Theriogenology 102 (2017) 126–138

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Characterizing the reproductive biology of the female pygmy hippopotamus (*Choeropsis liberiensis*) through non-invasive endocrine monitoring

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ARTICLE INFO

Article history:
Received 13 October 2016
Received in revised form
8 July 2017
Accepted 17 July 2017
Available online 19 July 2017

Keywords:
Enzyme-linked immunoassay (EIA)
Estrogen
Estrous cycle
Gestation
Pygmy hippo
Progestogens

ABSTRACT

The pygmy hippopotamus (*Choeropsis liberiensis*) is endangered in the wild and very little is known about its reproductive biology. In zoological facilities, this species experiences a number of reproductive issues that complicate breeding management, including a high rate of stillbirths and failure of many pairs to reproduce. We conducted a comprehensive study to evaluate reproductive cycles and pregnancy in this species using enzyme immunoassays (EIAs) for fecal hormone metabolite analysis. Fresh fecal samples were collected twice weekly for a one to three year period from 36 female pygmy hippos housed at 24 zoological institutions. Samples were analyzed in three separate laboratories. Three progestogen metabolite EIAs (*Pg-diol*: 5β-pregnane-3α,20α-diol 3HS:BSA; *PdG*: pregnanediol-3-glucuronide R13904; *mono-P4*: Quidel clone 425) and three estrogen metabolite EIAs (*E2a*: estradiol-17β-OH 17-HS:BSA; *E2b*: estradiol 17β R0008; *E2c*: estradiol 17β R4972) accurately reflected reproductive events. Average estrous cycle length was 31.8 ± 7.4 days based on estrogen metabolite peaks and 30.9 ± 7.3 days based on nadir to nadir progestogen metabolite concentrations. Cyclical patterns in both estrogen and progestogen metabolites were detected throughout the year, indicating a lack of seasonality. Estrogen metabolite peaks were also observed during pregnancy and lactation, suggesting that follicular development occurs during both reproductive states. Pregnancy was most reliably demonstrated by elevation in progestogen metabolites (Pg-diol or PdG) in the second half of gestation. Average gestation length based on breeding to calving date was 203 ± 4 days for 15 pregnancies. This comprehensive overview of the reproductive biology of the female pygmy hippo provides valuable data for guiding long-term breeding management for this endangered species and serves as a baseline for future studies addressing the potential influence of social structure, diet, body condition, and other husbandry factors on estrous cycling and reproduction.

1. Introduction

The pygmy hippopotamus (*Choeropsis liberiensis*) – hereafter referred to as pygmy hippo – is classified as endangered by the International Union for the Conservation of Nature [1] and is ranked 21st worldwide among mammals by Programme EDGE (www.edgeofexistence.org) as a priority for conservation action [2]. The pygmy hippo is endemic to the Upper Guinean Rainforest ecosystem in the West African countries of Côte d'Ivoire, Guinea, Liberia and Sierra Leone. Wild population size is uncertain but is

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http://dx.doi.org/10.1016/j.theriogenology.2017.07.017
0093-691X/

estimated at < 2500 and is thought to be declining due to ongoing habitat loss and poaching [1,3]. Our understanding of the biology of this species in the wild is limited and data pertinent for developing effective conservation strategies are lacking. The first Conservation Strategy Action Plan was developed by the IUCN Pygmy Hippo Specialist Group in 2010 [3] and one of several research priorities was to characterize basic reproductive biology for both sexes.

There is no information concerning reproduction in wild pygmy hippos, but some general aspects of this species' reproductive biology are known from animals under managed care [4]. For example, both males and females reach sexual maturity between three and four years of age and can remain reproductively active into their third decade. The length of the estrous cycle, based on behavioral observations, ranges from 28 to 40 days. The gestation period is approximately 200 days after which a single calf is born; twin births are rare. There are no external signs of pregnancy except for enlargement of the mammary glands within a few days of parturition. The female is assumed to be polyestrous as births occur throughout the year in both northern and southern hemispheres, and conception is possible within a few weeks of perinatal mortality [5]. Historically, certain pairs have reproduced readily and often, and are therefore genetically over-represented in the managed population [4,5]. This trend continues because several breeding pairs have failed to reproduce despite regular estrous behavior and/or mating being observed by husbandry staff. Other pairs have repeatedly experienced perinatal calf mortality or stillbirth [5]. These issues, together with a high neonatal mortality rate (>30%), have limited the success of captive breeding and could reduce the long-term genetic diversity of the managed population, especially as imports from the wild ceased after 1982.

Finding solutions to these problems is hampered by a poor basic knowledge of the reproductive biology in this species, particularly the absence of hormone patterns. A clear way forward is to use enzyme immunoassays (EIAs) for the non-invasive assessment of endocrine processes that present insights into reproductive biology and improved breeding management [6]. These assays were originally established to quantify native hormones in serum or urine and exhibit varying degrees of cross-reactivity with the multitude of hormone metabolites excreted in the feces. As a result, measuring fecal hormone metabolites using these assays can prove challenging for some taxa, such as different species of tapir (J. Brown, personal communication, 2015), due to limited cross-reactivity between available antibodies and that species' particular repertoire of metabolites. In these scenarios collaborations between institutions are essential, especially where endangered species with limited numbers in the *ex situ* population are concerned, because not all endocrine laboratories will have access to the same selection of EIAs.

Endocrine assessment of reproductive events in female pygmy hippos is limited to one study, with only two females, that used a radio-immunoassay to analyze progesterone in skin secretions and saliva over a six-month period; the authors reported an average estrus cycle length of 26 days [7]. For the common hippo (*Hippopotamus amphibius*), on the other hand, non-invasive methods for confirming pregnancy, monitoring the reproductive cycle, pinpointing estrus, and identifying the timing of puberty have already been established [8–10]. However, this information cannot be directly extrapolated to the pygmy hippo because the reproductive physiology and steroid hormone metabolites of even closely related species can differ markedly. For example, different species of rhinoceros, felids, and ursids exhibit different estrous cycle characteristics and produce variable types and amounts of fecal estrogen and progesterone metabolites [11]. Thus, non-invasive protocols for evaluating reproductive events in the pygmy hippo are needed to facilitate estrous cycle monitoring, pregnancy diagnosis and prediction of parturition, especially as repeated blood sample collection is difficult.

Therefore, our overall objective in the present study was to characterize the basic reproductive biology of female pygmy hippos under managed care using fecal hormone metabolite analysis to define endocrine profiles during the estrous cycle, pregnancy and lactation. Specific aims were to: 1) demonstrate biological relevance for measuring fecal metabolites of estrogen and progestogens via enzyme immunoassays; 2) establish a standardized method for pregnancy detection; 3) determine the length of the estrous cycle via physiologic rather than behavioral assessment; 4) test for seasonality of the estrous cycle.

2. Materials and methods

2.1. Animals and sample collection

Thirty-six female pygmy hippos from 13 European and nine North American zoological institutions were included in this study; 33 were sexually mature (≥3 y) at the time sampling commenced (Appendix I). Fecal samples were collected twice weekly and stored frozen at −20 °C until extraction and analysis. Occasionally, biweekly sampling was not possible and the interval between samples was up to two weeks. Reproductive events, including behavioral estrus, mating and parturition, were recorded in conjunction with the timing of fecal sample collection (Appendix I).

2.2. Gastrointestinal transit time

The lag time between patterns of steroid secretion in the blood and subsequent metabolite excretion in the feces is correlated with gastrointestinal transit time [12,13]. We determined gastrointestinal transit time for one male and one female pygmy hippo housed at the same facility and fed the same diet. We used an easily identifiable fecal marker (glitter; Sulyn Industries, Coral Springs, Florida, USA), as previously described in similar studies investigating transit time [14,15], mixed with grain and fed to each hippo. We recorded the time from ingestion until the first and last passage of glitter in the feces for both hippos.

2.3. Reproductive hormone metabolite analysis

Hormone analysis was conducted in three separate laboratories (designated Lab A, Lab B, and Lab C; see Appendix I) using previously established fecal hormone metabolite extraction and EIA techniques. Modifications to these techniques are described below.

2.3.1. Fecal hormone extraction

Lab A (Vienna, Austria) performed fecal extraction as previously described [16]. Briefly, 0.5 g wet fecal material and 0.5 mL water were mixed with 4 mL reagent grade methanol and vortexed for 30 min. After centrifugation to remove solid fecal material, 1 mL of the methanol solution was transferred to a clean vial and mixed with 0.25 mL of a 5% $NaHCO_3$ solution and 5 mL diethyl ether. The mixture was then vortexed for 30 s, centrifuged at 1200 g for 10 min, and the extract supernatant held at −20 °C for 30 min. Finally, the supernatant ether phase was placed in a clean vial, evaporated to dryness, and re-suspended in 0.5 mL assay buffer.

