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PERSPECTIVE

CHALLENGES IN BIRD CRYOPRESERVATION

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Abstract

Cryopreservation is a fundamental technique for preserving the structural and functional integrity of biological material, particularly for the conservation of genetic resources in avian species. Since its development in the 1940s, this technology has advanced significantly, although challenges persist, primarily due to the unique morphology of avian sperm, which complicates cryoprotectant penetration and increases the risk of structural damage. Overcoming these challenges is crucial for improving semen preservation, supporting the sustainability of avian species, and contributing to conservation efforts. In domestic production birds, cryopreservation is essential for maintaining genetic diversity. However, these species often exhibit low tolerance to the freezing process, primarily due to the high concentration of polyunsaturated fatty acids in their sperm membranes, making them susceptible to oxidative damage. This has driven research aimed at developing more effective cryoprotectants and techniques to enhance semen quality post-thaw. Wild birds, particularly endangered species, face additional challenges in cryopreservation. These species are often managed in captivity to prevent extinction, with artificial insemination serving as a valuable tool. However, artificial insemination is constrained by low post-thaw motility rates, even when advanced cryoprotectants are employed. Research indicates that certain cryopreservation media can improve sperm motility and fertility rates, although further optimization of these methods is required. The future of avian semen cryopreservation will concentrate on customizing extenders and cryoprotectants, optimizing freezing techniques, and improving post-thaw semen quality. These advancements are essential for enhancing commercial poultry production and for the conservation of endangered species. Research in this area is expected to evolve over the next decade, developing effective solutions to address both commercial and conservation needs.

Keywords: domestic birds; wild birds; raptors; cryoprotectants; semen extenders.

INTRODUCTION

Cryopreservation, the process of freezing biological material at extremely low temperatures, is a critical technique for halting biological and biochemical processes in cells and tissues, thereby preserving their structural and functional integrity over extended periods (1). Since its inception in the 1940s, when fertilization of chicken eggs was successfully achieved using cryopreserved rooster semen, this technology has undergone significant

advancements. Identifying glycerol as an effective cryoprotectant laid the foundation for further research in avian semen cryopreservation (2).

The significance of avian semen cryopreservation extends beyond assisted reproduction; it plays a pivotal role in conserving genetic resources, particularly for endangered species such as cranes, penguins and raptors. Additionally, in poultry production, cryopreservation is a strategic tool to mitigate risks associated with the emergence of infectious diseases, such as avian influenza, which can decimate entire poultry populations.

However, despite its advantages, achieving high-quality semen cryopreservation for artificial insemination (AI) remains a significant global challenge. This difficulty is primarily due to the unique morphology of avian sperm. The elongated, cylindrical heads reduce the efficiency of cryoprotectant osmosis, while the longer, more delicate tails are susceptible to

Table 1. Diluents and cryoprotectants used with best results in domestic avian semen cryopreservation.

Species	Semen-to-diluent + cryoprotectant ratio	Viability of thawed semen[1]	Thawed semen motility[1]	Stabilization cooling	Freezing	Thawing	Ref
Rooster, *Gallus gallus*	1:1 BPSE diluted 1:3 with 6% DMA + 0.5 recombinant RhoA protein and 1 µg / mL.	+20.71%	+20.93%	Cooling at 5°C for 2 h	From 5°C to -35°C (7°C/min) and from -35°C to -120°C (9°C/min)	Water-bath at 37°C	(3)
	1:1 (v/v) Lake + 8% Glycerol+ BSA (10 mg / mL).	-2.45%	+24.8%	Cooling at 5°C	From 5°C to -35°C at 7°C/min, and then from -35°C to -140°C 60°C/min	Water-bath at 5°C with contin-uous agitation for 3 min	(5)
	BPSE + 0.5% DMSO+ 0.04% phosphorus + B12 vitamin+ 3% glycerol + 0.5% DMFA	+5.3%	+4.6%	Loading into 0.25 mL French straw and incubation for 15 min in cooled tray	LN vapor for 7 min, then immersion in LN	Water-bath at 5°C for 3 min	(6)
	Lake + ficoll 10% + DMA 6%	-1.8%	-13.3%	25°C	Cooled at of 1.6°C/min, in nitrogen vapor (-70°C) for 10 min; then immersion in LN	Water-bath 37.5°C for 30 s	(7)
Turkey, *Meleagris gallopavo*	1:4 Tselutin + 8% DMA (v/v; 0.860 M)	-50%	-34%	Cooling at 4°C for 60 min	80 µL drops of semen directly into LN to form pellets	Cryovials with pellets immer-sed in water-bath at 75°C for 12 s	(8)
	1:1 Lake + 20% DMSO + ficoll 70 (1 mM)	-44.4%	-46.4%	Cooling at 4°C for 20 min	LN vapor for 10 min. Then immersion in LN	Water-bath at 50°C for 10 s	(9)

