Morphological characterization of spermatozoa in *Apis mellifera* and the effect of processing and semen storage

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Abstract

The aim of this study was to stablish a standardized approach for evaluating sperm morphology in the honey bee and to investigate the impact of processing techniques and semen storage on the incidence of sperm morphoanomalies. The first experiment was designed to characterize the sperm morphoanomalies in unaltered honey bee semen, examining if they are compatible with sperm motility and viability and determining their occurrence in the ejaculates of mature and healthy honey bee drones. The different forms of sperm morphoanomalies were described in detail. In the morphological analysis of fresh drone ejaculates, 7.77% of spermatozoa showed abnormal morphology on average, with 2.20%, 4.75% and 0.81% of head, tail and multiple defects, respectively. The method also allowed to determine the status of acrosome integrity. The second experiment was designed to assess the impact of smearing and air-drying on the occurrence of sperm morphoanomalies, showing a significant increase in the incidence of both head and tail sperm defects when comparing to wet samples. In the third and fourth experiments, the effect of semen storage at room temperature and semen cryopreservation, respectively, on the occurrence of sperm morphoanomalies were investigated. The preservation of semen at 22°C led to a significant increase in spermatozoa with coiled tails after day 1, whereas the remaining anomalies did not exhibit significant variations over time. The freezing-thawing process showed a more pronounced effect on the incidence of morphological defects, with an increase in the percentage of spermatozoa with deformed heads and coiled tails.

Keywords: Honey bee, semen, sperm quality, sperm morphology, semen storage, cryopreservation

Introduction

The crucial role of honey bees as efficient pollinators is of paramount importance in agriculture, as they significantly contribute to increased fruit and seed production, while also maintaining ecosystem balance and biodiversity (Potts et al. 2010). Despite their importance, honey bees have experienced a decline in the last few decades due to numerous factors such as habitat loss, pesticide use, climate change and diseases, posing significant challenges for agriculture and ecosystem sustainability (Hristov et al., 2020).

The queen bee plays a pivotal role in the colony success, as is responsible for laying eggs and maintaining the worker population. The quality of the queen, determined by factors such as genetics, health and mating success, significantly impacts the colony productivity and resilience (Tarpy and Olivarez 2014). Poor sperm quality may also influence the reproductive success of the queen (Pettis et al. 2016), the production and survival of the colony (Tarpy and Olivarez 2014) and the efficacy of artificial insemination techniques (Collins 2000, 2004). The investigation of drone sperm quality is also a subject of considerable research interest (Yaniz et al. 2020).

Among the techniques of sperm quality evaluation, the study of sperm morphology is regarded as a fundamental tool in mammals, in which poor sperm morphology has been linked to diminished fertility rates (Yániz et al. 2015). In contrast to mammals, there have been relatively few studies evaluating sperm morphological abnormalities in honey bee semen (Yaniz et al. 2020). There are also variations in the types of sperm morphological defects described in several studies, and there is a need of a more precise characterization and standardization. Head and tail sperm morphoanomalies have been documented. The former included variations in nucleus shape and size, flexed and curved acrosome, and its absence, and double heads (Tarliyah et al. 1999; Power et al. 2019; Bratu et al. 2022). Aberrant tail forms included coiledflipped-curled, frayed, double-ended, double tails and broken (Tarliyah et al. 1999; Lodesani et al. 2004; Power et al. 2019; Morais et al. 2022; Morais et al. 2023).

Conventional methods employed in the study of sperm morphology usually involve several steps of specimen preparation, including dilution/washing, smearing, fixing and staining (WHO 2010; Yániz et al. 2015). Some of these steps may have an effect on sperm morphology. As an example, when compared to spotted wet preparations, smear samples from mice showed a higher proportion of spermatozoa with damaged acrosomes (Lybaert et al. 2009). We have observed that honey bee spermatozoa exhibit high sensitivity to processing, although the significance of this aspect requires clarification.

On the other hand, the preservation of semen, associated with artificial insemination and the establishment of semen banks, is a fundamental tool for maintaining the genetic diversity and improvement of bees. However, the results obtained following artificial insemination with frozen-thawed semen are still suboptimal (Smilga-Spalvina et al. 2023), and there is a need of novel tools for the precise assessment of the impact of these techniques on sperm quality. In this regard, morphological analysis has been rarely employed to evaluate the effect of semen preservation (Morais et al. 2023), despite its potential to provide insights for enhancing the protocols.

