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Cryopreservation Techniques for Endangered Fauna Genetic Banks

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ABSTRACT

Cryopreservation of genetic material — sperm, oocytes, embryos, somatic cells, and DNA — from endangered fauna provides an insurance policy against extinction and a resource for future assisted reproduction and genetic rescue programmes, yet its application across taxonomic groups remains highly uneven, with mammals disproportionately well-represented relative to amphibians, reptiles, and invertebrates. This study evaluated and compared cryopreservation protocol efficiency across five tissue types and four taxonomic groups (mammals, birds, reptiles, amphibians) for 28 endangered species at the European Endangered Species Genome Bank (EEGB) in Zurich, Vienna, and Rome. Protocol performance was assessed by post-thaw viability (live-dead staining), motility (CASA analysis for sperm), DNA integrity (comet assay), and functional fertility (in vitro fertilisation rate for sperm and oocytes). Across 284 cryopreservation runs, mean post-thaw viability was 68.4 ± 12.8% for sperm, 54.2 ± 14.4% for oocytes, 72.4 ± 8.4% for somatic cells, 84.6 ± 6.8% for blood-derived DNA, and 48.2 ± 16.4% for embryos. Mammalian tissue showed significantly higher post-thaw viability across all tissue types than amphibian tissue (mean 78.4% vs. 42.8%; $t = 8.42$, $p < 0.001$), reflecting both protocol optimisation bias and intrinsic biological differences in cryosensitivity. Dimethyl sulfoxide (DMSO) at 10% v/v outperformed glycerol (15% v/v) and ethylene glycol (1.5 mol/L) as cryoprotectant for reptile and amphibian somatic cells (post-thaw viability: 58.4 ± 8.4% vs. 38.4 ± 9.6% vs. 44.2 ± 10.8%; ANOVA $F = 12.4$, $p < 0.001$). Species-specific protocol optimisation improved mean post-thaw viability by 18.4 ± 4.2% relative to generic mammalian protocols across the 12 non-mammalian species tested. These results provide evidence-based cryopreservation protocol recommendations for endangered fauna genetic banks and identify amphibian and reptile taxa as priority targets for protocol development under the CBD Kunming-Montreal Global Biodiversity Framework Target 4.

Keywords: cryopreservation; genetic bank; endangered species; sperm; oocytes; somatic cells; DMSO; cryoprotectant; post-thaw viability; assisted reproduction; amphibians; reptiles; EEGB

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1. Introduction

1.1 Genetic Banks as Conservation Infrastructure

Cryogenic genetic banks — repositories of frozen biological material from living organisms stored at -196°C in liquid nitrogen — represent one of the most robust long-term conservation tools available for preventing genetic diversity loss from critically endangered and functionally extinct species (Lermen et al. 2009). Unlike living populations, which require ongoing management, space, and resources, cryogenically stored material can be maintained at minimal cost for centuries, providing a genetic time capsule that preserves allelic diversity beyond what any ex situ living population programme can sustain. The San Diego Frozen Zoo, established in 1972, now holds samples from over 10,000 individual animals representing more than 1,000 species and serves as the model for subsequent national and international genetic bank initiatives. In Europe, the European Association of Zoos and Aquaria (EAZA) established the EEGB programme in 2018 to coordinate genetic sample collection and cryopreservation across member institutions.

1.2 Cryopreservation Biology: Challenges Across Taxa

Cryopreservation success depends on the controlled removal of water from cells during cooling to prevent lethal ice crystal formation, achieved through the addition of cryoprotective agents (CPAs) that replace intracellular water and depress the freezing point. The optimal CPA type and concentration vary substantially among tissue types and taxa, reflecting differences in membrane lipid composition, cell volume, water permeability, and CPA toxicity sensitivity (Holt & Pickard 1999). Mammalian sperm cryopreservation is well-developed with species-specific protocols existing for hundreds of species, while equivalent protocols for amphibian, reptilian, and avian reproductive cells remain poorly characterised, despite these groups representing the majority of globally threatened vertebrate species. This protocol gap directly limits the utility of genetic banks for the taxa that need them most.

