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Research article

Reproductive cycle and pregnancy monitoring in the common hippopotamus *Hippopotamus amphibius* through salivary steroid analyses and transabdominal ultrasonography

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Abstract

The use of an enzyme immunoassay (EIA) was validated to monitor progestogens in common hippopotamus *Hippopotamus amphibius* saliva and transabdominal ultrasonography was used to diagnose and monitor pregnancy in this species. Both faecal and saliva extracts contained elevated progestogens indicative of luteal phases and gestation. Faecal and saliva progestogen concentrations of six adult female hippos revealed a strong positive correlation between the two sample types (r=0.60–0.85). Salivary and faecal progestogen analysis revealed a cycle length of 31.8±6.9 days and 33.4±2.9 days and average non-pregnant luteal phase duration of 17.3±1.5 days and 14.0±1.5 days, respectively. Progestogen concentrations (faecal and saliva samples collected ~days 0 to 90) from a full-term pregnancy (saliva: 1,167.1±1,269.4 pg/ml; faecal: 2,812.4±1,657.5 ng/g) were higher on average than luteal phase concentrations (saliva: 335.0±358.1 pg/ml; faecal: 1,399.5±613.0 ng/g). Comparatively, progestogen concentrations (saliva samples collected days 0 to 181; faecal samples collected ~days 120 to 181) of a pre-term (premature live birth ~181 days) pregnancy (saliva: 244.3±155.2 pg/ml; faecal: 501.7±492.2 ng/g) were on average lower than concentrations observed during the full-term pregnancy and/or luteal phase. The nulliparous female that gave birth pre-maturely was trained for voluntary transabdominal ultrasound exams conducted weekly. Intrauterine fluid and foetal tissue were observed 79 days following last confirmed mating. Foetal spine, rib cage, beating heart and internal organs were visible at 156 days. In summary, salivary progestogen monitoring and transabdominal ultrasonography appear suitable for tracking reproductive activity and diagnosing and monitoring pregnancy in the common hippo.

Introduction

The common hippopotamus *Hippopotamus amphibius*, hereafter referred to as hippo, is an iconic species of mega-fauna indigenous to central, eastern and western Africa, south of the Sahara Desert (Lewison and Pluháček 2017). The International Union for Conservation of Nature's (IUCN) Red List categorises hippos as vulnerable to extinction due to widespread population decline likely caused by increased incidence of droughts due to climate change, habitat destruction and human–hippo conflict (Lewison 2007; Kanga et al. 2012; Lewison and Pluháček 2017). Such threats can disrupt reproductive cycles, resulting in lower reproductive rates that hinder population growth (Smuts and Whyte 1981; Lewison 2007). Informed reproductive management is crucial for maintaining genetically sound ex-situ populations, which could provide insurance against species loss if current in-situ population trends continue.

Non-invasive monitoring of reproductive hormones using biomaterials such as blood, urine, faeces, or saliva allows for the characterisation of the ovarian cycle, pregnancy and lactational anoestrus, offering insight into the timing of oestrus (receptivity) and conception, facilitating pregnancy diagnosis and estimation of parturition date. Faecal steroid analysis has been validated for measuring progestogens in many species including hippos, which has revealed an average cycle length of 35 days and pregnancy length of approximately 8 months (Graham et al. 2002). This method is popular for use with wildlife species due to its non-invasive nature but proves difficult in practice in some species, especially hippos, due to their behaviour (Blowers et al. 2010; Subalusky et al. 2014). Both wild and captive hippos spend at least half of every day submerged in or near water, making sample collection difficult (Blowers et al. 2010; Subalusky et al. 2014). Additionally, hippos often wag their tails during defaecation, mixing water

with the faecal matter which then disintegrates into small particles (Blowers et al. 2010; Subalusky et al. 2014). Salivary steroid analysis offers an alternative to faecal monitoring and has been validated for pygmy hippos *Choeropsis liberiensis* (Dathe and Kuckelkorn 1989) and many other mammalian species, including the false killer whale *Pseudorca crassidens* (Atkinson et al. 1999), Amazonian manatee *Trichechus inunguis* (Amaral et al. 2015), and black and greater one-horned rhinoceros, *Diceros bicornis* and *Rhinoceros unicornis*, respectively (Czekala and Callison 1996; Gómez et al. 2004).

