REVIEW

Comparative reproduction of the female horse, elephant and rhinoceros: implications for advancing assisted reproductive technologies

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Abstract

Recent loss of rhinoceros subspecies has renewed interest in using more advanced assisted reproductive technologies (ART) in rhinoceroses and elephants. Currently, only semen collection, semen preservation and artificial insemination (AI) have been used repeatedly with success in these species. Although ovum pick-up (OPU) and intra-cytoplasmic sperm injection (ICSI) have been reported recently in rhinoceroses, the techniques are not yet optimised. In contrast, multiple ART applications are routinely used in the horse. Since elephants and rhinoceroses share some reproductive features with equids, we postulate that procedures such as OPU, ICSI, *in vitro* fertilisation (IVF) and embryo transfer (ET), which are well established in the horse, may represent a basis to develop protocols for endangered pachyderms. In this review, we summarise current knowledge on reproductive physiology relevant to ART. We discuss the current state of ART in all three families and the requirements for the successful implementation of OPU, ICSI, IVF and ET in these species.

Lay summary

Wild rhinoceros and elephant populations are facing ongoing threats; therefore, additional measures are required to protect these species for future generations. Assisted reproductive technologies (ART) include the collection of semen to directly inseminate females or to fertilise oocytes (eggs) in a laboratory to produce embryos, which can be transferred into a recipient female at a later date. While these techniques are routinely used in humans and domestic animals such as the horse, more research is needed to incorporate such technologies into the breeding of elephants and rhinoceroses. As the horse is the closest related domestic species to the rhinoceros, it may serve as the best possible role model. We discuss the current state of ART in the horse, elephant and rhinoceros and the possibilities for future use of these techniques in breeding such endangered animals.

Keywords: ▶ artificial reproduction ▶ ovum pick-up ▶ embryo transfer ▶ elephantidae ▶ rhinoceridae

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Introduction

Captive breeding populations of endangered species need a certain number of fertile animals with genetic diversity to remain stable and/or become self-sustaining. However, natural breeding in captive animals often poses difficulties. For example, genetically diverse mating partners may be located at different facilities in other countries or continents, and fertility may be sub-optimal when the social needs of captive animals are not met. The use of assisted reproductive technologies (ART) would appear to be one solution to help overcome these problems (Comizzoli 2015) and preserve fertility beyond the reproductive lifespan of an individual. However, currently the use of ART in pachyderms is far from being a routine procedure that can be used to increase an individual's breeding prospects and/or increase numbers within a population.

The application of any ART to increase numbers and diversity in captive breeding programmes should never be viewed as the single answer. It is important to remember that many other factors that influence reproduction in these animals have not been studied in enough detail, including the physiology and hormonal control of reproduction, husbandry needs *per se* and how the structure of their societies and social relations affects breeding and fertility (Lueders & Allen 2020).

Hence, the current rather low success rate of ART in the rhinoceros and elephant is due, in part, to a lack of fundamental reproductive knowledge. Coupled with this are the intractability of these species compared to domestic animals, low numbers of individuals available to undertake research on and the substantial cost involved with developing these techniques in new species (Lueders & Allen 2020). To complicate the matter still further, there are several species, and even subspecies, of elephants and rhinoceroses, each with their own individual reproductive characteristics.

Nevertheless, for both the elephant and rhinoceros, ART has a role to play in captive wildlife breeding programmes (Hermes *et al.* 2007, 2013, Stoops *et al.* 2016). Most of the advancements in the modern reproductive technologies in pachyderms have been made in *ex situ* breeding of Asian and African elephants, primarily by establishing protocols for semen freezing and artificial insemination (AI).

Over a decade ago, Hermes *et al.* (2007) reviewed ART being undertaken in elephants and rhinoceroses. Since then, improvements in AI techniques in both families and developments in other more advanced reproductive technologies such as ovum pick-up (OPU) and *in vitro* embryo production in rhinoceroses have been made (Hermes *et al.* 2009*b*, Hildebrandt *et al.* 2012, 2018, Stoops *et al.* 2016, Pennington *et al.* 2020). However, the use of ART in pachyderms is far from routine. Since ART are more advanced in the horse (Hinrichs 2018), can we use this species as a model for the elephant and rhinoceros, despite variations in their reproductive anatomy and physiology?

Elephants, rhinoceroses and horses are all large terrestrial mammals. However, taxonomic classification of these three species varies. Elephants are classified in the order Proboscidea and family Elephantidae and consist of three existing species: African savannah (*Loxodonta africana*), African forest (*Loxodonta cyclotis*) and Asian elephant (*Elephas maximus*) (Roca *et al.* 2015) and are far removed from the rhinoceros and horse on the evolutionary tree. Two African species, the black (*Diceros bicornis*) and white rhinoceros (*Ceratotherium simum*), and three Asian species, the Indian (*Rhinoceros unicornis*), Sumatran (*Dicerorhinus sumatrensis*) and Javan rhinoceros (*Rhinoceros sondaicus*) (Amin *et al.* 2006), are classified in the same order as the horse, Perissodactyla, and although in different families (Rhinoceridae and Equidae), they share more reproductive characteristics than the horse and elephant. Nevertheless, certain reproductive similarities between the elephant and horse are present (Allen 2010).

Hence, the aim of this review is to highlight similarities and differences in the reproductive characteristics of the domestic horse mare, elephant cow and white rhinoceros cow and discuss how these relate to our ability to practically apply ART in pachyderms, and to what degree the horse is a suitable model.

Reproductive anatomy

Understanding the reproductive anatomy of a species is essential to undertake reproductive procedures, interpret clinical findings and allow the development of species-specific equipment.

Schematic drawings of the female reproductive tract of the horse, elephant and rhinoceros *in situ* and *ex situ* are compared in Fig. 1. Similarities, like a bicornuate uterus and ovarian functional structures, differences and special features are summarised in Table 1 and briefly described below.

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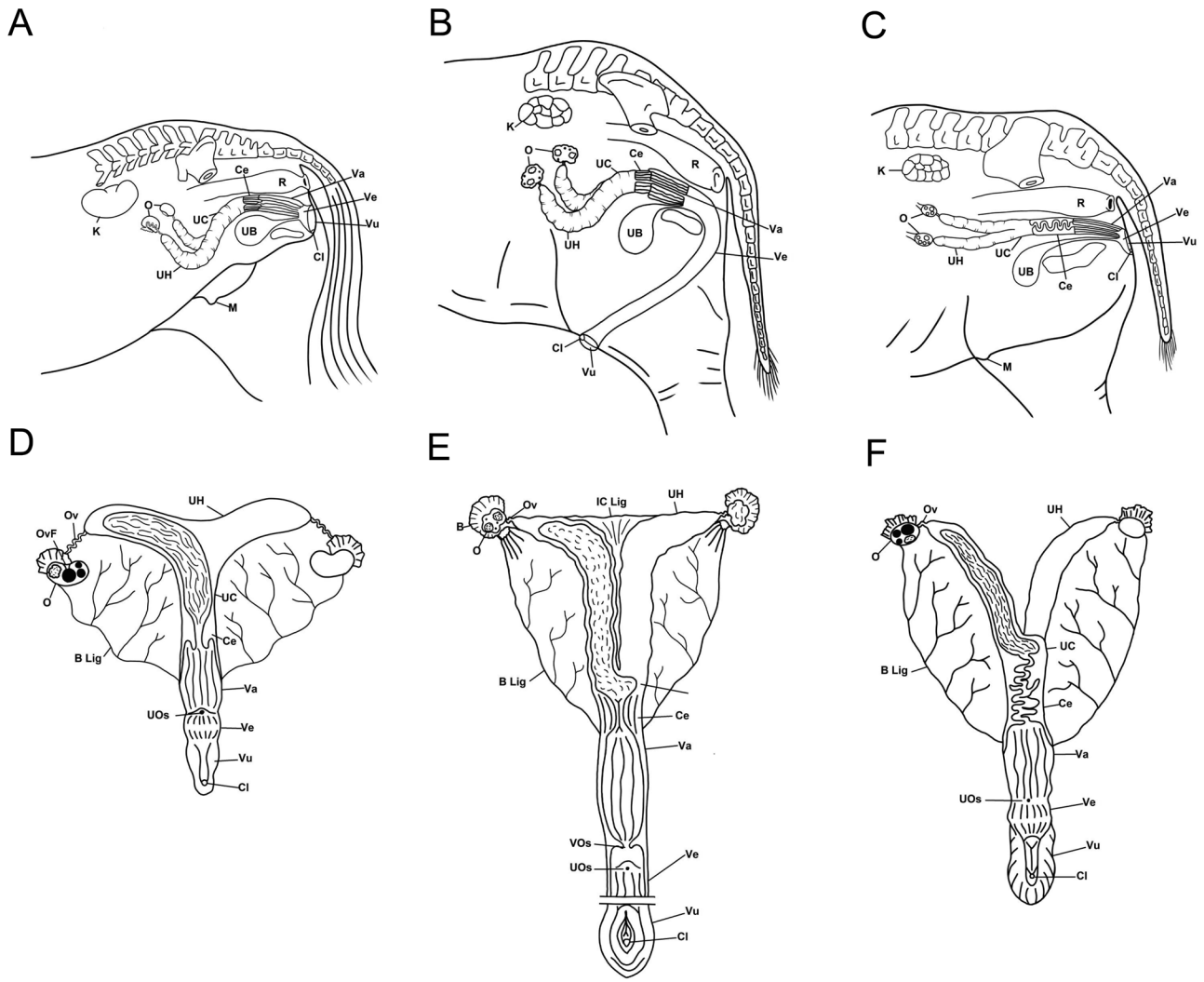

Figure 1 Schematic drawing of the female reproductive organs *in situ* (A, B, C) and *ex situ* (D, E, F) in the mare (A and D), elephant (B and E) and rhinoceros cow (C and F). B, bursa; B lig, broad ligament; Ce, cervix; Cl, clitoris; IC Lig, intercornuate ligament; K, kidney; M, mammary gland; O, ovary; Ov, oviduct; OvF, ovulation fossa; R, rectum; UB, urinary bladder; UC, uterine corpus; UH, uterine horn; UOs, urethral os; Va, vagina; Ve, vestibule; VOs, vaginal os; Vu, Vulva. Note that the full vestibule of the elephant *ex situ* (D) is not displayed due to the length. Adapted from Allen *et al.* 2003, Schaffer *et al.* 2001.

