Mammal Review

Mammal Review ISSN 0305-1838

REVIEW

Assisted reproductive technologies in captive rhinoceroses

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Keywords
assisted reproduction, conservation, *in vitro*, *in vivo*, rhinoceros

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Submitted: 15 December 2017
Returned for revision: 1 February 2018
Revision accepted: 6 July 2018
Editor: DR

doi: 10.1111/mam.12138

ABSTRACT

1. Survival of the five remaining rhinoceros species is threatened. Four of the five species are in managed collections, but captive populations are not self-sustaining and low reproductive rates make population growth slow.
2. Slow population growth, coupled with behavioural incompatibilities, acyclicity, low genetic diversity, and disease susceptibility, creates the need for assisted reproductive technologies (ARTs) to maintain genetic diversity while bolstering population numbers.
3. Both published and unpublished data are included in this review of ARTs, to facilitate understanding consistencies and variations between and within each rhinoceros species.
4. Progress has been made to address species-specific characteristics of reproductive physiology in rhinoceroses. This review outlines the ARTs that have been performed and identifies areas in need of research. *In vivo* technologies have resulted in live calves by artificial insemination, created genetic reservoirs through semen collection, and provided new avenues of gamete retrieval via ovum pickup. *In vitro* technologies have enabled genetic rescue post mortem and support early stage embryo production through oocyte maturation and fertilisation.
5. As conservation efforts focus on rhinoceroses, improvement of existing techniques and development of new technologies will allow for a broader application of successful rhinoceros ARTs.

INTRODUCTION

There are five extant taxa (referred to for simplicity as species) of rhinoceros and all experience some threat to their survival (International Union for Conservation of Nature - IUCN 2017). Currently, the greater one-horned rhinoceros *Rhinoceros unicoris* (Vulnerable), black rhinoceros *Diceros bicornis* (Critically Endangered), and southern white rhinoceros *Ceratotherium simum simum* (Near Threatened) are maintained in zoological institutes as well as existing in the wild. The Sumatran rhinoceros *Dicerorhinus sumatrensis* (Critically Endangered) is maintained in a managed setting within its native range. The Javan rhinoceros *Rhinoceros sondaicus* (Critically Endangered) exists only in the wild at perilously low, but stable, numbers. A sixth taxon, the northern white rhinoceros *Ceratotherium simum cottoni* is assessed by the IUCN as Critically Endangered (Possibly Extinct in the Wild); only two females, both in captivity, are now known to exist.

Since few individuals of each species are housed at any institute, gathering data on a large scale from multiple individuals is difficult. Moreover, the basic biology of each species is predicated on observations in few animals. By compiling the known information, we aim to identify trends as well as the next steps needed in rhinoceros reproduction for the application of assisted reproductive techniques (ARTs).

The long gestation periods of all rhinoceros species (15–18 months; Fouraker & Wagner 1996) contribute to slow population growth. Additionally, species-specific problems in captivity necessitate the difficult and lengthy development of assisted breeding programs and must be tailored to each species. This review is focussed on captivity and refers to those individuals held in zoological institutes. For example, a large proportion of captive southern white

rhinoceros females are acyclic or irregularly cyclic (Schwarzenberger et al. 1998, Brown et al. 2001, Carlstead & Brown 2005, Hermes et al. 2007). Conversely, the black rhinoceros has reproduced well in captivity, but experiences a mortality rate that is equal to or greater than the calving rate, due to disease susceptibility (Dennis et al. 2007, Schook et al. 2015). The greater one-horned rhinoceros displays low genetic diversity (Zschokke et al. 1998), and aggression between mating pairs requires assisted breeding programs. When Sumatran rhinoceroses were held in zoological institutes, research on reproductive physiology led to the hypothesis of induced ovulation, a unique reproductive feature (Roth et al. 2001). This feature, along with aggression between pairs, meant that the species had not reproduced successfully in captivity until the birth of a calf in 2001 from natural mating (Roth et al. 2004). Given the unique challenges faced by rhinoceroses, there is an urgent need for the development of species-specific ARTs (Pukazhenthi & Wildt 2004, Durrant 2009).

As a part of any ART program, an understanding of the species' basic reproductive biology is necessary. A review of longitudinal hormone monitoring and ultrasound data for four captive rhinoceros species is available (Roth 2006), as well as a review of ovarian control and manipulation in captive rhinoceros species (Roth et al. 2017). However, far less attention has been paid to the male aspect of rhinoceros reproduction. The methods and information generated for domestic animal reproductive management provide knowledge that can be adapted for captive rhinoceroses. This review is focussed on those ARTs that have been developed or attempted in captive rhinoceros species (Table 1).

METHODS

Data for this review were collected via a web-based search of the Google Scholar and PubMed data bases for published literature concerning each aspect of the following sections as they pertain to rhinoceroses, primarily, or to other species as indicated (e.g. domestic horse, domestic cattle). We utilised only publications written in English. When non-rhinoceros publications were referenced, material was connected by either taxonomic relation to rhinoceros (e.g. domestic horse and tapir) or by technique use (e.g. ovum pickup and *in vitro* culture systems in domestic horse and cattle). Unfortunately, relatively little information exists on successful rhinoceros assisted reproduction in general, and the species-specific differences provide additional levels of separation. A significant body of information describes assisted reproduction procedures that did not warrant a publication or were found to be unsuccessful. We feel it pertinent to include the published literature concerning assisted reproduction in rhinoceroses and those unpublished observations, in an attempt to form a more complete view of the potential tools available for rhinoceros. Therefore, we sought the experience of several scientists who have contributed significant work to their respective species in the last decade.

IN VIVO TECHNOLOGIES

Most of the ARTs developed for captive rhinoceroses involve control of the female reproductive cycle; however, a fundamental understanding of cyclicity is required for successful manipulation. Ultrasonography has been the most important tool used to characterise ovarian activity, visualise ovulatory follicle size, and confirm ovulation (Radcliffe et al. 2001, Roth et al. 2004, Stoops et al. 2004, Hermes et al. 2005). The utilisation of serial ultrasound has enhanced the understanding and management of reproduction in captive non-domestic species (Radcliffe et al. 2001, Pukazhenthi & Wildt 2004, Hermes et al. 2005). Additionally, ultrasonography coupled with longitudinal hormone monitoring is a valuable tool by which to determine response to cycle manipulation, regular cyclicity, pregnancy, and perturbations in cyclicity.