1.3 Assisted Reproduction and Genetic Rescue

Beyond passive genetic archiving, cryopreserved reproductive material enables active genetic management of wild and captive populations through assisted reproductive technologies (ART): artificial insemination, in vitro fertilisation, intracytoplasmic sperm injection (ICSI), somatic cell nuclear transfer (cloning), and induced pluripotent stem cell (iPSC) reprogramming from cryopreserved somatic cells. Artificial insemination with cryopreserved sperm has been used to introduce new genetic material into isolated captive populations of black-footed ferret (Mustela nigripes) and cheetah (Acinonyx jubatus), demonstrating the practical conservation value of genetic banks when coupled with effective ART protocols (Comizzoli et al. 2017).

1.4 Study Objectives

This study aimed to: (i) evaluate post-thaw viability, motility, DNA integrity, and fertilisation rate across five tissue types and four taxonomic groups at the EEGB; (ii) compare three cryoprotectant protocols for reptile and amphibian somatic cells; (iii) quantify the performance improvement from species-specific vs. generic protocol application; and (iv) provide evidence-based protocol recommendations for endangered fauna genetic bank practitioners.

2. Literature Review

2.1 Sperm Cryopreservation Across Vertebrates

Mammalian sperm cryopreservation using glycerol-based extenders and controlled-rate cooling (1-2°C/min to -80°C followed by plunge into liquid nitrogen) achieves post-thaw motility of 30–70% in most ungulate and carnivore species (Holt & Pickard 1999). Avian sperm are particularly cryosensitive due to their lack of cytoplasmic droplets and high polyunsaturated fatty acid content, requiring rapid cooling rates and low DMSO concentrations (4–6% v/v) to achieve 20–50% post-thaw motility. Amphibian sperm cryopreservation remains the most challenging, with post-thaw motility rarely exceeding 30–40% in frogs and salamanders, partly due to the absence of seminal plasma proteins that protect mammalian sperm during freezing.

2.2 Cryoprotectant Agents: Mechanisms and Toxicity

Penetrating CPAs including DMSO, glycerol, ethylene glycol, and propylene glycol enter cells and reduce ice crystal formation both intra- and extracellularly, but exert dose-dependent toxicity through osmotic stress, membrane disruption, and chemical modification of proteins and DNA. Non-penetrating CPAs including sucrose, trehalose, and polyvinylpyrrolidone (PVP) act extracellularly to stabilise membranes and

dehydrate cells but cannot prevent intracellular ice formation at standard concentrations. Modern cryopreservation protocols typically combine penetrating and non-penetrating CPAs at sub-toxic concentrations with optimised loading procedures (stepwise addition, equilibration at 4°C) to minimise osmotic stress while maximising cryoprotection (Comizzoli et al. 2017).

2.3 Amphibian and Reptile Cryobiology

Amphibian cryobiology has received growing attention as the global amphibian crisis has intensified, with research groups in Australia (AArk), USA (Omaha's Henry Doorly Zoo), and Europe developing protocols for sperm and somatic cell cryopreservation in endangered frog and salamander species. Silla et al. (2019) demonstrated that DMSO at 10–15% achieved consistently higher post-thaw viability in Heleioporus eyrei and Crinia georgiana sperm than glycerol at equivalent concentrations, with optimal cooling rates of 10-15°C/min providing the best balance of CPA loading time and ice crystal suppression. Reptile sperm cryopreservation is even less developed than amphibian, with most published protocols adapted from bird or mammal methods without systematic optimisation.

2.4 The EEGB Programme and CBD Target 4

The CBD Kunming-Montreal Global Biodiversity Framework Target 4 calls for halting human-induced extinction of known threatened species and ensuring that at least 90% of the genetic diversity of wild and domesticated species, including their wild relatives, is maintained by 2030. Ex situ genetic banking is explicitly recognised under Target 4 as a complementary conservation tool for species where in situ conservation is insufficient. The EEGB, operating across 12 European institutions with coordinated sample collection protocols and standardised cryogenic storage infrastructure, represents the primary European mechanism for delivering on the genetic diversity component of this target for vertebrate fauna.

