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A non-invasive identification of hormone metabolites, gonadal event and reproductive status of captive female tigers

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ABSTRACT

Putranto HD (2011) A non-invasive identification of hormone metabolites, gonadal event and reproductive status of captive female tigers. Biodiversitas 12: 131-135. As a non-invasive method, fecal sample provides some advantage for animal and collector. The purpose of the present study were to monitor the reproductive status of female Siberian tigers (*Panthera tigris altaica*) by assessing changes in fecal during natural ovarian activity and pregnancy and to identify whether progesterone (P4) exists and what kinds of P4 metabolites excreted into the feces. Two female tigers were fed a diet consisting of meat. Drinking water was available *ad libitum*. Feces were collected ones to twice a week. The fecal contents of P4 and estradiol-17β (E2) were determined by EIA and P4 metabolites were separated by a modified HPLC. The EIA results shown that during its natural ovarian activity the E2 contents showed cyclic changes at the average of 27.0 d interval, however, no distinct cycles were shown in fecal P4 contents of non-pregnant tiger. In contrary, the fecal P4 contents in pregnant tiger increased remarkably after copulation approximately 2- to 6-fold higher than the mean value. The HPLC results indicated that two peaks were primarily detected fraction 63- 64 min (identified metabolites) and fraction 85 min (not identified metabolite) in feces of pregnant tiger. However, P4 detected only small amount in feces. It is possible to assess non-invasively gonadal events such as luteal or follicular activity or ovulation of Siberian tigers by endocrine monitoring based on fecal P4 and E2 to understand reproductive status.

Key words: EIA, feces, HPLC, reproductive status, female tiger.

INTRODUCTION

Tiger, *Panthera tigris*, is classified into order Carnivora and family Felidae (MacDonald 2001). It is the largest species among Felidae family member. This species is divided into eight subspecies namely Bengal (*Panthera tigris tigris*), Siberian (*P. t. altaica*), Indochinese (*P. t. corbetti*), Sumatran (*P. t. sumatrae*), South China (*P. t. amoyensis*), Javan (*P. t. sondaica*), Bali (*P. t. balica*), and Caspian (*P. t. virgata*) tiger (Mazák 1981). Unfortunately, there are only five living subspecies tigers left on the world recently. The critically endangered subspecies, the Siberian tiger (*P. t. altaica*) is estimated at approximately less than 400 and 400-500 individuals in the wild (Putranto et al. 2006a; 2007a).

Reproduction in tigers remains poor and unclear. With its restricted population, it would be a challenge for scientists to learn and familiarize themselves with the reproductive potential of tigers to improve the breeding potential of captive tigers. There are limited scientific references on the reproductive physiology and endocrinology of tigers (Graham et al. 2006; Putranto et al. 2006a, b; 2007a, b). Gonadal steroid hormone analysis is a major point in reproduction, and assessing its changes could allow the enhancement of captive breeding programs.

Serum hormones are the most accurate reflection of gonadal activity, and it has been used widely to monitor the profile of reproductive physiology and endocrinology status in endangered animals. Serum collection process especially from endangered animal body, induced several injure side effects such as stressful, impractical and difficulties. Non-invasive method by using fecal sample becomes the best preference because fecal sample collection is providing some advantage such as non-stressful for animal and non-risk to the sample collector, and thus easy to collect without any timing or period limitation (Putranto et al. 2006a, b; 2007a, b; 2009). Fecal sampling for fecal hormone analysis has been available for non-invasive method with long-term advantages (Putranto et al. 2007a,b).

The food has been digested and excreted from the body through the digestive tract as waste. Feces contain many important physiological signals such as sex steroid hormones and these metabolites. Generally sex steroid hormones are synthesized from cholesterol and carried in the blood flow bound to specific carrier proteins such as sex hormone binding globulin or corticosteroid binding globulin. These hormones act on target organs and are converted to many metabolites by 5α- or 5β-hydrogenase and 3α- or 3β-OH steroid dehydrogenase, then they are excreted into urine through kidney and with the bile into feces (Putranto et al. 2009).