Transabdominal ultrasonography is a relatively non-invasive method to visualise reproductive anatomy and monitor development of a foetus in utero. Both rectal and transabdominal ultrasound techniques have been successfully used to monitor ovarian activity and diagnose pregnancies in numerous mega fauna species, including four rhinoceros species (Radcliffe et al. 1997; Radcliffe et al. 2001; Roth et al. 2001; Stoops et al. 2004), Asian and African elephants, *Elephas maximus* and *Loxodonta africana* and *L. cyclotis* (Hildebrandt et al. 2006), and beluga whales *Delphinapterus leucas* (Steinman et al. 2012). The use of ultrasonography to diagnose and monitor pregnancy in hippos is relatively unexplored, likely due to the hippos' size and aggression. There are reports of pregnancy diagnosis (Australian Broadcasting Corporation 2015) and characterisation of weekly foetal development of pygmy hippos by ultrasonography (Wenker et al. 2018), but the pygmy hippo is less than one-fifth the body mass of a common hippo. Restraints and sedation can be used to safely examine challenging animals but are not ideal for frequent monitoring of a foetus (Armeno et al. 2006). The ability, specifically within zoos, to employ operant conditioning to train and work cooperatively with larger mammals enables transabdominal ultrasonography to be safe and productive for both the animals and humans. Although ultrasonography is an effective technique for determining health and status of the dam and foetus prior to and post conception, it is often paired with endocrine monitoring for a more holistic understanding.

Salivary steroid analysis and transabdominal ultrasonography in hippos may offer alternatives to faecal monitoring for tracking reproductive events and determining fertility status. The goals of this project were to: 1) validate the use of an enzyme immunoassay (EIA) to monitor progestogens in common hippo saliva, 2) confirm that salivary progestogen profiles accurately reflect reproductive activity, 3) confirm that transabdominal ultrasonography can be used to diagnose pregnancy, and if so 4) monitor and characterise foetal development via weekly examinations.

Materials and methods

Animals

All procedures were reviewed and approved by the Cincinnati Zoo and Botanical Garden's Animal Care and Use Committee (protocol #16-135). Female hippos (n=6; 17 to 26 years old) were maintained at the Cincinnati Zoo and Botanical Garden (CZBG; Cincinnati, OH) and Disney's Animal Kingdom® (Lake Buena Vista, FL). Any unusual behaviours considered to be associated with oestrus, such as sniffing/snorting and interest in males, among others, were recorded. Animal care staff used operant conditioning with positive reinforcement to train the animals to voluntarily open their mouths and allow saliva collection. Saliva was collected prior to the morning feeding by creating a vacuum with a syringe attached to a small flexible tube placed within the pocket of the lower lip where saliva pooled. Faecal samples were collected, from the floor of the animals' enclosures, between 0500 and 0800 following observed defaecation. To capture approximately three cycles worth of data, saliva and faecal samples were collected 4–7 days and 2–7 days per week, respectively, for three months from five females. One of the females included in this group was discovered to be pregnant only after sample collection was complete and therefore the corresponding progestogen profile does not span the entire length of gestation. Saliva samples were also collected at the same frequency throughout pregnancy, plus one-month post parturition, from one female who had a pre-mature live birth. Faecal samples from this female were not collected during the first four months of gestation due to logistical challenges. Saliva and faecal samples were stored at -20°C until analysis.

