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)

Maxime Meunier, Franz Schwarzenberger, Baptiste Mulot

REVISED

Title: 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)

Authors: Maxime Meunier[a,b], Franz Schwarzenberger[c], Baptiste Mulot[a]

[a]Zooparc de Beauval & Beauval Nature, 41110, Saint-Aignan, France

[b]UMR Physiologie de la Reproduction et des Comportements, INRAE, CNRS, IFCE, Université de Tours, 37380 Nouzilly, France

[c]University of Veterinary Medicine, Department of Biomedical Sciences, Physiology – Endocrinology, Veterinärplatz 1, 1210 Vienna, Austria.

Corresponding author: Zooparc de Beauval & Beauval Nature, 41110, Saint-Aignan, France

E-mail address: maxime.meunier18@outlook.com (Maxime Meunier)

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.

Keywords: Enzyme immunoassay; Fecal analysis, Mini VIDAS®; Progestagens; Zoo species

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].

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

93 Fecal samples were collected two times per week for 6 months from African elephants (n = 2),
94 4 months from greater one-horned rhinoceros (n = 4), and 3 months from white rhinoceros (n = 2) and
95 hippopotamus (n = 2). Samples from okapis (n = 2) and giraffes (n = 4) were collected three to four
96 times per week for 3 months. Only one fecal sample per week could be collected from giant anteater (n
97 = 1) for 3 months. Keepers collected only fresh fecal samples (recently defecated) in freezer bags
98 before being stored at -20°C until the extraction process. For all females (except those collected in
99 blood), each fecal sample was divided into two aliquots. One was analyzed directly at the ZooParc de
100 Beauval clinic with mini VIDAS® PRG and the second was sent to the endocrine laboratory of the
101 Vetmeduni Vienna, Austria (Unit of Physiology, Pathophysiology and Experimental Endocrinology)
102 for a fecal progestagen assay (okapi: [11,12]; greater one-horned rhinoceros: [13]; data unpublished
103 for common hippopotamus and giraffes).
104 The fecal extraction protocol was set up by following an assembly of data collected in the
105 literature [14] in such a way as to be easily feasible in zoos. Frozen feces were thawed then crushed. A
106 0.5 g aliquot was then placed into a plastic tube and 5 mL of 80% EtOH was added to extract
107 progesterone metabolites. The mix was then vortexed with an automatic shaker for 30 min and then
108 centrifuged 15 min at 2000 rpm. An aliquot of the supernatant (containing progesterone metabolites)
109 was recovered and transferred into a plastic cryotube before being stored at -20°C until analysis.
110 **2.4 Enzyme immunoassays**
111 We used the automated instrument mini VIDAS® (bioMérieux) for Vitek ImmunoDiagnostic
112 Assay System to analyze serum and fecal extracts. Mini VIDAS® uses a VIDAS® Progesterone
113 (PRG) assay combining a competitive enzyme immunoassay method with a final detection by
114 fluorescence called ELFA (Enzyme-Linked Fluorescent Assay). This assay uses a single-use cone
115 (Solid Phase Receptacle (SPR)) as a solid phase and pipetting system. Each cone is sensitized with
116 mouse monoclonal anti-progesterone immunoglobulin at the time of manufacture. All the reagents
117 necessary for the immunological reaction are in the sealed reagent strips. 200 µL of the sample (serum
118 or fecal extract) were dispensed in the reagent strip's first well. Samples were identified and then
119 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 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.

