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# Assisted Reproduction in Female Rhinoceros and Elephants – Current Status and Future Perspective

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## Contents

Over the last few decades, rhinoceroses and elephants became important icons in the saga of wildlife conservation. Recent surveys estimate the wild Asian (*Elephas maximus*) and African (*Loxodonta africana*) elephant populations to be, at most, 50 250 and 637 600 respectively. For the five rhinoceros species, black (*Diceros bicornis*), white (*Ceratotherium simum*), Indian (*Rhinoceros unicornis*), Javan (*Rhinoceros sondaicus*) and Sumatran rhinoceros (*Dicerorhinus Sumatrensis*), the population estimates of 3610, 11 330, 2400, 60 and 300, respectively, are of even greater concern. Protected against habitat loss, poaching and left undisturbed, rhinoceros and elephants reproduce well in the wild. But small and decreasing populations make successful captive management of these taxa increasingly important. In captivity, however, most populations face possible 'extinction' because of historically poor reproductive performance.

From the first descriptions of the reproductive anatomy and the oestrous cycle (Laws 1969; Kassam and Lasley 1981; Balke et al. 1988a,b; Plotka et al. 1988; Godfrey et al. 1991) to the present use of advanced assisted reproduction technologies, researchers have strive to understand the function and dysfunction of the reproductive biology of these charismatic species. This paper reviewed the current knowledge on rhinoceros and elephant reproduction biology, reproductive cycle, gestation, dystocia, reproductive pathology, oestrous induction and artificial insemination, sperm sexing, IVF and contraception, and how this knowledge is or might be used to aid species conservation for maximal reproductive efficiency and enhancement of genetic management.

## Reproductive Biology

The elephant has the longest reproductive tract of any land mammal, 3 m from vestibule to ovary (Laws 1969; Balke et al. 1988a,b; Niemuller et al. 1998). In the rhinoceros, the reproductive tract length ranges between 0.8 and 1.5 m depending on the species (Godfrey et al. 1991). The size and topography of the reproductive organs has, for decades, hampered man's ability to evaluate the reproductive fitness and develop ART, like artificial insemination in elephants and rhinoceros (Hildebrandt and Schnorrenberg 1996; Hildebrandt et al. 1999). Today, transrectal ultrasound techniques are accustomed to enable longitudinal evaluations (Hildebrandt et al. 1997, 2000a,b,c, 2003a; Hildebrandt and Göritz 1999; Hermes et al. 2000). The unique anatomical features of the female elephant are the long vestibule and the permanent hymenal membrane in nulliparous females with the small vaginal os having two blind pouches of similar size on either side of the vaginal opening. Unique anatomical features of the rhinoceros are the hymeneal membrane, used as evidence of previous successful mating activity in aged animals during reproductive assessments (Hermes et al. 2006) and the long, convoluted cervix with extremely tight folds of the connective tissue (Godfrey et al. 1991).

## Reproductive Cycle

Despite being one taxon the oestrous cycle in rhinoceros is quite diverse. Oestrogen and progesterone profiles measured in the faeces, urine and serum using various hormone assays characterized the oestrous cycle with different cycle lengths in each rhinoceros species: 21–25 days in the Sumatran (Heistermann et al. 1998; Roth et al. 2001), 27 days in the black (Schwarzenberger et al. 1993, Berkeley et al. 1997, Brown et al. 2001; Lance et al. 2001; Radcliffe et al. 2001; Garnier et al. 2002), 30–35 or 65–70 days in the white (Hindle et al. 1992; Radcliffe et al. 1997; Schwarzenberger et al. 1998; Patton et al. 1999; Brown et al. 2001) and 43–48 days in the Indian rhinoceros (Kassam and Lasley 1981; Schwarzenberger et al. 2000; Gomez et al. 2004; Stoops et al. 2004). The reproductive cycle length seems to increase almost by the species size, the Sumatran being the smallest and the Indian being the largest. Individual differences of the cycle length occur in every species being most pronounced in the Indian rhinoceros, with recorded extremes ranging from 36 to 86 days. While the oestrous cycle is subject to individual variations in the Sumatran, black and Indian rhinoceros, the white rhinoceros exhibits more puzzling specifics. Two different oestrous cycle lengths were recorded in the white rhinoceros, of which only the shorter 30–35 days cycle is believed to be fertile. The longer cycle, which has yet not resulted in a pregnancy, is discussed as being associated with reproductive ageing in older females (Schwarzenberger et al. 1998; Patton et al. 1999; Brown et al. 2001). More troubling is the high incidence of white rhinoceros with long periods of anoestrous. Half of the females are determined in various studies to remain acyclic. Anoestrus is present in young and aged females (Brown et al. 2001; Hermes et al. 2006). Concurrent with endocrine data, ultrasound and morphological findings revealed that the anoestrous is associated with different, age-dependent ovarian activity. Young anoestric females exhibit regular follicular waves. Yet ovulatory-sized follicles do not ovulate but become atretic or form haemorrhagic follicles, which is also reported for the other species (Radcliffe et al. 1997, 2001; Roth et al. 2004; Stoops et al. 2004). In aged females, all follicular development ceased completely (Hermes et al. 2004, 2006). Anecdotal reports that the transport of females to other facilities or the introduction of new males initiated regular oestrous cycle activity (Patton et al. 1999, Fchwarzenberger, personal communication) indicate that the impact of behavioural aspects to initiate or resume the oestrous cycle activity are underestimated. All scientific data support the conclusion that anoestrous in young females is primarily the result of deficient animal husbandry/management, which fails to provide the behavioural needs to initiate the regular oestrous cycle activity. Over decades, this ultimately leads to depletion of oocyte, resulting in a secondary cessation of reproductive activity and premature senescence in mid-aged females (Hermes et al. 2004, 2006).

