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The effect of long non-reproductive periods on the genital health in captive female white rhinoceroses (*Ceratotherium simum simum*, *C.s. cottoni*)

Robert Hermes [a,*], Thomas Bernd Hildebrandt [a], Christian Walzer [b], Frank Göritz [a], Marilyn L. Patton [c], Sandra Silinski [d], Matt J. Anderson [c], Catherine E. Reid [a], Gudrun Wibbelt [a], Kristina Tomasova [e], Franz Schwarzenberger [f]

[a] *Institute for Zoo and Wildlife Research, PF 601103, D-10252 Berlin, Germany*
[b] *Institute of Animal Ecology, University of Veterinary Medicine, Savoyenstr. 1, A-1160 Vienna, Austria*
[c] *CRES Conservation and Research for Endangered Species, 15600 San Pasqual Valley Road Escondido, CA 92027-7000, USA*
[d] *Allwetter Zoo Münster, Sentruper Str. 315, D-48161 Münster, Germany*
[e] *Zoological Garden Dvur Kralové, Stefanikova 1029, Dvur Kralové n. L. 54401, Czech Republic*
[f] *Institute for Biochemistry, University of Veterinary Medicine, Veterinärplatz 1, A-1210 Vienna, Austria*

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Abstract

White rhinoceroses suffer from a low reproductive rate in captivity. Intensive efforts to propagate specifically the northern white rhinoceros have been very limited. The dismal outlook for this subspecies in the wild makes successful ex situ breeding programs paramount. In this context, this study examined 48 southern and 6 northern white rhinoceroses using ultrasound and faecal hormone analysis to elucidate causes for female reproductive failure and to determine whether long non-reproductive

* Corresponding author. Tel.: +49 30 5168604; fax: +49 30 5126104.
E-mail address: hermes@izw-berlin.de (R. Hermes).

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periods have a detrimental impact on genital health. Results showed that 76% of the nulliparous females had intact hymenal membrane indicating these females had never been bred, at an age when their wild counterparts have delivered multiple offspring. Fifty-six percent of the studied population had various reproductive pathology. Cystic endometrial hyperplasia; leiomyomas of the cervix, uterus and ovary, adenoma; para-ovarian cysts and hydromucometra represent the scope of lesions identified. The stages of the lesions in nulliparous females correlated with age ($r = 0.4$, $P < 0.05$). Due to the severity of the lesions, 28% of the study population was considered post-reproductive. Therefore, the reproductive life span in some individuals was 10–20 years shorter than expected. However, in parous females the incidence of pathological lesions was significantly lower ($P < 0.0001$). Seventy-eight percent females studied had erratic or absent luteal activity. The hormone data corresponded with two ultrasonographic levels of ovarian activity, active and inactive, occurring within an age range of 3–19 years and 15–38 years, respectively. This suggests the lack of ovarian activity by reproductive mid-life in non-reproducing females. The accuracy of the ultrasound findings was validated by necropsy in nine animals showing a strong positive correlation ($r^2 = 0.9$, $p < 0.001$). Our data suggests that the development of reproductive pathology and ovarian inactivity in white rhinoceros is an age-related consequence of long non-reproductive periods. This asymmetric ageing process of the genital organs can be prevented with the achievement of at least one pregnancy.

Keywords: White rhinoceros; Reproduction failure; Ultrasound; Pathology; Ageing

1. Introduction

The two subspecies of the white rhinoceros, the southern (*Ceratotherium simum simum*) and the northern (*Ceratotherium simum cottoni*), are the most recent of the rhinoceros species brought into captivity [1]. Despite a long history of black and Indian rhinoceroses in captivity and the abundant knowledge acquired about their husbandry and reproductive needs, the application of this knowledge in the white rhinoceros has been unsuccessful. Consequently, captive reproduction in white rhinoceros has been poor, since their arrival in modern Zoos during the middle of the last century [1,2]. Specifically, the F1 generation reproduction rate of 8% is of great concern [3]. In contrast to the demographic crisis of the captive population, the southern white rhinoceros wild population is growing due to rigorous conservation efforts. Decades of intensive wildlife protection efforts and an annual population growth of 8% has resulted in a current population totalling ~11.640 animals making the southern subspecies the most abundant of all rhino taxa [4–6]. While the population of the southern white rhinoceros appears viable and stable, the status of the northern subspecies is quite the opposite. A minimal number of four individuals in the wild located in the Democratic Republic of Congo and 10 maintained in captivity are the last remaining animals of their taxon on earth. Intense poaching pressure on the wild population in an instable geopolitical region in Garamba National Park (located in the North of the Congo adjacing to the border of Sudan) puts the survival of this rhinoceros taxon at high risk of extinction [5]. Limited breeding success in captivity greatly impairs efforts to maintain a viable supporting population [2]. Different from the Southern subspecies where an ageing captive population can be supplemented by new imports from the wild, understanding of reproductive failure in captivity is essential for the overall survival of the northern subspecies.

Driven by the low rate of reproduction of white rhinoceroses in captivity, basic knowledge on the reproductive endocrinology of rhinoceros has been compiled to characterize reproductive patterns and to identify underlying problems associated with reproduction [7,8]. In these studies, variable oestrous cycle lengths of 35 and 70 days are described. Some postulated causes for the multi-factorial reproduction failure in captivity are silent oestrous in females that are managed in sibling relationship with one male and conception and pregnancy failure in individual females [7–9]. Nonetheless, the dominating problem for the reduced rate of reproduction is absent or erratic oestrous cycle activity in over 50% of the females in the European and North American Species Survival Program [7,8].

In addition to the reported endocrine dysfunctions, it was widely assumed that females managed in captivity might develop reproductive pathology that remains undetected by means of hormone analysis. Ultrasonography has proven to be a valuable tool for exploring reproductive mechanisms and genital tract pathology in zoo and wildlife medicine [10]. Yet, ultrasonography remains under-utilized in rhinoceroses, used only sporadically for reproductive evaluation in individual animals. Regular ultrasound monitoring of genital organs has illustrated morphologic changes occurring during known reproductive events including the oestrous cycle and pregnancy in individual Sumatran, black and white rhinoceros. Ultrasound diagnosed idiopathic early embryonic death as the cause of reproductive failure in individual animals in all three species of rhinoceroses [9,11,12]. In Indian and Sumatran rhinoceroses genital leiomyomas were characterized as the cause for female infertility [10,13]. The dimensions of the described benign tumours suggest that they were long-standing indicating a progressive development of these alterations in captive females.

The current study was conducted to determine the incidence of reproductive pathology in the female captive southern and northern white rhinoceros population. The hypothesis that long non-reproductive periods may have detrimental effects on the female genital health was evaluated by the use of ultrasonography and faecal hormone analysis. Females of all ages representing different stages of the female-reproductive life were examined to provide basic information for the chronology of the presumed genital pathology. In females with advanced age and absent oestrous cycle activity, we evaluated the effectiveness of oestrous cycle induction protocols to further scrutinize the potential of returning non-reproducing individuals to the breeding population.

