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Prolactin enzyme-linked immunosorbent assay for rhinoceroses – Another tool for assessing reproductive function and dysfunction in this taxon

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ABSTRACT

For decades, progesterone and its metabolites have served as the primary biomarkers for monitoring reproductive activity in rhinoceroses, whereas protein hormones have received little attention despite their potential value in understanding reproductive function and dysfunction in this taxon. The goals of this study were to: 1) identify an enzyme-linked immunosorbent assay (ELISA) effective in measuring rhinoceros serum prolactin, 2) generate representative prolactin data in male and female rhinoceroses in diverse reproductive states, and 3) characterize prolactin throughout pregnancy in a white rhinoceros that exhibited mid-gestation lactation. Our results indicated that an equine prolactin ELISA by CUSABIO® is effective in measuring serum prolactin concentrations in white, black and Sumatran rhinoceroses. Preliminary data suggested that prolactin is lowest in males and acyclic females, but also appeared low during post-partum lactation. In contrast, prolactin concentrations were elevated in pregnant females and moderate in sexually mature females experiencing reproductive cyclicity. Prolactin increased following conception and generally continued to rise throughout pregnancy in the one pregnant white rhinoceros profiled herein. Spikes and dips in prolactin and progesterone, respectively, were documented around the time of mid-gestation mammary development and colostrum production in this individual and may provide some clues into the physiological triggers of this newly described aberrant reproductive condition. In conclusion, we have identified a new tool for studying reproductive activity in rhinoceroses, generated preliminary data, and revealed an intriguing relationship between prolactin fluctuations and premature lactation. This work provides the foundation for larger, focused studies on the role of prolactin in this taxon.

1. Introduction

Non-invasive hormone metabolite monitoring has been employed by numerous scientists and animal managers since the 1990s to better understand the reproductive biology of the rhinoceros and to guide breeding efforts. Although fecal samples have been the biological sample of choice for most studies [1–9], urine samples have been used on occasion, especially in the greater one-horned (GOH) rhinoceros [10–12], and saliva collected via minimally invasive methods also has been shown to contain measurable steroid hormone concentrations [13–15]. Because protein degradation is likely in all of these biological matrices, especially fecal samples, protein hormones associated with rhinoceros reproductive function have rarely been studied. An exception is characterization of the pre-ovulatory LH surge in urine from both Sumatran and GOH rhinoceroses [2,11,16].

Steroid hormone metabolite data has greatly enhanced our understanding of basic reproductive physiology while also exposing aberrant reproductive activity within our rhinoceros populations. However, more clarity has been achieved, and corrections have been made to early hormone-based conclusions by employing serial rectal ultrasound examinations in conjunction with steroid monitoring. The use of these two tools has revealed that rhinoceroses experience irregular cyclicity, acyclicity, anovulation, persistent anovulatory luteinized follicles, and failure to form a dominant follicle [2,3,9,11,17,18]. In addition, through ultrasonography, early embryo loss has been diagnosed on numerous occasions in clinically healthy rhinoceroses [2,6,18–21], and uterine pathology in the form of cysts and tumors has been repeatedly documented [22,23]. Recently, attentive animal care staff have described a new peculiarity in both black and white rhinoceroses - mid-gestation mammary development and lactation (Dr. Tara Reilly,

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Virginia Zoo, Dr. Priscilla Joyner, The Wilds, and Joseph T. Svoke, Zoo Miami, personal communications).

With many reproductive dysfunctions now identified in rhinoceroses, the next logical scientific steps are to determine why these events/conditions develop and/or persist, what physiological processes are driving them, and what can be done to reduce their prevalence. It is possible that little-studied protein hormones are involved and could shed some light on these questions. With the expansion of animal operant conditioning training in U.S. zoos, it now is possible to routinely collect blood samples voluntarily from many rhinoceroses, providing a biological sample in which protein quantification is likely to be more accurate and successful. One recent example is the assessment of anti-Müllerian hormone in rhinoceros serum [24]. Therefore, the opportunity now exists to expand our repertoire of rhinoceros reproductive hormones so that we are better armed with the tools we need to understand the complexities of these varied reproductive conditions.

