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Review

A review on reproductive biotechnologies for conservation of endangered mammalian species

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Abstract

This review describes the use of modern reproductive biotechnologies or assisted reproductive techniques (ART) including artificial insemination, embryo transfer/sexing, *in vitro* fertilization, gamete/embryo micromanipulation, semen sexing, genome resource banking, and somatic cell nuclear transfer (cloning) in conservation programs for endangered mammalian species. Such biotechnologies allow more offspring to be obtained from selected parents to ensure genetic diversity and may reduce the interval between generations. However, the application of reproductive biotechnologies for endangered free-living mammals is rarer than for endangered domestic breeds. Progress in ART for non-domestic species will continue at a slow pace due to limited resources, but also because the management and conservation of endangered species is biologically quite complex. In practice, current reproductive biotechnologies are species-specific or inefficient for many endangered animals because of insufficient knowledge on basic reproduction like estrous cycle, seasonality, structural anatomy, gamete physiology and site for semen deposition or embryo transfer of non-domestic species.

Keywords: Conservation; Reproductive biotechnologies; Assisted reproductive techniques; Endangered mammals

1. Introduction

Extinction of mammalian species is part of the natural process of evolution and is irreversible, but is now occurring at much higher rate than speciation because of human activities such as habitat destruction, over-hunting, or competition with introduced herbivores (Holt and Pickard, 1999).

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For some domestic species, extinction has been due to intensive selection of a few breeds imposed by management techniques and market demands. The magnitude of this phenomenon is illustrated by the *2006 Red List* launched by the Species Survival Commission (IUCN—World Conservation Union; http://www.iucnredlist.org/). This document catalogues 1528 animal species reported as critically threatened, including 162 species of mammals. This report also categorises 70 species of mammmalia as completely wiped out within last few years, and four species as extinct in their natural habitats with chances of rehabilitation through *ex situ* conservation programs.

The aim of animal conservation is to maintain biodiversity because removal of single species can affect the functioning of global ecosystems (Henson, 1992; Margules and Pressey, 2000; Myers et al., 2000). Habitat preservation is one of the best ways to conserve biodiversity (Wildt et al., 1997; Loi et al., 2001). Small population propagation is also part of a multi-disciplinary approach to conservation, including genetic and ecological characterizations and other strategies (Comizzoli et al., 2000). *In situ* conservation strategies enable live populations of animals to be maintained in their adaptive environments. However, these efforts are sometimes insufficient for the propagation of small populations and maintaining adequate genetic diversity. Hence, newer *ex situ* conservation strategies have been developed, aimed at establishing a viable population through cryopreservation of animal genetic resources (like gametes, embryos and other cell/tissue samples or DNA).

In situ and *ex situ* conservation programs for some endangered mammalian species can benefit from modern reproductive biotechnologies or assisted reproductive techniques (ART) including artificial insemination (AI), embryo transfer (ET), *in vitro* fertilization (IVF), gamete/embryo micromanipulation, semen/embryo sexing and genome resource banking (GRB). With more knowledge emerging on the basic biology of reproduction, cloning or somatic cell nuclear transfer (SCNT) have been suggested as a potentially integral part of wildlife conservation programs. To date, however, natural breeding coupled with traditional ART has been the preferred method for increasing endangered animal populations, due to the poor efficiency of SCNT. With future progress in the field of cloning, this technology will also become helpful for saving species at risk of extinction.

It is believed that modern biotechnologies or ART for mammalian species threatened with extinction will allow more offspring to be obtained from selected parents to ensure genetic diversity and may reduce the interval between generations. Therefore, this review will analyze the use and current status of ART for endangered mammalian species in the context of the broad variability in, and sparse knowledge of reproductive physiology between species.

2. Strategies for application of reproductive biotechnologies in endangered species

Within the past few decades, a powerful new approach has emerged for conservation of threatened wildlife species, through *in situ* and *ex situ* conservation programs. Hanks (2001) in his review on conservation strategies suggested that zoo-based captive breeding programs should also be regarded as a supplement rather than an alternative to *in situ* conservation activities. Also captive breeding programs should essentially be guided by rational priorities for *ex situ* conservation, ideally focusing on threatened species or groups with which zoos already have husbandry experience.

One of the major problems with the implementation of *in situ* and *ex situ* conservation programs is the lack of availability of the biological material which is required for a better understanding of reproductive patterns as well to maximize reproductive efficiency. This constraint arises from the strict procedures adopted for restraining or anaesthetizing free-living animals for collection

of biological/reproductive samples. However, this has been partially resolved by the development of viable methods for assessment of hormonal profiles from voided urine and faeces, also termed as non-invasive hormonal monitoring.

