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CRYOPRESERVED SEMEN IN THE EX SITU CONSERVATION OF EQUINE GENETIC RESOURCES

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Abstract

Ex situ conservation, alongside in situ conservation, is an important component of livestock genetic resources conservation. Cryopreserved biological material accumulated in gene banks creates a reserve gene pool that safeguards population biodiversity in emergency situations such as breed extinction or disease epidemics. In Poland, seven horse populations are covered by conservation programmes: Hucul, Silesian, Małopolski, Wielkopolski, Sokólski-type and Sztumski-type cold-blooded horses, and the Polish Konik. Although natural mating is required in the reproduction of horses included in genetic resources conservation programmes, in justified cases the collection and storage of frozen semen from selected stallions is recommended in order to maintain the continuity of breeding lines. For the stallion semen collection to be used effectively in the future as an element of breed conservation activities, attention must be paid not only to the quantity but also to the quality of the cryopreserved material. This article presents current research directions aimed at improving methods for protecting stallion semen against temperature-related damage during freezing and thawing, as well as insemination techniques that may be particularly useful in the reproduction of endangered horse breeds.

Keywords: horses, heritage breeds, stallion semen cryopreservation, biodiversity, ex situ conservation

Introduction

For the ex situ conservation of livestock genetic resources, the National Bank of Biological Material (NBBM) was established in 2014 at the National Research Institute of Animal Production. The key role of the NBBM is to collect and store cryopreserved biological material from breeds covered by the Genetic Resources Conservation Programme and from animals with outstanding production and breeding traits. The material accumulated at the NBBM constitutes a reserve gene pool in the event of factors threatening population biodiversity, such as breed extinction or animal disease epidemics. At present, seven horse populations in Poland are covered by genetic resources conservation programmes. These are the Hucul, Silesian, Małopolski, Wielkopolski, Sokólski-type and Sztumski-type cold-blooded horses, and the Polish Konik. Natural reproduction is mandatory in horse populations covered by genetic resources conservation programmes. Insemination with fresh or cooled semen, as well as insemination with

thawed semen, is permitted only in exceptional situations. However, in order to safeguard the continuity of male and female lines, in justified cases it is recommended to collect and store biological material in the form of frozen semen from selected stallions. For the stallion semen collection established at the NBBM to be used effectively in the future as part of in situ conservation activities, attention must be paid not only to the quantity but also to the quality of the cryopreserved material.

Although insemination with cryopreserved semen is routinely used in horse breeding and its advantages are widely recognized, its success is influenced by factors such as semen quality, the number of spermatozoa in the insemination dose, the selected insemination method, and preparation of the mare (Miller, 2008). The low cryotolerance of semen frequently observed in stallions, which causes a marked decline in post-thaw quality, limits the practical use of frozen semen, particularly in endangered horse breeds that are not selected for semen freezability (Smits et al., 2012).

Semen evaluation and preservation methods

The ejaculate of a mature warm-blooded stallion (over 6 years of age) has an average volume of 50–60 ml and can be divided into three fractions: the pre-ejaculatory fraction, the sperm-rich fraction, and the post-sperm fraction, whose function is to flush the preceding fractions from the urogenital tract. The daily amount of semen produced is influenced mainly by age, breed, testicular conformation and size, body condition, and the season in which semen is collected. In fully mature stallions at peak breeding condition (6–20 years of age), the number of spermatozoa per ejaculate reaches approximately 6.4 billion during the breeding season (May–August) and about 3.2 billion outside the season. Stallions reach peak seasonal sperm production in May and June. Stallions included in artificial insemination programmes usually provide semen every other day (Kosiniak-Kamysz, 2007; Tischner, 2010). According to Podico et al. (2023), the first and second ejaculates of the season, collected one hour apart, differed in sperm number but not in quality. Post-thaw semen quality also did not differ between the first and second ejaculates. Although mares were not inseminated, the results suggest that there is only a minimal difference between the first and second ejaculates collected from breeding-inactive stallions and that both are equally suitable for further processing for cooling or freezing.

The classical stallion Breeding Soundness Examination (BSE), introduced in 1975 in accordance with recommendations of the Society for Theriogenology, states that, for an individual to be considered a potentially satisfactory breeding stallion, at least one billion progressively motile, morphologically normal sperm (PMMNS) should be obtained in the second of two ejaculates collected one hour apart. Since these criteria were introduced, PMMNS has been a major component in assessing the potential suitability of ejaculates for insemination (Whitesell et al., 2020).

