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THE IMPORTANCE OF *IN SITU* AND *EX SITU* PROTECTION OF HONEY BEE (*APIS MELLIFERA*) IN POLAND WITH EMPHASIS ON DRONE SEMEN CRYOPRESERVATION – A REVIEW*

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Abstract

Human activities, new technologies and climate change may significantly affect the worldwide decline of livestock populations, including the honey bee (*Apis mellifera*) in the coming years. In Poland, animal conservation efforts with a particular emphasis on small populations of native breeds are focused on *in situ* protection programs, i.e., preserving animals in their natural environment, with limited use of breeding methods. A total of four lines/breeds of the Middle European bee subspecies (*Apis mellifera mellifera*), including Kampinoska, Augustowska, Asta and North bee, and one line/breed of the Carniolan honey bee subspecies (*Apis mellifera carnica*), designated as Carniolan Dobra, undergo the *in situ* protection. Whereas, the use of *ex situ* methods offers the possibility of indefinite storage of biological material from animals that exhibit unique functional traits, such as productivity, gentleness, as well as winter hardiness, hygienic behavior and adaptation of bee colony development to environmental conditions. The article presents the current state of knowledge and future goals related not only to the *in situ* protection program of honey bee native breeds in Poland, but also *ex situ* methods that can and should be implemented as a complement to *in situ* strategies. Opening up the possibilities for the purposes of short- and long-term conservation of drone semen in the future may become a way that will contribute to the effective management of honey bee genetic resources in Poland.

Key words: honey bee, biodiversity, reproductive biotechnology, sperm cells, cryogenic protection

Species biodiversity depends on ecosystem biodiversity, which, in turn, depends on climatic conditions. While it is a global challenge to counteract the impacts of climate change, there is the worldwide decline in biodiversity. Over the past several years, the number of species listed in the Red List of Threatened Species published by the International Union for Conservation of Nature (IUCN) has been growing exponentially and in 2025, 46,300 species were listed as threatened with extinction (https://www.iucnredlist.org/). Globally, numerous wild pollinator species are experiencing significant declines, leading to their inclusion on various Red Lists of threatened species. In Poland, many species of wild pollinators are endangered and have been included in national Red Lists. The most affected groups include solitary bees and bumblebees: *Hylaeus variegatus*, *Bombus ruderatus*, *Bombus pomorum*, *Bombus distinguendus*, *Xylocopa violacea*. Protecting these species is crucial for maintaining biodiversity and ensuring effective pollination in both natural and agricultural ecosystems.

The honey bee (*Apis mellifera*) is not listed on the IUCN Red List of Threatened Species because its global population is relatively stable, and it is not at risk of extinction. Unlike many wild pollinators, honey bees benefit from human intervention, as they are actively managed by beekeepers worldwide. This makes their situation significantly different from that of many solitary bees and other wild pollinators. However, deforestation, industrialization of the environment or the introduction of pesticides pose a lethal threat to their population levels. Honey bees are an integral part of the ecosystem, pollinating over 80% of plants (both cultivated and wild plants) (Gül et al., 2017; Kucharczyk et al., 2017). It is estimated that 1/3 of products consumed by humans depend to a greater or lesser extent on insects pollinating plants (Zych et al., 2018). In recent decades, there have been significant losses in honey bee colonies worldwide (Kucharczyk et al., 2017; Abdelkader et al., 2018). The reasons for this adverse phenomenon are numerous. Among them, the most important seem to be the destruc-

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tion of natural habitats, the incidence of infectious and transmissible diseases evoked by ecto- and endoparasites, bacteria, mycoplasms, fungi and viruses, morbidity and mortality prompted by pesticides or nutritional deficiencies (Kucharczyk et al., 2017; Ciereszko et al., 2017; Abdelkader et al., 2018; Fisher and Rangel, 2018; Liu et al., 2020; Smilga-Spalvina et al., 2023) and environmental degradation (Gül et al., 2017; Kucharczyk et al., 2017; Fisher and Rangel, 2018).

Given its importance for the preservation of biodiversity, the Convention on Biological Diversity was drawn up more than 30 years ago on June 5, 1992, in Rio de Janeiro. Poland ratified the Convention, and the Ministry of Agriculture and Rural Development entrusted the National Research Institute of Animal Production in Balice with coordinating efforts to protect animal genetic resources (Dz.U. no. 184, item 1532, 1992). The Convention provided the impetus to address issues related to environmental change in the context of its impact on food production, agriculture, and animal husbandry.