The amount of 85-95% of steroid hormones metabolites are excreted in feces of domestic cat (Brown 2006). Specifically, progesterone (P4) metabolites in domestic cat is metabolized and excreted primarily both conjugated and unconjugated metabolites into feces (Brown et al. 1993). In non domestic felids, leopard cat (*Felis bengalensis*) and clouded leopard (*Neofelis nebulosa*), it has been reported that the P4 metabolism may be similar between those two species and were almost one conjugated metabolite (> 90%). However in cheetah (*Acinonyx jubatus*), it has been identified as three immunoreactive metabolites fractionating with percentages of 42, 51 and 7%, respectively (Brown et al. 1993).

Previous reports were described about the combination of two methods (high performance liquid chromatography (HPLC) and enzyme immunoassay (EIA) to confirm the excreted hormone metabolites (Putranto et al. 2009). The HPLC method and the EIA method were applied to separate the specific metabolites and to confirm the presence of that steroid hormone metabolites. The purpose of the present study were to monitor the reproductive status of female Siberian tigers by assessing changes in fecal during natural ovarian activity and pregnancy and to identify whether P4 exists and what kinds of P4 metabolites excrete into the feces of pregnant female Siberian tiger by using the combination of HPLC and EIA methods.

MATERIALS AND METHODS

Animal and fecal sample collection

The animal monitored included two female Siberian tigers. Tiger No.1 (Japanese Studbook No. 179, 14 years of age at the beginning of this study) housed at Yokohama Zoological Gardens Zoorasia, Japan and it was a non-pregnant female. Tiger No.2 (International Studbook No. 4441, 7 years of age at the beginning of this study) housed at Tama Zoological Park, Japan and it was a pregnant female when this study conducted. Generally, they were fed a diet consisting of horse meat, chicken and rabbit meat. Drinking water was available ad libitum. They had free access to the natural photoperiod in an outdoor paddock during the daytime. Female tiger No.1 was kept alone in an individual indoor chamber during nighttime and have a free access to socialize with male in outdoor paddock during daytime, and tiger No.2 lived together with a male in a paddock. .

Feces were collected ones to twice a week during 14 months of collection period for both tigers. There were 190 fecal samples collected from tiger No.1 and 142 samples collected from tiger No. 2. Totally, there were 332 samples collected. Fresh sample collected from sleeping chamber within at least 18 h after excretion. The feces placed into a plastic bag size 200 x 140 x 0.04 mm (Uni Pack Mark Series-G, Seisan Nippon Co., Tokyo, Japan) and stored at -20°C immediately after collection.

Sample extraction and hormones analysis

The fecal extraction procedure was described in previous reports (Kusuda et al. 2006a, b, c; 2007; Putranto et al. 2007a, b, c). The fecal contents of P4 was determined by enzyme immunoassay (Kusuda et al. 2006a, b, c; 2007; Putranto et al. 2007a, b, c). P4 antiserum (LC-28; Teikoku hormone Mfg. Medical, Kanagawa, Japan) mainly cross-reacts with P4 (100%), 5α-pregnanedione (62.2%), pregnenolone (6.3%), 11-deoxycorticosterone (3.9%), 17α-hydroxyprogesterone (2.3%), and 11α-hydroxy-progesterone (1.2%). Estradiol-17β (E2) antiserum (QF-121; Teikoku hormone Mfg. Medical) mainly cross-reacts with E2 (100%), estrone-3-sulfate (8.0%), 16-epiestriol (5.3%), estrone (3.2%), and estriol (1.8%).

HPLC procedure

The HPLC method in this study was a modification of previous report (Schwarzenberger et al. 2000). Prior to HPLC method, pooled extracts randomly chosen from those twenty three samples during early to late pregnancy were cleaned up on a Sep-Pak C-18 cartridge. Progesterone metabolites were separated by a modified HPLC method with a reverse-phase Nova-Pak C-18 column using acetonitrile/water (40/60 v/v) mixture (Putranto et al. 2009). One hundred and twenty fraction samples (1 mL) were collected.