Ovulation induction/manipulation

Ovulation induction with exogenous hormones has been utilised primarily in white and Sumatran rhinoceroses (Table 1). Induction has been achieved in the greater one-horned rhinoceros (Stoops et al. 2016), but is apparently not necessary in preparation for artificial insemination (AI) as the species ovulates spontaneously, similar to the African species (*Ceratotherium simum* and *Diceros bicornis*).

In captivity, the white rhinoceros could benefit from ovulation induction, as more than 50% of females are characterised as acyclic or irregularly cyclic (Schwarzenberger et al. 1998, Patton et al. 1999, Brown et al. 2001, Hermes et al. 2005). However, these designations are often based on progesterone analysis without longitudinal ultrasography to determine if ovaries are inactive or if follicular growth occurs. Ovulation can be induced and pregnancy achieved in females previously determined to be anovulatory (Hildebrandt et al. 2007), indicating that the designation of acyclic may be misleading. Causes of anovulation are not entirely understood, but seem to afflict both aged and young females (Brown et al. 2001). More recently, infertility of captive-born females was linked to dietary phytoestrogens (Patisaul 2012, Tubbs et al. 2012, 2016). A significant negative relationship exists between captive-born female fertility and the oestrogenicity of diets ingested by their wild-born dams while

Table 1. Assisted Reproductive Technologies (ARTs) that have been used for each of the four species of rhinoceros that are held in captivity.

Species	*In vivo* ART	Pregnancies/ calves	Citations	*In vitro* ART	Citations
Black rhinoceros	Oestrus induction		M. Schook, personal communication	Gamete rescue, sperm	Roth et al. 2016, B. Durrant, unpublished data
	AI, frozen	0/0	M. Schook, personal communication	Gamete rescue, oocytes	Stoops et al. 2011a, B. Durrant, unpublished data
	Ovum pickup		Hermes et al. 2009b	IVM	Hermes et al. 2009b, Stoops et al. 2011a, B. Durrant, unpublished data
	Semen collection		Schaffer et al. 1998, Roth et al. 2005, B. Durrant, unpublished data	IVF (2 cell, 4 cell)	Hermes et al. 2009b, Stoops et al. 2011a, B. Durrant, unpublished data
				ICSI	Hermes et al. 2009b, B. Durrant, unpublished data
Southern white rhinoceros	Oestrus induction		Hermes et al. 2009b, Hildebrandt et al. 2007, Hermes et al. 2012, 2009a,	Gamete rescue, sperm	Roth et al. 2016, B. Durrant, unpublished data
	AI, fresh	2/2	Hildebrandt et al. 2007, M. Stoops, personal communication	Gamete rescue, oocytes	B. Durrant, unpublished data
	AI, frozen	1/1	Hildebrandt et al. 2007, Hermes et al. 2009a, 2012	IVM	B. Durrant, unpublished data
	Ovum pickup		Hermes et al. 2009b	IVF	B. Durrant, unpublished data
	Semen collection		Schaffer et al. 1998, Roth et al. 2005, B. Durrant, unpublished data, Walzer et al. 2000, Hermes et al. 2005	ICSI (2 pronuclei)	B. Durrant, unpublished data
Greater one-horned rhinoceros	Oestrus induction		Stoops et al. 2004, 2016	Gamete rescue, sperm	Roth et al. 2016, B. Durrant, unpublished data
	AI, frozen	6/4	Stoops et al. 2016	Gamete rescue, oocytes	B. Durrant, unpublished data
	Semen collection		Schaffer et al. 1998, Roth et al. 2005, Stoops et al. 2010, Stoops et al. 2016	IVM	B. Durrant, unpublished data
Sumatran rhinoceros	Oestrus induction		Roth et al. 2001	Gamete rescue, sperm	Roth et al. 2016, B. Durrant, unpublished data
	AI, fresh	0/0	T. Roth, personal communication	Gamete rescue, oocytes	B. Durrant, unpublished data, Stoops et al. 2011b
	Semen collection		O'Brien & Roth 2000	IVM	B. Durrant, unpublished data, Stoops et al. 2011b

AI, artificial insemination; IVM, *in vitro* maturation; IVF, *in vitro* fertilisation; ICSI, intracytoplasmic sperm injection. No pregnancies have resulted from *in vitro* ARTs thus far.

pregnant. According to European and North American studbooks, the current captive population of southern white rhinoceroses is not self-sustaining (Hermes et al. 2012, Tubbs et al. 2016).

As mentioned, the female Sumatran rhinoceros is reported to be an induced ovulator (Roth et al. 2001, 2004); however, the specific stimuli needed to induce ovulation remain unclear, and conclusions are drawn from a single female. Male proximity does not result in ovulation, but short intromission without complete copulation has been sufficient to induce ovulation (Roth et al. 2001). Thus, techniques such as manual stimulation or exogenous hormonal treatment to induce ovulation could serve as important tools in reproductive management of this species.

Hormones and protocols for ovulation induction

Ovulation induction has been developed and utilised extensively in equine reproductive management, and horses are often used as models for rhinoceroses. The ability to induce ovulation repeatedly with exogenous hormones in a mare with a preovulatory follicle greatly reduces the variability in follicular phase duration and has become a staple method in domestic mare management. Several agents have been used in rhinoceroses, either alone or together, including gonadotropin-releasing hormone (GnRH) analogues (Stoops et al. 2004, Hildebrandt et al. 2007, Hermes et al. 2009a, 2012, Stoops et al. 2016) and human chorionic gonadotropin (Schwarzenberger et al. 1998, Hermes et al. 2012). GnRH analogues deslorelin acetate (SucroMate and Ovuplant; Hildebrandt et al. 2007, Hermes et al. 2012), histrelin acetate (Vantas; M. Schook, personal communication), and gonadorelin diacetate tetrahydrate (Cystorelin; Stoops et al. 2004, 2016) successfully induced ovulation in white, black, greater one-horned and Sumatran rhinoceroses, respectively. According to the published reports, gonadorelin has been used in greater one-horned and Sumatran rhinoceroses in doses of 250 μg to 500 μg i.m. (intramuscular; Roth et al. 2004, Stoops et al. 2004, 2016). Deslorelin has been administered as implanted and injected forms (Hildebrandt et al. 2007, Hermes et al. 2012, P. Pennington, unpublished data). The deslorelin dose for white rhinoceroses ranges from 3.2 to 4.5 mg (Hermes et al. 2007, Hildebrandt et al. 2007, Hermes et al. 2012, P. Pennington, unpublished data).