On the one hand, the loss of biodiversity is particularly evident in the context of pollinating insects, including the honey bee, following the destruction of their natural habitats as a result of agricultural intensification (including the use of pesticides and fertilizers) (Bugin et al., 2022). On the other hand, the biocenotic divergence of pollinators can be drastically limited or ceased due to: (1) the urbanization and/or (2) depletion of the gene pool within a given species, breed/line, or population by displacing native breeds as a consequence of importing bee breeds/lines undergoing intensive genotypic selection for desirable phenotypic traits (Leclercq et al., 2023). Both these phenomena negatively impact physiological and reproductive health or even the adaptability of native honey bee breeds to the biotopic niches within agricultural ecosystems despite achieving high production efficiency (Hung and Yiin, 2023; Fine et al., 2023). To rebuild the populations and preserve the gene pools of indigenous bee breeds in Poland, the above-mentioned phenomena must be included in the programs intended to protect genetic resources, which simultaneously give rise to promoting/expanding breeding progress. The great importance of the honey bee (*Apis mellifera*) for the environment and human life has prompted scientists to work intensively to develop effective methods of preserving the biological material of this species (Oleksa et al., 2023).

The objective of this paper is, on the one hand, to provide a survey of the current activities undertaken to protect the genetic reserves of honey bee and perpetuate its genotypic diversity by using *in situ* methods in Poland. On the other hand, due to the growing necessity for the implementation of *ex situ* methods intended for the conservation of this insect species, the article also presents the state of the art in the field of the short- and long-term preservation of honey bee drone semen, including future recommendations aimed at development of comprehensive strategy useful for protection of honey bee genetic resources in Poland.

The *in situ* conservation in honey bee with emphasis on the Polish native breeds of this livestock species

In Poland, the ranges of two native subspecies of the honey bee are present: the Middle European bee *Apis mellifera mellifera* Linnaeus 1758 (the so-called "dark bee") (Madras-Majewska and Skonieczna, 2018) in the north-east and *A. mellifera carnica*, Pollmann 1879, commonly known as "krainka" (Bieńkowska et al., 2021) in the Island Beskids. The bees are adapted to living in Poland's specific climatic conditions and have been kept in wild beehives, logs, and later in hives for centuries (Madras-Majewska et al., 2021). However, in recent decades, the influx of other bee subspecies has significantly reduced both colony size and area of distribution. Based on the analyzed surveys, Buckfast honey bees first appeared in Polish apiaries during the 1990s. Their popularity increased significantly after 2007 and has since stabilized, fluctuating around 10% of the total bee population (Bieńkowska et al., 2021). Despite their many favorable traits, especially their higher resistance to disease, better wintering, and ability to take advantage of poor forage, native honey bees have been displaced due to their lower productivity and greater aggression (Kolasa and Molasy, 2022). In Poland, the honey bee, according to Article 2, paragraph 1b of the Law on the Organization of Breeding and Reproduction of Farm Animals (Journal of Laws 2021, item 36), is a livestock animal. As a result, it has been possible to include preserved local bee lines in genetic resources protection programs. Taking into consideration the programs of *in situ* conservation that are intended for the honey bee (*Apis mellifera*), four lines/breeds of the Middle European bee subspecies (*Apis mellifera mellifera* L.) including Kampinoska, Augustowska, Asta, and North Bee, and one line/breed of the Carniolan honey bee subspecies (*Apis mellifera carnica* P.), designated as Carniolan Dobra, undergo *in situ* protection in Poland (Figure 1). The phenotypic characteristics for the selected traits (including coloration) of bee breeding lines within individual breeds are contained in breeding standards developed to keep herd books and implement breeding and conservation programs. In addition, breed affiliation is verified based on morphometric studies in the Skrzydlak program (Madras-Majewska and Skonieczna, 2016).