P4 metabolites by EIA procedure

Those 120 fractions, by EIA method were assayed to confirm the presence of P4 metabolites (Kusuda et al. 2006a, b, c; 2007; Putranto et al. 2007a, b, c). The antiserum cross reaction was similar to first EIA method.

Data analysis

All fecal values are presented as the weight in dried feces. Data of first EIA procedure for hormone analyses (P4 and E2) are shown as the mean ± SEM. Beyond the second EIA procedure, the presence of P4 peaks was compared to the eluted position of some standards of P4 metabolites (Table 1, Figure 1).

Table 1. Elution time of steroid hormone and metabolites

Steroid metabolites	Elution time (min)
PdG	3
20α-OHP	31
5β-pa-3β, 20α-diol or 5α-pa-3α, 20α-diol	35
Pd	43
P4	51
5β-pa-3β-ol-20-one or 5β-pa-3α-ol-20-one	63-64
5α-pa-3β-ol-20-one	68
5α-pa-3α-ol-20-one	79
5α-DHP	98

The length of the ovarian cycle was calculated as the number of days of the peak fecal E2 interval for periods not exceeding 50 d (that is, longer than about twice the estimated cycle length) (Putranto et al. 2006a; 2007a). The peak fecal E2 contents were defined as those values that were greater than the mean of all values from an individual tiger (Putranto et al. 2006a; 2007a).

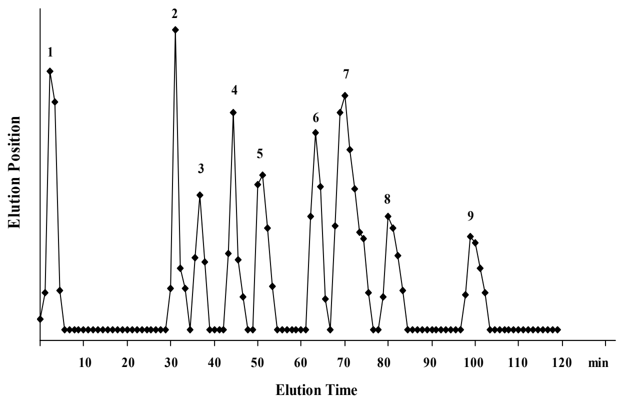

Figure 1. The chromatogram for elution position of some steroid metabolites. Numbers (1-9) indicate steroid hormone and metabolites presence. No.1 = pregnanediol-glucuronide, 2 = 20α-OHP, 3 = 5β-pa-3β, 20α-diol or 5α-pa-3α, 20α-diol, 4 = pregnanediol, 5 = progesterone, 6 = 5β-pa-3β-ol-20-one or 5β-pa-3α-ol-20-one, 7 = 5α-pa-3β-ol-20-one, 8 = 5α-pa-3α-ol-20-one, 9 = 5α-DHP.

RESULTS AND DISCUSSION

Serum hormones are the most accurate reflection of gonadal activity. However, the inconvenient method of serum collection could conduced several injure side effects such as stressful, impractical and difficulties. Lately, some studies have shown similar patterns in serum and fecal hormones, and utilization of fecal samples as a noninvasive tool is widely used to monitor gonadal activity in tigers (Putranto et al. 2006a, b; 2007a, b).

In this study, there were two different reproductive status of female Siberian tigers, non-pregnant and a pregnant female tigers. By using an EIA method, in this study also we can assess the fecal contents of steroid hormones such as P4 and E2 levels and understand about the gonadal events and its reproductive physiological status. Putranto et al. (2009) stated that the combination between EIA and HPLC method would allow us to identify whether P4 exists and what kinds of P4 metabolites excrete into the feces of female Siberian tiger.

