REVIEW

Resurrecting biodiversity: advanced assisted reproductive technologies and biobanking

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Abstract

Biodiversity is defined as the presence of a variety of living organisms on the Earth that is essential for human survival. However, anthropogenic activities are causing the sixth mass extinction, threatening even our own species. For many animals, dwindling numbers are becoming fragmented populations with low genetic diversity, threatening long-term species viability. With extinction rates 1000–10,000 times greater than natural, *ex situ* and *in situ* conservation programmes need additional support to save species. The indefinite storage of cryopreserved (−196°C) viable cells and tissues (cryobanking), followed by assisted or advanced assisted reproductive technology (ART: utilisation of oocytes and spermatozoa to generate offspring; aART: utilisation of somatic cell genetic material to generate offspring), may be the only hope for species' long-term survival. As such, cryobanking should be considered a necessity for all future conservation strategies. Following cryopreservation, ART/aART can be used to reinstate lost genetics back into a population, resurrecting biodiversity. However, for this to be successful, species-specific protocol optimisation and increased knowledge of basic biology for many taxa are required. Current ART/aART is primarily focused on mammalian taxa; however, this needs to be extended to all, including to some of the most endangered species: amphibians. Gamete, reproductive tissue and somatic cell cryobanking can fill the gap between losing genetic diversity today and future technological developments. This review explores species prioritisation for cryobanking and the successes and challenges of cryopreservation and multiple ARTs/aARTs. We here discuss the value of cryobanking before more species are lost and the potential of advanced reproductive technologies not only to halt but also to reverse biodiversity loss.

Lay summary

The world is undergoing its sixth mass extinction; however, unlike previous events, the latest is caused by human activities and is resulting in the largest loss of biodiversity (all living things on Earth) for 65 million years. With an extinction rate 1000–10,000-fold greater than natural, this catastrophic decline in biodiversity is threatening our own survival. As the number of individuals within a species declines, genetic diversity reduces, threatening their long-term existence. In this review, the authors summarise approaches to indefinitely preserve living cells and tissues at low temperatures (cryobanking) and the technologies required to resurrect biodiversity. In the future when appropriate techniques become available, these living samples can be thawed and used to reinstate genetic diversity and produce live young

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ones of endangered species, enabling their long-term survival. The successes and challenges of genome resource cryopreservation are discussed to enable a move towards a future of stable biodiversity.

Keywords: ▶ biobanking ▶ cryopreservation ▶ biodiversity ▶ assisted reproductive technology ▶ conservation

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Introduction

Humans are causing the sixth mass extinction, the largest predicted loss of biodiversity for 65 million years, with 41% of amphibians, 26% of mammals and 14% of bird species assessed by the International Union for the Conservation of Nature (IUCN) being threatened with extinction (Ceballos *et al.* 2015, Ceballos & Ehrlich 2018, IUCN 2021). The catastrophic decline in biodiversity is a global threat to our own existence, affecting our economies, societal equality and way of life, including the food we eat and our climate (WHO 2015). This current loss of species is estimated to be between 1000- and 10,000-fold higher than the natural extinction rate (Ceballos *et al.* 2015, Turvey & Crees 2019). Human activity is changing the environment too fast for organisms to evolve in response, resulting in extinction (Ceballos & Ehrlich 2018). Restoring habitats alone will not halt the decline in biodiversity as many species are now fragmented, resulting in unviable populations with low genetic diversity (Hoban *et al.* 2020).

Animal conservation aims to maintain populations large enough, and with enough genetic diversity, to be sustainable (Comizzoli *et al.* 2019). *Ex situ* breeding programmes are a vital insurance policy for preserving endangered species and for enabling research, for example, into their behaviour and physiology. In zoos, pedigree-based management typically aims to maintain 90% of genetic diversity over 100 years and minimise mean kinship and inbreeding in threatened populations to retain the evolutionary potential of the species of interest (Ballou *et al.* 2010). Using captive breeding programmes to maintain genetic diversity is not always successful due to lack of reproduction, for example, due to unnatural social structures resulting in reduced breeding behaviour, lack of mate choice or limited number of founders potentially leading to inbreeding (Lees & Wilcken 2009). Furthermore, transporting large animals between different locations for breeding purposes comes with logistical and welfare challenges with the addition of potential disease transmission (Pukazhenthi & Wildt 2004). For some species with unsuccessful breeding programmes (Lees & Wilcken 2009), cryopreserving (freezing cells with cryoprotectants enabling long-term viable cell and tissue storage), followed by cryobanking (indefinite storage of viable cells and tissue in liquid nitrogen at −196°C or ultra-low freezers) and assisted or advanced assisted reproductive technology (ART/aART) can save the genotypes that are being lost today (Mitchell & Williams 2022, for definitions please see Supplementary Table 1, see section on supplementary materials given at the end of this article). ART includes techniques that utilise oocytes and spermatozoa to generate offspring such as artificial insemination (AI), *in vitro* fertilisation (IVF) or intra cytoplasmic sperm injection (ICSI) (Brown *et al.* 2004, Howard *et al.* 2016, Briski & Salamone 2022). These techniques are used for a number of taxa, but species-specific protocols need honing for many endangered species. More recently, the use of aART, technologies that utilise genetic material from somatic cells to generate offspring, has been highlighted as a key technology to resurrect biodiversity such as somatic cell nuclear transfer (SCNT) or induced pluripotent stem cells (iPSC) (Gómez *et al.* 2004, Hikabe *et al.* 2016). Prior to ART or aART, a vital aspect of conservation is storing viable cells and tissues to enable the reintroduction of genes. As a result, genetic diversity can increase within a population, allowing species to recover. This is particularly important for aART, where technology needs further development. Storing viable samples in a biobank not only enables the technology to catch up but also prevents vital genetics from being lost.

A biobank is a repository of biological samples, that is, a searchable, organised collection of biological samples and associated data stored predominantly for research or management, for example, of captive populations (Agca 2012, Hewitt & Watson 2013). Biobanking is not new; conservationists have been collecting samples from wildlife for decades to save genetic diversity (Soulé *et al.* 1985, Montfort 2014). These samples are vital to improve understanding of the fundamental biology of rare and endangered species (Comizzoli & Wildt 2017). Cryopreserving (freezing cells with cryoprotectants enabling long-term viable storage using liquid nitrogen

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at −196°C or ultra-low freezers) then storing cells in a cryobank (biobank of viable, cryopreserved biological samples), to enable the maintenance or regeneration of a species for conservation purposes, is highly specialised in that it requires the application of several complex and novel ARTs working in harmony to be truly effective. Indeed, Comizzoli and Wildt (2017) quote that cryobanking is a 'crucial unfilled gap – offering a backup storage of the extant genomes of living species that are already under threat or are likely to be soon.' As the biodiversity crisis continues, there is an increasing need for global conservation management of endangered species and to interconnect all populations throughout the *ex situ–in situ* continuum to maximise the available genetic diversity. Depending on the species' specific needs, cryobanking could be deemed necessary in the conservation strategy for single or multiple species as part of the One Plan Approach (the One Plan Approach coined by the Conservation Planning Specialist Group of the IUCN; Lees & Wilcken 2011, Byers *et al.* 2013, Traylor-Holzer *et al.* 2018).