Ovulation in the rhinoceros, like in most mammals, is induced by a single, pre-ovulatory LH surge that occurs at the end of the follicular phase reported for the Indian and Sumatran species (Roth et al. 2001; Stoops et al. 2004). The Sumatran rhinoceros, different from the other species, is additionally described as an induced ovulator. In the Sumatran rhinoceros, ovulation following the LH surge is triggered when the female is mated and the vagina and cervix are mechanically stimulated (Roth et al. 2001). Similar to the diversity of the oestrous cycle length, the dimension of the ovulatory follicle deviates considerably between species measuring 20–25 mm in the Sumatran, 30–34 mm in the white, approximately 50 mm in the black and 120 mm in the Indian rhinoceros, the largest Graafian follicle reported in a mammal (Radcliffe et al. 1997, 2001; Roth et al. 2001, 2004; Stoops et al. 2004). Luteinization and formation of haemorrhagic follicles, which exceed the dimensions of the species-specific Graafian follicle by 10–30 mm, are known to occur in all four rhinoceros species. Except for the Sumatran rhinoceros as induced ovulator, these anovulatory structures are regarded as non-physiological. In the wild rhinoceroses are described as polyoestric, non-seasonal breeders (Garnier et al. 2002; Kretzschmar et al. 2004). The incidence of anovulatory and haemorrhagic follicles in the captive black and Indian rhinoceros population and possible consequence on the reproductive rate is yet unknown. However, in the white rhinoceros, these are common findings in young anoestrous females (Hermes et al. 2006).

With 13–17 weeks, the elephant exhibits the longest spontaneous oestrous cycle of any mammal studied to date. The luteal phase of 8–10 weeks is followed by a 4- to 7-week follicular phase with no differences between species (Hess et al. 1983; Plotka et al. 1988). The major circulating luteal steroid to describe the oestrous cycle in elephants is 5$\alpha$-reduced pregnanes (Heistermann et al. 1997; Hodges et al. 1997; Meyer et al. 1997; Schwarzenberger et al. 1997; Hodges 1998; Dehnhard et al. 2001). To date, measurements of circulating oestradiol have not reliably reflected the follicular activity. Only recently, urinary oestrogen patterns confirmed the presence of two follicular waves during the non-luteal phase, which were formally identified by repeated ovarian ultrasound (Hermes et al. 2000; Czekala et al. 2003).

Regarding the LH, the elephant is quite unique among the mammals, exhibiting two, quantitatively and qualitatively similar, precisely timed LH surges during the follicular phase. The first surge is observed within 12–21 days after progestins decline to baseline at the end of the luteal phase, and the second surge occurring 19–22 days later (Kapustin et al. 1996; Brown et al. 1999a,b). Only the second LH surge induces ovulation with ovulatory follicles measuring relatively small 20 ± 2 mm compared to the elephants body mass (Hermes et al. 2000; Hildebrandt et al. 2000a). Serial ultrasound studied the influence of the hormonal cycle on the morphological appearance of the endometrium (Hildebrandt and Göritz 1999; Hermes et al. 2000). Under the influence of oestrogens during the late follicular phase, the endometrium becomes enlarged (35.4 ± 2 mm). In the late luteal phase, the endometrium begins to decrease (23.7 ± 2 mm) due to the lack of embryonic signals in a nonpregnant female. During the early follicular phase, the endometrium regresses to the smallest measured thickness (17 ± 1.5 mm) due to a lack of oestrogens and progestins (Hermes et al. 2000). The non-ovulatory LH peak in the mid-follicular phase has little to no effect on the thickness of the endometrium, in contrast to the dramatic changes on the ovaries. These findings indicate that attempts to conceive during the first follicular wave/first anovulatory LH surge are futile because of the lack of endometrial development, which would prevents implantation. Different reasons are discussed to explain this yet unmatched mechanism of one anovulatory and one ovulatory LH surge. One claims it is an elephant-specific mating strategy to ensure reproduction occurs. With the longest interbirth interval from 3 to 7 years oestrus is a rare event in elephants. The first anovulatory LH surge is to attract males as an early advertisement of the impending oestrus. Others hypothesize that it serves as an endocrinological filter and ovarian function to ensure only a single follicle ovulates minimizing the risk of twin pregnancy, a rare event in elephants (< 1%) (Niemuller et al. 1998; Hermes et al. 2000).

## Gestation

The gestation period of rhinoceroses is the second longest in mammals after the elephant ranging from 15 to 18 months. Pregnancy can be diagnosed by elevated progesterone concentrations 3–5 months after conception (Ramsay et al. 1987; Hodges and Green 1989; Schwarzenberger et al. 1993, 1996, 2000; Czekala and Callison 1996; Berkeley et al., 1997; Patton et al. 1999; Brown et al. 2001; Lance et al. 2001; Roth et al. 2004) or by ultrasound 2–4 weeks post-breeding (Adams et al. 1991; Radcliffe et al. 1997; Roth et al. 2001, 2004). The embryonic vesicle is detectable by ultrasound as early as 15 days post-ovulation (Radcliffe et al. 1997).

