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A NON-INVASIVE TECHNIQUE TO MONITOR REPRODUCTIVE HORMONE LEVELS IN COMMON PALM CIVETS, *Paradoxurus hermaphroditus* Pallas, 1777

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ABSTRACT

Fecal steroid assays have been used to provide information on the estrous cycle, pregnancy, re-estrus, reproductive season and therapeutic treatments of an expanded list of species. This same method could be used in monitoring the reproductive status of the common palm civets (*Paradoxurus hermaphroditus)*, also called toddy cats, a nocturnal omnivorous mammal found in South east Asia in general and Vietnam in particular. To study its reproductive cycles, we have collected 2,635 fecal samples from 12 adult female civets in captivity at Dong Nai Biotechnology Center. Progesterone (P4) and Estradiol (E2) contents from fecal samples were analyzed using automatic ELISA Dynex DS2 and the Progesterone and Estradiol ELISA Kit. In non-pregnant civets, the concentrations of fecal E2 ranged from 0.05 to 7.01 μg/g dry feces (df), with the average of 1.07 ± 0.84 μg/g and a peak of 3.22 ± 0.64 μg/g. Fecal progesterone metabolites ranged from 0.15 to 12.32 μg/g, and the overall mean of the samples was 1.72 ± 2.16 μg/g. The average period of change in E2 content was 28.6 ± 2.29 days. During pregnancy, the P4 content in the stool ranged from 6.21 to 23.12 μg/g with the average of 15.17 ± 5.22 μg/g, which was approximately 5 to 7 fold higher than that of non-pregnant animals ($p < 0.05$). In pseudopregnancy civets, there were also significant changes in P4 after fertilization, but the duration was only a half of the pregnant length. The P4 concentration in the stool at this stage ranged from 8.02 to 11.47 μg/g, an average of 9.73 ± 1.73 μg/g. This index was also significantly higher in non-pregnant and statistically different from those in pregnant ($p < 0.05$). It was found that fecal hormone metabolite analysis is a useful and reliable indication to confirm estrous cycle, pregnancy, non-pregnancy or presumed pseudopregnancy of *P. hermaphroditus*, both in the wild and in captivity.

Keywords: Common palm civets, non-invasive, estrogen, progesterone, reproductive hormone, steroid.

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INTRODUCTION

Efforts in assisted reproductive technologies (artificial insemination, in vitro fertilization or embryo transfer) depend on the knowledge of reproductive physiology and the endocrine status of a particular species (Schwarzenberger et al., 1996). In case of artificial insemination, the exact time of fertilization is critical such that the animal's estrous cycle needs to be determined using a simple and non-invasive method for a successful fertilization.

Serotonin level is the most accurate representation of sexual activity. However, techniques for collecting this information through blood serum may affect animal welfare and are costly. Repeated blood sampling will cause stress, affect animal health. It is also difficult to perform such sampling in field conditions, such

as with wildlife. Even repeated blood sampling to measure steroids may be impractical for small animals, because sampling might interfere the daily life of animals or even lead to death.

Urine and faece sampling could be used as a better alternate non-invasive method to give accurate results. Although urine specimens and faeces can be collected to assess reproductive status in captive wildlife, collecting urine from free-ranging animals would be difficult, thus prevented routine use of urine as a monitor. In this regard, stool samples are the most practical choice. The methods of measuring the flow of steroid metabolites in faeces for the evaluation of endocrine status of animals was pioneered in the late 1970s for birds, and was established for mammals in the early 1980s. Evaluation of fecal estrogen has been used as a reliable indicator of pregnancy in some ungulates (Morden et al., 2011) and some primates (Shideler et al., 1993; Ziegler et al., 1996). They are also used to determine the pre-ovulation period in predators (Putranto et al., 2011). Over the past three decades this method has been applied to the increasing number of animal species to monitor sexual activity (Palme, 2005). Fecal steroid assays have been used to study and provide information on the estrous cycle, pregnancy, abortion, genital reproduction, re-estrus, reproductive season, effective monitoring of data, and fertility treatment (Kumar et al., 2013). Previously unknown or unclear information on female reproductive status has now been easily evaluated using steroid hormone analysis (Kumar et al., 2013). Steroids are metabolized in the liver and excreted into faeces and extraction of steroids from faeces can be done using a variety of diluents such as ethanol, methanol etc.