There have been a number of examples of the application of non-invasive endocrine monitoring techniques for *in situ* or zoo-based studies. The first non-invasive measurement of testosterone in the urine of free-ranging African elephants that were in or around the time of behavioural musth was by Poole et al. (1984). Other examples include Creel et al. (1991, 1992, 1993) who conditioned dwarf mongooses to urinate on a rubber pad during the course of scent marking. This approach provided hundreds of urine samples which were analysable for hormonal metabolites, allowing elegant examinations of behavioural and endocrine mechanisms of reproductive suppression in this species. Monfort et al. (1990) monitored ovarian function and pregnancy in the Eld's deer by evaluating urinary steroid metabolite excretion. Wasser (1995) analyzed faecal hormonal metabolites from baboons to understand how dominance is related to conception and reproduction. Brown et al. (1996) assessed faecal steroids in captive cheetahs to validate that the cheetahs are polyoestrus and ovulation is almost always induced. They also found new evidence suggesting that many female cheetahs inexplicably experienced periods of anoestrus unrelated to season. Ostner and Heistermann (2003) have characterized female reproductive status during breeding season and gestation in wild redfronted lemurs through analyzing immunoreactive 5α-pregnane-3α-ol-20-one and total estrogens in faeces. Czekala et al. (1994) and Robbins and Czekal (1997) measured gonadal steroids in protected mountain gorillas, including quantitating urinary glucocorticoid excretion to assess social and environmental impacts on animal well being. Faecal progesterone hormone monitoring has also revealed major differences in reproductive patterns, estrous cycles and seasonality among rhinoceros species (Heisterman et al., 1998; Schwarzenberger et al., 1998, 2000; Brown et al., 2001; Roth et al., 2001; Garnier et al., 2002). Non-invasive endocrine monitoring has also proved to be an essential tool for assisting in better understating of basic reproductive biology of elephants (Brown, 2000). In a very recent review, Pukazhenthi and Wildt (2004) refers to unpublished observations of Monfort and Brown on the possibility of detecting the ovulatory luteinising hormone (LH) surge in urine of giant panda and killer whale using radioimmunoassay or enzymeimmunoassay techniques. Also there is a recent study claiming accurate profiles of gonadal steroid metabolites by analyzing the faeces of giant panda (Kersey et al., 2003). These studies have highlighted the possibilities for non-invasive, remote monitoring of reproductive status in a number of endangered mammalian species. Further development of techniques that allows the instantaneous assessment of the endocrine status of animals living in nature would offer exciting opportunities to interrelate their physiology, especially that of reproduction, with their natural environment. This information would also help to apply available ART like AI and ET more efficiently for *in situ* or zoo-based conservation of endangered species.

Other developments have taken place on the collection of biological material, like semen from aggressive males, by the use of internal artificial vaginas or vaginal condoms (Bainbridge and Jabbour, 1998). Semen can also be collected from the epididymes following the death of an animal or during the rut period. Additionally, post-coital sperm recovery has been used successfully in marmoset monkeys (Morrell et al., 1998) and rhinoceros (O'Brien and Roth, 2000). For embryo recovery, non-surgical or less invasive methods like transcervical embryo collection have been applied in the oryx, bongo, eland, greater kudu (Schiewe et al., 1991) and Poitou donkey (Vendramini et al., 1997), and laparoscopic embryo collection has been used in an endangered swine breed (Ratky et al., 1997), the silver fox (Jalkanen and Lindeberg, 1998) and the bear (*Ursus americanus*; Boone et al., 1999).

Ultrasonography is another non-invasive technology that can be helpful for monitoring female ovarian function, reproductive tract morphology, pregnancy, foetal growth and assessing the male reproductive tract in many non-domestic species. For example, ultrsonography has been applied in the rhinoceros, elephant and killer whale to monitor ovarian follicular dynamics, time of ovulation, perform AI, study gestational events, and aid in the difficult task of semen collection (Radcliffe et al., 1996, 2001; Hildebrandt et al., 2000; Roth et al., 2001; Robeck et al., 2004; Stoops et al., 2004).

3. Current status of reproductive biotechnologies

A number of reproductive biotechnologies are available that are being widely applied for *in situ* and *ex situ* conservation of endangered species. These have been applied for animal conservation for almost two decades since ET was first used by Kraemer et al. (1976) to produce a baboon infant. The main technologies that have been used or considered are artificial insemination, embryo transfer (and its combination with *in vitro* fertilization) and sexing, gamete and embryo micromanipulation, sperm sexing, genome resource banking, and cloning.

3.1. Artificial insemination

One of the major applications of AI in conservation is to avoid genetic depression caused by fragmentation of groups in free-living species. Therefore, for some species living in small populations, it may be feasible to capture females for short periods for AI with sperm collected from zoo-maintained healthy males. The females could then be returned to the original habitat to give birth to a generation of genetically healthy young (Pukazhenthi and Wildt, 2004). Alternatively, for some species it would be more appropriate to capture free-ranging males for short time for semen collection, and AI captive females. Another possible application of AI, for *in situ* or zoo-based conservation, is to circumvent the poor natural mating behaviour in some species like giant pandas (Wildt et al., 2003). Additionally, AI can be applied in captive non-domestic species to avoid the challenges of translocation of the animals for breeding purposes (Pukazhenthi and Wildt, 2004).

