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短報

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

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Running head: SERUM LEPTIN CONCENTRATION IN FEMALE BEARS

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ABSTRACT. The assay of serum leptin concentrations in the Japanese black bear (*Ursus thibetanus japonicus*) was tested by canine-leptin-specific sandwich enzyme-linked immunosorbent assay (ELISA). A 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 and a gradual increase from September or October, followed by a remarkable increase in late November. We conclude that this method may be useful for measuring bear serum leptin concentrations and that serum leptin concentrations change annually with a peak in late November.

KEY WORDS: ELISA, Japanese black bear, leptin, progesterone, *Ursus thibetanus japonicus*

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 its correlation is higher in greater BMI [17]. In some captive and free-ranging wild animals such as raccoon dogs, blue fox, woodchuck and brown bears, peripheral leptin concentrations were measured, and effects of seasonality and fasting on the leptin levels have also been discussed [10, 16, 21, 27]. These studies demonstrated that there were correlated seasonal changes between blood leptin concentrations and body mass in woodchuck and brown bears while, in raccoon dogs and blue fox, there was no correlation between leptin concentrations and BMI, body mass or body fat mass. Recently it has been known that leptin does not always function effects of weight reducing because findings of high blood level of leptin during gestation in human lead interpretation pregnant women rather require more energy without food intake reducing [9], and furthermore that soluble leptin receptors (LRs) but not long-form and short-form LR_s modulate circulating leptin levels in baboons and rats [6, 12]. It has also been reported that leptin might play some roles for 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 one of terrestrial large mammals 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 bears enter hibernation in the wild. Bears reproduce cubs and then lactate using energy from only body fat accumulated before hibernation [20], although they do not eat anything during hibernation [31]. Thus, it is

important especially for pregnant female bears to accumulate body fat in autumn. It was suggested that the amount of fat store might influence implantation and fertility rate in bears [7]. Hence, their reproductive success is dependent upon maternal fat accumulation in autumn.

5 We focused on leptin secreted principally by the white adipose tissue to investigate annual changes of the serum concentrations and possible relationship between body weight and the serum leptin levels in the Japanese black bear. First, we examined whether or not serum leptin concentrations of the Japanese black bear can be measured using a sandwich enzyme-linked immunosorbent assay (ELISA) using
10 canine-leptin-specific kit [13]. Second, using this ELISA kit, we examined annual changes in serum leptin concentrations in adult female Japanese black bears. Additionally annual changes in serum progesterone (P₄) concentrations were determined in these animals.

Six captive, sexually mature female Japanese black bears managed at Ani
15 Mataginosato Bear Park, Akita, in northeastern Japan (40° N, 140.1° E) were used. 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, and had access to water but not to food during the hibernating period from December to the following March. Bears were sleeping at individual indoor
20 rooms throughout the whole hibernating period.

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,
25 no positive fetal images were recognized by ultrasonographic diagnosis on January 6,

and ultimately no bear gave birth. In the second group, two (Nos.59 and 60) of the three had been isolated completely from other animals at an indoor run throughout the year, while a single remaining female (No.33) had numerous opportunities to meet male bears through a fence during the breeding season when the animals in the first group had been allowed to copulate. She could touch the male bears but not mate physically.

Blood sampling was performed between 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. Animals which were not fed after evening (about 5:00PM) 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, bears were weighed and handled. Blood samples for hormone assays were collected from the jugular vein into vacuum tubes. Collected blood was centrifuged at $1200 \times g$ for 15-20 min and the separated serum was stored at -30°C until assay.

Serum leptin concentrations were measured by a sandwich enzyme-linked immunosorbent assay (ELISA) developed for the 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 indicates relative value to the canine leptin concentrations. The parallelism between dose-response curves of serially diluted reference standard and serial doses of bear serum was examined. The intraassay and interassay coefficient variations were 7.2% and 9.3%, respectively.

