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Use of a simplified non-invasive technic to monitor fecal progesterone metabolites and reproduction function in several zoo species: Efficacy of mini VIDAS® automate (bioMérieux)

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ABSTRACT

Developing the zoos' ability to assess the reproductive status of the individuals they house is essential to improve the husbandry and management of these species. The use of non-invasive techniques such as fecal hormone analysis has been proven to be a simple and effective way to achieve this. Designed by bioMérieux, mini VIDAS® instrument is used in human and veterinary medicine to evaluate different endocrinological parameters, including serum or plasma progesterone. This study evaluates VIDAS® Progesterone (PRG) assay's efficacy to monitor fecal progestagens using a simple sample extraction protocol adapted to the zoo environment. We compared (1) VIDAS® PRG fecal profiles with established assays specifically designed for fecal progestagens analysis at the VetmedUni (Vienna, Austria) for okapis (*Okapia johnstoni*), greater one-horned rhinoceros (*Rhinoceros unicornis*), giraffes (*Giraffa camelopardalis reticulata*) and hippopotamus (*Hippopotamus amphibius*) (2) VIDAS® PRG fecal profiles with VIDAS® PRG serum profiles for African elephants (*Loxodonta Africana*), giant anteater (*Myrmecophaga tridactyla*) and white rhinoceros (*Ceratotherium simum*). Spearman mean correlations were: 0.6748 for African elephants (n = 2 animals), 0.7969 for giant anteater (n = 1 animal), 0.7926 for okapis (n = 2 animals), 0.6072 for greater one-horned rhinoceros (n = 4 animals), 0.6062 for giraffes (n = 4 animals) and 0.5740 for hippopotamus (n = 2 animals). Fecal progestagens analysis revealed estrous cycles in several species: 12.5 ± 0.5 weeks for African elephants (n = 2 cycles), 15.3 ± 1.1 days for okapis (n = 6 cycles), 44 ± 2.1 days for greater one-horned rhinoceros (n = 4 cycles) and 15.5 ± 0.5 days for giraffes (n = 4 cycles). We observed pregnancies in a giant anteater, an okapi and a hippopotamus. We observed a strong positive Spearman correlation (r > 0.60) for individuals exhibiting estrous cycles. These first results indicate that the mini VIDAS® can be used for monitoring of the reproductive status of non-domesticated species and can be a useful tool for the reproductive management through fecal progesterone analysis. A simple extraction protocol was suitable for sample preparation of fecal progesterone metabolite analysis. Further studies using a larger number of individuals per species at different reproductive stages could confirm the relevance of mini VIDAS® in the zoo community.

1. Introduction

Zoos have a fundamental role in the *ex-situ* conservation of endangered species. These structures represent a genetic conservatory by allowing the reproduction of the species they house. Proximity to the animals enables researchers to better understand their biology, particularly at a reproductive level. Determining animals' reproductive status in zoos allows effective management and can facilitate the reproductive success of many species [1–3]. The most accurate and commonly used indirect method, in this case, is the assessment of endocrine status. Hormones are present in many biological matrices such as blood, urine, and feces [3,4]. This information is also crucial if assisted reproductive technologies such as artificial insemination or in vitro fertilization are used [1,2].

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Repeated measurements of blood progesterone appear to be the most accurate approach for monitoring animals' reproductive function. This method reflects variations in circulating progesterone concentrations at a given time with little or no latency. However, the capture or restraint of an animal, necessary for blood collection, is often accompanied by stress limiting a regular application of this procedure. As a result, non-invasive methods using urine and feces have been developed [2–5]. Indeed, steroid hormones such as progesterone are mainly metabolized by the liver and excreted in the urine or feces [6]. Nevertheless, urine collection remains difficult. Since urine collection remains difficult in most cases, fecal samples are preferred for non-invasive monitoring of zoo animals' reproductive status [1,4,5].

There is little or no native progesterone in feces. Fecal metabolites of progesterone (progestagens) are categorized in 5α- or 5β-reduced pregnanes, and these are further subdivided depending on the presence of either a 20-oxo, 20α- or 20β-OH group. Each species excretes several progesterone metabolites [1,6]. For several decades, immunoassays using broad-spectrum antibodies targeting progesterone and cross-reacting with progestagens have been developed [2,3,5,6]. Enzyme immunoassays (EIAs) are simple to use and less expensive than radioimmunoassays (RIAs). Therefore, EIAs are preferred in many settings, including zoos [4,7]. However, these techniques require qualified personnel and process time not necessarily available in zoos. Mini VIDAS® (bioMérieux) is an automatic bench-top instrument based on ELFA (Enzyme-Linked Fluorescent Assay), an EIA technology. This instrument can evaluate various endocrinological parameters. Regarding reproduction, VIDAS® Progesterone (PRG) assay is used in humans [8] and veterinary medicine [9,10] to assess serum or plasma progesterone. It represents an ideal candidate for regular monitoring of animals' reproductive status in zoos.

This study was conducted to verify whether VIDAS® Progesterone (PRG) assay can, to a certain extent, allow the assessment of the reproductive status of zoo animals via fecal samples. Here we compared (1) VIDAS® PRG fecal profiles with established assays specifically designed for fecal progestagens analysis at the VetmedUni (Vienna, Austria) for okapis (*Okapia johnstoni*), greater one-horned rhinoceros (*Rhinoceros unicornis*), giraffes (*Giraffa camelopardalis reticulata*) and hippopotamus (*Hippopotamus amphibius*) (2) VIDAS® PRG fecal profiles with VIDAS® PRG serum profiles for African elephants (*Loxodonta Africana*), giant anteater (*Myrmecophaga tridactyla*) and white rhinoceros (*Ceratotherium simum*). This comparison with blood samples or other validated non-invasive methods was performed to demonstrate VIDAS® Progesterone (PRG) assay efficacy in monitoring the reproductive status of various non-domestic species directly in the zoo.

