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The biotechnological potential for manipulating offspring sex in the rhinoceros and the elephant

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List of abbreviations

AfE	African elephant
AsE	Asian elephant
AfRSG	African Rhino Specialist Group
AI	artificial insemination
ART	assisted reproductive technology
AZA	Association of Zoos and Aquariums
BC	Blottner's Cryomedia
BR	black rhinoceros
BSM	Blottner's Sorting Medium
BTS	Beltsville thawing solution
CBSG	Conservation Breeding Specialist Group
CITES	Convention on International Trade in Endangered Species of Wild Flora and Fauna
cm	centimetre
DNA	desoxyribonucleic acid
DNS	Desoxyribonukleinsäure
EAZA	European Association of Zoos and Aquaria
EEP	European Endangered Species Breeding Programme
EGTA	ethylene glycol bis 2 aminoethyl ether
ESR	electron spin resonance spectroscopy
g	gravity, acceleration due to
G	giga hertz
GAG	glycosaminoglycan
GnRH	gonadotropin releasing hormone
GRB	genome resource bank
h	hour
H33342	Hoechst 33342
HCL	hydrochloric acid
ICSI	intracytoplasmic sperm injection
IEF	isoelectric focussing
Ip	isoelectric point
IPG	inositolphosphate glycan
IR	Indian rhinoceros
IRF	International Rhino Foundation
IU	international units
IUCN	International Union for the Conservation of Nature
IVF	*in vitro* fertilisation
KCl	potassium chloride

kDa	kilo Dalton
kg	kilogram
l	litre
lab	laboratory
LN	liquid nitrogen
M	molar
MALDI-TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MES	MES-HEPES based semen extender
Me_2SO	dimethyl sulfoxide
mg	milligram
$MgCl_2 \cdot 6H_2O$	magnesium chloride hydrate
ml	millilitres
MTG	multi-thermal gradient
MW	megawatt
m/z	mass: charge ratio
NaCl	sodium chloride
NaOH	sodium hydroxide
P250	rhinoceros seminal plasma protein with a molecular weight of 250 kDa
PBS	phosphate buffered saline
PBU250	bulbourethral gland secretion protein with a molecular weight of 250 kDa
pers. comm.	personal communication
pH	hydrogen ion concentration, -log 10 of
PM	progressive motility
PMF	peptide mass fingerprint
psi	pounds/square inch
RT	room temperature
s	second
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sek	Sekunde
SEM	standard error of the mean
SSI	sperm sorting index
TBP	tributyl phosphate
TCA	tris-citric acid based semen extender
TCM	traditional Chinese medicine
Tempo	2,2,6,6-tetramethyl-1-piperidinyloxy
TES	triethylsilane
TM	total motility
UV	ultraviolet

V	volt
vs	versus
v/v	volume: volume ratio
WR	white rhinoceros
WWF	World Wide Fund for Nature
w/v	weight: volume ratio
1D	one-dimensional
2D	two-dimensional
μ (prefix)	micro (x 10^{-6})
m (prefix)	milli (x 10^{-3})

Chapter I Introduction

1. Ecology and population dynamics of rhinoceroses and elephants

Rhinoceroses have populated the earth for millions of years and represent one of the most ancient extant mammalian genera. Nowadays five remaining rhinoceros species live on two continents: The white rhinoceros *(Ceratotherium simum*; Lydekker, 1908*),* the black rhinoceros *(Diceros bicornis*; Drummond, 1826*)*, the Indian rhinoceros *(Rhinoceros unicornis*; Linnaeus, 1758*)*, the Javan rhinoceros *(Rhinoceros sondaicus*; Desmarest, 1822*)* and the Sumatran rhinoceros *(Dicerorhinus sumatrensis*; Fischer, 1814*)*. Due to habitat loss, political unrest, failed conservation efforts and continuing illegal poaching, all of these five species have been driven to near extinction. The present thesis deals with three of them, of which two, the black and the white rhinoceros, occur on the African continent, and the third species the Indian rhinoceros lives in Asia.

Rhinoceroses belong to the odd-toed ungulates, the order Perissodactyla. The Indian rhinoceros is closely related to the Javan rhino and originates from a lineage of Asian rhinos which first emerged 2 – 4 million years ago, whereas the first ancestors of the African species appeared in the mid Miocene 12 – 14 million years ago (Groves, 1997). The two African species did not diverge until the early Pliocene (3.5 – 5 million years ago; Shoshani, 2006) and are still closely related enough to hybridise.

All rhinoceros species reach one ton or more in weight, comprising one of the few megafauna families still extant today. The word rhinoceros is derived from the Greek words *rhino* and *kera*, meaning nose and horn, respectively. This “horned nose” gives these megaherbivores their unique appearance and is the main reason for their threatened status. Both African species and the Sumatran rhinoceros have two horns while the Javan and the Indian rhinoceros have one only. Rhinoceros horns are unusual among the horns of ungulates in that they lack a bony horn core. The horn is an epidermal derivative, consisting of keratinised tubules of cells set in an amorphous keratinized matrix (Hieronymus et al., 2006). The two main uses of rhino horn by people are medicinal – as an ingredient in traditional Chinese medicine (TCM), and ornamental – as material for making handles of jambiyas (ceremonial curved daggers) worn in some Middle East countries as status symbols (Pui-Hay et al., 1990). Rhino horn, primarily used as a fever reducer, has been one of the most revered ingredients in the pharmacopoeia of TCM for centuries (Zhang, 1990). Rhino horn does have fever reducing properties if applied at high dosages of grams, and with similar effects achieved using gazelle horn (Pui-Hay et al., 1990).

The white rhinoceros belongs to the genus *Ceratotherium*. After the elephant and along with the Indian rhinoceros the white rhinoceros is the largest extant terrestrial animal worldwide. With its distinguishing wide mouth and flat-fronted lips, especially adapted to crop short or medium-length grass, it is a grazer and prefers open grassland and savannah habitats

(Emslie & Brooks, 1999). There are two subspecies of the white rhinoceros, the southern *(Ceratotherium simum simum)* and the northern *(Ceratotherium simum cottoni)* white rhinoceros. Once widespread in the bushveld of southern Africa, the total population of the southern white rhinoceros was reduced a century ago to approximately 20 animals through heedless poaching (Acocks, 1988). Due to intensive conservation efforts and various translocations, the species was rescued from near extinction. To date the southern white rhinoceros stands as one of the world's greatest conservation success stories, with the wild population now numbering 17,480 [Figure 1; International Union for Conservation of Nature (IUCN)/Species Survival Commission (SSC) African Rhino Specialist Group (AfRSG), 2008a]. Nevertheless, the southern white rhinoceros is still classified as "near threatened" in the IUCN Red List of Threatened Species, except for the population of South Africa and Swaziland, international trade is prohibited and it is listed in Appendix I[1] and II[2] of the Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES; IUCN/SSC AfRSG, 2008a). In contrast to the positive development of its southern relative, the situation of the northern subspecies of the white rhinoceros is highly critical. Once ranging in large numbers throughout north-central Africa, the population was reduced by poaching to only four confirmed animals in the Garamba National Park, Democratic Republic of Congo in 2003. Recent fieldwork has failed to find any presence of these animals (IUCN News, 2008). The two subspecies of the white rhinoceros can be distinguished by their slightly different phenotype. The northern white rhino is hairless whereas the southern subspecies is somewhat hairy over the whole of the body. They also differ in the form of the skull (Nowak, 1999). Despite genetic differences, cross-breeding between the two subspecies is possible (Ochs & Mercado, 2005a).

The second African rhino species, the black rhinoceros, is listed as "critically endangered" in the IUCN Red List of threatened Species (IUCN/SSC AfRSG, 2008b) and listed in Appendix I of CITES. It is smaller than the white, has a different mouth structure and belongs to the genus *Diceros*. There are four subspecies of black rhinoceroses: The most numerous south-central (*Diceros bicornis minor*), the south-western (*Diceros bicornis bicornis*), the East African (*Diceros bicornis michaeli*) and the West African (*Diceros bicornis longipes*). Historically, the black rhinoceros had a much broader distribution than the white, as it is adapted to survive in drier or cooler climates than its relative (Emslie & Brooks, 1999; Figure 1). Being a browser, it uses its characteristic pointed mouth with the prehensile upper lip to grasp stems, branches, twigs and leaves. Around 1900 there was an estimated several hundred thousand black rhinoceroses in Africa (IUCN/SSC AfRSG, 2008b). In the 20th century, the number of animals dropped to a fraction of that number, with the primary population reaching a low of 2,410 animals in 1995 [International Rhino foundation (IRF),

[1] Appendix I: CITES prohibits international commercial trade in the species listed in this appendix
[2] Appendix II: International trade in the species listed here is permitted if it is sustainable and the specimen are obtained legally

2008a]. Since then the continental population slowly increased to 4,180 individuals to date (IUCN/SSC AfRSG, 2008b) with the help of conservation programs. Nevertheless, in July 2006, the western subspecies *(Diceros bicornis longipes)* was declared as tentatively extinct (IRF, 2008b).

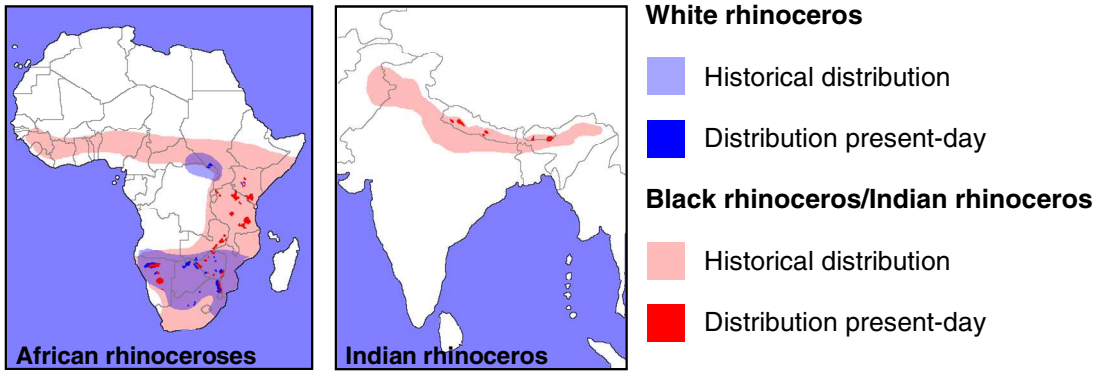

Figure 1: Historical and present-day distribution of the white, the black and the Indian rhinoceros (modified from www.rhinos-irf.org)

The Indian or greater one-horned rhinoceros (genus *Rhinoceros*) differs from its African relatives in its single horn and in its skin, which has a number of loose folds, giving the animal the appearance of wearing armour. The Indian rhinoceros feeds mainly on grass but the diet also includes fruit, leaves, branches of trees and scrubs and cultivated crops. It has a prehensile lip comparable to the black rhinoceros. The typical habitat is the grassy flooded areas at the foot of the Himalayas. An estimated 500,000 animals once existed from Pakistan to Bangladesh and Burma and may have also existed in Myanmar and China (Foose & van Strien, 1997). Today the Indian rhinoceros only remains in small populations situated in north-eastern India and in Nepal. The wild population recovered from very low numbers at the beginning of the 20th century to 2,619 individuals to date (Figure 1; IRF, 2008c). The greater one-horned rhinoceros is listed as “vulnerable” in the IUCN Red List of threatened Species and in Appendix I of CITES (Talukdar et al., 2008).

The Asian elephant (*Elephas maximus*; Linnaeus 1758) and the two African elephant species (*Loxodonta africana;* Blumenbach, 1797; *Loxodonta cyclotis*; Matschie, 1900) are the last three living species of the once diverse order of Proboscidae that proliferated from aquatic or semi aquatic ancestors in the Eocene. Only recently were the two subspecies of *Loxodonta* elevated to the level of two distinct species - the bigger savannah elephant (*Loxodonta africana*) and its smaller relative the forest elephant (*Loxodonta cyclotis*). This classification is not unanimously accepted (Roca et al., 2001). Elephants generally populate forests, savannahs, and river valleys in gregarious herds consisting of matriarchal family groups of mothers, daughters and sisters (Morris, 1965; Hanks, 1980). As in the rhinoceros female-male interactions are transient and occur typically during female oestrus. When the bulls are not associated with a herd they stay solitary or accompany small temporary all-male groups.

Asian and African elephants vary in their appearance (Nowak, 1999; Shoshani, 2006). The most apparent difference between the Asian *Elephas* and its African relative *Loxodonta* are the smaller ears of the Asian species along with a different shape of the forehead. The trunk of the African species has two finger-like processes at its tip, differing from the single tip of the Asian elephant. The African forest elephant and the Asian elephant have four or five toenails on the hind foot, whereas the African bush elephant only has three.

Nowadays, all extant elephant species are threatened. The increasing human population in Asia and Africa has led to a steady decline of elephant habitat, resulting in augmented contact and conflict of these megaherbivores with human settlements, leading to the death of several hundred people and elephants each year (Sukumar, 1989; WWF, 2006). After habitat fragmentation and conflicts with human settlements, the hunt for elephant ivory is still the main threat to the animals. With the introduction of firearms, poaching has taking place on a grand scale, especially in Africa (Morris, 1965; Kahumbu, 2004). The resulting decline of 90 % of several elephant populations let to the ban of international trade in ivory in 1989 by CITES. Nevertheless, a recent assessment of ivory markets in Africa and Asia estimated that more than 12,000 elephants are needed each year to satisfy the demand of these markets (WWF, 2006).

The Asian elephant is the most threatened elephant species. It is being pushed to extinction, although it is of great cultural significance in many Asian cultures. The charismatic giants once held sway over a vast region from the Tigris-Euphrates rivers system in Iraq eastwards through Persia into the Indian sub-continent, south and south-east Asia up to northern China. To date its distribution is limited to mainly small isolated woodland areas in the Indian sub-continent and south-east Asia (Figure 2; Sukumar, 1992). The Asian elephant is listed as endangered in the IUCN Red List of Threatened Species and in Appendix I of CITES (Choudhury, et al., 2008). Currently, the entire wild population consists of approximately 25,600 – 32,750 elephants (WWF, 2006).

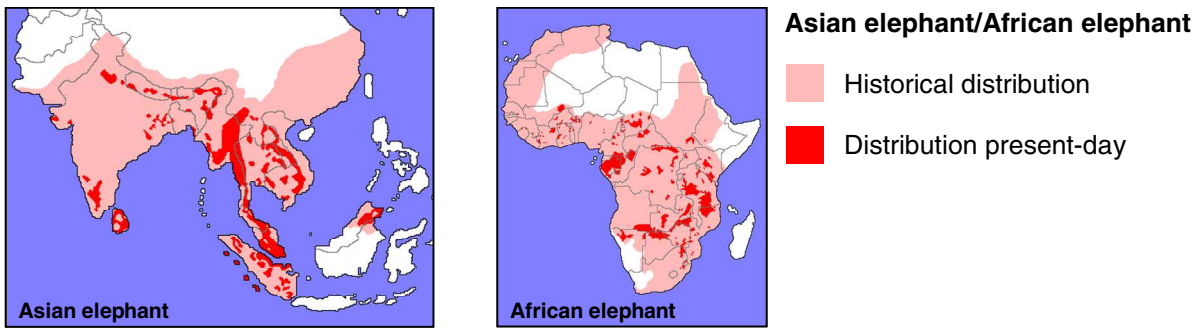

Figure 2: Historical and present-day distribution of the Asian and the two African elephant species (modified from www.species.net; www.waza.org)

Four subspecies of the Asian elephant are distinguished. The most widely distribute subspecies is the Indian elephant (*Elephas maximus indicus*). The Sumatran elephant (*Elephas maximus sumatrensis*) and the largest Asian elephant subspecies, the Sri Lankan elephant (*Elephas maximus maximus*) are only found on the island of Sumatra and south-western Sri Lanka, respectively. The small Borneo pygmy elephant *(Elephas maximus borneensis*) was only recently recognised as a genetically distinct subspecies (Fernando et al., 2003). A recent report suggests that the Borneo pygmy elephants may not be native to Borneo, but could be the last survivors of the previously thought extinct Javan elephant (Earl of Cranbrook, 2008).

The African elephant is listed as “near threatened” in the IUCN Red List of Threatened Species and in Appendix I and II of CITES (Blanc, 2008). Once numbering millions across the African continent, its current population size is estimated to lie between 470,000 and 690,000 (Figure 2; WWF, 2006). Animal numbers vary greatly over the 37 range states from dozens in some western African countries to 300,000 in southern Africa. Despite the threats to the African elephant, high densities in shrinking areas due to progressive habitat destruction lead to punctual over-population. If artificially concentrated in protected areas of limited size, elephants are unable to migrate and growing populations may damage their habitats (Botaha, 2005). For this reason, 16,210 elephants were culled in the Krueger National Park, the oldest National Park in Africa, from 1965 to 1994. Due to international public pressure, the culling was abandoned in 1994, but the problem remained. Just recently, in February 2008, the South African government announced the lifting of the moratorium on culling of elephants as a response to a steadily growing elephant population which threatens to destroy the habitat it lives in (Timberg, 2008).

2. Rhinoceroses and elephants in captivity

2.1. Population dynamics and breeding success

Captive breeding is a significant component of conservation to create self-sustaining populations that can be maintained as a genetic reservoir. Unfortunately, so far, only the Indian rhinoceros captive population and the European African elephant population have reached a level of being self-sustaining (Wiese, 2000; Wiese & Willis, 2004; Foose & Wiese, 2006).

Captive rhinoceroses were first seen in the arenas in Rome and at the courts of the Chinese rulers about 2000 years ago, as the earliest available records show. Since then, at least 2439 rhinoceroses have been exhibited around the world in 501 collections located in 79 countries (Rookmaker, 1998). Four of the five extant rhinoceros species are currently maintained in captivity: The white, the black, the Indian and the Sumatran rhinoceros. As in the wild, the southern white rhinoceros has the largest population of any rhinoceros species in captivity with a global population of 750 animals (Ochs & Mercado, 2005a). Unfortunately,

only very limited breeding successes has been observed and the population is far from being self-sustaining (Swaisgood et al., 2006). A rapidly aging captive population is supplemented by new imports from the wild (Hermes et al., 2006). 121 of a total of 240 animals housed in European zoological institutions were born in the wild (Ochs & Mercado, 2005a). The annual mortality rate of captive individuals of 14 % exceeds the birth rate of only 10.4 %, leading to an annual decrease of 3.6 % of the captive population (Reid, pers. comm.). Whereas the female wild caught F_0 population achieves a total reproductive rate of 30 %, reproduction among captive-born (F_1) females has been fairly low, with as few as 8 % reproducing in some populations [Schwarzenberger et al., 1999; American Zoo and Aquarium Association (AZA), 2004; Swaisgood et al., 2006]. Even less successful than in the southern white rhinoceros was the breeding management of the highly endangered northern subspecies, with only four recorded offspring in 57 years and only one birth in the past 22 years (Lacy, 1993). To date, three bulls and two females of the eight remaining individuals in captivity are still considered to be breeding candidates, representing the last chance of survival for the subspecies (Hermes, unpublished observations).

The global captive black rhinoceros population was estimated in 2005 to consist of 240 individuals (Ochs & Mercado, 2005b). 69 of these animals belong to the most common southern subspecies (*Diceros bicornis minor)* and 171 to the eastern subspecies (*Diceros bicornis michaeli*). The black rhinoceros has been bred relatively successfully in zoological institutions. From 1999 to 2001, 52 animals were born in captivity (Ochs & Mercado, 2005b). In total, 57 % of female rhinoceroses from the southern subspecies and 66 % of females from the eastern subspecies produced offspring (Hermes et al., 2006). However, black rhinoceroses in captivity display unusual disease syndromes not described in black rhinoceroses in the wild causing the mortality rate to balance the recruitment rate (AZA, 2005). These syndromes lead to increased morbidity and mortalities and are mostly characterised by haemolytic anaemia, hepatopathy, and ulcerative dermatopathy (reviewed in Dennis, 2007a). Additionally, a male-biased offspring sex-ratio poses another challenge to captive reproduction management (AZA, 2005; Dennis et al. 2007b).

The Indian rhinoceros generally reproduces well in captivity. To date, an estimated 154 Indian rhinoceros are currently housed in zoological institutions worldwide (Zschokke et al., 1998; Hlavacek, 2005). It is the only rhinoceros species that has reached a level of being self-sustaining (Zschokke et al., 1998). However, genetic composition is significantly skewed towards a few highly represented founders (Hlavacek, 2005), which is partly due to severe aggression sometimes exhibited between specifically designated breeding pairs, making breeding a challenge for animal managers (Roth et al., 2001). Another problematic trend for captive population management of the Indian rhinoceros is a 60 to 40 % skewed sex-ratio towards male offspring (Hlavacek, 2005).

Elephants have maintained a special relationship with people for more than 4,000 years (Lair, 1997). Used as beasts of burden, elephants have played important roles in war, agricultural colonisation, economic development, religious ceremonies, ecotourism, entertainment and education (Sukumar, 1992). By far the biggest population of elephants outside their range states live in captivity in Europe and the USA. This current captive population of Asian and African elephants consists of approximately 590 and 353 animals, respectively, from which about 50 % are housed in Europe and 50 % in North America (Keele & Lewis, 2005; Olson, 2006; Belterman, 2007; Terkel, 2007). Until the mid-1980s importation of elephants was easy and the need to develop self-sustaining populations was not recognised. At the moment 60 % and 75 % of Asian elephants in Europe and North America are born in the wild, respectively. In the African elephant the rate is even 81 % and 68 %, respectively (Wiese & Willis, 2006; Olson, 2006; Terkel, 2007). The husbandry of male elephants in zoological institutions is problematic and cost-intensive. Therefore, mostly females have been imported from the wild and about 80 % of the captive population of both species are elephant cows. Due to a constant decline of the *in situ* population, wild-caught elephants are now rarely available for imports and captive populations have become interesting for the conservation of the megaherbivores in terms of research, education and fund-raising to support habitat protection (Hildebrandt et al., 2006). But this important captive population suffers from a low reproductive rate, a trend that if not reversed, will probably lead to its extinction within three to five decades (Wiese, 2000). Demographic models require for an eight-fold and five-fold increase in reproductive rate of Asian and African elephants, respectively, if these captive populations are to become self sustaining (Olson & Wiese, 2000; Faust et al., 2006). The low reproductive rate of elephants in captivity is even more amplified due to a substantial juvenile mortality (Saragusty et al., 2009) and fatal cases of dystocia (Hermes et al., 2008). The only exception is the European African elephant population as it has been self-sustaining for the past few years and can remain so if there are six births per year (Terkel, 2004).

2.2. Reproductive disorders

During the last decades advances in endocrinology, endoscopy, and ultrasonography have elucidated some of the underlying causes for poor reproductive success in captive megaherbivores species. These technologies have detected reproductive pathologies in female elephants and rhinoceroses such as uterine, cervical and vaginal tumours and cysts (rhinoceros: Hermes et al., 2006; Roth, 2006; elephants: Hildebrandt et al., 2000a, 2003; Agnew et al., 2004). Hermes and co-workers reported that in about 56 % of the investigated captive female white rhinos reproductive pathologies were observed (Hermes et al., 2006), a number comparable to the elephant (Hildebrandt, pers. comm.). 80 % of the most common lesions found in non-reproducing female rhinoceroses and elephants are leiomyomas or cystic hyperplasia of the reproductive tract (Hermes et al., 2004). The incidence of

reproductive disorders is positively correlated with age and particularly occurs in nulliparous animals. This relationship is supposed to be a result of prolonged periods of exposure to fluctuations in the concentrations of sex steroids from continuous ovarian cycle activity resulting from a permanent non-pregnant state (Montali et al., 1997; Hildebrandt et al., 2000a; Hermes et al., 2004). Another presumed consequence of long-term, non-conceptive oestrus cycles is the utilisation of the follicular stock at a higher rate (Sopelak & Butcher, 1982; Hinrichs, 1997) probably leading to premature senescence 15 – 20 years prior to the expected reproductive life-span. Especially in the white rhinoceros this process results in a complete cessation of follicular development in affected females. One successful pregnancy is suggested to prevent genital tract lesions that are the consequence from the hormone-dependent syndrome, thus preserving fertility (Hermes et al., 2006). Apart from "prematurely-aged" acyclic female rhinoceroses, anoestrus has also been frequently monitored in young captive female rhinoceroses where it is primarily thought to be the result of deficient animal husbandry and management, failing to satisfy the behavioural needs required to initiate a regular oestrus cycle activity (Hermes et al., 2007).

Reproductive pathologies appear much less frequently in captive male rhinoceroses and elephants than in females (Hildebrandt et al., 2006; Roth et al., 2006), suggesting that limited opportunities for breeding do not have as significant an impact on the health of male sexual organs as it does on the female reproductive tract. Nevertheless, male rhinoceroses and elephants exhibit a larger range of reproductive failures caused by lack of libido or unsatisfactory ejaculate quality (Hildebrandt et al., 2000b; Hermes et al., 2005). Only 21 % of all male white rhinoceroses participating in the species survival programs have sired offspring so far (studbook analysis of the author; Table 1).

Table 1: Studbook analysis of current world population of male white rhinoceroses in captivity regarding its breeding performance

Rhinoceros species	Captive ♂ population (total number)	Captive ♂ proven breeders	Captive ♂ offspring*	Captive ♀ offspring*
Southern white rhinoceros (*Ceratotherium simum simum*)	334	70	442**	375**
Northern white rhinoceros (*Ceratotherium simum cottoni*)	4	1	0	3

*, **captive offspring from current living world populations of captive male rhinoceroses, male/female birth sex-ratio for the southern white rhinoceros in captivity (54 %/46 %)

According to the European Endangered Species Breeding Programme (EEP), 35 % of Asian and 22 % of African elephant bulls produced offspring (Belterman, 2004; Terkel, 2004).

Sperm samples collected from captive rhinoceros bulls exhibited a wide range of semen quality. Only 52 % of 21 examined males showed high sperm quality (progressive motility > 75 %; Hermes et al., 2005). A similar situation occurs in the captive elephant population, as the majority of semen samples obtained by manual stimulation of accessory sex glands were of poor quality (Hildebrandt et al., 2000b; Thongtip et al., 2001). The high percentage of rhinoceros and elephant bulls with reduced semen quality might be a consequence of the artificial social structure created in captivity. The husbandry of several bulls in one location is thought to have a negative influence on the reproductive status through social depression of subordinate males by dominant bulls, dominant females or even animal handlers (Hildebrandt et al., 2000b; Seror et al., 2002; Hermes et al., 2005). A similar behavioural subordination was previously observed in wild rhinoceroses (Owen-Smith, 1975) and horses (*Equus caballus*; McDonnel, 2000).

