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Citation	Japanese Journal of Veterinary Research, 50(1), 17-27
Issue Date	2002-05-31
DOI	10.14943/jjvr.50.1.17
Doc URL	<a href="http://hdl.handle.net/2115/2932">http://hdl.handle.net/2115/2932</a>
Type	bulletin (article)
File Information	50-1_p17-27.pdf



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Efficiency of fecal steroid hormone measurement for assessing reproductive function in the Hokkaido brown bear (*Ursus arctos yesoensis*)

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(Accepted for publication : April 16, 2002)

Abstract

The present study aimed to establish simple systems for measuring fecal steroid hormones in order to monitor the reproductive profiles of captive Hokkaido brown bears. The efficiency of fecal sample processing at the steps of dehydration and extraction and the correlation between steroid concentrations in matched fecal and blood samples were studied. Then, monthly changes in fecal estradiol-17 $\beta$  and progesterone in female bears, and testosterone in male bears were examined. The procedure was finalized as follows. Fecal samples were dried at 100°C for 3 hr and extracted with diethyl ether. The diethyl ether in the extracts was evaporated and residues were reconstituted in ethanol for the assays. Hormone concentrations were quantified using enzyme immunoassays. Concentrations of progesterone and testosterone in fecal and plasma samples were correlated in the systems. The changes in fecal progesterone and testosterone concentrations were similar to those in serum concentrations of bears as reported previously. In contrast, fecal estradiol concentrations did not correlate with plasma levels probably because of the time lag in excretion. However, the changes in estradiol-17 $\beta$  concentrations in feces in the present study were similar to those reported in serum. In conclusion, fecal progesterone and testosterone assay systems appear practical for monitoring ovarian and testicular activities without immobilization, though methodological improvements and further validation may be required. For the fecal estradiol-17 $\beta$  assay, there is a need to solve the problem of excretion time lag before the system can be used in the study of reproductive physiology.

Key words : enzyme immunoassay, estradiol-17 $\beta$ , progesterone, testosterone

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## Introduction

The Hokkaido brown bear (*Ursus arctos yesoensis*) is a subspecies of the brown bear (*Ursus arctos*). The brown bear is a seasonal breeder whose breeding season extends from May to July<sup>3)</sup>. Implantation reportedly occurs in brown bears between November and December<sup>22)</sup>, followed by parturition from January to February<sup>24)</sup>. A novel increase in serum progesterone concentrations at the estimated time of implantation was reported in pregnant brown bears<sup>23)</sup>. Seasonal patterns and peak concentrations of serum progesterone in non-pregnant bears were virtually identical to those in the pregnant bears. Male serum testosterone concentrations start to increase in February and peak around May and June<sup>21)</sup>. Similar characteristics to those of brown bears, such as seasonal breeding<sup>6)</sup>, delayed implantation<sup>30)</sup> and pseudopregnancy<sup>9)</sup> were found in American black bears (*Ursus americanus*), a species in which much information on reproductive events has been accumulated. However, the duration of estrus and mode of ovulation are unknown in both species due to a lack of detailed endocrinological information.

Fecal and urine steroid hormone measurements have been applied in zoo animals to study reproductive profiles such as the mode and length of the estrous cycle, gestation period and also testicular function<sup>1, 2, 7, 8, 15, 26, 29)</sup>. These techniques do not require chemical immobilization for blood sampling which is stressful to animals and limits the frequency of sampling in physiological studies when blood is used for hormone assays. Therefore, these noninvasive methods have the potential to provide more precise and detailed information on endocrinological events than the conventional method using blood samples. Urine can make for better material depending on the species and objectives of a study<sup>7, 15)</sup>.

However, feces appear to be the material of choice, since the collection of fecal samples is more feasible for relatively terrestrial animals for the assessment of steroid concentrations<sup>31)</sup>.

Fecal steroid hormone measurements can also prove an effective tool in bears to investigate ovarian and testicular activities in detail during the breeding season without immobilization. The present study aimed 1) to establish simple methods of measuring fecal estradiol-17 $\beta$ , progesterone and testosterone concentrations in the brown bear using enzyme immunoassays, and 2) to determine the longitudinal steroid hormone profiles in captive animals using fecal steroid hormone measurements. The present study documented the monthly changes in fecal steroid hormones as a preliminary application of non-invasive methods using feces in bears.