2. Materials and methods

2.1. Animals

Animals in this study included female African elephants (n = 2), white rhinoceros (n = 2), greater one-horned rhinoceros (n = 4), giraffe (n = 4), okapi (n = 2), hippopotamus (n = 2), and giant anteater (n = 1) housed at ZooParc de Beauval in France (Table 1). All the animals were adults except two juvenile greater one-horned rhinoceroses. In general, females were housed individually but close to males. For each species, males and females were put together during periods of interest for the opposite sex.

2.2. Blood sample collection and processing

Blood sampling was voluntarily performed through medical training implemented prior to this study. Blood samples were collected one time per week from female African elephants (n = 2), white rhinoceros (n = 2), and giant anteater (n = 1). Samples were collected in 5 mL BD Vacutainer® Serum tubes then centrifuged (15 min at 3000 rpm). The serum was stored at −20 °C until analysis.

2.3. Fecal sample collection and processing

Fecal samples were collected two times per week for 6 months from African elephants (n = 2), 4 months from greater one-horned rhinoceros (n = 4), and 3 months from white rhinoceros (n = 2) and hippopotamus (n = 2). Samples from okapis (n = 2) and giraffes (n = 4) were collected three to four times per week for 3 months. Only one fecal sample per week could be collected from giant anteater (n = 1) for 3 months. Keepers collected only fresh fecal samples (recently defecated) in freezer bags before being stored at −20 °C until the extraction process. For all females (except those collected in blood), each fecal sample was divided into two aliquots. One was analyzed directly at the ZooParc de Beauval clinic with mini VIDAS® PRG and the second was sent to the endocrine laboratory of the Vetmeduni Vienna, Austria (Unit of Physiology, Pathophysiology and Experimental Endocrinology) for a fecal progestagen assay (okapi: [11,12]; greater one-horned rhinoceros: [13]; data unpublished for common hippopotamus and giraffes).

The fecal extraction protocol was set up by following an assembly of data collected in the literature [14] in such a way as to be easily feasible in zoos. Frozen feces were thawed then crushed. A 0.5 g aliquot was then placed into a plastic tube and 5 mL of 80% EtOH was added to extract progesterone metabolites. The mix was then vortexed with an automatic shaker for 30 min and then centrifuged 15 min at 2000 rpm. An aliquot of the supernatant (containing progesterone metabolites) was recovered and transferred into a plastic cryotube before being stored at −20 °C until analysis.

2.4. Enzyme immunoassays

We used the automated instrument mini VIDAS® (bioMérieux) for Vitek ImmunoDiagnostic Assay System to analyze serum and fecal extracts. Mini VIDAS® uses a VIDAS® Progesterone (PRG) assay combining a competitive enzyme immunoassay method with a final detection by fluorescence called ELFA (Enzyme-Linked Fluorescent Assay). This assay uses a single-use cone (Solid Phase Receptacle (SPR)) as a solid phase and pipetting system. Each cone is sensitized with mouse monoclonal anti-progesterone immunoglobulin at the time of manufacture. All the reagents necessary for the immunological reaction are in the sealed reagent strips. 200 μL of the sample (serum or fecal extract) were dispensed in the reagent strip's first well. Samples were identified and then scanned by the instrument that automatically programmed the Progesterone (PRG) assay. The mini VIDAS controls all steps and temperatures of the test. This assay has been validated in-house on the serum of elephants, white rhinos and giant anteaters (unpublished results).

In the instrument, sample is diluted in 600 μL of dilution buffer (0.1 mol/L sodium phosphate, pH 7.5 + protein stabilizer + 1 g/L sodium azide). Washing steps (0.1 mol/L sodium phosphate + 0.3 mol/L NaCl, pH 7.5 + 1 g/L sodium azide) remove unbound compounds and 600 μL of conjugate (alkaline phosphatase labelled progesterone derivative + 1 g/L sodium azide) is added. Unbound conjugate is washed out (Tris-NaCl 0.05 mol/L, pH 7.4 + 1 g/L sodium azide). 300 μL of the substrate (4-Methyl-ombelliferyl phosphate 0.6 mmol/L + diethanolamine 0.62 mol/L, pH 9.2 + 1 g/L sodium azide) is added and hydrolyzed by alkaline phosphatase to a product (4-Methyl-ombelliferone) with emitted

Table 1
History of studied individuals.

Species	Name (studbook number)	Date of birth	Number of offspring (♂/♀)
African elephants	Ashanti (20014F)	Jan. 2003	0
	N'Dala (8908)	Jan. 1989	1/0
Southern white rhinoceros	Mafu (1463)	May 2001	0/1
	Satara (1307)	Feb. 1998	3/0
Greater one-horned rhinoceros	Saathi (360)	Nov. 2005	0/2
	Henna (432)	Jul. 2010	1/1
	Sananda (556)	Jan. 2018	0
	Anjali (574)	Aug. 2019	0
Reticulated giraffe	Chloé (4-4506)	Mar. 2013	0
	Baya (4-4509)	Mar. 2013	0/1
	Binti (4-4354)	Feb. 2012	0
	N'Zuri (4-4402)	Mar. 2012	0
Okapi	Ann (640)	Nov. 2008	0
	Tafari (701)	Oct. 2012	1/0
Common hippopotamus	Kiwi (T1374)	Jul. 2010	0
	Bolinhas (T1416)	Nov. 2014	0/1
Giant anteater	Aurora (0846)	Jul. 2007	2/3

fluorescence measured at 450 nm. Mini VIDAS® performs two fluorescence measurements in the reading cuvette for each test. The first one considers the background noise due to the substrate cuvette before contact with the cone. The second is performed after incubation of the substrate with the enzyme. The Relative Fluorescence Value (RFV) is the result of the difference between these two measurements. From a calibration curve, each result is expressed in ng/mL by the instrument. These results were then transformed into ng/g wet fecal weight for the fecal samples.

