CSIRO PUBLISHING
Foreword
www.publish.csiro.au/journals/rfd
Reproduction, Fertility and Development 2007, **19**, iii–vii

A perspective on the role of emerging technologies for the propagation of companion animals, non-domestic and endangered species

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Abstract. Assisted reproductive technologies (ART) have been used successfully in humans, domestic and laboratory species for many years. In contrast, our limited knowledge of basic reproductive physiology has restricted the application of ART in companion animal, non-domestic and endangered species (CANDES). Although there are numerous benefits, and in some cases a necessity, for applying ART for the reproductive and genetic management of CANDES, the challenges encountered with even the most basic procedures have limited the rate of progress. In this foreword we discuss the status of conventional ART, such as artificial insemination and *in vitro* fertilisation, as well as their benefits and inherent difficulties when applied to CANDES. It is upon these techniques, and ultimately our knowledge of basic reproductive physiology, that the success of emerging technologies, such as those described in this special issue, are dependent for success.

Development of assisted reproductive technologies (ART) for domestic species and humans has been ongoing for several decades and is routine practice in many situations. Well-developed technologies in domestic species include artificial insemination (AI), *in vitro* fertilisation (IVF) and embryo transfer (ET). Unfortunately, this wealth of knowledge developed for commercially important livestock species, biomedically relevant model species and human infertility treatment cannot be directly applied to other species, as significant species-specific challenges exist. Our knowledge of the basic reproductive physiology of many companion animal, non-domestic and endangered species (CANDES) is limited in comparison. This is partly due to scarce and costly animal resources, lack of economic demand and inadequate research funding. However, there is a need for reproductive knowledge in CANDES to meet an increased interest in breeding companion animals for pleasure and recreation, propagate valuable genetic models for biomedical research (Songsasen and Wildt 2007), and manage *ex situ* populations of endangered species (Pukazhenthi and Wildt 2004). Although ART have numerous potential applications, we must remind ourselves that the success of any ART is completely dependent on our knowledge of basic reproductive physiology, in any species. Thus, detailed descriptions of reproductive cycles (seasonal patterns, ovulatory mechanisms, etc.), structural anatomy, and gamete and embryo physiology must be collected in many, if not most, CANDES before advanced ART can be effectively used to significantly impact reproductive and genetic management. In general, the application of ART to CANDES is technically and biologically complex, representing many basic and applied scientific challenges. However, in looking to the future, we can envision a time when ART may be required to genetically manage threatened and endangered populations for their continued existence, as well as meet the varied demands for breeding horses, cats and dogs.

The technologies typically used with CANDES are AI, IVF/ET, intracytoplasmic sperm injection, sperm sexing, genome resource banking (cryopreservation of gametes, embryos, somatic and gonadal tissue), as well as cloning to some extent (Andrabi and Maxwell 2007). There have been multiple reports of the successful use of these ART in CANDES, resulting in the production of live, healthy offspring (recently reviewed by Pukazhenthi and Wildt 2004 and Andrabi and Maxwell 2007). However, there are many more failures than successes and, overall, the efficiency of ART in CANDES remains extremely low. Many of these reports represent the first, and often the only, instance when the procedure was successful in the species of question. All of the ART have applications that could benefit CANDES, but a distinction needs to be made between those techniques that are efficient enough for immediate application and those techniques that require extensive refinement before

10.1071/RD07091 1031-3613/07/060iii

they can be used routinely. It is also important to note that this distinction will vary dramatically between species. For example, offspring have already been produced by IVF/ET in several felids (Pope *et al*. 2006) and improvements in efficiency to the point of practical use may be achievable within the next 5 years. In contrast, the very first successful AI in a rhinoceros was only recently reported (Hildebrandt *et al*. 2007) and little or no work has been done on oocyte recovery, IVF, embryo culture or ET. Therefore, IVF/ET may be decades from routine or practical use in rhinos.