2. Materials and methods

2.1. Animals

The female reproductive health was assessed in 48 mature, clinically healthy Southern white rhinoceroses (*Cerothoterium simum simum*) and six Northern white rhinoceroses (*Cerathoterium simum cottoni*) (Table 1). The six Northern white rhinoceroses represent the entire female population of this subspecies in captivity. Twenty-one of all females were evaluated two to nine times over a period of 3 years to monitor the progression of detected pathological alterations and to evaluate the success of hormonal treatments. Three females (nos. 1193, 1194 and 1195) were examined every second day for period of 4–6 weeks

Table 1
Reproductive health assessments in 54 female white rhinoceroses (*Cerathoterium simum simum*, *Cerathoterium simum cottoni*) (Ordered by age the individual results of the clinical, endocrine and ultrasonographic examinations are shown)

Institution	Animal name	Studbook number	Age at examination	Generation	Group size	Offspring number	Exams number	Hymen	Ovarian status	Endocrine status	Endocrine category[b]	Cystic hyperplasia stages I–IV	Leiomyom number
L. Buena Vista, USA	Nande	1246	3	2	3.4	0	2	Intact	Active	Not cycling	n.d.		
Cambridge, Canada	Star	1193	4	0	2.4	0	40	Intact	Active	Not cycling	4		
Cambridge, Canada	Leni	1195	4	0	2.4	0	40	Intact	Active	Not cycling	4		
Omaha, USA	Marina	1243	4	1	2.3	0	1	Intact	Active	Not cycling	n.d.		
San Diego, USA	Utamu	1151	4	1	5.9	0	1	Intact	Active	Not cycling	n.d.		
Cambridge, Canada	Fire	1194	5	0	2.4	0	40	Intact	Active	Not cycling	4		
Erfurt, D	Temba	1208	6		1.3	0	3	Ruptured	Active	Cycling	2		
Perth, Australia	Katala	1282	6	0	1.2	0	4	Intact	Active	Not cycling	Plasma P4		
Jerusalem, IL	Tanda	1183	8	0	1.2	0	1	Intact	Active	Not cycling	4		
Ramat Gan, IL	Keren	1028	8	2	5.6	0	1	Intact	Active	Erratic cycle	3		
Muenster, D	Emmely	812	10	1	1.4	1	1	Ruptured	Active	Erratic cycle	3		
Jerusalem, IL	Shosa	1082	11	0	1.2	0	1	Intact	Active	Erratic cycle	3		
Ramat Gan, IL	Maia	949	11	1	5.6	0	1	Intact	Active	Erratic cycle/cycling	2–3		
Dubbo, Australia	Intombi	1431	11	0	2.3	1	Ruptured	n.d.	Pregnant	n.d.			
Muenster, D	Emmi	967	12	0	1.4	1	1	Ruptured	Active	Erratic cycle/cycling	2–3		
Dvur Kralove, CZ	Najin[a]	943	15	1	2.4	1	1	Ruptured	Active	Erratic cycle/cycling	2–3		
Omaha, USA	Mashile	1242	15	0	2.3	1	4	Ruptured	Active	Not cycling	n.d.		
Muenster, D	Vicky	854	15	1	1.4	0	1	Intact	Inactive	Not cycling	4	III	
San Diego, USA	Dumisha	819	18	1	5.9	0	1	Intact	Active	Erratic cycles	n.d.	I	
San Diego, USA	Sinyaa	822	18	1	5.9	0	1	Intact	Active	Erratic cycles	n.d.		
La Plamyre, F	Noelle	767	18	1	1.1	0	4	Intact	Inactive	Not cycling	4	II	
Dublin, Ireland	Suki	262	18	0	1.2	1	1	Ruptured	Inactive	Not cycling	n.d.	III	2
Dvur Kralove, CZ	Nabire[a]	789	18	1	2.4	0	3	Intact	Inactive	Erratic cycle	3	III	
Dubbo, Australia	Aluka	1428	18	1	2.3	1	Ruptured	n.d.	Pregnant	n.d.			
Salzburg, A	Kifaru	773	19	1	2.3	0	6	Intact	Active	Erratic cycle/not cycling	3–4	I	
Arnhem, NL	Petra	167	19	1	2.5	0	3	Intact	Inactive	Not cycling	4	II	
Schwerin, D	Dicke	583	19	1	1.1	0	3	Intact	Inactive	Not cycling	4		
Bratislava, Sk	Ada	1154	20	0	1.2	0	1	Intact	Active	Erratic cycle	3	II	
Ramat Gan, IL	Carnavella	641	20	1	5.6	6	1	Ruptured	Active	Erratic cycle/cycling	2–3		
Budapest, Hungry	Lulu	902	20	0	1.1	0	7	Intact	Inactive	Erratic cycle	3	II	
Bratislava, Sk	Sena	1155	20	0	1.2	0	1	Intact	Inactive	Not cycling	4		
Givskud, DK	Sophie	652	21	1	1.2	0	1	Intact	Active	Erratic cycle	3	III	

Table 1 (*Continued*)

Institution	Animal name	Studbook number	Age at examination	Generation	Group size	Offspring number	Exams number	Hymen	Ovarian status	Endocrine status	Endocrine category[b]	Cystic hyperplasia stages I–IV	Leiomyom number
Givskud, DK	Eva	653	21	1	1.2	0	1	Ruptured	Inactive	Not cycling	4		
Paris, F	Gaby	651	22	1	1.1	0	1	Intact	Inactive	Not cycling	n.d.	I	
Dvur Kralove, CZ	Nasi[a]	476	24	1	2.4	0	3	Intact	Inactive	Erratic cycle/not cycling	3–4	III	2
Dvur Kralove, CZ	Nesari[a]	377	26	0	2.4	0	3	Intact	Inactive	Erratic cycle/not cycling	3–4	I	2
Salzburg, A	Baby	361	27	0	2.3	0	4	Intact	Inactive	Not cycling	4	I	
San Diego, USA	Getrude	470	27	0	5.9	0	1	Intact	Inactive	Not cycling	n.d.	III	1
San Diego, USA	Hortense	471	27	0	5.9	0	1	Intact	Inactive	Not cycling	n.d.	III	1
San Diego, USA	Nola[a]	374	28	0	1.2	0	1	Intact	Inactive	Not cycling	n.d.	II	2
Arnhem, NL	Freya	230	29	0	2.5	1	1	Ruptured	Active	Cycling	2	I	
Salzburg, A	Kathi	362	29	0	2.3	0	2	Intact	Inactive	Not cycling	4	II	
Ramat Gan, IL	Ziona	241	30	0	5.6	7	1	Ruptured	Inactive	n.d.	n.d.	I	
San Diego, USA	Nadi[a]	376	30	0	1.2	0	1	Intact	Inactive	Not cycling	n.d.	I	
Ramat Gan, IL	Lola	243	30	0	5.6	0	1	Intact	Inactive	n.d.	n.d.	IV	
Ramat Gan, IL	Mazal	242	30	0	5.6	6	1	Ruptured	Inactive	n.d.	n.d.		
Arnhem, NL	Gea	174	31	0	2.5	4	1	Ruptured	Active	Not cycling	4	I	1
Arnhem, NL	Roelli	175	31	0	2.5	0	1	Intact	Active	Cycling	2	III	
Arnhem, NL	Ineke	171	31	0	2.5	0	3	Intact	Ianctive	Not cycling	4	II	1
Cambridge, Canada	May	366	31	0	2.4	0	8	Intact	Inactive	Erratic cycle	3	III	
Dublin, Ireland	Reni	89	32	1	1.2	0	1	Intact	Active	Cycling	2	II	
Madison, USA	Naida	696	32	0	1.1	0	2	Intact	Inactive	Not cycling	n.d.	II	2
Dubbo, Australia	Alexandra	614	35	1	2.3	6	1	Ruptured	Inactive	Not cycling	n.d.		
Omaha, USA	Henrietta	39	38	0	2.3	0	3	n.d.	Inactive	Not cycling	n.d.	III	

Ceratotherium simum simum.

[a] *Ceratotherium simum cottoni*.

[b] Schwarzenberger et al. [7].

during two subsequent years to determine follicular development on the ovaries. All animals were listed in International Studbook [2] and in the European, North American and Australian Species Survival Programs (SSP) – which are dedicated to the propagation of this species and to the provision of superior husbandry in captivity. These females were housed as a breeding pair or with one and more other females.

