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REVIEW

Advanced assisted reproduction technologies in endangered mammalian species

Thomas Bernd Hildebrandt[1,2] | Susanne Holtze[1]

[1]Leibniz Institute for Zoo and Wildlife Research (IZW), Berlin, Germany

[2]Faculty Veterinary Medicine, Freie Universitaet Berlin, Berlin, Germany

Correspondence
Thomas Bernd Hildebrandt, Leibniz Institute for Zoo and Wildlife Research (IZW), Alfred-Kowalke-Street 17, Berlin 10315, Germany.
Email: hildebrandt@izw-berlin.de

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Abstract

A new synergistic approach of classical conservation strategies combined with advanced assisted reproduction technologies (aART) allows for protection and rescue of endangered keystone species at the brink of extinction, which can help to safeguard complex ecosystems. Reproduction biology and management in mammal species is not only challenging in regards to their diverging sizes, anatomy, and often unknown physiology; it also requires customized training or chemical restraint protocols for safe handling. Besides these general challenges, there are several new assisted reproduction techniques (ART) specifically tailored to critically endangered mammals. The current portfolio of ART in these mammalian taxa is ranging from sexual cycle characterization and manipulation, semen collection and cryopreservation, artificial insemination, biobanking of living cells, oocyte collection, in vitro fertilization (IVF), and embryo production, embryo transfer as well as stem cell-derived in vitro gametogenesis for generating gametes in culture. The article covers advanced assisted reproduction technologies (aART), success and challenges, as well as ethical implications.

KEYWORDS
assisted wildlife propagation, conservation, fertility improvement, genetic enhancement, rewinding extinction

1 | INTRODUCTION

In light of the Earth's sixth great extinction due to anthropogenic exploitation of natural resources, poaching, as well as indirectly via climate change (Barnosky et al., 2011; Ceballos & Ehrlich, 2023; Kolbert, 2014), traditional conservation strategies such as habitat protection and ex-situ breeding combined with reintroduction programmes will be insufficient to stop or even slow down this process significantly. Currently, 22% of the mammals are at risk of extinction (http://www.iucnredlist.org).

It was recently evidenced that the loss of keystone species, such as species serving as significant landscape architects (Elephants, Rhinoceroses, and Mountain Gorilla) or taxa at the top of food chains, for example, Asiatic Lion (*Panthera leo leo*), Amur and Javan Leopard (*Panthera pardus orientalis* and *melas*), Sumatran and South Chinese Tiger (*Panthera tigris sumatrae* and *amoyensis*), and North Atlantic Right Whale (*Eubalaena glacialis*), can lead to the decline of entire species communities (Mills et al., 1993). In addition, charismatic umbrella species such as giant panda (*Ailuropoda melanoleuca*) or koala (*Phascolarctos cinereus*) can fulfil a similar function due to their emotional attractiveness, leading to indirect protection of their natural habitats, including the resident species communities (Roberge & Angelstam, 2004). Therefore, the most efficient way of maintaining functional complex ecosystems

