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Article

Non-Invasive Reproductive Hormone Monitoring in Endangered Pygmy Hog (*Porcula salvania*)

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Simple Summary: The pygmy hog is one of the world's rarest suids and classified as endangered species. Efforts are being made to breed them in captivity and reintroduce them into the wild. In this study, we examined reproductive hormones in captive pygmy hog using a non-invasive method by collecting 785 faecal samples from five females and two males for 12 months. High-pressure liquid chromatography was performed to examine the presence of immunoreactive progesterone and testosterone metabolites in the faecal samples. We have standardized and validated Enzyme immunoassays for faecal progesterone and testosterone metabolites. Using progesterone EIA, we could able to detect pregnancy in four females and estimate the gestation period. We also recorded 172 births from the captive breeding centre and found strong seasonality in births. In males, faecal testosterone metabolite concentrations were higher in the breeding season than the non-breeding season as evidenced by elevated testosterone concentrations during breeding season. A significant difference in faecal progesterone metabolites concentration was observed between non-pregnant and one-month-old pregnant females. This study would directly help in monitoring the reproductive status of reintroduced hogs in the wild and conservation breeding programs in India and elsewhere.

Abstract: The Pygmy hog *(Porcula Salvania*), till recently, classified as a critically endangered suid, is facing the threat of extinction globally due to habitat degradation. Efforts are being made to protect the pygmy hogs from extinction and breed them in captivity under Pygmy Hog Conservation Programme (PHCP). However, very little information is available on the reproductive physiology of pygmy hogs. Therefore, the present study aimed to standardize enzyme immunoassays (EIAs) for monitoring pregnancy and reproductive status using progesterone and testosterone metabolites. A total of 785 faecal samples were collected from five females and two males over a period of one year from PHCP Research and Breeding Centre, Guwahati, Assam. High-pressure liquid chromatography (HPLC) analysis revealed the presence of immunoreactive progesterone and testosterone metabolites in faeces. Mating was observed in all the five females and four of them gave birth successfully. We were able to detect pregnancy using faecal progesterone metabolites. Based on mating and parturition, the mean gestation period was estimated to be 153.25 days from four females. The breeding centre recorded 172 births between 1996 and 2000 and found strong seasonality in births and most of the births were between May and June. Faecal testosterone metabolites were significantly higher in the breeding season than the non-breeding season. This is the first study and will help in future breeding programs in other captive breeding centres and reproductive monitoring of reintroduced populations.

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Keywords: Pygmy hog; *Porcula salvania*; Progesterone; Testosterone; Faecal hormone; Pregnancy detection

1. Introduction

The Pygmy hog (*Porcula salvania*), the rarest and world's smallest wild suid belongs to the suidae family [1], was listed as critically endangered by the IUCN Red List till 2019; now it has been downgraded to endangered category [2] due to the conservation breeding and reintroduction efforts of the Pygmy Hog Conservation Programme (PHCP). It continues to be listed under the Schedule I of the Indian Wildlife (Protection) Act, 1972. Pygmy hog is considered an indicator species of the healthy grassland ecosystem and suffers from poor wildlife management practices, persistent burning and the other anthropogenic disturbances [3,4]. Once widespread across tall wet grassland in a narrow strip south of the Himalayan foothills from Uttar Pradesh to Assam (India) across Nepal and Bhutan, its populations declined in the last century. By early 1990s, it was reduced to a single global population of 400-500 individuals in the Manas National Park, India. The pygmy hog populations have declined due to degradation and loss of grassland, the rapid expansion of human settlements and agricultural encroachments, flood control schemes and improper management of grasslands ecosystem [5-7]. Further, planting trees in grasslands, indiscriminate use of fire to create an opening and to promote fresh grass are other major threats to pygmy hog's habitat [8]. Interestingly the pygmy habitats are shared by other endangered animals that include the one-horned Indian rhinoceros (*Rhinoceros unicornis*), tiger (*Panthera tigris*), hispid hare (*Caprolagus hispid*), water buffalo (*Bubalus arnee*), Bengal florican (*Houbaropsis bengalensis*) and Assam roofed turtle (*Kachuga sylhetensis*).