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

152 **2.5 Data analysis**

153 Statistical tests were performed on the Rstudio software. Data are presented as mean ± SEM.
154 Parallelism test was carried out by comparing the standard curve with serial dilutions of fecal extracts
155 from each species by a *t*-slope comparison test. A baseline progestagen was calculated using an
156 iterative process [15]. Thus, each value above mean plus 1.5 standard deviations (SD) was discarded.
157 The elimination process was repeated by recalculating the average until no value exceeded the mean
158 plus 1.5 SD. The beginning of the luteal phase (LP) was defined as the first point after an increase in
159 values above the baseline for at least three consecutive values. The end of the LP and the beginning of
160 the follicular phase (FP) was established as the first of the two values returning to the basal level [15].
161 Estrous cycle length was calculated from the beginning of one luteal phase to the beginning of the next
162 (or using the follicular phase if the kinetics start in the luteal stage). A period longer than twice the
163 length of a normal follicular phase between two luteal phases was considered as anoestrus (8 to 14
164 weeks for African savannah elephant [16,17]; 30 days for Southern white rhinoceros [15,18]; 30 days
165 for greater one-horned rhinoceros [13,19]; 15 days for giraffe [20,21]; 15 days for okapi [11,22]; 20
166 days for giant anteater [23]; and not determined for common hippopotamus). The normality of fecal
167 progestagen profiles was examined using the Shapiro-Wilk test. Significant differences in the non-
168 parametric data were evaluated using the Mann-Whitney test for two groups (e.g., basal fecal
169 progestagen excretion and luteal phase values). We performed Spearman rank correlation tests to
170 assess the correspondence between (i) the means of each fecal sample assessed with the mini VIDAS®
171 and its aliquot analyzed by the Vienna laboratory, (ii) the weekly means of fecal progestagens and
172 serum progesterone (both measured by the mini VIDAS®). Spearman rank correlation test is used to
173 determine whether there is a relationship between the rank of observations for two variables
174 (interpretations: $-1 \leq r \leq 1$; .00-.19 : “very weak”, .20-.39 : “weak”, .40-.59 : “moderate”, .60-.79 :

175 “strong”, .80-1.0 : “very strong”). Since fecal and blood samples were not necessarily collected
176 simultaneously, a weekly mean value was calculated to compare the individuals concerned (n = 5).

177 **3. RESULTS**

178 **3.1 Intra-assay and inter-assay CVs and parallelism test**

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

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

186 **3.2 Okapi**

187 Progestagens values measured with VIDAS® technology correlated strongly positively with
188 Pg-diol values measured for “Ann” ($r = 0.8737$, $P < 0.001$) and “Tafari” ($r = 0.7115$, $P < 0.001$).
189 Moreover, both females studied showed variations in fecal progestagens corresponding to estrous
190 cycles according to Pg-diol kinetics (Fig. 2A). Average estrous cycle length for female “Ann” was
191 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
192 days; n = 2 cycles) for female “Tafari” (Table 2). An anoestrus period started on Day 68 of the
193 sampling period for “Ann”. VIDAS® PRG values were 70.18 ± 3.73 ng/g and 571.49 ± 80.23 ng/g in
194 the follicular and in the luteal phase, respectively (Table 2). For “Tafari”, a variation in values
195 indicated a beginning pregnancy after Day 40 of the investigation and this was confirmed by Pg-diol
196 kinetics. Thus, baseline values of this female were 64.24 ± 5.12 ng/g while luteal values were 1111.51
197 ± 291.57 ng/g, and pregnancy values were 2408.13 ± 125.98 ng/g. For this species, luteal phase values
198 were higher than basal values (Mann-Whitney test, $P < 0.001$). Moreover, pregnancy values were
199 higher than basal values (Mann-Whitney test, $P < 0.001$) and luteal values (Mann-Whitney test, $P <$
200 0.001).

201 **3.3 Reticulated giraffe**

202 Progestagen profiles obtained with VIDAS® technology had a very strong positive correlation
203 with 20-oxo-P profile for female “Binti” ($r = 0.9293$, $P < 0.001$); this female had, according to 20-oxo-
204 P kinetics of Vienna laboratory, clear estrous cycles (Fig. 2B). The average estrous cycle length of
205 “Binti” was 15.5 ± 0.5 days (FP: 5 ± 0.58 days; LP: 10.5 ± 0.5 days; n = 4 cycles; Table 2). Basal
206 VIDAS® PRG values (71.14 ± 3.3 ng/g) were lower (Mann-Whitney test, $P < 0.001$) than luteal phase
207 values (775.77 ± 155.6 ng/g). For three other females, which had no luteal and thus no estrous cycle
208 activity, a weak correlation was observed (“Chloe”: $r = 0.3896$, $P = 0.0375$; “Baya”: $r = 0.3024$, $P =$
209 0.1177; “N’Zuri”: $r = 0.1574$, $P = 0.3801$). Baseline VIDAS® PRG values of these females are listed
210 in Table 2.