Mare

The internal reproductive tract of the mare is positioned horizontally, extending into the pelvic cavity and abdomen (Fig. 1A). The vulva forms the gateway to the vestibule which continues cranially as the vagina; a vestibule-vaginal ring is present at the junction of the two, and a hymen may be present in unmated females (Kainer 2011). The relatively straight cervix consists of a thick muscle layer and multiple longitudinal mucosal folds, but no transverse cervical rings, making it easy to pass catheters through for AI or embryo transfer (ET) (Stout 2020). The external os of the cervix protrudes into the cranial vagina and is surrounded by the vaginal fornix, an internal os opens into the uterus (Fig. 1D). The Y-shaped uterus consists of a prominent uterine body and two horns suspended within the pelvic cavity and abdomen by the broad ligament (Kainer 2011). The relatively long oviducts of 20–30 cm have a fimbriated infundibulum positioned over the ovulation fossa to guide the oocyte into the ampulla of the oviduct and are attached to the lateral surface of the ovaries (Kainer 2011). The ovaries can be readily palpated per rectum, are comparatively large and consist of an outer medulla and an inner cortex containing the functional structures, such as follicles and corpora lutea. Follicles can reach up to 50 mm prior to ovulation, which can only occur at the ovulation fossa as the rest of the ovarian surface is covered by the tough tunica albuginea (Kainer 2011).

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Table 1 Overview of special reproductive anatomy characteristics of mare, African elephant and white rhinoceros cow with average measurements.

	Horse	Elephant	Rhinoceros
Vestibule and vagina	Horizontally positioned, separated by vestibule-vaginal ring, vestibule 10-12 cm, vagina 15-20 cm long	Extremely long (100–150 cm) vestibule, opens between the hind legs; vagina (20–40 cm) has many longitudinal folds and opens into the vestibule through small vaginal os (0.5–2 cm) within the intact hymenal membrane in nulliparous females, additional vaginal opening(s) and /or blind pouches on either side of the vaginal os possible; vaginal os in parous females wide at 5–19 cm	Similar conformation to the mare, transversal, thin vaginal folds in the cranial vagina, vestibule 14–20 cm, vagina 20–30 cm long
Cervix	Short (5–7 cm), diameter: 3.5–4 cm; consists of a thick muscle layer and longitudinal folds	Short (6–10 cm), longitudinal folds, cranio-ventral direction of pelvis	Long (13–20 cm), tortuous, with firm, right angle folds of connective tissue, narrow lumen, prominent cervical os and fornix similar to mare
Uterus	Bicornuate; Y-shaped, prominent uterine body (18–20 cm), horns (20–25 cm)	Bicornuate; U-shaped, short body (5–15 cm); long horns (30–80 cm), connected for 50–70 cm up to the uterine bifurcation	Bicornuate; short body (3.5–7.5 cm); long, straight horns (40–50 cm) of equal diameter (3–4 cm) over the entire length
Oviduct	Relatively long (20–30 cm), fimbriated infundibulum positioned over the ovulation fossa	Short (10 cm); enveloped by the infundibulum of the oviduct which is incorporated in the ovarian bursa, forms serosal pouch around ovary	Short, fimbriated at the cranial opening nearest the ovaries
Ovaries	• Comparably large (6–8 × 3–4 cm) cranially of the tip of the uterine horns • Outer medulla and inner cortex • Ovulation fossa where cortical tissue extends to the ventral surface of the ovary	• Relatively small (7 × 5 × 2.5 cm), cranially of uterine horn • Inner medulla and outer cortex • Surface has irregular ridges • Entire ovarian surface is enveloped by ovarian bursa	• Comparably large (8 × 4 × 2cm), when active, cranially of the tip of the uterine horns, caudo-laterally to the kidneys • Inner medulla and outer cortex
Functional structures	• Antral follicles of various sizes always present • Pre-ovulatory follicle size: 3.5–5 cm • One CL from ovulation • Accessory CLs during pregnancy	• Tiny antral follicles only during follicular phase visible (0.1–1.5 cm) • Pre-ovulatory follicle size: 2 cm • One CL from ovulation (4–5 cm) • Accessory CLs formed from luteinised unovulated follicles prior to each ovulation (2–3.5 cm)	• Antral follicles of various sizes always present • Pre-ovulatory follicle size: 3–4 cm • Only one CL during pregnancy, no accessory CLs

Elephant cow

The reproductive tract of the female elephant, from vestibule to ovary, is the longest of the terrestrial animals, reaching a length of approximately 3.5 m in the African elephant (Balke *et al.* 1988). Some unique anatomical features (Figs. 1B and E) complicate the development and use of ART in these species (Balke *et al.* 1988, Hildebrandt *et al.* 1999). The extremely long vestibule curves from the caudal pelvis to just under the anus before arching downwards to open between the hind legs (Fig. 1B). The pelvic part of the vestibule is separated from the vagina through a hymen-like structure which breaks only during the first parturition, and not during copulations as in the mare and the rhinoceros. Unique to elephants is a small opening in the hymen and two blind pouches on either side of the vaginal opening (Hildebrandt *et al.* 2006), although variations are seen between individuals (i.e., two vaginal openings, no blind pouch; Balke *et al.* 1988 and I. Lueders, personal observation; Fig. 2D-F). A short cervix with longitudinal folds opens into a short uterine body. Similar to the horse, the two horns are initially positioned straight before diverging laterally from each other towards the ovaries (Fig. 1E). However, in contrast to the mare, the long horns are connected by a short intercornuate ligament for 0.5–0.7 m and aligned in an anterior direction (Allen *et al.* 2003, Hermes *et al.* 2006). The ovaries are relatively small, lying cranial to the uterine horn at the termination of short oviducts, and the entire ovarian surface is encapsulated by an ovarian bursa (Fig. 2). The

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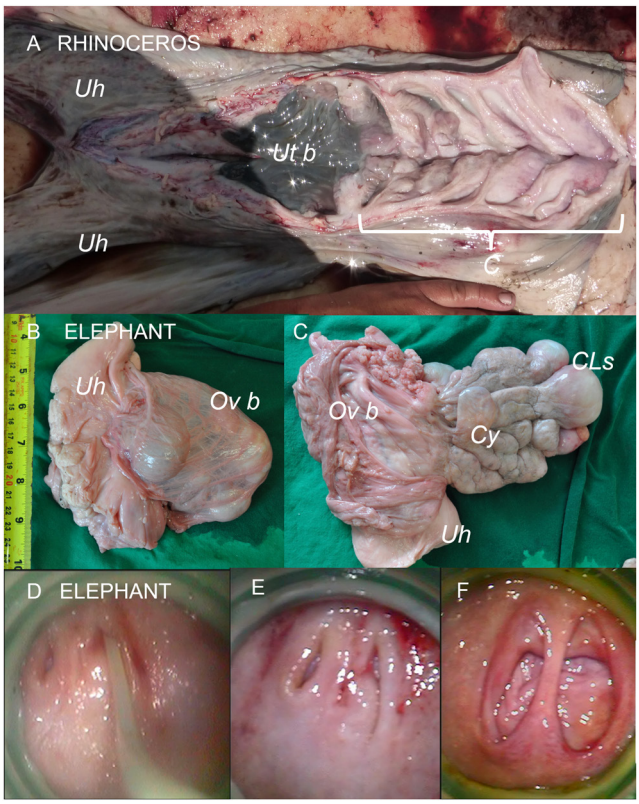

Figure 2 Photographs of special anatomical features of the rhinoceros (A) and elephant (B–F). (A) White rhinoceros cervix opened, exposing the folded structure. utb, uterine body; uh, uterine horn; (B) African elephant ovary within the ovarian bursa (ovb) and uterine horn tip (uh); (C) same ovary, ovarian bursa (ovb) removed and ovary turned over, note the corpora lutea (CLs) and a flaccid cystic structure (Cy); (D––F) endoscopic view (*in situ*, from vestibulum) onto the hymen-like structure separating vestibule and vagina in the Asian elephants: (D) with AI catheter placed through the vaginal os; (E) two small ostia opening into the vagina, (F) variation of the larger vaginal os with tissue bridge and vaginal mucosa shining through.

elephant ovary is composed of an inner medulla and outer cortex, meaning ovulation can occur at any location on the ovaries' surface (Hildebrandt *et al.* 2000). In contrast to the mare and rhinoceros cow, which have smooth plum-like ovaries, the surface of an elephants' ovaries show irregular ridges (Allen *et al.* 2003). Antral follicles are visible only during the follicular phase and at no other stage. Dominant follicles are considerably smaller than those of the mare and rhinoceros cow, reaching only about 2.0 cm prior to ovulation (Hildebrandt *et al.* 2011).