Efforts are being made to save the species from extinction which includes conservation breeding and reintroduction. The initial efforts during 1971 and 1976 failed to yield any success due to the nonscientific way of breeding [5]. Later, with Assam Forest Department, Durrell Wildlife Conservation Trust, and IUCN/SSC Wild Pig Specialist Group, the Eco-Systems-India set up a research and breeding centre in 1996 to breed pygmy hogs in captivity and release them into wild to replenish natural habitats. The program had successfully produced 683 individuals till 2020 from 10 wild-caught pygmy hogs. Between 2008 and 2018 a total of 116 captive-bred individuals were released periodically into three reintroduction sites at Sonai Rupai Wildlife Sanctuary, Rajiv Gandhi Orang National Park and Barnadi Wildlife Sanctuary in Assam [8]. In 2020, 14 hogs were released in the eastern range of Manas National Park, where less than only 100 hogs may now survive in the central range. Thus, 130 captive-born individuals have been released into the wild as part of the continuing recovery program, which is putting increased stress on the efforts to restore and manage suitable grasslands in its former range.

Pygmy hogs eat a wide range of food, including roots, tubers, shoots, insects, earthworms, eggs, and carrion. They are foragers and spent six to eight hours searching for food by digging and turning up litter and topsoil using their snout [4]. They live in group of 4-6 individuals, primarily adults with their young ones. Adult males weigh about 8-10 kg with the head-body length of 61-71 cm, while females weigh 6-8 kg with a head-body length of 55-62 cm [6]. Most of the matings in captivity were between December and February, and births were recorded before the monsoon (May to September). The litter size ranged between 2-7 but mostly in the range of 4-6 in captivity [6].

Reproductive seasonality is the characteristics of many mammalian species. Seasonality is a result of various intricate factors formed by physiological mechanisms due to environmental factors such as climate, temperature, humidity, photoperiod, nutrition, foraging conditions and social interactions between the conspecifics [9-11]. The physiological control of seasonal breeding is driven by the central circadian regulatory system situated in suprachiasmatic nucleus (SCN) which involves modulation of neuroendocrine mechanism using hypothalamus, pituitary and pineal glands to regulate the breeding season.

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Most species show strong seasonal reproductive variation evidenced by increasing levels of sex steroids, including long-tailed macaques (*Macaca fascicularis*) [12], Plains zebra (*Equus quagga*) and springbok (*Antidorcas marsupialis*) [13], Iberian red deer (*Cervus elaphus hispanicus*) [14], Père David's deer (*Elaphurus davidianus*) [15], coyote (*Canis latrans*) [16] and camels (*Camelus dromedarius*) [17]. The wild boar (*Sus scrofa*), a close relative of pygmy hog is seasonal polyestrous, while the domestic pig is known to breed throughout the year [18].

Understanding basic reproductive function is crucial for successful conservation breeding programs of endangered species, and it can be studied by monitoring circulating hormones [19-21]. Hormones can be measured in a variety of biological samples such as faeces [22-24], urine [25], blood [26], saliva [27], milk [28] and hair [29-31]. Circulating steroid hormone in the blood is the precise index of reproductive-endocrine relationships; however, blood collection in free-ranging animals is impractical. As an alternative method, estimating the hormone metabolites in faeces as non-invasive method is feasible since circulating hormone metabolize in liver and excrete through faeces. Moreover, the steroid metabolites in the faeces are known to accumulate over a period of time and provides as pooled values [20]. However, a little native hormone is present in the faeces therefore biological validation is required for each species before using faecal steroid metabolites for hormone monitoring. Faecal steroid analysis has been used to assess the reproductive status and endocrine function in various captive and free-ranging wild species including Asian elephants [32,33], musk deer [34], red panda [35], primates [36,37], big cats [22,38], birds [39] and chelonians [40].

Despite the successful breeding program, the reproductive physiology of this species is poorly understood, the ongoing conservation breeding program provides an exceptional opportunity to understand the reproductive biology of the species, particularly reproductive physiology of endangered suid. The present study aimed (1) to characterize faecal hormone metabolites using HPLC, 2) to biologically validate enzyme immunoassays for progesterone and testosterone metabolites, 3) to monitor the pregnancy and reproductive status in captive pygmy hogs using faecal steroid hormone analysis and 4) to examine the seasonality of reproduction. This is the first report of monitoring the reproductive status of pygmy hogs in India using a non-invasive method.