Stallion semen for BSE was initially evaluated using visual, subjective microscopic assessment. Stained preparations were examined with a conventional light microscope, whereas unstained smears were evaluated using phase-contrast microscopy. Sperm concentration was most commonly estimated with a spectrophotometer. With technological progress and the development of computerized systems, the possibilities for accurate and objective semen-quality assessment have increased substantially (Jasko et al., 1988; Varner, 2008).

Currently, the CASA system (computer-assisted sperm analysis) is widely used to analyse sperm motility, while differential interference contrast (DIC) microscopy is used to assess sperm morphology. Fluorescence-based technology and automated cell counters are used for precise concentration measurements (Whitesell et al., 2020). Flow cytometry is used to determine the degree of DNA fragmentation, cell-membrane integrity and intracellular enzyme ac-

tivity in spermatozoa, as well as for semen sexing (Bochenek, 2003). These methods help standardize semen-evaluation results and eliminate subjectivity on the part of the diagnostician. Applying the above methods when qualifying semen for freezing enables efficient work and repeatable results, which cannot be achieved with manual assessment (Egyptien et al., 2023).

Fresh, cooled, or frozen semen may be used for inseminating mares. Research is also being conducted on vitrification of stallion spermatozoa (Hidalgo, 2025). Although fresh semen provides the highest fertilization efficiency, in practice it is rarely used because of its short usable life and the resulting inability to transport it over long distances. Cooled semen is used much more frequently for insemination because storage time can be extended to as much as 72 hours. However, this is the most logistically demanding method. Only cryopreservation makes it possible to store semen for a very long, practically unlimited period and to use it for insemination even after the donor animal has died. This is of invaluable importance in biodiversity-conservation efforts involving ex situ protection of heritage breeds. Semen cryopreservation also helps prevent disease transmission between animals.

Because post-thaw survival of stallion spermatozoa depends on many factors and is difficult to predict, further work is needed to optimize freezing protocols and modify the composition of extenders and freezing media in order to obtain better results. This is difficult because stallions are selected for breeding on the basis of pedigree and their own or their offspring's performance rather than semen quality. In stallion semen, the main problem is low post-thaw sperm motility despite good fresh-semen quality before cryopreservation (Ponthier et al., 2012; Ponthier et al., 2013).

For many years, a popular line of research has been the development of tests predicting stallion semen quality after thawing (Ponthier et al., 2013; Ponthier et al., 2014; Martin-Cano et al., 2020; Gaitskell-Phillips et al., 2021a; Gaitskell-Phillips et al., 2021b; Dordas-Perpinya et al., 2022). Ponthier et al. (2013) and Pukazhenthi et al. (2014) consider that different extenders and freezing methods should continue to be tested to improve the quality of thawed semen from individuals whose semen shows poor tolerance to cryopreservation. According to Egyptien et al. (2023), when the recommended dose of progressively motile spermatozoa is used for insemination but pregnancy rates are unsatisfactory, semen diagnostics using advanced technologies such as flow cytometry or proteomic analysis are recommended.

Cryopreservation methods

Stallion semen is collected using an artificial vagina (open or closed), with the aid of a phantom or a teaser mare. Semen can also be collected by pharmacological induction or manual penile massage (Tischner, 2010). According to Egyptien et al. (2023), the collection method affects ejaculate volume and sperm concentration, but total sperm number depends on testicular production and collection frequency. Semen pH, peroxidative activity, and certain enzymes also affect semen quality and cryopreservation. Cells other than spermatozoa suspended in seminal plasma may also influence semen freezing.

Freshly collected stallion semen is usually filtered, initially evaluated, diluted and then subjected to further procedures as required. Centrifugation is routinely used before semen freezing, although stallion spermatozoa are highly sensitive to this procedure (Tischner, 2010). Semen is generally centrifuged after the addition of an extender designed to protect spermatozoa and prolong their viability. Centrifugation with an extender also makes it possible to use a higher g-force to maximize the percentage of sperm recovered after centrifugation without adversely affecting viability (Bradecamp, 2021). Iodixanol added to modern extenders is responsible for protecting spermatozoa during centrifugation. Recommendations for centrifuging stallion semen are 400 × g for 20 min without iodixanol and 1000 × g for 20 min with iodixanol (Kelley, 2023).