Lab B (Chester, UK) performed fecal extraction with methods adapted from Walker et al. [17]. Following manual homogenization of the sample, 0.5 g of wet fecal material was mixed with 4.5 mL methanol (reagent grade, Sigma-Aldrich, Dorset, UK) and 0.5 mL of distilled water. Samples were vortexed for 30 s, rotated continuously on an orbital shaker at room temperature overnight and centrifuged the following day for 20 min at 598 g. The supernatant

was air-dried in a water bath at 55 °C in a fume cupboard and then re-suspended in 1 mL of methanol.

Lab C (Yulee, Florida, USA) performed fecal extraction using previously described methods [18]. Briefly, after manual homogenization of the sample, 0.5 g wet fecal material was mixed with 4 mL methanol (reagent grade, Fisher Scientific, Fair Lawn, NJ, USA) and 1 mL reverse osmosis-purified water. Samples were then shaken in a Glas-Col Large Capacity Mixer (Glas-Col LLC, Terre Haute, IN, USA) for 20 min at 90 rpm followed by centrifugation for 10 min at 3100 rpm. Then 200 μL of the supernatant was transferred into new vials containing 800 μL assay buffer and stored at −20 °C until analysis.

2.3.2. Enzyme immunoassays (EIAs) & validation

Italicized terms are those we have used to refer to the individual hormone assays throughout the remainder of our study. Cross-reactivities for all of the assays are provided in Appendix II. All samples, controls, and standards were assayed in duplicate.

Lab A analyzed fecal extract aliquots using previously performed group-specific EIAs with rabbit-origin polyclonal antibodies against the following: *i*) 20-oxo-pregnane (5α-pregnane-3β-ol-20-one 3HS:BSA [19]); *ii*) pregnanediol (*Pg-diol*: 5β-pregnane-3α,20α-diol 3HS:BSA [20]); *iii*) total estrogen (*E2a*: estradiol-17β-OH 17-HS:BSA [21]). The intra- and inter-assay coefficients of variation for all assays were <10% and <15%, respectively. The minimum assay sensitivities were 2.6 pg/well for 20-oxo-pregnane, 5.5 pg/well for Pg-diol, and 0.5 pg/well for E2a. Serial dilutions of fecal extracts yielded displacements curves parallel to the standards curves for all three EIAs. Depending on the concentration in the sample, the dilution factors for the samples were between 1:10,000 and 1:125,000 for 20-oxo-pregnane, Pg-diol and E2a. As the data for the 20-oxo-pregnane assay were not biologically relevant, it was not used for further sample analysis.

Lab B analyzed all fecal extract aliquots using a monoclonal progesterone antibody (*mono-P4*: Quidel clone 425 (CL425)) and an estrogen metabolite antibody (*E2b*: estradiol 17β R4972) developed by Coralie Munro, University of California, Davis, USA [22]. The intra- and inter-assay coefficients of variation for all assays were <10% and <15%, respectively. The minimum assay sensitivities were 0.78 pg/well for mono-P4 and 1.25 pg/well for E2b. Assays were validated by demonstrating parallelism between standard curves and serial dilutions of fecal extracts: CL425 ($r^2 = 0.980$, $F_{1,7} = 293$, $P < 0.001$); E2b ($r^2 = 0.991$, $F_{1,7} = 809$, $P < 0.001$). Dilutions were run at 1:256, 1:512, 1:1024, 1:2048, 1:4096, 1:8192, 1:16,386 and 1:32,768 for mono P4 and E2b. There was no evidence of matrix interference, demonstrated by significant recovery of pooled fecal extract added to the standards: CL425 (observed = 4.13 + 0.98 [expected], $r^2 = 0.999$, $F_{1,7} = 8694$, $P < 0.001$); E2b (observed = 7.86 + 2.19 [expected], $r^2 = 0.986$, $F_{1,7} = 514$, $P < 0.001$). Both the mono-P4 and the E2b EIAs were biologically validated and used for sample analysis at Lab B.

Lab C analyzed fecal extracts using a monoclonal progesterone antibody (*mono-P4*: Quidel clone 425), a polyclonal progesterone antibody (*poly-P4*: R4859), a pregnanediol antibody (*PdG*: pregnanediol-3-glucuronide, R13904) and an estrogen metabolite antibody (*E2c*: estradiol 17β R0008), all developed by Coralie Munro, University of California, Davis, CA, USA. A double-antibody EIA protocol was used as previously described [18,23]. The intra- and inter-assay coefficients of variation for all assays were <10% (average 2.58%) and <20% (average 8.07%), respectively. The minimum assay sensitivities, determined at 90–95% binding, were 10.1 pg/well for mono-P4, 11.0 pg/well for poly-P4, 18.6 pg/well for PdG, and 4.04 pg/well for E2c. Serially diluted fecal extracts yielded parallel displacement curves to the standard curves in all antibodies tested: mono-P4 ($r^2 = 0.801$, $F_{1,7} = 14.1$, $P < 0.001$); poly-P4 ($r^2 = 0.844$, $F_{1,7} = 19.0$, $P < 0.001$); PdG ($r^2 = 0.874$, $F_{1,7} = 24.3$, $P = 0.001$); E2c ($r^2 = 0.831$, $F_{1,7} = 17.3$, $P = 0.002$). Dilutions were run at 1:30, 1:60, 1:120, 1:240, 1:480, 1:960 and 1:1920 for mono-P4 and poly-P4, and at 1:16; 1:32, 1:64, 1:128; 1:256, 1:512, and 1:1024 for PdG. An antibody accuracy check showed that recovery of known amounts of progesterone (0.19–100 ng/mL) added to pools of diluted extracted feces was 107% at 1:100 for PdG ($y = 1.030x + 0.002$, $r^2 = 0.998$, $F_{1,17} = 5397$). Recovery of known amounts of estrogen (0.039–10.0 ng/mL) was 119% at 1:50 for E2c ($y = 1.607x + 9.381$, $r^2 = 0.992$, $F_{1,15} = 714$). The mono-P4 and poly-P4 EIAs were not biologically validated as neither clearly demonstrated a rise in progestogen metabolites during pregnancy. Thus, recovery was not assessed and neither EIA was investigated further at Lab C.

2.4. Data analysis

We plotted hormone concentrations over the sampling period (1–3 y) for each pygmy hippo. We first calculated non-pregnant baseline values for each EIA by averaging the lowest 10% of the samples for each female. We subsequently calculated concentrations for each EIA as a percent of each female's baseline. We characterized progestogen metabolite profiles by examining luteal phases and estrogen metabolite profiles by observing estrogen peaks indicative of follicular phases. We defined a luteal phase as any period when progestogen immunoreactivity was at least 50% above baseline for >14 days. We defined the peak luteal values for each cycle as the highest progestogen metabolite level during the cycle, and the nadir luteal concentration as progestogen metabolite level closest to baseline on either side of the peak. We defined an estrogen metabolite peak as the highest value within a group of samples that was greater than 10 days apart and greater than 2.5-fold (250%) above baseline for E2a and greater than 2-fold (200%) above baseline for E2b and E2c. We then determined the length of the estrous cycle by *i*) the interval between two estrogen metabolite peaks or *ii*) the onset of one luteal phase until the onset of the next. For some profiles we used a 3-point moving averaging to reduce background noise and clarify peak and nadir patterns; however, the values for actual peak and nadir metabolite levels were calculated from the non-averaged data for each female. Due to the numerous sources of variability associated with measuring immunoreactive metabolites in fecal material, in some cases we made subjective observations to distinguish between true cyclic patterns and random fluctuations in the data. We also assessed estrous cycle length based on direct observations of mating or behavioral by husbandry staff and compared these data (when available) with the hormone metabolite profiles for each hippo.

We calculated the length of gestation from the date of a confirmed mating event until parturition; for some pregnancies, the date of mating was unknown and we were unable to determine the length of gestation. We evaluated both progestogen and estrogen metabolites before, during and after gestation to determine if there is follicular activity during pregnancy, if there is a period of lactational anestrus, and when the first post-partum estrus occurs. For some study females, we could only generate partial pregnancy profiles as the sampling periods did not overlap with the full length of gestation. We calculated the mean and standard deviation (±SD) for estrogen and progestogen metabolite concentrations during pregnancy, in the non-pregnant state overall, and during peak luteal phase by combining data for all females. The data for pregnancy comprised all hormone concentrations from mating until parturition; we calculated the overall mean as well as a separate mean for the first and the second half of gestation (E2a & Pg-diol: $n = 7$ pregnancies, $n = 256$ samples; E2b & mono-P4: $n = 3$ pregnancies, $n = 96$ samples; E2c & PdG: $n = 8$ pregnancies, $n = 341$

samples). The data for non-pregnancy included all hormone concentrations throughout the estrous cycle and during periods of acyclicity (E2a & Pg-diol: $n = 16$ hippos, $n = 1683$ samples; E2b & mono-P4: $n = 9$ hippos, $n = 719$ samples; E2c: $n = 8$ hippos, $n = 321$ samples; PdG: $n = 16$ hippos, $n = 1237$ samples). The data for peak luteal phase comprised the highest concentration of progestogen metabolites during each luteal phase for each female (Pg-diol: $n = 15$ hippos, $n = 174$ samples; mono-P4: $n = 7$ hippos, $n = 58$ samples; PdG: $n = 15$ hippos, $n = 120$ samples). We first used Shapiro-Wilk tests for normality and then used either paired *t*-test analysis (two groups) or ANOVA (more than two groups) to compare estrogen and progestogen immunoreactivity in the different reproductive states (pregnancy, non-pregnant, and peak luteal phase).