P. hermaphroditus is widely distributed in Central, South and Southeast Asia, Taiwan, Southern China, Nepal, Singapore, Sri Lanka, Vietnam and scattered in other places in the world (Duckworth et al., 2017).

Civets have been trapped and hunted for various uses, such as meat, fur, flavoring, weasel coffee production. Their habitat destruction is causing depletion of this animal in nature. Though not considered to be an endangered species, it is important to preserve its genetic resources to maintain healthy population in the long run. The development of sustainable genetic resources is directed towards the management and conservation of natural resources (FAO, 2007). It had been proven that conservation of genetic resources of livestock breeds, exploring and developing gene sources is an effective solution to maintain the species population (Duc, 2016). In Vietnam, many civet farms have been developed successfully. Raising civet would not only bring economic benefits to the farmers but also helps reducing civet hunting (Huynh et al., 2010). Growth characteristics, reproductive traits and physiological parameters of civets have been studied in captivity (Hien et al., 2017 a, b, c). The results of the use of gonadotropin in raising the herds in captivity and the efforts to protect civets in nature have yielded satisfactory results (Binh, 2015) and contributed to preserve the biodiversity. However, the lack of information on steroid hormone levels of females leads to the difficulties of reproductive status examination. As such there is a shortage of information when reference values are needed for comparative study. It is the authors' belief that data on reproductive steroid hormones in this study will serve as a basis for evaluating reproductive status and contributing to the efforts of assisted reproductive technologies for farm raised common palm civets.

MATERIALS AND METHODS

A total of 2,635 fecal samples were collected from 12 adult female civets. All of the animals were considered clinically healthy based on history and physical examination, and were not pregnant at beginning of the study. Each individual was tagged in a separate box for monitoring (Table 1).

The civets in this study were raised in the farm of Dong Nai Biotechnology Center in Xuan Duong commune, Cam My district, Dong Nai province. The farm was surrounded by a 2.5 m high wall, which helps to prevent the civets from escaping and keeps the civet safe from direct wind, and bright light. The farmhouse was washed with tap water daily. Extensive cleaning work was done once a month using BESTAQUAM-SR in water antiseptic solution 1/400 didecyl dimethyl ammonium bromide. The civets were vaccinated every 6 months.

All civets received the same good nursing and housing conditions during the study. Main

meal (18:00) is porridge cooked with different ingredients such as fish, viscera, chicken heads. As a supper (11:00), fruit of all kinds, mainly bananas, papaya, watermelon. The daily rations were metabolizable energy (ME): 450 Kcal, crude protein (CP): 18 gr, Lipid: 3 gr, dry matter (DM) 105 gr.

Clean drinking water was put in clean cups in cages for self-drinking. The cups were cleaned daily and replaced once a day. Utensils were taken out of the cage in the morning, washed and dried preparation for dinner.

Table 1. Data regarding 12 female civets examined in this study

ID	Age* (month)	Weight (kg)	Body length (cm)	Fecal sample
F1DN	18	3.17	67.41	210
F2DN[a]	24	3.32	68.64	224
F3DN	31	3.43	68.72	210
F4DN[a]	36	3.56	69.26	208
F5DN	40	3.51	69.18	227
F6DN	33	3.33	68.34	224
F1TD[a]	30	3.28	68.22	210
F2TD	32	3.55	68.41	231
F3TD[a]	32	3.48	68.45	231
F4TD[b]	32	3.38	68.56	230
F5TD	27	3.25	67.68	214
F6TD[b]	27	3.27	67.72	216

