General and Comparative Endocrinology 294 (2020) 113487

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Anti-Müllerian hormone in managed African and Asian rhino species

K.E. Pollock[a], J.K. O'Brien[b,1], T.L. Roth[a], J. Proudfoot[c], J. Niederlander[d], L. Micheas[d], T.R. Robeck[b], M.A. Stoops[a,*]

[a] Center for Conservation and Research of Endangered Wildlife, Cincinnati Zoo & Botanical Garden, 3400 Vine Street, Cincinnati, OH 45220, USA
[b] SeaWorld and Busch Gardens Reproductive Research Center, SeaWorld Parks and Entertainment, 2595 Ingraham St, San Diego, CA 92109, USA
[c] Indianapolis Zoo, 1200 W Washington St, Indianapolis, IN 46222, USA
[d] University of Missouri, Columbia, MO 65211, USA

ARTICLE INFO

Keywords:
Anti-Müllerian hormone
AMH
Rhinoceros
Porcine zona pellucida
pZP
Age

ABSTRACT

Serum collected across the lifespan of four managed rhino species: black (*Diceros bicornis*, $n = 16$), white (*Ceratotherium simum simum*, $n = 19$), greater one-horned (GOH, *Rhinoceros unicornis*, $n = 11$) and Sumatran (*Dicerorhinus sumatrensis*, $n = 6$) were validated and analyzed in an anti-Müllerian hormone (AMH) enzyme-linked immunoassay. Concentrations of AMH were examined over time, between sexes and throughout different reproductive states which included $n = 3$ female white rhinos immunocontracepted with porcine zona pellucida (pZP). Across species, males produced higher AMH concentrations compared to females. Among males, AMH concentrations varied by species aside from comparable values secreted between black and white rhinos. The GOH and Sumatran rhino secreted the highest and lowest male AMH concentrations, respectively. However, within each species, AMH concentrations were similar across male age categories. Preliminary insight into male AMH changes from birth to sexual maturity suggest its potential as a marker for onset of testicular maturation. Female black, GOH and Sumatran rhinos secreted comparable AMH concentrations which were higher than those in white rhino. Within each species, inter-individual variation in AMH secretion occurred among females of similar age. While AMH secretion did not differ across the ages sampled for female white (4- > 26 yr) and GOH (4–26 yr) rhinos, black and Sumatran rhinos > 26 and < 4 yr, respectively secreted lower AMH compared to conspecific females 7–26 yr of age. Two idiopathic infertility cases corresponded to low (outside species range) AMH values. The establishment of normative AMH concentrations in managed African and Asian rhinos provides an additional metric beyond traditional sex steroids to assess gonadal function. Further work is needed to determine if AMH can predict fertility potential in rhinos.

1. Introduction

Rhinos are faced with extinction in the wild. Managed populations of African white (*Ceratotherium simum simum*), black (*Diceros bicornis*), greater one-horned (GOH, *Rhinoceros unicornis*) and Sumatran (*Dicerorhinus sumatrensis*) rhinos exist, in part to help safeguard species survival. As zoological and conservation organizations strive to maintain these populations as self-sustaining, developing biomarkers useful for evaluating fertility potential is important to enhance natural and/or assisted breeding efforts. The glycoprotein anti-Müllerian hormone (AMH) has shown promise as a systemic measure to assess gonadal function across a wide range of species. In females, AMH is produced in recruited follicles prior to selection, and appears to be highly correlated with antral follicle counts (AFC) in mice (Kevenaar et al., 2006), cattle (Ireland et al., 2008), humans (Hansen et al., 2011) and horses (Claes et al., 2015). Females who no longer have a sufficient stock of primordial follicles have fewer (if any) recruited follicles and therefore lower AMH levels. In addition to its role in Müllerian duct regression in the male fetus, AMH produced by Sertoli cells contribute to normal testicular development and function (Lee et al., 2003). As AMH analysis only requires a single blood sample or a series of blood samples spread out over several years, if demonstrated to reflect fertility, it would be a relatively easy diagnostic test to carry out with managed rhinos.

Difficulty has been encountered in trying to establish self-sustaining managed rhino populations. The primary underlying causes for these difficulties include issues related to health and reproduction. This is particularly true for white rhinos in which a significant portion of individuals have never reproduced in managed settings. One contributing

* Corresponding author at: Omaha's Henry Doorly Zoo and Aquarium, 3701 S 10th St, Omaha, NE 68107, USA.
E-mail address: monica.stoops@omahazoo.com (M.A. Stoops).
[1] Present address: Taronga Institute of Science and Learning, Taronga Conservation Society, Bradleys Head Rd, Mosman, NSW 2088, Australia.

https://doi.org/10.1016/j.ygcen.2020.113487
Received 18 November 2019; Received in revised form 14 February 2020; Accepted 4 April 2020
Available online 09 April 2020

Table 1
Description of African (n = 35) and Asian (n = 17) rhinos used in the study.

Animal ID	Species[a]	Sex	Number of samples	Collection age (yrs) range	Animal ID	Species[a]	Sex	Number of samples	Collection age (yrs) range
1	Csi	F	46	8.21–21.53	27	Dbi	F	4	35.63–38.81
2*	Csi	F	26	2.46–10.7	28	Dbi	F	23	17.81–18.89
3*	Csi	F	33	7.76–21.61	29	Dbi	F	7	11.09–11.23
4*	Csi	F	25	4.19–9.49	30	Dbi	F	71	12.65–23.48
5	Csi	F	1	5.82	31	Dbi	F	9	0.5–2.16
6	Csi	F	1	10.29	32	Dbi	F	26	2.29–8.25
7	Csi	F	1	11.44	33	Dbi	F	3	4.47–5.12
8	Csi	F	34	8.0–21.01	34	Dbi	F	15	20.86–29.86
9	Csi	F	5	7.0–11	35	Dbi	M	5	22.56–26.59
10	Csi	F	7	30–37.26	36	Dbi	M	3	28.45–31.98
11	Csi	F	1	8	37	Dbi	M	3	1.09–4.91
12	Csi	F	2	32–33	38	Dbi	M	1	18.34
13	Csi	F	1	18	39	Dbi	M	91	9.58–31.51
14	Csi	M	12	8.32–21.38	40	Dbi	M	5	1.62–1.88
15	Csi	M	5	7.0–11	41	Dbi	M	8	1.05–2.97
16	Csi	M	8	19–26	42	Run	F	12	4.05–22.5
17	Csi	M	1	29.87	43	Run	F	26	5.28–11.37
18	Csi	M	1	10	44	Run	F	1	7.62
19	Csi	M	2	0.5–4	45	Run	F	1	5
20	Dsu	F	24	7.11–19.12	46	Run	M	4	11.98–15.27
21	Dsu	F	8	2.04–9.67	47	Run	M	16	23–34
22	Dsu	F	5	14.12–17.52	48	Run	M	4	7.07–7.27
23	Dsu	M	17	13.02–31.37	49	Run	M	2	7.28–8.05
24	Dsu	M	1	5.22	50	Run	M	2	34.87–35.94
25	Dsu	M	6	0.19–8.22	51	Run	M	1	34.05
26	Dbi	F	1	5.17	52	Run	M	1	10.63

[a]Csi, *Ceratotherium simum simum* (white rhino); Dsu, *Dicerorhinus sumatrensis* (Sumatran rhino); Dbi, *Diceros bicornis michaeli* or *minor* (black rhino); Run, *Rhinoceros unicornis* (greater one-horned rhino). *administered immunocontraceptive porcine zona pellucida (pZP).

factor for the low reproductive rate in white rhinos is that many females exhibit follicular waves without intervening ovulation as indicated by basal progesterone profiles over time (Hermes et al., 2004; Pennington et al., 2019; Roth et al., 2018; Stoops et al., 2017). Large variation exists among the different rhino species in normative estrous cycle length, follicular growth pattern and pre-ovulatory follicle size (Radcliffe et al., 1997, 2001; Roth et al., 2001; Stoops et al., 2004). Given that AMH in humans has been demonstrated to play a key role in regulating follicle recruitment (Sirsikar et al., 2016), it could prove useful to explore its secretion in relation to follicular dynamics and the ovarian aging process in the different rhino species. Additionally, understanding how AMH secretion in rhinos compares to its closest living domestic relative the horse will provide a wider perspective of its biologic functions.

