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Applications of emerging technologies to the study and conservation of threatened and endangered species

Budhan Pukazhenthi[A,C,D], *Pierre Comizzoli*[A], *Alexander J. Travis*[B] *and David E. Wildt*[A]

[A]Smithsonian's National Zoological Park, Conservation and Research Center, Washington, DC 20008, and Front Royal, VA 22630, USA.

[B]Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA.

[C]Present address: Department of Reproductive Sciences, Smithsonian's National Zoological Park, 3001 Connecticut Ave, NW, Washington, DC 20008, USA.

[D]Corresponding author. Email: pukazhenthib@si.edu

Abstract. Sustaining viable populations of all wildlife species requires the maintenance of habitat, as well as an understanding of the behaviour and physiology of individual species. Despite substantial efforts, there are thousands of species threatened by extinction, often because of complex factors related to politics, social and environmental conditions and economic needs. When species become critically endangered, *ex situ* recovery programmes that include reproductive scientists are the usual first line of defence. Despite the potential of reproductive technologies for rapidly increasing numbers in such small populations, there are few examples of success. This is not the result of a failure on the part of the technologies *per se*, but rather is due to a lack of knowledge about the fundamental biology of the species in question, information essential for allowing reproductive technologies to be effective in the production of offspring. In addition, modern conservation concepts correctly emphasise the importance of maintaining heterozygosity to sustain genetic vigour, thereby limiting the practical usefulness of some procedures (such as nuclear transfer). However, because of the goal of maintaining all extant gene diversity and because, inevitably, many species are (or will become) 'critically endangered', it is necessary to explore every avenue for a potential contributory role. There are many 'emerging technologies' emanating from the study of livestock and laboratory animals. We predict that a subset of these may have application to the rescue of valuable genes from individual endangered species and eventually to the genetic management of entire populations or species. The present paper reviews the potential candidate techniques and their potential value (and limitations) to the study and conservation of rare wildlife species.

Introduction

There are two major approaches to conserving threatened wildlife species. The first involves the preservation of habitat, generally on a large scale, thereby protecting the species within (Margules and Pressey 2000; Hanks 2001). The second concentrates on breeding and propagating individual species *ex situ* (in captivity; Holt and Pickard 1999; Wildt and Wemmer 1999; Wildt *et al.* 2001; Loskutoff 2003; Pukazhenthi and Wildt 2004). Although there has been debate about the relative efficacies of these strategies, both obviously have merit. In an ideal world, habitat preservation would always be the highest priority, helping to protect entire ecosystems and many species simultaneously while concurrently retaining the inherent 'wildness' of both nature and animals (and plants). However, the ability to conserve habitat long term is itself under constant threat from natural stochastic factors and local changes in human land use and natural resource management choices. In addition, increasing human population pressures have steadily resulted in the formation of 'islands' of wild space, some of which are too small to conserve viable wildlife populations (especially megavertebrates). The result can be loss of entire species or inbreeding depression that can trigger negative downstream effects on health and the composition of the entire island ecosystem (Terborgh *et al.* 2001). Such events are not only occurring in the undeveloped world, but also seem characteristic of the unrelenting fervour of modern society to promote urban sprawl.

Although the *ex situ* management of wildlife species is never preferred over sustaining truly natural places, captive populations are a hedge for their wild counterparts in nature and have served as a resource for animal re-introductions when practical. Moreover, animals in captivity can be an invaluable reservoir of individuals and entire populations for systematic research investigations, including generating data of value for managing wildlife conspecifics. Finally, there is no question that the presence of species held appropriately in

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captivity can be used to inspire the public to both appreciate nature and support *in situ* conservation initiatives. In short, it is our experience that there are high-priority reasons for sustaining viable wildlife populations *ex situ* and that both *in situ* and *ex situ* approaches can (and should be) complementary rather than competitive.

Preserving animals in wild habitats or in zoos can benefit from a variety of tools, ranging from 'low tech' traditional habitat analysis, wildlife surveys and animal husbandry to 'high tech' satellite imaging, geographical information systems and assisted breeding technologies. Within reproductive biology, researchers have developed artificial insemination (AI), non-invasive hormone monitoring, *in vitro* oocyte maturation and culture, *in vitro* fertilisation (IVF), embryo transfer and germplasm banking (for reviews, see Lasley *et al.* 1994; Pope 2000; Wildt *et al.* 2001; Donoghue *et al.* 2003; Hildebrandt *et al.* 2003; Monfort 2003; Roth and Obringer 2003; Pukazhenthi and Wildt 2004). More recently, cloning and stem cell-based technologies have kindled debate over the applicability and practicality of emerging technologies for the study and conservation of endangered species (Critser *et al.* 2003; Holt *et al.* 2004). Despite the abundant literature available, only a few reproductive technologies have been used routinely for genetically managing wildlife species, including non-invasive hormone monitoring, AI and germplasm (sperm) banking. Specific examples have been provided in a recent review (Pukazhenthi and Wildt 2004) and largely span small captive populations, but also include species that have been produced for release into nature (Howard *et al.* 2003).

Although some techniques have not yet been proven viable for pragmatic wildlife conservation, every technology has value for generating new data on these mostly unstudied species. Furthermore, when adequate basic data are available in the future, it may be possible to routinely use methods such as embryo transfer, oocyte and embryo cryopreservation and intracytoplasmic sperm injection (ICSI) to benefit wildlife genetic management. For this reason, it is necessary now to consider how we may take advantage of additional 'emerging tools' in the reproductive sciences. These technologies will certainly be of value in adding more fundamental knowledge, but may also serve a direct role in preserving and distributing genetic diversity, as well as conserving individuals, populations or entire species. One issue is certain: the amount of wild space will continue to decrease and, with it, wild populations. It appears inevitable that: (*1*) many species and subspecies will approach the 'critically endangered' criteria recognised by the IUCN-World Conservation Union (IUCN 2004); and (*2*) scientists and veterinarians (especially those in the conservation community) will be requested to participate in heroic attempts at salvage and rescue. The aim of the present paper is to address the potential contributions and pitfalls of emerging tools for the study, propagation and preservation of wildlife, especially those nearing extinction.