The primary objective of this study was to establish a standardized and reliable approach for evaluating sperm morphology in the honey bee. Additionally, the study aimed to investigate the impact of processing and semen storage on the incidence of sperm morphoanomalies.

Materials and methods

Animals and semen collection

The experiments were carried out during the beekeeping season (March-June 2023) and included drones reared in 10 honey bee (*Apis mellifera iberiensis*) colonies from two apiaries (5 colonies/apiary) in northeastern Spain. In order to increase variability, an attempt was made to minimize genetic relationships between the colonies used in the study.

Mature flying drones were manually collected in the afternoon of days with good weather on their return to the hive after blocking the entrance with a queen excluder. Subsequently, the collected drones were transported to the laboratory and kept in accordance with the protocol described by Yániz et al. (2023). Ejaculation was induced within the first 24 hours after drone collection using manual procedures (Yániz et al., 2023). An insemination syringe (Peter Schley, Lich, Germany) was used to collect semen in a capillary tube.

Experiment 1. Morphological characterization of honey bee spermatozoa

The first trial was designed to characterize the sperm morphoanomalies in honey bee semen, examining if they are compatible with sperm motility and viability and determining their occurrence in the ejaculate of healthy and mature honey bee drones.

Semen was collected individually from 81 healthy drones from 10 colonies and diluted in Kiev medium (Yániz et al., 2019). Sperm quality assessment included the analysis of sperm motility (Yániz et al., 2019; 2023), sperm viability (Yániz et al., 2023) and sperm morphology. Sperm morphology was assessed in the same aliquots used for sperm motility analysis without further processing to minimize iatrogenic morphological defects. For this purpose, 5 µl of the samples were placed between a slide and a coverslip in duplicate, and directly observed using an Olympus BX40 (Olympus Optical Co., Tokyo, Japan) microscope equipped with a heated stage (35 °C), and a 40X positive phase contrast objective.

For every individual sample, a random selection of 200 spermatozoa was made, and a visual assessment was conducted to document any morphological abnormalities. The classification of spermatozoa into specific morphological categories was determined based on a preliminary study of the samples, and these categories are described in the Results section. The compatibility of the different types of sperm morphoanomalies with sperm motility and viability was assessed, cell by cell, in 10 random samples.

To determine the minimum sample size needed to characterize the sperm morphology of the whole population and the effect-size measure, 200 spermatozoa from each drone were analyzed. The results obtained in subsets of 100, 150 and 200 ramdomly selected spermatozoa were compared.

Experiment 2. Effect of processing

The second experiment was conducted to assess the impact of smearing and air-drying on the occurrence of sperm morphoanomalies. In this trial, 56 ejaculates from 7 colonies were collected individually, diluted in Kiev-BSA and processed both as wet samples, following the procedure described in experiment 1, and as smears. The last were prepared by placing 5 µl of the specimens on the frosted end of a slide and then spreading the drop across the slide's surface using the edge of another slide. Subsequently, the prepared slides were left to air dry for a minimum of 2 hours and examined without any additional processing using the same optical setup as in experiment 1.

Experiment 3. Effect of semen maintenance at room temperature on the incidence of sperm morphoanomalies

The third experiment was devised to investigate the impact of semen storage at room temperature on the occurrence of sperm morphoanomalies. For this purpose, 54 semen samples from individual drones from 7 colonies were collected and diluted in 50 µl of Kiev, then kept at 22 ºC for two days. Sperm quality, encompassing motility, viability, and morphoanomalies, was assessed at 0, 24, and 48 hours after collection using similar methodologies to those employed in experiment 1.