BPSE: Beltsville Poultry Semen Extender; DMA: Dimethylacetamide; DMSO: Dimethyl sulfoxide; BSA: Bovine serum albumin; DMFA: Dimethylformamide; CSD: Crane semen extender; GPBM: Glutamate-polyvinylpyrrolidone-based medium. [1]: Compared to the values of fresh semen (prior to freezing).

breakage. Additionally, the high concentration of polyunsaturated fatty acids in the sperm membranes increases susceptibility to lipid peroxidation, heightening the risk of structural damage (2, 3, 4).

Traditional cryoprotectants like glycerol may be less effective in penetrating the elongated heads of avian sperm. This limitation, coupled with the fragility of the sperm tails, results in suboptimal post-thaw survival and fertility rates. Despite substantial advancements in avian semen cryopreservation, the current limitations in cryoprotectant penetration and sperm survival represent a major hurdle in achieving high-quality preservation. The search for more effective antioxidant solutions is a key area of research. Additionally, commonly used cryoprotectants (CPAs) such as glycerol and dimethylacetamide have shown contraceptive or even toxic effects on avian sperm, limiting their effectiveness. The survival of gametes after cryopreservation largely depends on the type and concentration of CPAs introduced before freezing. Although various protocols have been developed using compounds such as dimethyl sulfoxide (DMSO) and ethylene glycol (EG), these have not eliminated variations in post-thaw fertility (4). Thus, identifying less harmful cryoprotectants is crucial for advancing this field and to ensuring the long-term viability of avian genetic resources and supporting conservation efforts.

The cryopreservation of rooster sperm represents a significant challenge in the conservation of genetic diversity in both domestic and wild birds. Unlike other animals, freezing fertilized eggs is not viable in birds, making sperm cryopreservation the only available option for preserving sex cells. However, this process is associated with extremely variable fertility, highlighting the need to improve current techniques.

This perspective aims to explore the current challenges in semen cryopreservation in birds and discuss potential future directions to enhance the efficacy of these techniques, with the ultimate goal of ensuring the sustainability of avian species and supporting strategies for rescuing endangered species.

CRYOPRESERVATION OF DOMESTIC BIRDS

Rooster (Gallus gallus)

Recent research in rooster cryopreservation suggests that the addition of proteins such as bovine serum albumin (BSA) and egg yolk may mitigate some negative effects of cryopreservation, but these findings still require further validation in different experimental contexts (4, 5). On the other hand, the addition of recombinant RhoA protein, a GTPase involved in sperm capacitation, has shown promising results. This compound has demonstrated the ability to maintain sperm structural integrity and improve motility after thawing. However, its impact on acrosomal membrane integrity remains limited, suggesting that further research is needed to optimize its use (3).

Supplementation with phosphorus and vitamin B12 has been shown to improve sperm motility and viability post-thaw by maintaining crucial energy levels and protecting cell membranes. Phosphorus supports energy metabolism and membrane integrity, while vitamin B12 acts as an antioxidant, reducing oxidative damage. These benefits open the door to developing more effective semen extenders (6).

