Features of reproduction and assisted reproduction in the white (*Ceratotherium simum*) and black (*Diceros bicornis*) rhinoceros

Kenmerken van de voortplanting en de geassisteerde voortplanting bij de witte (Ceratotherium simum) en zwarte (Diceros bicornis) neushoorn

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ABSTRACT

Despite the worldwide increase of rhinoceros calf numbers, the growth of the population of white and black rhinoceros is slowing down mainly due to anthropogenic causes, such as poaching and habitat loss. Assisted reproduction is one of the methods of preserving the valuable genomes of these animals from being lost, and assists in breeding them in captivity to maintain the specie(s) numbers and provide an option for possible reintroduction into the wild. Since wild rhinoceros are difficult to handle and examine clinically, most of the current information available on their reproductive characteristics has been gained from captive rhinoceros populations. Nevertheless, very little is known about rhinoceros reproduction. Since the rhinoceros belongs to the odd-toed ungulates (Perissodactyls) group, like the horse and the tapir, the horse has been proposed as a suitable model to study reproduction and artificial reproductive techniques in the rhinoceros. In this review, the current knowledge of the reproduction of the rhinoceros is summarized.

SAMENVATTING

De populatie in het wild levende witte en zwarte neushoorns neemt drastisch af, hoofdzakelijk ten gevolge van stroperij en het verlies van de natuurlijke habitat. Geassisteerde voorplanting kan dienen om waardevolle bloedlijnen te behouden en om neushoorns te kweken met als doel de soort in stand te houden en eventueel zelfs terug in het wild te introduceren. Omdat neushoorns moeilijk te benaderen en te onderzoeken zijn, is er nog relatief weinig bekend over de natuurlijke en kunstmatige voortplanting. De neushoorn behoort tot de onevenhoevigen (Perissodactyla), waartoe ook het paard en de tapir behoren. Zodoende kan het paard wellicht het best als model dienen voor het bestuderen van de karakteristieken van de voortplanting en geassisteerde voortplantingstechnieken bij de neushoorn. In dit overzichtsartikel wordt de huidige stand van zaken weergegeven.

INTRODUCTION

Over the last decade, assisted reproduction and captive breeding have become more important for wildlife conservation. However, some species that are threatened do not breed well in captivity (Lueders, 2014). Extinction of a species may occur due to factors, such as habitat loss through human activity, poaching and effects of pollution on fertility and fecundity (Amin et al., 2006). The rate of man-induced deaths in rhino is increasing in Southern Africa as a result of poaching, with over three rhinoceros killed for their horn each day, in 2014. As a result, the population of wild white and black rhinoceros is being tremendously threatened. At present, the ongoing debate on whether to legalize the trade of wildlife products or not, i. e. horn in case of the rhinoceros, could provide a viable solution for species conservation (Biggs et al., 2013). To help ensure the survival of wildlife species, the experience and knowledge gained from wildlife breeding operations, such as those in zoological institutions, may serve to provide options for conservational biologists for application in situ. Similarly, assisted reproduction may also play an important role in the above mentioned applications. However, facilitating optimal conditions for natural mating and

breeding remains the central goal for increasing population numbers. Furthermore, the increased economic interest of keeping wildlife species will result in more intensive captive breeding operations in the future.

The rhinoceros belongs to the Perissodactyls group or odd-toed animals, which is an order of large herbivores that may be subdivided in the suborder of Hippomorpha, to which the Equidae belong (including the horse, donkey and zebra), and a common clade for the families of the tapirs (Tapiridae) and rhinoceros (Rhinocerotidae). The latter consists of five species: the black rhinoceros (*Diceros bicornis*), with its four subspecies, of which the D. b. longipes or Western black rhinoceros is considered to be extinct, and the white rhinoceros (*Ceratotherium simum*), with its two subspecies, which live in Africa, and the Indian (*Rhinoceros unicornis*), Javan (*Rhinoceros sondaicus*) and Sumatran rhinoceros (*Dicerorhinus sumatrensis*), which live in Asia. They differ in population numbers and level of endangerment, with some being listed as critically endangered (black, Javan, Sumatran rhinoceros) to vulnerable (Indian) and some being listed as near-threatened (white) (List, 2013).

It has been estimated that worldwide 20.405 white and 5.055 black rhinoceros are still living in the wild and that 750 white and 240 black rhinoceros exist in captivity (List, 2013). As mentioned above, the horse is one of the closest domestic relatives of the rhinoceros, and preferable over the donkey or zebra as a model since reproduction in the horse has been studied at a much profounder level than in other equids. Comparing the horse model with the current reproductive data on the (white and black) rhinoceros could hence provide useful information on how to improve the breeding techniques in rhinoceros. However, it should be kept in mind that equids and rhinoceros also differ in many aspects. Therefore, the horse is merely used as a basic model. Further research into rhinoceros reproduction as well as detailed knowledge on interspecies variation are warranted.

In this paper, an overview is given of the current information available on the reproduction and assisted reproduction techniques in the white and black rhinoceros and, if applicable, a comparison with the available knowledge of equine reproduction is provided.

