FULL PAPER *Theriogenology*

Annual Changes in Fecal Estradiol-17β Concentrations of the Sun Bear (*Helarctos malayanus*) in Sarawak, Malaysia

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ABSTRACT. Fecal estradiol concentrations were measured in three captive unmated female sun bears (*Helarctos malayanus*) from August 1998 to July 1999 in Sarawak, Malaysia and vaginal smears from one of the females was observed in August 1998 and March 1999. A single peak in fecal estradiol concentration was obvious for each bear in August or September 1998, and there was a much higher percentage of superficial vaginal anuclear cells in August 1998 than in March 1999. These results suggest that sun bears in Sarawak are likely to be a seasonal breeder associated with a peak of estrogen production in August or September.
KEY WORDS: estradiol, feces, *Helarctos malayanus*, seasonality, vaginal smear.

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The sun bear (*Helarctos malayanus*) is the smallest bear in the Ursidae family and is distributed throughout Southeast Asia, including Borneo and Sumatra [7]. The bear is listed under the 'Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES)', Appendix 1, and the 1996 IUCN 'Red List of Threatened Animals', as the population is declining due to habitat loss and excessive human-induced mortality [5, 9]. However, little is known about the behavior and ecology of sun bears in their original area of distribution. In our earlier report [6], we showed that ovulation of the sun bear occurs spontaneously and is followed by pseudopregnancy, and we suggested that the sun bears in Sarawak may breed seasonally in association with the rainy season. In the present study, fecal estradiol-17β (E_2) concentrations were quantified in three captive unmated female sun bears, and vaginal smears were examined in one of the bears to identify her estrus.

MATERIALS AND METHODS

Study site and animals: The study was conducted in Sarawak, Malaysia; the details of the site are described in our previous report [6]. Three females, 5, 7, and 11 years old, were used in the study. The bears were segregated from male bears during the study and held in captivity, as explained previously [6].

E_2 assay method: Fecal and serum E_2 was quantified by enzyme immunoassay using the IMx® System automated analyzer (Abbott Laboratories, U.S.A.) at the Advanced Diagnostic Laboratories (ADL) (Kuching, Sarawak, Malaysia). The assay procedure as follow, 200 μl of a sample (a fecal solution or a serum) is poured into the sample well of IMx® REACTION CELLS (Abbott Laboratories, U.S.A.). The sample is automatically mixed with anti-E_2 antibody coating microparticles and E_2-alkaline phosphatase conjugates. E_2 in the sample and E_2-alkaline phosphatase conjugates competitively bind to the anti-E_2 antibodies on microparticles. Then, the mixture is transferred to the glass fiber matrix and microparticles bind to the glass fiber matrix. Finally, 4-methylumbelliferylphosphate is added to the matrix and fluorescence intensity of 4-methylumbelliferone is measured at 365 nm. The standard curve is prepared using IMx® Estradiol CALIBRATOR and IMx® Estradiol CONTROL (Abbott Laboratories, U.S.A.). Under the system settings at ADL, the range of detectable levels of E_2 was 8.2 to 3,000 *p*g/m*l*. The antibody cross-reactivity was 100% with E_2, 2.4% with estradiol-3-glucuronide, below a detectable level with estradiol-17-sulfate, 0.3% with estrone, 0.05% with estriol, 0.0003% with testosterone, below a detectable level with progesterone and below a detectable level with cortisol. The E_2 concentrations were expressed as *n*g/g for a fecal sample, to indicate the quantity of E_2 (*n*g) in 1.0 g of wet feces and as *p*g/m*l* for a serum sample.

Fecal sample collection: Twenty-two fecal samples were collected from each bear every 10 to 21 days from 15 August 1998 to 9 July 1999. Samples were frozen (–20°C) until the E_2 was extracted. A total of 66 fecal samples were analyzed.