The mouse monoclonal anti-progesterone immunoglobulin cross reacts with 100% progesterone and to a lesser degree with several metabolites of progesterone such as 20α-hydroxyprogesterone (0.03%), 6β-hydroxyprogesterone (0.29%), 16α-hydroxyprogesterone (0.20%), 5β-dihydroxy progesterone (17.39%), 5α-dihydroxyprogesterone (12.95%) and 17α-hydroxyprogesterone (1.18%). Low cross-reactivity is observed with other steroids such as deoxycorticosterone (1.15%), corticosterone (0.09%), testosterone (0.01%), estrone (0.01%), estradiol and estrone (<0.01%). Assay sensitivity of VIDAS® Progesterone (PRG) is 0.25 ng/mL and can detect up to 80 ng/mL.

Assay validation was performed by comparing a serial dilution of a fecal extract of each species with the standard progesterone curve (see section 3.1). Extracts from standard and species samples were serially diluted (1:2 ratio). Besides, intra-assay variation (12 replicates of the same pool in same conditions) and inter-assay variation (2 replicates each day for 6 days) were calculated to be <10% and <15%, respectively.

Assays carried out by the endocrine laboratory of the Vienna University of Veterinary Medicine are directed towards pregnanediol or 20α–OH–pregnanes (Pg-diol) for okapi [11,12] and greater one-horned rhinoceros [13], and towards 20-oxo-pregnanes (20-oxo-P) for hippos and giraffes (assay described in Ref. [6]; assay results unpublished for these two species).

2.5. Data analysis

Statistical tests were performed on the Rstudio software. Data are presented as mean ± SEM. Parallelism test was carried out by comparing the standard curve with serial dilutions of fecal extracts from each species by a *t*-slope comparison test. A baseline progestagen was calculated using an iterative process [15]. Thus, each value above mean plus 1.5 standard deviations (SD) was discarded. The elimination process was repeated by recalculating the average until no value exceeded the mean plus 1.5 SD. The beginning of the luteal phase (LP) was defined as the first point after an increase in values above the baseline for at least three consecutive values. The end of the LP and the beginning of the follicular phase (FP) was established as the first of the two values returning to the basal level [15]. Estrous cycle length was calculated from the beginning of one luteal phase to the beginning of the next (or using the follicular phase if the kinetics start in the luteal stage). A period longer than twice the length of a normal follicular phase between two luteal phases was considered as anoestrus (8–14 weeks for African savannah elephant [16,17]; 30 days for Southern white rhinoceros [15,18]; 30 days for greater one-horned rhinoceros [13,19]; 15 days for giraffe [20,21]; 15 days for okapi [11,22]; 20 days for giant anteater [23]; and not determined for common hippopotamus). The normality of fecal progestagen profiles was examined using the Shapiro-Wilk test. Significant differences in the non-parametric data were evaluated using the Mann-Whitney test for two groups (e.g., basal fecal progestagen excretion and luteal phase values). We performed Spearman rank correlation tests to assess the correspondence between (i) the means of each fecal sample assessed with the mini VIDAS® and its aliquot analyzed by the Vienna laboratory, (ii) the weekly means of fecal progestagens and serum progesterone (both measured by the mini VIDAS®). Spearman rank correlation test is used to determine whether there is a relationship between the rank of observations for two variables (interpretations: $-1 \leq r \leq 1$; 0.00-0.19: “very weak”, 0.20-0.39: “weak”, 0.40-0.59: “moderate”, 0.60-0.79: “strong”, 0.80–1.0: “very strong”). Since fecal and blood samples were not necessarily collected simultaneously, a weekly mean value was calculated to compare the individuals concerned ($n = 5$).

3. Results

3.1. Intra-assay and inter-assay CVs and parallelism test

For all species, intra-assay CVs (high and low) are less than 10%. Inter-assay CVs for all species (high and low) are less than 15% (Fig. 1A). For the parallelism test, serial dilutions of fecal extracts of each species, except the southern white rhinoceros (t-slope test, $P < 0.05$), gave a curve shift parallel to the standard curve (Fig. 1B, $P > 0.05$).

Correlations between fecal progestagens results with VIDAS® technology and serum progesterone or validated methods for non-invasive monitoring of reproductive status are presented on a species-by-species basis.

