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Germany/Australia Index of Sperm Sex Sortability in Elephants and Rhinoceros

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Contents

Flow cytometric sexing of spermatozoa followed by application in artificial insemination or *in vitro* fertilization 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 rhinoceros. To obtain an indication of the sortability of spermatozoa from these species, the relative DNA differences 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 both elephant species, the Asian and the African 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 relative DNA differences and the head profile areas, the sperm sorting indices were 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 sorting 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 indices in rhinos indicate that sex sorting of spermatozoa from the rhinoceros will be more challenging than in elephants.

Introduction

Pre-determination 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 programmes 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 separation of X and Y chromosome bearing spermatozoa, using a flow cytometer, by means of their relative difference in DNA content (Johnson and Welch 1999; Seidel and Garner 2002). Since the first report of sex sorting mammalian spermatozoa (rabbit, 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 and De Grofft 2003) and the bottlenose dolphin (*Tursiops truncates,* O'Brien and Robeck 2006).

Various parameters influence the sorting ability of spermatozoa. Previous studies have revealed that the greater the relative DNA difference between X and Y chromosome, the better the resolution of the X and Y sperm population. The relative DNA difference of at least 23 mammals has been determined to date by means of flow cytometry, from which the Chinchilla showed the biggest difference in DNA content (7.5%) and the possum the smallest (2.3%, Garner 2001; Johnson 2000; Maxwell et al. 2004; O'Brien and Robeck 2006). In addition to the DNA difference, the ability to orient the gametes precisely 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 relative XY sperm DNA difference (%), combines these two influencing parameters (Garner 2006).

The aim of this study was to determine the relative DNA difference between X and Y chromosome bearing spermatozoa from the white (WR, *Ceratotherium simum*), black (BR, *Diceros bicornis*), Indian rhinoceros (IR, *Rhinoceros unicornis*) and both elephant species, the Asian elephant (AsE, *Elephas maximus*) and the African elephant (AfE, *Loxodonta africana*). Furthermore, the SSI of these species was calculated to provide an indication of the sex sortability of spermatozoa from rhinoceros and elephant species, with a view to possible application of sex-sorting as a tool for assisted reproduction and conservation management of these 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 electro ejaculation under general anaesthesia (Hermes et al. 2005) in several zoological institutions in Europe and Australia, contributing to Regional Endangered Species Programmes. Semen collection was performed in context with general fertility assessments. Additionally, one sperm sample was obtained by flushing the epididymis 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 et al. (2005).

Twelve ejaculates from one Asian and three ejaculates (n = 2) from African elephants were collected by manual rectal stimulation (Schmitt and 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 100×10^6 spermatozoa/ml for incubation at 37 or 15°C. Sperm samples of low concentration ($\leq 100 \times 10^6$ 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 one from an African elephant were diluted in modified KMT for incubation at 37°C. The extended semen was then processed for sperm sorting following the general procedure described by 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 100×10^6 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 over 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 aliquots of 2 ml were washed by extension in 8 ml modified KMT and centrifugation for 15 min at 500 ***g***. After removing the supernatant, the resulting pellet was resuspended in modified KMT to a concentration of 100×10^6 spermatozoa/ml. DNA staining was performed at 5°C for 4–6 h. 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 p.s.i. Sperm sex sorting was performed at room temperature (25°C).

The fluorescence intensity of X and Y chromosome bearing spermatozoa was collected from both the 0° and the 90° detectors and stored as frequency 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 population) were then calculated as described by Garner et al. (1983): $100(X - Y)/0.5(X + Y)$, where X and Y were the respective mean values for the two peaks (Parrilla et al. 2004; O'Brien and Robeck 2006).

