FULL PAPER *Theriogenology*

Serum Progesterone and Estradiol-17β Concentrations in Captive and Free-Ranging Adult Female Japanese Black Bears (*Ursus thibetanus japonicus*)

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(Received 7 July 1999/Accepted 28 December 1999)

ABSTRACT. Progesterone (P_4) and estradiol-17β (E_2) concentrations were measured in serum samples obtained from 23 captive and 23 free-ranging adult female Japanese black bears. We then determined the relationship between changes in these sex steroid hormones and pregnancy. In all captive bears, which included animals of both known and unknown reproductive status, serum P_4 concentrations were low from April to July, then tended to become higher after August. The levels then became much higher still in November and December, but returned to low levels in March. Serum P_4 concentrations in eight captive pregnant bears, which had parturitions the following spring, increased gradually from August (0.5–2.4 *n*g/m*l*) to October (0.9–3.6 *n*g/m*l*), and achieved significantly higher maximum levels in December (7.2–18.0 *n*g/m*l*). Thereafter, serum P_4 concentrations tended to decrease (3.5–6.4 *n*g/m*l* in January and 0.3–0.7 *n*g/m*l* in March). In all captive bears, serum E_2 concentrations varied from April to October but showed low levels in November and December, and became high in January. Serum E_2 concentrations in the eight pregnant bears were high in May (95.6–191.4 *p*g/m*l*) and varied from August to October (35.6–143.3 *p*g/m*l*). Subsequently, serum E_2 concentrations in December dropped to significantly lower minimum levels (5.3–11.9 *p*g/m*l*) and increased again in January (67.6–153.1 *p*g/m*l*). Among the free-ranging bears, the data on serum P_4 concentrations in eight bears led to expectations of pregnancy, whereas serum E_2 concentrations showed no distinct evidence related to pregnancy. These results, particularly in captive pregnant bears, indicate that a marked increase of P_4 in December might be accompanied by reactivation of the corpus luteum preceding implantation. Furthermore, changes in E_2 concentrations suggested the possibility that a decline in December and an increase in January are associated with implantation and parturition, respectively.—KEY WORD: bear, estradiol-17β, pregnancy, progesterone.

J. Vet. Med. Sci. 62(4): 415–420, 2000

The Japanese black bear (*Ursus thibetanus japonicus*), which inhabits the islands of Honshu and Shikoku in Japan, is one of the large mammalian species exhibiting seasonal breeding. Pregnant bears possess some fascinating reproductive features, such as obligate delayed implantation (embryonic diapause) and parturition in the middle of the denning period [8]. The mating season of captive Japanese black bears is from late June to early August, and the birth occurs from late January to early February [27]. Studies on the delayed implantation in other ursids, such as the American black bear (*Ursus americanus*) [26] and the Hokkaido brown bear (*Ursus arctos yesoensis*) [20–22, 24], have revealed that the development of fertilized eggs is arrested at the blastocyst stage until the blastocyst is implanted in late November or early December, and that parturition occurs about 60 days after implantation. However, few studies have provided information on the time of implantation in the Japanese black bear.

Changes in sex steroid hormones have generally been assessed to obtain information on reproductive status. In several species with delayed implantation, especially musterids, progesterone is regarded as an essential factor in the maintenance of embryonic diapause, although the corpus luteum seems considerably less active. In addition, implantation occurs in association with a sharp increase in progesterone, which suggests that the elevation of progesterone is involved in the reactivation of the dormant corpus luteum [3, 6, 7, 11, 13, 15, 16, 24]. On the other hand, the role of estrogen during gestation in which there is delayed implantation has not been well defined. However, there is evidence that estrogen levels are reduced together with renewed blastocyst development in the western spotted skunk [17] and the European badger [14].

The objective of the present study was to determine the progesterone (P_4) and estradiol-17β (E_2) profiles of the Japanese black bear, with particular emphasis on their changes related to pregnancy.

In addition, reproductive knowledge of free-ranging bears is very valuable information and a great concern for wildlife managers, because the Japanese black bear is threatened in some areas. However the difficulties in obtaining samples and evaluating data from free-ranging bears has resulted in a dearth of information on this subject. One of our goals in studying captive bears was to define their endocrinological features and to apply this knowledge to free-ranging bears. Therefore, in the present study, we also tried to evaluate data from free-ranging bears in comparison with those from captive bears.

