

CRYOGENIC PRESERVATION OF HONEY BEE *APIS MELLIFERA* DRONE SEMEN – SELECTED BIOTECHNOLOGICAL SOLUTIONS AND RESEARCH PERSPECTIVES

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Abstract

*In honey bee *Apis mellifera* breeding in Poland, interest in the use of artificial insemination for queen reproduction is steadily increasing. Selection and the choice of maternal and paternal material for mating take into account performance traits such as productivity, gentleness and low swarming tendency, as well as winter hardiness and the adaptation of colony development to the environmental conditions in which the bees are kept. Demand for high-quality queens is increasing each year, because their reproductive and productive traits affect colony population growth and condition. Artificial insemination technology is currently based mainly on fresh semen; however, in the future, the use of cryopreserved semen may accelerate breeding progress and contribute to increased genetic diversity in the Polish honey bee population. This article discusses key biotechnological solutions and research perspectives involving the use of cryogenically preserved drone semen in honey bee breeding.*

Keywords: drone semen, artificial insemination, honey bee

Introduction

Honey bees, by pollinating more than 80% of cultivated and wild-growing plants, are an integral part of the ecosystem (Gül et al., 2017; Kucharczyk et al., 2017). It is estimated that one-third of the food consumed by humans depends, to a greater or lesser extent, on plant-pollinating insects (Zych et al., 2020). In recent years, however, substantial losses of bee colonies have been reported worldwide (Kucharczyk et al., 2017; Ben Abdelkader et al., 2018). Major causes include infectious diseases and diseases transmitted by ecto- and endoparasites, bacteria, mycoplasmas, fungi and viruses, the use of fertilizers and pesticides in agriculture (Ciereszko et al., 2017; Ben Abdelkader et al., 2018; Fisher et al., 2018; Bugin et al., 2022; Smilga-Spaltvina et al., 2023), environmental degradation, intensive monoculture farming and the expansion of urbanized areas (Gül et al., 2017; Kucharczyk et al., 2017; Fisher et al., 2018). The rapid decline in pollinating insects is increasingly associated with Colony Collapse Disorder (CCD), in which most worker bees leave food stores and the queen with only a few workers and fly away from the hive (Smilga-Spaltvina et al., 2023).

Actions aimed at protecting the honey bee population in Poland in the coming years should include not only maintaining but also increasing its current size, while improving colony

condition and the performance value of bees. Introducing queens of high breeding value into apiaries helps consolidate desirable traits in the population, including productivity, gentleness, low swarming tendency, winter hardiness and adaptation of colony development to local environmental conditions. Consequently, the number of apiaries implementing breeding programmes based on artificial insemination of queens is increasing. This technique is particularly important because, during mating, a queen copulates with several to more than a dozen drones (Withrow and Tarpy, 2018; Tarpy and Page, 2000). Under natural conditions, approximately 10% of each male ejaculate is transferred to the queen's oviducts (Brutscher et al., 2019) and mixed (Haberl and Tautz, 1998), making controlled selection of paternal material impossible. Artificial insemination alone enables controlled queen–drone mating, allowing breeders to determine the genetic quality of colonies, including breed purity or controlled inter-line and inter-breed crosses, and to select for important performance traits transmitted to subsequent generations.

Artificial insemination in the honey bee was initiated by Lloyd Watson in the 1930s (Cobey, 2016). Subsequent studies have identified factors that negatively affect its effectiveness, such as infections, an inappropriate semen dose and failure of the colony to accept the queen (Woyke, 1960; Woyke and Jasiński, 1982, 1990), as well as positive factors including queen quality, age at insemination, an appropriate semen dose and housing the queen with workers after insemination (Woyke, 1960; Woyke and Jasiński, 1976). Recent research has focused not only on freshly collected drone semen, but also on semen stored short-term (Harbo, 1977; Collins, 2000; Özkök and Selcuk, 2020) and cryopreserved semen (Harbo, 1979; Hopkins and Herr, 2010; Hopkins et al., 2012).