3. Results

3.1. Gastrointestinal transit time

The time between consumption of the fecal marker and the first passage of glitter in the feces was 20 h for the female and 30 h for the male. Peak glitter excretion occurred between 30 and 46 h for the female and between 48 and 70 h for the male. Thus, gastrointestinal transit time for pygmy hippos can vary between individuals fed the same diet, but ranges from one to three days. Hormone metabolite levels measured in the feces are therefore likely to represent endocrine events that occurred in the previous 24–48 h. However, for simplification of results, reproductive events are reported as if they occurred on the same day as the feces were voided. Behavioral reports of estrus up to 72 h before endocrine events indicative of estrus were considered temporally associated.

3.2. Reproductive patterns – gestation and the post-partum period

Mean ± SD, minimum and maximum hormone metabolite levels for each EIA are presented in Table 1 for both pregnant and non-pregnant pygmy hippos. Mean progestogen metabolite concentrations were higher during pregnancy than in the non-pregnant state for all three progestogen EIAs (Pg-diol, $n = 16$ hippos, $P < 0.001$; PdG, $n = 16$ hippos, $P < 0.001$; mono-P4, $n = 9$ hippos, $P < 0.001$). Compared to baseline for each female, concentrations during pregnancy were up to 144-fold higher for Pg-diol, up to 9-fold higher for PdG, and up to 12-fold higher for mono-P4. Concentrations of Pg-diol and PdG excreted during the second half of pregnancy were significantly higher than those excreted during the first half (Fig. 1). Additionally, during the first half of gestation Pg-diol ($P = 0.143$) and PdG ($P = 0.131$) were similar to peak luteal phase concentrations, whereas during the second half of gestation they were significantly higher than peak luteal values for both Pg-diol (up to 9-fold higher, $n = 7$ pregnancies, $P < 0.001$) and PdG (up to 4-fold higher, $n = 8$ pregnancies, $P = 0.010$). On the other hand, no gestational difference was noted for mean concentrations of mono-P4 excreted throughout pregnancy, and mean values were significantly lower those excreted during peak luteal phase ($n = 3$ pregnancies, $P < 0.001$).

The average length of gestation for 15 pregnancies ($n = 12$ hippos) was 203 ± 4 days. Progestogen metabolite concentrations (Pg-diol and PdG) rose to more than 50% above baseline values within 17 ± 15 days after mating and conception. Concentrations fluctuated considerably during the first half of gestation but overall exhibited a slow, steady increase followed by a discernable decrease mid-gestation, around 90–100 days post-conception. This decrease was followed by a prominent rise in progestogen metabolites throughout the second half of gestation, reaching peak levels (mean = 31-fold above baseline values) just before parturition followed by a rapid return to baseline values within 13 ± 8 days after parturition (Fig. 1). These patterns were readily evident in some pregnancies (Fig. 2a), whereas in others there was considerable variation, complicating interpretation (Fig. 2b). In the latter situation a three-point moving average helped to clarify these patterns. The gestational patterns observed with Pg-diol and PdG were not as clearly discernable with mono-P4; however, this EIA was only used for two pregnancies (Fig. 3).

Estrogen values varied considerably during gestation, but some trends were evident. There was no difference between overall mean pregnant and non-pregnant concentrations of estrogen

Table 1
Mean (± SD) values for minimum, maximum, and baseline concentrations (per g feces) of reproductive hormone metabolites in female pygmy hippos during pregnancy and the estrous cycle. Results are for six different EIAs assayed in three separate laboratories (A, B and C).

	Lab A	Lab B	Lab C	Lab A	Lab B	Lab C
	Pg-diol µg/g	mono-P4 ng/g	PdG µg/g	E2a ng/g	E2b (R4972) ng/g	E2c (R0008) ng/g
Pregnant	14.3 ± 9.4	1137 ± 319	5.3 ± 1.6	1.3 ± 0.4	121 ± 13.9	611 ± 341
Range	0.19–59.8	313–4187	1.16–19.8	0.01–17.5	15.6–218	132–2922
n pregnancies	7	3	8	7	3	8
Non-pregnant	3.2 ± 1.7	917 ± 271	3.2 ± 1.1	1.3 ± 0.7	74.7 ± 24.2	388 ± 186
Range	0.02–26.9	50–7084	0.66–13.9	0.09–43.8	17.1–261	43–1052
n hippos	16	9	16	16	9	8
Cycle length (days)	30.2 ± 7.4	31.8 ± 5.9	30.7 ± 8.7	31.2 ± 7.1	32.6 ± 7.6	–
Range	14–46	17–42	14–49	14–48	17–46	
n cycles	157	50	80	147	34	
Peak luteal	6.2 ± 3.5	2150 ± 682	4.7 ± 1.1	–	–	–
Range	0.97–26.9	683–7084	2.3–9.9			
Nadir luteal	1.3 ± 0.8	498 ± 185	1.8 ± 0.5	–	–	–
Range	0.12–4.8	171–1180	0.66–3.18			
Peak follicular	–	–	–	4.5 ± 2.9	142 ± 47.7	–
Range				0.55–43.8	57.0–262	
Baseline	0.93 ± 0.61	354 ± 162	1.9 ± 0.6	0.30 ± 0.10	44.2 ± 14.6	252 ± 173
Range	0.27–2.8	152–733	1.1–3.2	0.14–0.56	25.5–62.1	99–618
n hippos	16	9	16	16	9	8