Early embryonic loss has been reported in three rhinoceros species (black: Berkeley et al., 1997, white: Radcliffe et al. 1997; Patton et al. 1999, Sumatran: Roth et al. 2001, 2004). Early pregnancy loss which is associated with uterine inflammation or pyometra in individual cases (Radcliffe et al. 1997; Patton et al. 1999; Roth 2006) has been discussed as one of the major reasons for reproductive failure in captivity. But a recent study on the assessment of the reproductive activity in black rhinoceros in the wild strongly suggested the occurrence of embryonic loss and abortion also in free ranging animals (Garnier et al. 2002). This placed embryonic loss, abortion and stillbirth in rhinoceros (Schwarzenberger et al. 1996; Berkeley et al. 1997, Hildebrandt et al. 2007a,b) in the perspective of an occasional individual reproductive failure, rather then a fundamental captive population management problem. Luteal insufficiency has been a suspected cause in animals with a history of embryonic resorption in captivity. One black and one Sumatran rhinoceros supplemented with synthetic progestin 1st subsequently carried their pregnancy to term (Berkeley et al., 1997; Roth et al. 2004). But whether luteal deficiency is occurring or whether the used synthetic progestin truly has a supplementing effect is controversial. When progesterone profiles during a normal and a supplemented pregnancy were compared in one Sumatran rhinoceros, no difference was detected (Roth 2006). These results question the existence of luteal deficiency in rhinoceros in general or at least the effect of the used synthetic progestin, which previously failed to initiate a response during oestrous induction attempts.

Elephants have the longest gestation period, lasting 20–23 months on average. In comparison to other terrestrial mammalian species, early embryonic development appears delayed in elephants (Hildebrandt et al. 2007a,b). During weeks 1–8, the ultrasonographic detection of embryonic structures is impossible. It is only after a transient progesterone drop in weeks 8–9 post-conception and subsequent recovery that an embryonic vesicle becomes detectable. The developing embryo doubles in size (crown-rump-length approximately 60 mm at 120 days and 120 mm at 150 days) between the fourth and the fifth month of pregnancy. Organogenesis is completed at 12 months of age, when the foetus reaches a length of approximately 400 mm. At that time, fetal sex determination is possible in Asian elephants by measuring the maternal testosterone levels in the peripheral maternal blood serum (Duer et al. 2002).

Luteal activity and pregnancy in the elephant can be monitored by analyzing $5\alpha$-reduced pregnanes in the blood or their relevant urinary and faecal metabolites (Heistermann et al. 1997; Hodges et al. 1997; Schwarzenberger et al. 1997; Hodges 1998; Fiess et al. 1999). There is a broad range of individual variation in gestation length (Asian, 623–729 days; African, 640–673 days) (Meyer et al. 2004). Prediction of parturition can be made on the basis of a drop in progestins in blood or urine that occurs 2–5 days before birth (Brown and Lehnhardt 1995; Carden et al. 1998; Fiess et al. 1999; Meyer et al. 2004). Recently, a quick analytical method that combines headspace solid-phase microextraction (SPME) and gas chromatography-mass spectrometry (GC/MS) for the analyses of luteal phase-specific urinary volatile $5\alpha$androst-2-en-$17\beta$-ol has been used to predict parturition in Asian elephants (Dehnhard et al. 2003). Concentrations of $5\alpha$androst-2-en-$17\beta$-ol began to drop approximately 5 days before parturition, and decreased to baseline levels on the day of parturition. One advantage of this method is its rapidity in generating results 4 h after sample arrival. One drawback with urine is that extremely low creatinine concentrations make a reliable parturition prognosis difficult.

## Dystocia

So far, dystocia has been reported only in the elephant where it appears to be more common in the Asian species (Dittrich 1985; Murray et al. 1996; Schaftenaar 1996; Foerner 1999; Lange et al. 1999, Olson et al. 2004, Hildebrandt et al. 2003b). Dystocia in the elephant ; Olson 2004can be associated with the long-term retention of the dead foetus until it is naturally expelled months or years later (Foerner 1999; Schaftenaar et al. 2001; Olson 2004). A high percentage of over 50% dystocia cases occurs in primiparous females $>20$ years of age. Dystocia results in stillborn calves, and in approximately 5% of the cases in the death of the cow due to retained conceptus and intoxication (Schaftenaar 1996; Lange et al. 1999; Hildebrandt et al. 2003b). The causes for the foetal retention in the elephant are unknown. Uterine inertia due to hypocalcaemia is a suggested cause for the initial failure of the second stage labour to progress, but there are no reports on serum calcium concentrations prior to or throughout parturition, neither for dystocia cases. Other additional causes for dystocia suggested are the intensified captive elephant breeding 1st programmes and the involvement of older but nulliparous females. Moreover, the endotheliotropic elephant herpes virus (EEHV), identified as one of the most common causes of foetal death and stillbirth in Asian elephants in captivity, contributes to the increasing number of dystocia cases (Richman et al. 1999, 2000; Fickel et al. 2001; Montali et al. 2001).

Episiotomy and caesarean section have been described as surgical methods of foetal delivery (Schaftenaar 1996; Lange et al. 1999; Olson 2004). Yet, all seven caesareans sections reported to date have resulted in the death of both the female and the calf (Hildebrandt et al. 2003b). Pore wound healing in the elephant as a common complication of any surgical procedure; a thick abdominal wall and the enormous intestinal weight and pressure on the post-surgical wound are fatal combinations when attempting abdominal surgery in the elephant. While small surgical accesses to the abdomen have shown to heal successfully, a large incision, as it is necessary to extract an 80–160 kg foetus, has been fatal (Hildebrandt et al. 2003b; Olson 2004; Stetter et al. 2005).

Repeated transrectal massage of the vagina combined with a preceding transrectal or dermal application of oestrogens 1st is regarded as the first method of choice to further stimulate ceased labour activity and to achieve cervix dilatation (Lange et al. 1999; Schaftenaar et al. 2001). The manual massage stimulates pelvic receptors and induces strong labour activity. Very different from the domestic animals, the use of oxytocin to enhance labour activity is very critical in the elephant. The elephant uterus seems hypersensitive to oxytocin, showing strong reactions, fast exhaustion with complete irresponsiveness. In at least three cases the administration of oxytocin resulted in uterus rupture because of insufficient cervix dilatation. Oxytocin should be used with care only when the foetus is present in the birth canal. Oxytocin, normally given as a bolus, may dramatically exhaust the contractibility of the uterus muscles as well as the general condition of the female. Concerns are raised that with the use of oxytocin, there might be the risk of reduced blood circulation in the umbilical chord, due to spasms in the myometrium (Schafternaar and Hildebrandt 2005; Schmitt 2006).

