GHENT UNIVERSITY
FACULTY OF VETERINARY MEDICINE

Academic year 2014 – 2015

Reproductive characteristics of female African Elephant and Rhinoceros and the possibilities of gamete cryopreservation.

By

Maaike DE SCHEPPER

Supervisor: Prof. dr. Peter E.J. Bols.
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Literature Review
as part of the Master's Dissertation

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Preface

First of all, I want to thank Prof. dr. Wouter van Hoven from the University of Pretoria, who lectured me about rhino and elephant poaching and made me think of new possible ways to preserve both species. Because of him I started to think further than rhino farms and the standard ways of saving animals.

Second of all, I want to thank my promotor Prof. dr. Peter Bols from the University of Antwerp, who jumped on the crazy rhino and elephant train with me. As well as I want to thank my co-promotor dr. Tom Rijsselaere, for reading my thesis and correct my English.

I also really want to thank veterinarian Cyriel Ververs, who helped me with my thesis without any hesitation and always had answers ready for all my questions. As well as veterinarian Margot Van De Velde, who corrected my thesis and gave me things to think about during the writing process.

Many thanks to Prof. dr. Ronald de Krijger for reading my (almost) final version and dot my I's and cross my t's!

And last but not least, I want to thank my roommates, friends and boyfriend for their motivational talks when I couldn't find light in all my articles and writing. But also my parents, especially my dad, who gave me some new insights when I was stuck with the writing process.

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Abstract

Poaching is the biggest treat to elephants and rhinoceroses nowadays in Africa. A possible way to preserve the female genes is cryopreservation, by vitrification or slow-cooling, of female genetic material such as embryos, oocytes and ovaries. The preferred method depends on the environment and the physical characteristics of the sexual cycle. In field environment vitrification is recommended, because there is no need for computer controlled cooling. Vitrification requires liquid nitrogen and a cryoprotectant for very fast cooling.

In contrast to horses, there are no studies have yet been conducted on embryo cryopreservation in rhinoceroses and elephants. To establish an embryo cryopreservation protocol for rhinoceroses the horse protocol can therefore be used as an example.

There are different opinions among scientists regarding oocyte cryopreservation. Some say it is better to preserve immature oocytes (Pereira and Marques, 2008; Aerts and Bols, 2010a), however, others say it is preferable to preserve mature oocytes (Lim *et al*., 1992; Ledda *et al*., 2000; Pereira and Marques, 2008). Another method is to harvest and cryopreserve the ovaries post-mortem from deceased or killed animals. A complete ovary can be cryopreserved as well as small ovarian pieces. Viable oocytes can be recovered from ovarian tissue after transplantation of cryopreserved ovaries.

To maximize success of assisted reproductive techniques (ARTs) a thorough understanding of the normal reproductive cycle is crucial. As for the elephant (*Loxodonta africana*) it displays an oestrous cycle of 13-18 weeks, with a double luteinising hormone (LH) surge. A non-ovulatory LH surge, 10-20 days after the progesterone decrease and an ovulatory LH surge, 19-22 days after the non-ovulatory surge (Hildebrandt *et al.,* 2010). Also the oestrous cycle of the black rhinoceros (*Diceros bicornis*) is fairly well defined. It takes 25 days with a single LH surge (Hindle *et al.,* 1992; Schwarzenberger *et al.,* 1993). However, scientist share different views about the cycle length of the white rhinoceros (*Ceratotherium simum*). Generally, the assumption is made that there are two different cycle types; type I, a regular cycle, taking 31-35 days and a type II, an irregular cycle, taking 65-70 days (Radcliffe *et al.,* 1997; Patton *et al.,* 1999; Brown *et al.,* 2001; Morrow *et al.,* 2008). On the other hand, Schwarzenberger *et al.* (1998) stated that a regular cycle takes 70 days while an irregular one takes 30-70 days. There happens to be only one LH surge to induce ovulation.

Keywords: Cryopreservation, Elephant (*Loxondota africana*), Fertility preservation, Oestrous cycle, Rhinoceros (*Ceratotherium simum and Diceros bicornis)*.

Samenvatting

De laatste decennia is het stropen van olifanten (*Loxondota africana*) en neushoorns (de witte neushoorn: *Ceratotherium simum* en de zwarte neushoorn: *Diceros bicornis*), om hun slagtanden en hoorn in Afrika exponentieel toegenomen. In de afgelopen honderd jaar is de olifantenpopulatie met 95% gedaald. Het aantal neushoorns dat slachtoffer is geworden van stropers was in 2014 in Zuid-Afrika alleen al 1,225 dieren. In 2015 zijn tussen 1 januari en 1 april, volgens onofficiële cijfers, 240 neushoorns gestroopt. Als het doden van deze dieren in dit tempo doorgaat zullen beide diersoorten in de nabije toekomst in het wild uitsterven. Om de stropers te ontmoedigen kunnen de neushoorns onthoornd worden. De hoorn, bestaande uit keratine, groeit terug aan. Ook kan er overwogen om ivoren slagtanden van olifanten te verwijderen of in te korten. Verder valt een translocatie van de dieren naar veiligere locaties te overwegen. Dit heeft als bijkomend voordeel dat er nieuwe genen in een bestaande groep worden geïntroduceerd, wat een vernauwing van de genetische basis kan voorkomen.

Daarnaast moet men ook het preserveren van vrouwelijk genetisch materiaal zoals embryo's, oocyten en ovaria als belangrijke strategie overwegen. Dit kan door middel van cryopreservatie, via slow-cooling of vitrificatie. Vitrificatie is aan te raden als de preservatie in het veld moet gebeuren omdat hiervoor geen computer gestuurde koeling bij nodig is. Hierbij wordt het te preserveren monster doordrongen met een cryoprotectant en vervolgens in vloeibare stikstof overgebracht. Door de super snelle koeling wordt een glasachtige staat gecreëerd binnen de cellen, de koelingssnelheid kan oplopen van 100° en 10.000° Celsius per minuut, afhankelijk van de procedure, het volume en de oplossing die gebruikt wordt (Saragusty and Arav, 2011). Embryo cryopreservatie is tot nog toe niet uitgevoerd bij olifanten of neushoorns. Bij het paard, het dier dat aanzien wordt als het best gerelateerde gedomesticeerd dier ten opzichte van de neushoorn (O'Brien and Roth, 2000; Stoops *et al*., 2011), is dit wel mogelijk. Het paardenprotocol zou mogelijk, in licht aangepaste vorm, voor het cryopreserveren van olifanten- en neushoorn embryos gebruikt kunnen worden. Over het cryopreserveren van oocyten bestaan verschillende opvattingen binnen de wetenschap. Zo zijn er wetenschappers die beweren dat het beter is om mature oocyten te cryopreserveren (Lim *et al*., 1992; Ledda *et al*., 2000; Pereira and Marques, 2008; Saragusty and Arav, 2011), terwijl andere meer voordelen zien in de cryopreservatie van immature eicellen (Pereira and Marques, 2008; Aerts and Bols, 2010a). Een andere mogelijkheid is het cryopreserveren van stukjes ovarieel weefsel. Deze techniek heeft het voordeel dat zowel gebruik gemaakt kan worden van weefsel van seksueel mature als immature dieren. Ook is het eenvoudiger om post-mortem de ovaria uit de dieren te verwijderen dan embryo's te kweken en/of oocyten te isoleren. De ovariële weefsel stukjes kunnen ter plekke, in het veld, worden gecryopreserveerd door middel van vitrificatie of individueel steriel verpakt worden in fosfaat-gebufferde zoutoplossing (PBS). Dit kan op kamertemperatuur vervoerd worden naar een beter uitgerust onderzoeks laboratorium (Stoop *et al.,* 2011). Na het ontdooien kunnen immature oocyten op een cultuurmedium gerijpt worden en later eventueel ingebracht worden bij een ander seksueel actief vrouwelijk dier.

