Seasonal Changes in the Immunolocalization of Steroidogenic Enzymes in the Testes of the Japanese Black Bear (*Ursus thibetanus japonicus*)

Takeshi KOMATSU, Toshio TSUBOTA[1)], Yoshio YAMAMOTO[2)], Yasuro ATOJI[2)], and Yoshitaka SUZUKI[2)]

United Graduate School of Veterinary Science, [1)]Laboratories of Theriogenology and [2)]Veterinary Anatomy, Faculty of Agriculture, Gifu University, Gifu 501–11, Japan

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ABSTRACT. Seasonal changes in sites of immunostaining of steroidogenic enzymes were examined in testes of the Japanese black bear, *Ursus thibetanus japonicus*. In addition, serum concentrations of testosterone and estradiol-17β were investigated by radioimmunoassay, and the seasonal changes were compared with the results of immunostaining. On the basis of morphological observations of spermatogenic activity, the reproductive cycle was divided 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. Serum concentrations of testosterone differed with season accompanied by differences in spermatogenic activity, with baseline levels in November and January, increasing levels in March and April, and high levels in May, and April and June of the next year. Immunoreactivities specific for cholesterol side-chain cleaving cytochrome P450, 17α-hydroxylase cytochrome P450 and 3β-hydroxysteroid dehydrogenase (3βHSD) were observed in Leydig cells throughout the year. Only the percentages of Leydig cells immunopositive for 3βHSD exhibited seasonal differences that correlated with serum concentrations of testosterone. Aromatase cytochrome P450 (P450arom) was immunolocalized in Leydig and Sertoli cells throughout the year, in spermatids in May, and April and June of the next year and in myoid cells in January and March. The percentages of Leydig cells immunopositive for this enzyme increased in May, and January, March and June of the next year. On the other hand, no pattern of seasonal change in serum estradiol-17β concentration was observed. These results suggest that 3βHSD is a key enzyme in the regulation of the testosterone production in Leydig cells. Furthermore, estrogen derived from Leydig and myoid cells seems to play a role in the regulation of Leydig cells by negative feedback as a paracrine and/or autocrine mediator. — KEY WORDS: bear testis, seasonal change, spermatogenesis, steroidogenic enzyme, testosterone.

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The testis of seasonal breeders including bear species exhibits morphological differences throughout the year [6, 7, 11, 14, 19]. It has been reported that spermatogenic activity exhibited seasonal changes accompanied by changes in peripheral concentrations of testosterone [11, 12, 14, 19, 20, 24, 38]. These findings strongly suggest the occurrence of seasonal changes in testicular steroidogenesis in seasonal breeders such as bears.

The Japanese black bear, *Ursus thibetanus japonicus*, is a seasonal breeder which mates in early summer. It has been reported that cellular associations of the seminiferous epithelium reflect eight stages during the mating season [16], while seminiferous tubules contain almost totally undifferentiated spermatogonia and Sertoli cells during the non-mating season [15]. However, seasonal changes in testicular morphology and endocrinology have not been demonstrated in this species.

It has been well documented that Leydig cells produce testosterone [9]. In the first step in the production of testosterone, cholesterol side-chain cleaving cytochrome P450 (P450scc) converts cholesterol to pregnenolone. Then 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (3βHSD) and 17α-hydroxylase/C_{17-20} lyase cytochrome P450 (P450c17) convert pregnenolone to progesterone/androstenedione and dehydroepiandrosterone/androstenedione through both the Δ^4 and the Δ^5 pathway, respectively. In the final step, 17-ketosteroid reductase converts androstenedione to testosterone. It has been reported previously that 3βHSD is a key enzyme in the regulation of the production of testosterone [18, 21, 22, 31, 35]. By contrast, there is the assertion that P450c17 is the predominant regulator of testosterone production [2, 26, 27, 42]. Thus, studies on the regulation of androgen synthesis in Leydig cells remain controversial.

Small quantities of estrogen are produced in the testes of some mammalian species [8]. Aromatase cytochrome P450 (P450arom) is the enzyme that converts androgen to estrogen. It has been reported that the interstitial tissues in the testes of adult human [25] and rat [36] and the Sertoli cell in the testes of prepubertal rat [5, 29, 30, 36] are a site of estrogen synthesis. However, there are few reports on the intratesticular synthesis of estrogen in other mammals.

Therefore, the purpose of the present study is to analyze the mechanisms that regulate spermatogenesis and testicular steroidogenesis by immunocytochemical and endocrinological observations in the Japanese black bear, a typical seasonal breeder.