2. Materials and Methods

2.1. Sample Collection

A total of 785 faecal samples were collected from seven captive pygmy hogs (two males and five females) from the Pygmy Hog Research and Breeding Center, Basistha, Guwahati, Assam. The males and females were caged separately and adjacent to each other. The males were allowed into female enclosures during the breeding season for mating. The captive pygmy hogs were fed daily with a balanced diet with wide range of variety of tubers, cereals, pulses, fruits, vegetables and eggs. Further, they were allowed to forage with natural vegetation, soil invertebrates such as earthworms, termites, ants and beetles within the enclosures. The enclosures are planted with *Saccharum narenga* and *Phragmities karka* grasses, which are known to occur pygmy hog habitats. The temperature in this region ranges from 11° C (January) to 33° C (July) and June to September months are rainy season with a peak rainfall during July.

Samples were collected three to four days in a week during one year (July 2015 to July 2016). Due to space restriction samples collection for some individuals were discontinued for some periods. Freshly collected faecal samples were dried in a hot air oven at 70°C, pulverized and stored in zip lock bags with date, individual ID etc. at 4°C until further extraction. Observations on mating and other reproductive behaviours (nudging, mounting, squeaking, soft grunting) were recorded if any on a daily basis during the sample collection period. Details of the age, sex, individual IDs and the number of samples collected are given in Table 1.

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2.2. Birth and gestation data

To examine the seasonality in births in pygmy hogs, the data on births from April 1996 to July 2020 at Pygmy Hog Research and Breeding Centre, Basistha, Guwahati, Assam were collected and analyzed. Data on mating observations and parturition have also been collected from the centre's records for estimating the length of gestation.

2.3. Extraction of faecal steroid metabolites

Faecal samples were extracted using the previously described procedure with minor modification [41,42]. The dried faecal powder was sieved and weight to 0.2 g in 15mL falcon tube, 2 mL of 80% methanol added and vortexed for 20 minutes. Furthermore, samples were then kept at 4°C for overnight, centrifuged at 2000 x *g* for 10 minutes and supernatants were stored in -20°C for further analysis.

2.4. Hormone assays

Faecal progesterone was measured using the monoclonal anti-progesterone antibody (CL425; provided by Dr. Coralie Munro, University of California, Davis, CA, USA). The progesterone antibody had cross-reactivity with progesterone 100% and other 5α and β reduced pregnane [43]. Faecal testosterone was measured using the polyclonal anti-testosterone antibody (R156/7; provided by Dr. Coralie Munro, University of California, Davis, CA, USA). The testosterone antibody cross-reacts with testosterone 100%, dihydrotestosterone 57.4%, <0.3% with androstenedione and <0.1% with androsterone, dihydroepiandrosterone, β-estradiol and progesterone [44].

2.5. Enzyme immunoassay procedure

Enzyme immunoassays (EIAs) for faecal progesterone and testosterone were performed as described previously [32,34]. The 96 well Nunc-Maxisorp microtiter plate was coated with 50 µl of antibody, diluted in coating buffer (0.05 M sodium bicarbonate buffer, pH 9.6) and kept at 4°C for overnight incubation. The plate was washed four times with washing buffer (0.15 M NaCl, 0.05% Tween 20). 50 µl of faecal extract diluted in EIA buffer (0.1 M PBS, pH 7, and 1% BSA) or standard was added in each well followed by 50 µl of conjugated HRP (Horseradish peroxidase), incubated at room temperature for 2 hrs. The plate was then washed 4 times with washing buffer, then 50µl of TMB (Tetramethyl benzidine/H2O2, Genei, Bangalore) was added in each well and kept in the dark for 5-10 mins for colour development. The reaction was stopped using 50µl of stop solution (1M Hydrochloric acid (HCL), and optical density (absorbance) was measured at 450 nm using ELISA reader (Thermo Multiskan Spectrum Plate Reader, version 2.4.2; Thermo Scientific, Finland).