## Materials and Methods

### Animals

We used Hokkaido brown bears maintained at Noboribetsu Bear Park (Noboribetsu, Japan) to collect fecal and blood samples. They were housed in separate male or female groups in indoor/outdoor enclosures. None of the females were pregnant. A balanced diet, comprising a mixture of commercial chow for bears and for dairy cows, beet pulp and alfalfa, was provided throughout the year.

### Fecal and blood sample collection

The matched fecal and blood samples were collected monthly from the rectum and the jugular vein, respectively, under immobilization with an intramuscular injection of atropine (Atropine sulfate injection, Tanabe Seiyaku Co., Ltd., Osaka, Japan) at a dose of 0.01 mg/kg body weight, xylasin HCl (Celactal, Bayer Ltd., Leverkusen, Germany) at 1 mg/kg and ketamine HCl (Ketalar, Sankyo Co., Ltd.,

Tokyo, Japan) at 5 mg/kg. Other fecal samples were collected from the floor of the enclosures immediately after voidance. When an animal defecated more than once on the same day, the first specimen was used for assays. Each fecal sample was well-mixed and divided into several portions of 10 cm<sup>3</sup> and frozen within 1 hr after collection. Blood plasma was obtained immediately after blood collection. Both fecal and plasma samples were stored at -20°C until analysis.

#### *Dehydration of fecal samples*

Fecal portions (10 cm<sup>3</sup>) were dried by heating for 3 hr at 100°C in an electric oven or lyophilization with a conventional freeze-dryer. Dried fecal samples were pulverized.

#### *Steroid extraction*

Fecal steroid extraction was carried out with either diethyl ether<sup>11)</sup> or ethanol<sup>28)</sup>. Briefly, for the extraction with ether, 0.25 g of fecal powder was placed in a 50 ml screw-capped plastic tube and shaken vigorously with 3 ml of distilled water for 10 min. Ten milliliters of diethyl ether was added and then the tube was shaken vigorously for another 10 min. The mixture was centrifuged at 250 x g for 10 min and cooled at -80°C for 15 min. The ether phase was decanted into another 50 ml plastic tube, evaporated to dryness at 45°C in a waterbath, and redissolved in 2 ml of ethanol. For the extraction with ethanol, 0.25 g of fecal powder was boiled twice in 5 ml of 90% ethanol for 20 min. The supernatants were combined, dried completely and redissolved in 0.5 ml of ethanol. Both fecal extracts prepared by ether and ethanol extraction were diluted (1:5-1:20 for estradiol-17 $\beta$  and progesterone; 1:100-1:200 for testosterone) in 0.04 M phosphate-buffered saline supplemented with 0.1% BSA (BSA-PBS, pH 7.2) and assayed. Plasma ster-

oids were extracted using diethyl ether as described by Mori and Kano<sup>17)</sup>. In brief, for progesterone and testosterone measurements, 200  $\mu$ l of sample was placed in a 1.5 ml tube and shaken with 1 ml of diethyl ether for 10 min. The mixture was allowed to stand at room temperature for 10 min and frozen at -80°C for 15 min. The ether phase was decanted into a 10 ml glass tube, evaporated as described above and redissolved in 200  $\mu$ l of BSA-PBS. For the estradiol-17 $\beta$  measurement, 2 ml of plasma was taken into a 15 ml screw-capped glass tube and shaken with 6 ml of diethyl ether for 10 min. The subsequent procedure till evaporation was the same as described above. The glass tube was rinsed with 1 ml of diethyl ether, again evaporated and redissolved in 100  $\mu$ l of BSA-PBS.