Experiment 4. Effect of semen cryopreservation on the incidence of sperm morphoanomalies

In the last trial, the effect of drone semen cryopreservation on the occurrence of sperm morphoanomalies was investigated. Semen samples were collected from 39 mature drones from 5 colonies and grouped into pools of three ejaculates. The 13 pooled samples were further divided into three aliquots: the first served as the control, and the other two were diluted in Kiev diluent to achieve a final concentration of 10% egg yolk plasma and 10% DMSO. One of these samples was used to evaluate the effect of dilution in the cryoprotective medium and the other was then packed into 0.25 ml straws and subjected to cryopreservation using a Freeze Control CL8800® (Cryologic, Victoria, Australia) programmable freezer. A conventional slow cooling treatments was employed, with rates of 0.2 °C/min from 20 ºC to 5 ºC and 3 °C/min from 5 ºC to -40 ºC, followed by immediate immersion in liquid nitrogen. After a minimum of 24 h of storage, cryopreserved semen samples were thawed in a water bath at 37 °C for 1 min. The assessment of sperm quality was performed on both fresh, diluted and post-thaw semen samples, using the same methods as described in Experiment 1.

Statistical analysis

Statistical analyses were performed using the SPSS package, version 25.0 (IBM SPSS Statistics, Chicago, IL, USA). Distribution normality and the homogeneity of variance of the median for each set were checked using the Kolmogorov–Smirnov and Levene tests respectively. In the first experiment, results of the different parameters of sperm quality were compared using the Spearman's correlation test. In the second to forth experiments, for samples that were normally distributed, differences in the sperm quality parameters between treatments were recorded by means of analysis of variance (ANOVA) using generalized linear models. When more than two different groups were compared, if the F value was significant, a Tukey test was used for the posteriori multiple comparisons between the groups. For non-normally distributed populations, the Kruskal–Wallis test, followed by the Mann–Whitney post hoc test, were used for multiple comparisons. The results of the main effects are expressed as mean ± standard deviation of the mean (SD). The statistical level of significance was set at $P < 0.05$.

Results

Experiment 1. Morphological characterization of drone spermatozoa

Following the analysis of the sperm morphology, it was possible to determine the following sperm types: 1) normal spermatozoa, characterized by being long and filamentous cells with tapered ends, with a relatively small and narrow head region, along with a long flagellum, with no abrupt bends, self-coiling, breakages, or thickening along its length (Fig. 1a). The sperm head comprises two consecutive parts that cannot be distinguished without staining: the acrosome, followed by a linear nucleus. The acrosome is composed of a conical and two-layered acrosomal vesicle, which covers the perforatorium, making this structure indistinguishable in unaltered sperm (Fig. 1a). 2) Spermatozoa with an abnormal head, including shape deviations from a normal spermatozoon (Fig. 1b), and damaged acrosomes, in which the perforatorium may be distinguished (Fig. 1c, d). 3) Spermatozoa with a defective tail, including coiled (Figs. 2a-2c), frayed (Fig. 2d), broken (Fig. 2e) and bent (Fig. 2f) tails. 4) Spermatozoa having any combination of the above mentioned morphological defects.

The sperm morphology study demonstrated that of the total number of spermatozoa analyzed (16,200 cells) 92.23% of them were normal. Head abnormalities were relatively infrequent (2.20%), with values ranging from 0.00% to 8.50%. Tail defects exhibited the highest prevalence (4.75%), and showed the highest variation between individuals, with values ranging between 1.00% and 21.00%. The prevalent forms of tail defects were primarily coiled (2.16%) and frayed (1.68%) tails, whereas bent and broken tails were less commonly observed. Multiple abnormalities observed were rare, with a mean value of 0.81% and values ranging from 0.00 to 3.00%. The majority of these multiple defects consisted of an abnormal head (abnormal head shape and/or damaged acrosome) together with a frayed tail, with other combinations less frequently observed.

Spermatozoa that displayed abnormal head shape, frayed, bent and broken tails showed no signs of motility and were always dead. However, coiled spermatozoa were often found to be motile and alive, and spermatozoa with damaged acrosomes were either immotile/dead or motile/alive.

No statistical differences were found among the subsets of 100, 150 and 200 sperm cells for the results of morphologic assessment. It suggests that the analysis of 100 spermatozoa is sufficient for the morphometric characterization of honey bee semen sample under these conditions.

There was a negative correlation between several sperm morphologic anomalies, particularly those associated to the sperm head, and sperm motility and viability (Table 1).

Table 1: Spearman's correlation coefficients between different sperm quality parameters in honey bee drones.