As AMH has been shown to help predict success of ovarian stimulation and embryo retrieval in some livestock species (Ireland et al., 2011; Lahoz et al., 2014; Monniaux et al., 2011; Rico et al., 2009; Rico et al., 2012) it may prove similarly useful in managed rhinos. Within the past two decades, assisted reproductive techniques (ART) have been applied to rhinos to augment population management concurrent with natural breeding. Exogenous hormone administration has proven useful for inducing follicular growth and ovulation in females where oocyte stock has not yet been depleted (Hildebrandt et al., 2007; Hermes et al., 2004; Hermes et al., 2012; Stoops et al.,2017; Pennington et al., 2019). Technologies for rhino oocyte-pick-up, *in vitro* fertilization and embryo transfer (Hermes et al., 2007; Hermes et al., 2009, Hildebrandt et al., 2018, Pennington and Durrant, 2018) are in their infancy. However, advancements are being made quickly as their applications are deemed necessary for survival of doomed populations, such as the Northern white rhino (*C. simum cottoni*; Saragusty et al., 2016). Physiologic tests, such as serum AMH concentrations, that help identify individuals with highest potential for reproductive success from ART, could help determine where such resources are best spent.

The reproductive management of animal populations not only involves implementing methods to promote breeding but also developing methods for suppressing reproduction in select individuals to meet species viability and sustainability goals. This is especially challenging in animals which do best in large social groupings, such as white rhino. A porcine zona pellucida (pZP) vaccine has been used to control reproduction in wild horse, elephant and deer populations whilst keeping the natural physiology and behavioral processes required for social/herd structure intact (Kirkpatrick et al., 2011). Many zoo species, including several white rhinos, have been contracepted using pZP. Numerous studies have examined the clinical, endocrine, and histological effects of pZP vaccination on equine ovarian function (Bechert et al., 2013; Joone et al., 2017; Liu et al., 1989; Powell and Monfort, 2001), but to date nothing has been reported regarding the application of pZP to rhino. Recently, AMH concentrations were used as an endocrine measure to assess ovarian function in mares during immunocontraception with the pZP vaccine (Joonè et al., 2018). Novel approaches such as these are needed to further understand pZP mechanisms of action given there is considerable species-specific variation in ovarian activity following immunization.

Objectives of this study were to 1) validate an AMH enzyme-linked immunoassay (ELISA) for Asian and African rhinos 2) establish normative AMH concentrations for managed male and female white, black, GOH and Sumatran rhinos 3) investigate whether AMH concentrations change with respect to rhino age 4) compare AMH across the estrous cycle of select females from each species and 5) document reproductive parameters, including AMH, prior to and following contraceptive treatment of white rhinos with porcine zona pellucida (pZP) vaccine.

2. Material and methods

2.1. Ethics of experimentation

The project was approved by the Cincinnati Zoo & Botanical Garden's (CZBG) Institutional Care and Use Committee 14-118, SeaWorld Parks and Entertainment RR2015-29 and by the individual zoo facilities where rhinos were housed and/or owned.

2.2. Animals and blood sample collection

Blood samples were drawn (Metrione and Eyres, 2014) under approved animal use protocols at each rhino housing institution and

serum fractions were briefly stored at −20 °C and/or −80 °C thereafter. As samples were banked for other purposes their use in this study were opportunistic with appropriate approval obtained by each rhino owning institution. Samples not originating from CZBG's collection were shipped frozen to the CREW lab for analysis. Serum samples (n = 618; 1–91 samples/rhino) were collected during 1994–2016 from 52 individual (n = 29 female, n = 23 male) rhinos representing four species maintained at 10 US zoological institutions (Table 1).

2.3. Age categories

Females were classified by age into one of five categories: < 4 yrs (n = 3), 4–6 yrs (n = 8), 7–15 yrs (n = 15), 16–26 yrs (n = 9) and > 26 yrs (n = 4). Males were similarly classified: < 7 yrs (n = 6), 7–9 yrs (n = 6), 10–19 yrs (n = 7), 20–30 yrs (n = 8) and > 30 yrs (n = 6). Age categories were based on breeding management recommendations (Metrione and Eyres, 2014).

2.4. Contraceptive treatment

Three female white rhinos maintained at a single facility were treated with the immunocontraceptive porcine zona pellucida (pZP). Two females aged 6.09 yr (#2) and 17.01 yr (#3) had conceived and given birth to one and four calves, respectively prior to treatment. The third female (#4) did not have any conceptions/pregnancies prior to treatment at age 4.94 yr. The primary vaccination (V1) incorporated Freund's Modified Complete Adjuvant and two booster (V2, V3) injections with Freund's Incomplete Adjuvant were administered 21 and 46 days after V1, respectively. Two females (#2, #4) received a yearly booster (V4) 388 days after V3. Thereafter, no additional treatments were given. Longitudinal serum samples collected prior to pZP differed among females ranging from 152 days up to 9 yr 9 months prior to V1. All females had serum samples collected up to 4 yr 4 months after V1. Also located at the same facility was a female (#1) of proven fertility not treated with contraceptive that served as a control. An adult breeding bull (#14; Table 2) of proven fertility was maintained on exhibit with all females with the exception of a 132-day period of time 1.5 yr after V1. Dates of breeding and/or breeding behavior were noted in the 5 yr following V1.

2.5. Reproductive monitoring

Reproductive status of female rhinos were determined by a combination of one or more monitoring techniques: hormone concentrations, ultrasound, observations of estrous behavior, and visual confirmation of breeding behavior/copulation. Specifically, progesterone (P4) was measured to detect cycle stage, cycle abnormalities (i.e. acyclicity, long/short cycles) and pregnancy. Rectal ultrasound was used in some instances to monitor follicular development/cycle stage. Weekly or twice weekly serum samples collected from a reproductively active female African white (n = 4; #1), black (n = 1; #28) and Sumatran (n = 1; #20) rhino were concurrently analyzed for AMH and P4 concentrations over two estrous cycles spaced 1 to 6.8 years apart. Similarly, serum AMH and P4 concentrations were examined throughout a single estrous cycle during years 3 and 5 after pZP immunization in white rhinos (n = 3; #2, #3, #4)

2.6. Hormone analysis

2.6.1. AMH

An equine anti-Müllerian hormone enzyme-linked immunosorbant assay (ELISA; AL-115, Ansh labs, Webster, TX, USA) was used in accordance with manufacturer's instructions to measure AMH concentrations. Undiluted (female rhino) or diluted (male rhino 1:10–1:100) serum samples in assay kit buffer and standards (0.06–14 ng/mL) were added (50 µL/well) to 96-well microtitre plates, followed by addition of 0.05 mL of assay kit buffer. After a 2 hr incubation (21 °C) on a plate shaker (600–800 rpm), well contents were emptied, plates were washed prior to addition of 0.1 mL/well of antibody-biotin conjugate solution. Following a 1 hr incubation on the plate shaker, wells were emptied and washed, then 0.1 mL/well of streptavidin-enzyme conjugate solution was added. After a 30 min incubation on the plate shaker, wells were emptied and washed a final time. A total of 0.1 mL/well of Tetramethylbenzidine (TMB) chromagen solution (in buffer with hydrogen peroxide) was added and incubated for 10 min (21 °C) before the addition of 0.1 mL/well of STOP solution (0.2 M sulfuric acid). Absorbance was measured within 20 min at 450 nm (VersaMax plate reader, Molecular Devices, Sunnyvale, CA, USA). Assay sensitivity was 0.009 ng/mL, with intra- and inter- assay CV's < 10%. The specific assay was validated for male and female African and Asian rhino serum by conducting tests of parallelism between serial dilutions of pooled individual sex serum and the AMH standard curve.

2.6.2. Progesterone

Progesterone concentrations in serum were determined following an extraction procedure that involved adding 2 mL of diethyl ether (Sigma) to 120 µL of serum in a 12 × 75 borosilicate glass tube (Fisher Scientific, Hampton, NH, USA) and vortexing for 45 s. The serum layer was frozen (−80 °C; 15 min) and the ether layer was immediately transferred to a new clean glass tube and dried under a stream of air. Dried samples were reconstituted in 120 µL assay buffer (0.1 M $NaPO_4$, 150 mM NaCL, 0.1% BSA) and vortexed briefly before P4 concentrations were determined in a single-antibody, direct enzyme

Table 2
Variation of female and male rhinoceros serum anti-Müllerian hormone (AMH) concentrations (mean ± SEM) according to species and age category.