Cryopreservation of drone semen and its use for queen insemination

Cryopreservation of drone semen is the only biotechnological method that enables long-term storage of male honey bee genetic material for an unlimited period and transport of biological material over long distances, thereby permitting the use of frozen semen for queen insemination both domestically and internationally. Cryogenically preserved semen also plays an important role in safeguarding desirable gene pools against unforeseen threats such as bee disease epidemics, climate change and other environmental factors that may adversely affect bee numbers. The use of frozen drone semen provides flexibility in breeding management and reduces dependence on the seasonal availability of high-quality semen from sexually mature drones. An effective cryopreservation technology makes it possible to establish genetic resource banks that may become a key component of biodiversity conservation strategies, especially for small populations of native Polish breeds. Semen stored in liquid nitrogen may help rebuild populations with desirable traits and counteract allelic losses caused by parasites such as *Varroa destructor*, viral infections (Topolska et al., 2008; Pohorecka et al., 2011) or CCD. The use of cryopreserved semen from genetically diverse males may improve colony vitality and adaptive capacity and increase tolerance to genetically determined diseases (Tarpy, 2003; Tarpy and Seeley, 2006; Mattila and Seeley, 2007). Literature data indicate that disease incidence and colony mortality are lower in more genetically diverse populations (Tarpy, 2003; Mattila and Seeley, 2007).

Cryopreserved drone semen was first used for insemination in the late 1970s and early 1980s (Harbo, 1977, 1983; Kaftanoglu and Peng, 1984). This approach can extend the beekeeping season and allow rational control of reproduction and selection of maternal and paternal material, thereby supporting breeding progress and maintaining desirable genetic diversity without inbreeding (Paillard et al., 2017). The use of frozen drone semen remains limited, however, because no sufficiently effective and repeatable method for cryogenic preservation of this genetic material has been established and post-insemination results remain unsatisfactory (Cobey, 2016; Smilga-Spalsvina et al., 2023).

The principal indicators of the fertilizing capacity of frozen-thawed drone semen are the proportion and survival of female brood produced by inseminated queens and the number of spermatozoa reaching the spermatheca. In 1984, Kaftanoglu and Peng used drone semen prese

lected for high post-thaw sperm motility and obtained 56% female brood and 290,000 spermatozoa in the spermatheca, whereas Hopkins et al. (2012) reported brood survival of 50%. It should be noted that these studies did not include post-cryopreservation semen quality selection. The physical condition and reproductive capacity of queens after insemination are also influenced by procedures required for artificial insemination itself, including separation of queens from workers, carbon dioxide anaesthesia and the use of freezing extenders for drone semen. Queens inseminated with frozen-thawed semen often show reduced survival and may be superseded within two months, indicating that too few spermatozoa reached the spermatheca or that components of the freezing extender adversely affected the queen (Wegener et al., 2014).

In recent years, progress has been made in improving queen insemination success and the quality of cryopreserved drone semen by modifying freezing-extender composition and optimizing cryopreservation technology (Hopkins et al., 2012; Wegener and Bienefeld, 2012). The process includes selection of sexually mature males, semen collection, semen quality assessment, dilution, addition of cryoprotectants, packaging, freezing and post-thaw evaluation (Smilga-Spalvina et al., 2023). New technological solutions aim to reduce cryogenic sperm damage caused by low temperature, thermal shock and intracellular ice-crystal formation (Pegg, 2002). For drone spermatozoa, controlled slow cooling to approximately 4–5°C is a prerequisite for satisfactory post-thaw quality (Taylor et al., 2009; Hopkins and Herr, 2010). Early attempts using slow cooling at 3–4°C/min had a positive effect on post-thaw motility, and queens inseminated with such semen produced female brood at a rate of 50% (Harbo, 1979; Hopkins and Herr, 2010). Available evidence indicates that, after preliminary cooling, semen is packaged in 0.25-mL straws or 22-μL capillaries, then gradually cooled at no more than 3°C/min to –120°C and finally immersed in liquid nitrogen at –196°C (Harbo, 1983; Alcay et al., 2019). Drone semen should be thawed at 25–40°C over a period ranging from several seconds to one minute (Wegener and Bienefeld, 2012).

Protective compounds and antioxidants as components of freezing extenders

Cryopreservation causes functional and structural damage to spermatozoa, manifested by reduced motility, impaired plasma- and acrosomal-membrane integrity, increased sperm chromatin damage and decreased mitochondrial potential (Pegg, 2002; Alcay et al., 2022). During freezing and thawing, reactive oxygen species (ROS) are generated as a result of lipid peroxidation in the cytoplasmic membrane. The resulting free radicals adversely affect post-thaw sperm motility and consequently reduce the effectiveness of artificial insemination.