211 **3.4 Greater one-horned rhinoceros**

212 Progestagens values measured with VIDAS® technology correlated strongly positively with
213 Pg-diol values measured for both adult females “Saathi” ($r = 0.8824$, $P < 0.001$) and “Henna” ($r =$
214 0.7848, $P < 0.001$). Furthermore, both adult females showed variations in fecal progestagens
215 corresponding to estrous cycles according to Pg-diol profile (Fig. 2C). Average estrous cycle length
216 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 ±
217 1.5 days (FP: 22.5 ± 4.5 days; LP: 24.5 ± 4.5 days; n = 2 cycles) for “Henna” (Table 2). Luteal and
218 baseline VIDAS® PRG values were respectively 68.95 ± 6.8 ng/g and 20.86 ± 1.28 ng/g for “Saathi”,
219 44.64 ± 4.01 ng/g and 20.01 ± 0.85 ng/g for “Henna” (Table 2). For both adult females, luteal
220 VIDAS® PRG values were higher than baseline (Mann-Whitney test, $P < 0.001$). For the two juvenile
221 females, there was a weak correlation (“Sananda”: $r = 0.4590$, $P < 0.01$; “Anjali”: $r = 0.3024$, $P =$
222 0.098). Young female “Anjali” exhibited several variations in fecal progestagens and Pg-diol as
223 opposed to “Sananda”. Baseline VIDAS® PRG values were 16.87 ± 0.53 ng/g for “Sananda” and
224 54.47 ± 2.4 ng/g for “Anjali” (Table 2).

225 **3.5 Common hippopotamus**

226 Progestagens profile measured with VIDAS® technology had a moderate positive correlation
227 with 20-oxo-P kinetics for both females “Bolinhas” ($r = 0.5992$, $P < 0.01$) and “Kiwi” ($r = 0.5488$, $P <$
228 0.01). Only the first one showed large variations in fecal progestagens with mini VIDAS® (Fig. 2D).
229 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 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],

285 which has been and is currently in use in a very large number of different animal species, is also based
286 on a monoclonal antibody [2,3,7]. In contrast to the mentioned monoclonal antibodies, the group-
287 specific assays established in Vienna are using antibodies specifically raised against defined functional
288 groups of steroid hormone metabolites [2,6,24]. It would be interesting to determine the cross-
289 reactivity of specific metabolites such as allopregnanolone [25], 20-oxo-P [26], and Pg-diol [11].
290 Nevertheless, these cross-reactions seem enough to indicate the reproductive status of the species
291 mentioned above.
292 Our study focused mainly on non-domesticated species whose reproduction in captivity is
293 complicated but crucial.
294 **4.2 Okapi**
295 We recorded a strong positive correlation of fecal progesterone variations with Pg-diol
296 variations for okapis. Both females had estrous cycles of about 15 days. These observations are
297 consistent with previous reports [11,22]. An extended period of anestrus was observed with one of the
298 two females and remains unexplained. The second started pregnancy from Day 40. It appears that
299 VIDAS® PRG can be used to monitor okapi pregnancy, although the entire pregnancy of 14 months
300 was not tested [11,12].
301 **4.3 Reticulated giraffe**
302 Using the VIDAS® PRG, we also recorded a strong positive correlation of fecal progesterone
303 variations with 20-oxo-P variations for giraffes (except non-cyclic individuals). Only one giraffe
304 exhibited estrous cycles. These cycles had a duration of about 15 days like those reported previously
305 [20,21,27,28]. Compared to this female, the other individuals did not show cyclic ovarian activity. One
306 of them ("Baya") was probably in postpartum anestrus (calving in July 2019), while a second
307 ("N'Zuri") was on contraception (450 ug im of Improvac 150 ug/mL every 2 to 3 months). The third
308 female acyclicity ("Chloé") is unexplained.
309 **4.4 Greater one-horned rhinoceros**
310 Concerning greater one-horned rhinoceroses, three individuals showed luteal activity. These
311 were two adult females ("Saathi" and "Henna") and a young female ("Anjali", aged 5 months at the
312 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 to 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, 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.