A number of commercial extenders for freezing stallion semen are currently available. Most are also used to prepare cooled semen and maintain ejaculate quality for 24 hours. After this period, sperm viability decreases (Batellier et al., 1997). Extenders usually contain sugars, electrolytes, buffers, egg yolk, milk, and dairy products (Ramires et al., 2015). Glucose in extenders increases sperm motility, while buffering agents prolong motility during storage (Dean et al., 2012). Egg yolk, milk and its components protect spermatozoa against cold shock (Manjunath, 2012). One of the most commonly used extenders is based on skim milk (Ramires et al., 2015). As a biological product with a rich, complex and non-uniform composition, milk contains components that are both beneficial and harmful to sperm survival. Milk components such as α-lactoglobulin negatively affect sperm motility, whereas β-lactoglobulin and caseinates have a positive effect (Pagl et al., 2006). Because of the variable effects of milk components, synthetic extenders with balanced, fully defined and constant compositions have been developed. They consist of substances clearly beneficial to spermatozoa and reduce possible batch-to-batch inconsistencies compared with skim milk (LeFrapper et al., 2010; Plante et al., 2015). The most commonly used commercial chemically defined extender is currently INRA 96. It is based on modified Hanks' salts supplemented with caseinates (Batellier et al., 1997; Hartwig et al., 2014; Ramires et al., 2015). New extenders have also been introduced in recent years, including the popular BotuSemen Gold, which contains sodium caseinate combined with cyclodextrin (Novello et al., 2020). Casein micelles are the active components intended to act as sperm protectants (Bergeron and Manjunath, 2006). Binding of seminal-plasma proteins and reduction of lipid loss from the sperm membrane create a mechanism that protects spermatozoa during storage in the extender (Manjunath, 2012). Cholesterol in BotuSemen Gold lowers the temperature at which the lipid phase transition occurs, maintaining fluidity at lower temperatures and consequently reducing damage to the plasma-membrane structure (Amann and Pickett, 1987).

Rečková et al. (2022) tested semen quality after using a milk-based extender and commercially available chemically defined extenders: Equi Pro, Equi Plus, INRA 96 and BotuSemen Gold. The best results were obtained with the skim-milk-based extender, INRA 96 and BotuSemen Gold, with the skim-milk-based extender performing better than some newer extenders.

After dilution and centrifugation, media containing cryoprotectants that allow spermatozoa to survive in liquid nitrogen must be used for cryopreservation. Skim milk, egg yolk and glycerol (2–5%) are the most commonly used cryoprotectants (Martin et al., 1979; Heitland et al., 1996; Sieme et al., 2016). In 2005, Alvarenga et al. carried out landmark studies that formed the basis for the development of a commercial stallion-semen cryopreservation medium, Botucrio. These studies showed that replacing glycerol with amides, which are less toxic to spermatozoa than glycerol, improved post-thaw motility and membrane integrity of stallion spermatozoa. Botucrio contains a relatively low concentration of glycerol (1%), as well as 4% methylformamide and 10% egg yolk. It is recommended as a suitable extender for semen with reduced freezability.

Because semen freezing is costly owing to the specialized equipment and qualified personnel required, as well as the need to transport stallions to centres where semen can be cryopreserved, specialized teams equipped with mobile laboratory freezing lines have appeared on the market. They provide on-site semen-freezing services at the stallion's location. Unfortunately, this alternative also generates high costs, which are borne by mare owners when paying for semen doses and often constitute a barrier to widespread use of frozen semen. Ferrer et al. (2020) proposed a solution that could reduce cryopreservation costs. Freezing semen transported under cooled conditions would allow ejaculates to be collected from stallions and the semen shipped to centralized facilities for further processing. They therefore tested three commercially available extenders for semen cooled before the freezing stage. The results showed

that once the cooled semen had arrived at the laboratory after transport, centrifugation and addition of freezing extender could be carried out at room or refrigeration temperature. Botucrio and EZ Mixin Cryomax LE were recommended for cryopreservation of semen prepared in this way.

Stallion semen can be frozen conventionally or in a fully automated manner. The conventional method involves freezing straws in liquid-nitrogen vapour over a nitrogen bath. It offers limited control over temperature decreases, which can damage spermatozoa if freezing is too rapid. In the automated method, programmable freezers are used to lower the temperature according to a defined time schedule. For example, IMV Technologies, L'Aigle, France, manufacturer of the Mini-Digitcool ZH 400 freezer, recommends cooling at 15°C per minute between +5°C and −10°C, followed by 10°C per minute between −10°C and −100°C. By contrast, the freezing protocol used by Aurich et al. (2020) recommends first cooling semen to 5°C at 0.3°C/min, then to −25°C within 3 minutes (10°C/min), and finally to −140°C at a cooling rate of 25°C/min. After removal from the freezing chamber, the straws should then be placed directly in liquid nitrogen.