MATERIALS AND METHODS

Animals: Three adult male Japanese black bears, kept at the Animatagi Bear Park (Akita, Japan), were used for this study. The bears (bear number 43, 44 and 47) were 7, 5 and 7 years old in May of 1994, respectively. The facility is located in the northern part of Japan (N 40°, E 140.4°) and consists of an outdoor run where the animals can

exercise freely and a den connected to the run. Thus, the bears that we studied experienced natural changes in day length and temperature throughout the year. Food and water were provided except when the bears were hybernating in their den during the winter. The bears remained in the den from December 17, 1994, to April 14, 1995. In this park, the bears mate from late June until early August (M. Suzuki, personal comm.).

Materials: For histological and immunocytochemical investigations, testicular biopsies were performed, with the three bears under general anesthetic, in May and November of 1994, and in January, March, April and June of 1995. Anesthesia was achieved either by the intramuscular administration of xylazine hydrochloride (1.0 mg/kg) and ketamine hydrochloride (15.0 mg/kg), or by the intramuscular administration of medetomidine hydrochloride (0.04 mg/kg) and ketamine hydrochloride (5.0 mg/kg) after intramuscular administration of atropine (0.025 mg/kg). Biopsied tissues were immediately fixed for 13 hr in Bouin's solution. To measure serum concentrations of testosterone and estradiol-17β, blood was taken from the jugular vein in the same months as those when biopsies were performed. The blood was centrifuged for 10 min to separate the serum from the hematocytes. The serum was stored at −80°C prior to assays.

Histology and immunocytochemistry: The biopsied specimens were dehydrated in an ethanol series and embedded in paraffin. Serial sections (4 μm) were mounted on slides coated with poly-L-lysine (Sigma, St. Louis, MO, U.S.A.). Some sections were stained with hematoxylin-eosin (HE) for observations of general histology. Adjacent sections were used for immunocytochemical staining with antibodies against four steroidogenic enzymes; P450scc, 3βHSD, P450c17 and P450arom. Deparaffinized sections were incubated with methanol that contained 0.3% H_2O_2 for 20 min to block endogenous peroxidase. Then the specimens were washed in 0.01 M phosphate buffered-saline (PBS) for 15 min and treated with 10% normal goat serum for 10 min. The sections were treated with polyclonal rabbit antibodies for 16 hr at 4°C. Characteristics and dilutions of the primary antibodies are shown in Table 1. After washing in PBS for 25 min, the sections were incubated with biotinylated antibodies raised in goat against rabbit immunoglobulin (1:50; Sigma) for 1 hr and then with ExtrAvidin peroxidase (1:50; Sigma) for 40 min at room temperature with washing in PBS for 25 min between the incubations. A final wash in PBS for 20 min was followed by immersion of the sections for 5 min in 0.05 M Tris-HCl (pH 7.4) that contained 0.56 mM 3,3'-diaminobenzidine and 0.005% H_2O_2. The sections were finally counterstained with hematoxylin and sealed under coverslips. For negative controls, either non-immune rabbit serum (1:500) or antibodies (anti-P450scc, anti-P450c17, anti-P450arom; see Table 1) that had been adsorbed with an appropriate amount of the respective purified antigen were used instead of the primary antibodies.

All Leydig cells that had immunoreacted with antibodies against P450scc, 3βHSD, P450c17 and P450arom were counted on each section. The percentage of immunopositive cells relative to the total number (range for total number in each section: 54–1010) of Leydig cells was calculated. The statistical significance of monthly differences was examined by Friedman's test.

Radioimmunoassays: Serum samples for assays of testosterone and estradiol-17β were double-extracted with diethyl ether and with a solution of hexane and 75% methanol [3].

The antibodies against testosterone (HAC-AA61-02-RBP81) were obtained from the Institute of Endocrinology, Gunma University (Maebashi, Japan). They were raised against a conjugate of testosterone, 3-carboxymethyloxime, and bovine serum albumin (testosterone-3-CMO-BSA). They cross-reacted 100% with 5α-dihydrotestosterone, 0.001% with progesterone and 0.003% with estradiol-17β. The antibodies against estradiol-17β (FD-121) were obtained from Medical System Service Kanagawa, Teikokuzoki (Kawasaki, Japan). They were raised against estradiol-6-CMO-BSA and cross-reacted 3.2% with estrone, 1.77% with estriol, 0.29% with testosterone and <0.08% with progesterone. Concentrations of testosterone and estradiol-17β were measured by a previously reported procedure [3] with slight modifications. We used Ready Safe™ (Beckman, Tokyo, Japan) as scintillation fluid. The interassay and intraassay coefficients of variation were 15.7% and 17.9%

Table 1. Characteristics and dilutions of primary antibodies

Antibody	Antigen	Dilution	Reference
Anti-cholesterol side-chain cleaving cytochrome P450 (P450scc)	Bovine adrenal P450scc	1:500	[13]
Anti-3β-hydroxysteroid dehydrogenase/isomerase (3βHSD)	Human placental 3βHSD	1:2,000	[4]
Anti-17α-hydroxylase/C_{17-20} lyase cytochrome P450 (P450c17)	Guinea-pig adrenal P450c17	1:500	[32]
Anti-aromatase cytochrome P450 (P450arom)	Human placental P450arom	1:500	[10]

All antisera were raised in rabbits.

for testosterone and 23.2% and 25.3% for estradiol-17β, respectively. The sensitivity of the assays for testosterone and estradiol-17β was 14.4 *p*g/m*l* and 6.4 *p*g/m*l*, respectively. The statistical significance of monthly differences was examined by Friedman's test.