2.6. High-performance liquid chromatography

To evaluate the immunoreactivity of faecal progesterone and testosterone with corresponding antibody and separation of faecal steroid metabolites, high-performance liquid chromatography was performed using Shimadzu CTO-10AS system (Shimadzu corporation, Japan). Steroid specific reverse-phase C-18 column was used (Waters column, Symmetry C-18, 4.6 3 20 mm, 3.5 mm, Intelligent Speed [IS] column) to identify the steroid metabolites from faecal samples. Before HPLC analysis, pooled faecal extracts were passed through Sep-Pak C18 cartridges (Waters, Milford, MA, USA) for purification and eluted with 3mL of absolute methanol. The purified faecal extracts were dried using nitrogen gas and resuspended in 100µl of absolute methanol as described previously [34,45]. The protocol running time was 8 mins using a gradient flow of 20%–64% acetonitrile (ACN): water (H2O) at a flow rate of 1 ml/min and steroid hormones were detected at 190 to 400 nm wavelength. Fractions were collected manually about 250µl every 15 seconds (4 fractions/minute) and vacuum dried. The dried fractions were resuspended in 100µl of EIA buffer and used in the assay.

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2.7. Data analysis

Data are represented as mean ± SEM. Correlation analysis for parallelism was carried out using Pearson's correlation analysis. The faecal testosterone metabolites data is presented using descriptive statistics because of the small sample size. Difference between mean progesterone metabolites concentrations of pregnant and non-pregnant was analyzed using Mann Whitney U test, as data were not normally distributed (using Shapiro-Wilk test). All statistical analyses were carried out using SPSS 17.0.

3. Results

3.1. Enzyme immunoassay validation

Progesterone and testosterone enzyme immunoassays were validated by demonstrating the parallel displacement curves between the pooled serial dilution of faecal extracts of pygmy hog and their respective standards to determine the immunological activity of faecal hormone and standard with the corresponding antibodies used in the assays (Figure.1). Assay sensitivity was calculated at 90% binding and found to be 0.39 pg. /well and 1.17 pg. /well, for progesterone and testosterone, respectively. The intra and inter-assay coefficient of variations (CV) were 6.6% and 11.6% for progesterone, 6.8% and 11.1% for testosterone. Recovery and accuracy of a known amount of unlabeled steroid hormones in faecal extracts were 81.4 ± 4.4% for progesterone and 83.1 ± 11.42% for testosterone. The correlation (r^2) and slope (m) values for the recovered exogenous steroids were r^2 =0.99, m= 0.87 and r^2= 0.98, m=0.80 for progesterone and testosterone, respectively. The presence of faecal progesterone and testosterone confirmed by HPLC profiles and eluted fractions showed the immunoreactivity with corresponding EIAs (Figure 2).

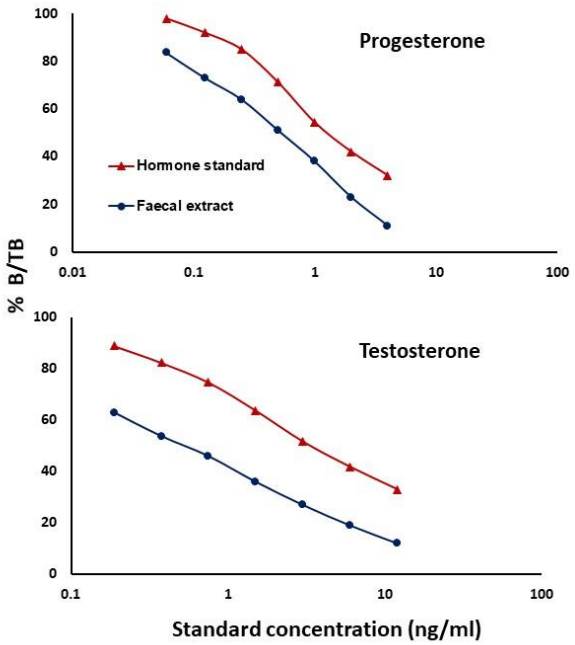

Figure 1. Parallelism between serial dilution of pooled faecal extracts of pygmy hogs (circles) and respective standards (triangles) of progesterone and testosterone.

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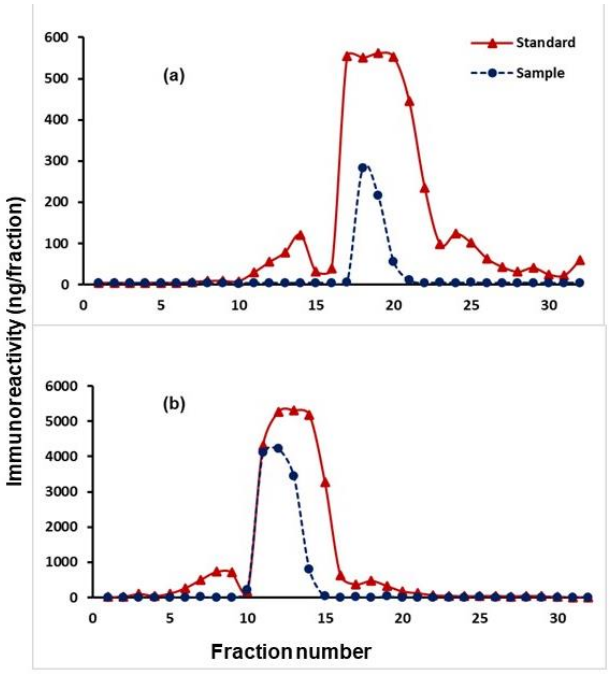