Prolactin is one protein hormone of interest given its role in many reproductive functions across species, including reproductive cyclicity and ovarian function, seasonality, pregnancy, lactation as well as emotional and physical stress [25–27]. In fact, prolactin has received significant scientific attention in elephants (another pachyderm) due to the species' propensity for acyclicity. Over 50% of female African elephants are acyclic, and about half of these acyclic females also exhibit hyperprolactinemia [28]. Hyperprolactinemia-induced anovulation and amenorrhea lead to infertility in a significant number of women [29], but high prolactin occurs following years of acyclicity in elephants [30]. Approximately 50% of female white rhinoceroses exhibit acyclicity [3], but any association with hyperprolactinemia is unknown. Furthermore, since prolactin is named for its primary role in lactation [31], it may figure prominently in the mid-gestation lactation condition recently identified. An ELISA effective in detecting rhinoceros serum prolactin would allow for gathering evidence regarding this hormone's involvement (or lack thereof) in these and other aberrant rhinoceros reproductive conditions. However, to date, efforts to analyze rhinoceros prolactin have been minimal and largely opportunistic relying on radioimmunoassays (RIA) designed for measuring the hormone in other species.

In 1991, Kock et al., [32] were the first to report serum prolactin concentrations in a rhinoceros species. They used a human RIA to analyze five serum samples from three pregnant female black rhinoceroses late in gestation and detected low levels of prolactin in two of these samples (2.1 and 8.0 ng/ml). The only other report of rhinoceros prolactin was in a pregnant Sumatran rhinoceros [33]; serum samples were analyzed with an equine prolactin RIA, and concentrations remained low throughout most of gestation (7.17 ± 1.69 ng/ml) but increased significantly two weeks prior to parturition (16 to 76 ng/ml). Together, these studies indicate that prolactin may increase during rhinoceros gestation, but the low concentrations reported suggest the assays were sub-optimal.

The goals of this study were to: 1) identify an enzyme-linked immunosorbent assay (ELISA) effective in measuring rhinoceros serum prolactin, 2) generate prolactin data representing both sexes and females in diverse, known reproductive states, and 3) characterize prolactin throughout pregnancy in a white rhinoceros experiencing mid-gestation lactation.

2. Materials and methods

2.1. Individual samples

Serum samples from 12 individual rhinoceroses (n = 1–6/individual) were strategically chosen for this study from the Center for Conservation and Research of Endangered Wildlife's rhino serum bank (male.female; 1.5 white rhinoceroses, 1.4 black rhinoceroses and 0.1 Sumatran rhinoceros). Rhinoceros age (range: 2–38 years) and reproductive status are detailed in Table 1. Samples were stored in a − 80 °C or − 20 °C freezer for 1–26 years, and most had been thawed previously for other analyses prior to their use in this study. However, freeze-thaw cycles should not significantly impact prolactin concentrations [34,35]. This study was approved by the Cincinnati Zoo & Botanical Garden's Institutional Animal Care and Use Committee (Protocol #19–154) and was conducted following guidelines in the National Research Council's Guide for the Care and Use of Laboratory Animals.

2.2. Pregnant white rhinoceros

In addition to the serum detailed above, serial blood samples (n = 73) recently collected prior to and throughout pregnancy/early lactation from a white rhinoceros were also included. The primiparous 6 y 10 m old female white rhinoceros at the Virginia Zoo was trained for voluntary, weekly blood sample collections and was observed mating with confirmed copulation on February 19, 2020. Subsequent matings were not observed later that year. On November 2, 2020, at approximately 8.5 months of gestation, it was noted that the female's mammary glands were swollen, and this swelling increased over the next couple of weeks. Voluntary trans-mammary ultrasonography (stall-side with behavioral cooperation) performed on November 18 and 20, 2020, revealed no evidence of hypoechoic fluid accumulation within the mammary tissue that would be consistent with lactation at that time. Milking was attempted, but no fluid was expressed. However, on December 12, 2020, animal care staff reported clear fluid actively dripping from both teats, and samples were easily expressed manually from each teat. By January 14, 2021, at 10.5 months of gestation, the rhino's vulva was relaxed, and the udder was filled and swollen. Several (n = 4) mammary secretion samples that grossly appeared to be colostrum were collected over consecutive days in mid-January and sent to the Colorado State University Veterinary Clinical Pathology Laboratory for protein electrophoresis analysis. After the fourth sample was collected on January 18, 2021, it became more difficult to extract secretions from the female's mammary gland, and only volumes < 1.0 ml could be recovered. The last reported sample was obtained on February 8, 2020. Nothing further could be expressed until July 9, 2021, two days prior to parturition. The female gave birth to a healthy male calf on July 11, 2021, following a 16-month, 3-week (508-day) gestation, and she successfully raised her calf.