composed of thousands of species is the protection of single keystone or umbrella species. Ironically, the latter are also most prone to extinction due to human impact. Given the enormous benefit of saving keystone and umbrella species, it is indicated, if not even mandatory, to utilize substantial resources for their protection. This requires the exploration of new avenues in innovative conservation strategies intertwined with basic research. In extreme cases such as the northern white rhinoceros (NWR, *Ceratotherium simum cottoni*), whose global population was reduced to only two infertile females, science in the form of advanced Assisted Reproductive Technologies (aART) in combination with living-cell biobanking plays a new vital role for restoring population numbers as well as genetic diversity (Hildebrandt et al., 2018, 2023; Hildebrandt, Hermes, et al., 2021; Hildebrandt, Holtze, et al., 2021). We here distinguish between (i) well-established conventional ART approaches such as hormone monitoring and administration, semen collection, artificial insemination (AI), transvaginal ovum pick-up (OPU), in vitro fertilization (IVF), transcervical embryo transfer (ET), and (ii) advanced ART (aART) methods that require extensive laboratory equipment and expertise that lie beyond those needed for the more "classical" ART methods. The latter comprise stem-cell associated techniques (SCAT), IVF using intracytoplasmatic sperm injection (ICSI), somatic cell nuclear transfer (SCNT), inner cell mass (ICM) exchange, and substantial adjustments of classical methods to match the anatomical constraints of megavertebrates such as transrectal OPU and ET in the rhinoceros (Hildebrandt et al., 2023). These aART methods are often tightly intertwined and combined with cryopreservation and biobanking. The efficiency of these future rescue approaches will be substantially enhanced by the emerging opportunities of Stem-Cell-Associated Techniques (SCAT) (Hayashi et al., 2021, 2022; Korody et al., 2021, Zywitza, Frahm, et al., 2022, Zywitza, Rusha, et al., 2022). This article summarizes novel and cutting-edge aART applications utilized in mammal conservation, their past successes and challenges, ethical implications, and future directions.

2 | THE PORTFOLIO OF aART IN ENDANGERED MAMMAL CONSERVATION

In general, the application of assisted reproduction techniques in free-ranging wildlife and wild animals in human care needs substantial taxon-specific customization due to the great variability in anatomy, morphology, chemical restraint protocols, and endocrine pathways in the various species. For example, studying reproduction in captive and wild terrestrial megavertebrates is not only challenging with regards to their exceptional size and defensiveness but also represents a challenge due to the time scale of the reproductive events such as onset of puberty, sexual cycle, and pregnancy length. Zoo animals can often be successfully trained for certain manipulations, like frequent blood sampling for hormonal analyses, ultrasound examinations, or even semen collections, whereas their wild counterparts always require general anaesthesia for any kind of intervention. Advanced ART comprises a variety of technologies tailored to overcome challenges such as complete infertility or geographic distribution linked to drastic population declines in endangered mammal species. Critically endangered species are generally characterized by (1) loss of genetic variability, (2) compromised health and immune system, (3) reproductive disorders, (3) habitat fragmentation leading to reduced encounters with potential breeding partners, (4) behavioural disorders, (5) loss of tradition, (6) malnutrition, or (7) accumulation of pollutants. One of the most relevant factors causing infertility in critically endangered species (Figure 1) is the phenomenon of asymmetric reproductive ageing affecting the female reproductive soundness (Hermes et al., 2004). Prolonged periods of futile sexual cycling due to the lack of pregnancy and lactation lead to infertility caused by severe reproductive disorders comprising uterine cyst and tumour formation, pyometra, hydrosalpinx, and/or ovarian dysfunction. This may result in a vicious cycle, ultimately causing severe health issues and premature death.

