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—Original Article—

Changes in the Immunolocalization of Steroidogenic Enzymes and the Androgen Receptor in Raccoon (*Procyon lotor*) Testes in Association with the Seasons and Spermatogenesis

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Abstract. The raccoon is a seasonal breeder with a mating season in the winter. In a previous study, adult male raccoons exhibited active spermatogenesis with high plasma testosterone concentrations, in the winter mating season. Maintenance of spermatogenesis generally requires high testosterone, which is produced by steroidogenic enzymes. However, even in the summer non-mating season, some males produce spermatozoa actively despite low plasma testosterone concentrations. To identify the factors that regulate testosterone production and contribute to differences in spermatogenetic activity in the summer non-mating season, morphological, histological and endocrinological changes in the testes of wild male raccoons should be known. In this study, to assess changes in the biosynthesis, metabolism and reactivity of testosterone, the localization and immunohistochemical staining intensity of four steroidogenic enzymes (P450scc, P450c17, 3βHSD, P450arom) and the androgen receptor (AR) were investigated using immunohistochemical methods. P450scc and P450c17 were detected in testicular tissue throughout the year. Seasonal changes in testosterone concentration were correlated with 3βHSD expression, suggesting that 3βHSD may be important in regulating the seasonality of testosterone production in raccoon testes. Immunostaining of P450arom and AR was detected in testicular tissues that exhibited active spermatogenesis in the summer, while staining was scarce in aspermatogenic testes. This suggests that spermatogenesis in the raccoon testis might be maintained by some mechanism that regulates P450arom expression in synthesizing estradiol and AR expression in controlling reactivity to testosterone.

Key words: Immunohistochemistry, Raccoons, Seasonal changes, Spermatogenesis, Steroidogenic enzymes

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Many mammalian species breed seasonally for effective reproduction. Seasonal changes in the gonads are generally controlled by the hypothalamic-pituitary-gonadal axis and directly regulated by sex steroid hormones. In many seasonal breeding mammals, males exhibit a seasonal cycle in testicular activity, with spermatogenesis and testicular steroidogenesis limited to a specific period of the year. Active spermatogenesis along with high testosterone concentrations have been detected in the mating season, and, in contrast, inactive spermatogenesis along with low testosterone concentrations have been detected in the non-mating season in many seasonal breeding males, such as Hokkaido sika deer (*Cervus nippon yesoensis*) [1], American black bears (*Ursus americanus*) [2] and Hokkaido brown bears (*U. arctos yesoensis*) [3].

The raccoon (*Procyon lotor*) is a long-day seasonal breeder [4], with mating in the winter, from January to March, in Hokkaido, Japan [5]. We previously found that adult male raccoons exhibited active spermatogenesis with high plasma testosterone concentrations in the winter mating season, as in other seasonal breeding mammals [6]. Steroidogenesis is accomplished by several steroidogenic enzymes. The immunolocalization of steroidogenic enzymes in testicular tissue has been determined in many mammals, and both localization and expression intensity have been observed to change seasonally in several seasonal breeders, such as raccoon dogs (*Nyctereutes procyonoides*) [7], Japanese black bears (*U. thibetanus japonicus*) [8] and ground squirrels (*Citellus dauricus* Brandt) [9]. The immunolocalization and the seasonal changes vary among species and have not yet been reported in raccoons. In particular, the relationship between testicular function and the role of steroidogenic enzymes remains unknown. To reveal factors that regulate testosterone production, expression sites and seasonal changes in steroidogenic enzymes should be investigated.

In a previous study, some male raccoons were found to produce spermatozoa actively despite low plasma testosterone concentrations in the summer non-mating season [6]. Maintenance of spermatogenesis generally requires high testosterone production from Leydig cells. In raccoons, there may be other mechanisms for maintaining spermatogenesis that are not coincident with peripheral testosterone concentration changes. There are at least two possible proximate factors. First, testosterone concentrations in local sites in testicular tissue may be independent of peripheral testosterone concentrations and relatively high in testes with active spermatogenesis. Second, metabolism and reactivity against testosterone in testicular tissue

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Table 1. Characteristics and dilutions of primary antibodies