MATERIALS AND METHODS

Captive bears: Between 1995 and 1997, 96 blood samples were collected from 23 captive adult female Japanese black bears, eight of which were confirmed to have given birth. However the reproductive status of all animals, except the pregnant bears, was unclear. All blood samples were collected for routine health examinations whenever we had the chance to immobilize animals, and the serum samples were stocked for hormone measurements. The captive animals were managed under natural conditions at Ani Mataginosato Bear Park, Akita, Japan (N 40°, E 140.4°). In this facility, male and female bears were placed together in outdoor runs (25 × 50 m) from mid-April through mid-December and moved indoors (3.47 × 4.88 m) for denning during the remaining winter period. All animals were fed corn meal mainly once a day, and water was available *ad libitum*, except during the denning period when they were not fed. All bears were considered adults because their ages were more than 4 years old based on Bear Park records [10]. The bears were anesthetized by blow dart injections with any one of the following three combinations: ketamin HCl (Ketaral, Sankyo, Japan) and xylazine HCl (Celactar, Bayer, Germany), ketamine HCl and medetomidine HCl (Domitor, Meiji, Japan), or Zolazepam HCl and Tiletamine HCl (Zoletil, Virbac, France). Blood samples were taken from the jugular vein after immobilization and the serum was separated by centrifugation (1,200 G, 15–20 min) and stored at −30˚C until assayed.

Free-ranging bears: Thirty-two blood samples were taken from 23 free-ranging female bears. The free-ranging bears were trapped using barrel traps between 1985 and 1997 in Tanzawa, Kanagawa and Wadayama, Hyogo, Japan. The ages of bears were determined by interpretation of tooth cementum annuli, and bears whose ages were more than 4 years were considered sexually mature animals. Immobilization and sampling were carried out following procedures similar to those described for captive bears.

Radioimmunoassays: Serum P_4 concentrations were measured by the radioimmunoassay method described by Palmer *et al*. [15], using a rabbit anti-serum against progesterone (HAC-AA63-06RBP84, provided by the Institute of Endocrinology, Gunma University, Maebashi, Japan) at a final dilution of 1:56,000, and [2,4,6,7,16,17-3H(N)]-progesterone (NET-1112, New England Nuclear Life Science Products, U.S.A.) as the radioligand. The assay has a sensitivity of 80 *p*g/m*l*, and intra- and inter-assay coefficient variations were 5.4 and 7.9%, respectively. Because of the low levels, E_2 was assayed using Palmer's procedure with minor modifications as follows: [2,4,6,7,16,17-3H(N)]-estradiol (NET-517, New England Nuclear Life Science Products, U.S.A.) was diluted to give an approximate concentration of 1,000 dpm/tube, about half the amount of the isotope in the progesterone assay, in order to raise sensitivity. A rabbit anti-serum against estradiol-17β (FD121, Medical System Service Teikokuzoki, Kanagawa, Japan) was used at a final dilution of 1:252,000. As a result of these improvements, the assay sensitivity was 5 *p*g/m*l*. The intra- and inter-assay coefficient variations were 13.0 and 9.0%, respectively.

Statistical analysis: The Mann-Whitney U test was used to determine differences in P_4 and E_2 concentrations in pregnant bears between December versus those in October. A p value less than 0.1 was considered statistically significant.

RESULTS

Captive bears: Serum P_4 concentrations in all captive bears, both pregnant and non-pregnant are shown in Fig. 1(a). Serum P_4 concentrations ranged from 0.04 to 3.6 *n*g/m*l* between April and October. Some animals exhibited high serum P_4 levels in November and December (7.2–18.0 *n*g/m*l*). The values ranged between 0.1 and 6.4 *n*g/m*l* in January and 0.3 and 0.9 *n*g/m*l* in March. The serum P_4 profiles of eight pregnant bears in captivity are shown in Fig. 1(b). Low concentrations of P_4 were maintained in May (0.5–1.2 *n*g/m*l*) and June (0.6 *n*g/m*l*). They increased gradually from August (0.5–2.4 *n*g/m*l*) to October (0.9–3.6 *n*g/m*l*), then reached significantly higher maximum levels in December (7.2–18.0 *n*g/m*l*: p=0.067). Thereafter, the values tended to decline gradually and fell to basal levels in March (0.3–0.7 *n*g/m*l*).