A

Species	CV intra						CV inter					
	HQC			LQC			HQC			LQC		
	Mean (ng/g)	SD (ng/g)	CV (%)	Mean (ng/g)	SD (ng/g)	CV (%)	Mean (ng/g)	SD (ng/g)	CV (%)	Mean (ng/g)	SD (ng/g)	CV (%)
Okapi	615,44	33,57	5,46	40,08	2,68	6,69	416,77	41,69	10,00	33,63	2,59	7,7
Greater one-horned rhinoceros	114,73	8,18	7,13	30,28	2,02	6,67	96,45	7,68	7,96	23,03	2,63	11,42
Giraffe	450,76	30,40	6,74	40,03	3,43	8,56	437,51	47,15	10,78	33,83	4,15	12,27
Hippopotamus	405,81	29,37	7,24	33,00	3,25	9,84	410,52	29,63	7,22	35,32	4,09	11,59
African elephant	189,60	12,02	6,34	21,96	1,72	7,85	143,07	10,75	7,51	30,80	3,07	9,95
White rhinoceros	63,07	3,74	5,93	28,61	1,49	5,20	62,55	5,59	8,94	36,27	2,78	7,67
Giant anteater	228,63	14,31	6,26	38,58	2,28	5,91	189,74	10,60	5,58	37,16	1,57	4,23

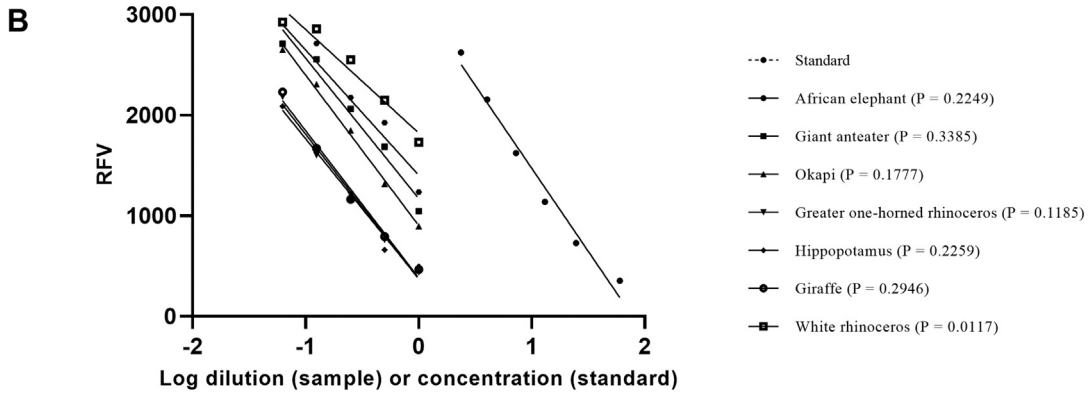

Fig. 1. (**A**) Intraassay and interassay CV results. CV, coefficient of variation; HQC, high-level quality control; LQC, low-level quality control. (**B**) Parallelism test of VIDAS® Progesterone (PRG) assay used with fecal samples. Comparison between progesterone standard and species samples from African elephant, giant anteater, okapi, greater one-horned rhinoceros, hippopotamus, giraffe and white rhinoceros. RFV, Relative Fluorescence Value.

3.2. Okapi

Progestagens values measured with VIDAS® technology correlated strongly positively with Pg-diol values measured for “Ann” ($r = 0.8737$, $P < 0.001$) and “Tafari” ($r = 0.7115$, $P < 0.001$). Moreover, both females studied showed variations in fecal progestagens corresponding to estrous cycles according to Pg-diol kinetics (Fig. 2A). Average estrous cycle length for female “Ann” was 15.5 ± 1.7 days (FP: 7.5 ± 1.71 days; LP: 8 days; n = 4 cycles) and 15 ± 1 days (FP: 5 ± 1 days; LP: 10 days; n = 2 cycles) for female “Tafari” (Table 2). An anoestrus period started on Day 68 of the sampling period for “Ann”. VIDAS® PRG values were 70.18 ± 3.73 ng/g and 571.49 ± 80.23 ng/g in the follicular and in the luteal phase, respectively (Table 2). For “Tafari”, a variation in values indicated a beginning pregnancy after Day 40 of the investigation and this was confirmed by Pg-diol kinetics. Thus, baseline values of this female were 64.24 ± 5.12 ng/g while luteal values were 1111.51 ± 291.57 ng/g, and pregnancy values were 2408.13 ± 125.98 ng/g. For this species, luteal phase values were higher than basal values (Mann-Whitney test, $P < 0.001$). Moreover, pregnancy values were higher than basal values (Mann-Whitney test, $P < 0.001$) and luteal values (Mann-Whitney test, $P < 0.001$).

3.3. Reticulated giraffe

Progestagen profiles obtained with VIDAS® technology had a very strong positive correlation with 20-oxo-P profile for female “Binti” ($r = 0.9293$, $P < 0.001$); this female had, according to 20-oxo-P kinetics of Vienna laboratory, clear estrous cycles (Fig. 2B). The average estrous cycle length of “Binti” was 15.5 ± 0.5 days (FP: 5 ± 0.58 days; LP: 10.5 ± 0.5 days; n = 4 cycles; Table 2). Basal VIDAS® PRG values (71.14 ± 3.3 ng/g) were lower (Mann-Whitney test, $P < 0.001$) than luteal phase values (775.77 ± 155.6 ng/g). For three other females, which had no luteal and thus no estrous cycle activity, a weak correlation was observed (“Chloe”: $r = 0.3896$, $P = 0.0375$; “Baya”: $r = 0.3024$, $P = 0.1177$; “N'Zuri”: $r = 0.1574$, $P = 0.3801$). Baseline VIDAS® PRG values of these females are listed in Table 2.