Besides the vast potential of AI in conservation programs there are also significant boundaries for AI technology in wildlife. Although it is generally possible to collect semen from most non-domestic mammals by artificial vagina, vaginal condoms, digital masturbation of penile bulb or electroejaculation (under anaesthesia), these processes remain difficult for some species, including the rhinoceros, non-domestic equids, certain great apes, canids and marsupials (Pukazhenthi and Wildt, 2004). This challenge needs more investigation, especially the impact of anaesthesia applied for electro-ejaculation on seminal release (Hildebrandt et al., 2000).

Another challenge is the poor knowledge on female reproductive physiology and anatomy in many non-domestic species, particularly a comprehensive understanding of female reproductive cycles and determination of the most suitable site for semen deposit in female tract, which are very necessary for successful AI. Although dynamics of female reproductive cycle can be determined by non-invasive hormonal monitoring or real-time ultrasonography, but most of the AI protocols for wildlife require induction and/or synchronization of ovulation through exogenous gonadotrophins (Howard, 1999). Pukazhenthi and Wildt (2004) in their review have highlighted the divergent results by the use of commercially available gonadotrophins in mammalian wildlife species and have suggested looking for alternative sources of gonadotrophins to get better AI results. Likewise highly variable results are reported for other hormones like prostaglandin F2α

and progesterone used for synchronization of ovarian activity in diverse non-domestic species (reviewed by Pukazhenthi and Wildt, 2004). For every non-domestic species there are also technical limitations linked to various gross anatomies. Genital tracts have anatomical species-specific characteristics, especially in marsupials that have two separate uteri, each connected to lateral vaginae by twin cervices (Mate et al., 1998). Similarly giraffe have a totally non-penetrating cervix (Loskutoff et al., 1995). Cervix of oryx, rhinoceros and elephant are also quite complicated usually by tortuous or bifurcated or excessively long vaginal vault (Pukazhenthi and Wildt, 2004). Therefore, the site of semen deposit through AI in uterus, cervix or vagina of the female reproductive tract is still unclear for most of the free-living mammals (Comizzoli et al., 2000). For this reason animals are inseminated transabdominally directly into the uterus by laparoscope (Howard, 1999; Roth et al., 1997; Pope and Loskutoff, 1999), however, this technique is again not applicable for many species.

The most significant advancements in AI for wildlife in terms of live births have involved African lion (Bowen et al., 1982), Persian leopard (Dresser et al., 1982), blackbuck (Holt et al., 1988), black-footed ferret (Howard et al., 1991), Eld's deer (Monfort et al., 1993), ocelot (Swanson et al., 1996), cheetah (Wildt et al., 1997), Poitou donkey (Trimeche et al., 1998), marmoset (Morrell et al., 1998), Asian and African elephants (Olson and Wiese, 2000, Brown et al., 2004), oryx (Morrow et al., 2000), eland (Bartels et al., 2001) and giant panda (Masui et al., 1989; Wildt et al., 2003). A full term pregnancy with AI has been reported in Mohor gazelle (*Gazella dama mhor*; Holt et al., 1996). More recently, AI conceptions have also been reported in gerenuks (Penfold et al., 2005), Pacific white-side dolphins and bottlenose dolphins (Steinman et al., 2003; Robeck et al., 2005). Live births with AI have also been achieved in, killer whales (Robeck et al., 2004), European mouflons (Berlinguer et al., 2005), Spanish ibexes (Santiago-Moreno et al., 2006), and wallabies (Paris et al., 2005). In most of the above examples, AI techniques and strategies have been significantly improved, however, there are large differences between species in insemination techniques and pregnancy rates using fresh or frozen semen.

3.2. Embryo transfer and in vitro fertilization

Despite some early successes ET like AI has not been fully applied to the genetic management of wildlife populations. The key conservation strategy in this regard has been to pave the way for interspecies ET. In this case, embryos from an endangered species are cryopreserved and/or transferred to a more common surrogate of a different species.

For a long period, the only success was the production of a gaur calf born to a Holstein cow (Stover et al., 1981), and later on an Armenian red sheep to a domestic sheep (Coonrod et al., 1994), and a Spanish ibex to a domestic goat (Fernandez-Arias et al., 1996) were also reported. There have recently been some more successful studies, for example a banteng born to a cow, a zebra to a domesticated horse (Loskutoff, 2003), an Indian desert cat born to a domestic cat (Pope, 2000; Loskutoff, 2003), and a European mink to a hybrid recipient (Amstislavsky et al., 2004). However, none of these accomplishments have been consistently repeatable. Two interspecies ET studies have been published involving a rescue mission for free-living European mouflon by transferring embryos to sheep (Moreno et al., 2001; Ptak et al., 2002). Very recently, a pregnancy has been reported after interspecies ET between an endangered urial (*Ovis vignei*) and a domesticated sheep (*Ovis aries;* Ullah et al., 2006).

