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Review of Sustainable Cryopreservation and Above-Freezing Storage Solutions of European Honey Bee *Apis Mellifera* Drone Semen

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***Abstract* – The European honey bee *Apis mellifera* is the main pollinator for most crops used for human consumption. However, a number of diseases, parasites, pesticides and other factors that generally result in the widely described colony collapse disorder weakens honey bee colonies. In order to maintain the existing honey bee germ lines and facilitate the creation of new disease-resistant lines, it is necessary to ensure consistent breeding work, which would also allow the long-term preservation of the unique germplasm lines. One of the most promising solutions for the preservation of honey bee germplasm is the storage of honey bee drone semen. In recent decades, there has been a renewed interest in the preservation of honey bee drone semen using both cryopreservation and above-freezing storage methods. This review summarizes the latest developments in novel sperm storage technologies and their assessment by sperm quality and queen fertility indicators. Additionally, this article analyses the advantages and disadvantages of the reviewed solutions and outlines directions in which additional research would be needed to develop effective, available and affordable drone semen storage solutions. To the best of the authors' knowledge this is the first review of cryopreservation and above-freezing storage solutions of European honey bee *Apis mellifera* drone semen in the scientific literature.**

***Keywords* – Above-freezing preservation; *Apis mellifera*; cryopreservation; honey bee drone semen; queen fertility; sperm quality.**

1. INTRODUCTION

One of the priorities of the European Green Deal is the sustainable development of agriculture. Its purpose is to stop the decline of biological diversity, to create a healthy and sustainable food production system and to reduce the risks related to food availability, which have been actualized by the Covid-19 crisis, which is related to the security of the logistics chains and worsened after the Russian invasion of Ukraine [1]–[3]. The Farm-to-Fork strategy states that food production should have a neutral or positive impact on the environment, help reduce the impact of climate change, prevent biodiversity loss and ensure mineral-rich and sustainable food available to all citizens [4]–[6]. One of the agricultural sectors that fit all the previously defined sustainability conditions is beekeeping, which is essentially a climate-neutral and residue-free agricultural sector. Climate neutrality is achieved indirectly

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by accumulating carbon in the produced beekeeping products. Plants use carbon dioxide from the air to produce carbon-rich compounds such as nectar, pollen and exudates, which honey bees use to produce honey, bee bread, propolis, beeswax and royal jelly. On the other hand, the use of wooden hives in beekeeping helps to store carbon for several decades. In 2020, approximately 19.6 million bee colonies were registered in Europe and 389 thousand tons of honey were produced [7].

The beekeeping industry also contributes to other agricultural sectors through pollination. According to Faostat [7] data, in Europe 10.6 % of the total produced plant origin production depends directly on pollinators (fruits, berries, buckwheat, rapeseed, etc. cultures) [8], [9]. It is estimated that honey bees, together with other pollinators, contribute 22 billion EUR annually to agriculture in Europe [10]. However, nowadays, due to various diseases, pesticides and climate change, bee colonies are weakened and it is more difficult to maintain them. Every year beekeepers are faced difficult to explain losses of bee colonies, this phenomenon is called colony collapse disorder (CCD), and as a result a number of pollinated areas as well as biodiversity can decrease. In the European Union, 50 % of agricultural areas dependent on pollinators already suffer from pollinator shortages [11].

The only sustainable solution for preserving bee colonies, restoring genetic material and ensuring genetic diversity is consistent breeding work by developing disease resistance and other economically important characteristics. In addition, the honey bee breeding work has its cornerstones. Queens usually live 3 to 4 years [12], in some cases even longer. Usually, the conclusions about the characteristics of a queen's bee colony can be made in the third year of the queen's life. It is because in the first year, the queen is purchased or reared and overwinters with her own worker bees and/or non-related worker bees. In the second year, data are obtained on the performance of the bee colony over the entire season [13]. If the assessed bee colony has an excellent performance, then in the third year a decision must be made to preserve the genetic material by raising the next generation of queens, whose life span will also be 3 years on average. The genetic material of the superior bee colony can be directly obtained from queens of the 1st generation (F1) by rearing drones (male honey bees), since they develop from unfertilized eggs and transmit only the genetic information of the grandmother's bee colony[14]. After drone rearing, controlled insemination of the queens can be carried out by setting up an isolated mating station or using instrumental insemination. When performing instrumental insemination, the age of the reproductive material must be taken into account, which significantly affects the quality of insemination. Respectively, virgin queen bees must be 5–14 days old, while drones must be 2–3 weeks old since birth [12], [15], [16]. Using older drones reduces the quality of sperm: less sperm enters the spermatheca (sperm reservoir) of the queen and increases the risk that the queen may die from the accumulation of sperm residues in the oviducts [12]. Due to the seasonal nature of beekeeping, the weather conditions and the specific mating mechanism, in order to ensure the preservation of the genetic material, it is necessary to evaluate the bee colonies, rear the reproductive material (drone rearing takes 40 days) and perform insemination in a relatively short period of time. Sometimes these tasks even overlap and must be done simultaneously. In addition, there is a risk of losing queens for various reasons, inbreeding can occur due to a lack of new genetic material, and the performance of new crosses may be drastically different than expected.

In order to preserve the valuable genetic material of honey bees and to carry out instrumental insemination of queen throughout the entire beekeeping season, it is necessary to implement long-term honey bee drone semen storage solutions in beekeeping industry. Research on the long-term storage of drone semen began in the 1970s [17]. Development of these methods became more rapid in the last 20 years, studying the reduction of the toxicity

of chemical reagents, various improvements of the cryopreservation process and analysing sperm quality and the fertility of queens. In 2021, the idea of creating Europe's first gene bank specifically for the preservation of the honey bee gene pool was also widely considered [18]. This topic is becoming more and more relevant every year. Therefore, the purpose of this review article is to provide an overview of the progress of research published in the last 20 years related to long-term storage solutions of honey bee drone semen, as well as to outline future trends. This article is written as a guide for anyone interested, and is intended to provide an overview of the development of storage methods, state-of-the-art techniques, their results, and to highlight recurring crucial factors that need to be taken into account when using current or developing novel storage methods.

The table (see Table 1 and extended table in the Annex A) summarizes information on all publications that have investigated long-term drone semen storage solutions. The Table 1 summarizes the purpose and objects of researches, but extended table in Annex A summarizes the following information: research design, the purpose of the research and description of experimental tests. Depending on the object of study, number, nature and results of the tests, some of the reported tests were not included in the table, e.g., if particular test resulted in low or no survival of drone semen then it was decided to not include it in the table. The extended table (see Annex A) also lists the factors that showed the best or the most promising results within each relevant study, such as: diluents, cryoprotectants, used storage packaging, sample cooling, freezing and thawing methods, sample storage durations, additional actions after thawing, determined sperm quality and queen fertility indicators and their results. At the beginning of the article, the importance of sperm quality and queen fertility indicators in the evaluation of the long-term storage methods is discussed. Additionally, the standard methods for determining quality indicators are presented. Afterwards, the main steps directly related to the storage itself were analysed: the used diluents, cryoprotectants and dilution rates for preparing samples of semen; storage temperature regimes, methods and tested durations of storage.

The extended table (Annex A) is available as electronic supplementary file.

To the best of the authors' knowledge this is the first review of cryopreservation and non-freezing storage solutions of European honey bee *Apis mellifera* drone semen in the scientific literature.

