+n4)$, the increase was not significant, probably because there was a small number of particles in P4. Other proportions were not significantly affected by the mixing (Table II), therefore, it can be concluded that mixing has not affected populations other than P1. The proportion of live spermatozoa $(n2+n3)/(n2+n3+n4)$ in mixed

Table I. Mean forward scatter and side scatter in four populations of particles (Figure 2) found in unmixed semen of one drone and mixed semen of two drones.

Population	Forward scatter			Side scatter		
	Unmixed	Mixed	P	Unmixed	Mixed	P
P1	176.9±69.0 a	175.9±52.9 a	0.644	351.6±14.9 a	354.0±12.7 a	<0.001
P2	246.2±61.6 bc	249.4±43.1 bc	0.282	375.0±20.9 b	378.3±18.3 b	<0.001
P3	232.7±72.3 b	234.9±52.7 b	0.539	377.2±12.2 b	380.5±11.4 b	<0.001
P4	267.5±113.8 c	274.6±89.8 c	0.199	392.5±17.3 c	398.5±12.9 c	<0.001

P values indicate significance of differences between unmixed and mixed samples (paired Student's t test). Within columns, means (±SD) followed by the same letters are not significantly different ($P>0.05$) using Duncan's multiple range test

Table II. Mean proportions of number of particles from four populations found in honey bee semen.

Proportion	Freeze-thawed	Fresh		Student's t test freeze-thawed vs. unmixed	Paired Student's t test unmixed vs. mixed
		Unmixed	Mixed		
n1/(n1+n2)	0.918	0.649	0.692	$P<0.001$	$P<0.001$
n1/(n1+n3)	0.962	0.605	0.649	$P<0.001$	$P<0.001$
n1/(n1+n4)	0.844	0.952	0.956	$P<0.001$	$P=0.580$
n2/(n2+n3)	0.282	0.550	0.550	$P<0.001$	$P=0.991$
n2/(n2+n4)	0.321	0.901	0.899	$P<0.001$	$P=0.499$
n3/(n3+n4)	0.176	0.918	0.915	$P<0.001$	$P=0.456$

The semen was analyzed immediately after collection (fresh) or after freeze-thawing. The fresh samples consisted of semen of one drone (unmixed) or semen of two drones mixed together. n1, n2, n3, and n4 correspond to number of particles in populations P1, P2, P3, and P4 (Figure 2), respectively

semen (0.952 ± 0.025) and unmixed semen (0.952 ± 0.048) did not differ significantly (paired Student's t test: $t_{(29)}=-0.53$, $P=0.599$). After mixing, each of P1, P2, P3, and P4 showed significantly higher side scatter (Table I). The effect of mixing on forward scatter was not statistically significant (Table I).

4. DISCUSSION

The results presented here show that mixing of sperm from different drones does not affect their viability. This is in agreement with some studies (Woyciechowski and Król 1996; Shafir et al. 2009) but not with the other work (den Boer et al. 2010). The difference in results may be related to the methods of semen collection. When the semen was collected from ejaculation (Woyciechowski and Król 1996; Shafir et al. 2009; this study), there was no difference in sperm mortality between mixed and unmixed semen. The expected higher mortality of sperm was found when the semen was collected from dissected seminal vesicles and mixed with content of accessory glands dissected from other drones (den Boer et al. 2010). It is not clear whether the second experimental setup (den Boer et al. 2010) reflects natural conditions. Immediately after copulation, there is usually only semen inside the queen's lateral oviducts (Woyke 1960). Mucus and whitish substance containing epithe-

lial membrane from the accessory gland is in the queen's bursa copulatrix (Ruttner 1956; Woyke and Ruttner 1958; Woyke 2010) and, together with parts of endophallus, it forms mating plug (Woyciechowski et al. 1994) called mating sign. Before the next drone is able to transfer its semen to the oviducts, it needs to remove the mating sign of the previous drone. There is little contact between the accessory gland secretion of one drone and the semen of another drone.

We cannot exclude the possibility that, in natural conditions, mortality of mixed sperm is higher. In the experiment presented here, the semen was diluted with Kiev buffer before it was mixed. It is possible that at low concentrations, hypothetical factors responsible for mortality of spermatozoa are not active.

Side scatter and proportion of particles in the populations changed significantly after the semen of different drones was mixed. At least some of the changes may be related to sperm competition. It is particularly interesting that after mixing the proportion of particles in P1 (believed to be nonviable particles) increased in relation to other populations, although in the other populations the proportions of particles remained similar (Table II). One possible explanation of this result is that the number of particles in P1 increased as a result of coagulation. In other insects, coagulation of seminal fluids is important for formation of the mating plug (Rogers et al. 2009). In *Drosophila melanogaster*, the mating plug con-

sists of PEB-me protein, which shows autofluorescence (Lung and Wolfner 2001). In primates, there is some evidence that coagulation of seminal fluids is related to sperm competition (Dixson and Anderson 2002). It is possible that honey bee proteins present in semen coagulate when in contact with the spermatozoa of another drone in order to immobilize them. The coagulation on the surface of spermatozoa could explain the differences in the side scatter between mixed and unmixed samples. It is not clear what the source of hypothetical factors causing the coagulation is. They can be present in seminal fluid filling the space between spermatozoa and their origin can be seminal vesicles. However more research is required to confirm the coagulation and identify source of the hypothetical coagulation factors.

Flow cytometry allowed us to detect in honey bee semen two different populations of live spermatozoa (P2 and P3). This suggests the presence of sperm polymorphism, which has been found in many systematic groups of invertebrates (Swallow and Wilkinson 2002). In honey bee, the morphological differences between sperm populations may be small and therefore overlooked despite a great many microscopic observations. Such small differences can easily be detected by flow cytometry, which can measure a large number of particles. This method has been used to detect subpopulations of sperm in semen of boar (Pena et al. 2005; Thurston et al. 2001; Abaigar et al. 1999), dog (Martinez et al. 2006), and gazelle (Abaigar et al. 1999). It is important to explain the differences between the populations of particles in honey bee semen; this can be achieved by combining flow cytometry analysis with fluorescence microscopy observations.

It is known from earlier studies based on microscopic observations that live spermatozoa are fluorescent green and the dead ones are fluorescent red when stained with SYBR-14/PI (Collins and Donoghue 1999). The same staining method combined with flow cytometry gives large samples and makes blind counting easier (Holman 2009). Flow cytometry has been used to study hemocytes of honey bee (de Graaf et al.

2002) and sperm of ants (Cournault and Aron 2008). The freeze-thawing experiment presented here confirmed that flow cytometry can be used to assess the mortality of spermatozoa. This method allows quantitative analysis of semen and permits detection of sperm polymorphism.

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Mise en évidence d'une compétition spermatique chez l'abeille (*Apis mellifera*) par la méthode de cytométrie en flux

Spermatozoïde / compétition spermatique / *Apis mellifera*

Mittel Durchflusszytometrie gewonnene Erkenntnisse zur Sperma-Kompetition bei der Honigbienene (*Apis mellifera*)

Honigbiene / Spermien / Sperma-Kompetition

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