2.3. Assisted reproductive technology

The role of assisted reproductive technology (ART) has been acknowledged as increasingly important for the success of captive breeding management, specifically in those species with a dismal outlook of survival in both captivity and the wild, such as the rhinoceros or elephant (reviewed in Hermes et al., 2007; Andrabi & Maxwell, 2007).

The greatest challenge in the evaluation of reproductive fitness and development of ART for rhinoceroses and elephants are the size and topography of reproductive organs. In the female rhinoceros the lengths of the reproductive tracts range between 0.8 and 1.5 m depending on the species (Godfrey et al., 1991). With 3 m from the opening of the vestibule to the outside to the ovaries, the elephant has the longest reproductive tract of all mammals (Laws, 1969). Despite these anatomical obstacles, transrectal ultrasound technologies have been successfully adapted to enable longitudinal evaluations of reproductive organs in megaherbivores (Hildebrandt & Goeritz, 1999; white rhinoceros: Hermes et al., 2005; 2006; black rhinoceros: Radcliffe et al., 2001; Indian rhinoceros: Stoops et al., 2004; elephants: Hildebrandt et al., 2000a,b). The latest developments even permitted the ultrasonographic determination of foetal age in elephants (Hildebrandt et al., 2007a; Drews et al., 2008). The use of ultrasonography together with endocrine monitoring using faecal, urine or blood samples allowed the description of reproductive fitness, reproductive cycles and ovarian function for each species during the last decade (rhinoceroses: reviewed in Roth et al., 2006; Hermes et al., 2007; elephants: reviewed in Hildebrandt et al., 2006; Schmitt, 2006; Hermes et al., 2007). Today these techniques are essential tools for captive reproduction management routinely used to monitor oestrus cycle activity and diagnose and observe pregnancy. In the male rhinoceroses and elephants, ultrasonography has been used to characterise testicles and accessory sex glands. The results demonstrate a positive association between size of glands and quality of semen (rhinoceroses: Hermes et al., 2005; elephants: Schmitt, 2006).

Besides screening reproductive status, a reliable and successful procedure for the collection of an adequate amount of intact spermatozoa must be developed to achieve the application of valuable ART such as sperm (cryo)preservation, AI or IVF. In rhinoceroses, numerous attempts of semen collection have been described in the literature. Studies using hot towel compression (compression of penis base and tip with hot towels) and artificial vaginas did not result in the ejaculation of sperm rich samples (Young, 1967; Goeltenboth, 1986; Schaffer et al., 1990; Hermes et al., 2005). Manual stimulation of the penis with or without support by rectal massage of accessory sex glands delivered motile spermatozoa in several individuals (Schaffer et al., 1990, 1998). In two studies manual stimulation was supported by detomidine-HCl and butorphanol, simplifying semen collection (Walzer et al., 2000; Silinski, 2003). The least invasive method for sperm collection is the post-coital sperm recovery from the female genital tract. However, so far it has only been successfully applied in one Sumatran rhinoceros (O'Brien & Roth, 2000). The general success rate of manual stimulation in rhinoceroses and the volume of obtained ejaculates is low, and the quality of collected samples shows major differences in all parameters [volume: 0.2 – 164.5 ml; sperm concentration: 0.1 – 217.3 x 10^6 spermatozoa/ml, intact sperm morphology: 10 – 90 % and sperm motility: 0 – 90 %; (Schaffer et al., 1990; Hermes et al., 2001; Silinski, 2003; Hermes et al., 2005)]. As a result, semen collection using electroejaculation is currently to be considered the only reliable procedure for sperm collection in rhinoceroses (Hermes et al., 2005; Roth et al., 2005). The technique is based on transrectal stimulation of accessory sex glands through electric impulses originating from a specially designed probe (Schaffer et al., 1998; Hildebrandt et al., 2002). Simultaneously, the penile and pelvic part of the urethra is massaged manually to transport the ejaculatory fluid through the urethra into the collection tube. Electroejaculation has been successfully applied for the white (Hermes et al., 2005; Roth et al., 2005), the black (Schaffer et al., 1998; Roth et al., 2005) and the Indian rhinoceros (Goeltenboth et al., 2000; Roth et al., 2005).

Not much is known about the physiology of rhinoceros spermatozoa. In the white rhinoceros morphometric measurements of sperm cells have been performed. Spermatozoa were demonstrated to be remarkably small, with a sperm head dimension of about 5.4 x 2.8 µm and a morphometry similar to the horse stallion (Silinski, 2003). In both African rhinoceros species, there was a high percentage of abaxially placed midpieces (Silinski, 2003; O'Brien et al., 2000).

In elephant, first reports described semen collection from the urogenital tract after copulation (Landowski & Gill, 1964) or passive semen collection after the bull was prevented from mounting the female (Jainudeen et al., 1971). Several studies on semen collection using electroejaculation in the wild and in captivity have been published (Howard et al., 1984; Mar et al., 1992; Schmidt 1993). To avoid the anaesthesia that is necessary for electroejaculation semen collection using manual rectal stimulation of accessory sex glands was also attempted. First reports of this technique in combination with an artificial vagina (Heath et al.,

1983) or a condom made from a palpation sleeve (Price et al., 1986) were followed by the description of a reliable ultrasound supported trans-rectal stimulation method for semen collection in the elephant a decade ago (Schmitt & Hildebrandt, 1998). Elephant sperm cells are rather smaller to most other mammalian species and exhibit some ultrastructural particularities. Compared to spermatozoa from domestic species the equatorial segment extends more distally on the ventral side of the head and dense bodies of unknown function not seen in other species are located near the top of the mitochondria (Schmidt, 2006). For the Asian elephant, sperm dimensions have been described with sperm heads being about 7.8 µm long and 4.7 µm at its widest. Midpiece and sperm tail combined have a length of about 66 µm (Heath et al., 1983). In association with sexual activity elephant bulls demonstrate a unique phenomenon, the so-called “musth”, that has been described as “breeding excitement“ (Hildebrandt et al., 2006). In this state, which may last for several months, aggressive behaviour, sexual behaviour and temporal drainage are heightened and androgen secretion is increased (e.g. Eisenberg et al., 1971; Poole & Moss, 1981; Schmidt, 1993; Yon et al., 2008).

After high quality semen collection, the next step towards the broad adoption of ART requires the successful cryopreservation of spermatozoa to enable the long-term storage of spermatozoa and their worldwide application by AI or IVF. In the rhinoceroses, a range of different freezing and collection methods for sperm cryopreservation have been tested (epididymidal or post-coital: Lubbe et al., 1999, 2000; ejaculated: Platz et al., 1979; Hermes et al., 2005; Reid et al., 2009) and varying post-thaw motilities (≤ 75 %). Most of these studies used liquid nitrogen (LN) vapour freezing methods. A recent study demonstrated that directional freezing technology is superior to conventional freezing techniques (Reid et al., 2009), as previously shown in other species (O’Brien & Robeck, 2006; Saragusty et al., 2007). This novel freezing technique is based on multi-thermal gradient (MTG) directional solidification (Arav, 1999). Using conventional freezing methods, ice grows at an uncontrolled velocity and shape and may therefore, break up cells in the sample. By moving a large volume (2.5 or 8 ml) cryogenic HollowTube™ (IMT Ltd., Ness Ziona, Israel) with the spermatozoa at a constant velocity through a linear temperature gradient, it is possible to control the ice crystal propagation, to achieve continual seeding and a homogenous cooling rate during the freezing process, thereby minimising cell damage. Apart from the rhinoceroses, directional freezing has already been successfully applied for a variety of species (Arav et al., 2002; Gracitua & Arav, 2005; Saragusty et al., 2006; Si et al., 2006).

For cryopreserved spermatozoa of African elephants, a post-thaw motility has reported ranging between 30 and 50 % (Jones, 1973; Howard et al., 1986). Only recently, similar results were achieved for cryopreservation of Asian elephant spermatozoa (Hermes et al., 2003; Thongtip et al., 2004; Sa-Ardrit et al., 2006). However, these reports were based on a small number of samples only and attempts to freeze-thaw spermatozoa from elephants with sufficiently high post-thaw motility for the application in ART, thus far, failed. Sperm from

Asian and African elephants differ greatly in their membrane fatty acid composition (Swain & Miller, 2000). Different to those from its African relative, spermatozoa from the Asian elephant contain a relatively low percentage of unsaturated, long-chained fatty acids (Swain & Miller, 2000), which is thought to be associated with the high sensitivity to cold-shock injuries and to spontaneous acrosome degenerations (Schmitt, 2006).

Recently, AI was added as a new and promising tool in the ART toolbox of megaherbivores. For AI there are severe anatomical obstacles to overcome such as the hymeneal membrane present in most nulliparous female rhinoceroses (Hermes et al., 2006), the very tight rhinoceros cervix (Godfrey et al., 1991; Hermes et al., 2007) or the long vestibulum of vagina and a hymen-like structure that does not rupture during mating of elephant cows (Hildebrandt et al., 2000a; Schmidt, 2006). To negotiate these obstacles, specific insemination catheters and insemination techniques were developed for each species (elephants: Hildebrandt et al., 1999, 2002; Brown et al., 2004; rhinoceroses: Hildebrandt et al., 2007b; Hermes et al., 2009). In the rhinoceroses, ovulation is routinely induced before AI using a gonadotropin-releasing hormone (GnRH) analogue (deslorelin) when a pre-ovulatory follicle is present. To date 12 white rhinoceros cows have conceived after non-surgical AI using a minimum of 500 x 10^6 fresh or cyropreserved spermatozoa (Hermes et al., 2009). In the Indian rhinoceros, one pregnancy was achieved by AI so far (Roth, pers. comm.). Two female and one male white rhinoceros calves and the Indian rhinoceros calf have already been born. The Indian and one white rhinoceros calf were stillborn (Hildebrandt et al., 2007b; Hermes et al., 2009; Roth, pers. comm.).

To overcome anatomical challenges in the elephant a surgical and a non-surgical AI method were developed. In the G□□□ical approach, the hymen-like structure and therefore the opening of the vagina is visualised through an incision below the anus accessing the vestibule before depositing the semen with an equine semen pipette (Schmitt, 2006). This technique involves 4 – 6 weeks of post-surgical wound management. More frequently applied is the non-surgical approach. It requires a well-trained elephant cow to accept the insertion of a large custom-made balloon catheter into the lower urogenital tract. Within this catheter a flexible endoscope and an AI catheter is passed through the vestibule to visualise the vaginal opening and cervix as desired sites of semen deposition (vagina, cervix or uterus; Hildebrandt et al., 1999; Brown et al., 2004). Success rate of AI in elephants varies between 30 – 40 % (Schmitt, 2006). Currently, 23 elephant calves were born after non-surgical AI with fresh semen (Saragusty, 2009).

3. Sex-preselection

3.1. Historical overview and approaches to sex-preselection

The desire to influence the sex of offspring seems to be present since time immemorial and certainly dates back to the first writings of Greek authors connected with veterinary science

(Smith, 1976; Betteridge, 1984). Historical instructions to manipulate sex of offspring included improvements on certain sexual techniques, diet, the removal or retention of boots prior to sexual activity, the placement of various household articles under the bed, or the confinement of intercourse to specific phases of the moon (Betteridge, 1984; Windsor et al., 1993). With the discovery of mammalian sex chromosomes (Guyer, 1910) and DNA as the carrier of genetic information (Avery et al., 1944), attempts to predetermine sex of offspring began to have a serious scientific background, focussing on the separation of X and Y-chromosome bearing spermatozoa (de Graaf, 2006). Since then, separation of X and Y-chromosome bearing spermatozoa was attempted by several different techniques. Sex selection was attempted by volume of spermatozoa (Shettles, 1960, 1961; Cui, 1997; Cui & Matthews, 1993; van Munster et al., 1999), differing sperm surface antigens (Hancock, 1978; Pinkel et al., 1985; Hendriksen et al., 1996), sperm surface charge (e.g. Gordon, 1957; Kaneko et al., 1984; Blottner et al., 1994), assumed higher motility of Y- spermatozoa (e.g. Ericsson et al., 1973; Goodall & Roberts, 1976; White et al., 1984) or cell density (Bhattacharya, 1962; Wang et al., 1994). None of these techniques has proved to be consistently reproducible (Amann, 1989; Catt, 1998). The only currently reliable method for high purity sex-sorting of mammalian spermatozoa – the flow cytometric Beltsville Sperm Sexing Technology – is based on the identification of the difference in DNA content of X and Y- chromosome bearing spermatozoa, first discovered in 1979 through measurements of differences in chromosome lengths (Moruzzi, 1979). After fluorescence staining of sperm DNA, this difference can be recognised in a modified flow cytometer which then separates X from Y- chromosome bearing spermatozoa.

Since the birth of the first offspring conceived after insemination with flow cytometrically sexed semen in 1989 (Johnson et al., 1989) more than two million offspring in several species were produced in 13 countries (Rath & Johnson, 2008). Most were cattle calves (*Bos taurus*), followed by offspring from domestic pigs (*Sus scrofa*), domestic sheep (*Ovis aries*) and domestic horses (*Equus caballus*).

3.2. Development and basic principles of sperm sorting technology

Developmental work on the technique of differentiation between X and Y- sperm populations by flow cytometry occurred during the 1970's and 1980's (Fulwyler, 1977; Dean et al., 1978; Pinkel et al., 1982a,b; Garner et al., 1983). During that period preparative methods for staining and analysing mammalian sperm involved removal of sperm tails and the membranes surrounding the nuclei prior to staining with the membrane impermeant dye, 40-6-diamidino-2-phenylindole, resulting in non viable cells (Pinkel et al., 1982a). It was not until the membrane permeant bisbenzimidazole DNA-binding dye (Hoechst 33342) was employed before accurate measurements of DNA content was achieved in viable sperm (Johnson et al., 1987). Through the introduction of this dye in 1989, the birth of healthy rabbits (*Oryctolagus cuniculus*) conceived with sex-sorted sperm after intrauterine insemination was

achieved for the first time (Johnson et al., 1989). The original method, capable of sorting up to 350,000 spermatozoa/h, was enhanced by the development of a cylindrical needle with an orienting nozzle (Rens et al., 1998), which substituted the original bevelled needle. A better orientation of sperm to the laser beam was achieved and sorting efficiency was increased to 6,000,000/h (Garner & Seidel, 2008), equivalent 1666.7/s. To date, between 6,000 and 8,000 X and Y- spermatozoa/s of high purity (≥ 90 %) can be sex-sorted in a modern high-speed sperm sorter, after further refinements and developments relating to the resolution of the sperm populations (Sharpe & Evans, 2009) and further technical improvements (Garner & Seidel, 2008).

In brief, sperm sample preparation for flow cytometric sex-sorting begins with DNA-staining with the fluorescence dye Hoechst 33342 at 34 – 37°C for 60 – 90 minutes after sample dilution in species specific semen extenders. Following DNA-labelling, spermatozoa enter the sperm sorter through a needle and are passed into the flow cell, surrounded by a carrier fluid, known as sheath fluid (Johnson, 2000). A flat, ribbon like stream of fluid is created and the spermatozoa exit through a vibrating nozzle tip, allowing orientation of an individual spermatozoon with an ultraviolet laser beam (de Graaf, 2006). As the spermatozoon passes two fluorescence detectors, each detector measures the intensity of fluorescence resulting from the excitation of the DNA-bound dye molecules by light (Seidel & Garner, 2002). The ability to differentiate X and Y- spermatozoa is determined by the positioning of the two photomultiplier tubes set at right angles to one another – one (at 0° to the laser) to determine the part of the population which is oriented at right angles to the incident laser beam and the other (at 90° to the laser) to recognise DNA differ ences within the population, oriented in this manner (Cran, 2006). After measurement of DNA content, an undulating piezo-electric crystal breaks the sheath fluid stream into individual droplets containing one labelled cell. Only X and Y- chromosome bearing spermatozoa with appropriate fluorescence are given an opposite charge and are electrostatically deflected into collection tubes. Spermatozoa with unresolved chromosome content and without classifiable fluorescence intensity are given no charge and are rejected to the waste (Figure 3).

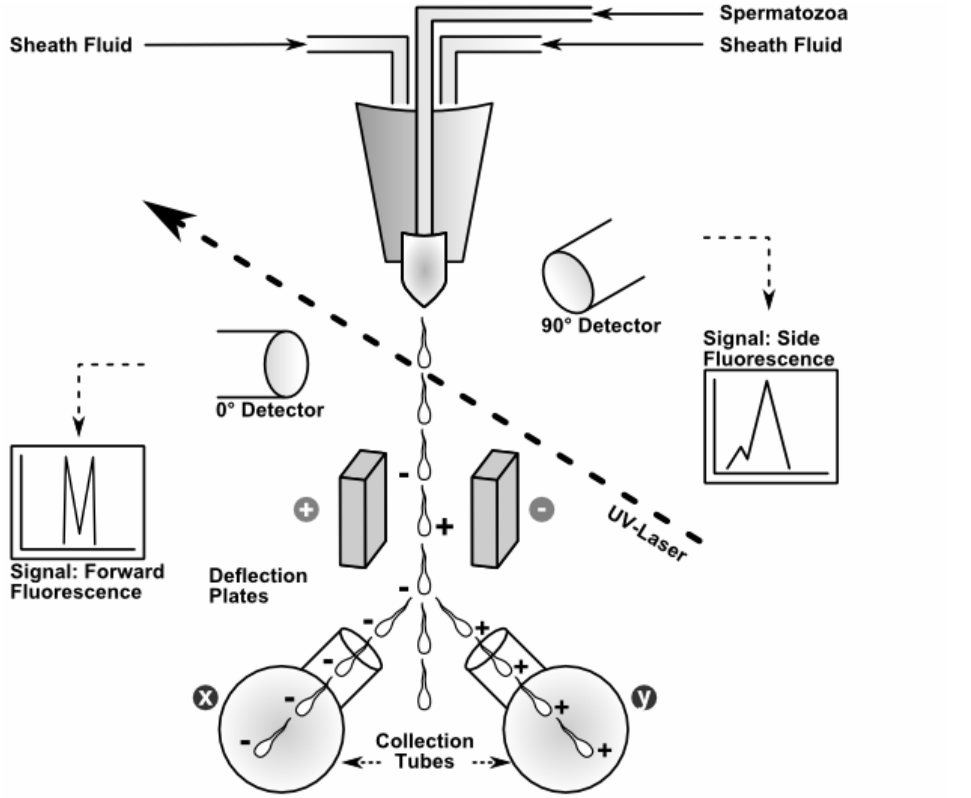

Figure 3: Principals of flow cytometric sperm sex-sorting (modified from Rath, 2001; de Graaf, 2006)

For verification of sorting success it is essential to have a reliable method to estimate the purity of sorted sperm samples. This is carried out by reanalysis in the sperm sorter. Sperm tails from an aliquot of the sorted sample are removed through sonication and the sperm cells are restained with a low concentration of Hoechst 33342 to ensure staining uniformity. The spermatozoa are analysed in the sorter and the purity of X and Y- chromosome bearing spermatozoa populations is determined on the basis of difference in DNA content (Johnson & Welch, 1999). The accuracy of this reanalysis technique has been confirmed by comparative studies with the fluorescence in situ hybridisation technique (FISH) using sex-specific probes [domestic boar: Kawarasaki et al. 1998; Parilla et al., 2003; cattle bull: Rens et al., 2001; baboon (*Papio hamadryas*), common marmoset (*Callithrix jachus*), common chimpanzee (*Pan troglodytes*): O'Brien et al., 2005a] and zinc finger allele polymerase chain reaction (domestic boar: Welch et al., 1995). Finally the sex bias of offspring after the application of sex-sorted semen demonstrates the success of this technique: Approximately 90 % accuracy for both male and female offspring from first field trials to recent studies (e.g. Cran et al., 1995; Seidel et al., 1999; de Graaf et al., 2007).

3.3. Parameters influencing the sortability of spermatozoa

The challenge of flow cytometric sperm sex-sorting is to obtain pure populations of X and Y-sperm without damaging their physiological function. Therefore, the sortability of spermatozoa highly depends on the difference of relative DNA content between X and Y-bearing spermatozoa – the higher the difference, the better the resolution of "male" and "female" populations (Garner, 2006). Until today, the difference in relative DNA content of male and female spermatozoa of at least 23 mammalian species has been determined by means of flow cytometry. The chinchilla (*Chinchilla lanigera*) showed the biggest difference in DNA content (7.5 %) of any examined mammalian species and the brushtail possum (*Trichosurus vulpecula*) the smallest (2.3 %). The difference in DNA content for domestic livestock (cattle, pigs, sheep and horses) varies from 3.6 to 4.2 % (Johnson & Welch, 1999).

In addition to the difference in DNA content the ability to precisely orient the gametes at the time of measurement in the flow cytometer strongly affects the efficiency of sperm sorting. Mammalian spermatozoa with flattened, oval heads tend to be more readily oriented by controlling the fluidity than those possessing more rounded or angular heads. The sperm sorting index (SSI) combines these two parameters (Garner, 2006) into a measure of sortability. SSI is calculated by multiplying sperm head profile area (μm^2) with the average difference in relative sperm DNA content between X and Y- spermatozoa (%).

3.4. Impact of sex-sorting on spermatozoa

It remains generally accepted that sex-sorted spermatozoa are functionally compromised by the sorting process and as a consequence are less fertile than non-sorted controls. As for the specific cause of alterations to the sexed sperm cell, the possibilities are many and varied (de Graaf et al., 2009). Successful sperm sexing must take into account the susceptibilities of gametes to a myriad of stressors, including high dilution, fluorochrome staining, exposure to 351 and 364 nm of ultraviolet laser light, elevated pressure and resistance to the changes in media composition that occur during the sexing process (Maxwell et al., 1998). Numerous studies have assessed the effects of these potentially cell damaging stressors on integrity and functionality of sorted spermatozoa. The primary conclusion regarding the impact of sorting on spermatozoa *in vitro* is that a higher proportion of membranes are partial acrosome reacted or pre-capacitated in sperm that have been sorted than in unsorted sperm (e.g. Gillan et al., 1997; Maxwell et al., 1998; Maxwell & Johnson, 1999; Hollinshead, 2003). A main cause for the detected membrane alterations may be the lack of protective substances present in seminal plasma because of the high dilution of semen samples during sorting. Addition of 1 – 10 % of seminal plasma to the staining buffer as well as the collection medium appears to be beneficial in preventing capacitation, or even cause decapacitation of sorted sperm (Maxwell et al., 1996). As a result, 1 % of homologue seminal plasma is

routinely added to both collection and conservation medium after sorting boar semen (Grossfeld et al., 2005).

Sorted spermatozoa also displayed lower viability than non-sorted spermatozoa. This may be caused by any stressor acting on sperm cells during sorting. Stepwise evaluations of stages of sorting using bovine spermatozoa revealed that the mechanical stress of transit through the machine (without staining or laser illumination) resulted in the highest increase in the percentage of damaged spermatozoa (18.6 % above non-sorted controls; Seidel & Garner, 2002). Exposure to UV-laser illumination, staining with H33342, or both stressors combined caused a further increase of damaged spermatozoa of 6.8 %, 3.6 % and 0.3 %, respectively. Different levels of laser exposure did not have any effect on sperm function (Guthrie et al., 2002).

Unlike capacitation status, the percentage of motile, viable and acrosome intact spermatozoa after sorting can be increased through additional staining of sperm cells with food dye directly prior to sorting (e.g. Maxwell et al., 2004; Hollinshead et al., 2003; O'Brien et al., 2003). The food dye penetrates spermatozoa with diminished viability and reduces the intensity of Hoechst 33342 fluorescence to make sure that only plasma membrane intact spermatozoa are selected for sorting (Welch & Johnson, 1999). The mechanical stress of sorting was reduced through reduction of the routine sorting pressure propelling spermatozoa through the sorter. Lowering the applied pressure from 3.51 kg/cm^2 [50 pounds/inch^2 (psi)] to 2.81 kg/cm^2 (40 psi) had been shown to be beneficial regarding sperm motility (34 .5 % vs 30.1 %) and viability (72.5 % vs 67.8 %) after sorting (Suh et al., 2005).

Does sorting have a cytotoxic or mutagenic effect on gamete cells, due to fluorochrome DNA staining and UV laser light exposure? Following reports of chromosome aberrations, DNA damage, cell-cycle perturbation and cytotoxic effects in somatic cells after exposure to H33342 (Durand & Olive, 1982; Libbus et al., 1987; Erba et al., 1988), several studies investigated the possible DNA damage and cytotoxic effect of Hoechst 33342 on sperm integrity. However, there was no genotoxic effect on chromatin of mammalian spermatozoa after exposure of gametes to high concentrations of the fluorescence dye and/or UV-light (cattle bull: Catt et al., 1997; domestic boar: Garner et al., 2001; Parilla et al., 2004). In one study, sperm sorting even improved sperm integrity in comparison to unsorted controls (Boe-Hansen et al., 2005). These results are consistent with the definded physico-chemical properties of the Hoechst 33342 stain. It permeates cell membranes and binds selectively to the minor groove of adenosine-thymine regions of the DNA helix by hydrogen bonding, van der Waals forces and electrostatic interactions, and is not intercalative[3], unlike many other DNA binding dyes (Johnson & Schulmann, 1994). Additionally, the extreme degree of condensation of DNA in the sperm by substitution of histones by protamines increases its

[3]DNA Intercalation: A ligand fits itself between two base pairs of DNA, wherefore the DNA has to open a space between its base pairs by unwinding (Römpp, 1995).

protection against foreign influences (Tanphaichtr et al., 1978; Rodriguez-Martinez et al., 1990).