In 2024, a total of 766 bee colonies occurring in six regions of Poland undergo *in situ* protection (Figure 2). The importation and hybridization of the bee population significantly contributed to the displacement of native breeds of the honey bee increasing the economic capacity, which decreased the adaptability of hybrids (Kolasa and Molasy, 2022). Reproducing the gene pool of native breeds under natural conditions in Poland is difficult due to, among others, the lack of isolated geographic areas enabling the controlled natural mating of queen bees with drones of the same breed/line to maintain intra-breed purity and prevent interbreeding or decrease the incidence of undesirable collapse of bee populations/colonies and, consequently, reduce inbreeding (Gromisz, 1997). The

conservative breeding of the Kampinoska line has been conducted in the Kampinos National Park, a closed region occupying an area of 40 km × 20 km, since the early 1980s. The apiary is traditionally run without controlling bee development. The natural population of bee colonies of this line is in the conservative breeding region. The natural habitat of the Augustowska line of bees has been the Augustów Primeval Forest since the 1970s. The conservation breeding region of this bee line is divided into a central zone with a radius of about 10 km and an isolation belt of 10 km. Bee colonies in the central zone constitute a natural "gene bank" from which the breeding (conservation) stock/flock can draw material to renew and select its own stock (Kolasa and Molasy, 2022). The apiary is run in a traditional system without controlling the development of the bees. The Northern M line bee population is derived from local bees, maintained and selected under the climatic conditions and forage activity of northern Poland. This line was created from the merger of two other Middle European honey bee lines, Mazurka and Pomorska (Madras-Majewska and Skonieczna, 2016). Work on the derivation of the Mazurka line was carried out in the 1960s by the Zootechnical Experimental Station of the National Research Institute of Animal Production in Siejnik, while the Pomorska line was derived in Szczecin by the District Animal Breeding Station in the 1990s (IZ-PIB, 2024 a) and the programs intended to breed the Asta line were initiated in 1937. The first *in situ* conservation programs for the Central European breed line were approved by the Polish Ministry of Agriculture and Rural Development in 2000 and the Carniolan Dobra line in 2014. The current conservation programs were introduced by Order of the Managing Director of the National Research Institute of Animal Production No. 12/22 of January 21, 2022 (IZ-PIB, 2024 a). The strategy of the conservation programs is to preserve the population by increasing its numbers, maintaining the phenotypic traits per the line's pattern as well as the typical biological and functional traits (such as early development, hardiness, good overwintering, ability to accumulate large amounts of pollen, increased nest defense, high wax production), and reintroducing queens into the wild and moderate improvement.

The population size increased by augmenting the abundance of mothers in existing conservation stocks and incorporating new cooperating conservation stocks. Breeding flocks are maintained by the Animal Breeding and Insemination Centre in Bydgoszcz (Augustowska, Kampinoska, and North Bee breeds) and the Mazovian Animal Breeding and Insemination Centre in Łowicz (Asta breed). Each of the four lines is comprised of one breeding flock, the primary role of which is to propagate and multiply the numbers of naturally and/or artificially inseminated bee queens (Madras-Majewska and Skonieczna, 2016). Moreover, the maintenance of breeding stock is indispensable to subject the bees to the evaluation of their functional and breeding value, and carry out/facilitate the selection programs. In native honey bee breeding programs, maintaining genetic diversity is crucial to prevent inbreeding and ensure colony health. One approach to achieve this is the periodic introduction of new genetic material into breeding populations. This strategy aims to incorporate desirable traits and mitigate the adverse effects of inbreeding (Kolasa and Molasy, 2022). However, such introductions are conducted with great caution, primarily by selecting superior individuals from existing populations to preserve the genetic integrity of native lines. The importation of foreign genotypes is avoided to maintain this integrity. These activities are overseen by appropriate breeding institutions, such as the National Animal Breeding Center, which maintain breeding records and monitor the implementation of conservation programs.