Successful maintenance and regeneration of a species are primarily dependent on genetic biodiversity (Choudhary *et al.* 2016). Heterozygosity of a population undoubtedly contributes to stabilisation and robustness of the effective population size (Soulé 1987). Without adequate genetic diversity, any species will inevitably become extinct (Ryder & Onuma 2018). For some species, such as the northern white rhinoceros (*Ceratotherium simum cottoni*), there are already too few individuals remaining to maintain genetic diversity for the long-term sustainability of the population (Korody *et al.* 2021). For these species, aART may be their only hope of long-term survival. For both ART and aART to be successful, there needs to be a knowledge of basic biology, which is lacking for many species (Herrick 2019). Indeed, there is an understanding of the reproductive physiology of only approximately 250 species, with a bias towards mammals and birds, while amphibians are most at risk of extinction (Comizzoli *et al.* 2019, see Case Study: Box 1, Fig. 1). This results in ART and aART developed for specific domestic animals being used as a 'model' for taxonomically similar wild species. One example of ART successfully being applied to selected endangered species is AI. Approaching 100 species of wild mammals and birds have been propagated by AI including giant panda (*Ailuropoda melanoleuca*), cheetah (*Acinonyx jubatus*), black-footed ferret (*Mustela nigripe*), Siberian crane (*Leucogeranus leucogeranus*) and Houbara bustard (*Chlamydotis undulata*) (Ballou 1984, Pukazhenthi & Wildt 2004, Andrabi & Maxwell 2007, Morrow *et al.* 2009, Herrick 2019, Penfold *et al.* 2021). In addition, it is important to highlight the successful utilisation of ART to coral species. To date, there are ~30 species of coral that have been cryobanked from coral reef populations around the world (Hagedorn *et al.* 2019). The frozen-thawed sperm has been used to fertilise eggs from the same spawn, successive spawns, and used for trans-regional IVF of corals (Hagedorn *et al.* 2012, 2017, 2018) separated by hundreds of miles, thereby increasing the heterozygosity of species. The integration of these technologies has helped mitigate the loss of heterozygosity from coral species and continued to aid in global coral conservation efforts (Hagedorn *et al.* 2019).

However, difficulties arise from the huge diversity of both reproductive physiology and behaviour between species of the same taxa, for example, among canids, domestic dogs (*Canis familiaris*) show spontaneous ovulation, whereas the island fox (*Urocyon littoralis*) only ovulates in the presence of a male (Asa *et al.* 2007). Furthermore, in the African wild dog (*Lycaon pictus*), dominant female behaviour regulates reproductive success to alpha females only (Van den Berghe *et al.* 2012). The complications of using techniques developed in the domestic industry for endangered species have resulted in birth rates that are significantly lower than those seen in domestic animals (Mastromonaco & Songsasen 2020). However, there are successful case studies including the now stable endangered black-footed ferret (*M. nigripe*) population (Santymire 2016). The lack of widespread application of ART across all taxa after more than 30 year of efforts highlights the need for alternative approaches. This includes gamete and somatic cell cryopreservation for genome resource banking (Mastromonaco & Songsasen 2020), and the application of aART, even if the production of offspring is many years away. There is also an increasing importance of developing species-specific protocols for ART/aART for endangered species to improve reproductive success rates in the future (Herrick *et al.* 2019, Mastromonaco & Songsasen 2020).

Both ART and aART may raise ethical issues which are rarely explored. The use of aART can remove the invasive manipulation of living animals as these techniques mainly use tissue from neutered or deceased individuals at the point of collection. However, many techniques require invasive manipulation as an end point, for example, surrogacy, cross fostering or tissue implantation and subsequent harvesting of gametes. It is important to note that these procedures must always be performed under general anaesthesia with included analgesia by a highly trained professional, minimising risk to the individuals

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Box 1 Case study preserving amphibians

Amphibians are arguably the class most at risk of extinction (Bishop *et al.* 2012, Ficetola *et al.* 2015) with populations declining faster than any other vertebrate class (Ceballos *et al.* 2015, IUCN SSC Amphibian Specialist Group 2017, Zimkus *et al.* 2018). Significantly challenged by chytridiomycosis (Van Rooij *et al.* 2015), climate change, declining resources, pollution, etc., (Cheng *et al.* 2011), many amphibians including the mountain chicken frog (Leptodactylus fallax) are on the brink of extinction (IUCN SSC Amphibian Specialist Group 2017, Fig. 1A). Amphibians also suffer from reduced research, investment and conservation advancement, including ARTs and aARTs (Kouba *et al.* 2013, Strand *et al.* 2020).

Amphibian IVF has been available since the 1950s; however, this technology has predominantly been used for non-conservation-based research (Clulow *et al.* 2019*a*,*b*). As amphibians utilise external fertilisation, IVF is relatively straight forward compared to that of mammals (Silla *et al.* 2021). After primary publication of frog IVF by Wolf and Hedrick in 1971, little additional research has been conducted (Silla & Byrne 2019). Gamete release can be induced by activation of the hypothalamic–pituitary–gonadal axis (Peter *et al.* 1988, Uteshev *et al.* 2015, Silla & Byrne 2021), and Waggener and Carroll (1998) demonstrated the first example of induced gamete release in Paraguay horned frogs (Lepidobatrachus species) with resultant fertilisation in vitro, thus validating the application of IVF to amphibian conservation. Since then, amphibian IVF has been conducted in several threatened species including Wyoming toad (Bufo baxteri) (Browne *et al.* 2006), corroboree frog (Pseudophryne corroboree) (Byrne & Silla 2010) and dusty gopher frog (Rana sevosa) (Kouba *et al.* 2012).

Amphibian semen can be refrigerated (4°C) for temporary holding or cryopreserved (−196°C) for long-term storage (Browne *et al.* 2002, 2019). For some species, semen has been held at 4°C for 30 days with retained viability (Browne *et al.* 2001), and refrigerated semen has been used in over 40 amphibian species, with outcomes including retrieval of motile sperm and fertilisation in vitro (Browne *et al.* 2001, Keogh *et al.* 2017, Gillis *et al.* 2021*a*). Refrigerated and cryopreserved semen are the two most successful ARTs for amphibians, with semen capable of storage in whole testes and sectioned testicular strips, as well as spermic urine (Poo & Hinkson 2019).

For semen cryopreservation, amphibian spermatozoa appear to be highly tolerant of prolonged exposure to cryoprotectants that other species' cells rarely are (Clulow *et al.* 2019*a*,*b*). However, while semen cryopreservation is generally successful, the theoretical understanding of why the methods work is lacking (Clulow & Clulow 2016). The ability to successfully cryopreserve amphibian oocytes would be a ground-breaking development for conservation (Lawson *et al.* 2013); however, the success of oocyte freezing is low due to the high yolk content and large diameter (Guenther *et al.* 2006). An alternative would be the cryopreservation of embryos; more success may be expected here as early embryonic cells are typically smaller and contain less liquid (Lawson *et al.* 2013).

Cryopreservation of amphibian somatic tissue for use in aART provides additional and vital conservation resources (Strand *et al.* 2020). To date, there has been little research into developing tissue preservation procedures for amphibians (Stand 2021). However, the San Diego Zoo Institute for Conservation Research Frozen Zoo® already holds a large collection of cryopreserved amphibian tissue and cell lines (Chemnick *et al.* 2009), and the IUCN amphibian specialist group have biobank and ART working groups, so advances are being made in this area. One main challenge with amphibian skin cryopreservation and cell culture is contamination from bacteria and fungi (Strauß *et al.* 2013). Strand *et al.* (2021) have shown that even with extensive washing, contamination can still be problematic, especially as liquid nitrogen is known to not fully inhibit the replication of microorganisms (Bajerski *et al.* 2020). Though much work is yet to be done, amphibian cryobanking, ART and aART are undeniably exciting and hold great promise as conservation safety nets.

involved. Nevertheless, ethical and welfare risk assessment should be mandatory prior to the use of them, especially as the welfare of an individual animal risks becoming a secondary consideration after the larger goal of saving a species (de Mori *et al.* 2021). In addition, it could be thought that the time required for successful sample collection, cryopreservation, thawing and use to make viable offspring, with all the research and development involved for species-specific optimisation, could be better spent on more traditional conservation methods (de Mori *et al.* 2021). But, with so many species being lost today, cryopreservation followed by viable cell and tissue cryobanking can fill the gap between permanently losing genetic diversity and the development of future technologies. This review will discuss the potential of cryobanking and the use of reproductive technologies to resurrect biodiversity.

Global prioritisation of species for cryopreservation

Only one aspect of biodiversity conservation, cryobanking, can make significant contributions to population management and species recovery, as seen in the black-footed ferret (*M. nigripes*) and giant panda (*A. melanoleuca*) (Howard *et al.* 2016, Santymire 2016, Comizzoli 2020). However, the immense resources required to sample, maintain and utilise biobanked samples, combined with the sheer number of threatened species requiring conservation intervention globally, mean that not every species can be sampled and conserved in this way (Hobbs *et al.* 2019). The current approach to the selection of species for cryobanking has been mainly opportunistic, with the collection of tissue samples on an *ad hoc* basis, resulting in the prioritisation of large charismatic species and missed

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Figure 1 Critically endangered mountain chicken frog (*Leptodactylus fallax*), photo © Chester Zoo, 2022; photo shared with permission. Chytridiomycosis, volcanic eruptions and habitat loss have resulted in a catastrophic decline in mountain chicken frog numbers, with less than 150 mature individuals now surviving (IUCN SSC Amphibian Specialist Group 2017). Fortunately, somatic tissue samples have been cryopreserved in living biobanks, and with poor *ex situ* breeding success, aART may be an additional conservation tool to prevent this species from going extinct.

conservation opportunities for others (Hobbs *et al.* 2019). If cryobanking is to be an effective and efficient biodiversity conservation tool, then it is important that the way in which we select and prioritise species for storage follows a clear, coordinated and transparent methodology (CPSG 2016, Mooney 2021).