Rhinoceros cow

The uterus, vagina and cervical os of the rhinoceros resemble those of the mare (Figs. 1C and F). In maiden females, a hymenal membrane is present (Hermes *et al.* 2006). The cervix is particularly long and has firm folds of connective tissue (Figs. 1F and 2) leaving a narrow, tortuous lumen (Godfrey *et al.* 1991, Hermes *et al.* 2007) which is challenging to catheterise. The uterine horns are straight with their tips pointing cranially and not diverging laterally as in the mare and elephant cow (Fig. 1F). Like those of the horse, the oviducts consist of a small tubular structure with fimbriae at the cranial openings nearest the ovaries. The ovaries are positioned within an ovarian bursa (Godfrey *et al.* 1991, Schaffer *et al.* 2001). Inactive ovaries are small and flat, while active ovaries are large and round with follicles, and/or a corpus luteum or corpus haemorrhagicum (Schaffer *et al.* 2001, Hermes *et al.* 2006). Unlike the mare, rhinoceros ovaries show an inner medulla and outer cortex. Despite this difference, antral follicles, present at any cycle stage develop similar to the horse with a single dominant follicle prior to ovulation (Radcliffe *et al.* 1997).

Reproductive physiology

A precondition for undertaking ART is a thorough understanding of the hormonal changes of the oestrous cycle and early gestation. The mare and rhinoceros cow share several hormonal oestrous cycle features, whereas elephants present some unique aspects (Fig. 3). While extensive literature is available for the mare (as reviewed by Satué & Gardon 2020), only relatively recently did we understand the reproductive physiology of the elephant (as reviewed by Hildebrandt *et al.* 2011) and rhinoceros (as reviewed by Roth 2006, Stoops *et al.* 2016) in more detail. The most relevant aspects are summarised in Table 2 and briefly described here.

Oestrous cycle

Mare

The mare is a long day, seasonally polyoestrous breeder with regular oestrous cycles of 20–23 days occurring between spring and autumn. Each cycle consists of 5–6 days of oestrus or sexual receptivity induced by oestrogens secreted by enlarging follicles in the ovaries and which is followed by 14–15 days of dioestrus under the dominance of progesterone (Fig. 3A) the source of which is a, usually single, corpus luteum (CL). Luteinising hormone (LH) levels rise during late oestrus and peak after, not before, ovulation (Ginther 1992, Aurich 2008). Regression of

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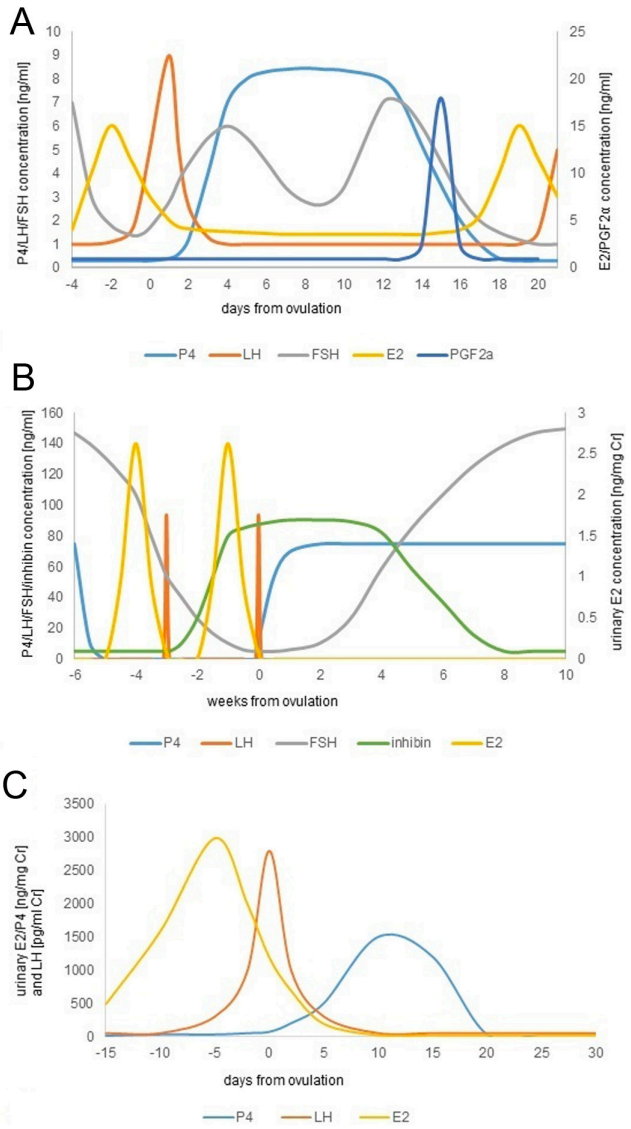

Figure 3 Model of changes in known reproductive hormones during the oestrous cycle of the mare (A), elephant (B) and (Indian) rhinoceros cow (C). Day 0, day of ovulation; P4, progesterone (5α-reduced metabolites in elephants); LH, luteinising hormone; FSH, follicle-stimulating hormone; E2, oestrogen; PGF2α, prostaglandin F2alpha. Adapted from Stoops *et al.* 2004, Ginther 2007, Aurich 2011, Hildebrandt *et al.* 2011, Lueders *et al.* 2011.

other follicles in a cohort occurs when one dominant follicle begins to secrete inhibin as it enlarges causing suppression of follicle-stimulating hormone (FSH) from the pituitary, helping to ensure only a single follicle matures and ovulates (Fig. 3A). Progesterone dominates until around day 14 of dioestrus when spike-like bursts of prostaglandin F2alpha (PGF2α), released from the lumenal epithelium of the endometrium, travel to the ovaries via the maternal circulation causing luteolysis of the corpus luteum (Ginther 1992).

Elephant cow

The female elephant exhibits a long oestrous cycle of 13–17 weeks, composed of a follicular phase of 6-8 weeks, followed by a luteal phase of 6–10 weeks (Lueders *et al.* 2010*b*). Instead of a single ovulation-inducing LH peak, the elephant expresses two LH peaks (Fig. 3B), of which only the second results in ovulation (Brown *et al.* 1999). During a first follicular wave, which commences when progesterone of the previous luteal phase reaches baseline levels, a cohort of follicles develop over the course of approximately 3 weeks (Lueders *et al.* 2010*b*). This first wave ends with a first, anovulatory LH peak in the absence of overt oestrous signs, although interest in the bull and even mating have been observed at this time (Lueders *et al.* 2010*b*, Hildebrandt *et al.* 2011). Typically, 1–10 follicles of >10 mm luteinise after the first LH peak but only become ultrasonographically visible as luteinised unovulated follicles (LUFs) about 10 days later. These LUFs form slowly growing accessory CLs in each oestrous cycle. A second wave of follicular growth then commences and a second LH peak occurs approximately 3 weeks (19–21 days) later (as reviewed by Hildebrandt *et al.* 2011). Only 12–24 h after the second LH peak does one single follicle of the second cohort mature and ovulate when the follicle reaches approximately 2.0 cm (Hildebrandt *et al.* 2000, Lueders *et al.* 2010*b*). The ovulated follicle develops into the largest CL, which along with the accessory CLs, produces metabolites of 5-alpha reduced progestagen (Lueders *et al.* 2012). The serum progestagen rise occurs within 24 h after the second LH surge (Fig. 3B). The ovulatory CL is significantly larger than the anovulatory CLs derived from LUFs and remains discernible into the next follicular phase (Lueders *et al.* 2010*b*). All CLs persist throughout the dioestrous period after which they regress in the absence of pregnancy during the new follicular phase (Brown *et al.* 1999, Hildebrandt *et al.* 2006, 2011, Lueders *et al.* 2010*b*).

The LUFs do not produce measurable progestagens in the peripheral circulation prior to the second LH peak but are a source of inhibin (Kaewmanee *et al.* 2011, Yamamoto *et al.* 2012), which rises measurably only after the first LH peak (Fig. 3B) and in parallel with LUF growth. Therefore, LUF formation may be a precondition for the selection of a single, dominant follicle at the second LH peak (Lueders *et al.* 2011).

Rhinoceros cow

The five living rhinoceros' species show slight variation in their oestrous cycle length, dominant follicle size and

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Table 2 Overview of key features of the reproduction physiology of the mare, elephant cow and the rhinoceros species.

	Horse	Elephant	Rhinoceros				
			White	Black	Indian	Sumatran	Javan
Female maturity (years)	1	6–10	5–6	4–7	6–7	4–6	5–6
Oestrous cycle length (days)	20–23	95–120	30–35 or 65–70	27	40–50	21–24	?
Oestrous duration (days)	5–6	2–4	1–3				
Repeated reproductive ultrasound	Yes	Yes	Yes	Yes	Yes	Yes	No
Ovulation type	Spontaneous	Spontaneous	Partially induced	Spontaneous	Spontaneous	Induced	?
Ovulatory follicle size (cm)	3.5–5.0	2.0	3.0–4.0	4.7–5.1	12.0–14.0	2.0–2.5	?
Pregnancy detection							
TU at	14 days	55 days	15 days				
Serum	eCG	Prolactin at 120 days	Progesterone				
Embryonic vesicle	Spherical, mobile	Spherical, immobile	Spherical, mobile (?)*				
Gestation length (days)	330	600–680	450–490	450–490	450–490	450–490	490–580
Intercalving interval (years)	1	4–8	2.5–3.5	2.5–3.5	3	3	?
Placenta type	Diffuse epitheliochorial	Endotheliochorial zonary	Diffuse epitheliochorial with villus-free areas*				

TU, transrectal ultrasound.
*this is for all sub-species of rhinoceros

hormone secretion, but overall they are akin to the mare with a 3- to 5-day period of oestrous behaviour (Radcliffe *et al.* 1997) that accompanies the growth and ovulation of one single dominant follicle (as reviewed by Roth *et al.* 2018). Ovulation of the dominant follicle (30–40 mm in white rhinoceroses) at the end of the follicular phase takes place within the cortex of the ovary and is induced by a single pre-ovulatory LH peak (Hermes *et al.* 2007, Radcliffe *et al.* 2001, Fig. 3C).