THE HORSE AND THE RHINOCEROS: TWO RELATED SPECIES

Anatomy of the female reproductive tract

In the cycling mare (*Equus caballus*), the reproductive tract measures between 67 and 76 cm from the vulva to the utero-tubal junction. The ovaries are large (28 cm^3), bean-shaped organs located in the sublumbar area. Ovulation of mature follicles only occurs in the ovulation fossa, because of tough and fibrous tunica albuginea covering the external surface of the ovary (Allen, 2005). This is unique compared to other animal species. The uterine tubes are long and tortuous ducts, 20 to 30 cm in length, and are divided in the infundibulum, the ampulla and the isthmus. The uterus of the mare is bicornuate, with a pronounced corpus and short horns, and the lumen is lined with prominent endometrial folds. The longitudinal folds of the thick-walled cervix are continuous with the endometrial folds. The cervix is large (5 - 7.5 cm length) and softens during estrus. There are no cervical rings present, which enables the cervical lumen to greatly expand and contract during the estrous cycle. The vertically oriented vulva is located ventral to the anus (Brinsko et al., 2011).

In the rhinoceros cow, the length of the reproductive tract ranges between 0.8 and 1.5 m, from the vulva to the tip of the uterine horn, depending on the species (Godfrey et al., 1991; Schaffer et al., 2001). The ovaries are very large (34.1 ± 4.3 cm^3 for white rhinoceros with regular cycles. In animals with less regular cycles, active ovaries are much smaller in size (29.2 ± 2.2 cm^3) and inactive ovaries are about half the size (14.7 ± 1.3^3) (Hermes et al., 2006), and are located caudo-laterally to the kidneys (Schaffer et al., 2001). The ovaries are positioned within the ovarian bursa. Germinal epithelium lines the surface of the ovary, enclosing the stroma within, like in most domestic species. The follicles, unlike the situation in the horse, can ovulate at any place over the entire surface (Godfrey et al., 1991). It has been reported that in rhinoceros, ovulations generally occur in only one of the ovaries. However, this data is based on only a few individual animals (Radcliffe et al., 1997). This same phenomenon has been reported in maiden mares (Ginther, 1983). In the rhinoceros, each uterine tube consists of a small tubular structure with fimbriae at the open cranial end. The uterus is bicornuate. The anatomy of the uterus of the rhinoceros is anatomically more similar to the uterus of the bitch and the sow than that of the mare, since female rhinoceros have large uterine horns and a relatively short uterine body (Godfrey et al., 1991; Schaffer et al., 2001). The cervix, with its long and convoluted morphology and extremely tight folds of connective tissue, may represent an obstacle for artificial insemination (AI) (Godfrey et al., 1991; Hermes et al., 2007). The presence of the hymen or hymeneal membrane during examination can be used to determine whether there has been a successful mating earlier or not. Persistence of the hymen indicates that proper penetration during mating did not occur. This is not unique to rhinoceros, but this finding may be used as such to indicate possible reproductive problems in the white rhinoceros (Hermes et al., 2006).

Estrous cycle: physiological changes and follow-up

Horses have a reproductive cycle of 22 days with 5-7 days of estrus (Aurich, 2011). They are long-day seasonal breeders. Puberty occurs around 12-18

months of age. Typically, there is no substantial post-reproductive life span. Estrous behavior is characterized by increased interest towards the stallion. Stallions are also attracted to the mare in heat. The mare turns her hindquarters towards the stallion and shows a typical posture with lowered pelvis and straddled hind limbs, accompanied by deviation of the tail with 'clitoral winking' (Aurich, 2011). One or two major follicular waves develop per cycle. The dominant follicle reaches an average size of 40 mm at ovulation, which can even go up to a pre-ovulatory size of 55 mm, also depending on the type of breed and time during the season (Ginther et al., 2008; Aurich, 2011). During estrus, progesterone levels are low (<1 ng per mL) but they reach high levels (often in excess of 10 ng per mL) three to four days after ovulation (Terblanche and Maree, 1981).

After fertilization, a single spherical blastocyst (sometimes two) migrates through the uterus until day 17, the time of fixation (Allen and Stewart, 2001). The placenta is of the endotheliochorial type. After 35-38 days of pregnancy, implantation starts and endometrial cups form in the placenta, which secrete a gonadotrophic hormone (eCG) (Allen, 2001). The eCG hormone stimulates the development of accessory corpora lutea by stimulating ovarian follicles to luteinize and secrete progesterone to maintain pregnancy. Horses have a gestation period of 330-350 days (Howell and Rollins, 1951; Satue et al., 2011).

For many years now, transrectal ultrasonography has been used to monitor the estrous cycle in the mare (Ginther and Pierson, 1983). It is a technique that can be applied in the standing, non-sedated horse. Examinations can be done repeatedly over many days (Griffin and Ginther, 1992). Peripheral blood progesterone concentrations can also be used to determine the stage of the cycle, due to the luteal phase being 14 days on average. During the first 14 days of pregnancy, the progesterone levels are similar to those of the luteal phase. During the follicular phase, the level of progesterone is at baseline (Allen and Hadley, 1974).