2.3. Interspecies prolactin gene sequence analysis

The prolactin genomic sequence (Gene ID 101397071) [36] derived from the NCBI annotation (Release 101) of the white rhinoceros genome assembly (RefSeq accession GCF 000283155.1) was used to search the genome assemblies for black, GOH, and Sumatran rhinoceros. Unpublished genome assemblies and sequencing data for black and GOH rhinoceroses were used with permission from the DNA Zoo Consortium (dnazoo.org [37]). Both draft assemblies were generated by the DNA Zoo team from short, insert-size, PCR-free, DNA-Seq data using w2rap-contigger [38], (see Dudchenko et al., [39] for details). Black and GOH rhinoceros genome assemblies were loaded into Anaconda [40], and packages conda and bioProg were used for decompression prior to blasting with the white rhinoceros sequence and extracting the identified similar genome sequences. Genbank's blastn tool was used to interrogate the Sumatran rhinoceros' assemblies (ASM284483v1 and NRM_Dsumatrensis_v1;[41,42]) and obtain the Sumatran rhinoceros prolactin genomic sequence. The retrieved rhinoceros sequences were uploaded into GenScan [43] to identify exon positions and extract the corresponding amino acid sequences. The signaling peptide portion of the sequences was removed prior to using the multiple alignment program for amino acid sequences (MAFFT ver 7; [44] to align the 4 rhinoceros' sequences with the human (NP_000939.1), pig (NP_999091), and horse (NP_001075365.1) mature prolactin sequences. The resulting alignment was subjected to analysis with the

Table 1
Serum prolactin concentrations in rhinoceroses of different species, sex, age, and reproductive status.

Sex	Species	ID	Age	Reproductive Status	Prolactin (ng/ml)
Male	Black rhino	1	16 y 6 m	Proven sire	14.9
	White rhino	1	9 y 10 m	Proven sire	12.7
Female	Black rhino	1	4 y 10 m	Young; sexually mature	37.9
		2	38 y 10 m	Geriatric; not pregnant	23.8
		3	14 y 8 m	Lactating; 13 m post-partum	17.2
		4	2 y 7 m	Sexually immature	17.2
	White rhino	1	5 y 4 m	Luteal phase	37.6
		2	13 y 2 m	Acyclic	13.9
		3	9 y 3 m	Pregnant; 10–11 m pre-partum	81.9 ± 26.8 (n = 4)[a]
		4	8 y 4 m	Pregnant; 8–9 m pre-partum	185.2 ± 43.7 (n = 6)[a]
		5	8 y 5 m	Pregnant; 9–10 m pre-partum	141.9 ± 35.5 (n = 4)[a]
	Sumatran rhino	1	14 y 5 m	Pregnant; 1 m pre-partum	1423.7
		1	14 y 6 m	Pregnant; 6 days pre-partum	806.3

[a] Values are means ± standard deviations.

Sequence Identity and Similarity (SIAS) tool (http://imed.med.ucm.es/Tools/sias.html).