There is still only limited interest in interspecies ET and knowledge on the kinetics of embryo development and feto-maternal recognition in many species is scanty (Hammer et al., 2001). It

is clear that the biological mechanisms that ensure survival of transplanted embryos in the uterus of a foreign species are more sophisticated and fragile than once appreciated (Fernandez-Arias et al., 1999; Demmers et al., 2000; Pukazhenthi and Wildt, 2004).

In wildlife ET, sexing pre-implantation embryos could be a useful conservation tool. Currently there are no references on endangered species, but techniques for sexing bovine and ovine embryos in breeding programs to manipulate the sex ratios of offspring could be modified for application in wildlife species of the same families. A recent study on sexing of *in vitro* produced sheep embryos describes the advancements and advantages with embryo sexing by duplex PCR (Mara et al., 2004). This, demonstrated the possibility of transferring fresh sexed embryos on the same day of biopsy, a feature extremely important when the animals are in synchronized oestrus and recipient dams are available for immediate ET. This could eliminate the tedious step of embryo cryopreservation. In cases where recipients are not available, sexed embryos could be frozen after selective retention of the most likely the female embryos and discarding the unwanted males (Mara et al., 2004).

In vitro fertilization has been also tried in various wildlife species but with sporadic success. The major studies involving IVF are in pumas (Miller et al., 1990), tigers (Donoghue et al., 1990), cheetahs (Donoghue et al., 1992), Indian desert cats (Pope et al., 1993), gaurs (Johnston et al., 1994), Armenian red sheep (Coonrod et al., 1994), llamas (Del Campo et al., 1994), African elephants (Kidson et al., 1995), gorillas (Pope et al., 1997), zebras (Meintjes et al., 1997), marmosets (Gilchrist et al., 1997), minke whales (Fukui et al., 1997), bongos (Pope et al., 1998a), addaxes (Hall-Woods et al., 1999), African wild cats (Pope et al., 2000), jaguars (Morato et al., 2000), sika deer (Comizzoli et al., 2001), ocelots, tigrinas (Swanson et al., 2002), blesboks, African buffaloes, springboks, black wildebeests (Herrick et al., 2004) and European mouflons (Ptak et al., 2002; Berlinguer et al., 2005). Live births have been only reported in tigers, Indian desert cats, Armenian red sheep, gorillas and European mouflons by using IVF followed by intra or interspecies ET, largely because of the scarcity of information on the fundamental biology on the species of interest.

Another potential application of IVF in endangered wildlife species is the use of *in vitro* oocyte maturation (IVM) to save the germplasm from females that die unexpectedly or accidentally (Pukazhenthi and Wildt, 2004). In a recent review by Galli et al. (2003) this strategy is termed "genetic recovery". The oocytes recovered from the ovaries of a dying individual would be matured *in vitro* and subsequently utilized for *in vitro* production of embryos. To date, IVM followed by IVF/ET has resulted in live offspring in the gaur (Johnston et al., 1994), Armenian red sheep and llama (Pope and Loskutoff, 1999), European mouflon (Ptak et al., 2002) and red deer (Locatelli et al., 2005). The success rate through IVM is highly dependent on culture supplements, as well as on the age and health of the oocyte donors (Christensen et al., 1993).

A relatively new reproductive biotechnology is also emerging based on IVM, IVF and ET techniques, essentially for shortening the generation interval, called juvenile *in vitro* embryo transfer (JIVET; Galli et al., 2003). To date JIVET has been mostly developed for farm animals of great genetic potential. Female calves of 2–3 months of age have been used as oocyte donors by ovum pick up conducted by laparotomy or laparoscopy and, following IVM and IVF, the embryos were grown to the blastocyst stage and then transferred effectively to recipients (Duby et al., 1996). JIVET also has potential as a powerful tool for saving endangered wildlife species, especially those belonging to the bovine or ovine family. The immediate outcome of JIVET would be a large number of embryos obtainable for propagation even from young females of the species under risk of extinction.

3.3. Gamete and embryo micromanipulation

The importance of intra-cytoplasmic sperm injection (ICSI) to wildlife or rare animal preservation appears limitless, as even non-viable sperm in cattle has resulted in the birth of live calves (Goto et al., 1991). There are some other studies and reviews detailing the use of ICSI in horses (Guignot et al., 1998; Squires, 2005), cattle (Rho et al., 2004), sheep (Catt et al., 1996; Tibary et al., 2005), rhesus monkeys (Sutovsky et al., 1996), goats (Keskintepe et al., 1997), dogs (Fulton et al., 1998), cats (Pope et al., 1998b; Gomez et al., 2000), cynomolgus monkeys (Ng et al., 2002) and European mouflons (Berlinguer et al., 2003). The development of ICSI in humans has lead to its application in non-human primate species, a number of which are endangered. Similarly, improvements in ICSI techniques in some domesticated animals could be useful for conservation programs of endangered species belonging to the same families. It should be noted that for the fertilization of feline and murine oocytes, ICSI has been less effective than sub-zonal insemination (SUZI; Pope et al., 1995; Yanagimachi, 1998), raising the possibility that ICSI, like most of the other reproductive biotechnologies, is also species-specific. Despite this, there is a preference for applying ICSI to wildlife conservation, rather than SUZI, because there is a total absence of polyspermy with ICSI (Pope, 2000).