3.4. Greater one-horned rhinoceros

Progestagens values measured with VIDAS® technology correlated strongly positively with Pg-diol values measured for both adult females “Saathi” ($r = 0.8824$, $P < 0.001$) and “Henna” ($r = 0.7848$, $P < 0.001$). Furthermore, both adult females showed variations in fecal progestagens corresponding to estrous cycles according to Pg-diol profile (Fig. 2C). Average estrous cycle length was 41.5 ± 3.5 days (FP: 19.5 ± 1.5 days; LP: 23.5 ± 0.5 days; n = 2 cycles) for “Saathi” and 46.5 ± 1.5 days (FP: 22.5 ± 4.5 days; LP: 24.5 ± 4.5 days; n = 2 cycles) for “Henna” (Table 2). Luteal and baseline VIDAS® PRG values were respectively 68.95 ± 6.8 ng/g and 20.86 ± 1.28 ng/g for “Saathi”, 44.64 ± 4.01 ng/g and 20.01 ± 0.85 ng/g for “Henna” (Table 2). For both adult females, luteal VIDAS® PRG values were higher than baseline (Mann-Whitney test, $P < 0.001$). For the two juvenile females, there was a weak correlation (“Sananda”: $r = 0.4590$, $P < 0.01$; “Anjali”: $r = 0.3024$, $P = 0.098$). Young female “Anjali” exhibited several variations in fecal progestagens and Pg-diol as opposed to “Sananda”. Baseline VIDAS® PRG values were 16.87 ± 0.53 ng/g for “Sananda” and 54.47 ± 2.4 ng/g for “Anjali” (Table 2).

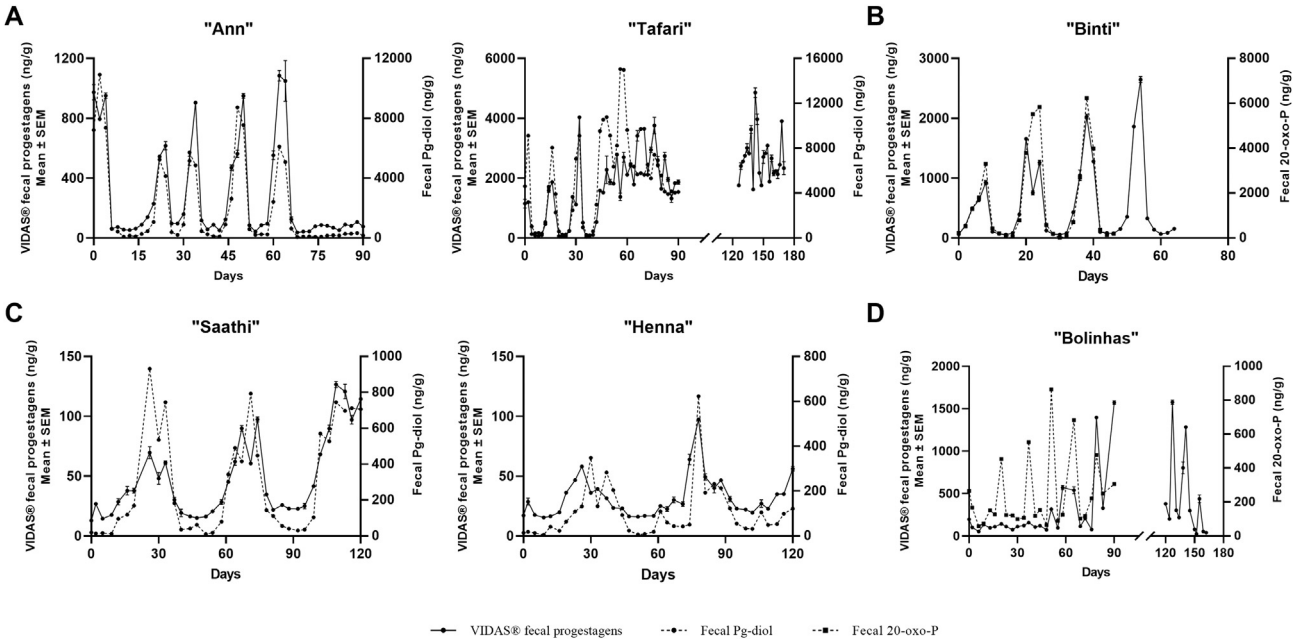

Fig. 2. Comparisons between VIDAS® fecal progestagens profiles and fecal Pg-diol profiles for (**A**) two okapi females called "Ann" and "Tafari", and (**C**) two greater one-horned rhinoceros females called "Saathi" and "Henna. Comparisons between VIDAS® fecal progestagens profiles and fecal 20-oxo-P profiles for (**B**) a reticulated giraffe female called "Binti", and (**D**) a common hippopotamus female called "Bolinhas". VIDAS® fecal progestagens plotted values are mean ± SEM (error bars) from n = 2 assay replicates for each data point.

3.5. Common hippopotamus

Progestagens profile measured with VIDAS® technology had a moderate positive correlation with 20-oxo-P kinetics for both females "Bolinhas" ($r = 0.5992$, $P < 0.01$) and "Kiwi" ($r = 0.5488$, $P < 0.01$). Only the first one showed large variations in fecal progestagens with mini VIDAS® (Fig. 2D). According to 20-oxo-P results and calving on Day 145, this female was pregnant. Thus, pregnancy values measured with mini VIDAS® were 362.77 ± 77.6 ng/g. The second female did not seem to emit any luteal activity during this study. Baseline VIDAS® PRG values were 23.5 ± 0.71 ng/g (Table 2). "Bolinhas" VIDAS® PRG pregnancy values were higher than "Kiwi" baseline values (Mann-Whitney test, $P < 0.001$).