RESULTS

Histology of testes: The testes contained germ cells at various stages of development (Fig. 1). In May, the seminiferous tubules contained populations of germ cells from spermatogonia to spermatozoa (Fig. 1a). In November, seminiferous tubules had a small diameter and contained Sertoli cells and spermatogonia, while luminal spaces were occupied by degenerating germ cells including spermatocytes (Fig. 1b). Spermatogonia were distinguished as undifferentiated or differentiated based on characteristics of their nuclei as previously reported by us [15, 16]; i.e., the former had a large pale nucleus and the latter a small dark nucleus. In January, only Sertoli cells and undifferentiated spermatogonia were found, although differentiated spermatogonia and degenerating germ cell masses were occasionally observed in tubules (Fig. 1c). In March, degenerating germ cell masses, Sertoli cells and spermatogonia were observed in tubules, as in November (Fig. 1d). However, the number of differentiated spermatogonia with a dark nucleus increased relative to January. In April, although round and elongating spermatids were present, no lumen was observed in any seminiferous tubule (Fig. 1e). In June, as in May of the previous year, active spermatogenesis was evident in the testes (Fig. 1f).

In May (Fig. 1a), April (Fig. 1e) and June (Fig. 1f), the Sertoli cells were tall and thin cytoplasm was slightly observed among the germ cells. However, seasonal differences in Sertoli cells were not evident by light microscopic observation.

In May (Fig. 1a) and June (Fig. 1f), the most prominent cells were Leydig cells in the interstitial connective tissues. Each Leydig cell had a large round nucleus and large cytoplasm that stained with eosin. In November (Fig. 1b), January (Fig. 1c), March (Fig. 1d) and April (Fig. 1e), the nucleus in each Leydig cell was small and contained condensed chromatin. Furthermore, connective tissues were more evident than in May and June.

Immunolocalization of steroidogenic enzymes: No immunostaining was detected in control sections that had been incubated with non-immune serum. Preabsorbed primary antibodies also did not immunostain any cells, with the exception that spermatocytes remained immunopositive for P450c17. Immunoreactivity specific for P450scc was observed in a granular pattern in the cytoplasm of Leydig cells at all times of the year (Fig. 2a). 3βHSD (Fig. 3) and P450c17 (Fig. 2b) were uniformly immunostained in the cytoplasm of Leydig cells at all times of the year. The percentage of immunopositive cells for P450scc and P450c17 was high throughout the year (Table 2). The percentage of immunopositive cells for 3βHSD decreased from November to March, and increased from April to June (Fig. 3, Table 2). These percentages were significantly different ($P<0.02$) in terms of month (n=3). P450arom was immunoreactive in the cytoplasm of spermatids in May (Fig. 2c), April and June, but was not observed in November, January and March (Fig. 2d) when spermatids were absent from the seminiferous tubules. By contrast, this enzyme was immunostained in myoid cells only in January and March (Fig. 2d). P450arom was also weakly immunoreactive in the cytoplasm of some Leydig and Sertoli cells at all times of the year. The percentages of immunopositive cells for P450arom were not significantly different in terms of month (Table 2). However, these percentages tended to increase during the active (May and June) and early-resumptive period (March).

Serum concentrations of testosterone and estradiol-17β: Seasonal changes in serum concentrations of testosterone and estradiol-17β are shown in Table 3. Serum concentrations of testosterone were significantly different ($P<0.05$) in terms of month (n=3). Serum testosterone levels were high in May, April and June (range for the three bears: 1,734.7–4,270.5 *p*g/m*l*, >40.2%) and low during November and January (20.0–171.2 *p*g/m*l*, <2.8%). Levels began to increase in March and April (239.3–977.1 *p*g/m*l*, 4.9–23.2%). Serum concentrations of estradiol-17β did not exhibit seasonal changes (12.2–26.5 *p*g/m*l*).

Relationship between immunoreactivity and steroid concentration: Relationships between the percentages of immunoreactivities for the steroidogenic enzymes and the serum steroid concentration of number 43 bear are shown in Figs. 4 and 5. Seasonal differences in serum testosterone concentrations were correlated with that in percentages of immunopositive cells for 3βHSD but not P450scc and P450c17 (Fig. 4). In Fig. 5, P450arom was immunostained in myoid cells in January and March. The percentages of immunopositive Leydig cells for this enzyme increased in May, and January, March and June of the next year. However, no pattern of seasonal change in serum estradiol-17β concentration was observed.

