NOTE *Theriogenology*

Annual Changes in Serum Leptin Concentration in the Adult Female Japanese Black Bear (*Ursus thibetanus japonicus*)

Toshio TSUBOTA[1)]*, Miho SATO[2)], Tsukasa OKANO[3)], Sachiko NAKAMURA[2)], Makoto ASANO[2,4)], Takeshi KOMATSU[6)], Haruki SHIBATA[6)] and Masayuki SAITO[7)]

[1)]*Laboratory of Wildlife Biology, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818,*
[2)]*United Graduate School of Veterinary Sciences,* [3)]*Wildlife Rescue Center,* [4)]*Laboratory of Zoo and Wildlife Medicine, Faculty of Applied Biological Sciences, Gifu University, Gifu 501–1193,* [5)]*The Institute of Japanese Black Bear in Ani, Kita-Akita 018–3392,*
[6)]*Morinaga Institute of Biological Science, 2–1–1 Shimosueyoshi 2, Tsurumi, Yokohama 230–8504 and*
[7)]*Department of Nutrition, School of Nursing and Nutrition, Tenshi College, Sapporo 065–0013, Japan*

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ABSTRACT. In the present study, assay of the serum leptin concentration of the Japanese black bear (*Ursus thibetanus japonicus*) was attempted using a canine-leptin-specific sandwich enzyme-linked immunosorbent assay (ELISA). The dose-response curve of the bear serum was linear and parallel to the canine leptin standard curve. In mated and unmated bears, the serum leptin concentration was stable at low levels from May to August or September, gradually increased from September or October, and then remarkably increased in late November. We conclude that this method may be useful for measuring bear serum leptin concentration and that the serum leptin concentration changes annually with a peak in late November.
KEY WORDS: Japanese black bear, leptin, *Ursus thibetanus japonicus*.

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Leptin, an obese gene product, is primarily synthesized and secreted by the white adipose tissue and regulates food intake and whole-body energy balance [8, 11]. Generally, the serum leptin concentration correlates with body fat mass or body mass index (BMI) [5, 17, 24, 28], and the correlation increases as BMI increases [17]. In some captive and free-ranging wild animals, such as raccoon dogs, blue foxes, woodchucks and brown bears, peripheral leptin concentrations have been measured, and the effects of seasonality and fasting on the leptin levels have been discussed [10, 16, 21, 27]. These studies have demonstrated that there are correlated seasonal changes in blood leptin concentration and body mass in the woodchuck and brown bear and that there was no correlation between the leptin concentration and BMI, body mass or body fat mass in the raccoon dog and blue fox. Recently it has been known that leptin does not always function reduction of body weight because pregnant women exhibit high blood level of leptin during gestation and require more energy without food intake reducing [9], and that soluble leptin receptors (LRs) in blood but not long-form and short-form LRs modulate circulating leptin levels in baboons and rats [6, 12]. It has also been reported that leptin might play some roles in reproductive events, such as puberty [4], oocyst maturation and early embryonic development [2, 3, 15] and implantation [18, 23].

The Japanese black bear (*Ursus thibetanus japonicus*), which inhabits the islands of Honshu and Shikoku in Japan, is a terrestrial large mammal exhibiting seasonal breeding and has unique reproductive physiology such as delayed implantation. The mating season is from mid-June to August, and implantation occurs between late November and early December [26] when the bears enter hibernation in the wild. Bears reproduce cubs and then lactate using energy from only the body fat accumulated before hibernation [20]; they do not eat anything during hibernation [31]. Thus, it is important, especially for pregnant female bears, to accumulate body fat in autumn. It has been suggested that the amount of fat store might influence the implantation and fertility rates in bears [7]. Hence, their reproductive success is dependent upon maternal fat accumulation in autumn.

We focused on leptin secreted principally by the white adipose tissue in order to investigate annual changes in the serum concentration and the possible relationship between body weight and the serum leptin level in the Japanese black bear. First, we examined whether or not the serum leptin concentration of the Japanese black bear can be measured using a sandwich enzyme-linked immunosorbent assay (ELISA) using a canine-leptin-specific kit [13]. Subsequently, using this ELISA kit, we examined annual changes in the serum leptin concentrations of adult female Japanese black bears. Annual changes in the serum progesterone (P_4) concentrations were also determined in these animals.

