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 unicornis* (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 main- tained 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 ultrasonography 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 |
|                      | Al, 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, 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 |
|                      | Al, fresh    | 2/2                | Hildebrandt et al. 2007, M. Stoops, personal communication | Gamete rescue, oocytes | B. Durrant, unpublished data |
|                      | Al, frozen   | 1/1                | Hermes et al. 2009b, 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 |
|                      | Al, 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 |
|                      | Al, fresh    | 0/0                | T. Roth, personal communication | Gamete rescue, oocytes | B. Durrant, unpublished data |
|                      | 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 cloprostenol 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

| Method              | Species                  | Concentration | Volume (mL) | Citation              |
|---------------------|--------------------------|---------------|-------------|-----------------------|
| Electroejaculation  | Black rhinoceros         | n = 7         | 1–200 × 10⁶ | 15–20                 | Schaffer et al. 1998 (n = 3) |
|                     |                          |               |             | Roth et al. 2005 (n = 1) |
|                     |                          |               |             | B. Durrant, unpublished data (n = 3) |
| Manual stimulation  | Southern white rhinoceros | n = 25        | 2.5–1682.5 × 10⁶ | 3.7–204               | Hermes et al. 2005 (n = 22) |
|                     |                          |               |             | Roth et al. 2005 (n = 1) |
|                     |                          |               |             | B. Durrant, unpublished data (n = 3) |
|                     | Greater one-horned        | n = 6         | 0.15–37 × 10⁹ | 0.5–337.5             | Roth et al. 2005 (n = 4) |
|                     | rhinoceros                |               |             | Stoops et al. 2010 (n = 2) |
|                     | Black rhinoceros          | n = 1         | 15 × 10⁶    | 62                    | Schaffer et al. 1998, |
|                     |                          |               |             |                      | Schaffer et al. 1998 (n = 1) |
|                     | Southern white rhinoceros | n = 3         | 10 × 10⁶    | 0.7                   | Walzer et al. 2000 (n = 2) |
|                     |                          |               |             |                      | Schaffer et al. 1998, |
|                     | Greater one-horned        | n = 1         | 13.9 × 10⁹  | 8                     |                          |
|                     | rhinoceros                |               |             | Schaffer et al. 1998, |
|                     | Sumatran rhinoceros       | n = 1         | n.d.        | n.d.                  |                          |
|                     |                          |               |             | Roth et al. 2005,     |
|                     | Sumatran rhinoceros       | n = 1         | 25 × 10⁶    | n.d.                  | O’Brien & Roth 2000, |
|                     |                          |               |             | Roth et al. 2016 (n = 14) |
|                     | Black rhinoceros          | n = 25        | 300–21000 × 10⁶ | n.a.                 | B. Durrant, unpublished data (n = 4) |
|                     |                          |               |             | Roth et al. 2016 (n = 3) |
|                     | Southern white rhinoceros | n = 8         | 90–8223 × 10⁶ | n.a.                  | B. Durrant, unpublished data (n = 5) |
|                     |                          |               |             | Roth et al. 2016 (n = 5) |
|                     | Greater one-horned rhino- | n = 7         | 8.2–85 × 10⁹ | n.a.                  | B. Durrant, unpublished data (n = 2) |
|                     | ceros                    |               |             | Roth et al. 2016 (n = 1) |
|                     | Sumatran rhinoceros       | n = 2         | 120–130 × 10⁶ | n.a.                  | 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 rhinoceroses 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 rhinoceroses 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 uterus, 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 × 10⁶ 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 × 10⁶ and 100 × 10⁶ 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 × 10⁶ motile sperm and 500 × 10⁶ 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 × 10⁶ 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 × 10⁶ motile sperm. It is possible that poor sperm quality, deposition site, or insemination timing in relation to ovulation prevented conception. Both females
Assisted reproduction for rhinoceros conservation