2.2. Endocrinology

Endocrine function of all females was monitored non-invasively by faecal progesterone metabolite analysis prior to the first ultrasound examination and between subsequent exams (Table 1) [7,8]. Luteal activity was classified into four groups based on the faecal pregnane analysis [7]. The period of routine endocrine monitoring varied between individuals from 6 months to 9 years.

A short-term protocol for ovulation induction was applied in selected females who did not exhibit luteal activity. Four females were treated multiple times (one to three) orally with the synthetic progestin chlormadinone acetate (CMA; 35 mg/36 h/45 days, Synchrosin®, Werfft-Syntex, Vienna, Austria) followed by an hCG injection (10.000 I.U. Chorulon®, Intervet, Boxmeer, Germany) to induce ovulation [7,14]. The success of the CMA + hCG induction protocol was evaluated by an ultrasound examination 72–96 h after hCG injection and by the measurement of faecal 20-oxo-pregnane concentrations [7].

Six females were treated with 14.1 mg GnRH agonist (Desloerlin, Peptech, Melbourne, Australia) as a long-term estrus induction protocol. The GnRH was released from long acting implants over a period of 6–8 months. The implants were applied subcutaneously behind the ear using a specialized blow dart (TS-ID 100, Telinject GmbH, 67352 Römerberg, Germany). In well-trained females, the implants were implanted manually using a plastic applicator. The area of application was anaesthetized locally with lidocaine-hydrochloride Gel (Xylocain® Gel 2%, AstraZeneca GmbH, 22876 Wedel, Germany) for 20 min. The use of the plastic applicator required a small surgical incision that penetrated the extremely thick rhinoceros dermis before the application of the implants. The success of the GnRH oestrous induction protocol was evaluated by an ultrasound examination 8–10 months after GnRH implant application and by the measurement of faecal 20-oxo-pregnane concentrations [7].

2.3. Anesthesia

Over 200 clinical and ultrasonographic examinations were performed at 21 different institutions. Physical and chemical restraint necessary for the reproductive assessment varied among these institutions according to the level of animal training, presence of a restraint chute and anxiety of the individual animal. Thirty females required full anaesthesia (Table 2). In these cases, anesthetics were injected into the neck muscles caudo-ventral to the ear using a dart pistol and 3.5 ml plastic darts with a 60-mm needle (Dan-inject International Gelsenkirchen, Germany). An additional IV injection of ketamine at a dose of 150 ± 100 mg (Narketan®, Chassot AG, Bern, Switzerland) injected into the ear vein was used to reduce the time to lateral recumbence. A heavy-duty inner tube placed beneath the shoulder and pelvis alleviated possible compressive trauma to the

Table 2
Different chemical restraints for the performance of reproductive assessments in white rhinoceros

Chemical restraint	Etorphine[a] (mg/animal)	Acepromazine[b] (mg/animal)	Detomidine[b] (mg/animal)	Butorphenol[c] (mg/animal)	Naltrexone[d] (mg/animal)	Atipamezole[e] (mg/animal)
Anaesthesia ($n = 30$)	3.1 ± 0.6	12.5 ± 2.5	12.0 ± 2.0	12.0 ± 2.0[a]	250	20
Sedation free standing ($n = 12$)	1.1 ± 0.3	5 ± 1.0	15 ± 2.0	15 ± 2.0	50	
Sedation standing in a restraint chute ($n = 3$)	–	–	15 ± 2.0	15 ± 2.0		
Chute trained for repro assessment ($n = 7$)	–	–	–	–	–	–

[a] Large Animal Immobilon® C-Vet Veterinary Products, Lancs, UK.
[b] Domosedan®, Orion Corporation, Farmos, Finland.
[c] Turbugesic®, Fort Dodge Animal Health, IA.
[d] Trexonil®, Wildlife Laboratories Inc., Fort Collins, CO.
[e] Antisedan®, Orion Corporation, Farmos, Finland.

downed side during lateral recumbence. Recumbent animals received supplemental oxygen at a rate of 15 L/min through a nasal tube. The animals were standing and alert approximately 2 min following administration of the antagonists [15].

Less anxious animals ($n = 12$) were sedated standing free in the stall with their head leaning against the wall or bars of the enclosure (Table 2). In three females, which were partially chute trained, a light standing sedation was sufficient to achieve tolerance for the reproductive assessment. For standing sedation, the sedatives were hand injected into the neck muscles. Two minutes after the partial reversal all 15 sedated animals were alert and walking. Seven animals were trained to tolerate rectal palpation in a restraint chute without the use of chemical restraint [16]. With reduced anxiety and increased training of the animals to tolerate rectal palpation, the amount of high potent, synthetic morphine was significantly reduced ($P < 0.001$) decreasing the anaesthetic risk.

Even when females were trained to tolerate rectal palpations on a regular basis the attempt of a clinical palpation of the vagina to determine the status of the hymenal membrane still induced strong defensive reactions. Therefore, lidocaine-hydrochloride Gel (Xylocain® Gel 2%, AstraZeneca GmbH, 22876 Wedel, Germany) was applied in those females, which were only sedated or examined without chemical restraint. The local anaesthetic was applied to the slightly parted vaginal labia. The palpation started minimum ten minutes after the application of the Gel.

2.4. Ultrasonography

Reproductive assessment consisted of a clinical examination of the vagina and an ultrasonographic examination of the genital organs.

To ensure acoustic coupling of the ultrasound waves, an enema was given prior to the transrectal ultrasound [17]. The vagina, cervix, uterine body and parts of the uterine horns were imaged in cross-sectional and longitudinal planes with a hand-held 2–5 MHz ultrasound probe (SonoSite 180 Plus, C60 5–2 MHz probe, L52 10–5 MHz Product Group International, Inc., Lyons, CO 80540). The exceptional length of the genital tract in the white rhinoceros compared with other rhinoceros species [18] required the use an ultrasound probe extension in 50% of the animals ($n = 26$) in order to visualize the cranial parts of the genital tract. When probe extensions were used the uterine horns and ovaries were visualized longitudinally only using a 2–5 MHz convex or a 10–5 MHz linear ultrasound probe fitted to a 45 cm S-shaped steel adapter (Schnorrenberg Chirugie-mechanik GmbH, 16352 Schönewalde, Germany). Length, weight and angle of this adapter was specifically designed for the use in female rhinoceroses to overcome the distance to the ovary and to facilitate acoustic coupling of the probe close to the laterally positioned organs. The genital organs were measured as still images during the actual examination with standard calibration software included in the ultrasound unit and were more precisely measured in retrospective analysis from recorded videotapes (Sony, Watchman, GV-D 900 E, Germany; Sony, DVM 60, Mini DV Cassette, Germany) using an image analysing program (analySIS PRO 2.10.100, Soft-Imaging System GmbH, Münster, Germany).

Measures of the length and the height of the ovary were taken from longitudinal still images. Since the ovary is elongated ellipsoid organ its approximate volume was

calculated, according to the volume of a rotation ellipsoid accommodating the symmetrical shape of this organ [17,19].

2.5. Ultrasonography validation and histology

Nine animals from the group died during this study and were subsequently necropsied (Studbook nos.: 89, 167, 171, 174, 175, 366, 470, 471 and 583). The genital tract of these females was extracted, dissected and evaluated for the presence of pathological lesions that had previously been diagnosed during ultrasonographic examination. The uteri, ovaries and any pathological lesions observed were then fixed in 10% formalin. After fixation, tissue samples were embedded in paraffin blocks. Tissue sections (4 μm) were cut and stained by routine methods with haematoxylin eosin (HE), Azan, Masson trichrome, Masson Goldner, van Giesson for histological examination.