Oestrous cycle length differs, and shorter or longer cycles have been reported in all captive species (Roth *et al.* 2018, Table 2). Unique to the white rhinoceros, two distinctive cycle lengths of 30–35 and 65–70 days have been recognised in captive animals (Patton *et al.* 1999, Hermes *et al.* 2012, Pennington *et al.* 2020). Although ovulations followed the follicular phase in cycles of both lengths, the longer cycle was originally thought to be associated with in- or subfertility (Schwarzenberger *et al.* 1998, Patton *et al.* 1999, Brown *et al.* 2001). However, more recently, ovulation induction and AI in rhinoceroses presenting with long luteal phases prior to the new cycle resulted in pregnancy (Pennington *et al.* 2020). In the white rhinoceros and its African relative, the black rhinoceros, longer cycles are associated with longer luteal phases which have not been identified in the Asian species. Instead, longer cycles in Asian rhinoceroses appear to be related to a delayed development of a dominant follicle in a follicular wave or ovarian cysts (Stoops *et al.* 2004).

As reported in the horse, follicles may become haemorrhagic (Pennington *et al.* 2019), frequently referred to as haemorrhagic anovulatory follicles (HAFs) in the mare (Ginther 2007). These follicles typically grow larger than the normal pre-ovulatory size and develop echogenic patterns and fibrinous, spider-web-like structures within the fluid-filled lumen. Like HAFs in mares, they may luteinise and secrete some progesterone or slowly organise and regress without any progestagen production, as noted in white rhinoceroses (Roth *et al.* 2018).

Oestradiol, progesterone and LH (Fig. 3C) all play a part in orchestrating the reproductive cycle in the white rhinoceros, but other reproductive hormone (especially proteohormones such as FSH, inhibin, prolactin, PGF2α) profiles have not been elucidated.

Placentation and the hormonal control of early gestation

A sound understanding of early embryonic development, maternal recognition of pregnancy (MRP) and the

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hormonal changes involved during early gestation is required in order to undertake procedures such as ET successfully. While the mare has been studied in detail (as reviewed by Allen 2001), and over the last decade much has been learnt about early pregnancy in the elephant (as reviewed by Allen & Stansfield 2021), there remains a paucity of information regarding the rhinoceros.

Mare

Pregnancy in the mare includes a number of fascinating and apparently equine-specific events. Only fertilised oocytes enter the uterus, with the early embryo secreting prostaglandin E2 (PGE2) from day 5 to day 6 (Weber *et al.* 1991) to cause relaxation and contraction of the muscles of the oviduct and, hence, passage through the utero-tubal junction into the uterus. Unfertilised oocytes remain within the oviduct and either degenerate over time or pass into the uterus in the presence of an embryo. The equine embryo has a long oviductal transport time of 144–156 h post-ovulation (Battut *et al.* 1998) and arrives in the uterus as a late morula or early blastocyst. Practically, this means embryo recovery for transfer, bisection or cryopreservation in the mare can only be undertaken from day 6.5, with optimum recovery rates from day 7.

The spherical equine embryo sheds the zona pellucida around day 7, underneath which is a tough acellular glycoprotein capsule, at least partly secreted by the trophectoderm cells, that will encapsulate the embryo until around day 25 (Betteridge 2000). The equine embryo also shows mobility during early pregnancy, continually moving through the uterine lumen until around day 16 when it lodges at the base of one of the uterine horns (Ginther 1983). This mobility is believed to be driven by prostaglandins secreted by the embryo (Stout & Allen 2001) and is considered essential to liberate the MRP signal over the uterine epithelial surface to prevent the cyclic release of PGF2α from the endometrium and a return to oestrus by the mare. In addition, copious amounts of oestradiol are produced by the early equine conceptus from at least day 12 of pregnancy, and although their precise role has not been elucidated, they have been proposed to play a role in the establishment of pregnancy (Heap *et al.* 1982).

Throughout the mobility stage, and even after the conceptus becomes stationary at the base of a uterine horn, it relies on protein-rich secretions from the endometrial glands, the so-called histotrophe, which contain numerous proteins (e.g. uterocalin and uteroferrin) (Ellenberger *et al.* 2008), to supply its nutritional needs. Interdigitation of the allantochorionic placenta does not commence until around day 45 and this is initially rudimentary (Samuel *et al.* 1974), making histotrophic nutrition very important in the horse. Indeed, secretions from the endometrial glands play an important role throughout gestation in the horse (Ellenberger *et al.* 2008).

The equine conceptus also produces a chorionic gonadotrophin (eCG) during early pregnancy with the only other mammals known to do so being humans and the higher primates. In the horse, a specialised band of tissue called the chorionic girdle develops around the conceptus. This invades the endometrium around days 36–38 of pregnancy to form the endometrial cups which secrete eCG into the maternal bloodstream between 40 and 120 days of gestation (Allen & Moor 1972).

Hence, following the development of the endometrial cups, progesterone levels in the mare's bloodstream, which had been falling steadily from around day 20, rise sharply and remain elevated thereafter until days 120–140 as a result of the continuing 'collaborative' actions of the pituitary FSH-stimulated follicular waves and luteinising properties of the eCG secreted by the endometrial cups causing accessory CL to develop. Interestingly, eCG also causes an increase in peripheral oestrogens which are secreted by luteal tissue within the ovaries (Daels *et al.* 1991).

Continuing interdigitation of the allantochorion from day 45 results in the formation of complex microcotyledons over the entire surface of the allantochorion which interdigitate with the endometrium in corresponding crypts with no breakdown of the maternal tissue (Samuel *et al.* 1974). Hence, placentation in the horse is described as diffuse, non-invasive and epitheliochorial. In addition to providing the means for haemotrophic nutrition during pregnancy, the fetoplacental unit also synthesises steroid hormones during the second half of pregnancy. To do so, it utilises fetal C-19 precursors produced by the enlarged fetal gonads to produce oestrogens from around day 80, peaking at 220 days before falling as parturition approaches. In addition, maternal C-21 precursors are synthesised by the placenta to 5α-reduced progestagens (Allen 2001).

In summary, principal endocrinological changes during pregnancy in equids are unusual with many being specific to this genus. Luteal progesterone production declines during the first 40 days but thereafter shows a secondary rise during the next 100 days due to the development of secondary or accessory CLs in the maternal ovaries stimulated by the combined actions of continuing 10- to 12-day releases of pituitary FSH and the

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LH-like properties of eCG secreted by the endometrial cups between 40 and 120 days of gestation. Thereafter maternal serum progestagen concentrations remain low and constant until a further rise occurs during the last 6–8 weeks of gestation as a result of secretion by the fetal adrenal glands. Oestrogens also play a role in equine pregnancy with initial production by the early embryo, then the luteal tissue and in later pregnancy synthesis by the placenta from precursors from the fetal gonads (Allen 2001).

Elephant cow

A number of aspects of the 590–680 days gestation in the elephant are of particular interest due to their similarity to events in the pregnant mare, while other features vary greatly (Allen & Stansfield 2021).

As mentioned previously, the LUFs from the first wave of follicular growth persist in the ovaries well beyond the second follicular wave and the associated ovulation that can result in pregnancy, if accompanied by a fertile mating. If conception has taken place, all CLs enlarge significantly around the time of implantation (40–50 days). These CLs persist in the ovaries for the remainder of gestation and secrete the metabolites of 5α-reduced progestagens needed to maintain pregnancy, since the placenta of the elephant does not synthesise progestagens (Lueders *et al.* 2012).

Implantation of the embryo occurs at the base of the uterine horn, always ipsilateral to the side of ovulation (Lueders *et al.* 2012) with no observable free embryonic migration as described for the mare. Consistent with the long oestrous cycle and gestation length, embryonic development is rather slow. Compared to the horse and rhinoceros, the embryonic vesicle and the embryo proper only become apparent ultrasonically at 40–50 days (Fig. 4C) (Lueders *et al.* 2010*a*) and 65 days (Fig. 4D), respectively, resembling the stages of day 14 (Fig. 4A) and day 21 (Fig. 4B) pregnancy in the mare. At this stage, a band of trophoblast in the equatorial region of the spherical conceptus extrudes 'fingers' of trophoblast cells, which undermine and dislodge the lumenal epithelium of the endometrium to replace it with a layer of trophoblast, which now lies on the lumenal surface of the endometrial stroma. The endotheliochorial zonary placenta will subsequently develop from increasingly elongated upgrowths of the trophoblast-lined stroma. The trophoblast begins to secrete placental lactogen (Yamamoto *et al.* 2012), which with its prolactin-like action appears to stimulate the enlargement of the all persisting CLs at around 40–50 days of gestation. Unlike the horse, no chorionic gonadotrophin (CG) has been detected during pregnancy in the elephant. The zonary-girdle placenta is unable to produce progestagens to support the pregnancy but probably contributes to its maintenance through the indirect effect of the prolactin/placental lactogens it produces (Yamamoto *et al.* 2017, Lueders *et al.* 2012).

The progesterone metabolite-secreting ability of the fetal gonads, which in parallel with the situation in equids, enlarge greatly during gestation, may add to the pool of progestagens and, hence, assist the long-lived maternal CLs to maintain the pregnancy state until term (Stansfield & Allen 2012). Thus, as in the horse, the elephant fetus appears to play a hormonal role in orchestrating the endocrinology of pregnancy (Allen *et al.* 2003).

Rhinoceros cow

There is a paucity of information on pregnancy in the rhinoceros compared to the horse and elephant. The gestation length averages 450–500 days for the four captive rhinoceros species (Hermes *et al.* 2007, Schwarzenberger & Hermes 2023).