Fecal E_2 extraction: Fecal E_2 was extracted with a modified version of the protocol described by Goeritz *et al.* [3]. In brief, 5 m*l* of 100% methyl alcohol was added to 0.5 g of wet fecal sample, agitated for 30 min at room temperature and then centrifuged (15 min, 112 × g). The supernatants were used as the fecal E_2 assay samples. Prior to the assay, the assay samples were diluted 10-fold with the specific sample-dilution buffer of the IMx® System (ESTRADIOL SPECIMEN DILUENT, Abbott Laboratories, U.S.A.). This procedure can extract unconjugated E_2 forms in feces.

Reliability of the assay method:

1) Reliability of the fecal E_2 assay. The recovery rate and intra- and inter assay coefficients of variation (CVs) were

determined to evaluate the reliability of the fecal E_2 assay method. To calculate the recovery rate of the assay method, between 1.6 and 14.4 *n*g of E_2 was added to five wet fecal samples that contained below a detectable level of E_2(< 0.1 *n*g/g). And two fecal samples were used to determine intra- and inter assay CVs. Five replications per sample were conducted.

2) Reliability of serum E_2 assay. Parallelism between the standard curve of IMx® System and that of sun bear serum was assessed. Serum samples were collected from four female sun bears. The sera were pooled and depleted of steroid hormones by stripping with charcoal-dextran (2% (w/v) charcoal and 0.2% (w/v) dextran), and then added known concentrations of E_2 to prepare four kinds of standards (10 *p*g/m*l*, 50 *p*g/m*l*, 100 *p*g/m*l* and 1,000 *p*g/m*l*). Each standard was assayed in duplicate to make a standard curves. In addition, two serum samples were used to determine intra- and inter assay CVs. Four replications per sample were conducted.

3) Investigation for the correlation between serum and fecal E_2 concentrations. Blood and rectum fecal samples were collected from the sun bears after immobilization with a mixture of 50 μg/kg medetomidine solution (Domitor®, 1 mg/m*l*, Orion Corp. FARMOS, Turku, Finland) and 2 mg/kg zolazepam-tiletamine solution (Zoletil 100® 100 mg/kg Laboratories Virbac B.P., France)(MZT mixture). Seven sample sets, each consisting of a serum and fecal E_2 extraction from the same individual, were analyzed to examine the correlation between serum and fecal E_2. A correlation between fecal E_2 and serum E_2 levels was assessed using a Pearson correlation test with Microsoft EXCEL97©.

Vaginal smear observation: The 7-year old bear was immobilized with MZT mixture in August 1998 and March 1999 to obtain vaginal smears. A cotton swab was inserted approximately 3 cm into the vagina and it was smeared onto two clean microscope slides, air-dried, fixed with 100% methyl alcohol and stained for 30 min with 3% Giemsa solution. Two hundred cells on each slide were classified as superficial anuclear, nuclear or other cells, and the percentage of superficial anuclear cells in each sample was calculated.

RESULTS

Reliability of fecal and serum E_2 assay by the IMx® System:

1) Reliability of the fecal E_2 assay. The mean recovery rate of E_2 added to wet feces was 86%, and there was a positive correlation between added and recovered E_2 (R^2=0.99, P<0.01, n=5) (Fig. 1).

The intra-assay CVs of two fecal steroid samples, with mean E_2 concentrations of 11.6 ± 0.6 (mean ± SE) and 79.1 ± 2.9 *n*g/g, were 6% and 7% (n=5), and the inter-assay CVs were 20%and 16% (n=5), respectively.

2) Reliability of serum E_2 assay. We confirmed that the standard curve of sun bear serum fitted with that of IMx® System (Fig. 2). Therefore, IMx® System standard was used to quantify E_2 in sun bear sera during the present study.