Episiotomy is recommended as the second method of choice for correcting dystocia in the elephant, but only if the foetal parts are present in the birth canal and frequent transrectal massage of the vagina remained insufficient to deliver the calf (Schaftenaar 1996; Lange et al. 1999; Hildebrandt et al. 2003b; Schafternaar and Hildebrandt 2005). If the episiotomy attempt fails due to the inability to extract a dead, oversized or malpositioned foetus, foetotomy remains as the last resort to save the females live (Hildebrandt et al. 2003b).

A common post-surgical complication when performing episiotomy has been a persistent vestibule fistula (Schaftenaar 1996; Lange et al. 1999; Schaftenaar et al. 2001; Schafternaar and Hildebrandt 2005). Despite different attempts to suture the different wound layers, the surgical incision never healed completely. This resulted in the general recommendation that elephant skin should not be sutured following episiotomy but instead left to heal by second intention, after the deeper tissue layers have been closed. However, a recent report on a successful wound management described an episiotomy wound, sutured in two layers, which healed uneventfully by second intention (Thitaram et al. 2006).

In general, it is a myth that pluriparous elephants do not need to be monitored and assisted during parturition. Many calves have been born dead or very weak because of the fact that parturition had started unnoticed and stopped unnoticed. Therefore, detailed guidelines for veterinary assistance around the elephant parturition have been published for the captive breeding programmes (Schafternaar and Hildebrandt 2005). Endocrine and ultrasound monitoring during pregnancy and different endocrine or behavioural criteria to predict the time of parturition are regarded equally important as the physical preparation of the female for parturition. Intensive exercise, optimization of the body weight with a pregnancy associated diet and training for safe handling in case of necessary veterinary intervention are recommended as precautions to prevent or to reduce the risk of dystocia in the elephant.

## Reproductive Pathology

So far, none of the captive elephant and none but the Indian rhinoceros propagation programmes reached the level of being self-sustaining (Olson and Wiese 2000; Wiese 2000; Wiese and Willis 2004; Foose and Wiese 2006). The consequence is that the zoo community is still dependent on imports from the range countries. Reproductive disorders are the main obstacle for successful captive management plans. However, until recently the diagnosis of reproductive disorders relied on postmortem examinations and on clinical signs, such as bloody vaginal discharge or tumour growth, which exceeded the genital opening after being already present for many years. Only the use of ultrasound adapted to the large anatomical dimensions in the larger species facilitated the *in vivo* diagnosis of reproductive pathology and evaluation of the reproductive potential (Adams et al. 1991; Schaffer et al. 1994; Radcliffe et al. 1997; Hildebrandt and Göritz 1999; Hildebrandt et al. 2000a,b,c; Roth et al. 2001; Hermes et al. 2006). In general, reproductive pathologies in female elephants and rhinos involve uterine, cervical and vaginal tumours, endometrial cysts, endometrial hyperplasia, and ovarian tumours and cysts (Schaffer et al. 1994; Hildebrandt and Göritz 1995; Brown et al. 1999b; Hildebrandt et al. 2000a, 2003a; Agnew et al. 2004; Hermes et al. 2006). Specific for the elephant are vestibular cysts and polyps with a high incidence of 70% in captive African elephants >30 years of age (Hildebrandt et al. 2006). The most dominant uterine tumour across species is the leiomyoma, but endometrial adenoma has also been documented to occur in the white rhinoceros (Hermes et al. 2006). The preferred location of leiomyomas within the genital tract is species specific. While in the white rhinoceros and the elephant these benign tumours occur mainly in the uterus, in the Indian rhinoceros, cervix and vagina are the most affected genital organs (Hermes, unpublished data). In general, there is a higher incidence of genital tract leiomyomas in the Asian variety, while cystic hyperplasia is the dominant finding in the African species (Hermes et al. 2006; Hildebrandt et al. 2006). Cystic formations, endometrial hyperplasia and neoplastic formations can become so extensive causing discomfort during oestrus and mating and preventing intromission or semen transport. Ovarian cysts occur frequently in the African elephant and less in the Asian elephant, and seem associated with acyclicity (Montali et al. 1997; Brown et al. 1999b; Hildebrandt et al. 2000b; Hildebrandt et al. 2003a). In white rhinoceros, ovarian cysts (2–15 cm) and tumours have been found to be associated with age (Hermes et al. 2004, 2006).

All these pathologies in advanced stages affect reproduction dramatically. It now is known that the incidence of reproductive disorders is positively correlated with age, and is greater in nulliparous than age-matched parous individuals (Hildebrandt et al. 2000b; Hermes et al. 2004, 2006). This phenomenon is also found in other nulliparous females of long-living species in captivity, and is an age-related consequence of long non-reproductive periods. As the reproductive pathology along with ovarian exhaustion renders the female irreversibly infertile early in her reproductive life, this phenomenon was termed ‘asymmetric reproductive ageing’ (Hermes et al. 2004).

