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ORIGINAL RESEARCH

Factors affecting in vitro oocyte maturation in Southern white rhinoceros (*Ceratotherium simum simum*)

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Background: Population numbers of free-roaming rhinoceros are dwindling while natural breeding in captive populations is challenged by sub-optimal fertility. When natural breeding programmes fail and population numbers continue to decline, the use of assisted reproduction technologies (ART) as a conservation tool to save wildlife species from extinction is imperative. Assisted breeding programmes in rhinoceros are challenging and attempts to develop species-specific ART beyond artificial insemination have been limited. This study evaluates various *ex-* and *in-situ* factors that could potentially affect *in vitro* oocyte maturation (IVM) in the Southern white rhinoceros (SWR).

Objectives: To identify predictors of oocyte maturation following ovum pick-up (OPU) procedures performed on a large number of rhinoceros under different management conditions.

Methods: Data from 85 OPU procedures between November 2018 and June 2023 were obtained for this study. A total of 23 factors (three animal, four environmental, 16 laboratory) were evaluated as potentially affecting IVM success.

Results: Of the 85 procedures performed on 59 SWR, a total of 265 oocytes were collected with a median of two oocytes (range 0–18) per OPU. Eighteen (18) of the procedures yielded 0 oocytes. The overall maturation rate was 30% (80/265), and six identified factors had significant univariate associations with maturation outcome, of which three remained in the multivariable model.

Conclusion: Farming system, season and oocyte density in maturation medium (> 100 ul/oocyte) were independently associated with improved maturation success ($p < 0.05$).

Keywords: assisted reproduction; oocyte pick-up; oocyte maturation; rhinoceros

Introduction

Rhinocerotidae is one of the world's most threatened large mammal families. The Javan (*Rhinoceros sondaicus*), Sumatran (*Dicerorhinus sumatrensis*) and black (*Diceros bicornis*) are all classified as critically endangered, while the greater one-horned rhinoceros (*Rhinoceros unicornis*) is considered vulnerable and the Southern white rhinoceros (*Ceratotherium simum simum*; SWR) near threatened (IUCN 2023). Anthropogenic actions, including habitat destruction and poaching for the illicit trade in rhinoceros horn are the major driving forces behind rhinoceros dwindling numbers (Durrant 2009; Hildebrandt et al. 2018; Hildebrandt et al. 2021a; Hildebrandt et al. 2021b). The Northern white rhinoceros (*Ceratotherium simum cottoni,* NWR) is functionally extinct with only two living females, both of which are non-reproductive. This highlights the need to develop alternative assisted reproduction methods to aid conservation efforts aimed at saving this subspecies from extinction.

Population numbers of free-roaming rhinoceros are dwindling while natural breeding in captive populations is challenged by sub-optimal fertility. Institutional diet oestrogenicity effects, as well as disruption of social needs of breeding animals may negatively impact fertility of captive populations (Meuffels-Barkas et al. 2023; Tubbs et al. 2016; Ververs et al. 2017), although this has been challenged recently by the finding that captive-born rhinoceros females in fact had better oocyte fertility outcomes following IVF procedures compared to those born in the wild, and housed under the same circumstances (Hildebrandt et al. 2023). Rhinoceros are considered non-seasonal breeders with calves born throughout the year in wild and captive populations. A gestation period of 15 to 16 months is followed by birth of a singleton that stays with its mother for approximately 24 to 36 months (Pennington and Durrant 2019). Bull calves reach reproductive age between seven and nine years of age while heifers reach sexual maturity around four to six years (Roth 2006; Van der Goot et al. 2015; Ververs et al. 2015).

While assisted reproduction technologies (ART) are routinely applied with success in humans and domestic animals, limited success has been reported in rhinoceroses (de Mori et al. 2024; Hildebrandt et al. 2018; Hildebrandt et al. 2021a). Some major challenges are species-specific optimisation and limited knowledge of the complex biological management and reproductive physiology of rhinoceros (Lueders and Allen 2020; Meuffels-Barkas et al. 2023; Pennington and Durrant 2019). First-time ART successes are often reported in literature, but repeated applications are limited (Lueders and Allen 2020; Pennington and Durrant 2019). Artificial insemination (AI) in rhinoceros is performed with semen collected by means of electro-

ejaculation, urethral catheterisation or manual stimulation by transrectal massage (Meuffels-Barkas et al. 2023). However, although live offspring were born through AI in SWR and Indian rhinoceros, exact numbers of births are unknown with no reports of successful AI in black, Sumatran or Javan rhinoceros (Lueders and Allen 2020; Roth 2023; Stoops et al. 2016).

Attempts to super ovulate SWR for recovery of oocytes were reported as early as 1990 (Godfrey et al. 1990). Following these attempts, the recovery of 29 oocytes from six transrectal ovum pick-up (OPU) procedures were reported in white and black rhinoceros resulting in a single cleaved black rhinoceros zygote that arrested at the four-cell stage (Hermes et al. 2009). Similar outcomes with low maturation and fertilisation success and a single cleaved black rhinoceros zygote that arrested at the two-cell stage were also reported in 2011 (Stoops et al. 2011a; Stoops et al. 2011b).

Reproductive characteristics (e.g. cycle length and pre-ovulatory follicle size) and hormone stimulation are important factors to consider for successful ART in rhinoceros species (Pennington et al. 2019; Roth 2006). Although cycle lengths of ~30 and ~70 days were originally described for SWR (Radcliffe et al. 1997; Roth 2006; Roth 2023; Ververs et al. 2015), a more recent study reported that cycles could range from 22 to 80 days and roughly cluster around 30 and 60 days for SWR (Pennington et al. 2019). This information has led to hormone stimulation protocols being administered to cows prior to OPU. Understanding reproductive hormones and their role in the breeding cycle of rhinoceros has been prioritised in attempts to develop species-specific ART protocols (Pennington et al. 2019). In short, treatment with gonadotrophin releasing hormone (GnRH) is commonly used as an ovulation induction agent in SWR (Pennington et al. 2019).