Importance of developing 'models' for wildlife species

Regardless of the specific technology to be explored, new tools will require the significant use of 'models' (usually domestic animals) for comparable wildlife species. This need has been recognised and adhered to for more than two decades (Wildt *et al.* 1986). It largely emerged from the rather naïve initial supposition that the reproductive mechanisms of species are highly conserved. Such an assumption is highly flawed, as shown by years of research demonstrating the remarkable differences among species between (or even within) taxonomic families. For example, cattle AI technology failed in the cheetah (Wildt *et al.* 2001), necessitating the need for species-specific research in felids. Bold experiments, however, cannot be performed on the cheetah (owing to its rarity), but rather must first be conducted in a model, such as the domestic cat. Even then, the cat may have inherent differences from the cheetah target, yet provide adequate basal information to allow safe application to the rarer counterpart. Thus, as we proceed to consider the value of emerging technologies, it will be essential to consider the practicality of initial testing and application, which will likely require exploration first in a taxonomically related 'model'. Even then, if a certain technique works efficiently in the model, it may require further modifications to be used effectively in the species of interest.

Traditionally, close relatives have been selected: for example, the domestic cat (for wild felids), domestic dog (for wild canids), red or white-tailed deer (for wild cervids), brushtail possum (for endangered marsupials) or common frog (for rare amphibians). Finally, there will be some species that are so specialised that models may be unavailable. Examples of these include the elephant, rhinoceros, giant panda and killer whale (among hundreds of others), all of which will most likely require direct studies, although based on best available knowledge or predictions from work performed in other species. And although these situations must be performed under strict guidelines to minimise any potential danger, some risks (such as anaesthesia) will be imposed and may well be necessary for creating new knowledge to promote the conservation of these species at the population level.

Technologies with application to producing, preserving and using male germ cells

Based on mature spermatozoa

The collection, preservation and use of mature sperm have been a major focus of assisted reproduction for studying and managing small populations of wildlife species. Specifically, the use of these cells allows moving male genetic information within or between populations without requiring the physical presence and/or mating activity of that animal (Howard *et al.* 2002). This permits introducing 'founder' genes without removing an individual from the wild or displacing a captive animal by requiring translocation between institutions.

Using mature sperm also can bypass sexual incompatibility challenges, such as male aggression or mate selection preferences. Moreover, mature spermatozoa can be used to preserve the breeding potential of a male even after its death if long-term storage methods are available (see below).

Collecting sperm from wildlife species is generally possible via electroejaculation, rectal or penile stimulation, massage technique or the use of an artificial vagina, although there are species that have special challenges (Howard 1993; VandeVoort 2004; Umapathy *et al.* 2005). Sperm can also be harvested from the ductus deferens/epididymis after castration or via microsurgical aspiration in cases of obstructive pathology (Wildt 1996). In terms of sperm recovery from within the tract, it is best to collect cells that have mostly undergone epididymal maturation. These, as well as ejaculated spermatozoa, have practical utility for AI, IVF or ICSI.

We predict that most emerging technologies associated with male gamete recovery from semen and the epididymis will be related to new approaches for preservation. Traditional methods for cryopreserving sperm from wildlife species have been reviewed (Pope and Loskutoff 1999; Agca and Critser 2002; Leibo and Songsasen 2002; Donoghue *et al.* 2003), with a similar conclusion: there continue to be vast needs in basic cryobiological studies of diverse species, especially comparing freeze/thaw rates and permeating (e.g. dimethylsulfoxide, propylene glycol) *v.* non-permeating (e.g. raffinose, trehalose) cryoprotectants. Past experiences have demonstrated irrefutably that sperm from different species vary remarkably in cryosensitivity, with this variation often extending to individual animals (Yu *et al.* 2002). Therefore, some 'traditional' methods of sperm preservation may never be adequate for certain species or individuals, thereby requiring the exploration of alternative approaches.

One such method is freeze-drying, first attempted using bull sperm 30 years ago (Larson and Graham 1976). To date, offspring have been produced in the mouse (Wakayama and Yanagimachi 1998; Ward *et al.* 2003), rabbit (Liu *et al.* 2004) and rat (Hirabayashi *et al.* 2005) using freeze-dried sperm by ICSI. Early stage embryos or blastocysts have even been produced in the hamster (Hoshi *et al.* 1994), cow (Keskintepe *et al.* 2002), pig (a species with significant reproductive challenges; Kwon *et al.* 2004) and the cat (a model for wild felids; Moisan *et al.* 2005) after ICSI of such stored spermatozoa. The major advantage of this approach is the lack of dependence on liquid nitrogen for cryopreservation or long-term storage. Specifically, this technology could allow the safe storage of male germplasm even in remote geographic areas, where the majority of critically endangered species occur, and where there is a lack of liquid nitrogen storage facilities. The current limitation of this technique is the need for efficient, established ICSI methods, which have currently been developed only for a few wildlife species, including the lion-tailed macaque (Cranfield *et al.* 2000), gorilla (Lanzendorf *et al.* 1992) and jaguarundi (Pope *et al.* 1998). In addition, this method depends extensively on the availability of all needed embryo transfer-related technologies (including culture, recipient synchronisation and embryo delivery into the uterus).