For example, a reproducing female white rhinoceros in captivity may produce up to nine calves (Ochs 2005). This female exhibits a calculated number of approximately 90 oestrous cycles during her reproductive life. Observations from the wild confirm that pregnancy and lactation are the dominating endocrine status. With as few as 30 cycles, oestrous remains a rare event during her reproductive life (Owen-Smith 1973, 1975; Kretzschmar 2001; Garnier et al. 2002). These numbers compare with up to 310 oestrous cycles in captive, non-reproducing female rhinoceros. By the age of 16 years, non-reproducing females have displayed 90 oestrous cycles, at an age when first reproductive lesions are detected (Hermes et al. 2006). Therefore, the reproductive organs of non-reproducing female rhinos and elephants are exposed to prolonged periods of sex steroids' fluctuations from continuous ovarian cycle activity. The central effect of this asymmetric reproductive ageing process includes progressive development of genital pathology with subsequent sub or infertility and, presumably, the utilization of the follicular stock at a higher rate (Hermes et al. 2004). As the incidence of reproductive disorders in parous females is significantly lower, the achievement of early pregnancy in young animals by either natural breeding or by artificial insemination is regarded as prophylaxis to reproductive disorders (Hermes et al. 2004, 2006; Hildebrandt et al. 2006).

## Hormonal Oestrous Induction in Rhinoceros

To address the problem of anoestrous, considered as a primary cause to the low reproductive rate in the white rhinoceros, over 15 oestrous induction protocols have been attempted in three different species. Various combinations of the synthetic progestin sometimes preceded by $PGF_{2\alpha}$ and followed by different combinations of FSH, PMSG, hCG and GnRH have long failed (Godfrey et al. 1990, Patton, personal communication). Results suggested a low species-specific receptor affinity of the primarily used altrenogest. The use of another synthetic progestin, chlormadinone acetate combined with hCG seemed to achieve better receptor affinity inducing the oestrous cycle in one white rhinoceros (Schwarzenberger et al. 1998). Further use of chlormadinone acetate (0–45 days Synchrosin®) and hCG (55 days, 10 000 IU, Chorulon®) in white and recently black rhinoceros confirmed the effectiveness of this protocol to induce the oestrous cycle (Hermes et al. 2006, Bryant et al. 2007). However, a recent study evaluated the effectiveness of the protocol treating 10 animals. Although 80% of the females developed a pre-ovulatory follicle, oestrous was induced in only 30%, exhibiting increased luteal concentrations indicating ovulation. Moreover, the goal to initiate a regular oestrous cycle pattern in those females was never achieved (Schwarzenberger et al. 1998, Hermes et al. 2006, Bryant, personal communication). Two reasons are regarded as responsible for the low response rate and not lasting effect of this protocol: (i) the treatment of aged females with inactive ovaries in the state of premature senescence and (ii) an underestimated influence of behaviour and hierarchy on the reproductive activity of females.

More promising is the recent use of synthetic GnRH analogue, deslorelin acetate to induce and time ovulation in accordance with artificial insemination in young, anoestrous white rhinoceros with active follicle development. This new approach combines both close endocrine and ultrasound monitoring. Analogue to the mare, where synthetic GnRH analogue is used to induce or hasten ovulation in the transitional phase or during the breeding season (Jöchle and Trigg 1994; McKinnon et al. 1996), deslorelin was given when ultrasound identified a pre-ovulatory follicle. So far ovulation was induced in 80% of the treated females ($n = 10$) resulting in four pregnancies by artificial insemination (Hermes, unpublished data).

## Artificial Insemination

The integration of AI as assisted reproduction technique to enhance captive rhinoceros breeding programmes has been discussed and desired for over 15 years (Godfrey et al. 1991, Schwarzenberger et al. 1998, Patton et al. 1999; Roth 2006). When considering the limited breeding success of both white rhinoceros subspecies in captivity, skewed birth sex ratios in the Indian and black rhinoceros (Zschokke et al. 1998; AZA 2005), and the disastrous situation of some rhinoceros subspecies in the wild (Foose and Wiese 2006; IUCN The World Conservation Union 2006) advanced assisted reproduction seem greatly underutilized to overcome these crisis.

Anatomical challenges for the AI in the rhinoceros are the hymeneal membrane, which was present in one study in 76% of the nulliparous females (Hermes et al. 2006) and, more difficult, the firm cervix with its extreme tortuous cervical canal between large folds of dense fibrous connective tissue (Godfrey et al. 1991). Another challenge is the accurate timing of ovulation or determination of the presence of a pre-ovulatory follicle in the anoestrous female. Few rhinoceros are conditioned to tolerate daily reproductive ultrasound evaluations. Therefore, follicle growth is calculated from few ultrasound examinations during the preceding week of AI, which require repeated standing sedation (Radcliffe et al. 1997; Hermes et al. 2006; Hildebrandt et al. 2007a,b).

It was only recently that three pregnancies were achieved from non-surgical artificial insemination in white rhinoceroses, using fresh or cryopreserved semen (Fig. 1) (Hildebrandt et al. 2007a,b, Hermes, unpublished data). Ovulation was induced in previously anoestrous and one postpartum females using GnRH analogue, deslorelin at a time when a pre-ovulatory-sized follicle was present. Fresh or cryopreserved semen was deposited in the uterine horn overcoming the hymeneal membrane and torturous cervical folds with a specific patented AI catheter.

Artificial insemination, as now demonstrated with fresh and cryopreserved semen in the rhinoceros, provides reproductive physiologists and conservationist with a potent instrument to re-establish viable, self-sustainable captive populations of imminently threatened rhinoceros species and to maximize the genetic diversity when using

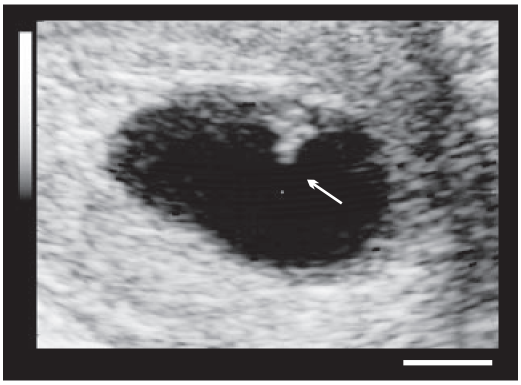

Fig. 1. Ultrasound image of embryonic vesicle (Ø 25 mm) and early embryo ( ← ) in a southern white rhinoceros on day 27 post-artificial insemination using cryopreserved sperm

unrepresented captive or wild semen donors (Hermes et al. 2005; Hildebrandt et al. 2007a,b).