Following advances in understanding the female reproductive tract (Meuffels-Barkas et al. 2023) and improvements in aspiration techniques used to harvest rhinoceros oocytes, recovery rates have improved (Hildebrandt et al. 2018; Hildebrandt et al. 2023). However, the development of maturation protocols is challenging due to limited availability of oocytes (Meuffels-Barkas et al. 2023; Pennington and Durrant 2019). Also, maturation success is significantly lower in *post-mortem*-derived oocytes than from OPU procedures. Stoops and colleagues reported *post-mortem* maturation rates of 7% and 10% in black and Sumatran rhinoceros respectively (Stoops et al. 2011a; Stoops et al. 2011b). Improved maturation rates of 35% and higher were reported more recently from NWR and SWR OPU procedures and noticeable improvements in ART protocols have led to increased successes in blastocyst development (Hildebrandt et al. 2018; Hildebrandt et al. 2021a; Hildebrandt et al. 2021b; Hildebrandt et al. 2023). The aim of this study was to investigate predictors of *in vitro* oocyte maturation (IVM) of SWR, with the view of improving IVM success in SWR in future.

Materials and methods

Animals and farming systems

Between November 2018 and June 2023, 265 SWR oocytes were recovered from 85 OPU sessions performed on 59 SWR and submitted to three different *in vitro* laboratories: one at San Diego Zoo Safari Park (SDZ) in California, USA, and two in the vicinity of Pretoria in South Africa (University of Pretoria, Faculty of Veterinary Science IVF Laboratory, Onderstepoort, Gauteng and EmbryoPlus, a privately-owned veterinary IVF laboratory in Brits, North West Province). Exact locations of OPU procedures are omitted to limit security risks to the rhinoceros. General suitability for immobilisation of each donor was initially determined by veterinarians performing the immobilisation and OPU, based on the visual appearance of the animal's health and breeding status and suitability (including age, where this was known). Since no exact scoring system was used to evaluate each parameter, it was not explicitly considered during the statistical analysis.

Sixteen (16) rhinoceros were super-stimulated prior to OPU with one of three treatments. Rhinoceros that were super-stimulated in South Africa received three deslorelin (Vetscript, South Africa) injections (4.5 mg) starting a week prior to OPU and given two days apart (Hermes et al. 2009). At SDZ, the super-stimulation protocol consisted of either 1.8 mg deslorelin acetate (Thorn Biosciences LLC, USA) between one and three days prior to OPU, or 30 mg chlormadinone acetate (CMA) (Sigma, USA) per day for 30 days followed by 3.6 mg deslorelin acetate, or CMA given for 35 days followed by 7 mg deslorelin acetate.

Farming systems were classified as either "intensive", where animals were managed either in zoo enclosures or on farms where they were kept in bomas and supplied with adequate nutrition and fresh water *ad libitum*; "semi-intensive" where animals were mostly free-roaming in a natural veld enclosure, but also had access to bomas with feed supplementation and fresh water; or "free-roaming" where animals were kept under natural conditions in a large game farm without human intervention (Vorster et al. 2024).

Anaesthesia and OPU procedures

At SDZ, anaesthesia was induced with a combination of etorphine, butorphanol, medetomidine and azaperone in doses scaled to the weight of each individual. Anaesthesia was reversed with atipamezole at a 5:1 ratio to medetomidine and naltrexone delivered at a 5:1 ratio to etorphine (Klohonatz et al. 2024). In South Africa, anaesthesia was induced with a combination of an opioid etorphine (Novartis, South Africa) and azaperone (VTech, South Africa) at a dose rate of 1.5–5 mg etorphine and 30–60 mg azaperone administered intramuscularly via 1.5 ml darts (Motsumi, South Africa) and a .22 blank 397 dart gun (Pneu-Dart Inc. Pennsylvania, United States). Anaesthesia was maintained at acceptable levels during OPU procedures through an intravenous combination of ketamine (VTech, South Africa), medetomidine (VTech, South Africa) and butorphanol (Kyron, South Africa). Anaesthesia reversal was achieved through intravenous administration of atipamezole hydrochloride (Zoetis, South Africa) at a 5:1 dosage ratio to medetomidine and naltrexone (VTech, South Africa) (Vorster et al. 2024).

Faecal removal, flushing and disinfection of the rectum was performed to avoid contamination and associated infection risks followed by a transrectal reproductive ultrasound examination to assess reproductive status, ovarian dimensions (length, width and height) and presence and size of follicles. Ovarian volume was estimated as length x width x height.

A customised OPU instrument and ultrasound transducer was used to perform OPU procedures (Vorster et al. 2024). Each pierced follicle was flushed several times with aspiration fluid (ABT 360, USA) containing 12.5 IU/ml heparin (Pfizer, USA). Aspirates were kept at 37 °C during the transfer (± 10 minutes) to the SDZ laboratory and the cumulus oocyte complexes (COCs) were washed with flushing solution (Vigro complete flush, Vetoquinol, USA). Follicle aspirates were rinsed through a 68 µm embryo filter (EZ Way filter, Pets Inc. USA) with ABT flush medium (ABT 360, USA) followed by locating COCs using a stereomicroscope at 20 x magnification. Recovered COCs were kept in holding media at 22 °C for 4–10 hours.