3.6. African elephant

Fecal progestagens and serum progesterone measured with VIDAS® technology had a strong positive correlation for both females "N'Dala" ($r = 0.6832$, $P < 0.001$) and "Ashanti" ($r = 0.6663$, $P < 0.001$). Moreover, both females studied showed variations in fecal progestagens corresponding to estrous cycles, which correlates to serum progesterone profile (Fig. 3A). The average estrous cycle length was 13 weeks (FP: 3 weeks; LP: 10 weeks; n = 1) for "N'Dala" and 12 weeks (FP: 4 weeks; LP: 9 weeks; n = 1) for "Ashanti" (Table 2). For "N'Dala", basal fecal values were 21,68 ± 0,37 ng/g and luteal values were 36,09 ± 2,20 ng/g. For "Ashanti", baseline fecal values were 25,66 ± 0,75 ng/g and luteal values were 54,55 ± 4,00 ng/g (Table 2). In both cases, luteal phase

Table 2
Reproductive characteristics of studied individuals.

Species	Animal name (ng/g)	Basal progestagens (ng/g)	Luteal progestagens (days)	Follicular phase (days)	Luteal phase	Estrous cycle (days)
African	Ashanti	25,66 ± 0,75	54,55 ± 4,00 ***	28	63	91 (n = 1)
elephants	N'Dala	21,68 ± 0,37	36,09 ± 2,20 ***	21	71	91 (n = 1)
White	Mafu	22,61 ± 0,87	ND	ND	ND	ND
rhinoceros	Satara	18,63 ± 0,57	32,13 ± 2,50 ***	21	21	42
Greater	Saathi	20,86 ± 1,28	68,95 ± 6,80 ***	19,5 ± 1,5	23,5 ± 0,5	41,5 ± 3,5 (n = 2)
one-horned	Henna	20,01 ± 0,85	44,64 ± 4,01 ***	22,5 ± 4,5	24,5 ± 4,5	46,5 ± 1,5 (n = 2)
rhinoceros	Sananda	16,87 ± 0,53	ND	ND	ND	ND
	Anjali	54,47 ± 2,40	ND	ND	ND	ND
Reticulated	Chloé	65,36 ± 1,69	ND	ND	ND	ND
giraffe	Baya	64,88 ± 1,58	ND	ND	ND	ND
	Binti	71,14 ± 3,30	775,8 ± 155,6 ***	5 ± 0,58	10,5 ± 0,5	15,5 ± 0,5 (n = 4)
	N'Zuri	59,99 ± 1,39	ND	ND	ND	ND
Okapi	Ann	70,18 ± 3,73	571,5 ± 80,2 ***	7,5 ± 1,71	8	15,5 ± 1,7 (n = 4)
	Tafari	64,24 ± 5,12	1112 ± 291,6 ***	5 ± 1	10	15 (n = 2)
Common	Kiwi	23,5 ± 0,71	ND	ND	ND	ND
hippopotamus	Bolinhas	ND	ND	ND	ND	ND
Giant anteater	Aurora	ND	ND	ND	ND	ND

Data are shown as the mean ± SEM.
ND, not determine in this study.
n, number of complete estrous cycles.
Median value differs significantly ($P < 0.001$).

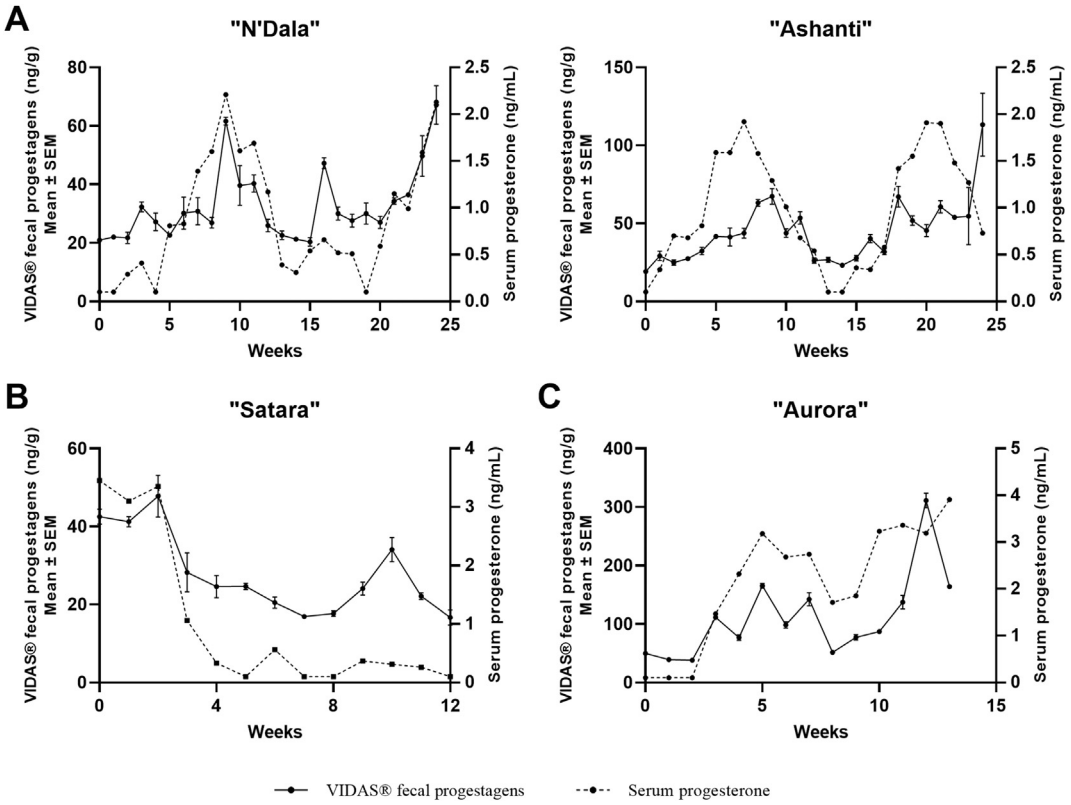

Fig. 3. Comparisons between VIDAS® fecal progestagens profiles and serum progesterone profiles for (**A**) two African elephant females called "N'Dala" and "Ashanti", (**B**) a white rhinoceros female called "Satara", and (**C**) a giant anteater female called "Aurora". VIDAS® fecal progestagens plotted values are mean ± SEM (error bars) from n = 2 assay replicates for each data point.

values were higher than basal values (Mann-Whitney test, $P < 0.001$).