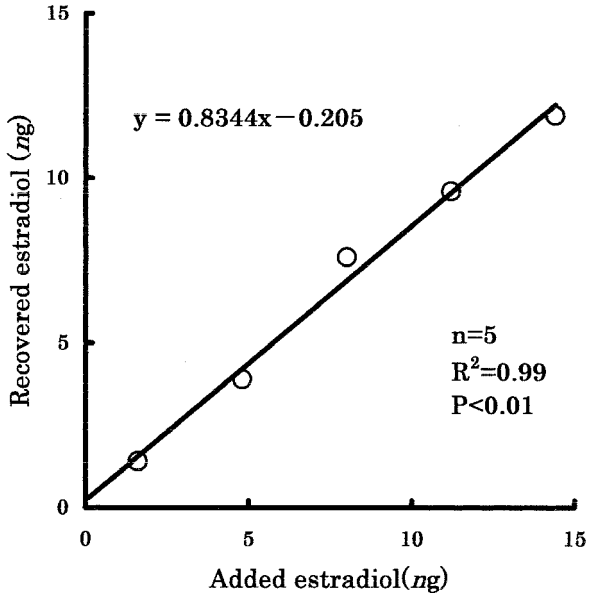

Fig. 1. The recovery of estradiol-17β added to wet feces. The results show a positive correlation and an 86% recovery rate.

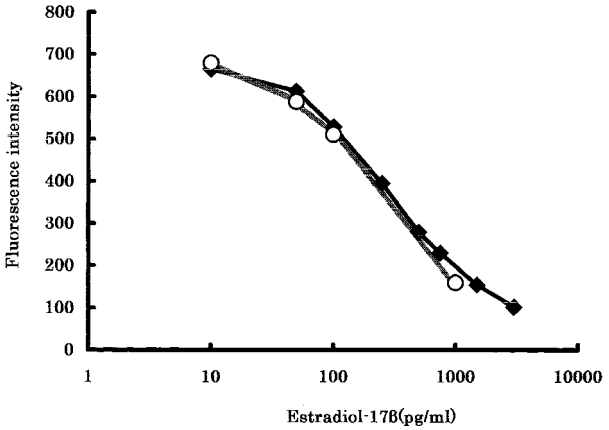

Fig. 2. Comparison between a standard curve for enzyme immunoassay of estradiol-17β in sun bear sera (○) and that of IMx® system (◆).

The intra-assay CVs of two serum samples, with mean E_2 concentrations of 31.7 ± 1.2 (mean ± SE) and 125.5 ± 4.8 *p*g/m*l*, were 2% and 4% (n=4), and the inter-assay CVs were 14% and 14% (n=4), respectively.

3) Investigation for the correlation between serum and fecal E_2 concentrations. The concentration of E_2 in fecal extractions was positively correlated with the concentration of E_2 in serum samples (R^2= 0.95, P< 0.01, n=7) (Fig. 3).

Annual changes of E_2 concentrations in feces: The bears showed similar changes in fecal E_2 concentration during the study period (Fig. 4). The E_2 concentrations ranged from <

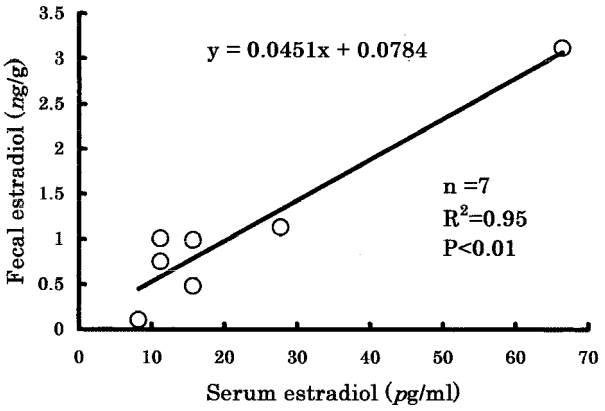

Fig. 3. Correlation between fecal and serum estradiol-17β concentrations measured by enzyme immunoassay using the IMx® system.

0.1 *n*g/g to 12.0 *n*g/g. A single distinct concentration peak was observed in each female during the study: 4.9 *n*g/g in September for the 5-year-old female; 8.7 *n*g/g in August for the 7-year-old female; and 12.0 *n*g/g in August for the 11-year-old female.

