Citation: Kaya A, Uysal **O.** In vitro spermatological parameters in drones. U. Arı D. / U. Bee J. 2023, 23(2):268-279 DOI: 10.31467/uluaricilik.1279779

DERLEME / REVIEW

IN VITRO SPERMATOLOGICAL PARAMETERS IN DRONES

Bal Arılarında In Vitro Spermatolojik Parametreler

Abdulkadir KAYA[1,2,*], Ongun UYSAL[3]

[1]Department of Reproduction and Artifical Insemination, Faculty of Veterinary Medicine, Kırıkkale University, Kırıkkale, TÜRKİYE. Yazışma Yazarı / Corresponding author E-posta: abdulkadirkaya@kku.edu.tr, ORCID No: 0000-0001-7903-4358

[2]Institute of Health Sciences, Ankara University, Ankara, TÜRKİYE

[3]Department of Reproduction and Artifical Insemination, Faculty of Veterinary Medicine, Ankara University, Ankara, TÜRKİYE, E-posta: ongun.uysal@veterinary.ankara.edu.tr, ORCID No: 0000-0001-5025-6512

Received / Geliş Tarihi: 09.04.2023 Accepted / Kabul Tarihi: 18.05.2023 DOI: 10.31467/uluaricilik.1279779

ABSTRACT

Honey bees are an indispensable element of the ecosystem, as they provide an important part of plant pollination beyond food production. The queen bee, which forms the core of the bee colony, is at the center of bee production in the beekeeping sector. Undoubtedly, the production of high-quality queen bees primarily depends on suitable drones and therefore, quality sperm. In addition, artificial insemination of queen bees is a successful production method, as in other species, due to its superior characteristics compared to natural mating. In many apiaries and research centers, artificial insemination is used routinely for queen production. One of the advantages of this method is that the sperm can be preserved for the short or long term. In this respect, it is very effective in protecting genetic resources, preventing bee diseases and facilitating bee transportation. Today, spermatological studies and parameters used in drones are very few compared to other species. With the increasing importance given to the subject in recent years, many spermatological parameters have started to be used in honey bees. However, the morphological and physiological differences of drone semen require the development and standardization of these parameters with further studies. In this review, the spermatological parameters used in drone semen, together with the working principles and materials used, were examined in general terms and presented to the readers.

Keywords: Beekeeping, Artificial insemination, Drone, Honey bee, Sperm

ÖZ

Bal arıları, gıda üretiminin ötesinde bitki tozlaşmasının önemli bir bölümünü sağladıkları için ekosistemin vazgeçilmez bir unsurudur. Arı kolonisinin çekirdeğini oluşturan ana arı, arıcılık sektöründe arı üretiminin merkezinde yer almaktadır. Kuşkusuz kaliteli ana arı üretimi öncelikle uygun erkek arılara ve dolayısıyla kaliteli spermaya bağlıdır. Ayrıca suni tohumlama doğal çiftleşmeye göre üstün özellikleri nedeniyle diğer türlerde olduğu gibi başarılı bir üretim yöntemidir. Birçok arıcılık işletmesinde ve araştırma merkezinde ana arı üretimi için suni tohumlama rutin olarak kullanılmaktadır. Bu yöntemin avantajlarından biri de spermanın kısa ya da uzun süreli saklanabilmesidir. Bu yönüyle genetik kaynakların korunmasında, arı hastalıklarının önlenmesinde ve arı taşımacılığının kolaylaştırılmasında oldukça etkilidir. Günümüzde erkek arılarda kullanılan spermatolojik çalışmalar ve uygulanan parametreler diğer türlere göre çok azdır. Son yıllarda konuya verilen önemin artmasıyla bal arılarında birçok spermatolojik parametre kullanılmaya başlanmıştır. Ancak erkek arı spermasının morfolojik ve fizyolojik farklılıkları, bu parametrelerin ileri çalışmalarla geliştirilmesini ve standardize edilmesini gerektirmektedir. Bu derlemede erkek arı spermasında kullanılan spermatolojik parametreler, çalışma prensipleri ve kullanılan materyaller genel hatlarıyla incelenmiş ve okuyucuların beğenisine sunulmuştur.

Anahtar kelimeler: Arıcılık, Suni tohumlama, Bal arısı, Erkek arı, Sperma

DERLEME / REVIEW

GENİŞLETİLMİŞ ÖZET

Amaç: Bal arıları, birçok bitki türünde tozlaşmayı sağlayıcı rolünden dolayı ekosistemin devamlılığını sağlayan önemli bir unsurdur. Bir meslek olarak arıcılık, düşük maliyetle yüksek kar getirisine sahip olması sebebiyle önemli bir hayvancılık koludur. Modern arıcılıkta ana arı üretimi kolonilerin çoğaltılması, devamlılığı ve verimini artırma amacıyla oldukça yaygın bir şekilde ve farklı metotlarla yapılmaktadır. Erkek arılardan uygun donanım ve metot ile toplanılan spermanın ana arıya nakli ile bal arılarında suni tohumlama yüz yıla yakın bir süredir uygulanmaktadır. Suni tohumlama tekniğinin kaliteli damızlık üretimi, ıslah çalışmalarının yaygınlaştırılması, spermanın muhafazası ve bulaşıcı hastalıkların önlenmesi gibi birçok faydası vardır. Bu sebeple suni tohumlamada kullanılan arı spermasının kalitesini belirlemeye yönelik ve spermanın muhafazasına yönelik çalışmalarda gün geçtikçe önem kazanmaktadır. Yapılacak araştırmanın hedefleri doğrultusunda kullanılacak parametrelerin seçilmesi ve bu parametrelerin uygun metotla uygulanabilmesi önem arz etmektedir. Bu derlemede arı spermasında kullanılan spermatolojik parametreleri geniş bir yelpazede ele almaya çalıştık. Böylece hem arı spermasının daha efektif değerlendirilebilmesi hem de konuyla ilgilen araştırmacılara yararlı olacağını düşünmekteyiz.

