Spermatogenesis, Serum Testosterone Levels and Immunolocalization of Steroidogenic Enzymes in the Wild Male Japanese Black Bear (*Ursus thibetanus japonicus*)

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ABSTRACT. Twenty-one wild male Japanese black bears (*Ursus thibetanus japonicus*) were captured in the summer-autumn of 1998–2000 in the vicinity of Neo Village, Gifu Prefecture. Testes were measured, and testicular samples were biopsied and observed histologically. Four steroidogenic enzymes, i.e., cholesterol side-chain cleavage cytochrome P450 (P450scc), 3β-hydroxysteroid dehydrogenase (3βHSD), 17-α hydroxylase cytochrome P450 (P450c17), and aromatase cytochrome P450 (P450arom) were immunolocalized. Serum testosterone concentrations were measured by radioimmunoassay. Testis size changed little from 1–3 years of age, increased rapidly at 4 years, and attained its peak at 5 years. Serum testosterone concentrations ranged from 0.05 to 1.78 *ng*/m*l*, and the mean ± standard deviation was 0.43 ± 0.48 *ng*/m*l*. Age of sexual maturation in wild male Japanese black bears was estimated to be 3–4 years. Seasonal changes in spermatogenesis were obvious; active in June, July and August, degenerated by September. Leydig cells, Sertoli cells and germ cells have the capability of synthesizing androgen, and Leydig cells, Sertoli cells, spermatids and spermatogonia have the capability of synthesizing estrogen in Japanese black bears.

KEY WORDS: Japanese black bear, spermatogenesis, steroidogenic enzyme, testosterone, *Ursus thibetanus japonicus*.

In order to enhance the conservation and management of wild animals, we have to know the population dynamics of breeding-age animals. For this purpose, age at sexual maturation and seasonal changes in reproductive activity such as gonadal morphology profiles and sexual behavior in the population need to be determined [11]. With this in mind ecological investigations and physiological examinations of the Japanese black bear (*Ursus thibetanus japonicus*) are being performed in many study areas, and reproductive physiology in the male Japanese black bear has been reported recently from this point of view [11, 12, 22].

The age of sexual maturation (puberty) in male Japanese black bears is estimated to be 2–4 years with some variations among studies based on the histology of testes, their size and weight, and the diameter of seminiferous tubules [11, 12, 16, 22]. It is known that the reproductive physiology of the male Japanese black bear shows notable seasonal changes [25] with mating occurring from June to August [29]. In relation to this, testes indicate physiological and morphological changes, and the period of high spermatogenic activity is limited to several months, including the mating season [13].

The aim of this study was to determine the age of sexual maturation and the seasonal changes in spermatogenesis and testicular steroidogenesis in wild male Japanese black bears.

MATERIALS AND METHODS

Capture and handling: Wild male Japanese black bears that inhabit Neo Village, Gifu Prefecture, were caught by barrel-type traps from 26 June 1998 to 13 November 1998, from 15 May 1999 to 12 November 1999, and from 15 July 2000 to 31 August 2000. Capture of bears was performed with the permission of the former Environment Agency, Japan. All the traps were checked twice a week for captured bears. After capture, bears were handled on the next day.

Trapped bears were immobilized by an intramuscular administration of ketamine HCl (Veterinary Ketalar 50 (10 mg/kg) and medetomidine HCl (Domitor: 0.04 mg/kg), or Zolazepam HCl and Tiletamine HCl (Zoletil: 9 mg/kg). After immobilization, bears were weighed and their physical condition was monitored for body temperature, pulse and respiration. Then blood samples were obtained from the jugular vein with vacuum blood drawing tubes. Testes were measured from outside the scrotum, and the testis size was calculated by the formula $\sqrt[3]{(length) \times (width) \times (depth)}$. Testicular tissues were biopsied surgically in 5 bears. The second *dentes premolare* from either the left or right upper side was extracted for age determination.

Microscopic observations: Testicular samples were fixed in Bouin's solution for 3–4 hr, followed by dehydration and embedding in paraffin (Merck, Germany). Then 4-μm sections were prepared and stained with haematoxylin and eosin. The Sertoli cells, spermatogonium, spermatocytes, and round and elongating spermatids were observed histologically.