Another technique worthy of note is the suspension of spermatozoa in solutions of various osmolalities. Van Thuan *et al.* (2005) have reported the birth of live mouse offspring after ICSI with unfrozen sperm stored in this fashion for 1 week at room temperature. The viability of such suspended sperm can also be extended by comparatively 'high' temperature cryopreservation. These same investigators demonstrated that such treated mouse sperm retained functional fertilisability for up to 3 months when stored only at −20°C. This technology could be particularly significant for the developing world and would allow sperm to be stored in conventional freezers rather than liquid nitrogen.

Based on spermatogonial stem cells

Wild animals die in captivity for many reasons, including male-to-male aggression, accidents, disease and maternal neglect, despite veterinary medical interventions. Deaths of neonatal and prepubertal animals are of special concern because these individuals are lost before contributing their genetic information to descendents and the population as a whole. The techniques described above for the collection, preservation and use of mature sperm are irrelevant for prepubertal males because spermatogenesis has not yet commenced or been completed. An alternative approach with potential for conservation involves exploiting male germline stem cells to preserve the genetic potential of immature individuals. Spermatogonial stem cells can be harvested from neonatal and immature males (as well as adults) and are self-replenishing while simultaneously producing daughter cells that undergo meiosis and differentiate into sperm during spermatogenesis. Thus, in theory, stem cell technologies could generate sperm continuously on a renewable basis. The practical benefits also extend to scholarly advantages. For example, the effective development of this technology may reduce the need to collect sperm from living animals for basic research. Rather, there could be a renewable supply of cells available for conducting fundamental sperm biology studies ranging from cryopreservation to contraceptive development.

Spermatogonial stem cell transplantation

Spermatogonial stem cell transplantation (SSCT) was first reported in the mouse in 1994 (Brinster and Zimmermann 1994). To date, this approach has produced sperm in the boar (Honaramooz *et al.* 2002*a*), goat (Honaramooz *et al.* 2003), bull (Izadyar *et al.* 2003) and cynomolgus monkey (Schlatt *et al.* 2002*a*), as well as the mouse and rat (Zhang *et al.* 2003). Spermatogonial stem cell transplantation is also being tested in the cat (Y. Kim, V. Selvaraj, I. Dobrinski, B. Pukazhenthi and A. J. Travis, unpublished observations). In this procedure, either enriched populations of spermatogonia or mixed

germ cell populations that include spermatogonia are placed within the lumens of a recipient's seminiferous tubules. Placement is performed by retrograde injection through the efferent ducts (rodents) or into the rete testis, often under ultrasound guidance (larger animal models).

Xenogeneic SSCT, in which the donor and recipient are heterologous species, has been performed using germ cells from several donor species, including the rat (Clouthier *et al.* 1996), dog, rabbit (Dobrinski *et al.* 1999), boar, bull, stallion (Dobrinski *et al.* 2000), baboon (Nagano *et al.* 2001) and cat (Y. Kim, V. Selvaraj, H. Lee, I. Dobrinski and A. J. Travis, unpublished data), with the mouse being the usual recipient. When xenogenic transplantation has occurred between closely related species (such as rat to mouse), it has successfully resulted in sperm production (Clouthier *et al.* 1996). However, it is clear that increasing phylogenetic distance between the donor and recipient compromises the success of this process, with non-rodent donor spermatogonia colonising the recipient mouse's seminiferous tubules but failing to undergo spermatogenesis (Dobrinski *et al.* 1999; Nagano *et al.* 2001). To overcome this limitation, it may be possible to develop common, domesticated species as recipient surrogates for their rare, wildlife counterparts of the same taxonomic family. In this regard, we are investigating the use of domestic cats as recipients for xenogenic SSCT from wild felids (Y. Kim, B. Pukazhenthi and A. J. Travis, unpublished data). Similarly, recent advances using the pig (Honaramooz *et al.* 2002*a*), goat (Honaramooz *et al.* 2003), bull (Izadyar *et al.* 2003) and non-human primate (Schlatt *et al.* 2002*a*) as recipients suggest that domesticated/laboratory species may be useful recipients for cells from endangered porcine, ungulate and primate species, respectively.

Two steps must be performed to maximise the potential use of a species as a recipient for SSCT. The first requires that the recipient have its endogenous germline stem cells depleted so that introduced spermatogonia have increased access to the appropriate 'niche' along the basement membrane. This depletion, which could be accomplished by irradiation (Meistrich *et al.* 1978; Ogawa *et al.* 1997), chemotherapy (Ogawa *et al.* 1997; Brinster *et al.* 2003) or cold ischaemia (Young *et al.* 1988), greatly enhances the yield of donor-derived sperm. External beam radiation treatment is particularly useful owing to its highly focal (as opposed to systemic) application. In addition, germ cells are radiosensitive (Dym and Clermont 1970; Huckins 1978) in contrast with Sertoli and Leydig cells, which are comparatively radioresistant (Dym and Clermont 1970; Joshi *et al.* 1990). Several radiation treatment protocols have been used successfully for SSCT recipient preparation (Schlatt *et al.* 2002*a*; Izadyar *et al.* 2003), including in the cat (Y. Kim, V. Selvaraj, I. Dobrinski, B. Pukazhenthi and A. J. Travis, unpublished data).

The second requirement for effective SSCT is the preparation of a germ cell suspension, including viable spermatogonia from the donor testis. Typically, enzymatic digestion protocols use a two-step approach, first isolating the seminiferous tubules from interstitial cells with collagenase and then separating the individual cells with trypsin (Bellve *et al.* 1977). This method can be modified to account for species-specific differences in the amount of connective tissue (Dobrinski *et al.* 1999). To maximise the number and relative yield of donor-derived sperm, the mixed germ cell population produced from enzymatic digestion should be sorted to enrich the stem cell population to be transplanted (Shinohara *et al.* 2000). A technical challenge sure to be encountered when trying to use new species as donors is that enrichment typically depends upon antibody recognition of stem cell-specific markers. We have recently discovered that there can be considerable species-specific variation when trying to detect antigens on spermatogonial stem cells (Y. Kim, V. Selvaraj and A. J. Travis, unpublished data).