The results of serum E_2 concentrations in all captive bears are illustrated in Fig. 2(a). Serum E_2 concentrations ranged from 22.2 to 191.4 *p*g/m*l* between April and October. In most animals, serum E_2 concentrations were relatively low in November and December (1.4–67.8 *p*g/m*l*) and high in January (42.2–153.1 *p*g/m*l*). Serum E_2 concentrations in eight bears, which were confirmed to be pregnant, are illustrated in Fig. 2(b). The changes in serum E_2 concentrations varied among individual animals between May and October. For example, the concentration in Bear 7 was high in May (191.4 *p*g/m*l*) and tended to decline noticeably afterward, while that in Bear 8 showed little change (83.3–95.6 *p*g/m*l*) between May and October. However, serum E_2 tended to reach significantly low minimum levels in December (5.3–11.9 *p*g/m*l*: p=0.067), and to rise in January (67.6–153.1 *p*g/m*l*).

Free-ranging bears: Serum P_4 and E_2 concentrations in free-ranging bears are presented in Fig. 3(a) and (b), respectively. Serum P_4 concentrations were between 1.1 and 1.3 *n*g/m*l* in June, then tended to increase in July and August (0.5–8.3 *n*g/m*l*). The values in September and October did not show further increases (0.5–3.4 *n*g/m*l*) over those between July and August. Serum E_2 concentrations varied erratically from June to October (0.1–106.1 *p*g/m*l*), with relatively low levels in September (16.4–32.8 *p*g/m*l*).

DISCUSSION

The sex steroid profiles of many carnivorous species exhibiting obligate delayed implantation have been examined by many investigators, and the roles of these sex steroid hormones throughout gestation, including the delay period, have been described. It has been hypothesized that delayed implantation may be due to the uncompleted activity