Serum P₄ concentrations were measured by the radioimmunoassay (RIA) method described by Palmer *et al.* [22] with some modification. The antiserum against P₄ (HAC-AA63-06RBP84) was used at a final dilution of 1:56,000. The radioligand used was [1,2,6,7,16,17-³H(N)]-progesterone (NET-1112, New England Nuclear Life Science Products, USA). The minimum detectable concentration was 0.04 ng/ml. The intraassay and interassay coefficient variations were 12.6% and 16.1%, respectively. All of values are presented as mean $\pm$ standard error of the mean (SEM).

Annual changes of body weights in female bears are presented in Fig. 1-A. The body weight of 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), and increased gradually until November 27 (86.0 ± 3.6 kg and 84.3 ± 5.0 kg), followed by gradual decrease of the body weight until April (60.3 ± 2.6 kg and 65.5 ± 0.4 kg) during hibernating period, respectively.

Annual changes of serum P₄ concentrations in mated and unmated bears are presented in Fig. 1-B. In mated bears that did not give birth, serum P₄ concentrations showed low levels from May to July (0.47 ± 0.07 ng/ml) and began to increase in August (1.92 ± 0.06 ng/ml). Subsequently, a marked elevation was observed on November 17 (7.27 ± 1.21) or November 27 (9.60 ± 0.37 ng/ml), and high levels were maintained until December 28 (13.28 ± 2.82) or January 6 (8.20 ± 1.58), when the concentrations began to decrease, and returned to basal levels by February 5 (1.09 ± 0.22 ng/ml). In an unmated animal, Bear No. 33, which had met male bears through a fence, had a marked elevation in P₄ concentrations on December 7 (12.30 ng/ml), which was consistent with the results in the mated bears. On the other hand, no remarkable P₄ increase was observed throughout the year (0.26-2.85 ng/ml) in the remaining 2 unmated bears, except for a transient elevation in January (7.65 ng/ml) in Bear No. 59.

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

Annual changes of serum leptin concentrations in mated and unmated bears are presented in Fig. 1-C. In mated bears, the serum leptin concentrations were stable at low levels from May to September (0.72 ± 0.03 ng/ml) and began to increase from October (5.18 ± 1.07 ng/ml) and exhibited a peak level on November 27 (12.21 ± 1.51 ng/ml), followed by a decrease until January 6 (0.41 ± 0.15 ng/ml). Subsequently, low levels were maintained until April (0.84 ± 0.02 ng/ml). In unmated bears, there is similar tendency of annual changes in serum leptin concentrations as in mated bears.

The serum leptin concentrations were low from May to August (0.84 ± 0.01), increased from September (2.35 ± 0.67) and peaked on November 27 (12.29 ± 1.87), followed by decrease of the concentrations at basal levels between January 6 (0.84 ± 0.37) and April (0.72 ± 0.04).

In some feral animals, including little brown bats (*Myotis lucifugus*) [16], European brown bears (*Ursus arctos arctos*) [10], raccoon dogs (*Nyctereutes procyonoides*) and blue fox (*Alopex lagopus*) [21], leptin concentrations has been measured by multi-species RIA kit with human leptin as a standard. In recent years, leptin cDNA has been cloned and species-specific leptin assay were established in cats, dogs and sheep [13, 14, 28]. Shibata *et al.* [27] also determined serum leptin concentrations of feral raccoon and bears using canine-leptin-specific ELISA kit, and suggested that this ELISA kit is useful for assays of blood leptin concentrations in these species. In this study, we measured serum leptin levels of the Japanese black bear using the same canine-leptin-specific ELISA kit. A dose-response curve of the bear serum was linear and parallel to the canine leptin standard curve (Fig. 2). Thus, we regard that this ELISA kit may be useful for measuring bear serum leptin

concentrations.

For the first time, annual changes in serum leptin concentrations were evidenced using the canine-leptin-specific ELISA kit in the Japanese black bear. The concentrations were maintained at low levels from May to August or September and increased gradually from September or October to mid November, associated 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*), plasma leptin concentrations also reached its maximum just prior to winter sleep, when fat reserves were greatest [10].