Figure 2. HPLC profiles of immunoreactive faecal progesterone (a) and testosterone (b) in pygmy hogs.

Table 1. Details of study animals, samples collection, mating, parturition and gestation period of pygmy hogs at Pygmy Hog Research and Breeding Center, Guwahati, Assam.

S. No	ID of the Animal	Sex	Age of the animal (years on Jan 2016)	No. of Samples collected	Mating dates	Date of Parturition	Gestation period (days)
1	PH 419	Female	04	141	23-12-2015	20-05-2016	148
2	PH 368	Female	4.3	124	26-12-2015	Died	
3	PH 295	Female	06	159	02-01-2016	16-06-2016	157
4	PH 401	Female	03	84	15-01-2016	18-06-2016	153
5	PH 425	Female	02	124	31-01-2016	03-07-2016	155
6	PH 418	Male	04	77			
7	PH 294	Male	06	76			

3.2. Reproductive monitoring

A total of 785 samples were collected from five adult females and two adult males for one year period. All five females were found mating with males between January and April and four of them delivered young ones, and one died due to unknown reason (PH368) (Table 1).

Overall, individual faecal progesterone metabolites concentrations ranged from 172 to 2590 ng/g (Figure 3). The pregnant females had significantly higher faecal progesterone metabolites concentrations compared to their non-pregnant values; M-W U test, $P<0.001$ for all five animals; Figure 4). All the pregnant females showed similar progestogen profiles during the pregnancy.

Based on observation of mating and parturitions of four females, the gestation period ranged from 148 to 157 days with an average of 153.25 days, however, the conservation breeding centre record showed that it ranged from 148 to 161 with a mean of 154.40 days (n=30).

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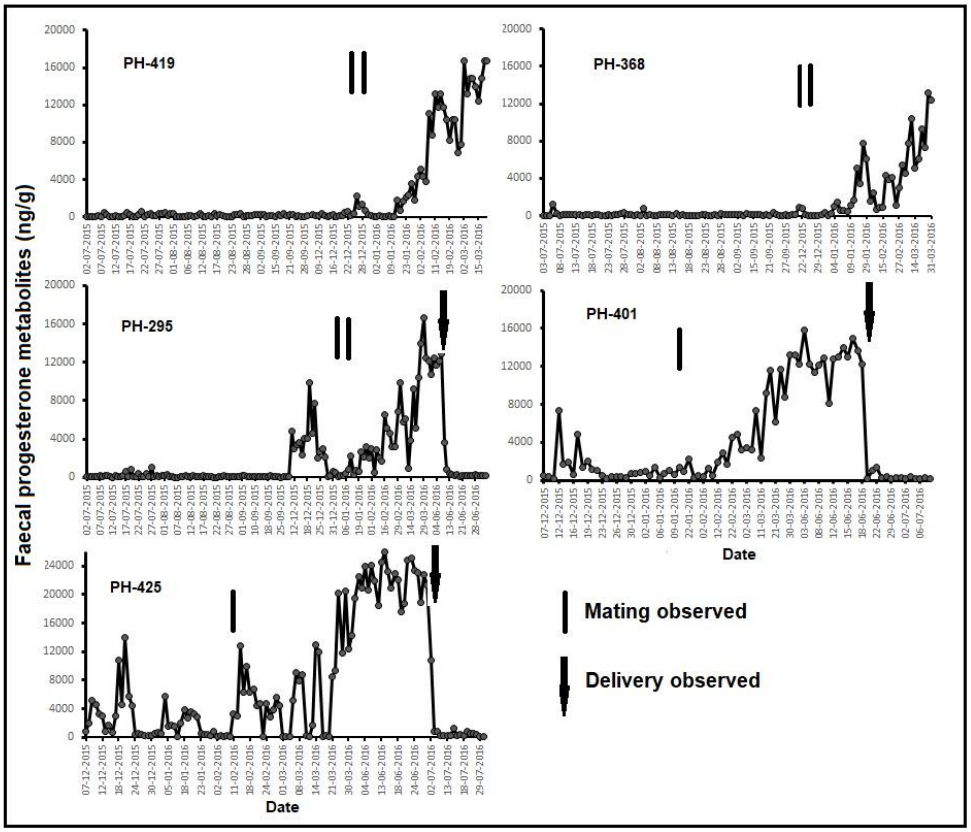