#### *Hormone assay*

Hormone concentrations were determined using competitive double antibody enzyme immunoassays (EIA). The primary antisera used for EIA for the estradiol-17 $\beta$ , progesterone and testosterone measurements were anti-estradiol-17 $\beta$ -6-CMO-BSA (Teikoku Hormone Mfg. Co. Ltd., Tokyo, Japan), anti-progesterone-3-CMO-BSA (7720-0504, Biogenesis Ltd., New Fields, England) and anti-testosterone-3-CMO-BSA (8680-1404, Biogenesis), respectively. The antiserum against estradiol-17 $\beta$  cross-reacted with estrone-3-sulfate, 8.0%; 16-epiestriol, 5.3%; and estrone, 3.2%. The antiserum against progesterone cross-reacted with pregnenolone, 20.0%. The antiserum against testosterone cross-reacted with dihydrotestosterone, 100%; and epitestosterone, 2.1%. The cross-reactivity of each antiserum with other steroids was less than 2.0%. The secondary antiserum was goat anti-rabbit serum (Seikagaku Co., Tokyo, Japan). All samples were assayed in triplicate. Assay sensitivity was

17.2 pg/well for estradiol-17 $\beta$ , 4.3 pg/well for progesterone and 1.1 pg/well for testosterone. When plasma was used as sample, intra- and inter-assay coefficients of variations were 4.9 and 6.8% for estradiol-17 $\beta$ , 3.9 and 6.5% for progesterone, and 7.1 and 8.9% for testosterone. Steroid recoveries from plasma were  $86.3 \pm 8.9\%$  for estradiol-17 $\beta$ ,  $86.1 \pm 7.7\%$  for progesterone and  $77.7 \pm 2.4\%$  for testosterone. The overall recovery rate of fecal steroid hormone in each assay was determined to estimate the actual steroid hormone concentration from the assay data. The mean recovery rates (R) calculated from the recovery rates at 10, 100, 1000 ng/g wet weight for estradiol-17 $\beta$ , progesterone and testosterone were  $39.5 \pm 5.2$ ,  $62.5 \pm 10.6$  and  $51.9 \pm 1.3\%$ , respectively. The steroid hormone concentration in each fecal sample was calculated using R, the dilution factor for each sample (D) and the volume of extract (V) as follows: Steroid hormone concentration in sample (ng/g dry weight) = concentration in assay sample (ng/ml)  $\times 100DV / 0.25R$ . Steroid hormone concentrations in wet materials were calculated us-

ing the concentrations in dry materials and the weights of the fecal samples before and after drying. The intra-assay coefficients of variation for estradiol-17 $\beta$ , progesterone and testosterone in fecal samples were 10.7, 12.1 and 7.2%, respectively. Similarly, the inter-assay coefficients of variation were 14.8, 19.9 and 6.3%, respectively. The parallelism between the curves for the reference standard of the steroid hormones and the serial dilution of plasma and fecal extracts containing known amounts of steroid hormones is shown in Fig. 1.

#### Experimental design

The animals used for fecal and blood sample collections in each experiment are listed in Fig. 2.

#### Experiment 1. Assessment of dehydration and extraction methods for fecal samples

Efficiencies of dehydration and extraction methods were compared by determining the recovered amounts of supplemented hormones at known concentrations. To compare

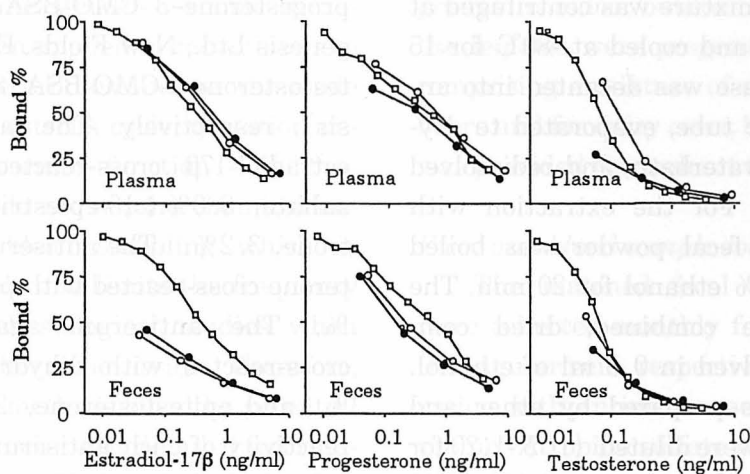


Fig. 1. Parallelism among curves for the reference standards ( $\square$ ) and those of plasma and fecal samples of estradiol-17 $\beta$ , progesterone and testosterone. Assay samples were prepared using plasma and fecal samples collected when levels of steroidogenic activity were expected to be high ( $\bullet$ ) and low ( $\circ$ ).