Insemination of mares with frozen semen – methods and recommended dose

The effectiveness of insemination with frozen semen is influenced by proper ejaculate evaluation, appropriate dilution, and a well-selected semen cryopreservation protocol. Equally important are accurate determination of the insemination time based on assessment of ovulation in the mare and the correct choice of insemination technique and procedure.

Straws (8 × 0.5 ml) containing approximately 800 million total spermatozoa and at least 250 million progressively motile spermatozoa have traditionally been considered an appropriate single insemination dose of cryopreserved stallion semen (Pasch et al., 2024). International commercial recommendations for equid semen cryopreservation specify progressive sperm motility of 50–70% before freezing and 30–35% after thawing. These minimum fresh-semen quality requirements in conventional equine semen-freezing programmes are needed to obtain good post-thaw semen quality, with initial sperm motility being the most important variable. However, it has repeatedly been shown that the relationship between post-thaw sperm motility and in vivo stallion fertility is weak (Müller, 1987; Samper et al., 1991; Wilhelm et al., 1996; Kirk et al., 2005; Kuisma et al., 2006; Barrier-Battut et al., 2017). Aurich et al. (2020) report that the quality of semen accepted for insemination after thawing differs not only among individual stallions but also among breeds. Logistic regression analysis demonstrated that age and breed were the variables with the greatest influence on post-thaw semen quality. This suggests that cryotolerance of equine semen may have a genetic basis.

As semen doses from valuable stallions have become more expensive and unsatisfactory numbers of progressively motile spermatozoa after thawing are frequently encountered, mare-insemination techniques have advanced considerably over the past two decades. Efforts were made to minimize the single insemination dose, which resulted in the development of two methods using reduced sperm numbers (Lindsey et al., 2001, 2002). The principal technique allowing insemination doses with a reduced sperm content is deep-horn artificial insemination (DHAI), also referred to as rectally guided deep-horn insemination (RI) (Samper and Plough, 2010). This technique modifies traditional insemination into the uterine body: the insemination catheter is guided through the uterine body into the uterine horn ipsilateral to the expected or confirmed ovulation (Pasch et al., 2024; Samper and Plough, 2010). Semen can also be delivered hysteroscopically (hysteroscopic insemination, HI), in which semen is deposited deep in the uterus near the uterotubal papilla with the aid of an endoscope. These methods allow the effective single insemination dose to be greatly reduced to between 1 and 25 million spermato-

zoa, although doses containing fewer than 5 million spermatozoa are recommended to be delivered hysteroscopically (Samper and Plough, 2010). These techniques are recommended when the amount and quality of frozen semen from a particular stallion are limited or when a genetically valuable sire does not produce the expected quantity of semen with the desired survival rate. They also enable maximum use of the potential of individual ejaculates (Samper and Plough, 2010; Pasch et al., 2024).

Mares may also be inseminated with sex-sorted semen. In such cases, according to Lindsey et al. (2002) and Gibb et al. (2017), a method allowing insemination with a reduced sperm dose is recommended because lower semen quality can be expected as a result of substantial external manipulation of the ejaculate and the longer interval between semen collection and insemination. Semen sorted by flow cytometry is particularly attractive to breeders and for the conservation of endangered breeds because the desired sex of the foal can be selected already at the time of insemination. In 2001, Lindsey et al. investigated pregnancy rates in mares inseminated with fresh sorted semen. The rate was the same as that obtained with unsorted cryopreserved semen (37.5%). For sorted and cryopreserved semen, the pregnancy rate was only 13%. In 2017, Gibb et al. obtained a pregnancy rate of 27% after insemination with sex-sorted and cryopreserved semen. Research is continuing to improve this promising method. More recently, Samper et al. (2025) developed an improved process for sex-sorting equine semen. The results showed improved motility and viability and reduced sperm DNA fragmentation after sorting. Although a small, consistent increase in lipid peroxidation was observed in sorted spermatozoa, sperm quality during the first 24 hours after sex sorting was comparable with that of non-sexed cooled semen. Fertility rates for fresh semen did not differ between sex-sorted and conventional spermatozoa.