Om met deze technieken van ‘geassisteerde voortplanting’ voldoende succes te kunnen behalen is het absoluut noodzakelijk een goed inzicht te krijgen in het verloop van de normale seksuele cyclus bij beide species. Voor de olifant (*Loxodonta africana*) is dit redelijk het geval. Hier is sprake van een 13-18 weken durende oestrale cyclus, waarvan de luteale fase 6-12 weken en de folliculaire fase 4-6 weken duurt. Gedurende de folliculaire fase zijn er twee verschillende luteïniserend hormoon (LH) pieken, de non-ovulatoire, 10-20 dagen na de daling van progesteron concentratie, en de ovulatoire LH piek, die 19-22 dagen na de non-ovulatoire volgt (Hildebrandt *et al.,* 2010).

De oestrale cyclus van de zwarte neushoorn (*Diceros bicornis*) is ook goed gekend. Deze duurt gemiddeld 25 dagen, waarvan de luteale fase 18 ± 1.1 dagen duurt en de folliculaire fase 5 ± 1 dagen duurt (Garnier *et al*., 2002), met één enkele LH piek om de ovulatie te induceren (Hindle *et al.,* 1992; Schwarzenberger *et al.,* 1993).

Over de oestrus cyclus van de witte neushoorn (*Ceratotherium simum*) bestaat enige controversie tussen de verschillende wetenschappers. Er wordt algemeen aangenomen dat er twee verschillende types cycli zijn, namelijk het type I dewelke regelmatig is en rond de 31-35 dagen duurt. Een type II cyclus is onregelmatig en duurt 65-70 dagen (Radcliffe *et al.,* 1997; Patton *et al.,* 1999; Brown *et al.,* 2001; Morrow *et al.,* 2008). Daarentegen beweren Schwarzenberger *et al.* (1998) dat de regelmatige 70 dagen duurt en dat de onregelmatige cyclus 30-70 dagen duurt. De ovulatie wordt geïnduceerd door één enkele LH piek.

Introduction

During a two month stay (November-December 2013), I took up an internship at the University of Pretoria, South Africa. During this trip, I spoke with several people, such as Prof. van Hoven and Ralf Kalwa, the former head of the Kruger National Park rangers, about the increase in the frequency of poaching of elephants and rhinoceroses. Those people made me aware about how big the poaching problem actually is. We discussed a lot about different options to preserve those precious species, varying from translocation, dehorning, legalizing the ivory and rhino horn trade and starting breeding farms. This experience made me realise that different solutions must be explored, such as genetic preservation.

Basically there are two different elephant species: the African (*Loxondota africana*) and the Asian elephant (*Elphas maximus*) (Fig 1).

Fig 1: African Elephant (*Loxodonta africana*) (© Maaike de Schepper) and Asian elephant (*Elphas maximus*) (from http://library.sandiegozoo.org).

The African elephant has two subspecies, namely the African bush elephant (*Loxodonta africana africana*) and the African forest elephant (*Loxodonta africana cylotis*). In this literature review, the African elephant as such will be discussed.

In 2013 and 2014, over 20,000 elephant poachings were recorded worldwide each year. Over the last 100 years, the world's elephant population declined with 95% (from: https://theguardian.com). The African elephant is stated 'vulnerable' on the International Union for Conservation of Nature (IUCN) red list of endangered species. Elephants are mainly poached for their ivory tusks. Those are worth $2.100 per kilogram on the Asian black market. The ivory tusks are mainly used to make jewellery, chopsticks, hair accessories and ornaments (from: http://theguardian.com)

There are five different rhinoceros species: the White (*Ceratotherium simum*), the Black (*Diceros bicornis*), the Greater one-horned (*Rhinoceros unicornis*), the Sumatran (*Dicerorhinus sumatrensis*) and the Javan (*Rhinoceros sondaicus*) (Fig 2).

Fig 2: From left to right: Sumatran rhinoceros (*Dicerorhinus sumatrensis*), Indian rhinoceros (*Rhinoceros unicornis*), White rhinoceros (*Creratotherium simum*), Javan rhinoceros (*Rhinoceros sondaicus*) and Black rhinoceros (*Diceros bicornis*) (from: http://rhinoresourcecenter.com).

Both the white and the black rhinoceros have several subspecies. The white rhinoceros can be divided in the Northern (*Ceratotherium simum cotoni*) and the Southern white rhinoceros (*Ceratotherium simum simum*). The black rhinoceros can be divided in the Eastern (*Diceros bicornis michaeli*), the South central (*Diceros bicornis minor*) and the South western black rhinoceros (*Diceros bicornis bicornis*). In this literature review the black and the white rhinoceros will be discussed in general.

The numbers of poached rhinoceros, in South Africa alone, increase yearly (Fig 3). In 2014, 1,225 recorded rhinoceroses where poached, which means one every eight hours! The real numbers might even be higher, and is underestimated because not all findings are recorded and not all killed animals are found. In 2015, the unofficial statistics stated that there are 240 poached rhinoceroses between January 1st and April 1st, in South Africa. The most recent official numbers are: 49 killed rhinoceroses until the January 22 2015 (Department of Environmental Affairs, South Africa).

The white rhinoceros is stated as to be ‘almost threatened’ and the black rhinoceros is stated ‘critically endangered’ on the IUCN red list however, the South western black rhinoceros is declared extinct since April 2015 by the IUCN. The rhinoceroses are poached for their horns. That contains only keratin, the same substance where human hair or nails are made of. However for one kilogram of rhinoceros horn, around $65.000 is paid for on the black market in Asia. The rhinoceros horn is mainly sold in China and Vietnam. Where it is used as a drug to cure hangovers, cancer and to stimulate the libido. However there is no scientific basis for any medical benefits. In addition the horn is also seen as a status symbol for wealthy people. Since 2013 the poaching rate is higher then the birth rate, which will lead in ultimately to extinction in the wild in the near future.

The battle against poachers has taken immense proportions. They are using advanced equipment, like helicopters, night vision binoculars and several types of machine guns and the poachers don’t hesitate to take a human life to reach their goal.

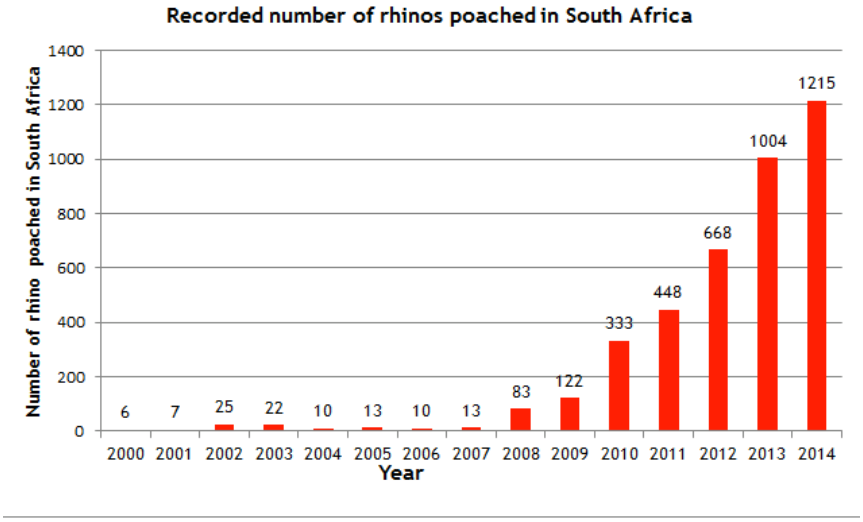

Fig 3: Number of rhinoceros poached in South Africa (from Save the rhino, South African Department of Environmental Affairs, 2015)

The shocking numbers mentioned above illustrate the overall failure of preventive measures, like translocation, dehorning and security of the animals. This means that preservation of both species needs to be taken to another level. Fertility preservation from animals that die unexpectedly or animals that are still alive but not sexually active might be a very valuable strategy. This way genetic material can be used to re-establish a sustainable population.

In this literature review I will focus on three different topics. Firstly I will review the basic characteristics of the oestrous cycle of the elephant and the rhinoceros. Secondly, fertility preservation and how it can contribute to preserve the elephant and the rhinoceros in the future will be discussed. Finally, several future perspectives will be briefly commented upon.

