JOURNAL OF EXPERIMENTAL ZOOLOGY 303A:527–533 (2005)

Seasonal Changes in Serum Leptin of the Feral Raccoon (*Procyon lotor*) Determined by Canine-Leptin-Specific ELISA

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ABSTRACT Several reports have been published on blood leptin concentrations in feral animals, including members of the *Carnivora*, using a commercially available multi-species radioimmunoassay (RIA) kit with anti-human leptin antibody. However, we observed weak immunoreactivity between recombinant canine leptin and anti-human leptin antibody, suggesting a limitation in the applicability of the RIA kit for leptin assays in *Carnivora* species. We tested the applicability of RIA and sandwich enzyme-linked immunosorbent assay (ELISA) with anti-canine leptin antibody to assay blood leptin in the dog (*Canis familiaris*) and the raccoon (*Procyon lotor*). When RIA was used for recombinant canine leptin and dog sera, values were much lower than those determined by ELISA at higher concentrations (>10 ng/ml), while rather higher at lower concentrations (<2 ng/ml). A similar discrepancy between the two methods was found for serum leptin concentrations in raccoons. Clear seasonal variations were observed by ELISA, but not by RIA, with high values in autumn (3.46 ± 0.45 ng/ml) and low values in spring and summer (0.71 ± 0.07 ng/ml). Serum leptin concentrations in raccoons correlated positively with their body weight ($r = 0.753$) and body mass index ($r = 0.755$), corroborating our previous findings of a strong positive correlation between serum leptin concentrations and body fat content in dogs. Thus, the canine leptin ELISA is useful for assays of dog and raccoon leptin, and blood leptin is a good marker of nutritional condition in the species of *Carnivora* assayed in this study. *J. Exp. Zool. 303A:527–533, 2005.*

Leptin is a protein synthesized and secreted primarily by adipose tissue and is a key molecule for the regulation of food intake and whole-body energy balance (Friedman and Halaas, '98; Houseknecht et al., '98). Since the first report on mouse and human leptin by Zhang et al. ('94), the molecular structure of leptin has been determined by cDNA cloning in various laboratory species, livestock and pets (Ji et al., '98; Ramsay et al., '98; Iwase et al., 2000a; Sasaki et al., 2001). In addition, studies on blood leptin by immunoassay using antibodies specific for recombinant leptin in various species revealed that circulating leptin concentrations were positively correlated with body fat content and were elevated in cases of obesity (Maffei et al., '95; Considine et al., '96; Appleton et al., 2000; Backus et al., 2000; Ishioka

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Received 23 August 2004; Accepted 10 March 2005
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jez.a.185.

et al., 2002; Sagawa et al., 2002; Shibata et al., 2003). These findings imply that blood leptin is a suitable marker for adiposity and that it could therefore be employed to infer long-term nutritional condition. Various attempts have consequently been made to assess the nutritional condition of feral animals from their blood leptin concentrations. For example, blood leptin concentrations were measured in three species of *Carnivora*: European brown bear (*Ursus arctos arctos*) (Hissa et al., '98), raccoon dog (*Nyctereutes procyonoides*) and blue fox (*Alopex lagopus*) (Nieminen et al., 2001). Although all these studies revealed seasonal variations in blood leptin concentrations, the amplitudes were relatively low, and the leptin concentrations in the latter two species did not appear to be correlated with body fat content. While the reasons for these anomalies are not immediately apparent, they might have arisen because the immunoassays used in these studies employed a commercially available multi-species radioimmunoassay (RIA) kit from LINCO Research Inc. (St. Charles, MO) with anti-human leptin antibody. At the time of writing, it is uncertain whether this RIA kit is in fact suitable for leptin assays in the aforementioned feral animal species.

