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Differences in drone sperm morphometry and activity at the beginning and end of the season

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Abstract: The sperm cells of the honey bee, like the sperm of most invertebrates, belong to the modified type of spermatozoa. In our own research, differences have been observed in the activity of sperm cells isolated from drones at the beginning and end of the season. On the basis of these observations, research was undertaken to determine whether the changes in drone activity are associated with morphological changes. The sperm cells collected from drones at the beginning of the mating season are longer than the sperm cells from the end of the season. Moreover, sperm cells from the beginning of the season exhibited more intensive rotational motion than those collected at the end of the season. Experimental $AgNO_3$ staining differentiated the sperm head into the acrosomal part (darkly stained) and the distal part (lightly stained). Staining in this manner clearly identifies the sperm nucleus and structurally differentiates the tail.

Key words: *Apis mellifera*, honey bee, drone, sperm morphometry

1. Introduction

During the summer, a bee colony consists of tens of thousands of workers, several hundred to several thousand drones, and one queen. During natural mating, the queens receive semen from about a dozen drones (1,2), so it is impossible to control the selection of pairs for mating. Each drone may produce up to 1.25 µL of semen with 10 million sperm (3). The queen bee retains about 4.5 to 5.7 million sperm in the spermatheca (4,5). This quantity is sufficient to fertilize egg cells for her entire life, as sperm can be stored in the spermatheca for several years (6,7). In the 1930s, the first successful artificial insemination of queen bees was carried out in the United States. Currently, artificial insemination of bees is used primarily for research purposes and in breeding work for the maintenance and selection of stocks with specific traits (6,8). During insemination the queen receives (depending on the method) from 2 to 10 mm^3 of semen in 1–3 doses. After mating, the queen transfers some of the semen to the spermatheca and expels the rest. Only about 6% of the dose (over 5 million sperm) remains in the spermatheca. The entire process lasts about 20 h (9,10). During the insemination procedure the inseminator takes into account the age and condition of the drones and the appearance and consistency of the semen. In our own research, differences have been observed in the activity of sperm cells isolated from drones at the beginning and end of the season. On the basis of these observations, research was undertaken to determine whether the changes in drone activity are associated with morphological changes.

2. Materials and methods

This study was carried out according to the guidelines of the III Ethical Committee in Warsaw (No. 37/2011 from 22 June 2011).

Semen was collected from 40 mature Carniolan drones at both the beginning (June) and end (August) of the mating season. Sexually mature drones were stimulated to ejaculate by pressing on the thorax. This usually resulted in partial eversion of the penis. The semen was collected directly into sterile Eppendorf tubes and diluted by adding 0.5 mL of 0.9% NaCl solution to the semen, mixed by gentle stirring. Microscope slides were prepared immediately following dilution. A preliminary assessment was made of the motility of the sperm cells, and then the slides were stained using two techniques. The sperm cells were

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stained in a routine manner commonly used for staining mammalian semen, with a complex of eosin and gentian violet according to a method described by Kondracki et al. (11). Staining with silver nitrate $AgNO_3$ in a colloidal gelatin solution was used experimentally according to Andraszek and Smalec (12). The experimental $AgNO_3$ staining differentiated the sperm head into the acrosomal part (darkly stained) and the distal part (lightly stained). Staining in this manner clearly identifies the sperm nucleus and structurally differentiates the tail.

The sperm cells were evaluated with an Olympus BX50 microscope (Tokyo, Japan) and the MultiScan image analysis system and measurement software from Computer Scanning Systems, Ltd. (Warsaw, Poland). In each ejaculate, morphometric measurements were made of 50 randomly selected sperm that were clearly visible in the field of view of the microscope. A total of 4000 sperm were evaluated. The data from the morphometric measurements of the spermatozoa were stored in a database and exported for further statistical analysis. Statistical differences between the samples were tested using Tukey's test (STATISTICA version 10.0; StatSoft Inc., Poland). The significance level was set at $P \leq 0.01$. Correlation coefficients were calculated between the morphometric traits of the drone sperm for the beginning and end of the season.