Six captive, sexually mature female Japanese black bears managed at Ani Mataginosato Bear Park, Akita, in northeastern Japan (40°N, 140.1°E) were used for the present study. All animals were fed primarily cornmeal with some fruits and commercial bear pellets as supplements, and water was provided *ad libitum* during the active season from April to November. They all had access to water but not to food during the hibernation period from December to the following March. The bears slept in individual indoor rooms throughout the entire hibernation period.

* CORRESPONDENCE TO: Dr. TSUBOTA, T., Laboratory of Wildlife Biology, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan.
e-mail: tsubota@vetmed.hokudai.ac.jp

The 6 female bears were randomly divided into two groups of three animals each. The animals (Nos. 35, 37 and 38) in one group were housed together in isolation at an indoor run (3.47 × 4.88 m) and were given chances to mate with several different males four or five times during the breeding season (July 6 to August 10). However, no positive fetal images were identified by ultrasonography on January 6, and ultimately none of the bears gave birth. In the second group, two (Nos. 59 and 60) of the three bears were isolated completely from the other animals in an indoor run throughout the year, and the single remaining female (No. 33) was given numerous opportunities to meet male bears through a fence during the breeding season when the animals in the first group were allowed to copulate. She could touch the male bears but not mate physically.

Blood sampling was performed between the 15th and 20th of each month from May 1998 to April 1999, except for the period from November 17 to February 15, when the blood sampling was performed at 10-day intervals. The animals, which were not fed after the evening (about 5:00 PM) of the previous day, were immobilized by a blow dart or spear injections with either a combination of ketamine HCl (Ketaral, Sankyo, Japan) and medetomidine HCl (Domitor, Meiji, Japan) at doses of 5 mg/kg and 0.04 mg/kg body weight (BW), respectively, or a mixture of zolazepam HCl and tiletamine HCl (Zoletil, Virbac, France) at a dose of 9 mg/kg BW. After immobilization, the bears were weighed and handled. Blood samples for hormone assays were collected from the jugular vein into vacuum tubes. The collected blood was centrifuged at 1200 × g for 15–20 min, and the separated serum was stored at –30°C until assay.

Serum leptin concentrations were measured using a sandwich enzyme-linked immunosorbent assay (ELISA) developed for canine leptin measurement (canine-leptin-specific ELISA kit, Morinaga, Japan) according to the manufacturer's instructions. Since the antibody and standards used in this kit was made from canine recombinant leptin, the bear leptin concentrations obtained from the assay indicate values relative to the canine leptin concentrations. The parallelism between dose-response curves of a serially diluted reference standard and serial doses of bear serum was examined. The intra-assay and interassay coefficients of variation were 7.2% and 9.3%, respectively.

Serum P_4 concentrations were measured by the radioimmunoassay (RIA) method described by Palmer *et al.* [22] with some modifications. The antiserum against P_4 (HAC-AA63-06RBP84) was used at a final dilution of 1:56,000. The radioligand used was [1,2,6,7,16,17-^{3}H(N)]-progesterone (NET-1112, New England Nuclear Life Science Products, U.S.A.). The minimum detectable concentration was 0.04 *n*g/m*l*. The intra-assay and interassay coefficients of variation were 12.6% and 16.1%, respectively. All of values are presented as means ± standard error of the mean (SEM).

Annual changes in the body weights of the female bears are presented in Fig. 1-A. The body weights of the mated and unmated bears decreased from May (51.7 ± 1.4 kg and 48.7 ± 2.8 kg) to June (49.0 ± 0.5 kg and 44.7 ± 2.0 kg), increased gradually until November 27 (86.0 ± 3.6 kg and 84.3 ± 5.0 kg) and then gradually decreased until April (60.3 ± 2.6 kg and 65.5 ± 0.4 kg) during the hibernation period, respectively.