In previous studies, we cloned leptin cDNA of the dog (Iwase et al., 2000a), another *Carnivora*, and found that the recombinant canine leptin produced in *Escherichia coli* had considerably different immunogenicity and immunoreactivity from rodent and human leptin (Iwase et al., 2000b). We also developed a sandwich enzyme-linked immunosorbent assay (ELISA) with anti-canine leptin antibody (Iwase et al., 2000b), because we failed to obtain reliable and reproducible values for blood leptin concentration in dogs with the RIA kit. Using our ELISA, we found that blood leptin concentrations correlated positively with body fat content in beagles and several other dog breeds (Ishioka et al., 2002; Sagawa et al., 2002), as well as in rodents and humans. These results suggest that our canine-leptin-specific ELISA may also be useful for the assay of blood leptin in feral animal taxa, especially in members of *Canidae* and other *Carnivora*, whose leptins are phylogenetically similar (Doyon et al., 2001). In the present study, we tested the applicability of our ELISA for the measurement of blood leptin concentrations in dogs and feral raccoons (*Procyon lotor*) and compared these results against concentrations of the same samples determined by RIA.

MATERIALS AND METHODS

Animals and serum samples

Sixteen raccoons (*P. lotor*) were caught using box traps for research or nuisance control in Hokkaido, Japan, and euthanized with ketamine (10–20 mg/head, intramuscular injection) and diethyl ether according to guidelines published by the American Veterinary Medical Association (Beaver et al., 2001). Blood samples were taken immediately, and body length and weight were measured (Asano et al., 2003). Ten Sika deer (*Cervus nippon*) were also caught in Hokkaido for nuisance control, and blood was collected immediately after sacrifice (Suzuki et al., 2004). Blood samples from six bears (*U. arctos*, $n = 5$; *U. thibetanus*, $n = 1$) were kindly supplied by Noboribetsu Bear Park (Noboribetsu, Hokkaido, Japan) and Dr. Toshio Tsubota (United Graduate School of Veterinary Sciences, Gifu University). Dog blood samples were obtained from 20 healthy dogs of 11 different breeds as described previously (Ishioka et al., 2002). The serum was separated and stored at −80°C until use.

Antigen-coated enzyme immunoassay (EIA)

Recombinant canine and feline leptin were prepared as described previously (Iwase et al., 2000b; Shibata et al., 2003) and recombinant human and murine leptin were purchased from CRYSTAL CHEM Inc. (Downers Grove, IL). The leptin samples were dissolved in phosphate-buffered saline at concentrations in the range of 12.5–800 ng/ml in 100 μl aliquots. These were incubated overnight in the wells of a 96-well microplate (Nunc, Tokyo, Japan) at 4°C. After washing twice with 20 mM Tris/HCl (pH 7.4) containing 150 mM NaCl and 0.05% Tween 20 (TBST), the wells were incubated with 300 μl of TBST containing 1% bovine serum albumin (BSA) for 2 hr at room temperature to block non-specific binding sites. The wells were washed thrice with TBST before incubating overnight at 4°C with 100 μl of anti-canine leptin antibody (10 ng/ml) prepared as previously described (Iwase et al., 2000b). The wells were washed six times with TBST and incubated with 100 μl of horseradish peroxidase-conjugated anti-rabbit IgG antibody ($\times$ 30,000 dilution of the original; Bio-Rad Laboratories, Hercules, CA) for 4 hr at 4°C. After washing six times, the wells were finally incubated with 100 μl of substrate solution (DACO TMB One-Step Substrate system, Daco Corporation,

Carpinteria, CA) for 30 min at room temperature. Sulfuric acid (1 N, 100 μl) was added to stop color development, and the absorbance readings at 450 and 630 nm were taken as reference values.