3.5. Sperm sex-sorting in domestic animals – the state of the art

Most offspring produced through the application of sex-sorted semen in AI or in IVF are born from domestic cattle, the only species in which the technique is used commercially to date. After the rabbit (*Oryctolagus cuniculus*; Johnson et al., 1989) and the domestic pig (Johnson, 1991), domestic cattle was the third species in which sex-sorting technologies resulted in offspring with predetermined sex. The first sex-sorted calf was produced in 1992 via IVF with fresh (Cran et al., 1993) and frozen-thawed semen samples (Cran et al., 1994). In 1997 the first report on offspring production of predetermined sex after deep intrauterine insemination of heifers with fresh semen was published (Seidel et al. 1997), followed by the successful application of sex-sorted frozen-thawed bovine sperm in deep intrauterine insemination two years later (Seidel et al., 1999). Currently, the recommended dose is 2 x 10^6 of sex-sorted cryopreserved bovine sperm per AI attempt (Sharpe & Evans, 2009). Fertility of heifers using this low dose AI with sex-sorted spermatozoa is at 60 – 90 % of non-sorted controls (Schenk et al., in press).

In other livestock and domestic species practical commercial application is not yet feasible. Key problems are the reduction of the number of sperm per inseminate owing to the inefficiency of processing (less than 30 – 40 % of viable spermatozoa from a fresh ejaculate remain in the final sorted population; O'Brien et al., 2009) and the high costs of this patented technology (Rath & Johnson, 2008). Furthermore, an important precondition for the routine application of sex-sorted semen is good freezability before and after sorting. Otherwise, the sexed semen would need to be used in immediate application after sorting, thereby constraining possible options. Satisfactory pregnancy rates were achieved in low dose insemination trials with fresh sorted or frozen-thawed sorted sperm in domestic pigs and domestic horses only when using sufficient numbers of sex-sorted spermatozoa for AI (domestic pig, deep intrauterine AI: 50 – 100 x 10^6; Rath et al., 2003; Vazquez et al., 2009; domestic horse, deep intrauterine or hysteroscopic AI: 5 – 25 x 10^6, Lindsey et al., 2002). Recent trials with sexed-frozen-thawed domestic sheep spermatozoa resulted in comparable fertility to that of non-sorted, frozen-thawed controls (intrauterine laparoscopic insemination with 5 – 10 x 10^6 sex-sorted spermatozoa; de Graaf, 2006b; de Graaf et al., 2007; Beilby et al., 2009). Sex-sorting sperm from the Mediterranean Italian buffalo (*Bubalus bubalis*) followed by deep intrauterine low dose insemination resulted in a proportion of pregnancies similar to controls (43 %) and no difference in blastocyst development between sorted and non-sorted spermatozoa was noticed when utilised in IVF (Presicce et al., 2005; Zhang et al., 2006). In domestic carnivores flow-cytometrically sexed semen was also applied. Recently, cat blastocysts of predetermined sex and offspring of a Labrador retriever female after

insemination with fresh sex-sorted semen have been successfully produced (Spinaci et al., 2007; Meyers et al., 2008).

3.6. Predetermined sex of offspring in wildlife species

In wildlife species, application of the sorting technique is more challenging as little is known about sperm physiology and only low numbers of animals are available for investigations. Often basic preconditions as the accessibility of high quality semen donors, knowledge of suitable semen diluents or essential requirements for semen handling are absent, which inhibits the development of a species-specific sex-sorting protocol. So far, the difference in relative DNA content between X and Y- chromosome bearing spermatozoa was determined by flow cytometry for at least 23 species (reviewed in Garner, 2006) and the general sex-sorting ability of fresh and frozen-thawed spermatozoa from several non-human primates was studied (O'Brien et al., 2003, 2005a,b). Sex-sorted offspring have been produced in two wildlife species. Sex-sorting of spermatozoa in context with AI was successfully applied in North American red deer (*Cervus elaphus*), where 11 out of 17 cows (64.7 %) were pregnant after insemination with sexed semen, a proportion similar to that of controls (Schenk & de Grofft, 2003). The second species was the bottlenose dolphin (*Tursiops truncates)*. The first calf was born at Sea World, California, after endoscopic insemination with liquid-stored sex-sorted spermatozoa (O'Brien & Robeck, 2006).

3.7. Manipulation of offspring sex – why in rhinoceroses and elephants?

The possibility to control sex of offspring in endangered species in captivity offers an important opportunity for conservation efforts, since the number of females is the limiting factor in efforts to enlarge a small population, especially in slowly reproducing, long-lived mammals (e.g. Holliday et al., 1994; Primack, 1995). In rhinoceroses and elephants inter-calving interval is long with at least 1.5 – 3 and 2.5 – 5 years, respectively, owing to long durations of gestation (elephants: 21 – 22 months; Meyer et al., 2004; rhinoceroses: 12 – 16 months; Keter & Rotich, 1997), long periods of offspring dependence, and long cycle intervals (reviewed in Hermes et al., 2007; Hildebrandt et al., 2006). Especially in highly endangered species like the northern white rhinoceros, offspring production biased in favour of female offspring would strongly improve the chance of population viability. Predetermination of offspring sex would help to balance asymmetric aging processes of the female reproductive tract which lead to early infertility of female rhinoceroses and elephants. Additionally, sperm sex-sorting would help to adjust the operational sex-ratio of captive populations which is highly skewed towards male calves in the black and the Indian rhinoceros and the Asian elephant (Zschokke et al., 1998; Hlavacek, 2005; Dennis et al., 2007b; Saragusty, et al., 2009), reducing the potential for population growth in these species.

Besides its unique potential for species conservation, preselection of offspring sex by sperm sexing and application of ART is a promising approach to improve management of captive wildlife: (1) In numerous captive settings, the environment is insufficient for maintaining socially cohesive groups, particularly for large long-lived terrestrial mammals such as elephants and rhinoceroses with complex female societies and/or transient female-male interactions during brief reproductive periods (O'Brien & Robeck, 2006). For elephants, international species conservation programs recommend husbandry in intact matriarchal family groups [IUCN/SSC Conservation Breeding Specialist Group, 2004; European Association of Zoos and Aquaria (EAZA), 2004; Lee, 2004] mimicking the sex-ratios of free-ranging social groups. These requirements for female-oriented social groups make designated elephant bull barns a must. (2) Behavioural suppression of male fertility in elephants and several rhinoceros species was observed in facilities with several adult bulls (Hildebrandt et al., 2000b; Seror et al., 2002; Hermes et al., 2005), demonstrating the need for appropriate management not only of female social groups but also of the solitary males. (3) Finally, periods of musth, during which bulls may become very aggressive complicate adequate housing and can be dangerous for husbandry staff and animal. As elephant populations in zoos will have to change from being maintained by individuals imported from free-ranging populations to self-sustaining groups maintained by captive breeding, there will be an increase in the number of bulls that need to be managed. Skewing the sex ratio of offspring in favour of female calves would help to overcome these problems to some extend.

3.8. Objectives

The objectives of this thesis are to investigate whether spermatozoa from rhinoceroses and elephants can be separated into X and Y- chromosome bearing spermatozoa populations using a high-speed flow cytometer and to estimate the feasibility of the sperm sex-sorting procedure. Assuming that spermatozoa from theses megaherbivores can be divided into X and Y- sperm populations, the second aim of this study was the exploration and establishment of sorting conditions suitable to produce viable sex-sorted spermatozoa for AI or IVF programs. As good freezability of spermatozoa is a prerequisite for the broad application of sex-sorted spermatozoa in AI programs, the third objective of this study was to improve the cryopreservation of Asian elephant spermatozoa.

Chapter II Summary of Articles

1. Index of sperm sex sortability in elephants and rhinoceroses

Contents

Flow cytometric sexing of spermatozoa followed by application in AI or IVF provides a unique opportunity to predetermine the sex of offspring and might enhance the conservation management of endangered species in captivity such as the elephant and the rhinoceros. To obtain an indication of the sortability of spermatozoa from these species, the differences in relative DNA content between X and Y- chromosome bearing spermatozoa (fresh, frozen-thawed, epididymal) from three rhinoceros species [white (*Ceratotherium simum*), black (*Diceros bicornis*), Indian (*Rhinoceros unicornis*)] and two elephant species, the Asian elephant and the African savanna elephant (*Elephas maximus, Loxodonta africana*), were determined through separation of spermatozoa into X and Y- sperm populations, using a modified high-speed flow cytometer. The head profile areas of spermatozoa from all five species were measured using light microscopy. By multiplying the differences in relative DNA content and head profile areas we calculated the sperm sorting index (SSI) as a measure of reliability of sorting. SSI was 47, 48 and 51 for white, black and Indian rhinoceros, respectively. The determined SSI for the Asian elephant was 66, in the African elephant 76. The results of this study indicate that flow cytometric sex-sorting of spermatozoa from the tested rhinoceros and elephant species is feasible. The lower SSI values in rhinos indicate that sex-sorting of spermatozoa from these species will be less reliable than in elephants.

2. Feasibility of sex-sorting sperm in rhinoceros species

Contents

The objective of these studies was to investigate the practicality of flow cytometrical sex-sorting for rhinoceros spermatozoa. In Experiment 1, four semen extenders were tested on their suitability for liquid preservation of spermatozoa before sorting. Dilution in MES-HEPES based semen extender followed by incubation generated the best sperm quality parameters (motility, viability and acrosome integrity). In Experiment 2, the effect of the staining method (15℃ for 4 – 6 h during transport or 37℃ for 1 – 1.5 h) on sorting efficiency and sperm quality was investigated. Staining at 15℃ during t ransport resulted in a higher percentage of sperm samples showing a resolution of X and Y- chromosome bearing spermatozoa populations (60 %) than staining at 37℃ after tran sport (33 %) and superior sperm integrity after staining (43.8 ± 11.3 % vs 19.6 ± 12.1 %). Sorting rate was 300 – 700 cells/s and sort purity was 94 % for X- chromosome bearing spermatozoa. In Experiment 3, the highly viscous component of rhinoceros seminal plasma, which complicates the process of sperm-sorting was examined by gel electrophoresis and mass spectrometry. Results suggested a 250 kDa glycoprotein (most likely originating from the bulbourethral gland) to be responsible for the characteristic viscosity of ejaculates. In Experiment 4 viscosity of seminal plasma, as measured by electron spin resonance spectroscopy, was significantly decreased after addition of α-amylase and collagenase (0.5 and 3 IU/100 µl seminal plasma) by 28 % and 21 %, respectively, with no negative effect on sperm characteristics. The results of this study demonstrate for the first time that rhinoceros spermatozoa can be successfully sorted into high purity X and Y- chromosome bearing spermatozoa populations. The successful liquefaction of viscous ejaculates provides a method to greatly improve sorting-efficiency in this species.

3. Sperm sex-sorting in the Asian elephant *(Elephas maximus)*

Contents

In captive Asian elephants, there is a strong need for the production of female offspring to enhance reproduction, prevent premature aging processes in the reproductive tract of female animals and improve the management and husbandry conditions posed by maintaining several bulls in one location. AI of flow cytometrically sex-sorted spermatozoa offers the possibility to predetermine the sex of offspring with high accuracy. The aims of this study were to determine basic parameters for flow cytometrical sex-sorting of Asian elephant spermatozoa and the development of a suitable semen extender. In total, 18 semen samples were collected by manual rectal stimulation from one bull. Sperm quality parameters and sex-sortability of spermatozoa were evaluated after dilution in three semen extenders (MES-HEPES-skimmed milk, MES-HEPES, TRIS–citric acid) and DNA staining. MES-HEPES-skimmed milk was the only semen extender suitable for sexing Asian elephant spermatozoa. Twelve out of 18 ejaculates were successfully sorted with a purity of 94.5 ± 0.7 % at an average sorting rate of 1,945.5 ± 187.5 spermatozoa/sec. Sperm integrity, progressive motility and total motility were 42.6 ± 3.9 %, 48.1 ± 3.3 %, 59.4 ± 3.8 % after DNA labelling, and 64.8 ± 3.2 %, 58.0 ± 5.0 %, 70.8 ± 4.4 % after sorting, respectively. After liquid storage of sorted spermatozoa for 12 h at 4℃, sperm integr ity, progressive motility and total motility were 46.4 ± 5.2 %, 32.2 ± 4.2 % and 58.2 ± 3.9 %, respectively. These results suggest that by selecting the appropriate semen extender it is possible to inseminate Asian elephants with sexed semen with a reasonable chance of success.

4. Successful cryopreservation of Asian elephant (*Elephas maximus*) spermatozoa

Contents

Reproduction in captive elephants is low and infant mortality is high, collectively leading to possible population extinction. AI was developed a decade ago and still relies on freshly-chilled semen. These usually can only obtained – within the geographic restrictions this imposes in terms of transporting ejaculates – from just a handful of bulls with variable sperm quality. AI with frozen-thawed sperm has never been described, probably, in part, owing to low semen quality after cryopreservation. The present study was designed with the aim to find a reliable semen freezing protocol. Screening tests included freezing semen with varying concentrations of ethylene glycol, propylene glycol, trehalose, dimethyl sulfoxide and glycerol as cryoprotectants and assessing the value of the procedure of cushioned centrifugation, rapid chilling to suprazero temperatures, freezing extender osmolarity, egg yolk concentration, post-thaw dilution with cryoprotectant-free Blottner's Cryomedia (BC) solution and the addition of 10 % (v/v) of autologous seminal plasma. The empirically determined optimal freezing protocol uses cushioned centrifugation, two-step dilution with isothermal 285 mOsm/kg BC with final glycerol concentration of 7 % and 16 % egg yolk, and freezing in large volume by the directional freezing technique. After thawing, samples were diluted 1:1 with BC solution. Using this protocol, post-thaw evaluation results were: motility upon thawing 57.2 ± 5.4 %; motility following 30 min of incubation at 37°C 58.5 ± 6.0 % and following 3 h of incubation 21.7 ± 7.6 %; intact acrosomes 57.1 ± 5.2 %, sperm with normal morphology 52.0 ± 5.8 % and viability 67.3 ± 6.1 %. With this protocol, good quality semen can be accumulated for future use in AI when and where needed.

Chapter III General Discussion and Conclusions

Compared to sex-sorting protocols in domestic species, that have been developed over many years using tens of thousands of ejaculates, sperm-sorting in rhinoceros and elephant species remains in its infancy. In this study, spermatozoa from these megaherbivores were sex-sorted for the first time into high purity X and Y- chromosome enriched sperm populations. The results mark the first step towards the successful application of sperm sex-sorting in conservation breeding programmes of endangered species. The general sortability and SSI (Garner, 2006) of spermatozoa from black, white and Indian rhinoceros, Asian elephant and African savannah elephant were determined flow cytometrically. While promising results were obtained for both elephant species, all three rhinoceros species performed poorly in comparison. Nevertheless, successful separation of X and Y-chromosome enriched sperm populations using fresh (elephants, white, black and Indian rhinoceros) and frozen-thawed (black, white and Indian rhinoceros) sperm samples demonstrated the general potential of sex-sorting in all examined species.

In the rhinoceroses, the problematic development of reliable methods for sperm-sorting and processing techniques resulted in a currently still relatively low number of sortable sperm samples, a low sorting rate and a moderate percentage of intact spermatozoa post-sorting. Apart from the small SSI and low general sortability reflected in a limited resolution of X and Y- chromosome bearing spermatozoa populations, several obstacles had to be overcome. These are explored in some detail in article II of appendix. The foremost important issue regarding the preparation of rhinoceros spermatozoa for sorting was the often lengthy transport (4 – 10 h) of collected spermatozoa to the laboratory. The results of accomplished semen extender trials for the transport of rhinoceros spermatozoa indicated an essential requirement of membrane protective egg yolk for chilling and conservation of rhinoceros spermatozoa, similar to observations in the Asian elephant (Graham et al., 2004; Saragusty et al., 2005). Quality parameters of spermatozoa decreased during transport regardless of the (egg yolk free) semen extender used. Dilution in a MES-HEPES based medium (MES) provided the most promising post-transport results regarding motility and membrane integrity characteristics of rhino spermatozoa. DNA staining of spermatozoa at 15°C during transport resulted in significantly better post-staining sperm quality parameters than staining and incubation at 37°C after transport at 4°C. Furtherm ore, a higher percentage of semen samples were successfully separated into X and Y- chromosome bearing spermatozoa populations after incubation with Hoechst 33342 at 15°C. The transport and incubation temperature of 15°C was chosen according to unpubli shed investigations on the thermotropic lipid phase transition of rhinoceros spermatozoa which is generally known to be associated with chilling injury (Drobnis et al., 1993; Saragusty et al., 2005). The critical point for chilling injuries lies somewhere between 12°C and 14°C (unpu blished data) for spermatozoa from white and black rhinoceros. It will be interesting to investigate in future whether further

improvement of sperm quality after staining and sorting can be achieved through transport in medium containing cryoprotective egg yolk. As it interferes with DNA staining and resolution of spermatozoa during the sorting procedure, the pre-sort removal of egg yolk by washing, density gradient centrifugation or glass wool filtration (Fugger et al., 1999; O'Brien et al., 2005a; Maxwell et al., 2007) will be necessary, followed by re-dilution in MES, staining and sperm-sorting. Regarding the substantial variation in sperm quality of rhinoceros ejaculates (Hermes et al., 2005), the additional removal of damaged and immotile spermatozoa by gradient centrifugation might prove beneficial to improve and standardise sortability of semen samples, as previously observed in the gorilla (O'Brien et al., 2005a) and human (Fugger, 1999). However, with this procedure a substantial loss in the total number of spermatozoa has been observed (Maxwell et al., 2007) that may lead to a further decrease of sex-sorting efficiency.

A remarkable common finding when performing sex-sorting in the Asian elephant and the African rhinoceroses was the severe damage of spermatozoa by staining with Hoechst 33342, unlike former records in other species (Schenk et al., 1999; Seidel & Garner, 2002; Hollinshead et al., 2003; O'Brien et al., 2005a; O'Brien & Robeck, 2006). The amount of fluorescent dye suitable for staining spermatozoa in the Asian elephant (133 – 221.7 µmol per 100 x 10^6 spermatozoa/ml) was comparable to the upper range of commonly used quantities for domestic species (Hollinshead et al., 2002; Buss, 2005; Klinc et al., 2007). Yet, the necessary time of incubation (1.5 – 2 h) for good staining results was longer than in most domestic livestock species. This unexpected result might be the consequence of interference of the high percentage of skimmed milk in the BSM semen extender with the staining process. Preliminary results of a study comparing skimmed milk containing semen extender with a clear diluent for sex-sorting stallion spermatozoa found a drastic decrease of staining time when the clear semen extender was used (Gibb, pers. comm.). In the rhinoceroses, the quantity of dye necessary for a good staining result (clear resolution of X and Y-chromosome bearing sperm populations), was with 399 – 665.2 µmol per 100 x 10^6 spermatozoa/ml surprisingly three times higher than in the elephant at an incubation time of 1 – 1.5 h. This may have been due to the high viscosity of semen samples, probably preventing a consistent staining of spermatozoa. However, as Hoechst 33342 is known to damage spermatozoa (Seidel & Garner, 2002), a possible reduction of the minimum effective stain concentration through improvement of the sorting protocol in rhinoceros and elephant sperm samples might enhance post-staining and post-sorting sperm quality.

Increased viscosity of semen samples, a direct result of gelatinous seminal plasma fractions, has been observed in camelids (Bravo et al., 1999, 2000) and several primate species (Hoskins et al., 1967) including the human (Bunge et al., 1954). In the experiments of this thesis this phenomenon was also noticed in rhinoceroses, especially the white rhino. High viscosity of semen samples prevented stable single cell suspensions and was detected as a major challenge for the development of a reliable sperm sex-sorting protocol. The gel fraction

in the ejaculates even prevented physical passage through the flow cytometer in one of four stained sperm samples, due to mechanical blockage of sorter capillaries by viscous samples. To resolve this problem the origin and composition of the gelatinous fraction in rhinoceros seminal plasma was investigated and its enzymatic liquefaction attempted. Through separation, display and further analysis of seminal plasma proteins from the white and the black rhinoceros by gel electrophoresis and mass spectrometry a protein (P250) of 250 kDa molecular weight and a pI ≤ 3 was discovered as the major component of the gel fraction in ejaculates. It most likely originates from the bulbourethral glands.

The size and low pI of the protein in conjunction with its dissolution not only by the peptidase collagenase, but also the carbohydrate-cleaving glycoside hydrolase α-amylase, indicate that P250 is either a glycoprotein of high molecular weight or a glycosaminoglycan (GAG). The latter suggestion may explain the inability to locate P250 in the database, despite the generation of good MS spectra, as GAGs are unlike glycoproteins not usually identified by MALDI-TOF MS (Tissot et al., 2007). Another possible explanation for the missing protein match is that P250 is a new, previously undescribed protein. However, further analysis will be required to test these hypotheses.

Several enzymes were used to cleave gelatinous components of seminal plasma of different species in order to reduce its viscosity. The enzymes α-amylase and collagenase used in the present thesis had been previously successfully utilised to dissolve coagulated ejaculates of several mammalian species including the human (α-amylase; Bunge et al., 1954) the rhesus monkey (*Macaca mulatta*; collagenase: Hoskins et al., 1967) the guinea-pig (*Cavia porcellus*; collagenase; Freund, 1958) and the alpaca (*Lama pacos*) and llama (*Lama glama*; Bravo et al., 1999, 2000). In this thesis a significant reduction of rhinoceros seminal plasma viscosity was achieved following the addition of both enzymes to the samples, having no significant impact on sperm motility or integrity (in terms of viability and acrosome integrity). Further investigation is required to elucidate whether the reduction of semen sample viscosity will have a positive impact on the sortability of rhinoceros spermatozoa and whether the enzymes affect the function and fertility of the spermatozoa. In initial trials of enzyme addition (α-amylase) to rhinoceros sperm samples that were too viscous for processing by the flow cytometer, viscosity was reduced sufficiently to sort, indicating that enzyme treatment might improve the use of rhino ejaculates for sex-sorting.

Another challenge in sorting rhino spermatozoa is the varying composition of collected ejaculates owing to the artificial method of semen collection. During electroejaculation, the accessory sex glands are stimulated in a non-natural way, which results in great variation of concentration, quality and maturity of collected spermatozoa and a variable composition of the seminal plasma produced during natural ejaculation. It is therefore unclear whether the highly viscous component is a natural part of the ejaculate or if electroejaculation increases the viscous seminal plasma component in comparison to a naturally ejaculated sample.

Variation in ejaculate composition influences the ability to stain and sort spermatozoa. As electroejaculation is currently the only reliable method to collect full ejaculates from rhinoceros species, the only possibility to minimise variation between ejaculates is to further optimize the electroejaculation procedure and restricting stimulation of the bulbourethral glands.

Similar to the domestic horse (Clulow et al., 2008) a skimmed milk containing semen extender was suitable for high purity and quality sperm sex-sorting in the Asian elephant, providing protective conditions during the sorting process. Within the scope of this thesis, a protocol for sex-sorting was developed that yielded an acceptable sorting rate, high population purity and post-sorting quality of spermatozoa as well as reasonable post-sorting liquid storage ability. However, the realisation of this protocol requires close proximity between the institutions housing the stock bull, the female designated for AI, and a flow cytometer modified for sperm sorting. To overcome long distances between sperm sorter and semen donor locations, liquid storage of semen samples (cooled or at room temperature) might be the method of choice. Its feasibility has already been documented for several species (domestic horse stallion: Lindsey et al., 2005; cattle bull: Seidel et al., 1997, domestic sheep ram: Hollinshead et al., 2004; domestic boar: Spinaci et al., 2005, gorilla: O'Brien et al., 2005a; bottlenose dolphin: O'Brien & Robeck, 2006). If necessary, there is also the option to perform DNA labelling at low temperatures during transport which proved practical in domestic boars (D. Rath, unpublished observation) and within the present – as the results of this thesis show – also in the rhinoceros.

Similar to rhinoceroses, dilution of sperm samples in egg yolk semen extender during transport to the laboratory followed by its removal prior sorting might be effective to avoid cold-shock damage of sensitive Asian elephant spermatozoa. Ongoing investigations into the pre-sorting storage of Asian elephant spermatozoa delivered promising preliminary results. The spermatozoa were diluted in 16 % egg yolk BC semen extender (Blottner, 1998; Hermes et al., 2005), stored at 4℃ for 4 h, followed by e gg yolk removal using density gradient centrifugation using Bovipure™ (Nidacon, Gothenburg, Sweden), dilution in BSM semen extender, staining, and sorting: Post-sorting motility of spermatozoa was 70 %, post-sorting sperm integrity 85 %, and sorting rate ≈ 2000 spermatozoa/s (unpublished data). These data suggest it may be possible to apply the aforementioned protocol when lengthy semen transport times are unavoidable.

Another method to bridge transport times, increase the number of sex-sorted spermatozoa available for ART and make sperm samples available where and when they are needed, is the cryopreservation of spermatozoa prior to or following sex-sorting. In general, a reliable protocol for cryopreservation of spermatozoa ensuring high post-thaw quality is a prerequisite for broad application of sperm sex-sorting in any species. This technique is available for the rhinoceros (Hermes et al., 2005; Reid et al., 2009) and sortability of frozen-

thawed rhinoceros spermatozoa was demonstrated in principle in this thesis. In the Asian elephant however, a comprehensive and successful study on sperm cryopreservation was missing not helped by the often low quality of collected ejaculates and the high sensitivity of spermatozoa to chilling (Arav et al., 2000). We therefore optimised the protocol for cryopreservating Asian elephant spermatozoa by adoption of directional freezing using the MTG apparatus (Arav, 1999). The results demonstrate that high post-thaw sperm survival of gametes is possible if species-specific requirements during the chilling and freezing process are considered. The detailed protocol and results are specified in article IV of appendix. These encouraging results form a promising base for the future development of a procedure to successfully cryopreserve sex-sorted spermatozoa from the Asian elephant or for the use of frozen-thawed samples for sorting. The availability of such a procedure would not only enable sorted semen to be banked to increase insemination doses, but would also allow spermatozoa to be sorted independent from time and place of semen collection.