1.1. A general overview: the concept of an oestrous cycle

In adult, female mammals, mature oocytes are released periodically from the ovaries until the female becomes pregnant (Sjaastad *et al.,* 2010). The time between two released oocytes is called the oestrous cycle *i.e.* the time interval between the start of one oestrous cycle until the next; in non-pregnant mammals, it is defined as the 'length' of an oestrous cycle. During the oestrus cycle the female is receptive to mating while her behaviour is changing, which can be used as an oestrus indicator in several mammalian species. Most of the mammals will ovulate during or shortly after oestrous, maximizing the change of fertilization.

An oestrous cycle can be divided into two different phases according to the ovarian activity: the follicular and the luteal phase. The follicular phase is the period when follicles are growing and eventually ovulate (Fig 4).

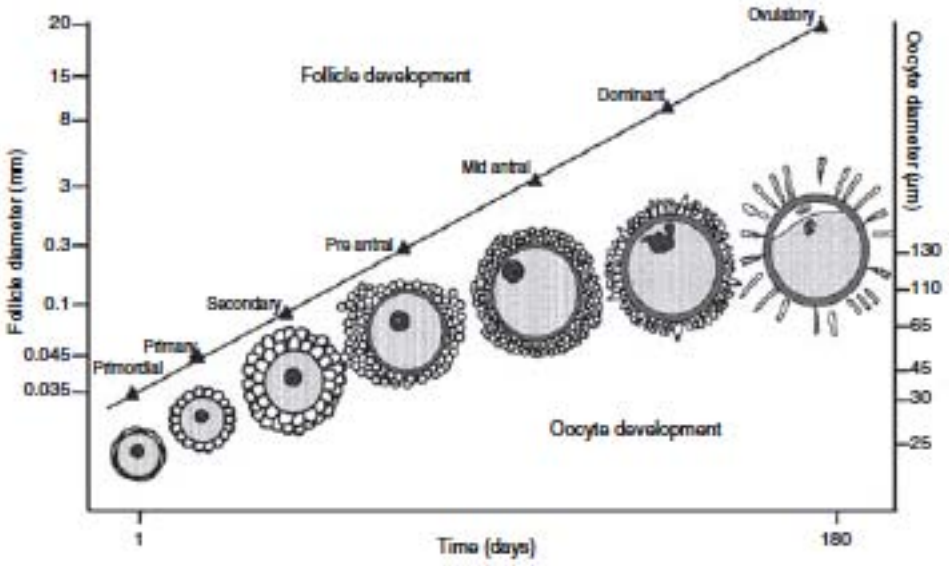

Fig 4: Relationship between follicle and oocyte development (modified from Lussier *et al.,* 1987; Fair, 2003; Aerts and Bols, 2010a).

During this phase the dominant hormone is oestradiol, secreted by developing follicles. The luteal phase (the period from ovulation until the regression of the corpus luteum), is dominated by progesterone, secreted by the Corpus Luteum (CL).

1.1.1. Oocyte formation

Cells divide and reproduce in two possible ways, mitosis and meiosis (Fig 5). During mitosis the complete DNA set is doubled and transferred into two daughter cells. Consequently, the number of cells rises to a large number of identical cells in the body.

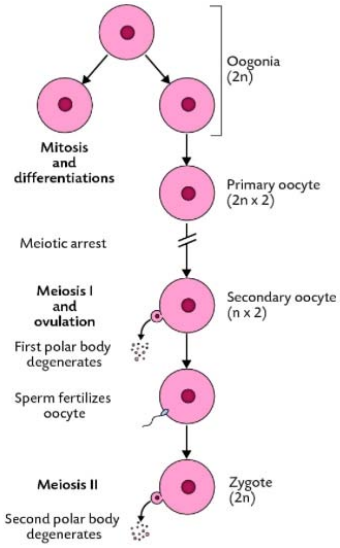

Fig 5: Development of oocytes (from Sjaastad, 2010).

The oocytes are formed during meiosis. From the moment the germ cells start the first stage of meiosis, at the end of embryonic life, they are called primary oocytes. The chromosomes are duplicated. In contrast to mitosis, in meiosis the division of cytoplasm, cytokinesis, does not take place.

The ovaries contain a large reserve of non-growing primordial follicles (Aerts and Bols, 2010a). Follicles can be divided in four different morphological stages: the primordial, primary, secondary and the tertiary follicle. A primordial follicle is surrounded by a single layer of flattened granulosa cells, meanwhile containing an immature oocyte. The development or activation from a primordial follicle to a growing follicle is a gradual process, continuous during the whole reproductive life. It is assumed that the primordial follicles first need to go in the first meiotic arrest before they can be activated (Yang and Fortune, 2008). Primordial follicle activation into a primary follicle is facilitated by the proliferation and differentiation of the granulosa cells. The rising number of granulosa cells influences the transformation form flattened into cuboidal granulosa cells. Additionally the volume expansion of the oocyte influences this transformation. The granulosa cells contain Follicle Stimulating Hormone (FSH) receptors and take part in the follicle growth. The greater number of the activated primary follicles develops into an antral follicle, detectable by the cavity or antrum. Along the way, most follicles regress and start apoptosis, including the growing follicles in a follicular growth wave (Aerts and Bols, 2010a). During the transition from the primary to the secondary stage the follicle expands and the zona pellucida is formed between the oocyte and the growing layers of granulosa cells. Tertiary follicles can be divided in small or large antral follicles, consisting of an antrum filled with follicular fluid. Mature antral follicles are called Graafian follicles. The mural granulosa cells within the mature antral

follicles display LH receptors. Their number increases with follicle maturation, which means the follicle is sensitive for the pre-ovulatory LH-peak.

1.1.2. The follicular phase

The follicular phase covers of only 20% of the duration of the oestrous cycle and can be divided into two stages: the pro-oestrous and the oestrous. The pro-oestrous starts when the luteolysis of the old corpus luteum starts. Pro-oestrous is known for the transition from progesterone to oestrogen dominance. This transition is covered by the rise of Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH). The anterior pituitary gland produces both FSH and LH, which is induced by Gonadotropin Releasing Hormone (GnRH), secreted by the hypothalamus (Fig 6). The granulosa cells in the growing follicle have specific hormone receptors in the cell membrane. FSH binds to these receptors and promotes follicle growth and proliferation. Due to LH secretion, the theca cells of the follicle produce testosterone, which is transfered to the granulosa cells. Due to FSH, testosterone is converted into oestrogen in the granulosa cells. Oestrogen stimulates follicular development and diffuses into the blood stream (Fig 6). During the pro-oestrous, follicles for ovulation are selected. During the follicular phase, oestradiol is the dominant hormone, exteriorising the oestrous behaviour and the willingness of the female tot copulate.

The selected, expanding follicle also produces the hormone inhibin. Inhibin has a negative feedback effect on the secretion of FSH in the anterior pituitary gland. Oestrogen has a negative feedback on the hypothalamus and both hormones cause a decrease in FSH secretion. Due to this decrease in FSH new follicles do not mature and atresia is triggered. The low levels of FSH are however, still sufficient to convert testosterone into oestrogen.

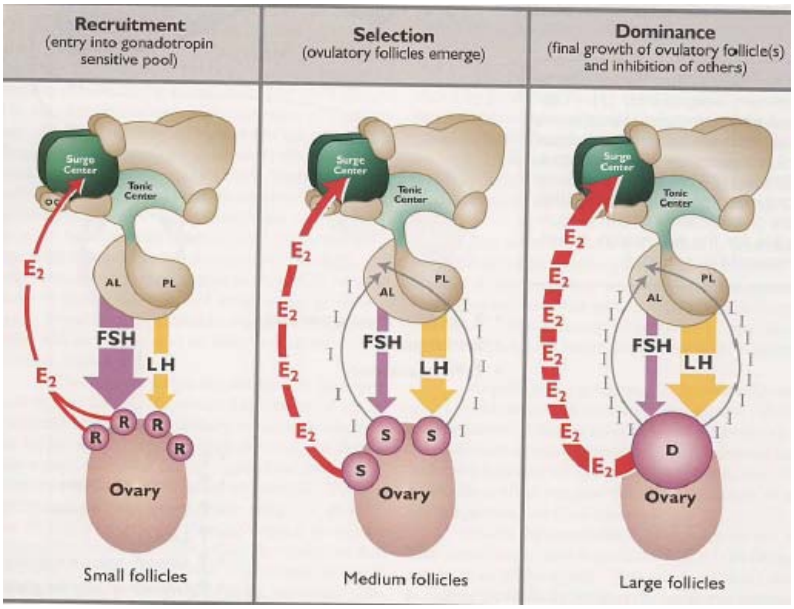

Fig 6: Hormone secretion during pro-oestrous (from Senger, Pathways to pregnancy and parturition, 2005)

When LH secretion increases, so does oestrogen, mainly due to the fact that there are more hormone-producing granulosa cells present in the developing follicle. At the end of the follicular phase the oestrogen concentration rapidly increases. When the oestrogen concentration reaches a threshold value, oestrogen initiates a positive feedback effect on the GnRH surge centre in the brain, causing a preovulatory LH surge (Sjaastad *et al*., 2010). Because of the inhibitory effect of inhibin the positive feedback effect is smaller on the FSH increase (Sjaastad *et al*., 2010).