Additionally, digital images (with scales) of the gross dissections were also taken (in three cases; studbook nos.: 89, 470 and 471) to test the accuracy of the ultrasonographic techniques. Image analysis of digital photographs of dissected reproductive tracts (using NIH image for Apple macintosh) were utilised and resultant measures (lengths and widths) compared to those made previously during ultrasonography examinations. Comparisons included measurements of left and right ovaries respectively, a hymenal cyst, uterine cysts and leimyomas, and several para-ovarian cysts.

2.6. Statistical analysis

All values are given as means ± S.E.M. The Mann–Whitney test (non-parametric) was used to examine the differences of the amount of uterine cystic hyperplasia among females with different parity status and age. The Mann–Whitney test was chosen because standard deviations were significantly different between means of categories for several different parameters (Bartlett's test). Differences between categories were examined by the unpaired *t*-test; the normality was confirmed by Kolmogorov–Smirnov-test. The Spearman rank correlation coefficient was calculated to characterize the relationships between the age of the animals and ovarian volume and the degree of pathological alteration. A major axis regression correlation was also undertaken by plotting measures of reproductive anatomies and pathological lesions (lengths and widths) taken during ultrasonographic examination against those made during necropsy (gross dissection of animals 89, 470 and 471). All calculations were performed using the SPSS 9.0 (SPSS Inc., Chicago, IL) statistical software package. The significance level was set to 5%.

3. Results

3.1. Clinical examination

The vagina of all nulliparous females was palpated during the reproductive examination ($n = 45$). In 76% ($n = 34$) of these females only the *vestibulum vaginae* was accessible. The intact hymenal membrane prevented the palpation of the cranial aspect of the vagina and

portio cervicale. In 24% of the nulliparous females ($n = 11$) the vagina and cervix were accessible, as the hymenal membrane had previously been ruptured during mating.

3.2. Ultrasound

Reproductive pathology was observed in 56% ($n = 30$) of the captive white rhinoceroses investigated. Cystic endometrial hyperplasia ($n = 30$), intra-mural leiomyoma in the uterus ($n = 8$) and cervix ($n = 1$), intra-luminal adenoma ($n = 3$) in the uterus, para-ovarian cysts ($n = 15$) and mesovarial tumours ($n = 3$) were alterations detected by ultrasonography in the genital organs of these females. Twenty-eight percent of these females ($n = 15$) were diagnosed with advanced stages of pathological alterations and therefore were considered post reproductive.

According to the ultrasonographic, macroscopic and histological findings ($n = 6$) different stages of cystic endometrial hyperplasia and leiomyoma were classified. Cystic endometrial hyperplasia was classified as follows: (I) single endometrial cysts ($n = 5$), (II) diffuse multi-focal and confluating cysts in the uterine body and horns ($n = 13$), (III) diffuse multi-focal and confluating cysts associated with increasing amounts of endometrial fibrosis ($n = 12$) and (IV) complete endometrial fibrosis ($n = 1$). Endometrial adenoma ($n = 1$) and hydromucometra ($n = 2$) with turbid fluid accumulations up to 60 litres was associated with the presence of cystic uterine hyperplasia in three females as the last sequela of this progressive uterine degeneration. The earliest detection of cystic endometrial hyperplasia was 15 years of age. The incidence of cystic hyperplasia was significantly lower in pluriparous females compared with nulliparous females ± 15 years of age ($P < 0.0001$). In nulliparous females, the age and the stage of the pathological alteration were positively correlated (Spearman $r = 0.4$, $P < 0.05$).

The ultrasonographic appearance of leiomyomas was classified as (I) small low echogenic, distinct intramural lesion with a diameter of 1–4 cm ($n = 3$) and (II) larger tumour masses >5 cm with detectable internal blood supply and high echogenic areas of tissue necrosis ($n = 6$).

3.2.1. Ovary

Absent or erratic luteal activity characterized the endocrine profile of forty females investigated. Despite similar hormone profiles in these non-cycling females, their ovarian activity status, i.e. the amount of follicular or luteal activity, varied considerably in the ultrasonographic assessment. In females with absent or erratic luteal hormone profile, active and inactive ovarian statuses were found. Active ovaries (29.2 ± 2.2 cm^3) were characterized by the presence of 4–10 follicles >10 mm and were documented in females 3–19 years of age ($n = 16$). Inactive ovaries (14.7 ± 1.3 mm^3) were characterized by absent follicle development ($n = 19$) or minimal follicular activity with 1–2 follicles <4 mm and were imaged in females 15–38 years of age ($n = 24$). In one female (no. 167), luteal spikes of about 1 week length in the hormone profile corresponded with the formation of persistent luteal structures on both ovaries. Active and inactive ovaries showed significant differences in size ($P < 0.001$). In comparison, ovaries of regular cycling females (34.1 ± 4.5 cm^3, $n = 9$) did not show a significant difference in size to active ovaries of non-cycling females ($p > 0.05$).

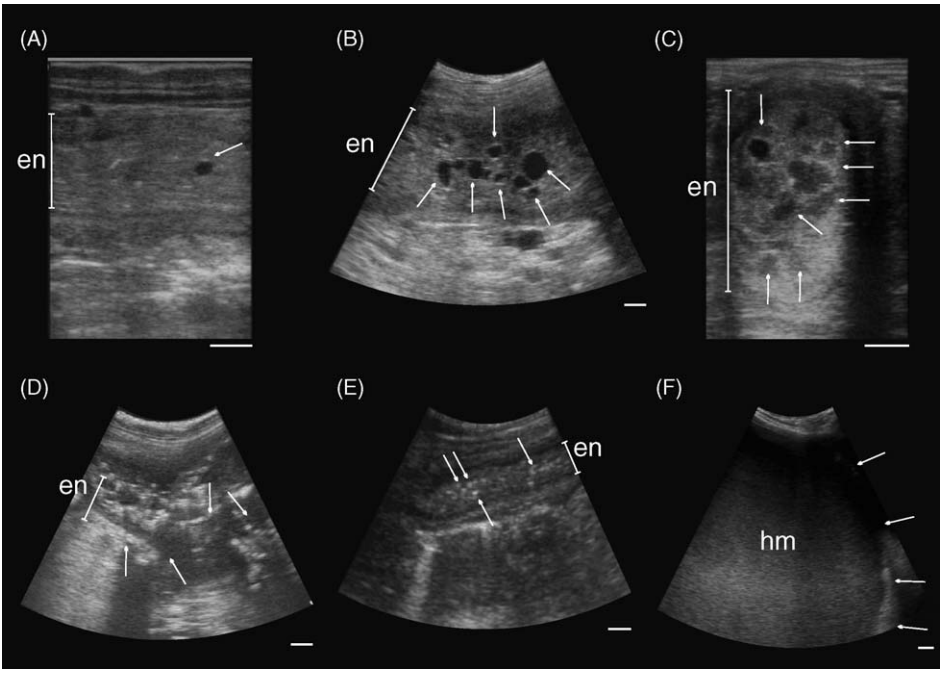


Fig. 1. Ultrasonographic images of the uterus in white rhinoceroses showing different stages of cystic hyperplasia in the endometrium (en). (A) A singular fluid-filled cyst (arrow) in a cycling, primiparous 30-year-old female, no. 230. (B) Multiple fluid-filled cysts (arrows) in a cycling nulliparous 20-year-old female, no. 652. (C) Multiple confluating cysts with fluid-filled and medium echogenic cavities (arrows) in a non-cycling, nulliparous 20-year-old female, no. 1154. (D) Multiple confluated cysts with increased amount of fibrotic areas (arrows) in a non-cycling nulliparous 25-year-old female, no. 476. (E) Endometrial fibrosis (arrows) in a senile non-cycling, nulliparous 30-year-old female, no. 243. (F) Hydromucometra (hm): fluid filled cavity of the uterus (arrows) in 30-year-old female, no. 366. Scale bars represent 1 cm.