There are multiple ways to integrate cryobanking with wildlife conservation: to support captive breeding programmes and/or to support *in situ* breeding programmes. Each country is likely to have its own set of priorities, and while some might favour the support of threatened populations *in situ*, others will focus on the support of species in *ex situ* populations. *Ex situ* genome banking will likely involve the international transport of cells, tissues and gametes and cryopreservation in facilities outside the home ranges of species. This poses some practical problems, including the risks of disease transmission via the stored samples and via the liquid nitrogen. However, even though the risk of contamination of samples preserved in liquid nitrogen (and subsequent disease transmission) is highly unlikely when appropriate techniques and safe practices are implemented (Penfold *et al.* 2021), it is not possible to move ungulate gametes or embryos in the United States due to the inherent disease transmission risk to the agricultural industry and associated economic threats (Joaquim *et al.* 2017). It is therefore important to guard against this potential disease transmission. Cryobanks set up to serve local species do not run the same risks, although the avoidance of bacterial and viral contamination is still important (Penfold & O'Brien 2012). There are a number of ways to mitigate the risk of disease transmission when transporting genetic material including disease screening of donor animals, high levels of biosecurity and using fresh, previously unused liquid nitrogen (Penfold *et al.* 2021). In the United States, disease transmission risk to the agricultural industry is lower for carnivores, and therefore, transportation has been achieved, for example, embryo transportation of the Brazilian ocelot (*Leopardus pardalis mitis*) (Conforti *et al.* 2009). In Europe, prior to transporation, samples from certain species may require CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) permits. However, within the European Union (EU) generally there is no need for CITES export or import permits. Outside the EU, CITES export permits are required unless the receiving biobank is a registered scientific institution with a CITES exemption.

The utilisation of existing conservation assessment schemes, such as the International Union for the Conservation of Nature (IUCN) Red List, has been suggested as one way to prioritise species for cryobanking efforts, with more threatened species receiving greater priority (Ryder & Onuma 2018). Similarly, considering multiple assessment schemes simultaneously (such as the EDGE (Evolutionarily Distinct and Globally Endangered) of Existence and Alliance for Zero Extinction) can help to identify the most at-risk and uniquely vulnerable species and provide more nuanced species recommendations and prioritisations (Mooney 2021). Prioritising and sampling species on the brink of extinction are invaluable for scientific studies, last-gasp conservation efforts and for any future de-extinction attempts, as seen in the Pyrenean ibex (*Capra pyrenaica pyrenaica*) (Folch *et al.* 2009). However, this also results in the selection of species which already lack genetic diversity within their populations and therefore have limited prospects for meaningful conservation intervention and recovery, ultimately resulting in a limited conservation value of cryobanking such species (Hobbs *et al.* 2019). However, it is possible that gene editing techniques, such as CRISPR-Cas9 (Doudna & Charpentier 2014), may help overcome these problems in the future; but, many ethical considerations will need to be observed (Johnson *et al.* 2016, Segelbacher *et al.* 2021).

To improve the chances of success for conservation intervention and population management, sample collection should focus on species which still have sufficient extant population sizes and genetic diversity available to sample from (Hobbs *et al.* 2019, Ryder & Onuma 2018). This involves a better understanding of species population sizes and genetic diversity and the use of existing assessment

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schemes, for example, the IUCN Red List, to investigate which currently non-threatened species might become threatened in the future and then prioritise these species for cryobanking efforts before they suffer population declines and genetic diversity loss. Prioritising and sampling species while genetic diversity still exists, and before they become threatened, means that early intervention and genetic restoration are possible once their populations begin to decline, improving the probability of successful species recovery (Hobbs *et al.* 2019). Although predicting which species will become threatened, and why, is difficult (Walker *et al.* 2021), studies such as that by Foden *et al.* (2013) have identified species which are most vulnerable to future climate change, even those not currently threatened with extinction, such as the griffon vulture (*Gyps fulvus*), and can provide potential new priorities for conservation and cryobanking efforts (Mooney 2021).

Similarly, opportunities for sample collection should be incorporated into the species prioritisation process, as many species are found in isolated or inaccessible locations, making sample acquisition and transport both difficult and expensive (Ryder & Onuma 2018, Houck 2019). However, the global zoo and aquarium community represent a unique resource for samples, either through existing collections such as, for example, the European Association of Zoos and Aquaria (EAZA) Biobank, or new sampling drives, and can provide easier access to populations of thousands of species which are currently threatened or likely to become threatened in the future (Mooney 2021). Additionally, many zoos and aquariums have active links and partnerships with *in situ* conservation projects, providing opportunities to collect additional genetic samples from already represented species and from species which are not currently maintained *ex situ* (AZA 2015). By capitalising on sampling opportunities and utilising *ex situ* collections and their partnerships, we can reduce sampling costs and increase the probability that biobanked samples can be employed to help conserve and manage both *in situ* and *ex situ* populations in the future (Benirschke 1984, Clarke 2009, Mooney 2021).

The process of prioritising *ex situ* managed species for cryobanking also needs to consider which individuals within the population are the most genetically valuable and suitable for future conservation efforts, maximising the conservation value of banked samples and limiting the loss of genetic diversity within a population (Clarke 2009). Many of the species found in zoos and aquariums are being actively managed to maintain genetic diversity through regional or international population management programmes (Che-Castaldo *et al.* 2021), and the availability of such pedigree managed and potentially also genotyped populations can help to identify the most genetically valuable individuals to sample. Such strategic cryobanking efforts have helped to reintroduce once lost genetic variation into extant populations of black-footed ferrets (*M. nigripes*), using samples collected in 1988 from an individual which had no living descendants and was no longer genetically represented in the population (Imbler 2021) and the endangered Przewalski's horse (*Equus przewalskii*) which was cloned in 2020 using samples cryopreserved in 1980 at the San Diego Zoo Institute for Conservation Research Frozen Zoo®. Unfortunately, for the many species which have yet to be sampled, such opportunities are not available, limiting the options open to conservation practitioners and population managers. Cryobanking needs to be seen as an integral part of the conservation toolkit and when used appropriately can even reduce the costs required to achieve genetic diversity retention targets compared to traditional *ex situ* breeding strategies (Howell *et al.* 2021). However, this will require the combining of both species and individual animal prioritisations to provide the most effective use of bio and cryobanking as conservation and population management tools.

Gamete and reproductive tissue cryopreservation

Spermatozoa cryopreservation is an example of just one ART that can facilitate a living cryobank capable of aiding in the genetic management of endangered species and has been achieved in many species (covered in-depth elsewhere, for example, amphibians: Browne *et al.* 2019; fish: Asturiano *et al.* 2017, Xin *et al.* 2017; mammals: Swanson *et al.* 2007, Rickard *et al.* 2022; avians: Asano & Tajima 2017, Cardoso *et al.* 2020). Storing genes in the form of spermatozoa is particularly beneficial as spermatozoa are continuously replenished haploid cells that can be collected from numerous genetically diverse representatives of a species. Furthermore, the cryopreservation of genes in the form of spermatozoa enables the application of ART to reintroduce genes back into populations. ART techniques such as AI, IVF, embryo transfer or intra cytoplasmic sperm injection (IVF, ET, ICSI) are currently the most efficient treatment modalities practised compared to alternative aART methods such as somatic nuclear transfer (Choudhary *et al.* 2016, Gouveia *et al.* 2020), with AI currently remaining the most efficient and commonly used ART technique (Holt & Lloyd 2009).