Figure 3. Showing faecal progesterone metabolite concentrations in five females monitored over 10 to 12 months at PHCP, Guwahati (Vertical bars – Mating observed; Down arrow – delivery of piglets observed). The PH-419 could not be sampled before and after the delivery due to restriction in space, while PH-368 had died due to unknown reason.

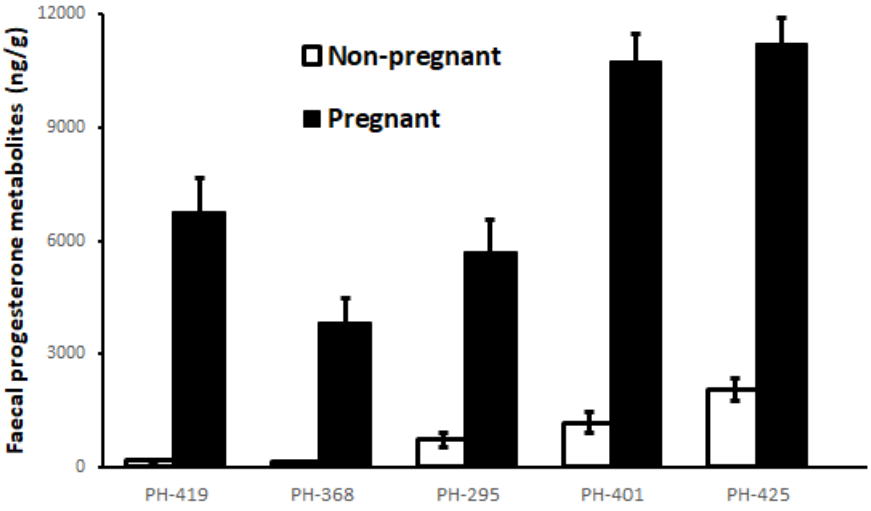

Figure 4. Faecal progesterone metabolite concentrations in pregnant and non-pregnant individuals (n = 5 females; 523 samples). The pregnant samples include two days after successful mating until the delivery, while the non-pregnant samples include non-pregnant period.

Two adult males, those involved in successful mating with these females were also monitored for faecal testosterone metabolites and they showed elevated faecal testosterone

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concentrations between September and December (Figure 5), which is about two to three months before the mating observations. Overall, the faecal testosterone metabolite concentrations ranged from 36 to 888 ng/g and the elevated values were recorded during the premating period (September - December).