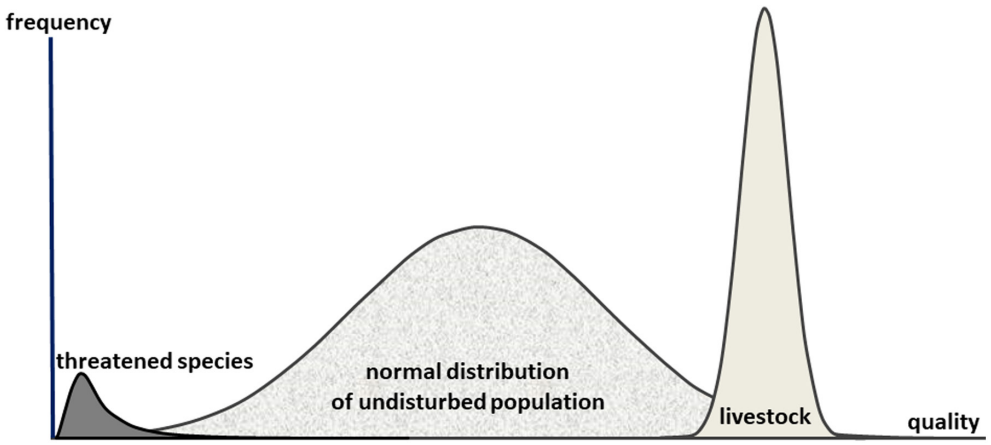

FIGURE 1 Reproductive capacity: Comparative distribution of the reproductive capacity in the population of critically endangered species (e.g., northern white rhinoceros, *Ceratotherium simum cottoni*), in an undisturbed population (e.g., brown rat, *Rattus norvegicus*) and in a livestock breeding population (e.g., cattle, dramatically enhanced during 2000 years of domestication).

In the following, we shortly summarize the relevant laboratory-based technologies for generating viable offspring in wildlife and for restoring genetically diverse and viable populations that go beyond the classical assisted reproduction techniques such as manipulation of sexual cycles or artificial insemination. Several new advanced assisted reproduction techniques (aART) have been specifically tailored to the targeted taxa. The current portfolio of aART in these mammalian taxa is ranging from (1) cryopreservation and biobanking of living cells, (2) oocyte retrieval, (3) IVF and ICSI, (4) In Vitro Embryo Production, (5) SCNT, (6) ICM, (7) ET, as well as the first attempts of (8) stem cell-derived in vitro gametogenesis for generating gametes in culture, and (9) gene editing for restoring genetic diversity using museum-derived reference material.

2.1 | Cryopreservation and biobanking of living cells

The pace of extinction leaves little room to develop customized conservation programmes for all threatened flora and fauna. Biobanking is a potent backup strategy to buy time for as many species as possible sharing currently the earth with us. While many biobanks collect dead materials such as blood serum for hormone or biochemical analyses, or tissue preserved in formalin, alcohol, or frozen without cryoprotectant for pathophysiological or genetic analyses living cells not only contain genetic information but the entire machinery and organelles necessary for sustaining and propagating life. Preserving living cells requires specific knowledge, effort, skills, and technical prerequisites such as, for example, specialized cryopreservation protocols, cell culture media or sperm extenders, sterile working conditions, and cryoprotectants, but offers further possibilities. While living gametes can be used to produce embryos, somatic cells represent an expandable source of biological material. With the advent of stem cell technology (see Section 2.8), this approach holds enormous potential for safeguarding the planetary biodiversity (Hildebrandt, Hermes, et al., 2021). Zoological institutions such as classical zoos, safari parks, or animal shelters play a crucial role as an ark for many species, providing relatively easy access to suitable biomaterials. Either during veterinary interventions, post-mortem or from little or non-invasively gathered biomaterials such as gingiva swabs, hair follicles, blood feathers, the cellular component in urine, the extracted buffy coat from whole blood, or even from the mucoid surface of faeces, it is possible to extract suitable cell material for culture. After successful culture, these living cells can be stored in liquid nitrogen (lN_2) at −196°C in central bioarchives for several hundred, maybe even thousand years. Biopsy material, cultured fibroblasts, embryonic stem cells (ES, Hildebrandt et al., 2018) or iPSC (Hayashi et al., 2022; Zywitza, Frahm, et al., 2022; Zywitza, Rusha, et al., 2022) require only simple, robust cryopreservation techniques using 10% DMSO using, for example, slow freezing over liquid nitrogen vapour. However, if it comes to oocytes, spermatozoa, and preimplantation embryos, there is a wide variation in the freezability of these sensitive biomaterials, which is often species-specific and even individual-specific (Hermes et al., 2013; Saragusty et al., 2009, 2010, 2011). The introduction of the vitrification technique mainly for embryos and Metaphase II (MII) oocytes (Rall & Fahy, 1985; Schiewe & Anderson, 2017) was a game changer in cryobiology and provided the new cryopreservation solution for the entire human IVF industry (Reinzi et al., 2017; Rienzi et al., 2012) with improved cryosurvival and clinical outcomes. Vitrification can use either open or closed systems, that is, with or without direct contact to lN_2. While cryosurvival rates are decreased in closed systems, by avoiding cross-contamination through liquid nitrogen they increase biosafety. However, the application of vitrification is still underutilized in wildlife reproduction (Simone et al., 2024; Zahmel et al., 2021).