The different procedures used for sperm insertion (ICSI and SUZI) will have an important role to play in future conservation efforts, particularly for endangered species in which males might develop a higher proportion of abnormal sperm and no other method except ICSI or SUZI would be available for successful IVF. This is particularly important for some feline species, such as the clouded leopard and cheetah, where a high level of abnormalities have been detected in the spermatozoa of animals in captive populations (Wildt et al., 1986; Roth et al., 1994, 1995).

Micromanipulation of embryos by treatments such as zona drilling or partial zona dissection could raise their chances of implantation (Edwards and Brody, 1995). Drilling holes on the zona pellucida aims to facilitate earlier hatching of embryos from the zona pellucida when it has been hardened by ovarian stimulation and/or embryo culture. Results obtained by Loskutoff et al. (1999) indicate that partial zona dissection improves the hatching frequencies of bovine blastocysts produced *in vitro* and co-culture conditions can affect survival after thawing. Hence, it is probable that the embryos of endangered wildlife species will also benefit from micromanipulated hatching techniques.

3.4. Sperm sexing

Sex pre-selection of offspring through the use of sexed spermatozoa has great potential as a captive population management strategy for endangered wildlife, particularly those species with single-sex dominated social structures (O'Brien et al., 2002). Moreover, unbalanced sex ratios, especially excessive male births, can play havoc with small population management of wildlife (Pukazhenthi and Wildt, 2004). Producing predominantly female offspring is advantageous in accelerating the re-population rate, especially in species that are notorious for slow reproduction (Maxwell et al., 2004). Thus, recent advances in sexing mammalian sperm on the basis of the differences in DNA content in X-compared with Y-chromosome bearing sperm deserves consideration for wildlife conservation (Pukazhenthi and Wildt, 2004).

Among wildlife species, sperm sorting methodologies for non-frozen and frozen semen of non-human primates (O'Brien et al., 2001, 2003, 2005) and bottlenose dolphin (O'Brien and Robeck, 2006) have been developed. Recently, an AI study of sexed and thawed elk sperm has

been conducted that produced 11 offspring, nine of which were of the predicted sex based on the use of predominantly X- or Y- bearing sperm in the inseminates (Schenk and DeGrofft, 2003).

A significant challenge in using the sperm sexing technique for controlling gender in wildlife breeding programs will be the often low sperm densities encountered and/or the tendency for males to produce pleiomorphic spermatozoa (Pukazhenthi and Wildt, 2004). In this connection, it was found in several non-human primates, such as the hamadryas baboon, marmoset, chimpanzee and gorilla, that high-purity sorting of frozen-thawed semen (containing a low concentration of spermatozoa), with recovery of progressively motile and acrosome intact spermatozoa, was made possible by processing to remove excessive cryodiluent (O'Brien et al., 2002,2003). Also for use of sex-sorted semen in AI, insemination close to the site of fertilization and time of ovulation is critical for successful fertilization and ongoing pregnancy (Maxwell et al., 2004). A more practical approach would be development of improved insemination tools for placing low numbers of sexed sperm in the upper regions of the uterine horns (Garner, 2006). An alternative is to use sexed spermatozoa in IVF and ICSI to produce embryos, and such was the case in a recent study in gorillas by O'Brien et al. (2002).

3.5. Genome resource banking

There have been a number of approaches proposed to slow or halt the rate of species decline. One suggestion is to undertake a program aimed at preserving genetic material or *ex situ* cryoconservation of germplasm, specifically spermatozoa, oocytes or embryos, and other cells/tissues or DNA from endangered species.

Often termed as genetic resource banking (GRB), the aim is to create depositories of germplasm as an interface between *in situ* and *ex situ* conservation programs (Holt and Pickard, 1999). Therefore, GRB can be tool for managing the exchange of genetic diversity among endangered species by facilitating the creation of a global gene pool (Hanks, 2001). The current GRB facilities worldwide are enlisted in Table 1. These groups/centres are working to collect and preserve as much biological material as possible, together with the development of databases, from threatened or endangered mammalian species before further genetic diversity is lost.