2. Assessment of the Long-Term Storage Methods – Sperm Quality and Queen Fertility Indicators

The honey bee queen is the only individual in the bee colony that lays fertilized eggs and ensures the progeny in the bee colony. Accordingly, female organisms or worker bees develop from fertilized eggs and male organisms or drones develop from unfertilized eggs. Worker bees perform the functions of nursing, cleaning and building cells, foraging, protecting the nest, and if necessary, they rear a new queen from a fertilized egg to replace the existing one. The lifespan of worker bees varies from 21 days in the active season to 6 months in the wintering period. The only task of the drones is to successfully mate with new queens and pass their genes on to future generations in other bee colonies. The drones of bee colony are reared and fed for only a few months, during the seasonal period of active mating of virgin queens. During this period the proportion of drones in a bee colony is usually 5 %, which can change depending on the age of the queen or the quantity and quality of sperm in the queen's spermatheca [14]. In order to maintain the functionality of the bee colony, the fertility indicators of the queen play an important role. Fertility indicators are the proportion of

fertilized eggs (brood) from the total number of eggs, the quality of laying and the duration of laying. All of these indicators are directly affected by the quality of the drone's sperm.

2.1. The Number of Sperm Cells in the Queen's Spermatheca

At the age of 5–14 days, the queen goes on 'wedding' flights to mate with several drones and store semen for the rest of her life for several years. The lifespan of the queen is directly dependent on the number of sperm cells in the spermatheca. If the queen begins to lay more unfertilized eggs that develop into drones, the existing worker bees perform queen replacement to maintain the colony's viability [12]. In naturally mated queens an average of 4 to 5 million sperm cells (may differ from 2 to 7 million) enter the spermatheca. On the other hand, an instrumentally inseminated queen can be considered of a good quality if at least 3 million sperm cells have entered the spermatheca [19], which would be a sufficient amount for the working life of the queen. If the number of cells in the spermatheca is less than 0.5 million cells, there is a risk that the queen will start laying only unfertilized eggs [20].

To determine the number of sperm cells in the spermatheca, queens are dissected no earlier than 48 h after instrumental insemination. This is mainly because during the first 40 h after insemination, drone sperm migrates from the oviducts to spermatheca [15]. To determine the number of sperm cells the spermatheca is usually ruptured and the sperm is diluted with a solvent, e.g. potassium ion buffer solution (Kiev solvent) [21] or other solutions of salts, nutrients, antibiotics intended for diluting sperm (see section 3.2.) [22]. Cell counting is carried out with a microscope at 400× magnification and a hemocytometer, which is filled with a sperm sample. Cells are counted and by taking into account the dilution of the sample, the number of cells in the spermatheca is calculated [19], [22].

The number of sperm cells in the spermatheca of the queen is an important indicator of the quality of the drone sperm (including insemination), on which the lifespan and performance of the queen depends, so it is worth using it as one of the parameters characterizing sperm quality.

2.2. Sperm Motility

Sperm needs to move in order to reach the queen's spermatheca and then later successfully fertilize the eggs, therefore sperm motility is an important indicator of sperm quality. Motility is one of the most frequently tested indicators of sperm quality in research. Motility is believed to provide more accurate conclusions of sperm performance than sperm viability assessment [19] (see section 2.3). The sperm of honey bee drones are characterized by both circular movements and sudden vibrations. Determination of motility is carried out in the same way as counting sperm cells. The semen sample can be diluted with either a buffer solution containing potassium ions [21] or with another semen solvent containing 0.05 g of glucose per 100 mL in a 1:1 ratio [23]. After that, the sample is carried on a glass slide and under a microscope at 400× magnification motility is assessed. Motility is expressed as a percentage or in points from 0–5 that indicate how much of the total number of sperm cells in the sample make circular movements and vibrate [17], [22]–[26]. Motility is usually determined both in semen samples before their use for instrumental insemination of queens, and after insemination of queens by determining motility in the spermatheca.

Sperm motility is also an important indicator of drone sperm quality that should be included in studies to give an idea of sperm functionality. It is very valuable to determine the motility just before the insemination of the queens, in order to avoid wasting a lot of work and time resources that are usually devoted to the insemination and monitoring of the queens' post-insemination performance.

2.3. Sperm viability (plasma membrane integrity)

The most commonly determined indicator of sperm quality is sperm viability. Collins [27], [28] using semen samples mixed in different proportions of fresh and dead sperm cells for insemination of queens, determined that at least 46 % of sperm cells in the semen samples should be alive in order to ensure that queen lays fertilized eggs for at least one season after instrumental insemination. The viability of fresh drone sperm can vary dramatically from 55 % to 99 %. When the sperm enters the spermatheca of the queen, its viability decreases by 10 %. It should be noted that viability indicators may change depending on the age of the drones, their feeding base, environmental factors, etc. The viability of the sperm in the spermatheca of the queen can also change, on average it is 80–98 %, but it can decrease as the queen's age increases [19].

Viability is determined by the permeability of the outer shell of sperm cells – the plasma membrane. If sperm cells are damaged, the permeability of cell membranes is greater than that of healthy cells. Damaged sperm cells are stained with a fluorochrome-propidium iodine (PI), which passes through damaged cell membranes, reaches the cell nucleus and stains the DNA in a red fluorescent colour. In order to stain living cells, additional staining substances can be used, e.g. SYBR-14 Hoechst 33342 or acridine orange, which can cross the membranes of healthy cells and emit green (SYBR-14 acridine orange) or blue (Hoechst 33342) fluorescent light in contact with living cells' DNA. After staining under a fluorescence microscope healthy and damaged cells are counted in the sample and their proportion is expressed as a percentage [20]–[22], [29], [30]. Auth & Hopkins [23] describes the sample preparation for this method in further detail.

However, determining sperm viability alone can give misleading information about sperm quality. For example, increasing the length of semen storage should decrease sperm viability rates, but in some studies, [20], [31], [32], they increase. Hopkins *et al.* [32] already mentioned a possible reason for obtaining inaccurate indicators. During storage, lysed cells are produced, i.e. old cells are purified or self-degraded, as a result of which proteins and DNA, which can be stained green, are extracted (separated). Due to the self-degradation of cells, in addition to the viability indicator, it is also necessary to determine the number of cells in the sample before and after storage [32]. Even if a true viability reading is obtained in the sample, there is no guarantee that the living cells are also functioning (motile) cells.

2.4. Other Indicators of Sperm Functionality – Acrosome Integrity, Mitochondrial Function, DNA Fragmentation, Malondialdehyde (MDA) Concentration

In relation to sperm cell functionality, other indicators such as acrosome integrity, mitochondrial function, DNA fragmentation and MDA concentration are also determined. The acrosome is the part of the head of the sperm cell, which, upon contact with the outer membrane of the egg (the vitelline membrane, which is accessed through the micropyle – the opening of the egg membrane), releases lytic enzymes so that the nucleus of the sperm cell enters the egg and successful fertilization occurs [19]. Acrosome integrity is determined by fluorescently labelled lectins (plant proteins) that bind to acrosome glycoproteins. PSA *(Pisum sativum agglutinin)* is used as a staining agent in honey bee studies, which is used for fixed dead sperm. Fully fluorescent cells are considered intact, while less fluorescent or speckled cells are considered damaged. Incorrect results can be obtained due to the presence of egg yolk in the semen sample if it has been used for semen storage [19], [25], [26].

Mitochondria are the energy provider for cells by utilizing ATP. Mitochondria are known to provide functionality and cellular longevity to mammalian sperm. Mitochondrial functionality is determined by fluorescent staining (PI, Rhodamine R123) followed by

determining the percentage of stained cells [25], [26]. The method has a low sensitivity because the staining depends on the number of active mitochondria [19].

DNA is the carrier of genes. Due to the concern that the long-term storage of semen is exposing it to chemical and physical processing that could damage cellular DNA, some studies [21], [25], [26] determined the proportion of DNA fragmentation in the sample, but did not observe statistically significant differences between fresh sperm and treated sperm. Various assays are available to determine DNA fragmentation: sperm chromatin structure assay (SCSA), terminal transferase dUTP nick-end labeling (TUNEL) assay, and sperm chromatin dispersion (SCD) assay [19].