Acknowledgments

This work was supported by the Beauval Nature association. We want to thank the ZooParc de Beauval's veterinary service and the animal keepers for blood sampling and fecal sample collection. We also thank BioMérieux for supplying the VIDAS® Progesterone (PRG) assay kits (7 boxes of 60 tests were offered). The mini VIDAS® used in this study was in the possession of the zoo. Finally, we thank the endocrine laboratory of the Vetmeduni Vienna, Austria (Unit of Physiology, Pathophysiology and Experimental Endocrinology) for its participation in the project.

References

[1] Schwarzenberger F, Möstl E, Palme R, Bamberg E. Faecal steroid analysis for non-invasive monitoring of reproductive status in farm, wild and zoo animals. Anim Reprod Sci

1996;42:515–26. https://doi.org/10.1016/0378-4320(96)01561-8.

[2] Schwarzenberger F, Brown JL. Hormone monitoring: An important tool for the breeding management of wildlife species. Wien Tierarztl Monatsschr 2013;100:209–25.

[3] Brown JL. Comparative ovarian function and reproductive monitoring of endangered mammals. Theriogenology 2018;109:2–13. https://doi.org/10.1016/j.theriogenology.2017.12.004.

[4] Hodges JK, Brown JL, Heistermann M. Endocrine Monitoring of Reproduction and Stress. Wild Mamm. Captiv. Princ. Tech. Zoo Manag., 2010, p. 447–67.

[5] Peter ID, Haron AW, Jesse FFA, Ajat M, Han MHW, Fitri WN, et al. Opportunities and challenges associated with fecal progesterone metabolite analysis. Vet World 2018;11:1466–72. https://doi.org/10.14202/vetworld.2018.1466-1472.

[6] Schwarzenberger F, Palme R, Bamberg E, Möstl E. A review of faecal progesterone metabolite analysis for non-invasive monitoring of reproductive function in mammals. Int J Mammal Biol 1997;62:214–21.

[7] Graham L, Schwarzenberger F, Möstl E, Galama W, Savage A. A versatile enzyme immunoassay for the determination of progestogens in feces and serum. Zoo Biol 2001;20:227–36. https://doi.org/10.1002/zoo.1022.

[8] Anckaert E, Mees M, Schiettecatte J, Smitz J. Clinical Validation of a Fully Automated 17β-Estradiol and Progesterone Assay (VIDAS®) for Use in Monitoring Assisted Reproduction Treatment. Clin Chem Lab Med 2002;40. https://doi.org/10.1515/CCLM.2002.143.

[9] Merkl M, Ulbrich SE, Otzdorff C, Herbach N, Wanke R, Wolf E, et al. Microarray Analysis of Equine Endometrium at Days 8 and 12 of Pregnancy1. Biol Reprod 2010;83:874–86. https://doi.org/10.1095/biolreprod.110.085233.

[10] Brugger N, Otzdorff C, Walter B, Hoffmann B, Braun J. Quantitative Determination of Progesterone (P4) in Canine Blood Serum Using an Enzyme-linked Fluorescence Assay. Reprod Domest Anim 2011;46:870–3. https://doi.org/10.1111/j.1439-0531.2011.01757.x.

[11] Schwarzenberger F, Patzl M, Francke R, Ochs A, Buiter R, Schaftenaar W, et al. Fecal progestagen evaluations to monitor the estrous cycle and pregnancy in the okapi (Okapia