It is also thought that banked reproductive material serves as an insurance policy for selected species in response to an unexpected catastrophic incident. For example, the meltdown of the nuclear reactor at Chernobyl in 1986 exterminated a large number of non-domestic species and destroyed their natural habitat leaving no chance for their rehabilitation (Skuterud et al., 2005; Sazykina and Kryshev, 2006). Similarly, during the civil war in Rwanda during the 1990s, the Akagera National Park lost about 90% of its large mammals (Kanyamibwa, 1998).

3.5.1. Semen banks

Systematic cryopreservation and storage of semen from endangered species can facilitate maintenance of genetic heterozygosity, while minimizing movement of living animals between captive areas/zoos/research centres or countries (Johnston and Lacy, 1995). Using frozen-thawed spermatozoa would facilitate the infusion of new genetic material across populations by AI. The use of frozen sperm from semen banks increases the generation interval indefinitely and allows fewer males to be held in captivity because some of the genetic diversity is maintained strictly as frozen spermatozoa.

Having sperm samples preserved from representative free-living males protects the existing diversity from unforeseen danger and eliminates the need to remove males from their natural habitats to support *in situ* or zoo-based breeding programs. Meanwhile, stored spermatozoa from

Table 1
List of current genome resource banking (GRB) facilities worldwide

GRB facility	Location
Ambrose Monell Laboratory, American Museum of Natural History	New York, USA
Animal Gene Storage Resource Centre, Monash University	Melbourne, Australia
Antwerp Zoo	Antwerp, Belgium
Centre for Cellular and Molecular Biology	Hyderabad, India
Centre for Reproduction of Endangered Species, The Zoological Society of San Diego	California, USA
Cheetah Conservation Fund	Otjiwarongo, Namibia
Conservation and Research Centre, National Zoological Park, Smithsonian Institution	Virginia, USA
Conservation Genome Resource Bank for Korean Wildlife, Seoul National University, College of Veterinary Medicine	Seoul, South Korea
Cryobiology Research Group, Institute of Research in the Applied Natural Sciences, The University of Luton	Luton, UK
Experimental de Zonas Aridas	Almeria, Spain
Giant Panda Breeding and Research Base	Chengdu, China
King Khalid Wildlife Breeding Research Centre	Thumamah, Saudi Arabia
Museum of Natural History	London, UK
Museum National d'Histoire Naturelle	Paris, France
North of England Zoological Society	Chester, UK
School of Biology, The University of Nottingham	Nottingham, UK
Metro Toronto Zoo	Toronto, Canada
University of Sassari	Sardinia, Italy
Wildlife Biological Resource Centre, Endangered Wildlife Trust	Pretoria, South Africa
White Oak Conservation Center	Florida, USA
Zoological Park of Buenos Aires	Buenos Aires, Argentina
Zoological Society of London	London, UK

free-living and captive individuals could be introduced back into existing populations immediately, a decade from now or even centuries into the future. Thus, the advantages of a semen bank as part of the GRB are profound and provide for potential future improvements in application technology.

Semen banks are currently more developed for rare domestic breeds (bovine, ovine, caprine and porcine) than for non-domestic species, but the concept of using them to facilitate the management and conservation of endangered species is being promoted extensively (Wildt et al., 1997). In order to maximize genetic diversity, a rare animal from the family bovidae could be saved with 1000 sperm doses collected from 25 different males (Comizzoli et al., 2000).

Successful cryopreservation has been carried out for spermatozoa of cynomolgus monkeys (Tollner et al., 1990), felidae (domestic and wild cats; reviewed by Jewgenow et al., 1997), marmosets (Morrell, 1997), chinchillas (Ponce et al., 1998), Poitou donkeys (Trimeche et al., 1998), Drill baboons (Durrant et al., 1999), Bottlenose dolphins (Durrant et al., 2002), canidae (wild dogs, silver fox, red fox, blue fox, red wolf; reviewed by Leibo and Songsasen, 2002), European mouflons (Ptak et al., 2002; Berlinguer et al., 2005), Assamese macaques (Li et al., 2004), blesboks, African buffaloes, springboks, black wildebeests (Herrick et al., 2004), Japanese black bears (Okano et al., 2004), hares (Kozdrowski et al., 2006) and different species of gazelles (Garde et al., 2003; Saragusty et al., 2006), but semen banks have been only initiated for Siberian tigers (Wildt et al., 1995), gazelles (Holt et al., 1996), bison (Sipko et al., 1997), giant pandas (Wildt et al., 1997), European mouflons (Ptak et al., 2002), black-footed ferrets (Howard et al.,

2003), gerenuks (Penfold et al., 2005), Gulf Coast Native sheep (Nel-Themaata et al., in press), Arabian leopards (de Haas van Dorsser and Strick, 2005), generic and Mexican gray wolves (Zindl et al., in press) and Namibian cheetahs (Crosier et al., 2006). Hence, the pragmatic decision may be that a semen bank program is not realistic for most of the endangered species because germplasm cryopreservation procedures for most of them have not been initiated or requires perfection in techniques.