Alcay *et al.* [25], [26] also examined the concentration of malondialdehyde in semen samples, which is defined as an indicator of oxidative cellular stress, but also did not observe statistically significant differences between the samples when testing this factor.

2.5. The Prediction of Sperm Quality Indicators: the Validation of in vivo and in vitro Quality Indicators

There are a relatively large number of tests that can be performed to determine different qualities of drone sperm. If there is a lot of time resources, all existing tests can be performed on a stored semen sample at any time in laboratory conditions. However, fertility results and sperm quality indicators after insemination of queens are much more important, as they directly indicate whether it will be possible to restore the genetic material by rearing new queens. The main purpose of long-term semen storage is to obtain fertile offspring and ensure the renewal of the genetic pool. In order to reach the fertility determination stage, additional human resources are needed for a relatively short, fixed period within the beekeeping season. Additional tasks include queen rearing, forming nuclei (small bee colony units), inseminating queens and monitoring nuclei. On the other hand, when determining sperm quality indicators after insemination of queens, dissecting the queen and obtaining semen from the spermatheca (sphere with a diameter of 1 mm) [15], another limiting factor appears - the amount of obtainable semen sample. The volume of the spermatheca is 0.52 microliters. This means that the most important sperm quality tests must be carefully selected, due to the limited volume of the available semen sample.

Research of the long-term storage of honey bee drone semen involves extensive *in vitro* testing, that is, results are obtained under laboratory conditions without the use of living organisms [17], [23]–[26], [29], [31], [33]. However, *in vivo* testing needs to be done as well, that is, the quality of the sperm needs to be tested using living organisms, i.e., queens are instrumentally inseminated and semen from spermatheca is collected and assessed. Due to labour intensive, time consuming, seasonal and resource limiting nature of *in vivo* testing, it is performed much less frequently [20]–[22], [30], [32], [34], [35]. *In vivo* testing is also much more difficult to perform than *in vitro* testing. In addition, *in vivo* testing is impractical, because resources are invested in rearing and inseminating queens, but shortly after that, the queens are dissected, without waiting for them to start laying and without obtaining a significant fertility indicator – the proportion of worker bee brood.

In order to facilitate the research process related to the performance of *in vivo* tests and to be able to predict sperm quality only with *in vitro* data, it is necessary to validate the data obtained from *in vitro* and *in vivo* testing or look for correlations of results that would ensure reliable prediction of fertility results. The search for such correlations was initiated by Wegener *et al.* [35], examining the relationships between *in vivo* results (proportion of brood of worker bees, number of sperm cells in the spermatheca, egg hatching rate, queen laying initiation rate) and *in vitro* results (circular cell movement, motility, viability, osmotic stress test, pH stress test, presence of enzymes from cytosol and mitochondria). Although the study

failed to test the correlation of fertility (proportion of broods of worker bees) with *in vitro* data, a very strong correlation with all *in vivo* indicators was found specifically for sperm motility. Thus, motility is a very important indicator to include as a factor in determining drone sperm quality [35]. On the other hand, Hopkins *et al.* [32] found that there is no direct correlation between the proportion of live cells in a semen sample and the proportion of fertilized eggs. Until now, the relationships between many qualitative indicators, for example, between the proportion of fertilized eggs, sperm motility in the spermatheca, the concentration of sperm cells in the sperm sample before insemination, etc., have not been tested. Qualitative indicators of sperm quality and fertility are a measure of the effectiveness of long-term storage methods of drone semen, so additional research is needed in finding correlation between *in vivo* and *in vitro* data.

3. METHODS OF LONG-TERM STORAGE OF HONEY BEE DRONE SEMEN

In connection with the long-term storage solutions of honey bee drone semen, in the last 20 years there has been a rapid progress in the improvement of methods, and also over time the focus has changed on different stages of the storage process. In this chapter, we will provide insight into each of the stages of long-term storage methods: drone rearing, collection of semen, sample dilution and preparation for freezing, temperature regimes and durations of cooling, freezing and thawing, and duration of storage. Additionally, the experimental designs are summarized in Table 1 (see extended Table 1 in the Annex A) in order to give an overview of the choices made by researchers at each stage of storage procedures, and also the main objectives and the obtained results are listed accordingly.

TABLE 1. SUMMARY OF DRONE SEMEN STORAGE SOLUTIONS STUDIES (SEE EXTENDED TABLE IN ANNEX A)

Ref.	Object of research
[31]	Determination of sperm viability limits at above-freezing temperatures: 9 storage durations (1, 2, 3, 6, 9, 12, 26, 39, 52 weeks); 2 storage temperatures (+12 °C, +25 °C).
[17]	Improvement of cryopreserved sperm post-thaw viability by changing diluents, cryoprotectants (CPA) and dilution ratios: 6 diluents; 3 cryoprotectants (DMSO, DMA, glycerol); 5 dilution ratios (1:1, 1:3, 1:6, 1:9, 1:12); 3 honey bee strains (*Buckfast, Rogers, Szabo*).
[29]	Improvement of cryopreservation process by examination of diluents, cryoprotectants, temperature sensitivity, freezing rate and cold shock: 5 diluents combined with 3 cryoprotectants (DMSO, glycerol, ethylene glycol); 2 cooling methods (slow, rapid); 2 freezing methods (programmable, vitrification); 2 thawing methods (in water bath, hot air); 2 storage durations (6 or 343 days).
[34]	Obtaining second-generation queens using frozen semen and back-crossing, Testing of egg yolk-free diluent for cryopreservation: 2 diluents with DMSO; 2 generations of queens.
[30]	Toxicity of cryoprotectants *in vitro* testing: 5 cryoprotectants (DMSO, N,N-dimethyl formamide, ethylene glycol, 1,3-propane diol, 2,3-butane diol); 2 final concentrations of cryoprotectants (5 %, 20 %).
[30]	Toxicity of cryoprotectants *in vivo* testing: 3 cryoprotectants (DMSO, ethylene glycol (EthGly), 1,3-propane diol); 3 final concentrations of cryoprotectants (5 %, 10 %, 20 %).

[30]	Toxicity of cryoprotectants *in vivo* testing by using single or combinations of cryoprotectants: 2 cryoprotectants (*DMSO, ethylene glycol* (*EthGly*)).
[21]	Addition and removal of cryoprotectant DMSO: 2 storage duration (2 days, 9 months); 2 dialysis #2 step cryoprotectant concentrations (13.5 % DMSO, 21 % DMSO); 2 washing methods (stirring, centrifugation).
[32]	Comparison of sperm viability and fertility using different above-freezing storage temperatures and antibiotic addition methods: 2 storage above-freezing temperatures (+4 °C, +14 °C); 3 storage durations (45, 99 or 439 days); 2 methods of antibiotics introduction (by diluent or coating capillaries).
[33]	Improvement of sperm quality by using egg yolk or lecithin containing diluents and a modified cryopreservation protocol: 3 diluents (20 % egg yolk, 0.5 % or 2 % soybean lecithin); 1 new freezing method (LN_2 vapor).
[22]	Evaluation of different diluents added for post-thaw sample washing and centrifugation with purpose to remove cryoprotectant for better sperm quality and fertility: 4 diluents for sample washing (glucose, ram semen plasma, drone semen plasma, salt solution).
[20]	Comparison of cryopreservation and above-freezing methods on sperm quality: 3 storage durations (90, 180 or 330 days); 2 storage temperatures (+16 °C, −196 °C).
[24]	The effect of using different concentrations of royal jelly in the solution on sperm quality in post-thaw samples: 4 concentrations of royal jelly (1 %, 2 %, 4 % or 8 %).
[26]	The effect of using different concentrations of rainbow trout seminal plasma in the solution on sperm quality in post-thaw samples: 3 concentrations of rainbow trout seminal plasma (2.5 %, 5 % or 10 %).
[25]	The effect of using different concentrations of L-carnitine in the solution on sperm quality in post-thaw samples: 3 concentrations of L-carnitine (2.5 mM, 5 mM or 10 mM).
[23]	Testing the efficacy of different freezing techniques, type of freezing packages, cryoprotectant concentrations and addition methods on post-thaw sperm quality: 3 freezing methods (vitrification, freezing with LN_2 vapor, programmable freezing); 2 type of packaging (CryoLock Vitrification device, microdialysis tube); 3 concentrations of cryoprotectant (10 %, 20 %, 30 %); 2 methods of cryoprotectant addition (with dilution, with dialysis).