For over two decades, attempts to impregnate elephants by AI were unsuccessful until the first success was achieved in two Asian and three African elephants (Hildebrandt et al. 1999; Brown et al. 2004). The description of the oestrous cycle, the unique mechanism of two LH surges, the improved ability to predict the time of ovulation accurately by ultrasound and a non-surgical insemination technique using endoscopy and ultrasound to guide semen placement were necessary innovations for these first elephant AI pregnancies (Hildebrandt and Schnorrenberg 1996; Hildebrandt et al. 1999).

The identification of the first anovulatory LH surge is instrumental to schedule artificial insemination to coincide with the second ovulatory LH surge 19–21 days later. Ultrasound, then, characterizes the pre-ovulatory follicle development and documents that ovulation has occurred. AI is ideally performed on the day of LH surge and the day after ovulation (Hildebrandt et al. 1999; Schmitt 2006).

Anatomical challenges in the elephant are the 1.5 m vestibule which leads into the vagina, the site of natural semen deposition. A hymen-like structure between vestibule and the vagina that does not rupture during mating and involutes to a diameter of < 1 cm after parturition is technically difficult for semen placement during AI. Two methods are used to overcome these anatomical challenges in the elephant, a surgical and a non-surgical method (Hildebrandt et al. 1999; Brown et al. 2004).

For the surgical AI, an incision is made below the anus accessing the proximal part of the vestibule. The hymen-like structure, thus the opening of the vagina, or the cervix is visualized with a speculum and a light source. The semen is deposited with an equine semen pipette (Schmitt 2006). Although this method is technically not as demanding, it involves 4–6 weeks of post-surgical wound management. Considering a success rate of 30–40% for each AI attempt this method is not ideal for repeated AIs over consecutive oestrous cycles (Schmitt 2006).

For non-surgical AI, a 3-m long, flexible endoscope is passed though the urogenital canal, the vestibule, to visualize the vaginal opening or cervix. Through the endoscope, a flexible AI catheter is passed into the vagina or uterus. Ultrasound verifies the correct position of the catheter and the semen deposition (Hildebrandt et al. 1999; Brown et al. 2004).

To date all pregnancies from AI derived from fresh semen collected on the day of AI (Hildebrandt et al. 1998, 2000b; Schmitt and Hildebrandt 1998). Despite the general ability to cryopreserve the African and Asian elephant sperm, frozen-thawed sperm has yet failed to produce a stable pregnancy in elephants (Howard et al. 1986; Hermes et al. 2003; Thongtip et al. 2004; Schmitt 2006).

## Sperm Sexing

In domestic species and a few wildlife species, the use of sex-sorted sperm is described as a tool to influence the sex of the offspring by using sex-biased sperm samples (Maxwell et al. 2004; Garner 2006). AI in rhinoceros and elephants now facilitates the development and implementation of protocols to use sex-sorted spermatozoa in these megavertebrates. Specifically, the potential of AI to encounter low reproductive rate in captivity was demonstrated recently in the Southern white rhinoceros. Successful AI in the anoestrous and the postpartum female overcame long non-reproductive periods, reducing the currently long inter-calving intervals to an absolute minimum of 19 months (Hildebrandt et al. 2007a,b). In rhinoceroses, the sex-sorted sperm might help to boost critically small captive rhinoceros populations by producing female offspring using X-chromosome bearing spermatozoa or to influence skewed birth sex ratios, such as in the Indian or black rhinoceros. In elephants, behavioural suppression of male fertility in facilities with multiple adult bulls and management challenges when housing multiple males ('bull-prove' housing, protected contact) could be addressed by producing predominantly female offspring.

To demonstrate the potential future role of advanced ART in conservation programmes, we modelled the impact of AI and AI with sex-sorted sperm on the population demography and survival probability of the highly endangered northern white rhinoceros, using individual based computer simulation software (VORTEX). The captive population of the critically endangered northern white rhinoceros subspecies consists of only five remaining viable breeding individuals, three males and two females (Hermes et al. 2004; Ochs 2005). In 57 years of captive management, only four captive births were recorded and in the past 22 years, only one birth occurred (Lacy 1993). With the current reproductive rate and the population size of the five potential breeders, the northern white rhinoceros population in human care is doomed for extinction. The current five reproductively sound animals of known sex and age served as starting population to calculate the population dynamics in human care in the next 50 years. Further simulation parameters for white rhinos in captivity were derived from figures in the international studbook (Ochs 2005) (Table 1). The predicted survival probability and population size as a function of time were modelled for three different scenarios: (i) conventional captive breeding with a current average inter-calving interval of 20 years, (ii) artificial breeding with an estimated average inter-calving interval of 3 years and (iii) artificial breeding using a 20/80 sex bias after 10 years. For conventional captive breeding, the survival probability of the population dropped to values below 20% and < 1% within 20 and 50 years respectively. Artificial breeding and artificial breeding with a 20/80 female sex bias increased the survival probability of the population after 50 years to 71% and 80%, respectively, thus increasing population size to numbers of 28 and 57 animals respectively. The combined application of unbiased semen samples and female biased semen for AI after 10 years proved the most promising for the population's survival rate and size. This model yielded both the highest survival probability (80%) and the largest population size (11/46 = 57 animals as opposed to 6/28 = 28 animals with artificial