Reproductive cyclicity and pregnancy in Siberian tigers

Range of fecal P4 and E2 during non pregnancy in female Siberian tigers is described in Table 2. The value for fecal P4 and E2 varied between 0.27 to 38.19 and 0.09 to 18.52 µg/g (n = 332, Table 2), respectively. In female Siberian tiger No. 1, the fecal P4 contents were an average of 0.78 ± 0.96 µg/g (n = 100, Figure 2). Although only two changes were recorded on February and March, no distinct cycles were shown in fecal P4 contents.

Table 2. The range of fecal P4 and E2 contents during non-pregnancy in female Siberian tigers

Female	Fecal P4	Fecal E2
	(µg /g)	
No. 1	0.27- 9.40	0.15- 3.76
No. 2	0.05- 32.32	0.04- 4.01

During its natural ovarian activity, non-pregnant female Siberian tiger No. 1 had no distinct cycles were shown in her fecal P4 contents. This value is smaller than the fecal P4 contents in female Sumatran tiger (12,17 µg/g) and its fecal P4 contents changed cyclically. The cycle length based on changes in fecal P4 in female Sumatran tiger was 58.3 ± 2.7 d (Putranto et al. 2007b). Furthermore, the value of fecal P4 contents in female Siberian tiger is also smaller than Bengal tiger which could reached 36.05 µg/g level and changed cyclically (Putranto 2008). It seems that female Sumatran and Bengal tigers excreted more fecal P4 into feces than female Siberian tiger.

The fecal E2 contents in female No. 1 and 2 were an average of 0.49 ± 0.61 µg/g (n = 107) with a peak of 1.69 ± 1.04 µg/g (n = 12, Figure 2) and 0.39 ± 0.55 µg/g (n = 100) with a peak of 1.37 ± 0.93 µg/g (n = 14, Figure 3), respectively. The ovarian cycle based on changes in fecal E_2 profiles of female Siberian tiger No. 1 and 2 are described in Table 2.

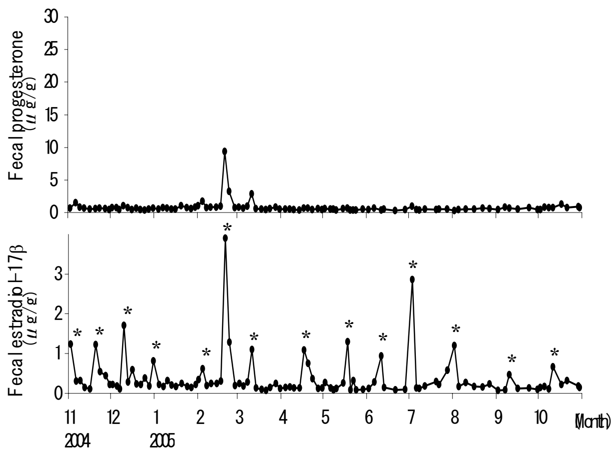

Figure 2. Changes in the fecal contents of P4 (upper) and E2 (lower) in female Siberian tiger (No.1, Yokohama Zoological Gardens). The 14 asterisks indicate the peaks of fecal estradiol-17β. (Putranto et al. 2007a).

The fecal E2 contents in these two female Siberian tigers were reached average of 0.39 to 0.49 µg/g level and changed cyclically. The value of female Siberian tigers fecal E2 contents were similar to female Bengal tiger (0.45 µg/g) (Putranto 2008), however it was smaller than female Sumatran tiger (2.36 µg/g) (Putranto et al. 2007b). The pattern of fecal E2 profiles from three tiger subspecies (Siberian, Bengal and Sumatran) were not similar. The Sumatran and Bengal tigers are shown no cyclical changes, however, Siberian tiger is shown a cyclical changes.

In two female Siberian tigers of this study, the fecal E2 contents showed cyclic changes at the average of 27.0 d interval. A similar finding was made in a previous report, which showed cyclic changes at 29.3 ± 4.4 d intervals in crossbreed Bengal-Siberian tigers (Graham et al. 2006). Our findings suggest that fecal E2 was probably excreted in parallel with follicular growth and that the remarkable changes in the fecal E2 contents indicate a regular ovarian cycle.