[a] Females became pregnant in this study as indicated in later experiment

[b]Females were presumed pseudopregnancy

*Age, weight and body length at the start of the study

Fecal sampling and extraction

Fecal samples were collected at around 18:00–20:00 with an average of 3 days per week for 16 months. Fresh faecal samples (5g) were collected in a plastic bag and stored at -20°C until analysis. After thawing, 0.2 g was weighed and placed in a glass jar containing 2 ml of 90% methanol. After shaking for 30 min, the suspension was centrifuged at 1,700 × g for 20 min on a shaker HS 260 control (IKA, Germany). After centrifuged, approximately 1 ml of the aqueous solution was extracted into a 1.5 ml eppendorf vial and freezed at (–)20°C until use (Frederick et al., 2010). The remainder was returned to a glass vial and dried to determine the dry weight of the stool.

Hormone assay processing

The amount of P4 and E2 in faeces was determinned with fully automated ELISA Dynex DS2 processing system (Dynex, USA). A progesterone ELISA Kit (DRG International, Inc., Germany) reacted with progesterone (P4) (100 %), with slight cross-reaction to 17α OH progesterone (0.30%), 11-desoxycorticosterone (1.1%), corticosterone (0.20%), pregnenolone (0.35%), 11-desoxycortisol (0.10%). The intra- and inter-assay coefficients of variation were 6.99% (mean = 4.67 ng/ml, n = 20) and 4.34% (mean = 4.55 ng/ml, n = 12), respectively. An estradiol ELISA Kit (DRG International, Inc., Germany) reacted with estradiol-17ß (100%) with cross-reaction to estriol (0.05%). The intra- and inter-assay coefficients of variation were 2.71% (mean = 198.05 pg/ml, n = 20) and 6.72% (mean = 197.21 pg/ml, n = 12), respectively.

Data analysis

All hormone levels were expressed in micrograms per gram of dried faeces (μg/g df). The length of the ovarian cycle was calculated as number of days with peak fecal E2 readings. The peak fecal E2 and P4 contents were defined as those values that were greater than mean values from an individual civet (Putranto, 2011).

The basic statistical parameters such as mean, standard deviation (SD), and T-test with a significance level α=0.05 were calculated using Microsoft Excel (Verson 2013 Microsoft, USA).

RESULTS AND DISCUSSION

The range of estradiol (E2) and progesterone (P4) of non-pregnant civets in this study are shown in table 2 below.

Faecal estradiol in non-pregnant civets

Estradiol is the primary and biologically active estrogen secreted both from theca and granulosa cells of growing ovarian follicles (Kumar et al., 2013). The concentrations of fecal E2 in non-pregnant civets in our study ranged from 0.05 to 7.01 μg/g df, with an average of 1.07 ± 0.84 μg/g df and a peak of 3.22 ± 0.64 μg/g df. Although faecal estradiol value of civets have not been published, faecal estradiol values in other animals have already been widely used to monitor their sexual activities. For example, faecal E2 of the Siberian tiger population ranged from 0.39 to 0.49 μg/g and the mean faecal E2 of Bengal tiger was 0.45 μg/g, and that of Sumatra tiger was 2.36 μg/g (Putranto, 2011).

Table 2. The range, peak and cycle of P4 and E2 during non-pregnancy in civets

Hormone	During (μg/g)		Peak (μg/g)		Cyclic change (day)	
	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD
E2	0.05–7.01	1.07 ± 0.84	1.13–7.01	3.22 ± 0.64	26.8–33.1	28.6 ± 2.29
P4	0.15–12.32	1.72 ± 2.16	6.03–12.32	7.26 ± 1.11	26.6–31.3	27.8 ± 2.80

In this study, the average number of E2 peaks of individual civet was 13–14 counts in 16 months (Fig. 1 below shows a representative pattern). Changes in E2 levels showed a cyclic fertilization. The duration of each cycle ranged from 26.8–33.1 days with the average of 28.6 ± 2.29 days. This period was comparable with that (27.0 days) of Siberian tigers (Putranto, 2011) and of Bengal tigers (29.3 days) (Putranto et al., 2007), but different from that of domestic cat (21 days) or leopard (10–20 days) (Brown, 2011). Putranto (2011) suggested that fecal E2 excretion probably paralleled with follicular growth and that the remarkable cyclic changes in the fecal E2 contents indicate a regular ovarian cycle.