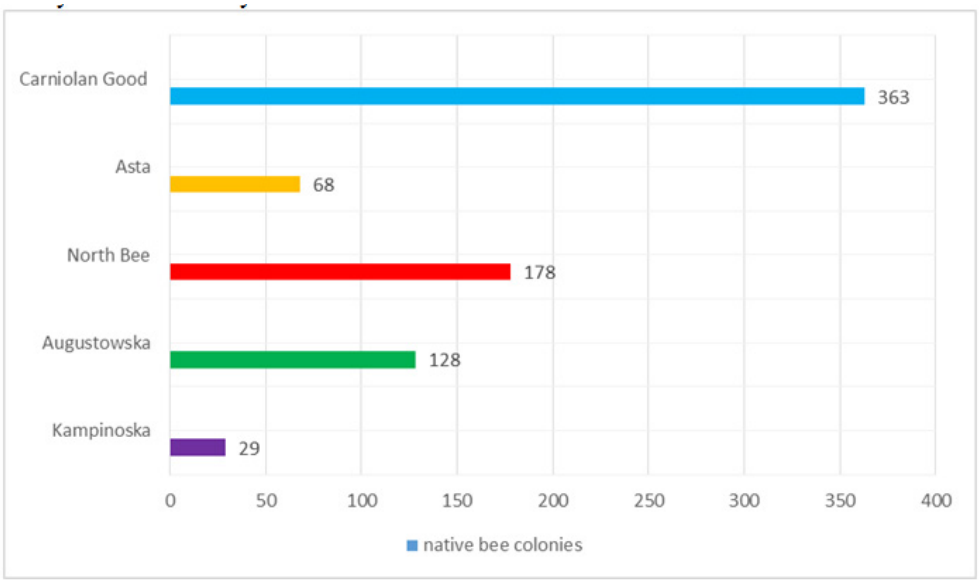


Figure 1. The numbers and the quantitative distribution of bee colonies within the framework of the populations of Polish native honey bee (*Apis mellifera*) undergoing the programs of *in situ* conservation in 2024, representing one line/breed of the Carniolan honey bee subspecies (*Apis mellifera carnica*) termed Carniolan Dobra and four lines/breeds of the Middle European bee subspecies (*Apis mellifera mellifera*), including Asta, North bee, Kampinoska, and Augustowska

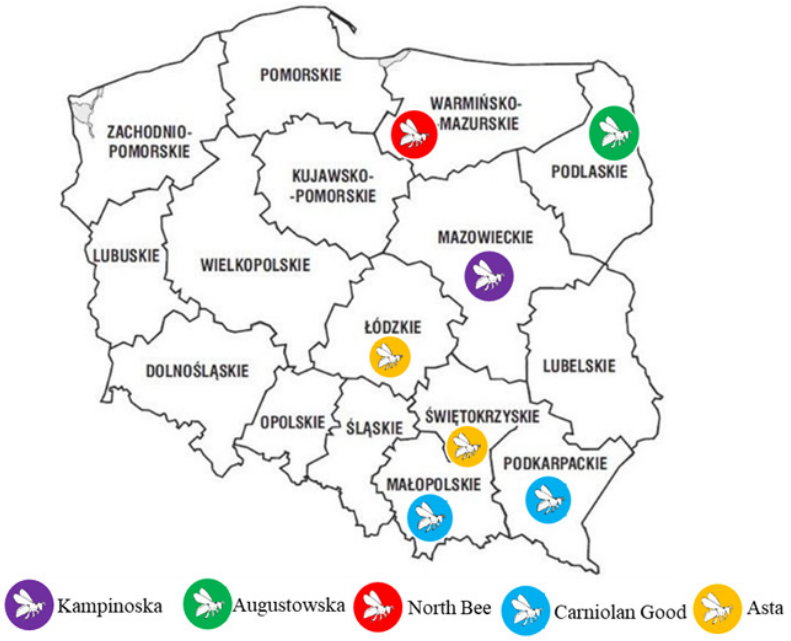


Figure 2. Territorial extent of the occurrence of Polish native honey bee colonies undergoing *in situ* protection

In addition to the breeding stocks, there are cooperative conservation flocks, aimed at perpetuating the population in the required numerical state, transferring queen bees to the breeding flock, making the apiary available for phenotypic evaluation and collection of polymorphic juvenile and parental specimens (larvae/broods and drones). The number of bee colonies included in the genetic resources conservation program is presented annually on the website of the National Research Institute of Animal Production (IZ-PIB, 2024 b). The number of bee colonies steadily increased from 118 to 1281 between 1999 and 2020. However, since 2022, a successive decline in the number of bee colonies has been observed, amounting to 1288, 846 and 766 in 2022, 2023 and 2024, respectively. The decline in the population of colonies within conservation programs may, in part, be attributed to winter losses, which can reach 10% to 30% annually. Moreover, diseases and parasites, particularly *Varroa destructor* infestations and *Nosema apis* infections, weaken bee populations before winter, significantly compromising their ability to survive into the next season. Accordingly, efforts should be strengthened on the long-term goal of *in situ* conservation programs intended for native breeds, that is, to systematically elevate their numbers and, consequently, perpetuate the inheritance of phenotypic traits characteristic of subpopulations within the same lineage. In addition, starting in 2023, extensive efforts have been made to expand conservation programs to include new elements related to the preparation of biotechnological methods for preserving drone semen. In particular, the development of an effective method for cryopreservation of drone semen will allow the expansion of existing efforts to preserve honey bee biodiversity in Poland.