Age Category*	1	2	3	4	5
Female ♀					
Black	3.566 ± 0.491[a]	2.993 ± 0.371[a,b]	2.752 ± 0.137[a]	3.454 ± 0.298[a]	0.751 ± 0.038[b]
White^	n/a	0.047 ± 0.000[a]	0.439 ± 0.061[a]	0.616 ± 0.061[a]	0.120 ± 0.031[a]
GOH	n/a	2.399 ± 0.789[a]	2.798 ± 0.429[a]	1.781 ± 0.488[a]	n/a
Sumatran	1.982 ± 1.051[a]	2.857 ± 0.466[a,b]	2.766 ± 0.17[b^^]	2.074 ± 0.359[b^^]	n/a
# ♀, # samples	(n = 3, n = 20)	(n = 8, n = 21)	(n = 15, n = 146)	(n = 10, n = 123)	(n = 4, n = 25)
Male ♂					
Black	110.65 ± 8.05[a]	147.91 ± 6.98[a]	143.17 ± 3.46[a]	125.01 ± 1.89[a]	111.16 ± 2.26[a]
White	81.27 ± 41.18[a]	99.79 ± 21.64[a]	65.66 ± 7.24[a]	82.953 ± 6.57[a^^^]	n/a
GOH	n/a	655.72 ± 44.51[a,b]	776.42 ± 47.68[a,b]	769.19 ± 62.80[a]	904.49 ± 131.4[b]
Sumatran	8.40 ± 1.62[a]	13.39 ± 0.00[a]	14.56 ± 0.41[a]	10.98 ± 0.91[a]	10.85 ± 1.67[a]
# ♂, # samples	(n = 6, n = 24)	(n = 6, n = 16)	(n = 7, n = 55)	(n = 8, n = 87)	(n = 6, n = 20)

*♀ age categories 1: < 4 yrs, 2: 4–6 yrs, 3: 7–15 yrs, 4: 16–26 yrs, 5: > 26 yrs; ♂ age categories 1: < 7 yrs, 2:7–9 yrs, 3: 10–19 yrs, 4: 20–30 yrs, 5: > 30 yrs. Within a row, means without a common superscript letter indicate significance at the 95% or higher level. ^ pZP-treated females #2,3,4 omitted from analysis; ^^include outlier #22, category 3, 2.539 ± 0.222[b], category 4, 1.189 ± 0.459[b]; ^^^ include outlier #17; 74.83 ± 9.98[a].

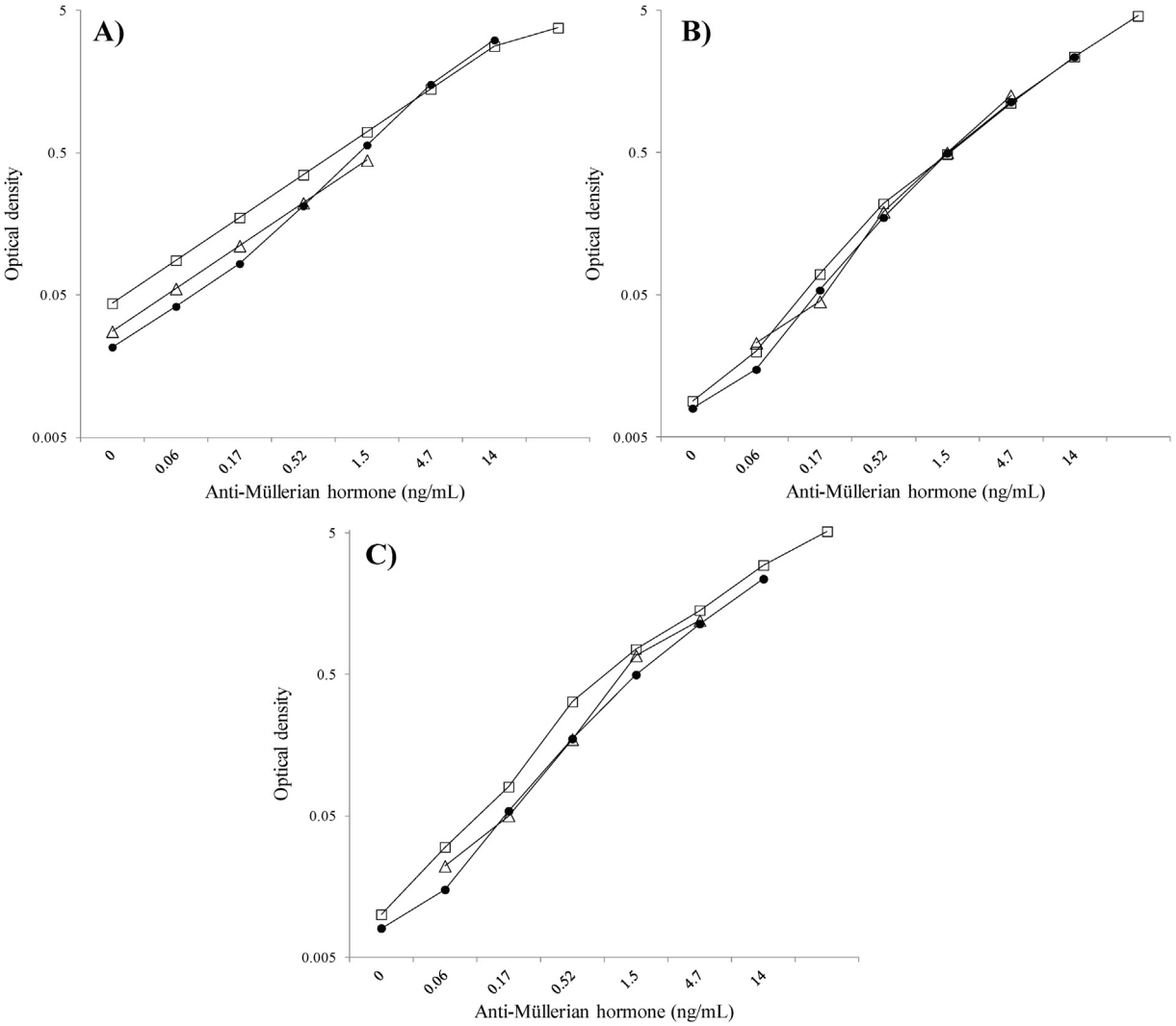

Fig. 1. Parallelism results for anti-Müllerian hormone (AMH) concentrations in rhinoceros serum; serial dilutions of pooled A) white, B) black and C) greater one-horned male (□) and female (Δ) rhino serum samples are parallel to the standard (●) curve.

immunoassay (EIA; Munro and Lasley, 1988) described in detail by Graham et al., 2001. For this assay, monoclonal antibody CL425 (C. Munro, University of California, Davis, CA, USA) was used at a 1:6,000 dilution with progesterone-horseradish peroxidase (HRP) added to each well (Nunc-Immuno Maxisorp, Fisher Scientific, Pittsburgh, PA) at a 1:75,000 concentration. Progesterone standards (50 μL, range 3.9–250 pg/well; P0130, Sigma Aldrich), samples (50 μL) and internal controls (50 μL) diluted in assay buffer were added in duplicate, combined with progesterone-HRP (50 μL), except for blank wells, and shaken (600–800 rpm) covered from light at RT for 2 hr. Plates were washed three time with wash buffer before adding 100 μL of substrate solution (0.05 M $C_6H_8O_7$, 0.4 mM ABTS). After incubation for 30–60 min at RT with shaking, the absorbance was read at 450 nM (VersaMax plate reader, Molecular Devices, Sunnyvale, CA, USA) once the optical density reached 0.9–1.0. Any results with > 10% CV between duplicates were re-analyzed. If sample results indicated binding was not within the linear portion of the standard curve, samples were diluted accordingly. Serum P4 concentrations were expressed in ng/mL and calculated based on an extraction efficiency of 85%. Intra- and inter-assay coefficients of variation (CV) for the P4 EIA's were < 10% and < 15%, respectively. Inter-assay CV's for two quality controls binding at 67% and 26% and a low biological control at 16% were 9%, 10%, and 12%, respectively.