Once SSCT has been performed and before transplantation success can be evaluated, the recipient requires an adequate period of recovery to allow multiple spermatogenic cycles to be completed. Assessment of transplantation efficacy also requires quantification of the relative percentages of sperm that were produced from the donor as opposed to those from the recipient that may have survived the depletion process. This can also be challenging because sperm from related species can be morphologically indistinguishable. In these cases, a polymerase chain reaction-based microsatellite analysis of single blastomeres taken from resulting embryos produced *in vitro* may be required to confirm the genetic origin of the paternal gamete. Although SSCT is burdened with these non-trivial technical challenges, the benefit of generating a self-renewing population of viable sperm is highly attractive. Furthermore, the application of this approach to rare species could be possible in the near future, especially for endangered rodents, should there be adequate compatibility with the laboratory mouse or rat. For example, SSCT from the South American short-tailed chinchilla (*Chinchilla brevicaudata*) or the Ethiopian water mouse (*Nilopegamys plumbeus*) may be used to produce viable sperm in laboratory mouse or rat recipients. Similarly, some fish species could benefit, especially because sperm cryopreservation is often suboptimal and the freeze–thawing of embryos has yet to be successful (Rana 1995; Tiersch 2001). Recently, germ cell transplantation in salmonids was conducted successfully by injecting primordial germ cells isolated from rainbow trout into the peritoneal cavity of newly hatched masu salmon embryos (Takeuchi *et al.* 2004; Yoshizaki *et al.* 2005). Male recipients produced donor-derived spermatozoa in milt that was subsequently used to fertilise trout eggs to produce normal offspring. Interestingly, male masu salmon reach sexual maturity at a younger age (1 year) compared with rainbow trout (2 years). As a result, this xenotransplantation permitted earlier sperm production, which improved overall reproductive efficiency, which, in turn,

has potential conservation implications (Takeuchi *et al.* 2004). However, in xenogeneic SSCT between the rat and mouse, the timing of the cell cycle of the donor species has controlled the timing of spermatogenesis (Franca *et al.* 1998), suggesting that the temporal benefit seen with the trout and salmon should not be expected routinely in other species.

Testis xenografting

Testis xenografting involves the grafting of small pieces (1–2 mm^3) of testis parenchyma from one species under the skin or testicular capsule of an orchidectomised immunodeficient mouse. The testicular tissue can originate from a biopsy specimen, a castrated testis or a testis from a recently deceased animal. Unlike attempts to reproduce spermatogenesis under *in vitro* conditions, this technique has the advantage of maintaining the complex architecture of the testis. The interstitial Leydig cells can produce testosterone immediately adjacent to the seminiferous tubules, in which all the complex intercellular bridges between germ cells and the junctions between germ cells and Sertoli cells have been left intact.

Because the mouse host is immunodeficient, the xenografts are not rejected, but become supported by the host. The xenotransplants develop and, after a species-specific time period, will produce spermatozoa that can be recovered by the surgical excision of all or part of a xenograft (Honaramooz *et al.* 2002*b*). Testicular xenografting into immunodeficient nude or severe combined immunodeficiency (SCID) mice has been successful using predominantly neonatal or early juvenile testicular tissue from the mouse (Honaramooz *et al.* 2002*b*), pig (Honaramooz *et al.* 2002*a*), goat (Honaramooz *et al.* 2003), Djungarian hamster, marmoset (Schlatt *et al.* 2002*b*), bull (Oatley *et al.* 2005), rabbit (Shinohara *et al.* 2002) and cat (Snedaker *et al.* 2004). To date, this type of xenografting has only been successful using testicular tissue from immature individuals or adult seasonal breeders (Djungarian hamster) that had been subjected to a non-breeding photoperiod to induce maximal regression of spermatogenesis (Schlatt *et al.* 2002*b*).

Interestingly, xenografting of prepubertal rhesus monkey testis tissue into mouse recipients accelerated testicular maturation and the production of fertilisation-competent sperm faster than if the testicular tissue had grown normally in the donor (Honaramooz *et al.* 2004). These observations hold promise for this technique to be useful in species that take several years to attain sexual maturity, such as elephants. Without an acceleration of spermatogenesis, this technique would not be useful for any species in which the time from donation until the anticipated time of puberty was longer than the typical life span of an immunodeficient mouse (approximately 1.5 years). In terms of potential uses for conservation, these findings suggest that testis xenografting may be able to produce mature spermatozoa in mouse recipients earlier than normal.

Conversely, Snedaker *et al.* (2004) demonstrated that completion of spermatogenesis in xenografted feline testis tissue is delayed until approximately 50 weeks after surgery with a typical onset of spermatogenesis at 20–28 weeks in normal males. Preliminary data from our laboratories have confirmed retarded spermatogenesis in testis xenografts of both the domestic cat and dog in recipient immunodeficient mice (Y. Kim, V. Selvaraj, B. Pukazhenthi, I. Dobrinski and A. J. Travis, unpublished data). Nonetheless, viable sperm are still produced in xenografts from these species. Collectively, these observations suggest that the immunodeficient mouse may, indeed, be a useful recipient capable of supporting the spermatogenesis of widely diverse species. However, differences in the interactions between the host mouse and donor tissue of different species will influence the kinetics of spermatogenesis and, possibly, the yield of overall sperm production. A high research priority will be determining how to manipulate the host environment to enhance spermatogenesis in xenografts.