DISCUSSION

Seasonal changes in the morphology of the testes and in serum concentrations of testosterone: Testes of the Ursidae, including those of the brown bear *Ursus arctos*, and the American black bear *Ursus americanus*, exhibit morphological and endocrinological differences throughout the year [12, 20, 24, 38]. Testis size and peripheral concentrations of testosterone are low in the non-mating season, increase in the pre-mating season, and reach maximum just before the mating season [12, 20, 24, 38]. In the present study, the spermatogenic activity differed with season accompanied by differences in serum concentrations of testosterone similar to other bear species; i.e., active in May and June, reduced in November and January, and resumptive in March and April. On the basis of these observations of spermatogenic activity, we defined the active

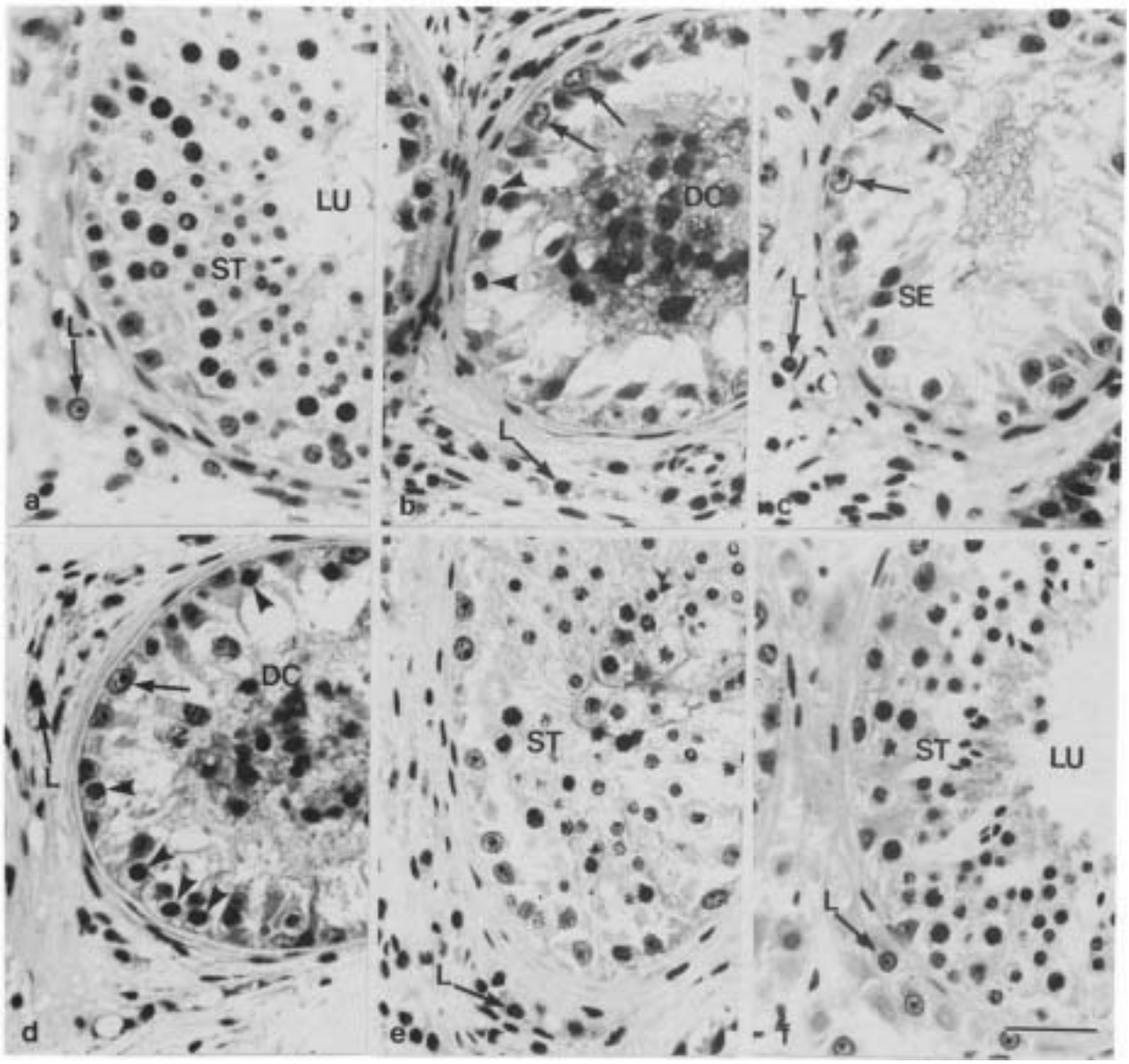

Fig. 1. General histology of the testis of the Japanese black bear during each season. (a) Seminiferous tubules contain germ cells ranging from spermatogonia to spermatids, and interstitial tissues are occupied by numerous Leydig cells, each of which has a large round nucleus in May. (b) Seminiferous tubules have a small diameter and contain the degenerating germ cells including spermatocytes, and undifferentiated (arrows) and differentiated (arrowheads) spermatogonia, and the nucleus in each Leydig cell is small and contains condensed chromatin in November. (c) Seminiferous tubules contain mostly Sertoli cells and undifferentiated spermatogonia (arrows) in January. (d) Seminiferous tubules contain degenerating germ cells including spermatocytes, and undifferentiated (arrows) and differentiated (arrowheads) spermatogonia in March. Differentiated spermatogonia increase in number relative to January. (e) Although round and elongating spermatids are present, no lumen is observed in any seminiferous tubule in April. (f) Active spermatogenesis occurs in the testes, and interstitial tissues are occupied by numerous Leydig cells in June, as in May of the previous year. LU, Lumen; ST, seminiferous tubule; L, Leydig cell; DC, degenerating spermatocyte; SE, Sertoli cell. Bar, 30 μm.

period as May and June, the degenerative period as November, the resting period as January, the early-resumptive period as March and the late-resumptive period as April, respectively (see Table 2).

Synthesis of androgen: Leydig cells produce testosterone from its precursors by the action of specific enzymes (see review, Hall [9]). In the testes of the American black bear, P450scc, 3βHSD and P450c17 were immunolocalized in Leydig cells throughout the year [37]. In the present study of Japanese black bears, these enzymes were present in Leydig cells at all times, as they are in the American black bear. However, there were seasonal differences in the number of immunopositive cells for 3βHSD. In interstitial tissues or Leydig cells of experimental animals such as the