Antibody	Antigen	Host	Type	Dilution	Source
Anti- cholesterol side-chain cleavage cytochrome P450 (P450scc)	Bovine	Rabbit	Polyclonal	1:1000	[36]
Anti- 3β-hydroxysteroid dehydrogenase (3βHSD)	Human	Mouse	Monoclonal	1:100	sc-100466 Santa Cruz Biotechnology, Inc, CA, USA
Anti- 17α-hydroxylase cytochrome P450 (P450c17)	Porcine	Rabbit	Polyclonal	1:1000	[37]
Anti- aromatase cytochrome P450 (P450arom)	Human	Rabbit	Polyclonal	1:100	[38]
Anti- androgen receptor (AR)	Human	Rabbit	Monoclonal	1:100	A9385 SIGMA-ALDEICH, St. Luis, USA

may vary among individuals in the summer season.

Testosterone influences spermatogenesis by binding to a specific nuclear receptor, the androgen receptor (AR). Detecting the types of cells that express the AR may assist identification of the cells that control spermatogenesis via a direct influence of testosterone. Additionally, seasonal changes in AR expression in testicular tissue have been reported in several seasonal mammals, such as bank voles (*Clethrionomys glareolus*) [10] and big fruit-eating bats (*Artibeus lituratus*) [11]. Thus, clarifying the localization and expression changes in steroidogenic enzymes and the AR can help in understanding the mechanisms that regulate spermatogenesis and testicular steroidogenesis in the raccoon testes.

In the present study, to assess changes in the biosynthesis, metabolism and reactivity of testosterone, the localization and immunohistochemical staining intensity of steroidogenic enzymes and AR were investigated according to season and spermatogenesis activity in wild raccoons. The goals of this study were to identify factors related to seasonal changes in testosterone production and differences in spermatogenetic activity in the summer non-mating season.

Materials and Methods

Sample collection

We collected carcasses of adult male raccoons that were euthanized for pest control in west-central Hokkaido from 2010 to 2013. In total, 15 raccoons were used in this study. Five were collected between February and early April, and 10 were collected in July; these 10 samples were divided into two groups according to spermatogenetic activity. Raccoon carcasses were weighed, and body length was measured; blood was obtained by cardiac puncture, and the testes were removed. Blood was placed in a 5 ml tube and centrifuged (1,050 *g*, 10 min). The plasma was removed and stored at –30 C until assayed. The testes were brought to the laboratory on ice within 1 h after euthanasia. The left testes were weighed, measured and immediately fixed for about half a day in 4% paraformaldehyde solution at 4 C for histological examination. The right testes were kept at –80 C until assayed.

All procedures were performed with methods approved by the Animal Care and Use Committee of Hokkaido University (approval no. JU13054).

Histological and immunohistochemical analyses

Testicular tissues were dehydrated through an ethanol series, embedded in paraffin wax, sectioned at 4 µm and stained with hematoxylin and eosin. From four fields of each testis section under a microscope, ten seminiferous tubules were chosen randomly. The "spermatogenetic score" (SS) was evaluated as the mean value of each seminiferous tubule chosen according to the most advanced spermatogenetic cells present [6]: 1 = spermatogonia, 2 = no cells beyond primary spermatocytes, 3 = some cells beyond secondary spermatocytes, 4 = round spermatids and 5 = elongated spermatids and/or spermatozoa. The presence of spermatozoa in the cauda epididymis was also checked.

For immunohistochemical analyses, testicular tissue was cut into 5×5-mm pieces and mounted on the same glass slide. To detect the immunohistochemical sites and expression intensity of AR and the four steroidogenic enzymes—cholesterol side-chain cleavage cytochrome P450 (P450scc), 17-α hydroxylase cytochrome P450 (P450c17), 3 β-hydroxysteroid dehydrogenase (3βHSD) and aromatase cytochrome P450 (P450arom)—sections of testicular tissue were immunostained. The primary antibody references are summarized in Table 1. The sections were activated in immunostimulatory with DakoCytomation Target Retrieval Solution (S3307, DakoCytomation, Carpinteria, CA, USA) at 105 C for 15 min and were then incubated with methanol containing 3% H_2O_2 at room temperature for 30 min. All sections were treated using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's protocol. Immunoreactivity was visualized with DAB solution (3,3'-diaminobenzidine tetrahydrochloride, 0.01 g, Tris buffer 50 ml, 20% H_2O_2 10 µl). Negative controls were treated with each normal serum or 0.01 M PBS instead of the primary antibody. The immunostaining intensity was evaluated when staining was detected strongly (++), detected (+) or scarce/absent (–).