Tartışma: Arı spermasında kullanılan parametreler oldukça sınırlı kalmaktadır. Bu sebeple arı spermasının *in vitro* değerlendirilmesindeki yaşanılan eksiklikler fertilizasyon yeteneğinin belirlenmesini zorlaştırmaktadır. Bu konuda yapılan çalışmaların artması ve arı üretiminin yaygınlaşması konuya olan ilgiyi artırmıştır. Ancak halen birçok parametre arı spermasında kullanım alanı bulmamakla beraber bazı parametreler ile yalnızca birkaç çalışmaya rastlanılmaktadır. Örneğin spermatozoon hareket yeteneğinin belirlendiği motilite tayini ile ilgili farklı metotlar ve değerlendirme kıstasları kullanılmaktadır. Memeli türlerinde ilerleyici tarzda bir spermatozoon hareketine karşın arı spermasında sirküler bir hareketin olması, spermatozoon baş kısmının ayırt edilememesi ve spermatozoon uzunluğunun yaklaşık 10 kat büyük olması sebebiyle memeli spermasında yaygın kullanılan Bilgisayar Destekli Sperm Analizi yöntemi arı spermasında efektif bir biçimde kullanılamamaktadır. Benzeri bir durum sperma hacminin oldukça az olmasından kaynaklı olarak oksidatif stres parametrelerin kullanımı ile alakalı da yaşanmaktadır. Bunlar ve benzeri sebeple kullanılan spermatolojik parametrelerin geliştirilmesine de ihtiyaç duyulmaktadır. Böylece arı spermasının fertilizasyon yeteneği hakkında daha kapsamlı bir değerlendirme mümkün olabilecektir.

Sonuç: Bal arılarında *in vitro* spermatolojik parametreler fertilizasyonun önemli bir göstergesidir. Yapılan birçok çalışmada sperma kalitesinin belirlenmesinde farklı parametrelerden yararlanılmıştır. Ancak arı spermasının memeli spermasına kıyasla morfolojik ve fizyolojik açıdan farklılıkları diğer türlerde yaygın olarak kullanılan birçok parametrenin kullanımını sınırlandırmaktadır. Ayrıca başka türlerde başarıyla uygulanan parametrelerin birçoğu halen arı spermasında kullanım alanı bulamamıştır. Bal arılarında spermatolojik parametrelerin daha yaygın kullanımı ve yeni yöntemlerin ve modifikasyonlar ile birlikte arı spermasının daha efektif bir şekilde belirlenebileceği düşünülmektedir. Yapılan bu derlemede arı spermasında yaygın olarak kullanılan ve henüz birkaç çalışmada kullanım alanı bulmuş birçok spermatolojik parametre birlikte ele alınmıştır. Böylece konu ile alakalı araştırma yapacak bilim insanlarına faydalı olacağı düşünülmektedir.

INTRODUCTION

Honey bees are the only animal in the world that provides 80% of the pollination of flowering plants (Falencka-Jabłońska 2022). For this reason, the decline in the population of honey bees due to factors such as global warming, pollution of the natural environment and diseases affects the ecosystem significantly (Papa et al. 2022). In terms of animal husbandry, beekeeping in the world is a business with high profitability due to low input costs and obtaining valuable bee products.

Queen bee production, which ensures the continuity of the colony in beekeeping, is very important and many apiaries either produce or buy quality queens. Artificial insemination is an innovative method that can be used for quality queen production. Compared to natural mating, this method provides similar or higher quality reproductive efficiency. More importantly, the cryopreservation of semen plays an important role in the transportation of drone semen to distant regions, the protection of genetic resources and protection against bee diseases (Ghramh et al. 2022). With the widespread use of this technique, in vitro studies on reproductive

efficiency have increased. In recent studies, spermatological methods, which are widely used in many mammalian species, are also applied in drone semen. Evaluation of sperm is important because, after mating or artificial insemination in honey bees, the queen will use the semen obtained from the male individuals (polyandry) in the production of fertile eggs throughout her life. Therefore, changes in spermatological parameters are one of the important factors affecting the success of fertilization. There are many factors that affect spermatological parameters, for example; drone age (Hayashi and Satoh 2019), body weight (Schlüns et al. 2003), genetic factors (Rhodes et al. 2011), temperature (Morais et al. 2022), nutrition (Stürup et al. 2013), colony management (Abdelkader et al. 2014), seasonal changes (Morais et al. 2022), diseases (Collins and Pettis 2001), insecticides (Ben Abdelkader et al. 2021), microbial contamination (Johnson et al. 2013, Rajamohan et al. 2020), cryopreservation of semen (Wegener and Bienefeld 2012), semen collection method (Collins 2004) and semen physiology (den Boer et al. 2010).

For this reason, spermatological analyses for many purposes allow more effective determination of semen quality and fertility. While semen quality was determined by in vivo parameters (spermatozoa concentration in queen’s spermatheca, brood area and queen acceptance rates) in the early periods (2000 and before), in vitro parameters have been used more widely with the development of many new and effective methods in recent years. This review aimed to comprehensively discuss the spermatological parameters used in drone semen. Thus, it is thought that it will help to provide information and develop procedures for future studies.

Spermatogenesis and struce of drone sperm

The reproductive organs of drones consist of testes, vas deferens, seminal vesicles, mucus glands, ductus ejaculatorius and endophallus (penis). Spermatogenesis in drones begins during the larval stage and takes place within the tubules of the testes. Spermatogonia cells, located at the upper end of approximately 200 tubules in the testes, divide through mitosis to produce more spermatogonia. Spermatogonia later divide to form primary spermatocytes, which in turn become secondary spermatocytes through meiosis. Since the germinal tissue of drones has haploid chromosomes, there is no reduction in the number of chromosomes during the meiotic stage. Sharma et al. appropriately described honey bee spermatogenesis as "abortive meiosis I and abnormal meiosis II": no reduction division occurs. Secondary spermatocytes then produce spermatids through meiosis, which eventually develop into mature sperm cells known as spermatozoa. Spermatogenesis is completed during the pupal stage, and the storage of sperm in the seminal vesicle continues until approximately the 12th day after emergence as an adult drone. The spermatozoa then pass through the vas deferens and collect in the vesicula seminalis. In adult drones, these sacs are filled with active sperm, while the testes become rudimentary. The vesicula seminalis opens into an accessory mucous gland, which is filled with a thick, white liquid that mixes with the sperm before they are discharged through the median ejaculatory duct and out of the endophallus (Sharma et al. 1961; Snodgrass 1956).