2.4. Prolactin ELISAs tested

Four prolactin ELISAs were tested for their ability to detect prolactin in rhinoceros serum samples. The DetectX Prolactin Immunoassay by Arbor Assays™ (Ann Arbor, MI, USA) was chosen early because the assay had been validated for Asian and African elephants despite its reliance on a monoclonal antibody to human prolactin. This competitive assay had the lowest sensitivity with a standard curve ranging from 15.6 to 1000 pg/ml. ABclonal Porcine Prolactin ELISA Kit (PRL) (RK03363; Woburn, MA, USA) was also tested. This sandwich assay with a standard curve ranging from 0.156 to 40 ng/ml employs a monoclonal antibody targeting the porcine prolactin sequence and was chosen after determining the relatively high sequence pairwise identity between porcine and rhinoceros prolactin. The third assay tested was Nori® Equine Prolactin ELISA Kit by Genorise (GR 106523–1; Glen Mills, PA, USA), a sandwich ELISA that utilizes a monoclonal antibody to equine prolactin and has a standard curve ranging from 47 to 3000 pg/ml. Finally, an assay by CUSABIO®, Horse Prolactin (PRL) ELISA kit (CSB – ELO18724HO; Houston, TX, USA) which relies on an equine prolactin polyclonal antibody was tested. This assay is a competitive ELISA with a standard curve ranging from 2.5 to 1000 ng/ml.

2.5. Prolactin assay validation

Initial assay validation testing was conducted using two separate pooled serum samples, one consisting of serum from several black rhinoceroses (n = 6) and another made from serum of white rhinoceroses (n = 4). These pooled samples were serially diluted (1:4–1:512 or neat–1:8) and tested for parallelism with the kit standard curves. All samples were processed and analyzed neat or diluted in accordance with each assay's instructions using the reagents provided in the kits.

Secondary biological validations were conducted for the CUSABIO assay by analyzing samples from both black and white rhinoceroses of known sex and, in the case of females, known reproductive status. Samples were strategically selected based on existing data from previous reproductive evaluations (luteal, acyclic, pregnant) or data gleaned from ZIMS [45] regarding age, stage of pregnancy, or lactation.

Finally, serial samples (1–4/month; n = 44) collected from a primiparous female that exhibited mid-gestation mammary development and secretions were analyzed to produce a complete prolactin profile from conception to parturition and early post-partum lactation. These samples were diluted 1:4 to 1:10 prior to analysis to ensure values landed on the standard curve since higher prolactin concentrations were detected in pregnant rhinoceros samples during assay validation.

2.6. Serum progesterone analysis

In addition to prolactin, progesterone was analyzed in the serial samples (~4/month; n = 73) from the pregnant, primiparous rhino. Most of the female rhinoceros serum samples listed in Table 1 were also analyzed for progesterone prior to this study, and the information related to that previous work was utilized when choosing samples from females in different reproductive states (i.e., luteal = elevated progesterone but not pregnant; acyclic = consistent baseline progesterone over 6 weeks or more; pregnant = high progesterone concentrations over 4–6 consecutive weeks followed by parturition within 12 months of sampling). The birth date of the calf was used to count the months backward when determining the stage of gestation (pre-partum) when samples were analyzed because breeding dates were unknown. The Arbor Assays Progesterone mini-kit (ISWE003) was used for all serum progesterone analyses. This kit utilizes an antibody previously validated for rhinoceros serum progesterone (CL425, Coralie Monroe, University of California – Davis, CA, USA [24,46]).

3. Results

3.1. Interspecies prolactin gene sequence analysis

Alignment of the mature prolactin sequences revealed that much of the protein is conserved across all species (Fig. S1A). Sequence pairwise identity ranged from 98.99% to 100% among the four rhinoceros species, and when the rhinoceros sequences were compared to those of the horse and pig, pairwise identity remained high at ~96% and ~95%, respectively (Fig. S1B). In contrast, pairwise identity was only ~81% between human and rhinoceros sequences.

3.2. Prolactin assay validations

Parallelism was not detected between serially diluted, pooled rhinoceros serum samples and the standard curves of the first three prolactin assays tested. Sample values were all very similar and fell below the lowest standard of each kit. In contrast, the CUSABIO horse prolactin assay proved effective in measuring rhinoceros serum prolactin. Linearity was observed for the serially diluted black rhinoceros pooled sample and white rhinoceros pooled sample (Pearson's $r > 0.98$). Parallelism with the standard curve (2.5 to 1000 ng/ml) was confirmed for both species (Comparison of slopes $P = 0.155$ with shared $R^2 = 0.96$, Fig. S2). Intra-assay coefficients of variability (CVs) were < 10% but too few assays were performed to accurately characterize inter-assay variability. Additionally, biological validation appeared to be achieved when individual samples were analyzed. Pregnant rhinoceros serum contained the highest prolactin concentrations. Males and acyclic or sexually immature females had much lower prolactin