The preovulatory LH surge induces an increased blood flow to the ovary resulting in an accumulation of fluid from the surrounding tissue in the mature follicle. Due to this accumulation, the hydrostatic pressure in the follicle increases. The enzyme collagenase is released and weakens the follicle wall. These changes finally result in a sudden burst of the follicle wall and a release of the follicular fluid and the oocyte into the peritoneal cavity: ovulation. The oocyte is surrounded by the granulosa cells (the corona radiata) at the moment of ovulation. It is captured by the fimbriae at the end of the tuba uterina. Once the released oocyte is present in the oviduct, it can be fertilized when mating has occurred. The remaining cells in the ruptured follicle undergo luteinisation and start to form the corpus luteum.

1.1.3. The luteal phase

The luteal phase covers approximately 80% of the oestrous cycle and can be divided into two different stages: the metoestrous and the dioestrous. During metoestrous the ovulated follicle is converted into a CL, a temporary intra-ovarian endocrine gland, which starts to secrete progesterone. During di-oestrous which is the longest period of the cycle, the CL is fully functional and secretes significant quantities of progesterone. The dioestrous ends when the CL starts to luteolyse due to the increased amount of circulating prostaglandin F2α that is produced by the non-pregnant uterus. Due to the regression of the CL, the concentration of progesterone decreases and its negative feedback on GnRH disappears. The secretion of FSH and LH by the anterior pituitary gland rise again and follicular development will start all over in the next oestrous cycle in continuous breeders.

1.2. Elephant

The sexual cycle as described above, describes the general pattern of the oestrous cycle of most mammals. However, the oestrous cycle of the elephant differs in several aspects. Elephants are continuous breeders. Female elephants reach sexual maturity approximately at 10-12 years of age. The oestrous cycle of the elephant cow lasts for 13-18 weeks, including a luteal phase with a duration of approximately 6-12 weeks and a follicular phase of 4-6 weeks (Plotka *et al.,* 1988). Consequently, the cow only has three to four oestrous cycles per year. During each of these reproductive cycles multiple corpora lutea are present in the ovaries.

During the follicular phase (the non-luteal phase), two different LH surges occur (Fig 7). The first surge is a non-ovulatory LH surge appearing 10-20 days after the progesterone decrease. During this follicular wave follicles grow reaching a maximum diameter of 13.7 ± 0.7 mm while none of them ovulate (Hildebrandt *et al.,* 2010).

The luteal cells of the accessory corpora lutea from the first, non-ovulatory LH surge start to produce inhibin, which is important for the selection of the dominant follicle during the second LH surge. Inhibin reaches its maximum concentration during the mid-luteal phase and is negatively correlated with FSH release (Brown, 2000). The second LH surge leads to the selection of a single dominant follicle and ovulation. The size of the dominant follicle varies between 21 ± 0.5 mm (Hildebrandt *et al.,* 2010). Dominant follicle rupture takes place 12-24 hours after the ovulatory LH surge. This ovulatory surge occurs approximately 19 to 22 days after the anovulatory LH surge.

Approximately 1 to 3 days after the ovulatory LH surge, the CL reaches a maximum diameter of 22.5 ± 0.8 mm (Hildebrandt *et al*., 2010) and starts to produce 5α-dihydroprogesterone and 5α-pregnane-3-ol-20-one. In some female elephants a preovulatory progesterone rise has been observed. This rise appears 1-3 days prior to the ovulatory LH surge and could be caused by the accessory luteinized follicles to support ovulation (Lueders & Hildebrandt, 2011).

Those accessory corpora lutea, varying from 2 to 10 in each ovary, can be found in female elephants that are cycling or pregnant. They have a different growth pattern as compared to the ovulatory CL. After 25-30 days the accessory CLs reach their maximum diameter, 10 days prior to the single CL from the ovulated follicle (Lueders & Hildebrandt, 2011).

Luteolysis of the anovulatory corpus luteum is initiated one week prior to the ovulatory CL. This regression occurs without any noteworthy changes of the progesterone concentration. After the progesterone concentration starts to decrease, luteolysis of the ovulatory corpus luteum is initiated. Remnants of luteinized corpora lutea, both anovulatory and ovulatory, can be observed in the ovaries during the next follicular phase.

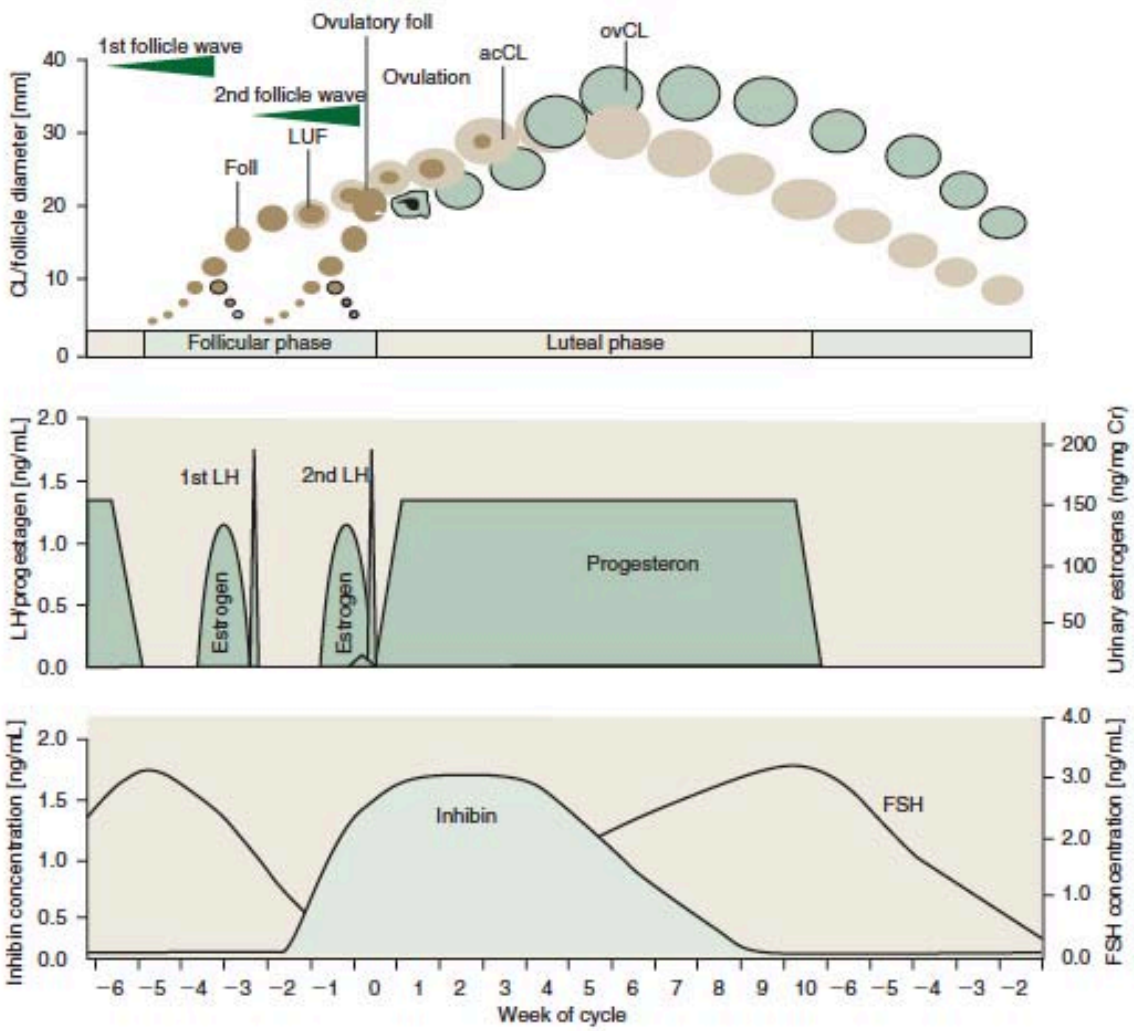

Fig 7: The relationship between the steroid hormones (oestrogen and progesterone), inhibin, gonadotropin and FSH secretion and the ovarian activity, development of follicles (*foll*), luteinizing follicles (*LUF*), accessory CLs (*acCL*) and ovulatory CL (*ovCL*) (Lueders and Hildebrandt, 2011).