The honey bee spermatozoon is long and filamentous, about 250-270 μm in length, with a conical acrosomal vesicle and a perforatorium. The nucleus is strongly electron dense, measuring 5 μm in length and has an eccentric nuclear cone. The tail consists of an axoneme, two mitochondrial derivatives, and two accessory bodies. The axoneme has the typical 9 + 9 + 2 arrangement of microtubules, and the mitochondrial derivatives are of unequal diameter and length. The spermatozoon also has two accessory bodies situated between the axoneme and each mitochondrial derivative (Lino-Neto et al. 2000).

Semen collection and diluting

In honey bees, drones that have reached the age of 14 days, which have become adults, are used for semen collection. Especially afternoon, it is preferred to collect drones returning from the flight. Eversion and ejaculation are stimulated by pressure on the abdomen with the hand, and semen is seen together with the mucus layer at the end of the endophallus. While artificial insemination device is used to obtain drone semen, Harbo and Scheley syringes are used in this regard. Before collecting the semen, the syringe should be filled with semen extender to facilitate the collection of semen (Cobey et al. 2013).

The diluent used has many benefits such as preventing the drying of the semen, creating an energy source for the semen, providing the ion balance and preventing oxidative stress. Many

extenders have been used to dilute semen until today. These are isotonic NaCl, Kiev solution, Hyes solution, Harbo solution, BSS (Bee Sperm Solution),Tris buffer, TL-Hepes and modifications of these extenders have been used successfully (Alçay et al. 2019; Cobey et al. 2013; Moritz 1984; Rajamohan et al. 2020; Wegener et al. 2014). Extenders are also used to preserve semen for short and long periods. Sperm can be stored at room temperature for a short time up to two weeks without any major changes. In a study, semen was preserved for up to 439 days without freezing (Hopkins et al. 2017). In addition, although many studies have been carried out on the cryopreservation of semen, the desired success has not been achieved yet, and the preservation of the genetic source for many years has been successfully achieved (Alcay et al. 2019; Auth and Hopkins 2021; Rajamohan et al. 2020; Wegener et al. 2014).

Semen volume

Semen volume is determined by marking the glass tip and using a Gilmont micrometer. In the studies, it was determined that the volume of drone semen was related to the age of the drone, body weight, the rice of the drone and the season. In addition, it was stated that the semen volume in drones varies between 0.1 µl and 3.6 µl (Table 1).

Table 1: Sperm Volumes Obtained in Different Studies

References	Minimum Semen Volume (µl)	Maximum Semen Volume (µl)	Average Semen Volume (µl)
(Woyke 1960)	0.7	3.0	1.5
(Woyke 1962)	1.5	1.7	1.7
(Collins and Pettis 2001)	-	-	0.9
(Rhodes et al. 2011)	0.1	3.6	1.0
(Gençer and Kahya 2011)	-	-	1.0
(Czekońska et al. 2013a)	-	-	0.8
(Czekońska et al. 2013b)	0.5	1.3	0.9
(Rousseau et al. 2015)	0.4	2.4	1.0
(Czekońska et al. 2015)	-	-	1.1
(Rousseau and Giovenazzo 2016)	-	-	1.0
(Kairo et al. 2016)	-	-	0.8
(Bratu et al. 2022)	0.5	0.9	0.7
(Kahya and Gençer 2023)	0.8	1.3	1.0

Spermatozoa concentration

The determination of spermatozoa concentration has been one of the most preferred spermatological parameters in drones for many years. This parameter is especially used in the in vitro evaluation of the effects that may occur in the process of semen production and drone maturation. Hematocytometric and spectrophotometric methods are generally used for determination of spermatozoa concentration (Ciereszko et al. 2017, Rhodes et al. 2011). Spermatozoa concentration was found to be 7-12 million per microliter in drones whose seminal vesicle was dissected. In addition, in many studies where semen concentration was determined, a wide range of sperm density was obtained (Table 2). This is an indication that semen concentration can change under the influence of many factors.

In addition, spermatozoa concentration in spermatheca is among the frequently used spermatological parameters. This method is based on the dissection of the spermatheca after mating or artificial insemination and the determination of the spermatozoon concentration in the spermatheca by emptying it in a low-volume extender (Collins 2000, Moreira et al. 2022, Woyke 1971).

Table 2: Spermatozoa Concentration Obtained in Different Studies

References	Minimum Spermatozoa Concentration (million per µl)	Maximum Spermatozoa Concentration (million per µl)	Average Spermatozoa Concentration (million per µl)
(Woyke 1960)	7.23	13.62	11.95
(Woyke 1962)	-	-	7.47
(Moritz 1981)	-	-	8.5
(Rinderer et al. 1985)	-	-	5.7
(Berg and Koeniger 1990)	-	-	7
(Rinderer et al. 1999)	-	-	8.6
(Collins and Pettis 2001)	-	-	9.15
(Duay et al. 2002)	-	-	7.54
(Schlüns et al. 2003)	1.09	30.31	9.18
(Taylor et al. 2009)	-	-	8.8
(Rhodes et al. 2011)	0	19.1	3.63
(Gençer and Kahya 2011)	-	-	7.25
(Rousseau et al. 2015)	0.008	7.77	1.8
(Czekońska et al. 2015)	-	-	5.45
(Rousseau and Giovenazzo 2016)	-	-	2.7
(Kaya ve Akyol, 2021)	1.9	8.5	5.9
(Bratu et al. 2022)	-	-	9.16
(Kahya and Gençer 2023)	5.19	8.28	7

Sperm motility

The method based on scoring drone semen between 1 and 5 with a method similar to mass movement as in ram and bull semen without any dilution was suggested by Kaftanoglu and Peng (Kaftanoglu and Peng 1984). In the following years, it has found use in a subjective method of individual observation of semen by diluting it at a certain rate (Wegener et al. 2012). However, the long flagellar structure of drone semen and its relatively low progressive movement compared to mammalian semen is one of the factors limiting this method.

In addition, there are some studies showing that drone semen has an ameboid movement contrary to the expected circular movement when it is taken into a narrow canal, and this may be effective during the transition from the spermatheca to the lateral oviduct (Tofilski 2014). In addition, the use of special preparations such as Leja slide and the use of anti-aggregation agents such as Bovine Serum Albumin (BSA) were recommended in a study (Yániz et al. 2019). It has been shown that drone spermatozoa marked with fluorescent dyes can be analyzed more effectively with the help of the Computer-Assisted Sperm Analyzed Device (CASA) (Murray et al. 2022).