Progesterone rises only 5–7 days after ovulation and is at luteal phase levels for the first 2–5 months with an increase to pregnancy levels thereafter (Berkeley *et al.* 1997, Patton *et al.* 1999, Brown *et al.* 2001, Pennington *et al.* 2020, Schwarzenberger & Hermes 2023). Only a single CL has been described in pregnant rhinoceroses. The rhinoceroses share some features of early pregnancy with the horse, as the early embryonic vesicle is spherical (Fig. 4) and appears to be mobile during the early stages (Radcliffe *et al.* 1997, Hermes & Hildebrandt 2011). Furthermore, the vesicle can be visualised by ultrasound from 15 days post-conception (Fig. 4E), the embryo itself around 23 days (Fig. 4F) and an embryonic heartbeat at 26 days (Radcliffe *et al.* 1997, Ververs *et al.* 2015), mirroring the timing of these stages in the equine conceptus (Fig. 4). Another similarity with the horse is the diffuse, epitheliochorial placenta, although the villus surface of the rhinoceros placenta has villus-free lines, sometimes referred to as 'streets' (Benirschke & Lowenstine 1995).

No CG nor other feto-placental hormones have been described in the rhinoceros that could play a role in maintaining the single CL, which appears to remain throughout pregnancy with no accessory CL formation (Sherman *et al.* 1997). However, between 60 and 120 days of gestation, there appears to be a transition from luteal to feto-placental progestagen secretion since faecal pregnane metabolite levels rise significantly above luteal phase concentrations. These faecal pregnanes are

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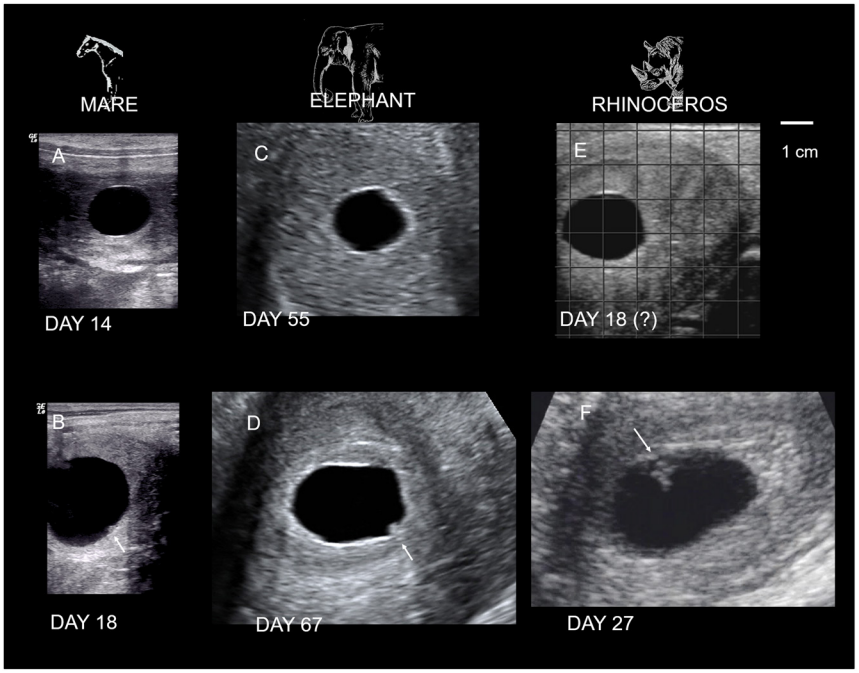

Figure 4 Ultrasonographic appearance of the early pregnancy of the mare (A, B), Asian elephant (C, D) and white rhinoceros (E, F) presented at similar stages of pronounced, spherical embryonic vesicle (EV) and appearance of the embryo proper (→). The EV appears very similar in all three families, although development in the elephant is considerably longer than that of the same stage embryos as in mare and rhinoceros are reached; DAY, day from ovulation; (?), day from observed last breeding, exact day of ovulation was not determined in this rhinoceros; sizes normalised to bar (= 1 cm).

similar to pregnanes in the plasma of pregnant mares, where the source is known to be the feto-placental unit (Schwarzenberger & Hermes 2023). An endocrinological difference with the horse is the low oestrogen level throughout gestation in at least three of the five rhinoceros species in which this hormone has been measured (Schwarzenberger *et al.* 2000, Stoops *et al.* 2004, Roth 2006).

ART – current state

The development, advancement and implementation of ART in many wildlife species, including elephants and rhinoceroses, is limited by several challenges, including restricted access to individuals, individual differences and intractability as well as low commercial drive (Lueders & Allen 2020). Additionally, some of the unique anatomical features of the rhinoceros and elephant, as described above, complicate routine implementation of ART into captive breeding programmes.

Reports on the successful application of ART in pachyderms are mostly limited to a few individuals, thus bringing the sustainability and repeatability of these techniques into question (as reviewed by Hermes *et al.* 2007, Ververs *et al.* 2015, Pennington & Durrant 2018). However, equine reproductive physiology and many of the ARTs in the mare have been studied for decades resulting in a wealth of knowledge and the ability to apply them routinely in clinical practice (Hinrichs 2018). To-date applied ARTs in the mare, elephant and rhinoceros species are indicated in Table 3.

Ultrasonography of the female reproductive tract

Ultrasonographic visualisation of the reproductive tract is a requirement to directly undertake ART and to manage or monitor associated reproductive cycles. Due to a thick rectal wall in both, elephant and rhinoceros, the ovaries are not palpable. Like the more tractable mare, both captive elephants and rhinoceroses can be trained to enter a restraining chute to facilitate transrectal manipulation and/or ultrasound of the ovaries and uterus (Fig. 5). Heavy-duty restraining chutes offer the safest option for repeated examinations in pachyderms. However, since the use of chutes requires financial investment and time-consuming training and not all zoos are equipped with these, a standing sedation is an alternative option (Hermes *et al.* 2005, 2007).

Useful descriptions of the female reproductive sono-anatomy are available for horses (Kähn & Volkmann 2004), elephants (Hildebrandt *et al.* 2000) and rhinoceroses (black: Schaffer *et al.* 2001, Sumatran rhinoceros: Roth 2001, white: Hermes *et al.* 2005, Indian: Roth *et al.* 2018).

In contrast to equine transrectal ultrasound, where a linear probe is commonplace, in elephants and rhinoceroses a convex, 3–7 MHz ultrasound probe is more

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Table 3 Summary of to-date reported and successfully performed assisted reproductive technologies (ART) in the mare, elephant and rhinoceros species.

	Horse	Elephant	Rhinoceros				
			White	Black	Indian	Sumatran	Javan
Semen collection	AV	TM, EE	EE, UC	EE, UC	EE	EE	None
Semen cryopreservation	Yes	Yes	Yes	Yes	Yes	Yes	No
Hormonal oestrous induction	Yes	No	Yes	No	Yes	No	No
Artificial insemination							
Fresh semen	Yes	Yes	Yes	No	Yes	No	No
Frozen–thawed semen	Yes	Yes	Yes	No	Yes	No	No
Sperm sex sorting	Yes	Yes	Yes	Yes	Yes	No	No
Successful AI with sex-sorted semen	Yes	No	No	No	No	No	No
Ovum pick-up	Yes (TV)	No	Yes (TR)	Yes (TR)	No	Yes (TR)	No
In vitro maturation	Yes	Yes (EM), no (LA)	Yes	Yes	No	Yes	No
IVF	Yes	No	No	2- and 4-cell	No	No	No
ICSI	Yes	No	Blastocyst	No	No	No	No
In vitro embryo production	Yes	No	Yes	Yes	No	No	No
Embryo cryopreservation	Yes	No	Yes	No	No	No	No
Successful embryo transfer	Yes	No	No	No	No	No	No

AV, artificial vagina; EE, electro-ejaculation; EM, *Elephas maximus* (Asian elephant); ICSI, intra-cytoplasmatic sperm injection; TM, transrectal massage; TV, transvaginal; TR, transrectal; UC, urethral catheterisation.

appropriate due to the size and depth of the reproductive organs in these substantially larger patients.

Artificial insemination (AI)

Collecting and maintaining semen are pre-requisites for successful AI. Improvements in semen collection in chemically restrained elephants and rhinoceroses using electro-ejaculation (Saragusty *et al.* 2016*b*, Hermes *et al.* 2018) and urethral catheterisation (rhinoceroses only: Meuffels 2022) or manual stimulation by transrectal massage (elephants only: Hildebrandt *et al.* 1998) as well as advances in semen cryopreservation protocols (African elephant: Hermes *et al.* 2013, Asian elephant: Arnold 2014, white rhinoceros: Hermes *et al.* 2018, and black rhinoceros: Meuffels 2022) have been made in the last decade. AI with chilled or fresh semen is the easiest approach to breeding mares and is also a practical option in captive elephants and rhinoceroses, if distances allow for the movement of semen within a restricted time frame (Hildebrandt *et al.* 1999, 2007).

The first report of an African elephant calf born following AI with fresh semen originates from the late 1990s (Hildebrandt *et al.* 1999), but it took the same group more than another decade to repeat this success using frozen–thawed semen (Hildebrandt *et al.* 2012).