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Semen can be collected from non-domestic species in many ways which include the use of an artificial vagina, transrectal massage (Schmitt & Hildebrandt 1998), electroejaculation (Roth *et al.* 1998), testicular sperm aspiration (Damiani *et al.* 2004), urethral catheterisation (Lueders *et al.* 2012) or post-castration dissection (ante-mortem or post-mortem) (Saragusty *et al.* 2010, Roth *et al.* 2016). The process of collecting and freezing spermatozoa from testes post-mortem is commonly referred to as 'gamete rescue' and is used by scientists to prevent the permanent loss of a male genetics from a population. Once spermatozoa are collected, they are diluted in a cryopreservation medium, which is formulated to mitigate damage inflicted by the cryopreservation process (Purdy 2006, Comizzoli *et al.* 2012). In general, cryopreservation mediums contain (1) energy substrates for spermatozoa to metabolise; (2) antioxidants to prevent the build-up of reactive oxygen species; (3) buffers to prevent harmful shifts in pH; (4) osmolytes to create an isosmotic solution; (5) plant or animal source proteins and/or lipids to stabilise the membrane; (6) antibiotics to mitigate potential risks of bacterial disease transmission and (7) a cryoprotectant (such as glycerol, DMSO, ethylene glycol, etc.), which slow down the kinetics of ice crystal formation, preventing the formation of lethal intracellular ice (Holt 2000, Fuller 2004). As each species' physiology is inherently unique, cryopreservation mediums must be formulated to meet species-specific physiologic requirements and mitigate that species' sensitivities to cryopreservation (Comizzoli *et al.* 2012).

Similar to cryopreservation media, methods to cryopreserve spermatozoa are highly diverse across taxa. As a sample is cooled and ice begins to form, the remaining solution becomes increasingly concentrated. The increased concentration of the solution results in the dehydration of the spermatozoa, preventing the formation of lethal intracellular ice. However, the increased concentration of the solution can also elicit toxic effects if the spermatozoa are exposed to the solution for too long. An optimal cooling rate varies between species and cell type, but in general, an optimal cooling rate is achieved when the rate is slow enough that spermatozoa can be dehydrated, preventing the formation of intracellular ice, but fast enough that the spermatozoa are not exposed to changes in the solution for too long. Once cryopreserved, viable spermatozoa can be stored almost indefinitely without decomposition or metabolism, typically beneath liquid nitrogen, in 'suspended animation' until thawing.

Even though there are some positive examples, like the black-footed ferret (*M. nigripes*, Howard *et al.* 2016) and certain coral species (Hagedorn *et al.* 2012, 2017), successful production of live offspring using frozen-thawed spermatozoa can be extremely variable and challenging for different species (Leibo & Songsasen 2002, Comizzoli *et al.* 2015). This can be observed most notably in marsupials (Taggart *et al.* 1996, Unwin & Pettit 2004). So far, it has not been possible to cryopreserve any marsupial spermatozoa successfully and the only successful artificial insemination in a marsupial was achieved in the koala (*Phascolarctos cinereus*) using chilled, but not frozen, spermatozoa (Johnston & Holt 2019). In this example, it is thought that dilution of koala semen for artificial insemination is complicated because koalas are induced ovulators, and it is thought that ovulating factors are present in the semen. Therefore, the extension of semen for preservation purposes, which involves significant dilution, might be anticipated to result in a failure to induce ovulation (Allen *et al.* 2008).

In other species where cryopreservation has been attempted but failed, it is likely that the failures are also due to complex underlying factors affecting viability or fertilising ability that are still poorly understood or, yet, unknown by researchers. These include factors relating to species that are relatively important from both commercial and conservation perspectives such as swine (Bailey *et al.* 2000, 2008), avian (Blesbois *et al.* 2005) and elasmobranch (Gillis *et al.* 2021*b*).

Establishing banks of frozen and viable semen from such species using conventional freezing methods is therefore not possible at present. However, it has been proposed that, on balance, spermatozoa is still worth freezing in the hope that techniques that can take advantage of the genetic material contained in currently non-viable cryopreserved gametes will become available sometime in the future (Rodger *et al.* 2019). Being able to quickly develop an optimal spermatozoa cryopreservation protocol for the variety of species selected as suitable candidates for cryobanking, and standardisation of these protocols, is the main challenge for researchers, especially when faced with extremely limited biological material to effectively develop a working protocol when an opportunity for spermatozoa collection arises. Furthermore, there are two important phenomenon that should also be considered. First, inbreeding depression can lead to poor spermatozoa morphology, resulting in poor fertility (Huffmeyer *et al.* 2022). Secondly, wild species subject to lower levels of spermatozoa competition (for instance, those limited by population size) may also result in an increase in the variability of spermatozoa morphology (Carballo *et al.* 2019). A morphologically homologous sperm

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population is a prerequisite for developing an optimal cryopreservation protocol; therefore, factors that lead to variability in morphology will significantly impede the likelihood of developing a successful protocol. However, more research is needed to ascertain the degree to which inbreeding depression and spermatozoa competition leads to poor semen freezing ability, and the precise evolutionary mechanism is yet to be explored.

Spermatozoa is commonly cryopreserved from domestic species for commercial breeding programmes with high levels of success, including domestic species of cattle, water buffalo, cats, rodents, horse, goat, deer, sheep, dog, rabbit and selected fish species (Curry 2000, Woelders *et al.* 2012, Kochan *et al.* 2019, Thongphakdee *et al.* 2020). It is prudent to utilise spermatozoa cryopreservation protocols already established in well-developed specimens as a reliable model for poorly understood specimens, including for species from the same genera or closely related species (Comizzoli 2015). This has been successfully demonstrated with numerous critically endangered species already, including the use of equine semen protocols as a model for rhinoceros species (Reid *et al.* 2009), bovine semen protocols as a model for gazelle species (Saragusty *et al.* 2006), domestic ferret as a model for black-footed ferret (*M. nigripes*) (Howard *et al.* 2016) and human spermatozoa cryopreservation protocols as a model for macaques (Si *et al.* 2010). However, for some, closely related species to use as semen cryopreservation models, do not exist. Protocols have been successfully using already established methods with slight modifications to take the variability in semen characteristics into account. Examples where this can be observed include Asian elephants (*Elephas maximus*) (Saragusty *et al.* 2009), giant panda (*A. melanoleuc*) (Martin-Wintle *et al.* 2019), bees (Comizzoli *et al.* 2019), killer whale (*Orcinus orca*) and bottlenose dolphin (*Tursiops truncatus*) (O'Brien & Robeck 2006, Robeck *et al.* 2011). A thorough understanding of the phylogenetic relationship of species is therefore important when planning an effective strategy for cryopreserving sperm from novel species. Developing forums that actively encourage knowledge transfer between cryobiologists, adequate data capture and sharing of proven spermatozoa cryopreservation protocols are also mission critical to creating an effective living biobank. Further research is also required to better understand the root causes why some species produce more cryo-sensitive spermatozoa than others. Innovative work in this field includes the successful application of novel ART such as control-rate freezers, directional cryopreservation (Saragusty *et al.* 2007, Reid *et al.* 2009), vitrification (Hunt 2017) and freeze drying of spermatozoa (Sherman 1963, Kaneko *et al.* 2014).

In addition, spermatozoa can be collected from the epididymis of testes following death or neutering of sexually mature individuals. However, if this technique fails, or the individual is immature, the testis remains a viable source of spermatozoa (Crabbé *et al.* 1997). Indeed, in domestic cats, a model for endangered wild felid species, spermatozoa has been successfully removed from cryopreserved testicular tissue by mincing thawed tissue. Via ICSI, embryos were then created, resulting in live kittens (Tharasanit *et al.* 2012). Furthermore, testicular spermatozoa has the potential of retaining higher viability (Chatdarong 2011). The cryopreservation of testicular tissue also increases the potential of saving important genetics from valuable animals that died unexpectedly. There are multiple techniques for the preservation of testicular tissue. Following cryopreservation, testicular fragments can be cultured *in vitro* to obtain viable spermatozoa, and techniques including ultra-rapid freezing have resulted in promising results (Sato *et al.* 2011). Effective preservation of testes is vital to maintain the functionality of retrieved spermatozoa (Pothana *et al.* 2017, da Silva *et al.* 2020). The cryopreservation of testicular tissue has been achieved for many wild mammalian species (Table 1), although this is more complex than cell cryopreservation due to increased requirements of permeation of cryoprotectant and increased heterogeneity of the tissue (Pothana *et al.* 2017).