Abnormal sperm morphology

Morphologically abnormal spermatozoon ratio in mammalian semen is an important spermatological parameter that can be differentiated, as head, body and flagella anomalies. After diluting and immobilizing the semen with diluents such as Hancock, the abnormal spermatozoon ratio can be determined by counting them in a microscope with a 100× lens (Uysal and Bucak 2009). Abnormal spermatozoon types in drones were determined as curled, damaged and double-ended tails (Lodesani et al. 2004, Power et al. 2019). However, drone

spermatozoon has a long flagellum and it is very difficult to distinguish between the head and flagellum without cellular staining methods. For this purpose, staining techniques have been developed to determine the length of the intracellular parts with different staining procedures (Banaszewska and Andraszek 2023).

Ultrastructure of the spermatozoon

The visualization of the internal and external parts (such as acrosome, nucleus, axoneme and mitochondria) of the spermatozoon with the help of an electron microscope is called ultrastructure (Lensky et al. 1979). A few years later, differences in the spermatozoon structure of drones with haploid and diploid chromosomes were investigated (Woyke 1984). In a study, the mitochondrial apparatus in the flagellar region was determined in detail (Lino-Neto et al. 2000). In a recent study, useful information was obtained on the structural effects of freezing-thawing (Gulov and Bragina 2022).

Plasma membran integrity

This method is based on the evaluation of the permeability of the cell membrane (plasmalemma). In addition to the incubation of spermatozoa with fluorescent dyes, the morphological change of spermatozoa in a hypoosmotic solution can be determined by HOST (hypo-osmotic swelling test) method (Alçay et al. 2019). Among the mentioned staining techniques, the most common ones are SYBR-14/PI (Collins and Donoghue 1999), Hoechst/PI (Locke et al. 1990) (Figure 1), AO/PI (Yániz et al. 2020) and non-fluorescent Eosin/ Nigrozine (Peng et al. 1990). In research, plasma membrane integrity in drone semen was found to be in the range of 80-98% on average (Kaya ve Akyol 2021, Yániz et al. 2020).

Acrosome integrity

The acrosome part of the spermatozoa can be stained with the help of dyes containing fluorescently labeled lectin protein that can bind to spermatozoa acrosomal glycoproteins. In a study with Pisum sativum agglutinin (PSA) lectin fluorescence dye, spermatozoon acrosome integrity was determined (Alçay et al. 2019). However, this dye is less specific to the acrosome than the others among lectin preparations. Although we achieved successful results with the acrosome-specific Arachis hypogaea (Peanut) agglutinin (PNA) fluorescence dye in our studies, we did not find any changes in acrosomal structure in drone pure semen (Unpublished results).

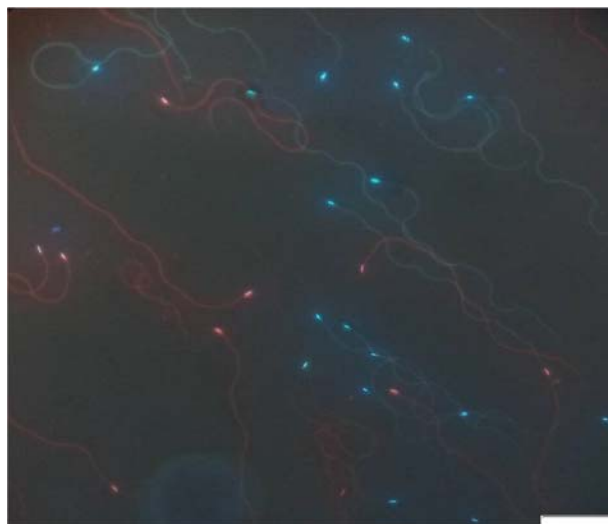

Figure 1: Assessment of plasma membrane integrity with Hoechst/PI dyes. (Red: Dead Spermatozoon, Blue: Live Spermatozoon, Scale bar: 50µm).

Mitochondrial membrane potential

Cell mitochondria have an important role in supplying the energy required to perform their glycolysis and oxidative phosphorylation functions and provide important clues about the continuity of motility. There area few studies in which the mitochondrial membrane potential was stained with Rhodamine 123 and flow cytometric analysis was performed (Alcay et al. 2021, Ciereszko et al. 2017).

In our study, 45 different samples were stained with JC-1 dye, which can more specifically detect mitochondrial membrane potential (Figure 2). According to the results, an average of 78% high mitochondrial membrane potential was determined (Kaya ve Akyol 2021). In addition, Abdelkader et al., in a study, investigated a commercial kit that showed luminescence by reacting with intracellular ATP. In the study, a difference was obtained in terms of ATP levels in drone semen produced under different conditions (Ben Abdelkader et al. 2014).

Deoxyribonucleic acid (dna) fragmentation

Detection of spermatozoa DNA fragmentation provides important information about fertilization ability. For this purpose, sperm chromatin structure assay (SCSA), the terminal transferase dUTP nick-end labeling (TUNEL) test, sperm chromatin dispersion (SCD) test and single cell gel electrophoresis assay (SCGE-COMET) tests can be applied. In a study using the SCD test, DNA fragmentation was found in the spermatozoa of

drones infested with *Nosema cerenae* (Borsuk et al. 2018). In another study, the TUNEL method was used, but no significant fragmentation was found in the samples (Wegener et al. 2014). In addition, in a study, the effect of freezing and thawing semen with different extenders on DNA fragmentation was investigated and DNA fragmentation was found to be quite low (Range 0.53-0.71%) (Alçay et al. 2022).

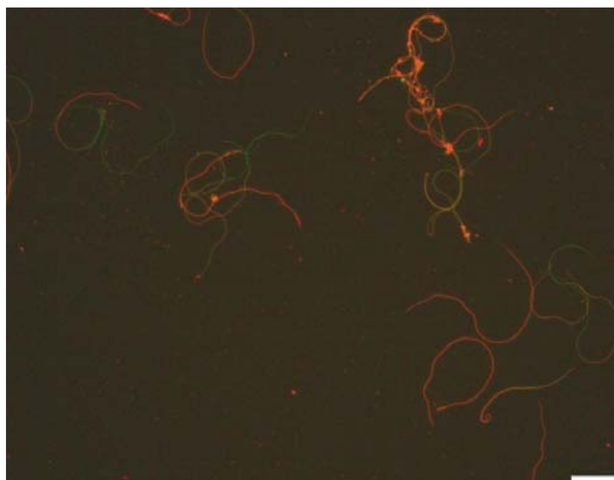

Figure 2: Assessment of mitochondrial membrane potential (Orange: High mitochondrial membrane potential, Green: Low mitochondrial membrane potential, Scale bar: 50µm).