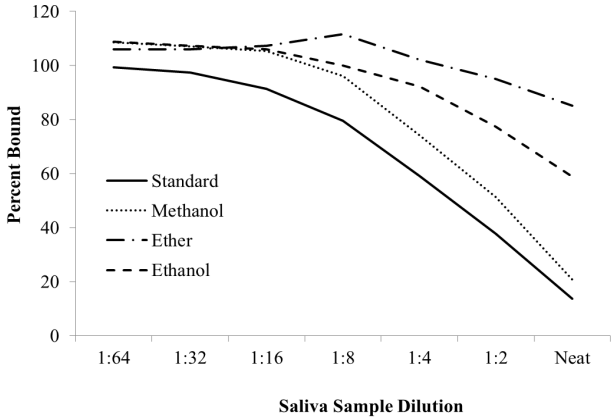

Figure 1. Parallelism between pooled hippopotamus saliva samples extracted using methanol, ether, or ethanol and progesterone standards.

Extraction

Faecal samples were lyophilised and crushed into a powder, which was then sifted through a fine mesh sieve to remove undigested material such as hay particles. Faecal samples were extracted using a modified version of the extraction protocols described by Graham et al. (2001, 2002). Briefly, 5 ml of 80% methanol were added to 0.1 g of faecal powder and samples were agitated overnight. Samples were centrifuged (1675×g) for 15 min, supernatants were poured off, and an additional 5 ml of 80% methanol were added to the pellet; this mixture was agitated for 30 min, centrifuged (1675×g) for 15 min, and the resulting supernatant was combined with the first one. The extracts (~10 ml) were placed under a stream of air and dried overnight. Samples were reconstituted in assay buffer (1 ml; Arbor Assays, Ann Arbor, MI 48108, USA), tubes were sonicated, and then stored at -20°C until analysis. Parallelism was confirmed between serially diluted, pooled faecal samples (r=0.99) and the standard curve. A pool of faecal extracts spiked with a known concentration of progesterone standard revealed a recovery of ~91%.

Three methods were tested for extraction of saliva samples: 80% methanol (Graham et al. 2001 and 2002) and 90% ethanol (Wojtusik et al. 2017) as described for faecal extractions and ethyl ether (Gómez at al. 2004) which was previously employed for rhinoceros saliva extraction. The 80% methanol extraction

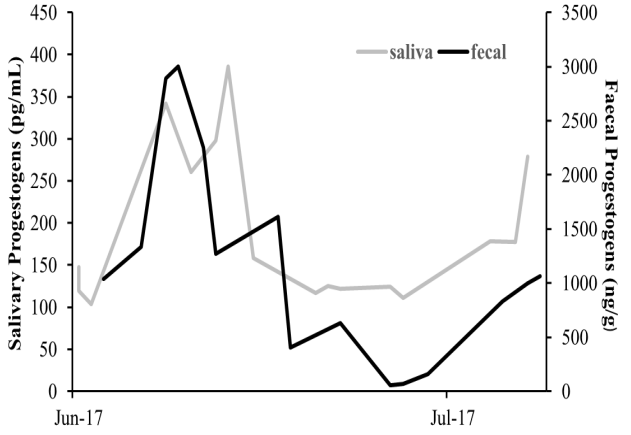

Figure 2. An example of faecal and salivary progestogen metabolite concentrations in one non-pregnant common hippopotamus female. Faecal concentrations (ng/g) are indicated with a solid black line and saliva concentrations (pg/ml) are indicated by a solid gray line.

was similar to the protocol described for hippo faecal extraction, except 1 ml of saliva was used in place of 0.1 g faecal powder and samples were reconstituted in 0.250 ml assay buffer instead of 1 ml. The extraction protocol employing 90% ethanol was conducted using 1 ml of saliva in place of faecal powder and slightly modified from Wojtusik et al. (2017). Briefly, 5 ml of 90% ethanol was added to 1 ml of saliva, the mixture was agitated for 30 min and then centrifuged (1675×g). The supernatant was poured off and the pellet was resuspended in 90% ethanol, agitated for 15 min, centrifuged (1675×g) and the resulting supernatant was combined with the first one. The mixture was dried overnight under a stream of air. Samples were then reconstituted in 1 ml methanol, sonicated, dried down for ~1 hr, and then reconstituted in 0.250 ml assay buffer. The ethyl ether extraction was conducted as described by Gómez at al. (2004). Diethyl ether (5 mL; Sigma Aldrich, St. Louis, MO 63146, USA) was added to 1 ml saliva, samples were vortexed for 1 min, and then placed in -80°C freezer for 2 hr. The ether fraction was collected and air dried (~1 hr). Samples were reconstituted in 1 ml methanol, sonicated, dried down under a stream of air, and reconstituted in 0.250 ml assay buffer.