Radioimmunoassay: Blood taken was centrifuged at

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1,000 g for 10 min to separate serum from haematocytes, and the serum was stored at –20°C until assay. Serum test-osterone concentrations were measured by radioimmunoassay using testosterone antiserum (T-3-CMO-BSA; Institute of Endocrinology, Gunma University, Gunma), standard testosterone (Nakalai Tesque, Tokyo), and [1, 2, 6, 7, 16, 17-^{3}H(N)] testosterone (NET-553, NEN Life Science Products, U.S.A.). The intra- and inter-assay coefficients of variation were 12.6% and 17.8%, respectively.

Immunohistochemistry: To determine the immunohistochemical sites of four steroidogenic enzymes [cholesterol side-chain cleavage cytochrome P450 (P450scc), 3β-hydroxysteroid dehydrogenase (3βHSD), 17-α hydroxylase cytochrome P450 (P450c17), and aromatase cytochrome P450 (P450arom)], testicular sections were immunostained by the Avidin-biotinylated-peroxidase complex (ABC) method with a Rabbit ExtrAvidin staining kit (Sigma, U.S.A.). Antibodies against these steroidogenic enzymes were all polyclonal antibodies raised in rabbits, and refined by the affinity column method. As the antigen, we used P450scc refined from the mitochondria fraction of a bovine adrenal cortex [23], 3βHSD refined from the microsome fraction of a human placenta [2], P450c17 refined from the microsome fraction of a guinea pig adrenal gland [14], and P450arom refined from the microsome fraction of a human placenta [8]. Control sections were treated with normal rabbit serum (Sigma, U.S.A.; 1:1,000) instead of the primary antiserum. Three (P450scc, 3βHSD and P450c17) of the 4 antibodies used in this experiment were same as those used previously and have been already proved to be specific to the corresponding antigens [13]. The antibody of P450arom was first used for bear testes in this study and its specificity has not been proven although its specificity for human placenta has been evidenced already [8].

RESULTS

Testis size: In 21 bears (1–6 years old), testis size ranged from 15.7 to 47.9 mm with a mean ± standard deviation of 30.5 ± 8.7 mm. The relation of testis size measured in the mating season (June - August, n=19) to age is shown in Fig. 1 and changed little during 1–3 years, but increased rapidly at 4 years, and peaked at 5 years. Mean testis sizes by month in bears over 4 years were 36.1 mm in June (n=4), 37.3 mm in July (n=5), and 37.1 mm in August (n=2).

Spermatogenesis: Spermatogenic activity was evaluated by histological observation of seminiferous tubules in 5 biopsied bears. In June, July and August, the seminiferous tubules contained the Sertoli cells and germ cells from spermatogonia to spermatozoa in all the bears except for No. 11 (Fig. 2A). Spermatogenesis was especially active with the largest cell numbers in the seminiferous tubles in June (No. 9). In September (No. 14), the seminiferous tubules contained Sertoli cells and germ cells from spermatogonia to round-spermatids. In No. 11 (July, 3 years old), the seminiferous tubules contained only Sertoli cells and undifferentiated spermatogonia (Fig. 2B).
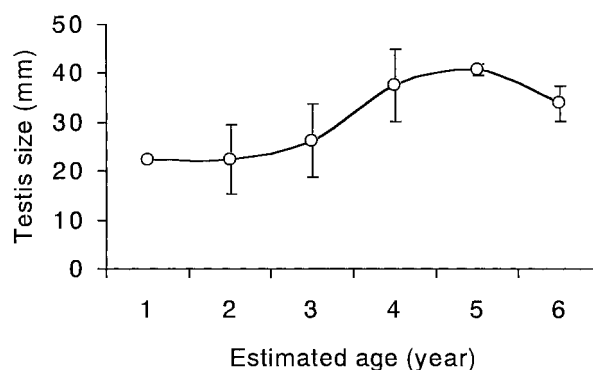

Fig. 1. Changes in the testes size according to age in wild Japanese black bears examined in the mating season (June to August, n=19). Testis size was calculated by the formula $\sqrt[3]{(lentgh) \times (width) \times (depth)}$, and age was estimated by dental examination.