The annual changes in the serum P_4 concentrations of the mated and unmated bears are presented in Fig. 1-B. In the mated bears that did not give birth, the serum P_4 concentrations were low from May to July (0.47 ± 0.07 *n*g/m*l*) and began to increase in August (1.92 ± 0.06 *n*g/m*l*). Subsequently, a marked elevation was observed on November 17 (7.27 ± 1.21 *n*g/m*l*) or 27 (9.60 ± 0.37 *n*g/m*l*), and the high levels were maintained until December 28 (13.28 ± 2.82 *n*g/m*l*) or January 6 (8.20 ± 1.58 *n*g/m*l*), when the concentrations began to decrease; the levels returned to basal levels by February 5 (1.09 ± 0.22 *n*g/m*l*). The unmated animal that was able to interact with male bears through a fence (Bear No. 33) had a marked elevation in its P_4 concentration on December 7 (12.30 *n*g/m*l*), and this was consistent with the results for the mated bears. On the other hand, no remarkable P_4 increase was observed throughout the year (0.26–2.85 *n*g/m*l*) in the remaining 2 unmated bears, except for a transient elevation in January (7.65 *n*g/m*l*) in Bear No. 59.

The displacement curve for serial dilutions of serum samples from the female bears paralleled the canine leptin standard curve (Fig. 2).

The annual changes in the serum leptin concentrations of the mated and unmated bears are presented in Fig. 1-C. In the mated bears, the serum leptin concentrations were stable at low levels from May to September (0.72 ± 0.03 *n*g/m*l*), began to increase from October (5.18 ± 1.07 *n*g/m*l*), exhibited a peak level on November 27 (12.21 ± 1.51 *n*g/m*l*) and then decreased until January 6 (0.41 ± 0.15 *n*g/m*l*). Subsequently, low levels were maintained until April (0.84 ± 0.02 *n*g/m*l*). In the unmated bears, there was a similar tendency in the annual changes in the serum leptin concentrations as seen in the mated bears. Their serum leptin concentrations were low from May to August (0.84 ± 0.01 *n*g/m*l*), increased from September (2.35 ± 0.67 *n*g/m*l*), peaked on November 27 (12.29 ± 1.87 *n*g/m*l*) and then decreased to basal levels between January 6 (0.84 ± 0.37 *n*g/m*l*) and April (0.72 ± 0.04 *n*g/m*l*).

In some feral animals, including little brown bats (*Myotis lucifugus*) [16], European brown bears (*Ursus arctos arctos*) [10], raccoon dogs (*Nyctereutes procyonoides*) and blue foxes (*Alopex lagopus*) [21], leptin concentrations have been measured using a multi-species RIA kit with human leptin as a standard. In recent years, leptin cDNA has been cloned and species-specific leptin assays have been established for cats, dogs and sheep [13, 14, 28]. Shibata *et al.* [27] determined serum leptin concentrations in feral raccoons and bears using a canine-leptin-specific ELISA kit and suggested that this ELISA kit is useful for assays of blood leptin concentrations in these species. In the present study, we measured the serum leptin levels of Japanese black bears using the same canine-leptin-specific ELISA kit. The dose-response curve of the bear serum was linear