In this special issue, a range of emerging assisted reproductive technologies, including sperm sorting, novel gamete storage, germ cell transplantation, stem cells, cloning, transgenesis and gonadal xenografting, will be reviewed and their current knowledge, advantages, disadvantages and complications will be discussed by experts in the area. For each emerging technology, a discussion of its potential application to CANDES is presented. In this introductory paper, we paint a brief picture of more conventional ART (such as AI and IVF) and their benefits and inherent difficulties when applied to CANDES. In reading the subsequent papers it is important to remember that all of the emerging technologies described are dependent on the more basic technologies in order to be successful. These more basic steps, required between the emerging techniques and offspring production, are often overlooked in light of the exciting applications of the more 'cutting-edge' technologies but are just as important in the successful application of the emerging techniques.

Advantages

In non-domestic and endangered species, the success of traditional captive breeding programs is influenced by numerous factors: space availability, animal health and welfare, behaviour, nutrition, genetic management and reproductive failure (Lasley *et al*. 1994). These limitations, along with the small numbers of individuals present in captive populations, often necessitate the use of ART for effective genetic management of captive, and ultimately wild, populations (Ballou 1992). Assisted reproductive technologies have made a tremendous impact on the general management practices of domesticated species (livestock and laboratory animals), but also for a variety of non-domestic species. The two most important advancements are the ability to control and manipulate reproduction, and the preservation and storage of genetic material in the form of gametes and embryos. These two factors have allowed better management practices, conservation of genetic diversity and improved methods for overcoming infertility (Woods *et al*. 2004).

Analysis of hormone levels forms the basis of many of the ART. Information gained by examining the reproductive hormone profiles of females and males is instrumental to the success of both natural and assisted breeding attempts. The ability to determine timing of oestrus, pregnancy status, sex, seasonality and overall reproductive status provides a powerful tool for breeders and wildlife managers. Development of non-invasive techniques for hormone monitoring (Hindle *et al*. 1992; Schwarzenberger *et al*. 1993) has facilitated sample collection in species where the acquisition of blood samples is difficult or counterproductive (e.g. stress-induced alterations in hormone levels). In companion animals, hormone analysis is not as widespread (with the exception of progesterone and luteinising hormone analysis to determine ovulation in dogs) due to the feasibility of applying behavioural observation, palpation and ultrasound techniques for oestrus and pregnancy detection. However, non-invasive hormone monitoring has been a prerequisite for assessing reproductive parameters in non-domestic species, particularly wild felids (Brown *et al*. 1996, 2001), canids (Walker *et al*. 2002; Songsasen *et al*. 2006) and cervids (Monfort *et al*. 1990; Kapke *et al*. 1999).

This leads to another important innovation, the manipulation of ovarian function (follicular development and ovulation). The application of exogenous gonadotrophins allows synchronisation of ovulation for AI and ET, the production of multiple offspring from one individual (i.e. a greater number of offspring than is generally produced in one breeding season) and, in some cases, the ability to overcome poor ovarian function. Control of follicular development is essential for genetic contribution by the female, via the oocyte. Unlike farm animal species, superovulation is used less frequently for companion animals and non-domestic species as many of these species are multiovulatory and production of supraphysiological numbers of oocytes is unnecessary for techniques other than IVF and ET. Oestrus synchronisation, however, is used extensively to time natural breeding or AI and as a method for inducing ovarian activity in anoestrous females. Ovulation induction protocols are available for domestic dogs (Kutzler 2005), cats (Pelican *et al*. 2006) and horses (Allen 2005); however, studies in cats have shown that the gonadotrophin regimen may perturb female reproductive function and lead to reduced fertility (Pelican *et al*. 2006). Species-specific hormone treatments have also been developed for numerous non-domestic species, including wild felids (Pelican *et al*. 2006), cervids (Asher *et al*. 2000) and bovids (Loskutoff *et al*. 1990; Thompson and Monfort 1999; Morrow *et al*. 2000).