3. Results

Tables 1–4 present the morphometric characteristics of sperm cells stained with eosin + gentian violet complex. The data in Table 1 indicate substantial variation in the morphometric dimensions of the drone sperm between the beginning and end of the season. The sperm cells collected from drones at the beginning of the mating season (Figure 1) are longer than the sperm cells from the end of the season (Figure 2). The experimental $AgNO_3$ staining is shown in Figure 3.

The acrosome of sperm cells and the nucleus from the beginning of the season were 0.07 and 0.09 μm longer, respectively, than these elements of the sperm structure at the end of the season ($P \leq 0.01$), resulting in larger dimensions of the heads of sperm from the beginning of the season. Very pronounced differences were noted in the dimensions of the sperm tail. The tails of sperm from the beginning of the season were nearly 16 μm larger than at the end of the season. Moreover, sperm cells from the beginning of the season exhibited more intensive rotational motion than those collected at the end of the season.

Correlations between the morphometric parameters tested in the sperm cells at the beginning and end of the season are presented in Tables 2–4. The length of the sperm head was found to be positively correlated with the length of the acrosome and cell nucleus, and the length of the flagellum was positively correlated with the total length of the spermatozoon. The length of the sperm head was found to increase with the length of the acrosome and nucleus, irrespective of whether the semen was collected at the beginning or the end of the season.

4. Discussion

In the present study, two staining techniques were used for morphometric observations of individual parts of the drone sperm structure. The eosin + gentian violet complex is an acidic stain. Silver nitrate is an alkaline stain, mainly used for identification of acidic chromatin proteins (12). This technique reveals more details in the morphological structure of the spermatozoon than the most commonly used acidic stains (12). The photographs included in this paper show that the drone spermatozoa are very well stained with eosin + gentian violet complex. The results of silver nitrate staining are also good, but the sperm cells are colored somewhat less distinctly. Both staining methods enable identification of the individual

Table 1. Morphometric traits of the drone spermatozoa at the beginning and end of the season.

Specification	Group I (beginning of the season, June)	Group II (end of the season, August)
Number of drones	40	40
Number of spermatozoa	1200	1200
Acrosome length (μm)	4.73 ± 0.32^a	4.66 ± 0.32^b
Sperm nucleus length (μm)	4.78 ± 0.25^a	4.69 ± 0.25^b
Head length (μm)	9.43 ± 0.38^a	9.35 ± 0.39^b
Flagellum length (μm)	264.07 ± 16.57^a	248.62 ± 16.38^b
Total length (μm)	273.50 ± 16.58^a	257.97 ± 16.37^b

Data are expressed as means ± SD. Means followed by different letters within rows are significantly different (a, b: $P \leq 0.01$).

Table 2. Phenotypic correlation (Pearson) coefficients between the morphometric traits of the drone spermatozoa at the beginning of the season.

Morphometric traits	Acrosome length	Sperm nucleus length	Head length	Flagellum length	Total length
Acrosome length	1.00	–0.07	0.78*	0.02	0.04
Sperm nucleus length	–0.07	1.00	0.57*	–0.01	0.01
Head length	0.78*	0.57*	1.00	0.02	0.04
Flagellum length	0.02	–0.01	0.02	1.00	0.99*
Total length	0.04	0.01	0.04	0.99*	1.00

*: $P \leq 0.05$.

Table 3. Phenotypic correlation (Pearson) coefficients between the morphometric traits of the drone spermatozoa at the end of the season.

Morphometric traits	Acrosome length	Sperm nucleus length	Head length	Flagellum length	Total length
Acrosome length	1.00	–0.08	0.77*	–0.05	–0.03
Sperm nucleus length	–0.08	1.00	0.57*	0.03	0.04
Head length	0.77*	0.57*	1.00	–0.02	0.01
Flagellum length	–0.05	0.03	–0.02	1.00	0.99*
Total length	–0.03	0.04	0.01	0.99*	1.00

*: $P \leq 0.05$.

Table 4. Phenotypic correlation (Pearson) coefficients between the morphometric traits of the drone spermatozoa.