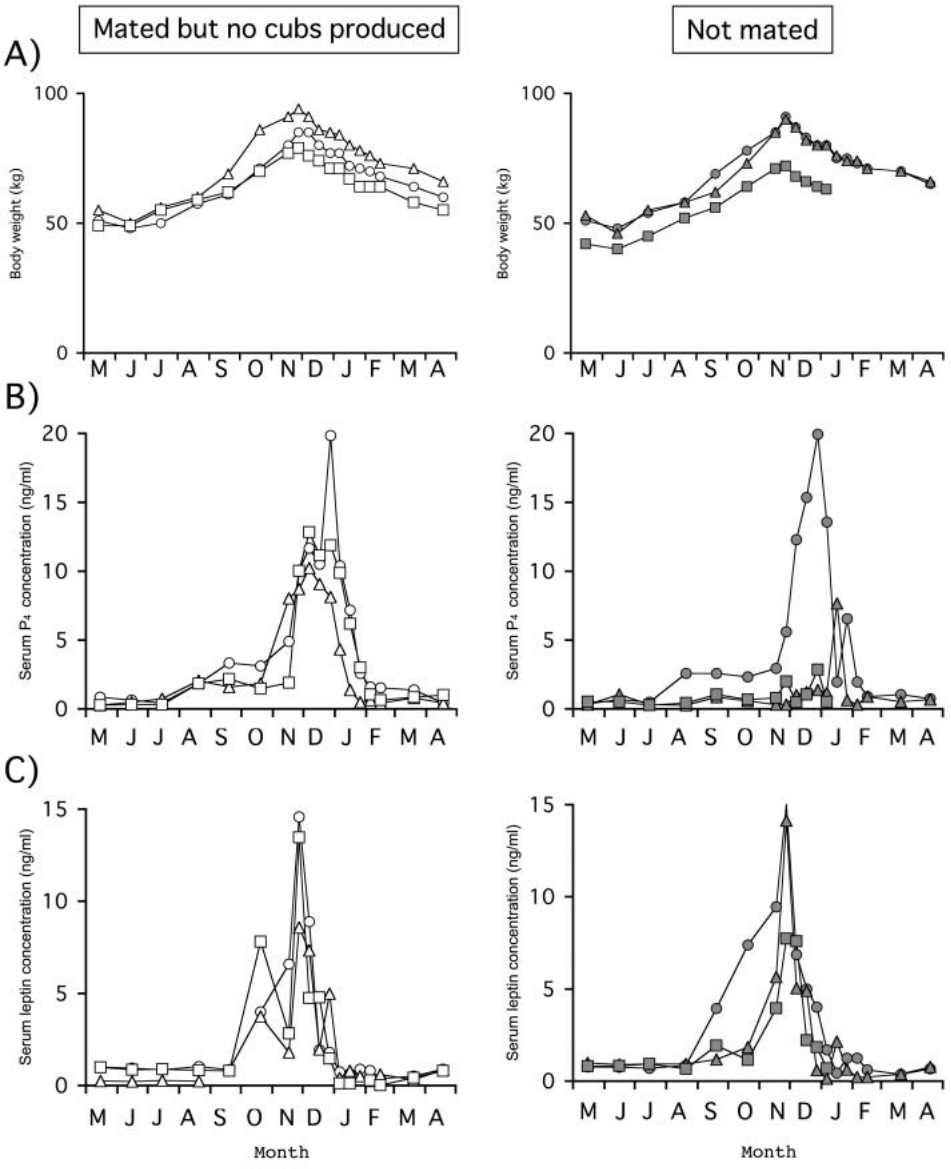

Fig. 1. Annual changes in the body weights (A), serum leptin concentrations (B) and serum P_4 concentrations (C) of the captive female Japanese black bears. The left-side panel is for the 3 mated bears, while the right-side panel is for the 3 unmated bears. The individual values for Bear Nos. 35 (○), 37 (△), 38 (□), 33 (●), 59 (▲) and 60 (■) are plotted in the figures. None of the mated female bears produced cubs. In the unmated bears, Bear No. 33 had contact with males through a fence, and Bear Nos. 59 and 60 were segregated completely from males.

and parallel to the canine leptin standard curve (Fig. 2). Thus, we believe that this ELISA kit may be useful for measuring the bear serum leptin concentration.

This is the first time that the annual changes in the leptin concentrations of Japanese black bears have been demonstrated using a canine-leptin-specific ELISA kit. The concentrations remained at low levels from May to August or September and increased gradually from September or October to mid November in association with body weight gain. In general, serum leptin levels exhibit high correlation with fat mass or BMI [5, 17, 24, 28]. In the European brown bear (*Ursus arctos arctos*), the plasma leptin concentration also reaches its maximum just prior to winter sleep, which is when fat reserves are greatest [10].