425 johnstoni). Zoo Biol 1993;12:549–59. https://doi.org/10.1002/zoo.1430120606.
426 [12] Schwarzenberger F, Rietschel W, Matern B, Schaftenaar W, Bircher P, Van Puijenbroeck B, et
427 al. Noninvasive reproductive monitoring in the okapi (Okapia johnstoni). J Zoo Wildl Med
428 1999;30:497–503.
429 [13] Schwarzenberger F, Rietschel W, Vahala J, Holeckova D, Thomas P, Maltzan J, et al. Fecal
430 Progesterone, Estrogen, and Androgen Metabolites for Noninvasive Monitoring of
431 Reproductive Function in the Female Indian Rhinoceros, Rhinoceros unicornis. Gen Comp
432 Endocrinol 2000;119:300–7. https://doi.org/10.1006/gcen.2000.7523.
433 [14] Palme R, Touma C, Arias N, Dominchin MF, Lepschy M. Steroid extraction: Get the best out
434 of faecal samples. Wiener Tierärztliche Monatsschrift Spec Issue 2013;100:238–46.
435 [15] Brown JL, Bellem AC, Fouraker M, Wildt DE, Roth TL. Comparative analysis of gonadal and
436 adrenal activity in the black and white rhinoceros in North America by noninvasive endocrine
437 monitoring. Zoo Biol 2001;20:463–86. https://doi.org/10.1002/zoo.10028.
438 [16] Hildebrandt TB, Lueders I, Hermes R, Goeritz F, Saragusty J. Reproductive cycle of the
439 elephant. Anim Reprod Sci 2011;124:176–83.
440 https://doi.org/10.1016/j.anireprosci.2010.08.027.
441 [17] Brown JL. Comparative Reproductive Biology of Elephants. In: Holt W V, Brown JL,
442 Comizzoli P, editors. Reprod. Sci. Anim. Conserv., vol. 753, New York, NY: Springer New
443 York; 2014, p. 135–69. https://doi.org/10.1007/978-1-4939-0820-2_8.
444 [18] Schwarzenberger F, Walzer C, Tomasova K, Vahala J, Meister J, Goodrowe KL, et al. Faecal
445 progesterone metabolite analysis for non-invasive monitoring of reproductive function in the
446 white rhinoceros (Ceratotherium simum). Anim Reprod Sci 1998;53:173–90.
447 https://doi.org/10.1016/S0378-4320(98)00112-2.
448 [19] Stoops MA, Pairan RD, Roth TL. Follicular, endocrine and behavioural dynamics of the Indian
449 rhinoceros (Rhinoceros unicornis) oestrous cycle. Reproduction 2004;128:843–56.
450 https://doi.org/10.1530/rep.1.00328.
451 [20] Bercovitch FB, Bashaw MJ, del Castillo SM. Sociosexual behavior, male mating tactics, and
452 the reproductive cycle of giraffe Giraffa camelopardalis. Horm Behav 2006;50:314–21.

https://doi.org/10.1016/j.yhbeh.2006.04.004.

[21] Lueders I, Hildebrandt TB, Pootoolal J, Rich P, Gray CS, Niemuller CA. Ovarian Ultrasonography Correlated with Fecal Progestins and Estradiol During the Estrous Cycle and Early Pregnancy in Giraffes (Giraffa camelopardalis rothschildi)1. Biol Reprod 2009;81:989–95. https://doi.org/10.1095/biolreprod.109.077743.

[22] Kusuda S, Morikaku K, Kawada K, Ishiwada K, Doi O. Excretion Patterns of Fecal Progestagens, Androgen and Estrogens During Pregnancy, Parturition and Postpartum in Okapi (Okapia johnstoni). J Reprod Dev 2007;53:143–50. https://doi.org/10.1262/jrd.18041.

[23] Patzl M, Schwarzenberger F, Osmann C, Bamberg E, Bartmann W. Monitoring ovarian cycle and pregnancy in the giant anteater (Myrmecophaga tridactyla) by faecal progestagen and oestrogen analysis. Anim Reprod Sci 1998;53:209–19. https://doi.org/10.1016/S0378-4320(98)00114-6.

[24] Palme R. Non-invasive measurement of glucocorticoids: Advances and problems. Physiol Behav 2019;199:229–43. https://doi.org/10.1016/j.physbeh.2018.11.021.

[25] Ghosal R, Sukumar R, Seshagiri PB. Prediction of estrus cyclicity in Asian elephants (Elephas maximus) through estimation of fecal progesterone metabolite: development of an enzyme-linked immuno-sorbent assay. Theriogenology 2010;73:1051–60. https://doi.org/10.1016/j.theriogenology.2010.01.004.

[26] Schwarzenberger F, Tomášová K, Holečková D, Matern B, Möstl E. Measurement of fecal steroids in the black rhinoceros (Diceros bicornis) using group-specific enzyme immunoassays for 20-oxo-pregnanes. Zoo Biol 1996;15:159–71. https://doi.org/10.1002/(SICI)1098-2361(1996)15:2<159::AID-ZOO6>3.0.CO;2-A.