2.7. Statistical analyses

Parallelism results for AMH were plotted as optical density versus standard mass. Differences between slopes of the binding curves for the serially diluted male and female rhino pools and the standard curve were assessed with *F* tests. AMH concentrations were considered outliers if > or < than mean AMH ± 2 s.d. within similar age category. To test the difference in AMH concentrations between age groups of rhinos (for each species separately) we ran repeated measures ANOVA model with age group as a within factor via a classical mixed random effect model (Fitzmaurice et al., 2004) with animal ID as the rand variable and alternatively a Bayesian MCMC simulation (Gelman et al., 2013) two level model (Kery 2010). The AMH measurement was natural log transformed to satisfy the normal distribution assumption of the two models. We decided to run MCMC simulation Bayesian since the small sample size affected the results of the classical mixed model ANOVA and research had demonstrated (O'Hagen and Stevens, 2001) that using informative priors with MCMC methods can boost the power of the statistical tests for small samples. SAS 9.4 proc mixed was used to run a classical mixed ANOVA and Winbugs and SAS 9.4 proc mcmc was used to run a simulation. The MCMC simulation results were averaged over 3 chains and the total number of iteration were 50000. The first 10,000 iterations were dropped from computation of summary statistics. Trace and density and autocorrelation plots, Gelman-Rubin Statics

(Winbugs) were used to monitor convergence. The informative normal priors were based on the results of the initial mixed model analysis and were used for parameter estimates and default vague inverse gamma prior for the errors.

As rhino species differ in length of the estrous cycle as well as follicular and luteal phase lengths, guidelines were established (based on normative species-specific cycle parameters) as to which serum samples would be included for documenting P4 and AMH secretion pattern throughout the estrous cycle. Specifically, day 0 in white rhino corresponded to day breeding and/or breeding behavior was observed. Samples obtained −7 days prior and through 26 days post-breeding/breeding behavior were included for white rhino. Whereas, anestrous white rhinos had samples collected over a 30 day timeframe included. Day 0 in black rhino corresponded to basal serum P4 (< 1 ng/mL) with samples obtained from 0 to 21 days included. As the only species known to experience induced ovulation, day 0 in Sumatran rhino aligned with pairing for natural breeding. Therefore, Sumatran serum obtained from −7 to 7 days post-breeding were included. Graphs were created using SigmaPlot (Systat Software Inc). The distribution of AMH concentrations by female age were represented with scatter plots in which linear regression analysis was performed for each species.

3. Results

3.1. AMH ELISA validation

There was no significant difference in slopes between AMH standards and serially diluted male (white, $F(1,13) = 0.318$, P = 0.582; black, $F(1,13) = 0.537$, P = 0.477; GOH, $F(1,13) = 0.921$, P = 0.355) and female (white, $F(1,10) = 1.395$, P = 0.265; black, $F(1,10) = 0.209$, P = 0.657; GOH, $F(1,10) = 0.172$, P = 0.687) rhino serum (Fig. 1a-c)

3.2. Male AMH

Significant species variability existed among male AMH concentrations, with the exception of the two African species which secreted similar concentrations (Table 2; Table S1). The highest and lowest AMH secretion occurred in GOH and Sumatran rhino bulls, respectively (Table 2). Within each species, no differences in AMH concentrations were documented across age categories for male black, white and Sumatran rhinos (Table 2; Table S2). While AMH concentrations were similar among male GOH 7 to > 30 yr of age, lower concentrations were produced from 20 to 30 yr (median, 712.05 ng/mL) compared to > 30 yr (median, 934.6 ng/mL; Table S2) bulls. The AMH concentration from one male white rhino (Table 1; #17) was flagged as an outlier at 9.86 ng/mL (Table 2).

The youngest male sampled was a 69-day old Sumatran rhino (Table 1; #25) whose serum AMH measured 13.96 ng/mL. Serum from this same male collected over the age range of 2–4 yr measured low AMH concentrations (5.86 ± 0.84 ng/mL) before increasing to 13.40 ng/mL when next sampled at 6 yr (Fig. 2a). Three young male black rhinos (Table 1; #37, #40, #41) sampled starting at one yr of age showed a pattern of increasing AMH concentrations over time that peaked (228.5 ng/mL) at 2.97 yr of age (Fig. 2b). Similarly, serum from a six month old male white rhino (Table 1; #19) had a lower AMH concentration (40.09 ng/mL) compared to his next sample at 4 yr of age (122.4 ng/mL; Fig. 2b).

3.3. Female AMH

Similar AMH concentrations were secreted by female black, GOH and Sumatran rhinos (Table 2; Fig. 3; Table S1). However, female white rhinos produced significantly lower AMH concentrations across the different age categories compared to the other three species (Table 2; Fig. 3; Table S1). Within each species, AMH concentrations did not differ across age categories for female white (4 yr - > 26 yr) and GOH (4 yr − 26 yr) rhinos (Table 2; Table S3). Female black and Sumatran rhinos > 26 yr and < 4 yr, respectively secreted lower AMH compared to conspecifics 7–26 yr of age (Table 2; Table S3). However, no AMH concentration differences were detected in either species for females ranging from 4 to 26 yr of age (Table 2; Table S3). The AMH concentrations (0.018 ± 0.008 ng/mL) secreted from one female Sumatran rhino (Table 1; #22) sampled over a 3 yr timeframe were identified as outliers (Table 2).

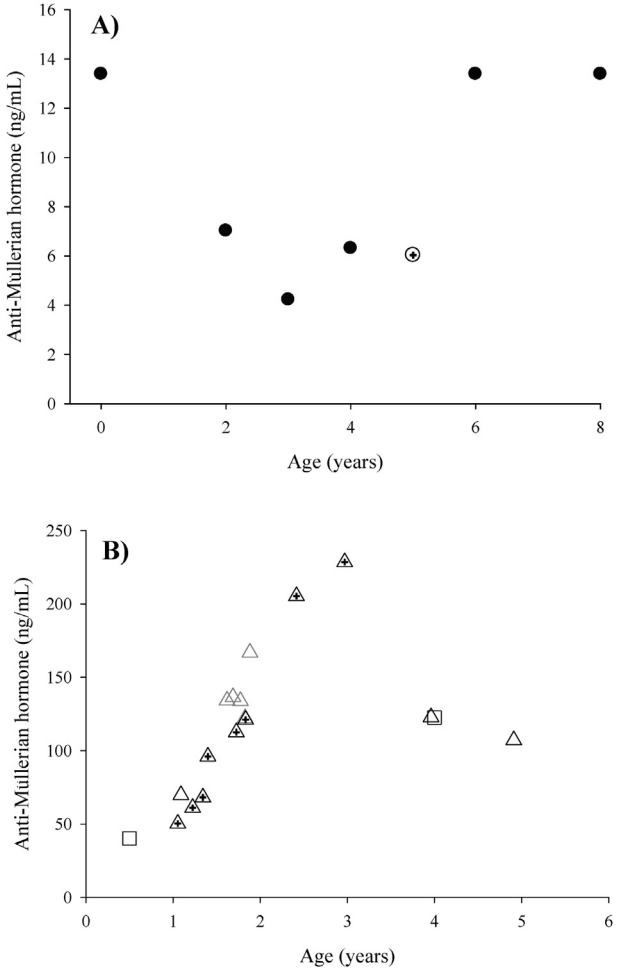

Fig. 2. Longitudinal serum anti-Müllerian (AMH) concentrations secreted from managed male A) Sumatran (n = 2; #24 ⊙, #25 ○), B) black (n = 3; #37 △, #40 △, #41 ◬) and white (n = 1; #19 □) rhinos throughout early life and transition to sexual maturity.