3.7. Southern white rhinoceros

Fecal progestagens kinetics had a strong positive correlation with serum progesterone kinetics for "Satara" ($r = 0.7744$, $P < 0.01$; Fig. 3B) and a moderate positive correlation for "Mafu" ($r = 0.5490$, $P = 0.0598$). According to serum progesterone profile, only "Satara" exhibited an estrous cycle of 42 days (FP: 21 days; LP: 21 days; $n = 1$). Basal fecal VIDAS® PRG values of "Satara" were lower than luteal phase values (Mann-Whitney test, $P < 0.001$; Table 2). The second female appeared to be in anoestrus throughout the study period.

3.8. Giant anteater

The fecal progestagens profile obtained with VIDAS® technology had a strong positive correlation with the serum progesterone profile of female "Aurora" ($r = 0.7969$, $P < 0.001$; Fig. 3C). According to serum progesterone and several ultrasound scans, this female was pregnant since Day 11. Thus, fecal progestagens values were 110.66 ± 19.36 ng/g (Table 2).

4. Discussion

This study is the first to describe the use of VIDAS® Progesterone (PRG) assay for non-invasive monitoring of zoo animals' reproductive status. Non-invasive monitoring of reproduction is possible in many species through regular measurements of fecal progesterone metabolites concentrations, however this usually involves specialized laboratories. In our study, the evaluation of fecal progestagens was carried out directly at the zoo's veterinary clinic. We have shown a positive correlation between fecal progestagen concentrations and progesterone serum levels (African elephant, giant anteater, and white rhinoceros) both determined by mini VIDAS®. Moreover, there is a positive correlation with fecal concentrations of Pg-diol (okapi and greater one-horned rhinoceros) and 20-oxo-P (giraffe and hippopotamus). Thus, these results indicate the possibility of monitoring several species' reproduction directly in the zoo using VIDAS® PRG.

4.1. Extraction protocol and assay validation

Our extraction protocol for fecal samples can be associated with enzyme immunoassay performed by the mini VIDAS® via the VIDAS® PRG. The accuracy of this assay has been verified. Indeed, intra-assay and inter-assay variations for each species were less than 10% and 15%, respectively. The parallelism test was validated for all species except white rhinoceros. For this species, fecal progestagen values obtained by the mini VIDAS® were relatively low (<80 ng/g). Thus, the highest dilutions carried out with white rhinoceros' sample are quickly close to the detection limit, explaining a linearity loss. We believe that values obtained in gestation (>300 ng/g, 2019 unpublished data) could validate the VIDAS® PRG parallelism test for white rhinoceros. Although reproductive status may explain low fecal progesterone concentrations, it is obvious that VIDAS® PRG assay quantitatively underestimates fecal progesterone metabolites. The cause of this underestimation is likely to be the high specificity of the anti-progesterone monoclonal antibody used in the VIDAS® PRG assay.

Prior to this study, it was not anticipated that a monoclonal antibody specifically generated against progesterone would have sufficient cross-reactivity with fecal progesterone metabolites. However, as demonstrated in this study, the antibody used in the VIDAS® PRG assay yielded reliable hormone profiles. This antibody is not the only described monoclonal progesterone antibody showing high cross-reactivity with progesterone metabolites. The assay described by Graham et al. (2001) [7], which has been and is currently in use in a very large number of different animal species, is also based on a monoclonal antibody [2,3,7]. In contrast to the mentioned monoclonal antibodies, the group-specific assays established in Vienna are using antibodies specifically raised against defined functional groups of steroid hormone metabolites [2,6,24]. It would be interesting to determine the cross-reactivity of specific metabolites such as allopregnanolone [25], 20-oxo-P [26], and Pg-diol [11]. Nevertheless, these cross-reactions seem enough to indicate the reproductive status of the species mentioned above.

Our study focused mainly on non-domesticated species whose reproduction in captivity is complicated but crucial.

4.2. Okapi

We recorded a strong positive correlation of fecal progesterone variations with Pg-diol variations for okapis. Both females had estrous cycles of about 15 days. These observations are consistent with previous reports [11,22]. An extended period of anestrus was observed with one of the two females and remains unexplained. The second started pregnancy from Day 40. It appears that VIDAS® PRG can be used to monitor okapi pregnancy, although the entire pregnancy of 14 months was not tested [11,12].

4.3. Reticulated giraffe

Using the VIDAS® PRG, we also recorded a strong positive correlation of fecal progesterone variations with 20-oxo-P variations for giraffes (except non-cyclic individuals). Only one giraffe exhibited estrous cycles. These cycles had a duration of about 15 days like those reported previously [20,21,27,28]. Compared to this female, the other individuals did not show cyclic ovarian activity. One of them ("Baya") was probably in postpartum anestrus (calving in July 2019), while a second ("N'Zuri") was on contraception (450 μg im of Improvac 150 μg/mL every 2–3 months). The third female acyclicity ("Chloé") is unexplained.