The same aspiration approach was used in South Africa except that COCs were searched for in either a mobile field laboratory or an on-site IVF laboratory (depending on the OPU location). Recovered COCs were washed five times with holding medium containing 40% M199 Earle's salts (Thermo Fisher, South Africa), M199 Hanks' salt with Hepes (Thermo Fisher, South Africa), 20% foetal bovine serum (FBS) (Merck, South Africa) and gentamicin (Merck, South Africa) or holding medium (ABT 360, USA) at 37 °C. Due to different transportation methods used by the different IVF laboratories, COCs were transported in either holding medium (ABT 360, USA) to the University of Pretoria IVF laboratory in an Equitainer® (Minitube, Germany) where it reached the laboratory between 22.0 °C and 27.0 °C, or in maturation medium in a portable incubator (WTA, USA) to EmbryoPlus where it reached the laboratory between 37 and 38.0 °C (Vorster et al. 2024). In the case of SDZ, OPU procedures were performed on site and COCs were directly transferred to the laboratory at 37 °C.

Laboratory procedures

Cumulus oocyte complexes were matured in different media. Eighty-five (85) oocytes were matured (between October 2019 and February 2021) in medium 1, containing M199 Earls (Thermo Fisher, South Africa), 2 µg/ml follicle stimulating hormone (FSH) (Merck, South Africa), 10 µg/ml luteinising hormone (LH) (Merck, South Africa), 1 µg/ml oestradiol (Merck, South Africa), 10 ng/ml epidermal growth factor (EGF) (Merck South Africa), 1 mIU/ml somatotropin (Merck, South Africa), 10% FBS (Merck, South Africa), 9% equine follicular fluid (Arroyo-Salvo et al. 2025) and 50 µg/mL gentamicin (Merck, South Africa) (Ruggeri et al. 2022). One hundred and nineteen (119) oocytes were matured (between January 2021 and June 2023) in medium 2, a modification of medium 1 with M199 Earls replaced by DMEM/F12 Glutamax (Thermo Fisher, South Africa), equine follicular fluid replaced by 1.65 uL/mL lactic acid (Merck, South Africa) and 5.5 uL/mL MEM non-essential amino acids (Merck, South Africa). Medium 2 was modified and formulated by EmbryoPlus. The remaining 61 oocytes were matured (between March 2020 and March 2023) in different formulations of medium, being variations and/or combinations of medium 1 and 2 (grouped for the purpose of our analysis as maturation medium 3). Designation of oocytes to a specific maturation medium was not randomised within or between laboratories.

At the SDZ laboratory, cumulus oocyte complexes were either cultured in 30 µl or 500 µl drops singly or in groups (2 to 5) under light mineral oil at 38.5 °C in a humidified atmosphere of 6% CO_2, for 36–38 h, or at 7% CO_2, 6% O_2 balance N_2 when DMEM was used as the base medium (Ruggeri et al. 2022). In South Africa, COCs were matured in either 400 µl or 35 µl drops of maturation medium overlaid by mineral oil (Cooper Surgical, South Africa) using a benchtop K-MINC-100 incubator at 37.0, 37.5 or 38 ˚C in 7% CO_2, 7% O_2 and 86% N_2 at EmbryoPlus. At the University of Pretoria, COCs were matured in a Thermo Forma Steri-cycle incubator (Labotec, South Africa) at 38.5 °C in 5.6% CO_2, 20% O_2, balanced N_2 atmosphere for 40–44 h. Following maturation, cumulus cells were mechanically removed by pipetting in 500 IU/ml hyaluronidase (Merck, South Africa) to determine maturation status of oocytes. Mature (MII) oocytes were identified, based on extrusion of a polar body and normal morphology (Fonte et al. 2024).

Fate of the matured oocytes

The 80 mature oocytes identified in this study were inseminated with frozen-thawed or fresh SWR semen by means of the intra-cytoplasmic sperm injection (ICSI) technique, following which only one of them cleaved by 24 h after ICSI and none progressed to the morula stage. All zygotes were therefore considered non-viable and were destroyed using the standard operating procedures of the relevant laboratory.

Statistical analysis

Due to the low cleavage rate, the statistical analysis focused on factors affecting maturation of the harvested COCs. Data were descriptively presented using scatter plots and bar charts generated using the ggplot2 package (Wickham 2009) within R (Team 2017). Quantitative data were categorised using natural breaks and percentiles prior to statistical modelling.

Season when the OPU procedure was performed, was categorised as summer (1 December to 28/29 February, or 1 June to 31 August), autumn (1 March to 31 May, or 1 September to 30 November), winter (1 June to 31 August, or 1 December to 28/29 February) or spring (1 September to 30 November, or 1 March to 31 May) in the southern and northern hemispheres, respectively. We defined season in a similar way to a previous publication that reported an improved blastocyst rate during the spring months (March to May in the northern hemisphere) compared to winter (Hildebrandt et al. 2023).

Super-stimulation was not randomised, some animals were aspirated more than once following super-stimulation, therefore, for the purpose of our analysis, super-stimulation prior to OPU was evaluated as a binary variable. Categorical data were grouped to make smaller numbers of levels when there were few observations in certain categories. Some rhinoceros were sampled by OPU multiple times and a cluster variable was defined so that all animals sampled only a single time were placed into one category and similarly, rhinoceros sampled twice, three or four times were placed into such categories. The ability of factors to predict oocyte maturation was evaluated using mixed-effect Poisson regression incorporating a term to account for extra-Poisson variation (negative binomial regression). Repeated sampling of some rhinoceros was modelled by adding the created cluster variables as a random effect with a variance components covariance structure.