The protocol developed for sorting Asian elephant spermatozoa allowed the application of sexed semen in four intrauterine inseminations (Hildebrandt et al., 2008). For each AI, 19.2 – 50.5 x 10^6 fresh X- chromosome bearing spermatozoa (sperm motility pre-insemination: 70 to 85 %; unpublished data) were used per oestrus. This dose is substantially higher than the average intrauterine insemination dosage of 2 x 10^6 frozen-thawed sex-sorted spermatozoa in cattle (Sharpe & Evans, 2009) and ought to be sufficient to produce a gestation. Successful gestations by intrauterine AI were also achieved with a similar dose in the domestic pig (Rath et al., 2003; Vazquez et al., 2005) and the domestic horse (Buchanan et al., 2000; Lindsey et al., 2002). Until now, AI with sex-sorted semen in the Asian elephant was unsuccessful. As the minimum number of (non-sorted) spermatozoa necessary for a successful AI is unknown for any elephant species, whole ejaculates were used as a matter of routine. Since not only sperm quality, motility and fertility affect the minimum sperm dose for successful AI but also the insemination procedure and the site of sperm deposition in the reproductive tract, further optimisation of the insemination techniques should lead to conceptions with fewer sex-sorted sperm if post-sorting fertility of spermatozoa is comparable with other species. Although fertility of sex-sorted elephant spermatozoa is not yet demonstrated by a gestation and the protocol was developed using ejaculates from one elephant bull, the protocol should be technically mature to permit the routine production of high quality sex-sorted spermatozoa.

In the African rhinoceros, intrauterine AI with 500 x 10^6 spermatozoa has resulted in successful gestations (Hermes et al., 2009). To date, as in the elephants, the minimum number of spermatozoa for successful conception is unknown and to reduce the required sperm dosage, the optimization of AI techniques concerning site of sperm deposition in the female reproductive tract needs to be conducted. Currently, sample quality and sorting efficiency in the rhinoceroses are not yet at a level to routinely use sex-sorted spermatozoa for AI. In order to increase output and quality of sorted rhinoceros spermatozoa in addition to

the use of viscosity decreasing enzymes in the semen might be the simultaneous operation of multiple sperm sorters and the cryopreservation of sex-sorted sperm for later use. By using a single sorter the protocol developed in this study easily produces adequate numbers of sex-sorted spermatozoa for intracytoplasmic sperm injection (ICSI) and in vitro embryo production. These procedures began with initial post-mortem collection of gametes and successful oocyte maturation and ICSI (Durant, unpublished data), followed by *in vivo* oocyte collection (Hildebrandt et al., 2007c), and *in vitro* maturation and fertilisation in black rhinoceroses (Hermes, unpublished data). Therefore, sperm sex-sorting technology as part of ART tool box will also become feasible in the rhinoceros.

Controlling offspring sex in rhinoceroses and elephants has a unique potential to improve the viability and management of captive populations. However, the implementation of sexing technology and its application in AI or IVF is still a challenge as seen in other wildlife species (O'Brien et al., 2009). Unlike many domesticated species, wild mammals were not selected for countless generations for high fertility, sperm quality or pedigree fitness suitable for cryopreservation, sex-sorting or AI. Therefore, sorting efficiency is likely to be lower. Even in the highbred domestic species, practical use of flow cytometry for sex-sorting is still limited to cattle, complemented by some human clinical situations. The main reasons for this limited range of application are low efficiency and the damage to spermatozoa caused by the technology, resulting in diminished fertility of sex-sorted spermatozoa (Vazquez et al., 2008). The significant resources and infrastructures, which are required to ensure successful integration of sperm sorting and ART into species management, may also complicate matters (O'Brien et al., 2009). Nevertheless, for critically endangered species like the northern white rhinoceros, predetermination of the sex of offspring is highly desirable as it drastically increases its chance it survival. AI with partial use of female-biased semen is likely to improve the chance of population survival (and increase population size) in the next 50 years to 80 % compared to < 1 % for conventional breeding (Hermes et al., 2007). Future studies that build on the promising results of this thesis are urgently required to help add sperm-sexing technology as soon as possible to the ART tool box for conservation breeding programmes of rhinoceroses and elephants.

Chapter IV Summary

THE BIOTECHNOLOGICAL POTENTIAL FOR MANIPULATING OFFSPRING SEX IN THE RHINOCEROS AND THE ELEPHANT

All extant rhinoceros and elephant species are endangered in the wild; yet urgently needed captive breeding to stabilise world populations of some species turns out to be a substantial challenge. One key issue in captive breeding is the unwanted high proportion of male offspring, a serious problem particularly in very small populations. If manipulating offspring sex was a feasible, successful and safe option, a higher number of females could be produced. This would accelerate population growth, thereby significantly improving the viability of populations and thus the conservation of these impressive animals. The ability to select sex would be especially useful in species close to extinction such as the northern white rhinoceros (*Ceratotherium simum cottoni*) with only two remaining female breeding candidates, worldwide. To date, the only reliable method to select offspring sex is the Beltsville sperm-sorting technology in combination with artificial insemination (AI) or *in vitro* fertilisation (IVF). This technique is based on flow cytometric separation of X and Y-chromosome bearing spermatozoa in relation to their DNA content.

The aim of this study was to determine the feasibility of spermatozoa from elephants and rhinoceroses for flow cytometrical sex-sorting and to conduct basic investigations on sperm sex-sorting in these megaherbivores by exploring and establishing species-specific sorting conditions. First, the theoretical sortability of the spermatozoa was established through determination of the sperm sorting index (SSI). This index is calculated by multiplying the difference in relative DNA content between X and Y- chromosome bearing spermatozoa (as calculated by the flow cytometer) with the profile area of the sperm head. The resulting SSI values indicated that spermatozoa from the African savannah elephant (*Loxodonta africana*) deliver best preconditions for successful sex-sorting in the flow cytometer, followed by those of the Asian elephant (*Elephas maximus)*. The general sortability of spermatozoa from the examined rhinoceros species [black (*Diceros bicornis*), white (*Ceratotherium simum*) and Indian rhino (*Rhinoceros unicornis*] was shown to be very similar among each other and lower when compared to the elephant species or to livestock.

Second, basic parameters for flow cytometric sex-sorting of spermatozoa from the black and the white rhinoceros and the Asian elephant were determined. Species-specific semen extenders, suitable for sex-sorting, and an appropriate DNA staining protocol (suitable amount of stain, incubation time and temperature) were developed. Sperm sex-sorting of rhinoceros spermatozoa thereby turned out to be challenging. Using the methods developed here, successful sex-sorting spermatozoa into specific X and Y- chromosome bearing populations produced high purity (94 %) but the sperm quality after sorting (sperm integrity: 42.0 ± 5.4 %; sperm motility: 11.5 ± 6.1 %) and the sorting efficiency (300 – 700 sperm/s) are

still low. The high viscosity of rhinoceros ejaculates strongly interferes with DNA staining and sorting. The main component of the viscous fraction, a glycosilated protein with a molecular weight of 250 kDa molecular weight (most likely originating from the bulbourethral gland), was characterised via gel electrophoresis and mass spectrometry. Investigating the liquefaction of the seminal plasma, the addition of the enzymes α-amylase and collagenase was shown to significantly decrease the viscosity without affecting sperm motility or integrity. In initial trials of enzyme addition to rhinoceros sperm samples that were too viscous for a processing by the flow cytometer, viscosity was reduced sufficiently enough to sort, indicating that enzyme treatment might allow better use of rhino ejaculates for sex-sorting.

The sex-sorting protocol developed for the Asian elephant provided very good results for post-sorting sperm quality (sperm integrity: 64.8 ± 3.2 %; motility: 70.8 ± 4.4 %) and purity (94.5 ± 0.7 %) and a reasonable sorting efficiency (1,945.5 ± 187.5 sperm/s). A successful protocol for cryopreservation of Asian elephant spermatozoa was also developed (post-thaw sperm integrity: 52.0 ± 5.8 %) by optimising the cryopreservation protocol (sperm handling pre- and post-cryopreservation, composition of cryomedium) and employing the directional freezing technology. Best post-thaw sperm quality was achieved using a two-step dilution of freshly collected and centrifuged spermatozoa in Blottner's Cryomedia (285 mOsmol/kg) containing 16 % of egg yolk and a final glycerol concentration of 7 % before freezing. Spermatozoa were slowly cooled to 4 – 5℃ and cryop reserved in large volumes of 2.5 or 8 ml at a concentration of 150 x 10^6 sperm/ml.

In the Asian elephant, the results of these studies have already permitted the use of sex-sorted spermatozoa for AI. The sorting protocol appears to be technically reliable. However, AI using sex-sorted Asian elephant sperm has not yet produced any gestation. Regarding all the rhinoceros species, the poor post-sort sperm motility and integrity as well as the sorting efficiency have not yet enabled the application of sex-sorted spermatozoa to be used for AI at present, but their use in IVF may still be feasible.

This is the first study to explore the potential of flow cytometric sex-sorting of spermatozoa from several species of rhinoceroses and elephants. In terms of its practical value, the developed protocols are ready to be applied in elephants, whereas in the rhinoceros species further research is likely to be required. The results demonstrate the potential of the developed techniques and provide a promising base for the future use of the technology in the conservation management of the endangered megaherbivores.

Kapitel IV Zusammenfassung

BIOTECHNOLOGISCHE MÖGLICHKEITEN ZUR BEEINFLUSSUNG DES NACHKOMMENGESCHLECHTS BEI NASHORN UND ELEFANT

Alle Nashorn- und Elefantenarten sind vom Aussterben bedroht und die dringend erforderliche Nachzucht in Menschenhand gestaltet sich schwierig. Ein Hauptproblem der Zuchtprogramme ist der ungewollt hohe Anteil männlichen Nachwuchses, der vor allem in kleinen Populationen ein ernsthaftes Problem darstellt. Eine erfolgreiche und sichere Methode zur Manipulation des Nachkommengeschlechts würde die vermehrte Nachzucht weiblicher Tiere und ein beschleunigtes Populationswachstum ermöglichen. Dadurch könnte die Lebensfähigkeit von Populationen signifikant gesteigert werden und somit auch die Chance auf Erhaltung der imposanten Tiere. Dies gilt insbesondere für hochbedrohte Arten, wie das nördliche Breitmaulnashorn (*Ceratotherium simum cottoni*) mit nur noch zwei verbliebenen weiblichen Zuchttieren, weltweit. Bis heute stellt die Beltsville sperm-sorting Technologie in Kombination mit künstlicher Besamung (KB) oder *in vitro* Fertilisation (IVF) die einzige verlässliche Methode zur Geschlechtbeeinflussung der Nachzucht dar. Diese Technik basiert auf der flowzytomterischen Trennung von X- und Y- Chromosomen tragenden Spermien anhand ihres Unterschiedes im relativen DNS-Gehalt.

Das Ziel dieser Arbeit war es die Eignung von Nashorn- und Elefantenspermien zur geschlechtsspezifischen Spermientrennung im Flowzytometer zu bestimmen und grundlegende Untersuchungen zur Spermien-Sortierung bei diesen Megaherbivoren durchzuführen. Speziesspezifische Sortierbedingungen sollten erforscht und erarbeitet werden. Zunächst wurde die theoretische Sortiereignung der Spermien durch Bestimmung des Spermien-Sortierindexes (SSI) ermittelt. Dieser errechnet sich durch Multiplikation des im Flowzytometer bestimmten Unterschiedes im relativen DNS-Gehalt zwischen X- und Y-Chromsomen tragenden Spermien mit der Spermienkopffläche. Demnach bringen die Spermien vom afrikanischen Savannen Elefant (*Loxodonta africana*) die besten Vorrausetzungen für eine erfolgreiche flowzyometrische Trennung in X- und Y-Chromosomen tragende Populationen mit, gefolgt vom asiatischen Elefanten (*Elephas maximus*). Die generelle Sortiereignung von Spermien der drei untersuchten Nashornarten [Breitmaulnashorn (*Ceratotherium simum*), Spitzmaulnashorn (*Diceros bicornis*) und Panzernashorn (*Rhinoceros unicornis*)] erwies sich als untereinander sehr ähnlich und als geringer im Vergleich mit Elefant sowie Nutz- und Haustierarten.

Weiterhin wurden elementare Parameter zur flowzytometrischen Sortierung von Breit- und Spitzmaulnashornspermien sowie von Spermien des asiatischen Elefanten bestimmt. Für den Sortiervorgang geeignete speziesspezifische Verdünnermedien wurden entwickelt und ein für die jeweilige Tierart passendes DNS-Färbeprotokoll (Farbmenge, Inkubationszeit und -temperatur) erstellt.

Die geschlechtsspezifische Sortierung von Nashornspermien erwies sich als problematisch. Zwar konnten die Gameten erfolgreich und mit hoher Reinheit (94 %) getrennt werden, die Spermienqualität nach Sortieren (Spermienintegrität: 42.0 ± 5.4 %; Spermienmotilität: 11.5 ± 6.1 %) sowie die Sortiereffizienz (300 – 700 Spermien/Sek) waren jedoch noch gering. DNS-Färbung und Sortiervorgang der Nashornspermien werden in hohem Maße durch die hohe Viskosität der Ejakulate beeinträchtigt. Als Hauptkomponente der störenden viskösen Fraktion wurde mittels Proteinanalyse von Nashornseminalplasma (via Gelektrophorese und Massenspektrometrie), ein 250 kDa schweres glykosiliertes Protein charakterisiert, das sehr wahrscheinlich aus der Bulbourethraldrüse stammt. Durch Untersuchungen zu einer möglichen Verflüssigung des Seminalplasmas wurde eine viskositätssenkende Wirkung durch Zusatz der Enzyme α-Amylase und Collagenase zu den Spermienproben festgestellt, ohne dass die Spermienmotilität oder deren Integrität negativ beeinflusst wurden. Vorläufige Ergebnisse von Enzymanwendungen zur Viskositätsminderung von Nashornejakulaten zeigten eine positive Wirkung auf deren Sortierbarkeit, was eine zukünftige Steigerung der Sortiereffizienz durch einen routinemäßigen Enzymzusatz erhoffen lässt.

Beim asiatischen Elefanten konnten mit Hilfe des im Rahmen dieser Arbeit entwickelten Sortierprotokolls sehr gute Ergebnisse hinsichtlich der Spermienqualität (Spermienintegrität nach Sortieren: 64,8 ± 3,2 %; Spermienmotilität: 70,8 ± 4,4 %) und Reinheit (94,5 ± 0,7 %) nach Trennung in eine X- und eine Y- Chromosomen tragende Population und der akzeptablen Sortiereffizienz (1945,5 ± 187,5 Spermien/Sek) erzielt werden. Parallel dazu wurde, durch Optimisierung des Spermien-Handlings vor und nach Kryokonservierung, der Zusammensetzung des Kryomediums und der Anwendung des direktionalen Gefrierverfahrens, ein erfolgreiches Protokoll zur Kryokonservierung der Spermien vom asiatischen Elefanten entwickelt (Spermienmotilität nach Auftauen: 58,5 ± 6,0 %). Die beste Spermienqualität nach Kryokonservierung wurde durch eine zweistufige Verdünnung der frisch gewonnenen und zentrifugierten Spermien in Blottner's Kryomedium (285 mOsmol/kg) erzielt, das 16 % Eigelb und eine Endkonzentration von 7 % Glyzerol enthielt. Die Spermien wurden langsam auf 4 – 5℃ gekühlt und in großen Volumina von 2.5 oder 8 ml bei einer Konzentration von 150 x 10^6 Spermien/ml kryokonserviert.

Beim asiatischen Elefanten erlauben die Ergebnisse dieser Studie schon heute den Einsatz sortierter Spermien in der KB. Das Sortierprotokoll erscheint technisch verlässlich, auch wenn bis jetzt noch keine Trächtigkeit erzielt werden konnte. Beim Nashorn ermöglichen die momentane Sortiereffizienz sowie die Integrität und Motilität der sortierten Spermien zwar anders als beim Elefant noch keinen Einsatz in Besamungen, wohl aber die Verwendung in Methoden der *in vitro* Embryoproduktion.

Diese Arbeit untersucht zum ersten Mal das Potential der geschlechtsspezifischen, flow-zytometrischen Spermientrennung von mehreren Nashorn- und Elefantenarten. Hinsichtlich der praktischen Anwendbarkeit sind die entwickelten Protokolle beim Elefanten einsatzbereit,

wobei beim Nashorn noch weitere Forschungsarbeit von Nöten ist. Die erzielten Ergebnisse zeigen die Möglichkeiten der entwickelten Methoden auf und bieten eine viel versprechende Basis für die zukünftige Anwendung der Technologie im Erhaltungszuchtmanagement der bedrohten Tierarten.

Chapter V References

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Appendix

Article I: Index of sperm sex sortability in elephants and rhinoceroses

Contents

Flow cytometric sexing of spermatozoa followed by application in artificial insemination or *in vitro* fertilisation provides a unique opportunity to predetermine the sex of offspring and might enhance the conservation management of endangered species in captivity such as the elephant and the rhinoceros. To obtain an indication of the sortability of spermatozoa from these species, the differences in relative DNA content between X and Y- chromosome bearing spermatozoa (fresh, frozen-thawed, epididymal) from three rhinoceros species [white *Ceratotherium simum*), black (*Diceros bicornis*), Indian (*Rhinoceros unicornis*)] and two elephant species, the Asian and the African savannah elephant (*Elephas maximus, Loxodonta africana*), were determined through separation of spermatozoa into X and Y-chromosome bearing populations, using a modified high speed flow cytometer. The head profile areas of spermatozoa from all five species were measured using light microscopy. By multiplying the differences in relative DNA content and the head profile areas, the sperm sorting index was calculated to be 47, 48 and 51 for white, black and Indian rhinoceros, respectively. The calculated sorting index for the Asian elephant was 66. In the African elephant we determined the highest index of 76. These results indicate the practicability of flow cytometric sex-sorting of spermatozoa from the tested rhinoceros species and both elephant species. The lower sorting index values in rhinos indicate that sex-sorting of spermatozoa from the rhinoceros will be more challenging than in elephants.

Introduction

Predetermination of sex by artificial insemination (AI) or *in vitro* embryo production using flow cytometrically sexed spermatozoa is an alternative population management strategy, that could be incorporated into captive breeding programs for endangered species with single-sex dominated social structures such as the rhinoceros and the elephant (O'Brien et al., 2005).

The technique of sex-sorting spermatozoa is based on the flow cytometrical separation of X and Y- chromosome bearing spermatozoa, by means of their difference in relative DNA content (Johnson & Welch, 1999; Seidel & Garner, 2002). Since the first report of sex-sorting mammalian spermatozoa [rabbit (*Oryctolagus cuniculus*); Johnson et al., 1989), almost 40,000 domestic animals of predetermined sex have been born (Maxwell et al., 2004). In wildlife, AI with sorted spermatozoa has produced offspring from two species, the elk (*Cervus elaphus nelsoni*; Schenk & de Grofft, 2003) and the bottlenose dolphin (*Tursiops truncates*; O'Brien et al., 2006).

Various parameters influence the sorting ability of spermatozoa. Previous studies revealed that the greater the difference in relative DNA content between X and Y- chromosome, the better the resolution of the X and Y- sperm population. The difference in DNA content of at least 23 mammals has been determined to date by means of flow cytometry, from which the Chinchilla (*Chinchilla lanigera*) showed the biggest difference in DNA content (7.5 %) and the brushtail possum (*Trichosurus vulpecula*) the smallest (2.3 %; Garner, 2001; Johnson, 2000; Maxwell et al., 2004; O'Brien et al., 2006). In addition to the DNA difference, the ability to precisely orient the gametes at the time of measurement in the flow cytometer strongly affects the efficiency of sperm sorting. Mammalian spermatozoa with flattened, oval heads tend to be more readily oriented using hydrodynamics than those possessing more rounded or angular heads. The sperm sorting index (SSI), calculated by multiplication of sperm head profile area (μm^2) and the X-Y sperm difference in DNA content (%), combines these two influencing parameters (Garner, 2006).

The aim of this study was to determine the difference in relative DNA content between X and Y- chromosome bearing spermatozoa from the white (WR**,** *Ceratotherium simum*), black (BR**,** *Diceros bicornis*), and Indian rhinoceros (IR, *Rhinoceros unicornis*) and from two elephant species the Asian elephant (AsE, *Elephas maximus*) and the African savannah elephant (AfE, Loxodonta africana). The SSI was calculated to provide an indication of the sex-sortability of spermatozoa from these rhinoceros and elephant species, with a view to possible application of sex-sorting as tool for assisted reproduction and conservation management of the endangered megavertebrates.

Materials and Methods

Nine sperm samples from WR bulls (n = 7), three from BR bulls (n = 2) and one from an IR were collected using electroejaculation under general anaesthesia (Hermes et al., 2005) in several zoological institutions in Europe and Australia, contributing to regional endangered species programs. Semen collection was performed in context with general fertility assessments. Additionally one sperm sample was obtained by flushing the epididymidis of a euthanized BR bull. Sperm samples from one bull of each rhinoceros species were cryopreserved in liquid nitrogen in straws (500 µl) as described by Hermes and co-workers (Hermes et al., 2005).

Twelve ejaculates from one Asian and three ejaculates from two African savannah elephants were collected by manual rectal stimulation (Schmitt & Hildebrandt, 1998, 2000). Fresh samples from WR and BR bulls and spermatozoa recovered from the epididymidis were diluted in MES/HEPES semen extender (Blottner et al., 1994*)* to a concentration of 100 x 10^6 spermatozoa/ml for incubation at 37°C or 15°C. Sperm samples of lo w concentration (≤ 100 x 10^6 spermatozoa/ml) were extended 1:1 (v/v). The sample from the IR was treated similarly, except dilution was in modified KMT medium (Kenney et al., 1975).

Sperm samples from the Asian and the African elephant were diluted in modified KMT for incubation at 37°C. The extended semen was then pro cessed for sperm sorting following the general procedure described by Johnson and co-workers (Johnson et al., 1989). One ml aliquots of extended spermatozoa were stained with 15 – 30 µl of a 26.7 mmol/l stock solution of Hoechst 33342 fluorophore (Sigma-Aldrich, Taufkirchen, Germany) in the three rhino species and of an 8.9 mmol/l stock solution in both elephant species. Staining concentration was adjusted *pro rata* for sperm samples less concentrated than 100 x 10^6 spermatozoa/ml. Rhinoceros semen samples were incubated with Hoechst 33342 for at least 1 h at 37°C or 4 – 6 h at 15°C, when transported ov er longer distances. Elephant semen samples were incubated with Hoechst 33342 for at least 90 min at 37°C.

Cryopreserved rhinoceros sperm samples were thawed by agitation in a water bath for 30 s at 37°C. To remove the freezing extender semen aliq uots of 2 ml were washed by extension in 8 ml modified KMT and centrifugation for 15 min at 500 x g. After removing the supernatant, the resulting pellet was resuspended in modified KMT to a concentration of 100 x 10^6 spermatozoa/ml. DNA staining was performed at 5°C for 4 – 6 hours. The DNA stain concentration was the same as for fresh sperm sample treatments.

The DNA-stained spermatozoa were passed through a high-speed flow cytometer (MoFlo SX®; Dako Colorado Inc., Fort Collins, CO, USA), equipped with an argon UV-laser set to 200 MW output, operating at 40 psi. Sperm sex-sorting was performed at room temperature (25°C). The fluorescence intensity of X and Y- bear ing spermatozoa was collected from both the 0° and the 90° detectors and stored as frequenc y distributions (histograms). Double records were performed with a minimum of 20,000 spermatozoa. Differences in DNA content (percentage separation of the fluorescent peaks representing the two populations) were then calculated as described by Garner and co-workers (Garner et al., 1983): 100 (X - Y) / 0.5 (X + Y), where X and Y were the respective mean values for the two peaks (O'Brien et al., 2006; Parrilla et al., 2004).

Aliquots of fresh spermatozoa collected from rhinoceroses and elephants were fixed and stained on slides with a modified Kovács-Foote sperm staining procedure (Kovács et al., 1992; Kútvölgyi et al., 2006) to measure the sperm head profile area using oil immersion and a 100 x objective lens on a standard bright-field microscope (analySIS™ Image Processing software, n = 200).

Results

Ejaculate volume and sperm concentration (mean ± SD) averaged 17.5 ± 6.2 ml, 175.9 ± 75.7 x 10^6 spermatozoa/ml for WR, 34 ± 26 ml, 63.5 ± 11.5 x 10^6 spermatozoa/ml for BR, 50 ml, 510 x 10^6 spermatozoa/ml in the IR, 60.8 ± 23.4 ml, 664.0 ± 95.4 x 10^6 spermatozoa/ml for AsE and 5 ml, 105 x 10^6 spermatozoa/ml in the sex-sorted sperm sample of AfE. Sperm integrity (viable, acrosome and tail membrane intact spermatozoa) and total motility of fresh samples from rhinoceroses and AsE were ≥ 50 % and ≥ 60 % after collection. Sperm integrity and total motility of the fresh sample from AfE were 37 % and 0 %, respectively. Sperm integrity and total motility of frozen-thawed rhinoceros sperm were ≥ 35 % and ≥ 50 %, respectively.

Head profile areas were similar for the different rhinoceros species (mean ± SD; n = 200): 12.8 ± 1.5 μm^2, 13.2 ± 1.3 μm^2 and 13.1 ± 1.4 μm^2 for WR, BR and IR, respectively. Measurements of sperm head area of epididymidal spermatozoa from one BR bull did not reveal any significant differences compared to fresh spermatozoa (13.2 ± 1.3 μm^2 vs 12.9 ± 1.2 μm^2). The head profile areas of AsE and AfE spermatozoa were 19.8 ± 1.7 μm^2 and 19.1 ± 1.8 μm^2.

Resolution into X and Y- populations was successful for all three rhinoceros species and both elephant species using fresh spermatozoa. A dot plot representing the fluorescence detected by the 90° and 0° detectors was used to id entify the properly stained sperm population in the flow cytometer. A gate (R1) was placed to include properly stained and correctly orientated spermatozoa to achieve the best resolution of the X and Y- sperm populations (Figure 1). In the resulting histogram, two species-specific overlapping distinct peaks were visible, corresponding to the X and Y- chromosome bearing sperm population. In all three rhinoceros species additional data was generated from frozen-thawed spermatozoa. The percentage of properly stained and correctly oriented spermatozoa in the flow cytometer (R1, Figure 1) was lower after cryopreservation (WR: 40.2 %, BR: 16.2 %, IR: 13.7 %) when compared with fresh samples (WR: 42.3 %, BR: 38.1 %, IR: 38.2 %) in all three rhinoceros species.