	Sperm motility	Sperm viability
Sperm motility		0.25^{*}
Abnormal Sperm	-0.39^{**}	-0.38^{**}
Head defects	-0.39^{**}	-0.33^{**}
Head shape defect	ns	-0.30^{**}
Damaged acrosome	-0.40^{**}	-0.27^{*}
Multiple abnormalities	-0.34^{**}	-0.34^{**}

ns: not significant. Significant correlations at $^{*}P < 0.05$ and $^{**}P < 0.01$.

Experiment 2. Effect of processing

The application of smearing and air-drying techniques resulted in a significant increase in the incidence of sperm abnormalities. Smeared-dried samples exhibited a significant increase in spermatozoa displaying tail defects and, specially, head morphoanomalies when compared to wet samples (Table 2).

Table 2. Percentage (mean ± SD) for normal and morphological sperm defects in honey bee drone semen (n=56 ejaculates), using wet and smearing/air-drying processing methods.

Sperm characteristics (%)	Processing method	
	Wet preparation	Smearing/air drying
Normal Sperm	92.37 ± 4.84^{a}	77.04 ± 14.12^{b}
Head defects	2.39 ± 2.08^{a}	11.48 ± 11.54^{b}
Head shape defect	0.69 ± 0.99^{a}	8.23 ± 10.84^{b}
Damaged acrosome	1.71 ± 1.67^{a}	3.45 ± 2.97^{b}
Tail defects	4.40 ± 3.64^{a}	9.62 ± 5.95^{b}
Coiled tail	2.04 ± 2.65^{a}	3.63 ± 3.87^{b}
Frayed tail	1.58 ± 1.38^{a}	4.48 ± 3.50^{b}
Bent tail	0.66 ± 0.86^{a}	0.80 ± 0.89^{a}
Broken tail	0.13 ± 0.26^{a}	0.71 ± 0.94^{b}
Multiple abnormalities	0.83 ± 0.95^{a}	1.66 ± 1.27^{a}

Different superscripts (a,b) within a row means significant differences at $P<0.01$.

Experiment 3. Effect of semen maintenance at room temperature on the incidence of sperm morphoanomalies

The preservation of semen at 22°C for two days led to a deterioration in sperm quality parameters, with an increase in total morphological abnormalities starting on day 1, as well as a decrease in motility and viability on day 2 (Table 3). Among sperm morphological abnormalities, head morphoanomalies were not affected by semen storage, but tail defects clearly increased with time. In particular, a significant increase in spermatozoa with coiled tails was observed on days 1 and 2 of maintenance at room temperature, and of spermatozoa with frayed tails on day 2, whereas the remaining anomalies did not exhibit significant variations over time (Table 3).

Table 3. Percentage (mean ± SD) for normal and morphological sperm defects in honey bee drone semen (n=54 ejaculates) during semen storage at 22ºC.

Sperm characteristics (%)	Time of semen storage at 22ºC (day)		
	0	1	2
Sperm motility	80.48±8.98^{a}	71.31±19.08^{a}	37.80±31.89^{b}
Sperm viability	77.73±9.35^{a}	65.73±18.12^{ab}	47.93±13.85^{b}
Normal Sperm	91.73±8.77^{a}	70.80±24.27^{b}	55.08±26.76^{b}
Head defects	2.19±2.16^{a}	2.20±2.25^{a}	2.36±2.20^{a}
Head shape defect	0.58±1.02^{a}	0.88±1.36^{a}	0.56±0.71^{a}
Damaged acrosome	1.60±1.75^{a}	1.32±1.65^{a}	1.80±1.79^{a}
Tail defects	5.45±8.37^{a}	25.80±24.02^{b}	41.39±26.43^{b}
Coiled tail	2.85±8.14^{a}	21.79±23.64^{b}	36.17±25.87^{b}
Frayed tail	1.59±1.43^{a}	3.02±3.39^{a}	4.06±3.58^{a}
Bent tail	0.89±1.22^{a}	0.82±1.09^{a}	1.12±1.22^{a}
Broken tail	0.12±0.27^{a}	0.18±0.38^{a}	0.05±0.19^{a}
Multiple abnormalities	0.63±0.85^{a}	1.20±1.56^{a}	1.17±1.07^{a}

Different superscripts (a,b) within a row means significant differences at P<0.05.