3.4. pZP immunization

Serum AMH concentrations measured 5–8 months prior to V1 in females #2 (5.2 yr), #3 (16.2 yr), and #4 (4.2 yr) were 0.125 ng/mL, 0.084 ng/mL and 0.422 ng/mL, respectively. All females exhibited lower AMH concentrations (#2, 0.052 ng/mL; #3, 0.059 ng/mL; #4, 0.294 ng/mL) when sampled 1–1.5 yr after V1. Regular breeding behaviors were observed from female #3 throughout the study but not observed for females #4 and #2 in the two years following treatment. Both females started exhibiting regular breeding behaviors starting in the third year at 7 (#4) and 8 (#2) yr of age. However, no breeding behaviors were recorded in year five for female #4. Intermittent ultrasound exams confirmed all females were growing follicles throughout the study with observations of corpus luteum or corpus hemorrhagicum formation on the ovaries. Mean estrous cycle lengths

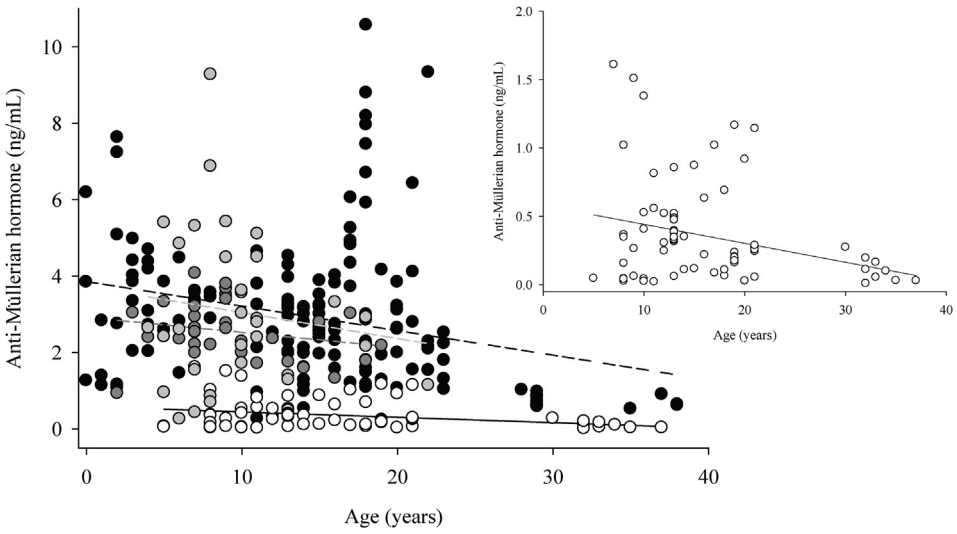

Fig. 3. Serum concentrations of anti-Müllerian hormone (AMH) in female A) African white (○), black (●), greater one-horned (◍) and Sumatran (◍) rhinos according to age. Inset shows adjusted Y-axis scale to provide additional clarity for the white rhino dataset. With the exception of white rhino, linear regression lines (dashed) of AMH concentrations by age correspond to each species symbol color. The regression line for white rhino is solid black.

based on duration between breeding behaviors were 68.13 ± 13.97 days (range 28–130; median, 62.5 days), 41.06 ± 6.55 days (range 19–224; median, 31 days), and 45.78 ± 7.8 days (range 19–149; median, 30 days) for females #4, #3, and #2, respectively. The single untreated female (# 1), when not pregnant, exhibited an estrous cycle length of 32.36 ± 1.45 days. Despite multiple confirmed copulations with the resident bull none of the treated females conceived.

3.5. Estrous cycles

An increase in P4 secretion was documented following day 0 in all cycles, with exception of the 2nd monitoring period in female #4 who had been confirmed anestrous (Fig. S1; Fig. S2). AMH concentrations and pattern of secretion varied between and within individual female estrous cycles (Fig. S1; Fig. S2; Table S4).

4. Discussion

In the present study, an assay for measuring AMH in serum of African and Asian rhino species was validated. Normative AMH concentrations now exist for managed African white, black, GOH and Sumatran rhinos. Decades of research conducted on human, companion animal, laboratory and livestock species have expanded the understanding of AMH's involvement in sexual differentiation and growth (Holst, 2017; Mossa et al., 2017; Sirsikar et al., 2016). Concentrations of AMH in the closest domestic relative of rhino, the horse, correlate to antral follicle count (Claes et al., 2015) and can reflect reproductive senescence in mares > 20 years of age (Uliani et al., 2019). Similar utility of AMH as a biomarker of gonadal function in other Perissodactyla has not been explored up until now. Examining AMH concentrations and patterns of secretion in different sexes, ages and across rhino taxa enabled establishment of endocrine metrics beyond traditional sex steroids for assessing reproductive competence based on a single blood sample.

Determining relevant hormone value limits for a given species requires sampling from a large number of individuals. Managed rhino populations do not reach the numbers found in domestic livestock for which comparable AMH data have been reported. The most numerous managed rhino population in North America is the white rhino (< 300), whereas the worldwide managed population for Sumatran rhinos is < 10 individuals. This comprehensive study involved serum collections from 29 female and 23 male rhinos over 0.19–38.81 years of age. The AMH assay validated for rhino serum was the same used in a recent large scale study of domestic mares (Uliani et al., 2019). Given it is commercially available and the primary assay used in some service-based clinical equine veterinary labs provides opportunity for zoo veterinarians and researchers to assess additional rhinos moving forward.

Concentrations of AMH were markedly higher in male than female rhinos of all species. Similar sex differences have been observed in west Indian manatee (Wilson et al., 2011), African and Asian elephants (Dow et al., 2011), cattle (Rota et al., 2002) and beluga whale (Montano et al., 2017). Additionally, species differences were documented, with GOH bulls secreting 5 to 70 times higher levels compared to equivalent aged (7–30 yr) bulls from the other three species. This is an interesting finding given that comparable systemic testosterone concentrations are secreted by mature bulls across the different rhino species (Christensen et al., 2009; Santymire et al., 2016; Seal et al., 1976; Stoops et al., 2010). Within each species, AMH concentrations remained similar across the lifespan of managed adult male rhinos. While GOH males > 30 yr secreted higher AMH concentrations compared to those 20–30 yr, more individual variation in AMH values were documented in the latter age category. Age-related changes in the regulation of Sertoli cell output have been suggested for the similarly widespread variation in AMH secretion seen in elderly men (Chong et al., 2013).

Similar to reports for male Asian and African elephants (Dow et al., 2011), serum AMH concentrations were not statistically different pre- and post-sexual maturity in rhinos. However, it is likely some males in our youngest category (< 7 yr) were already sexually mature. Age categories were based on average age of breeding in managed settings. However, it is generally thought that male rhinos attain physiological sexual maturity before achieving the behavioral maturation required to achieve successful breeding. Our study included samples collected early in life and through the expected or known transition to sexual maturity of six males representing three species. Measurable shifts in AMH secretion at key developmental transitions including the fetal/neonatal period, the time prior to puberty and at time of sexual maturity have been noted in male humans (Rey et al., 1993), mice (Al-Attar et al., 1997), cattle (Rota et al., 2002), horses (Claes et al., 2013) and beluga whales (Montano et al., 2016). AMH remains elevated throughout the early life of the male human (Rey et al., 1993), mouse (Al-Attar et al., 1997) and horse (Claes et al., 2013) with large declines coincident with the onset of puberty. Male Asian and African rhino calves demonstrated the reverse pattern whereby a notable and steady AMH increase occurred immediately prior to or at the time of established (Roth et al., 2013) or suspected sexual maturity for the different species. Serum AMH is inversely correlated to testosterone secretion during normal male sexual development in humans (Rey et al., 1993), mice (Al-Attar et al., 1997), cattle (Rota et al., 2002) and horses (Claes et al., 2013) but not elephants (Dow et al., 2011), and it appears neither the case in

rhinos. The AMH secretion pattern over time in the Sumatran rhino appeared to parallel fecal testosterone results confirming age of sexual maturity at 6–6.5 yr in this species (Roth et al., 2013). Conclusions regarding attainment of sexual maturity for the African rhinos at the young ages proposed by AMH data (3–4 yr) are speculative given concurrent fecal or serum testosterone concentrations were not available for comparison. Studbook data does confirm African rhinos have successfully sired offspring at ages even younger than our data suggest. Taken together, these results point to AMH as a potential marker for the onset of testicular maturation in rhinos.

Females from three of the four rhino species examined secreted similar AMH concentrations throughout peak reproductive years (4–15 yr) that were comparable to those produced by domestic mares using the same assay (Uliani et al., 2019). However, African white rhinos produced AMH concentrations much lower across all age categories than those measured in the other three species. The reason for this could be species-specific, underlying the need to establish the extent to which AMH reflects ovarian reserve and fertility. AMH is influenced by follicle population distribution as well as existing numbers within each stage at time of collection in mares (Uliani et al., 2019), and higher AMH concentrations have been documented in mares with a greater number compared to a lower number of growing follicles (Vernunft et al., 2011). Considerable species differences exist among rhinos in regard to follicular phase length and associated follicle growth pattern (Radcliffe et al., 1997; Radcliffe et al., 2001; Roth et al., 2001; Stoops et al., 2004) that may contribute to the variation in AMH observed. However, the rhino species producing the lowest AMH concentrations is known to exhibit significant modulation in reproductive activity in managed settings (Hermes et al., 2004; Pennington et al., 2019; Roth et al., 2018; Stoops et al., 2017). Many managed female white rhinos display an extended flat-line progesterone secretion/excretion pattern (Brown et al., 2001; Patton et al., 1999). This state has been associated with reduced fertility, shortened reproductive lifespan and formation of significant and irreversible pathology within the reproductive tract (Hermes et al., 2004). It has been determined via ultrasound that many of these females continue to develop small follicles and if pre-ovulatory sized follicles are attained, they fail to ovulate (Hermes et al., 2006; Stoops et al., 2017; Pennington et al., 2019). AMH regulates the rate at which primordial follicles leave the resting state and initiate growth, which in turn affect the rate of follicle depletion and ovarian senescence (Durlinger et al., 2002). Among pubertal elephants, AMH concentrations were shown not to differ between cyclic and acyclic females (Dow et al., 2011). However, the impact of repetitive follicular waves without intervening ovulation and/or luteal formation on AMH secretion in white rhinos remains unknown and may benefit from further inquiry.