2.2 | Oocyte retrieval in live and deceased animals

Oocytes derived from wildlife species can be collected in two principle ways: post-mortem or in vivo. The post-mortem recovery requires a much simpler technical approach. However, the quality of such post-mortally collected oocytes is often poor, especially if the donor has suffered from chronic illness or was of advanced age. In addition, the time period from the physiological death to the time of recovery of the ovaries should not exceed 30 minutes; otherwise, there will be an increased risk of microbial contamination and/or enzymatic degradation of the cumulus-oocyte complexes inside the antral follicles. The opportunity to access wildlife ovaries by using wildlife abattoirs is very rare (Zahmel et al., 2021) or nearly impossible, especially in the case of critically endangered mammals. Oocyte collection in live endangered mammals can be performed in different ways and is highly dependent on the species and the situation of the donor. Under zoo conditions, ovaries can be retrieved during surgical castration performed due to medical reasons or population management decisions. The oocyte pickup (OPU) technique in live female donors requires chemical immobilization in the form of general anaesthesia or standing sedation. The OPU can be carried out with two different imaging modalities, by endoscopy as laparoscopy (Jorge-Netoa et al., 2023; Miller et al., 1990) or by ultrasonography using the transvaginal (Loskutoff et al., 2003; Wirtu et al., 2009), the transrectal (Hildebrandt et al., 2018, 2023), or the transabdominal approach. The transabdominal, ultrasound-guided follicular needle aspiration was successfully applied by the authors in Sumatran tiger, Asian lion, Javan leopard, and giant panda (unpublished data). The developmental status of the collected oocytes derived from antral follicle aspiration can be mature (Metaphase-II) or immature (Germinal Vesicle). The matured oocyte, characterized by an expanded cumulus cell complex and the presence of a polar body, can be directly fertilized. However, the majority of oocytes collected in wildlife species are immature and require an in vitro maturation (IVM) step prior to fertilization. Immature oocytes are resilient to

long-distance transportation to specialized laboratories in a portable incubator for up to two days.

2.3 | In vitro fertilization (IVF) or intracytoplasmic-sperm-injection (ICSI)

The IVF protocol involves temporary culture (maximum 12 hours) of the matured, haploid ovum (M-II) together with ≥10^4 capacitated spermatozoa. One competent sperm cell will penetrate the zona pellucida and vitelline membrane, the oolemma. Fertilization occurs by karyogamy, and if successful, the resulting zygote starts cleavage 24 to 36 hours later.

ICSI refers to the injection of a single sperm cell into a MII oocyte using a micromanipulator and requires a substantially lower sperm number and semen quality, which may be useful for endangered species or subfertile males. The single sperm is selected by the embryologist based on the morphological and motility parameters of the available sperm cells. After immobilization of the sperm cell by inducing a tail injury, it is aspirated into the injection capillary. The micromanipulator consists of a high-resolution inverted microscope and two hydraulic actuator arms, with one holding and one injection capillary, respectively. While the classical ICSI capillaries generally used by human IVF laboratories are sharp, needle-like glass tubes with an inner diameter of 4–5 µm, the piezo drill technique uses a blunt glass capillary of usually 5 µm inner diameter (Simone et al., 2023) that penetrates through the zona pellucida and vitelline membrane using micro-vibrations. The latter is mainly used in equine IVF laboratories and for murine ICSI.

