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Morphological and morphometric analysis of the Italian honeybee (*Apis mellifera ligustica*) spermatozoa: a preliminary study in Campania region

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Abstract

Background: Since few decades, the world is facing important losses in the number of honeybees, with great threat to the agro-zootechnic economics and to the global biodiversity. It is well known that stressors can affect the female and male reproductive system, impairing queens' and drones' fertility. To date, still very little is known about drones' physiological characteristics and possible alterations of the reproductive activity. This study focused on describing the morphological features and morphometric parameters of the Italian honeybee (*Apis mellifera ligustica*) spermatozoa in order to define its standard characteristics.

Findings: The following morphometric values (mean ± standard deviation) were measured: sperm total length (230,81±17,22 µm), tail length (222,96±17,15µm), head length (7,85±0,65µm), nucleus length (4,44±0,61µm) and perforator length (3,58±1,21µm). Moreover, 7% of the spermatozoa exhibited alterations such as broken and double tails.

Conclusions: The results obtained, allow a preliminary definition of measures of *A. mellifera ligustica* spermatozoa using an easy and economical protocol that could help to study the morphology and the morphometry of drone spermatozoa and to point out possible alterations.

Keywords: Apis mellifera, drone, spermatozoa, standard measurements

Introduction

Over the past few decades, the influence of various stressors such as pathogens, agrochemicals, environmental changes, pollution, monocultures, poor flowering habitats has caused the reduction in the number of honeybees [1]. It has been shown that some of these factors are able to directly or indirectly reduce fertility in honeybees [2]. To date, studies on honeybees' reproduction have focused on the female aspect of reproduction, as the hypofertility of the queen appears evident thanks to signs such as: decrease of brood; abnormal pattern of the brood; excessive number of drones; early replacement of the queen and presence of orphan colonies [3]. Less attention has been paid to drones and even less to alterations of their fertility, although it has been shown that male hypofertility/infertility is as complex as that of females [4]. In order to study any possible alteration in the fertility of drones, it is important to establish as a starting point what is normal. The aim of this study was to describe the morphological characteristics and morphometric parameters of the Italian honeybee (*Apis mellifera ligustica*) spermatozoa in order to define standard features that could be used in further studies to show possible alterations.

Materials and methods

100 mature drones (16-18 days old) of *A. mellifera ligustica* (Hymenoptera, Apidae) were captured in different apiaries located in Campania (Italy) during March-June 2017, when drones are mostly produced. After being sacrificed, the dissection of the abdomen was performed under a dissecting microscope (Optika, Italy) as described by Carreck et al. [5]. The reproductive system (testes, seminal vesicles and mucus glands) was then

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removed and placed in a 1.5 ml tube (Eppendorf, Germany) containing 500 µl of 0.9% sodium chloride solution (Galenica Senese, Italy). The tubes containing the samples were then centrifuged (Centrifuge 5424, Eppendorf, Germany) at 250g for 10 minutes; a drop of pellet was swiped on a slide, stained with hematoxylin and eosin and observed with a Nikon ECLIPSE 80i (Nikon, Tokyo) microscope (100X objective, 10X ocular).

Morphological analysis was performed on 10 spermatozoa per sample (1000 total spermatozoa), while morphometric analysis was performed only on healthy spermatozoa. Total length of the sperm, tail length, nucleus length, head and perforator length were measured using image analysis software (Nikon NIS Elements 4.00.02, Nikon, Tokyo). Starting and end point of measurement are reported in **Table 1**. Mean values and standard deviation were calculated for each parameter.

Table 1. Morphometric parameters of *A. mellifera ligustica* spermatozoa.

Parameter	Starting and end point of measurement	mean value± standard deviation (µm)
Total sperm length	Anterior tip of the perforator-caudal tip of the tail	230.81±17.22
Tail length	point of insertion of the tail in the nucleus -caudal tip of the tail	222.96±17.15
Head length	Anterior tip of the perforator- point of insertion of perforator in the nucleus	7.85±0.65
Nucleus length	Caudal tip of the perforator- point of insertion of the tail in the nucleus	4.44±0.61
Perforator length	Anterior tip of the perforator-point of insertion of perforator in the nucleus	3.58±1.21

Results and discussion

The spermatozoa of the Italian honeybee, stained with hematoxylin-eosin, are characterised by a long tail and an elongated head, formed by a nucleus and a perforator (**Figure 1**). Morphometric parameters (mean values and standard deviation) are summarized in **Table 1**. Moreover, 7% (n=70) of the samples revealed visible defects, such as split and broken tails (**Figure 1**).

To date, many of the studies have focused on the vitality and motility of sperm and little importance has been given to the study of morphometrics, morphology and possible alterations, although considered of predictive value of infertility in many species [**6**-**7**].