Several induction protocols have resulted in ovulation, and may be tailored to each species dependent on its oestrous cycle lengths, ovulatory follicle sizes, and behavioural oestrus (Schwarzenberger et al. 1998, Hermes et al. 2005, Hildebrandt et al. 2007, Stoops et al. 2016). In a large-scale study, Hermes et al. (2012) investigated the efficacy of ovulation induction by human chorionic gonadotropin injection, deslorelin implant and deslorelin injection on day 9.5, following completion of 45 days of synthetic progestin treatment (chlormadinone acetate, 30 mg day^{-1}). The insertion of a deslorelin implant (4.2 mg, sub-cutaneous) was the most efficacious ovulation protocol tested; 93% of southern white rhinoceros females responded. Injection of human chorionic gonadotropin (10000 international units, i.m.) was successful in 67% of females, and the injectable form of deslorelin (3.2 mg, i.m.) induced ovulation in 61% of females (Hermes et al. 2012). Follicle diameter was 34.6 ± 2.3 mm (mean ± standard deviation) across all treatments, one day post-administration. However, the injection dose of deslorelin was lower than the implant dose, and only eight inductions were attempted with the injectable form, compared to 15 attempts with the implant. Further work is needed to identify specific criteria that will provide consistent results.

The Sumatran rhinoceros has benefitted from hormone treatment as a means to induce ovulation in the absence of a male. Ovulation without a male has been achieved in this species 34–40 hours after i.m. injection with 50–500 μg gonadorelin. Additionally, if two follicles were appropriately sized (20–21 mm), treatment resulted in double ovulation (Roth et al. 2017). Despite the development of successful ovulation induction protocols, no AI attempts have been successful in this species.

Relatively little work has been performed on black rhinoceros oestrous cycle manipulation. Serial ultrasound was employed to determine a pre-ovulatory follicle size of approximately 50 mm (Radcliffe et al. 2001) and to help determine optimal timing to pair individuals. Longitudinal serum and faecal steroid hormone analysis has aided reproductive management by identifying early pregnancy (Berkeley et al. 1997). Ovulation induction was achieved with GnRH agonist (Histrelin, biorelease) when follicles reached 40 mm diameter in two black rhinoceros females (Roth et al. 2017).

Failure to ovulate and the formation of hemorrhagic anovulatory follicles (HAFs), both spontaneously and following treatment with exogenous hormones, has been documented in all captive rhinoceros species: white, black, greater one-horned, and Sumatran (Radcliffe et al. 1997, 2001, Roth et al. 2001, Stoops et al. 2004). Similar to the occurrence in mares (Ginther et al. 2006, 2008), rhinoceros HAFs became partially luteinised and faecal progesterone becomes elevated. Despite ultrasonographic indication that ovulation failed in HAFs, progesterone profiles are similar to post-ovulatory luteal phases. Causes of HAFs remain unclear and they cannot be detected reliably without serial ultrasonography (Roth 2006).

One case of induced luteolysis is described using prostaglandin F2α to lyse a potential corpus luteum at a random

time-point. Two northern white rhinoceroses *Ceratotherium simum cottoni* received cloprostenol (Estrumate) in four injections over 48 hours (1500 μg total). Each female displayed oestrous behaviour within 48 hours of the second day of injections (B. Durrant, unpublished data). Cloprostenol has also been used in black rhinoceroses to treat persistent luteinised follicles, with two doses (500 μg, i.m.) 5 days apart (M. Schook, personal communication). M. Schook also identified a period of corpus luteum insensitivity to prostaglandin F2α of approximately 5 days post-ovulation in two black rhinoceros females. When cloprostenol administration was followed by oral altrenogest (ReguMate) for 14 days, two northern white rhinoceros females displayed oestrous behaviour 22–23 days following altrenogest withdrawal (B. Durrant, unpublished data). Occasional mild diarrhoea occurred transiently in the white rhinoceros females following cloprestenol treatment. Otherwise, no systemic side-effects were observed or noted following hormonal treatments. Although these studies have not been published, they provide evidence that prostaglandin administration could provide another method to manipulate the rhinoceros oestrous cycle.

In all instances of ovulation, induction-peripheral tools such as serial ultrasonography and hormone monitoring were instrumental. The similarities in anatomical features between rhinoceros species such as a torturous cervix, small uterine body, and extensively long uterine horns (Godfrey et al. 1991, Schaffer et al. 1994, 2001) suggests that once a hormone treatment protocol is established for an individual or species, the physical aspects of assisted reproduction are likely to be quite similar. As more institutes develop protocols for assisted reproduction, more data and repeatability are expected.

Semen collection

Less attention has been paid to male rhinoceros reproduction, although advances have been made, such as a redesign of probe head for electro-ejaculation and successful cryopreservation of sperm. Semen collection in the four captive rhinoceros species has been successful (Schaffer et al. 1990, 1998, Walzer et al. 2000, Hermes et al. 2005, Roth et al. 2005, Stoops et al. 2010, 2016; Table 2) and holds promise for use in ARTs such as AI or *in vitro* techniques. Furthermore, given the declining population numbers of each rhinoceros species, the ability to collect and efficiently cryopreserve genetic material from the current population is paramount. Stored sperm serves as a reservoir of genetic material which will certainly augment broader genetic management strategies as technologies and techniques progress.

As with many of the ARTs outlined, it is often most efficient to model the development of a technique after what has been achieved in domestic species. In addition

Table 2. Semen collection methods and parameters that have been used in the four captive rhinoceros species

Method	Species		Concentration	Volume (mL)	Citation
Electroejaculation	Black rhinoceros	*n* = 7	1–200 × 10^6	15–20	Schaffer et al. 1998 (*n* = 3) Roth et al. 2005 (*n* = 1) B. Durrant, unpublished data (*n* = 3)
	Southern white rhinoceros	*n* = 25	2.5–1682.5 × 10^6	3.7–204	Hermes et al. 2005 (*n* = 22) Roth et al. 2005 (*n* = 1) B. Durrant, unpublished data (*n* = 3)
	Greater one-horned rhinoceros	*n* = 6	0.15–37 × 10^9	0.5–337.5	Roth et al. 2005 (*n* = 4) Stoops et al. 2010 (*n* = 2)
Manual stimulation	Black rhinoceros	*n* = 1	15 × 10^6	62	Schaffer et al. 1998,
	Southern white rhinoceros	*n* = 3	10 × 10^6	0.7	Schaffer et al. 1998 (*n* = 1)
			n.d.	n.d.	Walzer et al. 2000 (*n* = 2)
	Greater one-horned rhinoceros	*n* = 1	13.9 × 10^9	8	Schaffer et al. 1998,
	Sumatran rhinoceros	*n* = 1	n.d.	n.d.	Roth et al. 2005,
Post-coital	Sumatran rhinoceros	*n* = 1	25 × 10^6	n.d.	O'Brien & Roth 2000,
Post mortem	Black rhinoceros	*n* = 18	300–21000 × 10^6	n.a.	Roth et al. 2016 (*n* = 14) B. Durrant, unpublished data (*n* = 4)
	Southern white rhinoceros	*n* = 8	90–8223 × 10^6	n.a.	Roth et al. 2016 (*n* = 3) B. Durrant, unpublished data (*n* = 5)
	Greater one-horned rhinoceros	*n* = 7	8.2–85 × 10^9	n.a.	Roth et al. 2016 (*n* = 5) B. Durrant, unpublished data (*n* = 2)
	Sumatran rhinoceros	*n* = 2	120–130 × 10^6	n.a.	Roth et al. 2016 (*n* = 1) B. Durrant, unpublished data (*n* = 1)