Univariate screening models were fit evaluating each potential predictor individually. Collinearity between predictors was assessed using Spearman's rho and variables were considered collinear when the correlation was greater than 0.7 in absolute value. All variables with a Wald $p < 0.2$ in univariate screening were selected for multivariable models unless collinearity was present and then only the single variable with the strongest association was selected. Multivariable models were fit using a manual backwards stepwise approach starting with all selected variables from the univariate screening models and removing variables one-by-one based on the largest Wald p value until all remaining variables were $p < 0.05$.

Effect sizes were estimated by calculating the risk ratio (RR) and its corresponding 95% confidence interval (CI). All statistical analyses were performed within commercial software (IBM SPSS Statistics Version 28, International Business Machines Corp., Armonk, New York, USA) with significance set as $p < 0.05$.

Ethical approval

This study was approved by the University of Pretoria Research Ethics- and Animal Ethics Committees (REC 053-19) and by the San Diego Zoo Institutional Animal Care and Use Committee (IACUC 21-016).

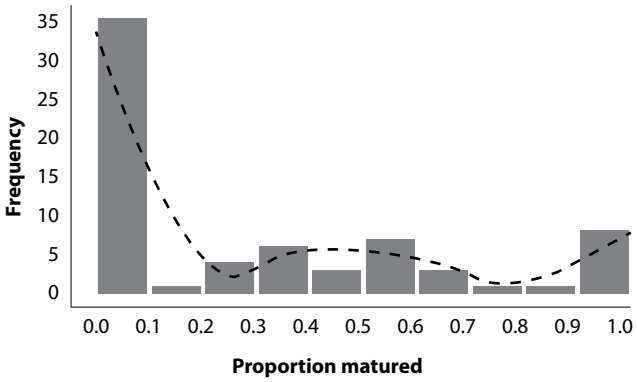

Figure 1: Frequency distribution for the proportion of successful oocyte maturation within 67 ovum pick-ups performed in 59 Southern white rhinoceros between November 2018 and June 2023. Dashed line represents the predicted probability of success based on locally-weighted scatterplot smoothing.

Results

Seventy three percent (n = 43) of rhinoceros were aspirated once over the 56-month study period. Fifteen (n = 9) and seven percent (n = 4) of the rhinoceros were aspirated two and three times, respectively. The remaining five percent of cows (n = 3) were aspirated four times each. Of the 85 OPU procedures performed, 79% (n = 67) resulted in at least one oocyte retrieved for culture. The majority of OPU procedures were performed in

Table I: Univariate associations between potential predictors and maturation success for 265 oocytes collected from 67 OPU procedures performed in 59 Southern white rhinoceros (*Ceratotherium simum simum*): *in situ* factors

Variable	Level	Parameter estimate (β)	Risk ratio (95% CI)	*p* value
Age	4–6 years	-0.208	0.81 (0.13, 5.26)	0.825
	7–9 years	-0.277	0.76 (0.14, 4.14)	0.746
	≥ 10 years	-0.342	0.71 (0.13, 3.94)	0.691
	Unknown*			
Super-stimulation	Yes	0.967	2.63 (0.75, 9.26)	0.130
	No*			
Total ovarian volume	> 120 cm^3	0.363	1.44 (0.36, 5.83)	0.605
	≤ 120 cm^3*			

CI = confidence interval *Referent

Table II: Univariate associations between potential predictors and maturation success for 265 oocytes collected from 67 OPU procedures performed in 59 Southern white rhinoceros (*Ceratotherium simum simum*): *ex situ* factors unrelated to the laboratory

Variable	Level	Parameter estimate (β)	Risk ratio (95% CI)	*p* value
Year	2018–2019*			
	2020	3.945	51.7 (0.19, 13853)	0.164
	2021	4.819	124 (0.48, 31902)	0.088
	2022–2023	3.928	50.8 (0.18, 14611)	0.171
Location	Limpopo	0.099	1.10 (0.25, 4.94)	0.896
	North West	1.208	3.35 (0.70, 16.1)	0.129
	Other location*			
Season	Autumn	1.835	6.27 (0.69, 56.9)	0.102
	Winter	2.087	8.06 (1.15, 56.4)	0.036⁺
	Spring	1.269	3.56 (0.61, 20.8)	0.156
	Summer*			
Farming system	Free-roaming*	Referent		
	Semi-intensive	3.853	47.1(5.13, 433)	< 0.001
	Intensive or zoo	2.994	20.0 (2.26, 177)	0.008⁺

CI = confidence interval *Referent ⁺Indicates significant difference from the referent

Table III: Univariate associations between potential predictors and maturation success for 265 oocytes collected from 67 OPU procedures performed in 59 Southern white rhinoceros (*Ceratotherium simum simum*): *ex situ* factors related to the laboratory