Sandwich ELISA for canine leptin

The serum leptin concentrations of dog, raccoon, bear and Sika deer were measured by sandwich ELISA using an anti-canine leptin antibody as described previously (Iwase et al., 2000b) with minor modifications. Briefly, each well of a 96-well microplate (Nunc) was incubated with 100 μl of purified anti-canine leptin antibody (2 μg/ml) for 2 hr at room temperature and BSA as described above. After washing the wells, a 20 μl solution of serum or canine leptin as a standard was added and incubated overnight in a total volume of 100 μl at 4°C. The wells were washed six times with TBST, and incubated with 100 μl of horseradish peroxidase-conjugated anti-canine leptin antibody (0.2 μg/ml) for 4 hr at 4°C. Spectrophotometric measurements were performed as described above.

Concentrations of serum leptin from dog, raccoon and Sika deer, as well as recombinant canine leptin protein, were also estimated using a multi-species leptin RIA kit (LINCO) with anti-human leptin antibody and human leptin as a standard according to the instructions provided.

Data analysis

Regression analysis was performed on the leptin concentrations estimated by ELISA and RIA, and also for ELISA and body weight and/or body mass index (BMI) of raccoons. Seasonal differences in serum leptin concentrations of raccoons were determined statistically by Student's *t*-test with a *P* value of less than 0.05 considered statistically significant. Data were expressed as means ± SEM.

RESULTS

To determine the cross-reactivity of the anti-canine leptin antibody to a native form of recombinant protein, we used an antigen (leptin)-coated EIA method with native-form leptin. As shown in Fig. 1, the antibody reacted poorly against human, murine and feline leptins, with cross-reactivities of only 6.0%, 4.6% and 11.0% of canine leptin, respectively. These findings are comparable to results obtained using Western blot analysis in which leptin molecules were denatured in the presence of the detergent, sodium dodecyl-sulfate (Iwase et al., 2000b).

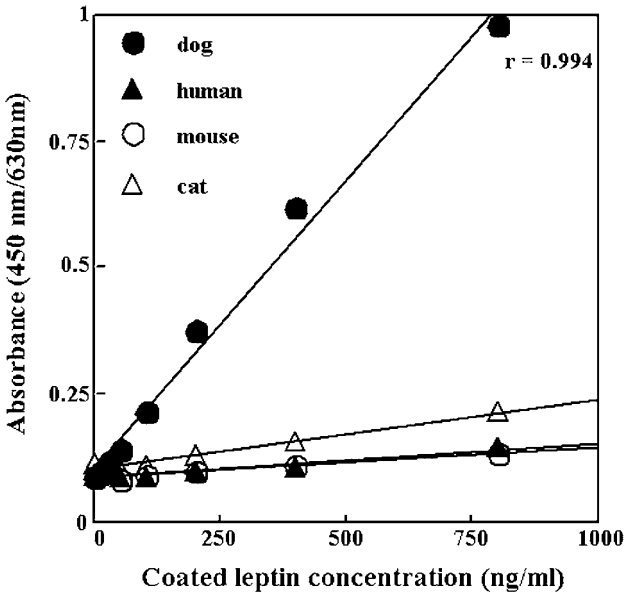

Fig. 1. Cross-reactivity of anti-canine leptin antibody to undenatured canine, human, murine and feline leptin proteins. Increasing concentrations of four species of recombinant leptin proteins were adsorbed onto a 96-well microplate, and detected by anti-canine leptin antibody with enzyme-linked second antibody, as described in the Materials and Methods.

Using this canine-specific antibody and recombinant canine leptin, we developed an ELISA (Iwase et al., 2000b) that was sufficiently sensitive for assays of leptin of dog serum in the range of 0.5–32 ng/ml with an intra- and inter-assay variation of less than 4%. The recovery of recombinant canine leptin added to canine sera was 96.5 ± 4.0%. We then tested the multi-species RIA kit from LINCO to determine whether it could also be used to assay canine leptin. The estimated human equivalent for recombinant canine leptin was much lower than that obtained by ELISA (Fig. 2A). Leptin concentrations in the sera of 20 dogs ranged between 1.7 and 5.1 ng/ml by RIA, whereas it ranged between 0.3 and 13.8 ng/ml by ELISA (Fig. 2B). Although the leptin concentrations obtained using the two methods were positively correlated ($r = 0.805$, $P < 0.001$), concentrations of the same samples estimated by RIA were less than those estimated by ELISA by a factor of three at the higher leptin concentration ranges (> 10 ng/ml), whereas it was apparently higher at the lower concentration ranges (< 2 ng/ml).