2.4 | In vitro embryo production

The embryo culture to the blastocyst stage is usually performed in special embryo culture media, which can follow a one-step or a two-step protocol, that is, without or with renewing the media during embryo culture from zygote to blastocyst stage. Media exchange removes metabolic products, re-supplying beneficial compounds while potentially also removing beneficial metabolic products such as growth factors. The embryo culture time to an early blastocyst stage depends on species-specific parameters and can range from 4 to 11 days. Culture is usually performed in a classical 5%-CO2 incubator with reduced oxygen. However, the use of time-lapse incubators such as the Embryoscope TM (VitroLife) or GERY (Merck) allows the embryologist to modify individual culture conditions based on the embryo performance during the incubation. This option is especially useful if there is not much experience with the species cultured. At the early blastocyst stage (unhatched), usually the embryos are cryopreserved in lN_2 (Hildebrandt et al., 2018, 2023) or used freshly in an embryo transfer programme. Negative epigenetic effects such as the large calf syndrome caused by the in vitro embryo production were described (Nava-Trujillo & Rivera, 2023) and should be avoided by optimization of the species-specific culture conditions.

2.5 | Somatic cell nuclear transfer (SCNT)

Somatic cell nuclear transfer is a method of cloning to generate genetic copies of an individual by transferring a somatic cell nucleus into an enucleated oocyte, followed by mitosis activation via applying an electric shock. It contributed to several species rescue programmes; for example, for the black-footed ferret (*Mustela nigripes*, Sandler et al., 2021) and the Przewalski's horse (*Equus ferus przewalskii*, Novak et al., 2023), SCNT may be combined with gene editing, for example, for improving specific traits, for the resurrection of extinct species, or for controlling invasive species (Teem et al., 2020). In interspecies SCNT (iSCNT), oocytes and somatic cells derive from different species. These can be used to produce ESC-like cell lines (Chen et al., 2003) from the blastocyst inner cell mass (ICM) or for species conservation. However, the low success rates in generating live offspring is mainly owed to mitochondrial heteroplasmy. So far live progeny was achieved in only 11 species, Yak (*Bos gaurus*), African Wildcat (*Felis lybica*), Sand Cat (*Felis margarita*), Bucardo (*Capra pyrenaica pyrenaica*), Bactrian Camel (*Camelus bactrianus*), Grey Wolf (*Canis lupus*), Coyote (*Canis latrans*), Mouflon (*Ovis aries musimon*), River Buffalo (*Bubalus bubalis bubalis*), and caracal (*Caracal caracal*) (for a review see Adams et al., 2024).

2.6 | Inner cell mass (ICM) exchange

For species with very few individuals, closely related species may be considered as surrogate mothers. To overcome the expected developmental failure due to incompatibility between trophoblast and uterus and/or immunological rejection, the donor inner cell mass may be transferred into the empty trophoblastic vesicle of the surrogate using micromanipulation to create an inter-species trophoblast-ICM chimera for inter-species embryo transfer. This has been achieved, for example, for goat and sheep (Meinecke-Tillmann & Meinecke, 1984). Although merging in vitro gametogenesis with ICM holds promise for critically endangered species, this has so far not been successfully applied.

2.7 | Embryo transfer (ET)

There are numerous techniques described for the embryo transfer in endangered mammals. The most common procedure applied is the transcervical ET. The placement of the transfer catheter into the ipsilateral uterine horn can be monitored manually through the rectum in larger mammals or by transrectal or transabdominal ultrasound in smaller species. The preferred embryonic stage transferred is the early, unhatched blastocyst (Loskutoff et al., 1995; Schiewe et al., 1991; Vendramini et al., 1997). The second technique of ET is performed via laparoscopy, which offers two options for the embryo placement: (i) into the upper ipsilateral uterine horn (blastocyst) by injection or (ii) non-invasively into the oviduct via the infundibulum pathway (2 to 4 cell embryo). These techniques have been applied in big cat species (Swanson, 2012). The embryo transfer in megavertebrates such as rhinoceroses and Asian and African elephants (*Elephas*

maximus and *Loxodonta africana*) requires a different approach due to their size and anatomical particularities. The ultrasound-guided transrectal transfer into the ipsilateral uterine horn close to the ovary with the *Corpus luteum graviditatis* has been proven to be the most minimal-invasive and successful ET technique in megavertebrates (unpublished data).