Table 1. Model parameters for population viability analysis scenarios using advanced ART in northern white rhinoceros captive breeding programme

| Parameter | Captive | Artificial breed. No sex bias | Artificial breed. Sex bias 20/80 | Artificial breed. No sex bias until year ≤ 10, sex bias 20/80 (year > 10) |
|---|---|---|---|---|
| Carrying capacity | 500 | 500 | 500 | 500 |
| Inbreeding depression | Yes | Yes | Yes | Yes |
| Annual mortality, age < 1 | 14.6%* | 14.6%* | 14.6%* | 14.6%* |
| SD mortality, age ≤ 1 | 4.4 | 4.4 | 4.4 | 4.4 |
| Annual mortality, 1 < age ≤ 7 | 3.6%* | 3.6%* | 3.6%* | 3.6%* |
| SD mortality, 1 < age ≤ 7 | 1.1 | 1.1 | 1.1 | 1.1 |
| Annual mortality, age > 7 | 3.6%* | 3.6%* | 3.6%* | 3.6%* |
| SD mortality, age > 7 | 1.1 | 1.1 | 1.1 | 1.1 |
| Age of first offspring (M) | 13 | 7 | 7 | 7 |
| Age of first offspring (F) | 7 | 7 | 7 | 7 |
| Max. breeding age | 40 | 40 | 40 | 40 |
| Calving interval | 20 years* | 3 years | 3 years | 3 years |
| Sex ratio | 50/50 | 50/50 | 20/80 | 50/50 (year ≤ 10), 20/80 (year > 10) |

*Calculated according to figures in (studbook white rhinos 2001–2004).

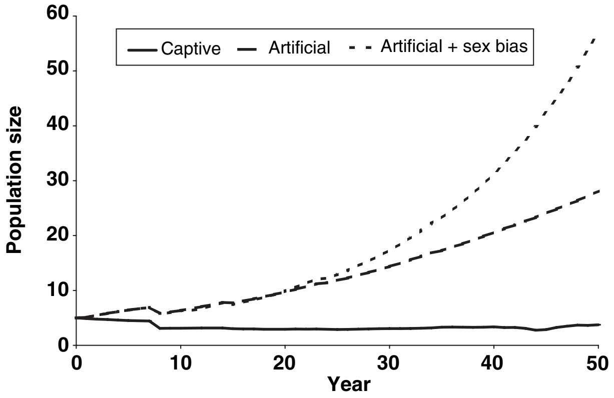

Fig. 2. Population size and growth of the northern white rhinoceros (*Ceratotherium simum cottoni*) in captivity in the next 50 years. Graphs compare natural breeding management vs assisted reproduction using AI, AI with sex-sorted X-chromosome bearing sperm after 10 years of AI with unbiased semen

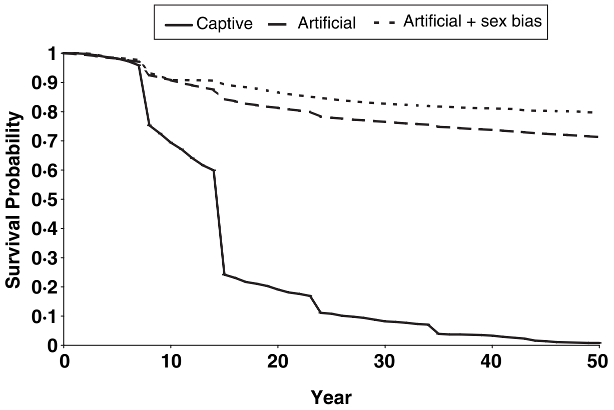

Fig. 3. Survival rate of the northern white rhinoceros population (*Ceratotherium simum cottoni*) in captivity in the next 50 years. Graphs show the impact when using natural breeding management vs assisted reproduction using AI and AI with female-biased sex-sorted sperm after 10 years of AI with unbiased semen

breeding without sex bias after 50 years (Figs 2 and 3). Obviously, the advantage of the sex bias approach becomes more prominent, the larger the population gets. While there is some doubt that captive management and natural breeding need to be further improved to increase the reproductive rates of captive white rhinoceros, this model demonstrates how AI or the use of sex-sorted sperm as additional tool to captive conservation programmes can help to recover critically small populations in captivity.

## IVF, ICSI, Embryo Transfer

IVF, ICSI and embryo transfer are well established ART in domestic species and the human for the production of embryos and as solution to infertility (Carnevale et al. 2001; Galli et al. 2002; Hardy et al. 2002). In infertile female rhinoceros and elephants with a long history of reproductive lesions, oocyte collection and *in vitro* assisted reproduction technologies represent the only remaining option to preserve the female genetic material and to contribute to the diversity of a population. The most alarming example in this regard is the captive population of the northern white rhinoceroses. Out of the seven female northern white rhinoceroses kept in captivity, five females are infertile due to asymmetric reproductive aging. These females will not contribute to the genetic pool of this extremely compromised population. Female gamete rescue from infertile animals is of tremendous importance for the survival of this rhinoceros subspecies to reduce the immense inbreeding factor. If *in vitro* technologies fail, the future population of this subspecies will be based on two related females only, mother and daughter.

