Cryopreservation of Reproductive Material and Cell Lines: Background, Benefits and Challenges

This statement presents the challenges and uses of employing cryopreservation techniques to store samples of reproductive material and cell lines. This document should be read in conjunction with the 'Cryopreservation Glossary of Terms' and the 'EAZA Position Statement on the use of cryopreserved materials and biotechnology'.

Background
Cryopreservation, or cryobanking, seeks to preserve intact or living cells from somatic and reproductive material (germplasm) and in vitro developed cell lines, for potential future revival and use. This is done by halting metabolic processes via specific, multi-step cooling, freezing and storage protocols which can vary between sample type and species. Samples are stored at temperatures of -196 °C and this ultra-low temperature is achieved using liquid nitrogen (usually in LN2 vapor phase).

Benefits
Preservation of reproductive material such as germplasm (oocytes/ova or spermatozoa), embryos, but also ovarian or testicular tissue, can be a useful tool for population management and can be extremely valuable for protecting species from threats of extinction by maintaining genetic diversity or even reviving lost genetic lines. For population management programmes like the EAZA Ex situ Programmes (EEP), it has the potential to increase their chance of success considerably, especially when they have roles that require long-term persistence (e.g. insurance population). Furthermore, it may allow additional EEPs with important conservation roles to be established, which currently may not be feasible to manage without preservation of genetic material for future use.

Common, recognized assisted reproductive technologies that are utilizing cryopreserved reproductive material are for example artificial insemination (AI), in-vitro fertilization (IVF) and embryo transfer (ET) (Prieto et.al., 2014).

Cell lines, however, are established cultures of cells, which when provided an appropriate environment and growth medium, can proliferate indefinitely. When preserved or frozen in a way that maintains their cellular viability, they can later be thawed and used for research purposes. This eliminates the need for constant maintenance of the living, replicating cells.

Application and use
There are various technologies for the application and use of cryopreserved materials, some of which fairly well established and more commonly used, and other more recent available of under development. Although new technologies offer new possibilities, their use needs to be balanced against concerns over any possible harmful consequences. EAZA does not endorse all applications and use (in any circumstances) as outlined in the 'EAZA Position Statement on the use of cryopreserved materials and biotechnology'.

Assisted reproductive technologies

There are already established assisted reproductive techniques such as artificial insemination, in vitro fertilisation and embryo transfer, that utilise cryopreserved reproductive material (e.g., semen, oocytes) to generate offspring. There are also advanced assisted reproductive technologies (aART) such as somatic cell nuclear transfer or induced pluripotent stem cells that utilise genetic material from cells other than gametes to generate offspring. Tissue or cell lines can be cryobanked for use in creating pluripotent stem cells. As such, cryopreservation of suitable material can help in the conservation of numerous species.

Cloning

Cloning organisms produces genetically identical copies of individuals via a process called somatic cell nuclear transfer (SCNT). Here, diploid somatic cell nuclei (non-gametes) are harvested from tissue and their nuclei are inserted into a denucleated oocyte to produce offspring that is genetically identical to the nucleus donor. While use of cloning may introduce new possibilities for species restoration or recovery, it is resource-intensive and a more detailed assessment and consideration of the technical, biological, and ethical aspects of this work needs to be done before its routine use in species conservation can be realised. Currently, cloning is primarily limited to use in biomedicine and research in the European Union and its use in species conservation has not, to date, proven beneficial.

Gene editing / Genome engineering

Gene editing, or genomic engineering, involves modification of an organism's DNA, and can be applied to add or enhance certain traits or to replace deleterious alleles. This technology has the potential to complement current efforts to halt biodiversity loss and aid biodiversity conservation by, for example, improving disease resistance, climate adaptability, and overall species resilience, as in improving amphibian resistance to chytrid fungus, or blight resistance in the American chestnut. Additionally, genetic engineering can provide synthetic alternatives to organic products, thereby reducing pressure on species threatened with extinction. However, introducing genetically modified organisms carries risks of unintended ecological consequences, such as disrupting existing ecosystems or outcompeting native species. These interventions should be thoroughly evaluated for their ethical and ecological impacts before being implemented in biodiversity conservation efforts. Additionally, gene editing might impact on animal welfare and this equally needs thorough evaluation before considering its application to support biodiversity conservation efforts.