Follicle stimulating hormone is secreted by the pituitary gland, together with LH. FSH reaches a maximum concentration at the end of the luteal phase, declines during the follicular phase and reaches the lowest concentration four days before the second LH surge. Fowler and Mikota (2006) stated that due to the high FSH concentration early in the follicular phase there is no selection of dominant follicles is possible. During the follicular phase the concentration of oestrogens and inhibin increase which hampers the secretion of FSH. When the FSH concentration reaches baseline values, the second follicular wave results in the selection of a dominant follicle and, ultimately in ovulation.

1.3. Rhinoceroses

In contrast to the elephant's cycle, the oestrous cycle of the rhinoceros corresponds more to the general cycle of most mammalian species as stated above. Female rhinoceroses are defined as continuous breeders (Garnier *et al.,* 2002). Ovulation in rhinoceroses is induced by a single, pre-ovulatory LH surge occurring at the end of the follicular phase (Hermes *et al.,* 2007). However, the white and black rhinoceros species do differ in certain aspects of their cycle.

1.3.1. White rhinoceros

Several different opinions exist about oestrous cycle length of the white rhinoceros, as summarized in Table 1. Two different oestrus cycle patterns can be distinguished for animals in captivity: cycle type I lasting 31-35 and type II lasting 65-70 days (Fig 8). There is no difference in the inter-luteal phase length between the two different cycles. The noted difference is associated with the variation in luteal phase length (Patton *et al.,* 1999).

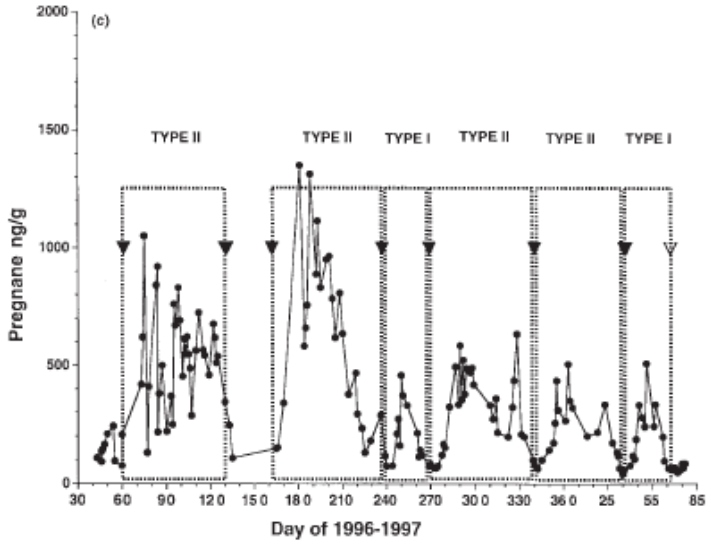

Fig 8: Concentrations of faecal progesterone in the white rhinoceros illustrating type I and type II cycles. Darkened triangle indicates copulation, open triangle indicates mounting (Patton *et al.* 1999).

There are different causes for the extended luteal phase during the type II cycle. Radcliffe *et al.* (1997) reported on a female rhinoceros with uterine inflammation that expressed a type II cycle. Another female rhinoceros in the same study experienced two times an embryonic loss, both one month post-conception and showed the type II cycle as well. Foetal resorption, as diagnosed by ultrasonic examination, also resulted in an extended luteal phase. In contrast, Schwarzenberger *et al.* (1998) stated that the 'normal' oestrous cycle takes up to 10 weeks in the white rhinoceros (Fig 9), instead of four weeks. This discrepancy can be caused by the length of the observation period, as Schwarzenberger *et al.* (1998) monitored different female animals for more than four years while

Hindle *et al.* (1992) and Radcliffe *et al.* (1997) observed the animals for only one to two months which means that these studies were only based on one or two cycles per female.

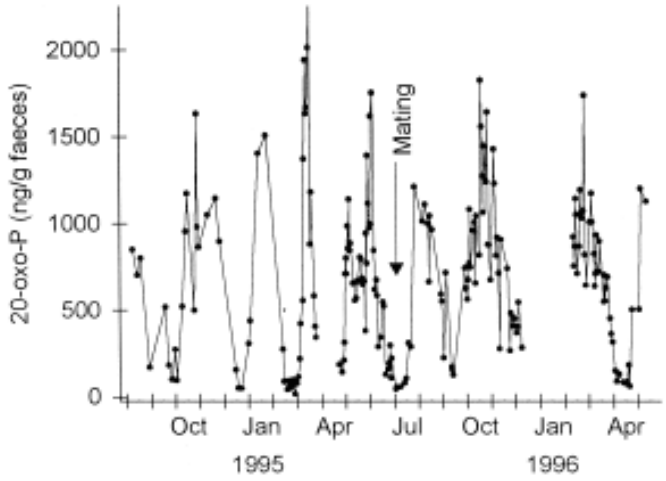

Fig 9: Concentrations of faecal 20-oxo-Progesterone in a white rhinoceros (Pistol), classified as category I (Schwarzenberger *et al.* 1998).

MacDonald *et al.* (2008) stated that wild female rhinoceroses usually show the 35 day cycle. This differs from the cycle in females in captivity that display a low reproductive efficiency. In wild rhinoceroses pregnancy and lactation are dominating the endocrine status (Hermes *et al.,* 2004). Pregnancies take 16 months and the calf is weaned after a lactation of 12 months. This means that a regular oestrous cycle in wild females is an uncommon occurrence, with around 30 full cycles in the entire reproductive life span, comparing to 310 oestrous cycles in captive non-reproducing females (Hermes *et al.,* 2004; Hermes *et al.,* 2007) (Fig 10).

Due to recurring influences of the steroid hormones, asymmetric aging processes in the reproductive organs occur, as well as a reduction of the follicular stock on the ovaries. Also, steroid hormone dependent tumours can develop due to the recurring concentrations of oestrogens and progesterone. This could explain why captive females show a 65-70 day cycle.

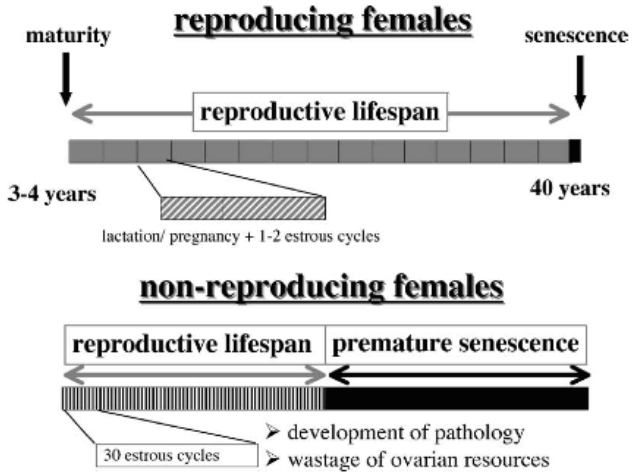

Fig 10: The reproductive aging process in reproducing and non-reproducing female rhinoceros (Hermes *et al*, 2004).