Experiment 4. Effect of semen cryopreservation on the incidence of sperm morphoanomalies

Dilution in the cryopreservation medium reduced sperm motility and increased the percentage of spermatozoa with coiled tails, but the other sperm quality parameters were not affected (Table 4). However, freezing-thawing process had a pronounced effect on sperm quality, with a noticeable decrease in sperm motility and viability, and an increase in the incidence of both head and tail morphological abnormalities (Table 4). Among these, there was an increase in the percentage of spermatozoa with deformed heads and coiled tails.

Table 4. Sperm quality parameters (mean ± SD) in honey bee drone semen (n=13 pooled samples) before and after freezing.

Sperm characteristics (%)	Determination		
	Fresh semen sample	After dilution	After freezing-thawing
Sperm motility	82.11 ± 6.07^{a}	71.30 ± 11.97^{b}	51.72 ± 7.54^{c}
Sperm viability	68.53 ± 6.80^{a}	63.87 ± 7.93^{a}	40.91 ± 9.99^{b}
Normal Sperm	91.02 ± 3.34^{a}	82.93 ± 9.72^{a}	46.28 ± 14.83^{b}
Head defects	2.23 ± 1.45^{a}	1.34 ± 1.45^{a}	10.88 ± 4.91^{b}
Head shape defect	1.08 ± 0.98^{a}	1.19 ± 1.22^{a}	9.50 ± 4.16^{b}
Damaged acrosome	1.15 ± 1.08^{a}	0.15 ± 0.32^{a}	1.38 ± 0.94^{a}
Tail defects	6.68 ± 2.62^{a}	15.61 ± 9.10^{a}	42.57 ± 15.72^{b}
Coiled tail	2.42 ± 1.10^{a}	11.81 ± 9.73^{a}	36.72 ± 15.36^{b}
Frayed tail	3.11 ± 1.72^{a}	3.26 ± 2.08^{a}	4.44 ± 2.01^{a}
Bent tail	1.11 ± 1.00^{a}	0.50 ± 0.35^{a}	1.26 ± 1.21^{a}
Broken tail	0.04 ± 0.14^{a}	0.04 ± 0.14^{a}	0.15 ± 0.55^{a}
Multiple abnormalities	1.00 ± 0.82^{a}	1.50 ± 0.54^{a}	3.49 ± 0.64^{b}

Different superscripts (a-c) within a row means significant differences at $P<0.05$.

Discussion

Morphological analysis is regarded as a crucial element of the spermiogram in mammals (Rijsselaere et al. 2004), but in the case of bees, there is limited research available on this subject (Yaniz et al. 2020). This study establishes the fundamental framework for a standardized and reliable analysis of morphological abnormalities in drone spermatozoa. Furthermore, an investigation into the factors influencing the occurrence of morphological abnormalities has also been conducted.

The types of sperm morphoanomalies observed differ from those of mammals, particularly in relation to sperm head morphology. Various head shapes have been described in mammals including, among others, pear-shaped, circular, elongated, macro- and micro-cephalic, which are compatible with sperm motility. In contrast, the honey bee shows a high uniformity in sperm head morphology, which might be associated with a high degree of sperm competition (Harbo 1990; Woyciechowski and Król 1996; Franck et al. 2002; Shafir et al. 2009; Tofilski et al. 2012; Gencer and Kahya 2020). In this species, we have only observed deformed heads shape anomaly, which were exclusively found in immotile and membrane-damaged spermatozoa, suggesting that they are a consequence of cell death. In fact, cell dead induced by liquid nitrogen immersion caused changes in sperm head morphology similar to those described in the present study (Paynter et al. 2014).