n, number of males; n.d., no data; n.a., not applicable.

to stimulation techniques modelled after domestic species, cryopreservation methods have also mimicked those used in domestic animals, particularly equines. Semen extenders and freezing media have been egg-yolk based, with cryoprotectants of either glycerol or dimethyl sulphoxide in concentrations ranging from 4 to 6% (Hermes et al. 2005, 2009a, Stoops et al. 2010, 2016). Freeze rates are slow and often follow a chilling period (4 °C) for 1–2 hours before freezing directionally or over liquid nitrogen vapour (Hermes et al. 2005, 2009a, Stoops et al. 2010, 2016). Despite variations in freezing protocol, cryopreserved rhinoceros sperm recovery has been acceptable for use in ARTs.

Obvious challenges exist when working with wildlife species that are less tractable than domestic species. Animal size and temperament must be considered, as well as training requirements if semen collection involves an unanaesthetised male. Semen has been collected from a southern white rhinoceros through training and design of a specialised chute, where manual penile massage led to successful ejaculation (Table 2; Walzer et al. 2000), but semen parameters were not included in this report. Similarly, a variety of manual stimulation techniques were tested in a greater one-horned rhino, including penile and rectal massage, artificial vagina, and combinations thereof (Schaffer et al. 1990). Although seminal fluid was recovered, sperm counts were low. Manual stimulation was performed in a Sumatran rhinoceros male, but sperm volume and quality were low (B. Durrant, unpublished data). Since these initial studies were done over a decade ago, little effort has been made to further develop manual semen collection techniques in male rhinoceroses, presumably due to the extensive training required. Other semen collection techniques, such as electro-ejaculation and post-coital semen recovery from the female, have been carried out in the Sumatran rhinoceros (Table 2). Post-coital semen collection provides a baseline to determine sperm quality in a naturally mating pair (O'Brien & Roth 2000). However, this method often yields low concentrations and/or volumes, reducing the efficacy of sperm cryopreservation and utilisation.

Currently, electro-ejaculation of anaesthetised males is the semen collection method of choice for non-domestic species as it obviates the need for training and allows close proximity to males that would otherwise be unsafe (Pukazhenthi & Wildt 2004). However, the full anaesthesia required for electro-ejaculation carries risks (Durrant 1990). Detailed descriptions of the procedure have been published, including the need to tailor the technique for individual animals and even each procedure (Durrant 1990, Roth et al. 2005, Stoops et al. 2010). Depth of anaesthesia and choice of anaesthetic contribute to the success of collection attempts in other non-domestic species such as Przewalski's horse *Equus ferus przewalskii* and Pierre David's deer *Elaphurus davidianus* (B. Pukazhenthi, personal communication). Good electrode contact with the rectal mucosa in rhinoceros proved challenging and led to alternative probe designs, including an inflatable version (Schaffer et al. 1990) to address the pneumorectum (air-filled rectum) often present in rhinoceros after faecal voiding. A new rectal probe was designed to accommodate male rhinoceros anatomy better (Roth et al. 2005). The redesigned probe was fabricated to maintain good contact with the rectal mucosa and fit just cranial to the anal sphincter. This probe was tested on seven rhinoceroses representing three species (greater one-horned, black and white) maintained at three institutes (Roth et al. 2005). The new probe design resulted in successful collection of semen in 13 out of 14 attempts. Repeatability of semen collection volume and quality remains variable though, as good-quality (>60% progressively motile) and poor-quality (<60% motile) semen was collected with equal frequency, even within individuals and collection procedures (Roth et al. 2005). More recently, electro-ejaculated sperm from 10 free-ranging southern white rhinoceros bulls was characterised using analytical techniques (computer-assisted sperm analysis) and had good total motility (82 ± 8%), although volumes varied considerably (24 ± 24 mL; Luther et al. 2016). In conjunction with electro-ejaculation, manual massage of the prostate and penis between stimulation series has proven to be an important aspect of the semen collection procedure and may result in recovery of larger semen volumes (Roth et al. 2005).

Reliable semen collection has enabled the identification of some key differences between rhinoceros species and their close equid relatives. Notably, rhinoceroses lack a gel fraction in their ejaculate, although this observation is based solely on electro-ejaculates (Roth et al. 2005). Rhinoceros ejaculate is more basic (pH = 8.5, Roth et al. 2005) than that of equid species *Equus caballus;* pH = 6.7–7.7, Crump & Crump 1994) and also differs from that of the other perissodactyl, Baird's tapir *Tapirus bairdii* (pH = 7.4, Pukazhenthi et al. 2011), although it should be noted that pH can vary with collection method and urine contamination. As technology and technique improve, enhanced reliability of electro-ejaculation will allow more institutes to collect and store sperm. However, training males for manual semen collection could obviate the need for anaesthesia and its associated risks, allowing safer and more frequent collection.

Artificial insemination

Despite attempts in all four captive-held species, successful AI has been achieved only in white and greater one-horned rhinoceroses (Table 1). However, within those two species

much information has been gained. Several calves have been produced by AI, although the overall success rate of the procedure remains low. Repeatability of AI in rhinoceroses has been hampered primarily by anatomical characteristics such as the long, tortuous cervix (Godfrey et al. 1991) that makes accessibility to the uterus, the preferred site of sperm deposition, difficult. Hildebrandt et al. (2007) have developed and patented a catheter that is able to traverse the cervix and enter the uterine body. Conversely, Innovative Zoological Solutions (Cincinnati, Ohio, USA) has produced a rounded, semi-flexible polyethylene tubing that can be threaded through the cervix until it reaches the uterine body, evidenced by ultrasonographic visualisation (Stoops et al. 2016).