Sex	Status (n)	1997		1998		1999		2000		Experiment no.
		Dec.	Feb.	Jul.	Dec.	Jan.	Dec.	Jan.		
Female	Adult (19)		▲							1 & 3
	Adult (2)								↔	2 *
	Adult (2)								↔	2 & 3
	Young (1)								↔	2
Male	Adult (32)		▲							1 & 3
	Adult (2)								↔	2 & 3
	Young (1)								↔	2

Fig. 2. Animals and samples used for the experiments.

←·····→ : Voided fecal samples were collected from groups of 17 females and 34 males once a month. However, samples could be obtained from only a limited number of animals due to restrictions of sampling time. Thus, the number of samples and individuals from which they were obtained differed every month.

↔ : Paired blood and fecal samples were collected once a month from each animal.

▲ : Fecal samples collected from 4 females in February and from 3 males in December were used to compare dehydration and extraction efficiencies in experiment 1, respectively, and also in experiment 3.

\* : The sample sets of blood and feces were used only for progesterone measurements.

the two drying methods, each of 3 steroid hormones was added at 100 ng/g wet weight to 2 portions (10 cm<sup>3</sup>) of fecal samples. One portion of the fecal samples was dried by heating and the other was lyophilized. Samples were extracted with diethyl ether and subjected to hormone assay. The same fecal samples without hormone supplementation were dried by heating or lyophilization and used as blanks. To compare the two extraction methods, each of 3 steroid hormones was added to the heat-dried samples at 1 µg/g dry weight. The samples were extracted with diethyl ether or ethanol. The same fecal sample without hormone supplementation was subjected either to ether or ethanol extraction and used as a blank.

#### Experiment 2. Correlation between fecal and

#### plasma steroid hormone concentrations

The correlations between the fecal and plasma concentrations of estradiol-17β and progesterone were determined using the matched fecal and blood samples of females (Fig. 2). The correlation between fecal and blood testosterone concentrations was determined using the matched fecal and blood samples of males (Fig. 2). Fecal samples were heat-dried and steroid hormones were extracted using diethyl ether for hormone assays.

#### Experiment 3. Longitudinal monitoring of fecal steroid hormone concentrations

The monthly changes in fecal concentrations of estradiol-17β and progesterone for females, and of testosterone for males were examined using the fecal samples shown in Fig. 2. Dehydration and extraction of fecal samples for steroid hormone assays were conducted as described in Experiment 2.

#### Statistical analysis

All statistical analyses were performed using the computer software Statview<sup>®</sup> 4.02 (Abacus Concepts Inc., Berkeley, CA, USA). The rates of recovery after the two drying and two extraction procedures were compared using Student's *t*-test and Welch's *t*-test, respectively. The correlation between fecal and plasma concentrations of each steroid hormone was determined using Pearson's correlation coefficient analysis. For longitudinal monitoring, steroid hormone concentrations of fecal samples were averaged monthly, and the differences among months were examined by one-way analysis of variance with Fisher's protected least significant difference as a post-hoc test. Statistical values were considered significant when the *p* value was smaller than 0.05.

## Results

### Experiment 1. Assessment of dehydration and extraction methods for fecal samples

The moisture content of the fecal samples was consistent ( $84.7 \pm 2.8\%$ ; mean  $\pm$  SD,  $n = 144$ ) throughout the study. There was no significant difference in efficiency between the two methods of drying (Table 1) and extraction of fecal samples (Table 2).

### Experiment 2. Correlation between fecal and plasma steroid hormone concentrations

As shown in Fig. 3, fecal progesterone and testosterone concentrations were correlated with plasma concentrations ( $r = 0.72$ ,  $p <$

$0.0001$  and  $r = 0.64$ ,  $p < 0.01$ , respectively). However, the estradiol- $17\beta$  concentrations in feces showed a poor correlation with those in plasma ( $r = 0.06$ ,  $p = 0.82$ ).