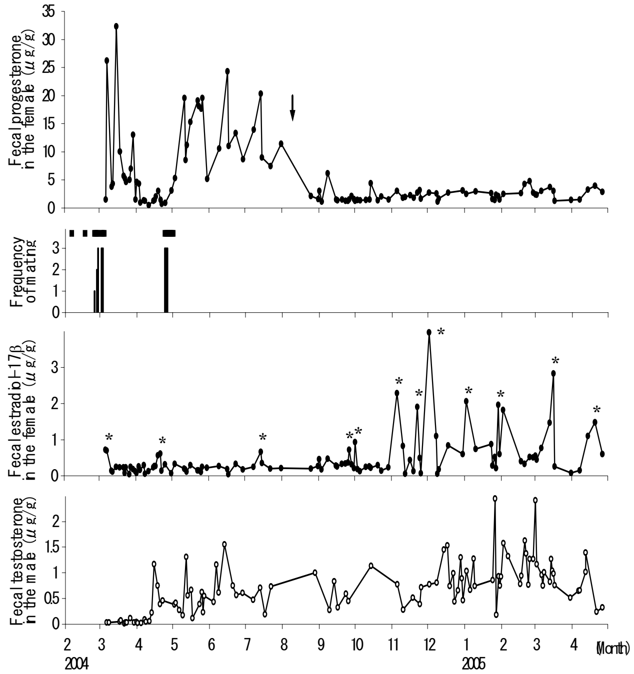

Figure 3. Changes in fecal contents of P4 and E2 in female Siberian tiger (No. 2, , which was kept at Tama Zoological Park). The arrow indicates parturition. A higher score for copulation means higher frequency. Horizontal bars indicate the periods when the female was housed together the male. The 12 asterisks indicate the peaks of E2 (Putranto et al. 2007a).

The fecal P4 contents of female No. 2, which was kept with male, increased remarkably after copulation on March 3 and April 24 (Figure 3). The P4 contents were approximately 2- to 6-fold higher than the mean value (5.20 ± 6.13 μg/g, n = 107) but the first copulation did not result in pregnancy. During pregnancy, the fecal P4 contents varied from 0.94 to 24.29 μg/g. However, the highest level of fecal P4 contents (24.29 μg/g) in this pregnant female is smaller than in pregnant female Bengal tiger (104.17 μg/g) (Putranto 2008). The length of the pregnancy in female Siberian tiger was 106 d, and this number is similar to the length of pregnancy in female Bengal tiger which was approximately 105 d (Putranto 2008).

In the case of pregnancy, the fecal contents of female Siberian tiger increased remarkably after copulation. From the beginning of pregnancy, the fecal P4 content increased and remains high during 106 days of pregnancy; however the fecal P4 contents decreased to baseline after parturition (Figure 3). The duration of the increased P4 contents after copulation differed between non-pregnancy and pregnancy. The P4 steroid hormone has a role function to maintain the pregnancy of female mammals (Brown et al. 2006). Therefore, the differences between non-pregnancy and pregnancy female tiger's fecal P4 contents in this study are strengthen the previous report result.

In leopard cats, clouded leopards, snow leopards, and cheetahs, it has been reported that the duration of increased fecal P4 contents during presumed pseudopregnancy is about half the pregnancy length (Putranto 2008). The main indicator to distinguish between pregnant and pseudopregnant conditions is the duration of increased fecal P4 content. Thus, fecal P4 monitoring can be used to confirm pregnancy in Siberian tigers.

The fecal E2 contents of pregnant female Siberian No. 2 began to change remarkably from November 2004. Female No. 2 and this cub were reared in the outdoor paddock on October 2, 2004. The remarkable changes in fecal E2 contents probably reflect recurrence of estrus, because weaning and natural light stimulation might affect the ovarian cycle.