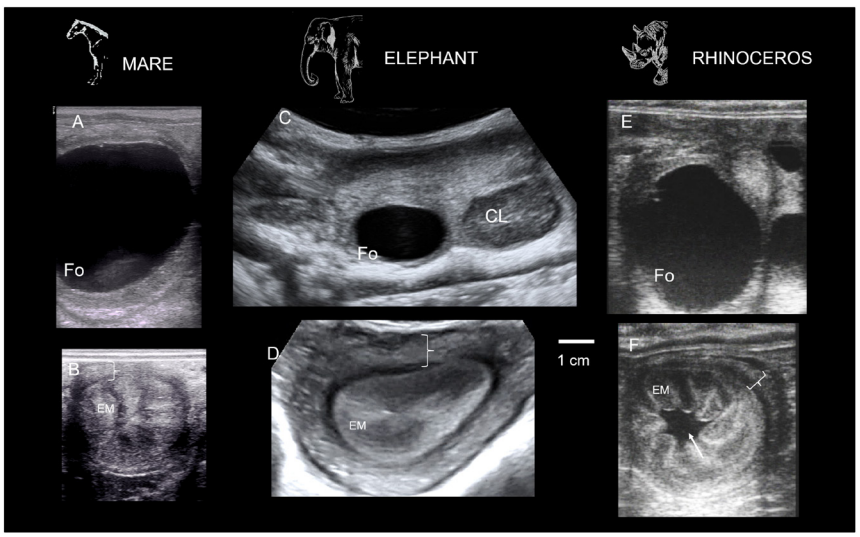

Figure 5 Ultrasonographic appearance of the dominant, pre-ovulatory follicle and concurrent uterine horn in cross-section of mare (A, B), Asian elephant (C, D), white rhinoceros (E, F); this kind of ultrasonic appearance (large oval follicle, uterine horn with oedema, slight intraluminal fluid accumulation maybe present) would indicate oestrus and the correct timing for AI with fresh semen. CL, corpus luteum; EM, endometrium; Fo, pre-ovulatory follicle; { = myometrium; → = fluid within uterine lumen; sizes normalised to bar (= 1 cm).

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In Asian elephants, at least one successful conception has occurred following frozen–thawed semen AI (Thongtip *et al.* 2009), although this did not result in the birth of a live calf. However, results using fresh semen AI are more encouraging and >40 Asian and African elephant calves have been born worldwide (Saragusty *et al.* 2009) using a non-surgical endoscopic AI method whereby semen is placed intravaginally (Brown *et al.* 2004).

The first living white rhinoceros' calves after successful AI with fresh semen were reported in 2007 (Hildebrandt *et al.* 2007), and frozen–thawed semen has since successfully been used in white (Hermes *et al.* 2009*b*) and Indian rhinoceroses (Stoops *et al.* 2016). However, the exact number of white and Indian rhinoceros calves born following AI is not known, and we estimate only a handful worldwide. No reports of successful AI in black, Sumatran or Javan rhinoceros were found by the authors.

Timing of AI

The detection of oestrus and prediction of ovulation is crucial for successful AI particularly with frozen semen. Knowledge on the longevity of spermatozoa in the female tract is also helpful although this is lacking in the elephant and rhinoceros.

Behavioural observations, endocrine measurements and ovarian ultrasonography (Fig. 5) all play a role in oestrous detection in horses, elephants and rhinoceroses. Similar to the mare, female rhinoceroses may urinate small volumes, lift the tail and accept the sexual behaviour of males by showing a standing reflex which allows mounting (Jenikejew *et al.* 2021). However, behavioural signs may be misleading in captive animals, and even in the domesticated horse, the absence of social cues can make detection of oestrus difficult.

Non-invasive monitoring of faecal or urinary progestagen (P4) levels may retrospectively define when oestrus has occurred. For elephants and rhinoceroses, ovulation takes place around 3 to 1 days (Brown *et al.* 2001, Lueders *et al.* 2010*b*) and at least 1 week prior to P4 rising from the baseline (Stoops *et al.* 2004).

Another endocrine monitoring option is the determination of the LH peak which, in rhinoceroses and elephants, occurs approximately 24 h prior to the ovulation as opposed to the mare, where ovulation frequently takes place up to 24 h before LH peaks (Fig. 3). However, daily collection of serum is required to measure LH in elephants (Brown *et al.* 1999, 2001) and rhinoceroses; in the latter species, urine LH determination alone is also an option (Stoops *et al.* 2004). Since elephants express two quite exactly spaced LH peaks, the identification of the first, anovulatory LH surge allows prediction of timing of the second, ovulatory LH surge 19–21 days later and therefore of ovulation (Brown *et al.* 1999, Thitaram *et al.* 2008, Hildebrandt *et al.* 2012). The ability to measure LH in elephants is, in part, the reason for the relatively good success rate following AI. If only LH determination is feasible in the training routine but not ultrasound, the best results are obtained using fresh semen AI on the day of the LH peak, since fresh semen is better placed into the vagina/uterus well in advance to ovulation (I. Lueders, personal observation). In both rhinoceroses and elephants, a commercial in-house serum LH snap test for cats and dogs (Witness®LH, Zoetis) may be used with qualitative results within 20 min. In a report of successful frozen–thawed semen AI in an African elephant, this in-house LH test was performed every 12 h to optimise the timing (Hildebrandt *et al.* 2012). Daily inseminations for 2–4 days around the day of ovulation with fresh or frozen semen have resulted in pregnancies in elephants (Brown *et al.* 2004*a*, Thongtip *et al.* 2009, Hildebrandt *et al.* 2012). One may speculate that similar to the mare, frozen–thawed semen AI in elephant and rhinoceros cows may yield the highest chances of success when the semen in placed no earlier or later than 6 h prior to or after ovulation.

In the mare, the standard procedure to judge the timing for insemination is monitoring follicular development during oestrus via transrectal ultrasound and, since most mares are easily restrained and tractable, this may be repeated daily, or more frequently when frozen semen is used, to measure the size of the pre-ovulatory follicle. Fresh semen AI in mares is usually performed when a follicle of >3.5 cm is present on an ovary in combination with uterine oedema (Fig. 5A and B). Ovulation inducing drugs (e.g. GnRH agonists or human choronic gonadotrophin (hCG)) are usually administered to ensure ovulation occurs some 36–40 h later. The timing of fresh semen AI within this time window is often operator preference and is, in part, dictated by the availability and/or expected longevity of the semen (Aurich 2012).

As the longevity of frozen equine semen is decreased, AI is usually undertaken as close as possible to ovulation. The increasing use of low doses of frozen semen has led to most mares being inseminated post-ovulation, with semen deposition at the tip of the horn ipsilateral to ovulation.

For precise determination of follicular development and ovulation for AI in rhinoceroses and elephants, daily ultrasound is the only feasible option in trained pachyderms in captivity. Like in the mare, the pre-ovulatory follicle changes shape from round to more

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flaccid (Fig. 5A, C and E), and obvious uterine tone (convoluted uterine appearance) and endometrial oedema (Fig. 5B, D and F) sometimes with intraluminal fluid accumulation occur close to ovulation. Fresh semen AI has been performed when a dominant follicle was >2.0 cm in Asian and African elephants (Brown *et al.* 2004*a*, Thongtip *et al.* 2009) and >3.5 cm in white rhinoceroses (Hermes *et al.* 2009*b*).

Control of the oestrous cycle via exogenous hormones has not yet been described for elephants and appears challenging given the two LH peaks necessary to prepare the ovary with sufficient luteal structures to maintain pregnancy and with the first LH peak needed to provide inhibin secretion from the LUFs. An attempt to induce the second LH peak by administering a GnRH agonist intravenously during the late, anovulatory follicular phase, resulted in a low non-ovulatory LH peak and a natural, second LH peak and/or ovulation 15–22 days later (Thitaram *et al.* 2008).

In rhinoceroses, particularly white rhinoceroses, oestrous induction or synchronisation may be carried out with a combination of oral progestagen supplementation for at least 30 days followed by GnRH injections or implants (Hermes *et al.* 2006, Pennington & Durrant 2018, Roth *et al.* 2018).

Practicalities of AI

During natural mating in elephants, the semen is deposited intra-vaginally, whereas in the mare most of the ejaculate ends up in the uterus, even though the tip of the stallion penis does not penetrate the cervix. In the rhinoceros, due to the shape of the penis and the structure of the cervix, only a fraction of the ejaculate may reach the cervical canal.

For AI, the mare's short cervix is easily passed during oestrus making intra-uterine insemination easy and straightforward. In contrast, the white rhinoceros' cervix is quite long and consists of three to five tight interdigitated folds of fibrous connective tissue leaving a very thin lumen (Fig. 1F and 2A), which can only be passed during oestrus using a species-specific semi-rigid AI-catheter to allow intra-uterine semen deposition under standing sedation or general anaesthesia. Reported sperm doses were 5800 and 10,560 × 10^6 total sperm for fresh (Hildebrandt *et al.* 2007) and approx. 500 × 10^6 motile sperm for frozen–thawed semen (Hermes *et al.* 2009*b*).

In elephants, the unique anatomical features complicate AI. Although the cervical canal is short with folds like those in the mare, the long vestibule and the hymen-like structure, which narrows the vestibule-vaginal opening to <1 cm in nulliparous females (Figs. 2C, D, E and F), have to be passed to deposit semen as close as possible to the cervix (Hermes *et al.* 2007). Initially, a flexible tube was guided through the curvature of the urogenital canal (Balke *et al.* 1988), but endoscopic guidance to visualise the vaginal os (Fig. 2C, D, E and F) with confirmation of AI catheter placement into the vagina by trans-rectal ultrasound is now commonplace (Brown *et al.* 2004, Thongtip *et al.* 2009, Hildebrandt *et al.* 2012). In African elephants, AIs with frozen–thawed semen were successful when total doses of 720 × 10^6 motile sperm/AI were used (Hildebrandt *et al.* 2012).

Surgical elephant AI has also been described, whereby an incision is made ventral of the anus into the proximal part of the vestibule through which a speculum with a light source is inserted and an equine insemination pipette guided through the vaginal os (Schmitt 2006). Bypassing the long vestibulum vaginae by directly entering the pelvic part gives straight access to the vagina and cervix at arms-length. The necessary postsurgical care of the incision wound leaves the use of this technique for routine, fresh semen AI impractical and questionable. For deep intrauterine AI with small doses of frozen–thawed semen, embryo recovery or ET, it may provide an interesting option.