Rhinoceros are non-seasonal, poly-estrous breeders (Garnier et al., 2002). Follicular activity starts at puberty, at the age of three to four years. Follicular waves are present in these pubertal animals but ovulation does not yet occur at this age. Ovulation is mainly seen in females starting from four to five years of age. Social hierarchy might be one of the triggers to inhibit ovulation at this age, as suppression of subordinates might lead to increased social stress, which can have a negative impact on reproductive performances (Hermes et al., 2006; Metrione et al., 2007). As in the horse and most other mammals, ovulation occurs after a single pre-ovulatory LH peak. The size of the pre-ovulatory follicle varies between the different rhinoceros species. In captive white rhinoceros, Graafian follicles reach a preovulatory size of 30-34 mm (Radcliffe et al., 1997; Hermes et al., 2007; Hermes and Hildebrandt, 2011). Follicular growth rates, measured in two induced estrous cycles, are calculated at ~0.2 mm per day (Hildebrandt et al., 2007). Graafian follicles of captive black rhinoceros reach a preovulatory size of 47-51 mm. Once the dominant follicle reaches 35 mm in diameter, the follicular growth rate is ~3 mm per day (Radcliffe et al., 2001). The formation of anovulatory, hemorrhagic follicles, which exceed Graafian follicle size by 10 to 30 mm, are known to occur in all four captive rhinoceros species and may have a negative effect on reproduction (Hermes and Hildebrandt, 2011). In estrus, female rhinoceros start to urinate small volumes, show tail-lifting and accept male rhinoceros in their close vicinity. Some of the females show a standing reflex to be mounted (Radcliffe et al., 1997). Local changes are also seen in the genital tract of the females during estrus. Vulvar swelling is classified as an indicator of estrus in the female rhinoceros. The swelling is not diffuse but more 'bubble-like' (Carter, 2007). There is also a color change of the vaginal mucosa during estrus: it is hyperemic and red during estrus and pale pink during anestrus. Color changes are seen from two days before until three days after estrus (Radcliffe et al., 1997). This is a fairly reliable, non-invasive technique.

Within the different rhinoceros species, different estrous cycle lengths have been observed, i. e. in the white rhinoceros 28 (Bertschinger, 1994) and 30-35 days (Hindle et al., 1992; Brown et al., 2001), and in the black rhinoceros 27 days (Schwarzenberger et al., 1993; Garnier et al., 2002). Only in the shorter 30-35 days cycle, the captive white rhinoceros appears to be fertile; the longer cycle of 65-70 days, which is also seen in captive rhinoceros, has been associated with reproductive aging in older females (Schwarzenberger et al., 1998; Patton et al., 1999; Brown et al., 2001).

The gestation length varies between the rhinoceros species, with the average being 15-16 months for the white, Sumatran, Indian and the black rhinoceros and the Javan at 16-19 months (Hermes et al., 2007). Pregnancy in captive rhinoceros can be diagnosed by the measurement of elevated progesterone levels in blood, urine and/or feces (Radcliffe et al., 1997) at about 3-5 months after conception. In free-roaming rhinoceros, the non-invasive method of fecal sampling is preferred. In pregnant animals, the progesterone metabolite levels (5α-pregnan-3ß-ol-20-one) are significantly higher than in non-pregnant and postpartum animals (Goot et al., 2013). The collection of freshly produced feces as predictive tool of the estrous cycle is the most recommended method. Fecal extracts can be measured for immune-reactive progesterone metabolites using an enzyme immunoassay (EIA) for 5α-pregnan-3ß-ol-20-one. These metabolites have been shown to provide reliable information about the reproductive steroid hormone pattern by reflecting total progestagens in different mammalian species (Szdzuy et al., 2006; Ahlers et al., 2012). Feces samples need to be freshly collected, stored frozen or lyophilized afterwards, and can be analyzed eventually or stored for a long time. Only very few labs of-

fer these hormone analyses of feces. In addition, early pregnancies of 1-3 months are more difficult to detect with fecal progestagen metabolite measurements, because they sometimes level luteal phase values, and there is no clear cut-off value (Hildebrandt et al., 2007; Goot et al., 2013). In some cases, inconsistency in plasma and fecal hormone levels is present until 4-5 months of gestation, which makes it more difficult to differentiate early pregnancy from normal cyclicity (Hermes et al., 2009b).

Blood and urine sampling in wild animals are more invasive and can only be performed in restraint or immobilized animals in the wild or well-trained animals in captivity. Therefore, these methods are not commonly used.

Ultrasound has also been used to detect pregnancy in captive rhinoceros. Pregnancy can be diagnosed starting from 15 days post-ovulation by visualization of the spherical embryonic vesicle. The embryo can be visualized as early as day 23 post-ovulation followed by heartbeat detection at day 26 (which is in accordance with the timing of the development in domestic horses). Mobility of the embryo during early pregnancy is seen both in the rhinoceros and in the domestic horse (Radcliffe et al., 1997; Hermes and Hildebrandt, 2011). The placenta is, like in the horse, of a diffuse epithelio-chorial type with large areas of villus-free areas (Benirschke and Lowenstine, 1995).

Anatomy of the male genital tract

The stallion has two ovoid testes, the size of which is influenced by season, and which are located in the scrotum between the hind legs. The testicular diameter varies between 10.3 cm and 12.7 cm, depending on age and season (Burns et al., 1984) or average 11 to 12 cm in length by 5 to 7 cm in width, with an average weight of 225 g (Amann, 1981). The testes are positioned horizontally, with the caput epididymidis cranially and the cauda caudally, while the corpus epididymidis is located along the dorsomedial margin of the testis. The stallion has a musculocavernous type of penis. Accessory sex glands in the stallion are the bulbo-urethral glands, the prostate, the seminal vesicles and the ampullae ductus deferentes (Little and Woods, 1986).

In the male rhinoceros, both testes are positioned horizontally in the scrotum, comparable to their orientation in the stallion. Based on 24 ultrasounds of 21 male white rhinoceros, the mean testicular and epididymal diameter is 6.5 ± 0.3 cm and 2.8 ± 0.1 cm, respectively (Hermes et al., 2005). Due to the thick scrotal skin and the dense tunica, it is difficult to examine the testicles by palpation. Identical to the stallion, the rhinoceros has a penis of the musculocavernous type. In rest, it is pointed in caudal direction, even when the animal is urinating. Hence, the urine stream is always directed caudally. The preputial orifice is located just caudal to the umbilicus. Only when erect, the penis points in a cranial direction. Identical to the tapir but different to the stallion, the rhinoceros has penile flaps on both lateral sides of the penis. The glans penis has a typical, mushroom-like shape. This shape together with the penile flaps suggests that the male rhinoceros is a cervical ejaculator (Hermes and Hildebrandt, 2011). Accessory sex glands are present in the male rhinoceros. The bulbo-urethral glands, seminal vesicles and prostate are comparable to those of the stallion (Schaffer et al., 1990). In rhinoceros, there are no ampullae at the end of the deferent ducts (Hermes et al., 2005; Hermes and Hildebrandt, 2011).