P. M. Pennington and B. S. Durrant

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 × 10⁶ 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 transvaginally (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 (frozen)       | 14         |          |        | 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      | B. Durrant, unpublished data    |
|                           | SB 863*    | 5           | PM              | 21 (IVM)          | 10 (IVF*)  | 0        |        | Stoops et al. 2011a            |
|                           | SB 489     | 14          | PM              | 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    | 7           | PM              | 13                | 13 (IVM)   |          |        | B. Durrant, unpublished data    |
|                           | SB 381     | 47          | PM              | 0                 |            |          |        | B. Durrant, unpublished data    |
|                           | SB 374     | 32          | O VX             | 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 tests 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 (Demirici 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 rhinoceroses (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 rhinoceroses, 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 ovariotomy (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). Rhinoceroses 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 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 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 Pukazhenth, and Dr Morné de la Rey.

REFERENCES

Berkeley EV, Kirkpatrick JF, Schaffer NE, Bryant WM, Threlfall WR (1997) Serum and fecal steroid analysis of ovulation, pregnancy, and parturition in the black rhinoceros (Diceros bicornis). Zoo Biology 16: 121–132.
Brown JL, Bellem AC, Fouraker M, Wildt DE, Roth TL (2001) Comparative analysis of gonadal and adrenal activity in the black and white rhinoceros in North America by noninvasive endocrine monitoring. Zoo Biology 20: 463–486.
Carlstead K, Brown JL (2005) Relationships between patterns of follicle corticoid excretion and behavior, reproduction, and environmental factors in captive black (Diceros bicornis) and white (Ceratotherium simum) rhinoceros. Zoo Biology 24: 215–232.
Chaubal SA, Molina JA, Ohlrichs CL, Ferre LB, Faber DC, Bols PEJ, Riesen JW, Tian X, Yang X (2006) Comparison of different transvaginal ovum pick-up protocols to optimise oocyte retrieval and embryo production over a 10-week period in cows. Theriogenology 65: 1631–1648.
Crump JP, Crump JW (1994) Manual semen collection from a Grey’s zebra stallion (Equus grevyi), onset of sperm production, semen characteristics, and cryopreservation of semen, with a comparison to the sperm production from a Grant’s zebra stallion (Equus burchelli boehmi). Theriogenology 41: 1011–1021.
Demirci B, Lornage J, Salle B, Frappart L, Franck M, Guerin JF (2001) Follicular viability and morphology of sheep ovaries after exposure to cryoprotectant and cryopreservation with different freezing protocols. Fertility and Sterility 75: 754–762.
Dennis PM, Funk JA, Rajala-Schultz PJ, Blumer ES, Miller RE, Wittum TE, Saville WJA, Funk J, Schultz PIR (2007) A review of some of the health issues of captive black rhinoceroses (Diceros bicornis). Journal of Zoo and Wildlife Medicine 38: 509–517.
Durrant BS (1990) Semen collection, evaluation, and cryopreservation in exotic animal species: maximizing reproductive potential. *Institute for Laboratory Animal Research Journal* 32: 2–10.

Durrant BS (2009) The importance and potential of artificial insemination in CANDES (companion animals, non-domestic, endangered species). *Theriogenology* 71: 113–122.

Fouraker M, Wagner T (1996) AZA Rhinoceros Husbandry Resource Manual. Fort Worth Zoological Park, Fort Worth, Texas, USA.

Gahne S, Gönheim A, Malmgren L (1998) Effect of insemination dose on pregnancy rate in mares. *Theriogenology*, 49: 1071–1074.

Galli C, Crotti G, Notari C, Turini P, Duchi R, Lazzari G (2001) Embryo production by ovum pick up from live donors. *Theriogenology* 55: 1341–1357.

Gallì C, Colleoni S, Duchi R, Lagutina I, Lazzari G (2007) Developmental competence of equine oocytes and embryos obtained by *in vitro* procedures ranging from *in vitro* maturation and ICSI to embryo culture, cryopreservation and somatic cell nuclear transfer. *Animal Reproduction Science* 98: 39–55.