Sperm apoptosis

Apoptosis is a mode of programmed cell death with a genetic mechanism that leads to a series of morphological and biochemical changes in the cell. The pushing of phosphatidylserine in the phospholipid structure found in the cell membrane structure to the outer part of the cell membrane, loss of mitochondrial membrane potential and DNA fragmentation are considered important signs of apoptosis. Annexin-V fluorescent staining technique, which can bind phosphatidylserine, has been used for many years in mammalian spermatozoa for the determination of spermatozoa apoptosis (Glander and Schaller 1999, Muller et al. 1999, Peña et al. 2003). Apart from this, the rate of cellular apoptosis can be determined by the Hoechst 33342 fluorescent staining method (Rowland et al. 2003, Yu et al. 2005). In a study investigating the effect of insecticides on drone semen, the rate of apoptosis was determined by the Annexin V/PI staining method, but apoptic spermatozoa were not found (Ciereszko et al. 2017).

Oxidative stress parameters

Mitochondrial functions are highly developed for the continuity of sperm motility and vital activities. During the energy cycle, reactive oxygen species whose amount increases in the environment tend to interact with the phospholipids in the cell membrane. This results in oxidative damage and causes lipid peroxidation and DNA damage in the spermatozoon. There are very few studies that determine the effectiveness of antioxidant enzymes such as catalase, glutathione peroxdase and superoxide dismutase, which are involved in the removal of reactive oxygen species by spermatozoa (Abdelkader et al. 2014, 2019). In another study, malondialdehyde level, which is one of the oxidative stress indicators, was determined (Alcay et al. 2021).

Microbial contamination of semen

During mating or artificial insemination, it is possible for the queen to receive bacterial, viral or parasitic agents from drones belonging to different colonies through semen and transmit the disease to the colony (Andere et al. 2011, Yue et al. 2006). In addition, contamination of semen with the feces of the drone during artificial insemination, sepsis and death can be seen in the queen bee if the necessary asepsis-antisepsis rules are not followed (Locke and Peng 1993, Mackensen 1951). Antibiotics added to semen extenders can adversely affect spermatological parameters (Rajamohan et al. 2020). In addition, it may not provide complete protection against viral, parasitic and fungal infections. In a study, it has been shown that Nosema spores can be transferred to the queen bee with semen (Borsuk et al. 2018). For these reasons, microbial contamination of semen is an important spermatological parameter.

Conclusion

Apart from the above-mentioned spermatological parameters, there are different parameters that are used more frequently in other species and that are likely to be applicable in drone semen. These include seminal fluid biochemicals, semen agglutination, intracellular organic and inorganic substances, spermatozoa surface proteins and spermatozoa endurance tests. In vitro parameters to be used can provide faster, cheaper and more comprehensive data than in vivo markers. Therefore, it is likely that these parameters will be developed and become widespread in future studies. Thus, it will be possible

to evaluate drone semen more effectively.

Authors' contributions: All authors contributed equally to the current manuscript. All authors read and approved the final manuscript.

Conflict of interest: The authors declare that they have no conflict of interest.

Ethics approval/declarations: Not Applicable.

Funding: Not Applicable.

REFERENCES

Abdelkader F, Kairo G, Bonnet M, Barbouche N, Belzunces LP *et a*l. Effects of Clothianidin on Antioxidant Enzyme Activities and Malondialdehyde Level in Honey Bee Drone Semen. J. Apic. Res. 2019;58 (5):740-745. http://dx.doi.org/10.1080/00218839.2019.1655182

Abdelkader F, Kairo G, Tchamitchian S, Cousin M, Senechal J *et al*. Semen Quality of Honey Bee Drones Maintained from Emergence to Sexual Maturity Under Laboratory, Semi-Field and Field Conditions. Apidologie, 2014;45(2):215-223. http://dx.doi.org/10.1007/s13592-013-0240-7.

Alcay S, Cakmak S, Cakmak I, Aktar A, Yilmaz M et al. L-Carnitine Supplemented Extenders Improve Post-Thawing Quality of Honey Bee Drone (*Apis mellifera*) Spermatozoa. Kafkas Univ. Vet. Fak. Derg. 2021;27(4):489-493. http://10.9775/kvfd.2021.25756

Alçay S, Çakmak S, Cakmak I, Aktar A, Yılmaz M et al. Honey Bee Drone (*Apis mellifera*) Sperm Cryopreservation with Rainbow Trout Seminal Plasma Supplemented Extenders. J. Hell. Vet. Medical Soc. 2022;1(73):3745-3750. http://dx.doi.org/10.12681/jhvms.25651

Alcay S, Cakmak S, Cakmak I, Mulkpinar E, Gokce E *et al*. Cryobiology Successful Cryopreservation of Honey Bee Drone Spermatozoa with Royal Jelly Supplemented Extenders. Cryobiology, 2019;87(October 2018):28-31. https://doi.org/10.1016/j.cryobiol.2019.03.005.

Alçay S, Çakmak S, Çakmak İ, Mülkpinar E, Toker MB et al. Drone Semen Cryopreservation with Protein Supplemented TL-Hepes Based Extender. Kafkas Univ. Vet. Fak. Derg. 2019;25(4):553-557. htttp://10.9775/kvfd.2018.2131.