There are numerous reasons why natural breeding may not be possible. An animal may be sick, deceased, located elsewhere or infertile, but has been selected for breeding. The opportunity for this individual to be included in breeding recommendations becomes possible with the use of AI or ET. These technologies permit the transfer of genetic material, as spermatozoa or embryos, into a recipient female without the need for the donors to be available at the time of transfer. AI is used routinely for breeding companion animals to eliminate the need for shipping animals while maintaining access to desired genetics. As natural habitats for many species are disappearing, intense species management becomes essential for many non-domestic and endangered species. Similarly, the use of ART may facilitate efforts to maintain critical genetic diversity in both wild and captive populations of endangered species. Technologies such as oestrus synchronisation, AI, IVF, ET in combination with gamete and embryo cryopreservation have the potential to greatly contribute to species management programs (Pukazhenthi and Wildt 2004). According to Wildt (1992), the benefits of genome resource banking in wildlife include: facilitating the distribution of genetic material between populations, improving the efficiency of breeding programs (eliminating the transport of live animals, problems with mate preference, behavioural or sexual incompatibility, large population sizes), extending the generation interval of individuals indefinitely, and providing insurance against the loss of diversity. In non-domestic species, there have been numerous documented successes using AI (reviewed by Pukazhenthi and Wildt 2004 and Andrabi and Maxwell 2007). More importantly, the technique has already been used successfully as a breeding-management tool in several species, such as the black-footed ferret and the cheetah (Wildt *et al*. 2001). ET is used less frequently due to its reliance on upstream technologies, including embryo or oocyte retrieval, IVF and *in vitro* embryo culture techniques, which are yet to be developed in many species. However, the ability to preserve and distribute both female and male genetics makes this a necessary technology to pursue. Lack of successful sperm or embryo cryopreservation techniques in certain species has resulted in the use of fresh or chilled samples, limiting transport and storage times. In the domestic dog and horse, sperm cryopreservation is possible; however, chilled samples are preferred because of the long lifespan, lower rate of damage and ease of shipping ice packs versus dry shippers (Iguer-Ouada and Verstegen 2001; Allen 2005). Further, in the horse, pregnancy rates do not differ between freshly transferred embryos and embryos transported at 4°C for up to 24 h, compared to 50–60% pregnancy rates at best after conventional embryo cryopreservation (Stout 2006). In cases where cryopreservation is applicable, the possibilities are endless as the sample may be stored for use at a later date or shipped over large distances. A recent study reported two live births in humans using spermatozoa that had been cryopreserved for 21 and 28 years (Feldschuh *et al*. 2005).

Disadvantages and complications

Many challenges have been encountered during development of ART for CANDES. In most cases, extensive animal conditioning, training, sedation or anaesthesia is required, even for routine procedures such as blood sampling or ultrasound monitoring, and sometimes complications with anaesthesia are encountered (Pope and Loskutoff 1999). Anaesthesia inhibits sperm transport after vaginal AI in several felids, canids and mustelids (Howard 1999). The deposition of semen into the uterus can be troublesome, and may need endoscopy in smaller hoofed species (Monfort *et al*. 1993). Development of appropriate species-specific tools to visualise male and female reproductive anatomy by ultrasonography presents unique difficulties (Hildebrandt *et al*. 2000). Other species present specific problems due to size (e.g. in rhinos), anatomy (e.g. the reproductive tract in elephants; Brown 2000), or embryo physiology (e.g. failed formation of vital glycoprotein-rich membranes in equine embryos *in vitro* (Tremoleda *et al*. 2003) or formation of shells around marsupialia early embryos (Pukazhenthi and Wildt 2004)). However, the lack of basic reproductive knowledge remains the primary dilemma for each new target species. For example, Paris *et al*. (2005) recently developed AI in the tammar wallaby (*Macropus eugenii*) using natural cycles and a unique method of synchronising oestrus in females by the removal of pouch young. However, this success was based on an extensive body of knowledge of the reproductive biology of this species, including studies to determine: (i) the time of reactivation and birth of the diapausing embryo after removal of pouch young (Merchant 1979); (ii) the time of oestrus post partum (Rudd 1994); (iii) the time of ovulation post coitum and the dynamics of sperm transport to the site of fertilisation (Paris *et al*. 2004); and even (iv) the time of birth more accurately (and hence better predict oestrus and ovulation) based on the superoxidative skin pigment changes that occur in neonates (Paris *et al*. 2005). The development of AI in the tammar wallaby can therefore not simply be transposed to a different macropodid where these stages are not known. Although studies on male and female reproduction in CANDES have been carried out in a large number of species (reviewed by Pukazhenthi and Wildt 2004; Andrabi and Maxwell 2007), this number represents only a small fraction of the species currently in existence and is dominated by mammals, one of the least diverse taxonomic groups.