Advanced art – future perspectives

Embryo recovery and transfer

In the mare, embryos are generally flushed from the uterus on day 7 or 8 post-ovulation (day 0 = ovulation). Embryos do not enter the uterus until late day 6 and rapid expansion of the blastocoele means that by late day 9 or 10, embryos are more fragile and easily damaged using commercial flushing equipment. Embryo recovery is undertaken using a sterile flexible two-way 24Fr embryo flushing catheter which is inserted through the dioestrous cervix. The uterus is infused with 1–2 L of a physiological flushing medium (e.g. lactated Ringer solution) and following gentle trans-rectal massage of the uterus, the flushing medium is recovered by gravity through an in-line embryo filter. Searching for the embryo is done using a binocular dissecting microscope. Equine embryos are spherical (200–800 µm diameter at days 7 and 8) at this stage and easily visualised. Recovered embryos can be transferred trans-cervically using a non-surgical technique into a synchronised surrogate mare with a >80% success rate (Oguri & Tsutsumi 1972, Wilsher & Allen 2004).

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The mare also boasts an unusually wide window for synchrony between donor and recipient with +1 to −5 days with respect to the donor achieving acceptable pregnancy rates (Stout 2006).

The timing of entry of the embryo into the uterus in the elephant and rhinoceros can currently only be speculated upon, so if embryo recovery, either for transfer or cryopreservation, is to progress in pachyderms, the timing of entry of the embryo into the uterus will need to be determined for these two species. As mentioned previously, in the white rhinoceros female, a clear embryonic vesicle (EV) is detectable at a similar time (15 days) to the mare (Radcliffe *et al.* 1997), although it is possible to visualise the EV ultrasonographically in some mares as early as day 9 post-ovulation. In elephants, an ultrasonographically detectable, tiny EV appeared no earlier than 42 days post-ovulation when elephants were scanned every other day (Lueders *et al.* 2010*a*, 2012).

From maturation of rhinoceros oocytes *in vitro,* we know that early embryonic development is slower than in the horse (Galli *et al.* 2007), but this may not translate to a longer oviductal transport time in this species as the timing of first cleavage, subsequent cell divisions and oviductal transport varies between species in general. Trials for embryo flushing would probably need to take place on days 7 and 8 post-ovulation in the rhinoceros and not before day 14 post-ovulation or potentially even later in elephants.

In pachyderms, close and repeated monitoring of the reproductive cycle and/or hormonal manipulation to synchronise recipients will only be practical in captive animals. In the rhinoceros family, different cycle lengths, the occurrence of long and short oestrous cycles in white rhinoceros individuals (Pennington *et al.* 2019) as well as endocrine differences, complicate the development of reliable and universal synchronisation protocols. In the elephant, the few cycles that occur throughout a year and two LH peaks with an exact timing make synchronisation with exogenous hormones challenging to say the least. However, the double LH peak in elephants does on the other hand allow for accurate timing of natural heat and ovulation, meaning that cryopreservation of recovered embryos would appear to be a more promising option, with subsequent transfer to a recipient elephant that has ovulated following a natural oestrous cycle.

Embryo transfer equipment for rhinoceros cows could involve using Wilsher forceps developed for the non-surgical mare ET (Card 2018) to fixate and straighten the cervix and a specially designed semi-rigid catheter to overcome the long folded cervix through which a smaller diameter ET catheter may be passed to dispose fresh or frozen–thawed embryos. In the elephant, a minimally invasive access through the cranial part of the vestibulum as described previously for AI (Schmitt 2006) may be an option to ensure access to the cervix and placement of the embryo into the ipsilateral horn of ovulation.

Oocyte recovery and ovum pick-up (OPU)

In the mare, oocytes can be harvested from ovaries recovered *post-mortem* (genetic salvage). This method has been used to good effect to produce live offspring in the horse and protocols for recovery and transport of ovaries are well established. For example, Hatzel *et al.* (2021) reported a 19% blastocyst rate from 620 oocytes that reached metaphase II from 1524 oocytes harvested from 168 sets of ovaries. Results are usually lower than when oocytes are recovered via follicle puncture and aspiration in live animals, because compromised mares (e.g., those with colic that are euthanised) are invariably the majority in which genetic salvage is undertaken. Likewise, *post-mortem* oocyte recovery has been reported in black and Sumatran rhinoceroses (Stoops *et al.* 2011*a*,*b*), and in the Asian (Hermes *et al.* 2007) but not in the African elephant, although the African elephant ovarian cortex and folliculogenesis have been described in detail (Stansfield *et al.* 2011) and ovarian tissue preservation has been tested successfully (Gunasena *et al.* 1998).

Oocyte recovery from live animals requires specific equipment and skills. Transvaginal OPU in the horse was first reported in the early 1990s (Brück *et al.* 1992) and has gained interest in large commercial breeding programs over the last decade (Stout 2020). Due to the lack of commercially available gonadotropins to stimulate the development of multiple preovulatory follicles, ultrasound-guided transvaginal aspiration of immature oocytes is performed in the standing sedated mare. Immature equine cumulus–oocyte complexes (COCs) in the mare are anchored to the follicle wall (Hawley *et al.* 1995), so successful recovery of oocytes requires vigorous scraping of the follicular wall and repeated flushing of the follicle with a double-lumen needle (12 gauge). Even with this approach, the oocyte recovery rates for punctured follicles are considered good if ≥50% (Galli *et al.* 2007).

OPU by laparoscopy or transvaginal ultrasound guidance is impractical in white rhinoceroses and elephants due to their body size and anatomical features, including the distance from the cranial vagina to the cranial located ovaries (1.2–1.4 m from the anus in rhinoceroses – Hermes *et al.* 2007, Hildebrandt *et al.* 2018).

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To overcome the anatomical problems of the transvaginal approach, transrectal ultrasound-guided OPU has been used with success in black and white rhinoceroses leading to the recovery of multiple oocytes (Hermes *et al.* 2007, 2009*a*). Different devices have been developed to facilitate the procedure (Hildebrandt *et al.* 2007, 2018, Hermes *et al.* 2009*a*). One device was described as 1.5 m long with a double-lumen needle system at an angle to the instrument axis, which could be guided by ultrasound through the cleaned and disinfected rectal wall into the ovarian tissue. Follicles identified ultrasonographically with a diameter of at least 1 cm (Hildebrandt *et al.* 2018) are aspirated and flushed by repeated refilling of the follicle with a commercial bovine or equine flushing media through the outer needle channel, and aspiration of fluid through the inner channel (Hildebrandt *et al.* 2018). The aspirated fluid is collected into sterile tubes and kept at 37°C with the excess passed through a nylon mesh filter before the remainder is searched for recovered oocytes with a stereomicroscope. Recovered immature oocytes are washed and placed in holding media prior to undergoing *in vitro* maturation (IVM). Recovery rates of <30% per aspirated follicle have been reported (Hildebrandt *et al.* 2018), which is lower than in the horse but may improve with further modification of the technique.

Successful OPU has not been described for elephants to date and comes with additional challenges. Similar to the rhinoceros cow, the reproductive tract of the female elephant is too large to undertake OPU as in the mare, and a vaginal approach is unfeasible due to the anatomy of the vestibulum. Therefore, transrectal, ultrasound-guided follicle aspiration would be the method of choice using a similar set-up to that described for rhinoceroses (Hildebrandt *et al.* 2018). OPU in elephants may be complicated by the thick rectal wall, the ovarian bursa covering the ovary (Fig. 2B) and the small size and number of antral follicles (1–15 mm), which are only present during the follicular phase prior to and between the two LH surges (Lueders *et al.* 2010*b*).

Superovulation protocols as used in cattle, but ineffective in the mare, may be worth investigating to potentially produce more/larger follicles for aspiration. In one report, white rhinoceroses were treated with a GnRH analogue to boost follicle growth. Intramuscular injections of 3.0 mL Histrelin (0.5 mg/mL BioRelease®, Bet Pharm LLC) were given every other day three to four times and OPU performed 24 or 48 h after the last injection (Hildebrandt *et al.* 2018). Superovulation protocols for elephants have not been considered and it is not known which exogenous hormone would stimulate elephant follicular development.

In vitro maturation (IVM)

In humans, where adequate superstimulation and superovulation allow for the collection of mature oocytes, IVM is not routinely used. However, in the mare, IVM of oocytes is the first step required prior to undertaking IVF or intra-cytoplasmic sperm injection (ICSI) and the production of an embryo. With the significant increase in the popularity of ICSI in horses and, hence, the need for IVM (Claes & Stout 2022), commercial IVM media are now available and may prove suitable for the rhinoceros or elephant. In the horse, shipping of immature oocytes collected via OPU at 20–22°C to specialised ICSI centres can be easily accomplished. One review gives the average maturation rate of equine oocytes shipped to an ICSI lab as 59% (Claes & Stout 2022), which usually involves culture for 24–30 h for viable oocytes to reach metaphase II (attain nuclear maturation).

Developing effective protocols and maturation media for IVM in new species, like the rhinoceros, has been described as difficult, especially when there is limited availability of oocytes (Pennington & Durrant 2018). Nevertheless, cell culture media (e.g. Medium 199; Dulbecco's Modified Eagle's Medium -F12) supplemented with porcine or ovine luteinising hormone FSH, oestradiol, and insulin-like growth factor 1 (Stoops *et al.* 2011*b*), southern white rhinoceros follicular fluid (Pennington & Durrant 2018) and white rhinoceros oestrus serum (Hermes *et al.* 2009*a*, Hildebrandt *et al.* 2018, Ruggeri *et al.* 2022), have been used as IVM media. In a recent study, a media containing FSH, LH, oestradiol, somatotropin and horse follicular fluid achieved better maturation results compared to white rhinoceros oestrous serum (Ruggeri *et al.* 2022).