Serum testosterone concentrations: In 14 bears (2–6 years old), serum testosterone concentrations ranged from 0.05 to 1.78 ng/ml with a mean ± standard deviation of 0.43 ± 0.48 ng/ml. Seasonal changes in serum testosterone concentrations are shown in Fig. 3. From June to July, serum testosterone values were relatively high (> 0.5 ng/ml), but showed no significant increase.

Immunohistochemistry: Immunolocalization of steroidogenic enzymes in the testicular biopsies of 5 bears is shown in Table 1. P450scc, 3βHSD and P450c17 were immunolocalized in the Leydig cells of all bears and in the Sertoli and germ cells in some bears.

The Sertoli cells of mature bears (Nos. 8, 9, 12 and 14) were stained positively for P450scc (Fig. 4A). The spermatogonia of immature bears (No. 11) were also stained positively for P450scc (Fig. 4E). Spermatogonia which had more highly condensed nuclei were more intensely stained for P450scc. The Golgi region of spermatocytes in No. 9 (June) was stained positively for P450scc (Fig. 4A). The spermatocytes and spermatids of mature bears (Nos. 8, 9, 12 and 14) were positively stained for P450c17 (Fig. 4C). P450c17 showed positive staining in the spermatocytes just like P450scc. The Golgi region of round and elongating spermatids was stained positively for P450c17. P450arom was localized in the spermatids and Sertoli cells of mature bears (Nos. 8, 9 and 12) (Fig. 4D). Round spermatids were stained positively for P450arom just like P450c17. Areas surrounding the nuclei of elongating spermatids were stained for P450arom. In an immature bear (No. 11), P450arom was localized in the cytoplasm of spermatogonia showing condensed nuclei (Fig. 4F). No immunostaining was detected in control sections that had been incubated with non-immune serum.

DISCUSSION

Sexual maturity: The process of precise sexual maturity begins in puberty and ends in what is generally called sexual maturity. Puberty (narrowly defined as sexual maturity) in
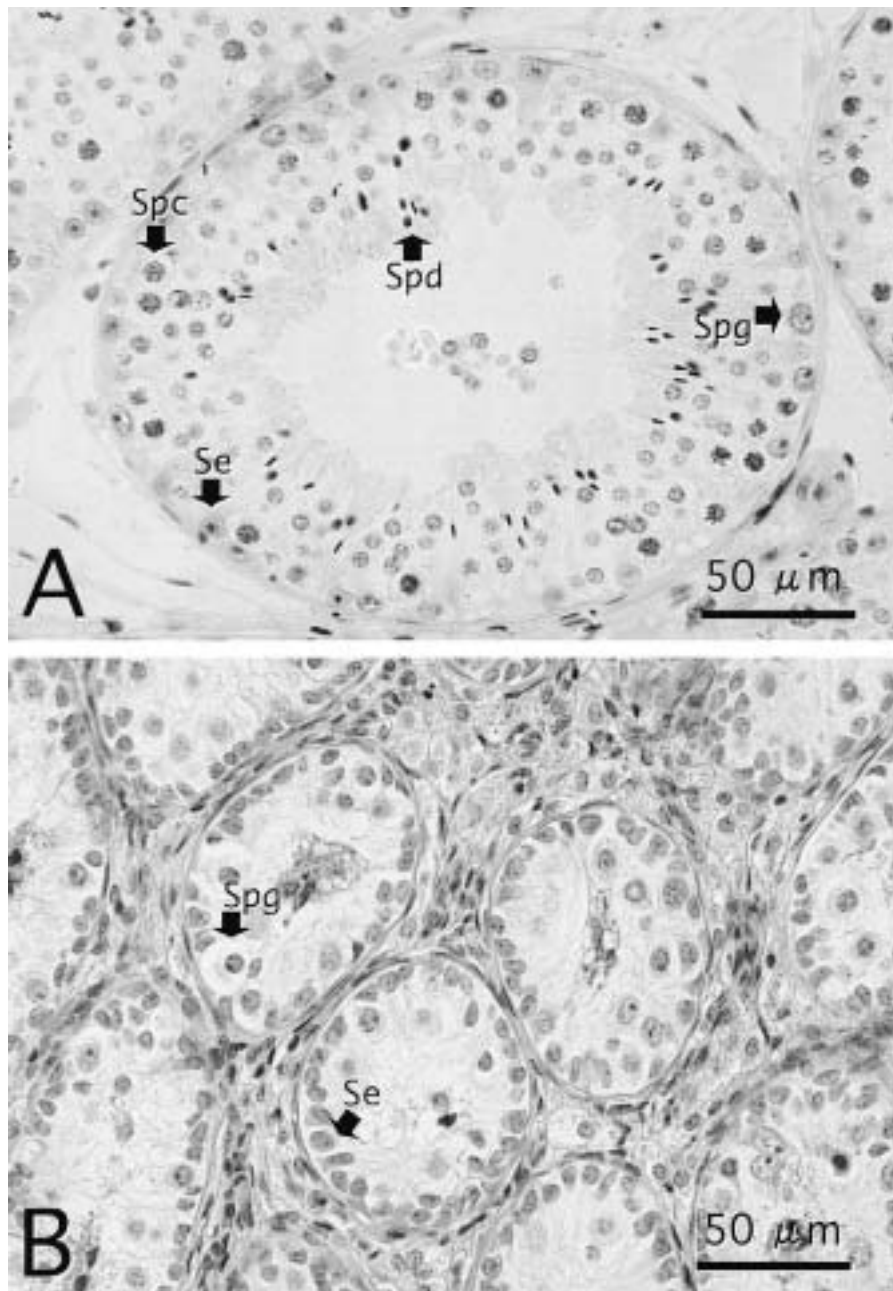
Fig. 2. Axial section of seminiferous tubules. A. Mature Japanese black bear (June). Sertoli cells (Se), spermatogonia (Spg), spermatocytes (Spc), and spermatids (Spd) are present. Spermatogenesis is active and lumen of the tubule is broad. B. Immature Japanese black bear (July). Only Sertoli cells (Se) and spermatogonia (Spg) are present. Lumen of tubule is not visible. Hematoxylin-eosin (HE) stain.