Andere CI, Monteavaro C, Palacio MA, Catena M, Rodríguez EM et al. *Apis mellifera* Semen: Bacterial Contamination and Susceptibility to Antibiotics. Apidologie 2011;42(5):551-559. http://dx.doi.org/10.1007/s13592-011-0051-7

Auth CA, Hopkins BK. Nitrogen Vapor Immersion : An Accessible Alternative for Honey Bee (*Apis mellifera* L .) Semen Cryopreservation. Cryobiology, 2021;100(April):12-18. https://doi.org/10.1016/j.cryobiol.2021.04.006

Banaszewska D, Andraszek K. Identification of Honey Bee Sperm Structures Following The Use of Various Staining Techniques. J. Vet. Res. 2023. http://dx.doi.org/10.2478/jvetres-2023-0001

Ben Abdelkader F, Çakmak İ, Çakmak SS, Nur Z, İncebıyık E *et al*. Toxicity Assessment of Chronic Exposure to Common Insecticides and Bee Medications on Colony Development and Drones Sperm Parameters. Ecotoxicology, 2021;30(5):806-817. http://dx.doi.org/10.1007/s10646-021-02416-3

Berg S, Koeniger N. Larger Drones (*Apis mellifera*) Have More Offspring. Proc. German Zoological Society, 83rd Meeting, Frankfurt Am Main, Gustav Fischer Verlag, 1990;614

Borsuk G, Kozłowska M, Anusiewicz M, Paleolog J. Nosema ceranae Changes Semen Characteristics and Damages Sperm DNA in Honeybee Drones. Invertebr. Surviv. J. 2018;15:197-202. https://doi.org/10.25431/1824-307X/isj.v15i1.197-202

Bratu IC, Igna V, Simiz E, Dunea IB, Pătruică S. The Influence of Body Weight on Semen Parameters in *Apis mellifera* Drones. Insects 2022;13(12):1141. http://dx.doi.org/10.3390/insects13121141

Ciereszko A, Wilde J, Dietrich GJ, Siuda M, Bąk B et al. Sperm Parameters of Honeybee Drones Exposed to Imidacloprid. Apidologie 2017;48 (2):211-222 http://dx.doi.org/10.1007/s13592-016-0466-2.

Cobey SW, Tarpy DR, Woyke J. Standard Methods

for Instrumental Insemination of *Apis Mellifera* Queens. J. Apic. Res., 2013;52(4):1-54. http://dx.doi.org/10.3896/IBRA.1.52.4.09

Collins AM. Relationship Between Semen Quality and Performance of Instrumentally Inseminated Honey Bee Queens. Apidologie, 2000;31(3):421-429. http://dx.doi.org/10.1051/apido:2000132

Collins AM. Sources of Variation in The Viability of Honey Bee, *Apis mellifera* L., Semen Collected for Artificial Insemination. Invertebr. Reprod. Dev. 2004;45(3):231-237. https://doi.org/10.1080/07924259.2004.9652594

Collins AM, Donoghue AM. Viability Assessment of Honey Bee, *Apis Mellifera*, Sperm Using Dual Fluorescent Staining. Theriogenology 1999;51(8):1513-1523. http://dx.doi.org/10.1016/S0093-691X(99)00094-1

Collins AM, Pettis JS. Effect of Varroa Infestation on Semen Quality. Am. Bee J. 2001;141(8): 590

Czekońska K, Chuda-Mickiewicz B, Chorbiński P. The Effect of Brood Incubation Temperature on The Reproductive Value of Honey Bee (Apis Mellifera) Drones. J. Apic. Res, 2013a;52(2):96–105. http://dx.doi.org/10.3896/IBRA.1.52.2.19

Czekońska K, Chuda-Mickiewicz B, Chorbiński P. The Influence of Honey Bee (*Apis mellifera*) Drone Age on Volume of Semen and Viability of Spermatozoa. J. Apic. Sci, 2013b;57:61–66. http://dx.doi.org/10.2478/jas-2013-0007

Czekońska K, Chuda-Mickiewicz B, Samborski J. Quality of Honeybee Drones Reared in Colonies with Limited and Unlimited Access to Pollen. Apidologie, 2015;46(1):1-9. http://dx.doi.org/10.1007/s13592-014-0296-z

Den Boer SPA, Baer B, Boomsma JJ. Seminal Fluid Mediates Ejaculate Competition in Social Insects. Science 2010;327(5972):1506-1509. https://doi.org/10.1126/science.1184709

Duay P, De Jong D, Engels W. Decreased Flight Performance and Sperm Production in Drones of The Honey Bee (*Apis mellifera*) Slightly İnfested By Varroa Destructor Mites During Pupal Development. Genet. Mol. Res, 2002;1(3):227-232

Falencka-Jabłońska M. Bees in The City. Studia Ecologiae et Bioethicae, 2022;20(3):25–31. http://dx.doi.org/10.21697/seb.2022.20

Gençer HV, Kahya Y. Are sperm traits of drones (*Apis mellifera* L.) from laying worker colonies noteworthy? J. Apic. Sci, 2011;50(2):130-137. http://dx.doi.org/10.3896/IBRA.1.50.2.04

Ghramh HA, Iqbal A, Rafique MK, Mahmood R, Ahmed AM et al. Instrumental Insemination: A Nontraditional Technique to Produce Superior Quality Honey Bee (*Apis mellifera*) Queens. J. King Saud Univ. Sci. 2022;34(5):102077. https://doi.org/10.1016/j.jksus.2022.102077

Glander H-J, Schaller J. Binding of Annexin V to Plasma Membranes of Human Spermatozoa: A Rapid Assay for Detection of Membrane Changes After Cryostorage. MHR Basic Science of Reproductive Medicine 1999;5(2):109-115. http://dx.doi.org/10.1093/molehr/5.2.109

Gulov AN, Bragina YE. Morphofunctional Changes in Frozen-Thawed Sperm of *Apis mellifera* Linnaeus Drones. Russ. Agric. Sci. 2022;48(6):512-520. http://dx.doi.org/10.3103/S1068367422060040

Hayashi S, Satoh T. Sperm Maturation Process Occurs in The Seminal Vesicle Following Sperm Transition From Testis in Honey Bee Males. Apidologie, 2019;50(3):369-378. http://dx.doi.org/10.1007/s13592-019-00652-5

Hopkins BK, Cobey SW, Herr C, Sheppard WS. Gel-Coated Tubes Extend Above-Freezing Storage of Honey Bee (*Apis mellifera*) Semen to 439 Days with Production of Fertilised Offspring. Reprod. Fertil. Dev. 2017;29(10):1944-1949. https://doi.org/10.1071/RD16087

Johnson RM, Dahlgren L, Siegfried BD, Ellis MD. Effect of In-Hive Miticides on Drone Honey Bee Survival and Sperm Viability. J. Apic. Res. 2013;52(2):88-95. http://dx.doi.org/10.3896/IBRA.1.52.2.18