To further elucidate follicular dynamics in females with active ovaries three females were examined every other day for 6 weeks during two subsequent years. During a 20 ± 1.8 day period a dominant pre-ovulatory sized follicle (45.9 mm $\pm$ 1.4) developed from a population of smaller follicles. Following the point of largest diameter, this follicle decreased in size until it was non detectable; whereas at the same time, a population of small follicles indicated the development of a new dominant follicle on the contra-lateral ovary. A spider web-like structure within the follicular cavity formed when the dominant follicle grew beyond the pre-ovulatory size up to 59.4 mm. The spider web-like structure persisted on the ovary even during the development of the next dominant follicle on the contra-lateral ovary.

Thin-walled, unilocular para-ovarian cysts were imaged in all nulliparous northern ($n = 5$) and 10 southern white rhinoceroses located between the ovary and the uterus. Average age of females displaying para-ovarian cysts was 26 ± 1.5 years. The endocrine profile in 87% of these females ($n = 13$) was characterized by the absence of luteal activity. The para-ovarian cysts attained sizes from a few centimetres in the southern (2.3 ± 0.4 cm)

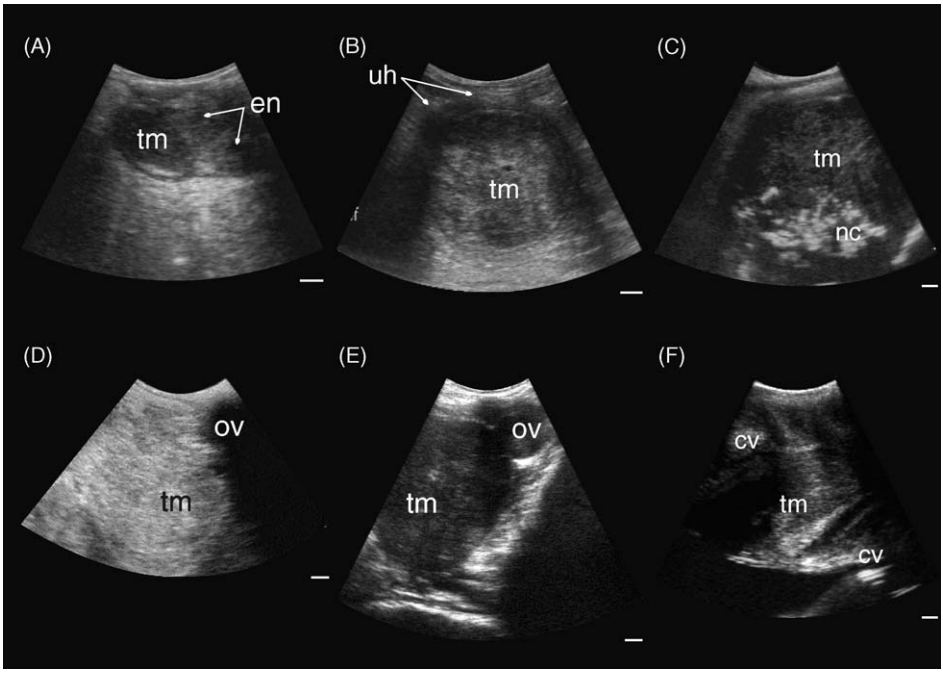


Fig. 2. Ultrasonographic images of uterine (A–C), ovarian (D and E) and cervical (F) tumours (tm) in white rhinoceroses: (A) A 4 cm × 2 cm mural tumour in left uterine horn. The tumour is lower echogenicity compared with the endometrium (en) in a nulliparous female, no. 374. (B) A 7 cm mural tumour compressing the uterine horn (uh) in a nulliparous female, no. 471. (C) A 12.5 cm tumour with central areas of necrosis (nc), no. 476. (D) A 5 cm para-ovarian tumour in cycling, multiparous, 30-year-old female, no. 174. (E) A 12 cm para-ovarian tumour a non-cycling, uniparous, 32-year-old, no. 696. (F) Longitudinal view: the low echogenic base of a polyp (arrows) in the cervix in a nulliparous female, no. 476. The tumour is located between the cervical folds (cv) with its growths directed towards the vagina. Scale bars represent 1 cm.

to 5–15 cm (9.6 ± 1.4 cm) in the northern white rhinoceros demonstrating a significant difference in size of this pathological alteration between the two subspecies ($P < 0.0001$). In the southern subspecies, no differences of the ovarian size were noted between the affected (21.0 ± 4.2 cm) and unaffected ovary (22.5 ± 4.0). In the northern white rhinoceros, the affected ovary (7.5 ± 1.4 cm) was significantly smaller compared with the inactive, unaffected contra-lateral ovary (30.5 ± 4.5 cm) ($P < 0.008$). The appearance and size of the cysts in the both subspecies remained static ($n = 6$) over the period of three years, with the exception of one female who formed septa within the cavity of a cyst that increased by 5 cm. Histology determined ($n = 3$) the origin of these cysts from mesovarium and oviductal serosa (Figs. 1–4).

3.2.2. Ultrasound validation

Measurements taken during ultrasound examinations were found to be highly accurate when compared with actual measurements made during necropsy (using digital