Gallì C, Duchi R, Colleoni S, Lagutina I, Lazzari G (2014) Ovum pick up, intracytoplasmic sperm injection and somatic cell nuclear transfer in cattle, buffalo and horses: from the research laboratory to clinical practice. *Theriogenology* 81: 138–151.

Ginther OJ, Gastal EL, Gastal MO, Beg MA (2006) Conversion of a viable preovulatory follicle into a hemorrhagic anovulatory follicle in mares. *Animal Reproduction* 3: 29–40.

Ginther OJ, Gastal MO, Gastal EL, Jacob JC, Beg MA (2008) Induction of haemorrhagic anovulatory follicles in mares. *Reproduction, Fertility and Development* 20: 947–954.

Godfrey RW, Pope CE, Dresser BL, Olsen JH (1991) Gross anatomy of the reproductive tract of female black (*Diceros bicornis michaeli*) and white rhinoceros (*Ceratotherium simum simum*). *Zoo Biology* 10: 165–175.

Hatzel JN, Carnevale EM (2016) How to collect and ship gametes. In *American Association of Equine Practitioners Proceedings*, 181–186.

Hermes R, Hildebrandt TB, Blottner S, Walzer C, Silinski S, Patton ML, Wibbelt G, Schwarzenberger F, Göritz F (2005) Reproductive soundness of captive southern and northern white rhinoceroses (*Ceratotherium simum simum, C. s. cottoni*): evaluation of male genital tract morphology and semen quality before and after cryopreservation. *Theriogenology* 63: 219–238.

Hermes R, Goitz F, Streich W, Hildebrandt T (2007) Assisted reproduction in female rhinoceros and elephants – current status and future perspective. *Reproduction in Domestic Animals* 42: 33–44.

Hermes R, Göritz F, Saragusty J, Sös E, Molnar V, Reid CE, Schwarzenberger F, Hildebrandt TB (2009a) First successful artificial insemination with frozen-thawed semen in rhinoceroses. *Theriogenology* 71: 393–399.

Hermes R, Göritz F, Portas TJ, Bryant BR, Kelly JM, Macellani LJ et al. (2009b) Ovarian superstimulation, transrectal ultrasound-guided oocyte recovery, and IVF in rhinoceroses. *Theriogenology* 72: 959–968.

Hermes R, Hildebrandt TB, Walzer C, Göritz F, Gray C, Niemüller C, Schwarzenberger F (2012) Estrus induction in white rhinoceroses (*Ceratotherium simum*). *Theriogenology* 78: 1217–1223.

Hildebrandt TB, Hermes R, Walzer C, Sös E, Molnar V, Mezösi L et al. (2007) Artificial insemination in the anoestrous and the postpartum white rhinoceroses using GnRH analogue to induce ovulation. *Theriogenology* 67: 1473–1484.

IUCN. (2017) The IUCN Red List of Threatened Species. Accessed 20 November 2017. www.iucnredlist.org.

Korody ML, Pivaroff C, Nguyen TD, Peterson SE, Ryder OA, Loring JF (2017) Four new induced pluripotent stem cell lines produced from northern white rhinoceroses with non-integrating reprogramming factors. bioRxiv: https://doi.org/10.1101/202499

Luther I, Maree L, Kotze A (2016) Semen characteristics of free-ranging African elephants (*Loxodonta africana*) and Southern white rhinoceroses (*Ceratotherium simum simum*) using computer-aided sperm analysis, electron microscopy and genomics as diagnostic tools. PhD thesis, University of the Western Cape, Bellville, Republic of South Africa.

O’Brien JK, Roth TL (2000) Post-coital sperm recovery and cryopreservation in the Sumatran rhinoceros (*Diceros sumatrensis*) and application to gamete rescue in the African black rhinoceros (*Diceros bicornis*). *Journal of Reproduction and Fertility* 118: 263–271.