Multiple saliva samples were pooled, divided, and subjected to each of the three extraction methods. Extracted samples were serially diluted to test for parallelism between extraction methods and standard curve. The samples subjected to methanol extraction demonstrated the highest correlation to the standard curve (r=0.99) followed by the ethanol extraction (r=0.95) and then the ether extraction (r=0.84; Figure 1). Therefore, the 80% methanol protocol was used to extract saliva samples in preparation for enzyme immunoassay. A pool of saliva extracts spiked with a known concentration of progesterone standard revealed a recovery of ~85%.

Saliva and faecal samples were diluted with assay buffer (1:2 to 1:10 and 1:25 to 1:500, respectively) and progestogen metabolite concentrations were analysed using a commercially available ISWE progesterone mini-kit enzyme immunoassay (EIA; Cat. #: ISWE003; Arbor Assays) which employs a monoclonal antibody against 4-pregnen-11-ol-3, 20-dione hemisuccinate: bovine serum albumin (CL425; Arbor Assays). Cross-reactions are described in Graham et al. (2001). The assay was completed as described in Wojtusik et al. (2017). Briefly, 96-well microtiter plates (Nunc-Immuno; Thermo Scientific, Waltham, MA 02451, USA) were pre-coated with 150 µl of a goat-anti-mouse IgG secondary antibody (10 µg/ml; Arbor Assays) diluted in coating buffer (Arbor Assays), blocked with 250 µl blocking solution (Arbor Assays), then air dried and stored in a desiccator cabinet (Dry Keeper; Sanplatec Corp, Osaka, Japan) at room temperature (RT) and <20% humidity until use. Diluted samples, progesterone standard (1.25–160 pg/well; Arbor Assays), and internal controls (50 µl) were added in duplicate. Progesterone horseradish peroxidase (HRP; 25 µl; 1:50; Arbor Assays) was added, followed by 25 µl of the primary antibody. Plates were agitated for 2 hr in the dark at RT then washed three times with wash buffer (Arbor Assays), and excess fluid removed. 1-StepTM Ultra TMB-Elisa (3,3', 5,5;-tetramethylbenzidine; 100 µl; Thermo Scientific) was added and plates were agitated in the dark for 30 min at RT. Following incubation, 50 µl of 1N HCl was added to each well and optical densities were measured at 450 nm (Versa Max; Molecular Devices, San Jose, CA 95134, USA). Sensitivity of the assay at maximum binding was: 1.25 pg/well. The intra-assay coefficient of variation was <15% (n=50 assays) and the inter-assay coefficient of variation for the two internal controls was 10.7% (mean binding: 10.4%) and 9.8% (mean binding: 78.4%).

Transabdominal ultrasonography

One nulliparous female housed at the CZBG was trained for weekly voluntary transabdominal ultrasound exams. An Ibex Pro portable ultrasound machine (E.I. Medical Imaging, Loveland, CO 80537, USA) with curvilinear probe (5–2.5 MHz) was used at a scanning depth of 17.8 and 23.4 cm for exams conducted weekly beginning three weeks after the last observed breeding. The probe was positioned caudal to the maternal umbilicus, medial to the hind leg, and proximal to the mammary.