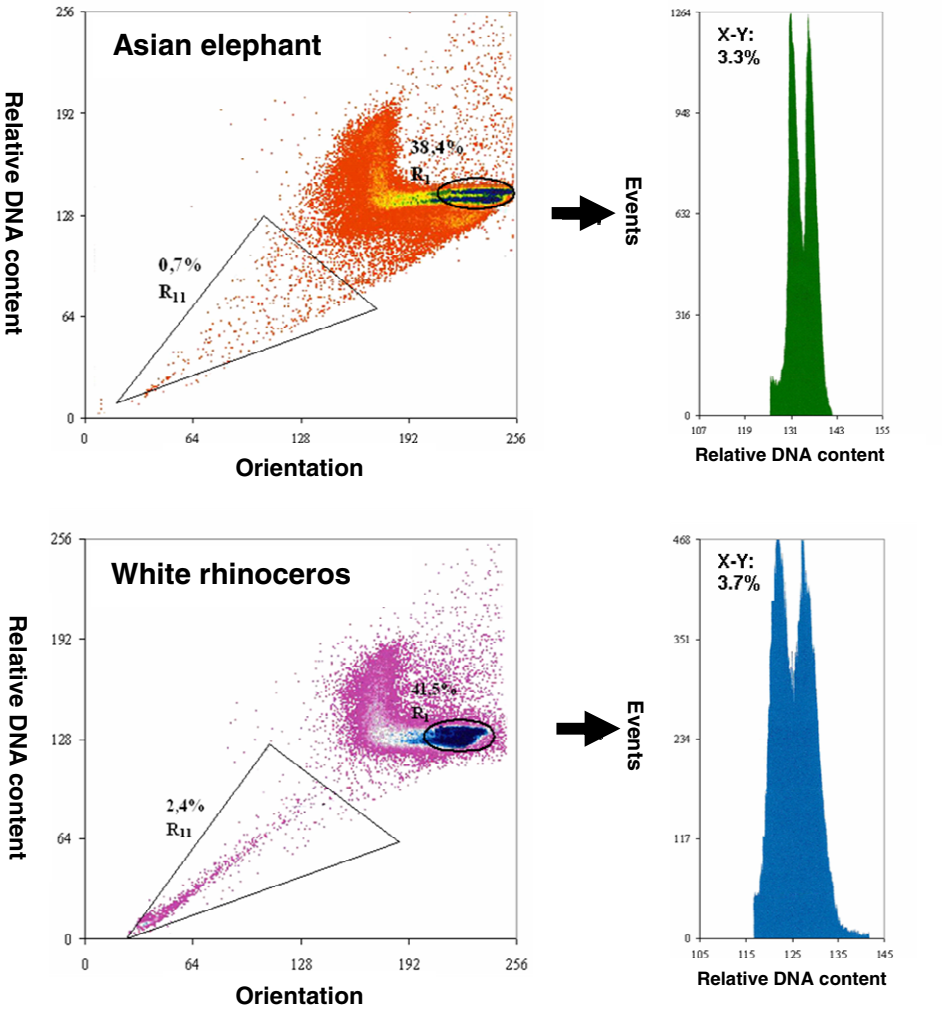

Figure 1: Flow cytometric dot-plot and histogram outputs showing fluorescence signals from DNA-stained spermatozoa from Asian elephant and white rhinoceros. Fluorescence signals of properly stained and correctly oriented spermatozoa in Region 1 (R1; AsE: 37.5 ± 3.1 %, WR: 33.3 ± 5.1 %, mean ± SEM) appear in the histogram output. Region 11 (R 11; AsE: 7.1 ± 2.4 %, WR: 12.4 ± 7.6 %, mean ± SEM) contains spermatozoa with reduced fluorescence intensity because of plasma membrane damage

By means of the difference in fluorescence intensity, the difference in relative DNA content (mean ± SEM) between “male” and “female” spermatozoa was calculated: 3.7 ± 0.05 %, 3.7 ± 0.03 % and 3.9 ± 0.04 % for WR (n = 9), BR (n = 3) and IR (n = 1). The difference in relative DNA content in the elephant species was 3.3 ± 0.05 % and 4.0 % for the AsE (n = 12) and AfE (n = 3), respectively.

The AfE had the highest SSI of 76, followed by the AsE with 66. The SSI values from the rhinoceros species were similar among each other with 47, 48 and 51 for WR, BR and IR, respectively.

Discussion

Flow cytometric sexing of spermatozoa followed by AI or IVF provides a unique opportunity to predetermine the sex of offspring from endangered, captive species such as the elephant and the rhinoceros. This technique opens up the potential for establishing self-sustaining populations by compensating premature female aging processes as well as balancing skewed sex-ratios. Additionally, sex-preselection can help to resolve difficult management situations such as problematic bachelor groups by avoiding the production of surplus males.

In this study, resolution of X and Y- sperm populations was performed using fresh spermatozoa collected from three rhinoceros species and two elephant species. Separation of X and Y- sperm populations using frozen-thawed samples demonstrated the general potential of sex-sorting after cryopreservation in rhinoceroses. Yet, due to mechanical stress and subsequent damage imparted on the spermatozoa during the freezing and thawing process the percentage of properly stained and correctly oriented spermatozoa in the flow cytometer was lower after cryopreservation compared with fresh samples.

The difference in DNA content between X and Y- chromosome bearing spermatozoa from WR, BR, IR, AsE and AfE was determined. From the AfE, so far only one sample was available for resolution in an X and Y- sperm population. However the calculated difference in relative DNA content of 4 % corresponded with former results from O'Brien and co-workers (O'Brien et al., 2002). Additionally the head profile areas of spermatozoa from all five tested species were measured. Head profile areas were calculated from fresh sperm samples only, as the freezing and thawing process is meant do cause sperm head volume regulation in response to varying osmotic pressure during the cryopreservation process. The head profile area of fresh spermatozoa from the WR corresponded with former computer assisted measurements using HTM IVOS (Silinski, 2003). The SSI, as combination of the difference in DNA content between X and Y- chromosome bearing spermatozoa and head profile are provides an appropriate indication of species-specific sperm sortability. The AfE had the highest SSI (76), followed by the AsE (66) and the three rhinoceros species (48, 49 and 51). The determined SSI values from successful sex-sorted livestock and domestic species vary between 59 for domestic stallion *(Equus caballus*) and 131 for cattle bull (*Bos taurus*; Welch & Johnson; 1999, Johnson 2000; Garner 2001; Garner & Seidel, 2003; Garner 2006). Compared to indices of these domestic species, the result for the AfE lies in the same range as domestic dog (*Canis familiaris*; 82) and cat (*Felis catus*; 80), while the AsE surpasses the domestic stallion (59). The SSI values for the examined rhinoceros species range between domestic stallion (59) and man (31, Johnson et al., 1993, Garner et al., 2006). The low SSI of human spermatozoa (31) is reflected in poor resolution of X and Y bearing spermatozoa (as indicated by the depth of the split on the histogram output) compared to other species (O'Brien et al., 2005). However, sex-sorting of human spermatozoa, followed by IVF has

resulted in the birth of healthy children (Fugger et al., 1998; Fugger, 1999; Levinson et al., 1995; Vidal et al., 1999).

Corresponding to the SSI differences between rhinoceros and elephant spermatozoa, better resolution of the X and Y- chromosome bearing spermatozoa was obtained in elephant compared to rhinoceros semen (Figure 1). The sperm sortability is influenced by the general quality of ejaculates and differs strongly even in one bull. In this study, used extenders were selected due to their positive influence on sperm motility, integrity, sortability and on cell aggregation, evaluated in preliminary tests. However, the variable composition and high viscosity of ejaculates interfered with the requirement of stable single cell suspensions for the sorting process, especially in the rhinoceroses. Therefore the separation of total ejaculates was not possible in each case. Nevertheless, the results of our study do indicate the feasibility for flow cytometric sperm sex-sorting of all tested rhinoceros and elephant species. The results are encouraging for the development of a protocol for sex-sorting spermatozoa suitable for AI in both elephants and rhinoceroses (Brown et al.; 2004; Hildebrandt et al.; 2007a). Moreover, *in vivo* ovum pick up has recently been achieved in the white and black rhinoceros (Hermes et al., 2007; Hildebrandt et al., 2007b). However, IVF or intracytoplasmic sperm injection in the rhinoceros needs further development. Given the limited sortability of rhinoceros spermatozoa, these techniques would offer great potential for the application of sex-sorted rhinoceros spermatozoa, as only small sperm numbers are required. Thus, the combination of assisted reproduction methods with sperm-sexing technique could considerably increase the efficiency of breeding programmes in the studied endangered species.

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Article II: Feasibility of sex-sorting sperm in rhinoceros species

Contents

The objective of these studies was to investigate the practicality of flow cytomerical sex-sorting for rhinoceros spermatozoa. In Experiment 1, four semen extenders were tested regarding their suitability for liquid preservation of spermatozoa before sorting. Dilution in MES-HEPES based semen extender followed by incubation generated best sperm quality parameters (motility, viability and acrosome integrity). In Experiment 2, the effect of staining method (15℃ for 4-6h during transport or 37℃ for 1-1.5h) on sort efficiency and sperm quality was investigated. Staining at 15℃ during t ransport resulted in a higher percentage of sperm samples showing a resolution of X and Y- chromosome bearing populations (60 %) compared to staining at 37℃ after transport (33 %) and in superior sperm integrity after staining (43.8 ± 11.3 vs 19.6 ± 12.1 %). Sort rate was 300 - 700 cells/sec and sort purity was 94 % for X-chromosome bearing spermatozoa, determined for one sorted sample. In Experiment 3, the highly viscous component of rhinoceros seminal plasma which complicates the process of sperm-sorting was examined by gel-electrophoresis and mass spectrometry. Results suggested a 250 kDa glycoprotein (most likely originating from the bulbourethral gland) to be responsible for the characteristic viscosity of ejaculates. In Experiment 4 viscosity of seminal plasma, as measured by electron spin resonance spectroscopy, was significantly decreased after addition of α-amylase and collagenase (0.5 and 3 IU per 100 µl seminal plasma) by 28 and 21 %, respectively, with no negative effect on sperm characteristics. The results of this study demonstrate for the first time that rhinoceros spermatozoa can be successfully sorted into high purity X and Y- chromosome bearing populations. Furthermore, the successful liquefaction of viscous ejaculates provides the means to greatly improve sort-efficiency in this species.

Introduction

Sex-sorting of spermatozoa by modified flow-cytometry has been applied to a range of species and numerous offspring were born after artificial insemination (AI), in vitro fertilisation (IVF) and intra cytoplasmic sperm injection (ICSI) with sorted spermatozoa (Rath & Johnson, 2008). In exotic and endangered species such as the rhinoceros, the possibility of producing a larger number of females and hence accelerating population growth makes the implementation of sex-sorting technology particularly appealing (O'Brien et al., 2003; Hermes et al., 2007). In addition, sex-preselection by sperm-sorting has the potential to offset breeding losses from premature female aging (Hermes et al., 2004) as well as balancing skewed sex ratios in the black (AZA, 2005; Dennis, 2004) and Indian rhinoceros (Zschocke, 1998; Hlavacek, 2005).

Semen from rhinoceroses has been routinely collected by electroejaculation (Hermes et al., 2005; Roth et al., 2005), successfully cryopreserved (Hermes et al., 2005; Reid et al., 2009)

and artificially inseminated either fresh or frozen (Hildebrandt et al., 2007; Hermes et al., 2009). While a theoretical sex-sorting index for rhinoceros spermatozoa has been established (Behr et al., 2009), the development of a sperm-sorting protocol for this species remains in its infancy. Spermatozoa of domestic species such as sheep and cattle have benefited from almost two decades of protocol optimisation and diluent development in an effort to maximise protection against the myriad of stressors to which they are exposed (Maxwell et al., 1998; Seidel & Garner, 2002). Sperm physiology is partly species-specific and often the available knowledge is very limited or absent in wildlife species, resulting in the need to develop new protocols for sperm handling prior, during and following the sorting process (O'Brien & Robeck, 2006).

A major challenge in handling and processing ejaculates from African rhinoceroses for flow cytometric sex-sorting, especially those from the white rhinoceros, is the high viscosity of semen samples. Upon ejaculation spermatozoa are mixed with secretions from the accessory sex glands, entrapping the spermatozoa in a highly viscous gel (unpublished observations). Spermatozoa can not be separated from the gelatinous material by filtration or centrifugation as routinely applied in the boar or the stallion, nor does the gel fraction liquefy after a short period of time (human: Tauber & Zaneveld, 1981) or releases spermatozoa by contraction [rhesus monkey (*Macaca Mulatta*): Fordney & Hendrickx, 1974]. These limitations are also evident in the semen samples of camelids such as the alpaca (*Lama pacos*) and llama (*Lama glama*: San-Martin et al., 1968; Garnica et al., 1983).

The main source of coagulating substrates in most mammals, including the human, is the vesicular glands (Mann & Lutwak-Mann, 1981). The African rhinoceros has three accessory sex glands, which may contribute to the high viscosity of seminal plasma, (i) the paired long-shaped bulbourethral glands, (ii) the prostate and (iii) the paired vesicular glands (Schaffer et al., 2001; Hermes et al., 2005). A number of different methods have been explored to characterize and reduce the viscosity of seminal plasma in several mammalian species. In the human a chymotrypsin-like prostatic specific antigen was determined to cause liquefaction of semen through cleavage of semenogelin I, the major component of the coagulum, which is expressed exclusively in the vesicular glands (Chaistitvanich & Boonsaeng, 1983; Lilia et al., 1984, 1985). Additionally, proteolytic enzymes were detected and isolated in the seminal plasma of different species such as a collagenase-like metalloproteinases in human, common rat (*Rattus norvegicus*) and domestic bull (*Bos taurus*) semen (Koren & Milkovic, 1973) and trypsin in semen from the guinea-pig (*Cavia porcellus*) and rabbit (*Oryctolagus cuniculus*; Freund, 1958; Stambaugh & Buckley, 1970). Based on these findings several enzymes have been used to cleave the gelatinous components of seminal plasma in order to reduce its viscosity. Trypsin and collagenase have been used as additives to reduce the viscosity of rhesus-monkey (Hoskins & Patterson, 1967), guinea-pig (Freund, 1958) and, more recently, alpaca and llama ejaculates (Bravo et al., 1999, 2000). While reducing the viscosity of seminal plasma, Bravo and co-workers

claimed that collagenase had no impact on functional parameters of spermatozoa in the alpaca and llama (Bravo et al., 1999) and no effect on cleavage and embryo development rates after *in vitro* fertilization in llamas was observed (Conde et al., 2008). The glycoside hydrolase enzyme α-amylase was successfully used to liquefy coagulated human semen (Bunge & Sherman, 1954) but failed to reduce the viscosity of guinea-pig or rhesus-monkey semen (Hoskins & Patterson, 1967). In these investigations the influence of amylase on sperm fertility was not determined. However, a study on bull semen extenders showed that the addition of α-amylase to bull spermatozoa had a significantly positive effect on pregnancy rates following AI (Kirton et al., 1968).

The aim of the present study was twofold. We first attempted to determine optimal techniques for semen extension during chilled transport and staining of spermatozoa preliminary to sex-sorting (Experiments 1 and 2). Second, we tried to investigate the origin and composition of the gelatinous fraction in the rhinoceros seminal plasma, with the ultimate goal of enzymatic liquefaction of this fraction without compromising the functional integrity of spermatozoa (Experiment 3 and 4).

Materials and Methods

Nineteen semen samples from 13 white rhinoceros bulls (WR) and seven ejaculates from four black rhinoceros bulls (BR) were collected using electroejaculation under general anaesthesia (Hermes et al., 2005). Unless otherwise mentioned, all materials were of reagent grade or higher and were purchased from Sigma Aldrich Chemie GmbH, Taufkirchen, Germany.

1.1 Experiment 1: Effect of extender on quality of chilled rhinoceros spermatozoa

Seven semen samples from seven WR bulls were used for this experiment. After semen collection, aliquots were immediately diluted 1:1 (v/v) with four different, pre-warmed (37℃), semen extenders: 1) Blottner's sorting medium, a MES-HEPES and skim milk based medium (BSM; Hermes et al., 2009), 2) MES-HEPES semen extender (MES; Blottner et al., 1998), 3) Andromed™ (Minitüb, Tiefenbach, Germany), 4) Beltsville thawing solution (BTS; Graham et al., 2004). All extenders were supplemented with sodium pyruvate (1.0mM; as an antioxidant) and EGTA (2.0mM; to prevent sperm agglutination). Smears from each sample were prepared for assessment of morphology and viability of spermatozoa. Samples were then chilled slowly during transport by placing the test tube with the semen inside an isothermal water bath stored at 4℃. Chilled sample s were transported to the lab over 4 – 10 h, incubated at room temperature (RT) for 30 min then at 37℃ for 15 min before evaluating sperm quality parameters. These included total motility (TM), progressive motility (PM), viability, and acrosome integrity of spermatozoa.

1.1.1 Assessment of spermatozoa

Motility, viability and morphology of spermatozoa were evaluated after collection, transport to the laboratory, DNA staining and incubation, and sex-sorting. PM and TM of spermatozoa were determined as described by Hermes and co-workers (Hermes et al., 2005). Evaluation of sperm motility was complicated by high viscosity of semen samples. As viscosity of semen samples was not homogenous, it was attempted to evaluate motility parameters in parts of preferably low viscosity. The viability and acrosome integrity of spermatozoa were assessed using a modified Kovács-Foote staining technique (Kovács & Foote, 1992; Nagy et al., 1999; Kútvölgyi et al., 2006). Sperm sample aliquots (10 µl) were diluited 1:5 (v/v) with phosphate buffered saline (PBS). 10 µl of diluted samples were stained with 10 µl of a 0.16 % Chicago Sky Blue solution diluted in PBS by mixing the drops on a slide and preparing smears. The smears were fixed for 2 min in a fixative composed of 86 ml of 1.0 N HCL plus 14 ml formaldehyde solution (37 % w/w) and 0.2 g neutral red. The acrosome stain was a 7.5 % Giemsa stock solution (GS-500) diluted in distilled water. Smears were stained for 5 h at 35°C. A total of 200 stained spermatozoa were count ed in duplicate using oil immersion and at 1,000 x magnification on a standard bright-field microscope. Spermatozoa were categorized as “viable” (head membrane intact; posterior part of the sperm head white to light blue) or “dead” (head membrane damaged; posterior part of the sperm head dark blue). Acrosomes were classified as intact (anterior part of the sperm head pink to purple), modified (anterior part of the sperm head dark lavender) or reacted (anterior part of the sperm head light lavender) including completely detached acrosomes (anterior part of the sperm head white to grey).

1.2 Experiment 2: Effect of DNA staining method on sort efficiency and post-sort quality of rhinoceros spermatozoa

Only spermatozoa extended in MES extender were subjected to DNA staining. Aliquots from 10 ejaculates were stained following chilled transport (4°C; 4 – 10 h) to the lab at 37°C for 1-1.5 h after re-warming to RT for 30 min (10 WR). Spermatozoa from 10 different ejaculates were stained during transport for 4-6 h at 15°C (7 WR, 3 BR). For DNA staining at 37°C only spermatozoa with a TM ≥ 35 % after transport were used. Aliquots of 0.5 ml from each treatment were labelled with Hoechst 33342 DNA stain at three different concentrations (7.5, 10 and 12.5 µl of 26.7 mM stock solution). Stained spermatozoa (100 x 10^6 spermatozoa/ml) were assessed as described in 2.1.1 and sorted on a high speed flow cytometer (MoFlo SX, Dako Colorado, Fort Collins, CO) equipped with an argon laser (200 mW) and operating at 40 psi with a HEPES-buffer based sheath fluid (Buss, 2005). Sortability of labelled samples was estimated in the flow cytometer through optic purity of the resolution between X and Y-chromosome bearing sperm populations, visible in the dot plot output. Samples with good resolution between the X and Y- chromosome bearing spermatozoa were sorted into two 10 ml conical plastic tubes (Greiner, Nürtingen, Germany) containing 500 µl of TEST-yolk

collection extender with an egg yolk concentration of 2 % (Johnson, 1991). After sorting, samples were centrifuged at 840 x *g* at room temperature for 20 min to remove the sheath fluid. The supernatant was discarded and the pellet was resuspended in 100 µl of Blottner's Cryomedia (BC) semen extender (Blottner et al., 1998; Hermes et al., 2005). An aliquot of the X and Y- sorted sample from one bull was re-stained to determine the proportions of X and Y- bearing spermatozoa (Welch & Johnson, 1999).

1.3 Experiment 3: Characterisation of viscous seminal plasma fraction

1.3.1 Rating and preparation of seminal plasma and secretions of the accessory sex glands

The viscosity of collected semen was macroscopically evaluated based on the ability to raise a 10 µl semen aliquot in a 10-100 µl pipette (ABIMED, Langenfeld, Germany). Viscosity was subjectively divided into three groups. I: not viscous (unhindered filling), II: viscous (decelerated filling) and III**:** highly viscous (filling not feasible). Seminal plasma aliquots from 10 ejaculates (6 WR, 4 BR) were stored for further assessment. When possible, seminal plasma was separated from spermatozoa by centrifugation for 20 min at 1000 x *g*. The supernatants were transferred into 1.5 ml tubes, re-centrifuged for 25 min at 4000 *g* to eliminate the remaining cells and stored at -80°C. When separation of spermatozoa and seminal plasma was not possible due to high viscosity, aliquots of semen were stored at -80°C without treatment. Additionally, secretions of post-mortem extracted bulbourethral glands, prostate and vesicular glands from two WR were ejected and stored at -80°C.

1.3.2 Gel electrophoresis

All reagents and apparatus used in the preparation and separation of the protein samples were from Bio-Rad (Hercules, CA, USA) unless otherwise specified. Thawed seminal plasma aliquots were centrifuged at 10,000 x *g* for 10 min to remove cellular components. Proteins of seminal plasma samples and secretions from the bulbourethral glands, prostate and vesicular glands were comparatively separated by one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) following determination of quantitative protein content using bicinchoninic acid (Smith et al., 1985). Samples were subjected to the SDS-PAGE as described by Laemmli (Laemmli, 1970) using a 12 % polyacrylamide gel. Twenty µl samples containing 28 µg of total protein were mounted to the gel. Molecular mass was estimated by comparison with Page Ruler™ Unstained Protein Ladder standards (10 µl) or the Precision Plus Protein Standard (3 µl). Electrophoresis was run at 120 V for 90 min. After electrophoresis, gels were stained with Coomassie Blue G250 for 2h and washed for 1h in 10 % ethanol (v/v) and 2 % ortho-phosphoric acid (v/v).

For further characterization of seminal plasma proteins two-dimensional gel electrophoresis was performed. Protein samples were mixed with a 2-DE compatible buffer containing 5 M urea, 2 M thiourea, 2 % (w/v) CHAPS, 40 mM Tris–HCl, 2 mM TBP, 2 % (w/v) sulfobetaine

3–10, 1 % (v/v) Bio-Lyte 3–10 carrier ampholytes and 0.002 % (w/v) bromophenol blue, followed by centrifugation (16,000 x *g*, 1min, 25℃) to remove insoluble material. Samples (100 µg of protein, 250 µL) were loaded onto an 11 cm pH 3–10 linear pH gradient IPG gel strip. After overnight in-gel rehydration, isoelectric focusing was carried out by a IEF Cell apparatus using a five-step program (2 h at 200 V, 2 h at 500 V, 2 h at 1 kV, 2 h at 3 kV and 50 kVh at 5 kV) for a total of 55-60 kVh. After focusing, the IPG strips were equilibrated for 10 min in 6 M urea, 2 % (w/v) SDS, 20 % (v/v) glycerol, 5 mM TBP, 2.5 % (v/v) acrylamide and 350 mM Tris–HCl (pH 8.8), then transferred onto a second dimension sodium SDS-PAGE gel (4–12 % linear gradient) and sealed in place with 0.5 % (w/v) agarose in MES buffer with a trace of bromophenol blue. Second dimension electrophoresis was carried out using a Criterion system at 180 V for 55 min in MES buffer. Gels were stained with Sypro Ruby overnight and washed in 10 % (v/v) methanol and 7 % (v/v) acetic acid for 1 h before being visualized on a Molecular Imager® FX Pro Plus. Gels were post-stained with Coomassie Blue G250 as described above.

1.3.3 In-gel digestion and mass spectrometry

Selected bands displayed in the one-dimensional SDS-PAGE from seminal plasma and accessory gland secretions were analyzed using mass spectrometry (Henrich et al., 2007). Briefly, selected protein bands were de-stained, the gel pieces then dried in a rotor evaporator and re-hydrated with 15 µl of porcine sequencing grade trypsin at 125 ng/µl. After 1 h at 4℃ any excess trypsin was removed and 15 µl of 20 mM ammonium bicarbonate solution added and the samples incubated at 37℃ overnight. For matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis 1 µl of sample was spotted onto a target plate with an equal volume of matrix solution and air-dried at room temperature. Mass spectra were acquired in the mass-charge range of 800–3500 u on a QSTAR XL mass spectrometer equipped with a MALDI source (Applied Biosystems Inc., Foster City, CA, USA). Mass calibration was performed using Fibrinopeptide B as an external calibrant. The generated monoisotopic peak masses were subjected to database searching against the MSDB comprehensive non-redundant database using MASCOT vr. 2.0 (Matrix Science, London, UK). The peptide mass fingerprinting (PMF) data were also searched using the MS-Fit program (Protein-Prospector, UCSF Mass Spectrometry Facility). Each PMF spectrum was manually inspected, and peptides were obtained by manual annotation of the spectra. Parameters for protein identification included searching with a mass error tolerance of 65 ppm per peptide, 1 missed tryptic cleavage, and allowing oxidation of methionine as an optional modification. No Ip (isoelectric point) or mass restrictions were included. Confident matches were defined by the MASCOT score and statistical significance ($p < 0.05$), the number of matching peptides and the percentage of total amino acid sequence covered by those matching peptides.