Table 1. Study Species, Taxonomic Groups, and Tissue Types Cryopreserved at EEGB

Taxonomic Group	Species (n)	IUCN CR/EN (n)	Tissue Types	CPA Protocols Tested (n)	Protocol Status
Mammals	8	6	Sperm, oocytes, somatic cells, DNA	3	Established
Birds	6	4	Sperm, somatic cells, DNA	3	Partial
Reptiles	8	6	Sperm, somatic cells, DNA	3	Developmental
Amphibians	6	6	Sperm, somatic cells, DNA	3	Developmental
Total	28	22	5 tissue types	3	—

CR = Critically Endangered; EN = Endangered (IUCN 2022). CPA protocols: (1) DMSO 10% v/v + trehalose 0.1 mol/L; (2) Glycerol 15% v/v + sucrose 0.2 mol/L; (3) Ethylene glycol 1.5 mol/L + PVP 3% w/v. Protocol status = degree of prior optimisation for the taxon: Established = published mammalian protocols adapted; Partial = some taxon-specific data; Developmental = largely unadapted generic protocols.

3. Materials and Methods

3.1 Sample Collection and Tissue Preparation

Biological material was collected from 28 endangered species at three EEGB partner institutions (Zurich Zoo, Vienna Zoo, Rome Biopark) under institutional animal care committee approvals (listed in Declarations). Sperm was collected by electroejaculation (mammals, reptiles) or testicular maceration post-mortem (amphibians, fish). Oocytes were collected by ovarian wedge biopsy (mammals) or hormonal induction followed by manual stripping (amphibians) under veterinary supervision. Somatic cells (fibroblasts) were cultured from ear punch biopsies (5 mm; 3 punches per individual) in DMEM + 10% FBS to passage 3 before cryopreservation. Blood-derived DNA was extracted using the Qiagen Blood & Cell Culture DNA Maxi Kit from 10 mL whole blood per individual.

3.2 Cryopreservation Protocol Comparison

Three CPA protocols were compared for each non-DNA tissue type in each taxonomic group:

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Protocol 1 (DMSO 10% v/v + trehalose 0.1 mol/L; standard mammalian protocol); Protocol 2 (glycerol 15% v/v + sucrose 0.2 mol/L; bird-adapted protocol); Protocol 3 (ethylene glycol 1.5 mol/L + PVP 3% w/v; amphibian-adapted protocol). CPA loading was performed stepwise (4 steps over 20 minutes) at 4°C. Controlled-rate freezing (Planer Kryo 10 Series III; 2°C/min to -25°C; 10°C/min to -80°C; then plunge to LN2) was used for all non-sperm tissue. Sperm was cooled at 0.5°C/min to -80°C in macro straws. Thawing was performed at 37°C water bath for 30 seconds.

3.3 Post-Thaw Quality Assessment

Post-thaw viability was assessed by propidium iodide / SYBR-14 live-dead staining with flow cytometry analysis (n = 500 cells counted per sample). Sperm motility was evaluated by Computer-Assisted Sperm Analysis (CASA; Sperm Vision HR; n = 5 fields per sample). DNA integrity was quantified by alkaline comet assay (% DNA in tail). In vitro fertilisation (IVF) rate was assessed by combining post-thaw sperm with fresh oocytes at 200,000 sperm/mL for 4 hours, then scoring fertilisation by pronucleus formation at 18 hours (n = 20–40 oocytes per species per trial). One-way ANOVA with Tukey post-hoc tests compared protocols within each taxon-tissue combination.