3.5.2. Embryo or oocyte banks

Embryo cryopreservation and storage allows conservation of the full genetic complement of the sire and dam and thus has enormous potential for protecting and managing species and population integrity and heterozygosity. However, the success of applying this technology to wildlife will be dictated by the uniqueness of the embryo of each species (Pukazhenthi and Wildt, 2004). Furthermore, the differences among embryos in cryosensitivity are substantial, as demonstrated by the variance between the freezable bovine compared with the difficult to freeze swine embryos (Nieman and Rath, 2001).

Currently cattle embryo freeze-thaw protocols have been fairly effective in the few studies conducted in non-domestic bovidae like gaur, banteng, bongo and eland, including the production of living young in each species (reviewed by Loskutoff et al., 1995). Also there is a raw estimate for maximizing genetic diversity and saving a rare bovine breed, is through freezing and banking 300 non-sexed embryos collected from 90 unrelated donors (Comizzoli et al., 2000). In contrast, embryo cryopreservation protocols for non-ungulates are still superficial, with evidence that the hurdles ahead will be substantial (Pukazhenthi and Wildt, 2004). In the case of carnivores, there has been some progress in that a baseline protocol for embryo freezing and thawing has been established for the domestic cat (Pope, 2000), and healthy offspring have been produced following the same freeze-thaw protocol for embryos in three non-domestic felid species (ocelot, African wild cat and cercal; Pope, 2000; Swanson, 2001; Swanson and Brown, 2004).

Conventional freezing and thawing procedures for embryos are time-consuming and require the use of biological freezers and a microscope. Complicated embryos freezing procedures may soon be replaced by a relatively simple procedure called vitrification. However, its greatest advantage is its simplicity, because to date vitrification is only used experimentally in embryos from domestic animal breeds like cattle *et cetera* (reviewed by Vajta, 2000). Therefore, the biggest challenge is to establish a standardized vitrification method, which can be successfully applied for cryopreservation of embryos at different developmental stages of endangered mammalian species.

The situation for oocyte cryopreservation from non-domestic species is not different from that of embryos. However, there has been significant progress in the cryopreservation of oocytes from laboratory animals (Nakagata, 1992; Frydman et al., 1997; Shaw et al., 2000; Vajta, 2000; Stachecki and Cohen, 2004), non-human primates (Parks and Ruffing, 1992), horses (Maclellan et al., 2002) and farm animals (Otoi et al., 1996; Abe et al., in press; Albarracin et al., 2005; Cetin and Bastan, 2006). These studies have made substantial progress in using ultrarapid freezing protocols for retaining the stability of oocyte cytoskeleton and, therefore, could be applied for oocyte cryopreservation of endangered species particularly from same family or genera.

Finally for making the embryo or oocytes banks a practical reality, there is a need to understand the fundamental cryobiological factors that determine embryo and oocyte viability and functionality before and after cryopreservation for virtually every individual endangered species.

3.5.3. Tissue graft banks

Although little research has been directed towards wildlife species, the cryopreservation and subsequent use of gonadal tissue offers fascinating opportunities. This has particularly been the case since the news of a live birth following orthotopic transplantation of cryopreserved ovarian tissue in humans (http://news.bbc.co.uk/go/pr/fr/-/2/hi/health/3685174.stm; published 23/09/2004 22:55:51 GMT).

Recent developments in the autografting and xenografting of ovaries and testes clearly demonstrate the potential value of cryopreserving gonadal tissue (Oktay and Yih, 2002; Tibary et al., 2005). Thawed ovarian tissue has been transplanted into conspecific recipients in the mouse, sheep (Demirci et al., 2003), and more recently humans (Donnez et al., 2004), resulting in the birth of normal young.

The aim of ovarian and testicular tissue cryopreservation is to store primordial follicles and spermatogonial cells, respectively (Demirci et al., 2003; Pukazhenthi and Wildt, 2004). Xenografting the thawed ovarian tissue from the marmoset monkey (Candy et al., 1995), African elephant (Gunasena et al., 1998), cat (Bosch et al., 2002), and cow (Herrera et al., 2002) to immunodeficient mice has resulted in antral follicle development. A similar phenomenon has occurred in immunodeficient (nude) rats receiving transplants of thawed wombat ovarian tissue (Wolvekamp et al., 2001). Likewise, spermatogonia from rats have been xenografted to the testes of nude mice having destroyed endogenous germ cells, which in turn produced viable sperm (Russell and Griswold, 2000). However, the major current obstacle limiting widespread application of this technology is the low efficiency of sperm production in many species (Paris et al., 2004).

It has been also reported that transplantation of germ cells from phylogenetically more distant species like rabbit, dog, pig, bull, horse and primate fails to establish spermatogenesis in the nude mouse testis (Honaramooz et al., 2002). Therefore, instead of xenografting, autografting may be more useful. This can be carried out orthotopically on the ovarian/testicular tissue or can be performed heterotopically on another physiological site. Furthermore, oocytes or spermatocytes harvested from grafts could also be used for IVM/IVF or ICSI procedures to produce offspring.