1.4 Experiment 4: Liquefaction of viscous fraction using α-amylase and collagenase and evaluation of the enzyme-influence on sperm characteristics

1.4.1 Electron spin resonance spectroscopy

0.5 IU of α-amylase and 3 IU of collagenase dissolved in MES semen extender (1 mg/ml) were mixed with 100 µl aliquots of undiluted seminal plasma from 7 white rhinoceros bulls. In this experiment MES semen extender was used instead of PBS for dosslving the enzymes to avoid a possible damaging influence of diluent medium on sperm quality. As a control, 100 µl samples of undiluted seminal plasma were mixed with 10 µl MES semen extender. Samples were incubated for 30 min at RT. The viscosity of enzyme treated and control seminal plasma samples were characterized by using electron-spin resonance spectroscopy (ESR) to measure the rotation of the paramagnetic substance 2,2,6,6-Tetramethyl-1-piperidinyloxy (Tempo; Hermann & Mueller, 1985). This assay has the advantage of allowing measurement of sample volumes as low as 50 µl. The rotation of Tempo in aqueous solution depends on the solution viscosity and can be estimated from the shape of the ESR spectrum. Five µl of a 1 mM Tempo solution were added to the seminal plasma samples incubated for 30 min at RT. Subsequently, ESR spectra were recorded at 4℃ on a Bruker ECS 106 spectrometer (Bruker, Karlsruhe, Germany) with the following settings: modulation amplitude, 1 G; power, 10 mW; scan width, 100 G; accumulation, once. To quantify solution viscosity, a rotational correlation time (τ_c) was estimated from the ESR spectra (Keith et al., 1970; Davis et al., 1976).

1.4.2 SDS-PAGE

SDS-PAGE was used to evaluate the effect of α-amylase (31.8 IU/mg) and collagenase (196 IU/mg) on the protein profile of rhino seminal plasma. 3 enzyme concentrations (α-amylase: 3 IU, 1.5 IU, 0.5 IU; collagenase: 18.5 IU, 9.25 IU, 3 IU) diluted in 10 µl PBS were added to the seminal plasma sample mounted to the gel. In the control sample only 10 µl PBS was added. After 30 min incubation at RT samples were subjected to the SDS-PAGE as described above.

1.4.3 Quality parameters of spermatozoa after treatment with α-amylase and collagenase

Directly after collection aliquots from ejaculates were diluted 1:1 (v/v) in MES semen extender. Three concentrations of enzymes [α-amylase (n = 12, 8 WR, 4 BR): 0.16 IU, 1.6 IU, 4 IU; collagenase (n = 8, 4 WR, 4 BR): 24.5 IU, 9.8 IU, 0.98 IU] dissolved in pre-warmed (37℃) MES semen extender (1 mg/ml, 5, 50, 125 µl of enzyme solution) were added to 0.5 ml semen samples. As a control 125 µl of MES semen extender without any enzyme addition was added to 0.5 ml semen aliquots. Sperm PM and TM, viability and acrosome integrity were evaluated following dilution and 1h after addition of the enzyme, respectively.

1.5 Statistical analyses

Statistical analyses were performed using SPSS v. 16.0 (SPSS Inc., Chicago, IL). Due to small number of samples, none parametric tests were performed. Differences in viscosity and quality parameters of spermatozoa between enzymes treated and control groups as well as between semen samples diluted in different extenders after transport to the laboratory were analysed using rank variance analysis (exact Friedman Test). A multiple paired comparison (Wilcoxon and Wilcox Test; Sachs & Hedderich) was used to further define differences between the groups. Quality parameters of spermatozoa were compared between samples after collection, transport and after staining and incubation with Wilcoxon Signed Ranks Test with continuity correction or exact Mann-Whitney U Test. For all analyses, the level of significance was set to $p \leq 0.05$.

Results

The average concentration of spermatozoa in collected ejaculates was 248.0 ± 470.2 x 10^6 spermatozoa/ml (mean ± SD; n = 26). The median was 80 x 10^6 spermatozoa/ ml. Of the 26 samples evaluated, there were two extremes (1,500 and 2,000 x 10^6 spermatozoa/ml), defined as cases with values more than three box lengths from the upper or lower edge of the box in the boxplot option The box length is the interquartile range (boxplot not shown). Mean sperm concentration ± SD excluding these extremes was 117.6 ± 112.3 x 106 spermatozoa/ml. The mean volume of ejaculates collected was 20.8 ± 15.7 ml. PM, TM and sperm integrity (viable, intact acrosome) were 38.5 ± 24.4 %, 73.5 ± 16.0 % and 73.3 ± 12.0 %, respectively. The high viscosity of the seminal fluids in most ejaculates made the evaluation of PM and TM difficult, as most spermatozoa were ‘trapped’ in the gel fraction.

2.1 Experiment 1: Effect of extender on quality of chilled rhinoceros spermatozoa

The quality parameters of spermatozoa after collection and after transport are presented in Table 1.

After transport to the laboratory, semen samples extended in MES had a significantly higher PM and TM than those in Andromed™ (Wilcoxon and Wilcox Test; PM: df = 3, $p \leq 0.01$, TM: df = 3, $p \leq 0.05$). PM and TM were also higher in MES than in BTS and BSM, but these differences were not significant. There was a decrease of the quality parameters of spermatozoa during transport to the laboratory regardless of the extender used, even if not all declines were significant (Wilcoxon Signed Ranks Test with continuity correction).

Table 1: Sperm quality characteristics (mean ± SEM) after collection and after transport to the laboratory (4 – 10 h, 4℃), comparatively displayed after dilution in four semen extenders (MES, Andromed™, BTS, BSM; n = 7).

Extender	Quality parameter (%)	After collection	After transport
MES	Progressive motility	57.3 ± 4.2[a]	19.4 ± 7.1[b,c]
	Total motility	74.9 ± 5.2[a]	56.9 ± 6.9[b,c]
	Intact*	68.3 ± 7.1	53.4 ± 9.3
	Viable	74.1 ± 5.9	61.9 ± 8.4
	Intact acrosomes	70.1 ± 6.9	54.7 ± 9.6
Andromed™	Progressive motility	37.6 ± 11.2[a]	3.6 ± 1.8[b,d]
	Total motility	68.1 ± 8.7[a]	34.6 ± 9.4[b,d]
	Intact*	69.0 ± 8.3	50.1 ± 6.4
	Viable	75.0 ± 7.5	62.6 ± 6.1
	Intact acrosomes	71.6 ± 8.3	52.9 ± 6.8
BTS	Progressive motility	53.1 ± 10.8[a]	14.1 ± 7.9[b]
	Total motility	71.1 ± 8.2[a]	51.6 ± 10.9[b]
	Intact*	68.7 ± 7.5[a]	51.3 ± 23.6[b]
	Viable	77.3 ± 5.9	64.1 ± 8.7
	Intact acrosomes	71.1 ± 7.9	50.7 ± 9.4
BSM	Progressive motility	35.0 ± 10.7	14.7 ± 8.5
	Total motility	60.0 ± 9.5	47.0 ± 10.4
	Intact*	69.6 ± 7.5[a]	53.5 ± 7.5[b]
	Viable	77.0 ± 6.0	64.7 ± 7.1
	Intact acrosomes	71.7 ± 7.6	54.8 ± 7.6

[a-d]within lines (a,b) and rows (c,d) values of same parameters with different superscript differ significantly ($p \leq 0.05$), *intact: viable, intact acrosome.

2.2 Experiment 2: Effect of DNA staining method on sort efficiency and post-sort quality of rhinoceros spermatozoa

The quality parameters of spermatozoa after staining and incubation are presented in Table 2 [n = 10 (15°C/ 37°C)].

All measured sperm quality parameters decreased during staining and incubation, regardless of whether incubation was performed at 15°C or 37°C [Wilcoxon Signed Ranks Test: TM, sperm integrity, sperm viability, acrosome integrity: $p = 0.002$ (15°C/ 37°C)]. However, after staining with Hoechst 33342 and incubation at 15°C for 4 – 6 h all sperm quality parameters were significantly higher than after incubation of the different samples at 37°C for 1 - 1.5 h (Mann –Whitney U Test: TM: $p = 0.023$; sperm integrity: $p = 0.001$; sperm viability: $p = 0.005$; acrosome integrity: $p = 0.0004$). The initial quality parameters of ejaculates after collection did not differ between the two groups (Mann –Whitney U Test: $p \geq 0.238$).

After incubation at 37°C high sample viscosity (n = 3) and the absence of a split into two populations in the flow cytometer (n = 4) hindered the sortability of the samples. Only three samples could be separated into X and Y- sperm-bearing populations. After incubation at 15°C, six samples were resolved into X and Y- sperm populations from which two could not be sorted due to low sample volume. High viscosity (n = 2) and the absence of resolution in two populations in the flow cytometer (n = 2) again prevented testing for sortability. The quality parameters of spermatozoa after stating and incubation at 37°C or 15°C and sex sorting are presented in Table 2. Due to low number of sorted samples (37°C: n = 3; 15°C: n = 4), no statistical testing was performed.

The event rate ranged from 1,000 – 15,000 spermatozoa/sec and the sort rate was 300 - 700 spermatozoa/sec. The mean percentage of correctly oriented cells in sortable samples was 30.8 ± 13.8 %. Determined sort purity was 94 % and 75 % for X and Y- chromosome bearing populations, respectively.

Table 2: Sperm quality characteristics (mean ± SEM) comparatively displayed after collection/ dilution in MES semen extender and after staining and incubation at 15℃ or 37℃ [n = 10 (15℃/ 37℃)].

Quality parameter (%)	After collection	After incubation at 37℃	After sorting
Total motility	70.6 ± 6.4[a]	13.3 ± 6.9[b,c]	11.7 ± 7.6
Intact*	67.7 ± 4.5[a]	19.6 ± 3.8[b,c]	30.0 ± 8.2
Viable	72.5 ± 3.7[a]	28.5 ± 4.9[b,c]	46.3 ± 12.9
Intact acrosome	70.4 ± 4.5[a]	21.3 ± 3.6[b,c]	33.3 ± 8.3
Quality parameter (%)	After collection	After incubation at 15℃	After sorting
Total motility	72.7 ± 3.0[a]	32.2 ± 4.1[b,d]	11.5 ± 6.1
Intact*	74.6 ± 2.0[a]	43.8 ± 3.6[b,d]	37.7 ± 6.7
Viable	78.5 ± 2.1[a]	49.9 ± 3.7[b,d]	49.3 ± 8.4
Intact acrosome	76.1 ± 1.9[a]	46.4 ± 3.5[b,d]	42.0 ± 5.4

[a-d]within lines (a,b) and rows (c,d) values of same sperm parameter with different superscript differ significantly ($p \leq 0.05$), *intact = viable, intact acrosome.

2.3 Experiment 3: Characterization of viscous seminal plasma fraction component

From 19 ejaculates collected from WR bulls, 12 were classified as viscosity grade II, 5 as viscosity grade III and 2 were not viscous (grade I). Regarding the seven ejaculates collected from BR bulls, two were classified grade II and the other five showed no noticeable viscosity (grade I). Characterization of proteins from six rhino seminal plasma samples of different viscosity (I-III) by SDS-PAGE and 2D-gelelectrophoresis, showed a highly acidic (Ip ≤ 3) 250 kDa protein spot (P250), whose occurrence and intensity strongly correlated with the grade of viscosity (Fig. 1, 2). One and 2-dimensional (1D, 2D) electrophoresis gels displaying the protein composition of secretions from the bulbourethral glands, the prostate and the vesicular glands were searched for a 250 kDa protein band to determine the origin of P250. The only suitable protein spot that was detected originated from the bulbourethral gland secretions (PBU250).

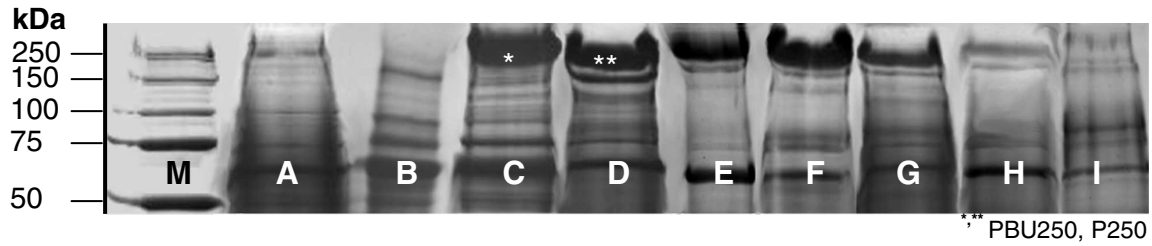


Figure 1: SDS-PAGE pattern of accessory sex glands secretions and whole seminal plasma from ejaculates of different bulls and of different viscosity. Lane **M**: protein marker. Lane **A**: vesicular gland secretion. Lane **B**: prostate secretion. Lane **C**: bulbourethral gland secretion. **D – F**: seminal plasma viscosity grade III. Lane **G**: seminal plasma viscosity grade II. Lane **H + I**: not viscous seminal plasma.

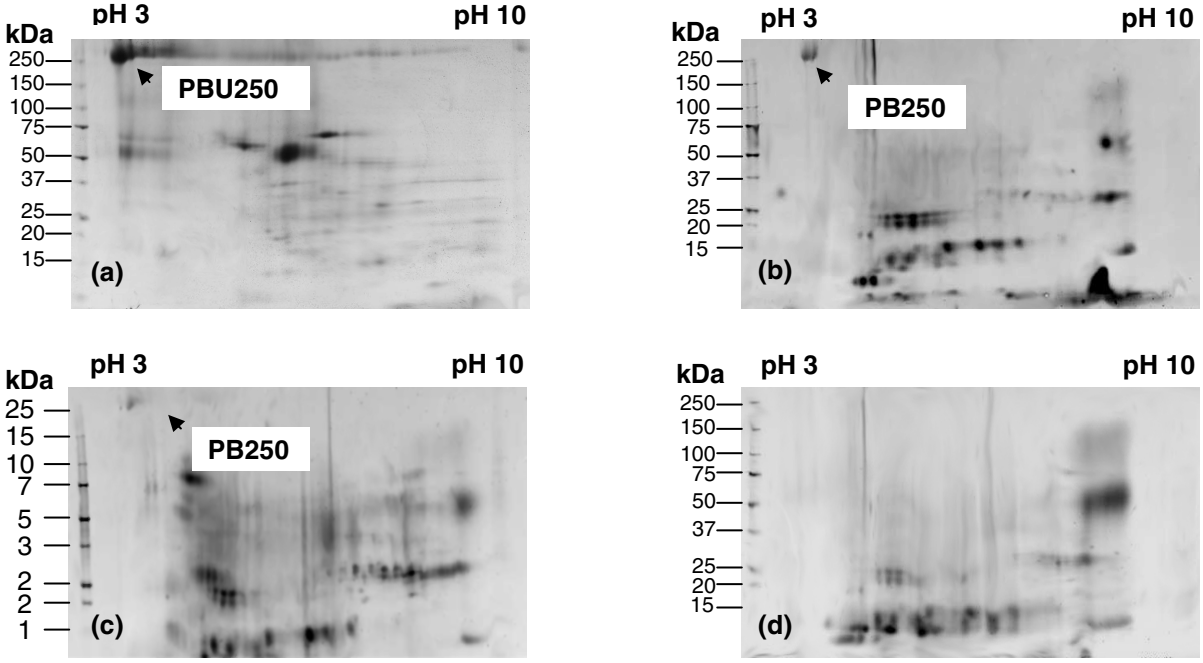


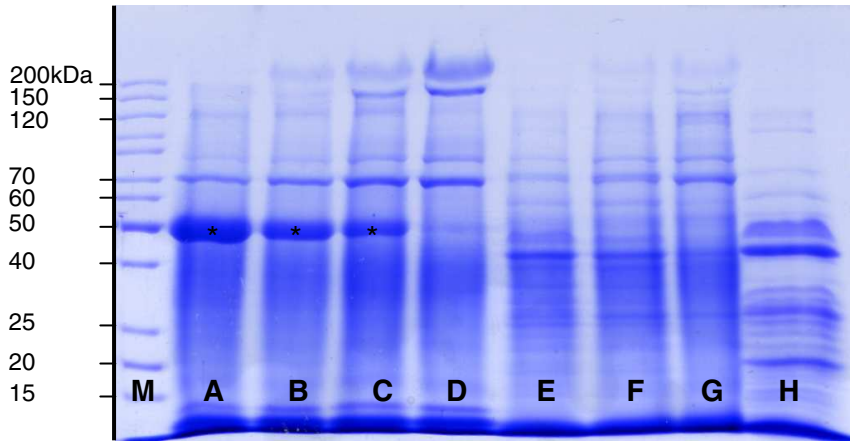
Figure 2a – d: 2D-gel electrophoresis of (a) bulbourethral gland secretion displaying protein PBU250 as highly acidic protein spot, (b) viscous rhinoceros seminal plasma (III) displaying P250 as highly acidic protein spot, (c) viscous rhinoceros seminal plasma (II) displaying P250 as weak acidic protein spot, (d) not viscous rhinoceros seminal plasma (I) not displaying P250.

To test analogousness of P250 (black and white rhinoceros) and PBU250, and for further characterization, the protein spots were analyzed by MALDI-TOF MS followed by data searching using MASCOT. Although good MS spectra were generated, no matching protein was found in the database. However, both spots are likely to represent the same proteins as 11 out of 12 peptides (+/- 0.1 m/ z) were seen in the spectra of both proteins.

2.4 Experiment 4: Liquefaction of viscous fraction using α-amylase and collagenase and evaluation of the enzyme-influence on sperm characteristics

2.4.1 SDS-PAGE

The concentration of P250 decreased in the SDS-PAGE when different concentrations of α-amylase or collagenase dissolved in 10 µl PBS were added to seminal plasma of viscosity grades II and III. The extent of protein decrease depended on the amount of enzyme added. Addition of 3 IU α-amylase and 18.5 IU collagenase caused a complete removal of the P250 band. Figure 3 shows a SDS-PAGE gel displaying seminal plasma samples with and without addition of α-amylase and collagenase.



*Protein band (50 kDa) displaying α-amylase

Figure 3: SDS-PAGE pattern of seminal plasma from one white rhino bull with and without addition of three concentrations of α-amylase or collagenase diluted in PBS. Lane **M**: protein marker. Lane **A – C**: seminal plasma with addition of 3 IU, 1.5 IU and 0.5 IU α-amylase, respectively. Lane **D**: untreated seminal plasma. Lane **E – G**: seminal plasma with addition of 18.5 IU, 9.25 IU and 3.1 IU collagenase, respectively. Lane **H**: collagenase (40 IU).

2.4.2 Effect of α-amylase and collagenase on viscosity of seminal plasma

Viscosity was quantified by estimating a rotational correlation time (τ_c) from ESR spectra of untreated, α-amylase and collagenase treated seminal plasma (0.5 and 3 IU per 100 µl seminal plasma; Fig. 4). Figure 4 reveals that viscosity was significantly reduced upon addition of both enzymes compared to the controls (exact Friedman Test, Wilcoxon and Wilcox Test; df = 2, $p \leq 0.01$, n = 7). The τ_c-times of control seminal plasma varied between 12.9 x 10^{-12} and 26.5 x 10^{-12} sec with a mean of 18.7 x 10^{-12}sec. After addition of α-amylase and collagenase τ_c values were in the range of 5.81 x 10^{-12} to 14.70 x 10^{-12}sec (mean 13.2 x 10^{-12}) and 10.30 x 10^{-12} to 16.42 x 10^{-12}sec (mean 14.5 x 10^{-12}), respectively. Compared to untreated samples addition of α-amylase or collagenase decreased viscosity of seminal plasma by 28 and 21 %, respectively.

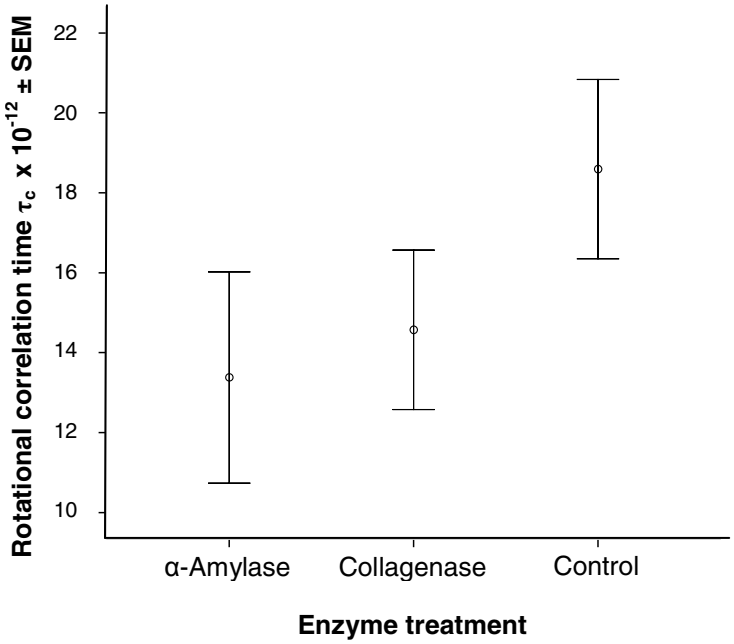


Figure 4: Mean viscosity of α-amylase (0.5 IU) or collagenase (3 IU) treated and of control seminal plasma samples from white rhino bulls, evaluated through determination of τ_c-times. Data are means ± SEM, (n = 7).

2.4.3 Impact of α-amylase and collagenase on quality parameters of spermatozoa

The influence of enzymes on PM, TM and integrity (viability, acrosome integrity) was tested for α-amylase in 12 ejaculates (WR = 8, BR = 4) and for collagenase in 8 ejaculates (WR = 7, BR = 1). No significant effect on sperm motility or integrity was found through addition of enzymes to semen samples compared to control samples after incubation for 1 h at RT (exact Friedman Test: df = 4, $p \geq 0.187$).

Discussion:

Sex-sorted spermatozoa suitable for AI or IVF might become an important management tool for captive wildlife populations as unbalanced sex ratios create difficulties with management of small populations, particularly in large, slow reproducing mammals (Belterman, 2007; Hermes et al., 2007). The methods developed in this study mark a substantial step towards the successful application of sex-preselection technology in the rhinoceros.

The first challenge was to retain sperm function during transport (4 - 10 h) from collection site to sorting facility. Blottner's cryomedia (BC; Blottner, 1998; Hermes et al., 2005) is routinely used for chilled storage of rhinoceros spermatozoa as motility is uniformly maintained over time periods similar to that used in this experiment (Hermes et al., 2005). Unfortunately, the high percentage of egg yolk in this medium (16 %) interferes with DNA staining and resolution of spermatozoa during the sorting procedure, making this extender not suitable for sex-sorting. Unlike chilling of rhinoceros semen diluted in BC, which maintains almost

constant motility of spermatozoa, the decline in PM and TM observed in experiment 1 for all non-egg yolk extenders indicates the importance of egg yolk in the protection of rhinoceros spermatozoa and maintenance of their motility during low-temperature liquid storage. In any case, of the egg yolk-free media tested, MES was best to maintain the motility of spermatozoa after transport and was classified suitable for sperm sorting in the rhinoceros, as reasonable resolution of X and Y- chromosome bearing sperm populations was achieved using this semen extender.

Staining during transport to the laboratory at 15℃ resulted in better quality of spermatozoa than staining at 37℃ after transport at 4℃. Despi te the sensitivity to chilling of rhinoceros spermatozoa this finding might be due to an aversion to temperatures changes or to exposure to high temperatures further complication sperm sex-sorting in this species.

As wildlife have not been selected for high fertility and sperm production, sorting efficiencies in most exotic species are likely to remain below those observed in livestock species. Considering the sperm sorting index of the rhinoceros, general sortability sits in the range between the human and the stallion, consistent with the medium degree of resolution of X and Y- sperm populations (Behr et al., 2009). However, sorting rates in the rhinoceros were low in the present study, with a maximum of 700 cells/sec compared to a mean of 3500 - 4500 cells/sec in the stallion (Buss, 2005) and only 30 % (incubation at 37℃) and 60 % (incubation at 15℃) of stained sperm samples were separable into X and Y- sperm populations. These findings may be due to the great variance in semen quality between ejaculates resulting in a relatively low mean percentage of viable and intact spermatozoa after DNA staining, and the well established differences in sortability of spermatozoa between individual males (Clulow et al., 2008). Additionally, the low concentration of spermatozoa in some ejaculates complicated the staining procedure and reduced the mean event and sort rates.

High viscosity of semen samples interfered with the requirement of stable single cell suspensions and even prevented the handling of 25 % of stained sperm samples in the flow cytometer. A glycoprotein of 250 kDa molecular weight and an Ip of ≤ 3 was discovered, which is suggested to be the major component of the highly viscous fraction in seminal plasma of the African rhinoceros. The results of peptide mass fingerprint analysis provided strong evidence that P250 originates from the bulbourethral gland secretions. The main functions of the secretion from these accessory glands are lubrication and neutralization of the urethra prior to ejaculation (Mann & Lutwak-Mann, 1981; Moré, 1991). However, in the boar, glycoproteins derived from the bulbourethral glands form a gelatinous plug in the uterine cervix of the sow, which is believed to prevent backflow of the semen (Dyce et al., 1999). In camelids, the viscous seminal plasma entraps the spermatozoa in the uterus after ejaculation, possibly steadily releasing spermatozoa to allow fertilisation for an extended period after the induction of ovulation (Morton et al., 2007). In the rhinoceros, it is difficult to

specify which task the bulbourethral gland secretion performs. To date, the only way to collect full ejaculates has been by electroejaculation. Therefore, it is possible that the highly viscous component is not a natural part of the ejaculate, but rather an artefact of the artificial method of semen collection.

Bulbourethral glands are generally known to secrete mucin-like glycoproteins (Boursnell et al., 1970). The dissolving effect of α-amylase suggests that P250 may be a glycoprotein, as the glycoside hydrolase α-amylase breaks down long-chain carbohydrates. However, P250 could not be identified and no matching protein was found in the database, although good MS spectra were generated. Therefore, further investigation of protein structure is necessary.

The viscosity of analysed semen samples was higher for the WR than the BR. The reasons for this are not yet fully understood as anatomical features of reproductive tracts from these two closely related species are similar. Nevertheless, the bulbourethral glands of the WR are proportionally bigger in size and more elongated compared to the BR (Schaffer et al., 2001). To find a way of decreasing the viscosity of semen without damaging the spermatozoa the impact of the enzymes α-amylase and collagenase was investigated. By employing an ESR approach, a significant decrease of a rotational correlation time of the spin label Tempo was observed in seminal plasma treated with α-amylase or collagenase in comparison to control plasma. Although a detailed calculation of the solution viscosity from τ_c values depends on several molecular parameters (size of the label compared to that of surrounding solvent molecules, interaction between label and solvent molecules; Hermann & Mueller, 1985), these data clearly show that upon addition of the enzymes the viscosity of seminal plasma was decreased. No negative impact of enzymes on sperm quality parameters (sperm motility, viability, acrosome integrity) was observed. Nevertheless, prior to a routine addition of the enzymes to viscous rhinoceros ejaculates their impact on function and fertility of spermatozoa has to be further investigated. Both enzymes completely dissolved P250 when added at the highest concentration tested (Figure 3). Thereby, despite P250 also further high molecular proteins were degraded (≥ 70 kDa; Figure 3) rendering an impact of these proteins on seminal plasma viscosity possible next to the dominant P250. To verify a potential involvement of these proteins for instance in the form of an interaction of several seminal plasma components as described in the domestic boar (Boursnell et al., 1970) and the common rat (Beil & Hart, 1973), further investigations on the fine structure of rhinoceros seminal plasma are required.