Slow freezing methods for testicular tissue have shown promising results with tissue showing maintenance of spermiogenesis after cryopreservation for nonhuman primates including white-headed marmoset (*Calllithrix geoffroyi*), mandrill (*Mandrillus sphinx*) and chimpanzee (*Pan troglodytes*) (Pothana *et al.* 2016). Following cryopreservation, conditions need to be met to enable the thawed tissue to resume spermatogenesis. One option is the autologous grafting of the thawed tissue (autografting: grafting tissue from original location to elsewhere in the same individual). This has been achieved for rhesus macaques (*Macaca mulatta*) where grafted testicular tissue produced spermatozoa, which was retrieved and used to fertilise oocytes by ICSI, resulting in embryos and a successful graft-derived baby (Fayomi *et al.* 2019). While complete spermatogenesis after testicular tissue cryopreservation and xenografting (grafting tissue from a donor animal into a recipient of another species) has not been achieved for adults of wild or domestic species, it has been achieved for immature individuals of ovine and swine (Arregui *et al.* 2008, Silva *et al.* 2020). However, autografting and xenografting of testicular tissue have little application for endangered species (Silva *et al.* 2020). An alternative method is *in vitro* culture of testicular tissue to

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Table 1 Species for which cryopreservation of testicular tissue has been achieved.

Species	Reference
Primates	Poels *et al.* (2012), Pothana *et al.* (2016), Fayomi *et al.* (2019)
Rhesus monkey (*Macaca mulatta*)	
Mandril (*Mandrillus sphinx*)	
Chimpanzee (*Pan troglodytes*)	
White-headed marmoset (*Callithrix geoffroyi*)	
Cervids	Thuwanut *et al.* (2013), Pothana *et al.* (2015, 2017)
Indian spotted mouse deer (*Moschiola indica*)	
Indian hog deer (*Hyelaphus porcinus*)	
Barking deer (*Muntiacys muntjak*)	
Sambar deer (*Rusa univolor*)	
Rusa deer (*Rusa timorensis*)	
Fea's muntjac (*Muntiacus feae*)	
Bovids	Thuwanut *et al.* (2013)
Sumatran serow (*Caprivornis sunatraensis*)	
Felids	Thuwanut *et al.* (2013)
Jungle cat (*Felis chaus*)	
Lion (*Panthera leo*)	
Leopard (*Panthera pardus*)	
Canids	Andrae *et al.* (2021)
Grey wolf (*Canis lupus*)	
Suids	da Silva et al. (2019)
Collard peccary (*Dicotyles tajacu*)	

initiate spermatogenesis (Lee *et al.* 2013, Richer *et al.* 2020). The success of *in vitro* testicular tissue culture is reliant on specific methodologies which are still to be established for endangered species making it vital to cryopreserve and biobank this tissue (Lima *et al.* 2020). Biobanking of tissue maintains genetic variability across time and space providing the opportunity to first develop and optimise the necessary technologies (Hildebrandt *et al.* 2021).

Mature oocytes can be harvested from ovarian follicles, and immature oocytes can be collected from ovarian tissues. Due to the low surface area-to-volume ratio of the oocyte, increased levels of intracellular ice formation during freezing makes cryopreservation more challenging; however, it has been attempted in a number of species (Table 2) (Borini & Bianchi 2012). This damage during the cryopreservation process is exacerbated by the low and variable membrane permeability to cryoprotectants (dependent on oocyte development state) (Leibo 1980, Arav 2014, García-Martínez *et al.* 2021), resulting in cellular disruption and death and leading to generally poor fertilisation rates from frozen-thawed oocytes (Tharasanit & Thuwanut 2021). Furthermore, with reference to cryopreserving oocytes from endangered animal species, the oocyte membrane permeability to cryoprotectant agents varies among species, again leading to theoretical models being used to predict likely optimal freezing protocols (Tharasanit & Thuwanut 2021). Oocyte cryopreservation is particularly challenging in fish due to the large cell volume, multiple compartments, the presence of a chorion, the low membrane permeability to cryoprotectants and a high chilling sensitivity (Asturiano *et al.* 2017, Diwan *et al.* 2020). Therefore, for those species, alternatives are intensively investigated and germ cell surrogacy via germ cell transplantation looks like one of the most promising methods (Rivers *et al.* 2020). Technical difficulties confronted during fish oocyte cryopreservation were already ominous for amphibian oocyte handling. The same large diameters, volumes and high yolk content can be observed in both taxa and are barriers in efficiently applying cryopreservation methods (Clulow *et al.* 2019*a*,*b*) (also see case study Fig. 1). In some species, such as the domestic cat, oocytes contain high levels of lipid droplets that become disrupted during slow freezing procedures resulting in cellular injury (Okotrub *et al.* 2018). This may well apply to endangered felines too. As an alternative, vitrification, which avoids ice formation by using high concentrations of cryoprotectants and very rapid freezing, resulting in solidification without ice formation (Rall & Fahy 1985), has been successfully employed in oocyte cryopreservation, and, despite some problems (Prentice & Anzar 2010), the evidence suggests that this is the preferred method, at least for some species (Rienzi *et al.* 2017, Whaley *et al.* 2021). Cryopreservation of feline oocytes, domestic and non-domestic species, however, remains in an experimental phase (Jewgenow & Zahmel 2020). Post-thawing viability and developmental competence are seriously impaired and until now, none of the existing techniques could significantly improve

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Table 2 Examples of mammalian species for which oocyte cryopreservation has been conducted.

Species	Method	Reference
Bovine	Vitrification	Fuku *et al.* (1992), Hamano *et al.* (1992), Hurtt *et al.* (2000), Chian *et al.* (2004), Vieira *et al.* (2008), Nakayama *et al.* (2020)
Equine	Slow freezing	Otoi *et al.* (1995), Suzuki *et al.* (1996)
	Vitrification	Hurtt *et al.* (2000), Maclellan *et al.* (2002), Ortiz-Escribano *et al.* (2018), Clérico *et al.* (2021)
Ovine/caprine	Slow freezing	Bhat *et al.* (2014)
	Vitrification	Purohit *et al.* (2012), Moawad *et al.* (2013), Bhat *et al.* (2014), Quan *et al.* (2016)
Porcine	Slow freezing	Yang *et al.* (2012)
	Vitrification	Vallorani *et al.* (2012), Appeltant *et al.* (2017), Jia *et al.* (2019), López *et al.* (2021)
Canine		
Domestic	Vitrification	Abe *et al.* (2010), Turathum *et al.* (2010)
Mexican grey wolf (*Canis lupus baileyi*)	Vitrification	Boutelle *et al.* (2011)
Blue fox (*Alopex lagopus*) farmed	Vitrification	Zhou *et al.* (2009)
Feline		
Domestic	Slow freezing	Luvoni and Pellizzari (2000)
	Vitrification	Fernandez-Gonzalez and Jewgenow (2017), Nowak *et al.* (2020), Sowińska *et al.* (2020), Fernandez-Gonzalez *et al.* (2021)
Non-human primate		
Lowland gorilla (*Gorilla gorilla gorilla*)	Slow freezing	Lanzendorf *et al.* (1992)
Macaque (*Macaca mulatta*)	Slow and rapid freezing	Vandevoort *et al.* (2008)

freezing (Jewgenow & Zahmel 2020). Since there is scarcity in wild feline samples, ART protocols are being developed in the domestic cat and seem to be working out well as a model for the wild species (Fernandez-Gonzalez *et al.* 2021) although more research is required to verify gamete rescue methods in exotic felids (Jewgenow & Zahmel 2020).