[27] del Castillo SM, Bashaw MJ, Patton ML, Rieches RR, Bercovitch FB. Fecal steroid analysis of female giraffe (Giraffa camelopardalis) reproductive condition and the impact of endocrine status on daily time budgets. Gen Comp Endocrinol 2005;141:271–81. https://doi.org/10.1016/j.ygcen.2005.01.011.

[28] Dumonceaux GA, Bauman JE, Camilo GR. Evaluation of progesterone levels in feces of captive reticulated giraffe (Giraffa camelopardalis reticulata). J Zoo Wildl Med 2006;37:255–

481 61. https://doi.org/10.1638/04-081.1.
482 [29] Houwald F von, Pagan O, Rieches R. International studbook for the greater one-horned or
483 Indian rhinoceros, Rhinoceros unicornis. Basel: Basel Zoo; 2019.
484 [30] Graham LH, Reid K, Webster T, Richards M, Joseph S. Endocrine patterns associated with
485 reproduction in the Nile hippopotamus (Hippopotamus amphibius) as assessed by fecal
486 progestagen analysis. Gen Comp Endocrinol 2002;128:74–81. https://doi.org/10.1016/S0016-
487 6480(02)00066-7.
488 [31] Fieß M, Heistermann M, Hodges JK. Patterns of Urinary and Fecal Steroid Excretion during
489 the Ovarian Cycle and Pregnancy in the African Elephant (Loxodonta africana). Gen Comp
490 Endocrinol 1999;115:76–89. https://doi.org/10.1006/gcen.1999.7287.
491 [32] Wasser SK, Papageorge S, Foley C, Brown JL. Excretory Fate of Estradiol and Progesterone in
492 the African Elephant (Loxodonta africana) and Patterns of Fecal Steroid Concentrations
493 throughout the Estrous Cycle. Gen Comp Endocrinol 1996;102:255–62.
494 https://doi.org/10.1006/gcen.1996.0067.
495 [33] Schwarzenberger F, Strauss G, Hoppen H-O, Schaftenaar W, Dieleman SJ, Zenker W, et al.
496 Evaluation of progesterone and 20-oxo-progestagens in the plasma of Asian (Elephas
497 maximus) and African (Loxodonta africana) elephants. Zoo Biol 1997;16:403–13.
498 https://doi.org/10.1002/(SICI)1098-2361(1997)16:5<403::AID-ZOO3>3.0.CO;2-E.
499 [34] Patton ML, Swaisgood RR, Czekala NM, White AM, Fetter GA, Montagne JP, et al.
500 Reproductive cycle length and pregnancy in the southern white rhinoceros (Ceratotherium
501 simum simum) as determined by fecal pregnane analysis and observations of mating behavior.
502 Zoo Biol 1999;18:111–27. https://doi.org/10.1002/(SICI)1098-2361(1999)18:2<111::AID-
503 ZOO3>3.0.CO;2-0.
504 [35] Radcliffe RW, Czekala NM, Osofsky SA. Combined serial ultrasonography and fecal progestin
505 analysis for reproductive evaluation of the female white rhinoceros (Ceratotherium simum
506 simum): Preliminary results. Zoo Biol 1997;16:445–56. https://doi.org/10.1002/(SICI)1098-
507 2361(1997)16:5<445::AID-ZOO7>3.0.CO;2-A.
508

Figure captions

Figure 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.

Figure 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.

Figure 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.