Statistical analysis

To account for the slow metabolite clearance of the hippo digestive system, the profiles generated using faecal data were shifted backward on average ~3 days (range: 1-6 days depending upon individual) to align with the saliva profiles based on known long ingesta retention times in hippos and animals of similar size (Clauss et al. 2004; Amaral et al. 2009; Flacke et al. 2017). Day of gestation for the female that delivered prematurely was calculated based on last observed breeding. For the female that experienced a full-term pregnancy, day of gestation was estimated based on delivery date. Salivary and faecal metabolite concentrations (pg/ml and ng/g, respectively) were analysed using descriptive statistics including mean, standard deviation (SD), and standard error of the mean (SEM). For the full term pregnancy, average concentrations were calculated using only the first three months of gestation (days ~0 to 90) and averages for the pre-term pregnancy concentrations were calculated using saliva samples from the full length of the gestation (days ~0 to 181) and faecal samples from the final two months (days ~120 to 181), due to sample availability.

A mean baseline was calculated using an iterative process that removed all values above the mean ±1.5 SD (Brown et al. 2001). Concentrations were considered elevated when they exceeded the baseline by 1.5 SD for at least two consecutive days. Non-pregnant luteal phase was defined as elevated concentrations lasting more than four consecutive days. Oestrous cycle length was calculated from the start of one luteal phase to the next (n=10 cycles; 4 females). Pearson product-moment correlation coefficient was calculated to determine degree of relationship between sample