3.1. Sexual Maturity of the Drones

The purpose of rearing drones is to procure sexually mature drones of known origin, from which semen is obtained for storage and later, if necessary, used for the insemination process. From the start of drone rearing, i.e., since drone brood is laid in the combs, it takes an average of 40 days for drones to reach sexual maturity [13]. It is easier to record the date when the drone eggs were laid in the comb than to record the date of drone birth. Nevertheless, since both counting methods are used, both will be mentioned here as well. Drones are born on the 24^{th} day after the egg is laid in the drone cell. They reach sexual maturity in day 36^{th}–38^{th} after eggs are laid (or 12–14 days after birth) [15], [16]. It is only possible to collect semen from drones that have reached sexual maturity. In young drones, semen is yellowish-cream, liquid and can be easily collected in glass capillaries for instrumental insemination of queens. As the drones get older, the semen become thick and takes on an orange-brown colour, making it much more difficult to collect. For the instrumental insemination it is not recommended to use drones after day 45th after egg is laid (or longer than 21^{st} day after birth), because due to the thickness of the semen, the accumulation of semen residues in the oviducts of the queen increases [16], which can also cause premature death of the queen. To ensure good quality sperm for long-term storage, it is necessary to collect it within period of one week, i.e., from 38^{th} to 45^{th} day since the drone egg was laid (14–21 days since birth). This short period of

time to collect quality sperm is one of the main reasons why there is a need for long-term semen storage solution, so that it can be used in a targeted manner later when needed, rather than rushing the insemination within this period of one week.

3.2. Collection and Dilution of Drone Semen

Depending on the methods of drone rearing and maintenance, drones can be collected manually in cages or on the previous day in the afternoon by installing a special cage on the entrance of the hive. Once inside the cage drones cannot get out, but worker bees can access them to ensure they are being fed. Before using drones to collect semen, they should be allowed to fly for at least 10 minutes to defecate. Flying can be carried out in a specially prepared box (40×40×40 cm). Defecation will reduce the risk of contaminating the semen during the collection process. Matured drones are stimulated by applying physical pressure on the drone's body, using the thumb and forefinger to gradually transfer pressure from the drone's chest to the abdomen. With such a technique, the drone takes out the copulatory organ on which the semen is ejaculated. The process of drone stimulation and semen collection is described in more detail by Cobey *et al.* [15].

Semen is collected from several drones with glass capillaries, most often using a Harbo [17], [20], [34], [36] or Schley [22], [24], [30] syringe. If semen is used for insemination of queens on the same day, usually a simple semen solvent (0.9 % NaCl and antibiotics) is used during the collection process [15], while more complicated solutions are used for longer storage. In the reviewed studies, the composition of solvents for long-term storage is significantly different in terms of type and quantity of ingredients. In order to maintain the functionality of sperm cells in the long term and to avoid cellular oxidative stress, to prevent risks with bacterial contamination of the semen sample, the following ingredients are used in solutions: various potassium and sodium-containing salts (KCl, NaCl, $NaHCO_3$, NaH_2PO_4, Na_2HPO_4, sodium citrate), nutrients (glucose, sucrose, trehalose, chicken egg yolk), amino acids (L-glutamic acid, L-lysine, L-arginine, glycine, proline), antibiotics (penicillin, dihydrostreptomycin sulfate, streptomycin, amoxicillin, kanamycin, tylosin, gentamicin), enzymes (catalase) etc. [17], [20]–[26], [29]–[34]. In addition, to protect sperm cells from the formation of ice crystals in the cells during the freezing process, cryoprotectants or substances that provide this protective function are added.

The choice of solvent composition is one of the initial objects of research [17], [29]. But the most frequently used solvent in studies of honey bee semen freezing, with good sperm quality indicators [20], [22], [23], [29], [30], [34] is Harbo's solution [37]. Harbo's solution is consisting of 500 microliters of buffer solution, 250 microliters of fresh hen egg yolk, and 250 microliters of the cryoprotectant DMSO (dimethyl sulfoxide). The buffer solution is prepared from 239 mg NaH_2PO_4 and 112 mg Na_2HPO_4 in 25 mL H_2O. The pH of the Harbo's solution is adjusted to 7.2 using a 6 M NaOH. Usually, semen is diluted 3:2 with Harbo's solution, resulting in 10 % DMSO concentration in the final sample.

Various options have been tested regarding the choice of semen solvents and cryoprotectants, concentrations and semen dilution ratios. If the semen is stored without freezing, various combinations of potassium and sodium salts, amino acids, antibiotics, monosaccharides, enzymes, etc. containing solutions are used to which the pH is adjusted to 7.2–8.5 and the semen is diluted 1:1 [20], [31], [36]. If the semen is planned to be frozen, then the semen is diluted either 1:1 [20], [22], [34] or 3:2 [23], [29] or 3:2 [23], [29]. Taylor *et al.* [17] examined several semen-solvent dilution ratios (1:1, 1:3, 1:6, 1:9, 1:12) and assessed their effect on sperm quality after freezing, concluding that greater viability of the sperm can be achieved at higher dilution ratios. However, too high dilution ratio makes the storage of the sample inefficient, i.e., the sample has a lower concentration of sperm cells,

the use of such semen sample for insemination requires centrifugation, which can damage the sperm, and the storage of highly diluted sample requires a larger container capacity.

Long-term storage of fresh semen without freezing or diluting it with a solvent is also possible, e.g. by collecting fresh drone semen in glass capillaries previously coated with an antibiotic and antifungal gelatine solution [32].

3.3. Cryoprotectants for semen freezing

An important component for the freezing solution is a cryoprotectant, which protects sperm cells from the formation of ice crystals in the cells during the freezing process. Several cryoprotectants have been tested in research: dimethyl sulfoxide (DMSO) [17], [20]–[26], [29], [30], [34], glycerol [17], [29], dimethylacetamide (DMA) [17], ethylene glycol [29], [30], 1,3-propanediol, N.N-dimethylformamide, 2,3-butanediol [30]. The main concern about cryoprotectants is their toxicity or negative effect on sperm cells and the queens. Correlation has been observed in that increasing the concentration of cryoprotectant in samples decreases the motility and viability of sperm cells [30]. On the other hand, if the semen sample is frozen quickly, higher concentration of cryoprotectant is needed in the samples to protect the sperm cells from cold shock or to prevent them from dying due to rapid freezing [23]. The type of cryoprotectant and its concentration also affects the viability and motility of the sample [17], [29], [30]. Currently, DMSO is the most optimal cryoprotectant with the relatively miniscule negative effects on sperm cells [17], [20]–[26], [29], [30], [33]–[35]. Several studies have also tested different concentrations of cryoprotectants (from 0 %–30 %), clearly showing that the sperm cells in the samples are best protected from cold shock during the freezing process, if the concentration of the cryoprotectant in the sample is 10 % [23], [29], [30], [35].