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

Table 2. Reproductive characteristics of studied individuals

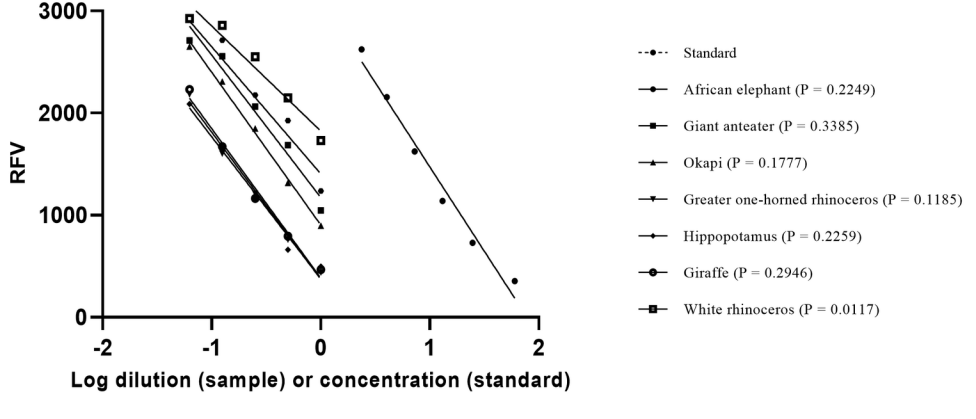
Species	Animal name (ng/g)	Basal progestagens (ng/g)	Luteal progestagens (days)	Follicular phase (days)	Luteal phase	Estrous cycle (days)
African	Ashanti	25,66 ± 0,75	54,55 ± 4,00 ***	28	63	91 (n=1)
elephants	N'Dala	21,68 ± 0,37	36,09 ± 2,20 ***	21	71	91 (n=1)
White	Mafu	22,61 ± 0,87	ND	ND	ND	ND
rhinoceros	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

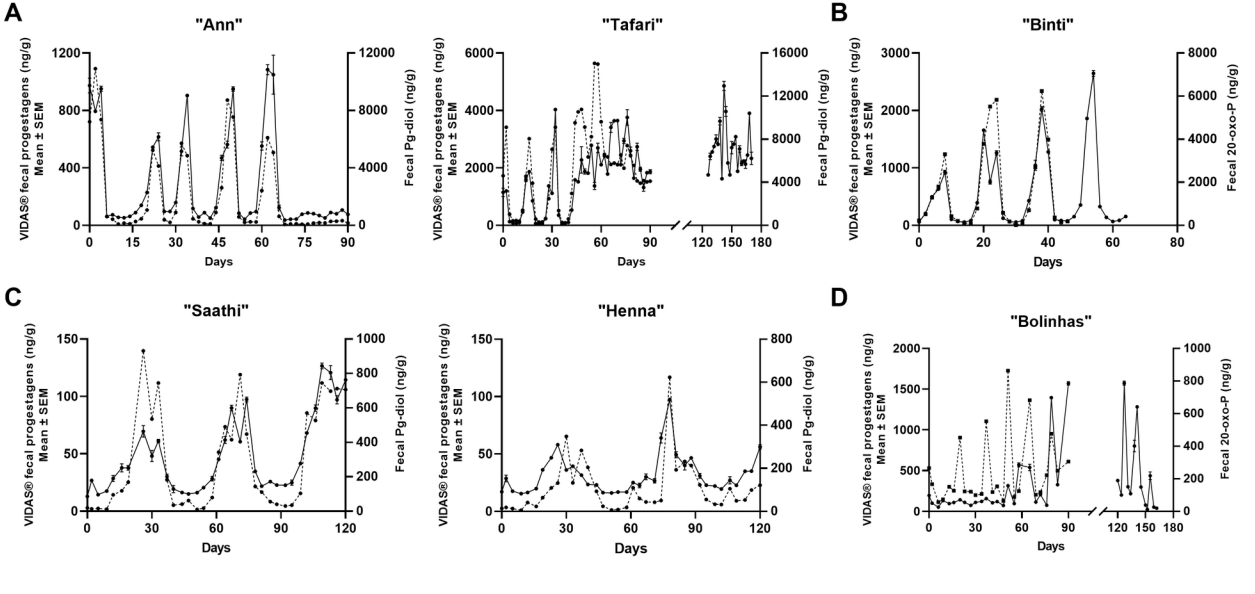
Data are shown as the mean ± SEM.
ND, not determine in this study.
n, number of complete estrous cycles.
*** Median value differs significantly ($P < 0.001$).