Semen

In rhinoceros, there is a positive correlation between sperm quality (viability, morphology and motility) and pregnancy rates (Hermes and Hildebrandt, 2011). Sperm quality is species-dependent, but is also affected by the semen collection method (Schaffer and Beehler, 1988; Hermes and Hildebrandt, 2011) (Table 1). Inbreeding and increased age of the male cannot be ruled out as a possible negative influence on sperm quality (Hermes et al., 2005). Apparently, semen quality in rhinoceros is also influenced by the social status of the bull in the group. If the bull switches from a subdominant to a dominant position in another herd, this can have a clearly positive effect on its fertility (Hermes et al., 2005). Libido, spermatogenesis

Table 1. Semen parameters of the rhinoceros and the horse (Hermes et al., 2005; Juhász et al., 2000; Miller and Fowler, 2011; Roth et al., 2005)

Parameter	White rhinoceros (*Ceratotherium simum*)	Black rhinoceros (*Diceros bicornis*)	Horse (*Equus caballus*)
Volume (mL)	0.7- 80	17 - 62	30 - 70
Concentration (x10^6/mL)	7 - 165	15 - 58	178 - 335
Abnormal cells (%)	31	71	33 - 53
Progressive motility (%)	Cat 1: > 75 (n=21) Cat 2: 50 - 75 (n=5) Cat 3: < 50 (n=8)	40 - 50	53 - 76
pH	8	8	6.7 - 7.5

The progressive motility in the white rhinoceros was divided into different categories of quality by Hermes et al., (2005) (34 (n) ejaculates of 21 animals).

and mating behavior of bulls are positively correlated with testosterone levels. In the wild, territorial bulls have higher testosterone levels than non-territorial bulls (Rachlow et al., 1998; Kretzschmar et al., 2004).

REPRODUCTIVE PATHOLOGIES IN THE RHINOCEROS

The breeding of captive rhinoceros provides a gene pool of valuable animals as a back-up source for wild populations. However, the natural breeding of rhinoceros in zoos continues to be problematic (Foose and Wiese, 2006). One of the main problems of captive breeding of rhinoceros is the high incidence of prolonged periods of anestrus in females, with more than half of these females remaining acyclic. This occurs both in old and young females (Hermes et al., 2004; Hermes et al., 2006; Hermes et al., 2007). Such young females show regular follicular waves but have no ovulation of the pre-ovulatory sized follicles (Radcliffe et al., 1997; Roth et al., 2004; Stoops et al., 2004). Another cause of the prolonged infertile cycle might be early embryonic death. Embryonic death can be associated with prolonged maintenance of the corpus luteum. This feature has also been described in the domestic cow (Kastelic et al., 1988) and horse (Bergfelt et al., 1992).

One of the approaches to improve fertility is to increase exposure to other individuals of the same species. When female rhinoceros are translocated to other facilities, the effect of transportation as well as encountering new rhinoceros can be sufficient to initiate regular estrous cycles. Likewise, the introduction of new males in a group can induce cyclicity in previously anestrous females (Rachlow et al., 1998; Kretzschmar et al., 2004; Hermes et al., 2005). This points out that socio-behavioral dynamics and/or pheromones influence the estrous cycle and ovulation in the rhinoceros. Poor management and animal husbandry often contribute to irregular or inactive cycles (Carlstead et al., 1999; Hermes et al., 2004). However, it should be kept in mind that moving animals between different facilities cannot be used as a routine management tool in captivity. Other reproductive pathologies are anovulatory and hemorrhagic follicles, which are common findings both in the white rhinoceros and in the domestic horse (Hermes et al., 2007; Cuervo-Arango and Newcombe, 2012). Sometimes only subtle, clinical signs of a possible reproductive pathology, such as vaginal discharge, are present in rhinoceros despite a prominent reproductive pathology of the uterus diagnosed later on necropsy. Recently, the increased application of ultrasound has facilitated earlier diagnosis and treatment of reproductive pathological conditions in the living animal. Pathologies, such as tumors and cysts of the endometrium and ovary, muco- and pyometra, and uterine leiomyoma, endometrial adenoma and adenocarcinoma have been reported in rhinoceros (Hermes and Hildebrandt, 2011). Reproductive pathologies in male rhinoceros are less well-known. Penile edema with penile prolapse, which prevents normal mating, has been described. Testicular fibrosis, testicular neoplasia and epididymal cysts have been incidentally discovered in male rhinoceros, in zoological institutions; in some cases with a negative influence on fertility (Portas et al., 2005; Portas et al., 2010; Hermes and Hildebrandt, 2011).

ROLE OF ASSISTED REPRODUCTIVE TECHNIQUES IN THE RHINOCEROS

Assisted reproductive technologies (ART) have been applied with great success in several domestic species. However, despite the good results achieved in domestic species, researchers are repeatedly faced with the problem that the implementation of these techniques in wildlife is hampered by the diversely distinct traits of each species, which precludes the easy transfer and application of this technology, despite it having been refined in domestic animals, over the past decades. In order to apply techniques, such as ovum pick-up (OPU), in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI), it is very important to have good knowledge of the female estrous cycle and of the anatomy of the reproductive tract of that specific species (Hermes et al., 2009a). This remains a problem in wild species, such as the rhinoceros, in which characteristics of estrous cycles are less well-defined and even differ between captive and free-ranging rhinoceros (Hermes et al., 2004; Hermes et al., 2006; Goot et al., 2013). To increase basic research efforts in these wild animals, more financial support is needed. Since wild animals generate less economic interest worldwide and because their wild state of being complicates easy collection of data, this presents as an ongoing problem.