Kaftanoglu O, Peng Y-S. Preservation of Honeybee Spermatozoa in Liquid Nitrogen. J. Apic. Res. 1984;23(3):157-163. http://dx.doi.org/10.1080/00218839.1984.11100625

Kahya Y, Gençer HV. Reproductive Quality in Drones Bred From Caucasian (*A. m. caucasica*) and Italian (*A. m. ligustica*) Honey Bee Colonies. Apidologie, 2023;54(1):1. http://dx.doi.org/10.1007/s13592-022-00973-y

Kairo G, Provost B, Tchamitchian S, Ben Abdelkader F, Bonnet M *et al*. Drone Exposure to The Systemic Insecticide Fipronil Indirectly Impairs Queen Reproductive Potential. Sci. Rep. 2016;6(1):1-12. http://dx.doi.org/10.1038/srep31904

Kaya A, Akyol N. Comparative Analysis of Spermatological and Morphometric Parameters in Honey Bee Samples Collected From Some Beekeeping Located in Kırıkkale Region. MSc Thesis. Kırıkkale University, Institute of Health Science, 2021

Lensky Y, Ben-David E, Schindler H. Ultrastructure of The Spermatozoon of The Mature Drone Honeybee. J. Apic. Res. 1979;18(4):264-271. http://dx.doi.org/10.1080/00218839.1979.11099981

Lino-Neto J, Báo SN, Dolder H. Sperm Ultrastructure of The Honey Bee (*Apis mellifera*) (L)(Hymenoptera, Apidae) with Emphasis on The Nucleus-Flagellum Transition Region. Tissue Cell, 2000;32(4):322-327. http://dx.doi.org/10.1054/tice.2000.0119

Locke SJ, Peng Y. The Effects of Drone Age, Semen Storage and Contamination on Semen Quality in The Honey Bee (*Apis mellifera*). Physiol. Entomol. 1993;18(2):144-148. http://dx.doi.org/10.1111/j.1365-3032.1993.tb00461.x

Locke SJ, Peng Y, Cross NL. A Supravital Staining Technique for Honey Bee Spermatozoa. Physiol. Entomol. 1990;15(2):187-192. http://dx.doi.org/10.1111/j.1365-3032.1990.tb00506.x

Lodesani M, Balduzzi D, Galli A. Functional Characterisation of Semen in Honeybee Queen (*A m ligustica*) Spermatheca and Efficiency of The Diluted Semen Technique in Instrumental Insemination. Ital. J. Anim. Sci. 2004;3(4):385-392. https://doi.org/10.4081/ijas.2004.385

Mackensen O. Viability and Sex Determination in The Honey Bee (*Apis mellifera* L.). Genetics 1951;36(5):500. http://dx.doi.org/10.1093/genetics/36.5.500

Morais LS, Araujo Neto ER, Silva AM, Marinho DEL, Bezerra LGP et al. Sperm Characteristics of Africanized Honey Bee (*Apis mellifera* L.) Drones During Dry and Wet Seasons in The Caatinga Biome. J. Apic. Res. 2022;1-8. http://dx.doi.org/10.1080/00218839.2022.2113328

Moreira DR, de Souza THS, Galhardo D, Puentes SMD, Figueira CL et al. Imidacloprid Induces Histopathological Damage in The Midgut, Ovary, and Spermathecal Stored Spermatozoa of Queens After Chronic Colony Exposure. Environ. Toxicol. Chem. 2022;41 (7):1637-1648. http://dx.doi.org/10.1002/etc.5332

Moritz RFA. Der Einfluss der Inzucht auf die Fitness der Drohnen von *Apis mellifera carnica*. Apidologie, 1981;12(1):41-55. http://dx.doi.org/10.1051/apido:19810104

Moritz RFA. The Effect of Different Diluents on Insemination Success in The Honeybee Using Mixed Semen. J. Apic. Res. 1984;23(3):164-167. http://dx.doi.org/10.1080/00218839.1984.11100626

Muller K, Pomorski T, Muller P, Herrmann A. Stability of Transbilayer Phospholipid Asymmetry in Viable Ram Sperm Cells After Cryotreatment. J. Cell. Sci.1999;112(1):11-20. http://dx.doi.org/10.1242/jcs.112.1.11

Murray JF, van der Horst G, Allsopp M, Kotzé RCM. A New Fluorescent Method to Determine Honey Bee Sperm Motility Parameters with Computer-Aided Sperm Analysis. J. Apic. Res. 2022;62(4):944-952. http://dx.doi.org/10.1080/00218839.2022.2090729

Papa G, Maier R, Durazzo A, Lucarini M, Karabagias IK et al. The Honey Bee *Apis mellifera*: An Insect at The Interface Between Human and Ecosystem Health. Biology (Basel), 2022;11 (2):233. http://dx.doi.org/10.3390/biology11020233

Peña FJ, Johannisson A, Wallgren M, Rodríguez-Martínez H. Assessment of Fresh and Frozen–Thawed Boar Semen Using An Annexin-V Assay: A New Method of

Evaluating Sperm Membrane Integrity. Theriogenology, 2003;60(4):677-689. https://doi.org/10.1016/S0093-691X(03)00081-5

Peng Y, Locke SJ, Nasr ME, Liu TP, Montague MA. Differential Staining for Live and Dead Sperm of Honey Bees. Physiol. Entomol. 1990;15 (2):211-217. http://dx.doi.org/10.1111/j.1365-3032.1990.tb00509.x

Power K, D'Anza E, Martano M, Albarella S, Ciotola F et al. Morphological and Morphometric Analysis of The Italian Honeybee (*Apis mellifera ligustica*) Spermatozoa: A Preliminary Study in Campania Region. Vet. Med. Sci. 2019;6(2):0-61. http://dx.doi.org/10.7243/2054-3425-6-2

Rajamohan A, Danka RG, Hopkins BK, Rinehart JP. A Non-Activating Diluent to Prolong in Vitro Viability of *Apis mellifera* Spermatozoa: Effects on Cryopreservation and on Egg Fertilization. Cryobiology 2020;92(September 2019):124–129. http://dx.doi.org/10.1016/j.cryobiol.2019.11.045

Rhodes JW, Harden S, Spooner-Hart R, Anderson DL, Wheen G. Effects of Age, Season and Genetics on Semen and Sperm Production in *Apis mellifera* Drones. Apidologie, 2011;42 (1):29-38. http://dx.doi.org/10.1051/apido/2010026