Rhinoceros oocytes have been typically incubated at 37.5-38.5°C in a humidified atmosphere containing 5% CO_2 for 32–44 h prior to being denuded and checked for a polar body to confirm if they had reached metaphase II (Hermes *et al.* 2009*a*, Stoops *et al.* 2011*b*, Hildebrandt *et al.* 2018, Pennington & Durrant 2018). In the few existing reports on IVM of rhinoceros oocytes, maturation rates were low (<30%) for *post-mortem* collected Sumatran and black rhinoceros oocytes after culture times of 24–32 h (Stoops *et al.* 2011*a*,*b*) and <40% for oocytes recovered by OPU from White rhinoceroses after culture times of 36–44 h (Hildebrandt *et al.* 2018).

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In the Asian elephant, IVM of *post-mortem* collected oocytes with a success rate of 60% was reported (Hermes *et al.* 2007).

IVF and ICSI

IVF and ICSI of oocytes for embryo production have been described with success in many species including the horse (Hinrichs 2018).

In the horse, ICSI as opposed to IVF is the method of choice for *in vitro* production of embryos, as only very recently has conventional IVF produced efficient and repeatable results following prolonged sperm incubation to induce its capacitation (Felix *et al.* 2022). IVF has been attempted in black and white rhinoceroses for more than a decade, but to date no blastocysts have been produced (Pennington & Durrant 2018). In black rhinoceroses, IVF of *post-mortem* collected oocytes with frozen–thawed epididymal semen collected *post-mortem*, resulted in a two-cell embryo (Stoops *et al.* 2011*b*), while a four-cell embryo was obtained from an oocyte collected via trans-rectal OPU fertilised with fresh, chilled semen (Hermes *et al.* 2009*a*).

After maturation, COCs were rinsed and incubated in a humidified atmosphere of 5% CO_2 in air for 1–4 h at 39°C or for 6.5–48 h at 38.6°C before co-incubation with sperm in IVF media (synthetic oviduct fluid supplemented with heparin (20 mg/mL) and 5% (white) rhinoceros oestrous serum (Hermes *et al.* 2009*a*)) or IVF–TALP (Stoops *et al.* 2011*b*), respectively. Homologous and heterologous (Indian rhinoceros) frozen–thawed sperm were capable of binding, penetrating and fertilising black rhinoceros oocytes recovered from *post-mortem* ovaries (Stoops *et al.* 2011*b*).

Results from ICSI in horses do vary between laboratories, but laboratories with skilled technicians can produce good results. For example, from a total of 515 OPU session in 2021, in which recovered oocytes were shipped overnight to a specialist laboratory, there was a 78% chance of obtaining one or more blastocysts per session following ICSI, with a mean of 2.1 embryos per session. Approximately 60% of transferred embryos result in a live foal (Claes & Stout 2022).

In rhinoceroses, ICSI was performed by injecting a single-frozen–thawed motile sperm of northern and southern white rhinoceroses, with normal morphology and selected through the swim-up procedure, into the cytoplasm of southern white rhinoceros' oocytes using a piezo-driven micromanipulator (Hildebrandt *et al.* 2018). COCs were treated with hyaluronidase and the cumulus cells were mechanically removed after 36 h of culture prior to ICSI. Injected oocytes were cultured in a modified SOF medium supplemented with BSA and MEM amino acids until blastocyst development after 9–12 days. Of 32 injected oocytes, 12 underwent cleavage and 7 developed to blastocysts (Hildebrandt *et al.* 2018).

Oocyte and embryo cryopreservation

Although live offspring have been produced from cryopreserved oocytes in the mare (De Coster *et al.* 2019), blastocyst rates remain poor and the technique is not routinely undertaken in clinical practice.

Oocyte cryopreservation in rhinoceroses and elephants is likely to be equally challenging, and current protocols are insufficient for routine application (Pereira & Marques 2008). African elephant ovarian tissue has been successfully cryopreserved (Gunasena *et al.* 1998), but to date oocytes have only been obtained at *post-mortem*. Although it was recently reported that *intravitam*-aspirated oocytes of rhinoceroses were frozen using a conventional freezing method (Pennington & Durrant 2018), information on their post-thaw quality and viability has not been published.

Embryos, although sensitive to chilling, freezing and thawing injury (Pereira & Marques 2008), offer more prospect as a means to cryopreserve genetics than oocytes do. Historically, embryo cryopreservation in the horse was problematic. The reasons typically cited for this were the late entry of the embryo into the uterus when it has already begun to blastulate, the quick development of a large blastocoele cavity and inhibition of the passage of cryoprotectants by the equine capsule. Hence, only morulae or early blastocysts ≤300 µm were originally a prospect for slow freezing or vitrification with any degree of success (Eldridge-Panuska *et al.* 2005). More recently, considerable success has been achieved in vitrifying larger embryos by micro-manipulator-assisted or manual puncture of the blastocoel cavity and its collapse (Wilsher *et al.* 2019, 2021) with pregnancy rates upon warming and transfer only marginally below or equivalent to those reported for transfer of fresh embryos. For *in vivo*-produced equine embryos, vitrification would appear to be the method of choice, whereas *in vitro* (ICSI)-produced ones, with their smaller blastocoele cavity and poorly developed capsule, have excellent survival rates when slow freezing or vitrification methods are used (Claes & Stout 2022, Spanner *et al.* 2022).

To date, no early elephant embryos have been recovered/produced and thus, their cryopreservation

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properties remain unknown. However, in recent years, equine reproduction specialist Dr Cesare Galli succeeded in producing white rhinoceros blastocysts from *in vivo* derived oocytes and frozen–thawed rhinoceros semen which were subsequently cryopreserved using equine-based protocols (Hildebrandt *et al.* 2018). Blastocysts with a clear detectable inner cell mass (11–13 days after successful ICSI), were cryopreserved in 0.25 mL straws after equilibration in media containing glycerol (5% for 5 min followed by 10% for 10 min). A slow-freezing vitrification protocol developed for cattle and horse embryos was followed using a freezing machine that allowed a controlled freezing curve to be achieved, prior to their long-term storage in liquid nitrogen (Hildebrandt *et al.* 2018).

The recovery of *in vivo*-derived embryos from both rhinoceroses and elephants will require the development of embryo recovery techniques in these species. The challenge in doing this lies with catheterising the cervix and, as previously mentioned, determining the day of entry of the embryo into the uterus. If these hurdles can be overcome, the technique should, in theory, be achievable. Furthermore, given the spherical nature of the rhinoceros and elephant embryonic vesicle, it is interesting to speculate that an embryonic capsule may also be present in these species. If this is indeed the case, the work already undertaken in the horse to successfully cryopreserve capsulated embryos could potentially be feasible for rhinoceros and elephant embryos, too.

Tissue and stem cell preservation and cloning

Cryopreservation of ovarian tissue has been suggested as an alternative for oocyte and embryo preservation, since primordial follicles respond better to the challenges of cryopreservation and therefore more generic protocols appear to be successful in freezing ovarian tissue (Gunasena *et al.* 1998, Santos *et al.* 2010). In African elephants, pieces of ovarian tissue (1–2 cm^3) were preserved under field conditions and xenografted into mice where antral follicles developed in the elephant ovarian tissue graft (Gunasena *et al.* 1998).

In vitro culture of fibroblasts from tissue can be used to establish and cryopreserve somatic cell lines and establish induced pluripotent stem cells (iPSCs), which has been achieved for the northern white rhinoceros (Ben-Nun *et al.* 2011, Saragusty *et al.* 2016*a*, Hildebrandt *et al.* 2018, Korody *et al.* 2017). Like embryonic stem cells, iPSCs have the potential to differentiate into gametes, which could then be used for IVF or ICSI (Saragusty *et al.* 2016*a*). In white rhinoceroses, stem cell derivation from embryos (Hildebrandt *et al.* 2018) and the differentiation of spermatogonial stem cells, isolated from testicular tissue, into mature spermatozoa, have been reported (Gomez *et al.* 2018). Cloning by interspecies somatic cell nuclear transfer (iSCNT), is especially interesting for (functionally) extinct species, like the northern white rhinoceros or the mammoth (Saragusty *et al.* 2016*b*). iSCNT was successfully used to produce blastocysts from Asian elephant's fibroblast cell lines, established from ear tissues and porcine oocytes. However despite containing the elephant genome, when transferred into sows these embryos appeared non-viable (Nguyen *et al.* 2022) and it appears questionable if these blastocysts were truly elephants.

Conclusion

Although the complex problems wild elephant and rhinoceros populations are facing cannot be solved with ART alone, these techniques certainly have a role to play in contributing to captive breeding programmes. Main advantages of implementing these techniques are the ability (i) to extend the individual reproductive lifespan by gamete preservation, (ii) to enhance genetic diversity by introducing individuals that are not reproducing naturally and (iii) to exchange genetics without moving entire animals which may come with welfare issues and disease risks.

In horses, ART has advanced tremendously in recent years and rhinoceros and elephant ART are likely to profit from the establishment of new protocols and the successes in equids. Basic applications, such as semen collection and preservation, and AI are currently applicable to elephants and rhinoceroses, whereas more advanced methods need further research. The recent achievements in rhinoceros OPU, oocyte maturation, fertilisation and embryo cryopreservation show that even in large pachyderms, these techniques can be successfully implemented. Although we are far from achieving consistent results, in white rhinoceroses the mare has served as a model, specifically for laboratory protocols. In elephant advanced ART, we may need to overcome additional obstacles.

Problems with availability of study animals, the handling of large, and thus potentially dangerous mammals, and sufficient funding remain challenging. Clever adaptations to routine equine protocols and equipment are necessary and can be feasible, as discussed and shown in this review. We need to attract a larger community and colleagues from the domestic animal

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reproductive disciplines to assist in this elephant-sized task.

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