### Experiment 3. Longitudinal monitoring of fecal steroid hormone concentrations

Fecal estradiol- $17\beta$  concentrations were low during the winter (December to February) and tended to increase from February to June (Fig. 4). Fecal progesterone concentrations were high in December and remained low from February to July (Fig. 5). There was no significant change in male fecal testosterone concentrations throughout the year ( $p = 0.58$ , Fig. 6).

Table 1. Comparison of the recovery rates of exogenous steroid hormones for two methods of drying fecal samples.

Methods	Recovery rate (%) <sup>a)</sup>		
	Estradiol- $17\beta$	Progesterone	Testosterone
Heating <sup>b)</sup>	$44.1 \pm 8.3$	$54.2 \pm 10.3$	$84.1 \pm 25.1$
Lyophilization	$40.5 \pm 3.9$	$56.5 \pm 10.0$	$86.6 \pm 15.2$

Values are means  $\pm$  SD of 4 replicates.

<sup>a)</sup> All the steroid hormones were added to the frozen-thawed fecal samples at 100 ng/g wet weight. Recovery rates were calculated as follows: recovery rate (%) = (recovered amount of steroid hormone from hormone supplemented samples - that from the sample without hormone supplementation) / amount of steroid hormone supplemented  $\times 100$ .

<sup>b)</sup> Fecal samples were dried at 100°C for 3 hr.

Table 2. Comparison of the recovery rates of exogenous steroid hormones for two methods of extraction.

Methods	Recovery rate (%) <sup>a)</sup>		
	Estradiol- $17\beta$	Progesterone	Testosterone
Snap-freezing with ether	$34.7 \pm 0.8$	$56.6 \pm 24.1$	$56.1 \pm 23.8$
Boiling with ethanol	$37.5 \pm 5.4$	$41.1 \pm 5.6$	$44.4 \pm 3.3$

Values are means  $\pm$  SD of 3 replicates.

<sup>a)</sup> All the steroid hormones were added to the dried fecal samples at 1  $\mu$ g/g dry weight. Recovery rates were calculated as shown in Table 1.

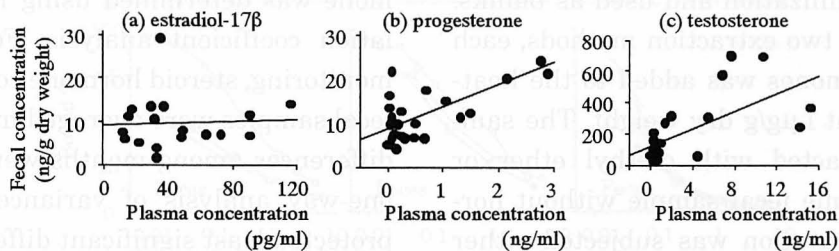


Fig. 3. Correlation in steroid hormone concentrations between plasma and fecal samples. Samples were collected to cover the months when levels of steroidogenic activity were expected to be high and low. (a) estradiol- $17\beta$  ( $r = 0.06$ ,  $p = 0.82$ ), (b) progesterone ( $r = 0.72$ ,  $p < 0.0001$ ), (c) testosterone ( $r = 0.64$ ,  $p < 0.01$ ).

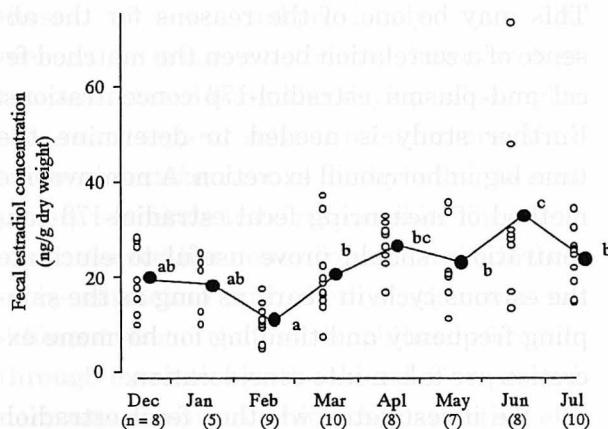


Fig. 4. Monthly changes in fecal estradiol-17 $\beta$  concentrations in non-pregnant females. Closed circle (—●—) represents the mean value. The means with different letters differ significantly ( $p < 0.05$ ). Numbers in parentheses indicate the number of animals from which fecal samples were collected. The number of samples and individuals from which they were obtained differed every month due to restrictions of sampling time.