males is characterized by rapid growth of the testis and the appearance of spermatozoa in the seminiferous tubules. On the other hand, male animals that ejaculate fertilizable spermatozoa are broadly regarded as having attained sexual maturity. If sexual maturity is defined according to the latter description, fertility tests would have to be carried out on

the ejaculated semen of each animal or the rate of impregnation determined for each wild male. Such tests are extremely difficult in wild animals. Therefore, we equate sexual maturity with puberty in this paper.

In this study, the testes were classified into two types by histological observation. One had Sertoli cells and sper-
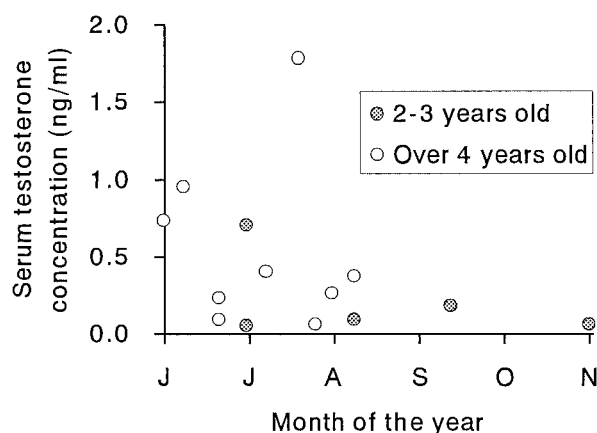
Fig. 3. Seasonal changes in serum testosterone concentrations in wild male Japanese black bears.

matogonia in the seminiferous tubules (3-year-old bear, No. 11), and the others (all but No. 11) had Sertoli cells, spermatogonia and spermatocytes and occasionally spermatids and spermatozoa in the tubules. Testes of immature bears clearly differ from those of mature ones based on histological observation [26]. Even the most degenerating testes during the seasonal changes contained germ cells from spermatogonia to spermatocytes in mature Hokkaido brown bears [24]. Thus bear No. 11 was regarded as immature and the others as mature. Accordingly, 50% of 3-year-old bears (n=1/2) and 100% of 4-year-old bears (n=3/3) were classified as mature. This result confirmed presumptions of the age of sexual maturity in male bears in other areas of Japan [11, 12, 17, 22].