The leptin concentration exhibited a peak on November 27, which is the most interesting findings in this study. This phenomenon of a peak in late November was not observed in the previous report on the European brown bear [10]. This difference might be yielded because of sampling frequency with 10-day intervals from November 17 to February 15 in this study while just 17 blood samples from March to November were used in the previous study. Although we cannot describe the definite mechanism that serum leptin concentrations were elevated drastically, one possibility is that the soluble LRs which were recently found and different type from long-form and short-form LRs previously identified, might modulate leptin abundance likely as reported in baboons [6].

This significant and drastic increase of serum leptin concentrations may indicate that leptin plays some roles as physiological signals at this time (late November) in Japanese black bears. In Hokkaido brown bear, implantation occurs between late November and early December when serum P₄ levels greatly elevated [30]. Serum P₄ concentration of the Japanese black bear also dramatically increased at the suspected time of implantation [25]. Sato *et al.* [26] suggested that a peak of serum P₄

concentrations in December might reflect endocrine function of the corpus luteum at implantation. In this study, leptin concentrations of both mated and unmated bears exhibited a peak (Fig. 3) at the approximately same time when serum P₄ concentration remarkably increased (Fig. 4) in 3 mated females and an unmated female which did not give birth actually and possibly were in the pseudopregnancy status [25]. This may mean that leptin concentrations increased drastically at the time of implantation regardless of positive or negative for pregnancy in the bear. Recent publications evidenced that leptin has roles for reproduction in some mammals based on findings of this protein and its receptor expression in the reproductive tissue [18, 23]. These facts indicate that leptin could be implicated in several processes of reproduction. Malik *et al.* [18] described that there is a requirement of leptin in implantation. Leptin was 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 peak of leptin concentration in late November is possible to be one of signals on implantation in the Japanese black bear.

After the peak, leptin concentration abruptly decreased to lowest levels as the same ones in summer regardless the body fat was still remained for energy during hibernation. After November 27, bears got into hibernation and ate nothing during the hibernation period. It was reported that no significant correlation was found between serum leptin concentrations and BMI or body mass about human (obese patients) [1], raccoon dogs (*Nyctereutes procyonoides*) and blue fox (*Alopex lagopus*) during fasting [19, 21]. Serum leptin levels in these species may not be determined only by the amount of fat deposit in the body. Especially in raccoon dogs which exhibit torpor and experience fasting reducing body temperature and metabolism to some degree during winter period, peripheral leptin levels were increased during autumn due to a signal for

entering torpor and decreased at the time of torpor in winter. A similar mechanism may be applicable to the bear at beginning of hibernation (December) in the present study.

There are some unsolved matters in the present study as following: 1) We did not
5 identify homology of leptin mRNA sequences between bears and dogs from which the
antiserum and standard was made for the leptin ELISA kit used in this 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 dose-response curve of the bear serum but not Western
10 blotting analysis. 3) We obtained blood samples from female bears under the
anesthetized condition and did not know effects of anesthesia for the leptin
concentrations by the possibly secreted hormone such as corticosteroid. These
problems should be solved in the future.

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

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Figure legends

Fig. 1. Annual changes of body weights (A), serum leptin concentrations (B) and serum P4 concentrations (C) in captive female Japanese black bears. Left-side panel is for 3 mated bears, while right-side panel is for 3 unmated bears. Individual values (○: No.35, △: No.37, □: No.38, ●: No.33, ▲: No.59, ■: No.60) were plotted in figures. All mated female bears produced no cubs consequently. In unmated bears, Bear No. 33 had contact with males through a fence but Bear Nos. 59 and 60 were segregated completely from males.