Finally, adjusting the viscosity of the extender for species-specific needs helps create a more stable environment for the sperm, reducing their free movement and minimizing the risk of mechanical damage, such as tail breakage or head deformation. Additionally, it reduces energy expenditure and helps buffer the osmotic and mechanical changes that can harm the sperm during the cryopreservation process (7).

Turkey (*Meleagris gallopavo*)

Rooster and turkey sperm share a similar morphology, characterized by a filiform shape, a long tail, and a condensed nucleus. However, turkey sperm exhibit differences in their composition, including a higher proportion of cholesterol/phospholipids in the membrane, which reduces its fluidity and permeability, making them less resistant to the hypoosmotic conditions encountered during cryopreservation.

A study employing the pellet freezing technique, using a 1:4 dilution in Tselutin diluent with 8% dimethylacetamide (DMA), revealed a significant decrease in semen quality after thawing. Moreover, changes in amino acid

composition were observed: the concentrations of alanine, isoleucine, leucine, phenylalanine, tyrosine, and valine were reduced in frozen and thawed sperm, while levels of glycine, citrate, and acetyl-carnitine increased. These metabolic changes may be linked to oxidative stress and cellular damage processes induced during cryopreservation (8).

In contrast, a study using Lake medium with dimethyl sulfoxide (DMSO) and Ficoll achieved better results in turkey semen cryopreservation. Semen frozen under these conditions exhibited improved progressive motility, and with a concentration of 400 × 10^6 spermatozoa/mL, a fertility rate of 87.2% was achieved after AI. These results highlight the importance of medium composition and freezing techniques in improving cryopreservation efficiency in turkeys (9). The semen extenders, their composition and the main results regarding sperm parameters during cryopreservation of sperm of production birds are shown in Tables 1 and 4.

CRYOPRESERVATION OF WILD BIRDS

Cranes (*Grus americana*; *Grus vipio*)

Table 2. Diluents and cryoprotectants used with best results in wild avian semen cryopreservation.

Species	Semen-to-diluent + cryoprotect-ant ratio	Viability of thawed semen[1]	Thawed semen motility[1]	Stabiliz-ation cooling	Freezing	Thawing	Ref
Cranes, *Grus americana, Grus vipio*	1:2 CSD + DMSO	-38.5%	-41.5%	Cooled to 4°C for up to 30 min	From 4°C to -30°C (at a cooling rate of 7°C / min) and from -30°C to -110°C (at a cooling rate of 9°C / min)	Water bath at 37°C	(2)
Cantabrian capercaillie, *Tetrao urogallus cantabricus*	1:1 Lake + 8% Glycerol	-13%	-60%	Cooled to 5°C for 10 min	From 5°C to -35°C at a rate of 7°C / min, and then from -35°C to -140°C at a rate of 60°C / min	Water bath at 5°C for 30 s	(10)
Pheasants, *Phasianus colchicus mongolicus*	1:3 Lake +6% DMA	-79.8%	-85.2%	Cooled to 5°C for 10 min	80 µL drops in a liquid nitrogen bath, then stored 6 to 7 pellets each	One pellet at a time on a heating plate at 50°C	(11)
Arabian bustard, *Ardeotis arabs*	1:1 Lake 7.1+ 6% DMA	-37.4%	-22.2%	Cooled to 4°C for 24h	The semen was frozen drop by drop in liquid nitrogen to form pellets	The pellets were placed on a temper-ature-controlled heating plate at 60°C	(12)
Black-footed penguins, *Spheniscus demersus*	1:1 GBPM	+9.81%	-26.54	Cooled to 5°C for 10 min	Straws in nitrogen vapor 5 cm above the surface of a liquid nitrogen bath for 10 min. Slow freezing: from 5°C to -85°C at a rate of 10°C / min	Water bath at 5 °C for 3 min	(13)
Gentoo penguins, *Pygoscelis papua*		+2.49%	-31.19			Water bath at either 37 °C for 30 s, or 5 °C for 3 min	

DMA: Dimethylacetamide; CSD: Crane semen extender; GPBM: Glutamate-polyvinylpyrrolidone-based medium. [1]: Compared to the values of fresh semen (prior to freezing).