Reproductive biotechnology-assisted approaches to the *ex situ* conservation in honey bee

Artificial insemination of bee queens

The physiological health and fertility of bee queen are one of the conditions for the proper development of a bee colony. The phenotypic value of a queen is determined using three basic criteria: physical efficacy expressed as the abundance of secreted maternal pheromones, fertility/fecundity and prolificacy measured with the number of eggs laid and the numbers of spermatozoa in the spermatheca, and the inheritance/heritability of genotypic traits. Mother bees lay up to 2,000 eggs per day during the peak season (Özkök and Selçuk, 2020). A queen bee must be inseminated before she can start laying eggs. Two main ontogenetic stages are required to attain the reproductive maturity of queen bees. They encompass the first 16-day phase indispensable to reaching bee brood and subsequently imago and the second 5–8-day phase critical to reaching sexual maturity. Therefore, the full developmental cycle of a queen bee oscillates between 21 and 24 days. In natural reproduction, one or two mating flights of a bee queen occur most frequently, during which she often copulates with many drones (from a few to even several dozen) (Withrow and Tarpy, 2018; Tarpy and Page, 2000; Schluns et al., 2005). After the termination of her mating flight, the queen bee returns to the hive. Over the next 1–2 days, the resumption of her mating flight may occur (Tarpy and Page, 2000), but once she starts laying eggs, she never leaves the hive again. The natural mating of a queen bee with multiple drones ensures a high extent of genetic diversity in her offspring (Oldroyd and Fewelly, 2007). In queen bees that self-inseminate physiologically, a negligible pool of drone spermatozoa migrates within 24–48 hours from the middle

compartment of oviducts directly into spermatheca. The overabundance of spermatozoa ranging from 85 to 95% is removed out of the reproductive tracts of a queen bee. The remaining sperm cells are subjected to long-term intracorporeal storage within the anatomohistological structures of spermatheca. The eggs are laid by the queen bee from early spring to late fall. It is worth highlighting that the eggs can undergo *in vivo* fertilization, followed by the development of diploid female specimens (worker bees), whereas the lack of fertilization leads to the development of haploid male specimens (drones) (Özkök and Selçuk, 2020). Instrumental insemination of queen bees is a controlled process that allows beekeepers to precisely select drone genetics for breeding. The procedure involves several steps, requiring specialized equipment and skilled handling. The queen is anesthetized using carbon dioxide (CO_2), which induces a physiological response that facilitates sperm retention. Then, the queen is restrained in an insemination apparatus using a queen holder, keeping her abdomen accessible for insemination. During artificial insemination in bees, it is necessary to use both the right number of insemination treatments and the right volume of diluted semen. Woyke and Jasiński (1990) showed that queen bees that are expected to brood for as long as possible should be inseminated twice with two consecutive doses of semen (each at a volume of either 4 µL or 6 µL) instead of one-step insemination with single semen dose at a volume of 8 µL. Queen bees artificially inseminated with lower doses of semen can sequestrate the semen from the oviducts to their spermathecae more easily and quickly, which results in an earlier onset of oviposition (Woyke, 1960; Moritz, 1984; Harbo, 1990; Skowronek et al., 1995).

The artificial insemination of queens is a method that allows controlling the process of mating (Cobey et al., 2013) and is an indispensable tool in implementing programs for the genetic improvement of bees and their crossbreeding. The insemination procedure allows the bee queen to be inseminated with semen collected from one or more drones and, in some cases, with semen collected from the bioreservoirs (spermathecae) of brooding queens (Cobey, 2016). The optimal age of drones for semen collection is considered to be between 14 and 21 days of age, corresponding to the attainment of full sexual maturity (Woyke and Jasiński, 1978). After 30 days of life, drones exhibited a significant decrease in sperm count (Metz and Tarpy, 2019). When collecting semen, care should be taken not to collect the mucus along with the semen. The results of the study by Woyke and Jasiński (1978) confirmed that drone derived anal/rectal mucus, which has been deposited in the reproductive tracts of artificially inseminated queen bees, clogs the entire oviductal lumina and can trigger the maternal mortality and morbidity.