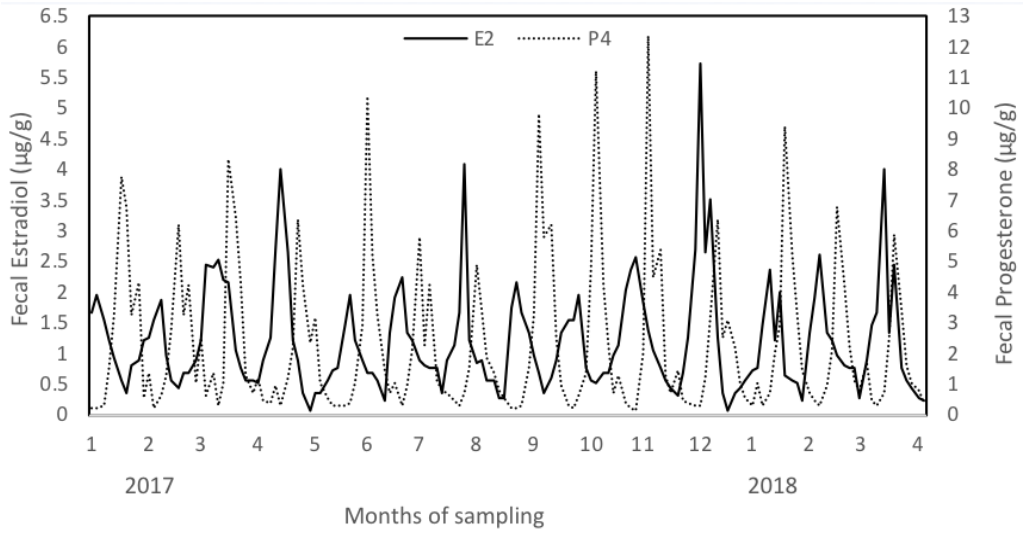

Figure 1. Changes in the fecal P4 and E2 levels of a common palm civet (No1: F1DN- non-pregnancy)

Faecal progesterone in non-pregnant civets

Progesterone is a principal steroid hormone secreted mainly from the corpus luteum (CL) that regulates estrous cycle and maintains pregnancy in all mammals. Adrenal cortex and placenta also secrete a certain amount of progesterone during certain physiological stages like pregnancy (Kumar et al., 2013).

In this study group, non-pregnant female civets' fecal progesterone (P4) metabolites levels ranged from 0.15 to 12.32 μg/g with the overall mean of 1.72 ± 2.16 μg/g (Table 2). For comparison, faecal progesterone level of Siberian tigers varies from 0.27 to 38.19 μg/g and that of the Sumatra tigers ranged from 0.09 to 18.52 μg/g, and the level in Bengal Tigers was 36.05 μg/g (Putranto, 2011).

Faecal progesterone levels of civets also change over time. The peak of faecal progesterone level ranged from 6.03-12.32 (μg/g) with an average of 7.26 ± 1.11 (μg/g). The cycle of change in progesterone level ranged from 26.6 to 31.0 days with an average of 27.8 ± 2.80 days.

We also noticed that, in the same period of the cycle, the peak of E2 usually occurs 3–5 days before the peak of P4. See Figure 1. According to Brown (2011), there are four phases of the estrous cycle: proestrus, estrus, diestrus and anestrus. Estrus accompanies with advanced follicular development and peak concentrations of estradiol. During diestrus, one or more corpora lutea (CL) produced progesterone that maintains a elevated level for some lengths of time.