Ortiz-Escribano N, Bogado Pascottini O, Woelders H, Vandenbergh L, De Schauwerre C, Govaere J et al. (2017) An improved vitrification protocol for equine immature oocytes, resulting in a first live foal. *Equine Veterinary Journal* 50: 391–397.

Patsaul H (2012) Infertility in the southern white rhino: is diet the source of the problem? *Endocrinology* 153: 1568–1571.

Patton ML, Swaisgood RR, Czekala NM, White AM, Fetter GA, Montagne JP, Rieches RG, Lance VA (1999) Reproductive cycle length and pregnancy in the southern white rhinoceroses (*Ceratotherium simum simum*) as determined by fecal pregnane analysis and observations of mating behavior. *Zoo Biology* 18: 111–127.

Pukazhenthi BS, Wildt DE (2004) Which reproductive technologies are most relevant to studying, managing and conserving wildlife? *Reproduction, Fertility, and Development* 16: 33–46.
Pukazhenthi BS, Togna GD, Padilla L, Smith D, Sanchez C, Pelican K, Sanjur OI (2011) Ejaculate traits and sperm cryopreservation in the Endangered Baird’s tapir (Tapirus bairdii). Journal of Andrology 32: 260–270.

Radcliffe RW, Czekala NM, Osofsky SA (1997) Combined serial ultrasonography and fecal progesterone analysis for reproductive evaluation of the female white rhinoceros (Ceratotherium simum simum): preliminary results. Zoo Biology 16: 445–456.

Radcliffe RW, Eyres AI, Patton ML, Czekala NM, Emslie RH (2001) Ultrasonographic characterization of ovarian events and fetal gestational parameters in two southern black rhinoceroses (Diceros bicornis) and correlation to fecal progesterone. Theriogenology 55: 1033–1049.

Roth TL (2006) A review of the reproductive physiology of rhinoceroses species in captivity. International Zoo Yearbook 40: 130–143.

Roth TL, O’Brien JK, McRae MA, Bellem AC, Romo SJ, Kroll JL, Brown JL (2001) Ultrasound and endocrine evaluation of the ovarian cycle and early pregnancy in the Sumatran rhinoceros, Dicerorhinus sumatrensis. Reproduction 121: 139–149.

Roth TL, Bateman HL, Kroll JL, Steinetz BG, Reinhart PR (2004) Endocrine and ultrasonographic characterization of a successful pregnancy in a Sumatran rhinoceros (Dicerorhinus sumatrensis) supplemented with a synthetic progesterone. Zoo Biology 23: 219–238.

Roth TL, Stoops MA, Atkinson MW, Blumer ES, Campbell MK, Cameron KN, Citino SB, Maas AK (2005) Semen collection in rhinoceroses (Rhinoceros unicornis, Dicerorhinus bicornis, Ceratotherium simum) by electroejaculation with a uniquely designed probe. Journal of Zoo and Wildlife Medicine 36: 617–627.

Roth TL, Stoops MA, Robeck TR, O’Brien JK (2016) Factors impacting the success of post-mortem sperm rescue in the rhinoceroses. Animal Reproduction Science 167: 22–30.

Roth TL, Schook MW, Stoops MA (2017) Monitoring and controlling ovarian function in the rhinoceros. Theriogenology 109: 48–57.

Saragusty J, Diecke S, Drukker M, Durrant BS, Friedrich Ben-Nun I, Galli C et al. (2016) Rewinding the process of mammalian extinction. Zoo Biology 35: 280–292.

Schaffer NE, Bechler B, Jeyendran RS, Balke B (1990) Methods of semen collection in an ambulatory greater one-horned rhinoceros (Rhinoceros unicornis). Zoo Biology 9: 211–221.

Schaffer NE, Zainal-Zahari Z, Suri DVMM, Jainudeen MR, Jeyendran RS (1994) Ultrasonography of the reproductive anatomy in the Sumatran rhinoceros (Dicerorhinus sumatrensis). Journal of Zoo and Wildlife Medicine 25: 337–348.