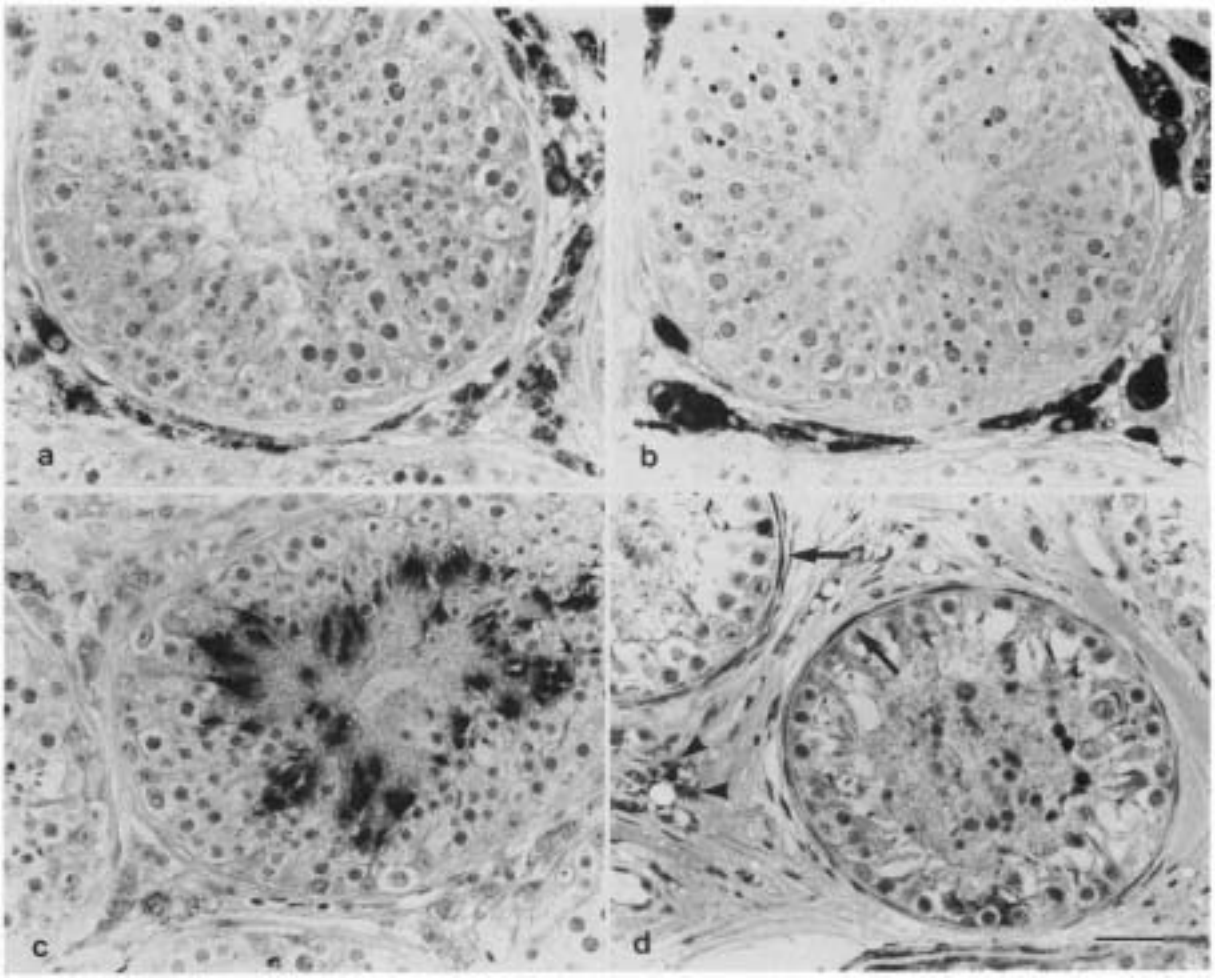

Fig. 2. Immunoreactivity specific for P450scc is distributed in a granular pattern in Leydig cells in May (a). Immunoreactivity specific for P450c17 is uniformly distributed in Leydig cells and non-specific reactions are observed in spermatocytes in May (b). Immunoreactivity specific for P450arom is detected in spermatids and Leydig cells in May (c) and is also observed in myoid (arrows) and Leydig (arrowheads) cells in March (d). Bar, 30 μm.

rat and hamster, the activity of 3βHSD was reduced by hypophysectomy or photoinhibition, and was restored by injection with hCG, LH alone or in combination with FSH *in vivo* [18, 21, 22, 31]. These findings suggest that 3βHSD is the predominant regulator of testosterone production in Leydig cells [18, 21, 22, 31]. In the present study, seasonal differences in serum testosterone concentrations correlated with those in percentages of immunopositive cells for 3βHSD (see Fig. 4). Furthermore, no seasonal differences in percentages of immunopositive cells for P450scc and P450c17 were observed. These findings suggest that 3βHSD is a key enzyme for the regulation of the production of testosterone in Leydig cells of the Japanese black bear.

Synthesis of estrogen: In the present study, immunoreactivity specific for P450arom was observed in Leydig and Sertoli cells throughout the year, in spermatids in May, and April and June of the next year, and in myoid cells in January and March (see Fig. 5). Aromatase activity has been observed in the Leydig cells of adult and in the Sertoli cells of prepubertal rats [5, 29, 30, 36]. In adult American black bears, immunoreactivity specific for P450arom was observed in Leydig and Sertoli cells of the spermatogenically active testis, and this enzyme was intensively immunostained in Sertoli cells during periods when spermatogenic activity had resumed [37]. However, in the present study, immunostaining for P450arom was weak and did not exhibit a seasonal difference in Sertoli cells.

It has been previously reported that estrogen dose not stimulate myoid cells to synthesize testicular paracrine factor PModS and Sertoli cells to synthesize androgen binding protein (ABP) *in vitro* [33, 34]. Other studies currently suggest that estrogen inhibits Leydig cell number or volume and secretion of LH and testosterone [1, 28, 40]. In the present study, P450arom was strongly immunostained in myoid cells in January and March, when serum concentrations of testosterone were low or began to increase (see Figs. 4 and 5). Furthermore, the percentages of