The negative effect of cryoprotectants on sperm cells is assessed by determining the motility and viability of sperm cells in the sample after its treatment. Based on the studies done by Collins *et al.* [27], [28], it is assumed that a sperm sample should have at least 46 % viable sperm cells in order for the sample to be worth using for insemination of queens and to be able to obtain fertilized eggs. Wegener & Bienefeld [30] observed that a 15-minute treatment at room temperature, regardless of the type and concentration of cryoprotectant (5 % or 20 %), had no statistically significant effect on sperm cell viability in samples compared to fresh, untreated sperm. After 1 h of treatment, significant changes in sperm viability and motility results begin to appear, depending on the choice of cryoprotectant [29]. For example, using 10 % DMSO, approximately 95 % of the cells in the samples were alive and motile after one hour, while using 8 % glycerol, 35 % of the cells remained viable and no movement was observed [29]. Taylor *et al.* [17] tested the effect of a cryoprotectant on sperm cells by treating semen samples with 10 % DMSO, DMA or glycerol, and freezing and thawing the samples. These tests resulted in significantly higher sperm viability in samples with DMSO (45.5 %) than with DMA or glycerol (15.3 % and 15.5 %). In order to reduce the effect of one type of cryoprotectant on semen samples, it has been attempted to combine two different cryoprotectants (DMSO, ethylene glycol), while not exceeding their total concentration of 10 % in the final sample. As a result, it is possible to obtain higher sperm viability indicators in the sample containing two cryoprotectants than in cases where only one cryoprotectant was used [30]. For example, queens were inseminated with unfrozen semen samples treated with one or two cryoprotectants. After spermatheca testing, semen samples containing only ethylene glycol had statistically significantly lower sperm viability (approximately 30 %) than samples containing combination of ethylene glycol and DMSO (viability approximately 80 %). The number of sperm cells in the spermatheca was also significantly higher when the semen samples were treated with two cryoprotectants. However,

no statistically significant differences were observed between the cryoprotectant combinations and 10 % DMSO [30].

It should be noted that this result of combining cryoprotectants was obtained without freezing the semen samples, in order to rule out the effect of cold shock on sperm viability. But to understand whether combining cryoprotectants better protects cells from cold shock than using a single cryoprotectant it would be necessary to evaluate the results also after freezing and thawing of the samples. Over time, scientific research related to the use of cryoprotectants in semen freezing has revealed that there are still no equivalent or better alternatives to the use of 10 % DMSO in the freezing process. 10 % DMSO best fulfills the main role of a cryoprotectant - it protects sperm cells from cold shock and the formation of ice crystals in cells, which causes cell death. However, long-term exposure to cryoprotectant negatively affect sperm cells and queen's organism. Therefore, after freezing, storing and thawing of the sample, cryoprotectant needs to be removed as much as possible, before it can be used to inseminate the queens. Several approaches have been explored to reduce the negative effects of cryoprotectant.

3.4. Methods to Reduce the Negative Effects of Cryoprotectant on Sperm and Queens

An innovation to reduce the negative effects of cryoprotectant on sperm cells is the application of a dialysis process to add the cryoprotectant to the semen sample, as well as to remove it after the sample is frozen and thawed. In a wide variety of experiments, Wegener *et al.* [21] tested the efficiency of the dialysis process and Auth & Hopkins [23] successfully repeated it. The process of dialysis in the processing of drone semen manifests itself as follows: a semen sample without a cryoprotectant is placed in a special dialysis tube in a cryoprotectant hypertonic (higher concentration) solution. Due to the different concentration of the cryoprotectant, the cryoprotectant molecules create pressure on a permeable membrane (osmotic pressure) and move through it to the semen samples where the cryoprotectant concentration is lower. It continues until the cryoprotectant concentration in the semen sample and its external environment is the same. The goal of the dialysis process is to gradually add a cryoprotectant to the sample to reduce the osmotic stress on the sperm cells membranes. Osmotic stress in sperm is related to the pressure exerted by water molecules on the cell membrane when the concentration of water outside and inside the cell is different [38]. If a cryoprotectant of high concentration (contains less water than the cell) is added rapidly to the semen sample, the cell will lose water inside the cell much faster and shrink. If distilled water is added to the semen sample, which has a significantly lower concentration of salts than inside the cell, the cell will absorb water and swell. Such rapid changes in osmotic pressure on cell membranes can cause cell death.

Wegener *et al.* [21] added the cryoprotectant to the semen sample via dialysis, by immersing the semen sample in a 21 % (v/v) DMSO/ BSS (bee sperm solution) solution for 32 min at +22.5 °C, thus obtaining the 10.5 % DMSO concentration required for freezing the semen sample. After freezing, thawing and insemination of the queens, it resulted in high sperm motility and viability (85–90 %) and high corresponding proportion of worker bee brood (fertilized eggs) when the queens started laying after insemination. If the semen were frozen in small portions for 48 h, it resulted in 47.5 % fertilized eggs. Semen frozen in one portion for several queens for 48 h resulted in 79.4 % fertilized eggs. And semen frozen and stored for 9 months resulted in 59.4 % fertilized eggs [21]. Auth & Hopkins [23] added cryoprotectant via dialysis by placing the semen sample in 2 baths of different DMSO concentrations for 15 minutes each at +20 °C, starting with a lower concentration, i.e. 10 %, 20 % or 30 %, and then placing the sample in baths containing double the DMSO concentrations, i.e. 20 %, 40 % or 60 %. Higher rates of sperm motility and viability were

found in all samples where dialysis was used [23]. Thus, the use of dialysis to reduce the osmotic shock to sperm cells helps to maintain higher viability and motility in semen samples after freezing and thawing than samples where dialysis was not used. The promising results of these studies point to the need to consider the inclusion of the dialysis process in future semen freezing and storage research protocols.

In order to prevent the long-term negative effect of cryoprotectant exposure on the queens and on the sperm in the spermatheca, various available methods have been tested to remove the cryoprotectant after freezing and thawing the semen sample. Cryoprotectant removal methods before insemination the queens are the following: sample washing, centrifugation and dialysis process. Wegener *et al.* [21] diluted the sample with BSS (bee sperm solutions) after thawing and then centrifuged it at 1300 RCF for 12 min to wash out the cryoprotectant and reconcentrate the semen. The use of centrifugation resulted in reduced sperm cell motility (65 %) in the spermatheca, which was also reflected in lower queen fertility (proportion of worker bee brood was only 27 %). Simply diluting and lightly mixing the sample by hand, resulted in higher sperm cell motility (80 %) and proportion of worker bee brood – 65.8 %. Gul *et al.* [22] washed and centrifuged the semen sample (2 min 2068 RCF) after thawing not only with semen diluent, but also with glucose solution, ram semen plasma and drone semen plasma. But statistically significantly better result for queen fertility – proportion of worker bee brood, was not obtained by washing the sample with either solution compared to a frozen semen sample control. After washing the sample with drone semen plasma, centrifuging and inseminating the queens, they laid 48 % fertilized eggs, while 47 % fertilized eggs were observed for the control. It should be mentioned that the number of sperm cells entering the sperm reservoirs of queens in the centrifuged samples was less than half than in the control [22]. In a study by Paillard *et al.* [20], using centrifugation for 10 min at 1000 RCF, there were no statistically significant differences in queen fertility, sperm cell count and viability in spermatheca compared to non-centrifuged samples. Auth & Hopkins [23] partially removed cryoprotectant in a semen sample via repeated dialysis, by placing the semen sample in a bath containing half the concentration of DMSO in Harbo's buffer than the semen sample for 15 min and then in a second bath containing only Harbo's buffer for 15 min. After that, the samples were only centrifuged for 10 min at 1000 RCF. Considering the significantly different experimental design done by Auth & Hopkins [23], the highest result of sperm viability of 73.51 % and motility of 80 % using 10 % DMSO after thawing, without inseminating queens is not comparable with other studies mentioned above, as others did apply dialysis prior to centrifugation. In the studies described above, different centrifugation speeds and durations have been chosen, which are directly influencing the quality of sperm and subsequently also the fertility of queens, because the centrifugation speed of 250 RCF for 10–20 min starts to damage sperm cells [15].