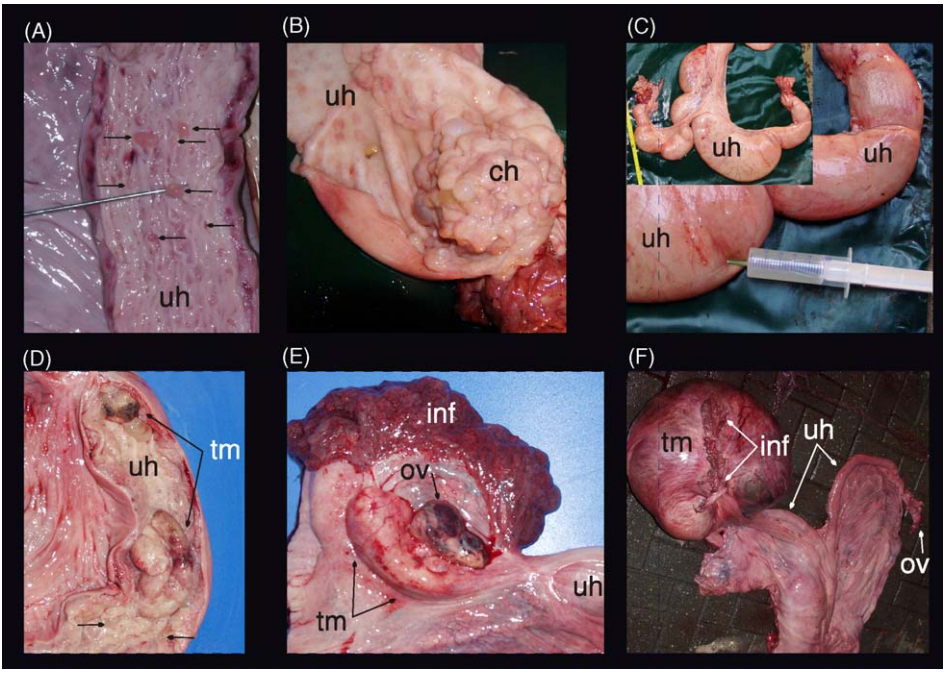
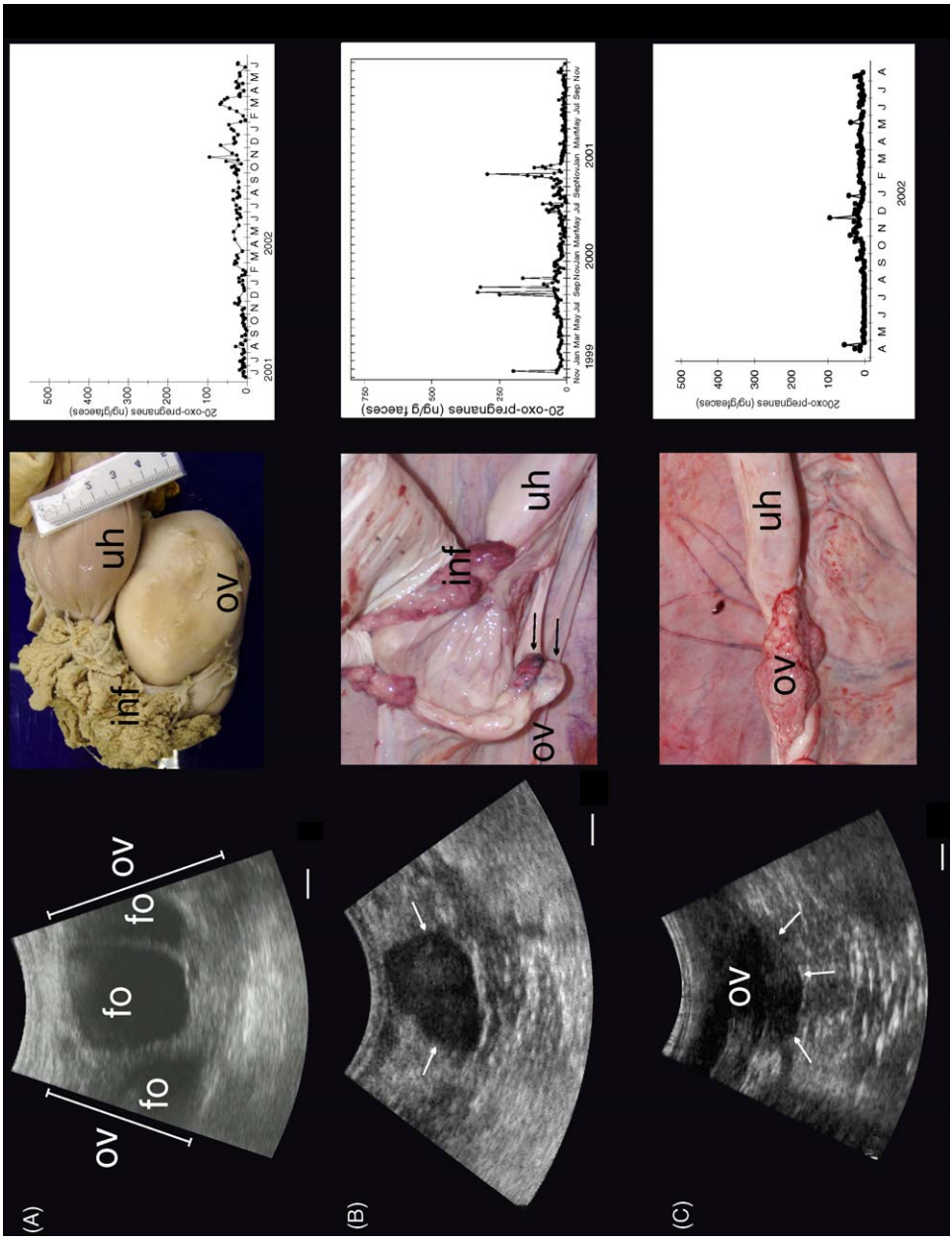


Fig. 3. Post mortem preparations of the pathological alterations in the genital organs from white rhinoceroses. (A) Multiple endometrial cysts (arrows) in the uterine horn, no. 174. (B) Multifocal cystic endometrial hyperplasia at the end of the uterine horn (uh) covering the opening of the oviduct, no. 175. (C) Hydromucometra: uterine body and horns (uh) filled with 3 l of fluid, no. 175. (D) Focally extensive cystic endometrial hyperplasia (arrows) and adenomatous polypoid intraluminal protrusions (tm) in the uterine horn (uh), no. 179. (E) A leiomyoma (tm) in the mesovarium located closely to the ovary (ov) and opposite from the infundibulum (inf). *Note*: The uterine horn (uh) is intact, thus, suggesting a mesovarian origin of this leiomyoma, no. 179. (F) A para-ovarian leiomyoma weighing 35 kg (tm) in a pluriparous cycling female, no. 174. The intact end of the uterine horn and the infundibulum (inf) stretched over the tumour surface suggested a mesovarian origin of this leiomyoma.

photography and image analysis). A major axis regression plot revealed a very high correlation (Fig. 5: $n = 38$, $r^2 = 0.9$, $p < 0.001$) between ultrasonographic and post-necropsy measures. Specifically, lengths and widths of left and right ovaries and pathological lesions (including a hymenal cyst, uterine cysts and leimyomas, and para-ovarian cysts) were observed in three separate female rhinoceroses.

Fig. 4. Ultrasonographic images and post mortem preparations of the ovary from anoestrus females as indicated by baseline luteal activity in the endocrine profiles. (A) Sonogram of pre-ovulatory sized follicles (fo) on the ovary of a 6-year-old female, no. 1195. Despite the absent luteal activity, the ovary is active and well sized measuring 6 cm × 10 cm, similar to the ovarian dimensions of 7 cm × 5 cm post mortem in a 9-year-old female, no. 840. (B) Sonogram of two permanent luteal structures in an 18-year-old female, no. 167 with erratic spikes of luteal activity. The post mortem shows similar luteal structures in a non-cycling, 30-year-old female, no. 175. (C) Sonogram and post mortem cross-section of a small inactive ovary (ov, arrows) without detectable functional structures in a non-cycling 30-year-old female, no. 171. Note the difference of ovarian dimensions and activity between (A), (B) and (C) despite similar baseline luteal activity. Scale bars represent 1 cm.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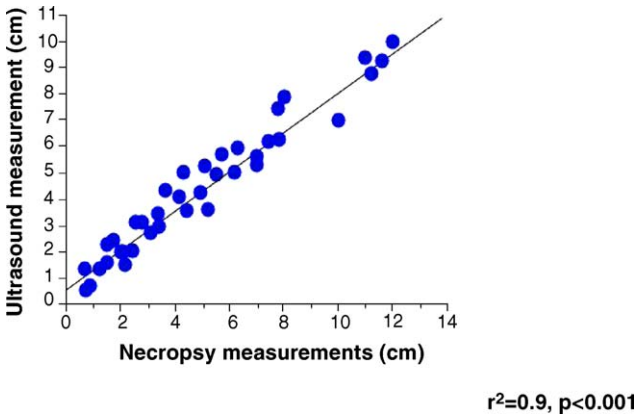

Fig. 5. Accuracy of ultrasound characterization and necropsy findings in two white rhinoceroses.

Such findings further validate the use of high resolution trans-rectal ultrasonography in the assessment of reproductive health (and disorders) in female rhinos. Indeed, the examination and comparison of the reproductive tracts from several rhinoceroses pre- and post-mortem has also shown that ultrasonography was not only accurate, but also an invaluable tool for the diagnosis of pathological reproductive conditions.

3.2.3. Ovulation induction

We investigated the effectiveness of chlormadinone acetate + hCG ($n = 10$) as a short-term treatment to stimulate ovulation.

In 80% ($n = 8$) of the CMA + hCG inductions ultrasound characterized an ovulatory-sized follicle (36.8 ± 0.3 mm) 72–96 h after hCG injection. However, in follicles >40 mm the beginning formation of intra-follicular septa was noted. Despite the presence of ovulatory-sized follicles following the CMA + hCG oestrous induction protocol elevated 20-oxo-pregnane concentrations were measured in only 30% of the inductions ($n = 3$).

3.2.4. Oestrous induction

In an attempt to resume oestrous cycle activity in acyclic female white rhinoceroses, long-acting GnRH implants ($n = 6$) were used.