2.8 | Stem cell-derived in vitro gametogenesis (IVG) for generating gametes

Takahashi and Yamanaka (2006) developed the basics to reprogramme living cells to induced pluripotent stem cells (iPSC). The first wildlife iPSC lines were generated by Ben-Nun et al. (2011), followed by the first successes to differentiate white rhinoceros (*Ceratotherium simum*) iPSC to primordial germ cells (PGC) for establishing future in vitro gametogenesis systems (Hayashi et al., 2021, 2022). IVG is a complex process that replicates embryonic stages from gastrulation to puberty, starting with the development of primordial germ cells (PGCs). PGCs develop early in the embryo, around gastrulation, and migrate to the gonadal ridge, where they develop into the gametes. Our understanding of PGC specification is the most complete in mouse (*Mus musculus*), where embryos are accessible across developmental stages. The generation of in vitro-derived mouse PGCs was first shown by Hayashi et al. (2011) and has since progressed to maturation of gametes in culture for females (Hikabe et al., 2016) and males (Ishikura et al., 2021). When combined with a natural gametes these IVG-derived gametes can produce live offspring. PGCs have been successfully generated in a few wild species, including cynomolgus monkey (*Macaca fascicularis*, Sakai et al., 2020), marmoset (*Callithrix jacchus*, Seita et al., 2023), and northern white rhino (Hayashi et al., 2022). However, to date, fully mature oocytes or sperm have only been achieved in the mouse model. The complete IVG requires the aggregation of the PGCs with gonadal matrix cells (rOvary/rTestis). For endangered species, it is necessary to generate these supporting cells from the iPSCs since embryonic tissue is not available. Differentiation of iPSCs to ovarian somatic-like cells has already been achieved in mouse (Yoshino et al., 2021). An exciting new possibility is also the generation of female gametes from a male cell line (Murakami et al., 2023). However, the practical application of IVG in critically endangered mammal species will take time and require substantial resources.

2.9 | Gene editing for restoring genetic diversity

In critically endangered mammal species with a very narrow extant gene pool, it might be a future solution to utilize preserved museum material to identify the genetic constitution of the species prior to its extinction crisis. The identification of the lost genetic patterns present in former extinct populations could be used as a blueprint for the genetic reconstitution of the extant population. The genetic code of lost single nucleotide polymorphisms (SNPs) or even entire haplotype patterns can be incorporated into growing cells of fibroblast cultures by gene editing techniques such as CRISPR-Cas9 (Doudna & Charpentier, 2014). The genetically modified cells can be transformed into iPSC and provide the future basis for IVG procedures. Finally, culture-derived gametes could carry lost genetic patterns previously identified in museum material. To our knowledge, this has so far not been applied to wildlife species.

3 | ETHICAL CONSIDERATIONS

The use of aART in species conservation comes with various ethical challenges and controversially discussed topics, which can be only briefly mentioned here. For example, the scientific rescue programme of the northern white rhino (NWR) – a practically extinct megavertebrate taxon – besides important natural scientific, technical, and logistical issues, raises relevant ethical questions (Biasetti et al., 2022; Callender, 2021; Ryder et al., 2020). For example, the older one of two remaining oocyte donors was retired from OPUs following repeated failure to produce viable embryos after transparent, thorough consideration of the relevant factors and involving all stakeholders, especially weighing anaesthesia risk versus expected benefit, using the methods of a decision tree and Bateson cube (https://izw-berlin.de/files/biorescue/FINAL_Report_Najin_October_2021.pdf).