In connection with the use of centrifugation in the processing of semen samples, there are considerable benefits and risks here. Centrifugation helps to wash the cryoprotectant from the sample, which has a negative effect on the sperm cells and the queen in the long term, but the sperm cells will be damaged due to centrifugation, which will manifest in lower mobility, viability, and fewer cells will enter the spermatheca of the queen. Centrifuging the samples for 12 minutes, increasing the revolutions from 1000 to 1600 RCF, decreases the sperm viability in the samples by 9 % and motility by 17 % [39]. If the chosen centrifugation speed is too low, it will not be possible to re-concentrate the semen in order to introduce enough semen in one pass (optimal is 8 microliters) [15] to inseminate the queen so that the queen can potentially lay fertilized eggs. At least 0.5 million sperm cells should end up in the spermatheca [20]. Thus, instrumental insemination of one queen will have to be done several times, which increases the risk of semen contamination and infection of queen. Therefore,

Wegener *et al.* [39], taking into account the effect of centrifugation on sperm cells, chose 1300 RCF for 12 min as the optimal intensity, because only at this speed it is possible to reconcentrate the semen to almost the original amount and ensure that the queens will have to be inseminated only once. Assessing the risks and balancing the methods remains up to each researcher.

3.5. Temperature regimes used for cooling, freezing and thawing semen samples

Drone semen samples are prepared for freezing in either 0.250 mL cryofreezing Cassou straws (IMV Technologies) [17], [20], [22], [24]–[26], [29], [34], in 0.15 mL micro glass cryostraws [29], 4 cm long polyethylene tubes inserted into punctured cryotubes [21], in 0.25 mL PETG semen straws (Cryo Bio Systems), S-CryoLock® Vitrifcation Device with loading area width – 0.36 inches, loading area thickness – 0.01 inches (BioTech) or Microdialysis tubes from the Pierce™ 96-well Microdialysis Plate kit (Thermo Scientific) [23].

After the preparation of the semen sample cooling follows. Hopkins & Herr [29] widely tested temperature regimes for cooling, where, after testing rapid and slow cooling, slow cooling proved to be more suitable for drone semen, because sperm cells are very sensitive to cold shock. In rapid cooling, the sample from room temperature (+20 degrees Celsius) was placed in an ice bath for 4 seconds to reach approximately 0 degrees Celsius. Slow cooling was performed by placing the sample in a water bath at room temperature (+20 °C), which was placed in the refrigerator for 2 h to reach the sample temperature of +4 °C. Then the sample was placed in an ice bath for 4 sec to reach a temperature of about 0 degrees Celsius. Such slow cooling from room temperature to +4 °C or 0 °C was used in several studies [20], [22]–[26], [29], [34]. Slow cooling can be done by placing a water bath in the refrigerator, or by using a programmable freezer [17], [21], e.g., cooling the sample at a rate of 3 °C per minute.

Cooling is then followed by freezing. In recent years, programmable freezers are most often used for freezing [17], [20]–[26], [29], [34], as such equipment enables to set slow freezing curves that protect sperm cells from cold shock. Researchers use different refrigeration equipment: IceCube, SY-LAB [17], [21]; CL-3000 CryoLogic PL [29], [34]; Crysalys Cryo Controller PTC-9500 [22]; Cell Freezer R204, Diamond Lab Supplies Inc [20]; Nicol Plus PC, Air Liquide [24]–[26]. The most commonly used freezing mode is to freeze the sample from +4/5 or 0 °C to −40 °C at a rate of 3 °C/min [20]–[23], [29], [34]. Other used freezing modes are: freezing from +5 °C to −80 °C in 2 minutes and then holding the sample at −80 °C for 10 minutes [17]; freezing from +5 °C to −8 °C at a rate of 3 °C/min and then freezing from −8 to −120 °C at a rate of 15 °C/min [24]–[26]. In all freezing modes, upon reaching the selected temperature of −40, −80 or −120 °C, the sample is placed in a liquid nitrogen (LN_2) container and stored at −196 °C.

Hopkins *et al.* [23], [29] has used vitrification or ultra-rapid freezing with liquid nitrogen LN_2 to freeze semen. In this case, the semen samples are placed in LN_2 for 2 min to rapidly freeze them and transfer them to the LN_2 storage tank. By using vitrification, the viability of drone sperm in thawed samples was lower than using programmed freezing, 75.46–77.48 % and 92.94 % respectively [29]. But the use of LN_2 is a cheaper, less time-consuming, and more mobile freezing method. Looking for a cheaper and more accessible alternative to programmed freezing, Auth & Hopkins [23] tested both different packages for freezing (S-CryoLock® Vitrifcation Device, which increases the freezing surface, and Microdialysis tubes) and two approaches of freezing with LN_2 (placing the sample immediately in LN_2 or first cooling it by holding sample on a metal mesh 2.5 cm above the LN_2 in LN_2 vapour for 5 min and only then completely immersing the sample in LN_2). A promising result similar to

the programmed freezer control was obtained by freezing stepwise in LN_2 vapour, reaching a temperature of approximately −120 °C, and then placing the sample in an LN_2 container using microdialysis tubes and dialysis baths with a 20 % DMSO concentration for the sample. Microdialysis tubes had slightly slower sample freezing rate. In order to develop a cheap, mobile, easy-to-use and effective freezing solution, freezing in liquid nitrogen vapour at different heights and durations, as well as choosing different packages in the process of freezing semen samples, is one of the directions that is worth exploring in future research. Perhaps it would be worth to evaluate the possibilities of using phase materials in the freezing process, which could ensure that the freezing mode in LN_2 vapour is similar to programmable freezing.

After a certain period of storage in the LN_2 tank at −196 °C, the semen samples are thawed using one of the following options: in a water bath at +25 °C for 30 s [17]; circulating water bath at +37 °C for 3 s [29] or 30 s [20]; in hot air at +40 °C for 7–10 s [29], [34]; in a water bath at +35 °C for 10 s [21] or 20 s [22]; in a paraffin bath at +35 °C for 15 s [21] or 30 s [23]; in Harbo's buffer at +35 °C for 30 s [23]. There is no consensus on thawing temperature regimes and their duration, so it is up to researchers to determine the most appropriate thawing method, which could also vary depending on the packaging of the semen sample and the size of the sample. However, it is recommended to take into account the most important condition that the thawing temperature should not exceed +40 °C in order not to damage the semen sample [29].

3.6. Tested storage durations for frozen semen

In the studies, semen were stored frozen in LN_2 tanks from 48 h up to 343 days. Pailard *et al.* [20] studied how sperm viability indicators change depending on storage temperature and duration of storage. Frozen semen samples were tested for sperm viability after using programmable freezing and thawing at 90 days, 180 days (25 wk) and 330 days (47 wk), yielding 61 %, 64 %, 76 % sperm viability in the samples, respectively. Viability indicators in the Pailard *et al.* [20] study increase atypically. It can be explained by the fact that during storage lysed cells start to appear, that is, the purification or self-degradation of old or damaged cells takes place, as a result of which proteins and DNA are extracted (released separately) and can be stained green in the same way as living cells using a fluorescent staining method to determine viability [32]. Therefore, it would be necessary to link the viability indicator with some other sperm quantitative indicator. When queens were inseminated with semen stored for 330 days, 5000 to 142 000 sperm cells with a viability of 10–68 % were observed when collected from queens' spermatheca [20]. Considering the small number of sperm cells, it would have been valuable to check also the fertility of the queens by determining the proportion of broods of worker bees, in order to conclude whether such a result can be used to obtain fertilized eggs for the reproduction of the F1 generation. Wegener *et al.* [21] stored semen for 9 months using programmable freezing as well as cryoprotectant addition via dialysis method. Sperm stored in this way had maintained high motility of 77 %. After inseminating the queens with such a semen sample, an average of 59 % (42.7–98.2 %) of the worker bee brood was observed. Hopkins & Herr [29] with two different freezing methods, storing semen for 343 days, reported on a sperm viability of 54.26 % in samples frozen with a programmable freezer and 41.46 % in samples frozen using vitrification. As other indicators, such as sperm motility or insemination of queens were not determined, it is not possible to draw conclusions about the quality of the samples. If it is taken into account that the samples must contain at least 46 % viable sperm cells [27], [28], it is possible to successfully maintain higher viability rates for up to 343 days with programmable freezing. However, in order to get a clearer picture of the quality of long-term

stored samples, it is necessary to determine other quality indicators in addition to viability, such as sperm motility.