Variable	Level	Parameter estimate (β)	Risk ratio (95% CI)	*p* value
Laboratory	University of Pretoria	-0.734	0.48 (0.09, 2.51)	0.379
	San Diego Zoo	-0.275	0.76 (0.16, 3.62)	0.726
	EmbryoPlus*			
Flushing medium	ABT	1.433	4.19 (0.60, 29.1)	0.145
	Other media*			
Flushing medium time	> 30 minutes	1.581	4.86 (1.13, 20.9)	0.035+
	≤ 30 minutes*			
Holding medium	SDZ	0.005	1.01 (0.20, 5.03)	0.995
	ABT*			
Arrival temperature	≥ 37 °C	2.057	7.83 (0.95, 64.2)	0.055
	< 37 °C*			
Time in holding medium	Holding	1.186	3.27 (0.91, 11.8)	0.070
	Transport time*			
Maturation medium	Medium 1[a]	-0.598	0.55 (0.10, 3.00)	0.485
	Medium 2[b]	0.666	1.95 (0.41, 9.18)	0.394
	Medium 3[c]*			
Maturation medium hours	31–41	0.378	1.46 (0.33, 6.53)	0.617
	42–44	0.515	1.67 (0.38, 7.36)	0.490
	45–66*			
Drop size	30 ul*			
	50–100 ul	1.956	7.07 (0.89, 56.1)	0.064
	400–500 ul	1.745	5.73 (1.24, 26.4)	0.026+
Oocytes per drop	1	0.275	1.32 (0.32, 5.44)	0.700
	2	0.892	2.44 (0.58, 10.2)	0.218
	3–9*			
Oocyte density	< 0.005 /ul	1.397	4.04 (0.73, 22.4)	0.108
	0.005–0.009 /ul	1.416	4.12 (0.89, 19.1)	0.069
	0.010–0.030 /ul	-0.172	0.84 (0.19, 3.82)	0.821
	0.033–0.133 /ul*			
Incubation temperature	37 °C*			
	37.5 °C	3.851	26.6 (2.49, 285)	0.001+
	38 °C	2.663	14.3 (0.97, 238)	0.063
	38.5 °C	3.282	47.0 (4.69, 471)	0.007+
CO_2 (%)	< 6*			
	6–6.5	3.372	29.1 (1.11, 766)	0.043+
	7	3.331	28.0 (1.43, 548)	0.029+
O_2 (%)	< 20*			
	20	-0.653	0.52 (0.12, 2.33)	0.387
Humidity (%)	Unknown*			
	73–73.9	-0.085	0.92 (0.13, 6.49)	0.931
	74–95	-3.359	0.04 (0.00, 0.67)	0.027+
Medium pH	< 7.3	-0.976	0.38 (0.06, 2.41)	0.296
	7.3–7.35	-0.585	0.56 (0.11, 2.95)	0.483
	Unknown*			

CI = confidence interval *Referent +Indicates significant difference from the referent

[a]Medium 1 is M199 Earls (Thermo Fisher, South Africa), 2 µg/ml FSH (Merck, South Africa), 10 µg/ml LH (Merck, South Africa), 1 µg/ml oestradiol (Merck, South Africa), 10 ng/ml EGF (Merck South Africa), 1 mIU/ml somatotropin (Merck, South Africa), 10% FBS (Merck, South Africa), 9% equine follicular fluid (Arroyo-Salvo et al. 2025) and 50 µg/mL gentamicin (Merck, South Africa) (Ruggeri et al. 2022).

[b]Medium 2 is a modification of medium 1 with M199 Earls replaced by DMEM/F12 Glutamax (Thermo Fisher, South Africa), equine follicular fluid replaced by 1.65 uL/mL lactic acid (Merck, South Africa) and 5.5 uL/mL MEM non-essential amino acids (Merck, South Africa).

[c]Medium 3 represents different formulations of maturation medium, being variations and/or combinations of medium 1 and 2 (grouped for the purpose of the analysis).

Limpopo (44/85) and North West (23/85) provinces. Rhinoceros were also sampled in Mpumalanga (3/85), Gauteng (2/85), KwaZulu-Natal (1/85) and the SDZ (12/85). In total, 265 oocytes were recovered with a median of 2 oocytes (range: 0–18) per OPU. Forty-five cultures were performed at EmbryoPlus (67%), 12 at SDZ (18%), and 11 at the University of Pretoria (16%) with one OPU split and cultured at both EmbryoPlus and University of Pretoria. The median age of the rhinoceros at OPU, as determined by farm and/or animal records, was eight years (range: 4–22 or more). The age of rhinoceros was categorised as follows: 4–6 years old (n = 16), 7–9 years old (n = 19), ≥ 10 years old (n = 23), or unknown (n = 15; Table I) (Hillman-Smith et al. 1986).

Within the univariate associations analyses, age, super-stimulation and total ovarian volume were not significantly associated with maturation success (Table I). However, season, farming system, time in flushing medium during OPU procedure, culture drop size, incubator temperature, CO_2 concentration and humidity % were significantly associated with maturation success (Tables II and III). Date of culture was excluded from further evaluation in the multivariable model seeing that "time" per se is unlikely to be causally associated with maturation success, though time is likely to be correlated with factors that are the true cause for improvement. For example, experience in performing laboratory methods or refinement of the methods themselves. For this reason, time was not evaluated further as adjusting for time might have reduced the apparent effects of the factors that might truly be the source of the change. The percentage humidity and medium pH was not evaluated further due to extensive missing data.

Thirty percent (n = 80) of the oocytes matured successfully with 49% of all OPUs resulting in at least one mature oocyte. The median maturation success for each OPU was 9% (range: 0–100%) with the probability of culture success being non-linear (Figure 1). There was a significant difference in maturation rate between oocytes harvested from free-roaming rhinoceros, when compared to those from semi-intensive or intensively managed rhinoceros (4/42 [9.5%] vs. 27/84 [32.1%] or 49/139 [35.3%], p = 0.006 and p = 0.001 respectively). There was no difference in maturation rate between oocytes harvested from semi-intensively and intensively managed rhinoceros (p = 0.636).

The final multivariable model identified breeding season, farming system and lower oocyte density as independent predictors of successful maturation (Table IV).

Discussion

This study forms part of a bigger project where the main focus is to develop ART protocols towards conservation efforts of rhinoceros species using the SWR as a model. The aim of this study was to identify (independent) predictors of rhinoceros oocyte maturation success following OPU performed on a large number of rhinoceros across a wide geographical area.