Effectiveness of frozen semen – pregnancy rates

There are few available data directly comparing fertilization success in horses according to the type of semen used – fresh, cooled, or frozen. Squires et al. (2006) reported pregnancy rates of 60% for fresh semen, 44% for cooled semen and 46% for frozen semen. Fresh stallion semen is considered the most effective for insemination. Mean pregnancy rates per cycle generally range from 60% to 76%, and values below 60% may indicate fertility problems in the stallion (Kelly, 2022). Similarly, an article published on The Horse website reported a mean pregnancy rate of 65–75% per cycle after insemination with fresh semen. Below this range, stallion fertility may be considered reduced (Vigouroux, 2024). Comparing results from three studies published in 1989, 2004 and 2014, respectively, pregnancy rates after insemination with fresh semen ranged from 76% to 83% (Vogelsang et al., 1989; Blanchard et al., 2004; Morrell et al., 2014). Haadem et al. (2015), in turn, showed that 77% of mares inseminated with fresh semen became pregnant in the first cycle. By comparison, effectiveness for cooled semen was 43%. Gáspárdy et al. (2020) obtained a 75.7% pregnancy rate per cycle using cooled semen, whereas Tanner and Barrell (2023) reported a lower pregnancy rate of 44% for cooled semen. These findings are consistent with earlier observations, in which the effectiveness of insemination with cooled semen ranged from 43% to 80% (Squires et al., 2006).

Pregnancy rates following the use of frozen semen are reported relatively infrequently in current scientific studies. According to Squires et al. (2006) and Tanner and Barrell (2023), they generally range from 40% to 60% per oestrous cycle in breeding programmes. A 2022 review described factors affecting the effectiveness of insemination with frozen semen, including semen quality, sperm number, timing of ovulation and technician experience. Effectiveness usually ranged from 30% to 50% per cycle (Kelly, 2022).

Summary

The dynamic development of reproductive techniques has enabled the creation of many advanced procedures that meet the expectations of horse breeders. Continued research on optimizing stallion semen cryopreservation protocols appears particularly necessary because of marked individual variation, although well-proven media and cryopreservation procedures are already available commercially. Biodiversity conservation of breeds covered by ex situ conservation programmes should be based on the most advanced methods capable of addressing most threats arising from declining populations of protected breeds. The best understood, most fundamental and universal of these methods is the storage of cryopreserved semen, which can safeguard genetic resources to a substantial extent for an unlimited period. Each of the reproductive biotechnology methods discussed may contribute to genetic-resources conservation, depending on its application to specific individual cases and needs.

References

Alvarenga M.A., Papa F.O., Landim-Alvarenga F.C., Medeiros A.S.L. (2005). Amides as cryoprotectants for freezing stallion semen: A review. Anim. Reprod. Sci., 89(1–4): 105–113.

Amann R.P., Pickett B.W. (1987). Principles of cryopreservation and a review of cryopreservation of stallion spermatozoa. J. Equine Vet. Sci., 7(3): 145–173.

Aurich J., Kuhl J., Tichy A., Aurich C. (2020). Efficiency of semen cryopreservation in stallions. Animals (Basel), 10(6): 1033; DOI: 10.3390/ani10061033.

Barrier-Battut I., Kempfer A., Lemasson N., Chevrier L., Camugli S. (2017). Prediction of the fertility of stallion frozen-thawed semen using a combination of computer-assisted motility analysis, microscopical observation and flow cytometry. Theriogenology, 15(97): 186–200.

Batellier F., Magistrini M., Fauquant J., Palmer E. (1997). Effect of milk fractions on survival of equine spermatozoa. Theriogenology, 48(3): 391–410.

Bergeron A., Manjunath P. (2006). New insights towards understanding the mechanisms of sperm protection by egg yolk and milk. Mol. Reprod., 73(10): 1338–1344; DOI: 10.1002/mrd.20565.

Blanchard T.L., Thompson J.A., Brinsko S. (2004). Mating mares on foal heat: A five-year retrospective study. Proceedings of the 50th Annual Convention of the American Association of Equine Practitioners; Denver, CO, USA. 4–8 December 2004, pp. 525–530.

Bochenek M. (2003). Sex sorting of semen and sperm quality assessment – the latest achievements of flow cytometry. Biotechnologia, 1(60): 106–115.

Bradecamp E.A. (2021). Centrifugation of semen: cushion technique. In: Equine Reproductive Procedures. Dascanio J.J., McCue P.M. (eds), pp. 557–560.

Dean C.J., Hobgood A.M., Blodgett G.P., Love C.C., Blanchard T.L., Varner D.D. (2012). The addition of ticarcillin-clavulanic acid to INRA 96 extender for stallion semen cooling. Equine Vet. J. Suppl., (43): 95–99.

Dordas-Perpinya M., Yanez-Ortiz I., Sergeant N., Mevel V., Bruyas J.F., Catalan J., Delehedde M., Briand-Amirat L., Miro J. (2022). ProAKAP4 concentration is related to sperm motility and motile sperm subpopulations in frozen-thawed horse semen. Animals (Basel), 12(23): 3417.