The preovulatory follicle reaches a diameter of 30 ± 2 mm within 48 hours before oestrus (Radcliffe *et al.,* 1997). During the 48 hours before ovulation the shape of the mature follicle changes from spherical to pear shaped. The progesterone metabolite 20α-dihydroprogesterone concentration is low during the follicular phase and increases after ovulation with a peak concentration at 8 to 12 days post-ovulation (called the mid-luteal phase). Oestradiol-17β, an oestrogen metabolite, increases during the basal levels of 20α-dihydroprogesterone; the highest concentration of oestradiol is reached on the day before or the day of the 20α-dihydroprogesterone rise (Hindle *et al.,* 1992).

Table 1: An overview of different cycle lengths in different studies for the White Rhinoceroses (*Ceratotherium simum*)

Author	Year	Method	Cycle length (days)	Number of animals (n)	Wild or Captive
Wagner	1986	Rectal palpation, Vaginal cytology, Urine hormone analysis (Progesterone, Oestrogens)	38-58, average 40-42	1	Captive
Hindle *et al.*	1992	Urine hormone analysis (Oestrogen, Progesterone) Behaviour analysis	North: 24 South 32	North: 1, South: 1	Wild
Bertschinger	1994	Behaviour analysis	±28	5	Wild
Radcliffe *et al.*	1997	Faecal hormone analysis (Progesterone), Echography	31-33	1	Captive
Schwarzenberger *et al.*	1998	Faecal hormone analysis (Progesterone)	Regular: 70 Irregular: 30-70	21	Captive
Patton *et al.*	1999	Faecal hormone analysis (Progesterone), Behaviour analysis	Type I: 35,4±2,2 Type II 65,9±2.4	13	Captive
Strike and Pickard	2000	Faecal hormone analysis (Progesterone), Urine hormone analysis (Progesterone)	32±1,85	5	Captive
Brown *et al.*	2001	Faecal hormone analysis (Progesterone)	Regular: 32,8±1,2 Irregular: 70,1±1,6	13	Captive
Morrow *et al.*	2008	Faecal hormone analysis (Progesterone), Behaviour analysis	Regular: 31,6±0,6 Irregular: 31,9±0,9-67,2±1,3	3	Captive

1.3.2. Black rhinoceros

Black rhinoceroses have individually different cycle lengths, but the average oestrus cycle is about 25 days. Garnier *et al*. (2002) described in a 9-month field study with 6 wild female black rhinoceroses using faecal progesterone analysis, three different types of oestrous cycles (Fig 11). Type I cycles were most frequently observed and were characterized by a total duration of 26.8 ± 1 days, with a period of high concentrations of faecal 20α-dihydroprogesterone of 18 ± 1.1 days (Garnier *et al.*,

2002). The second cycle type, referred to as a type IIa cycle, was detected during the first half of the hormonal profiles of two different animals. Both animals were observed to be mating before faecal sample collection started. In 3 additional animals in early pregnancy a variation in the concentration of 20α-dihydroprogesterone was observed comparable to the characteristics of a type IIa cycle (total duration of ≥40 days and a period of low 20α-dihydroprogesterone concentrations ≤15 days); they were all associated with a significantly longer period of high 20α-dihydroprogesterone concentrations compared to type I cycles (P<0.001) (Garnier *et al*., 2002). Moreover, the type IIb cycles had a longer period of low 20α-dihydroprogesterone concentrations compared to type I.

Follicular growth of the dominant follicle occurred at a rate of 3 mm/day once it reached a diameter of 35 mm (Radcliffe *et al.,* 2001). Follicles smaller than 35 mm will not become dominant. Preovulatory follicles were observed to reach a mean follicular diameter of 49.5±2.6 mm, changing from spherical to pear-shaped before ovulation (Radcliffe *et al.,* 2001). After ovulation a luteal structure is formed with a diameter of approximately 38.3±13.4 mm. Ovulation occurs 48-72 hours after the start of oestrous (Radclife *et al.,* 2001).

The baseline of the faecal progestagen concentration is 1.43±0.41µg/g, with the luteal phase concentrations ranging from approximately 2 to 20 µg/g and the follicular phase taking two to five days (Brown *et al.,* 2001).

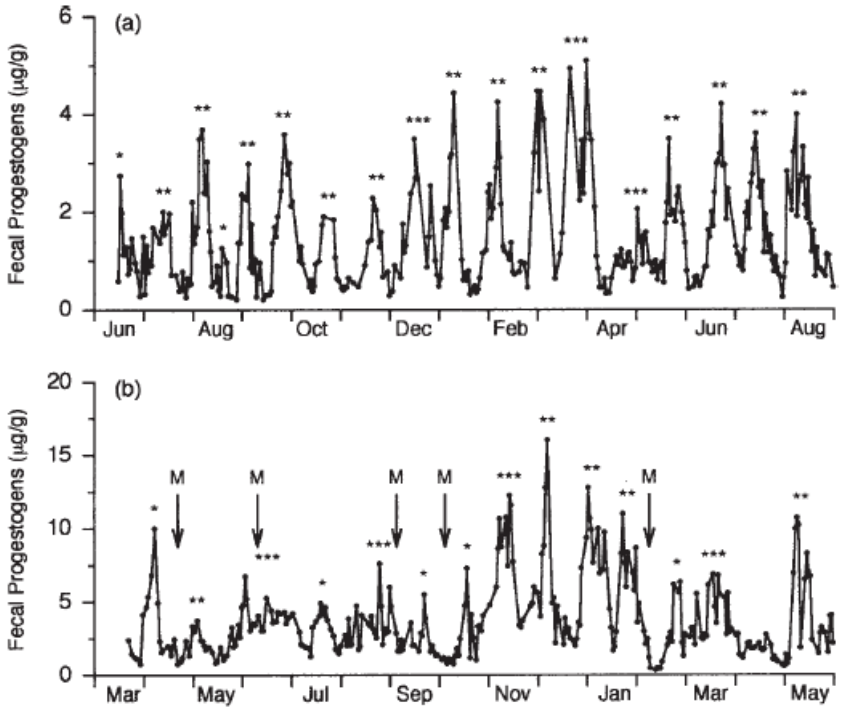

Fig 11: Individual profiles of faecal progestagen concentrations in two representative black rhinoceros females. Reproductive cycles are designated by an asterisk (*short, <20 days; **normal, 20-30 days; ***long >30 days) (Brown *et al.,* 2001).

Table 2: An overview of different cycle lengths as determined by different studies in the Black Rhinoceros (*Diceros bicornis*)

Author	Year	Method	Cycle length (days)	Number of animals (n)	Wild or Captive
Hitchins and Anderson	1983	Behaviour analysis	26-46 average of 35	5	Wild
Hindle *et al.*	1992	Urine hormone analysis (Oestrogen, Progesterone), Behaviour analysis.	25	2	Wild
Schwarzenberger *et al.*	1993	Faecal hormone analysis (Progesterone)	24-26,5	3	Captive
Berkeley *et al.*	1997	Faecal hormone analysis (Progesterone), Ultrasound, Behaviour analysis	26	14	Captive
Brown *et al.*	2001	Faecal hormone analysis (Progesterone)	26,8±0,5 (18% cycles <20, 21% cycles >32)	16	Captive
Lance *et al.*	2001	Faecal hormone analysis (Progesterone)	26-27	1	Captive
Radcliffe *et al.*	2001	Faecal hormone analysis (Progesterone), Ultrasound	25	2	Captive
Garnier *et al.*	2002	Faecal hormone analysis (Progesterone)	Type I: 26,8±1 Type II: >40 Type III: 53,0±6,6	6	Wild

2. Fertility preservation through cryopreservation

Due to the excessive amount of poached animals during the last decades, the genetic diversity in the elephant and rhinoceros population is decreasing rapidly. A narrow genetic basis results in an increase in homozygosity, accompanied by deleterious effects, caused by recessive genes (Demirci *et al.,* 2003), and resulting in lower reproductive efficiency and survival rates.

Several ways to preserve female genes and reproductive tissue have been described such as embryo, oocyte or gonadal tissue cryopreservation. The method of choice depends on the particular situation of the animals: living in a zoo or in the wild, cycling or anoestrous, young or old. 'Fertility Preservation' (FP) as such can be described as methods for an individual animal, male or female, to reproduce notwithstanding iatrogenic or pathological loss of fertility.