Despite the potentially problematic species variation and the difficulty of implementing ART in captive breeding management, there are several potential advantages. Unnecessary risks during long transportation of living animals to increase population numbers are no longer required, and there is a reduced risk of disease transmission. With the aid of reproductive techniques, there might also be an increased chance of breeding with sub- or infertile animals as well as with animals that are physically or socially unable to mate naturally. Even post-mortem material from recently deceased animals can be used (epididymal semen) as a source for ART (fresh, cooled or cryopreserved). However, since so little is known about these species, any interfering factor like sub- or infertility is bound to compromise any ART procedure to some degree.

Most of the assisted breeding techniques used in wild animals have been extrapolated from techniques used in domestic animals. Techniques, such as preg-

nancy diagnosis by ultrasonography, AI, embryo transfer (ET) and OPU can be applied in wild species too, provided that they are adapted to the anatomical configuration and reproductive characteristics of the species in question.

Assisted reproduction in the rhinoceros

Animal restraint

Husbandry systems used in horses are very well accessible for routine use in ART. In wild animals such as the rhinoceros, this is more complicated. These animals often need to be heavily sedated or immobilized to be able to work with them. In trained animals, chute conditioning with food may help the animals entering and remaining in a free-stall chute (Radcliffe et al., 1997). Other types of cage restraints may interfere and restrict the way of working (Schaffer et al., 1998b). In case of more invasive ART, such as ovum pick-up, the animals need to be fully restrained and anesthetized to facilitate handling (Hermes et al., 2009a).

Regulation of the estrous cycle

Regulation of the estrous cycle might be used for estrus induction, ovulation induction or estrus synchronization. A major impediment to the use of AI is the detection of estrus for the optimum timing of breeding. Good observation of the animals assists in detecting natural estrus, but estrus synchronization might be a very important tool as well. In domestic species, protocols have been developed to synchronize and regulate follicle development, luteal regression and the time of ovulation (Bisinotto and Santos, 2012). By controlling ovulation via synchronization, assisted reproductive procedures, like AI and ovum pick-up, can be timed.

In the domestic horse, ovulation synchronization programs are widely used. Estrus synchronization is used as part of the insemination strategy (timed insemination) on larger stud farms or AI centres (Allen and Cooper, 1975; Bergfelt et al., 2007; Handler et al., 2007; Squires and McCue, 2007), and as such, a single ejaculate from the same stallion can be applied to inseminate several synchronized mares. To gain high success rates in equine ET, it is necessary that the recipient mare is synchronized according to the moment of ovulation of the donor mares (Raz et al., 2011). In this way, pregnancy rates of >70 % can be achieved (Carnevale et al., 1987; McKinnon and Squires, 1988; Jacob et al., 2012; Leemans et al., 2012; Vandenberghe et al., 2012). As for wildlife, general information about the reproductive cycle of wild animals is collected by non-invasive hormonal analysis (feces, urine) and by observation of behavioral changes. But more invasive techniques, such as estrus synchronization and ovulation induction, might provide aid for the successful implementation of AI programs in wildlife. Although little data were available on programs developed specifically for wildlife species (Pukazhenthi and Wildt, 2004), the application of such programs has been described in captive rhinoceros (Schwarzenberger et al., 1998; Hildebrandt et al., 2007; Hermes et al., 2012).

Up till now, estrus synchronization, and estrus and ovulation induction have not been performed in the wild rhinoceros. Various combinations of follicle-stimulating hormone (FSH), equine chorionic gonadotropin (eCG), human chorionic gonadotropin (hCG) and gonadotropin-releasing hormone (GnRH) have failed in the past to induce ovulation in the captive rhinoceros (Godfrey et al., 1990; Hermes et al., 2012; Hildebrandt and Hermes, 2014). Other possibilities, although perhaps more complicated, are being investigated, i. e. hormone treatments based on the long-term downregulation of ovarian activity using long acting GnRH implants combined with an injection of hCG at the end, or using synthetic progesterone (Chlormadinone Acetate; Synchrosin®, Werfft-Syntex, Vienna, Austria) followed by an injection of hCG (Chorulon®, Intervet, Boxmeer, the Netherlands) (10000 IU). Doses and treatment intervals have been extrapolated from those used in domestic horses (Schwarzenberger et al., 1998). Although continuous cyclicity could not be induced, ovulation induction has been successful in white rhinoceros (Schwarzenberger et al., 1998; Hermes et al., 2012). In a study by Hildebrandt et al. (2007), a more simplified protocol induced ovulation during anestrus with a GnRH analogue, deslorelin (Ovuplant®, Peptech, Melbourne, Australia). This short-term GnRH analogue was implanted subcutaneously on the day of insemination, in two estrous cycles, resulting in ovulation and pregnancy (Hildebrandt et al., 2007). Due to the thick skin, it is difficult to remove the deslorelin implant. It is often left behind under the skin. Nowadays, the use of injectable hCG (Chorulon®, Intervet, Boxmeer, the Netherlands) (10000 IU) one day prior to AI is suggested instead of the deslorelin implant (Hermes et al., 2012; Hildebrandt and Hermes, 2014).