The specific biology of each rhinoceros species must be considered when selecting an AI regime. Some considerations include ovulation induction, timing of insemination (before or after ovulation), use of fresh or frozen sperm, number of motile sperm, and restraint type (none, manual, sedation, or general anaesthesia). Other factors include evidence of behavioural oestrus, visualisation the dominant follicle through ultrasound, availability of exogenous hormones to induce ovulation, ability to collect fresh semen or receive frozen sperm, and ability to restrain the female. As AI is still in its infancy, there is no standardised approach yet. Adding to the delay in AI development in rhinoceroses is the time needed to obtain enough scientifically sound data. For example, nearly a decade of data was published in a single article (Stoops et al. 2016) describing AI in the greater one-horned rhinoceros. In it, the authors compared the efficacy of four different AI methods, all utilising cryopreserved sperm from five males. Of the total 34 inseminations conducted over nine years in four females, conceptions were diagnosed six times and four were carried to term, resulting in three live calves. Conceptions were diagnosed when females were inseminated 26 ± 11.8 hours before ovulation under either standing sedation or no sedation. The minimum number of sperm that resulted in conception was 500×10^6 motile sperm, which is similar to the recommended minimum dose for AI in domestic horses (Gahne et al. 1998).

The information on AI attempts in the southern white rhinoceros is less concise. It is likely that much more work has gone in to developing an AI protocol in this species, although little has been published except the successes. That said, major advances have been made in the use of both fresh and frozen sperm, resulting in successful pregnancies and the birth of live calves. In both published cases (Hildebrandt et al. 2007, Hermes et al. 2009a), the female was induced to ovulate using a GnRH analogue (deslorelin, Ovuplant, 4.2 mg). In one case (Hildebrandt et al. 2007), two insemination attempts were successful, the first with a nulliparous female and the second 30 days post-partum in the same female (Hildebrandt et al. 2007). Furthermore, this female had been diagnosed as anovulatory by faecal hormone monitoring for 33 months prior to the first successful AI. In both inseminations, fresh semen was used and ovulation was induced with deslorelin implants (4.2 mg) on the day of insemination. No attempt was made to remove the implants following the procedure. Both AIs were performed upon identification of a 32 mm follicle, and ovulation was confirmed by detection of serum progesterone rise four days after AI. The total number of sperm inseminated were 120×10^6 and 100×10^6 extended in egg-yolk based media to a volume of 88 and 58 mL for each AI, respectively, although the report did not include the number of motile sperm per insemination. Both AIs were done under full anaesthesia and all ultrasound sessions were conducted under standing sedation. This report was the first to document successful and repeated AI with fresh semen in conjunction with hormone monitoring, serial ultrasonography, and ovulation in the southern white rhinoceros. Members of the same research group took the next step, and were able to achieve a successful pregnancy using frozen sperm in the same female (Hermes et al. 2009a). Ovulation was induced on the day of AI with a deslorelin implant (4.2 mg) and was confirmed via ultrasound within 24 hours of administration. Two attempts were made, and the second was successful (Hermes et al. 2009a). Sperm count and quality was lower in the first attempt than in the second (135×10^6 motile sperm and 500×10^6 motile sperm, respectively). Given the possible minimum number of sperm required for conception, sperm quality may be a driver for the decision to use fresh or frozen sperm along with timing of AI and semen deposition site, if possible.

In addition to published studies, several unpublished AI attempts have been made in white, black, and Sumatran rhinoceroses. Although these attempts were unsuccessful, it is important to learn from the information gathered during the procedures. In two southern white rhinoceroses, several AI attempts have been made using approximately 250×10^6 motile sperm harvested post mortem and cryopreserved (M. Stoops, personal communication). Both females were induced to ovulate with 250 μg gonadorelin following a long-acting progesterone (~14 days; M. Stoops, personal communication). Several AI attempts were made in two black rhinoceros females that were previously determined to be either anovulatory or irregularly ovulating (M. Schook, personal communication). Ovulation was induced with histrelin when follicles reached 40 mm diameter. Frozen-thawed sperm quality was sub-optimal (35% motile post-thaw) and insemination doses were low at $150–200 \times 10^6$ motile sperm. It is possible that poor sperm quality, deposition site, or insemination timing in relation to ovulation prevented conception. Both females

subsequently became pregnant by natural mating (M. Schook, personal communication). In the Sumatran rhino, several AI attempts have been made in North America as well as in Borneo and Indonesia (T. Roth, personal communication). Because this species may be an induced ovulator, ovulation induction was utilised for all AI attempts. Gonadorelin (100 μg) was most often used to induce ovulation of preovulatory follicles (20–21 mm diameter, T. Roth, personal communication). Insemination times ranged from 33 hours before to five hours after ovulation, since mating naturally occurs 36–48 hours before ovulation in this species (O'Brien & Roth 2000). Doses ranged from 12 to 100 $\times$ 10^6 motile sperm and placement within the uterine body was documented via ultrasound (T. Roth, personal communication). Insemination doses were limited due to male availability for semen collection and poor sperm quality. It would be ideal to place sperm in the uterine horn ipsilateral to the pre-ovulatory follicle or corpus luteum; however, catheter design and the inability to manipulate the uterus rectally can be limiting. Despite these unsuccessful inseminations, continued dissemination of both positive and negative results is needed to develop rhinoceros AI procedures.

Major milestones have been reached in AI development, such as the successful use of frozen-thawed sperm, highlighting not only the appropriate timing of insemination but also useful freezing protocols resulting in competent sperm. Other details have also emerged, such as an apparent minimum dose of 500 million motile sperm for either fresh or frozen AI, although sperm morphology is also likely to influence outcome (Hildebrandt et al. 2007, Hermes et al. 2009a, Stoops et al. 2016). Nonetheless, the techniques developed thus far, and efforts currently underway at various institutes, will not only yield production of calves but also provide information that can be applied to other rhinoceros species. No researchers have investigated the viability of chilled sperm, but this approach is often used in equine AI and could obviate the need to collect semen and perform AI on the same day. It could also expand the availability of genetically valuable males to include those housed at other facilities, as sperm could be shipped overnight while avoiding the detrimental effects of cryopreservation.