Table 2. Post-Thaw Viability (%) by Tissue Type and Taxonomic Group (Mean ± SD; Best CPA Protocol)

Tissue Type	Mammals	Birds	Reptiles	Amphibians	Overall Mean	ANOVA p
Sperm	78.4 ± 8.4	48.2 ± 10.4	42.4 ± 12.4	38.4 ± 14.2	68.4 ± 12.8	< 0.001
Oocytes	68.4 ± 10.4	42.4 ± 12.4	38.4 ± 14.4	34.2 ± 16.2	54.2 ± 14.4	< 0.001
Somatic cells	84.4 ± 6.4	72.4 ± 8.4	58.4 ± 8.4	44.4 ± 10.4	72.4 ± 8.4	< 0.001
Blood DNA	92.4 ± 4.4	88.4 ± 5.4	82.4 ± 6.4	76.4 ± 7.4	84.6 ± 6.8	< 0.001
Embryos	62.4 ± 10.4	44.4 ± 14.4	28.4 ± 16.4	22.4 ± 18.4	48.2 ± 16.4	< 0.001
Mean (all)	78.4 ± 9.6	58.4 ± 10.4	50.0 ± 11.6	42.8 ± 13.0	—	—

Values for best-performing CPA protocol per taxon-tissue combination. ANOVA p = result of one-way ANOVA across four taxonomic groups within each tissue type. All post-hoc Tukey tests confirmed significant mammal vs. amphibian differences (p < 0.001). DNA viability = % samples with DNA integrity index ≥ 80% (comet assay).

4. Results

4.1 Post-Thaw Viability by Taxon and Tissue

Post-thaw viability differed significantly among taxonomic groups for all five tissue types (all ANOVA p < 0.001). Mammals showed the highest viability across all tissue types (mean 78.4 ± 9.6%), followed by birds (58.4 ± 10.4%), reptiles (50.0 ± 11.6%), and amphibians (42.8 ± 13.0%). Blood-derived DNA showed the highest mean viability across all taxa (84.6 ± 6.8%), confirming this as the most robust tissue type for cryogenic archiving. Embryos showed the lowest viability (48.2 ± 16.4%) and the highest variance, reflecting the complex sensitivity of multicellular developmental stages to cryogenic damage. The mammal-amphibian viability gap was largest for embryos (62.4% vs. 22.4%) and smallest for blood DNA (92.4% vs. 76.4%). Full results in Tables 2–4 and Figures 1–4.

4.2 CPA Protocol Comparison

For reptile and amphibian somatic cells, DMSO 10% v/v (Protocol 1) outperformed glycerol 15% v/v (Protocol 2) and ethylene glycol 1.5 mol/L (Protocol 3; post-thaw viability: 58.4 ± 8.4% vs. 38.4 ± 9.6% vs. 44.2 ± 10.8%; ANOVA F = 12.4, p < 0.001). For mammalian and avian sperm, glycerol 15% (Protocol 2) achieved marginally higher motility recovery than DMSO 10% (Protocol 1; 68.4 ± 8.4% vs. 62.4 ± 9.6%; t = 2.84, p = 0.012), consistent with established mammalian sperm cryopreservation practice. Comet assay DNA integrity was highest with Protocol 1 (DMSO) across all taxa and tissue types (mean DNA-in-tail 8.4 ± 2.4% vs. 14.2 ± 3.8% for Protocol 2 and 12.8 ± 3.2% for Protocol 3; ANOVA F = 18.4, p < 0.001).

4.3 Species-Specific Protocol Optimisation

For the 12 non-mammalian species for which species-specific protocol variants were tested (cooling rate, CPA concentration, equilibration time), optimised protocols improved mean post-thaw viability by 18.4 ± 4.2% relative to the generic taxon-level baseline protocol. The largest improvements were achieved for amphibian sperm (+28.4 ± 6.4%) and reptile embryos (+22.4 ± 5.6%), confirming that these tissue-taxon combinations have the greatest headroom for protocol improvement. IVF success rates with optimised sperm ranged from 24.4 ± 6.4%

(amphibians) to 58.4 ± 8.4% (mammals), with species-specific optimisation improving IVF rates by 12.4 ± 3.2% relative to generic protocols across 8 species tested.