Observation of vaginal smears: The percentage of superficial anuclear cells in the 7-year old female was 87% in August 1998, whereas it was 20% in March 1999 (Fig. 5A, B).

DISCUSSION

The single peak in fecal E_2 concentration in each bear during August or September supports our earlier suggestion [6] that sun bears in Sarawak, Malaysia, may have a single breeding season that is associated with the rainy season. The estrus of the sun bears in the present study appeared to peak from August to September, and if the bears would be successful for breeding at that time, delivery would have been in December to January, as inferred from an average gestation period of 96 days [7].

The timing of the estimated delivery season corresponds to the rainy season in Sarawak, when fruiting occurs [1, 10]. The abundance of fruit at this time would make it easy for the bears to find high-energy food, which is necessary for bears during lactation [8]. For example, the American black bear (*Ursus americanus*) invests 0.3 kcal/kg$^{0.75}$/day in their litters during pregnancy, compared to 58 kcal/kg$^{0.75}$/day at the time of peak lactation [8]. It is therefore likely that the sun bear delivers during the rainy season in order to obtain sufficient energy for lactation. If this suggestion is correct, the annual period of reproductive activity should vary with the start of the rainy season. However, only non-pregnant females were observed for 12 months in the present study. Pregnant sun bears will therefore need to be observed for more than 12 months to confirm this assumption. Moreover, a study involving pregnant sun bears is essential for examining the occurrence of delayed implantation in the sun bear.

Vaginal cytology, similar to that used for the giant panda (*Ailuropoda melanoleuca*) [2], could be used as an index for detecting estrus in the sun bear. We found a high percentage of superficial anuclear cells in the vaginal smear taken in August 1998, when fecal E_2 concentrations were high. By contrast the percentage of these cells decreased and nucleotide cells appeared in March 1999, when the E_2 concentrations were below detectable levels. However larger sample

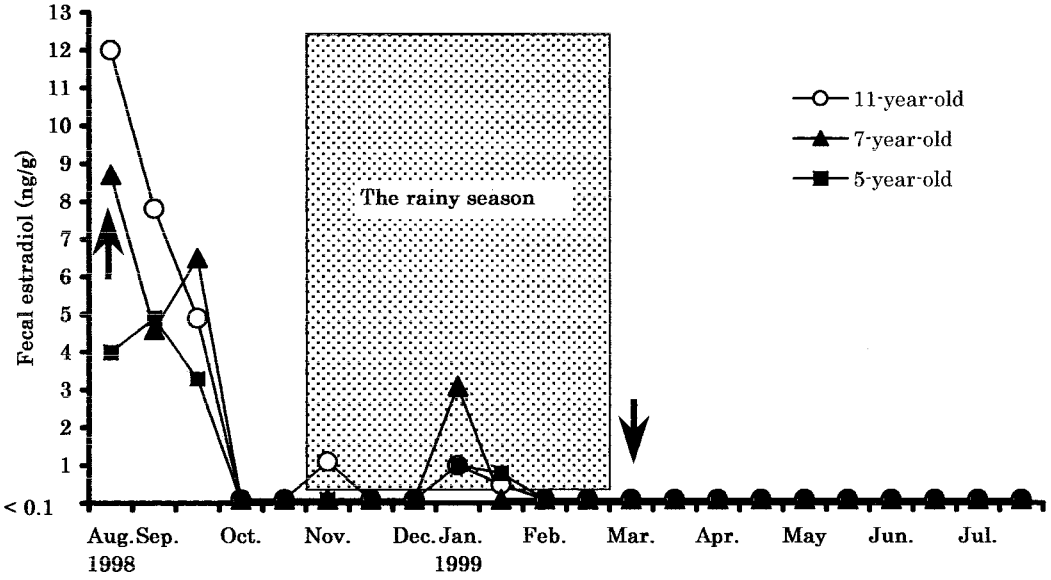

Fig. 4. Annual changes in fecal estradiol-17β concentrations in three non-pregnant sun bears, from August 1998 to July 1999. The two arrows indicate the time of immobilization of the 7-year-old female to obtain the vaginal smears.