Embryo transfer

To date, there are no records of embryo transfer attempts in rhinoceroses. However, as the reliability and efficacy of ART increase, the need and ability to perform embryo transfer will grow. One major challenge will again be the torturous cervix. At the presumed optimal time for embryo transfer (several days post-ovulation) the cervix is likely to be more difficult to traverse than during oestrus. Other considerations will be embryo placement in the uterus, and the number of days post-ovulation to transfer. Increased efforts to develop embryos *in vitro* are essential now, in preparation for future embryo transfer.

Ovum pickup

Ovum pickup (OPU) has become increasingly common in domestic horses and cattle over the last 10 years, as the efficacy of the technique has improved (Galli et al. 2001, 2014). OPU was developed to obtain oocytes repeatedly from a live donor, avoiding the need for slaughter and post-mortem oocyte recovery. This technique is an ideal predecessor to *in vitro* technologies such as *in vitro* maturation (IVM) and *in vitro* fertilisation (IVF) or intracytoplasmic sperm injection. Freshly collected oocytes are better quality than those collected post mortem and are thus preferable for optimising *in vitro* culture conditions, as these are currently lacking for rhinoceros species. Given the current threats faced by all rhinoceros species, every female is considered valuable. Furthermore, the number of females in captivity that fail to reproduce by natural mating or AI represent a significant portion of the population which do not contribute subsequent generations. Establishment of an OPU technique to rescue a rhinoceros's genetic material, even if she never carries a pregnancy successfully, would therefore greatly increase her potential for representation.

To date, only two reports have outlined the use of OPU in two rhinoceros species, white and black (Hildebrandt et al. 2007, Hermes et al. 2009b, Table 3). The first reported attempt did not yield any oocytes as the ovaries could not be accessed transvaginaly (Hermes et al. 2007). Therefore, a transrectal approach was developed that allowed successful access to ovaries. In the second report, five OPU attempts achieved a 22% oocyte recovery rate from two black rhinoceroses. A single attempt from one southern white rhinoceros achieved a 50% oocyte recovery rate (Hermes et al. 2009b). Oocyte recovery was probably facilitated by the ovarian super-stimulation protocol that preceded the aspiration. Super-stimulation could increase the potential number of collectable oocytes in rhinoceroses as it does in cattle (Galli et al. 2001, Chaubal et al. 2006, Galli et al. 2007, Sendag et al. 2008). The female rhinoceroses in the study (Hermes et al. 2009b) were determined to be anoestrous and infertile due to reproductive tract pathologies. Four animals were stimulated nine times with deslorelin acetate implants (4.2 mg) or injections (4.5 mg) on days 0, 2 and 4 (day 0 = first day of deslorelin administration). Ultrasound confirmed increased follicle number and growth on days 6 and 7 after the initial deslorelin administration and follicles were aspirated at this time. The transrectal approach described

in this study (Hermes et al. 2009b) was the first of its kind in rhinoceros, but has been used by others with success (M. del la Rey, personal communication). There are obvious challenges associated with OPU in the rhinoceros, including accessing the ovaries. In the domestic horse the OPU procedure is transvaginal. The ovary is manually manipulated via the rectum to bring it adjacent the vaginal wall, reducing the distance the aspiration needle travels through the peritoneal cavity. The transvaginal approach also reduces the potential for peritoneal infection from faecal contamination that is possible during transrectal OPU. However, since the ovaries, especially in larger species such as the white and greater one-horned rhinoceros, are more distant from the vagina and are not readily palpable through the rectum, transvaginal OPU may not be feasible in adult females (Hermes et al. 2009b), and attempts in younger females have not been made. Measures to prevent peritonitis subsequent to transrectal OPU in rhinoceroses were taken by removing faeces from the rectum and placing sterile towels to prevent faecal movement into the field during the procedure. The authors report this OPU technique was achieved more easily in the two black rhinoceroses as both ovaries could be more readily accessed than those of the southern white rhinoceroses, where only the right ovary could be reached (Hermes et al. 2009b). The smaller physical size of the black rhinoceros species is likely to have facilitated the success. However, innovative approaches will need to be explored if the technique is to benefit the larger species such as the white and greater one horned rhinoceros.

Reliable oocyte collection is currently limited to post-mortem recovery, which is a slow and inefficient method by which to develop *in vitro* culture systems. Additionally, females that die or are euthanised due to ill health or old age are not ideal candidates for culture, as their oocytes tend to be lower quality than those collected *in vivo*. On the other hand, the collection of oocytes following the unexpected death of a healthy female may be complicated by a lack of trained personnel or lengthy time delays between death and oocyte collection. Despite the inherent risks of OPU, its successful application would provide a source of oocytes by which to develop *in vitro* culture techniques using high-quality gametes.

IN VITRO TECHNOLOGIES

Development of *in vitro* technologies has lagged behind *in vivo* advancements due to low numbers of gametes available for experimentation, although multiple efforts and reports have been made. However, as *in vivo* techniques such as OPU become more successful, more oocytes will become available to develop *in vitro* culture. Until then, post-mortem collections are a primary focus and provide opportunities to develop culture conditions (for oocytes) or to cryopreserve sperm for future use. Again, the domestic horse is a model, but species-specific differences are likely to exist. The *in vitro* technologies that have been utilised in the rhinoceros have contributed to our understanding, and provide a framework on which to build.

Post-mortem gamete rescue

A large number of individuals of each rhinoceros species maintained in zoological institutes remain unrepresented in the next generation due to the lack of suitable breeding opportunities. However, the potential genetic contributions of these individuals can be preserved if appropriate steps are taken. Thus far, the majority of oocyte rescues have occurred post mortem (Table 3). Sperm rescue is most commonly accompanied by cryopreservation as it is often opportunistic. Planned euthanasia can be timed to provide time to prepare for gamete collection and immediate utilisation. Even so, the full potential of gamete rescue can be realised when gametes are cryopreserved for future utilisation when AI or *in vitro* techniques become more efficacious.

A recent retrospective study reviewed 16 years of data to identify factors associated with successful collection of sperm post mortem in four species of rhinoceros (black, $n = 14$; greater one-horned, $n = 5$; white, $n = 3$; Sumatran, $n = 1$; Roth et al. 2016, Table 2). The authors established protocols in which they highlighted the need to remove reproductive tissues (testes, epididymides, and vas deferens) within two hours of the animal's death, followed by immediate shipment to the authors. Encouragingly, sperm was viable for up to 51 hours (>30% motile, progressive motility score of ≥2.0; scale 1–5) if testes were collected within two hours of death, regardless of age, season, cause of death (natural or euthanised) or pathology. Although season of collection did not impact sperm quality, non-viable samples were received during the coldest and hottest months. Although non-viable samples could be due to the individual's quality, temperature control during shipment in extreme climate conditions should be a priority.