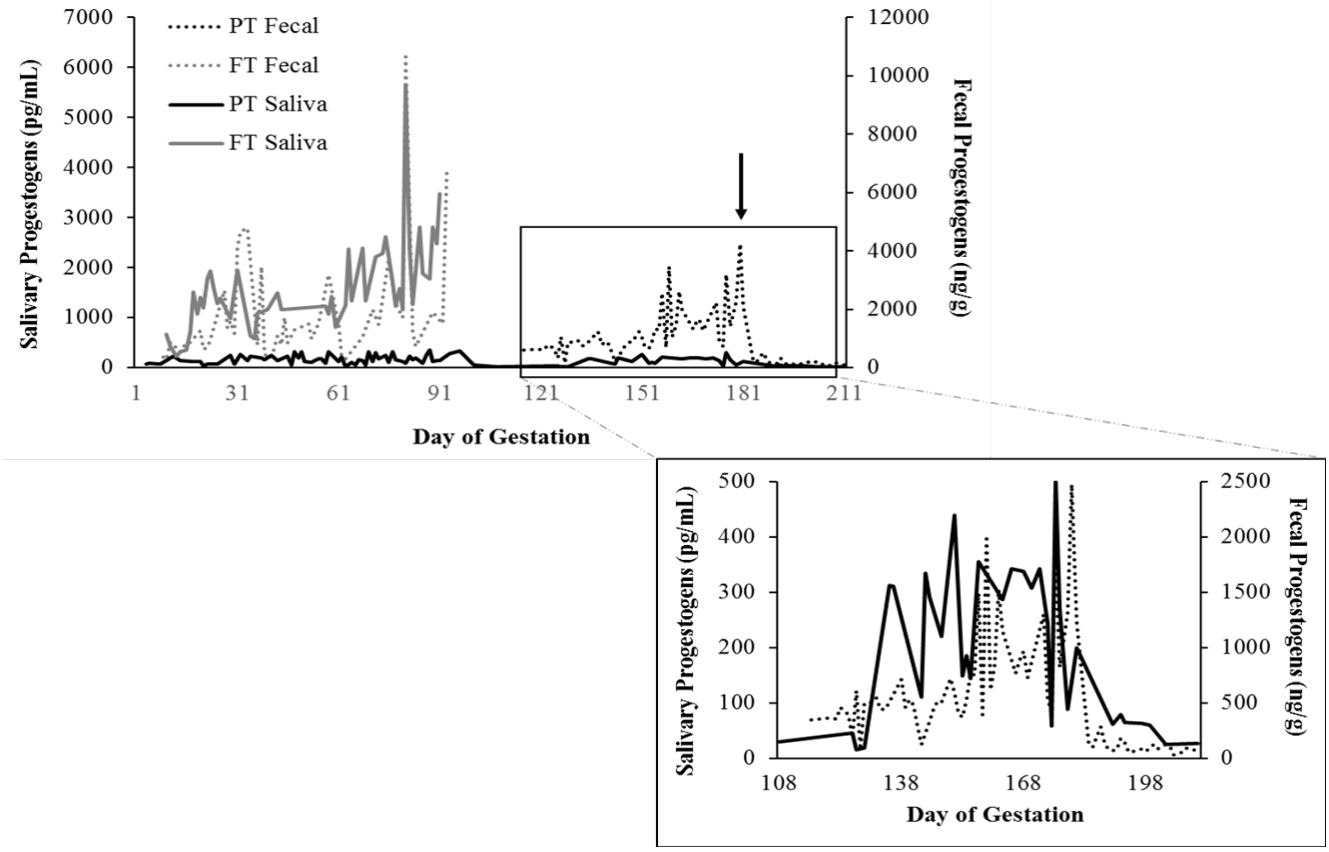

Figure 3. Faecal and salivary progestogen metabolite concentrations (ng/g and pg/ml, respectively) during a full-term pregnancy (FT; gray lines) and a pre-term pregnancy (PT; black lines) in common hippopotami. Samples from the full-term pregnancy. Day of pre-term parturition indicated by arrow. Inset shows data from PT with adjusted scale.

types. Analyses were conducted using SPSS for Windows (Version 24; IBM Corporation, Armonk, NY 10504, USA). Data is reported as mean ±SEM.

Results

Salivary and faecal steroid analysis

Salivary and faecal progestogen concentrations revealed a strong positive correlation (r=0.60 to 0.85; n=6 individuals). All non-pregnant females (n=4) displayed a cyclical pattern of progestogen excretion in both sample types (an example profile is provided in Figure 2) and exhibited elevated progestogen concentrations during luteal phases (saliva: 335.0±35.1 pg/ml; faecal: 1,399.5±72.2 ng/g) as compared to inter-luteal phases (saliva: 122.6±8.4 pg/ml; faecal: 355.4±34.4 ng/g). Average cycle length was 31.8±2.4 days (range: 23–39 days) and 33.4±2.2 days (range: 21–38 days), as calculated based on data from saliva (n=8 cycles) and faecal samples (n=7 cycles), respectively. The duration of the average luteal and inter-luteal phase were 17.3±1.5 days and 14.0±1.5 days as determined using saliva samples, and 19.0±1.7 days and 13.4±1.4 days, respectively, using faecal samples.

Progestogen concentrations during the full-term pregnancy (n=1; Figure 3) were greater (saliva: 1,167.1±183.2 pg/ml; faecal: 2,812.4±227.7 ng/g) than average luteal phase concentrations (saliva: 335.0±358.1 pg/ml; faecal: 1,399.5±613.0 ng/g) lower for the pre-term (parturition at ~181 days) pregnancy (saliva: 244.3±16.2 pg/ml; faecal: 501.7±55.4 ng/g).

Transabdominal ultrasonography

Intrauterine fluid and foetal tissue were observed 79 days following the last observed mating, at a depth of 23.4 cm (Figure 4A). Foetal spine, rib cage, beating heart and fluid-filled internal organs were first observed at a depth of 17.8 cm on ~156 days of gestation (Figure 4B and C). Two additional ultrasound procedures were conducted over three weeks, prior to the premature birth of a female calf.

Discussion

The strong correlation between saliva and faecal progestogen concentrations demonstrates saliva offers a comparable sample alternative for monitoring progestogens in the common hippopotamus. Given the challenges of obtaining faeces and the ease at which saliva can be collected from this species, these results have the potential to greatly facilitate the reproductive management of hippos in human care. Characterisation of the oestrous cycle and gestation via analyses of salivary hormones in hippos herein, produced patterns that mirrored previously published data from faecal hormone monitoring (Graham et al. 2002). Salivary and faecal progestogen concentrations were cyclical, and estimated cycle length and non-pregnant luteal phase duration were comparable to previously published data for common hippo (Graham et al. 2002) and closely related pygmy hippo (cycle length; Flacke et al. 2017). Progestogen concentrations reflected reproductive activity by increasing after common oestrous behaviours were observed in all non-pregnant females.