The GnRH suppression of gonadotropin release via pituitary desensitization resulted in a continued absence of luteal activity during the 6 months of hormone release. Following the GnRH agonist treatment a follicular activity was re-initiated in all treated females ($n = 6$) displaying two to four large follicles (15–20 mm). The follicular activity was enhanced when compared to the pre-treatment ovarian status with no follicles ($n = 3$) or presence of small follicle only (<10 mm, $n = 3$). In one female, the resumed follicular activity resulted in the initiation of a regular oestrous cycle activity (Fig. 6). The para-ovarian cysts detected in five of the treated females remained unaffected by the hormone treatment.

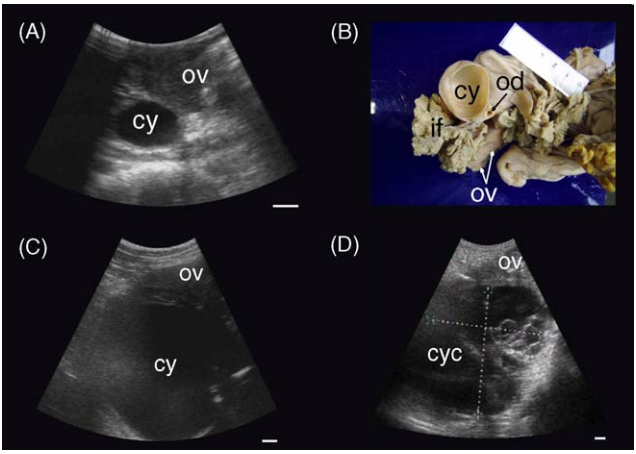

Fig. 6. Ultrasonographic images and post mortem preparation of para-ovarian cysts in female white rhinoceroses. (A) A 3 cm para-ovarian cysts (cy) closely associated to the ovarian parenchyma (ov) in a non-cycling, southern white rhinoceros, no. 1242. (B) Cross-section of an ovarian cyst (cy) located in the serosa of the oviduct (od) and infundibulum in 9-year-old nulliparous female, no. 840. (C and D) Three-year development of a 9.5 cm para-ovarian cyst into a para-ovarian cystic complex (cyc) containing multiple septa in a nulliparous northern white rhinoceros, no. 789. (C) The small inactive ovary (ov) appears atrophic due to the mechanic pressure of the already large cystic structure. (D) After 3 years the cyst has further increased in size and complexity into a polycystic structure (pcy) of 12 cm. The atrophic ovarian parenchyma now shows areas of fibrosis. Scale bars represent 1 cm.

4. Discussion

The data and conclusions provided in this study regarding the reproductive status of 54 female white rhinoceroses represent the first substantial database of reproductive soundness and disorders of this species in captivity. With this large dataset, it will be possible to more effectively scrutinize the significance of individually reported events and the validity of past and current theories on the low rate of reproduction in captive white rhinoceros, which in the past were usually based on only low numbers of individuals. For example, regular cycling of nulliparous female rhinoceroses was originally, thought to indicate normal reproductive function and was regarded as a sign of fertility. However, our large data set implies that the consequence of this long non-fertile cycling periods is hormone-dependent and age-related development of pathologic lesions in nulliparous white rhinoceroses. It is suggested that together with ovarian inactivity, this non-fertile cycling attributes to the low rate of reproduction in captivity. As observed in other exotic species in captivity and humans with long non-reproductive periods, progressive genital pathology develops over time, the ovarian stock depletes and thus the reproductive potential lost and premature senescence occurs within what is normally the reproductive age range [20–23].

In the sub-adult rhinoceroses the hymenal membrane seals the upper genital tract from the vestibule until it is ruptured during first copulation. The rupture is an evidence of successful penile intromission during mating activity regardless of the reproductive

success [24]. Thus, it can be concluded that 76% of the females examined in this study with intact hymenal membranes despite their advanced breeding age have no evidence of participation in mating events. These females were within an age range in which their counterparts in the wild have already reproduced a number of offspring [25,26]. Furthermore, the hymen functioning as a protective barrier rules out the possibility of infertility due to post-copulatory infections. We conclude that the extensive pathology found in non-reproducing females is not of an infectious origin but primarily is a result of continuous, prolonged non-reproductive periods.

The predominant pathological finding in non-reproducing female white rhinoceros was cystic hyperplasia, which was more prevalent and more severe in older nulliparous individuals. The faecal hormone assay indicated that there was little to no detectable luteal activity in the majority of the affected females suggesting there was no functional corpus luteum. However, ultrasonography illustrated continued follicular waves implying continued oestrogen production and constant in utero exposure presumably leading to a steroid-dependent progressive cystic hyperplasia that dominated the pathological findings. A similar scenario for the development of endometrial cysts, first appearing singly, then over time and with advancing age appearing as multiple cysts and clusters representing an accumulation of secretions within the endometrial glands, has been described for humans, the mare, other domestic species and a number of exotic species [20,22,27–29]. In humans, the WHO classifies endometrial hyperplasia in four forms. Stages comprise simple hyperplasia with cystic dilation of the endometrial glands as well as simple hyperplasia with endometrial atypia, the latter being recognized as a precancerous lesion. Both stages can advance to complex hyperplasia with adenomatous changes, where atypical hyperplasia has an up to 30% risk to develop into carcinoma [30]. In addition to the cystic form of endometrial hyperplasia endometrial adenoma were documented in white rhinoceroses in this study. Thus, it is suggested that the endometrial hyperplastic illustrated in the white rhinoceros parallels the same disorder defined for humans with potential as tumour precursor.

Throughout the literature, simple endometrial hyperplasia has been related to a hyperoestrogenic state or an imbalance between oestrogen and progesterone. In the female white rhinoceros, this condition appears to be associated with prolonged periods of sex steroid exposure from continuous ovarian activity without conception indicating states of oestrogen overexposure and/or oestrogen-progesterone imbalances. However, concurrent hormonal assays for oestrogen as confirmation are not available because oestrogen analysis from the faeces is not possible in the white rhino and blood sampling is not feasible in most individuals [7].

Hydromucometra in three rhinoceros 30+ years of age was defined by the presence of large amounts of turbid, viscous fluid in a distended uterus. The hydromucometra occurred in nulliparous and parous females with luteal structures on the ovary and absent mating activity. In the mare, hydromucometra is also correlated with age, absent breeding activity and additionally, a history of endometriosis [31]. Luteal insufficiency is the proposed aetiology demonstrating a hormonal imbalance, further supporting our hypothesis of age-related, hormone-dependent reproductive pathology in the rhinoceros. Pseudo-pregnancy, embryonic loss and foetal resorption are other aetiologies described for hydrometra in sheep and goats [32–33]. Since the rhinos were not sexually active, miscarriage as possible

aetiologies was discarded and we conclude that the observed hydromucometra in the rhinoceros rather patterns the hydromucometra of the horse.

Complementary to cystic hyperplasia, development and growth of leiomyomas are also steroidhormone-dependent based on an oestrogen–progesterone imbalance [21,34–36]. Contrary to the Asian rhinoceroses and Asian elephant where age-associated leiomyomas are the most frequently found reproductive lesion, the white rhinoceros showed a lower affinity for leiomyoma development [20,37–39]. In the development of benign reproductive tumours, captive megavertebrates are equivalent to humans and animal models [21,40]. The risk of uterine leiomyomas in parous and multiparous women is reduced by half or even greater, as compared to nulliparous women [41,42]. Similar to the human, the size of these lesions in white rhinoceros was greater in aged animals, presumably reducing fertility over time.