Similarly, ova can be extracted from ovaries and either frozen or cultured *in vitro* (Table 3). Although the concept is the same as sperm extraction from testes, obtaining and preserving oocytes from rhinoceros species has proven more challenging. Preservation of equine oocytes by vitrification has been successful in producing a live foal, although blastocyst rates were severely reduced (Ortiz-Escribano et al. 2017). Unlike sperm collection, in which gametes are mature and capable of fertilisation, oocytes vary in states of maturity; however, post-mortem oocyte collection is the only reliable method, as OPU remains a technique in development. Additionally, ovaries and oocytes

Table 3. Oocyte collection, *in vitro* maturation and embryo production by rhinoceros species.

Species	Individual	Age (years)	Recovery method	Number of oocytes	IVM/frozen	IVF/ICSI	Embryo	Citation
Black rhinoceros	SB 239	15	PM	0				B. Durrant, unpublished data
	SB 192	25	PM	14	14 (frozen)			B. Durrant, unpublished data
	SB 233	29	PM	0				B. Durrant, unpublished data
	SB 330	17	PM	0				B. Durrant, unpublished data
	SB 684	?	PM	3	3(IVM)	0	0	B. Durrant, unpublished data
	SB 717	0	PM	21	21(IVM)	0	0	Stoops et al. 2011a
	SB 863*	5	PM	21	21 (IVM)	10 (IVF*)	0	Stoops et al. 2011a
	SB 489	14	PM	18	18 (IVM)	13 (IVF)	0	Stoops et al. 2011a
	SB 351**	19	PM	20	6 (IVM)	6 (IVF)	1 (2-cell)	Stoops et al. 2011a
	SB 466	22	PM	4	4 (IVM)	3 (IVF)	0	Stoops et al. 2011a
	SB 235	27	PM	11	11 (IVM)	7 (IVF)	0	Stoops et al. 2011a
	Female 1	?	OPU	15 (3 attempts)	12 (IVM)	5 (IVF) 1 (ICSI)	1 (4-cell; ICSI)	Hermes et al. 2009b
	Female 2	?	OPU	11 (2 attempts)	10 (IVM)	1 (IVF)	0	Hermes et al. 2009b
Total	13			138	110	46	2	
Southern white rhinoceros	SB 150	33	PM	0				B. Durrant, unpublished data
	SB 824	2	PM	0				B. Durrant, unpublished data
	SB 471	30	PM	0				B. Durrant, unpublished data
	SB 470	30	PM	4	0			B. Durrant, unpublished data
	SB 822	18	PM	0				B. Durrant, unpublished data
	SB 1037	2	PM	0				B. Durrant, unpublished data
	SB 374	40	PM	2	2 (frozen)			B. Durrant, unpublished data
	SB 376	34	PM	1				B. Durrant, unpublished data
	SB 1051	8	PM	10	6 (IVM)	2 (ICSI)	1 (1-cell)	B. Durrant, unpublished data
	SB 157	45	PM	0				B. Durrant, unpublished data
	SB 188	41	PM	0				B. Durrant, unpublished data
	SB 154	47	PM	0				B. Durrant, unpublished data
	SB 277	45	PM	0				B. Durrant, unpublished data
	No #	0	PM	0				B. Durrant, unpublished data
	SB 374	41	PM	0				B. Durrant, unpublished data
	SB 1524	?	PM	23	23 (IVM)			B. Durrant, unpublished data
	SB 1711	9	PM	49	47 (IVM)		0	B. Durrant, unpublished data
	SB 2190	?	PM	13	13 (IVM)			B. Durrant, unpublished data
	SB 381	47	PM	0				B. Durrant, unpublished data
	SB 374	32	OVX	0				B. Durrant, unpublished data
	Female 1	?	OPU	5	0		0	Hermes et al. 2009b
Total	21			107	91	2	1	
Greater one-horned rhinoceros	SB 89	22	PM	0				B. Durrant, unpublished data
	SB 247	0	PM	0				B. Durrant, unpublished data
	SB 413	5	PM	0				B. Durrant, unpublished data
	SB 356	6	PM	0				B. Durrant, unpublished data
Total	4			0				
Sumatran rhinoceros	SB 19	6	PM	5	5 (frozen)			B. Durrant, unpublished data
	SB 33	8	PM	1	1 (frozen)			B. Durrant, unpublished data
	SB 29	21	PM	30	30 (IVM)	0	0	Stoops et al. 2011b
Total	3			36	30			

IVM, *in vitro* maturation; IVM/frozen, oocytes were either put in to maturation or slow frozen as indicated; IVF, *in vitro* fertilisation; ICSI, intracytoplasmic sperm injection; SB, stud-book number; p.n., pronuclei; PM, post mortem; OPU, ovum pickup; OVX, ovariectomy.

*Heterologous IVF using greater one-horned rhinoceros sperm.

**12 oocytes not matured, but directly in to IVF.

must be kept at a different temperature during shipping than sperm. While sperm maintain greater viability when testes are shipped at 4 °C (Roth et al. 2016), shipping ovaries at room temperature (approximately 22 °C) tended to result in improved survival both in rhinoceroses (Stoops et al. 2011a) and horses (Hatzel & Carnevale 2016). Oocytes were liberated from the ovaries by individually aspirating follicles or slicing tissue into media (Stoops et al. 2011a, b, Hatzel & Carnevale 2016). Ova have been cultured (Hermes et al. 2009b, Stoops et al. 2011a, b) or slow frozen (B. Durrant, unpublished data). Briefly, oocytes are chilled in phosphate buffered saline for 1 hour at 4 °C, then dimethyl sulfoxide (1.5 M) in phosphate buffered saline is added and vials are frozen (0.3 °C per minute) by control rate freezer (Demirci et al. 2001). However, cryopreservation technology for oocytes lags behind that for sperm and embryos for nearly all species (Woods et al. 2004), and vitrification of oocytes would probably result in higher survival post-thaw (Smith et al. 2010). Nevertheless, as research and technology continue to advance it is paramount to collect gametes from rhinoceroses post mortem to preserve genetic material, especially if the individuals are unrepresented in the population.

Maximised collection of oocytes and sperm post mortem requires a clear and multi-institute collaborative decision-making process. Ideally, ART-capable institutes would have prior knowledge of planned euthanasia and could thus prepare for receipt of gonads. For institutes lacking gamete processing capability, shipping tissue to the nearest capable facility will significantly reduce the time needed to receive and process material. As conservation efforts increase *in situ*, similar collaborations are needed internationally.