Semen collection

Stallions can be trained to mount a phantom; this is obviously much more difficult in the rhinoceros. Male rhinoceros are either shy or aggressive, which can both be dangerous as they become unpredictable, and hence might be difficult to handle or to train (Young, 1967; Schaffer et al., 1990). Some researchers have tried to develop a modified artificial vagina to collect semen, based on the artificial vaginas used in stallions. Due to the size and anatomy of the rhinoceros penis and due to the lack of appropriate sexual stimulation, the collection of a good-quality ejaculate has been very difficult, resulting in ejaculates with a low concentration of spermatozoa and in most cases, a low progressive motility (Schaffer et al., 1990). Penile

massage is a more ancient technique that can be used to induce ejaculation in primates, pigs and carnivores. In other large mammals, it is usually ineffective, although it can be used for semen collection in stallions (Crump and Crump, 1989). In rhinoceros, it has been used to collect semen, although it is not the most effective way. This technique is often combined with rectal stimulation (massage or electro ejaculation). The semen quality after penile massage is usually not as good as when semen is collected by other means, such as electroejaculation combined with rectal massage (Schaffer et al., 1990; Hermes et al., 2005).

Electroejaculation is another commonly used method for semen collection, especially in wild species. In horses, this invasive and painful method is rarely used since stallions are easy to train to mount a dummy for collection with an artificial vagina. In some cases however, electroejaculation is used as terminal sperm cell collection method, that is when stallions are no longer able to mount physically or stand up (Cary et al., 2004). Because it is difficult to work with wild animals without sedation or anesthesia, electroejaculation with concurrent sedation is an acceptable method for semen collection in the rhinoceros species (Schaffer et al., 1990; Roth et al., 2005). Probes are specially designed and adjusted to the different rectal anatomy of rhinoceros (Hermes et al., 2005; Roth et al., 2005). Typical rectal probes have longitudinal electrodes. The reproductive glands of the male rhinoceros, which need to be stimulated by electroshocks are adjacent to the neck of the bladder and lie ventral to the rectum (Schaffer et al., 1998a; Hermes et al., 2005). Electroejaculation in the rhinoceros is often combined and followed by manual massage of the pelvic and penile parts of the urethra (Schaffer et al., 1990; Schaffer et al., 1998a), which may result in urine contamination. As described in horses, there might be a chance of retrograde ejaculation during electroejaculation when attempted under general anaesthesia (Cary et al., 2004). This might interfere with semen quality.

As an alternative for electroejaculation or to apply electroejaculation in the gentlest possible way, the administration of hormones, which induce contractions of smooth muscle, such as oxytocin or prostaglandines, can be used. Oxytocin treatment to enhance smooth muscle activity in the ductus deferens, prior to electroejaculation has resulted in increased numbers of spermatozoa in the ejaculate of bulls (McDonnell et al., 1987a; Berndtson and Igboeli, 1988). Prostaglandin F2α used prior to semen collection may also influence smooth muscular contraction and consequent semen collection. In stallions however, the administration of prostaglandin F2α has been linked to inconsistent results (McDonnell et al., 1987a). In rhinoceros, this treatment has not been attempted yet.

Imipramine, a tricyclic antidepressant of the dibenzazepine group, with both central and peripheral effects on neurotransmission, has been used in stallions with ejaculatory dysfunction to induce ex copula ejaculation. With variable results, positive effects on erection and ejaculation were achieved in the stallion (McDonnell et al., 1987a; McDonnell, 2001). It is important that the stallion is kept quiet and undisturbed. In some cases, the imipramine treatment has been combined with injections of xylazine; in other cases ejaculation has been induced with xylazine alone (Mcdonnell and Love, 1991). The induction can be preceded by sexual prestimulation. These induced ejaculates are of lower total volume, higher concentration, lower gel volume, higher total numbers of spermatozoa and lower pH than normal ejaculates after copulation (Mcdonnell et al., 1987b; Mcdonnell and Odian, 1994). It remains to be determined if this method is equally suitable for the male rhinoceros.

Post-coital semen collection could be a good alternative in cases where animals are difficult to train to mount a phantom. Although it is not the best (semen is often mixed with other vaginal fluids/cells) or most practical method, post-coital semen collection can be used in some cases, more specifically when the female is kept in a closed environment and can be darted. The semen samples collected by this method represent a sample of a natural ejaculate, whereas the small volumes of fluid emitted during methods like manual stimulation or electroejaculation may not consist of the appropriate mixture of seminal fluids (O'Brien and Roth, 2000).

Collection of epididymal spermatozoa is a terminal procedure in animals with irreparable conditions, such as complicated fractures, soon after (natural or induced) death or directly after castration. It might also be of great value to maintain genetic diversity in a gene bank for endangered species.

The two main techniques of epididymal sperm collection, i.e. retrograde flushing and floating method, are commonly used in domestic horses (Cary et al., 2004; Roels et al., 2014). These techniques have also been used in other species, such as goats, dogs, cows and humans (Marks et al., 1994; Sharma et al., 1997; Martins et al., 2007).

In rhinoceros, it has also been used as a semen collection technique, especially in cases where the animal has died or has been euthanized (Williams et al., 1995; O'Brien and Roth, 2000). Testes are removed 1-3 hours after death and semen is collected approximately 3-30 hours post-mortem (O'Brien and Roth, 2000). In this specific study, sperm motility of 60% (in black rhinoceros) could be achieved by warming samples to 37°C. More than 80% contained cytoplasmic droplets and 60% had an abaxially placed midpiece. This last characteristic has also been seen in stallions (O'Brien and Roth, 2000). Williams et al. (1995) recovered 80-85% motility in white rhinoceros. However, the number of reported cases is too small to set a standard for this method, but with the rising increase of man-induced deaths, such as poaching, this method may become more important in the future.