Egyptien S., Deleuze S., Ledeck J., Ponthier J. (2023). Sperm quality assessment in stallions: How to choose relevant assays to answer clinical questions. Animals (Basel), 13(19): 3123; DOI: 10.3390/ani13193123.

Ferrer M.S., Canisso I.F., Ellerbrock R.E., Podico G., Lister B.N., Hurley D.J., Kline K., Palomares R.A. (2020). Optimization of cryopreservation protocols for cooled-transported stallion semen. Anim. Reprod. Sci., 221: 106581; DOI: 10.1016/j.anireprosci.2020.106581.

Gaitskell-Phillips G., Martin-Cano F.E., Ortiz-Rodriguez J.M., Silva-Rodriguez A., Gil M.C., Ortega-Ferrusola C., Pena F.J. (2021a). In stallion spermatozoa, superoxide dismutase (Cu-Zn) (SOD1) and the Aldo-keto-reductase family 1 member b (AKR1B1) are the proteins most significantly reduced by cryopreservation. J. Proteome Res., 20(5): 2435–2446.

Gaitskell-Phillips G., Martin-Cano F.E., Ortiz-Rodriguez J.M., Silva-Rodriguez A., Gil M.C., Ortega-Ferrusola C., Pena F.J. (2021b). Differences in the proteome of stallion spermatozoa explain stallion-to-stallion variability in sperm quality post-thaw. Biol. Reprod., 104(5): 1097–1113.

Gáspárdy A., Renkó E., Somoskői B., Bába A., Cseh S. (2020). Practical experience with artificial insemination (AI) using fresh chilled and frozen semen in mares. Acta Vet. Hung., 68(1): 85–90; DOI: 10.1556/004.2020.00007.

Gibb Z., Grupen C.G., Maxwell W.M.C., Morris L.H.A. (2017). Field fertility of liquid stored and cryopreserved flow cytometrically sex-sorted stallion sperm. Equine Vet. J., 49(2): 160–166; DOI: 10.1111/evj.12583.

Haadem C.S., Nødtvedt A., Farstad W. Thomassen R. (2015). A retrospective cohort study on fertility in the Norwegian Coldblooded trotter after artificial insemination with cooled, shipped versus fresh extended semen. Acta Vet. Scand., 14: 57–77.

Hartwig F.P., Lisboa F.P., Hartwig F.P., Monteiro G.A., Maziero R.R.D., Freitas-Dell'Aqua C.P., Alvarenga M.A., Papa F.O., Dell'Aqua Jr J.A. (2014). Use of cholesterol-loaded cyclodextrin: an alternative for bad cooler stallions. Theriogenology, 81(2): 340–346.

Heitland A.V., Jasko D.J., Squires E.L., Graham J.K., Pickett B.W., Hamilton C. (1996). Factors affecting motion characteristics of frozen-thawed stallion spermatozoa. Equine Vet. J., 28(1): 47–53; DOI: 10.1111/j.2042-3306.1996.tb01589.x.

Hidalgo M. (2025). Sperm vitrification in horses and donkeys. J. Equine Vet. Sci., 145, 105340; DOI: 10.1016/j.jevs.2024.105340.

Jasko D.J., Little T.V., Smith K., Lein D.H., Foote R.H. (1998). Objective analysis of stallion sperm motility. Theriogenology, 30(6): 1159–1167; DOI: 10.1016/0093-691x(88)90291-9.

Kelley D.E. (2023). Processing stallion semen for on farm use and cooled transport. Equine Veterinary Education, 36(1): 12–41

Kelly G. (2022). Breeding with frozen semen: what are the considerations? UK-Vet Equine. 6(2): 68–71; DOI.10.12968/ukve.2022.6.2.68

Kirk E.S., Squires E.L., Graham J.K. (2005). Comparison of in vitro laboratory analyses with the fertility of cryopreserved stallion spermatozoa. Theriogenology, 64(6):1422–1439.

Kosiniak-Kamysz K. (2007). Biology and preservation of stallion semen. In: Biological determinants of male reproductive value, Strzeżek J. (ed.), pp. 99–129.

Kuisma P., Andersson M., Koskinen E., Katila T. (2006). Fertility of frozen-thawed stallion semen cannot be predicted by the current laboratory methods. Acta Vet. Scand., 48: 14.

LeFrapper L., Walston L., Whisnant C.S. (2010). Comparison of various extenders for storage of cooled stallion spermatozoa for 72 hours. J. Equine Vet. Sci., 30(4): 200–204.

Lindsey A.C., Bruemmer J.E., Squires E.L. (2001). Low dose insemination of mares using non sorted and sex-sorted sperm. Anim. Reprod. Sci.; 68(3–4): 279–289; DOI: 10.1016/s0378-4320(01)00165-8.