In the present study rhinoceros spermatozoa were sex-sorted for the first time into high purity X and Y- populations. Nevertheless, sample quality and sorting efficiency prevented the utilization of the sex-sorted samples for deep intrauterine AI. However, adequate numbers of spermatozoa could be easily sex-sorted for ICSI or IVF. The latter has been successfully achieved with non-sorted spermatozoa after *in vivo* collection and *in vitro* maturation of ooctyes from the BR (Hermes et al., unpublished data). Furthermore, a glycoprotein of 250

kDa molecular weight, most likely originating from the bulbourethral glands, was identified and correlated with the viscosity of ejaculates. Its liquefaction was achieved using the enzymes α-amylase and collagenase without any effect on the quality parameters of spermatozoa. Such enzyme treatment may allow better utilization of rhinoceros ejaculates for sex-sorting by flow cytometry but further studies are required to confirm this.

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Article III: Sperm sex-sorting in the Asian elephant (*Elephas maximus*)

Abstract

In captive Asian elephants, there is a strong need for the production of female offspring to enhance reproduction, prevent premature aging processes in the reproductive tract of female animals and improve the management and husbandry conditions posed by maintaining several bulls in one location. Artificial insemination of flow cytometrically sex-sorted spermatozoa offers the possibility to predetermine the sex of offspring with high accuracy. The aims of this study were to determine basic parameters for flow cytometrical sex-sorting of Asian elephant spermatozoa and the development of a suitable semen extender. In total, 18 semen samples were collected by manual rectal stimulation from one bull. Sperm quality parameters and sex-sortability of spermatozoa were evaluated after dilution in three semen extenders (MES-HEPES-skimmed milk, MES-HEPES, TRIS–citric acid) and DNA staining. MES-HEPES-skimmed milk was the only semen extender suitable for sexing Asian elephant spermatozoa. Twelve out of 18 ejaculates were successfully sorted with a purity of 94.5 ± 0.7 % at an average sorting rate of 1,945.5 ± 187.5 spermatozoa/s. Sperm integrity, progressive motility and total motility were 42.6 ± 3.9 %, 48.1 ± 3.3 %, 59.4 ± 3.8 % after DNA labelling, and 64.8 ± 3.2 %, 58.0 ± 5.0 %, 70.8 ± 4.4 % after sorting, respectively. After liquid storage of sorted spermatozoa for 12 h at 4℃, sperm integr ity, progressive motility and total motility were 46.4 ± 5.2 %, 32.2 ± 4.2 % and 58.2 ± 3.9 %, respectively. These results provide a promising base to inseminate Asian elephants with sexed semen.

Introduction

The captive population of Asian elephants suffers from an insufficient reproduction rate to maintain self-sustainable populations (Faust et al., 2006). Many zoological institutions refrain from keeping elephant bulls due to the husbandry complications and high costs involved. Matriarchal social group requirements on the one hand and the need to protect animals and staff on the other hand, make designated bull barns a must in modern zoos. Furthermore, the husbandry of several bulls in one institution has a negative influence on the reproductive status through social depression (Hildebrandt et al., 2000). After developing a method to collect semen manually (Schmitt & Hildebrandt, 1998) artificial insemination (AI) was added as a new and promising supplement to the artificial reproductive technology (ART) toolbox for elephants (Brown et al., 2004). AI resulted in the birth of 23 elephant calves to date, giving great hopes to enhance the reproductive rate in captivity. Particularly in slow reproducing long-lived species, females are the limiting factor concerning the effort to enlarge the captive population, and are greatly affected by an asymmetric aging process of the reproductive tract (Hermes et al., 2004; Faust et al., 2006). Therefore, it is rather more important to breed female offspring. In recent years a reliable flow cytometric method has been introduced to influence the sex-ratio of offspring, utilising the difference in relative DNA

content between X and Y- chromosome bearing spermatozoa (Johnson et al., 1987). Since its first report, more than two million animals of the desired sex were born through the use of this technique (Rath & Johnson, 2008). Sperm physiology is species-specific and often the available knowledge is very limited or absent in wildlife, resulting in the need to develop new protocols for sperm handling prior, during and following the sorting process (Maxwell et al., 2004). However, two crucial characteristics for the attempt to sex-sort spermatozoa in any species, the difference in relative DNA content between X and Y- chromosome bearing spermatozoa and the head profile areas of spermatozoa indicated recently that general sortability parameters of Asian elephant sperm are comparable to the domestic stallion (Garner, 2006; Behr et al., 2009). The aim of the present study was to determine semen extenders and basic parameters for flow cytometrical sperm sorting of Asian elephant spermatozoa to dispose viable and motile sex-sorted spermatozoa suitable for the use in AI.

Materials and Methods

Eighteen ejaculates were collected from one Asian elephant (*Elephas maximus*). Semen samples were collected by rectal manual stimulation of the accessory sex glands (Schmitt & Hildebrandt, 1998). Only samples with total motility ≥ 65 % were used for the experiment and processed as previously described (Hermes et al., 2005). Smears from each sample were prepared for assessment of sperm morphology and viability. The combined samples were subdivided into treatment groups A and B. Treatment group A was under-laid with 1.5 ml of 60 % iodixanol (OptiPrep™; Axis-Shield Poc AS, Oslo, Norway) to act as a cushion to minimize centrifugation damage (Saragusty et al., 2006) and centrifuged immediately at 1000 x g at RT for 20 min (centrifugation treatment). Resuspension of the pellet was performed in one of the extenders as described below. The remaining half constituted the non-centrifugation group B.

Aliquots of both groups were diluted stepwise to a final concentration of 100 x 10^6 spermatozoa/ml with three different, pre-warmed (37°C), semen extenders. These included: (1) MES-HEPES and skimmed milk based medium, composed of 35 % buffer solution containing MES (10.1 mM), HEPES (10 mM), NaCl (87.2 mM), NaOH (11.6 mM), $MgCl_2 \cdot 6H_20$ (0.8 mM), KCl (25.1 mM) and 65 % sugar solution containing glucose (271.2 mM) and skimmed milk (2.4 g). This extender composition was named Blottner's Sorting Medium (BSM). (2) MES-HEPES semen extender (MES; Blottner et al., 1994) and (3) TRIS - citric acid based extender medium (TCA; Graham et al., 2004). Na-Pyruvat (1.0 mM) as antioxidant and EGTA (2.0 mM) to prevent sperm agglutination were added to all extenders. DNA labelling with Hoechst 33342 DNA stain was performed with three different concentrations (15, 20 and 25 µl of 8.91 mM stock solution) followed by incubation for 1.5 – 2 h at 37°C and addition of 2 µl food dye (FD&C #40; Warner Jenkinson, St. Louis, MO, USA; Welch & Johnson, 1999) per 1ml aliquots. Controls were treated identically, but without staining.

Total motility (TM) and progressive motility (PM) of semen samples were evaluated after collection, DNA-staining and incubation, sorting and chilling for 12 h at 4°C as well as of non-stained and non-sorted controls. Sperm characteristics including viability, acrosome integrity and tail membrane integrity were assessed using a modified Kovács-Foote staining technique (Kovács & Foote, 1992; Nagy et al., 1999; Kútvölgyi et al., 2006).

Sorting was performed on a high-speed flow cytometer (MoFlo SX, Dakocytomation, Fort Collins, CO) equipped with an argon laser (200 mW) and operating at 40 psi with a HEPES-buffer based sheath fluid (Buss, 2005). Samples with over 25 % of correctly oriented spermatozoa and good resolution between the X and Y- chromosome bearing spermatozoa were sex-sorted and collected in 500 µl of TEST-yolk collection extender (Johnson et al., 1991), enriched with 1 % of homologous seminal plasma. After centrifugation for 20 min at 840 x g the pellet was resuspended in 200 µl of Blottner's Cryomedia (BC); Blottner, 1998; Hermes et al., 2005) + 1 % of seminal plasma. Samples were chilled to 4°C over 4 h at a rate of 0.1°C/min and stored for 12 h before incubation at 37°C for 30 min. An aliquot of the X and Y- sorted sample was restained to determine the proportions of X and Y- bearing spermatozoa (Welch & Johnson, 1999).

Statistical analyses were performed using SPSS v. 10.0.7 (SPSS Inc., Chicago, IL). In all cases, the Kolmogorov-Smirnov-test was applied to test for normality[4]. Differences between treatment groups and tested extenders were calculated with sequential Bonferoni-adjusted two-tailed paired-sample t-test. For all analyses, the level of significance was set to $p \leq 0.05$.

Unless otherwise mentioned, all materials were of reagent grade or higher and were purchased from Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany.

Results

The average concentration (mean ± SD) of the collected sperm-rich fractions was 825 x 10^6 ± 94.5 spermatozoa/ml. Total collected volume was 39.0 ± 6.6 ml. PM and TM were 62.4 ± 4.0 % and 75.8 ± 3.0 %, respectively.

The influence of centrifugation on sperm characteristics was tested on six ejaculates following dilution in semen extenders and DNA-staining. No significant differences ($p \geq 0.074$) or positive impact were found between the centrifugation and control groups (data not shown). Data did not differ significantly from a normal distribution ($p \geq 0.143$).

Parameters of initial semen samples after dilution in three extenders (BSM, MES, TCA), after staining and incubation and of unstained controls are comparatively displayed in Table 1. Samples diluted in BSM reached best results regarding all evaluated sperm quality

[4] Due to low power of testing for normality with small sample sizes, the Friedman-Test or a generalised mixed linear model approach instead of paired-sample t-test could account for a non-normal (possibly binomial) data structure

parameters after DNA labelling, even though differences were not always significant. Data did not differ significantly from a normal distribution ($p \geq 0.094$).

Article III: Sperm sex-sorting in the Asian elephant (Elephas maximus)

Table 1: Characteristics of fresh collected and DNA-stained/incubated Asian elephant spermatozoa diluted in three extenders (BSM, MES, TCA); unstained/incubated controls (CON).

Sperm parameter (%)	BSM initial (n = 18)	Stained (n = 18)	CON (n = 17)	MES initial (n = 11)	Stained (n = 8)	CON (n = 7)	TCA initial (n = 8)	Stained (n = 6)	CON (n = 8)
Progressive motility	66.6 ± 2.9^{a,b,c,d}	48.1 ± 3.3^{a,g}	59.7 ±1.8^{h}	67.5 ± 3.3^{e,f}	14.8 ± 6.4^{b,e,g,h}	41.3 ± 7.1	58.1 ± 7.7	23.3 ± 7.4^{d,f}	37.5 ± 7.7^{c}
Total motility	79.5 ± 2.1^{a,b,c}	59.4 ± 3.8^{a,d}	71.8 ± 1.8^{a,e}	76.9 ± 3.3^{f,h,j}	20.4 ± 8.1^{b,d,e,h,g}	60.3 ± 2.9^{c,h,i}	71.1 ± 6.3^{g,i}	46.0 ± 8.1^{j}	58.0 ± 7.0
Intact	70.6 ± 3.0^{a,b,c}	42.6 ± 3.9^{a}	58.6 ± 4.1	62.1 ± 3.3^{d,e}	29.0 ± 4.7^{b,d}	57.1 ± 5.7	61.4 ± 4.5^{f}	21.7 ± 5.3^{c,e,f}	48.6 ±6.8
Viable	76.4 ± 3.0^{a,b}	55.9 ± 3.8^{a}	70.1 ± 4.1	76.5 ± 2.6	48.1 ± 6.1	67.9 ± 3.3^{b}	71.1 ± 3.6	42.5 ± 7.1	60.3 ± 5.3
Intact acrosome	73.8 ± 3.2^{a,b,c}	50.2 ± 4.3^{a}	68.0 ± 3.7	69.1 ± 3.4^{d,e}	40.4 ± 4.6^{b,d}	66.3 ± 5.5	72.8 ± 3.4	41.5 ± 5.0^{c,e}	59.8 ± 7.2

Sperm quality parameters are expressed as means ± SEM. Within a line, groups with common superscript letters are significantly different ($p \leq 0.05$).

Only semen samples diluted in the BSM semen extender were successfully sex-sorted (Table 2). Dilution of spermatozoa in MES or TCA semen extender did not resolve in reasonable and repeatable separation in X and Y- chromosome bearing spermatozoa populations of samples. In 12 out of the 18 trials, a very good resolution between the X and Y- chromosome bearing sperm populations was achieved. The percentage of correctly oriented and recognized spermatozoa (R1) varied between 25.1 and 57.0 %. The sorting rate averaged at 1,945 ± 187 (mean ± SEM) spermatozoa per second, giving a sorting purity of 94.5 ± 0.7 %. Chilling and storage of sorted samples for 12 h at 4°C led to a significant decrease in PM, sperm integrity and viability (Table 2). Data structure did not differ significantly from a normal distribution ($p \geq 0.212$)

Table 2: Characteristics of initial (n = 18), sorted (n = 12), sorted-chilled (n = 12; in BC semen extender) Asian elephant semen samples; non-stained, non-sorted (CON-1; n = 11) and non-stained, non-sorted chilled (CON-2; n = 8) control samples, (in BSM semen extender).

Sperm parameter (%)	Initial	Sorted	CON-1	Sorted-chilled[a]	CON-2[a]
Progressive motility	66.6 ± 2.9[a,b]	58.0 ± 5.0[c]	50.6 ± 4.3	32.3 ± 4.2[a,c]	26. 9 ± 4.3[b]
Total motility	79.5 ± 2.1[a,b]	70.8 ± 4.4	65.6 ± 3.4	58.2 ± 3.9[a]	47.5 ± 8.1[b]
Intact	70.6 ± 3.0	64.8 ± 3.2[a]	62.1 ± 5.2	46.4 ± 5.2[a]	40.5 ± 8.0
Viable	76.4 ± 3.0[a]	73.8 ± 3.7[b]	71.9 ± 4.5	57.6 ± 4.3[b]	45.5 ± 7.9[a]
Intact acrosome	73.8 ± 3.2	69.7 ± 4.3	71.2 ± 4.8	54.3 ± 5.5	52.8 ± 7.5

Sperm quality parameters are expressed as means ± SEM. [a] Chilled for 12 h at 4°C in refrigerator and then incubated for 30 min at 37°C before evaluation. Within a line, groups with common superscript letters differ significantly ($p \leq 0.05$).

Discussion

Flow cytometrical sperm sex-sorting can help to establish self-sustained populations by compensating premature female aging processes, balancing skewed sex-ratios and allowing intact social groups through predetermination of offspring sex. In this study Asian elephant spermatozoa were sex-sorted for the first time into X and Y- chromosome bearing sperm populations of high motility and integrity. The first challenge was the gentle preparation of semen samples for sex-sorting. Unlike the enhancement of post-thawing motility by removal of seminal plasma after semen collection (Saragusty, unpublished results), centrifugation of

fresh elephant semen showed no positive impact on sperm quality after DNA-staining. This diagnosis prevents unnecessary stress for elephant spermatozoa. Sperm quality parameters were superior after sorting than before sorting (after staining), reflecting the selective enrichment of viable (membrane intact) spermatozoa during the sorting procedure (Welch & Johnson, 1999). The realisation of the sorting protocol developed in this study requires proximity between zoological institutions housing the stock bull, the female designated for AI, and a flow cytometer modified for sperm sorting. However, an adequate preservation ability of samples after sorting is indispensable for the application in AI. In this study sex-sorted elephant spermatozoa were stored in a semen extender containing 16 % egg yolk (Blottner, 1998; Hermes et al., 2005) for at least 12 h at 4°C with an acceptable loss in quality parameters. In the future, the production of an adequate amount of sex-sorted spermatozoa for AI in a reasonable period of time must be put into practice. The general sperm sortability of Asian elephant spermatozoa indicates a sorting rate comparable or even superior to the domestic stallion (*Equus caballus*; Behr et al., in 2009). An explanation for the so far relatively low sort rate in the elephant might lie in the low mean percentage of correctly oriented spermatozoa (37 %), resulting from a decline in sperm viability during DNA-staining. As quality of non-stained control elephant sperm samples was not comparably diminished after parallel incubation at 37°C, the fluorescence dye Hoechst 33342 seems to severely damage the Asian elephant spermatozoa. Further adoption of preparatory stages and staining procedure to the requirements of Asian elephant spermatozoa might increase the sorting rate significantly.

Conclusions

In this study we showed the practicability of sex-sorting Asian elephant spermatozoa. An acceptable sorting rate, high population purity and reasonable post-sorting and post-liquid storage quality indicate the feasibility of utilising sex-sorted semen in AI. Further studies are called for to improve sorting rate and to test the procedure on additional bulls as sperm sex-sortability underlies strong inter-animal variability.

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Article IV: Successful cryopreservation of Asian elephant (*Elephas maximus*) spermatozoa

Contents

Reproduction in captive elephants is low and infant mortality is high, collectively leading to possible population extinction. Artificial insemination was developed a decade ago; however, it relies on freshly-chilled semen from just a handful of bulls with variable sperm quality. Artificial insemination with frozen-thawed sperm has never been described, probably, in part, owing to low semen quality after cryopreservation. The present study was designed with the aim to find a reliable semen freezing protocol. Screening tests included freezing semen with varying concentrations of ethylene glycol, propylene glycol, trehalose, dimethyl sulfoxide and glycerol as cryoprotectants and assessing cushioned centrifugation, rapid chilling to suprazero temperatures, freezing extender osmolarity, egg yolk concentration, post-thaw dilution with cryoprotectant-free BC solution and the addition of 10 % (v/v) of autologous seminal plasma. The resulting optimal freezing protocol uses cushioned centrifugation, two-step dilution with isothermal 285 mOsm/kg Berliner Cryomedium (BC) with final glycerol concentration of 7 % and 16 % egg yolk, and freezing in large volume by the directional freezing technique. After thawing, samples were diluted 1:1 with BC solution. Using this protocol, post-thawing evaluation results were: motility upon thawing: 57.2 ± 5.4 %, motility following 30 min incubation at 37°C: 58.5 ± 6.0 % and following 3h incubation: 21.7 ± 7.6 %, intact acrosome: 57.1 ± 5.2 %, normal morphology: 52.0 ± 5.8 % and viability: 67.3 ± 6.1 %. With this protocol, good quality semen can be accumulated for future use in artificial inseminations when and where needed.

Introduction

The Asian elephant (*Elephas maximus*) worldwide population is estimated to be at around 40,000 – 48,000 elephants, of which approximately 15,000 are in captivity (Taylor & Poole, 1998; Wiese, 2000; Sukumar, 2006). Unfortunately, its population in the wild, and to a greater extent in captivity, is not reproducing at a sufficient rate to at least maintain the current population size (Taylor & Poole, 1998; Faust et al., 2006). With the continuous aging of the captive population, low fertility, high infant mortality rate and an elevated occurrence of reproductive tract pathologies, this population might drive itself to extinction within the next few decades (Wiese, 2000). Preserving natural diversity can be done *in situ* by protecting the habitat of elephants or *ex situ* by creating a genome resource bank (GRB; Wildt, 1992; Johnston & Lacy, 1995). However, it is also imperative to find ways to improve fertility and fecundity of captive elephants.

Despite the close relationship between the Asian and the African elephants, their sperm seem to differ greatly in many aspects, including membrane fatty acid composition (Swain & Miller, 2000), size of the head and length of the tail (Jainudeen et al., 1971; Gilmore et al.,

1998) and sensitivity to chilling (Leibo & Songsasen, 2002). Finding a reliable protocol to cryopreserve Asian elephant spermatozoa will open the way to a variety of possibilities. It will enable us to establish a semen bank that can act as a GRB. It will provide breeding programs with a reliable source of semen where and when it is needed, overcoming the need to collect semen shortly before using it and the risk of quality inconsistency that we face today (Hildebrandt et al., 2000). Cryopreservation of the spermatozoa from the African savannah elephant has been reported with post-thawing motility ranging between 30 and 50 % (Jones, 1973; Howard et al., 1986). We and others were able to achieve similar results with the Asian elephant spermatozoa (Hermes et al., 2003; Thongtip et al., 2004; Sa-Ardrit et al., 2006). However, attempts to freeze elephant spermatozoa and thaw it with sufficiently high motility as is achieved in many other species, thus far, failed. Throughout the years, the conventional wisdom was that, unlike most other species, the elephant spermatozoa freezes better when using dimethyl sulfoxide (Me_2SO) as cryoprotectant (Watson, 1995). However, using Me_2SO, only moderate and largely insufficient success has thus far, been achieved. To tackle this problem we adopted a systematic approach to develop an optimal protocol for the cryopreservation of Asian elephant spermatozoa. A novel freezing technology was used in these experiments. This technology is based on multi-thermal gradient (MTG) directional solidification (Arav, 1999). In the conventional freezing methods, ice grows at an uncontrolled velocity and morphology and may, therefore, disrupt and kill cells in the sample. By moving the special large volume (2.5 or 8 ml) cryogenic HollowTube™ (IMT Ltd., Ness Ziona, Israel) with the semen at a constant velocity through a linear temperature gradient using the MTG apparatus, we are able to control the ice crystal propagation and optimize its morphology. Continual seeding and homogenous cooling rate is achieved during the whole freezing process, thereby minimizing damages to the cells. Several attempts using this technique in a variety of species has proved it to be successful (Arav et al., 2002b; Gacitua & Arav, 2005; Saragusty et al., 2006; Si et al., 2006) and superior to conventional freezing techniques (O'Brien & Robeck, 2006; Saragusty et al., 2007; Reid et al., 2009).

Materials and Methods

1. Materials

Unless otherwise mentioned, all materials were of reagent grade or higher and were purchased from either:

Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Carl Roth GmbH + Co., Karlsruhe, Germany
Merck KGaA, Darmstadt, Germany

2. Animals

Thirty ejaculates were collected from six Asian elephants (*Elephas maximus*) and one from an African savannah elephant (*Loxodonta africana*). Of these, semen freezing experiments were conducted on 10 ejaculates from one Asian bull. This elephant is a proven breeder and an excellent semen donor. It is housed in the Hannover Zoo, Germany, under protected management system. Attempts to collect from the other Asian elephant bulls provided samples that were not suitable for freezing or freezing could not be performed at the time of collection.

3. Semen collection and evaluation

Semen samples were collected by rectal manual stimulation of the accessory sex glands (Schmitt & Hildebrandt, 1998, 2000). Only samples with an initial total motility ≥ 65 % and little or no agglutination were used for the experiments, a requirement that has considerably restricted the number of usable ejaculates. Same day collections with comparable motility were combined. For each sample, total volume was noted and sperm concentration was estimated using improved Neubauer haemocytometer. Sperm motility was subjectively evaluated using a dark field microscope equipped with a heating stage (37°C; Olympus CH 40, Olympus, Hamburg, Germany). Smears from each sample were prepared for assessment of acrosome integrity, sperm morphology and viability using a modified Kovács-Foote staining technique (Kovács & Foote, 1992). A total of 100 stained spermatozoa were counted per slide. Spermatozoa were categorized as “viable” (head membrane intact) or “dead” (head membrane damaged). Acrosomes were classified as intact versus modified or reacted (including completely detached acrosomes; Kútvölgyi et al., 2006). Unless otherwise stated, suitable samples were underlaid with 2 ml of isothermal 60 % iodixanol (OptiPrep™) and then centrifuged at room temperature (~ 20°C) for 20 min at 1000 x g. Seminal plasma and OptiPrep were then aspirated and the pellet was resuspended in the freezing extender according to the specific experiment for which it was used.

4. Freezing extender

Unless otherwise specified, the BC solution (Blottner, 1998; Hermes et al., 2005) was used for making the various freezing extenders. To make this solution 2.41 g TES, 0.58 g Tris, 0.1 g fructose and 5.5 g lactose are mixed in 100 ml double distilled water to achieve osmotic strength of 330 mOsm/kg. To make the 285 mOsm solution 2.0 g TES, 0.58 g Tris, 0.1 g fructose and 5.5 g lactose are mixed in 100 ml double distilled water. Except when the experimental design required otherwise, 16 % egg yolk (v/v), 1 ml of Orvus ES Paste (Minitüb, Tiefenbach, Germany), 1 ml of Gentamycin and 25 IU α-tocopherol/ml were then added and the total mixture is centrifuged for 20 min at 10,000 x g. The supernatant is used and is referred to herein under as BC basic solution.

5. Semen freezing and thawing

After dilution with the freezing extenders to a final concentration of 150×10^6 cells/ml, the semen was cooled to 4 – 5℃ at ~ 0.3℃/min by subme rging the tubes in isothermal water bath and placing it into a refrigerator, packaged into 2.5 ml pre-chilled HollowTubes™ (IMT Ltd., Ness Ziona, Israel) and frozen in a directional freezing machine (MTG-516, IMT Ltd.) as previously described (Arav et al., 2002a,b; Gacitua & Arav, 2005; Saragusty et al., 2006, 2007; Reid et al., 2009). All frozen samples were stored under liquid nitrogen pending evaluation. HollowTube samples were thawed by first holding them in the air, at room temperature (22 – 23℃), for 90 s and then plunging them into a water bath at 37℃ for 60 s. Following thawing, samples were held in the water bath at 37℃ pending evaluation.

6. Post-thawing evaluations

Subjective motility evaluations were conducted on all samples by the same person. Evaluations were conducted by putting a 10 µl drop of thawed sample on a pre-warmed microscopic slide (37°C), covering with pre-warmed cover slip and immediately evaluating under dark field microscope equipped with a warm stage (37°C) at 1000 x magnification (Axiolab; Karl Zeiss Microscopy, Jena, Germany). All samples were evaluated immediately after thawing and following 30 min and 3 h incubation at 37°C. An aliquot of 0.5 ml from each sample was diluted immediately after thawing in 37°C BC basic solution at a ratio of 1:1, incubated at 37°C and evaluated 30 min and 3 h post-thawing. In some of the experiments (as will be indicated), 50 µl of autologous seminal plasma at 37°C was added to 450 µl of thawed sample, incubated at 37°C and evaluated 30 min and 3 h post-thawing.