We then compared the serum leptin concentrations determined using ELISA and RIA in 16 raccoons (*P. lotor*). Leptin concentrations measured in the sera of eight raccoons caught from February to June (four males, four females)

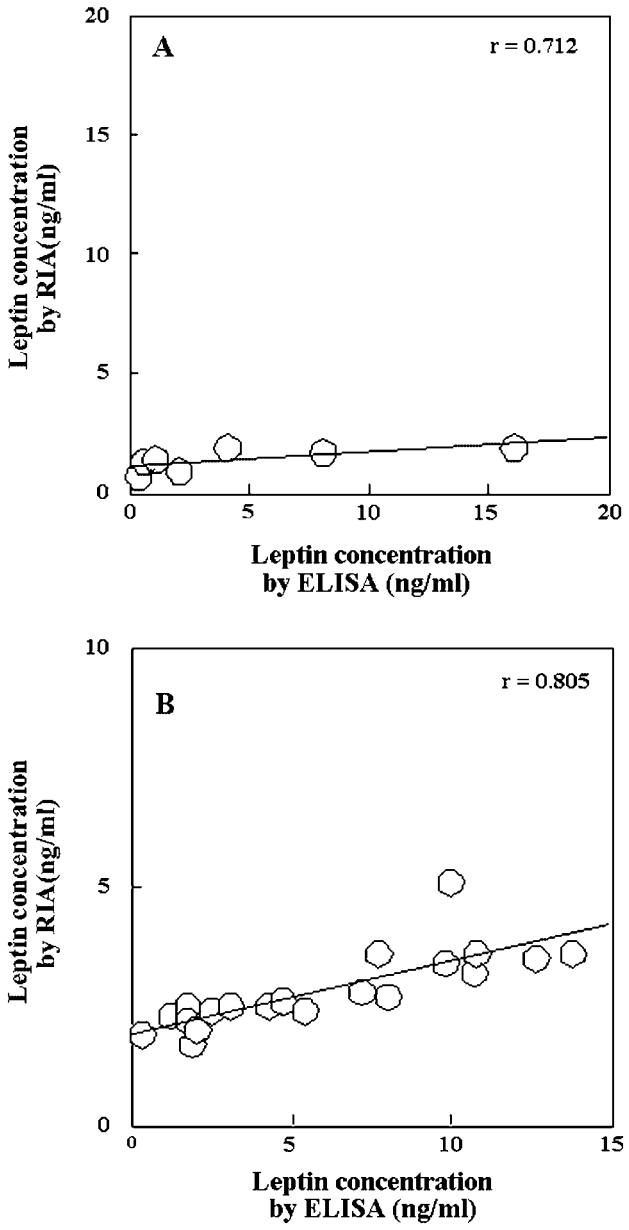

Fig. 2. Comparison between ELISA and RIA for the measurement of canine leptin. Recombinant canine leptin (0–16 ng/ml) (**A**) and sera from 20 clinically healthy dogs (**B**) were assayed by sandwich ELISA developed with anti-canine leptin antibody and also by a commercially available multi-species leptin RIA kit with anti-human leptin antibody (LINCO).