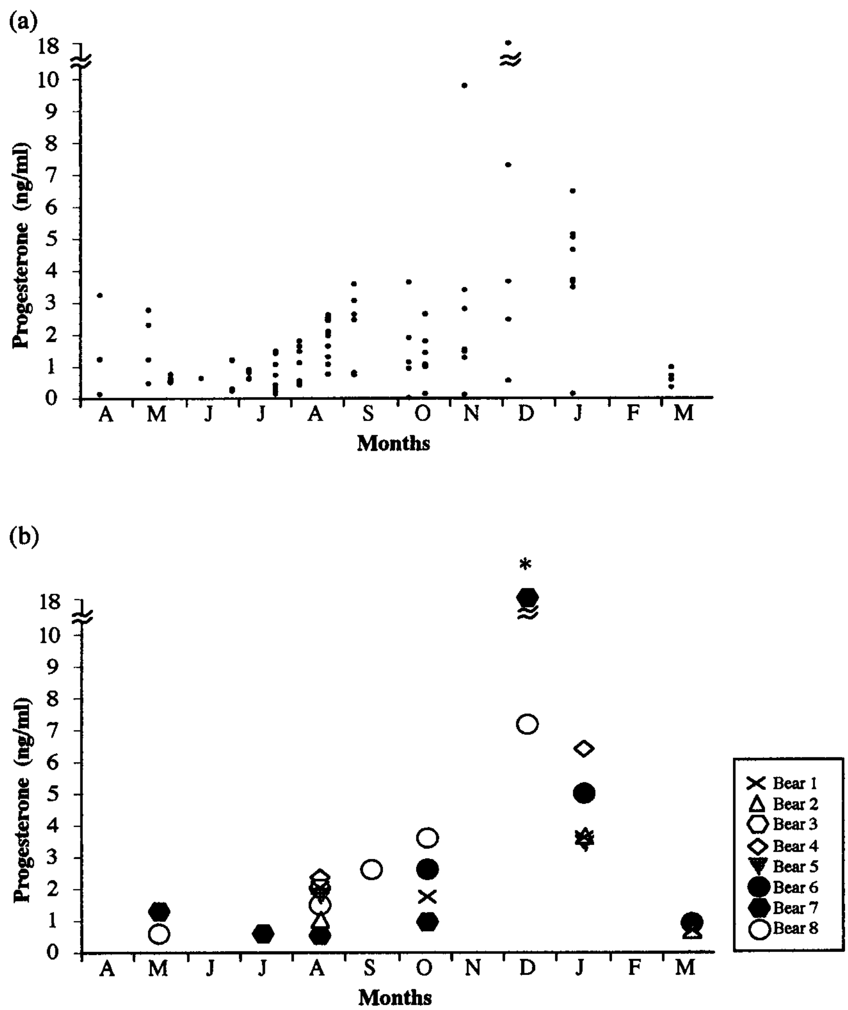

Fig. 1. Annual changes in serum progesterone concentrations in 23 captive female black bears. (a) The reproductive status of all bears was unknown, except 8 pregnant bears (n=96). (b) Eight bears all of which gave birth the following spring (n=23). *Values in December were significantly different from those in October (p=0.067). In both figures, the peaks which occur around the time of implantation are observed in November or December.

of the corpus luteum, while luteal cells maintain capability for steroidogenesis and sufficient secretion of P_4 to keep blastocysts viable in the uterine lumen [11]. In the present study, serum P_4 concentrations of captive pregnant bears began to rise in August and September, and reached significantly high levels in December. This finding is consistent with those reported previously for the mink [13, 16], the western spotted skunk [12, 18], the European badger [3], the stoat [7], the American black bear [6, 9, 15, 23], the polar bear [15], and the Hokkaido brown bear [21, 24], in which P_4 concentrations are maintained low or increase little by little during delayed implantation, and then increase significantly at the time of implantation, reflecting the initiation of full luteal activity. Thus, our results also suggest that a P_4 peak in December may reflect the reactivation of the corpus luteum at implantation in the Japanese black bear.

The present data showed that serum E_2 concentrations were at a significantly low minimum in December. This was similar to the findings of previous investigations in several species with delayed implantation: no increase in estrogen levels was reported in the mink [16], and tendencies to decreasing levels were observed in the European badger [14], the spotted skunk [17], the American black bear [15, 23] and the polar bear [15] as the time of implantation approached. Ravindra and Mead [17] described the relationship in the spotted skunk between changes in estrogen levels and blastocyst development *in vivo*, indicating that estrogen levels tended to decrease as the diameter of the blastocyst increased. Similar evidence was reported in an *in vitro* study of the mink [19]. However, it has not been determined precisely what the decrease in E_2 signifies with regard to embryo development. The results of this study suggest the possibility that a reduction in E_2