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

成熟雌ニホンツキノワグマ (*Ursus thibetanus japonicus*) における血清レプチン濃度の周年変化

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イヌレプチン特異的酵素抗体測定法により、ニホンツキノワグマ（以下クマと略）の血清レプチン濃度測定を試みた。交尾群および非交尾群の血清レプチン濃度は、5～8 または 9 月に低レベルで推移し、9 または 10 月から徐々に増加し 11 月下旬に顕著に上昇した。本測定法はクマ血清レプチン濃度測定に有効性が示唆され、レプチン濃度は 11 月下旬にピークを有する明瞭な周年変化を示すことが明らかとなった。

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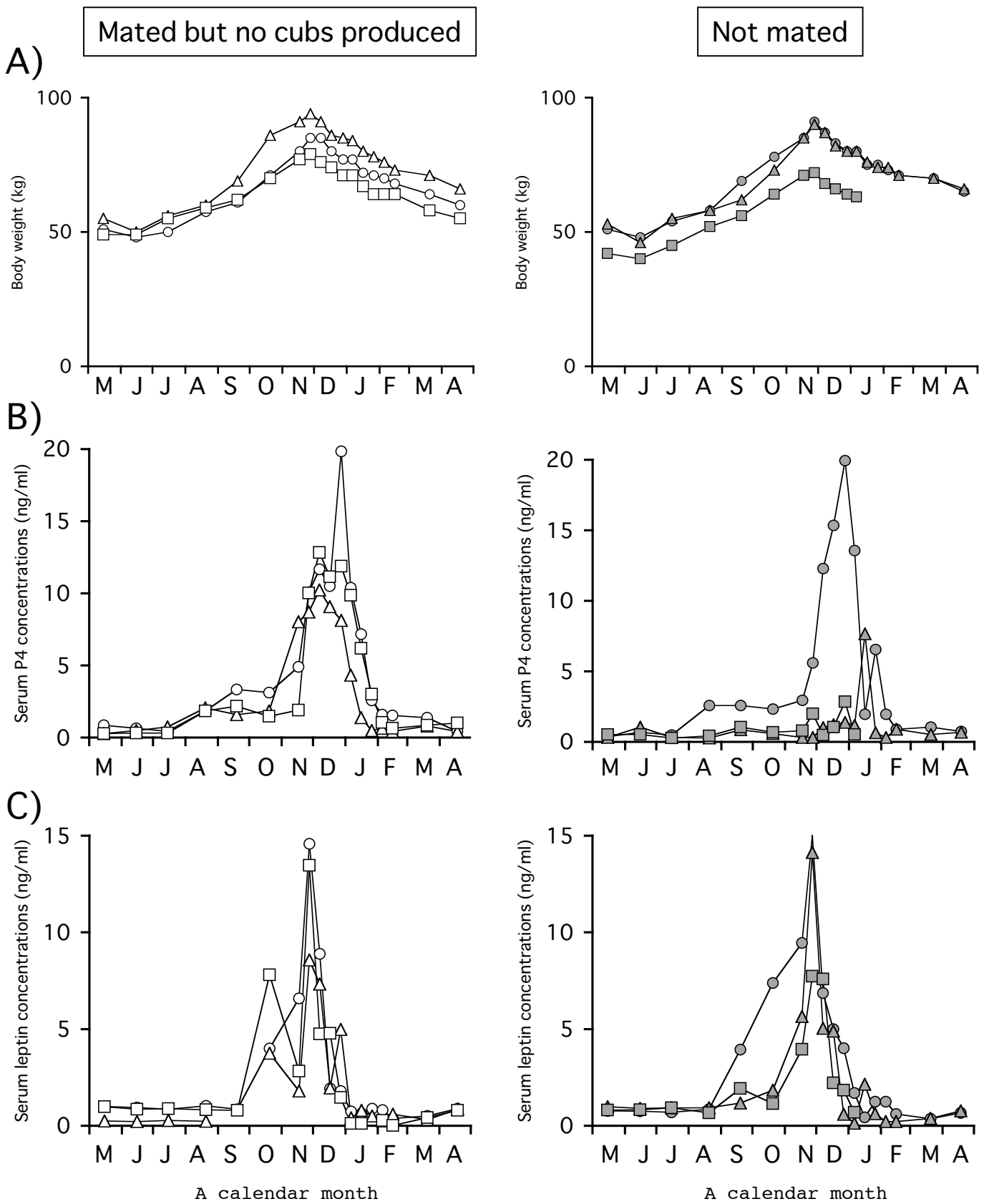


Fig. 1 Tsubota et al.