Some tail abnormalities (frayed, broken, and bent flagella) were also associated to immotile and dead spermatozoa. On the contrary, morphoanomalies compatible with sperm motility and viability included damaged acrosomes and coiled tails. The presence of coiled tails is indicative of proper functionality of the plasma membrane in response to osmotic changes, so cells exhibiting this anomaly are typically expected to be alive (Nur et al. 2012). The acrosome consists in a cone- shaped acrosomal vesicle that covers the perforatorium (Peng et al. 1993). The acrosomal vesicle forms an independent compartment, and thus, its damage is compatible with the integrity of the sperm plasma membrane. It is important to highlight that the occurrence of acrosome morphological defects was linked to acrosomal damage in the present study. The preservation of acrosomal integrity represents a significant and autonomous facet of sperm quality assessment, and the development of a straightforward and dependable method for its evaluation holds substantial importance (Yaniz et al. 2020). In the honey bee, *Pisum sativum* agglutinin lectin staining has been used for acrosomal integrity assessment, but this procedure has several disadvantages and there is a need for more research about this subject (Yaniz et al. 2020). We have described here that acrosomal integrity may be easily assessed through the use of wet preparations and phase-contrast microscopy, where the visualization of the perforatorium is a clear sign of acrosomal damage. Phase contrast microscopy of wet preparations has also been used in mammals for the evaluation of acrosome integrity, although there is a difficulty in clearly discerning the sperm acrosome in most animal species (Yániz et al. 2021).

While certain studies have mentioned the existence of double heads or flagella in bees (Tarliyah et al. 1999), we have not encountered these abnormalities in the numerous samples examined (more than 82,000 total spermatozoa). It is possible that double flagella were erroneously identified as the frayed ones described in this study, as they can occasionally appear quite similar.

With regard to the minimum number of spermatozoa required to be counted in each analysis, the findings indicated that a sample size of 100 spermatozoa effectively represented the entire population, and this may be considered the minimum number of spermatozoa to be evaluated in a morphological analysis. These results are coincident with the minimum number typically recommended for sperm morphology analysis in other species (Gago et al. 1998; Buendia et al. 2002; Hidalgo et al. 2005; WHO 2010).

The predominant techniques for assessing sperm morphology involve sample smearing, air drying and subsequent staining. Nonetheless, only a limited number of studies have investigated morphology through wet preparations (Soler et al. 2015; Soler et al. 2016). This poses a significant challenge since traditional processes of dehydration, rehydration, and staining introduce artifacts that distort sperm morphology in mammals (Katz et al. 1986; Yeung et al. 1997). Results of the present study evidenced that the common air-drying techniques induced dramatic changes in drone sperm morphology, suggesting that they should be replaced by examination of wet preparations, as proposed for mammals (Soler et al. 2016). Furthermore, it should be noted that certain fixation and staining protocols can result in the complete removal of the acrosomal membrane, rendering only the perforatorium visible and impeding the assessment of acrosomal damage (see for example the images in (Gontartz et al. 2016; Bratu et al. 2022; Banaszewska and Andraszek 2023).

The incidence of morphoanomalies in fresh semen has consistently remained below 10% on average in all conducted experiments. The high uniformity of sperm morphology might be related to the selection associated with a high sperm competition and the absence of female remating in this species (Baer 2005). However, previous studies have reported a higher incidence of morphological defects (Tarliyah et al. 1999; Bratu et al. 2022; Morais et al. 2022; Morais et al. 2023). This discrepancy can be attributed to the use of protocols involving the smearing, air-drying, fixation and staining of semen samples and/or to the semen dilution in media with low osmolarity, that increases the incidence of coiled tails (Yaniz et al. 2019; Morais et al. 2022; Morais et al. 2023). It is also important to highlight that in this study mature drones from healthy and wellnourished colonies during the reproductive season were used, which exhibited high sperm quality parameters in the majority of cases. The incidence of morphological anomalies is likely to be higher when colonies are in more unfavorable conditions, as has previously observed (Morais et al. 2022).

Semen preservation had a great effect on the incidence of sperm morphoanomalies. A gradual decrease in sperm viability along with an increase in morphological abnormalities, especially spermatozoa with coiled tails were evidenced during storage at room temperature. Tail coiling is generally associated with sperm exposure to hypo-osmotic conditions (Nur et al. 2012). Drone spermatozoa appear to be particularly sensitive to changes in osmolarity (Yaniz et al. 2019), but in this assay, the same medium (modified Kiev medium) was used throughout storage. The osmolarity of the drone semen and of the seminal plasma is between 467 and 325 mOsmol/L, respectively (Verma 1973). The modified Kiev diluent used in this study (Collins 2005) has an intermediate value between this range (384 mOsmol), and consequently was selected as the basis diluent in this research work. However, results of the present study suggest that this diluent may be slightly hypotonic, causing a clear increase of tail coilings only during long term semen storage.