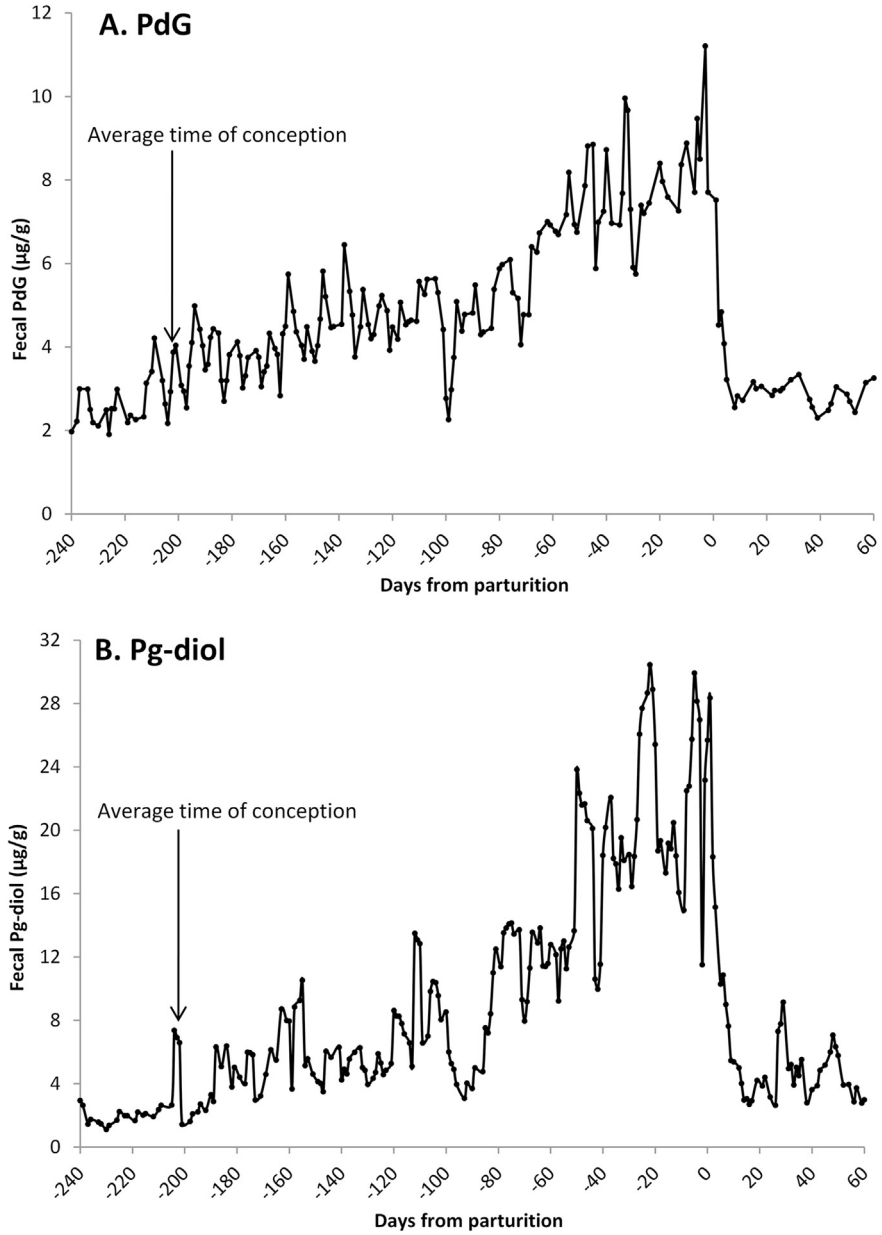

Fig. 1. Mean fecal progestogen metabolite concentrations before, during, and after gestation in the pygmy hippo; standard errors are not shown for clarity. Each data point comprised samples for all females spanning a 3 d time frame (between five and eight samples per mean). The data are aligned with parturition (Day 0); mating and conception occurred at an average of −203 d. **A.** PdG (pregnanediol-3-glucuronide); mean values for $n = 8$ pregnancies. **B.** Pg-diol (5β-pregnane-3α,20α-diol 3HS:BSA); mean values for $n = 7$ pregnancies. A nadir is noted at mid-gestation for both profiles, followed by a marked increase through the second half of gestation until parturition.

metabolites for E2a ($n = 16$ hippos, $P = 0.319$) whereas for E2b and E2c, overall concentrations were higher during pregnancy than in non-pregnant hippos ($n = 10$ hippos, $P < 0.001$). There was evidence of follicular activity as demonstrated by clear peaks in estrogen metabolite concentrations, especially during the first half of gestation, with a final peak just before or at the time of parturition in several females (e.g. Fig. 4a and b). In other females, clear peaks were not discernable, but values remained above baseline for extended periods during gestation, although in some cases the sampling interval was greater than twice weekly (e.g. Fig. 3b) and thus may not accurately reflect estrogen metabolite patterns. An estrogen metabolite peak prior to mid-gestation was evident in four of the five females with E2a data that spanned the mid-gestation time frame (e.g. Fig. 4a). For E2c pregnancy profiles, a similar peak just prior to mid-gestation was noted for three of seven females (e.g. Fig. 4c). Only two pregnancy profiles were analyzed with E2b and neither showed a clear mid-gestation peak (Fig. 3).

Average estrogen metabolite concentrations during gestation were not statistically different for male versus female calves for the E2a EIA ($P = 0.087$); however, concentrations tended to be lower for pregnancies with male calves. Concentrations were significantly different during gestation for the E2c EIA ($P = 0.002$); lower for male calves, higher for female calves. The sex ratio and number of samples during pregnancy for male and female calves was similar for E2a (3 ♂, 4♀) and slightly skewed toward females for E2c (3 ♂, 5 ♀).

The first post-partum estrus, demonstrated by an estrogen metabolite peak indicative of follicular activity (e.g. Fig. 4b), was observed from 22 to 92 days (mean 40 ± 21 d) after parturition with

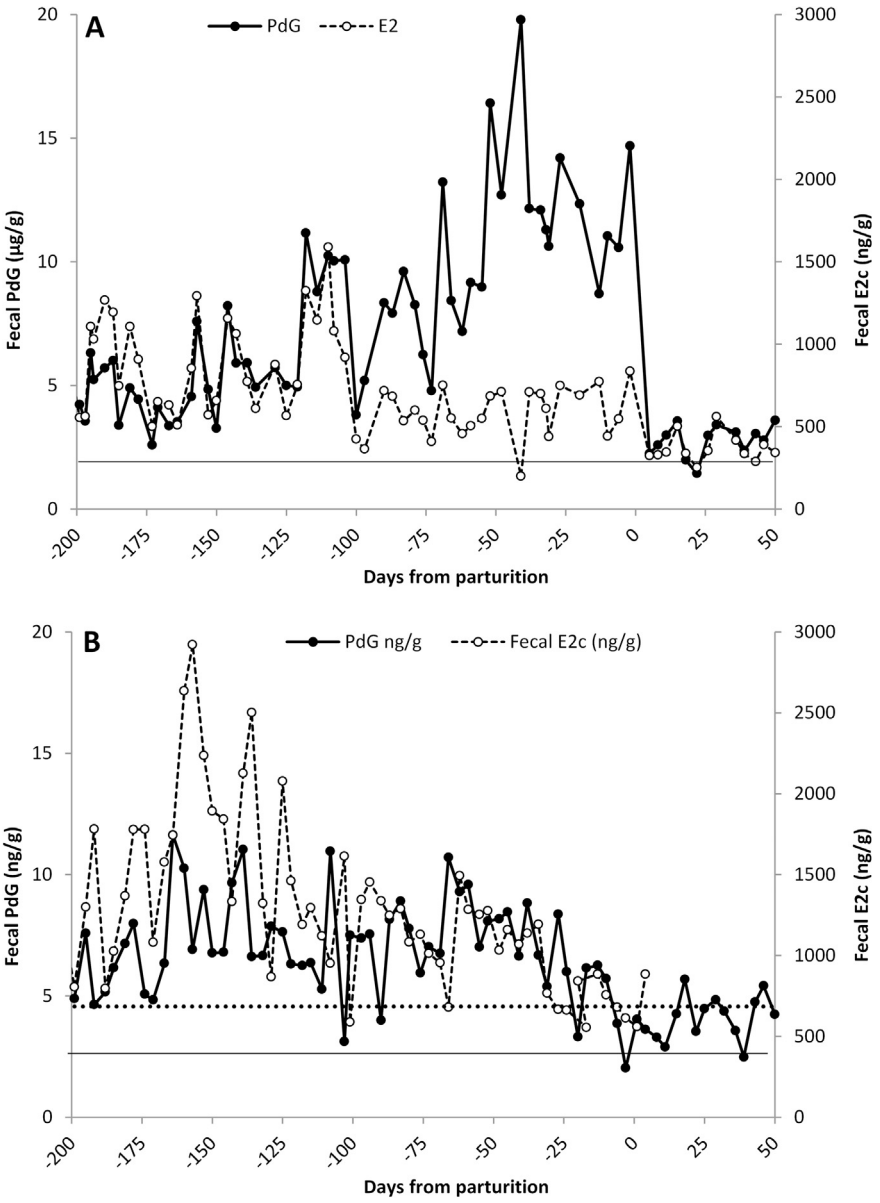

Fig. 2. Individual profiles for fecal PdG (pregnanediol-3-glucuronide; ●) and E2c (Estradiol 17β R0008; ○) immunoreactivity for two pygmy hippos; the data are aligned with parturition (Day 0). **A.** SB No. 1063, "Chomel." Example of a profile that demonstrates a gradual increase in progestogen metabolite concentrations after conception with a visible decline around mid-gestation (−100 d from parturition) followed by a substantial increase during the second half of gestation and a subsequent return to baseline shortly after parturition. Several estrogen metabolite peaks are also evident during the first half of gestation. The baseline for both PdG and E2c are indicated by the solid horizontal line. Gestation was 199 days. **B.** SB No. 1169, "Violet." Example of a pregnancy profile that does not demonstrate clear patterns in progestogen metabolites. Estrogen metabolite peaks are again noted during the first half of gestation. The baseline for PdG is indicated by a solid horizontal line; the baseline for E2c is indicated by a dotted horizontal line. Gestation was 210 days.

viable calves ($n = 10$). However, only seven of these post-partum follicular peaks were followed by a luteal phase indicative of ovulation. It is unknown whether these were fertile cycles as the male was always kept separate from the female and calf. For two females, the first post-partum estrogen metabolite peak was followed by an additional period of lactational anestrus (61 d and 99 d); the other eight females continued to cycle after the first post-partum estrus event. Hormonal evidence of estrous cycling resumed well before the calf was weaned for all 10 females. Two calves experienced perinatal mortality; in these cases estrus and mating occurred at 14 and 16 days post-partum and neither female conceived.