Currently, there are 15 crane species worldwide, of which 11 are categorized as vulnerable or endangered by the International Union for Conservation of Nature (IUCN). All these species are under captive management due to increasing threats to their natural habitats and wild populations. This *ex situ* management has become an essential strategy for preserving genetic diversity and protecting against extinction. AI using fresh semen has been a valuable tool to overcome pairing incompatibility issues in species such as the whooping crane (*Grus americana*) and the white-naped crane (*Grus vipio*). However, cryopreservation of semen from these birds presents significant challenges. The incorporation of DMSO as a cryoprotectant has improved sperm behavior during cryopreservation. Despite these advancements, whooping crane sperm shows low tolerance to freezing and thawing, with post-thaw motility ranging between 6% and 15%. Although cryopreserved sperm from both species has retained functionality, the low post-thaw motility remains a significant limitation.

These challenges highlight the need to optimize cryopreservation techniques and cryoprotectants to improve crane semen viability. Future research should focus on developing more effective methods to preserve semen quality and increase the success rate of AI in these endangered species. Innovation in cryopreservation is crucial not only for the conservation of cranes in captivity but also for the restoration of wild populations and the protection of global biodiversity.

Cantabrian capercaillie (Tetrao urogallus cantabricus)

The Cantabrian capercaillie is an endangered species due to its small population size, loss of genetic diversity, predator pressure, climate change, and hunting. This bird is found in the deciduous forests of the Cantabrian Mountains in Spain. Research on its cryopreservation has evaluated the use of common cryoprotectants used in domestic birds, finding that a combination of Lake medium with 8% glycerol improved sperm motility and reduced morphological abnormalities (10).

Mongolian pheasant (Phasianus colchicus mongolicus)

The Mongolian pheasant is another endangered species whose reproduction is managed in captivity in zoos. The pellet freezing technique has been used for this species' semen; however, this method has shown a low survival

Table 3. Diluents and cryoprotectants used with best results in raptor semen cryopreservation.

Species	Semen-to-diluent + cryoprotectant ratio	Viability of thawed semen[1]	Thawed semen motility[1]	Stabilization cooling	Freezing	Thawing	Ref
Peregrine falcons. *Falcon peregrinus peregrinus*	1:1 Lake 7.1+ 8% DMSO	-34.3	-3.6	Cooled to 5°C for 10 min	From 5 to -35 °C at a rate of 7 °C / min; then from -35 °C to -140 °C at 60° C / min.	Water bath at 37°C for 30 s or at 5°C for 1 min	(14)
Golden eagle. *Aquila chrysaetuos*	BPSE + 7% DMSO	-17.6%	-27%	Cooled to 5°C for 10 min	Liquid nitrogen vapor at -70°C for 10 min, and immersed directly in liquid nitrogen at -196°C	Water bath at 25°C for 30 s.	(15)
Red-tailed hawk, *Buteo jamaicensis*		-13.3%	-36.6%				
Harris's hawk, *Parabuteo unicinctus*		-15.5	-29.5				

BPSE: Beltsville Poultry Semen Extender; DMSO: Dimethyl sulfoxide. [1]: Compared to the values of fresh semen (prior to freezing).

rate of only 29% of spermatozoa (11).

Arabian bustard (Ardeotis arabs)

The Otididae family, which encompasses 26 species of bustards, includes eight classifieds as endangered according to the International Union for Conservation of Nature (IUCN). Among the subspecies of the Arabian bustard *Ardeotis arabs*, four are recognized: *Ardeotis arabs butleri*, *Ardeotis arabs lynesi*, *Ardeotis arabs stieberi*, and *Ardeotis arabs arabs*. Some of them extinct in certain regions.