An alternative approach to freezing oocytes is the cryopreservation of immature-oocyte-containing ovarian tissue, which has been conducted in a number of species (Table 3) (Martinez 2017). Since the first mouse was born in 1996 following *in vitro* growth of primordial follicles (Eppig & O'Brien 1996), there have been many published studies on this technique in a number of species including white-tailed deer (*Odocoileus virginianu*) (Gastal *et al.* 2018), domestic cat (*Felis catus*) (Mouttham & Comizzoli 2016), collared peccary (*Pecari tajacu*) (Lima *et al.* 2019), yellow-toothed cavies (*Galea musteloides*) (Praxedes *et al.* 2017), brown trout (*Salmo trutta*) (Lujić *et al.* 2017), donkey (*Equus asinus*) (Lopes *et al.* 2018) and domestic cattle (Figueiredo *et al.* 1993, 1994*a*,*b*, Hulshof *et al.* 1995, Vasconcelos *et al.* 2013). Following death, euthanasia or neutering, a portion of the ovary can be cryopreserved; the immature follicle-containing cortex is dissected and cut into small strips under sterile conditions before slow freezing (Benesova & Trefil 2016, Hinkle *et al.* 2021). As the ovary contains many follicles, there is a potential to produce large numbers of oocytes from the tissue within a laboratory. Harvested oocytes can be matured *in vitro* and used in IVF. Teams, including the Rhino Fertility Project, are developing the *in vitro* tissue culture technique to safeguard critically endangered species, including the northern white rhino (*C. simum cottoni*), of which there are only two individuals left, again highlighting the critical importance of viable cell cryobanking for resurrecting biodiversity. Several initiatives such as the Hemmersbach Rhino Force Cryovault (South Africa), Rhino Repro (South Africa), the Frozen Zoo (San Diego Zoo Wildlife Alliance, USA) and BioRescue (Germany) are storing rhinoceros tissue and genetic material that can be utilised once methods to produce rhinoceros calves *in vitro* will be established.

Somatic cell cryopreservation and advanced assisted reproductive technology

Reproductive cloning involves the transfer of genetic material from a somatic cell into an enucleated oocyte, SCNT, ultimately resulting in an animal that has a genome sequence within the nucleus identical to that of the donor of the somatic cell used. The ability of differentiated adult cells to produce viable offspring following SCNT was first demonstrated in the African clawed frog (*Xenopus*

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Table 3 Examples of ovarian tissue cryopreservation: domestic/laboratory and wild animal species.

Species	Freezing methodology	Cryoprotectants	Outcome	Reference
Domestic/laboratory				
Murine	Vitrification	Ethylene glycol and DMSO	Melatonin improved outcome post-thaw	Wu *et al.* (2019)
Porcine	Vitrification	Ethylene glycol		Jia *et al.* (2020)
Canine	Slow freezing	DMSO and propanediol	DMSO more effective as a cryoprotectant	Lopes *et al.* (2016)
Feline	Vitrification	DMSO and ethylene glycol	Use of metal (titanium) freezing tubes proved advantageous	Fernandez-Gonzalez *et al.* (2021)
Caprine	Slow freezing	DMSO and propanediol		Rodrigues *et al.* (2004)
Ovine	Slow freezing Vitrification	DMSO and sucrose DMSO, ethylene glycol and sucrose	No significant differences between techniques	Locatelli *et al.* (2019)
Bovine	Vitrification	DMSO and ethylene glycol	Leucosporidium ice-binding protein reduced post-thaw damage	Kong *et al.* (2021)
Wild				
Agouti (*Dasyprocta*)	Slow freezing	DMSO, ethylene glycol and propanediol		Wanderleya *et al.* (2012)
African lion (*Panthera leo*)	Slow freezing	Ethylene glycol and sucrose		Wiedemann *et al.* (2012)
Zebu (*Bos indicus*)	Slow freezing	Glycerol, DMSO, ethylene glycol and propanediol	DMSO and propanediol were the most effective cryoprotectants	Lucci *et al.* (2004)
Amur leopard (*Panthera pardus orientalis*), black-footed cat (*Felis nigripes*), Geoffroy's cat (*Leopardus geoffroyi*), northern Chinese leopard (*Panthera pardus japonensis*), oncilla (*Leopardus tigrinus*), serval (*Lupus cervarius*), sumatran tiger (*Panthera tigris sondaica*)	Slow freezing	Ethylene glycol and sucrose		Wiedemann *et al.* (2013)
Mexican grey wolf (*Canis lupus baileyi*)	Vitrification			Boutelle *et al.* (2011)

laevis) by Gurdon *et al.* (1975) who showed that nuclei from keratinised skin cells transplanted into enucleated oocytes could develop into viable tadpoles, establishing the principle that cell nuclei do not undergo irreversible changes as the cell specialises to form adult tissues. The importance of this fundamental finding has been recognised by the award of the Nobel Prize for Physiology or Medicine jointly to Gurdon in 2012. The successful application of nuclear transfer using adult somatic cells to a mammalian species, the sheep (*Ovis aries*), was reported by Wilmut *et al.* (1997) resulting in the first cloned mammal.

SCNT can be performed either by removing the nucleus from the somatic cell and introducing it into the enucleated oocyte by microinjection (Wakayama *et al.* 1999), or, alternatively, the entire somatic cell can be fused with the enucleated oocyte using electrical fusion (Liu *et al.* 2015), of which, the latter appears to be the preferable approach (Qu *et al.* 2020). Oocyte maturation is required prior to fusion, with the optimum conditions varying between species (Borges & Pereira 2019). Following artificial activation, for example, with ionomycin and 6-dimethylaminopurine, which has been successful in many different species including bovines (Bhak *et al.* 2006), camelids (Wani *et al.* 2017), porcines (Borges *et al.* 2020) and primates (Liu *et al.* 2018), the egg develops to an early embryo *in vitro* and is then implanted into the uterus of suitable recipient female. Identification and optimisation of the three critical procedures with the greatest impact on the

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development of oocytes and early embryos, namely oocyte micromanipulation, electrofusion, and the *in vitro* culture of early embryos, have recently been reviewed (Ma *et al.* 2021).

The possibility of using reproductive cloning in the conservation of endangered animal species has been widely discussed (e.g., Holt *et al.* 2004, Shapiro 2017, Borges & Pereira 2019). For any endangered species, it is unlikely that sufficient oocytes will be available for SCNT. Thus, cells of a separate but related species will need to be used: interspecific SCNT (iSCNT, Fig. 2). If successful, the result is the birth of an animal with the nuclear genome of the endangered individual but with the mitochondrial DNA derived from the donated oocyte. As both nuclear and mitochondrial genes regulate mitochondrial development and function (Mrowiec *et al.* 2021), the more closely related the species of the donor nucleus and recipient enucleated oocyte, the less likely there will be nuclear-mitochondrial incompatibility (Lagutina *et al.* 2013). Species or individuals created by means of interspecific cloning are considered by IUCN as 'proxies' which are the functional equivalents of an extinct species able to restore ecological functions or processes that might have been lost because of the extinction of the original species (IUCN 2016). However, because of, for example, microbiome differences and inheritance of the mtDNA of the donor oocyte, proxies result in a species that differs from the extinct one (IUCN 2016). Besides technical and biological hurdles, legal and ethical considerations need to be taken into account when approaching de-extinction efforts based on proxies (Seddon & King 2019).

Low success rates have been reported for reproductive cloning resulting in only 5–10% of reprogrammed embryos yielding viable offspring, with many factors affecting this success rate (Long *et al.* 2014). These factors include DNA damage, which can be improved by upregulating modulators of the DNA damage response (Lee *et al.* 2021), the cell type used for nuclear donation (Inoue *et al.* 2005, Liu *et al.* 2015, Lee *et al.* 2019) and the mismatch of mitochondrial DNA between donor cell and recipient oocyte (Takeda 2019, Mrowiec *et al.* 2021). Epigenetic processes may also affect DNA replication and transcription (Gouveia *et al.* 2020). Many of these problems occur early on in embryonic development and result from incomplete reprogramming of the donor cell nuclei and the subsequent developmental failure of the cloned embryos (Zuo *et al.* 2014). Choice of oocyte donor species to ensure compatibility with the somatic cell donor is also likely to be an important factor (Jeon *et al.* 2016).