3.7. Tested Storage Durations for Semen Samples at Above-Freezing Temperatures

If not frozen, drone semen is usually diluted with a solvent containing salts, nutrients, antibiotics, and stored in sealed glass capillaries in a dark, cool place. Several studies have been conducted regarding the storage temperature and duration of an unfrozen sample [20], [31], [32]. Collins [31] determined sperm viability for samples stored both at room temperature at +25 °C and in a refrigerator at +12 °C for up to one year. At both temperatures, it was possible to maintain >80 % viability for up to 6 weeks. Statistically significant drop in viability was observed directly from the 6th to the 9th week of storage, which dropped from 80 % to 66.5 % at a temperature of +25 °C, and from 80 % to 58 % at a temperature of +12 °C. The second significant decrease in sperm viability indicators was observed from 39 to 52 weeks at +25 °C from 51.5 % to 18.9 %, while at +12 °C it decreased from 62.2 % to 42.2 %. From this data, it was concluded that when storing fresh semen for longer than 39 weeks (273 days), significantly higher viability preservation will be possible at a lower temperature, i.e., +12 °C. Paillard *et al.* [20] also tested long-term storage of fresh semen at +16 degrees Celsius and managed to achieve good viability rates (69 %) when storing samples for up to 180 days (26 weeks), but when testing semen after 330 days (week 47) no living sperm cells were observed. A promising result was obtained by Hopkins *et al.* [32], by determining the viability indicators of semen samples after 45, 99, and 439 days, storing fresh semen at +4 °C and +14 °C, followed by insemination of the queens, and determining the proportion of fertilized eggs in laid brood. Higher sperm viability and the proportion of fertilized eggs in laid brood were observed when samples were stored at +14 °C compared to storage at +4 °C. In order to protect the fresh semen from contamination, two ways of adding antibiotics were also tested, either by dissolving the semen in a solution of salts, nutrients and antibiotics, or by coating the glass capillaries with antibiotics and antifungal substances in gelatin before semen were collected. Surprisingly, it was the undiluted semen stored at +14 °C for up to 439 days that showed the highest viability rates of 73.64 %, which also resulted in fertile queens with an average worker bee brood proportions of 71 % [32]. These studies indicate that it is possible to store fresh semen for long periods of up to 439 days at above-freezing temperatures and obtain fertile queens. The main condition is that the storage temperature should be above +10 °C [36], because sperm viability and motility decrease faster at lower temperatures [32], [36]. Meanwhile, the upper storage temperature is +25 °C. Accordingly, in this temperature range from +10 °C to +25 °C, it is possible to store semen from 2 weeks to up to 439 days. By storing fresh semen without freezing in closed capillaries makes it easier to schedule insemination of queens within the beekeeping season and opens up the possibility to inseminate queens in early spring with semen collected in the previous season. However, for a longer period of time, semen must be prepared for storage using one of the freezing methods.

4. CONCLUSIONS

Consistent breeding work plays an essential role in preserving bee colonies, restoring genetic material and ensuring genetic diversity. In order to increase the efficiency and productivity of breeding work, it is necessary to develop a reliable method of long-term storage of genetic material, which is at the same time cheap, widely available and easy to perform. Reliability of storage methods is ensured by predictable and reproducible high rates of sperm quality and queen fertility.

By far the most frequently used solvent in studies of honey bee semen freezing, with good sperm quality indicators is Harbo's solution. The best cryoprotectant that fulfills the main role to protect sperm cells from cold shock and the formation of ice crystals in cells is DMSO with a concentration of 10 % in the final sample. To reduce the risks associated with sperm cell osmotic stress and the negative side effects of the cryoprotectant, dialysis is beginning to be introduced into freezing protocols. The most commonly used freezing method is the programmable freezing with the mode to freeze the sample from +4/5 or 0 °C to −40 °C at a rate of 3 °C/min. After freezing, long-term storage is carried out in a liquid nitrogen (LN_2) container and stored at –196 degrees Celsius. In order to develop a cheap, mobile, easy-to-use and effective freezing solution, freezing in liquid nitrogen vapour and using various packaging solutions in the process of freezing semen samples are tested. So far, frozen sperm samples have been stored for up to 343 days (by using programmable freezing), while unfrozen sperm samples have been stored with good results in temperature range from +10 °C to +25 °C from 2 weeks to up to 439 days (by using different sealed capillary fillings). Most often, *in vitro* tests were used to determine the quality of stored sperm samples. But there are also some attempts to look for correlations between *in vitro* and *in vivo* tested indicators. So far, a strong correlation with all *in vivo* indicators was found specifically for sperm motility.

In order to be able to predict the potential queens' fertility indicators (rate of fertilized eggs, number of sperm cells in the queen's spermatheca ect.) based on the quality indicators of semen samples that were stored for a long term, further research of *in vitro* and *in vivo* data validation and correlation is necessary. Such correlation would enable to predict the relevant queens' fertility indicators without spending large amounts of material and time resources in testing of the samples, and insemination and monitoring of queens. In order to determine the quality of the semen samples, it is necessary to include the following indicators: the number of sperm cells; cell concentration; sperm cell motility; viability of sperm cells. These indicators need to be analysed together, because the analysis of a single indicator can provide insufficient and misleading information about the quality of the sample, for example, sperm viability does not guarantee that the living cells are also functioning (motile) cells.

In the last 22 years there has been rapid progress in the development of long-term storage methods. Innovations have been implemented with the aim of improving the quality and fertility indicators of semen samples, reducing the negative effects of chemical and physical treatment on sperm cells and queens. Innovations are also being sought for easy-to-use and low-cost methods that would be widely available in the beekeeping industry.

In order to improve the semen freezing process, in the future the following aspects regarding the wider utilization of LN_2 vapour freezing should be explored: (1) the freezing process in LN_2 vapour should be adapted to better mimic the optimal freezing curves of programmable freezers; (2) various sample packaging should be evaluated for their applicability; (3) different sample distances from LN_2 vapour should be tested; (4) application of various phase transition materials should be tested for reduced and more controlled freezing rates of the samples. Another valuable research direction that should be explored further is the testing of the longest possible and optimal storage period, as it is likely that there is a soft (method dependent) and/or a hard (universal) limit on the maximum time period that honey bee semen samples could be stored for. In addition, long period storage testing should be tested not only at −196 °C, but also at higher temperatures, e.g., −120, −80 or −40 °C, as these temperature modes could offer more alternatives to equipment that is usable for honey bee semen storage.