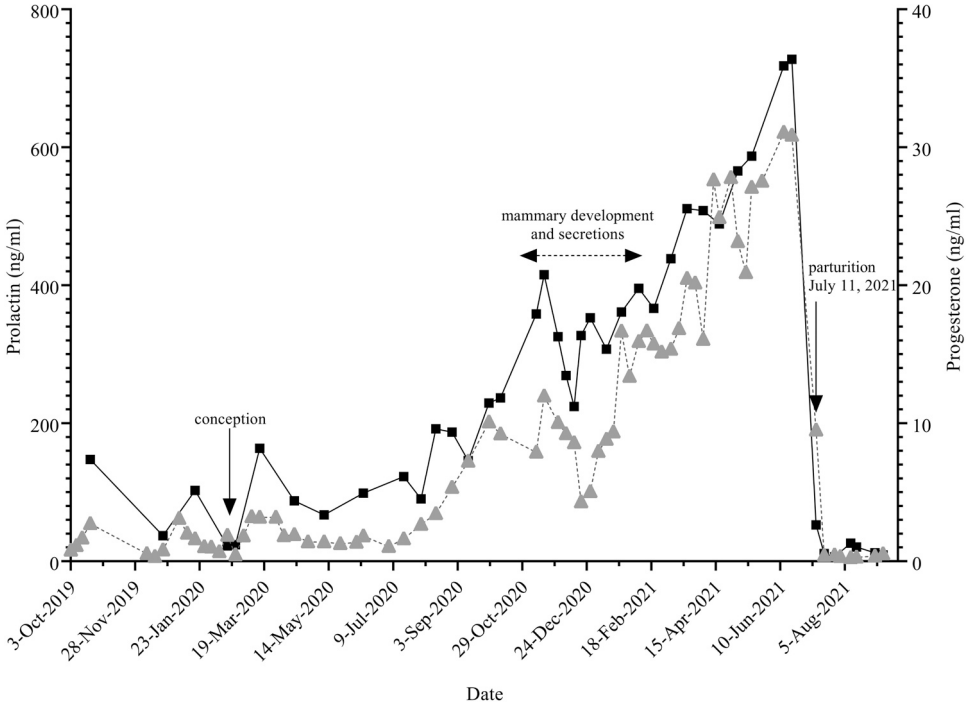

Fig. 1. Serum progesterone (grey dashed line; triangle symbols) and prolactin (black solid line; square symbols) concentrations (ng/ml) throughout successful breeding, conception, gestation, parturition, and early lactation in a white rhinoceros that exhibited mid-gestation mammary development and colostrum production.

concentrations, and sexually mature females experiencing reproductive cyclicity had moderate prolactin concentrations (Table 1). A single sample analyzed from a lactating female 13 months post-partum was also low in prolactin, with a value similar to those observed in male and acyclic female samples (Table 1). Finally, the two samples from the pregnant, near-term Sumatran rhinoceros contained very high prolactin concentrations, similar to, or even higher than those recorded near the end of gestation in the white rhinoceros profile.

3.3. Prolactin profile of pregnant white rhinoceros

The prolactin concentrations varied from 22.5 to 148.8 ng/ml (mean ± SD; 66.9 ± 55.9 ng/ml) in the five samples collected during reproductive cyclicity immediately prior to conception (Fig. 1). The lowest prolactin concentration measured in any serum sample collected post-conception and prior to parturition was 67.5 ng/ml. Prolactin increased sharply at seven months of gestation and then stayed elevated above 200 ng/ml throughout the rest of pregnancy, falling to 52.9 ng/ml on the day of parturition (Fig. 1). Notably, eight months prior to parturition (Nov. 2020) prolactin concentrations spiked upwards (values ranged from 325 to 415 ng/ml) for about one month before dropping by almost 50%. Afterward, prolactin concentrations gradually increased throughout the rest of gestation to a high of 727.6 ng/ml in the sample collected three weeks before delivery. During the period of mammary development and secretions, 6–8 months prior to parturition, mean prolactin was 322.6 ± 58.1 ng/ml. Prolactin concentrations during the first two months of post-partum lactation ranged from 6.5 to 26.5 ng/ml (n = 6; 14.4 ± 7.6 ng/ml).