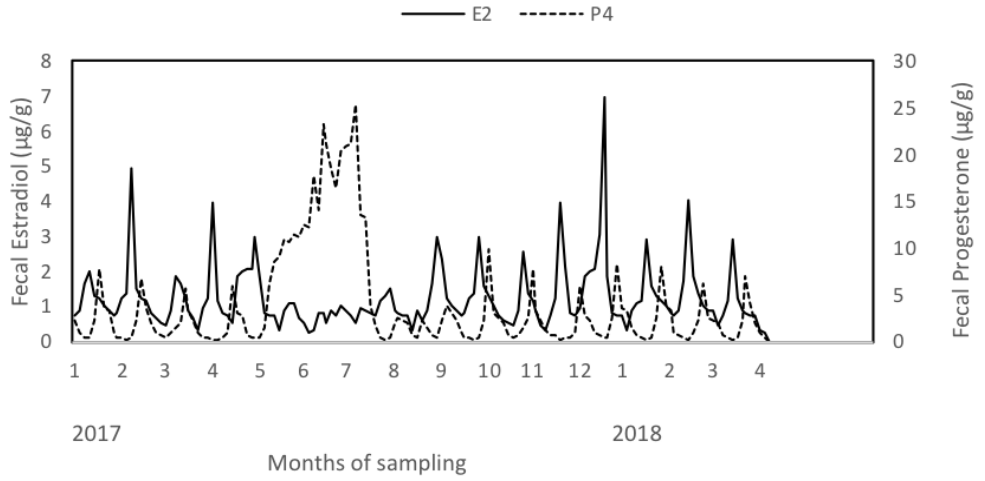

Figure 2. Changes in the fecal contents of P4 and E2 in common palm civets (No1: F4DN- became pregnant in 6/2017)

Pregnant and nonpregnant luteal phases in civets

During this study period, 12 female civets were mated with male civets. However, only 4 animals became pregnant while 6 remained non-pregnant, and 2 were assumed to be pseudopregnant. Changes Faecal P4 and E2 levels of individuals after fertilization are presented in table 3.

During pregnancy, the civets' faecal P4 level ranged from 6.21 to 23.12 μg/g with an average of 15.17 ± 5.22 μg/g. This value is approximately 5 to 7 times higher ($p < 0.05$) than non-pregnant and post-fertilization periods. In individuals with conception, P4 increased significantly for a period between 60 and 63 days after fertilization (Fig. 2). This result is consistent with the previous study that showed the average gestation period of civets is 60.9 days (Hien et al., 2017b).

Table 3. Faecal P4 and E2 levels of civets during pregnancy and pseudopregnancy after mating

Hormone	Non-Pregnant (n = 6)		Pregnant (n = 4)		Pseudopregnancy (n = 2)	
	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD
P4 (μg/g)	0.27–10.72	2.12 ± 1.86[a]	6.21–23.12	15.17 ±5.22[b]	8.02–11.47	9.73 ± 1.73[c]
E2(μg/g)	0.18–6.18	1.14 ± 0.78[a]	0.22–1.05	0.74 ± 0.23[b]	0.35–1.99	1.34 ± 0.57[a]

Note: Differences in characters (a, b, c) in the same row, the difference was statistically significant ($p < 0.05$), According to T-test with a significance level of $\alpha = 0.05$

In previous studies, the peak fecal P4 level in pregnant Siberian tiger was 24.29 μg/g and that of pregnant Bengal tiger was 104.17 μg/g, that were approximately 2 to 6 times higher than the mean value of the same species of tigers (Putranto, 2011). According to Brown et al. (2006), the P4 steroid hormone plays a critical role in maintaining the pregnancy of female mammals.

Faecal E2 concentration of a juvenile fennel during pregnancy is relatively lower than that in other periods. Fecal E2 ranged from 0.22 to 1.05 μg/g with an average of 0.74 ± 0.23 μg/g. After parturition, E2 increased and marked the recovery of ovarian activity from 25–30 days. See Fig. 2. In contrast, in fetal predisposed individuals, there was a negligible E2 change (range of 0.35–1.99 μg/g) compared to non-pregnant ones ($p > 0.05$), and distinctly lower than that of the pregnancy period ($p < 0.05$). This result is similar to that observed in the Pallas' cat, the clouded leopard, tiger, in these an increase in estrogen excretion in faeces was not observed during pregnancy (Brown, 2011).