Although technically easier than SSCT, testis xenografting has significant limitations. There can be no epididymal maturation with this technique, so the use of xenograft-derived sperm for breeding must be via ICSI, again mostly undeveloped for all but a few wildlife species. Second, the fact that successful xenografts have occurred with the use of neonatal or juvenile testicular tissue may limit the usefulness of the technique in captive management, largely because testes are much more readily available from adults. Therefore, another priority to optimise the value of this technique for conservation is exploring the feasibility of testis xenografting with adult tissue. A third disadvantage is that xenografts produce sperm only for the life span of the recipient mouse, thus requiring perpetual generation of sperm-producing mice as long as these genes are required (in the absence of technologies for the long-term storage of testis tissue; see below). Finally, on a practical note, maintaining an immunodeficient mouse colony requires costly housing and surgeries conducted under pathogen-free conditions. Therefore, most zoos interested in this approach will likely need to have university or other specialised partnerships that allow access to such resources.

Cryopreservation of testicular tissue

There is a growing need for the cryopreservation of testicular tissue to use in conjunction with germ cell transplantation or xenografting. Cryopreservation of testicular tissue has received substantial attention in humans experiencing both obstructive and non-obstructive azoospermia, as well as cancer (Gil-Salom *et al.* 1996; Park *et al.* 2003; Oehninger 2005). Live offspring have been produced following *in vitro* microinsemination with spermatozoa isolated from cryopreserved murine, rabbit (Shinohara *et al.* 2002) and human testicular tissue (Hovatta *et al.* 1996; Shinohara *et al.* 2002; Park *et al.* 2003) after ICSI. If the cryopreservation of testicular tissue samples could be optimised for different species of wildlife,

then testes from every genetically valuable male could be stored until a need to increase that animal's genetic representation in the population arises. Currently, when animals in zoological collections die or are castrated, the reproductive potential contained in their testicular tissue is discarded. The ability to salvage this material based on cryopreservation research could represent a valuable addition to genome resource banking. However, this approach could also be very important in the field, where there is growing interest in organised and legal hunting to generate revenues for supporting conservation. An additional, positive by-product of hunting could be the harvesting of testes from killed individuals, biomaterials that are currently wasted. Not only would that individual's genetic information be maintained, but it could be used to introduce new 'founder' genetics into captive populations without having to remove an animal from the wild or incur the expense and risks associated with the transport of live animals.

Testicular tissue has been cryopreserved using glycerol (human; Hovatta *et al.* 1996; Shinohara *et al.* 2002) or dimethylsulfoxide (mouse, hamster and marmoset; Schlatt *et al.* 2002*b*). In brief, this involves equilibrating 0.5–1.0 mm^3 pieces of testicular tissue in a cryoprotectant solution at room temperature, transferring the tissue into cryovials and subjecting it to cryopreservation using a programmable freezer (Schlatt *et al.* 2002*b*). Although this technique is effective in cryopreserving testicular tissue from the mouse, hamster and marmoset, there seems to be significant species variance in tissue cryosensitivity. For example, we have found that testicular tissue of the domestic cat has poor viability after cryopreservation, suggesting the need for substantial basic research on requirements for each species. For now, virtually no studies have been conducted in any wildlife species, so there is unlimited opportunity for generating new knowledge and, hopefully, practical applications. Similar to other emerging tools described above, the ultimate usefulness of this approach will depend on the successful development of ancillary technologies for *in vitro* embryo production, such as ICSI, embryo culture and transfer to recipients.

Technologies for producing, preserving and using female germ cells and embryos

Despite the significant use of oocyte- and embryo-related technologies for enhancing reproduction in humans, livestock and laboratory animals (for reviews, see Farstad 2000; Sharkey *et al.* 2001; Squires *et al.* 2003; Baldassarre and Karatzas 2004; Devroey and Van Steirteghem 2004; Wheeler *et al.* 2004; Wolf *et al.* 2004), IVF and embryo transfer have so far had a negligible impact on the genetic management of wildlife species (Loskutoff *et al.* 1995; Pope 2000; Pukazhenthi and Wildt 2004). Indeed, a few offspring have been produced in several wildlife species, including the baboon, rhesus macaque, marmoset, gorilla, Indian desert cat, ocelot, tiger, African wild cat, Armenian red sheep, water buffalo, gaur, red deer, llama and caracal (for a review, see Pukazhenthi and Wildt 2004). These successes have been mere demonstrations of the potential of each technology, with no species being routinely propagated by any oocyte or embryo method. Thus, even the now rather basic tools of IVF, oocyte or embryo culture, transfer and oestrous cycle synchronisation (mostly routine in common species) need to 'emerge' for wildlife species. Such advances will lay the foundation for some of the more sophisticated concepts and tools presented below.

Based on in vivo- *and* in vitro-*matured oocytes*

It is possible to harvest oocytes from living donors (by transvaginal or transabdominal laparoscopic ovum pick-up) or directly from the ovaries after ovariectomy or death (Comizzoli *et al.* 2000). *In vivo*-matured oocytes consistently appear to be developmentally more competent than their *in vitro* counterparts after IVF or ICSI. However, successful collection of *in vivo*-matured oocytes relies on: (*1*) the ability to induce folliculogenesis with exogenous hormones; and (*2*) recognition of the optimum time for collecting oocytes from preovulatory follicles. In contrast, recovering immature oocytes from antral follicles circumvents these limitations and enables the collection of gametes even from prepubertal, pregnant or dead individuals ('gamete rescue').