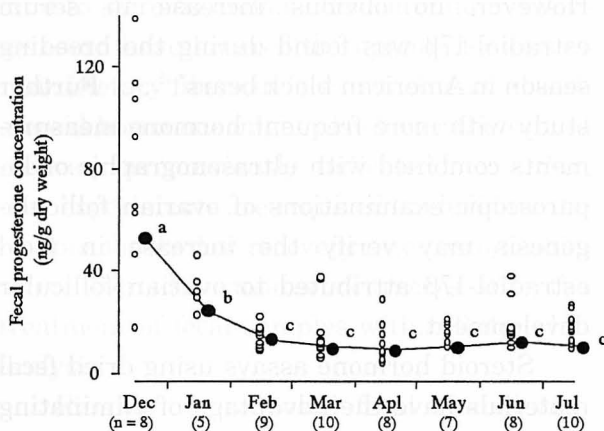


Fig. 5. Monthly changes in fecal progesterone concentrations in non-pregnant females. Closed circle (—●—) represents the mean value. The means with different letters differ significantly ( $p < 0.05$ ). Numbers in parentheses indicate the number of animals from which fecal samples were collected. The number of samples and individuals from which they were obtained differed every month due to restrictions of sampling time.

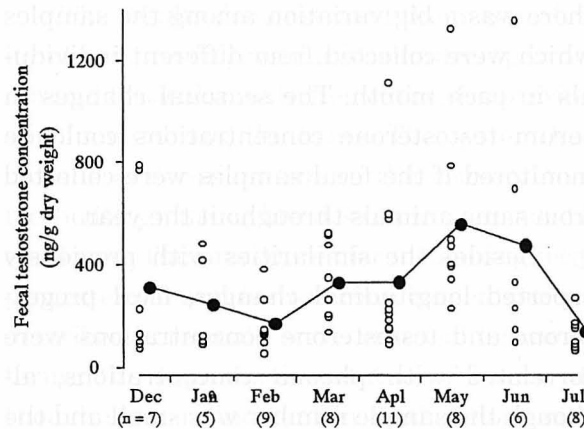


Fig. 6. Monthly changes in fecal testosterone concentrations in males. Closed circle (—●—) represents the mean value. Numbers in parentheses indicate the number of animals from which fecal samples were collected. The number of samples and individuals from which they were obtained differed every month due to restrictions of sampling time.

## Discussion

Serum progesterone concentrations in captive Hokkaido brown bears gradually increase after the breeding season (May to July) and peak during the estimated period of implantation (November to December)<sup>23</sup>. They fell around the parturition period between January and February and were then maintained at basal levels from March to early July. The annual changes of serum progesterone concentrations in non-pregnant females were indistinguishable from those of pregnant females (pseudopregnancy)<sup>23</sup>. A similar monthly change was observed in the fecal progesterone concentrations of captive non-pregnant female brown bears. Serum testosterone concentrations in male brown bears begin to increase in February when spermatogenesis starts, and reach a high level in May and June<sup>21</sup>. In the present study, the profile of the testosterone concentrations in feces was similar to that found in serum<sup>21</sup>; however,



there was a big variation among the samples which were collected from different individuals in each month. The seasonal changes in serum testosterone concentrations could be monitored if the fecal samples were collected from same animals throughout the year.

Besides the similarities with previously reported longitudinal changes, fecal progesterone and testosterone concentrations were correlated with plasma concentrations, although the sample number was small and the results were preliminary. The fecal steroid hormone concentrations vary according to the volume of feces even if the same amount of steroid hormones was excreted. There may be a better correlation between blood steroid hormone concentrations and total amounts of fecal steroid hormones during certain period (i.e., 24 hr). Considering the advantages of a sampling method that does not require chemical immobilization for sampling which is stressful to animals and limits the frequency of sampling, the present assay systems for fecal progesterone and testosterone will provide detailed information on longitudinal endocrine changes which is important to evaluate the reproductive performance in captive animals.