3.3. Reproductive patterns – estrous cycle

Mean (±SD) values for nadir and peak luteal concentrations of progestogens and peak follicular concentrations of estrogens are presented in Table 1. The overall mean estrous cycle length based on the interval between follicular peaks was 31.8 ± 7.4 days ($n = 185$ cycles; $n = 20$ hippos). Peak estrogen metabolite levels compared to baseline varied considerably between and among individual hippos. The overall mean estrous cycle length based on the interval between luteal phases was 30.9 ± 7.3 days ($n = 287$ cycles, $n = 30$ hippos). The overall mean estrous cycle length based on behavioral observations was 32.4 ± 6.3 days ($n = 42$ cycles, $n = 13$ hippos). There was hormonal evidence of estrous cycling

during all months of the year. Consecutive, year-round estrous cycles were observed for 13 females (age range 3–37 y), 10 from temperate climates where the animals were housed indoors during the colder months and three from sub-tropical climates where they were housed outdoors year-round. For the remaining 23 females in this study these trends could not be assessed, either because a portion of the data was collected during gestation and lactation, or the sampling time frame(s) did not include a contiguous 12-month period.

Follicular estrogen metabolite peaks or luteal progestogen metabolite nadirs were associated with observed estrous behavior in 31/46 cases (67%) when behavioral estrus was reported by the husbandry staff (e.g. Fig. 5b). In the other 15 cases, behavioral estrus was reported but hormone metabolite levels did not indicate estrus (e.g. Fig. 5c). Behavioral estrus was not reported for the majority of physiologic estrous cycles demonstrated by follicular (139/185) or luteal phases (241/287). In most cases, we were unable to determine if this disagreement was due to a lack of reporting or an actual absence of behavioral signs of estrus because behavioral data were not provided for most females (e.g. Fig. 5d). However, for some females, the husbandry staff ensured us they had not noted any signs of behavioral estrus yet patterns for estrogen and/or progestogen metabolites indicated clear estrous cycling (e.g. Fig. 5a).

Our study included four hippos aged 30 to 38 y at the start of the sample collection period; all four showed evidence of either intermittent or continuous estrous cycling. There were three

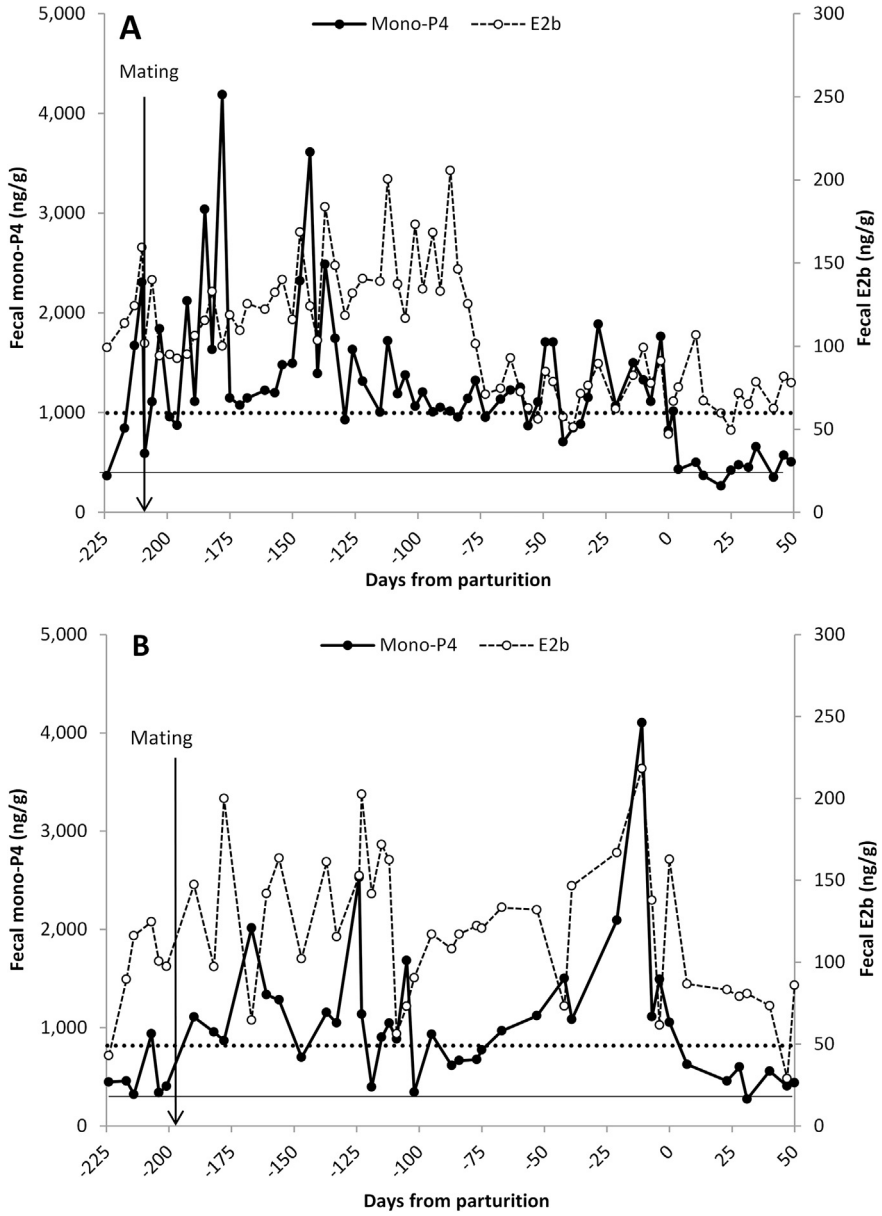

Fig. 3. Individual profiles for fecal mono-P4 (Quidel clone 425; ●) and E2b (estradiol-17β R4972; ○) for two pygmy hippos before, during and after pregnancy; the data are aligned with parturition (Day 0). The baseline for mono-P4 is indicated by a solid horizontal line and for E2b by a dotted horizontal line. **A.** SB No. 993, "Debby." Progestogen metabolite concentrations rise above baseline immediately after mating with several initial peaks and remain elevated until parturition followed by an immediate return to baseline. Estrogen metabolite concentrations are already well above baseline at the time of mating and continue to increase until mid-gestation (−90 d) followed by a rapid decline and fluctuating lower levels until parturition. Gestation was 210 days. **B.** SB No. 938, "Wendy." Progestogen metabolite concentrations also begin to rise above baseline immediately after mating but return to baseline at mid-gestation (−125 d and −100 d), then remain elevated until parturition followed by a return to baseline within 25 days. Estrogen metabolite concentrations fluctuate throughout gestation but generally remain above baseline with a peak just before (−11 d) and at parturition (Day 0). Gestation was 200 days.