For this reason, they have been given a high conservation priority, ranking ninth among 102 species. Studies on the cryopreservation of its semen have shown promising results, with a fertility rate of approximately 83% through AI, representing a significant advancement for conserving this species (12).

Black-footed penguins (Spheniscus demersus) and Gentoo penguins (Pygoscelis papua)

Of the 18 penguin species, 11 are threatened with extinction. The black-footed

Table 4. Extenders used for avian semen dilution before cryopreservation.

Extender	Chemical composition	Ref
CSD (Crane semen extender)	63.8 mM D-fructose, 112.1 mM sodium glutamate, 8.3 µM poylinylpyrrolidone, 13.0 mM glycine, and 50.9 mM potassium acetate.	(2)
BPSE (Beltsville Poultry Semen Extender) pH = 7.4	Solution of sodium glutamate (0.867 g / 100 mL), D-fructose (0.5 g / 100 mL), potassium dihydrogen phosphate (0.065 g / 100 mL), potassium hydrogen phosphate (1.27 g / 100 mL), sodium acetate (0.26 g / 100 mL), magnesium chloride hexahydrate (0.034 g / 100 mL), citric acid potassium (0.064 g / 100 mL), and water.	(3)
Lake extender (pH=7.08)	1.92 g sodium L-glutamate monohydrate, 0.5 g potassium acetate, 0.08 g magnesium acetate tetrahydrate, 0.8 g glucose, 0.3 g polyvinylpyrrolidone (Mr10000), and 100 mL of water (343 mOsm/kg, pH 7.08).	(5)
Modified BPSE	Sodium glutamate (8.67 g / L), sodium acetate (0.43 g / L), magnesium chloride (0.34 g / L), potassium citrate (0.64 g / L), dipotassium phosphate (12.7 g / L), monopotassium phosphate (0.65 g / L), TES [n-tris (hydroxymethyl) methyl 1–2 amino ethane sulfonic acid] (1.95 g / L), trehalose (1.9 g / L), fructose (5 g / L) with pH of 7.5 and osmolarity of 366 mOsm / kg.	(6)
Lake extender (pH=7.2)	Fructose 0.6%, sodium glutamate 1.92%, magnesium acetate 0.08%, sodium acetate 0.51%, potassium citrate 0.128%, pH of 7.2 and osmolarity of 330 mOms / kg	(7)
Tselutin extender	Glucose (44.4 mM), sodium glutamate (128.0 mM), dipotassium phosphate (20.0 mM), magnesium acetate (7.0 mM), glycine (13.3 mM), glutamic acid (7.68 mM), inositol (11.1 mM), with a pH of 6.65. + 1 µL of SYBR-14 diluted 1:100 into DMSO.	(8)
Lake extender (pH=7.0)	Fructose (44.4 mM), sodium glutamate (102.6 mM), potassium acetate (50.9 mM), magnesium acetate (4.91 mM), polyvinylpyrrolidone (0.3 mM), with a pH of 7.00.	(9)
GPBM (Glutamate-polyvinylpyrrolidone-based médium)	Sodium-L-glutamate (1.92 g), glucose (0.8 g), magnesium acetate $4H_2O$ (0.08 g), potassium acetate (0.5 g), polyvinylpyrrolidone (PVP, relative molecular mass=10,000; 0.3 g), and H_2O (100 mL) (final pH 7.08, final osmolality 343 mOsm / kg).	(13)
Lake extender (pH=7.1)	0.08 g of magnesium acetate, 0.128 g of tripotassium citrate, 1.52 g of sodium glutamate, 0.6 g of glucose, 3.05 g of BES, and 5.8 mL of sodium hydroxide, diluted in 100 mL of distilled water (370 mOsm / kg,pH = 7.1)	(14)

penguin (*Spheniscus demersus*), endemic to southern Africa, has been classified as endangered since 2018 by the IUCN. On the other hand, the gentoo penguin (*Pygoscelis papua*), distributed circumpolar, is categorized as "least concern" but faces significant threats such as tourism, maritime traffic, and environmental changes affecting its reproduction. Studies on the cryopreservation of semen from both species have identified that thawing temperature is a critical factor affecting the specific characteristics of thawed semen for each species (13).