To our knowledge this is the first study to analyze mo phological and morphometric characteristics of the Italian honeybee

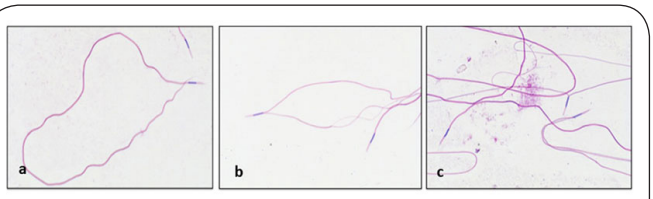

Figure 1. *Apis mellifera ligustica* spermatozoa.
a) normal spermatozoa showing no morphological alterations (haematoxylin and eosin staining, x100).
b) abnormal spermatozoa showing double and broken tails (haematoxylin and eosin staining, x100).
c)abnormal spermatozoa showing broken tails (haematoxylin and eosin staining, x100).

spermatozoa and reveal the presence of morphological abnormalities.

Hymenoptera spermatozoa have an elongated nucleus that can be straight and rigid, spiraled, or irregular and more flexible.

The acrosome is of variable size and structure depending on the species. Most hymenopteran spermatozoa presents the perforator, a granular structure that stretches from the acrosome to the front of the nucleus [**8**].

The spermatozoa of *A. mellifera*, as described by Peng [**9**] are long and filamentous, with a total length of about 250-270 µm, the head measures 8-10 µm and the total length of the acrosomal complex measures 5 µm. A study by Gontarz et al. [**10**] has focused the attention on the analysis of *Apis mellifera carnica* spermatozoa and the results are shown in **Table 2**.

In our study Italian honeybee spermatozoa appeared smaller with a mean total length of 230,81 (SD±17,22) µm. It has been shown that the production of short sperm cells consumes less energy allowing the production of a greater number of spermatozoa and the reduction of the resources allocated to the production of the spermatozoa themselves [**11**]. Furthermore, shorter sperm cells allow more sperm to be stored in the queen's spermateca, resulting in greater long-term fertilization potential [**12**]. However, according to Rhodes et al. [**13**] sperm volume and sperm concentration in drones depend on their genotype and to a lesser extent to their age and season of the year.

Our results show the presence of a high variability of the total length of spermatozoa, as underlined by the SD values

Table 2. Morphometric parameters of *A. mellifera carnica* spermatozoa [14].

Parameter	Mean value±Standard deviation (µm)
Total sperm length	273.50±16.58
Tail length	264.07±16.57
Head length	9.43±0.38
Nucleus length	4.78±0.25
Perforator length	Not assessed

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(SD±17,22). The reasons for this high degree of variability in sperm morphometry is not known, but has already been highlighted by previous studies and is probably linked to sperm competition [14-15].

Morphological analysis revealed visible morphological defects such as broken tails, double tails and double heads. It is known that some elements during the collection and handling of sperm, such as temperature, centrifugation or freezing, can influence sperm viability and induce morphological changes.

In our experiment, the sperm manipulation was minimal since the collection of spermatozoa was performed by washing and centrifugation of the testes and seminal vesicles, ensuring a high percentage of vital and morphologically normal spermatozoa. Moreover, centrifugation was performed at 250g x 10 min, this combination falls within the range suggested by Collins [16], so we can be sure that no damages and changes to sperm morphology have been caused by sperm manipulation.

It is more likely that the presence of morphologically altered spermatozoa could be determined by genetic and / or environmental factors, which could have modified the normal gametogenesis process. Conversely to mammals, drones emerge from their comb cells with the entire pool of sperm formed [17], so we suggest that if any alterations are found in the spermatozoa of mature drones they are likely to occur during larval stages, therefor morphology and morphometry should not be influenced by age. Furthermore, alterations were observed in the semen collected by drones of the same apiary, making it possible to hypothesize that there may be an external factor that strongly influenced the formation of morphologically altered sperm. Unfortunately, in this case, it was not possible to identify the actual cause of the alterations.

Conclusion

In conclusion, the results obtained, despite the low number of samples analysed, the limited area and time of sampling, allow a preliminary definition of the standard measures of the Italian honeybee spermatozoa. Further studies are necessary to collect more data in order to build a standard that could be used to outline any alterations.

Competing interests

The authors declare they have no competing interests.

Authors' contributions

Authors' contributions	KP	ED	MM	SA	FC	VP	PM
Research concept and design	✓	✓	--	--	--	--	--
Collection and/or assembly of data	✓	✓	--	✓	--	--	--
Data analysis and interpretation	--	--	✓	✓	✓	--	--
Writing the article	✓	--	✓	--	--	--	--
Critical revision of the article	--	--	--	--	--	✓	✓
Final approval of article	✓	✓	✓	✓	✓	✓	✓
Statistical analysis	--	--	--	--	✓	--	--

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