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

Results

Ejaculate volume and sperm concentration averaged 17.5 ± 6.2 ml, 175.9 ± 75.7 × 10^6 spermatozoa/ml for WR, 34 ± 26 ml, 63.5 ± 11.5 × 10^6 spermatozoa/ml for BR, 50 ml, 510 × 10^6 spermatozoa/ml in the IR, 60.8 ± 23.4 ml, 664.0 ± 95.4 × 10^6 spermatozoa/ml for AsE and 5 ml, 105 × 10^6 spermatozoa/ml in the sex sorted sperm sample of AfE. Sperm integrity (viable, acrosome and tail membrane intact) 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: 12.8 ± 1.5, 13.2 ± 1.3 and 13.1 ± 1.4 µm^2 for WR, BR and IR respectively. Measurements of sperm head area of epididymidal spermatozoa from one black rhino bull did not reveal any significant differences compared with fresh spermatozoa (13.2 ± 1.3 vs 12.9 ± 1.2 µm^2). The head profile areas for AsE and AfE spermatozoa were 19.8 ± 1.7 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 identify 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 (Fig. 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 were generated from frozen-thawed spermatozoa. The percentage of properly stained and correctly oriented spermatozoa in the flow cytometer (R1, Fig. 1) was lower after cryopreservation (WR: 40.23%, BR: 16.22%, IR: 13.67%) when compared with fresh samples (WR: 42.33%, BR: 38.13%, IR: 38.21%) in all three rhinoceros species.

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

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

Discussion

Flow cytometric sexing of spermatozoa followed by AI or *in vitro* fertilization provides a unique opportunity to predetermine the sex of offspring from endangered, captive species such as the elephant and rhinoceros. This technique opens up the potential for establishing self sustaining populations, by compensating premature female ageing processes as well as balancing skewed sex ratios and therefore ensuring maintenance of the gene pool. Additionally, sex pre-selection can help