Other than what was expected, within the univariate analyses, donor age was not identified as a predictor of oocyte maturation success. The younger age group had slightly less animals and therefore the statistical tests would have slightly less statistical power when compared to the unknown category (which probably represents an average age category if we assume that unknown age was randomly distributed). The fact that all known age groups had descriptively lower success than the referent (average group), in conjunction with the lack of statistical significance, suggests that at least within this population of rhinoceros, age was not an important predictor of maturation success. This finding might be surprising since one would expect a decrease in the quality of oocytes and maturation potential due to decreased oocyte developmental competence associated with aging. Considering that the SWR is classified in the same order as the horse, Perissodactyla, while sharing some reproductive characteristics, the horse may serve as a model for the rhinoceros (Meuffels-Barkas et al. 2023). In a recent study performed on mares, a significant increase in blastocyst numbers was reported from younger mares due to a decline in oocyte and embryo metabolic activity that potentially contributed to impaired developmental competence and fertility in aged females (Catandi et al. 2021). Similarly, Hildebrandt et al. (2023) reported a negative association between age and blastocyst formation following *in vitro* fertilisation of oocytes from SWR cows aged between 7.2 and 31.4 years (median, 20.0 years). The reasonable explanation for our findings is that the median age (range) of rhinoceros at OPU was eight years (4, 22+) with just four cows known to be over 20 years old. Therefore, older rhinoceros were under-represented in our data. Furthermore, in our data there were 10 OPU procedures performed on cows of unknown age. Although ovarian senescence in rhinoceros cows is dependent on their reproductive history, the age at which they reach ovarian senescence is currently unknown (Appeltant et al. 2023). Further research is needed to accurately determine the optimal age for OPU in rhinoceros, although such

Table IV: Multivariable associations between potential predictors and maturation success for 265 oocytes collected from 67 OPU procedures performed in 59 Southern white rhinoceros (*Ceratotherium simum simum*)

Variable	Level	Parameter estimate (β)	Risk ratio (95% CI)	*p* value
Farming system	Free roaming*			
	Semi-intensive	3.271	26.3 (2.80, 248)	0.005⁺
	Intensive or zoo	2.200	24.5 (2.80, 215)	0.005⁺
Season	Autumn/winter	1.281	3.60 (1.14, 11.4)	0.030⁺
	Spring/summer*			
Oocyte density	> 100 ul/oocyte	1.558	4.75 (1.51, 14.9)	0.009⁺
	≤ 100 ul/oocyte*			

CI = confidence interval *Referent ⁺Indicates significant difference from the referent

research opportunities are limited because the age of individual rhinoceros is often unknown.

The time that COCs spent in flushing medium was identified as an independent predictor of maturation success, with shorter exposure times resulting in higher maturation success. A possible explanation could be the prolonged exposure of oocytes to the flushing medium due to the necessity for repeated flushing and scraping to loosen and recover COCs from the follicular wall. This time-consuming procedure has also been reported for the recovery of equine COCs due to the tight connections between the cumulus and the membrana granulosa cells and the follicle wall (Cremonesi et al. 2010). Due to the phylogenetic proximity of the rhinoceros with equines (Hildebrandt et al. 2023; Meuffels-Barkas et al. 2023), this could explain why the recovery of rhinoceros COCs is also troublesome. Other factors that may also influence the prolonged exposure time of COCs to the flushing medium may include the experience of the team performing the OPU procedure, the number of follicles per ovary available to aspirate and unforeseen glitches experienced with the aspiration system/equipment during the OPU procedure. Also, maintaining a stable temperature of the flushing medium during the exposure time could affect oocyte maturation. This temperature effect was shown in a study where rhinoceros oocytes recovered from donors that received a warmed enema (31 °C) prior to oocyte retrieval were 2.3 times more likely to mature *in vitro* compared to oocytes from donors that did not (Vorster et al. 2024).

Another independent predictor of oocyte maturation success in our study was the time that oocytes spent in holding medium following the OPU procedure, with shorter exposure times resulting in higher maturation rates. In our study, COC transport times were determined by the distance between OPU sites and the laboratories. Although the aim would be to limit the time that COCs are kept in holding medium before being transferred to maturation medium, maturation rates of 38.6% following 24–36 hours transport at 22 °C for rhinoceros have been reported (Hildebrandt et al. 2018). In equines, maturation rates of ≥ 60.6% were reported following 18–24 hours of transfer in holding medium at 18–24 °C (Lazzari et al. 2020). In our study, the type of holding medium used to hold recovered COCs had no significant influence on the maturation success outcomes, although in the univariate analysis a shorter time and a higher temperature (≥ 37 °C) in holding medium seemed to improve the maturation outcomes (Table III). Additional controlled research methods/assays are required to determine the effects on rhinoceros oocyte maturation and subsequent embryo developmental outcomes.

Incubator temperatures significantly impacted maturation outcomes with more oocytes reaching MII stage at temperatures ≥ 37 °C , compared to lower temperatures. These findings are supported by other reports on rhinoceros oocyte maturation protocols where temperatures of ≥ 37.5 °C were used during oocyte maturation in SWR (Hildebrandt et al. 2018; Hildebrandt et al. 2023; Ruggeri et al. 2021), black rhinoceros (39 °C) (Stoops et al. 2011a) and Sumatran rhinoceros (38.6 °C) (Hermes et al. 2009). Although all the rhinoceros studies reported relatively low maturation rates, temperatures for oocyte maturation ≥ 37 °C seem to be the consensus for other mammalian species. In domesticated species such as equine (Lazzari et al. 2020), porcine (Martin 2000) and cattle (Smuts et al. 2019) maturation is generally performed at ≥ 37 °C , and temperatures of ≥ 41 °C seeming to have a negative effect on oocyte maturation (Lenz et al. 1983). The findings from this study suggest that oocyte maturation could be performed at ≥ 37 °C to ≤ 39 °C .