In pseudopregnancy civets, there were also significant changes in P4 after fertilization, but the change duration was only 26–30 days. See Figure 3. The faecal P4 level at this stage ranged from 8.02 to 11.47 μg/g with an average of 9.73 ± 1.73 μg/g. This value was significantly higher than that of non-pregnant civets but was significantly lower than those of pregnant animals ($p < 0.05$). In other studies, leopard cats, clouded leopards, snow leopards and cheetahs have been reported to have increased duration of P4 contents during presumed pseudopregnancy (Putranto, 2011). Thus, the main indicator to distinguish between pregnancy and fake pregnancies is both the duration and the degree of the increase in the fecal P4. Thus, the fecal P4 contents of female civets can be used to discriminate pregnancy and pseudopregnancy. From a standpoint of reproductive management, it is technically possible to diagnose pregnancy based on fecal progesterone levels that remain elevated over the normal length of a non-pregnant luteal phase (Brown, 2011).

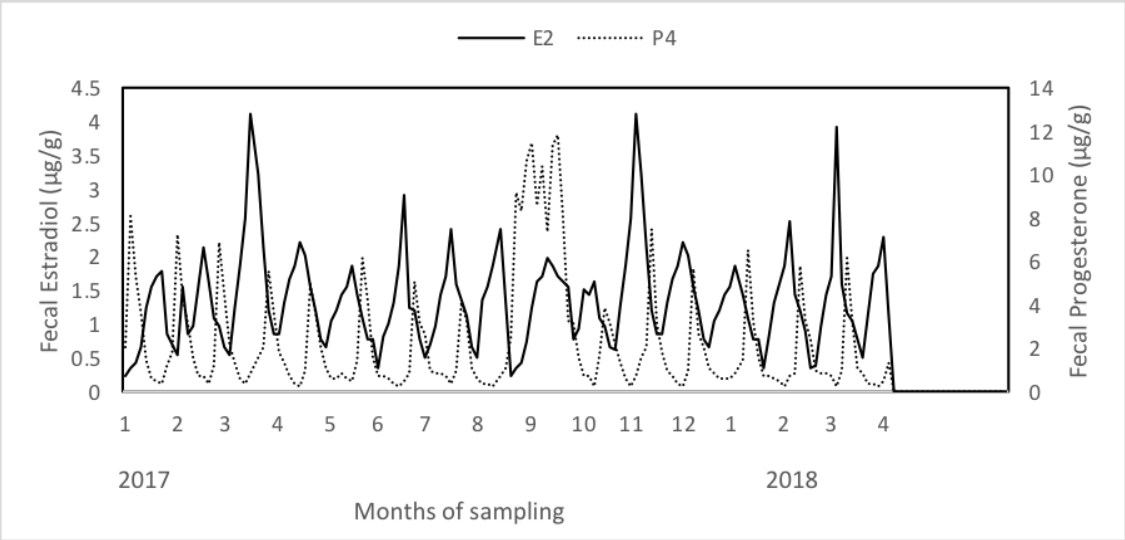

Figure 3. Changes in the fecal contents of P4 and E2 in common palm civets (No1: F4TD-presumed pseudopregnancy)

CONCLUSION

In conclusion, faecal estrogen and progesterone metabolites measurements are the wel1-established approaches for monitoring of reproductive functions in a variety of mammalian species. In common palm civets, fecal hormone metabolite analysis is a useful method to confirm estrous cycle, pregnancy, non-pregnancy or presumed pseudopregnancy. Since no such comprehensive study of the steroid hormone levels in civets, the present results provide a reliable reference value in the study

and contributing on efforts in assisted reproductive technologies in farming conditions.

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