The first successful research targeted at the artificial insemination of bee queens was initiated by Lloyd Watson in the 1930s (Woyke, 1960; Cobey, 2016) and continued in the decades that followed, allowing for the accurate identification of factors impacting both negatively (infections, inadequate sperm dosage, lack of family acceptance) (Woyke, 1960; Woyke and Jasiński, 1982, 1990) and positively (quality of the bee queen, her age at the time of insemination, insemination with the right dose of semen, placement of the mother with worker bees after insemination) (Woyke, 1960; Woyke and Jasiński, 1976; Cobey, 2007, 2016; Liu et al., 2020) on the effectiveness of this reproductive biotechnology.

Short- and long-term storage of drone semen

The initiation of research into developing methods for short- and long-term storage of drones' semen stemmed from the need to extend the queen production season (in temperate climates, it is not possible to raise queens in early spring, as this time of year is also limited by the lack of drones, and colonies usually begin to leave the hive only in the second half of April) and to create the possibility of transportation of semen. This research was inspired by the time-consuming nature of collecting semen from many drones, preparing the insemination dose, and performing the insemination procedure on the same day.

Semen is commonly diluted in various liquids after collection and then stored at positive temperatures (Locke and Peng, 1993; Skowronek et al., 1995; Ciereszko et al., 2017; Yániz et al., 2019). The best results are obtained by storing semen for two days in sealed glass capillaries. Mothers inseminated with such semen accumulate a similar number of sperm to mothers inseminated with fresh semen (Skowronek and Szymula, 1998). On the other hand, experiments conducted by Collins (2000) showed that storing drone sperm at 12°C for a 6-week period enables sperm viability to be maintained at 80%, while extending the storage time to 39 weeks reduces sperm viability to 65%, which, however, does not adversely affect maternal fertility results (Cobey, 2007, 2013). This allows for the extension of the queen bees reproductive season. However, only the cryopreservation of drone semen enables rationally controlled reproduction, the preservation of the desired genetic diversity and reduces the risk of spreading pests and pathogens associated with live bee transport (Paillard et al., 2017). Work on the cryopreservation of drones' semen began in the 1970s and continues to this day (Podlewski et al., 2000, 2003; Wilde at al., 2001; Cobey, 2016, Smilga-Spalvina et al., 2023). Various cryoprotectants are used to protect sperm from cryogenic damage. For the cryopreservation of drones' semen, the following have been tested: dimethylsulfoxide (DMSO), glycerol, dimethylformamide, 1,3-propanediol, 2,3-butanediol, and ethylene glycol (Hopkins and Herr, 2010; Wegener and Bienefeld, 2012; Wegener et al., 2014 b; Alcay et al., 2022). However, glycerol, commonly used as an additive to freeze thinner for livestock semen, does not apply to bees. The research conducted by Harbo (1979) shows that glycerol damages the cell membranes of drone sperm during the cryopreservation

process. Studies using DMSO have indicated that it is an effective cryoprotectant (Wilde et al., 2001; Taylor et al., 2009), and its addition to the freezing diluent at a concentration of 10% allows an adequate percentage of live sperm drones to be obtained (Hopkins and Herr, 2010). Previous studies have also shown that the addition of 10% DMSO to the freezing diluent does not adversely affect the percentage of sperm reaching the mother's spermatheca (Harbo, 1977). 10% DMSO in the semen-diluent mixture used for insemination had no effect on this parameter. On the other hand, Hopkins and Herr (2010) observed that a 15% concentration of DMSO reduces sperm viability after thawing. Performing insemination with cryopreserved semen with a diluent containing DMSO can furthermore be toxic to the queen bee (Smilga-Spalvina et al., 2023; Wegener and Bienefeld, 2012, Harbo and Hered, 1986). To reduce the effect of DMSO on sperm quality and/or on the inseminated queen bee, centrifugation of sperm is proposed to remove the cryoprotectant after freezing-thawing. However, the use of centrifuged sperm for insemination, on the one hand, does not affect the production of fertile eggs by queen bees (Kaftanoglu and Peng, 1984; Moritz, 1984; Harbo, 1990; Skowronek et al., 1995), on the other hand, there is evidence that the centrifugation protocols caused sperm damage. A study by Collins (2004 b) found that only 34.1% of sperm subjected to centrifugation showed a normal cell membrane integrity. Also, Fischer (1987) reported a significant reduction in the number of spermatozoa reaching the spermatheca when they were centrifuged before insemination of the mother bee. Wegener et al. (2014 b), on the other hand, showed that most queens inseminated with centrifuged semen produced normal brood, and the number of sperm reaching the mother's spermatheca was not significantly different from that of queens receiving untreated semen. In addition, the study showed no difference between the percentage of motile spermatozoa and spermatozoa with intact plasma membranes inside in the spermatheca of queen bees after insemination with centrifuged semen compared to the control.