For domesticated mammals studied to date, there have been common findings relative to oocyte *in vitro* maturation (IVM) that, no doubt, will be relevant to similar wildlife studies. For example, it now is well established that the initial quality of the immature oocyte influences subsequent embryo developmental competence *in vitro* or *in vivo* (Coticchio *et al.* 2004; Krisher 2004; Combelles and Racowsky 2005). Strict selection criteria are also useful in ensuring future developmental success and fertilisability. For instance, the morphological aspects of the oocyte (colour and cytoplasm homogeneity, number of cumulus cell layers surrounding the oocyte) are important markers and, recently, oocyte metabolism (cow, Krisher and Bavister 1999; cat, Spindler *et al.* 2000; pig, Brad *et al.* 2003) and follicle size (Kauffold *et al.* 2005; Songsasen and Wildt 2005) have been shown to be good predictors of developmental competence. These same 'tools' are a likely good first step for evaluating oocyte quality in most wildlife species.

For zoos, we expect IVM to be particularly worthwhile for dealing with increasingly aged animal populations, a by-product of modern and improved veterinary health care. Previous studies of humans have established a clear relationship between oocyte ageing and the non-disjunction of bivalent chromosomes during meiosis (Dailey *et al.* 1996). Although largely unstudied in zoo animals, ageing populations present some usual problems, especially in species that are challenging to propagate. One example is the cheetah, where early observations have revealed low pregnancy rates in females

older than 8 years after AI (B. Pukazhenthi and J. G. Howard, unpublished observations). It is unclear whether the failure of pregnancy arises from compromised oocyte quality or uterine pathologies. However, such findings help redirect previous assumptions of 'what is old,' because many captive-held cheetahs can routinely live to more than a dozen years of age or longer. Perhaps certain wild species maintained in captivity have the physiological ability to live to ages that far exceed the early onset of reproductive senescence. Given that scenario, then such species could provide interesting new insight into the origins of early oocyte ageing. Regardless, such older animals (although not normally cycling) may serve as a source of immature oocytes for emerging technologies, especially for species where the rudiments of IVF and embryo transfer are available. However, it is also now clear that oocytes isolated from older mice (Liu and Keefe 2004) and human donors (Volarcik *et al.* 1998) are compromised in their ability to complete meiotic maturation and support embryo development. Furthermore, it has been demonstrated that oocytes from ageing females (mice and human) are more developmentally sensitive to mitochondrial damage and exhibit a higher incidence of aneuploidy (Eichenlaub-Ritter *et al.* 2004; Thouas *et al.* 2005). As a result, recent studies have focused on identifying corrective solutions to this process. For example, microinjection of an isolated germinal vesicle from oocytes or the nuclei from somatic cells into enucleated abnormal oocytes, followed by electrofusion, supported normal chromosomal segregation (Palermo *et al.* 2002). The impact of this technique on gene imprinting and fidelity of chromosome segregation remains unstudied. Ooplasm transfer has also been shown to mitigate age-related effects in the human oocyte, even resulting in successful IVF and living offspring (Levy *et al.* 2004). In these cases, cytoplasm from immature, mature, fresh or cryopreserved donor oocytes was injected *in vitro* into those of older patients. Cytoplasm transfer is believed to correct putative imbalance between anti- and pro-apoptotic factors and/or defective mitochondrial membrane potential (Levy *et al.* 2004). Although relatively successful in the human, the long-term implications of introducing mitochondrial DNA from another individual within the same species (and the resulting heteroplasmy) pose several risks. These include severe pathologies in humans associated with the A3243G mitochondrial tRNA leu (UUR) point mutation that causes mitochondrial myopathy, encephalopathy, lactic acidosis, stroke-like episodes (MELAS) syndrome and maternally inherited diabetes and deafness syndrome (MIDD; Bai and Wong 2004). Despite potential application in species, such as the cheetah, with ageing captive populations, the potential risks to the offspring that would be produced demand significant further investigations before such technologies are used routinely.

New IVM approaches may also be particularly relevant to wild taxa because many species are seasonal in reproduction. In terms of maturational ability, oocytes collected during the quiescent season(s) of the year are likely to be resistant to development *in vitro*. Examples of this phenomenon are already available from the domestic cat (Spindler *et al.* 2000; Comizzoli *et al.* 2003) and red deer (Berg and Asher 2003), wherein oocytes recovered in the non-breeding season are compromised compared with those evaluated during the peak breeding time of the year. The result is lower or non-existent embryo production during most of the year. However, there is recent evidence that seasonal impositions on oocyte quality can be circumvented by modifying the *in vitro* culture system. For example, in the cat (a model for wild felids), supplementing the IVM medium with anti-oxidants and increased gonadotropin concentrations overcomes these seasonal compromises, enhancing overall embryo production efficiency throughout the year (Comizzoli *et al.* 2003). This knowledge could, potentially, be applied to the reproductive management of a host of wild species that exhibit distinct seasonal variations in reproduction.

Ovarian tissue xenografting

Ovarian tissue grafting has been studied in a host of species, including the mouse (Shaw and Trounson 2002*b*), cat (Bosch *et al.* 2004), dog (Metcalfe *et al.* 2001), pig (Kaneko *et al.* 2003), sheep (Gosden *et al.* 1994), cow, rhesus monkey (Lee *et al.* 2004), wombat (Wolvekamp *et al.* 2001), wallaby, elephant and human (Paris *et al.* 2004). In all cases, it has been possible to obtain normal-appearing antral follicles from these grafts. When inseminated *in vitro*, oocytes from such 'foreign' follicles fertilise and form viable embryos. Live offspring have also been produced in the mouse, sheep (Gosden *et al.* 1994) and macaque monkey from oocytes derived from ectopically (in the same animal) transplanted ovarian tissue (Lee *et al.* 2004).

The benefits of xenografting ovarian tissue would be similar to those of using testis tissue transplants. There would be a particular advantage for genetically valuable females that die unexpectedly. For example, in such cases it appears feasible to transplant ovarian tissue from a tiger or giant panda to an immunodeficient mouse recipient. In addition to rescuing valuable genotypes, this approach would offer an innovative way to study follicular dynamics and responsiveness to exogenous gonadotropins in previously unstudied species while characterising oocyte morphotype and culture and fertilisability *in vitro*.