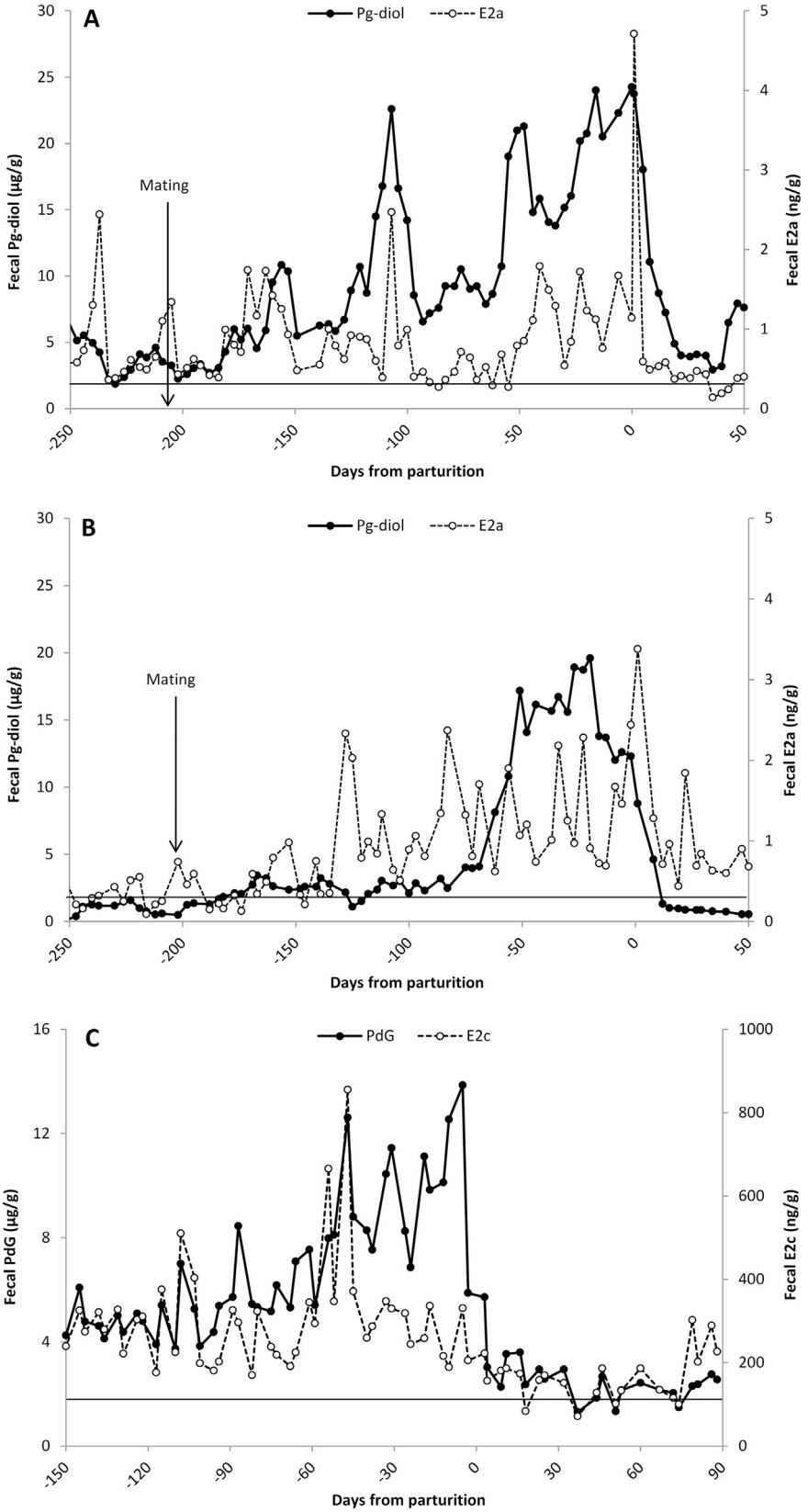

Fig. 4. Individual profiles for fecal progestogen and estrogen metabolite profiles during gestation for three pygmy hippos. The baseline for both progestogen and estrogen metabolites is indicated by the horizontal line; the data are aligned with parturition (Day 0). **A.** SB No. 993, "Debby." Pg-diol (pregnanediol-3-glucuronide; ●) and E2a (estradiol-17β-OH 17-HS:BSA; ○); progestogen metabolite concentrations remain above baseline from early gestation until parturition. An estrogen metabolite peak is noted at the time of mating (consistent with estrus), at parturition, and at various points throughout gestation, A peak occurs just prior to mid-gestation (−107 d) that temporally correlates with a marked decline in progestogen metabolites. **B.** SB No. 1033, "Torpedo." Pg-diol (pregnanediol-3-glucuronide; ●) and E2a (estradiol-17β-OH 17-HS:BSA; ○); a significant increase in progestogen metabolite concentrations above baseline does not occur until the second half of gestation. Estrogen metabolite peaks are again noted at the time of mating, throughout gestation including just before parturition, and at approximately at 22 days post-partum, indicative of post-partum estrus. **C.** SB No. 1143, "Fitri." PdG (pregnanediol-3-glucuronide 13904; ●) and E2c (Estradiol 17β R0008; ○); progestogen metabolite concentrations begin to rise at mid-gestation (−101 d) and remain elevated through parturition followed by a return to baseline by nine days post-partum. Estrogen metabolite concentrations fluctuate throughout gestation, with several prominent peaks.

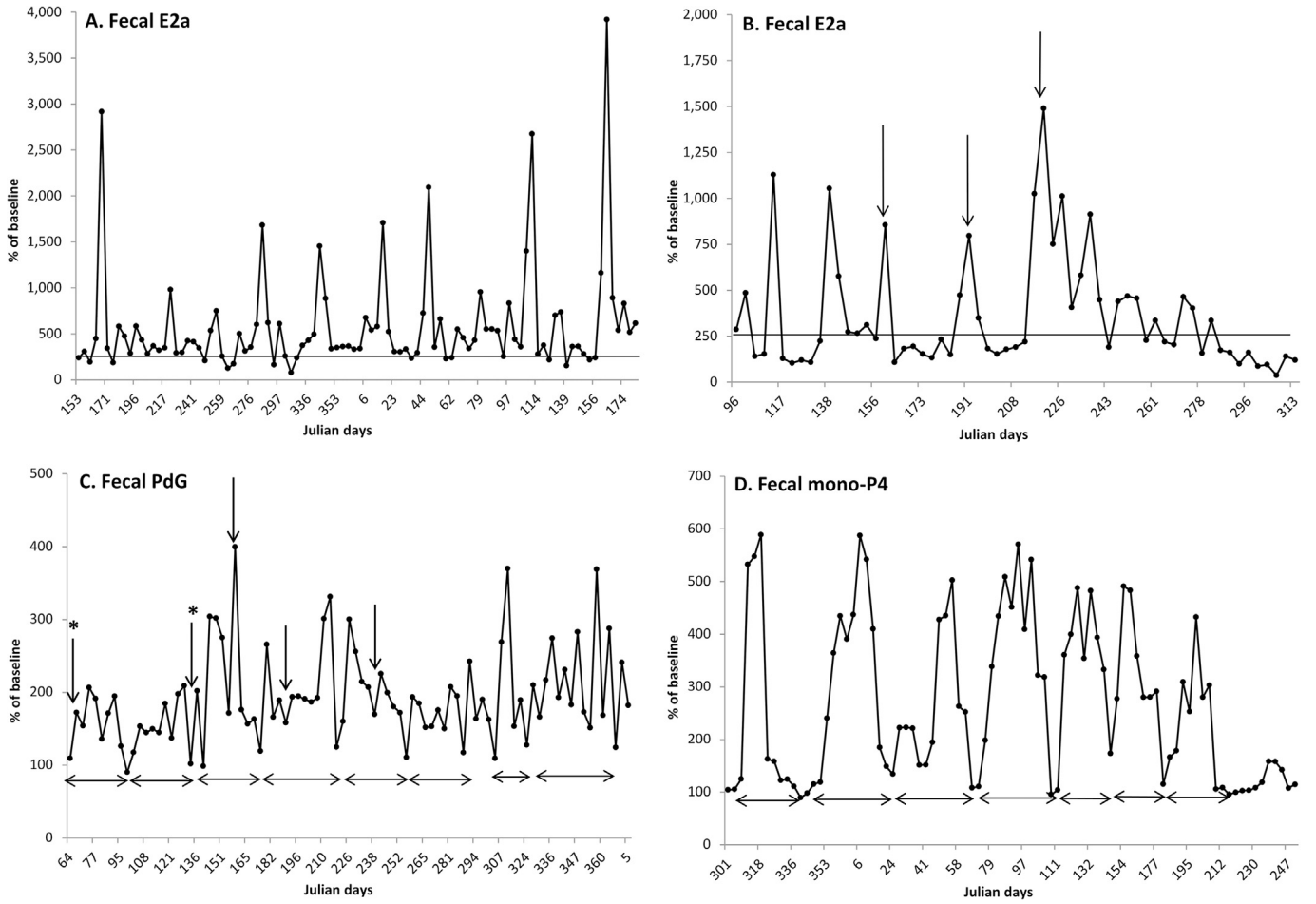

Fig. 5. Representative profiles from individual pygmy hippos demonstrating estrous cycles via peak estrogen metabolite concentrations or nadir progestogen metabolite concentrations. Data are presented as percentage of baseline (100%). The horizontal line indicates 250% baseline (2.5-fold increase) for estrogen metabolites; vertical arrows denote behavioral estrus events; horizontal arrows denote a luteal phase. **A.** SB No. 1182, "Ashaki." Estrous cycling indicated by peaks in E2a (estradiol-17β-OH 17-HS:BSA) immunoreactivity; there was no behavioral evidence of estrus during the sampling period. **B.** SB No. 548, "Tana." Estrous cycling indicated by peaks in E2a immunoreactivity; there are three points where behavioral estrus corresponds with an estrogen peak. **C.** SB No. 864, "Kelsey." Luteal phases indicated by nadirs in PdG (pregnanediol-3-glucuronide 13904) immunoreactivity; there are two behavioral/hormonal matches (arrow with *) and the remaining behavioral estrus events do not correlate with a luteal nadir. **D.** SB No. 1148, "Krakunia." Luteal phases indicated by nadirs in mono-P4 (Quidel clone 425) immunoreactivity; no data were provided concerning behavioral estrus. This female was housed at a zoo without a male; the data support spontaneous ovulation in the absence of a male.

juvenile hippos aged 2 to 2.5 y at the start of the study period and each showed evidence of intermittent cycles from the age of three onward. Only five adult females were housed at a facility without a male; the remaining females were housed at least adjacent to a male and in many cases the male was continuously together with the female (Appendix I). In all five females housed in complete absence of a male, there was clear evidence of estrous cycling as indicated by luteal phases (e.g. Fig. 5d).