It is particularly important to take the low success rates of reproductive cloning into consideration in the context of endangered species, where the production of viable offspring is the top priority. Although iSCNT has been applied to many species resulting in the birth of offspring, by no means all of these were viable in the long term for several reasons including morphological abnormalities, premature delivery, lung immaturity, stillbirths, placental separation and septicaemia (Table 4). Furthermore, cloning results in offspring genetically identical to the somatic cell donor and needs careful consideration where the gene pool of an endangered species is limited. In the future, it may be possible to use genome editing with CRISPR/Cas9 to address this issue (Sheets *et al.* 2016), albeit it also raises ethical concerns. Cryopreservation of somatic cells taken from as many different tissues as possible from each

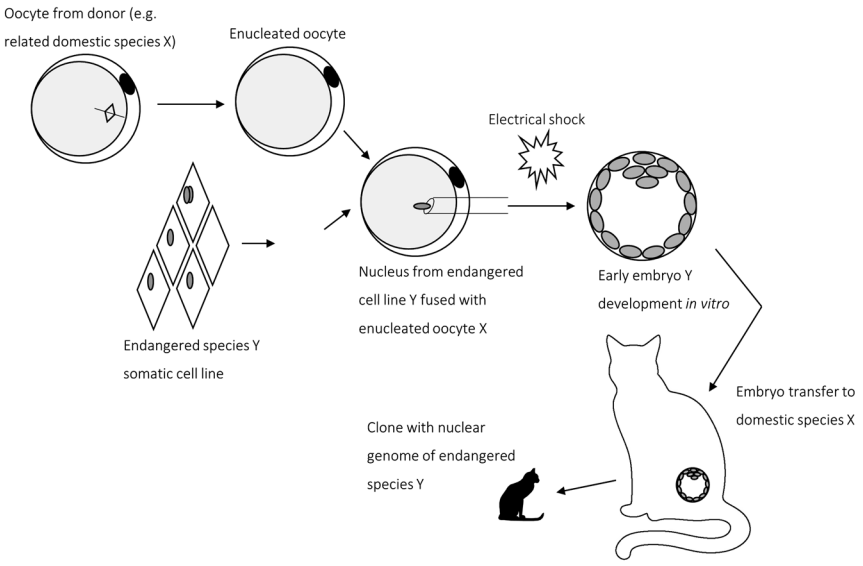

Figure 2 Outline of the interspecific somatic cell nuclear transfer (iSCNT) procedure. The nucleus from an endangered species' somatic cell (species Y) is fused with the enucleated oocyte from a closely related, domestic species (species X). Following electrical or chemical activation, an early embryo of species Y developed *in vitro*. The early embryo is transferred into a surrogate mother of domestic species X, resulting in a clone containing the nuclear genome of endangered species Y.

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Table 4 Examples of interspecific somatic cell nuclear transfer (iSCNT) of mammalian species including oocyte and nuclear donor. The International Union for the Conservation of Nature (IUCN) red list status of the nucleus donor species is also included. Many of the resulting offspring did not show long-term survival, and outcome is noted where available.

Oocyte donor	Nucleus donor	IUCN red list status of nucleus donor	Outcome	Reference
Domestic cat (*Felis catus*)	African wild cat (*Felis silvestris lybica*)	Least concern (subspecies unclear)	17 kittens, 2 survived long term	Gómez *et al.* (2004)
Domestic cat (*F. catus*)	Sand cat (*Felis margarita*)	Least concern	1 of 14 kittens born survived 2 months	Gómez *et al.* (2008)
Domestic cat (*F. catus*)	Cheetah (*Acinonyx jubatus*)	Vulnerable	Incomplete nuclear reprogramming	Moro *et al.* (2015)
Domestic cat (*F. catus*)	Kodkod (*Leopardus guigna*)	Vulnerable	Embryos only developed to the morula stage	Veraguas *et al.* (2020)
Domestic cow (*Bos taurus*)	Banteng (*Bos javanicus*)	Endangered	2 calves, 1 survived long term	Janssen *et al.* (2004)
Domestic sheep (*Ovis aries*)	Mouflon (*Ovis orientalis musimon*)	Near threatened	1 lamb, 'apparently normal'	Loi *et al.* (2001)
Domestic sheep (*O. aries*)	Esfahan mouflon (*Ovis orientalis isphahanica*)	Vulnerable	2 lambs, both died shortly after birth	Hajian *et al.* (2011)
Spanish Ibex (*Capra pyrenaica*)	Pyrenian ibex, Bucardo (*Capra pyrenaica pyrenaica*)	Least concern (subspecies extinct)	1 kid, died shortly after birth	Folch *et al.* (2009)
Dromedary (*Camelus dromadarius*)	Bactrian camel (*Camelus bactrianus*)	Critically endangered	1 calf, died on day 7 post-partum	Wani *et al.* (2017)
Domestic dog (*Canis familiaris*)	Grey wolf (*Canis lupus*)	Least concern	4 pups, 3 survived long term	Oh *et al.* (2008)
Domestic dog (*C. familiaris*)	Coyote (*Canis latrans*)	Least concern	8 pups, all viable	Hwang *et al.* (2012)
Domestic ferret (*Mustela furo*)	Black-footed ferret (*Mustela nigripes*)	Endangered	1 pup, survived long term	Sandler *et al.* (2021)
Macaque monkey (*Macaca mulatta*)	Crab-eating macaque (*Macaca fascicularis*)	Vulnerable	2 young, healthy	Liu *et al.* (2018)

endangered animal should be conducted and stored until significant advances have been made in our understanding of the reproductive biology of individual species. This will maximise the potential of reproductive cloning in the conservation of endangered animal species, for example, the black-footed ferret (*M. nigripes*) and Przewalski's horse (*E. przewalskii*). If we fail to collect and store these tissues now, they are gone forever.

In addition, the cryopreservation of tissues from endangered species, capable of differentiation to germ cells, could provide another way of improving genetic diversity, particularly where only small numbers of individuals remain. Embryonic stem cells can differentiate into all cell types of the body including oocytes (Hübner *et al.* 2003) and spermatozoa (Toyooka *et al.* 2003). As these cells are derived from the early embryo that is destroyed in the process, their use for endangered species is inappropriate. Induced pluripotent stem (iPSCs are derived from adult somatic cells by genetic reprogramming to an embryonic stem cell-like state (Takahashi & Yamanaka 2006) and have been used in attempts to regenerate endangered species (Fig. 3). However, some iPS cell clones differ from embryonic stem cells in several ways including gene expression, DNA methylation and cell differentiation (reviewed by Yamanaka 2012) resulting in a number of potential problems including increased immunogenicity (Okita *et al.* 2011). Such factors need to be taken into account when considering the use of iPS cell technology.

For the cryopreservation and storage of samples from endangered animals, the choice of tissue is an important consideration. The tissue chosen should yield viable cells following freezing and thawing and should be readily reprogrammable to generate iPS cells. The viability of fibroblasts obtained from a wide range of taxa including domestic (e.g. pig (Liu *et al.* 2014), sheep (Na *et al.* 2010) and cow (Li *et al.* 2009) and endangered species (e.g. Bengal tiger (*Panthera tigris tigris*) (Guan *et al.* 2010), brown brocket deer (*Mazama pandora*) (Magalhães *et al.* 2017) and jaguar (*Panthera onca*) (Mestre-Citrinovitz *et al.* 2016) has been demonstrated post freeze/thaw. Along with this demonstrable viability, the ability to successfully reprogramme fibroblasts to iPS cells render this cell type an obvious choice. Indeed, a number of biobanks, including the San Diego Zoo Institute for Conservation Research Frozen Zoo® and The Leibniz Institute for Zoo and Wildlife Research, maintain a collection of frozen

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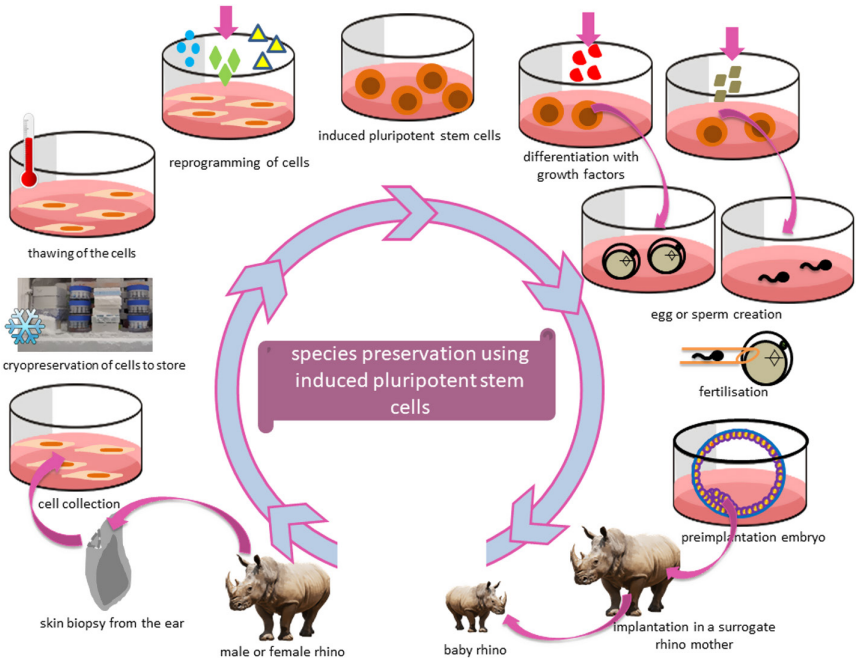