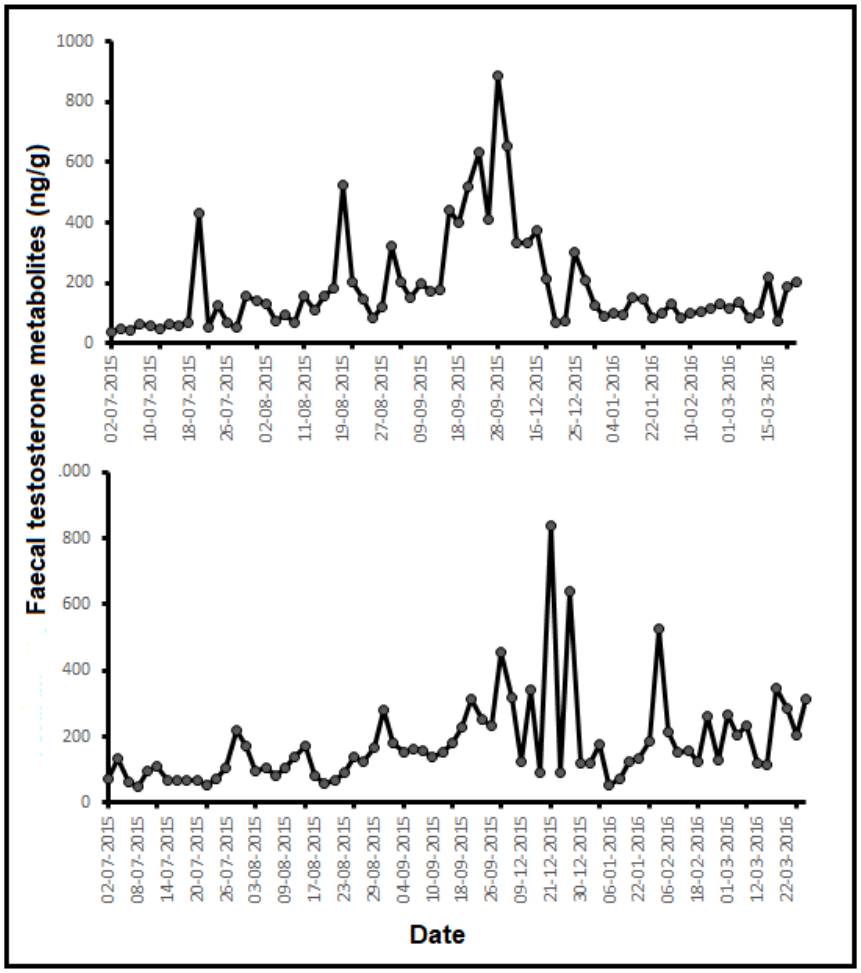

Figure 5. Faecal testosterone metabolite concentrations of two males monitored at PHCP, Guwahati.

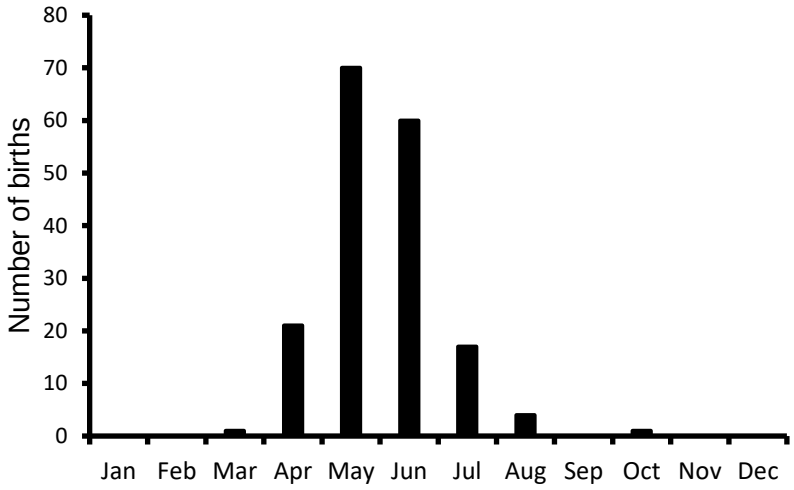

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Figure 6. Distribution of births of pigmy hogs in PHCP, Guwahati. About 172 births were recorded between April 1996 and July 2020 including three wild-caught animal's delivery (bar indicates the number of births, while the line indicates the percentage of births per month).

A total of 172 births were recorded between 1996 and 2020 in the PHCP, Guwahati. All the births were recorded between March and October, and about 74.71% of births were observed in May and June, showing a strong seasonality in the births (Figure 6). Interestingly, these are the pre-monsoon months in this geographic region.

4. Discussion

The present study reports on the standardization of enzyme immunoassays (EIAs) for faecal progesterone and testosterone metabolites and the endocrine patterns of reproductive hormones in endangered captive pygmy hogs using a non-invasive method. For the first-time long-term monitoring of reproductive hormones in pygmy hog was undertaken in a captive population. As expected, we could find immunoreactive progesterone and testosterone metabolites in faecal samples of pygmy hogs using HPLC analysis. The progesterone metabolites in the faecal extract could be monitored using monoclonal antibody EIA (CL425) developed against progesterone (UC Davis, USA). This antibody reported high cross-reactivity with 5 alpha and beta pregnane metabolites excreted in faeces of a variety of species [43]. The progesterone EIA (CL425) has been previously standardized to detect the pregnancy in a wide range of animals such as Himalayan musk deer (*Moschus chrysogaster*) [34], dugongs (*Dugong dugon*) [46], maned wolf (*Chrysocyon brachyurus*) [47], black rhinoceros (Diceros bicornis) and white rhinoceros (*Ceratotherium simum*) [48], giant anteater (*Myrmecophaga tridactyla*) [49], giraffes (*Giraffa camelopardalis rothschildi*) [50], Nile hippopotamus (*Hippopotamus amphibius*) [51] and red brocket deer (*Mazama americana*) [52]. In this study, we could successfully use and monitor pregnancies in pygmy hog and also able to distinguish between pregnancy and non-pregnancy values using faecal progestogen. This finding would provide direct implication on the successful breeding and monitoring of reproduction in one of the most endangered mammals in the world.