De-extinction

De-extinction refers to the process of generating an organism that closely resembles a species that has gone extinct (e.g., ecologically, phenotypically, behaviourally). The term is used to describe any attempt to create proxies of extinct species or subspecies using methods like selective back breeding, cloning, and genome engineering. The proxy will not result in a true replica of any extinct species due to genetic, epigenetic, behavioural, physiological, and other differences, but only serve as a substitute. While controversially discussed, de-extinction efforts have the potential to revive species that have recently gone extinct, thereby restoring or enhancing biodiversity. However, the ecological impact of reviving formerly extinct species should be carefully evaluated and underlying threats to the species and its habitat that potentially contributed to their extinction, should be addressed. The impact of animal welfare of de-extinct species need important consideration and hard to predict. Welfare of surrogate parent animals equally requires consideration. Without integrating de-extinction into a broader, more comprehensive environmental and species recovery strategy that addresses the original causes of extinction, de-extinction cannot be considered a viable conservation tool.

Challenges of cryopreservation

Cryopreservation, in contrast to biobanking, requires sample collection, transport and preservation to be done in a specific window of time (typically within 24-48 hours for spermatozoa or tissues, but

less for oocytes) in order to maintain viability of the cells. Similarly, samples need to be collected from live animals, or shortly after the time of death. Prior to storage in liquid nitrogen, gametes, cell lines or embryos need to be protected by specialized media, called cryoprotectant agents (CPAs), in order to avoid structural damage such as that from ice crystals formed during the freezing process (Pegg, 2007). Therefore, protecting and freezing these samples requires specialized and often species-specific reagents, techniques and expertise. Storage requires appropriate facilities with dedicated liquid nitrogen tanks and monitoring systems, as the liquid nitrogen needs to be periodically replenished.
Most protocols associated with these techniques have been developed and tested extensively specifically for domestic or farm animal species and need to be adapted or transposed for the purposes of use in threatened species. There are distinct challenges when attempting to apply the protocols of cryopreservation or cell-line development to wildlife, including species-specific reproductive knowledge and needs. When attempting to establish effective protocols, differences in taxon, species and tissue sample may impact variables such as CPA type and concentration as well as timing of the steps in the cooling process.

However, technology and methods are evolving rapidly, and the more species- or taxon-specific work that can be undertaken, the more information we can gain, both on the types and methods used for preservation, as well as the forms of assisted reproduction utilizing preserved materials.
Any insights gained now may one day lead to benefits for threatened species. Even “temporary” or inefficient methods can be employed now in attempts to preserve these limited, finite resources, while the science and research catches up (Leibo and Songsasen, 2002).

EAZA Cryopreservation Interest Group (CIG)
Given that questions remain regarding the effectiveness of some protocols for threatened or nondomestic species, it can be challenging for institutions who wish to use cryopreservation as a conservation tool. However, any attempts to preserve samples, when possible, should be encouraged, especially in collaboration with researchers who are working to develop or improve protocols, or other methods of biomaterial preservation. This could potentially allow for better and more comprehensive preservation of genetic material of more species in our member institutions.

As genetic samples such as blood and tissue are also distinctly important for population management and conservation research needs, the EAZA Biobank will continue urging members to contribute samples of blood, serum and tissue for long-term storage, but it cannot physically accept reproductive materials for cryopreservation at this time. To provide EAZA Members with cryopreservation services a Cryopreservation Interest Group (CIG) has been established under the EAZA Biobank. The CIG exist of a small group of representatives from the Biobank Working Group, EAZA Member and Biobank partners and the EEO that are interested and committed to progress the cryopreservation activities.

The CIG aims to provide expertise and guidance in cryopreservation to the EAZA membership, and realise that all EAZA Members will have access to cryopreservation biobanking facilities to support the objectives and goals of the EAZA population management programmes (EEP’s) and Long-term Management Plans (LTMP’s). To achieve this, a Cryopreservation Network is currently being facilitated by the CIG, in collaboration with the EAZA Reproductive Management Group (RMG), EAZA Population Management Centre (PMC) and EAZA Biobank WG. The Cryopreservation Network is comprised of EAZA and non-EAZA cryobank partners which have liquid nitrogen capacity and expertise in cryopreservation, and who prioritize conservation, specifically *ex situ* conservation and population management.

For any inquiries or more information regarding cryopreservation or cryopreservation services, please contact the EAZA Biobank Coordinator.

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