Enzyme immunoassay

Plasma testosterone concentrations (Plasma T) were measured by applying a competitive enzyme immunoassay [12], which was performed using blood samples. Testosterone-3-CMO-HRP (FKA101, Cosmo Bio, Tokyo, Japan) was diluted 600,000-fold with assay buffer. Testosterone standard (Cayman, Ann Arbor, MI, USA) was diluted in assay buffer. Anti-testosterone serum (first antibody, FKA102-E, Cosmo Bio) was diluted 1,200,000-fold with assay buffer. Anti-rabbit γ-globulin serum (Seikagaku, Tokyo, Japan) was used as the secondary antibody. The minimum detectable level of testosterone was 4.9 pg/well, and the intra-assay coefficient of variation was 3.15%.

High-performance liquid chromatography-electrospray-ionization tandem mass spectrometry (HPLC/ESI-MS/MS)

Testosterone concentrations in testis tissue (Tissue T) were measured by applying HPLC/ESI-MS/MS [13], which was performed using tissue samples. For steroid extraction, testicular tissues were homogenized in 3 ml phosphate buffer (0.01 M, pH 7.5). Homogenate aliquots were extracted three times with 5 ml diethyl ether, and then after centrifugation (1,100 *g*, 10 min, 4 C), the supernatant was evaporated at 47 C under a gentle stream of N_2 gas. The residue was reconstituted in 200 µl of acetonitrile:water (50:50, v/v) and centrifuged (15,000 *g*, 15 min, 4 C).

Tissue T was measured using HPLC/ESI-MS/MS (HPLC with a Shimadzu LC-20 series HPLC system; ESI-MS/MS with an LCMS-8030). Extracted samples (50 µl) were injected onto a Hypersil GOLD column (Thermo Scientific, Tewksbury, MA, USA) with a temperature at 45 C. The flow rate was 0.3 ml/min. Mobile phase A was 0.1% formic acid water, and mobile phase B was methanol. The gradient conditions were set as B concentration of 50% for 0–2 min, 50–95% for 2–5 min, 95% for 5–6.5 min and 50% for 6.5–8 min. The parent/product ion pairs of *m/z* 289.2 to 97.2 (positive ion mode) were used for analysis. Mass spectrometer parameters were optimized for the strongest product ion signal intensities; the optimized Q1 PreBias, collision energy and Q3 PreBias were –14, –30 and –19 V, respectively. Other mass spectrometry parameters were as follows: nebulizing gas flow of 3 l/min, dry gas flow of 15 l/min and electro ionspray voltage of 4,500 V at an ion source temperature of 350 C.

Statistical analysis

Results were analyzed using a one-way ANOVA and Scheffe's *F* test for pair-wise comparisons between each pair of groups with Microsoft Excel 2003. SS was not subjected to a statistical analysis because the samples from July were allocated to two groups based on the SS. All values are presented as means ± SEM.