Morphometric traits	Acrosome length	Sperm nucleus length	Head length	Flagellum length	Total length
Acrosome length	1.00	–0.05	0.78*	0.03	0.05
Sperm nucleus length	–0.05	1.00	0.58*	0.08*	0.10*
Head length	0.78*	0.58*	1.00	0.04	0.06
Flagellum length	0.03	0.08*	0.04	1.00	1.00*
Total length	0.05	0.10*	0.06	1.00*	1.00

*: $P \leq 0.05$.

structures of the sperm cell. The boundaries between the acrosome, the postacrosomal part of the head, and the tail are clearly visible. Staining with silver nitrate also shows the heterogeneous structure of the sperm tail, which is probably linked to variation in the activity of mitochondria or mitochondrial derivatives. The use of silver nitrate staining to identify the sperm midpiece in other species has been described by Andraszek et al. (13,14). A study by Collins and Donoghue (6) confirmed that it is useful to stain drone sperm cells using various techniques, because each technique can reveal different details of the sperm ultrastructure or defects in its morphological structure. Combinations of stains can be used to assess the sperm response to stresses such as cryopreservation and osmolality of diluents (15,16).

Traits differentiating the ultrastructure of sperm can depend on a number of factors. According to Rhodes et al. (7), sperm characteristics in drones depend on their genotype, and to a lesser degree on their age and the season of the year. The present study indicates that sperm characteristics are to some extent dependent on environmental factors, including the season of the year. Differences were found in morphological parameters and in the tail structure of sperm in the semen collected at the beginning and the end of the season. In the study by Rhodes et al. (7), the volume of the ejaculate collected in

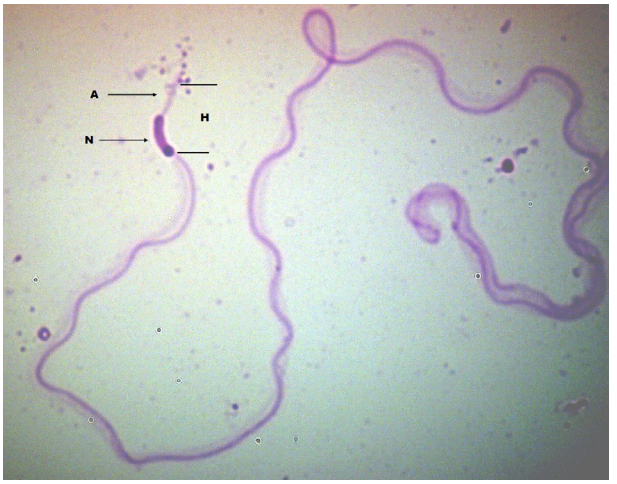

Figure 1. Sperm cell collected from drones at the beginning of the mating season (sperm cell stained with eosin + gentian violet complex). A – acrosomal region, N – nucleus, H – head.

the spring was considerably greater than in the summer or autumn, and there were over twice as many sperm cells in the semen of one drone in the autumn than in the spring. The study indicates the seasonality of sperm production in drones (7).

The spermatozoa of *Apis mellifera*, as in most insects (17), are fairly long and filamentous, with a length of about 250–270 µm. The total length of the acrosomal complex is 5 µm (18). In a study by Tarliyah et al. (19), honey bee spermatozoa were shorter than the sperm cells examined in the present study. The mean total length of the sperm cell was 217.57 µm, and the length of the sperm head