Several guidelines need to be followed to successfully preserve mammalian reproductive tissue. Firstly, the tissue needs to be cooled and stored at a temperature of –196 degrees Celsius so that all intracellular chemical reactions are inhibited (Demicri *et al.,* 2003). Cell survival during and following

freezing is determined by the risk of intracellular ice crystal formation and/or extreme dehydration, as well as mechanical and/or physical damage (Saragusty *et al.,* 2011). In general there are two different freezing methods: controlled-rate freezing and vitrification (Table 3).

2.0.1. Controlled-rate freezing or slow cooling

Controlled-rate freezing uses a computer device that controls gradual temperature changes during the freezing and thawing process. The cooling rate differs among cells of different sizes and water permeability. The tissue that needs to be frozen is stored in small volume straws and cooled to -5° to -7° Celsius and kept at this temperature for several minutes to equilibrate. Subsequently, it is cooled at a rate of -0.3°/-0.5° Celsius per minute to around -30° and -65° Celsius (Jewgenow *et al.,* 2010; Saragusty and Arav, 2011). When the correct temperature is reached, the straws are transferred to and stored in liquid nitrogen. This method ensures freezing takes place outside the cells due to the extracellular water crystallizing and the osmotic pressure changes, drawing intracellular water extracellular, resulting in gradual cell dehydration and leaving the intracellular matrix vitrified.

Directional freezing is a type of controlled-rate freezing which ensures the temperature decreases rapidly from 5° to -50° Celsius, avoiding intracellular crystal growth, and moving the cold front through the specimen in stead of working its way from the outside to the inner part.

2.0.2. Vitrification

Vitrification is a cryopreservation method that can be used in the field because there is no need for computer controlled cooling. Vitrification requires liquid nitrogen and a cryoprotectant for very fast cooling, creating a glass-state within the cells. The cooling rate can be around 100° to 10.000°Celsius per minute, depending on the container, the volume, the thermal conductivity and the solution (Saragusty and Arav, 2011). The tissue samples are loaded with high concentrations of cryoprotective agents, which can be toxic and need to be removed very quickly after warming the samples (Jewgenow *et al.,* 2010). Due to the rapid cooling much less ice-crystals are formed.

Table 3: Oocyte and embryo cryopreservation methods (Pereira and Marques, 2008)

Freezing procedures	
Conventional slow-freezing method	Vitrification
1. Standard 0.25 ml straws	1. Several devices for loading embryos and oocytes (conventional straws, OPS, cryoloop, cryoleaf…)
2. Low cryoprotectant concentrations	2. High cryoprotectant concentrations/reduced volume and time with vitrification solution
3. Seeding at −5 to −7°C, controlled slow cooling (0.1 to 0.3°C/min)	3. Ultrarapid cooling rates (−2500°C/min or 20000°C/min using OPS or cryoloop)
4. Plunging at −30 to −70°C and storage in liquid nitrogen (−196°C)	4. Plunging into liquid nitrogen (−196°C)

2.0.3. The role of cryoprotectant agents (CPA)

The aim of CPAs is to reduce the formation of ice-crystals due to an increase of the solution of the H_2O phase in the mammalian cells. There are two categories of cryoprotectants: permeating and nonpermeating. Permeating CPAs are small molecules that readily penetrate the membranes of the cells, form hydrogen bonds with intracellular water molecules and lower the freezing temperature of the resulting mixture, minimizing ice crystallization (Pereira and Marques, 2008). The most commonly used permeating cryoprotectant is propylene glycol (1,2-propanediol), which is usually at a concentration of 1.5 M. At this point the toxicity is low but it interferes only poorly with the ice crystal formation. Several other permeating cryoprotectants are commonly used as well, like ethylene glycol, glycerol and dimethyl sulfoxide (DMSO). Nonpermeating CPAs are used for the osmotic effect; they draw the intracellular water out of the cell, resulting in cell dehydration. Sucrose, galactose and threalose are used as nonpermeating cryoprotectants. Both cryoprotectant classes are used in combination to increase the net concentration of the permeating cryoprotectant inside the cell and also to prevent ice crystal formation (Pereira and Marques, 2008).

2.1. Embryo cryopreservation

The main advantage of embryo cryopreservation is the preservation of the genome of both parents in one embryo. However, embryo cryopreservation is no cure for inbreeding if the stock of the gametes is limited.

In contrast to horses, there are no studies conducted yet on embryo cryopreservation in rhinoceroses and elephants. The horse is considered to be the closest domestic relative of the rhinoceros (O'Brien and Roth, 2000; Stoops *et al*., 2011). To establish an embryo cryopreservation protocol for rhinoceroses the horse protocol can therefore be used as an example. Working with horse embryos showed that smaller, six day old embryos are better permeable for the CPAs than the larger seven day old embryos. Between day six and seven a cellular capsule is formed, which may impair movement of the cryoprotectant into the embryo (O'Brien and Roth, 2000). Meira *et al*. (1993) concluded that glycerol was superior to 1,2 propanediol as CPA for equine embryo cryopreservation. Squires *et al*. (1999) stated that the step-down equilibration method using vitrification is the most promising method for preservation of large equine embryos.

To improve the survival of cryopreserved embryos, integrated FP strategies need to be put in place. The most sensitive embryonic structures are the cellular membrane, the cytoskeleton, intracellular lipids, intracellular water and manipulations to *in vitro* culture conditions (Saragusty and Arav, 2011).

2.2. Oocyte cryopreservation

Oocytes are the largest mammalian cells. Therefore they have a small surface-to-volume ratio and a correspondingly higher sensitivity to chilling and intracellular ice formation (Songsasen and Comizzoli, 2009). Moreover, due to the delicate cytoskeleton of the oocyte the volumetric resistance is reduced.

Both the zona pellucida, a thick and protective wall around the oocyte, and the plasma membrane, have a low permeability coefficient and prevent the movement of cryoprotectant and water into the oocyte (Songsasen & Comizzoli, 2009). Additionally, oocytes are highly delicate to osmotic damage and the meiotic spindle, formed during metaphase II, is temperature-sensitive (Pereira and Marques, 2008; Saragusty *et al*., 2011; Comizzoli *et al*., 2012). In addition, problems arise post-cryopreservation in fertilization and embryonic development, due to hardening of the zona pellucida, preventing sperm penetration as well as changes in the organization of the organelles. The cytoskeleton is often damaged. An alternative approach is cryopreservation of immature (germinal vesicles) oocytes because at this stage the oocytes have lower microtubular chilling-sensitivity due to a smaller size, their lower metabolic rate, no zona pellucida and no meiotic spindle (Pereira and Marques, 2008; Aerts and Bols, 2010a). However, several studies reported immature oocytes to be more sensitive to cryopreservation than matured ones (Lim *et al*., 1992; Ledda *et al*., 2000; Pereira and Marques, 2008), probably due to a lower cell membrane stability, their particular cytoskeletal formation, the damage and/or interruption of cumulus cell projections, that control the intercellular communication between cumulus cells and the maturing oocyte (Ledda *et al*., 2000).

Cold tolerance studies of oocytes in elephants and rhinoceroses are however, limited due to the low availability of oocytes for research purposes.

2.3. Gonadal tissue cryopreservation

A possible solution to preserve genetic material from sexually mature or immature female elephants and rhinoceroses is to harvest and cryopreserve the ovaries post-mortem. Cryopreserved gonadal tissue can be used for post-thawing oocyte retrieval (Jewgenow *et al.,* 2010). A complete ovary can be cryopreserved as well as small ovarian pieces. Viable oocytes can be recovered from ovarian tissue after transplantation of cryopreserved ovaries. Another technique to cryopreserve ovaries is ‘directional freezing’. This technique is based on thermodynamics whereby an ovarian tissue piece or a whole ovary moves through a preprogrammed temperature gradient at a speed that determines the cooling rate (Maffei *et al.,* 2013). Due to the moving cold front the ovary or ovarian sample is cooled precisely and in a uniform way. The cryopreserved tissue can be stored in a tissue bank. These banks can be used as a source for germ cells that can be cultured *in vitro* or grafted into a host. After maturation the cells can be used for IVF (*in-vitro* fertilisation). However, an alternative way to develop and recover oocytes from ovarian tissue could be xenografting ovarian tissue to an immune compromised animal, unable to reject xenografts (Gunasena *et al*., 1998).