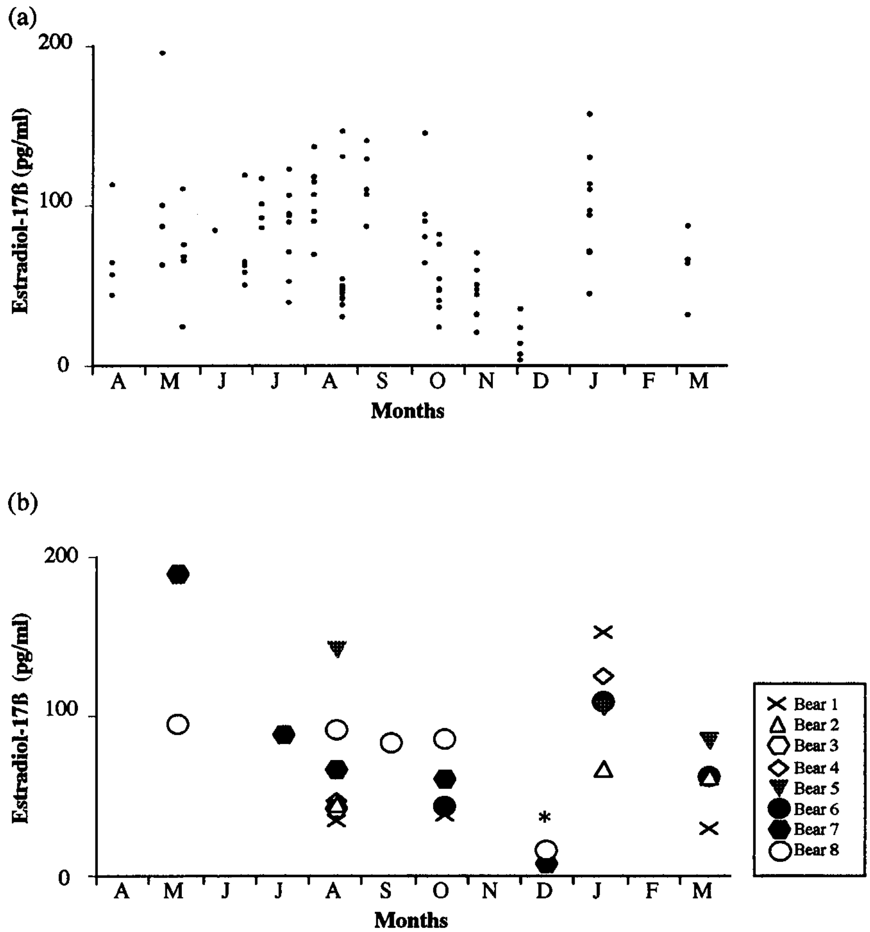

Fig. 2. Annual changes in serum estradiol-17β concentrations in 23 captive female bears. (a) The reproductive status of all bears was unknown except 8 pregnant bears (n=96). (b) Eight bears all of which gave birth the following spring (n=23). *Values in December were significantly different from those in October (p=0.067). In both figures, a depression in December and an increase in January are observed, which may be related to the times of implantation and parturition, respectively.

levels in December is associated with certain events that occur during the peri-implantation period which are responsible for implantation, and that this is an important component of the maternal condition of the Japanese black bear.

Serum E_2 concentrations after implantation tend to rise again before parturition, which generally occurs about 60 days after implantation [6, 21]. It is widely known that E_2 contributes to the inducement of parturition in various animals [4]. E_2 changes in this period are, therefore, suspected to be involved in parturition. A prepartum rise in E_2 was reported in the domestic dog [5], the domestic cat [25], the western spotted skunk [17] and the puma [1], but not in the red fox [2] and the mink [16]. However, this discrepancy among carnivore species has not been explained well.

In all captive bears in the present study, including both pregnant and non-pregnant bears, serum P_4 concentrations were markedly high in November and December, around the time of implantation, in some animals but not in others, while E_2 levels were low in most of them. Previous studies showed that bears which were neither pregnant nor lactating had similar serum P_4 changes to those of pregnant bears, suggesting the possibility of a pseudopregnancy [9, 24]. On the basis of our results with pregnant bears and previous studies, it may be considered that animals with markedly high P_4 levels must be pregnant or pseudopregnant. On the other hand, bears with no remarkable elevation in serum P_4 concentrations at this time might be interpreted as being not pregnant, although non-pregnant bears were not identified in this study. With E_2, in contrast, the tendency in November and December seems to be toward consistently low levels in most animals. This finding may indicate that E_2 levels undergo a decrease during this period regardless of reproductive status.

It is difficult to interpret data from free-ranging bears, as described by Hellgren *et al.* [9]. Tsubota *et al.* [24] reported that free-ranging Hokkaido brown bears could be classified

