Serum Progesterone Concentrations using P-EIA Kit in Captive and Free-Ranging Hokkaido Brown Bears, *Ursus arctos yesoensis*

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ABSTRACT. Serum progesterone (P) concentrations using P-EIA kit (Ovucheck, Cambridge Life Science Co., Ltd.) were examined in 8 captive and 7 free-ranging female Hokkaido brown bears (*Ursus arctos yesoensis*). The intra- and inter-assay coefficients of variation were 8.9%, 12.6% and 16.6%, 22.7%, respectively, based on 2 serum samples. There was a significant correlation between EIA and radioimmunoassay results based on 64 serum samples (r=0.725; p<0.01). Serum P concentrations were examined in 5 pregnant, 2 solitary non-pregnant bears and a lactating non-pregnant bear in captivity. Annual changes of P levels in pregnant bears were observed as a small elevation during the mating season (May–June), a re-elevation in September–October and a sharp elevation in November–December. The sharp elevation was suspected to reflect changes when implantation occurred. Annual changes of P levels in solitary non-pregnant bears were similar to those in pregnant bears. An annual change of P levels in a lactating non-pregnant bear maintained levels under 5 ng/ml. Two of 7 free-ranging bears exhibited P levels over 1 ng/ml and the birth of cubs was confirmed in the following year in 1 of the 2 bears. P concentrations of other free-ranging bears exhibited less than 1 ng/ml, and these bears were considered to be non-pregnant. It was concluded that P-EIA kit was available for measuring P concentrations in Hokkaido brown bears.—KEY WORDS: bear, delayed implantation, EIA-kit, progesterone, *Ursus arctos yesoensis*.

Hokkaido brown bears are the largest wild terrestrial mammal in Japan and are characterized by their winter sleep in dens. Delayed implantation should be noted especially from the standpoint of reproductive physiology in bears. The examination of serum progesterone (P) concentrations by radioimmunoassay and fetal growth curve by ultrasonography [9], the embryo recovery from uterine horns under the anesthetized condition [10] and the morphological observation of ovaries, uteri and embryos during the delay period (Tsubota et al., in preparation) have been performed in Hokkaido brown bears. Those results suggest that the unimplanted embryos are free into the uterine lumen for 5–6 months after mating, the implantation occurs in late November-early December, and the actual period of fetal growth is approximately 60 days. It was reported that P levels sharply elevated also in American black (*U. americanus*) and polar bears (*U. maritimus*) when the implantation occurred [3, 7].

Recently, enzyme immunoassay of P concentrations instead of radioimmunoassay is often carried out in many domestic animals (cows: [4, 8]; horses, sheep and dogs: [2]). However, there has been no report on this assay method for bears.

The objective of this study was to examine the availability of P-EIA method for the measurement of P concentrations of Hokkaido brown bears and to determine the change of serum P levels in relation to the reproductive cycle and pregnancy in captive and free-ranging animals.

MATERIALS AND METHODS

Eight captive and 7 free-ranging female Hokkaido brown bears were employed in this study. Eight captive bears were kept in Noboribetsu Bear Park (Noboribetsu), and five of them gave a birth in winter. The other 3 bears were non-pregnant; 2 bears were segregated from other bears around the mating season (solitary non-pregnant bears) and another was accompanied by her cubs (lactating non-pregnant bears). These bears were immobilized with 0.5 mg/kg suxamethonium chloride (Succin®, Yamanouchi Pharmaceutical Co., Ltd., Japan) and 5.0 mg/kg ketamine hydrochloride ( Ketalar, Sankyo-Park-Davis & Co., Inc., Japan) or 0.9 mg/kg xylazine hydrochloride (Celacar, Bayer, West Germany) and 4.0 mg/kg ketamine hydrochloride. The
blood samples were obtained every month and 3–5 times per year in 5 and 3 bears, respectively. The blood of 30–50 ml extracted from jugular, cephalic or saphenous vein was centrifuged at 3,000 rpm for 30 min and serum was stored at −80°C until assay.

Blood samples were obtained in 7 free-ranging bears. Those bears were captured by barrel traps [5] and immobilized with a combination of xylazine hydrochloride and ketamine hydrochloride [11] with a metallic poke stick syringe or blowpipe, for the attachment of radio-transmitters for ecological research and some physical examinations. Six of those 7 bears were thought to have attained sexual maturity. The remaining one was 1-year-old. The methods of the blood extraction and serum storage were similar to those in the case for captive bears.