Lindsey A.C., Morris L.H., Allen W.R., Schenk J.L., Squires E.L., Bruemmer J.E. (2002). Hysteroscopic insemination of mares with low numbers of nonsorted or flow sorted spermatozoa. Equine Vet. J., 34(2): 128–132; DOI: 10.2746/042516402776767178.

Manjunath P. (2012). New insights into the understanding of the mechanism of sperm protection by extender components. Anim. Reprod., 9(4): 809–815.

Tischner M. (2010). Veterinary and breeding aspects of horse reproduction – Stallion. University of Agriculture in Kraków Publishing House, pp. 41–57, 109–130, 148–159.

Martin-Cano F.E., Gaitskell-Phillips G., Ortiz-Rodriguez J.M., Silva-Rodriguez A., Roman A., Rojo-Dominguez P., Alonso-Rodriguez E., Tapia J.A., Gil M.C., Ortega-Ferrusola C., Pena F.J. (2020). Proteomic profiling of stallion spermatozoa suggests changes in sperm metabolism and compromised redox regulation after cryopreservation. J. Proteom., 221: 103765.

Miller C.D. (2008). Optimizing the use of frozen–thawed equine semen. Theriogenology, 70(3): 463–468; DOI:10.1016/j.theriogenology.2008.04.037.

Morrell J.M., Richter J., Martinsson G., Stuhtmann G., Hoogewijs M., Roels K., Dalin A.M. (2014). Pregnancy rates after artificial insemination with cooled stallion spermatozoa either with or without single layer centrifugation. Theriogenology, 82(8): 1102–1105; DOI: 10.1016/j.theriogenology.2014.07.028.

Müller Z. (1987). Practicalities of insemination of mares with deep-frozen semen. J. Reprod. Fertil. Suppl., 35: 121–125.

Novello G., Podico G., Segabinazzi L., Lima F.S., Canisso I.F. (2020). Stallion semen cooling using native phosphocaseinate-based extender and sodium caseinate cholesterol-loaded cyclodextrin-based extender. J. Equine Vet. Sci., 92: 103104; DOI: 10.1016/j.jevs.2020.103104.

Pagl R., Aurich J.E., Müller-Schlösser F., Kankofer M., Aurich C. (2006). Comparison of an extender containing defined milk protein fractions with a skim milk-based extender for storage of equine semen at 5 degrees C. Theriogenology, 66(5): 1115–1122.

Pasch L., Stefanovski D., Dobbie T., Lewis G., Turner R.M. (2024). Factors affecting pregnancy rates in mares bred with cryopreserved semen. J. Equine Vet. Sci., 141: 105167; DOI: 10.1016/j.jevs.2024.105167.

Plante G., Lusignan M.F., Lafleur M., Manjunath P. (2015). Interaction of milk proteins and Binder of Sperm (BSP) proteins from boar, stallion and ram semen. Reprod. Biol. Endocrinol., 13: 92.

Podico G., Spencer K.M., Magalhaes H.B., Canisso I.F. (2023). Semen quality of the first and second ejaculates collected from breeding inactive stallions after cooling and freezing. Vet. Sci., 10(3): 173; DOI: 10.3390/vetsci10030173

Ponthier J., Desvals M., Franck T., de la Rebiere de Pouyade G., Spalart M., Palmer E., Serteyn D., Deleuze S. (2012). Myeloperoxidase in equine semen: Concentration and localization during freezing processing. J. Equine Vet. Sci., (32): 32–37.

Ponthier J., Teague S.R., Franck T.Y., de la Rebiere G., Serteyn D.D., Brinsko S.P., Love C.C., Blanchard T.L., Varner D.D., Deleuze S.C. (2013). Effect of non-sperm cells removal with single-layer colloidal centrifugation on myeloperoxidase concentration in post-thaw equine semen. Theriogenology, 80(9): 1082–1087; DOI: 10.1016/j.theriogenology.2013.08.009.

Ponthier J., Franck T., Parrilla-Hernandez S., Niesten A., de la Rebiere G., Serteyn D., Deleuze S. (2014). Concentration, activity and biochemical characterization of myeloperoxidase in fresh and post-thaw equine semen and their implication on freezability. Reprod. Domest. Anim., 49(2): 285–291.

Ramires N.C., Sancler -Silva Y.F.R., Resende H.L., Guasti P.N., Monteiro G.A., Papa P.M. et al. (2015). Control methods and evaluation of bacterial growth on fresh and cooled stallion semen. J. Equine Vet. Sci., 35(4): 277–282.