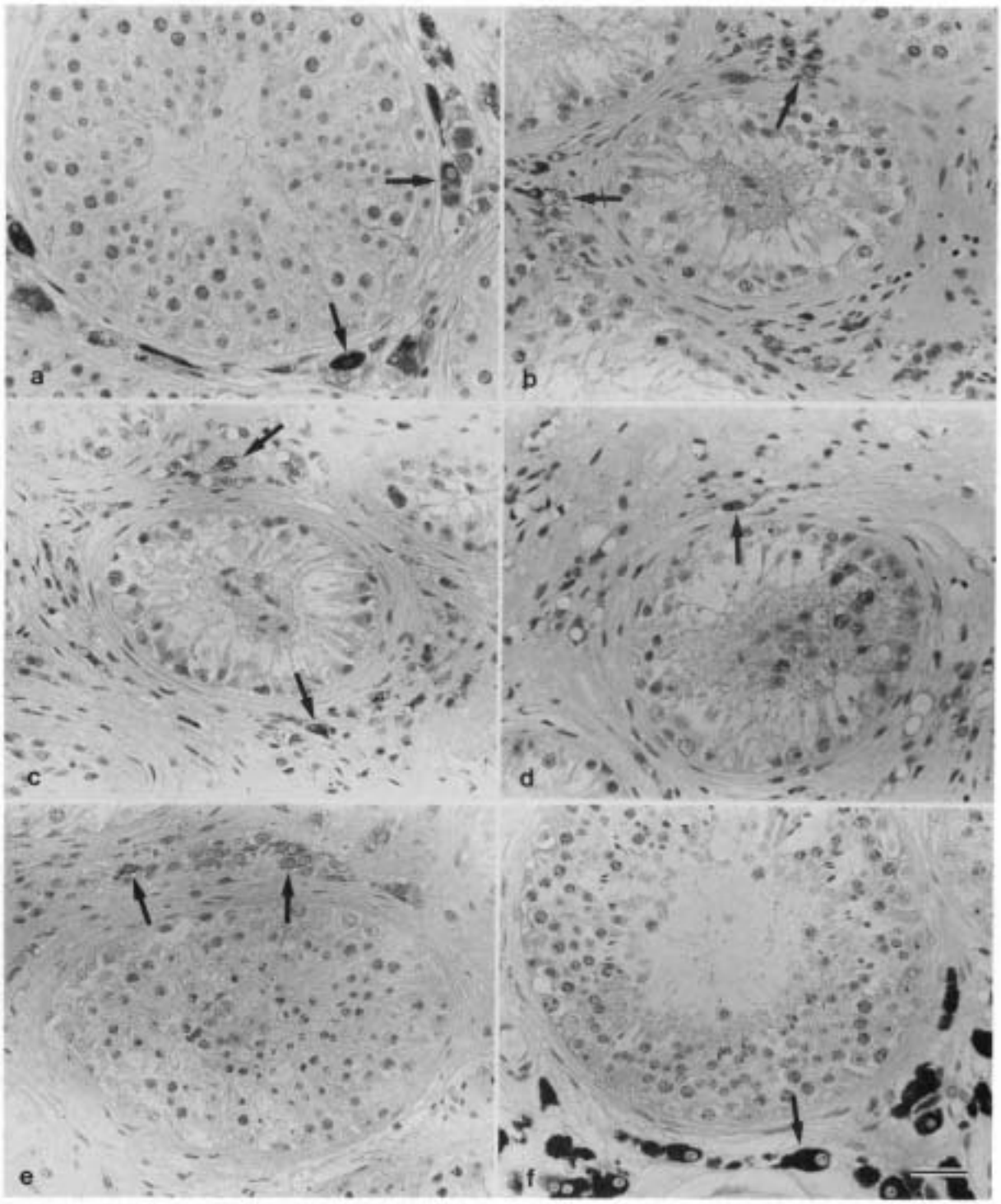

Fig. 3. Immunoreactivity specific for 3βHSD during each season. Immunoreactivity for 3βHSD is uniformly distributed in almost all Leydig cells in May (a) and June (f). Although the profile of immunoreactivity is similar at all times, the number of immunoreactive cells decreases during the non-mating season, namely, November (b), January (c), March (d) and April (e). Arrows indicate Leydig cells that are immunopositive for 3βHSD. Bar, 30 μm.

immunopositive Leydig cells for this enzyme increased in May, and January, March and June of the next year. These findings suggest that the Leydig and myoid cell-derived estrogen provides negative feedback to the Leydig cell but not myoid or Sertoli cells. However, no pattern of seasonal change in serum estradiol-17β concentration was observed.

Table 2. Seasonal changes in percentages of Leydig cells that were immunopositive for P450scc, 3βHSD, P450c17 and P450arom

Behavior		Pre-mating	Non-mating	Denning		Pre-mating	Mating
Sampling period		'94, May	Nov	'95, Jan	March	April	June
Spermatogenic activity[a)]		Active	Degenerative	Resting	Early-resumptive	Late-resumptive	Active
Percentage of immuno-positive cells (%)[b)]							
P450scc							
Bear number	43 (Age: 7)	92.4 (607)[c)]	91.6 (762)	90.1 (253)	92.9 (423)	88.5 (312)	95.6 (1,002)