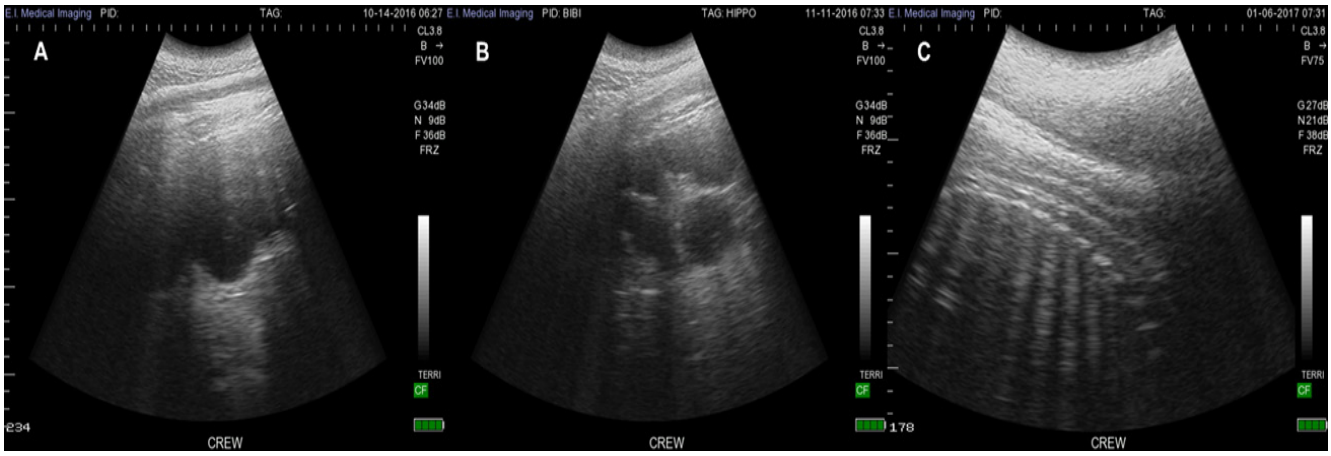

Figure 4. Common hippopotamus transabdominal ultrasound images during a pre-term pregnancy. Images depict A. foetal tissue (indicated with arrow) surrounded with fluid at ~79 days, B. fluid-filled foetal organs (indicated with arrows; ~156 days), and C. foetal spine and ribs (indicated with arrow) at ~156 days of gestation.

Additionally, salivary and faecal progestogen levels were elevated during pregnancy and dropped immediately prior to parturition, further demonstrating biological relevance. To accurately align the generated profiles for each sample type in this study, the faecal data were adjusted backwards approximately three days to account for the slow clearance of the hippos' digestive tracts. Hippos are foregut fermenting pseudoruminants (Owen-Smith 1988) and common and pygmy hippos have relatively long ingesta retention times (1–4.5 days; Clauss et al. 2004; Flacke et al. 2017). Similarly, in the Amazonian manatee (a hind-gut fermenter of similar body size), there was a five-day delay between matched androgen peaks observed in saliva and faecal samples (Amaral et al. 2009) which also was attributed to long ingesta passage time.