To protect spermatozoa against cryogenic damage, freezing extenders are supplemented with compounds that protect plasma membranes. Dimethyl sulfoxide (DMSO), glycerol, dimethylformamide, 1,3-propanediol, 2,3-butanediol and ethylene glycol have been tested in drone spermatozoa (Hopkins and Herr, 2010; Wegener and Bienefeld, 2012; Wegener et al., 2014; Alcay et al., 2022). Glycerol, commonly used in freezing extenders for semen of many livestock species, has not proved suitable for honey bee drone sperm. Harbo (1979) showed that glycerol damages drone sperm cell membranes during cryopreservation. DMSO, in contrast, has strong cryoprotective properties (Taylor et al., 2009); at 10% in the freezing extender it allows a high proportion of motile drone spermatozoa to be obtained (Hopkins and Herr, 2010). Stucky et al. (2008) reported more than 50% live sperm after thawing when DMSO was used. DMSO also did not adversely affect the proportion of sperm reaching the queen spermatheca after insemination. Hopkins and Herr (2010), however, observed that 15% DMSO reduced post-thaw sperm viability. Artificial insemination with cryopreserved semen in DMSO-containing extender may also be toxic to queens (Wegener and Bienefeld, 2012; Smilga-Spalvina et al., 2023). Some studies used hen egg yolk in addition to a cryoprotectant. Hopkins et al. (2012) showed that sperm motility was lower in extender without egg yolk and queens inseminated with such semen produced only drones. When semen was cryopreserved in extender containing both egg yolk and 10% DMSO,

post-thaw motility reached 90% (Hopkins and Herr, 2010). Because egg yolk is of biological origin and has a non-uniform composition, however, its use may contaminate semen, reduce repeatability of the technology and increase the risk of reduced queen fertility through oviduct obstruction and infection (Hopkins et al., 2012).

Recent work has focused intensively on modifying extenders with antioxidant compounds and substances protecting sperm plasma membranes. Such additives reduce the adverse effects of ROS and may improve cryopreservation efficiency and post-thaw semen quality. Available studies have examined catalase; supplementation at 1.59 mM/L increased the proportion of spermatozoa with intact cell membranes (Taylor et al., 2009). Alcay et al. (2021), however, found that catalase did not significantly affect post-thaw motility regardless of concentration, whereas supplementation with 5 mM L-carnitine increased both motility and the proportion of spermatozoa with intact plasma membranes and high mitochondrial potential. Other compounds tested in drone semen include trehalose, which minimizes cryogenic damage during freezing and thawing by increasing extender osmolarity (Nur et al., 2020), and royal jelly, which helps maintain sperm plasma-membrane stability (Alcay et al., 2019a). Nur et al. (2020) showed that supplementation with 0.1 or 0.05 M trehalose and 12% DMSO resulted in a higher proportion of motile sperm with high membrane integrity after thawing than a control extender without antioxidant. Alcay et al. (2019a) demonstrated beneficial effects of 1% royal jelly on post-thaw motility and plasma- and acrosomal-membrane integrity. Extenders supplemented with bovine serum albumin (BSA) (Alcay et al., 2019b) or soybean lecithin (Dadkhah et al., 2016) increased the proportion of motile spermatozoa to 50% and improved structural-functional parameters such as plasma- and acrosomal-membrane integrity compared with unsupplemented extenders. Comparing recent results (Taylor et al., 2009; Dadkhah et al., 2016; Alcay et al., 2019a,b; Nur et al., 2020; Alcay et al., 2021), the highest post-thaw motility values were reported with L-carnitine and trehalose supplementation.

Assessment of drone semen quality

One factor limiting successful honey bee reproduction is drone semen quality. Because a queen stores spermatozoa in the spermatheca for several years after artificial insemination, assessment of semen used for this procedure is particularly important: it determines the queen's reproductive success and, consequently, colony survival and productivity (Pettis et al., 2016). Factors affecting drone semen quality include drone age (Rhodes et al., 2011; Stürup et al., 2013; Rousseau et al., 2015), male body size (Schluns et al., 2003), breeding line (Rhodes et al., 2011; Rousseau et al., 2015), ambient temperature (Stürup et al., 2013; Czekonska et al., 2013), diet (Stürup et al., 2013), bee diseases (Collins and Pettis, 2001), insecticides (Ciereszko et al., 2017) and the preservation technology used (Taylor et al., 2009; Hopkins and Herr, 2010; Wegener et al., 2012; Hopkins et al., 2017).