Measurement of serum P concentrations using P-EIA kit: P-EIA kit (Ovucheck, Cambridge Life Science Co., Ltd., U.K.) was used for measuring P concentrations. The measurement procedures were as follows: Ten µl standards of 4 kinds (0.5, 1, 5, and 10 ng progesterone/ml) and serum samples were put in microtitre wells, and 200 µl progesterone-alkaline phosphatase conjugate was added to every well. Microtitre wells were covered and left for about 30 min at room temperature, after which they were emptied and washed by filling with cold water. These procedures were repeated twice. Thereafter 200 µl substrate reagent was added to all empty wells. Wells were covered and left for about 30 min at room temperature. Stopping solution was added to all wells. Serum P concentrations were measured using spectrophotometer (Microreader, Denka Pharmaceutical Co., Ltd., Japan) at 405 nm absorbance. When serum P concentrations were over 10 ng/ml, serum samples were diluted by serum of male bears (P concentrations: <0.5 ng/ml) and again assayed.

Examination of reliability:
1) Sensitivity of assay. The sensitivity of assay using P-EIA kit was determined as the lowest P level from 0.5 mg progesterone/ml standard. This was the P concentration which was two standard deviations above the mean of this sample.
2) Precision of assay. In each of eight assays performed by EIA, two samples were included as inter-assay controls and in one assay the same two samples were included 8 times each to estimate the intra-assay precision.
3) Correlation of EIA with radioimmunoassay results. Sixty-four serum samples of Hokkaido brown bears were used for analysis of EIA correlation with the radioimmunoassay results [9].

Statistical analysis: Student’s t test was used for all the statistical analysis in this study.

RESULTS

Reliability of assay using P-EIA kit:
1) Sensitivity of assay. A standard curve from the assay using P-EIA kit was shown in Fig. 1. The sensitivity of the assay was 0.98 ng/ml.
2) Precision of assay. Two samples (7.40±0.66 ng/ml, 16.01±2.01 ng/ml) gave a coefficient variation of 8.9% and 12.6% for intra-assay, and 16.6%

![Fig. 1. Standard curve for P-EIA kit (Mean ± SEM, n=4).](image1)

![Fig. 2. Correlation between progesterone concentrations in female Hokkaido brown bears measured by EIA kit and radioimmunoassay.](image2)
and 22.7% for inter-assay, respectively.
3) EIA correlation with radioimmunoassay results. There were significant correlations (r=0.725: p<0.01) between EIA and radioimmunoassay results. The regression equation was y=1.28x + 1.802 (Fig. 2).

Annual changes of serum P levels in captive bears: Annual changes of serum P levels in 5 pregnant bears were shown in Fig. 3. Three (Nos. 1, 2 and 3) of 5 bears showed similar changes in serum P levels through the year (Table 1); P levels were almost negligible in May (0.58 ± 0.21 ng/ml), rose to a relatively high level in June-July (July: 5.88±1.35 ng/ml; p<0.05), re-elevated in October (10.19±0.65 ng/ml; p<0.05), and sharply elevated in November-December (December: 16.74±0.60 ng/ml; p<0.01). It began to decrease in January (3.87±1.05 ng/ml; p<0.01) and was negligible in February (0.43±0.20 ng/ml). Bear No. 4 exhibited a similar change to Bear Nos. 1, 2 and 3, except a notable elevation from September to October (October: 21.87±1.83 ng/ml). In Bear No. 5, serum P levels did not increase till September (2.08±0.33 ng/ml), became slightly high in October (4.23±1.05 ng/ml) and increased again in December (8.25±1.45 ng/ml).

Annual changes of serum P levels in 2 solitary non-pregnant bears were shown in Fig. 4. These changes were similar to those of pregnant bears; the P level in Bear No. 6 gradually elevated from June and peaked in November (25.87±3.31 ng/ml). In Bear No. 7, it rose to 10.80±0.18 ng/ml in October and attained the highest level in December.

![Graph showing changes in serum progesterone levels](image)

**Fig. 3.** Annual changes of serum progesterone levels (Mean ± SEM) and dates of mating and parturition in 5 captive pregnant bears.

**Fig. 4.** Annual changes of serum progesterone levels (Mean ± SEM) in 2 captive non-pregnant bears, which were segregated from males during breeding season.

