



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

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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: 5 β -pregnane-3 α ,20 α -diol 3HS:BSA; PdG: pregnanediol-3-glucuronide R13904; mono-P4: Quidel clone 425) and three estrogen metabolite EIAs (E2a: estradiol-17 β -OH 17-HS:BSA; E2b: estradiol 17 β R0008; E2c: estradiol 17 β R4972) accurately reflected reproductive events. Average estrous cycle length was 31.8 $\pm$ 7.4 days based on estrogen metabolite peaks and 30.9 $\pm$ 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 $\pm$ 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.

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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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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 still-birth [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 progesterone 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

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