The immature bear of 3 years (No. 11) weighed less than the others. Bears that forage for food in garbage produce their first cubs earlier than those that eat natural foods, but captive bears do so even earlier [21]. Thus, the time required to reach sexual maturity is affected by food supply. We speculate that the sexual maturity of No. 11 might have been delayed due to lack of nourishment.

In this study, testis size tended to be almost unchanged from 1–3 years but then increased thereafter. Komatsu *et al.* [12] reported that the testis size of an immature bear differs significantly from that of a mature one, and increase very rapidly from 2–3 years, a feature that can generally be used to distinguish immature from mature bears. If the above is taken into consideration, it is reasonable to conclude that there are many bears that reach sexual maturity from 3–4 years in the Neo Village region.

Seasonal reproductivity: Japanese black bears, as well as the other Ursidae described in previous reports, exhibit seasonal changes in reproductive function [25]. The mating season of wild Japanese black bears is estimated to be from June to August based on the observation of sexual behavior in captive bears [29]. On the basis of morphological observations of spermatogenic activity, Komatsu *et al.* [13] divided the Japanese black bear's reproductive cycle into five periods: an active period in May and June; a degenerative period in November; a resting period in January; an early-resumptive period in March; and a late resumptive period in April. Komatsu [11] reported that testis size and weight in bears over 3 years old increased from January to April, and decreased from November to December. In the present study, spermatogenenic activity in bears over 3 years peaked from April to July, degenerated from August to November, and exhibited a baseline from December to February.

In this study, testis size in bears over 4 years of age hardly

Table 1. Immunolocalization of each steroidogenic enzyme in the testes of wild Japanese black bears

Date of collection Bear No. Age (year)[a]	P450scc	3β-HSD	P450c17	P450arom
8. Jun No. 9 4	Leydig cell Sertoli cell Spermatocyte	Leydig cell	Leydig cell Spermatocyte Spermatid	Leydig cell Sertoli cell Spermatid
1. Jul No. 11 3	Leydig cell Spermatogonia	Leydig cell	Leydig cell	Leydig cell Spermatogonia
8. Jul No. 12 5	Leydig cell Sertoli cell	Leydig cell	Leydig cell Spermatocyte Spermatid	Leydig cell Sertoli cell Spermatid
9. Aug No. 8 6	Leydig cell Sertoli cell	Leydig cell	Leydig cell Spermatocyte Spermatid	Leydig cell Sertoli cell Spermatid
13. Sep No. 14 3	Leydig cell Sertoli cell	Leydig cell	Leydig cell Spermatocyte Spermatid	Leydig cell