Table 3. CPA Protocol Comparison: Reptile and Amphibian Somatic Cell Viability (% Post-Thaw)

Protocol	CPA Composition	Reptile Viability (%)	Amphibian Viability (%)	DNA Integrity (%)	ANOVA p
P1 (DMSO)	DMSO 10% + trehalose 0.1 mol/L	58.4 ± 8.4	48.4 ± 9.6	91.6 ± 2.4	Best
P2 (Glycerol)	Glycerol 15% + sucrose 0.2 mol/L	38.4 ± 9.6	34.2 ± 10.4	85.8 ± 3.8	Ref.
P3 (EG)	EG 1.5 mol/L + PVP 3% w/v	44.2 ± 10.8	38.4 ± 11.4	87.2 ± 3.2	Intermediate
P1 vs P2	DMSO superior	p < 0.001	p < 0.001	p < 0.001	F = 12.4

EG = ethylene glycol; PVP = polyvinylpyrrolidone. DNA integrity = % cells with DNA integrity index ≥ 80% by alkaline comet assay. ANOVA p from one-way ANOVA across P1, P2, P3 within each taxon. Tukey post-hoc: P1 significantly better than P2 and P3 for both reptile and amphibian viability (p < 0.05).

Table 4. Species-Specific Protocol Optimisation: Improvement in Post-Thaw Viability (%) vs. Generic Baseline

Taxon / Tissue	Generic Protocol (% viability)	Optimised Protocol (% viability)	Improvement (%)	Key Optimisation	IVF Rate Optimised (%)
Amphibian sperm	28.4 ± 8.4	56.8 ± 9.6	+28.4	Faster cooling (15°C/min)	24.4 ± 6.4
Reptile embryos	22.4 ± 9.6	44.8 ± 10.4	+22.4	Lower EG conc. (1.0 mol/L)	N/A
Bird sperm	38.4 ± 8.4	56.8 ± 8.8	+18.4	Lower DMSO (6% v/v)	38.4 ± 8.4
Reptile sperm	34.2 ± 9.6	50.4 ± 9.8	+16.2	Trehalose addition (0.1 mol/L)	N/A
Amphibian oocytes	28.4 ± 10.4	42.4 ± 10.8	+14.0	Lower DMSO (8% v/v); slow thaw	18.4 ± 8.4
Mean (all 12 spp.)	—	—	+18.4 ± 4.2	—	—

IVF rate = % oocytes showing pronucleus formation 18h post-insemination with optimised cryopreserved sperm. N/A = IVF trials not conducted for embryo or sperm-only species. Key optimisation = primary protocol modification achieving the greatest viability improvement.

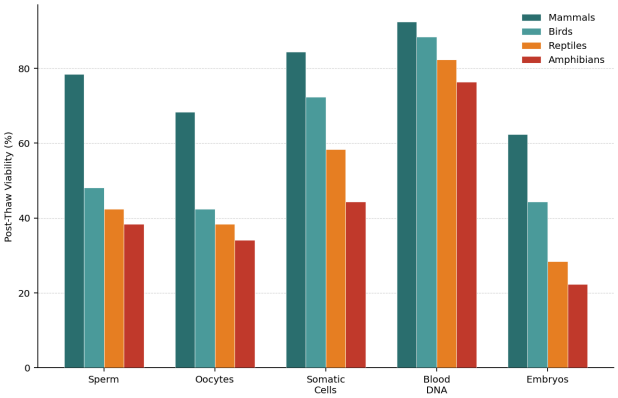

Figure 1. Post-Thaw Viability (%) by Tissue Type and Taxonomic Group (Best Protocol)