Semen processing

The use of frozen semen in domestic animals like horses and cattle has been successful for years. Cryopreservation with an adequate extender for semen has been used successfully in cattle and horses since the fifties of the last century (Barker and Gandier, 1957; Foote, 2002; Allen, 2005). The possibility to freeze semen made it possible to spread valuable male genes from excellent sires during and also after their sports career. In addition, AI with either fresh, cooled and frozen semen results in a reduced transmission of venereal diseases (Foote, 2002).

In rhinoceros however, the application of frozen semen has only been successful since the beginning of the 21st century. In the rhinoceros, cryopreservation of semen is quite similar to that in the stallion. It has been found that rhinoceros sperm may survive the cryopreservation process as evaluated by motility and membrane integrity. In a study by Hermes et al., (2005), after collection of semen, the samples were immediately diluted (1:1) with pre-warmed (37°C) cryoextender BC (Berliner Cryomedium). Semen extender BC is based on a buffer solution containing TES, TRIS, fructose and lactose, and supplemented with egg yolk (~16%), DMSO (~6%) and α-tocopherol (20 IU/ml). BC extender was chosen in these experiments, because of its proven efficiency in preserving semen from a large variety of endangered species. In this study, 12 white rhinoceros were included and in total 14 ejaculates were frozen (motility ≥ 50%). Samples diluted 1:1 were centrifuged (800 x *g*) for 10 minutes at room temperature (20-23°C) to eliminate seminal plasma from the ejaculate. After removal of the supernatant, the samples were re-extended with BC to four times the native sample volume. The samples were equilibrated for 2 hours at 4°C, frozen in 0.5 mL straws, 2 cm over liquid nitrogen vapor for 15 minutes before being plunged into liquid nitrogen. For evaluation and usage, straws were thawed in a 38°C water bath for 60 seconds and evaluated after 10-15 minutes of incubation at 37°C. In a comparative trial, ejaculates of five males were treated as described above but diluted with four different extenders, i.e. BC, Biladyl (supplemented with egg yolk and DMSO, Gent (egg yolk-based) and Kenney (supplemented with egg yolk and DMSO). BC maintained sperm quality better than the other extenders (Hermes et al., 2005).

The first successful AI in rhinoceros was reported in 2007, using fresh semen (Hildebrandt et al., 2007; Hermes et al., 2009b). Only in 2009, AI with frozen semen resulted in the birth of the first living rhinoceros calf (Hermes et al., 2009b). In this particular case, the directional freezing technique was used (Hermes et al., 2009b; Reid et al., 2009). Two inseminations were necessary to obtain pregnancy. In the first (unsuccessful) insemination, a dose of ~135 x 10^6 motile sperm cells was used, whereas in the second (successful) attempt, the dose was increased to ~500 x 10^6 motile sperm cells. In the latter case, the semen was collected using electroejaculation (customized probe, 125mm long with a diameter of 105mm and three longitudinal, slightly raised electrodes). The semen was immediately extended with isothermal BC at a ratio of 1:1. The extended semen was chilled slowly over ~2 hours inside an isothermal water bath stored at 4°C. The chilled semen was packaged into 8 mL and 2.5 mL HollowTubes and frozen using the MTG-516 apparatus (IMT Ltd., Nes Ziona, Israel). The frozen samples were kept under liquid nitrogen till the moment of insemination. For thawing, the samples were first held in the air at room temperature (22-23°C) for 60 seconds, and then plunged into a water bath at 37°C for 30 seconds.

In a study by Reid et al. (2009), liquid nitrogen vapor (LN vapor) freezing was compared to multithermal gradient directional freezing in ejaculates of sixteen white rhinoceros. All of them were electroejaculated and semen was diluted with cryoextender (Tris, lactose, egg yolk, DMSO). Directional freezing resulted in a higher semen viability of 5.6% and progressive motility of 34.7% than LN vapor freezing.

Artificial insemination

To date, fresh and cooled semen for AI is used in most domestic animal species, such as horses, donkeys, swine, cattle, dogs, sheep and goats; the cat being an exception. Up till now, in breeding programs, better results have been obtained by natural breeding than by AI. Besides studbook regulations and restrictions, sometimes, it is geographically not possible to bring the male and female animal together.

In zoos and game farms, breeding often happens naturally, which yields the best results but is never without the risk of injuries. Breeding healthy, fertile animals usually leads to the best breeding results. However, sometimes, assisted breeding techniques might be of use to increase the breeding results of less fertile animals (Blyde, 1997), as it is often the case in rhinoceros. Regarding genetic transfer of possible causes of infertility, these animals are usually not the best to breed with. However, because of the low number of white and black rhinoceros, it might also be considered to breed with animals that show a certain degree of subfertility.

Artificial insemination in the rhinoceros represents an anatomical challenge due to the firm tortuous cervix of the female rhinoceros. To overcome this problem, a rhinoceros-specific AI-catheter has been developed (Hermes and Hildebrandt, 2011). Artificial insemination with fresh semen is done in captive rhinoceros within zoological institutions (Hildebrandt et al., 2007). This is possible when both fertile male and female are on the same location, or within an acceptable geographical distance allowing preservation of semen quality during transport. Electroejaculation is usually followed by AI, when the female is anesthetized, examined and prepared for the insemination.

In case of wide geographic distances and spread

of genetic variety, AI can be done with frozen semen too. Semen collected after electroejaculation is diluted before cryopreservation. The use of frozen semen has already led to a successful pregnancy after two inseminations in two different cycles (Hermes et al., 2009b).