This emerging technology has even more potential when combined with cryopreservation. The ability to freeze ovarian tissue and then recover viable oocytes has already been successful in the mouse, rat, sheep, marmoset, macaque, human, elephant, wombat and wallaby (Lee *et al.* 2004; Paris *et al.* 2004). Typically, this involves cutting ovarian tissue into sufficiently small pieces to allow cryoprotectant permeation and cryopreservation using slow cooling. This technique provides high survival of the ovarian tissue in most species studied to date (Shaw and Trounson 2002*a*; Paris *et al.* 2004; Snow *et al.*

2004). Although most investigators have evaluated controlled rate freezers for tissue freezing, one study demonstrated the usefulness of a low-cost cryodevice that could permit routine ovarian tissue preservation even under field conditions (Cleary *et al.* 2001). This would have the same benefits as testis cryopreservation discussed above, taking advantage of germplasm that is normally wasted during legal wildlife hunting or culling for population control.

Oocyte cryopreservation

Investigations in recent years have vastly improved our knowledge and abilities to cryopreserve mammalian oocytes (Shaw *et al.* 2000; Maclellan *et al.* 2002; Murakami *et al.* 2004; Abe *et al.* 2005; Cai *et al.* 2005). However, it is clear that optimal methods tend to be species specific, due largely to variations in oocyte size, permeability and sensitivity to cryoprotectants and cooling/thawing rates. Compared with mature counterparts, immature oocytes appear to be more resistant to cryostress (Agca 2000) because these cells, at the germinal vesicle or germinal vesicle breakdown stage, do not have a temperature-sensitive meiotic spindle. Recently, both mature and immature oocytes have been cryopreserved using ultrarapid protocols, such as freezing on electron microscope grids and cryoloops (Stachecki and Cohen 2004). Live offspring have also resulted from cryopreserved oocytes in the mouse and human (Porcu *et al.* 1997; Tucker *et al.* 1998).

As with other technologies, there is a severe lag in progress in this area for wildlife species, largely due to the lack of access to good-quality oocytes from diverse species (Leibo and Songsasen 2002). One helpful approach would be to develop networks that link interested investigators with zoos willing to provide fresh ovarian tissue after an animal's death or after ovarian excision for medical reasons. Regardless, progress for wildlife will continue to be linked with parallel studies of taxonomically related domestic animal models. Certainly, continued advancements with the common cow, sheep, goat, cat, dog and white-tailed deer would have relevance to more rapid progress with wild bovids, small ruminants, felids, canids and cervids, respectively. However, this will not benefit the thousands of birds, reptiles, amphibians or fish for which there is little or no information about basic oocyte biology or the sensitivity of female gametes to cryopreservation. Interestingly, of these taxonomic groups, fish have received a fair amount of attention owing to the substantial commercial benefits that could be derived from freezing embryos, thereby allowing year-round farming. However, several studies have demonstrated the four factors that complicate successful fish oocyte cryopreservation: (*1*) large oocyte size; (*2*) the presence of vast amounts of yolk; (*3*) unique permeability in a complex membrane structure; and (*4*) extreme susceptibility to chilling injury (Routray *et al.* 2002; Isayeva *et al.* 2004). Therefore, a considerable amount of work is still needed to better understand the cryobiology of immature and mature oocytes in numerous species. When these technologies are optimised, it would allow the long-term preservation of the female genome.

In vitro *embryo production and cryopreservation*

The challenges to applying IVF and embryo cryopreservation technologies to wildlife have been reviewed recently (Leibo and Songsasen 2002; Loskutoff 2003; Pukazhenthi and Wildt 2004), with many of the obstacles related to the total absence of fundamental embryology for most species. Such data are of first-order priority before considering the pragmatic use of embryo collection, production and/or embryo transfer. At the same time, there are several emerging technologies that could likely aid *in vitro* embryo production from such species. For example, techniques that rely on microfluidic channels to promote the mixing of the male and female gametes (by IVF or ICSI) have improved developmental efficiency, imparted less stress on the embryo and appear highly promising (Beebe *et al.* 2002; Wheeler *et al.* 2004). Another example is the pre-ovulatory recovery of intrafollicular oocytes for *in vitro* culture before *in vivo* fertilisation. Such advances have proven beneficial for the domestic horse, a species in which IVF embryo production is challenging. Rather, *in vitro*-cultured oocytes are transferred to the recipient's oviduct, after which an intrauterine insemination is performed. A slight modification of this process is gamete intrafallopian transfer (GIFT), whereby both sperm and oocytes are transferred into the surrogate's oviduct (Carnevale 2004). Both these oocyte culture/transfer approaches could have potential for wild species, especially in instances of limited sperm numbers.

Successful embryo cryopreservation has been achieved in a wide array of domesticated mammals, essentially relying on the same basic cryobiological principles of using one of a few cryoprotectants and generally slow rates of controlled cooling (Rall *et al.* 2000; Shaw *et al.* 2000). Especially exciting have been advances in vitrification (Rall and Fahy 1985), because these approaches seem particularly attractive for the wildlife world owing to the need for minimal equipment and their applicability to remote field conditions. Vitrification involves a direct transition from a liquid to a glass phase, avoiding the formation of damaging ice crystals. Results to date have largely revealed a similarity in overall results between conventional embryo cryopreservation and vitrification for most species tested (domestic). Thus, as embryo preservation studies are considered for wild species studies, it would be prudent to explore vitrification, which could be applied widely at low expense, especially in developing countries. Similar to other aspects of studies on reproduction, there has been little effort directed at the cryopreservation of bird, reptile and amphibian embryos. As with oocytes, fish embryos certainly have been studied more extensively, largely due to commercial applications. Despite a significant number of studies, fish embryos have not been successfully frozen/thawed owing to extreme cryosensitivity and the complexity of embryonic membrane

permeabilities. These challenges are now being addressed by testing the feasibility of inserting transient water channels to enhance cryoprotectant permeation (Hagedorn *et al.* 2002), a technique that may be relevant to improving embryo cryopreservation for various taxa with large-sized embryos (including rare fish and amphibians).