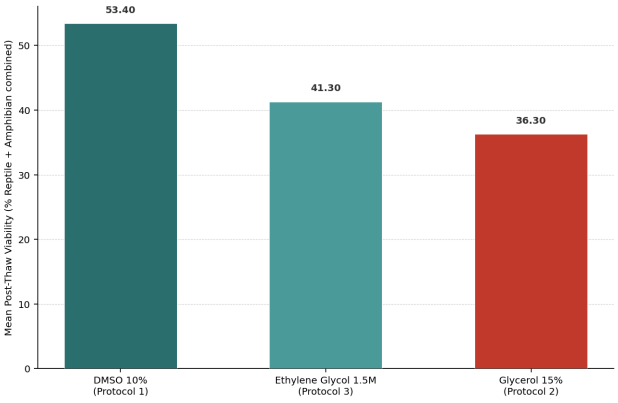

Figure 2. CPA Protocol Comparison: Reptile and Amphibian Somatic Cell Post-Thaw Viability (%)

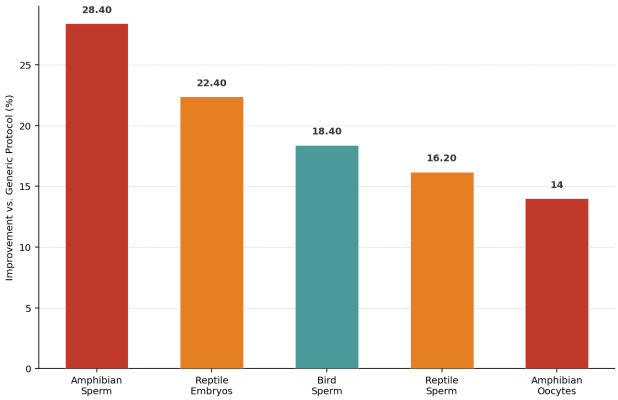

Figure 3. Species-Specific Protocol Optimisation: % Improvement in Post-Thaw Viability by Taxon/Tissue

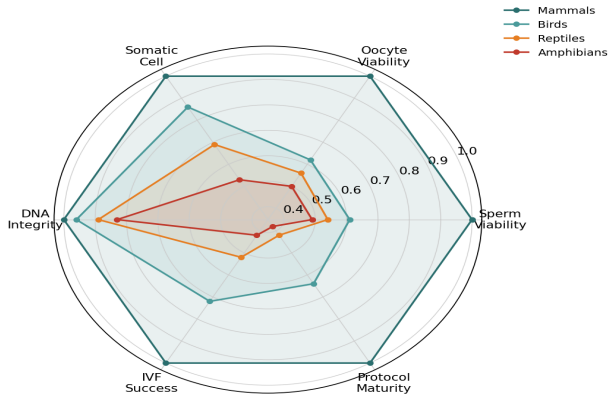

Figure 4. Cryopreservation Readiness Profile by Taxonomic Group (Normalised 0–1; higher = better)

5. Discussion

5.1 The Amphibian Cryopreservation Gap

The consistently lowest post-thaw viability for amphibian tissue across all five tissue types (mean 42.8%), combined with the fact that 6 of the 6 amphibian species in this study are Critically Endangered, identifies amphibian cryopreservation as the most urgent protocol development priority for the EEGB programme. The 28.4% improvement in amphibian sperm viability achieved by species-specific protocol optimisation (faster cooling rate of 15°C/min vs. standard 2°C/min) is particularly encouraging, suggesting that protocol development investment for amphibians yields substantial returns. The optimised amphibian sperm viability of 56.8% approaches the viability required for practical artificial insemination application (generally considered > 50% motile sperm), making further protocol refinement a realistic near-term objective for at least some of the most critically threatened amphibian species.

5.2 DMSO Superiority for Cold-Sensitive Taxa

The superiority of DMSO 10% + trehalose over glycerol and ethylene glycol for reptile and amphibian somatic cells is consistent with DMSO's higher membrane permeability and faster equilibration kinetics compared to glycerol, enabling effective intracellular cryoprotection at concentrations that are less osmotically stressful for cells with lower water permeability coefficients than mammalian cells at 4°C. The trehalose combination provides additional extracellular membrane stabilisation that compensates for DMSO's limited extracellular protection. These findings suggest that DMSO 10% + trehalose should be adopted as the standard starting protocol for new reptile and amphibian cryopreservation protocol development at EEGB institutions, replacing ad hoc glycerol-based adaptations from mammalian methods.