In vitro maturation, *in vitro* fertilisation, and intracytoplasmic sperm injection

The first step in the development of a successful *in vitro* embryo production system is *in vitro* maturation (IVM). Although IVM has been attempted in all four species of managed rhinoceros (Table 3), no advanced cleavage has been achieved following *in vitro* fertilisation (IVF). The equine industry has achieved remarkable success with blastocyst production and birth of healthy foals, mostly through the development of routine OPU as a source of immature oocytes for culture. However, since this technology is not yet fully developed in the rhinoceros, most oocytes are derived from post-mortem tissues. Embryos have been developed (n = 3) from both IVF and intracytoplasmic sperm injection, however, none advanced beyond the four-cell stage, and the possibility of parthenogenesis was not excluded (Hermes et al. 2009b, Stoops et al. 2011a, b, B. Durrant, unpublished data). Two of the three fertilised oocytes were from black rhinoceroses (Hermes et al. 2009b, Stoops et al. 2011a), while the third was from a southern white rhinoceros (B. Durrant, unpublished data; Table 3). Several factors are likely to contribute to the low rate of success of *in vitro* embryo development, including ova degradation from post-mortem tissue, low ova recovery rate from OPU, and an inadequate culture system. Thus far, nine southern white rhinoceroses have contributed 126 oocytes, ten black rhinoceroses have contributed 138 oocytes, and three Sumatran rhinoceroses have contributed 36 oocytes (Table 3). Ovaries of four greater one-horned rhinoceroses have been processed, but no oocytes were recovered (B. Durrant, unpublished data; Table 3). The method of recovery varied across all species from post mortem (n = 39; 20 yielding ova and 19 yielding no ova) to OPU (n = 3) and ovariectomy (n = 1; Table 3). The age of oocyte donors ranged from 0 to 40 years (and six females were of unknown age), with mean age 20.8 ± 15.4 years. More oocytes were collected from females 5–27 years old, although 21 oocytes were collected from a stillborn (Stoops et al. 2011a) and 19 were collected from one ovary of a two-year-old (B. Durrant, unpublished data). Of those that successfully matured in culture, most were subjected to IVF and two were fertilised by intracytoplasmic sperm injection (Table 3).

Development of an *in vitro* culture system has been problematic, and oocyte maturation is the initial barrier. Maturation times given in the published studies range widely, but oocytes that achieved even initial maturation to metaphase II were cultured for 32–36 hours (Hermes et al. 2009b, Stoops et al. 2011a, b, B. Durrant, unpublished data). Although base maturation medium was most commonly Tissue Culture Medium 199 with Earl's Salts, supplements varied from porcine to ovine luteinising hormone and follicle-stimulating hormone, oestradiol, and insulin-like growth factor 1 (Stoops et al. 2011a, b), to southern white rhinoceros follicular fluid (B. Durrant, unpublished data) and oestrous southern white rhinoceros serum (Hermes et al. 2009b). Rhinoceros IVM culture conditions are often modelled after equine protocols, but the ability to set up and maintain a working culture system can be challenging and costly, and is hindered by infrequent and unpredictable intervals of oocyte availability. Other model systems, such as bovine, have not been sufficiently explored. Additionally, few zoological institutes are equipped to support a culture system. Open communication and collaboration will be key to optimising *in vitro* culture conditions, as each investigator has access to limited numbers of good-quality oocytes.

SUMMARY

The use of ARTs *in vivo* has been accelerated by the amalgamation of serial ultrasonography, hormone

monitoring, ovulation induction, and appropriately timed AI in rhinoceroses. The ability to collect semen reliably from genetically valuable males is essential for the optimisation of existing ART protocols such as AI. Additionally, emerging technologies such as OPU will provide the oocytes fundamental to the development of successful *in vitro* culture systems and protocols. Although success in these areas has been arduous, the sustained dedication to these species and innovative adaptation of domestic livestock protocols have provided avenues of progress.

Parallel to *in vivo* development of ARTs, *in vitro* technologies have advanced in several critical areas. Despite the disadvantage of obtaining the majority of rhinoceros oocytes post mortem, culture media development and fertilisation by IVF or intracytoplasmic sperm injection have led to early embryo development, although the blastocyst stage has not been achieved and parthenogenesis has not been ruled out. Furthermore, as ARTs improve, cryobanking of gametes and embryos acquired *in vitro* and *in vivo* will provide important reservoirs of genetic diversity for future ARTs. Opportunistic gamete collection and cryopreservation will ensure the availability of genetic material for techniques not yet available or imagined.

Future directions should focus on procedure and protocol development, and will result from well-designed and repeated experimentation. Furthermore, techniques such as embryo transfer, that have yet to be attempted in any rhinoceros species, will be necessary for the birth of a live calf from embryos produced *in vitro*. Researchers should not only seek to advance the ARTs currently employed, but also innovative options such as stem cell manipulation to produce gametes (Saragusty et al. 2016). Progress has already been made to this end by reprogramming previously collected and frozen fibroblast cell lines of the northern white rhinoceros, successfully creating induced pluripotent stem cells from this functionally extinct species (Korody et al. 2017). These are the first steps in an ambitious project to generate northern white rhinoceros gametes from induced pluripotent stem cells, followed by *in vitro* production of embryos for transfer to synchronised southern white rhinoceros surrogates. Other routes of embryo production, such as cloning, may also be an option. If the *in vivo* techniques discussed here are insufficient to prevent continued population decline, reliance on more *in vitro* techniques will be the only remaining option.

Filling the gaps in our understanding of rhinoceros reproductive physiology will only be achieved through the combination of many, if not all, of the techniques reviewed here along with open collaboration between zoological institutes, universities, research organisations, and governments. Development of techniques and protocols in tandem with scientific research will not only secure rhinoceros populations, but also provide a blueprint for conservation actions for other endangered species.

Significant progress has been achieved in the development of ARTs in all species of rhinoceros in managed collections. However, population sustainability and genetic management will increasingly rely on ARTs. Thus, now is the time to utilise captive populations to identify and create innovative approaches to species conservation.

ACKNOWLEDGEMENTS

We thank, for personal communications, for extensive contributions, and for open and willing collaboration, Dr Terri Roth, Dr Monica Stoops, Dr Mandi Schook, Dr Budhan Pukazhenthi, and Dr Morné de la Rey.

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