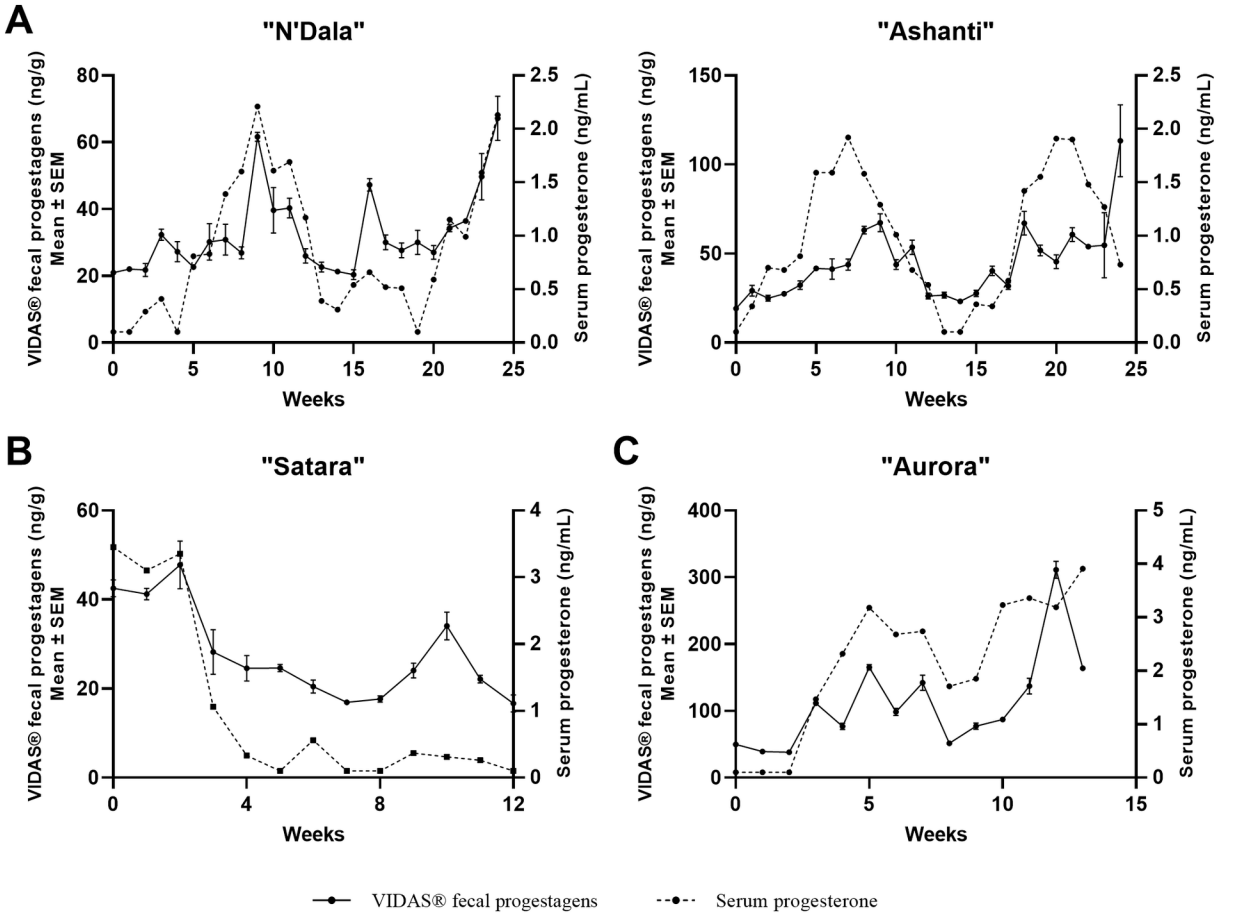
A

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

B




A
"Ann"
"Tafari"
B
"Binti"
C
"Saathi"
"Henna"
D
"Bolinhas"
VIDAS® fecal progestagens (ng/g) Mean ± SEM
Fecal Pg-diol (ng/g)
Fecal 20-oxo-P (ng/g)
Days
VIDAS® fecal progestagens
Fecal Pg-diol
Fecal 20-oxo-P


A
"N'Dala"
VIDAS® fecal progestagens (ng/g)
Mean ± SEM
Serum progesterone (ng/mL)
Weeks
"Ashanti"
B
"Satara"
C
"Aurora"
VIDAS® fecal progestagens
Serum progesterone