Major progesterone steroid metabolites excreted in feces

The results were shown that two peaks were primarily detected fraction 63- 64 min (might be 5β-pregnane-3β-ol-20-one or 5β-pregnane-3α-ol-20-one) and fraction 85 min (not identified metabolite) in feces of pregnant female Siberian tiger (Table 3, Figure 4). However, in this study P4 detected only small amount in feces of pregnant Siberian tiger.

Table 3. Major P4 and steroid hormone metabolites excreted in feces of pregnant female Siberian tiger

Steroid metabolites	Elution time (min)
PdG	3
5β-pa-3β-ol-20-one or 5β-pa-3α-ol-20-one	63- 64
Unknown	85

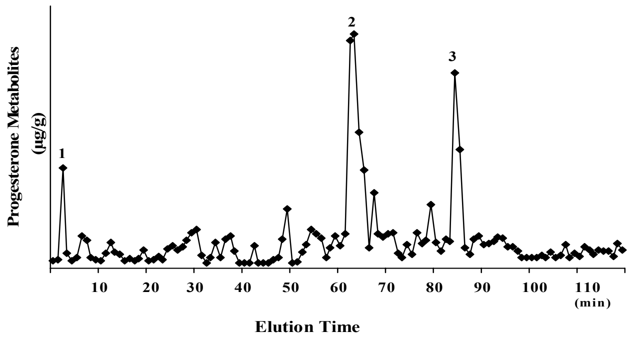

Figure 4. The chromatogram for major P4 metabolites excreted in feces of pregnant female Siberian tiger (No.2, Tama Zoological Park). Numbers indicate P4 metabolites presence in feces consecutively. No.1 = pregnanediol-glucuronide, 2 = 5β-pa-3β-ol-20-one or 5β-pa- 3α-ol-20-one, 3 = unknown.

Previous studies of steroid metabolites composition in feces of several felids has been conducted by international researchers. However, this study would be the first description and preliminary data of major metabolites of progesterone steroid excreted in feces of pregnant female Siberian tiger.

The chromatogram of this study is shown that progesterone (P4) detected only small amount in feces of Siberian tiger. The major P4 steroid metabolites excreted were presence in the form of conjugated progesterone which was 5β-pregnane-3β-ol-20-one or 5β-pregnane-3α-ol-20-one. Another peak eluting in fraction 3 min could be pregnanediol-glucuronide (PdG). The previous report confirmed that the presence of several progestogens in the feces of felids (Graham et al. 1995). Almost 90% of P4 metabolites in clouded leopard and leopards cat was the form of conjugated progesterone (Brown et al. 1993). In pregnant female Siberian tiger, probably a great portion of circulating P4 are metabolized into some pregnants and excreted into feces. Our result proved that during pregnancy in female Siberian tiger, the reproductive organs produced a great portion of a conjugated P4 and excreted into feces.

The tigers in this study had no seasonal reproductive pattern. Although tigers in the wild can breed all year, they typically breed during the winter or spring (about the end of November to April) (MacDonald 2001). Furthermore MacDonald (2001) stated that Bengal tigers in the wild seem to breed all year; however, the peak of breeding activities is from November to February. Furthermore, tigers are known as induced ovulators and rare individuals are spontaneous ovulators (Brown 2006; Graham et al. 2006). The relationships between successful breeding and hormone patterns should be clarified and the reproductive/physiological mean of these ovulation patterns in tigers should also be understood to improve the captive breeding of tigers in the future.

CONCLUSION

It was concluded that it is possible to assess non-invasively gonadal events such as luteal or follicular activity or ovulation of Siberian tigers by endocrine monitoring based on fecal P4 and E2 contents. The utilization of fecal steroid hormones is also useful to confirm pregnancy in female Siberian tiger by a continuous fecal P4 contents analyses. We can also assess non-invasively the pregnancy in female Siberian tiger by continuous fecal P4 analyses combining HPLC and EIA methods. In pregnant female Siberian tiger, a great portion of circulating P4 is probably metabolized into some pregnants and excreted into feces.

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