Similar to the horse (Claes et al., 2015; Uliani et al., 2019), elephant (Dow et al., 2011) and cheetah (Place et al., 2017), AMH concentrations varied among female rhinos of the same species and age. AMH secretion did not differ across the age ranges sampled for female white (4- > 26), GOH (4–26 yr) or Sumatran (< 4–26 yr) rhinos. Whereas, female black rhinos sampled in the oldest age category (> 26 yr) secreted lower AMH compared to conspecifics 7–26 yr of age. Several reproductively active females from both African and one Asian rhino species had serum AMH concentrations measured over the course of the estrous cycle. While serum AMH does not change significantly throughout the estrous cycle of mares (Almeida et al., 2011; Claes et al., 2015) conflicting results have emerged regarding AMH secretion during the human menstrual cycle. There is research to support a decline in AMH at the end of follicular and early luteal phase in spontaneously ovulating women (Wunder et al., 2008) and those subjected to controlled ovarian hyperstimulation (Franchin et al., 2005). Other studies failed to find significant variation in AMH within or between menstrual cycles (Van Disseldorp et al., 2010). In this study, preliminary evidence emerged to support AMH variation during the rhino estrous cycle. With the exception of the Sumatran rhino, ovulation outcome of cycles were not confirmed by ultrasound. Progesterone measurement is supportive, but not definitive in verifying successful ovulation has occurred in rhinos, as anovulatory hemorrhagic follicles can secrete similar concentrations of progesterone and of the duration typical of the species-specific luteal phase (Radcliffe et al., 1997; Radcliffe et al., 2001; Roth et al., 2001; Stoops et al., 2004). Research that simultaneously measures AMH and conducts ovarian ultrasonography throughout the estrous cycle of the different rhino species could help expand upon initial findings from this study.

This study afforded a unique opportunity to track reproductive parameters in white rhinos immunized against pZP. In its domestic relative the horse, pZP has been shown to induce intermittent to persistent anestrous in a majority of mares starting from one month after their second vaccination through six months (Joonè et al., 2018). Two of three pZP treated rhinos failed to show breeding behavior in the two years following initial vaccination. However, that may have been due to their young ages, management and/or herd social dynamics as ultrasound documented some follicular growth on their ovaries.

Two rhinos in our dataset presented outlier AMH concentrations for their species with unusually low values. They included a female Sumatran rhino and male white rhino sampled in the age categories of 16–26 yr and 20–30 yr, respectively. The female had already been deemed reproductively inactive based on basal serum progesterone, high basal LH and a lack of any ovarian structures formed over time (Roth et al., 2001). The male had a history of confirmed copulation with multiple females of proven fertility with no resulting conceptions/pregnancies. An elective electroejaculation procedure conducted at the time of serum sampling resulted in the collection of a large volume aspermic ejaculate (personal observation). As electroejaculation can produce inconsistent results (Roth et al., 2005) a definitive classification of infertile could not be made at the time. AMH is known to reflect Sertoli cell function (Iliadou et al., 2015; Sharpe et al., 2003) and extreme variation outside the range of established normative values have been used to diagnose lack of functional testicular tissue in cryptorchid humans (Lee et al., 2003), cattle (Kitahara et al., 2012), and stallions (Claes et al., 2013). While the male white rhino did not suffer from this condition, his low AMH result and that of his female counterpart suggest potential utility of this hormone in assessing rhino gonadal function that should be explored further.

This comprehensive analysis facilitated the first study of age related circulating AMH across the lifetime of managed male and female African and Asian rhinos. Furthermore, it represents an important first step in expanding the comparative understanding of AMH production across Perissodactyla. More work is needed to understand the full potential of AMH to as a biomarker of fertility in rhinos. However, these results expand our knowledge of the basic reproductive biology of the taxon while providing information that can be used to interpret how an individual rhinos' serum AMH concentration compares with managed population norms. Through further research, AMH may also prove valuable when implementing ART to increase population viability and sustainability goals.

CRediT authorship contribution statement

K.E. Pollock: Supervision, Methodology, Investigation, Data curation, Resources, Writing - review & editing. **J.K. O'Brien:** Project administration, Funding acquisition, Resources, Writing - review & editing. **T.L. Roth:** Resources, Writing - review & editing. **J. Proudfoot:** Resources, Writing - review & editing. **J. Niederlander:** Resources, Writing - review & editing. **L. Micheas:** Software, Formal analysis, Visualization. **T.R. Robeck:** Resources, Writing - review & editing. **M.A. Stoops:** Conceptualization, Project administration, Funding acquisition, Resources, Validation, Writing - original draft, Visualization.

Acknowledgements

This research was funded through an Institute of Museum and Library Services National Leadership Grant MG-30-14-0072-14. The authors wish to thank the numerous Veterinary, Animal Care and Management Staff at Bronx Zoo, Buffalo Zoo, Busch Gardens, Tampa (in particular Dr. Mike Burton and Mr. Jason Green), Cincinnati Zoo & Botanical Garden, Columbus Zoo and Aquarium, Great Plains Zoo, Indianapolis Zoo, Milwaukee County Zoo, White Oak Conservation Foundation, and the Wilds for their contributions to this research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2020.113487.

References

Al-Attar, L., Noel, K., Dutertre, M., Belville, C., Forest, M.G., Burgoyne, P.S., Josso, N., Rey, R., 1997. Hormonal and cellular regulation of Sertoli cell anti-Mullerian hormone production in the postnatal mouse. J. Clin. Invest. 100 (6), 1335–1343.

Almeida, J., Ball, B.A., Conley, A.J., Place, N.J., Liu, I.K.M., Scholtz, E.L., Mathewson, L., Stanley, S.D., Moeller, B.C., 2011. Biological and clinical significance of anti-Müllerian hormone determination in blood serum of the mare. Theriogenology 76 (8), 1393–1403.

Bechert, U., Bartell, J., Kutzler, M., Menino, A., Bildfell, R., Anderson, M., Fraker, M., 2013. Effects of two porcine zona pellucida immunocontraceptive vaccines on ovarian activity in horses. J. Wildl. Manage. 77, 1386–1400.

Brown, J.L., Bellem, A.C., Fouraker, M., Wildt, D.E., Roth, T.L., 2001. Comparative analysis of gonadal and adrenal activity in the black and white rhinoceros in North America by non-invasive endocrine monitoring. Zoo Biol. 20, 463–486.

Christensen, B.W., Troedsson, M.H.T., Young, L.J., Oliva, M., Penfold, L.M., 2009. Effects of sociosexual environment on serum testosterone in captive male African rhinoceros. Theriogenology 71, 1105–1111.

Chong, Y.H., Dennis, N.A., Connolly, M.J., Teh, R., Jones, G.T., van Rij, A.M., Farrand, S., Campbell, A.J., McLennan, I.S., 2013. Elderly men have low levels of anti-Müllerian hormone and inhibin B, but with high interpersonal variation: a cross-sectional study of the sertoli cell hormones in 615 community-dwelling men. PloS One 8 (8), e70967. https://doi.org/10.1371/journal.pone.0070967.

Claes, A., Ball, B.A., Almeida, J., Corbin, C.J., Conley, A.J., 2013. Serum anti-Mullerian hormone concentrations in stallions: developmental changes, seasonal variation, and difference between intact stallions, cryptorchid stallions, and geldings. Theriogenology 79, 1229–1235.

Claes, A., Ball, B.A., Scoggin, K.E., Esteller-Vico, A., Kalmar, J.J., Conley, A.J., Squires, E.L., Troedsson, M.H.T., 2015. The interrelationship between anti-Mullerian hormone, ovarian follicular populations and age in mares. Equine Vet. J. 47, 537–541.