In addition to uterine tumors, mesovarial leiomyomas were found in pluriparous females and were associated with age. These are described as very rare tumours in the bitch, the sow and humans deriving from smooth muscle cells in the mesovarium [43,44]. Mesovarial leiomyomas have an unknown aetiology, however, experiments in rats have shown the development of mesovarium tumours in beta-adrenergic receptor stimulant-treated rats indicating that mesovarium tumours appear to be adrenergic steroid hormone responsive [44].

In this study based on erratic or absent luteal activity in the faecal hormone profile female rhinoceros aged from 3 to 38 years were subdivided into two groups of ultrasonographic-determined ovarian activity, active and inactive. The active ovarian status group had an age range of 3–19 years and the inactive ovarian status group had an age range of 15–38 years. Thus, there appears to be a transitional range between ages 15 and 19 years in which non-rerpoducing females start to cease their ovarian activity; the ovaries progressing towards an irreversible acyclicity with the follicular stocks nearly depleted. The animals presumably entering premature senescence due to ovarian inactivity as demonstrated in mares and the female rat [45,46]. Comparable to mares and the Indian rhinoceros [47–49] the follicular activity in the white rhinoceros was documented to start at the age of 3–4 years. The follicular waves without ovulation and the formation of hemorrhagic follicles continued in animals monitored over a time period of 5 years and may continue indefinite in sexually mature females. Despite the fact that the white rhinos as a spontaneous ovulator [7,8] should start to ovulate and show luteal activity following puberty (age 4–5 years), our results demonstrate post-pubescent females remain acyclic; the trigger to induce ovulations and regular luteal activity in mature females remains unknown. In wild stallions and male rhinoceros, it has been illustrated that social hierarchy induces sexual suppression [49,50]. We speculate that females when managed as a group display aggressive behaviour and establish a social hierarchy, which perhaps inhibits the initiation of oestrous cycle activity. This hypothesis is based on observations in institutions, which housed two and more females. The more aggressive and dominant female – not necessarily the prime reproductive candidate – suppressed reproduction in younger more reproductively healthy individuals. Further investigations are necessary to illuminate a possible correlation of social behaviour, female reproductive activity and the presence or absence of reproductive pathology.

Para-ovarian cysts occur in the older mares, humans and have been described in detail in captive cheetahs [51–54]. Although their aetiology is unknown, in the southern white

rhinoceros these structures were associated with age, potentially pathognomonic for reproductive senescence. Para-ovarian cysts detected in the northern subspecies were significantly larger in southern subspecies, representing considerable mechanical obstruction for the transduction of the oocyte to the oviduct and inducing ovarian atrophy by applying pressure to the neighbouring ovary. Ovarian atrophy presumably contributed to the cessation of luteal activity as in comparison rats demonstrated a complete ovarian shutdown with a significant loss of ovarian volume [45]. From the mesovarian/oviductal serosa origin in addition to a non-response to the long-term GnRH agonist treatment, it was concluded that these para-ovarian cysts in the rhinoceros were non-functional. In the mare and human, non-functional para-ovarian cysts are also reported as not responsive to hormonal treatment but surgical removal only [53,54]. Yet, the difficult surgical accessibility of the rhinoceros ovary and the anaesthesia risk involved outweigh the benefit of such a procedure in aged, post reproductive females [55,56].

The spectrum of the described pathological changes summit in the diagnosis of reproductive lesions in over 50% of females and a post-reproductive status in 28% of the females primarily marked by chronic uterine degeneration with subsequent infertility and, presumably, the utilization of the follicular stock at a higher rate causing erratic luteal activity and acyclicity [7,8,45,46]. We define this process as multifaceted asymmetrical reproductive ageing, in which females end their reproductive life 10–15 years earlier compared to reproducing females in captivity or females in the wild. The asymmetric ageing can be regarded as a continuum in nulliparous females first evident with detectable lesions at 15 years of age.

This asymmetric aging process may have great consequences for the management and survival of captive rhinoceros populations. These consequences became evident particularly with the northern white rhinoceros. Poaching and political instability have reduced the remaining wild northern white rhinoceros population to probably less than 10 animals remaining alive in Garamba and surrounds [57]. Because such low numbers diminish the genetic diversity, the success of the captive breeding program is critically important for the species survival. However, only four offspring have been produced during 57 years of captive management of this subspecies [2]. Five out of six captive females representing about 50% of the entire female population worldwide were post-reproductive, with only one reproducing pair of animals left in captivity as a consequence of asymmetric ageing [50].

The occurrence of asymmetric reproductive ageing in other rhinoceros species and elephants is supported by past reports on reproductive pathology, which did not characterize the significance of these findings in the context with the entire reproductive lifespan of an individual [13,20,38]. Consequently, counter measures to prevent asymmetric ageing are increasingly important in the management of endangered species in captivity [50]. The selection of healthy breeding partners based on ultrasonographic genital examinations, further studies into reproductive activity in relation to social behavior, establishment of retirement facilities for post reproductive animals and means of assisted reproduction are a selection of measures to counteract a rapid reproductive ageing of a captive populations in mega-vertebrates [58,59].

Evidence among several species including results for the white rhinoceros illustrates the potential of pregnancy to prevent or reverse uterine lesions occurring from reproductive hormone-dependent syndromes, thus preserving fertility [50]. The fact that parous female

white rhinoceros showed a significantly lower prevalence for cystic hyperplasia suggests pregnancy as a preventative. Compared to what occurs in captive mega-vertebrates, the positive effects of pregnancy in humans seem similar. Pregnancy in young women significantly reduces the risk of breast cancer and the risk of uterine leiomyomas is greatly reduced in multiparous women and in rats from >70 to 10%, both of which represent sex steroid hormone-dependent disease [21,60]. Pregnancy has also been observed to regress symptoms and lesions of endometriosis [61]. The achievement of early initial pregnancy in young animals by either natural breeding or, if necessary, by artificial insemination can be regarded as prophylaxis to asymmetric ageing.

Asymmetrically-aged female white rhinoceroses with minimal uterine lesions that demonstrated erratic or absent luteal activity were chosen for oestrous cycle re-initiation and ovulation-induction trails in an attempt to return these females to the breeding population by down regulating endogenous steroid hormone production for extended periods [7]. For these purposes, two separate protocols were chosen: (1) the short-term (1–2 months) suppression of LH secretion via feedback to the hypothalamus using oral application of synthetic progestin followed by hCG to induce ovulation; (2) the long-term (6 months) suppression of endogenous reproductive steroid hormones using GnRH agonist that bind to pituitary receptors of gonadotrophs and trigger an acute increase in LH release followed by a decline due to chronic agonistic exposure [62–65] to induce oestrous. The results of reproductive examinations conducted with ultrasound and concurrent faecal hormone analysis before, during and after both treatments verified the effectiveness of these protocols to decrease endogenous steroid hormone levels in white rhinoceroses.

Reproductive down regulation with synthetic progestin and subsequent ovulation induction thereafter using hCG resulted in the initiation of an ovulation in only 30% of the study subjects. In these subjects, ultrasonography demonstrated the production of a single pre-ovulatory-sized follicle. Whereas, long acting GnRH agonist down regulated endogenous steroid hormone production for periods of over 6 months and ovarian activity was re-initiated after removal of implant in all study subjects. Ultrasonography revealed that this protocol stimulated the development of populations of follicles as opposed to a single follicle. However, no ovulations occurred with the exception of one acyclic female, who was later inseminated and is currently maintaining normal pregnancy [66]. In the context of asymmetric aging, these study animals represented a subpopulation with only minimal pathologic manifestations of the asymmetric aging process, nonetheless the results revealed reduced responsiveness to hormonal triggers. This could suggest the pre-mature irreversible ovarian inactivity in these asymmetrically aging females or could reflect the ineffectiveness of these protocols for white rhinoceroses. Although, the successful return of the one subject to the breeding population, is optimistic proof that the reproducing potential can be salvaged in at least some of the captive white rhinoceros population.

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