3.4. Progesterone profile of pregnant white rhinoceros

Progesterone remained slightly elevated above baseline two weeks following the February 19, 2020 mating, never dropping below 1.0 ng/ml; then it increased markedly in August at ~6 months of gestation (Fig. 1). Progesterone remained elevated but did exhibit a substantial dip in concentration in December 2020 at ~10 months of gestation. It increased again by 12 months of gestation and continued to rise, reaching 31.0 ng/ml at 16 months, 3 weeks of gestation before dropping precipitously to 9.5 ng/ml at parturition on July 11, 2021.

3.5. Mammary secretion analysis

Results of the mammary secretion analysis were valuable in ruling out mastitis or other health-related issues that cause mammary gland swelling, and for confirming the presence of colostrum (evidence of true lactation). Total protein in the four samples of mammary gland secretions collected at ~10.5 months of gestation ranged from 11.5 g/dl to 29.2 g/dl (mean: 22.7 ± 7.8 g/dl; Fig. 2). All four samples exhibited a distinct pre-albumin band expected to contain predominantly lipoproteins. A broad and tall gamma globulin region accounted for most of the sample protein 57.7 – 63.0% (mean: 61.5 ± 2.6) and was likely comprised predominantly of IgG. In contrast, total protein in the comparative serum sample was 7.9 g/dl with only 28.8% of that represented by the gamma globulin fraction.

4. Discussion

The results of this study demonstrate the efficacy of the CUSABIO horse prolactin ELISA in detecting and quantifying prolactin in black and white rhinoceros serum. The ability to measure rhinoceros prolactin via this simple ELISA creates a new avenue for expanding our knowledge of the reproductive physiology of this taxon and may provide insight into the reproductive dysfunctions experienced by these species. Key to the assay's success was its reliance on a polyclonal antibody generated against equine prolactin, a result in accordance with two previous rhinoceros studies that also employed polyclonal anti-equine prolactin antibodies, one that measured Sumatran rhinoceros prolactin in a RIA [33] and another that detected black and white rhinoceros pituitary prolactin via western blot [47]. The ≥ 96.48% homology between the horse prolactin genetic sequence and that of the rhinoceroses confirmed a strong relationship between the species but does not guarantee antibody cross-reactivity as evidenced by the failure of the Nori Equine Prolactin ELISA that relies on a monoclonal anti-equine prolactin antibody. Likewise, the monoclonal pig antibody-

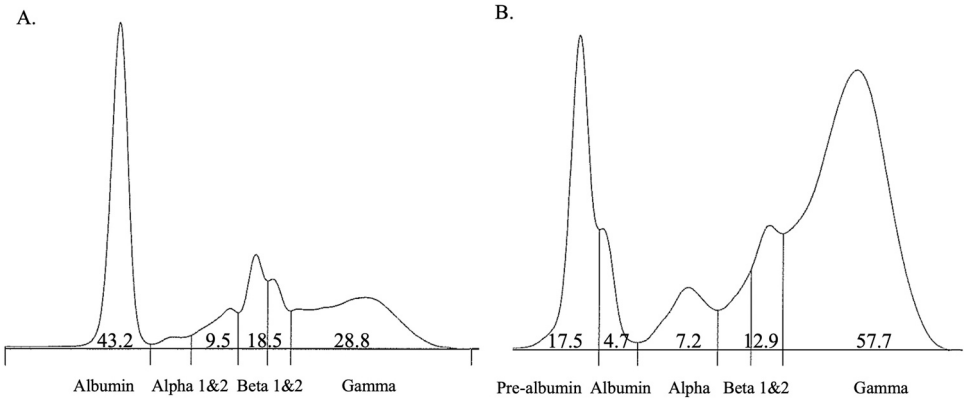

Fig. 2. White rhinoceros serum (A) and colostrum (B) protein electrophoresis results. Numbers represent the percentage of total protein. Total protein concentrations were 7.92 g/dl in serum (A) and 29.2 g/dl in colostrum (B). (Colorado State University Veterinary Clinical Pathology Laboratory).