Dow, T.L., Roudebush, W., Parker, F.N., Brown, J.L., 2011. Influence of age and gender on secretion of anti-Müllerian hormone in Asian (*Elephas maximus*) and African (*Loxodonta Africana*) elephants. Theriogenology. 75 (4), 620–627.

Durlinger, A., Visser, J. and Themmen, A., 2002. Regulation of ovarian function: the role of anti-Mullerian hormone. Reproduction 124, 601-609.Fitzmaurice, G.M., Laird, N. M., Ware, J.H., 2004. Applied longitudinal analysis. Wiley.

Franchin, R., Louafi, N., Mendez Lozano, D.H., Frydman, N., Frydman, R., Taieb, J., 2005. Per-follicle measurements indicate that anti-mullerian hormone secretion is modulated by the extent of follicular development and luteinization and may reflect qualitatively the ovarian follicular status. Fertil. Steril. 84 (1), 167–173.

Gelman, A., Carlin, J.B., Stern, H.S., Rubin, D.B., 2013. Bayesian data analysis. CRC Press.

Graham, L., Schwarzenberger, F., Mostl, E., Galama, W., Savage, A., 2001. A versatile enzyme immunoassay for the determination of progestogens in feces and serum. Zoo Biol. 20, 227–236.

Hansen, K.R., Hodnett, G.M., Knowlton, N., Craig, L.B., 2011. Correlation of ovarian reserve tests with histologically determined primoridal follicle number. Fertil. Steril. 95, 170–175.

Hermes, R., Goritz, F., Portas, T.J., Bryant, B.R., Kelly, J.M., Maclellan, L.J., Keeley, T., Schwarzenberger, F., Walzer, C., Schnorrenberg, A., Spindler, R.E., Saragusty, J., Kaandorp, S., Hildebrandt, T.B., 2009. Ovarian superstimulation, transrectal ultrasound-guided ooctye recovery, and IVF in rhinoceros. Theriogenology 72, 959–968.

Hermes, R., Goritz, F., Streich, W., Hildebrandt, T., 2007. Assisted reproduction in female rhinoceros and elephants- current status and future perspective. Reprod. Domest. Anim. 42 (2), 33–44.

Hermes, R., Hildebrandt, T.B., Göritz, F., 2004. Reproductive problems directly attributable to long-term captivity–asymmetric reproductive aging. Animal Reprod. Sci. 82, 49–60.

Hermes, R., Hildebrandt, T.B., Walzer, C., Göritz, F., Gray, C., Niemuller, C., Schwarzenberger, F., 2012. Estrus induction in white rhinoceros (*Ceratotherium simum*). Theriogenology 78 (6), 1217–1223.

Hildebrandt, T.B., Hermes, R., Coleoni, S., Diecke, S., Holtze, S., Renfree, M.B., Stejskal, J., Hayashi, K., Drukker, M., Loi, P., Goritz, F., Lazzari, G., Galli, C., 2018. Embryos and embryonic stem cells from the white rhinoceros. Nature Commun. 9, 2589.

Hildebrandt, T.B., Hermes, R., Walzer, C., Sos, E., Molnar, V., Mezosi, L., Schnorrenberg, A., Silinski, S., Streich, J., Schwarzenberger, F., Goritz, F., 2007. Artificial insemination in the anoestrous and the postpartum white rhinoceros using GnRH analogue to induce ovulation. Theriogenology 67, 1473–1484.

Holst, B.S., 2017. Diagnostic possibilities from a serum sample-clinical value of new methods within small animal reproduction, with focus on anti-Mullerian hormone. Reprod. Domes. Anim. 52 (2), 303–309.

Iliadou, P.K., Tsametis, C., Kaprara, A., Papadimas, I., Goulis, D.G., 2015. The Sertoli cell: novel clinical potentiality. Hormones (Athens) 14, 504–514.

Ireland, J., Scheetz, D., Jimenez-Krassel, F., Themmen, A., Ward, F., Lonergan, P., Smith, G.W., Perez, G.I., Evans, A.C., Ireland, J.J., 2008. Antral follicle count reliably predicts number of morphologically healthy oocytes and follicles in ovaries of young adult cattle. Biol. Reprod. 79, 1219–1225.

Ireland, J.J., Smith, G.W., Scheetz, D., Jimenex-Krassel, F., Folger, J.K., Ireland, J.L.H., Mossa, F., Lonergan, P., Evans, A.C.O., 2011. Does size matter in females? An overview of the impact of high variation in the ovarian reserve on ovarian function and fertility, utility of anti-Mullerian hormone as a diagnostic marker for fertility and causes of variation in the ovarian reserve in cattle. Repro. Fertil. Dev. 23, 1–14.

Joone, C.J., Bertschinger, H.J., Gupta, K., Fosgate, G.T., Arukha, A.P., Minhas, V., Dieterman, E., Schulman, M.L., 2017. Ovarian function and pregnancy outcome in pony mares following immunocontraception with native and recombinant porcine zona pellucida vaccines. Equine Vet. J. 49, 189–195.

Joonè, C.J., Schulman, M.L., Fosgate, G.T., Claes, A.N.J., Gupta, S.K., Botha, A.E., Bertschinger, H.J., 2018. Serum anti-Müllerian hormone dynamics in mares following immunocontraception with anti-zona pellucida or-GnRH vaccines. Theriogenology. 106, 214–220.

Kery, A.P., 2010. Introduction to WinBUGS for ecologists: Bayesian approach to regression, ANOVA, mixed models and related analyses. Academic Press.

Kevenaar, M.E., Meerashahib, M.F., Kramer, P., van de Lang-Born, B.M., de Jong, F.H., Groome, N.P., Themmen, A.P., 2006. Serum anti-Mullerian hormone levels reflect the size of the primordial follicle pool in mice. Endocrinol. 147, 3228–3234.

Kirkpatrick, J.F., Lyda, R.O., Frank, K.M., 2011. Contraceptive vaccines for wildlife: a review. Am. J. Reprod. Immunol. 66 (1), 40–50.

Kitahara, G., El-Sheikh, H.A., Sato, T., Kobayashi, I., Hemmi, K., Shirao, Y., Samimura, S., 2012. Anti-Mullerian hormone profiles as a novel biomarker to evaluate the existence of a functional cryptorchid testis in Japanese black calves. J. Reprod. Dev. 58 (3), 310–315.

Lahoz, B., Alabart, J.L., Cocero, M.J., Monniaux, D., Echegoyen, E., Sanchez, P., Folch, J., 2014. Anti-Mullerian hormone concentration in sheep and its dependence of age and independence of BMP15 genotype: an endocrine predictor to select the best donors for embryo biotechnologies. Theriogenology 81, 347–357.

Lee, M.M., Misra, M., Donahoe, P.K., MacLaughlin, D.T., 2003. MIS/AMH in the assessment of cryptorchidism and intersex conditions. Mol. Cell Endocrinol. 211, 91–98.

Liu, I.K., Bernoco, M., Feldman, M., 1989. Contraception in mares heteroimmunized with pig zonae pellucidae. J. Reprod. Fert. 85, 19–29.

Metrione, L., Eyres, A., 2014. (Eds.). Rhino Husbandry Manual. International Rhino Foundation. Fort Worth, TX. p.32.

Monniaux, D., Baril, G., Laine, A.L., Jarrier, P., Poulin, N., Cognie, J., Fabre, S., 2011. Anti-Mullerian hormone as a predictive endocrine marker for embryo production in the goat. Reprod. 142, 845–854.

Montano, G.A., Robeck, T.R., Steinman, K.J., O'Brien, J.K., 2017. Circulating anti-Müllerian hormone concentrations in relation to age and season in male and female beluga (*Delphinapterus leucas*). Reprod. Fertil. Dev. 29 (8), 1642–1652.

Mossa, F., Jimenex-Krassel, F., Scheetz, D., Weber-Nielsen, M., Evans, A.C.O., Ireland, J.J., 2017. Anti-Mullerian hormone (AMH) and fertility management in agricultural species. Reprod. 154, 1470–1626.

Munro, C.J., Lasley, B.L., 1988. Non-radiometric methods for immunoassay of steroid hormones. Prog. Clin. Biol. Res. 285, 289–329.

O'Hagen, A., Stevens, J.W., 2001. Bayesian assessment of sample size for clinical trials of cost-effectiveness. Med. Decis. Making 21, 219–230.