Rinderer TE, Collins AM, Pesante D, Daniel R, Lancaster V et al. Comparison of Africanized and European Drones: Weights, Mucus Gland and Seminal Vesicle Weights, and Counts of Spermatozoa. Apidologie, 1985;16(4):407-412. http://dx.doi.org/10.1051/apido:19850405

Rinderer TE, De Guzman LI, Lancaster VA, Delatte GT, Stelzer JA. Varroa in The Mating Yard: I. The Effects of Varroa Jacobsoni and Apistan on Drone Honey Bees. Am. Bee J. 1999;139:134-139. 134–139

Rousseau A, Fournier V, Giovenazzo P. *Apis mellifera* (Hymenoptera: Apidae) Drone Sperm Quality in Relation to Age, Genetic Line, and Time of Breeding. Can. Entomol. 2015;147(6):702-711. http://dx.doi.org/10.4039/tce.2015.12

Rousseau A, Giovenazzo P. Optimizing Drone Fertility with Spring Nutritional Supplements to Honey Bee (Hymenoptera: Apidae) Colonies. J. Econ. Entomol. 2016;109(3):1009-1014. http://dx.doi.org/10.1093/jee/tow056

Rowland SC, Jacobson JD, Patton WC, King A, Chan PJ. Dual Fluorescence Analysis of DNA Apoptosis in Sperm. Am. J. Obstet. Gynecol. 2003;188(5):1156-1157. http://dx.doi.org/10.1067/mob.2003.295

Schlüns H, Schlüns EA, Van Praagh J, Moritz RFA. Sperm Numbers in Drone Honeybees (*Apis mellifera*) Depend on Body Size. Apidologie, 2003;34(6):577-584. http://dx.doi.org/10.1051/apido:2003051

Sharma GP, Gupta BL, Kumbkarni CG. Cytology of Spermatogenesis in The Honey Bee, *Apis indica* (F.). J. R. Microsc. Soc. 1961;79(4):337-351. http://dx.doi.org/10.1111/j.1365-2818.1961.tb05227.x

Snodgrass RE. Anatomy of The Honey Bee. Cornell University Press. 1956, p.130-134

Stürup M, Baer-Imhoof B, Nash DR, Boomsma JJ, Baer B. When Every Sperm Counts: Factors Affecting Male Fertility in The Honeybee *Apis mellifera*. Behav. Ecol. 2013;24(5):1192-1198. http://dx.doi.org/10.1093/beheco/art049

Taylor MA, Guzmán-Novoa E, Morfin N, Buhr MM. Improving Viability of Cryopreserved Honey Bee (*Apis mellifera* L.) Sperm with Selected Diluents, Cryoprotectants and Semen Dilution Ratios. Theriogenology, 2009;72(2):149-159. https://doi.org/10.1016/j.theriogenology.2009.02.012

Tofilski A. A Scientific Note on Amoeboid Movement of Honey Bee Semen. Apidologie, 2014;45:637–640. http://dx.doi.org/10.1007/s13592-014-0269-2

Uysal O, Bucak MN. The Role of Different Trehalose Concentrations and Cooling Rates in Freezing of Ram Semen. Ankara Univ. Vet. Fak. Derg. 2009;56(2):99-103. https://doi.org/10.1501/Vetfak_0000002176

Wegener J, Bienefeld K. Toxicity of Cryoprotectants to Honey Bee Semen and Queens. Theriogenology, 2012;77(3):600-607. http://dx.doi.org/10.1016/j.theriogenology.201

1.08.036

Wegener J, May T, Bienefeld K, Kamp N. New Methods and Media for The Centrifugation of Honey Bee (Hymenoptera: Apidae) Drone Semen. J. Econ. Entomol. 2014;107(1):47-53. http://dx.doi.org/10.1603/EC13159

Wegener J, May T, Kamp G, Bienefeld K. A Successful New Approach to Honeybee Semen Cryopreservation. Cryobiology 2014;69(2):236-242. http://dx.doi.org/10.1016/j.cryobiol.2014.07.011

Wegener J, May T, Knollmann U, Kamp G, Müller K, Bienefeld K. In Vivo Validation of In Vitro Quality Tests for Cryopreserved Honey Bee Semen. Cryobiology, 2012;65(2):126-131. http://dx.doi.org/10.1016/j.cryobiol.2012.04.010

Woyke J. Naturalne I Sztuczne Unasienianie Matek Pszczelich. Pszczelnicze Zeszyty Naukowe, 1960;4(3/4):183-275

Woyke J. Natural and Artificial Insemination of Queen Honeybees. Bee World, 1962;43(1):21-25. http://dx.doi.org/10.1080/0005772X.1962.11096922

Woyke J. Correlations Between The Age at Which Honeybee Brood was Grafted, Characteristics of The Resultant Queens, and Results of Insemination. J. Apic. Res. 1971;10(1):45-55. http://dx.doi.org/10.1080/00218839.1971.11099669

Woyke J. Ultrastructure of Single and Multiple Diploid Honeybee Spermatozoa. J. Apic. Res. 1984;23(3):123-135. http://dx.doi.org/10.1080/00218839.1984.11100621

Yániz JL, Silvestre MA, Santolaria P. Sperm Quality Assessment in Honey Bee Drones. Biology (Basel), 2020;9(7):1-16. http://dx.doi.org/10.3390/biology9070174

Yániz J, Palacín I, Santolaria P. Effect of Chamber Characteristics, Incubation, and Diluent on Motility of Honey Bee (*Apis mellifera*) Drone Sperm. Apidologie, 2019;50(4):472-481. http://dx.doi.org/10.1007/s13592-019-00659-y

Yu W-H, Li Z-H, Yang W-M, Li G-Z, Zhou X-M et al. Comparison Between Two Methods for Staining DNA of Apoptotic Spermatozoa. Zhonghua nan ke xue = National Journal of Andrology, 2005;11 (2):101-103

Yue C, Schröder M, Bienefeld K, Genersch E. Detection of Viral Sequences in Semen of Honeybees (*Apis mellifera*): Evidence for Vertical Transmission of Viruses Through Drones. J. Invertebr. Pathol. 2006;92 (2):105-108. http://dx.doi.org/10.1016/j.jip.2006.03.001