3.1 | Ethical Dilemmata

The two most critical topics in public opinion regarding aART approaches for conservation are (i) the use of cloning and (ii) gene editing to create genetically modified objects (GMO), e.g. for enriching genetic diversity in the targeted population. Their implementations in current or future conservation projects require sound ethical discourse with the public and authorities.

- *Animal welfare*: Various aART techniques involve procedures that may impact the welfare of the targeted individuals undergoing ART procedures, or their offspring. They may be affected e.g. by the large calf syndrome, an overgrowth disorder in ruminants conceived using ART, which has been linked to in vitro embryo culture (see Section 2.4), asynchronous embryo transfer into an advanced uterine environment, nuclear transfer and maternal exposure to excessively high urea diets. These implications need to be taken into consideration, monitored for, and prevented if possible. However, aART techniques may further also affect the welfare of unrelated individuals deriving from different taxa potentially needed in crucial elements of the rescue program, e.g. interspecific surrogacy, laboratory animals used to develop basic biomedical pathways such as in vitro gametogenesis. A general public discussion supported by scientifically sound ethical investigations should be implemented to analyse and openly discuss relevant ethical issues, develop tools to monitor aART and biobanking

procedures (e.g. de Mori et al., 2021, 2024), and to structure transparent and accountable decision-making processes (Biasetti et al., 2022). Due to the inherent complex scenarios involving multiple values of conservation projects, non-complementary interests, and several occasions for conflict (Biasetti & de Mori, 2021) conservation projects, this emerging and novel application of ethical analysis should become standard practice.

- *High costs and resource demands*: Involving complex and specialized technologies as well as scientific innovations, aART techniques require substantial financial resources. This often provokes a ceaseless discussion about resource allocation for advanced scientific versus classical conservation projects. However, value and consequences of a lost species within the ongoing planetary biodiversity degradation (Anthropocene) are nearly impossible to assess as the long-term ecological impact and ultimately consequences for human existence (novel pathogens, loss of bioservices) are too complex for modelling. Climate change and biodiversity loss are the greatest threats for the future of humankind. Thus, ultimately, a holistic conservation approach comprising all available measures will be crucial: (i) Education to achieve a global change towards a sustainable human lifestyle, (ii) protection of habitat and biodiversity, and (iii) aART in critically endangered keystone species conservation programs need to be synergistically interlinked to ensure future planetary balance.

4 | FUTURE DIRECTIONS

Advanced ART combined with traditional conservation efforts will enhance biodiversity protection for future generations. Expected upcoming developments and innovations, especially in the fast evolving field of SCAT combined with gene editing technologies, will help to pave the road to genetically sound, self-sustaining populations of critically endangered mammals. In addition, this field will benefit substantially from international collaboration as well as the sharing of data, knowledge, and biological materials. The latter requires the implementation of more simplified regulatory pathways for sample exchange in context with CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora; https://cites.org/), national customs and animal health regulations, IATA (International Air Transport Association), and the Nagoya protocol.

5 | CONCLUSION

To counteract the accelerated global loss of biodiversity causing increasingly instable ecosystems, the application of advanced assisted reproductive technologies can provide new solutions that are urgently needed to retain and repair planetary health. This process should always be linked to a thorough practical and ethical assessment as well as a broad, transparent public discourse of the involved technologies and their benefits for biodiversity restauration, as well as their potential risks and offsets. The new synergistic strategy – combining classical conservation approaches with state-of-the-art reproductive science – will allow for effectively saving practically extinct keystone species. Despite requiring substantial financial and human resources, the long-term impact of regaining stable ecosystems cannot be overestimated.

AUTHOR CONTRIBUTIONS

TBH and SH jointly contributed to the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

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

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

ORCID

Thomas Bernd Hildebrandt https://orcid.org/0000-0001-8685-4733

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