A recent study in mice demonstrated that in contrast to mature oocytes and embryos, ovarian tissues can be collected irrespective of age, reproductive cycle and even following recent death (Cleary et al., 2001; Snow et al., 2001). This could have a huge impact in wildlife conservation, as ovarian tissues could even be collected and preserved from young females who had died due to unknown etiology.

The stage has probably not been reached where cloning technology is ready for application to maintain population viability or conserve species but in the future tissue samples (somatic cells) collected and stored from endangered species could be exploited by nuclear transfer. Research is required now to identify suitable sources of cells which could be exploited for banking and future cloning-based conservation programs.

Therefore, the establishment of worldwide tissue graft banks, to store reproductive/somatic tissue and cells collected opportunistically from threatened wildlife species, could be a milestone in conservation planning. This is particularly the case in situations where population numbers are critically low, other options have failed and conservationists are faced with the need to rescue all extant genetic diversity, including from dying neonates.

3.6. Cloning

Somatic cell nuclear transfer (SCNT) is a process by which the nucleus (DNA) is moved from a donor cell to an enucleated recipient cell to create an exact genetic match of the donor. If this

happens to be a viable embryo that proceeds to term, the resulting offspring has the same genetic complement as the original donor, except for the mitochondrial DNA, which is derived from the recipient (Wolf et al., 2001; Yang et al., 2004).

Conservation has been highlighted recently as an area where SCNT may be useful (Lanza et al., 2000; Latham, 2004). Transfer of a somatic cell nucleus into the enucleated egg of a genetic stock, a closely related species or another subspecies can potentially allow the recovery of the entire nuclear genetic complement of the donor without the genetic dilution that would occur in producing biparental hybrids (Corley-Smith and Brandhorst, 1999). Moreover, SCNT may preserve and propagate endangered species that reproduce poorly in captivity until natural habitats can be restored and populations reintroduced to their ecological units (Tong et al., 2002), and may even allow the resurrection of extinct species from appropriately preserved tissue. A first successful demonstration of SCNT in conservation attempt of an endangered Enderby Island cow that resulted in surviving offspring was by Wells et al. (1998). Attempts have also been made with the giant panda (Chen et al., 1999,2002), Argali sheep (White et al., 1999), and gaur (Lanza et al., 2000; Vogel, 2001) but these efforts have failed to produce viable offspring. However, an apparently normal mouflon sheep (Loi et al., 2001) and African wild cat (Gomez et al., 2004) have been born after successful *trans*-species SCNT. More recently, Sansinena et al. (2005) has reported pregnancies in banteng by interspecies nuclear transfer. There is also a highly publicized plan to clone a Tasmanian tiger from a single alcohol-fixed museum specimen (Anonymous, 2002), for which little progress has been reported to date.

A major practical objection to using cloning technology in wildlife conservation is the fundamental lack of information about the basic physiology of endangered species. While it is obvious that the species requiring most urgent protection and conservation are those that are considered endangered, it may be less obvious to some that these are the very same species for which the least background biological information exists (Holt et al., 2004).

A significant shortcoming of nuclear transfer technology in its current state is the prospect that resultant offspring will suffer from some degree of abnormality (Holt et al., 2004). Since the first sheep was produced by SCNT (Wilmut et al., 1997) and, more recently, a dog (Lee et al., 2005) and ferret (Li et al., 2006) using cultured cells as sources of nuclei, many studies have revealed that cloned mammals suffer from gestational and neonatal developmental abnormalities (reviewed by Eckardt and McLaughlin, 2004; Piedrahita et al., 2004). It is relevant to mention that many of the problems associated with SCNT embryos particularly large offspring syndrome have also been found with conventional IVF and ET procedures (Young et al., 1998), however the frequency and severity of the syndrome appears to be much higher with cloning (Wells, 2003). Hence, for the present, it has been suggested that SCNT should be only considered as a useful tool for basic research for the investigation of cell biology and reprogramming (Van Heyman, 2005).

In present circumstances, where rapid advances in cloning technology are being made, perhaps it is more appropriate to focus on developing realistic strategies for using these methods in wildlife conservation and ensuring that scarce resources are deployed where they will be most effective (Holt et al., 2004).

4. Conclusions

Application of reproductive biotechnologies for endangered free-living animals is rarer than for endangered domestic breeds. Progress in ART for non-domestic species will continue at a slow pace due to limited resources, but also because the management and conservation of endangered species is biologically quite complex. In practice, current reproductive biotechnologies

are species-specific or inefficient for many endangered animals, and this is because of insufficient knowledge on basic reproduction like estrous cycle, seasonality, structural anatomy, gamete physiology and site for semen deposition or embryo transfer in non-domestic species.

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