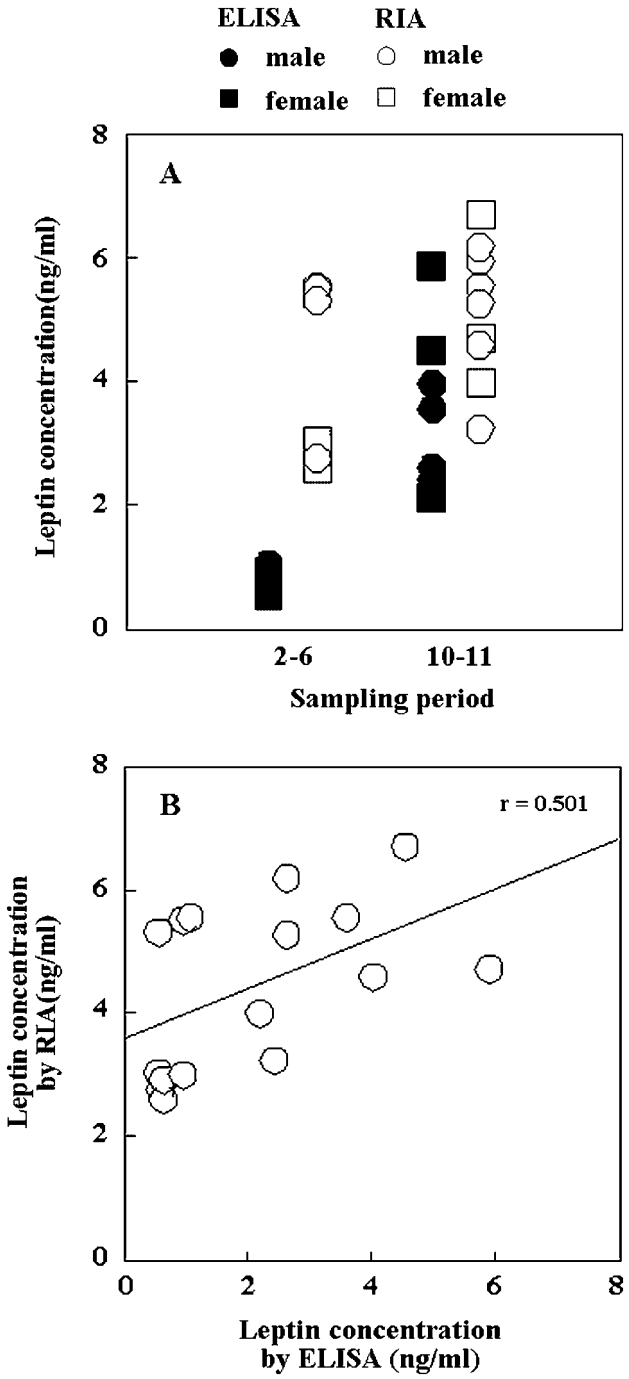

Fig. 3. Serum leptin concentration of raccoons. Sera were obtained from 16 wild raccoons trapped from October to November (four males and four females) and from February to June (five males and three females). Serum leptin concentrations were determined by ELISA and also by RIA. Estimated concentrations by the two methods were plotted against sampling seasons (**A**) and each other (**B**).

ranged between 0.52 and 1.04 ng/ml (mean = 0.71 ± 0.07 ng/ml), which was significantly lower ($P < 0.001$) than the levels obtained for eight animals caught from October to November (five males, three females) that ranged between 2.14 and 5.87 ng/ml (mean = 3.46 ± 0.45 ng/ml) (Fig. 3A). There were no apparent differences between the sexes in both sampling periods. However, when measured by RIA, leptin concentrations ranged between 3.23 and 6.72 ng/ml for

the animals caught from February to June and between 2.59 and 5.55 ng/ml for those caught from October to November. No significant difference was apparent between the two seasons ($P = 0.14$) (Fig. 3A). Although the values obtained by the two methods were positively correlated ($r = 0.501$, $P < 0.05$) (Fig. 3B), concentrations of the same samples estimated by RIA were apparently higher than those estimated by ELISA at the lower concentration ranges (<2 ng/ml). The same finding was made for leptin concentrations in dog serum. Figure 4 shows the positive correlation between serum leptin concentrations estimated by ELISA and body weight ($r = 0.753$, $P < 0.001$) and BMI calculated as weight/length^2 ($r = 0.755$, $P < 0.001$), respectively. The leptin concentrations estimated by RIA were also correlated with body weight ($r = 0.706$, $P = 0.001$) and BMI ($r = 0.734$, $P < 0.001$) (figure not shown).