The semen extenders, their composition and the main results regarding sperm parameters during cryopreservation of sperm of wild birds are shown in Tables 2 and 4.

CRYOPRESERVATION OF RAPTORS

Peregrine falcon (Falco peregrinus)

Captive peregrine falcon breeding often faces significant challenges, such as individual incompatibility or environmental stress, which can lead to unsuccessful pairings. Semen cryopreservation offers a viable solution to overcome these obstacles by allowing the storage and use of semen through AI at optimal times or with individuals in different geographic locations (14). This approach not only facilitates captive breeding but also contributes to the conservation and genetic management of the species.

Golden eagle (Aquila chrysaetos), Red-tailed hawk (Buteo jamaicensis) and Harris's hawk (Parabuteo unicinctus)

The reproductive biology of raptors, both in the wild and in captivity, remains poorly understood. Approximately 10% of these species are endangered, making ex situ reproductive techniques, such as AI, crucial for conservation programs. However, they face specific challenges related to the size and production of spermatozoa in these birds of prey.

For example, the ejaculate volume in *Buteo jamaicensis* and *Parabuteo unicinctus* is relatively similar, whereas *Aquila chrysaetos* produces a considerably larger volume. Additionally, differences in ejaculate quality parameters have been observed among the three species, with a notable reduction in motility to approximately 40% after thawing (15).

These challenges highlight the need to develop and refine cryopreservation and AI techniques specific to each raptor species. Tables 1 and 2 present some of the most successful extenders and cryoprotectants used in birds.

To maintain avian sperm in good condition during cryopreservation, the diluent (extender) must include antioxidants to minimize oxidative damage, cryoprotectants to prevent ice crystal formation, and osmotic regulators to maintain cell membrane stability. As presented in Table 4, there are several versions of diluents for both domestic and wild bird sperm. These diluents include various energy substrates such as glucose or fructose and other compounds that provide energy, such as citrate, glutamate, or acetate (16). The semen extenders, their composition and the main results regarding sperm parameters during cryopreservation of sperm of raptors are shown in Tables 3 and 4.

CONCLUSIONS

Various factors are crucial in avian semen cryopreservation to achieve optimal fertilization rates through AI. Key variables include the composition of seminal plasma, which is essential for optimizing cryopreservation techniques across different avian species, and the collection methods, which must be conducted carefully to prevent contamination with feces and urine. The choice of extender, its diverse components, the cold storage conditions, the addition of cryoprotectants and the semen thawing method are fundamental aspects of the process. Additionally, a higher concentration of spermatozoa may improve fertilization rates. Although semen quality generally declines after freezing, all reviewed studies show that viable sperm capable of fertilization can be recovered.

This field continuously evolves, and research on various avian species provides valuable insights for developing species-specific assisted reproduction strategies. This is crucial for both genetic improvement in the production of animals, aiming to increase food production, and for the *ex-situ* conservation of endangered species, particularly given the rising number of birds at risk due to pollution, predation, hunting, and infectious diseases. In conclusion, despite progress, semen cryopreservation in birds remains a challenge due to species-specific characteristics. The future of this technique will

focus on the customization of extenders, cryoprotectants, the optimization of freezing techniques and the improvement of post-thaw semen quality. These efforts will be crucial for both commercial application in poultry production and for the preservation of endangered species. This research process is expected to continue over the next decade, developing effective solutions for both contexts.

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