There was marked individual variation among hormonal patterns for non-pregnant, non-lactating, sexually mature females ($n = 26$ hippos). Seventeen of these animals exhibited near-continuous estrous cycling throughout the study period. The remainder exhibited variable periods of acyclicity (8/26); one adult female (age 7 y) did not show any patterns consistent with estrous cycling throughout the 12-month sampling period and neither behavioral estrus nor mating was observed.

4. Discussion

We have demonstrated biological relevance for a number of EIAs for measuring fecal metabolites of estrogen and progestogens in pygmy hippos and have employed these techniques to characterize several important aspects of female reproductive biology.

4.1. Gestation and the post-partum period

Pregnancy diagnosis was possible from mid-gestation onward (approximately 100 days before parturition) using an EIA that cross-reacts with 5β-pregnanediol metabolites of progesterone (Pg-diol, PdG). Progestogen metabolite concentrations significantly exceeded peak luteal values throughout the second half of gestation, in contrast with the first half of gestation where values fluctuated considerably and were similar to luteal values. It is therefore necessary to analyze fecal samples for at least four months after an observed mating event to avoid a false negative pregnancy diagnosis. With the mono-P4 EIA, progestogen metabolite concentrations did not rise above peak luteal levels at any point during gestation and only exhibited a sustained rise above baseline in one female, so we do not recommend using this assay to diagnose pregnancy in pygmy hippos. This finding is in contrast to the common hippo, where mono-P4 immunoreactivity was elevated throughout gestation at levels significantly greater than during luteal phases [8]. We noted a general trend of increasing estrogen metabolite concentrations during the second half of gestation, but

there was considerable variation between individuals. Based on our results, fecal estrogen metabolites cannot be used to reliably diagnose pregnancy in the pygmy hippo.

The decrease in progestogen metabolite concentrations around mid-gestation followed by a marked increase above luteal values until parturition suggests a change in the dynamics of progestogen secretion. There are two possible explanations for these patterns, although both need verification as a priori hypotheses. First, the follicular activity noted during gestation for several pygmy hippos in our study, especially during the first 100 days before the decline and subsequent rise of progestogen metabolite concentrations, may indicate the formation of accessory corpora lutea (CLs), similar to the domestic mare [24]. A fluctuating pattern in serum estrogen concentrations during the first half of gestation, possibly indicative of accessory follicle formation, has also been observed in some lowland tapirs (*Tapirus terrestris*) [25]. Second, the marked increase in progestogens in the second half of pregnancy may be indicative of a luteal-placental shift. This phenomenon has been documented via non-invasive endocrine monitoring in a number of other large mammalian species, including the domestic mare [26]. Other well-studied examples are the three rhinoceros species most commonly kept in zoos (black *Diceros bicornis*, white *Ceratotherium simum* and greater one-horned *Rhinoceros unicornis*), and the okapi (*Okapia johnstoni*), where fecal progestogen metabolites remain at luteal phase concentrations during the initial part of pregnancy but increase dramatically between three and five months after conception, signifying a luteal-placental shift [16,20,27–30].

Estrogen levels during gestation tended to being higher for pregnancies with female calves, although we cannot conclusively state if there is a sex specific difference, as has been reported in other species such as orcas (*Orcinus orca*) [31] and red-fronted lemurs (*Eulemur fulvus rufus*) [32]. In contrast to the trend in pygmy hippos, estrogen levels during gestation were higher in mother's carrying male calves for both of these species.

There were 10 females in our study with viable calves and sufficient data to determine timing of the first post-partum estrous cycle; seven of these females had hormonal evidence of an estrous cycle approximately 30 days after calving. Although weaning age in the wild is unknown, if the female can become pregnant approximately one month after parturition and carry a second calf during lactation, then weaning in wild pygmy hippos may occur around eight months of age (one month post-partum estrus followed by a seven month gestation). Calving intervals from studbook data indicate that the female is able to conceive on the first post-partum estrus, within one month of parturition, when the calf experiences neonatal mortality [5]. In the common hippo, lactational anestrus averaged 34 weeks; however it was not always observed and was inconsistent between and among individuals, possibly indicating the influence of external factors, such as photoperiod or body condition, on the timing of the first post-partum estrus [8].

4.2. Estrous cycle

All of the estrogen metabolite EIAs we used in this study detected patterns indicative of follicular development. Similarly, all three progestogen assays (Pg-diol, PdG, mono-P4) demonstrated hormone metabolite patterns consistent with luteal activity. However, the mono-P4 EIA most clearly demonstrated luteal phases with minimal background fluctuations. Mean estrous cycle length was 31–32 days, slightly shorter than the mean cycle length of 35 days reported for the common hippo [8]. Cycle length ranged from 14 to 49 days and exhibited considerable variability both between and among individual animals.

For animals with longer cycles, our biweekly sampling protocol may have missed some estrogen peaks indicating follicular development. Although we were able to identify 185 follicular phases with this protocol, we could not determine the precise duration of these phases as the majority were represented by a single estrogen metabolite peak. However, the follicular phase likely spans at least three to four days or else biweekly sampling would not have detected the associated endocrine patterns. Nadir luteal concentrations may likewise have been missed with biweekly sampling as the progestogen metabolite nadir was typically also short, with only one or two points at or near baseline before the next luteal phase. On the other hand, the pygmy hippo may actually exhibit both short and long cycles, similar to the Malayan tapir (*Tapirus indicus*) [25], the white rhino [29,33,34] and the black rhino [34–36]. The only previous reproductive hormone study for the pygmy hippo reported an average cycle length of 26 days based on salivary progestogen metabolites; the data were for a total of 10 cycles from two females [7]. However, these authors noted hormonal oscillations every nine days, leading them to hypothesize a normal cycle length of 18, 27, or 36 days.

Our ability to detect estrous cycles has allowed us to determine several aspects of the basic reproductive biology of the female pygmy hippo under managed care. We noted hormonal evidence of estrous cycling between 2.5 and 3 y for the three juvenile females, indicating that the onset of sexual maturity occurs within this time frame. This is slightly earlier than the 3 to 4 y reported for the onset of sexual maturity in common hippos based on non-invasive endocrine monitoring [10]. Our data also indicate that females continue to exhibit hormonal evidence of estrous cycles well into their 30s. Behavioral estrus and mating was observed for one aged (>30 y) pygmy hippo on several occasions and hormone profiles also provided evidence of estrous cycles; however, she never conceived. Within the overall Studbook population, the oldest multiparous female to successfully reproduce was 37 years old at the time of conception, but generally reproduction in females is uncommon beyond the age of 30 [5].

In accordance with the Studbook [5], and similar to the common hippo [8], our results also indicate the pygmy hippo to be non-seasonally polyestrous in both subtropical as well as temperate climates. Additionally, similar to domestic ruminants [37], pygmy hippos in the managed population exhibit spontaneous ovulation, as demonstrated by clear hormonal evidence of estrous cycles in females housed in facilities without a male. Although we have no comparative information from wild populations, the ecologic life history traits of the pygmy hippo largely support non-seasonally polyestrous reproduction. The species is endemic to a very limited region of tropical, equatorial West African rainforest where temperature and photoperiod remain relatively constant throughout the year, although there is a seasonal difference in rainfall [38].

4.3. General discussion

Interestingly, the mono-P4 EIA was able to detect luteal phases but not pregnancy. This assay predominately cross-reacts with 20-oxo-progestogen metabolites, so its spectrum of detection is notably different to the Pg-diol and PdG EIAs that primarily detect metabolites with 20α-hydroxyl groups (Appendix II). In order for two EIAs with distinctly different cross-reactivities to accurately reflect two separate phases of gestation, namely the post-ovulatory CL and the feto-placental unit, the predominant progestogens produced by each must be structurally dissimilar. This phenomenon has most clearly been demonstrated in the mare where Δ4 progestogens are produced in the first part of gestation by the primary and accessory CLs, as described by Legacki et al. [39]. Thereafter, an increase in 5α-dihydroprogesterone (a 5α-reduced pregnane) marks the luteo-placental shift, and metabolites of 5α-

reduced pregnanes, produced by the feto-placental unit, predominate in mid to late gestation [39]. A similar transition in the principal type of progestogen produced by the CL versus the feto-placental unit in pygmy hippos may explain why Pg-diol and PdG immunoreactivity increased in the second half of gestation and mono-P4 did not, remaining at or below luteal levels.

Temporally-associated estrous behavior was not reported for the majority of estrous cycles identified via endocrine monitoring. Dathe and Kuckelkorn [7] also noted a lack of behavioral signs during periods of physiologic estrus for the two females in their study. These findings indicate that behavioral evaluation, even when performed by experienced zoo staff, is not an infallible method of estrus detection. A comparable phenomenon of 'silent' estrus has been reported in other species, for example the greater one-horned rhino [40] and the Baird's tapir (*Tapirus bairdii*) [41], further emphasizing the value of endocrine monitoring as an important tool to guide breeding programs for endangered species. Additionally, data from two sets of females ($n = 4$ hippos) at the same zoo ($n = 2$ zoos) suggest that pygmy hippos housed in close proximity possibly synchronize their cycles as follicular activity exhibited temporal association on a number of occasions. Thus, behavioral signs of estrus may be more difficult to identify in subdominant females when multiple females are housed together or in close proximity.