Reproduction control and contraception

One of the most formidable challenges facing zoo and wildlife managers is overly abundant wildlife populations. Zoo genetic management programmes dictate the breeding of the most valuable individuals, but also help to ensure that animals of unknown origin or those already well represented are prohibited from reproducing. Certainly, the most effective contraceptive in the zoo world is to separate individuals into same-sexed groups. However, this is fraught with problems, especially severe aggression among adults housed together, thereby requiring significant additional space to place 'excess' individuals. As a result, there is an urgent need to develop safe and effective reproduction control measures relevant to the management of wildlife. This requirement extends beyond the zoo world to wild habitats, where native landscapes are being destroyed by wild horses, burros and white-tailed deer (in North America) to elephants (in Africa) to brush-tailed possums (in New Zealand).

Current evidence indicates that there are no simple solutions. Progestagen implants (largely melengestrol acetate) placed subcutaneously have been used in a wide array of zoo-held species ranging from rodents to elephants. This implant invariably prevents pregnancy with minimal financial cost, but is also associated with serious side effects in some taxa, especially those that are exquisitely sensitive to exogenous steroids. Particularly well documented is an increased incidence of reproductive tract neoplasms in progestagen-implanted felids (Munson *et al.* 2002). More recently, the efficacy of another progesterone derivative, namely levonorgestrel, has been evaluated in white-tailed deer (Plotka and Seal 1989; White *et al.* 1994), the male bonnet monkey (Rajalakshmi *et al.* 2000), kangaroo and tamer wallaby (Nave *et al.* 2000, 2002). Overall, these results demonstrated variable, inconsistent responses among the species examined.

These findings have motivated zoo scientists to examine alternatives, including the gonadotropin-releasing hormone (GnRH) agonist leuprolide acetate, which down-regulates ovarian function in species such as the wapiti (Baker *et al.* 2002) and the female mule deer (Baker *et al.* 2004). When administered, leuprolide acetate usually causes ovarian hyperstimulation and oestrus, which are followed by prolonged ovarian quiescence and, therefore, effective contraception. Other studies have examined a GnRH agonist (deslorelin) in a variety of male and female non-domestic species, including the tammar wallaby (Herbert *et al.* 2004, 2005) and wild African carnivores (Bertschinger *et al.* 2002). Similar to leuprolide, deslorelin also initially stimulates gonadal function, but then down-regulates testicular and ovarian functions. Specifically, deslorelin administration induced contraception in lionesses for 12–18 months and in female cheetahs and leopards for a minimum of 12 months after treatment. In male cheetahs, no viable sperm were observed until 21 months after treatment. Although the response in wild dogs was less consistent, mating was delayed in nine bitches after treatment with deslorelin (Bertschinger *et al.* 2002).

Immunocontraception also has been considered as a potential management tool for zoos. Many animals representing many species have been injected with porcine zona pellucida antigen to mount an antibody titre against zona pellucida proteins (Kirkpatrick *et al.* 1996; Brown *et al.* 1997; Shideler *et al.* 2002). Results have been mixed and questionable, because the measure of success has largely been whether offspring were produced rather than carefully examining gonadal function. Despite variable success, immunocontraception probably holds the most promise for reducing reproductive success in wild populations. Recent developments in this area have been summarised elsewhere (Cowan *et al.* 2003; Mate and Hinds 2003; Roger 2003). Significant progress has been made in the development of viral vector vaccines expressing zona pellucida anitigens, microsphere or liposome delivery systems and plant-based systems (Mate and Hinds 2003). However, the development of cost-effective delivery systems and the public acceptability of such vaccines will determine the applicability of these technologies in the future.

Conclusions

Reproductive technologies were once perceived as a possible 'quick fix' for propagating endangered species, at least until scientists realised that there were no easy answers to producing young from wildlife species (Wildt *et al.* 2001). In the face of actual trials, failures to apply domestic animal techniques successfully to wild counterparts far exceeded successes. The reason for this was due to differences in the animals themselves, even among closely related species, all of which were found to vary remarkably in phenotype, genotype and reproductive mechanisms. Without fundamental knowledge of the latter, no technique will be worth much for improving reproductive efficiency. In addition, there has been the gradual realisation that the quality of offspring (in terms of genetic value) is as important as numbers of young produced. Unlike the situation in livestock, wildlife managers are not selecting for specific traits but, rather, are attempting to maintain all existing gene diversity. That goal itself markedly limits the usefulness of certain technologies, such as nuclear transfer (cloning; Critser *et al.* 2003; Holt *et al.* 2004). Furthermore, all the reported successful applications of nuclear transfer in wildlife have used ooplasts from domestic species counterparts and the resulting heteroplasmy is suspected to influence

certain traits in the resulting offspring, which could also be detrimental for efforts of species preservation.

Although examples of how reproductive techniques have promoted wildlife breeding are limited, these technologies can (and are) vastly improving our collective basic knowledge about biological processes. Based on new mechanistic information from studies on closely related domestic species when possible, these technologies could well have more practical propagative applications in the future, including helping to establish sustainable captive populations by preserving and introducing valuable genetic information as needed. The maintenance of adequate genetic heterozygosity will continue to be the best path towards ensuring reproductive and health fitness for species and populations long into the future.

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