	44 (Age: 5)	90.3 (400)	92.3 (690)	93.7 (543)	52.5 (544)	89.5 (152)	86.2 (442)
	47 (Age: 7)	91.6 (535)	95.3 (533)	95.8 (457)	91.8 (366)	86.2 (275)	85.9 (489)
3βHSD							
Bear number	43	70.2 (416)	61.5 (257)	7.9 (190)	5.5 (236)	30.1 (133)	85.8 (260)
	44	56.6 (249)	28.5 (361)	15.4 (356)	9.6 (240)	24.1 (54)	76.5 (358)
	47	67.2 (119)	41.8 (239)	30.4 (289)	3.8 (130)	17.6 (176)	76.6 (261)
P450c17							
Bear number	43	94.0 (700)	94.2 (703)	77.3 (317)	83.2 (471)	86.0 (292)	93.0 (1,007)
	44	93.7 (397)	89.4 (710)	88.0 (675)	92.2 (677)	83.4 (157)	90.4 (571)
	47	94.2 (468)	88.9 (523)	92.0 (613)	83.8 (400)	90.2 (254)	81.8 (456)
P450arom							
Bear number	43	93.4 (467)	15.1 (259)	32.1 (156)	51.5 (266)	24.0 (154)	12.3 (1,010)
	44	78.5 (260)	35.0 (406)	65.8 (653)	78.5 (489)	29.4 (85)	26.5 (298)
	47	36.4 (113)	13.5 (237)	47.9 (353)	78.7 (197)	11.2 (143)	46.6 (234)

a) All ocation of spermatogenic activity: see Discussion.
b) Statistical significance of monthly differences was tested by Friedman's test. Percentages of cells immunopositive for 3βHSD are significantly different ($P<0.02$) in terms of month (n=3). Percentages of cells immunopositive for P450scc, P450c17 and P450arom do not differ in terms of month (n=3).
c) The total number of Leydig cells is shown in parentheses.