ANNEXES

Available at: https://zenodo.org/record/7866650

REFERENCES

[1] European Commission. The new common agricultural policy: 2023–27, *Agric. Rural Dev.* 1–7. 2022. [Online]. [Accessed: 11.12.2022]. Available: https://agriculture.ec.europa.eu/common-agricultural-policy/cap-overview/new-cap-2023-27_en
[2] European Commission. Reinforcing Europe's resilience: halting biodiversity loss and building a healthy and sustainable food system. 2020. [Online]. [Accessed: 11.12.2022]. Available: https://ec.europa.eu/commission/presscorner/detail/lv/ip_20_884
[3] European Commission. CAP Strategic Plans and Commission observations Summary overview for 19 Member States. 2022.
[4] European Commission. Farm to Fork Strategy. *DG SANTE/Unit 'Food Inf. Compos. food waste'*. DG SANTE/Unit 'Food Inf. Compos. food waste'. 2020. [Online]. [Accessed: 11.12.2022]. Available: https://ec.europa.eu/food/sites/food/files/safety/docs/f2f_action-plan_2020_strategy-info_en.pdf
[5] Bumbiere K., Diaz Sanchez F. A., Pubule J., Blumberga D. Development and Assessment of Carbon Farming Solutions. *Environmental and Climate Technologies* 2022:26(1):898–916. https://doi.org/10.2478/rtuect-2022-0068
[6] Locmelis K., Blumberga A., Bariss U., Blumberga D., Balode L. Industrial energy efficiency towards green deal transition. Case of Latvia. *Environmental and Climate Technologies* 2021:25(1):42–57. https://doi.org/10.2478/rtuect-2021-0004
[7] The Food and Agriculture Organization of the United Nations. FAOSTAT statistical database, 2020.
[8] Klein A. M. *et al.* Importance of pollinators in changing landscapes for world crops. *Proc. R. Soc. B Biol. Sci.* 2007:274(1608):303–313. https://doi.org/10.1098/rspb.2006.3721
[9] Sammataro D., Avitabile A. Pollination and Bee Plants. The beekeeper's handbook. Cornell University Press, 1998.
[10] Nieto A. *et al.* European Red List of Bees. Luxembourg: Publication Ofce of the European Union, 2014.
[11] European Commission. Green Deal targets for 2030 and agricultural production studies. February, 2021.
[12] Cobey S. W. Comparison studies of instrumentally inseminated and naturally mated honey bee queens and factors affecting their performance. *Apidologie* 2007:38(4):390–410. https://doi.org/10.1051/apido:2007029
[13] Büchler R. *et al.* Standard methods for rearing and selection of Apis mellifera queens. *Journal of Apicultural Research* 2013:52(1):1–30. https://doi.org/10.3896/IBRA.1.52.1.07
[14] Oxley P. R., Oldroyd B. P. Chapter 3 - The Genetic Architecture of Honeybee Breeding. *Advances in Insect Physiology* 2010:39:83–118. https://doi.org/10.1016/B978-0-12-381387-9.00003-8
[15] Cobey S. W., Tarpy D. R., Woyke J. Standard methods for instrumental insemination of Apis mellifera queens. *J. Apic. Res.* 2013:52(4):1–18. https://doi.org/10.3896/IBRA.1.52.4.09
[16] Woyke J., Jasinski Z. Influence of Age of Drones on the Results of Instrumental Insemination of Honeybee Queens. *Apidologie* 1978:9(3):203–212. https://doi.org/10.1051/apido:19780304
[17] Taylor M. A., Guzmán-Novoa E., Morfin N., Buhr M. M. 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
[18] Viert V., Wegener J., Bienefeld K. Europe's First Gene Bank for Honey Bees. *Bee World* 2021:98(4):110–114. https://doi.org/10.1080/0005772x.2021.1927576
[19] Yániz J. L., Silvestre M. A., Santolaria P. Sperm quality assessment in honey bee drones. *Biology (Basel)* 2020:9(7):174. https://doi.org/10.3390/biology9070174
[20] Paillard M., Rousseau A., Giovenazzo P., Bailey J. L. Preservation of Domesticated Honey Bee (*Hymenoptera: Apidae*) Drone Semen. *J. Econ. Entomol.* 2017:110(4):1412–1418. https://doi.org/10.1093/jee/tox149
[21] Wegener J., May T., Kamp G., Bienefeld K. A successful new approach to honeybee semen cryopreservation. *Cryobiology* 2014:69(2):236–242. https://doi.org/10.1016/j.cryobiol.2014.07.011
[22] Gül A., Şahinler N., Onal A. G., Hopkins B. K., Sheppard W. S. Effects of diluents and plasma on honey bee (*Apis mellifera* L.) drone frozen-thawed semen fertility. *Theriogenology* 2017:101:109–113. https://doi.org/10.1016/j.theriogenology.2017.06.020
[23] Auth C. A., Hopkins B. K. Nitrogen vapor immersion: An accessible alternative for honey bee (*Apis mellifera* L.) semen cryopreservation. *Cryobiology* 2021:100:12–18. https://doi.org/10.1016/j.cryobiol.2021.04.006
[24] Alcay S. *et al.* Successful cryopreservation of honey bee drone spermatozoa with royal jelly supplemented extenders. *Cryobiology* 2019:87:28–31. https://doi.org/10.1016/j.cryobiol.2019.03.005
[25] Alcay S. *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. https://doi.org/10.9775/kvfd.2021.25756
[26] Alcay S. *et al.* Honey bee drone (Apis mellifera) sperm cryopreservation with rainbow trout seminal plasma supplemented extenders. *J. Hell. Vet. Med. Soc.* 2022:4(4):527–534.
[27] Collins A. M. Relationship between semen quality and performance of instrumentally inseminated honey bee queens. *Apidologie* 2000:31(3):421–429. https://doi.org/10.1051/apido:2000132
[28] Collins A. M. Functional longevity of honey bee, Apis mellifera, queens inseminated with low viability semen. *J. Apic. Res.* 2004:43(4):167–171.

[29] Hopkins B. K., Herr C. Factors affecting the successful cryopreservation of honey bee (*Apis mellifera*) spermatozoa. *Apidologie* 2010:41(5):548–556. https://doi.org/10.1051/apido/20010006

[30] Wegener J., Bienefeld K. Toxicity of cryoprotectants to honey bee semen and queens. *Theriogenology* 2012:77(3):600–607. https://doi.org/10.1016/j.theriogenology.2011.08.036

[31] Collins A. M. Survival of honey bee (Hymenoptera: Apidae) spermatozoa stored at above-freezing temperatures. *J. Econ. Entomol.* 2000:93(3):568–571. https://doi.org/10.1603/0022-0493-93.3.568

[32] Hopkins B. K., Cobey S. W., Herr C., Sheppard W. S. 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

[33] Dadkhah F., Nehzati-Paghaleh G., Zhandi M., Emamverdi M., Hopkins B. K. Preservation of honey bee spermatozoa using egg yolk and soybean lecithin-based semen extenders and a modified cryopreservation protocol. *J. Apic. Res.* 2016:55(4):279–283. https://doi.org/10.1080/00218839.2016.1243292

[34] Hopkins B. K., Herr C., Sheppard W. S. Sequential generations of honey bee (*Apis mellifera*) queens produced using cryopreserved semen. *Reprod. Fertil. Dev.* 2012:24(8):1079–1083. https://doi.org/10.1071/RD11088

[35] 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. https://doi.org/10.1016/j.cryobiol.2012.04.010

[36] Harbo J. R., Williams J. L. Effect of above-freezing temperatures on temporary storage of honeybee spermatozoa. *J. Apic. Res.* 1987:26(1):53–55. https://doi.org/10.1080/00218839.1987.11100735

[37] Harbo J. R. Survival of Honey Bee (Hymenoptera: Apidae) Spermatozoa after Two Years in Liquid Nitrogen (−196 °C). *Ann. Entomol. Soc. Am.* 1983:76(5):890–891. https://doi.org/10.1093/aesa/76.5.890

[38] Esteki M. H., Malandrino A., Alemrajabi A. A., Sheridan G. K., Charras G., Moeendarbary E. Poroelastic osmoregulation of living cell volume. *iScience* 2021:24(12):103482. https://doi.org/10.1016/j.isci.2021.103482

[39] Wegener J., May T., Kamp G., Bienefeld K. New methods and media for the centrifugation of honey bee (Hymenoptera: Apidae) drone semen. *J. Econ. Entomol.* 2014:107(1):47–53. https://doi.org/10.1603/EC13159