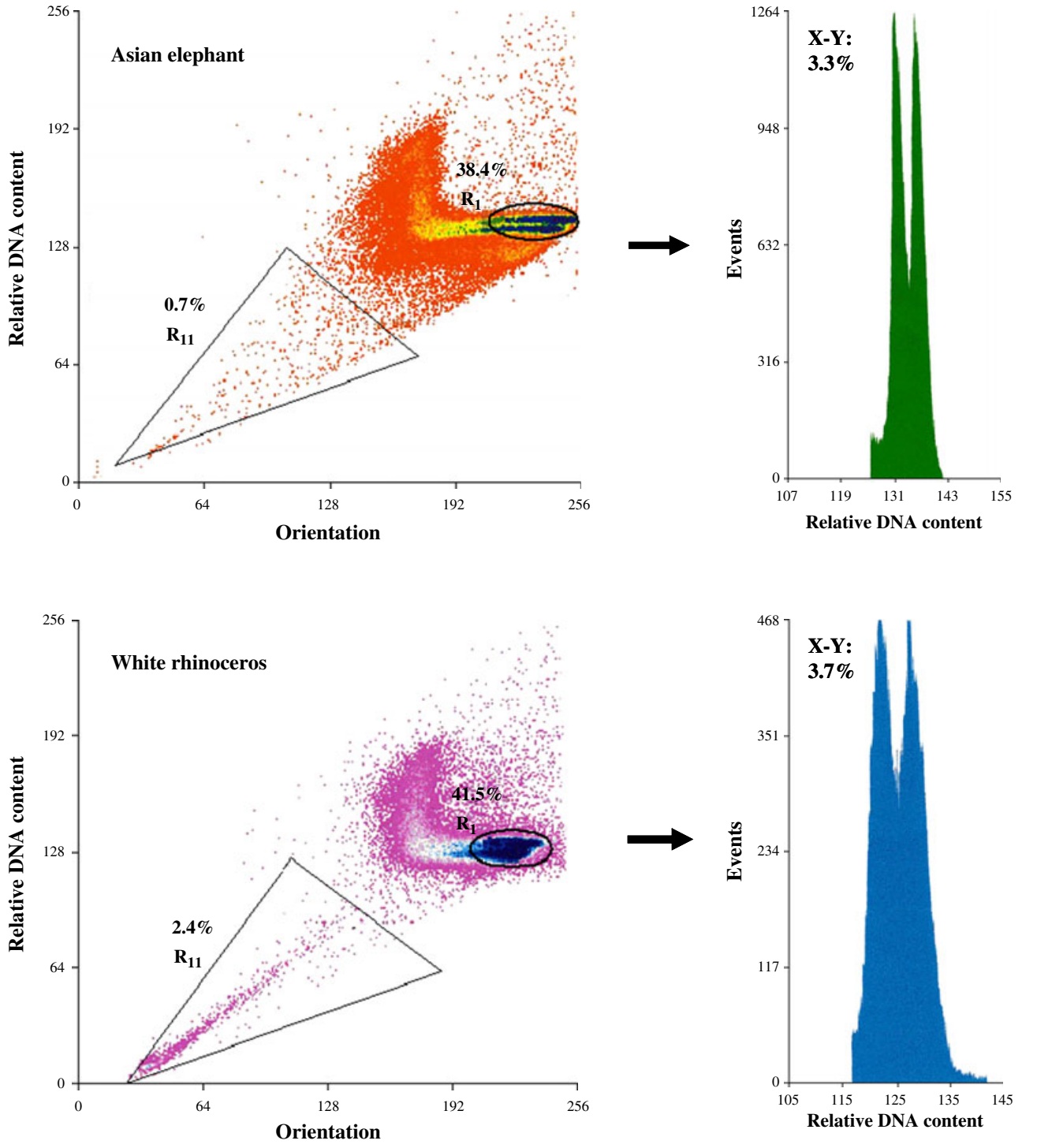

Fig. 1. Flow cytometric dot-plot and histogram outputs showing fluorescence signals from DNA-stained spermatozoa from Asian elephant and white rhinoceros. Fluorescence signals of properly stained and correctly oriented spermatozoa in Region 1 (AsE: 37.5 ± 3.1%, WR: 33.3 ± 5.1%, mean ± SEM) appear in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

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, because of 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 relative DNA difference between X and Y chromosome bearing spermatozoa of 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 relative DNA difference of 4% corresponded with former results from O'Brien et al. (2002). Additionally, the head profile areas of spermatozoa from all five tested species were measured. Head profile areas were measured from fresh sperm samples only, as the freezing and thawing process is meant to 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 corresponds with former computer assisted measurements using HTM IVOS (Silinski 2003) in the WR. The SSI as the combination of these parameters for all tested species provides an appropriate indication of the sperm sex sortability. The AfE had the highest SSI (76), followed by the AsE (66) and the three rhinoceros species (48, 49 and 51). The determined SSIs from successful sex sorted livestock and domestic species lie between 59 for the stallion and 131 for the bull (Welch and Johnson 1999; Johnson 2000; Garner 2001, 2006; Garner and Seidel 2003). Compared with indices of these domestic species, the result for the AfE lie in the same range as dog (82) and cat (80), while the AsE surpasses the stallion (59). The SSI for the rhinoceros species ranges between stallion (59) and man (31, Johnson et al. 1993; Garner 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 with other species (O'Brien et al. 2005). However, sex sorting of human spermatozoa, followed by *in vitro* fertilization, has resulted in the birth of healthy children (Levinson et al. 1995; Fugger et al. 1998; Fugger 1999; Vidal et al. 1999).

Corresponding to the evaluated differences in SSI between rhinoceros and elephant spermatozoa in this study, a better resolution of the X and Y chromosome bearing spermatozoa was obtained in elephant compared with rhinoceros semen (Fig. 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 because of their positive influence on sperm motility, integrity, sortability and low cell aggregation, evaluated in preliminary tests. However, the variable composition and high viscosity of ejaculates interfere with the requirement of stable single cell suspensions for the sorting process, especially in the rhinoceros. Therefore, the separation of total ejaculates is not possible in each case. Nevertheless, the conclusions of our study indicate the practicability of flow cytometric sex sorting of sperm from all tested rhinoceros and elephant species and therefore demonstrate the possibility of applying sex sorted spermatozoa from the megavertebrate species in captive breeding programmes. 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. 2006). Moreover, in the rhinoceros ovum pick up has recently been achieved (Hermes et al. 2007; Hildebrandt et al. 2007). However, *in vitro* fertilization or intra-cytoplasmic sperm injection in the rhinoceros needs further development. Given the limited sortability of rhinoceros spermatozoa reported in the present study, these fertilization techniques would offer a great potential for the application of sex sorted rhinoceros spermatozoa, as only small numbers are required for *in vitro* fertilization. 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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