We also measured seasonal variations in serum leptin concentrations from six bears by ELISA. The serum leptin concentration of a bear (*U. thibetanus*) sampled at the end of November was 14.4 ng/ml, while a sample obtained in June was lower than the detection limit (0.5 ng/ml). In addition, leptin concentrations of five sera samples from two bears (*U. arctos*) taken during winter from December to January and three samples from three bears (*U. arctos*) taken from March to July were 4.4–8.4 ng/ml and lower than 0.5 ng/ml, respectively. We also tried to assay serum leptin in ten Sika deer by ELISA, but failed to detect leptin at significant levels. In contrast, RIA gave values that ranged between 0.58 and 4.18 ng/ml, which were comparable to previously reported levels in this species (Suzuki et al., 2004) and in cows and sheep (Soliman et al., 2001, 2002).

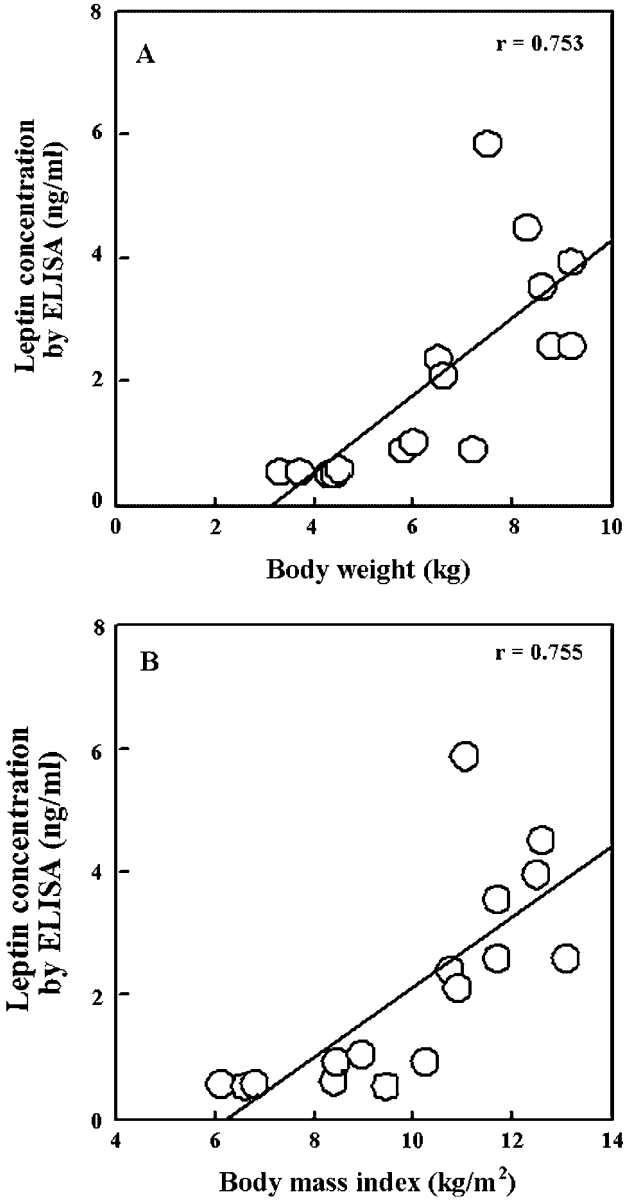

Fig. 4. Correlation of serum leptin concentration with body weight and body mass index (BMI) of raccoons. Serum leptin concentration estimated by ELISA in Fig. 3 was plotted against body weight (**A**) and BMI (**B**) calculated as body weight in kg divided by (body length in m)^2.