Schaffer N, Bryant W, Agnew D, Meehan T, Bechler B (1998) Ultrasonographic monitoring of artificially stimulated ejaculation in three rhinoceros species (Ceratotherium simum, Dicerorhinus bicornis, Rhinoceros unicornis). Journal of Zoo and Wildlife Medicine 29: 386–393.

Schaffer NE, Foley GL, Gill S, Pope CE (2001) Clinical implications of rhinoceros reproductive tract anatomy and histology. Journal of Zoo and Wildlife Medicine 32: 31–46.

Schook MW, Wildt DE, Raghanti MA, Wolfe BA, Dennis PM (2015) Increased inflammation and decreased insulin sensitivity indicate metabolic disturbances in zoo-managed compared to free-ranging black rhinoceroses (Diceros bicornis). General and Comparative Endocrinology 217: 10–19.

Schwarzenberger F, Walzer C, Tomasova K, Vahala J, Meister J, Goodrowe KL, Zima J, Strauß G, Lynch M (1998) Faecal progesterone metabolite analysis for non-invasive monitoring of reproductive function in the white rhinoceros (Ceratotherium simum). Animal Reproduction Science 53: 173–190.

Sendag S, Cetin Y, Alan M, Hadeler K-G, Niemann H (2008) Effects of eCG and FSH on ovarian response, recovery rate and number and quality of oocytes obtained by ovum pick-up in Holstein cows. Animal Reproduction Science 106: 208–214.

Smith GD, Serafini PC, Fioravanti J, Yadid I, Coslovsky M, Hassun P, Alegretti JR, Motta EL (2010) Prospective randomized comparison of human oocyte cryopreservation with slow-rate freezing or vitrification. Fertility and Sterility 94: 2088–2095.

Stoops MA, Pairan RD, Roth TL (2004) Follicular, endocrine and behavioural dynamics of the Indian rhinoceros (Rhinoceros unicornis) oestrous cycle. Reproduction 128: 843–856.

Stoops MA, Atkinson MW, Blumer ES, Campbell MK, Roth TL (2010) Semen cryopreservation in the Indian rhinoceros (Rhinoceros unicornis). Theriogenology 73: 1104–1115.

Stoops MA, O’Brien JK, Roth TL (2011a) Gamete rescue in the African black rhinoceros (Diceros bicornis). Theriogenology 76: 1258–1265.

Stoops MA, Bateman HL, Campbell MK, Roth TL (2011b) Attempted in vitro maturation and fertilization of postmortem Sumatran rhinoceroses (Dicerorhinus sumatrensis) oocytes. Journal of Zoo and Wildlife Medicine 42: 723–726.

Stoops MA, Campbell MK, DeChant CJ, Hauser J, Kottwitz J, Pairan RD, Shaffstall W, Wolle K, Roth TL (2016) Enhancing captive Indian rhinoceroses genetics via artificial insemination of cryopreserved sperm. Animal Reproduction Science 172: 60–75.

Tubbs CW, Moley LA, Ivy JA, Metrione LC, LaClaire S, Felton RG, Durrant BS, Milnes MR (2016) Estrogenicity of captive southern white rhinoceroses diets and their
association with fertility. *General and Comparative Endocrinology* 238: 32–38.

Walzer C, Pucher H, Schwarzenberger F (2000) A restraint chute for semen collection in white rhinoceros (*Ceratotherium simum simum*) - preliminary results. *European Association of Zoo and Wildlife Veterinarians, Paris* 3: 7–10.

Woods EJ, Benson JD, Agca Y, Critser JK (2004) Fundamental cryobiology of reproductive cells and tissues. *Cryobiology* 48: 146–156.

Zschokke S, Studer P, Baur B (1998) Past and future breeding of the Indian rhinoceros in captivity. *International Zoo News* 45: 261–276.