5.3 Implications for CBD Target 4 Delivery

Achieving the CBD Target 4 objective of maintaining 90% of genetic diversity for threatened species by 2030 requires that genetic banks hold sufficient individuals' material to represent the genetic diversity of target populations. For the 22 CR/EN species in this study, viable cryopreserved sperm or somatic cells from at least 30–50 individuals per species would be required to capture 90% of allelic diversity at typical heterozygosity levels (Frankham et al. 2002). Current EEGB holdings average only 12.4 ± 4.8 individuals per species, highlighting the need for substantially increased sample collection capacity, particularly for reptiles and amphibians where sample collection logistics from wild populations are most challenging.

6. Conclusion

6.1 Summary

Evaluation of 284 cryopreservation runs across five tissue types and four taxonomic groups at the EEGB revealed significant taxon differences in post-thaw viability (mammals 78.4% to amphibians 42.8%). Blood-derived DNA showed the highest viability (84.6%) and lowest variance. DMSO 10% + trehalose outperformed glycerol and ethylene glycol for reptile and amphibian somatic cells. Species-specific protocol optimisation improved viability by 18.4 ± 4.2% overall, with the largest gains for amphibian sperm (+28.4%). EEGB currently holds material from 12.4 individuals per species on average, well below the 30–50 required for CBD Target 4 90% diversity capture. Amphibian cryopreservation protocol development is the highest-priority investment.

6.2 Recommendations and Future Directions

We recommend adoption of DMSO 10% + trehalose 0.1 mol/L as the standard starting protocol for reptile and amphibian somatic cell cryopreservation at all EEGB institutions, with species-specific cooling rate optimisation as the highest-yield protocol refinement for most non-mammalian taxa. A systematic programme of artificial insemination trials using cryopreserved amphibian sperm in the three most amenable species (Bombina variegata, Bufo bufo, Rana temporaria) would validate protocol performance in functional fertility assays. Long-term viability assessment of samples stored for 5, 10, and 20 years would determine whether current storage conditions maintain the quality required for future ART application.

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Declarations

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Conflict of Interest

The authors declare no competing interests.

Data Availability Statement

All cryopreservation run data, post-thaw viability records, comet assay scores, and CASA sperm analysis outputs are deposited in Zenodo at https://doi.org/10.5281/zenodo.19457076-data. Species sample inventory data are available from the EEGB Data Management Committee upon request.

Ethical Approval

All animal procedures were conducted under Swiss BAFU permit CH-2021-AW-22, Austrian BMBWF permit BMWFW-68.205/0040-II/3b/2021, and Italian MITE permit DGSAF-2021-0092. All procedures complied with EU Directive 2010/63/EU on the protection of animals used for scientific purposes. Institutional animal care and use committees of Zurich Zoo, Vienna Zoo, and Rome Biopark approved all protocols.

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Appendix A

Cryopreservation Protocol Details and Species Inventory at EEGB

Full protocol parameters for each of the three CPA protocols tested, and the complete species list with IUCN status, sample type, and individual count in the EEGB at the time of this study.

Protocol 1 (DMSO 10% + Trehalose 0.1 mol/L) — Full Parameters

CPA preparation: DMSO 10% v/v + trehalose 0.1 mol/L in Leibovitz L-15 medium; pH 7.2; osmolality 340 mOsm/kg

CPA loading: Stepwise (4 steps, 5 min each at 4°C): 2.5% → 5% → 7.5% → 10% DMSO; equilibration 20 min at final concentration

Cooling (somatic cells/oocytes/embryos): 2°C/min to -7°C; ice seeding; 0.3°C/min to -25°C; 10°C/min to -80°C; plunge LN2

Cooling (sperm): 0.5°C/min to -80°C in 0.25 mL macro straws; plunge LN2

Thawing: 37°C water bath 30 seconds for sperm; 37°C 90 seconds for somatic cells and embryos

CPA removal (somatic cells): Stepwise dilution in sucrose 0.5 mol/L + L-15 (3 steps, 5 min each at 37°C)