In recent years, work has been carried out on modifying the composition of freeze–thaw dilution based on the addition of substances with antioxidant activity and shielding of sperm plasma membranes (Amidi et al., 2016; Sharafi et al., 2022). The addition of compounds with antioxidant activity reduces the negative effects caused by reactive oxygen species (ROS), which contributes to increasing the efficiency of cryopreservation and the quality of semen after thawing. Until now, the effects of antioxidants such as catalase (Taylor et al., 2009) and L-carnitine (Alcay et al., 2021) have been studied in relation to semen from drones. Studies have shown that the addition of catalase has no significant effect on drone sperm motility after thawing, while higher rates of sperm motility, plasma membrane integrity, and mitochondrial function were observed when 5 mM L-carnitine was added to the freezing extender.

Attempts are also being made to use compounds that protect sperm plasma membranes from cryogenic damage occurring during the cryopreservation process. The compounds used so far include (1) trehalose, which minimizes the degree of cell damage during the freeze–thaw process by increasing the osmolarity of the diluent solution (Nur et al., 2020), and (2) royal jelly, which contains about 50%–70% water, 9%–18% protein, 7%–18% carbohydrates, 3–8% fatty acids and lipids, and 1.5% minerals (Alcay et al., 2019 a). Therefore, they play a protective role in maintaining the stability of sperm plasma membranes. Experiments conducted by Nur et al. (2020) proved that adding 0.1 or 0.05 M trehalose to a freezing diluent containing 12% DMSO achieved higher motility and integrity of the plasma membranes of drone spermatozoa post-freeze–thaw compared to the control diluent. At the same time, studies by Alcay et al. (2019 a) have shown a beneficial effect of adding 1% royal jelly to the freezing diluent on drone quality parameters such as motility, integrity of plasma, and acrosome membranes after thawing. Taking into account the efforts undertaken to elaborate efficient technology for the cryopreservation of drones' semen, supplementing the freezing diluent with either bovine serum albumin (BSA) (Alcay et al., 2019 b) or soybean lecithin (Dadkhah et al., 2016) has also been attempted, which contributes to enhancing sperm motility and other quality parameters, such as the incidence of plasma membrane and acrosome integrities after thawing, compared to thawing without any of these additives. Considering the comparative analysis of the results achieved within the framework of the aforementioned studies (Taylor et al., 2009; Dadkhah et al., 2016; Alcay et al., 2019 a, b; Nur et al., 2020; Alcay et al., 2021) and presented in Table 1, it is noteworthy that the highest quality parameters (based on the assessment of post-thawed sperm motility) have been noticed for the cryopreservation of drone spermatozoa in the freezing diluents/extenders supplemented with either L-carnitine (Alcay et al., 2021) or trehalose (Nur et al., 2020).

The efficiency of cryopreservation of drone semen is finally measured with the production by each inseminated queen of an adequate number of worker bees, necessary for the proper functioning of the bee colony. According to the study by Collins (2004 a), a cryopreservation technique that ensures the preservation of at least 50% viable sperm would allow breeders to raise queen daughters with desirable genotypes from a breeding point of view. Stucky et al. (2008) showed that the addition of DMSO to the thinner freezing diluent makes it possible to achieve post-thaw sperm viability of more than 50%. Research by Dadkhah et al. (2016) confirmed that the cryopreservation of drone semen in a diluent containing chicken egg yolk and soy lecithin favorably affects the quality of sperm after thawing and allows 50% of live sperm to be obtained. Hopkins et al. (2012) found that the motility of sperm cryopreserved in diluent without a chicken egg was lower, and bee queens inseminated with such semen produced only drones. When drones' se-

men was cryopreserved using a diluent with both chicken egg yolk and 10% DMSO, a high sperm survival rate of 90% was achieved (Hopkins and Herr, 2010). However, the fertilization capacity of cryopreserved sperm drones was not evaluated in this study. In addition, in the cryopreservation of semen, the correct rate of cooling must be identified, which should take place at the optimal rate for the species; otherwise, sperm damage can occur (Harbo, 1979; Taylor et al., 2009; Hopkins and Herr, 2010). In the case of honey bee sperm, the controlled slow cooling rate is a prerequisite for obtaining adequate sperm quality after thawing. The first attempts to cryopreserve sperm of drones via slow cooling diluted semen (3–4°C/min) demonstrated a positive effect on sperm motility after thawing. However, mothers inseminated with such semen produced less than 50% of bee workers (Harbo, 1979; Hopkins and Herr, 2010).