The level of CO_2 during *in vitro* maturation varied between 5.5% and 7%, with improved maturation rates at 7% CO_2. Factors that influence gas exchange and equilibration time for the diffusion of CO_2 in culture medium are the association/combination of compounds in the solution, the volume of the medium, surface area, oil overlays, type of culture dishes used and altitude (Swain 2010). Partial pressure of CO_2 (being dependent on variations in atmospheric pressure, mostly as a result of altitude) affect the level of CO_2 that will dissolve in the culture medium. Once dissolved, CO_2 combines with water to form carbonic acid (H_2CO_3) that dissociates into HCO_3 and H^+ ions (Boshoff 2017; Swain 2010). Elevated levels of CO_2 in incubators result in higher levels of H_2CO_3 and increased H^+ levels. Since pH is a measure of acidity, an increase in H^+ levels mean a decrease in pH level (Boshoff 2017; Michl et al. 2019; Vajta et al. 1997). Extracellular pH (pH_e) is the result of a balance between CO_2 concentrations in the cell culture incubator and the amount of bicarbonate in the medium while the intra-cellular pH (pH_i) of gametes and embryos are affected by variations in the pH of *in vitro* culture medium; ranging between ~ 7.1–7.2 in cattle, mice, hamsters and humans (Swain 2012). Since cellular metabolic functions like cell differentiation and protein synthesis are regulated by pH_i, slight changes in pH_i might impact oocyte metabolism and maturation rates. Although we showed improved maturation rates at 7% CO_2 in air levels, other studies reported better maturation in black, Sumatran and SWR oocytes by maintaining the CO_2 levels at 5% (Hildebrandt et al. 2018; Hildebrandt et al. 2023; Stoops et al. 2011a; Stoops et al. 2011b). A possible explanation for this could be the influence of all the above-mentioned factors that affect the pH of culture medium and the pH_e and pH_i of oocytes in various laboratories. Unfortunately, we did not have consistent medium pH data to investigate the effect of pH on oocyte maturation accurately and further work is prudent in this regard. By standardising the *in vitro* maturation protocol, the investigation of factors like medium pH and type of medium needs further investigation in future, as there are unknown interactions between medium formulations and optimal pH.

Our results emphasise the significant effect of farming system on maturation rate of oocytes harvested from rhinoceros. Although our overall data reports a significantly lower maturation rate when compared to that of another recent study (80/265 [30.2%] vs. 150/393 [38.2%], $p = 0.035$) (Hildebrandt et al. 2023) this difference was in fact only the case because of the inclusion of oocytes from free-roaming rhinoceros in our data. In the case of the study reported by Hildebrandt et al. (2023), all oocytes reported originated from intensively and semi-intensively managed rhinoceros (according to our definition). There was no difference in maturation rate of oocytes harvested from semi-intensively and intensively managed rhinoceros in our data when compared to that of this previous study (76/223 [34.1%] vs.

150/393 [38.2%], $p = 0.311$). Furthermore, this significant effect of farming system was confirmed in our data to be independent of other effects (Table IV). We are not aware of previous publications investigating the effect of farming system on rhinoceros oocyte maturation outcome. Intensive farming systems or zoo enclosures and semi-intensive farming systems supported improved oocyte maturation success compared to free-roaming farming. Semi-intensive systems allowed rhinoceroses to move freely and interact with one another in their large enclosures. Rhinoceros were more accustomed to human interaction and had access to an abundance of food and water supporting good nutrition and health. These conditions are favourable for development of good quality oocytes and are in line with results of a previous study in which adequate serum albumin levels (≥ 36.0 g/L), an indicator of good nutrition, significantly predicted increased *in vitro* developmental competence of bovine oocytes (Smuts et al. 2019). Animals in intensive farming systems also had access to sufficient feed and water but they were unable to move between enclosures or engage in natural behaviour. Signs of increased stress in these environments included stereotypical pacing of female rhinoceros alongside the fences prior to darting and one male rhinoceros continuously mock-charging the fence while spraying urine in the direction of the OPU team during the OPU procedures. Although admittedly anecdotal, this was not observed in semi-intensive farming sites where the rhinoceros paid little attention to the researchers until darting had occurred (Marais 2022).

Rhinoceros in free-roaming farming systems mainly roamed without human intervention. Chemical immobilisation through darting was performed from helicopters or vehicles in free-roaming areas leading to a flight response with presumed increase in cortisol levels and other stress hormones that are known to be associated with reduced oocyte nuclear maturation and subsequent embryonic development (Carlstead and Brown 2005; Mahdy 2018; Menargues et al. 2013). Another factor that might influence the maturation success of oocytes harvested from rhinoceros under different management systems is the anaesthetic protocol used for immobilisation of the animal for the OPU procedure. The impact of anaesthetic agents on blood pH and blood gas (O_2 and CO_2) has been documented (Meyer et al. 2018) and might affect the oocyte prior to aspiration with potential detrimental effects. Therefore, the impact on rhinoceros oocyte maturation could be related to stressors caused by different farming systems and immobilisation protocols on blood and therefore follicular fluid pH, CO_2 and O_2 concentrations. Unfortunately, we did not have complete or reliable data on this aspect to include in this study. The effect of farming system on oocyte maturation is a significant finding though when considering the efforts to use ART as a conservation tool to preserve the rhinoceros species, seeing that by far the majority of rhinoceros in the world currently are free-roaming, and obtaining viable genetic material from them for future conservation efforts, is essential.