Modern ART

Other techniques, like OPU, ICSI and cloning, have also been used in rhinoceros, but so far, with limited success (Hermes et al., 2007; Hermes et al., 2009a). Although resources are limited in endangered animals, transfer of modern ART from domestic animals can lead to positive results.

FURTHER RESEARCH IN RHINOCEROS REPRODUCTION

Due to the increasing pressure on the small number of free-roaming rhinoceros, it will be important to perform further research on the reproduction of these species. Genetic variety will become very important to maintain a sustainable rhinoceros population in the future. Trying to stop the poaching might be the biggest challenge, but optimizing the breeding of these animals and increasing knowledge of their reproduction might compensate for current losses and be a solution to maintain a strong gene pool. Breeding rhinoceros in captivity remains a challenge.

Since pregnancy diagnosis by hormone analysis of feces is not always accurate within the first five months of pregnancy, ultrasound is the method of choice to determine early pregnancy. Pregnant females are of higher conservation priority and higher economic value. However, for ultrasound, the animal needs to be sedated, anesthetized or trained and it is not known yet what the influence of (frequent) anesthesia or sedation might be on early pregnancies. Although the rhinoceros is considered to be a non-seasonal breeder, there is some evidence that season might influence rhinoceros reproduction after all. It may be important to consider these factors for future breeding within zoological institutions. Due to the small number of animals used in most studies, it is sometimes difficult to find the true causes of reproductive failure.

Several aspects of rhinoceros reproduction have been studied. However, most of the research and above discussed techniques have been reported in captive rhinoceros in zoological settings. Breeding rhinoceros in their natural habitat, under natural circumstances might lead to different results. A lot of reproductive problems as described above are mainly seen in captive born male and female rhinoceros. Groups kept in most zoological institutions are small and stable when compared to animals living in the wild. Animals are often limited to breed only with certain other animals within the zoological institution. Males and females are often kept together in the same group, and translocations and new introductions are rare. Studies have already shown that subadult mature bulls are often suppressed by an older dominant male in the group, which may affect semen quality. Evaluation of different group settings will be necessary in further research, as reproductive improvement has already been proven after management changes (Hermes et al., 2005). In wild rhinoceros, strong ´friendships´ have been described between different animals (Smith and Norman, 1975). A better reproduction rate would be expected when individual animals have the opportunity to choose. Could extended sexual maturity of animals in the wild be beneficial for future reproductive success? Adult females in the wild tend to keep their calf on foot until the next calf is born. In zoological institutions, it is often more difficult for subadults to voluntary move into a separate (bachelor) group, as they are mostly kept within the same enclosure. Could this be the reason for the early occurrence of sexual puberty in captivity, due to a lack of competition? Could this be the reason why certain sub- or infertile males and females become fertile (again) when transferred to another institution or back into their natural habitat? All these questions, as well as the breeding successes so far, prove that breeding rhinoceros in captivity is challenging. Creating a better understanding of rhinoceros reproductive health would be of great help, especially, when more and more reproductive techniques will be applied.

CONCLUSION

Up till now, the domestic horse, being from the same order of Perissodactyla, has been demonstrated to be a good comparative model for the rhinoceros and can be used to gain knowledge for the improvement of reproductive parameters and reproductive techniques.

At present, AI with fresh and frozen semen is more often used than before. However, the results are inconsistent in reproductively healthy females. Therefore, the technique should be further developed. To improve semen analysis and semen (cryo)preservation, semen quality should be increased and semen collection methods improved for future species conservation (cf. frozen zoos).

For the future, investigation into other techniques, like ET, ICSI or even cloning, might be warranted. These techniques have already been used in horses and other domestic animals, with better-developed protocols (Smits et al., 2012).

Unfortunately, most of the research done so far has been based on only a small number of animals. Due to the non-domesticated origin of the rhinoceros, it is difficult to repeat certain examinations on a regular basis. Due to these small numbers and differences between captive and wild animals, zoological conditions and data derived from them might be problematic to use as an example for the whole species. The data de-

rived from these institutions can therefore only be extrapolated for the use in their wild counterparts, starting with semen collection, freezing of semen and AI. Using reproductive techniques on a larger scale, in a larger number of animals, might give a better idea of their reproductive rates. Implementing equine knowledge into this species might contribute to augment species numbers in the face of the severe implications of poaching. The use and development of assisted reproductive techniques are growing more rapidly in domestic species than in wildlife species. Zoological institutions might be challenged to keep on aiming at future rhinoceros breeding programs by working towards reproductive goals based on the analysis of the current reproductive parameters.

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Uit het verleden

FOSFORVERGIFTIGING BIJ VARKENS IN HET FRONTGEBIED (1935)

Enkele zeugen en jonge varkens met buitenbeloop op een weide stierven in één dag tijd aan bloederige maag- en darmontsteking. Bij het opensnijden van de maag werd een sterke fosforgeur waargenomen. Toxicologisch onderzoek bevestigde de diagnose fosforvergiftiging. Hoe de dieren die opliepen, werd duidelijk nadat het boerengezin midden in de weide een vlam uit de grond zag slaan. Die bleek afkomstig van een door de varkens boven gewoelde, kapot geroeste granaat. Zodra onvermengde fosfor droogt, ontvlamt het spontaan in contact met lucht.

Notities van inspecteur De Jonckheere, destijds werkzaam in de West-Vlaamse frontzone.
(Schenking Roland Vandermeersch aan de Museumcollectie Diergeneeskundig Verleden Merelbeke)