Of the five females, four were observed with successful mating and conceived as evidenced by the delivery of litters (size = 3-4). One of the hogs (PH 368) died during the study period and found to be pregnant as four fetuses were observed during post-mortem. The mean gestation period estimated to be 153.25 days based on mating and delivery observations. During pregnancy, the faecal progesterone metabolite concentrations were elevated in all females until parturition. The faecal progesterone concentrations dropped to baseline values within a few days of parturition. The observed gestation period is also within the range of overall records of the breeding centre, which is between 148 to 161 days from 30 females. However, previous reports suggested that the mean gestation period was 120 ± 5 days and it ranged between 110 and 130 days based on the behavioural observations [53-55]. Interestingly, the gestation periods in Suidae family ranges widely from 115 days in wild boar (*Sus scrofa*) to 170 days in common warthog (*Phacochoerus africanus*) [56]. The present observation is within the range of suidae family's gestation period. Furthermore, the present study showed that pygmy hogs are seasonal breeder as evidenced that most of the births were recorded within a few months and before the monsoon, while its related species breeds throughout the year.

Previously, testosterone EIA (Polyclonal antibody, R156/7) has been reported for monitoring faecal testosterone metabolites in a wide range of animals including pronghorn (*Antilocapra americana peninsularis*) [57], red river hogs (*Potamochoerus porcus*) [58], Polar Bears (*Ursus maritimus*) [59]. In this study, we have shown immunoreactive testosterone metabolites in the faecal samples and found immunoreactivity with the antibody (Munro, University of California, Davis, CA, USA). Faecal testosterone metabolite levels of the two monitored pygmy hogs did not show any clear cycle; however, there were elevated concentrations during September to December for both males. Most of the mating were observed between December and February, which is two to three months after the

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elevated faecal testosterone metabolites in males. Faecal testosterone metabolites elevation in mammals is directly related to reproductive preparedness and sperm production. Overall, the elevated testosterone metabolite concentrations were related to male fitness in breeding, as evidenced by mating with the females during December and January.

Previous studies have shown that faecal steroid metabolites analysis could be monitored in other of members of suidae family including red river hog (*Potamocherus porcus*), common warthog (*Phacochoerus africanus*), babirusa (*Babyrousa babyrussa*) [60] wild boar (*Sus scrofa*) [18] and collared peccary (*Pecari tajacu*) [61]. However, this is the first report of validation and standardization of enzyme immunoassays (EIAs) for reproductive monitoring in pygmy hogs using non-invasive methods. Since the pygmy hog is considered one of the most endangered mammals globally, this study would directly help in breeding management in captivity. Furthermore, this methodology could be used as fertility monitoring and pregnancy detection in pygmy hogs in captivity, in the wild and reintroduced populations.

5. Conclusions

This is the first study on reproductive hormones (progesterone and testosterone) monitoring in endangered pygmy hog using a non-invasive method. Faecal progesterone and testosterone EIAs can be used to detect the pregnancy and fertility status in pygmy hogs. This study would further facilitate in reproductive monitoring of breeding programs in captivity and also in the management of wild and reintroduced population

Author Contributions: Conceptualization, G.U., P.J.D. and G.N; methodology, V.K., S.B.; validation, V.K.; formal analysis, G.U., V.K.; investigation, V.K., S.B.; resources, G.U.; data curation, G.U., V.K.; writing—original draft preparation, V.K., G.U.; writing—review and editing, V.K., G.U., P.J.D., G.N.; visualization, G.U.; supervision, G.U.; project administration, G.U., P.J.D. and G.N.; funding acquisition, G.U. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which studies were conducted.

Data Availability Statement: All relevant data are presented in the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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