In contrast, there was no significant correlation between the matched fecal and plasma estradiol-17 $\beta$  concentrations in the present study. The estradiol-17 $\beta$  concentration increases markedly in 1 or 2 days during estrus, a phenomenon known as the estrogen surge, while progesterone and testosterone show long-term changes in most mammalian species. It takes 1 to 4 days for plasma steroid hormones to be excreted in the feces of the cat<sup>20)</sup>, African wild dog<sup>18)</sup>, Asian elephant<sup>4)</sup>, white rhinoceros<sup>10)</sup>, and scimitar-horned oryx<sup>16)</sup>. A time lag of 1 to 4 days may be critical for monitoring the changes in estradiol-17 $\beta$  but not progesterone and testosterone.

This may be one of the reasons for the absence of a correlation between the matched fecal and plasma estradiol-17 $\beta$  concentrations. Further study is needed to determine the time lag in hormonal excretion. A noninvasive method of measuring fecal estradiol-17 $\beta$  concentrations should prove useful to elucidate the estrous cycle in bears, as long as the sampling frequency and time lag for hormone excretion are taken into consideration.

We investigated whether fecal estradiol-17 $\beta$  concentrations changed seasonally in brown bears reflecting the follicular activities of estrogen production as suggested previously. A trend toward a gradual increase in fecal estradiol-17 $\beta$  during the breeding season (May to June) was observed in the present study. The results were supported by the previous findings that serum estradiol-17 $\beta$  elevated before (March) and during the time of mating (June) in American black bears<sup>25)</sup>. However, no obvious increase in serum estradiol-17 $\beta$  was found during the breeding season in American black bears<sup>9,19)</sup>. Further study with more frequent hormone measurements combined with ultrasonographic or laparoscopic examinations of ovarian folliculogenesis may verify the increase in fecal estradiol-17 $\beta$  attributed to ovarian follicular development.

Steroid hormone assays using dried fecal materials have the advantage of eliminating variations in the fecal moisture concentration, and of facilitating mixture of the fecal sample with extraction solvents<sup>5)</sup>. In the present study, although the moisture content of fecal samples remained constant due to the controlled diet, dried materials were used for extraction with a view toward field applications where moisture concentrations can vary to a great extent.

The recovery rates for steroid hormones in the present study were lower (40-60%) than

those (>80%) reported previously<sup>14,26,27,29</sup>. The reasons for this are not clear; however, high temperatures during the drying of samples, the type of organic solvent used for steroid extraction, and the ratio of diethyl ether to sample are not likely to be responsible. The recovery of steroid hormones from heat-dried feces or by extraction with diethyl ether was not different from that of lyophilized feces or through extraction with ethanol. Our preliminary experiment in which the extraction efficiencies of estradiol-17 $\beta$  were compared at various ratios of diethyl ether to fecal sample also showed that the ratio used in the present study was reasonable (data not shown).

The low recovery rates in the present study can be attributed, at least in part, to the influence of the contents of fecal extracts, since serum interference in the assays lowers the recovery rates evaluated using EIA systems. Substances in the samples that may bind to steroids and/or affect immunoreactivity could have lowered the apparent recovery rate. Dietary fibers in feces which may absorb steroid hormones might also lower the rate of recovery as previously indicated<sup>12,28</sup>. Further experiments are required to elucidate the reasons for the low recovery rates using radioactive steroid hormones as tracers after the treatment of fecal samples with cellulose hydrolysis.

Finally, the proposed fecal steroid hormone assays can be refined further by taking into account the metabolites of steroid hormones. Steroid hormones are excreted into feces partially as conjugated metabolites, such as glucuronides and sulfates, and the ratio of free to conjugated metabolites differs among animal species<sup>13,20</sup>. The extraction procedure described here could not extract conjugated metabolites; thus it remains necessary to evaluate the ratio of free to conjugated metabolites in bear feces.

The results demonstrated the practicability of the present system in monitoring ovarian and testicular activities without capturing and immobilizing the bears. The system has various applications where the conventional methodology using blood samples can not be employed. The procedures for measuring fecal progesterone and testosterone concentrations described here could serve as a tool for monitoring the reproductive status of bears, although some refinement in methodology may be required. For the fecal estradiol-17 $\beta$  assay, further validation of the assay procedure and sampling method should make the system of practical use in monitoring estrus and ovulation.

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