Collectively, on the one hand, the search for methods of cryogenically preserving drone semen stems from the need to make the usefulness of drone semen for artificial insemination (AI) of particularly valuable queens wider and longer and to extend the queen production season. On the other hand, the elaboration of efficient approaches to cryogenic protection of honey bee spermatozoa results from the necessity of prolonged assisted sexual reproduction and augmented fertility/fecundity of drones derived from haploid (unfertilized) ova, honey bee larvae (brood) and pupae originating from high-genetic-merit mother queens. This can contribute to not only reproductive biotechnology mediated rescue of the *Apis mellifera* species in Poland and worldwide, but also increase in zoocenotic diversity among bee colony populations through perpetuating sustainability of biotopic and fitocenotic niches in the habitats of honey bee occurring in anthropogenic and agricultural ecosystems.

Conclusions and future targets

In Poland the number of bee colonies included in the genetic resources protection program steadily increased between 1999 and 2020, nevertheless, this number has been gradually decreasing year by year. Therefore, the implementation of an *ex situ* program based on the cryopreservation of drone semen for the conservation of native honey bee breeds becomes a necessity. The effective cryoconservation technique that allows the preservation of high-quality semen after the freeze–thaw procedure will facilitate/expedite the use of a controlled paternal component in the artificial insemination of queens, which will enable breeders to raise queen daughters with desirable genotypes (Paillard et al., 2017; Alcay et al., 2019 b).

Despite intensive research into developing an effective method of cryopreserving drones' semen, there are still no fully satisfactory results that meet the conditions for the practical application of this method. According to a comparative analysis of the results obtained in a variety of research (Taylor et al., 2009; Dadkhah et al., 2016; Alcay et al., 2019 a, b, 2021; Nur et al., 2020) it is worth highlighting that the highest quality parameters (related to the evaluation of post-thawed sperm motility) have been pinpointed for the cryoconservation of drone spermatozoa in the freezing diluents enriched either with L-carnitine (Alcay et al., 2021) or with trehalose (Nur et al., 2020).

The elaboration of optimal procedures for securing biological material is of particular importance in terms of the *in situ* protection program of native breeds in Poland. The current program encompasses monitoring and protecting honey bees in their natural enclaves, where they have resided thus far due to their genetic distinctiveness and unique characteristics of adapting to natural climatic and forage conditions (Leclercq et al., 2023; Fine et al., 2023).

Developing an effective method of cryopreserving drone semen will enable the creation of semen collections as an *ex situ* genetic reserve pool of honey bee populations in Poland. As the only biotechnological method allows storage for an indefinite period of time, ensuring the preservation of the genetic reserve and the transport of biological material over long distances, and thus the use of frozen semen for the insemination of queen bees, both at home and abroad.

Table 1. The approaches to cryopreservation of semen in honey bees

Supplementation of freezing diluents with:		The assessment of post-thawed sperm quality parameters	The efficiency of cryopreservation measured by sperm quality parameters	Reference
antioxidants (concentration)	cryoprotectants (concentration)			
Catalase (1.59 mM/L)	DMSO (10%)	Plasmalemma integrity		Taylor et al., 2009
L-Carnitine (5 mM/L)		Motility; plasmalemma, acrosome and DNA integrities, mitochondrial activity	Improvement of assessed parameters	Alcay et al., 2021
Trehalose (0.1 M/L)	DMSO (12%)	Motility; plasmalemma integrity		Nur et al., 2020
Royal jelly (1%)	DMSO (10%)	Motility; plasmalemma and acrosome integrities		Alcay et al., 2019 a
Bovine serum albumin (5.0 mg/mL)		Motility; plasmalemma and acrosome integrities		Alcay et al., 2019 b
Soybean lecithin (0.5%; 2%)		Plasmalemma integrity	Lack of improvement in assessed parameter	Dadkhah et al., 2016

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