based assay (ABclonal Porcine Prolactin ELISA Kit) failed to detect rhinoceros prolactin despite a ≥ 94.97% pairwise identity between the porcine and rhinoceros prolactin sequences, so it is possible that a polyclonal porcine prolactin antibody would be successful. In contrast, the prolactin sequence comparison between rhinoceroses and humans revealed just 80.9–81.9% pairwise identity which could explain the failure of Arbor Assay's prolactin ELISA since it relies on a monoclonal antibody to human prolactin. Bach et al., 2021 [48], briefly reported a lack of parallelism when rhinoceros serum was analyzed in this assay, and our negative findings agree with their results. No GOH rhinoceros serum samples were tested in this study but given its prolactin sequence similarity when compared to the other three rhinoceros species tested (98.99%), GOH rhinoceros prolactin will likely also be detectable by the CUSABIO assay.

Biological validation of prolactin in a new species is challenging due to prolactin's highly variable biological function across diverse species [25,27], but because the horse is the closest domestic relative of the rhinoceros and they share some reproductive similarities, it is the model often used. Not surprisingly, the highest prolactin concentrations were in samples from pregnant rhinos. Just 5–6 months into their 16–17-month gestation, they were producing prolactin concentrations 5X higher than those of non-pregnant, cycling rhinoceroses (Table 1). Though originally named for its essential role in preparing mammals for lactation [31], prolactin tends to be elevated in most mammals during pregnancy and can play a role in many adaptations required during the maternal pregnant state. However, patterns of secretion vary broadly across species. The pregnant rhinoceros prolactin values in Table 1, in conjunction with the complete pregnancy prolactin profile in Fig. 2, demonstrate increasing prolactin throughout white rhinoceros pregnancy in a pattern more closely resembling that for humans or sheep [27] rather than that for mares in which prolactin only spikes significantly during the last week of gestation [49]. These findings are in contrast with the previous report of serum prolactin in a pregnant Sumatran rhinoceros that exhibited a pattern similar to that of the horse [33]. However, peak values just prior to parturition in that previous report were substantially lower (16 to 76 ng/ml) than values determined herein using the CUSABIO assay to measure prolactin in samples from that same pregnancy 1 month or 6 days prior to delivery (1424 and 806 ng/ml, respectively). Therefore, it seems plausible that the previous assay was detecting only a fraction of the prolactin in the samples and may have lacked the sensitivity necessary to document less substantial increases in prolactin that may have occurred earlier in gestation. Alternatively, Sumatran and white rhinoceros prolactin production throughout pregnancy may differ.

Lower prolactin concentrations in male rhinoceroses and females that were acyclic or sexually immature is likely related to the fluctuations in prolactin that can be associated with ovarian activity, estrogen concentrations, luteolysis, and/or prostaglandin secretion in reproductively active females [27]. These relationships differ across species, and our data were very preliminary and insufficient to speculate on any specific role for prolactin in these capacities in the rhinoceros. One unexpected result was the low prolactin concentrations measured in samples of lactating females. The initial sample tested (Table 1; 17.2 ng/ml) was collected late in lactation (13 months post-partum) in a black rhinoceros, but comparable results (6.5 to 26.5 ng/ml) were observed in the samples collected during early lactation in a white rhinoceros (1–8 weeks post-partum; Fig. 2), despite successful lactation and calf rearing. It is relatively well-documented that bouts of nursing stimulate pulses of prolactin during lactation [27], and it is highly unlikely that blood was collected from this female rhinoceros within minutes of calf suckling, but even if such associated peak concentrations were missed, some elevation in circulating prolactin would be expected if prolactin plays a role in post-partum lactation. Although prolactin is essential for lactation in some lab species [50,51], its role is less clear in ruminants since secretion can be blocked and lactation does not necessarily cease [52,53]. In horses, prolactin drops to baseline during the first 1–2 months of lactation [49,54] suggesting a similarly limited or temporary role in equine lactation.