To our knowledge, this is the first reported use of transabdominal ultrasonography to diagnose pregnancy in the common hippo, which can be detected as early as day 79 of gestation. Walzer et al. (2014) used transcutaneous ultrasonography to locate testes and facilitate surgical castration of male common hippos under anaesthesia, but otherwise ultrasonography of common hippos remains limited. Ultrasonography has been effective in visualising female reproductive organs of the beluga (Steinman et al. 2012), killer whale *Orcinus orca* (Robeck et al. 2004), and bottlenose dolphin *Tursiops truncatus* (Robeck et al. 2005), species closely related to hippopotami (Lihoreau et al. 2015) and similar in size in the case of the beluga and killer whale. Many practitioners report benefits of the use of ultrasonography in reproductive monitoring of wildlife species and to facilitate assisted reproductive technologies (Radcliffe et al. 1997; Hildebrandt et al. 2000). This study has laid the ground work for the use of transabdominal ultrasonography for monitoring pregnancy in the common hippo and perhaps will inspire wide-spread use of operant conditioning training needed to progress to transrectal ultrasonography for tracking ovarian changes, a technique well established and in use for reproductive management of other pachyderms (Radcliffe et al. 1997; Hermes et al. 2000; Hildebrandt et al. 2000; Radcliffe et al. 2001; Roth et al. 2001). Images obtained prior to the pre-term birth suggest general measures of growth, such as heart diametre, may be possible and could facilitate foetal age determination and parturition date predictions. However, as this study only monitored one female with an abnormal pregnancy, further data are needed to establish reliable and accurate foetal growth profiles and endocrine patterns.

The pre-term pregnancy described herein terminated early at 181 days, approximately 6–8 weeks prior to estimated term calving date. Normal gestation in the common hippo is approximately 232 days (Graham et al. 2002). Prevalence of premature births in common hippos is unknown; reports in captivity are few and incidence has not been investigated in wild populations. Vevers (1926) reported a six-week premature birth in a young (6 years old) female that was presumed to be induced by the stress of being confined and resulted in the calf's death. Graham et al. (2002) described a female hippo that repeatedly miscarried; initial faecal progestogen levels were consistent with full-term pregnancies, though a decrease in concentrations occurred prior to miscarriage; exact cause of the miscarriage was unclear.

In this study, both salivary and faecal progestogen concentrations in the pre-term pregnancy were 5–6 times lower than levels observed in months 1–4 of a full-term pregnancy and were 1.5–3 times lower than average luteal levels. The female was immediately treated with a contraceptive regimen following the calf's birth so data regarding this individuals' luteal progestogen concentrations are unavailable for comparison. While individual variation cannot be discounted, the obvious discrepancy between this individual's concentrations and average values for the study population suggests low progesterone may have played a role in the resulting pre-term birth. Hippos have an epitheliochorial placenta with diffuse villous coverage (Amoroso et al. 1957); however, little is known about the role of the placenta in progesterone production in this species. Placentitis, caused by an infectious agent and resulting in decreased placental synthesis of progesterone, late-term abortions, and pre-term deliveries (Smith and Hughes 1974; LeBlanc 2010), was a potential cause. However, gross examination

of the placenta did not reveal any abnormalities. Histopathological assessment of the placental tissue revealed multiple large cysts filled with fibrinoid material. In humans, microscopic chorionic pseudocysts of the placental membrane are associated with intra-uterine hypoxia, placental infarction (Stanek and Weng 2007), and intrauterine growth restriction (Brown et al. 2002) which can lead to birth deformities and miscarriage (Out et al. 1991; Webster and Abela 2007). Exact cause of the pre-term calving remains unclear but immediate intervention by animal care staff ensured the survival of the calf, which was unable to stand and nurse following its birth. To date, the hand-reared female calf is demonstrating growth rates equivalent to conspecifics that have been parent-raised.

Conclusion

In conclusion, salivary progestogen monitoring offers an alternative to faecal monitoring for tracking reproductive activity and gestation in the common hippopotamus. This alternative may ease management efforts as faecal sample collection can be challenging due to hippos' natural behaviour of defaecating in water. Furthermore, transabdominal ultrasonography now is confirmed as a useful tool for diagnosing pregnancy in the common hippo and is likely suitable for monitoring foetal development during later stages of full-term pregnancies. The application of these tools may facilitate reproductive management of the species, thereby promoting maintenance of sustainable populations in managed care.

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