There are several advantages in cryopreserving ovarian tissue instead of oocytes or embryos. First of all, the ovaries contain a large amount of oocytes enclosed in primordial follicles. Secondly, ovarian tissue can be collected from animals of different ages, dead or alive (Santos *et al.,* 2010). Thirdly, due to the rather inactive metabolism, the low amounts of lipids, the lack of a zona pellucida, cortical granules and a meiotic spindle, primordial follicles might be more resistant to cryopreservation.

2.3.1. Elephant

Gunsasena *et al.* (1998) conducted a study on xenografted, cryopreserved ovarian tissue of three female elephants from the Kruger National Park, South Africa. The goal of this study was to investigate if an immune compromised nude mouse model would be suited as a host to develop antral follicles originating from cryopreserved elephant ovarian tissue. The three adult female elephants were culled and their ovaries were examined and cryopreserved. On the ovaries of two elephants several corpora lutea were visible and the weight of the ovaries was between 28-188 grams (Gunsasena *et al*., 1998). The ovaries were frozen in liquid nitrogen vapour and cryovials. After thawing their cortical section it was minced (1 mm x 1-1.5 mm) to fit in the bursa ovarica of the athymic nude mouse that was used as a host (Gunsasena *et al*., 1998). The ovarian tissue of the elephant was placed in the pocket of the bursa ovarica in the mouse and covered by the membrane. About 10-11 weeks after transplantation, well developed antral follicles and several small follicles were observed in different grafts. Several oocytes were retrieved despite the fact that they had a poor morphological appearance.

2.3.2. Rhinoceros

Stoops *et al*. (2011) conducted a 10-year study on post-mortem ovaries in captive African black rhinoceroses. In this study the ovaries were shipped at the recommended transport temperature (22°C) for the horse, (Carnevale *et al*., 2004) and in vitro maturation was conducted for 32 to 36 hours (Stoops *et al*., 2011). Ovaries obtained from five adult African black rhinoceros yielded 74 oocytes, 14.8 ± 7.2 per female (Stoops *et al*., 2011). The oocyte quality was classified inyo three different grades. Grade 1: oocytes that were medium to darkly pigmented and completely surrounded by expanded cumulus cells; grade 2, dark to lightly pigmented oocytes surrounded by several layers of compact cumulus cells; and grade 3, dark to lightly pigmented oocytes with either no cumulus or corona radiata cells or only a single layer of corona radiata cells (Stoops *et al*., 2011). By grading the oocytes the chance on successful maturation and embryo formation after fertilisation can be predicted.

Because of to the high number of retrieved oocytes, the ovaries it can be considered as a good source for female gametes. Transported at the recommended temperature will result in oocytes with a distinct chromatin and in this study at least one oocyte per female achieved nuclear maturation (Stoops *et al*., 2011). Many of the oocytes reached the MI phase and several displayed further development in culture. The researchers concluded that rhinoceros oocytes can remain viable 24 to 48 hours post-mortem (Stoops *et al*., 2011).

3. Genome Resource Bank

A genome resource bank (GRB) refers to the collection, processing, storage and use of gametes, embryos and other biological material with the intention to use them in a future breeding programme (Comizzoli *et al*., 2000). A GRB can be used to manage the global gene pool of elephants and rhinoceroses, due to exchange of genetic diversity. In a Genome Resource Bank spermatocytes, oocytes, embryos or tissue grafts can be stored and a database with important biological information can be created. GRB can be used in combination with assisted reproductive techniques for *in situ* (in nature) and *ex situ* (in managed captive programs) conservation.

Cloning

A complimentary alternative to a genome resource bank is cloning or so-called somatic cell nuclear transfer (SCNT) of living elephants and rhinoceroses. During this process the nucleus (DNA) is moved from a donor cell to an enucleated recipient cell (Andrabi and Maxwell, 2007). If cloning results in a viable embryo, the genome of the embryo is a copy of the genome of the donor, apart from the mitochondrial DNA. This DNA is copied from the animal that was used as a recipient. By transferring a somatic cell nucleus into the enucleated egg of a genetic stock, a closely related species or another subspecies, the recovery of the complete genome of the donor can be obtained without the genetic dilution that would occur in producing biparental hybrids (Corley-Smith and Brandhorst, 1999).

This means that the genome of non-cycling, but healthy, elephants and rhinoceroses, or animals in anoestrous can be used to create offspring that in turn can reproduce itself. In this way, no vital DNA is lost and inbreeding in the population or the creation of a small founder population is prevented.

A downside of cloning is that it leads to a stagnation or decline of genetic diversity, especially if non-healthy animals will be cloned. In addition, the production of viable offspring using cloning uses a lot of germ cells, that are not available in elephants and rhinoceroses.

4. Conclusion and future perspectives

Nowadays the interest in preserving genetic material of endangered species is rising, mostly due to the rapid decline in numbers of wild living elephants and rhinoceroses because of the intensive poaching. With each poached animal a considerable amount of important genetics is irreversibly lost. Cryopreservation, through vitrification of ovarian tissue of recently killed animals and the development of a genome resource bank could be a solution to prevent lost of genetic diversity.

However, regardless of the source of the germ cells, oocytes, embryos or ovarian tissue, a thorough knowledge of the fundamental reproductive cycle of both animals is necessary to be able to produce viable offspring (Comizzoli and Wildt, 2014). The oestrous cycle length and the time of ovulation are two important aspects. The reproductive cycle is well known for the elephant, and characterized by an oestrous cycle length of 13-18 weeks, with an anovulatory LH surge 19-22 days before the ovulatory LH surge (Plotka *et al.,* 1988; Hildebrandt *et al.,* 2010). As for the black rhinoceros, the oestrous cycle covers 25 days with a single ovulatory LH surge (Hindle *et al.,* 1992; Schwarzenberger *et al.,* 1993). However, there are different opinions among scientists regarding the white rhinoceros reproductive cycle length, and differences between captive and wild animals.

To increase the overall success rate with cryopreserving elephant and rhinoceros genetic material, more studies on this topic should be conducted. Retrieving oocytes from *in-vivo* donor rhinoceros and elephant is a sensitive issue, due to the high value of the animals and the lack of knowledge on this topic. It is conducted in one study; Hermes *et al.* (2007) performed a trans-rectal ultrasound-guided follicle aspiration in an infertile black rhinoceros. Multiple follicles were punctured, but it did not result in an embryo yet. This can be promising in a future perspective. Another strategy could focus on the optimal use of ovaries from deceased animals in zoos and sanctuaries. A cryopreserving protocol should be made specifically for the two different animal species. In this protocol the most suited cryoprotectant, the optimal method of freezing, the cooling rate and the size of the ovarian tissue samples should be determined. In addition a universal scenario should be agreed upon by which scientists all over the world can recover gonads from freshly slaughtered or poached possible donors under the most optimal conditions. After the veterinarian conducted all these steps the ovaries could be transported to a central laboratory where further research can be performed.

Another solution could be composing a GRB including different biological products, like blood, tissue and DNA. These biomaterials can be used in phylogenetic, systematic and disease studies related to conservation (Wildt, 2000). They also can give insight in gene flow, selection and mating.

A difficulty in this solution is that in Europe all rhinoceroses and elephants are owned by the EAZA (European Association of Zoos and Aquaria) or privately owned. Also, it is prohibited to transport biological materials over long distances, due to the possible risk of spreading infectious diseases throughout Europe. In Africa, the animals that are living in wild parks or around lodges are mostly privately owned. The government owns all the animals that are living in the national parks. It could be difficult to come close to recently poached animals in the national parks, this because the deceased or

poached animal and it surroundings is treated as an official crime scene. All traces that can lead to the poachers are secured.

To solve both problems a central umbrella organisation should be founded. This organisation needs to keep track of all poached animals, and therefore work closely whith the police, the government, the wild parks and national parks in Africa. On the other hand, in Europe the organisation must keep track of all deceased animals in zoos and work closely with the house veterinarians and the laboratories. If all this can be established, there is an opportunity to save those animals.

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