Figure 3 Species preservation using induced pluripotent stem (iPS) cells, which can be differentiated into oocytes or spermatozoa using the rhinoceros as an example. After a biopsy of a recently deceased animal, cells can be cryopreserved until the moment they are needed to produce oocytes or spermatozoa. Those differentiated cells will first need to be reprogrammed to obtain pluripotency. Afterwards, growth factors can re-differentiate the cells into the desired cell population (oocytes or spermatozoa). *In vitro* fertilisation will result in an embryo, which can be transplanted into a surrogate mother leading to offspring.

fibroblasts from multiple endangered species. In the context of cryobanking, iPS cells were first developed from frozen fibroblasts from the endangered primate the drill (*Mandrillus leucophaeus*) and the critically endangered northern white rhinoceros (*C. simum cottoni*) using viral vectors carrying the human sequences of reprogramming factors Oct4, Sox2, cMyc and KLF4 (Ben-Nun *et al.* 2011). Perhaps somewhat surprisingly, the rhinoceros responded to the human reprogramming factor sequences, suggesting that the reprogramming mechanism is highly conserved between different species, which bodes well for the potential application of this approach to a range of species. One caveat is that reprogrammed iPS-like cells from certain species, for example bovines, do not appear to yield sustainable cell lines (Pillai *et al.* 2019). The *in vitro* development of gametes from iPS cells, and the subsequent generation of viable embryos, is key to the application of this technology to the successful prevention of extinction of endangered species. The reconstitution of gametes from pluripotent stem cells, both oocytes (Hikabe *et al.* 2016, Hamazaki *et al.* 2021) and spermatozoa (Li *et al.* 2013, Ishikura *et al.* 2021), has been achieved in the mouse, and primordial germ cells have been developed from iPS cells in the northern white rhinoceros (*C. simum cottoni*) (Korody *et al.* 2021). iPS cells have now been derived from a variety of species (Table 5).

Before iPS cells or even SCNT techniques being used to produce viable offspring, a much greater knowledge of the reproductive biology of both the embryo and the surrogate dam will be vital. Pregnancy is a major challenge for the mammalian maternal immune system with specific mechanisms including the induction by the decidua of regulatory M2 macrophages and Treg cells to elicit immune tolerance at the foetal–maternal interface to prevent rejection of the semi-allogeneic (sharing only half the genes of the mother) foetus (Lindau *et al.* 2021). The increased rate of spontaneous abortion seen in cattle pregnancies produced following SCNT has been attributed to the upregulation of inflammatory cytokines resulting from abnormal expression of major histocompatibility complex class 1 proteins on the trophoblast of these conceptuses (Rutigliano *et al.* 2022). Rejection is likely to be considerably more problematic with a xenogeneic (from a different species) foetus. Furthermore, this may be a particular problem for those species with a haemochorial placental structure, including primates and some rodents, where maternal blood comes into direct contact with the foetal chorion. It has been proposed that separating the inner cell mass (ICM) from an endangered species' early embryo and injecting this into a trophoblast vesicle derived from the putative surrogate dam may overcome such problems of incompatibility (Saragusty *et al.* 2020). However, interactions between the trophoblast and ICM may pose challenges if these are derived from different species (Girgin *et al.* 2021). Furthermore, other potential issues of developing viable offspring cross-species include the role of exosomes (endosomal-derived membrane nanovesicles involved in intercellular communication (Zhang *et al.* 2019)) in implantation and early embryo development (Shi *et al.* 2021) and the acquisition of a suitable microbiome

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Table 5 Examples of mammalian species from which induced pluripotent stem (iPS) cells have been generated and their International Union for the Conservation of Nature (IUCN) red list status.

Species	IUCN red list status	Reference
Snow leopard (*Panthera uncia*)	Vulnerable	Verma *et al.* (2012)
Tiger (*Panthera tigris*)	Endangered	Verma *et al.* (2013)
Jaguar (*Panthera onca*)	Near threatened	Verma *et al.* (2013)
Serval (*Leptailurus serval*)	Least concern	Verma *et al.* (2013)
Somali wild ass (*Equus africanus somaliensis*)	Critically endangered	Ben-Nun *et al.* (2015)
Northern white rhinoceros (*Ceratotherium simum cottoni*)	Critically endangered	Ben-Nun *et al.* (2011)
Banteng (*Bos javanicus javanicus*)	Endangered	Ben-Nun *et al.* (2015)
Sumatran orangutan (*Pongo abelii*)	Critically endangered	Ramaswamy *et al.* (2015)
Drill (*Mandrillus leucophaeus*)	Endangered	Ben-Nun *et al.* (2011)
Chimpanzee (*Pan troglodytes*)	Endangered	Marchetto *et al.* (2013)
Bonobo (*Pan paniscus*)	Endangered	Marchetto *et al.* (2013)
Western gorilla (*Gorilla gorilla gorilla*)	Critically endangered	Wunderlich *et al.* (2014)
Prairie vole (*Microtus ochrogaster*)	Least concern	Manoli *et al.* (2012)
Naked mole-rat (*Heterocephalus glaber*)	Least concern	Lee *et al.* (2017)
Tasmanian devil (*Sarcophilus harrisii*)	Endangered	Weeratunga *et al.* (2018)
Little brown bat (*Myotis lucifugus*)	Endangered	Mo *et al.* (2014)
Platypus (*Ornithorhynchus anatinus*)	Near threatened	Whitworth *et al.* (2019)
Quail (*Coturnix*)	Least concern	Lu *et al.* (2015)
Zebra fish (*Danio rerio*)	Least concern	Peng *et al.* (2019)

for the development of immune function of the neonate (Macpherson *et al.* 2017). These and many other problems will need to be overcome in the future, and the fundamental importance of long-term cryobanking of live cells from endangered species needs to be underlined.

Conclusion

ART and aART have a huge potential for wildlife conservation, and therefore, many advances have been made in the last few decades. However, for these techniques to be applicable and therefore useful, prompt global cooperation and action are imperative. Knowledge sharing, data and sample inventory sharing and creating international networks of biobanks are paramount. Indeed, it is vital that protocols for all techniques are standardised, and for this, global collaboration is required. This is particularly important when research groups/biobanks only have access to very limited biological material to develop protocols on an *ad hoc* basis. Furthermore, viable tissue cryobanking should be considered in all future conservation strategies as a source of genetically diverse material that may be required in the future to combat the extinction crisis (Comizzoli & Holt 2019). Great progress is already being made to save endangered species by using ART and aART, as demonstrated by the successful captive propagation and reintroduction using ART of endangered Mississippi gopher frogs in the United States (Watt *et al.* 2021), efforts to breed cheetahs and other endangered felids via ART and aART (Wildt & Roth 1997) and the northern white rhino project to name just a few. However, despite the above example, the current focus is still primarily on mammalian taxa, and there is an urgent need for allowing these technologies to catch up across all others. These technologies open the way for more innovative conservation strategies and integration of traditional conservation methods with biologically based safety nets for species in danger. Focus needs to be placed on less charismatic taxa and the development of cryopreservation and storage protocols for tissues from avian, amphibian, reptilian, piscine and even invertebrate species in addition to the development of ART and aART for these taxa. This field of science is fast moving and vital to future biodiversity conservation efforts and will be a 'hot topic' as the ethical debate surrounding these technologies comes to the fore with the advent of new techniques and possibilities. We need to start banking samples before we lose more species, populations and genetic diversity; we do not know what will be needed in the future.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/RAF-22-0005.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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