Maintenance at room temperature can also facilitate bacterial proliferation and increase the presence of reactive oxygen species (ROS), which might have deleterious effects on cellular integrity and morphology (Yaniz et al. 2010; Silvestre et al. 2021). However, little is known about the effect of oxidative damage on sperm morphology and more research is needed on this topic.

The effect of cryopreservation on the incidence of sperm morphoanomalies was even more intense, with a marked increase of both head and tail defects, particularly deformed heads and coiled tails. These findings are in agreement with a recent study, although the incidence of sperm morphoanomalies after cryopreservation was dependent on the diluent used (Morais et al. 2023). The single dilution with the cryopreservation medium induced an increase of spermatozoa with coiled tails, despite being in hyperosmotic conditions. In any case, the results of the present and other previous works demonstrate the utility of morphological study for improving cryopreservation protocols and more studies are needed to determine the effect of the diluent composition and freezing protocols on the incidence of sperm morphoanomalies. An important aspect of the sperm evaluation protocol after freezing-thawing is the need to use the cryopreservation medium (or a medium of similar osmolarity) for semen dilution after cryopreservation, since otherwise the incidence of sperm with tail coiled defects increases (data not shown).

Conclusion

It was concluded that the method based on the study of wet semen samples with phase contrast microscopy allowed a reliable estimation of sperm morphoanomalies in the honey bee, with a precise characterization of the types of morphological defects and their compatibility with sperm motility and viability. On the contrary, traditional processing methods, based on smearing and air-drying induced changes in drone sperm morphology, suggesting that they should be replaced by examination of wet preparations. Given that semen storage had a marked effect on the incidence of morphological anomalies, the study of this parameter may help to improve semen conservation protocols in this species. Further research in this area is needed for understanding the mechanisms underlying these anomalies and its relationship with reproductive outcomes.

Acknowledgements

The authors would like to acknowledge the use of the Servicio General de Apoyo a la Investigación-SAI, Universidad de Zaragoza.

Disclosure statement

The authors declare no conflict of interest.

Funding

This work was supported by the Spanish AEI-MICIN (grant PID2020-112673RB-I00/AEI/ 10.13039/501100011033), and the DGA-FSE (grant A07_23R).

Data availability statement

The authors confirm that the data supporting the findings of this study are available upon reasonable request from the corresponding author.

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Figure legends

Figure 1. Representative micrographs of *Apis mellifera* drone sperm head morphoanomalies. Normal sperm (a), spermatozoa with an abnormal head shape (b), and damaged acrosomes (c, d). Scale Bar: 10 µm.

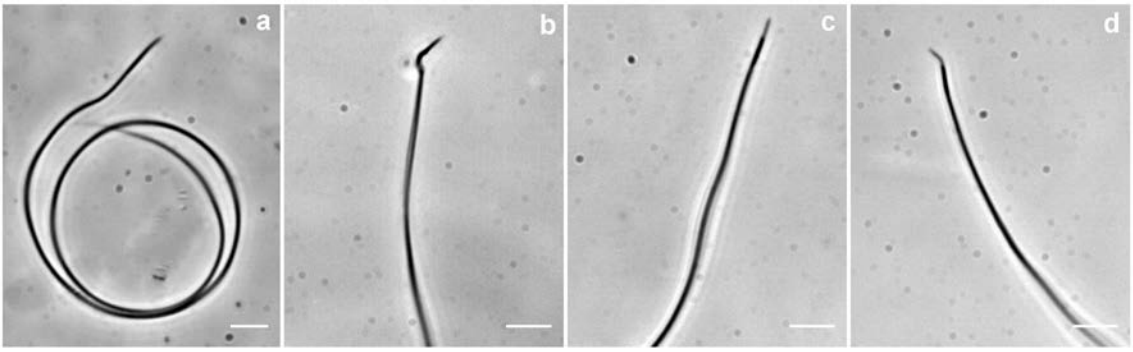

Figure 2. Representative micrographs of *Apis mellifera* drone sperm tail morphoanomalies. Spermatozoa with coiled tail (a-c), frayed tail (d), bent tail (e), and broken tail (f). Scale Bar: 10 µm.