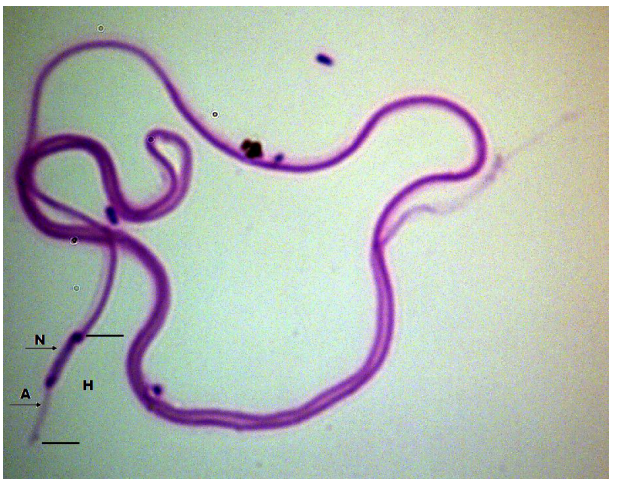

Figure 2. Sperm cell collected from drones at the end of the mating season (sperm cell stained with eosin + gentian violet complex). A – acrosomal region, N – nucleus, H – head.

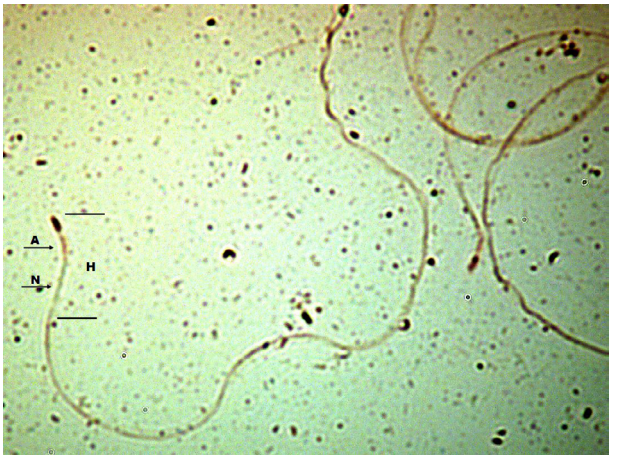

Figure 3. Sperm cell collected from drones (experimental $AgNO_3$ staining). A – acrosomal region, N – nucleus, H – head.

was 7.52 µm. Moreover, the authors noted a fairly large percentage of sperm with morphological defects, such as flipped tails (19.83%), broken tails (12.75%), double tails (6.42%), and double heads (2.25%) (19). In the present study, we also observed a substantial percentage of sperm cells with double tails, particularly in the spring (Figure 1). The present study also showed that the dimensions of drone spermatozoa differed between the beginning and end of the season. Sperm cells of drones from the spring were somewhat longer, and the individual elements of their structure were longer as well. According to Gomendio and Roldan (20), longer sperm cells in mammals may be an adaptation that increases their competitiveness. This also applies to the sperm of birds (21).

Research on competitiveness in spermatozoa has also been conducted on insects (22). Mechanisms acting in the genital tract of queen bees are conducive to intraoviductal competitiveness in sperm (23). One of the mechanisms of sperm competition is sperm incapacitation, whereby substances present in the semen of one male may cause the sperm of other males to die (24). A study by Gomendio and Roldan (20) showed that length is positively correlated with maximum sperm velocity. Our study did not evaluate sperm motility, but observations made during the initial assessment of the semen indicated that sperm collected from drones at the beginning of the season moved considerably faster than the sperm from the end of the season. This would confirm the hypothesis of the cited authors, as the sperm cells from the spring had larger dimensions. Sperm head dimensions have also been found to be associated with fertility. Mammalian sperm cells with high fertility have smaller and shorter heads than those of males with a lower fertilization capacity (25). It has also been suggested that sperm dimensions may be linked to the number of sperm, expressed as sperm concentration

in the ejaculate. There is a certain reproductive potential whereby the size of the sperm cell may be compensated for by the number of sperm, which has been confirmed in research on insects.

In summary, depending on the time of the year, drone spermatozoa differ in their morphometric characteristics and, based on observations, in their morphology and motility as well. Drone spermatozoa from the spring were somewhat longer, as were the individual elements of their structure. In addition, the use of different staining techniques in the study is a timely topic in light of literature on differential staining of sperm cells for morphological evaluation. Attention has been drawn to the need to determine or develop a staining technique enabling unambiguous and precise analysis of the morphology and morphometry of the sperm cell. This would allow comparison of results between laboratories, which would increase the value of morphological analysis of sperm cells in predicting and evaluating male fertility.

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