When behavioral signs of estrus are evident, they are likely to be a true indication of physiologic estrus as the majority (67%) of behavioral reports temporally coincided with an estrogen metabolite peak or progestogen metabolite nadir. Thus, husbandry staff should be consistently trained in recognizing and correctly classifying these behaviors, which include male vocalization, increased activity level, and direct interest in the female; females may exhibit a deep, audible breathing pattern [7,42]. Possible explanations for the 33% of cases where estrus-associated behaviors were reported but expected endocrine patterns were not detected include incorrect classification of behaviors or sampling frequency. The lack of an estrogen metabolite peak before all luteal phases could also be due to the biweekly sampling schedule occasionally missing these peaks. Reproductive behavior and mating in the absence of endocrine evidence of estrus has been reported in the white rhino [29] and was noted for one female in our study.

There were occasional estrogen metabolite peaks during luteal phases; these may represent follicular waves, similar to the patterns seen in domestic animals [37]. Alternatively, they may be an indication of estrogen production by the CL; however this phenomenon has only been described in humans and non-human primates [43,44]. We also intermittently noted temporally coinciding estrogen and progestogen metabolite peaks; this finding may represent secretion of progestogens from a non-ovulatory follicle undergoing atresia. Some individuals had periods of regular estrous cycling with clear follicular peaks and luteal phases followed by periods where physiologically relevant patterns were not discernable. This phenomenon may represent periods of normal cyclicity interspersed with irregular ovarian activity, possibly associated with environmental factors, captivity-associated stress [45], underlying reproductive pathology, or persistent luteal activity, as recently characterized in the domestic mare [46].

Estrous cycle patterns exhibited considerable variability both among and between individuals. A similar phenomenon has been reported for other pachyderms, including elephants and rhinoceroses, with potential links to social stress and other captivity-related factors [11,35,47]. Certain husbandry variables, including diet, body condition, and social grouping might also influence estrous cycling and reproduction in pygmy hippos. Dietary differences not only affect the amount of undigested fiber and the water content of the feces, but phytoestrogens and soy-based pelleted feeds could also influence hormone levels and cause significant reproductive abnormalities, especially in herbivores [48]. Diet also has a direct influence on body condition, and a number of pygmy hippos in zoological collections are overweight [4]. In Asian elephants (*Elephas maximus*), obesity has been proposed as a primary cause of reproductive problems in captivity, including a high incidence of stillbirth [49]. In zoo-housed black rhinos, higher body condition may play a role in reduced reproductive success for some females [35]. Overweight pygmy hippos may also experience impaired reproductive function.

Direct and indirect (i.e., camera trap photos, footprint tracking) observation of wild pygmy hippos has repeatedly demonstrated these animals to be predominately solitary [50–54]. Separate housing except during breeding therefore more closely reflects this species' natural biology; however, logistical constraints often preclude this arrangement and many zoos keep their pygmy hippo pair together continuously (see Appendix I). Abnormal social structure in zoo environments has been linked to reproductive abnormalities in several species, for example the cheetah (*Acinonyx jubatus*) [55,56], African elephant (*Loxodonta africana*) [47], and red river hog (*Potamochoerus porcus*) [57]. Chronic stress is hypothesized to be the primary link between unnatural social structure and resulting reproductive abnormalities and has been shown to negatively influence reproductive parameters in dairy cows [22], domestic ewes [58] and male cheetah [59]. Poor reproductive success and abnormal estrous cycling in zoo-born white rhinos is hypothesized to be associated with stress and undetermined captivity-associated factors [11,29,34,60,61]. These findings raise concern for captivity-induced modulation of reproductive performance, a phenomenon that may also impact pygmy hippos.

4.4. Future research and recommendations

Additional investigations are warranted to address a number of unanswered questions concerning the reproductive biology of the female pygmy hippo. We recommend performing ultrasound examination in conjunction with endocrine monitoring to correlate reproductive events with fecal hormone metabolite profiles and to further validate physiologic relevance. Ultrasound can also be used to determine if additional CLs are formed during gestation and to identify the timing of CL regression as an indication of luteal-placental shift. Further research is also needed to establish if there is more than one distinct estrous cycle length in the pygmy hippo, if longer periods of luteal activity are abnormal, and if the failure of some pairs to reproduce despite endocrine evidence of (often irregular) ovarian activity is indicative of underlying reproductive pathology as has been documented for the elephant and white rhino [62,63]. Additionally, future studies can employ the techniques we have described for monitoring reproductive hormones to investigate potential links between zoo-specific husbandry variables, obesity, chronic stress, and reproduction in pygmy hippos.

Saliva sampling, piloted in two pygmy hippos by Dathe and Kuckelkorn [7] has been successfully employed for endocrine monitoring in numerous other wildlife species [28,64–66] and further research may demonstrate it to be a valuable tool in pygmy hippos as well. Determining the biochemical structure of immunoreactive metabolites of estrogen and progestogen in the feces would help further refine which EIAs most accurately reflect reproductive hormones for this species; possibilities include high performance liquid chromatography (HPLC) and mass spectrometry [67]. Radio-label studies would also be useful to determine what percentage of reproductive hormone metabolites are excreted via the urinary and fecal route and to more precisely determine lag

time. However, this approach is not always practical or safe, and is often not justified in rare and endangered species. Thus, non-invasive endocrine monitoring paired with ultrasound validation of physiologic events is the next-best approach for tracking reproductive events.

5. Conclusions

This landmark collaborative study sets a significant milestone in elucidating reproductive endocrinology of the female pygmy hippo and provides essential data for future research. We describe endocrine patterns during pregnancy, lactation and the estrous cycle and have identified several EIAs with biological relevance for non-invasively monitoring reproductive hormone metabolites in the feces. Additionally, we have identified two progestogen metabolite EIAs (Pg-diol & PdG) that can detect pregnancy in the second half of gestation. We have also established that the pygmy hippo under managed care is a spontaneous ovulator and a non-seasonally polyestrous species. The length of the estrous cycle as determined by endocrine analysis was slightly shorter than the length reported from behavioral observations. Results were repeatable and consistent between laboratories using slightly different EIAs and techniques. Our data provide a vital resource for further investigations of reproductive physiology in the pygmy hippo and demonstrate how non-invasive endocrine monitoring can be a valuable tool for characterizing normal biology. These same technologies can potentially help diagnose reproductive abnormalities and improve future breeding management of this endangered species.

Funding

This work was supported by the American Association of Zoo Veterinarians Wild Animal Health Fund; the Center for Conservation of Tropical Ungulates; Dr. Mervyn Jacobson; Givskud Zoo; Omaha's Henry Doorly Zoo and Aquarium; the Royal Zoological Society of Scotland; Zodiac Zoos; Zoo Basel; the University of Western Australia, Convocation Postgraduate Research Travel Award; UWA Postgraduate Student's Association Fieldwork and Data Collection Award; and a UWA Graduate Research School PhD Completion Scholarship.

Acknowledgements

We would like to express our gratitude to all of the institutions that participated in this multi-year study: Aalborg Zoo; Arnhem Royal Burger's Zoo; Baton Rouge Zoo; Bristol Zoo; Chicago Zoological Society, Brookfield Zoo; Colchester Zoo; Dierenpark Wissel, Epe; Dvůr Králové Zoo Safari; Edinburgh Zoo; Gaia Zoo, Kerkrade; Gladys Porter Zoo, Brownsville; Jackson Zoo; Louisville Zoo; Marwell Zoo; Omaha's Henry Doorly Zoo and Aquarium; Paris Zoological Park; Parken Zoo, Eskilstuna; Rotterdam Zoo; Rum Creek Center for Conservation of Tropical Ungulates; Tampa's Lowry Park Zoo; Zoo Basel; Zoo Berlin; Zoo Miami; and ZooParc Overloon. Our special appreciation goes out to the husbandry staff that collected the samples; without their help and dedication we would not have been able to conduct this research. Additionally, we would like to thank Cayman Adams, Sarah Allred, Kim Daly-Crews, Thijs van den Houten, Saleha Khan, Elke Leitner, Lara Metrione, Erich Möstl, Paige Pickering, Kayla Weller, and the staff at the University Medical Center Groningen, The Netherlands for technical support.

Appendix A. Supplementary data

Supplementary data related to this chapter can be found at http://dx.doi.org/10.1016/j.theriogenology.2017.07.017.

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