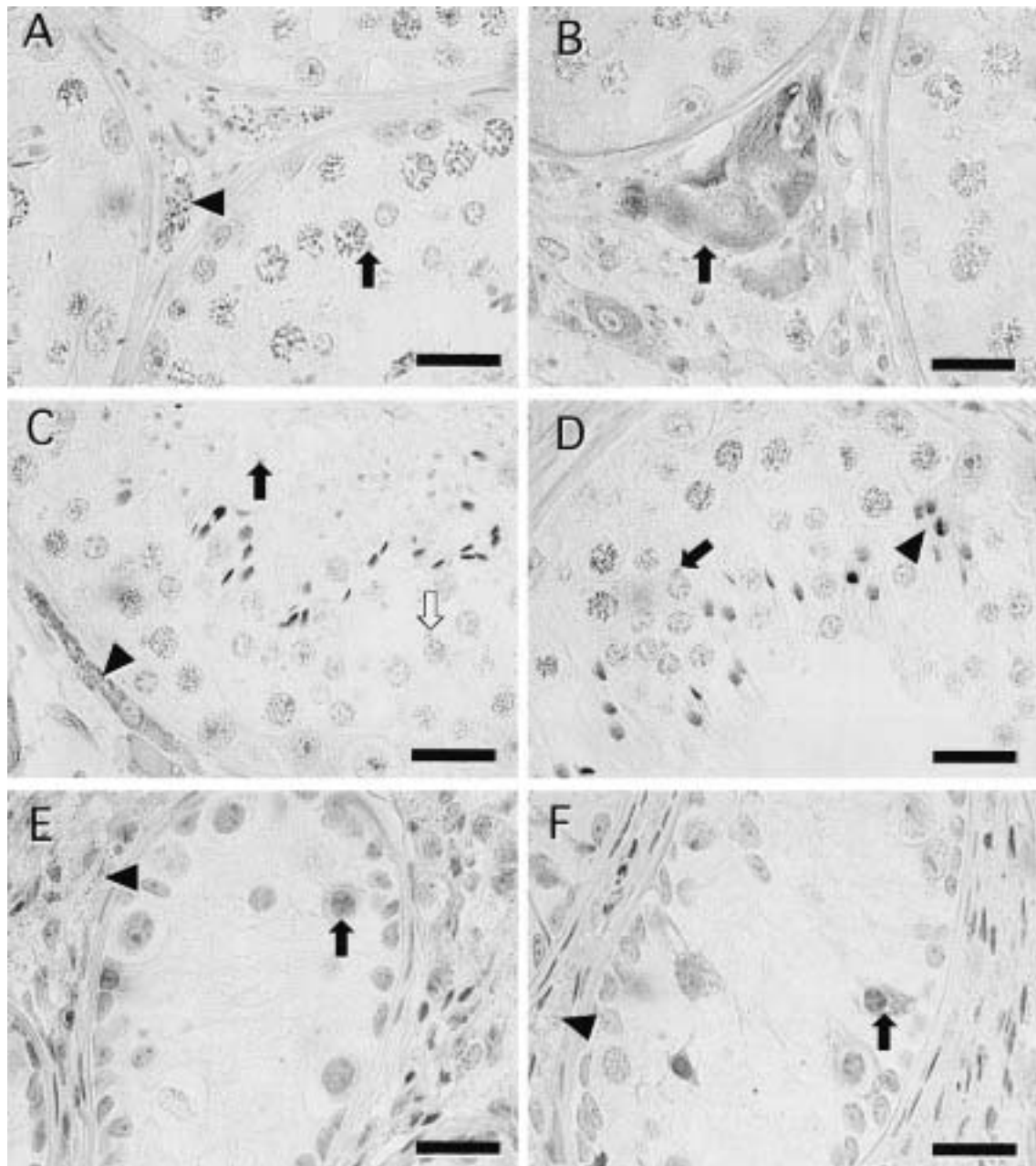a) Age was estimated by dental examination.

Fig. 4. Immunolocalization of steroidogenic enzymes in the testes of mature and immature Japanese black bears. Bar, 20 μm. A. Cholesterol side-chain cleavage cytochrome P450 (P450scc), June, mature. Leydig cells (arrow head), Golgi region of spermatocytes (arrow) and Sertoli cells are stained positively. B. 3β-hydroxysteroid dehydrogenase (3βHSD), June, mature (same bear as in A). Leydig cells (arrow) are stained positively. C. 17-α hydroxylase cytochrome P450 (P450c17), June, mature (same bear as in A). Leydig cells (arrow head), body adjoined spermatids (arrow) and Golgi region of spermatocytes (white arrow) are stained positively. D. Aromatase cytochrome P450 (P450arom), June, mature (same bear as in A). Golgi region of round spermatids (arrow), areas surrounding the nuclei of elongating spermatids (arrow head) and Sertoli cells are stained positively. E. P450scc, July, immature. Leydig cells (arrow head) and Spermatogonia (arrow) are stained positively. F. P450arom, July, immature (same bear as in E). Leydig cells (arrow head) and Spermatogonia (arrow) are stained positively.

changed from June to August. Komatsu [11] reported that the testis size and weight in wild Japanese black bears, when examined during their non-mating season, changed season-ally. Based on the data from the above references and the present study, the annual changes in testis size appear to be as follows: testis size increases from January to April, peaks

in May or June, hardly changes at all from June to August, and decreases from September to December. Testis size in the mating season reach about twice that in the non-mating season in black and brown bears [4, 5]. Testis size of captive brown bears exhibited high values from March to August, but low values from September to February [24].