Rečková Z., Filipčík R., Soušková K., Kopec T., Hošek M., Pešan V. (2022). The efficiency of different types of extenders for semen cooling in stallions. Anim Biosci., 35(5): 670–676; DOI: 10.5713/ab.21.0300.

Samper J.C., Plough T. (2010). Techniques for the insemination of low doses of stallion sperm. Reprod. Domest. Anim., 2: 35–39; DOI: 10.1111/j.1439-0531.2010.01632.x.

Samper J.C., Hellander J.C., Crabo B.G. (1991). Relationship between the fertility of fresh and frozen stallion semen and semen quality. J. Reprod. Fertil. Suppl., 44: 107–114.

Samper J.C., Hernandez Aviles J.C., Ramirez-Agamez L.F., Love C.C., Gonzalez-Marin C., Fleury P., Dini P., De La Fuente A., Foss R., Campos F.L., Ross P.J. (2025). The use of sex-sorted semen in horses. J Equine Vet. Sci., 145:105251; DOI: 10.1016/j.jevs.2024.105251.

Sieme H., Oldenhof H., Wolkers W.F. (2016). Mode of action of cryoprotectants for sperm preservation. Anim. Reprod. Sci., 169: 2–5. DOI: 10.1016/j.anireprosci.2016.02.004.

Smits K., Hoogewijs M., Woelders H., Daels P., Van Soom A. (2012). Breeding or assisted reproduction? Relevance of the horse model applied to the conservation of endangered equids. Reprod. Domest. Anim., 4: 239–248; DOI: 10.1111/j.1439-0531.2012.02082.x

Squires E., Barbacini S., Matthews P., Byers W., Schwenzer K., Steiner J., Loomis P. (2006). Retrospective study of factors affecting fertility of fresh, cooled and frozen semen. Equine Vet. Educ., 18(2): 96–99.

Tanner J.C., Barrell GK. (2023). Reproductive performance of a cohort of Standardbred mares under a commercial breeding system. Equine Vet. J., 56(4): 776–785; DOI: 10.1111/evj.13989.

Tischner M. (2010). Weterynaryjne i hodowlane aspekty rozrodu koni – Ogier. Wyd. URK, ss. 41–57, 109–130, 148–159.

Varner D.D. (2008). Developments in stallion semen evaluation. Theriogenology, 70(3): 448–462; DOI:10.1016/j.theriogenology.2008.04.023.

Vigouroux L. (2024). The Horse. Improving Equine Fertility; https://thehorse.com/1108873/improving-equine-fertility

Vogelsang M.M., Vogelsang S.G., Lindsey B.R., Massey J.M. (1989). Reproductive performance in mares subjected to examination by diagnostic ultrasound. Theriogenology, 32(1): 95–103; DOI: 10.1016/0093-691X(89)90525-6.

Whitesell K., Stefanovski D., McDonnell S., Turner R. (2020). Evaluation of the effect of laboratory methods on semen analysis and breeding soundness examination (BSE) classification in stallions. Theriogenology, 142: 67–76. DOI: 10.1016/j.theriogenology.2019.09.035.

Wilhelm K.M., Graham J.K., Squires E.L. (1996). Comparison of the fertility of cryopreserved stallion spermatozoa with sperm motion analyses, flow cytometric evaluation, and zona-free hamster oocyte penetration. Theriogenology, 46(4): 559–578

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THE USE OF CRYOPRESERVED SEMEN IN THE *EX SITU* CONSERVATION OF EQUINE GENETIC RESOURCES

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SUMMARY

Ex situ conservation, alongside *in situ* conservation, is a crucial element of livestock genetic resources conservation. Cryopreserved biological material collected in gene banks constitutes a reserve gene pool that safeguards the biodiversity of populations in emergency situations, such as breed extinction or disease epidemics. In Poland, seven horse populations are protected: Małopolski, Wielkopolski, Silesian, Sokólski, Sztumski, Hucul and Polish Konik. Although natural mating is required for the reproduction of these breeds, in justified cases it is recommended to obtain and store frozen semen from selected stallions in order to maintain the continuity of breeding lines. In order for the collection of stallion semen to be effectively used in the future as part of breed conservation efforts, it is necessary to pay close attention not only to the quantity but also to the quality of the cryopreserved material. This article presents current research directions in improving cryogenic preservation of stallion semen and the development of insemination techniques that may be particularly useful in the reproduction of endangered horse breeds.

Keywords: horses, heritage breeds, stallion semen cryopreservation, biodiversity, *ex situ* conservation