Two smears from each sample were prepared for assessment of sperm morphology and viability using the modified Kovács-Foote staining technique.

7. Experiment 1: Semen osmolarity

Osmolarity of seminal plasma of 30 ejaculates, collected from six Asian elephants and one from an African savannah elephant was evaluated. The measurements were performed on samples obtained from the supernatant after centrifugation of native sperm at 1000 x g for 20 min immediately after collection. Osmolarity of the samples was measured in triplicate with automatic osmometer (Roebling, Berlin, Germany). Samples were evaluated on the day of collection or kept at - 20^{o}C pending evaluation.

8. Experiment 2: Cushioned centrifugation and freezing extender osmolarity and temperature

This experiment was designed to evaluate three variables. Fast vs slow chilling rate was evaluated by re-suspending the semen pellet in either isothermal 7 % glycerol in 285 mOsm/kg BC basic solution or in the same extender at 4^{o}C and then keeping the samples at 4^{o}C till freezing. The effect of cushioned centrifugation was evaluated by conducting centrifugation with and without underlaying the semen with 2 ml of isothermal OptiPrep. The extender's osmolarity was evaluated by comparing the original BC solution with osmolarity of 330 mOsm/kg to reduced osmolarity BC solution with osmolarity of 285 mOsm/kg. Extender solutions A and B were added as described in the glycerol concentration experiment (experiment 6). The effect of adding 10 % (v/v) autologous seminal plasma after thawing was also evaluated.

9. Experiment 3: Ethylene glycol and propylene glycol concentrations

A Tris-citric acid-egg yolk freezing extender (2.18g Tris, 1.23g citric acid monohydrate and 20 % egg yolk with bi-distilled water to complete to 100 ml) was used, to which either ethylene glycol or propylene glycol were added at final concentrations of 4 % and 8 % (v/v), each. After cushioned centrifugation, the semen was resuspended in each of the four (isothermal) freezing extenders, chilled and frozen. The effect of adding 10 % (v/v) autologous seminal plasma after thawing was also evaluated.

10. Experiment 4: Trehalose concentration

Freezing extender was prepared by adding trehalose to water to achieve final concentration of either 0.375 M or 0.1 M. Osmolarity was adjusted with Tris to 375 mOsm/kg and pH 7.0. To these 20 % egg yolk was added and then split into two parts. To one part of each trehalose concentration, 4 % glycerol was added. After cushioned centrifugation, the semen was resuspended in each of the (isothermal) freezing extenders, chilled and frozen.

11. Experiment 5: Egg yolk concentration

Egg yolk concentration with dimethyl sulfoxide (Me_2SO): The 330 mOsm/kg BC basic solution, containing 6 % Me_2SO (v/v) and 10, 15 or 20 % egg yolk (v/v) were used as freezing extenders. After cushioned centrifugation, the semen was resuspended in each of the (isothermal) freezing extenders, chilled and frozen.

Egg yolk concentration with glycerol: The BC basic solution with osmolarity of 285 mOsm/kg was used. To this, 7 % glycerol (v/v) and either 10, 15 or 20 % egg yolk (v/v) were added. After cushioned centrifugation, the semen was resuspended in each of the (isothermal) freezing extenders, chilled and frozen.

12. Experiment 6: Glycerol concentration

Semen was frozen in 285 mOsm/kg BC basic solution containing final glycerol concentrations of 2.5, 5, 7, 10 and 15 %. The freezing extenders were divided into solution A and solution B. Solution A contained 10 % of the final glycerol volume and solution B contained the balance. Sperm pellet was suspended in isothermal solution A and chilled slowly to ~ 4 – 5°C. Isothermal solution B was then added to achieve the final desired glycerol concentration. The suspension was kept for another 15 min at 4°C, transferred into HollowTubes and frozen.

13. Experiment 7: Cryoprotectant – glycerol and Me_2SO

This experiment was designed based on the data obtained from the results of all the preliminary screening experiments described above. Semen was frozen in BC basic solution containing 6 % Me_2SO or 5, 7 or 10 % glycerol. Osmolarity of the basic solution was 285 mOsm/kg. After cushioned centrifugation, the semen was resuspended in each of the (isothermal) freezing extenders, chilled and frozen. The glycerol was added in two steps (solution A and B) as described above.

14. Statistical analysis

Comparison between treatments was performed with the SPSS software for Windows, version 10.0.7 (SPSS Inc., Chicago, IL). One-way ANOVA[5] was used to evaluate variances between treatments with specific individual comparisons done by post hoc tests. Comparisons between tests were done by paired sample t-test[6]. Descriptive analysis of seminal plasma osmolarity and normality (Lilliefors & D'Agostino–Pearson) and symmetry/skewness (Bowley's skewness coefficient & Randles–Fligner–Policello) tests were

[5] A generalised mixed linear model approach instead of one-way ANOVA would allow for an inclusion of sperm samples as random factor. Thus, pseudo replication caused by dependent samples in different treatments would be avoided.

[6] With a generalised mixed linear model approach post-hoc comparisons between treatments could account for a non-normal (possibly binomial) data structure.

done by WINPEPI, version 6.0 (Abramson, 2004). Differences were assumed significant at $p < 0.05$.

Results

1. Experiment 1: Semen osmolarity

The average osmolarity of all samples was 257.2 mOsm/kg with SEM = 6.8 and standard deviation of 37.6. The median was 270 mOsm/kg with median deviation of 9.0. Of the 31 samples evaluated, there were 6 outliers (5 low and 1 high), defined as measurements that are more than five median deviations larger or smaller than the median. Mean excluding these outliers was 268.5 mOsm/kg. Excluding the outliers, statistical tests showed the data to be of normal distribution ($p \geq 0.1$ in both Lilliefors and D'Agostino–Pearson tests) and without skewness (Bowley's skewness coefficient = 0.09 and $p > 0.2$ for Randles–Fligner–Policello test). Osmolarity of the sample obtained from the African savannah elephant was 273 mOsm/kg. Three of the outliers and two of the rest of the samples were turbid and yellow/brownish in colour with very poor semen quality.

2. Experiment 2 – 6: Summary of preliminary screening tests

The ejaculates used for the freezing experiments ranged between 20 and 100 ml, 26 – 480 × 10^6 cells/ml and 80 – 95 % motility on collection. Results of these preliminary screening experiments are presented in Tables 1 and 2. Here, we summarise the main findings of these tests. Cushioned centrifugation was clearly better than centrifugation without OptiPrep, and isothermal extender was better than cold extender (Table 1).

Table 1: Osmolarity, extender temperature, cushioned centrifugation and glycerol concentration experiments.

Exp.	Test / Parameter	Motil fresh	Motil chilled	Motil 0 h	Motil 30 min	Motil 3 h	Morphol intact	Viabil
Chilling rate, osmolarity and cushioned centrifugation								
	Native sperm	80 %					57 %	71 %
	330 mOsm, cold	80 %	5 %	1 %	2 %	0 %	22 %	20 %
	330 mOsm, cold + BC				5 %	0 %		
	330 mOsm, Iso	80 %	15 %	5 %	5 %	0 %	17 %	24 %
	330 mOsm, Iso + BC				10 %	0 %		
	285 mOsm, cold	80 %	10 %	3 %	2 %	0 %	17 %	23 %
	285 mOsm, cold + BC				2 %	0 %		
	285 mOsm, Iso	80 %	30 %	5 %	3 %	0 %	15 %	19 %
	285 mOsm, Iso + BC				5 %	0 %		
	285 mOsm, Iso, cushioned	80 %	90 %	40 %	40 %	1 %	30.5 %	60 %
	285 mOsm, Iso, cushioned + BC				50 %	40 %		
Glycerol concentration								
	Native sperm	95 %					88 %	85 %
	2.5 % Gly	95 %	80 %	42.5 %	43.3 %	9.2 %	66.3 %	58.7 %
	5 % Gly	95 %	85 %	63.3 %	60 %	10 %	60.3 %	66.3 %
	7 % Gly	95 %	85 %	68.3 %	73.7 %	18.3 %	77.3 %	68.3 %
	7 % Gly + BC				70 %	50 %		
	10 % Gly	95 %	70 %	64.2 %	68.3 %	3.3 %	70 %	50.3 %
	10 % Gly + BC				55 %	15 %		
	15 % Gly	95 %	60 %	13.3 %	5 %	0 %	53.3 %	43 %
	15 % Gly + BC				7.5 %	0 %		

Effect of various freezing extender osmolarities, chilling velocity, glycerol concentrations as well as cushioned centrifugation on post-tha*w* in vitro *p*arameters. Grey-shade rows are the basic treatment and white row that follow are of the same treatment with additional manipulation. Fresh, chilled and 0h motility in the white rows, as well as post-thaw morphology and viability are identical to those listed in the grey-shaded row above it. Cold = suspending semen in 4°C freezing extender, Iso = suspending semen in isothermal freezing extender, BC = BC solution added at a ratio of 1:1 after thawing, Exp.: experiment, Gly: glycerol, motil: motility, viabil: viability.

Evaluations of glycerol concentration indicated that the 2.5 % glycerol concentration was too low and the 15 % was too high. Neither freezing with ethylene glycol nor with propylene glycol resulted in any useable post-thawing samples (Table 2).

Both trehalose concentrations resulted in complete loss of motility even before freezing. The inclusion of glycerol in the extender did not help in this respect. However, it did help in maintaining cell viability in both trehalose concentrations (Table 2). When testing egg yolk concentration, it was evident that the freezing solution with glycerol better protected the cells than that with Me_2SO and that the 15 % egg yolk resulted in higher post-thawing viability and 3 h motility when diluted in BC basic solution after thawing (Table 2).

In all experiments, the dilution of the post-thawing sample with BC solution preserved its motility over extended period of time and proved to be better than the non-diluted sample. The addition of 10 % autologous seminal plasma after thawing did not result in any improvement. Attempts to remove the freezing extender by centrifugation and to re-suspend the pellet in BC basic solution without glycerol did not improve the results, even when the removal of the extender was gradual (data not shown).

Table 2: Ethylene glycol, propylene glycol, egg yolk and trehalose experiments.

Exp.	Test / Parameter	Motil fresh	Motil chilled	Motil 0 h	Motil 30 min	Motil 3 h	Morph intact	Viabil
Ethylene glycol/ propylene glycol								
	Native sperm	95 %					77 %	58 %
	EG - 4 %	95 %	90 %	7 %	9 %	8 %	31 %	40 %
	EG - 4 % + BC				20 %	10 %		
	EG - 8 %	95 %	75 %	6 %	4 %	0 %	29 %	27 %
	EG - 8 % + BC				5 %	0 %		
	PG - 4 %	95 %	80 %	1 %	0 %	0 %	26 %	36 %
	PG - 8 %	95 %	70 %	1 %	1 %	0 %	28 %	23 %
Egg yolk								
Glycerol	Native sperm	95 %					78 %	76 %
	10 % EY+7 % Gly	95 %	90 %	60 %	70 %	35 %	43.5 %	63 %
	10 % EY+7 % Gly+BC				65 %	35 %		
	15 % EY + 7 % Gly	95 %	90 %	70 %	70 %	22.5 %	44.5 %	75.5 %
	15 % EY+7 % Gly+BC				65 %	42.5 %		
	20 % EY+7 % Gly	95 %	80 %	65 %	70 %	5 %	39.5 %	62.5 %
	20 % EY+7 % Gly+BC				67.5 %	35 %		
Me_2SO	Native sperm	95 %					71 %	79 %
	10 % EY+6 % Me_2SO	95 %	90 %	17.5 %	15 %	0.5 %	31 %	69.5 %
	10 % EY+6 % Me_2SO+BC				10 %	5 %		
	15 % EY + 6 % Me_2SO	95 %	90 %	17.5 %	17.5 %	0 %	27.5 %	66 %
	15 % EY+6 % Me_2SO+BC				12.5 %	0 %		
	20 % EY + 6 % Me_2SO	95 %	90 %	20 %	12.5 %	0 %	39 %	66 %
	20 % EY+6 % Me_2SO+BC				10 %	1 %		

Trehalose								
	Native sperm	95 %					71 %	54 %
	0.375 M	95 %	0 %	0 %	0 %	0 %	26 %	18 %
	0.375 M+BC				0 %	0 %		
	0.375 M+4 % Gly	95 %	0 %	0 %	0 %	0 %	22 %	69.5 %
	0.375 M+4 % Gly+BC				0 %	0 %		
	0.1 M	95 %	0 %	0 %	0 %	0 %	72 %	41 %
	0.1 M + BC				0 %	0 %		
	0.1 M+4 % Gly	95 %	0 %	0 %	0 %	0 %	54.5 %	63.5 %
	0.1 M+4 % Gly+BC				0 %	0 %		

Effect of various cryoprotectants – ethylene glycol, propylene glycol and trehalose as well as varying egg yolk concentrations on post-thawing *in vitro* parameters. Grey-shade rows are the basic treatment and white rows that follow are of the same treatment with additional manipulation. Fresh, chilled and 0 h motility in the white rows, as well as post-thaw morphology and viability are identical to those listed in the grey-shaded row above it. EG: ethylene glycol, PG: propylene glycol, BC: BC solution added at a ration of 1:1 after thawing. EY: egg yolk, Gly: glycerol.

3. Experiment 7: Cryoprotectant - glycerol and Me_2SO

Based on the data collected from all the preliminary screening experiments described above, we have proceeded with this main study in which we have evaluated freezing extenders with the three promising glycerol concentrations and compared them to the one that has previously been used by us which contained Me_2SO as cryoprotectant (Hermes et al., 2003). To increase statistical power, results from the screening tests of identical groups were included in the analysis. Results presented here are based on Me_2SO: n = 6, 5 % glycerol: n = 7, 7 % glycerol: n = 8 and 10 % glycerol: n = 6. Comparison between the freezing extenders, done by one-way ANOVA test, indicated significant differences between groups in 0 h motility ($p = 0.014$), 30 min motility ($p = 0.003$), 3 h motility ($p = 0.003$), 30 min motility after dilution with BC basic solution ($p = 0.017$) and viability ($p = 0.014$; Figure 1). A strong tendency for difference was also found in 3 h motility after dilution with BC ($p = 0.081$). Specific post hoc comparisons between the extenders indicated that the extender with 7 % glycerol was superior to the one with Me_2SO at 0 h, 30 min and 30 min motility after dilution with BC basic solution ($p = 0.009$, $p = 0.002$ and $p = 0.011$, respectively). The 5 % glycerol group was also superior to the Me_2SO group in the 30 min motility evaluation and tended to be significant at 30 min motility following dilution with BC basic solution ($p = 0.014$ and $p = 0.054$, respectively). The 10 % glycerol group did not differ from the Me_2SO group but had significantly lower viability when compared to the 5 % and 7 % glycerol groups ($p = 0.028$

and p = 0.018, respectively). Although there was no difference between the 5 % and 7 % groups, numerically the 7 % group performed better on almost all evaluations (Fig. 1). To evaluate the usefulness of dilution with BC basic solution after thawing, two-tailed paired-sample t-test was conducted. When motility 30 minutes after thawing was evaluated, no difference was noted between the diluted and non-diluted samples. However, following 3 h of incubation at 37°C, the motility of the samples diluted with BC basic solution was superior to that of those that were not (p = 0.02). This difference was primarily in the 7 % glycerol group (p = 0.048).

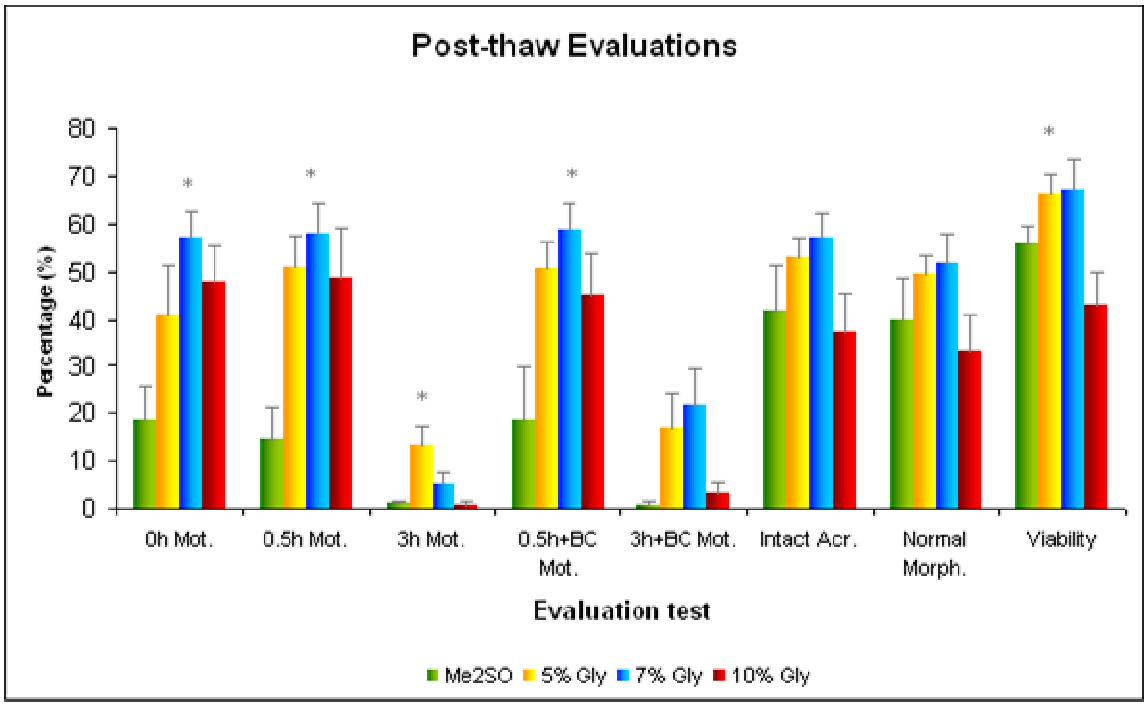

Figure 1: Post-thaw evaluations of four freezing extenders: 6 % Me_2SO and 5, 7 and 10 % glycerol in BC basic solution. Data is presented as means (bars) and standard error (error bars). Asterisk (*) above an evaluation method indicate significance between groups variance (ANOVA; $p < 0.05$). Mot: motility, Acr: acrosome, Morph: morphology, Gly: glycerol.

Discussion

In this study, we have conducted thorough and comprehensive screening experiments with the determination to further improve freezing of Asian elephant semen so that it can be preserved for extended periods of time and used when and where it is needed. While aware of the limitations inflicted by the scarcity of elephant semen collection opportunities and inconsistent semen quality, we are happy to report here that our efforts seem to have been successful in terms of post-thawing *in vitro* evaluations.

To the best of our knowledge, the osmolarity of the elephant seminal plasma was never reported before and it was assumed to be in the range commonly found in other mammals

and slightly higher than that of the serum. The freezing extenders were originally designed with osmotic strength similar to that in domestic species under this assumption. It was therefore interesting to find that the osmolarity of Asian elephant seminal plasma was in the range of 270 mOsm/kg which is similar to the normal serum osmolarity range in elephants (250 – 280 mOsm/kg; Mikota, 2006). The single African savannah elephant seminal plasma sample available for this study had an osmotic strength of 273 mOsm/kg which may suggest similarity between these species in this respect. Based on osmolarities (270 – 280 mOsmol/kg) of semen samples from the Asian bull that was used for the freezing experiments, the osmolarity of the BC basic solution was changed to 285 mOsm/kg. This was not reduced later since it provided consistently good results. With the range of osmolarities reported here, it may be advisable to evaluate each individual elephant bull and custom-design the freezing extender to suit its needs.

The next step in our study was to evaluate the need for cushioned centrifugation. Several previous studies showed that by underlying the semen sample with iodixanol aqueous solution, both recovery rate and sperm survival can be increased (Saragusty et al., 2006, 2007; Matas et al., 2007). Although, the general sperm survival and freezability of the collection used for this experiment was low, the difference between the sample that was underlaid with iodixanol and those that were not was obvious. Iodixanol was therefore used in all future centrifugations.

Most freezing protocols call for the addition of isothermal extender and then gradual, slow, cooling to 4°C before freezing. This design is aimed to reduce chilling injury as fast cooling rates result in a reduction in post-thawing motility recovery (Fiser & Fairfull, 1986). However, in experiments conducted on human spermatozoa the addition of cold freezing extender was tested to facilitate fast cooling (Clarke et al., 2003). These experiments showed significantly higher post-thawing motility recovery and sperm velocity when 4°C extender was added as compared to extender at 30°C. Although it was previously demonstrated that the spermatozoa from Asian (Saragusty et al., 2005) and African (Gilmore et al., 1998) elephants are chilling sensitive, we decided to test this method of fast chilling to suprazero temperatures. Despite the fact that collection used for this experiment was not of good quality, a sizeable decrease in motility was noted in the fast chilling group as compared to the slow chilling group, even before freezing.

In the field of sperm cryopreservation, a variety of cryoprotectants are employed in different species. To tackle the elephant sperm cryopreservation problem, we decided to run a screening test through multiple options with the hope that something that worked elsewhere will also work for the elephants. Both ethylene glycol and propylene glycol are penetrating cryoprotectants that probably also interact with either the cell membrane or extracellular water, through the two OH groups they both have, by forming hydrogen bonds. They are used as cryoprotectants in oocyte (Arav et al., 1993) and embryo (Jelinkova et al., 2002)

vitrification as well as for semen cryopreservation in both domestic (Kundu et al., 2000) and wildlife (Loskutoff et al., 1996) species. While they work well for some species, our results suggested that neither of them in none of the concentrations tested was suitable for elephant semen. Freezing with ethylene glycol produced similar results to those achieved in domestic goat cauda epididymal sperm, where a much higher concentration of 1.29 M was used (Kundu et al., 2000). However, up to 20 % motility and 30 – 40 % in viability is not satisfactory, at least not for use in elephant AI.

During the process of chilling and freezing the cells, the plasma membrane lipid bilayer is altered in both composition and structure and the freezing of extracellular water results in a hyperosmotic unfrozen fraction that draws water out of the cells. These processes lead to altered membrane permeability, cell dehydration and shrinkage and often to cellular death. Increasing the membrane fluidity may help the cells withstand such stresses as we have previously showed in the elephant (Saragusty et al., 2005). In domestic goats, trehalose was shown to have fluidizing influence (Aboagla & Terada, 2003) and trehalose has also been in use for semen cryopreservation in a variety of other species. This led us to try it on elephant sperm as well. Cells lost motility completely at both concentrations tested, even though morphology stayed intact in a high percentage of the cells. The addition of 4 % glycerol to the freezing solution resulted in even higher intact morphology, suggesting that glycerol protected the cells through a different mechanism from that provided by trehalose. Another component that helps in protecting the cells and increases the membrane fluidity is the egg yolk that is often added to freezing extenders. It was previously reported that the fatty acids composition of plasma membranes of spermatozoa from the Asian and African elephants are different (Swain & Miller, 2000). The lower proportion of polyunsaturated fatty acids in the Asian elephant sperm membrane suggested that they are less fluid. We therefore, decided to test a higher egg yolk concentration (20 %) than that found in the BC basic solution and, for comparison a lower one (10 %). Although there were only slight differences between the three concentrations, when either glycerol or Me_2SO were used as cryoprotectants, it seemed that viability was higher with the 15 % egg yolk in combination with glycerol. It was therefore decided to stick to the original BC basic solution's egg yolk concentration (16 %).

Glycerol is probably the most common cryoprotectant in use for freezing sperm cells. The benefits of glycerol as a cryoprotectant are multiple. It belongs to a group of penetrating cryoprotectants which increase the intracellular osmolarity and by that decrease cellular dehydration and shrinkage. It also has three OH groups which may form hydrogen bonds with oxygen in the membrane's phospholipids or with extracellular water, limiting the amount of water molecules available to form ice crystals (Dashnau et al., 2006). Finally, it has high viscosity at subzero temperatures, about 100 times higher than Me_2SO (Eto et al., 1993; Maltini & Anese, 1995). This high viscosity may help in hindering the movement of water molecules out of the cells. However, previous experiments with glycerol failed to achieve post-thawing spermatozoa survival results higher than about 40 % in Asian elephants

(Thongtip et al., 2004; Sa-Ardrit et al., 2006). For many years it was assumed that Me_2SO, another penetrating cryoprotectant, was the material of choice when elephants are concerned (Watson, 1995). However, even with Me_2SO post thawing motility was 40 % or lower (Hermes et al., 2003). To test these assumptions we evaluated several glycerol concentrations to see if a different concentration (low or high) will provide the expected results. It was surprising to find that glycerol concentrations often used in other species also worked for the Asian elephant and were better than Me_2SO. However, glycerol seems to be toxic to the Asian elephant spermatozoa as is evident from the low survival and motility of the 10 and 15 % glycerol groups and from the fact that cells diluted in BC basic solution after thawing survived better over time when compared to cells from the same sample that were not diluted.

In conclusion, we have demonstrated in this series of experiments that cryopreservation of the Asian elephant spermatozoa with high post-thawing sperm survival is possible. The optimal protocol arising from this study includes the following steps:

1. Underlying the sperm sample with OptiPrep for cushioned centrifugation at 1000 x g for 20 min.

2. Re-suspension of the sperm pellet in isothermal 285 mOsm/kg BC basic solution containing 16 % egg yolk and 1.48 % glycerol (solution A) to 320×10^6 cells/ml, followed by slow cooling to 4 – 5°C.

3. Dilution of the chilled, extended semen in isothermal 285 mOsm/kg BC basic solution containing 16 % egg yolk and 11.93 % glycerol (solution B) to 150×10^6 cells/ml, followed by 15 min incubation at 4°C for equilibration. Final glycerol concentration following these dilutions is 7 %.

4. Transferring the sample into isothermal 2.5 or 8 ml HollowTubes and freezing by the directional freezing technique.

5. After thawing, sample should be diluted in BC basic solution (devoid of cryoprotectant) at a ratio of 1:1 to reduce the glycerol concentration and extend sperm post-thawing survival.

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Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 9. Februar 2009

Britta Behr