In Ursidae, serum testosterone concentrations of male bears were low during the non-mating season, and peaked shortly before the mating season [9, 15, 20, 24]. Komatsu *et al.* [13] reported that serum testosterone concentrations in captive male Japanese black bears differed seasonally accompanied by differences in spermatogenic activity, with baseline levels (0.02–0.17 *ng/ml*) in November and January, rising levels (0.24–0.98 *ng/ml*) in March and April, and high levels (1.7–4.3 *ng/ml*) in May, and in April and June of the next year. Kojima *et al.* [10] found that serum testosterone concentrations in captive male Japanese black bears in the mating season (May-July) averaged 0.86 *ng/ml*, and were 0.72–1.49 in May, 0.82–2.76 in June, and 0.07–2.64 in July. Thus previous studies showed higher levels of blood testosterone around June compared with other periods during the year. In this study, serum testosterone concentrations of some bears were relatively high (> 0.5 *ng/ml*) in June and July, whereas those of others were low (< 0.5 *ng/ml*) in August-November even in bears judged to be mature and, although no peak was apparent, testosterone levels tended to decrease gradually from June to November. These results were in part in agreement with the general changes noted in other reports.

Sex steroid hormone synthesis: In the testes of mammals, Leydig cells are the main synthetic site for androgen, a sex steroid hormone for males; and the activities of some steroidogenic enzymes have been detected in the Leydig cells of various animals [6]. For example, Leydig cells contain P450scc, 3βHSD, and P450c17 in the brown bear [28], rat [19], mouse [18] and human [7].

As a result of immunohistochemical observation, P450scc, 3βHSD and P450c17 were detected in the Leydig cells of all the bears in this study. This strongly suggests that testosterone is synthesized in the Leydig cells of the Japanese black bear. Komatsu *et al.* [13] detected P450scc, 3βHSD, and P450c17 in the Leydig cells of captive Japanese black bears throughout the year, a result corroborated by the present study. However, in our study, these enzymes existed in germ cells and Sertoli cells as well as in the Leydig cells, which suggests that these cells have the ability to synthesize androgen. The physiological role of androgen for each cell is unknown, and further research is required.

In the present investigation, P450arom was detected in the Sertoli cells of all bears except No. 14 (September), as well as in the Leydig cells of all bears. Similar to our findings, Komatsu *et al.* [13] detected P450arom in the Leydig and Sertoli cells in captive Japanese black bears.

P450arom was also detected in the spermatogonia of an immature bear (No. 11), suggesting that spermatogonia may be a synthesis site of estrogen in the immature Japanese black bear. A positive reaction for P450arom in this study was detected in degenerating spermatogonia: the reaction was stronger according to the advancing stages of degeneration. In general, degenerated cells tend to show non-specific reactions and this possibility cannot be ruled out in this study. There is no report of P450arom activity in the spermatogonia except in the northern fur seal [27]. But as seen in the study by Komatsu *et al.* [13], our investigation found P450arom in the spermatids of mature bears, suggesting that spermatids may produce estrogen in Japanese black bears. In rodents in which the localization of estrogen receptor is well known, estrogen receptor α (ERα) and/or β (ERβ) were detected in spermatogonia (ERβ), pachytene spermatocytes (ERα and ERβ), round spermatids (ERα and ERβ) and spermatozoa (ERα and ERβ; not determined) [1]. It is thought that spermatogenesis is partially regulated by estrogen with the respect to the stem germ cell number and the spermatid maturation [1]. The physiological role of the estrogen potentially produced by spermatogonia as well as spermatids should be clarified in mammals, including bears, in the future.

In conclusion, the age of sexual maturation in wild male Japanese black bears was estimated to be 3–4 years. Spermatogenesis was obviously active in June, July and August, and degenerated in September. Leydig cells, Sertoli cells and germ cells have the capability of synthesizing androgen, while Leydig cells, Sertoli cells, spermatids and spermatogonia can synthesize estrogen in Japanese black bears.

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