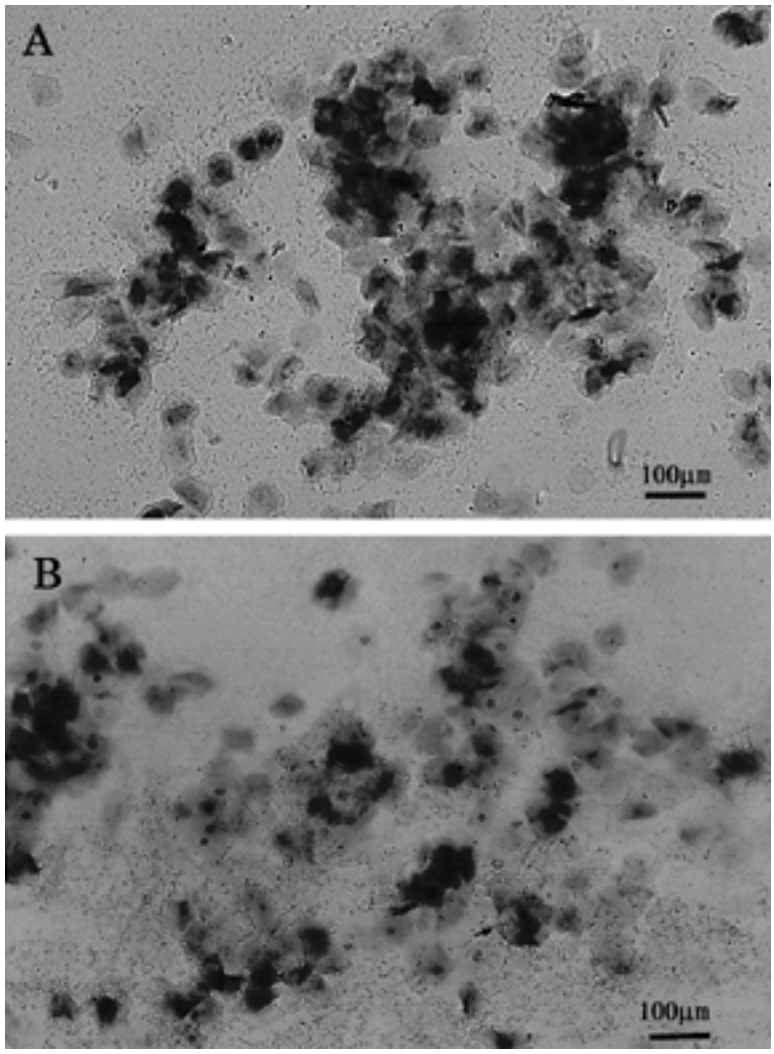

Fig. 5. The vaginal smears obtained from the 7-year-old female sun bear in August 1998 (A) and March 1999 (B). The percentages of superficial anuclear cells from 200 observed cells were 87and 20%, respectively.

sizes are needed to validate the potential use of vaginal cytology as an index for detecting estrus.

The concentration of fecal E_2 was below the minimum detectable level (0.1 *n*g/g) in 51 of 66 samples tested in this study. However, the fecal E_2 peak is important for detecting estrus, and the peak was readily detectable using the assay method, methanol extraction and IMx® System. In order to improve the detectable level of fecal E_2 with the assay method, an effective E_2 extraction technique is required to increase the E_2 concentration. In the present study, fecal E_2 was extracted from wet feces, which contains approximately 80% water. This means that on a weight-for-weight basis, dry feces would contain five times more E_2 than wet feces. As a reliable protocol for extracting fecal steroids from dried feces has been established [4], using dried feces is a practical method for improving the fecal E_2 assay with the IMx® System.

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