One initial study reported on the successful maturation and ICSI of oocytes in the white rhinoceros (CRES, 2004). In the Asian elephant, oocyte characterization and IVM with a success rate of 60% have been shown (Jewgenow and Hildebrandt 1995, Jewgenow, personal communication). However, these results relied on gametes collected incidental at postmortems. Further development of these ART in rhinoceros and elephants will depend on the development of new technical approaches for repeated harvesting of oocytes, collected from live donors. In domestic species, repeated follicle aspiration is either performed by laparoscopy, as in small ruminants or by transvaginal ultrasound-guidance as in cattle, horse and humans. However, these techniques are non-functional in mega vertebrates due to their anatomical dimensions. The anatomical features remain the main challenge for the development of IVF, ICSI and embryo transfer in rhinoceros and elephants. The long reproductive tracts, difficult laparoscopic accessibility of the ovaries through their thick integument/abdominal wall and absent superovulation/hormone stimulation protocols hamper *in vivo* oocyte harvesting. Although laparoscopic accessibility of the abdomen has been demonstrated in both, rhinoceros and elephants (Radcliffe et al. 2000; Stetter et al. 2005), pore skin healing abilities, high abdominal pressures and risk of post-surgical peritonitis specifically in elephants, exclude this surgical approach for repeated oocyte collection in mega vertebrates.

Transvaginal, endoscopic oocyte collection has been attempted in the black and the white rhinoceros ($n = 3$) using custom-made, flexible endoscopy, but failed (Hildebrandt, unpublished data). The transvaginal approach through a minimal incision in the vaginal fornix healed uneventful by second intention. However, technical problems, such as insufficient abdominal insufflation of the large abdominal cavity, difficult visualization of the ovaries with the long flexible endoscope and the resorption of $CO_2$ into the tissues with subsequent emphysema of the intestine and the abdominal wall during one procedure, render this approach as non-practical.

A new approach for oocyte retrieval seems to possibly overcome these anatomical obstacles and technical problems. Transrectal ultrasound-guided follicle aspiration in one infertile, black rhinoceros produced the first oocytes collected from a live donor. This new transrectal approach to the ovary for follicle aspiration using a 100-cm long, flexible puncture needle system made a critical surgical intervention unnecessary. Multiple follicles were aspirated with one single puncture of the rectal wall under direct ultrasound control and guidance. Successful IVM and IVF did not yet result in the formation of an embryo but marked the first step towards *in vitro* produced embryos from live infertile oocyte donors (Hermes, unpublished data).

Embryo transfer has yet not been attempted in rhinoceros or elephants. However, the double LH peak in elephants and ovulation hastening protocol in rhinoceros as currently used in AI programmes set ideal endocrine preconditions, which facilitate exact timing for embryo transfer protocols into recipients. With the slight modification of current AI techniques deep intrauterine deposition of *in vitro* produced embryos is technically feasible in both taxa (Brown et al. 2004; Hildebrandt et al. 2007a).

## Contraception

Under certain managed situations, the control of reproduction is necessary to prevent overpopulation in restricted areas without disrupting herd dynamics (Delsink et al. 2001) or to avoid mating in females with reproductive disorders (Lange et al. 1999). Control of African elephant populations has become an absolute necessity in a number of game reserves in southern Africa with annual population increases of 3–6%. There is an urgent need to implement an efficient population management protocol to keep the number of elephants at an ecologically acceptable level. The most widely applied and practical contraceptive agent in free ranging elephants is an immunogenic preparation, a porcine Zona Pellucida (pZP) vaccine (Delsink et al. 2001; Fayrer-Hosken et al. 2001; Bertschinger et al. 2006). Harvested from swine ovaries the immune response targets ideally mature elephant oocytes only. Although there are promising results increasing the intercalving period in semi-free ranging females (Bertschinger et al. 2006), the greatest concern with this immunological contraceptive, whether the ovarian tissue remains unaffected by the immune response and its reversibility, have so far not been addressed.

Oestrogen implants in combination with melengestrol acetate implants (MGA) are the second contraceptive method used on wild and captive elephants. Because circulating oestrogen concentrations are very low, small concentrations of oestradiol (315 $\mu$g/day, slow release implants) are effective to inhibit ovulation prolonging the inter-calving period (Göritz et al. 1999). The efficacy of the slow release melengestrol acetate (MGA) implants has been tested in one preliminary trial in African elephants. The implant achieved concentrations of 20–30 pg MGA/ml serum, a complete inhibition of luteal progestin, but no inhibition of oestrogen. The results suggest that the human 'mini-pill' strategy can be applied to elephants. In both these hormonal contraceptives, ultrasound documented no adverse effects on the uterus or ovaries (Göritz, unpublished data).

## Conclusion

Through advances in endocrine monitoring and ultrasonographic imaging, we have gained fundamental insight into the mechanisms controlling reproductive function. Several reproductive characteristics appear to be unique to each taxon or species: different oestrous cycle lengths and follicular development patterns in all captive rhinoceros species, unique pituitary gonadotrophin secretion with a two LH surges, long gestational length and a delayed early embryonic development in the elephant. Some reproductive disorders identified with breeding captive rhinoceroses and elephants are logistic like the absence of males in a facility, but others, like anoestrous, ovarian and uterine pathologies or maternal aggression have management-related aetiologies.

Future challenges involve, first, the broad implementation of established reproductive assessment protocols and further use of artificial insemination to improve captive breeding management of elephants and rhinoceroses. The reliable application of cryopreserved sperm in AI programmes will require further investigation into the improvement of the post-thaw semen quality and advances in AI techniques and semen placement to compensate for post-thaw sperm quality loss. Research into hormone treatments to induce the oestrous cycle activity, to hasten ovulation in captive populations or the contraception of wild elephants are further applied areas of ART research. The development of advanced ART in these mega vertebrates, such as the use of sex-sorted sperm in AI programmes, IFV, ICSI and embryo transfer or GIFT are an extreme technical challenge. Very limited animal numbers are, in contrast, with the large amount of gamete material normally necessary to establish a species-specific protocol for any advanced ART. Yet, for some rhinoceros species or subspecies with only a few individuals left, AI, gamete rescue and advanced ART represent the last hope to prevent genetic loss and eternal extinction.

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