Patton, M.L., Swaisgood, R.R., Czekala, N.M., White, A.M., Fetter, G.A., Montagne, J.P., Rieches, R.G., Lance, V.A., 1999. Reproductive cycle length and pregnancy in the southern white rhinoceros (*Ceratotherium simum simum*) as determined by fecal pregnane analysis and observations of mating behavior. Zoo Biol. 18, 111–127.

Pennington, P.M., Durrant, B.S., 2018. Assisted reproductive technologies in captive rhinoceroses. Mammal Rev. 49 (1), 1–15.

Pennington, P.M., Marshall, K.L., Capiro, J.M., Felton, R.G., Durrant, B.S., 2019. Ovulation induction in anovulatory southern white rhinoceros (*Ceratotherium simum simum*) without altrenogest. Conserv. Physiol. 7 (1) coz033, doi: 10.1093/conphys/coz033.

Place, N.J., Crosier, A.E., Comizzoli, P., Nagashima, J.B., Hafele, H., Schmidt-Kuntzel, A., Marker, L.L., 2017. Age-associated and deslorelin-induced declines in serum anti-Mullerian hormone concentrations in female cheetahs, *Acinonyx jubatus*. General Comparative Endocrinol. 250, 54–57.

Powell, D.M., Monfort, S.L., 2001. Assessment effects of porcine zona pellucida immunocontraception on estrous cyclicity in feral horses. J. Appl. Anim. Welf. Sci. 4, 271–284.

Radcliffe, R.W., Czekala, N.M., Osofsky, S.A., 1997. Combined serial ultrasonography and fecal progestin analysis for reproductive evaluation of the female white rhinoceros (*Ceratotherium simum simum*): preliminary results. Zoo Biol. 16 (5), 445–456.

Radcliffe, R.W., Eyres, A.I., Patton, M.L., Czekala, N.M., Emslie, R.H., 2001. Ultrasonographic characterization of ovarian events and fetal gestational parameters in two southern black rhinoceros (*Diceros bicornis minor*) and correlation to fecal progesterone. Theriogenology 55, 1033–1049.

Rey, R., Lordereau-Richard, I., Carel, J.C., Barbet, P., Cate, R.I., Roger, M., Haussain, J.L., Josso, N., 1993. Anti-Mullerian hormone and testosterone serum levels are inversely related during normal and precocious pubertal development. J. Clin. Endocrinol.

Metab. 77, 1220–1226.
Rico, C., Drouilhet, L., Salvetti, P., Dalbies-Tran, R., Jarrier, P., Touze, J.-L., Pillet, E., Ponsart, C., Fabre, S., Monniaux, D., 2012. Determination of anti-Mullerian hormone concentrations in blood as a tool to select Holstein donor cows for embryo production: from the laboratory to the farm. Reprod. Fertil. Dev. 24 (7), 932–944.
Rico, C., Fabre, S., Medigue, C., di Clemente, N., Clement, F., Bontoux, M., Touze, J.-L., Dupont, M., Briant, E., Remy, B., Beckers, J.-F., Monniaux, D., 2009. Anti-Mullerian hormone is an endocrine marker of ovarian gonadotrophin-responsive follicles and can help to predict superovulatory responses in the cow. Biol. Reprod. 80, 50–59.
Rota, A., Ballarin, C., Vigier, B., Cozzi, B., Rey, R., 2002. Age dependent changes in plasma anti-mullerian hormone concentrations in the bovine male, female, and freemartin from birth to puberty: relationship between testosterone production and influence on sexual differentiation. Gen. Comp. Endocrinol. 129, 39–44.
Roth, T.L., O'Brien, J.K., McRae, M.A., Bellem, A.C., Romo, S.J., Kroll, J.L., Brown, J.L., 2001. Ultrasound and endocrine evaluation of the ovarian cycle and early pregnancy in the Sumatran rhinoceros, *Dicerorhinus sumatrensis*. Reprod. 121, 139–149.
Roth, T.L., Reinhart, P.R., Romo, J.S., Candra, D., Suhaery, A., Stoops, M.A., 2013. Sexual maturation in the Sumatran rhinoceros (*Dicerorhinus sumatrensis*). Zoo Biol. 32, 549–555.
Roth, T.L., Schook, M.W., Stoops, M.A., 2018. Monitoring and controlling ovarian function in the rhinoceros. Theriogenology 109, 48–57.
Roth, T.L., Stoops, M.A., Atkinson, M.W., Blumer, E.S., Campbell, M.K., Cameron, K.N., Citino, S.B., Maas, A.K., 2005. Semen collection in rhinoceroses (*Rhinoceros unicornis*, *Diceros bicornis*, *Ceratotherium simum simum*) by electroejaculation with a uniquely designed probe. J. Zoo Wildl. Med. 35, 617–627.
Saragusty, J., Diecke, S., Drukker, M., Durrant, B., Ben-Nun, I.F., Galli, C., Goritz, F., Hayashi, K., Hermes, R., Holtze, S., Johnson, S., Lazzari, G., Loi, P., Loring, J.F., Okita, K., Renfree, M.B., Seet, S., Voracek, T., Stejskal, J., Ryder, O., Hildebrandt, T.B., 2016. Rewinding the process of mammalian extinction. Zoo Biol. 35, 280–292.
Seal, U.S., Barton, R., Mather, L., Gray, C.W., 1976. Baseline laboratory data for the white rhinoceros (*Ceratotherium simum simum*). J. Zoo Anim. Mec. 7, 11–16.
Santymire, R.M., Misek, S., Gossett, J., Kamhout, M., Chatroop, E., Rafacz, M., 2016. Male behaviours signal the female's reproductive state in a pair of black rhinoceros housed at Lincoln Park Zoo. J. Zoo Aquarium Res. 4 (1), 30–37.
Sharpe, R.M., McKinnell, C., Kivlin, C., Fisher, J.S., 2003. Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. Reprod. 125, 769–784.
Sirsikar, M., Pinnelli, V.B.K., Mohanty, S., Jayashankar, C.A., 2016. Anti-Mullerian hormone in health and disease: a review. Int. J. Res. Med. Sci. 4 (7), 2514–2520.
Stoops, M.A., Atkinson, M.W., Blumer, E.S., Campbell, M.K., Roth, T.L., 2010. Semen cryopreservation in the Indian rhinoceros (*Rhinoceros unicornis*). Theriogenology 73, 1104–1115.
Stoops, M.A., O'Brien, J.K., Niederlander, J., Metrione, L., Pootoolol, J., Delk, K., Niemuller, C., Hagen, D., Douglas, R.H., Proudfoot, J., 2017. Administration of biorelease progesterone and estradiol or GnRH to induce estrous cycles and breeding in anestrous African white rhinoceroses (Ceratotherium simum simum). Proceedings 6th International Society of Wildlife Endocrinology Conference, Orlando, FL, USA Aug 14-16. P. 111.
Stoops, M.A., Pairan, R.D., Roth, T.L., 2004. Follicular, endocrine and behavioural dynamics of the Indian rhinoceros (*Rhinoceros unicornis*) oestrous cycle. Reprod. 128 (6), 843–856.
Uliani, R.C., Conley, A.J., Corbin, C.J., Friso, A.M., Maciel, L.F., Alvarenga, M.A., 2019. Anti-Müllerian hormone and ovarian aging in mares. J. Endo. 240 (2), 147–156.
Van Disseldorp, J., Lambalk, C.B., Kwee, J., Looman, C.W., Eijkemans, M.J., Fauser, B.C., Borekmans, F.J., 2010. Comparison of inter- and intre-cycle variability of anti-Mullerian hormone and antral follicle counts. Hum. Reprod. 25, 221–227.
Vernunft, A., Schneider, F., Tuchscherer, A., Becker, F., Kanitz, W., 2011. Anti-Mullerian hormone (AMH) can help predict follicular growth in mares. Pferdeheilkunde 27, 293–296.
Wilson, R.C., Reynolds, J.E., Wetzel, D.L., Schwierzke-Wade, L., Bonde, R.K., Breuel, K.F., Roudebush, W.E., 2011. Secretion of anti-Mullerian hormone in the Florida manatee *Trihechus manatus*, with implications for assessing conservation status. Endang. Species Res. 14, 107–112.
Wunder, D.M., Bersinger, N.A., Yared, M., Kretschmer, R., Birkhauser, M.H., 2008. Statistically significant changes of antimullerian hormone and inhibin levels during the physiologic menstrual cycle in reproductive age women. Fertil. Steril. 89 (4), 927–933.