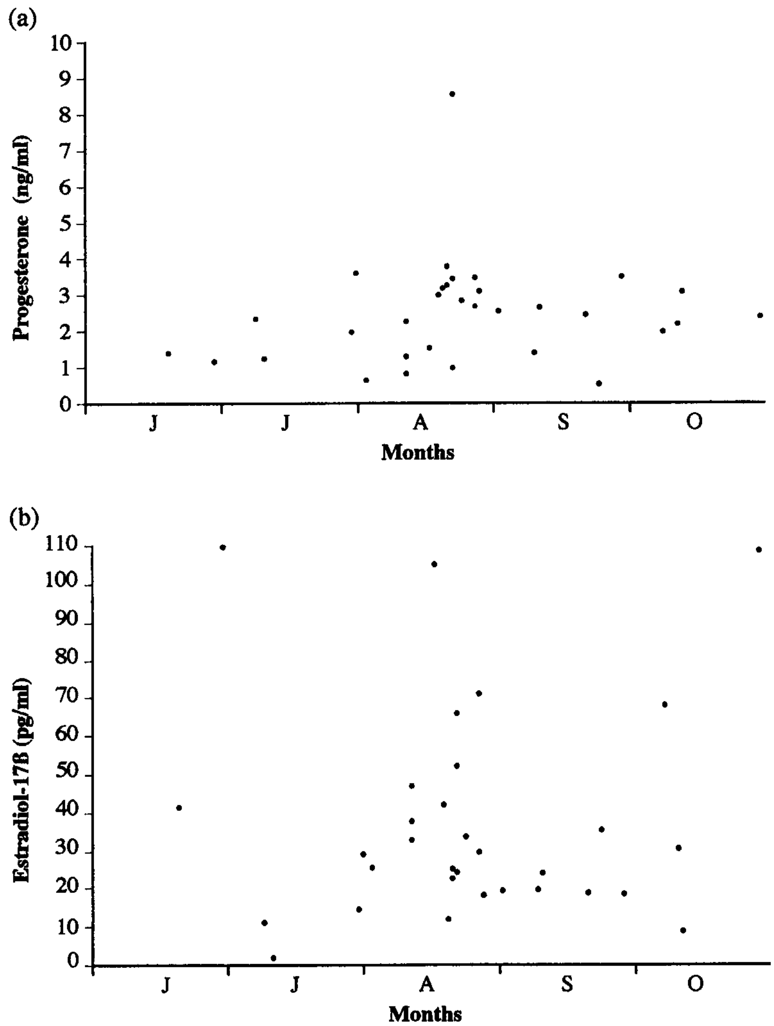

Fig. 3. Serum progesterone and estradiol-17β profiles in 23 free-ranging bears from June to October. Data on (a) serum progesterone concentrations and (b) serum estradiol-17β concentrations provide no evidence relating to pregnancy. But, for 8 bears captured in September and October, the data on serum P_4 concentrations (>1 *n*g/m*l*) led to expectations of pregnancy based on comparison with data from captive bears and previous reports.

as pregnant if serum P_4 concentrations were over 1 *n*g/m*l*. Other previous studies revealed that lactating or non-pregnant bears had low progesterone concentrations (<1 *n*g/m*l*) regardless of time of the year [9, 15]. In this study, in a comparison of the data for free-ranging bears with those for captive pregnant bears between September and October, corresponding to the period of delayed implantation, P_4 levels in all free-ranging bears (1.34–3.4 *n*g/m*l*) except one (0.5 *n*g/m*l*) were within the range of those in pregnant captive bears (0.94–3.65 *n*g/m*l*). Thus, the eight free-ranging bears with P_4 levels over 1 *n*g/m*l* might have been pregnant, although we cannot state this with absolute certainty, because we do not know whether or not these free-ranging bears gave birth after release, and from the present study cannot provide any information to identify differences in P_4 profiles between pregnant and non-pregnant bears. It seems to be difficult to detect pregnancy using only P_4 levels as a criterion, especially in free-ranging bears, because the availability of samples and information such as the reproductive history of individual animals is limited. Moreover, the presence of delayed implantation makes it difficult to form an accurate interpretation of pregnancy based on hormone changes. Therefore, the development of techniques other than hormone measurement to evaluate pregnancy in bears is much awaited.

This is the first study to investigate profiles of sex steroid hormones in blood samples from the female Japanese black bear. The results reported here indicate that implantation in the Japanese black bear occurs around December, when there is evidence of a significant elevation in P_4

concentrations and a significant depression in E_2 concentrations. In addition, E_2 levels underwent an elevation in January that was possibly associated with parturition. On the basis of previous studies of other species and the present study of bears, both sex steroids appear to play synergistic roles in maintaining adequate maternal circumstances throughout the gestation period rather than in inducing implantation. Further detailed investigations should be performed in order to define the roles of sex steroids during pregnancy, especially in the pre- and postimplantation periods, in the Japanese black bear.

ACKNOWLEDGMENTS. The authors thank the Institute of Endocrinology at Gunma University for a donation of the progesterone antiserum and Ms. Aiko Hamasaki for performing the age determination. This work could not have been conducted without the kind cooperation of all the staff at Ani Mataginosato Bear Park and the Institute of Japanese Black Bear in Ani, Akita. This study was supported in part by a Grant-in-Aid for Scientific Research No. 10306020 from the Ministry of Education, Science, Sports and Culture of Japan.

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