DISCUSSION

Recent findings that circulating leptin concentrations are positively correlated with body fat content (Maffei et al., '95; Considine et al., '96; Appleton et al., 2000; Backus et al., 2000; Ishioka et al., 2002; Sagawa et al., 2002; Shibata et al., 2003) in various species indicate that blood leptin can be used as a marker for adiposity. This has stimulated interest in the application of leptin assays to assess the nutritional condition of feral animals (raccoon dog (*N. procyonoides*), blue fox (*A. lagopus*) (Nieminen et al., 2001), European brown bear (*U. arctos arctos*) (Hissa et al., '98), little brown bat (*Myotis lucifugus*) (Kronfeld-Schor et al., 2000) and Sika deer (Suzuki et al., 2004)). In these studies, the multi-species leptin RIA kit with anti-human leptin antibody used for leptin assays can, according to the manufacturer's instructions, be applied to many species, including the cow, sheep, pig, horse, cat, bat and ground squirrel. However, the results of this study indicate that this RIA kit was less effective for assaying leptin concentrations in dogs, compared with our ELISA kit using anti-canine leptin antibody. That is, for both recombinant canine

leptin and dog sera samples, the estimated values by RIA were much lower than those by ELISA (Fig. 2). For example, RIA gave considerably higher values for dog serum leptin concentrations when very low concentrations were obtained by ELISA (Fig. 2B). This could be due to the presence of molecules other than leptin that reacted with the antibody in the RIA kit. This might also apply to the results obtained for raccoon sera (Fig. 3B). These results imply lower and less selective cross-reactivity of the antibody used in the RIA kit to canine and raccoon leptins. In contrast, the antibody raised against recombinant canine leptin cross-reacted strongly to canine leptin as expected, but weakly to murine, human and feline leptins (Fig. 1). These findings indicate that canine leptin has immunogenic regions that are quite different from other species, despite having approximately 80% identity of its amino acid sequence (Iwase et al., 2000a). It is also likely that raccoon leptin has immunogenic regions that are similar to those of canine leptin, although its amino acid sequence has not been determined. This seems possible given the closer phylogenetic relationship that exists between raccoon and canine leptin than between raccoon and feline leptin (Doyon et al., 2001). Taken together, our canine-leptin-specific ELISA is more useful for blood leptin assays, not only in dogs but also in raccoons and probably in other members of the *Carnivora* such as the bear.

Using the canine-leptin-specific ELISA, we found clear seasonal variations in blood leptin concentrations in raccoons, with low levels from February to June and higher levels from October to November (Fig. 3A). Furthermore, a significant positive correlation between blood leptin concentrations and body weight, as well as BMI was found (Fig. 4). Considering that BMI can be used as a general marker for adiposity in most mammals, higher blood leptin concentrations during the fall season may reflect increased accumulation of body fat in preparation for the energy demands of winter. While similar seasonal variations in blood leptin concentrations were also observed in the bear samples assayed in this study, the extent of seasonality was less marked or not detectable by RIA. We demonstrated that our canine-leptin-specific ELISA was useful for assays of blood leptin in members of *Canidae*, *Ursidae* and *Procyonidae* of the order *Carnivora*, and that the commercial multi-species RIA was well suited for assays of leptin in *Felidae* (Shibata et al., 2003). Given these findings, it may be necessary to re-evaluate several previously reported studies on blood leptin in feral animals, particularly among members of *Carnivora* assayed to date.

ACKNOWLEDGMENTS

This study was carried out in association with the Management Plan for the Feral Raccoon Population in Hokkaido. We are grateful to the local government offices, EnVision and the Hokkaido Forest Management Corporation for providing us with raccoons. We also thank Dr. Tsutomi Mano (Hokkaido Institute of Environmental Sciences) and Dr. Toshio Tsubota (United Graduate School of Veterinary Sciences, Gifu University) and the staff of Noboribetsu Bear Park for collecting bear blood.

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