**Table 1.** Monthly serum progesterone (P) concentrations (Mean±SEM) in 3 pregnant bears, which exhibited similar annual changes of progesterone

<table>
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</tr>
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<tbody>
<tr>
<td>No. of bears</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>P conc. (ng/ml)</td>
<td>±0.21</td>
<td>±0.71</td>
<td>±5.88</td>
<td>±5.35</td>
<td>±5.64</td>
<td>10.19</td>
<td>9.97</td>
<td>16.74</td>
<td>3.87</td>
<td>0.43</td>
<td>0.55</td>
<td>0.36</td>
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</tbody>
</table>
Table 2. Serum progesterone (P) levels in 7 free-ranging Hokkaido brown bears

<table>
<thead>
<tr>
<th>Bear No.</th>
<th>Age (year)</th>
<th>Body weight (kg)</th>
<th>Sampling month</th>
<th>Serum P level (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11</td>
<td>50</td>
<td>August</td>
<td>0.31 ± 0.07</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>63</td>
<td>May</td>
<td>0.38 ± 0.08</td>
</tr>
<tr>
<td>3</td>
<td>?</td>
<td>124</td>
<td>October</td>
<td>2.58 ± 0.36</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>40</td>
<td>September</td>
<td>0.63 ± 0.04</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>115</td>
<td>September</td>
<td>6.60 ± 0.98</td>
</tr>
<tr>
<td>6</td>
<td>?</td>
<td>101</td>
<td>July</td>
<td>0.49 ± 0.09</td>
</tr>
<tr>
<td>7</td>
<td>?</td>
<td>82</td>
<td>September</td>
<td>0.41 ± 0.18</td>
</tr>
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</table>

DISCUSSION

The typical change of serum P levels through the year, which relates to the phenomenon of delayed implantation, was observed in 3 pregnant Hokkaido brown bears. The change was characterized by a small elevation after the mating season, a re-elevation in September-October and a sharp elevation in November-December. These results agreed rather closely with those obtained from radioimmunoassay [9]. It is thought that the small elevation and the sharp one of P levels are changes accompanied with luteinization after mating and implantation, respectively. The P levels elevated when implantation occurred in the European badger [1], the spotted skunk [6] and marsupials [12]. In Hokkaido brown bears, the unimplanted embryos were taken by flushing into the uterine lumen under the captive condition in September [10] and from free-ranging animals in September, October and November (Tsubota et al., in preparation). These results lead to conclusions that Hokkaido brown bears maintain delayed implantation at least till late November. A small re-elevation of P levels in September-October was observed in this study, although it was not clear in the previous results obtained by radioimmunoassay [9]. This change suggests that the activity of corpus luteum may advance as access to implantation period. It was noted that corpora lutea were active during the second half of the delay period in black bears [3]. Similar results are obtained in our observations (Tsubota et al., in preparation).

The present study revealed a different change of P levels in comparison to the earlier study [9], in which P levels elevated markedly in October in one bear while they did not increase in another bear till October. But such discrepancies might be simply due to individual differences.

Two solitary non-pregnant bears exhibited the similar annual change of serum P levels to pregnant

(16.60±1.38 ng/ml).

The change of serum P level in a lactating non-pregnant bear was exhibited in Fig. 5. All the 3 samples obtained gave a low level under 5 ng/ml.

Serum P concentrations in free-ranging bears: Serum P concentrations in 7 free-ranging bears were shown in Table 2. Seven bears were divided into two groups with respect to serum P concentration below (5 bears) and above (2 bears) 1 ng/ml. Bear No. 3 (2.58±0.36 ng/ml) of the latter group was confirmed to give birth in the following year but it was not clarified that another (No. 5: 6.60±0.98 ng/ml) had cubs because the bear was not tracked after being released to permit free-ranging.
bears. This result corroborates the hypothesis of Tsubota et al. [9], that corpora lutea, which were formed independently of fertilization and secreted a slight progesterone, became functional in the appropriate season, and that all of those phenomena were part of inherent mechanisms in this species. This may correspond to the pseudo-pregnancy, which has been reported to often occur in dogs and cats. P levels of a lactating non-pregnant bear maintained a low level till December in the present study, corresponding to the result of Foresman and Daniel [3]. In this case, it is considered that the inhibitory mechanism functions endocrinologically and that the ovulation and luteinization in ovaries do not occur during lactation.

Two of 7 free-ranging brown bears showed P levels over 1 ng/ml. One bear was acertained to have been pregnant at the examination time because she was accompanied by her cubs in the following year. On the other hand, pregnancy could not be established in another bear. It cannot be concluded that this bear was pregnant, simply because a pseudo-pregnancy-like phenomenon was observed under captive conditions. Further investigation in this respect will be needed. P concentrations of other 5 bears gave negligible levels. The blood in 1 (No. 2) of these bears was possibly sampled before the mating season, while the other bears were considered not to be pregnant. No pseudo-pregnancy-like phenomena, which were suggested for the captive bears, may always occur in free-ranging animals and the activity of ovaries may pause throughout the year in these bears. More detailed examinations should be carried out after collecting larger sample size.

In this study, there were no problems with the sensitivity and precision of assay, and the correlation with radioimmunoassay results for measuring serum P levels using P-EIA kit. It is concluded that its simplicity and the fact that no special facilities are required make this method available for measuring serum P levels of Hokkaido brown bears.

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