The prolactin and progesterone profiles of the white rhinoceros that exhibited mid-gestation lactation revealed three specific patterns of potential interest. First, progesterone and prolactin concentrations trended together throughout gestation, parturition, and lactation (with a few periods of brief divergence), and prolactin concentrations were 10–20X higher than those for progesterone. Second, prolactin remained elevated post-conception, even more prominently than progesterone, a pattern not unlike that of rodents in which prolactin is responsible for rescuing the post-ovulatory corpus luteum and stimulating progesterone production in early pregnancy [27]. Maternal recognition of pregnancy in the rhinoceros remains a mystery since the presence of chorionic gonadotropin was ruled out years ago [55,56], so it is possible that prolactin plays a role via its luteotropic properties. However, the similarity between the rat and white rhinoceros disappears by mid-gestation since prolactin declines in the rat but continues increasing in the white rhinoceros as it does during human and sheep pregnancies [27]. The third pattern of interest was a significant spike in prolactin that occurred in conjunction with reports of mammary development eight months prior to parturition. Prolactin ranged from 325.7 to 415.6 ng/ml in November compared to a range of 90.4 to 236.7 ng/ml in the three preceding months.

Following the November spike in prolactin, mammary secretions were first collected from the teats in mid-December and collections continued through mid-January during which prolactin decreased slightly and plateaued. These mammary secretions initially were collected for analysis to rule out mastitis, a staff concern at the time, and secondarily to confirm colostrum production which would warrant preparations for an imminent birth. The test results were consistent

with colostrum based on a high total protein content comprised primarily of gamma globulin (61.5%) and the semblance to black rhinoceros mammary secretions (54% gamma globulin) collected 10 days prior to calving [57]. Interestingly, progesterone concentrations also appeared to drop sharply from 12.0 to 4.3 ng/ml in December before recovering in mid-late January and proceeding to increase until the day of parturition. Although notable, exactly how, or even if, one or both of these hormone fluctuations catalyzed the unusual mammary development and mid-gestation colostrum production cannot be concluded without more data. Prolactin concentrations for three additional pregnant white rhinoceroses are reported in Table 1 and appear to be lower. Only one of these rhinoceroses (white rhinoceros #4) was sampled eight months pre-partum, and her prolactin concentrations ranged from 101.0 to 197.8 ng/ml, considerably lower than the 325.7 to 415.6 ng/ml measured in serum from the female that experienced mid-gestation lactation profiled herein. Although female #4 did not exhibit early lactation, female #5 did (personal communication, Dr. Priscilla Joyner, The Wilds), and the timing of her mammary development and secretions was similar to that of our profiled rhino. Female #5's prolactin concentrations at 9–10 months pre-partum are not as high as those of our profiled rhinoceros, but samples were not collected during that critical 8-month pre-partum time period, so we cannot determine if prolactin spiked as it did in our profiled rhinoceros prior to colostrum secretion. If a prolactin surge, or a progesterone dip, or the combination of the two are involved in this newly described aberrant reproductive state, it is possible the degree of change, rather than a specific hormone concentration, initiates the physiological chain of events leading to untimely colostrum production. Therefore, complete serum profiles for additional pregnant rhinoceroses that do and do not experience mid-gestation lactation will be necessary to better understand the role of these hormones in this dysfunctional phenomenon.

5. Conclusions

With an effective, commercially available ELISA now identified for measuring prolactin in rhinoceroses, we have a new tool for expanding knowledge of the basic reproductive physiology of this taxon. Our results indicated that prolactin is significantly elevated throughout white rhinoceros pregnancy but did not support a role for it during postpartum lactation. Additionally, our findings suggest that further investigation into the relationship between gestational prolactin fluctuations and premature mammary development and lactation is warranted. Although unusual, this reproductive phenomenon of colostrum production during gestation should not cause animal caregivers excessive alarm as all three of the cases known to the authors ended with the successful delivery of healthy, full-term calves. Admittedly, this study was not a large-scale evaluation of prolactin in rhinoceros populations, but it does set the stage for more robust and targeted studies to better understand prolactin's role in normal reproductive function while determining any involvement in aberrant reproductive conditions experienced by rhinoceroses in human care. The study of prolactin is made possible by the outstanding efforts of so many animal care staff training their rhinos via operant conditioning for voluntary blood collection; however, we acknowledge that this is an invasive procedure, and the transport of samples across international borders for rhinoceros research can be challenging due to regulatory requirements that don't apply to noninvasively collected fecal samples.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.therwi.2023.100035.

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