Drone semen quality studies focus mainly on semen volume, sperm concentration and/or viability, and morphology (Wegener et al., 2012). The volume collected from a single drone ranges from 0.4 to 2.4 μ L. Sperm concentration is usually 2–9 million spermatozoa per 1 μ L and is assessed using a haemocytometer (Collins and Pettis, 2001; Rousseau et al., 2015) or spectrophotometer (Ciereszko et al., 2017). Concentration measurements show substantial variability because it is difficult to obtain a homogeneous and repeatable sperm dilution in both the sample and observation chamber (Collins, 2000; Koeniger et al., 2005).

Morphological assessment of drone spermatozoa has shown only a small proportion of abnormal forms. Reported defects include coiled, frayed or double-ended flagella (Lodesani et al., 2004). Only after freezing and thawing may the percentage of morphological abnormalities increase, mainly in the head region of dead spermatozoa (Paynter et al., 2014).

Motility is the basic semen-quality parameter in livestock. In honey bees it is assessed only sporadically (Yaniz et al., 2019), although Hopkins et al. (2017) showed that the proportion of motile spermatozoa is positively correlated with the effectiveness of queen artificial insemina-

tion. Most honey bee methods use a subjective 4–6-point scale corresponding to the proportion of motile sperm (Taylor et al., 2009; Nur et al., 2012; Dadkhah et al., 2016) or classify the percentage of spermatozoa showing a specific movement pattern (Wegener et al., 2012, 2014; Ciereszko et al., 2017; Yaniz et al., 2019). The most important indicator of high-quality drone semen is the proportion of motile spermatozoa exhibiting circular movement (Wegener et al., 2012; Ciereszko et al., 2017).

Another parameter is sperm viability, assessed on the basis of plasma- and acrosomal-membrane integrity. Methods use fluorescent dyes such as propidium iodide (PI), SYBR-14, Hoechst 33342 or acridine orange (Dadkhah et al., 2016; Alcay et al., 2015), or the hypo-osmotic swelling test (HOST) (Nur et al., 2012; Alcay et al., 2019). Acrosome integrity has also been evaluated using *Pisum sativum* agglutinin (PSA) lectin staining on fixed spermatozoa; intensely fluorescent acrosomes were considered normal, whereas weak or heterogeneous fluorescence indicated damage (Alcay et al., 2019a).

A limited number of reports indicate that objective assessment of drone semen motility and kinetic parameters is possible using computer-assisted sperm analysis (CASA) (Murray et al., 2022; Divasón et al., 2024). Flow cytometry can also be used to assess membrane and acrosome integrity, DNA fragmentation, mitochondrial potential and apoptotic changes (Peng et al., 2014; Paynter et al., 2014). Despite ongoing research, knowledge of these quality parameters remains much more limited than for other livestock species. The specific morphology and movement pattern of drone spermatozoa require customized protocols that define structural and functional parameters for this species and provide reliable, repeatable assessments.

Summary and research perspectives

The development of optimal procedures for preserving biological material is particularly important for the in situ protection of genetic resources of native breeds in Poland. The current programme includes monitoring and protecting honey bees in their natural enclaves because of their genetic distinctiveness and unique adaptation to local climatic and food conditions (Leclercq et al., 2023; Fine et al., 2023). Maintaining small honey bee populations using artificial insemination alone, however, creates a need for crossing with other bee lines (Gül et al., 2017). Developing an effective, repeatable drone-semen cryopreservation method and meeting the conditions for its practical application would enable the creation of semen collections as a unique ex situ genetic reserve for honey bee populations (Rajamohan et al., 2020; Gül et al., 2022; Oleksa et al., 2023). Effective cryopreservation would also maintain high semen quality after freezing and thawing and allow a controlled paternal component to be used in queen artificial insemination, enabling breeders to obtain daughter queens with desirable genotypes (Paillard et al., 2017).

In 2024, the Department of Reproductive Biotechnology and Cryopreservation of the National Research Institute of Animal Production initiated project No. 04-19-15-11 to develop both the composition of a freezing extender and a cryopreservation technology enabling effective cryogenic preservation of drone semen. The project will also develop customized protocols for automated post-thaw honey bee drone semen quality assessment using computer-assisted sperm analysis (CASA) and flow cytometry. These activities will define basic sperm-quality parameters, including the type and characteristics of sperm movement, ejaculate concentration, kinetic parameters, the proportion of motile spermatozoa, morphological and morphometric changes, as well as structural parameters including viability, plasma-membrane integrity, mitochondrial transmembrane potential and the degree of sperm chromatin fragmentation.

Objective assessment of cryopreserved honey bee drone semen based on a broad panel of structural and functional parameters will make it possible to determine its fertilizing capacity and suitability for artificial insemination.

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