As mentioned earlier, we defined season in a similar way to a previous publication that reported an improved blastocyst rate during the spring months (March to May in the northern hemisphere) compared to winter, where they attributed this potentially to cooling of oocytes during winter (Hildebrandt et al. 2023). In our study we found an independent improved oocyte maturation rate during autumn and winter when compared to spring and summer, which differs from the previous finding potentially because Hildebrandt et al. (2023) only analysed data from Northern Europe which may differ substantially in seasonal temperature ranges from South Africa and the Southern USA. Reported seasonal effects in rhinoceros natural breeding patterns differ under different circumstances from being non-existent to having outspoken seasonality (Radeke-Auer et al. 2022; Ververs et al. 2017).

It follows that various seasonal effects (ambient temperature variations, husbandry and availability of food, and photoperiod) might contribute in different ways to natural and assisted reproduction success (Radeke-Auer et al. 2022). Our findings are useful in that they demonstrate improved outcomes for artificial reproduction during the colder months in the circumstances under investigation, which potentially hold welfare benefits for rhinoceroses that have to be chemically immobilised for prolonged periods for these procedures i.e., the risk of hyperthermia is likely reduced during colder months.

Lower oocyte density per maturation medium volume (> 100 µl of maturation medium per oocyte cultured) independently increased the maturation success of oocytes in this study. No previous reports were found describing the effects of individual versus group culture on rhinoceros oocyte maturation. Information on the number of rhinoceros oocytes matured per maturation drop size is also limited. In a previous study, an attempt was made to test the efficacy of equine protocols for *post-mortem*-derived black rhinoceros oocytes when ≤ 6 oocytes were matured in 200 µl drops of maturation medium resulting in 3.6% (3/83 following chilled oocyte transport) and 5.9% (3/51 ovaries shipped at room temperature) MII oocytes (Stoops et al. 2011a). The same researcher reported slightly improved maturation success (10%; 3/30) by maturing two to six *post-mortem* Sumatran rhinoceros oocytes in 500 µL drops of maturation medium (Stoops et al. 2011b). Although significantly improved maturation success of 25% to 38.2% were reported from OPU-derived rhinoceros oocytes, it is not clear how many oocytes were cultured per drop of maturation medium in these reports (Galli et al. 2016; Hildebrandt et al. 2023).

In another study, gene expression in granulosa cells from SWR COCs was evaluated while comparing culture medium and oocytes from donors treated with or without gonadotropin stimulation prior to ovum recovery. While maturing oocytes singly or in pairs, this study reported maturation outcomes of 45% (5/11) using SDZ (M199 Earls, 5 mU/ml FSH, 10.6 µg/ml LH, 1 µg/ml Estradiol, 10 ng/ml EGF, 1 mU/ml Somatotropin, 10% FBS, 9% horse follicular fluid, 50 µg/ml gentamicin) and 9% (1/11) using IZW (DMEM/F12, 10% rhino estrus serum, 50 µg/ml gentamicin) maturation medium (Hildebrandt et al.,2018). Interestingly, these results showed no difference in the glucose consumption rate of rhinoceros oocytes, irrespective of culture medium or whether cultured singly or in pairs. Even though on average, COCs in one maturation medium consumed 68% of the total glucose available and 28% in the other, they also

found that COCs from super stimulated animals consumed more glucose (Ruggeri et al. 2021).

In the current study univariate analysis indicated that larger medium drop sizes might benefit oocyte maturation. However, this could easily be confounded by oocyte group culture effects, although this has not been well described in the rhinoceros species to our knowledge. We therefore considered medium drop size, the number of oocytes in the drop, as well as oocyte density (total volume of culture medium available per oocyte cultured) as independent variables in a multiple regression model. We found that oocyte density had the independent effect on maturation success. Considering that previous authors did not explicitly investigate oocyte density during maturation, one could speculate that if the maturation medium has sufficient energy sources to meet oocyte requirements, less culture medium might be required per oocyte cultured than what we report here. However, care should be taken not to overpopulate culture medium drops which could restrict oocyte maturation due to lack of nutrient requirements.

Conclusion

It is concluded that farming systems (semi-intensive and intensive or zoo, vs. free roaming), season (autumn and winter vs. spring and summer) and oocyte density in maturation medium (> 100 ul/oocyte) are independently associated with improved maturation success of SWR oocytes. Careful consideration should therefore be given to when and where rhinoceros OPU procedures are performed and potential effects that laboratory culture conditions will have on maturation and embryo development outcomes. Findings of this study are important for the development of assisted reproduction and IVF protocols for the support of conservation efforts for the SWR and the family Rhinocerotidae in general.

Outcomes of this study further highlighted some limitations that should be addressed through additional controlled research methods/assays. These would include investigations into the interactions between ovarian super stimulation, oocyte quality and group vs. individual culture. Also, the effects of donor age, as well as repeated flushing to recover oocytes on the actual time required for successful harvest and additional studies on the specific components of the culture mediums and effects of pH in order to improve maturation and subsequent embryo developmental outcomes are needed.

Conflict of interest

The authors declare they have no conflicts of interest that are directly or indirectly related to the research.

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Ethical approval

This study was approved by the Research and Animal Ethics Committees of the University of Pretoria, Faculty of Veterinary Science (REC 053-19), the Beckman Center for Conservation Research San Diego Zoo Wildlife Alliance, United States of America (21-016) and the necessary permits granted by the South African Department of Forestry, Fisheries and the Environment (permit S65757).

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