Although the development of methods to monitor reproduction in specific CANDES is usually the first step in the application of ART, this relatively simple (compared to cloning or transgenesis) step has often been tedious, time consuming and costly to optimise and validate a specific method. For example, to develop a radioimmunoassay for a specific species, an antibody often needs to be produced, which in itself can be very costly and time consuming. Frequent blood sampling has historically been a major obstacle for mapping the reproductive hormonal patterns in a wildlife species, but was a necessity until methods were developed to monitor these hormones non-invasively in faeces or urine (Schwarzenberger *et al*. 1998; Brown *et al*. 2001; Paris *et al*. 2002). The availability of non-invasive monitoring methods has revolutionised the field. Current challenges include improvement of limitations in existing methodology, such as simplification of faecal extraction for hormone monitoring, which is currently labour intensive, and development of antibodies that crossreact across species (Pukazhenthi and Wildt 2004).

The use of IVF/ET requires that gametes and early embryos are maintained in an unnatural environment *in vitro* for at least a short period of time. The environment encountered by the embryo during these early stages can have dramatic effects on pre- and postimplantation development, as well as impacting the health and behaviour of the offspring after birth (Ecker *et al*. 2004; Fleming *et al*. 2004; Gardner and Lane 2005). Described problems with *in vitro*-produced embryos include: (i) occurrence of cytogenetic and ultrastructural changes; (ii) increased abortions, perinatal loss or fetal abnormalities; and (iii) skewed sex ratio in favour of males (Loskutoff 2003; Fleming *et al*. 2004). For example, conventional protocols for gonadotrophin stimulation, oocyte recovery and IVF in the tiger resulted in abnormal endocrine profiles and oocyte and embryo morphology (Crichton *et al*. 2003). A better understanding of and insight into regulatory processes is essential when using ART to create optimal conditions during several critical time points in development. In this respect, the careful stepwise development of a feline embryo culture medium in domestic cats, resulting in improved embryo development and viability, was successful when applied to *in vitro* embryo culture of non-domestic small cat species (Herrick *et al*. 2006*a*, 2006*b*, 2007).

The International Embryo Transfer Society (IETS) CANDES Research Subcommittee has made it their mission to compile available ART methods in CANDES in a detailed resource manual that can be accessed via the frequently updated website http://www.omahazoo.com/iets/ResourceManual2006.pdf (verified 11 June 2007), in the hope that this will facilitate the responsible use of ART and its continued development in such species. Development of technologies specifically for CANDES is a high priority, and many of these have been listed by the IETS CANDES Technology Subcommittee on the following website: http://www.omahazoo.com/iets/NAPTlist.htm (verified 11 June 2007). Working with CANDES often means that one has limited access to animals; thus, harvesting gametes and reproductive tissues for banking and later use is very important. Several studies are addressing these research priorities in CANDES.

Summary and conclusions

In conclusion, significant challenges remain despite some progress in applying conventional ART to CANDES. Species-specific challenges remain enigmatic, necessitating slower species-by-species approaches. New concerns arise, such as a recently reported decline in mammalian reproductive health due to exposure to environmental chemicals (Gross *et al*. 2002; Baillie *et al*. 2004). As outlined in the reviews that follow, several newly emerging ART may offer opportunities to bypass problems encountered using more conventional ART. We have at our disposal technologies that offer the promise of solving complex animal management problems. However, to take full advantage of this promise, much more basic physiological information is required. For many CANDES, we may never have the opportunity to build a repertoire of knowledge that will allow us to apply advanced ART techniques to such an extent that we can significantly impact animal management. We must take care not to rely too heavily on ART to 'rescue' threatened and endangered species at the last possible moment. Rather, we must be diligent now to continue basic scientific research to gather reproductive information, as well as prevent further habitat fragmentation and destruction, to prevent CANDES from reaching the point of no return. If we are careful to focus our efforts in this direction, we may be able to begin to utilise advanced ART in CANDES in the future, in species-appropriate and meaningful ways.

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