4.4. Greater one-horned rhinoceros

Concerning greater one-horned rhinoceroses, three individuals showed luteal activity. These were two adult females ("Saathi" and "Henna") and a young female ("Anjali", aged 5 months at the beginning of the study). Mini VIDAS® fecal progestagen profiles and Vienna laboratory Pg-diol profiles of both adult females showed strong positive correlations. Besides, estrous cycle lengths of about 40 days described above [13] are consistent with our results on these adult females. However, the erratic luteal activity related to the young female did not show a significant correlation. At the moment, no study has yet described luteal activity in juvenile greater one-horned rhinoceros females before sexual maturity at around 3 years of age [29]. This 2-year-old female does not yet appear to have reached sexual maturity.

4.5. Common hippopotamus

The correlation between mini VIDAS® fecal progestagen and Vienna laboratory Pg-diol profiles was more moderate for hippopotamus. Although this species' reproductive physiology has been well described by Graham et al. (2002) [30], particularly highlighting cycles of 30–35 days, no estrous cycle was observed in our study. One female did not exhibit luteal activity ("Kiwi") while the second ("Bolinhas") achieved pregnancy Day 145. Unfortunately, "Bolinhas" pregnancy had begun before our study. We cannot confirm that VIDAS® Progesterone assay effectively monitors the entire pregnancy of approximately 8 months for common hippopotamus [7,30]. Nevertheless, we have noticed a drop in fecal progestagen (close to basal level) after calving.

4.6. African elephant

Using the VIDAS® PRG, we recorded a positive correlation between serum progesterone levels and fecal progestagen levels in African elephants. Both females studied had estrous cycles of 12 and 13 weeks, consistent with previous reports [31,32]. Of interest are the progesterone concentrations in the plasma of elephants determined in the VIDAS® PRG. The maximum values of 2.5 ng/mL are significantly higher than the usually reported plasma luteal phase progesterone levels of 0.8 ng/mL [17,33]. Similarly high luteal phase concentrations were determined in the plasma of African and Asian elephants with the 20-oxo-P assay established in Vienna [33]. These results clearly indicate high cross-reactions of the antibody used in the VIDAS® PRG with the 5α-pregnane-3,20-diones found in plasma of elephant [33].

4.7. Southern white rhinoceros

One white rhinoceros female appears to have a cycle of approximately 42 days closer to the short cycles than the 65–70 days long cycles described above [15,18,34,35]. However, we cannot confirm this observation because this species did not validate the parallelism test. Therefore, we cannot yet recommend the use of the VIDAS® PRG assay on fecal samples from southern white rhinoceros.

4.8. Giant anteater

Our study did not cover the entire pregnancy period of approximately 5–6 months of giant anteater [23]. Although there is an encouraging positive correlation, it does not allow us to affirm the VIDAS® PRG efficacy in this case.

4.9. Using mini VIDAS® to monitor the reproductive status of zoo animals

The advantage of the VIDAS® system is the possibility to analyze progesterone in blood samples as well as its metabolites in fecal samples. For some species kept in zoos, such as elephants, rhinos and anteaters, regular blood sampling is possible through medical training. In these species, analysis of progesterone from blood samples rather than fecal samples will be preferred. Overall, the VIDAS® PRG system seems to be particularly well suited for species with large differences in concentration between the follicular and luteal phases, such as okapis or giraffes. In okapis, fecal pregnanes are present in a ratio of 1: 10: >100 during the follicular, the luteal phase, and late pregnancy, respectively [11].

Consequently, monitoring the reproductive status directly on site seems to be the most rigorous method to control non-domesticated captive species' breeding management. Although hormone levels are underestimated by VIDAS® PRG assay, the profiles obtained reflect the same variations (cyclicity in particular) as assays performed by established laboratories. Moreover, the mini VIDAS® is easy to use and allows automated testing. This automaton does not require the use of personnel specifically qualified for

performing immunological assays. Besides, VIDAS® PRG can be combined with an extraction protocol that is simple to perform and does not require a lot of equipment. Therefore, the mini VIDAS® is an ideal candidate for the evaluation of this reproductive status within the zoo community. Ultimately, species-specific testing will be necessary to establish the VIDAS® system for its use. The most important type of application will be the establishment of hormone profiles over a period of time, and not so much the determination of absolute hormone concentrations. Nevertheless, it is essential not to neglect the involvement of external laboratories. Established laboratories will help in particular cases, such as the confirmation of a pregnancy or the presence of atypical luteal activity profiles (e.g. the young Indian rhino female “Anjali”).

5. Conclusions

In conclusion, the present study results give a first insight into the use of VIDAS® Progesterone (PRG) assay in non-invasive reproduction monitoring of non-domesticated species directly in zoos. Although the number of animals studied per species was limited, results indicate that this assay, coupled with an easy and inexpensive extraction protocol, is a useful tool for non-invasive assessment of estrous cyclicity of okapis, giraffes, and Indian rhinoceros. It is not easy to assert this protocol's total effectiveness on other species, although results are promising. For a certain species, more in-depth studies are needed to prove VIDAS® Progesterone's relevance over extended sampling periods with a larger number of individuals at different reproductive stages.

CRediT authorship contribution statement

Maxime Meunier: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Franz Schwarzenberger:** Investigation, Writing – review & editing. **Baptiste Mulot:** Conceptualization, Methodology, Investigation, Resources, Visualization, Supervision, Writing – review & editing.

Declaration of competing interest

The authors report no declarations of interest.

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