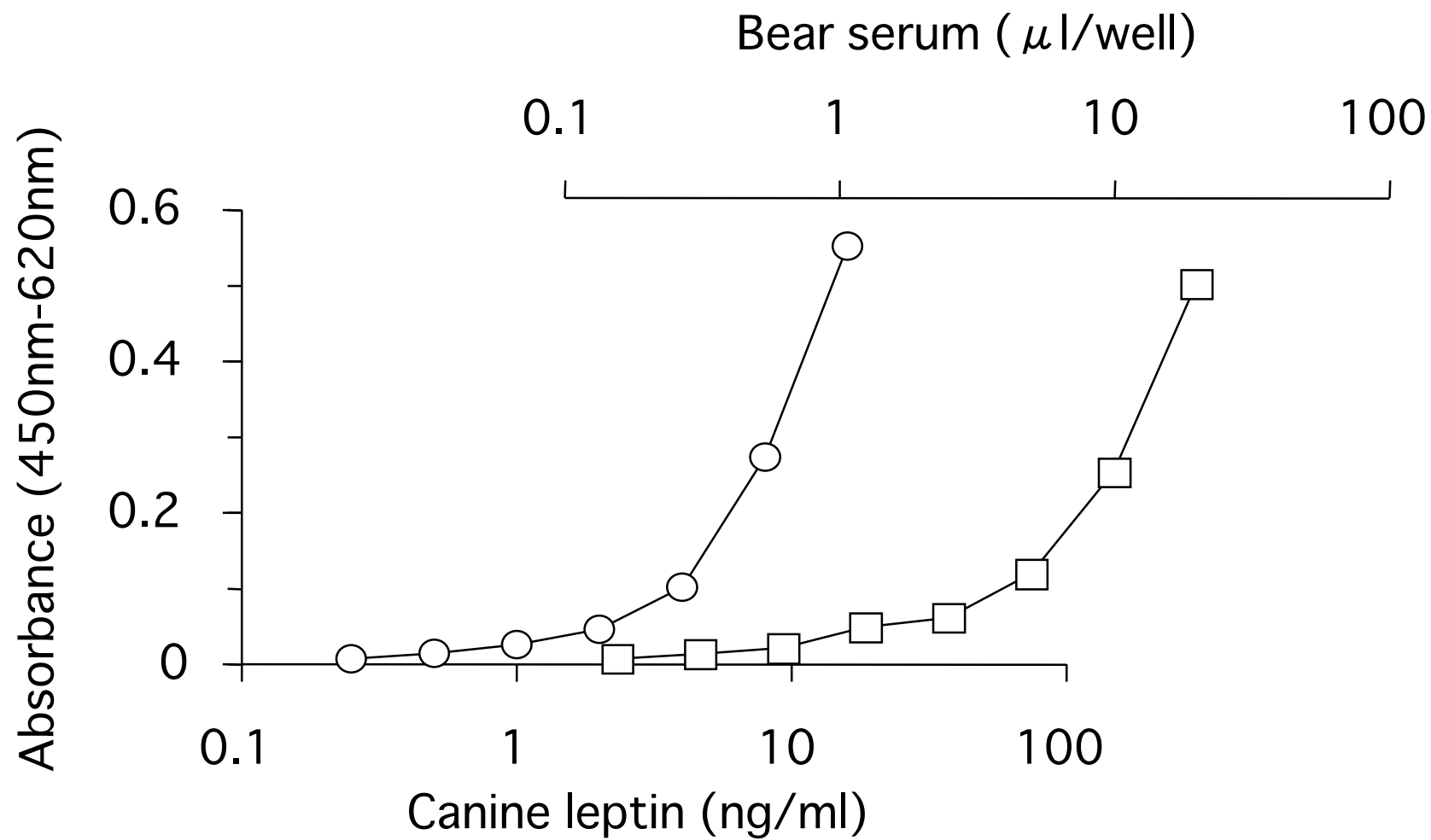


Fig. 2 Tsubota et al.