The leptin concentration exhibited a peak on November 27, which is the most interesting finding in the present study. This phenomenon of a peak in late November was not observed in a previous report concerning the European

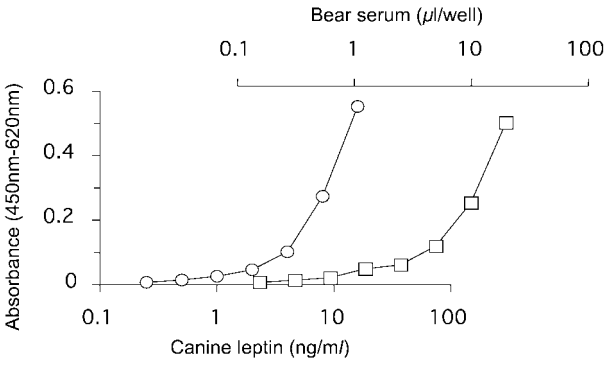

Fig. 2. Dose-response curves for canine leptin as a reference standard (○) and female bear serum (□) in an ELISA using canine antibody.

brown bear [10]. This difference might have resulted from the sampling frequency in the present study, with 10-day intervals from November 17 to February 15, while just 17 blood samples from March to November were used in the previous study. Although we cannot describe the definite mechanism of the drastic elevation in the serum lepin concentration, one possibility is that recently discovered soluble LRs and previously identified long- and short-form LRs might modulate leptin abundance, as reported in baboons [6].

This significant and drastic increase in the serum leptin concentration may indicate that leptin plays roles as a physiological signal at this time (late November) in Japanese black bears. In the Hokkaido brown bear, implantation occurs between late November and early December when the serum P_4 level is greatly elevated [30]. The serum P_4 concentration of the Japanese black bear also dramatically increases at the suspected time of implantation [25]. Sato *et al.* [26] suggested that a peak in the serum P_4 concentrations in December might reflect the endocrine function of the corpus luteum at implantation. In the present study, the leptin concentrations of both the mated and unmated bears peaked (Fig. 1-C) at approximately the same time, that is, at the time when the serum P_4 concentrations of the 3 mated females and one of the unmated females, which did not give birth and were possibly pseudopregnant [25], were remarkably increased (Fig. 1-B). This may mean that the leptin concentration increases drastically at the time of implantation regardless of whether the bear is pregnant. Recent publications have shown that leptin has roles for reproduction in some mammals based on findings for this protein and its receptor expression in reproductive tissue [18, 23]. These facts indicate that leptin may be implicated in several processes of reproduction. Malik *et al.* [18] described that leptin is a requirement of implantation. Leptin is also regarded as one of the primary factors that initiates and regulates the cascade system of molecules that promote the development of endometrial receptivity and successful implantation [23]. Thus, the leptin concentration peak in late November is possibly an implantation signal in the Japanese black bear.

After the peak, the leptin concentration abruptly decreased to the lowest levels as seen in the summer regardless of whether body fat remained for energy during hibernation. After November 27, the bears entered hibernation and ate nothing during the hibernation period. It has been reported that no significant correlation is found between serum leptin concentrations and BMI or body mass in humans (obese patients) [1], raccoon dogs (*Nyctereutes procyonoides*) and blue foxes (*Alopex lagopus*) during fasting [19, 21]. The serum leptin levels in these species may not be determined by only the amount of fat deposit in the body. In particular, the raccoon dog, which exhibits torpor and reduced body temperature and metabolism due to fasting to some degree during the winter period, has an increased peripheral leptin level in autumn due to a signal to enter torpor and a decreased peripheral leptin level during torpor in winter. A similar mechanism may be applicable to the bears at the beginning of hibernation (December) in the present study.

The following are some unsolved matters in the present study: 1) We did not identify homology of leptin mRNA sequences between bears and dogs from which the antiserum and standard were produced for the leptin ELISA kit used in the present study. The sequences of bear lepin mRNA should be clarified in the near future. 2) We confirmed the reliability of the assay using the canine-leptin-specific ELISA kit only by paralleism between the standard curve and the dose-response curve of the bear serum, but not by Western blotting analysis. 3) We obtained blood samples from female bears under anesthetized conditions and did not know the effects of anesthesia on the leptin concentration as a result of potentially secreted hormones, such as corticosteroid. These problems should be solved in the future.

In summary, the canine-leptin-specific ELISA kit may be useful for measuring the serum leptin concentration of the Japanese black bear. The serum leptin concentration exhibits annual changes with a gradual increase from September and a peak in late November. The leptin concentration peak in late November might be associated with a phenomenon such as implantation during pregnancy.

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