Table 3. Seasonal changes in the serum concentrations of testosterone and estradiol-17β

Behavior		Pre-mating	Non-mating	Denning		Pre-mating	Mating
Sampling period		'94, May	Nov	'95, Jan	March	April	June
Spermatogenic activity[a)]		Active	Degenerative	Resting	Early-resumptive	Late-resumptive	Active
Testosterone concentration *p*g/m*l* (%)[b)]							
Bear number	43 (Age: 7)	2,456.1 (77.2)[c)]	171.2 (2.4)	97.4 (0)	129.2 (1.0)	638.3 (17.7)	3,152.1 (100)
	44 (Age: 5)	161.7 (3.4)	20.0 (0)	136.0 (2.8)	977.1 (23.2)	4,140.5 (100)	1,024.6 (24.4)
	47 (Age: 7)	1,734.7 (40.2)	29.8 (0)	43.2 (0.3)	239.3 (4.9)	2,070.3 (48.1)	4,270.5 (100)
Estradiol-17β concentratin *p*g/m*l* (%)[b)]							
Bear number	43	20.0 (0)[c)]	20.2 (3.7)	25.1 (94.4)	25.4 (100)	20.1 (1.9)	23.8 (70.4)
	44	15.4 (22.4)	18.9 (46.9)	26.5 (100)	14.7 (17.5)	12.2 (0)	15.5 (23.1)
	47	21.2 (70.4)	20.6 (62.0)	16.2 (0)	23.3 (100)	18.4 (31.0)	17.2 (14.1)

a) Allocation of spermatogenic activity: see Discussion.
b) Statistical significance of monthly differences was tested by Friedman's test. Serum concentrations of testosterone are significantly different ($P<0.05$) in terms of month (n=3). Serum concentrations of estradiol-17β do not differ in terms of month (n=3).
c) The percentage of values I in individual bears are given in parentheses; maximum value 100%.

This result suggests that estrogen acts as a paracrine and/or autocrine rather than an endocrine mediator.

The present study demonstrated the localization of P450arom in spermatids in May, and April and June of the next year. It has been previously reported that developing spermatids and epididymal sperm contain P450arom [17, 23, 37, 39]. In addition, it has been reported that estrogen receptors are present in the epididymis of the dog and monkey [41, 43]. These studies suggest that estrogen in seminiferous tubules plays a role in the maturation of sperm

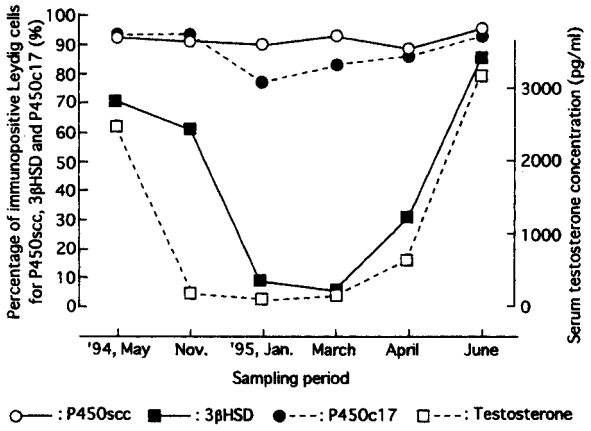

Fig. 4. Seasonal changes in the percentage of Leydig cells that were immunopositive for P450scc, 3βHSD and P450c17 and in the concentration of serum testosterone in bear number 43.

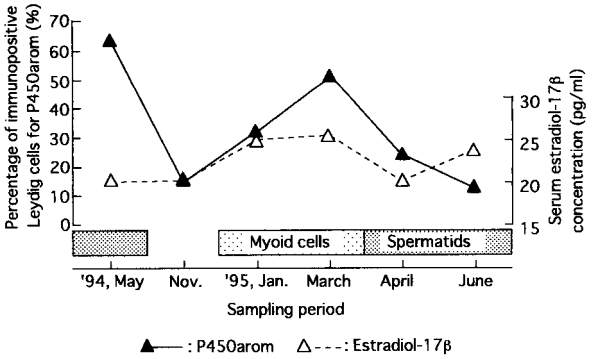

Fig. 5. Seasonal changes in the percentage of Leydig cells that were immunopositive for P450arom and in the concentration of serum estradiol-17β in bear number 43. The other cell types immunolocalized for P450arom are shown at the bottom of the figure.

in the epididymis.

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