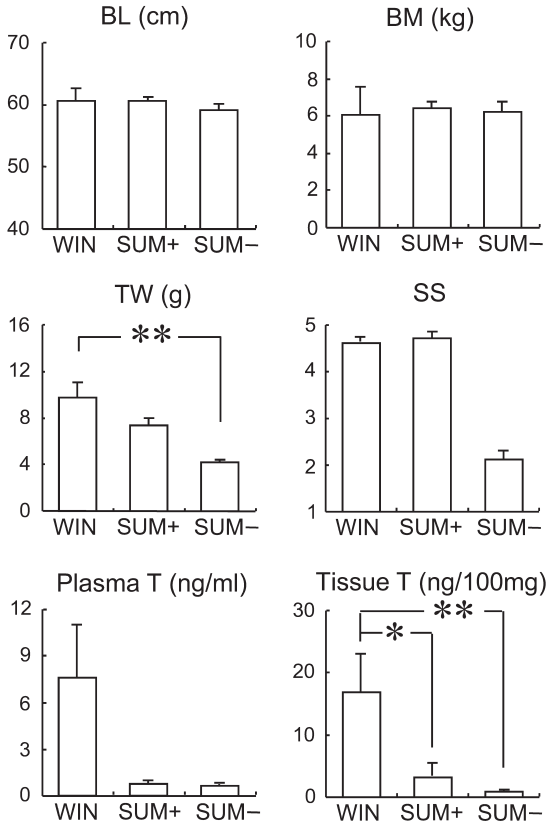

Fig. 1. Differences in body length (BL; cm), body mass (BM; kg), testis weight (TW; g), spermatogenetic score (SS), plasma testosterone concentration (Plasma T; ng/ml) and testis tissue testosterone concentration (Tissue T; ng/100 mg testis tissue) among the WIN, SUM+ and SUM– groups. *P < 0.05; **P < 0.01. The SS was not subjected to statistical analysis. All values are presented as means ± SEM.

Results

Anatomical and histological evaluations

The 15 male adult raccoons were classified into three groups according to the season and level of spermatogenesis. Group WIN contained samples from the winter (n = 5). Samples from the summer in which we observed spermatozoa in the cauda epididymis with active spermatogenesis (SS > 4) in the seminiferous tubules were classified into group SUM+ (n = 5). Samples from the summer in which we observed inactive spermatogenesis (SS ≤ 4) in the seminiferous tubules without spermatozoa in the cauda epididymis were classified into group SUM– (n = 5).

There was no significant difference in body length or body mass among the three groups (P = 0.64 and 0.95, respectively; Fig. 1). Testes weights in WIN were significantly heavier than those in SUM– (P < 0.01; Fig. 1).

Testosterone concentrations in plasma and testicular tissue

Regarding Plasma T, although there was no significant difference between any pair of two groups by Scheffe's *F* test, variability among the three groups was detected by ANOVA (P < 0.05), and the WIN value (7.57 ± 3.42 ng/ml) was higher than those of other two groups (0.76 ± 0.13 ng/ml in SUM+ and 0.62 ± 0.13 ng/ml in SUM–; Fig. 1). Regarding Tissue T, although only three fresh testes samples were collected in the winter, the WIN value (16.8 ± 6.17 ng/100 mg tissue) was significantly higher those of the other two groups (3.18 ± 2.13 ng/100 mg tissue in SUM+, P < 0.05, and 0.86 ± 0.10 ng/100 mg tissue in SUM–, *P* < 0.01; Fig. 1). There was no significant difference between the SUM+ and SUM– values.

Immunohistochemical localization and expression differences in enzymes and AR

Immunolocalization of the four steroidogenic enzymes was observed using the winter samples. Immunostaining for P450scc and P450c17 was present only in the cytoplasm of Leydig cells. 3βHSD was detected in the cytoplasm of Leydig cells and the cytoplasm in parts of Sertoli cells. Immunostaining for P450arom was detected in the cytoplasm of Leydig cells and parts of the cytoplasm in Sertoli cells and spermatids (Fig. 2). AR was detected in nuclei of Leydig cells and Sertoli cells. No immunostaining was detected in control sections (NC in Fig. 3). Immunostaining for some of the enzymes

became scarce or was absent in summer samples. Differences in immunohistochemical localization among groups are shown in Fig. 3, and immunostaining intensity is shown in Table 2. All immunopositive positions in summer samples were also detected in winter samples, in other words, there was no immunopositive site that was detected only in summer. P450scc was detected strongly in all groups. P450c17 was detected in all groups and strongly, especially in WIN and SUM+. Immunostaining of 3βHSD was detected strongly in WIN, and the intensity in SUM+ was weaker, especially in Leydig cells. Immunostaining in the cytoplasm of Sertoli cells also became weaker; however, it was still observed in some SUM+ samples. In SUM–, 3βHSD was detected in two samples, and no immunohistochemical reaction was observed in three samples. P450arom was detected in WIN and SUM+ but was weak in SUM–. Immunostaining of AR was strong in WIN and SUM+ samples and relatively weak in SUM– samples, while the intensities varied in two samples, and no immunohistochemical reaction was observed in three samples.

Discussion

In testes collected during the winter mating season, active spermatogenesis was observed with high testosterone concentrations in both plasma and testicular tissue. In the summer non-mating

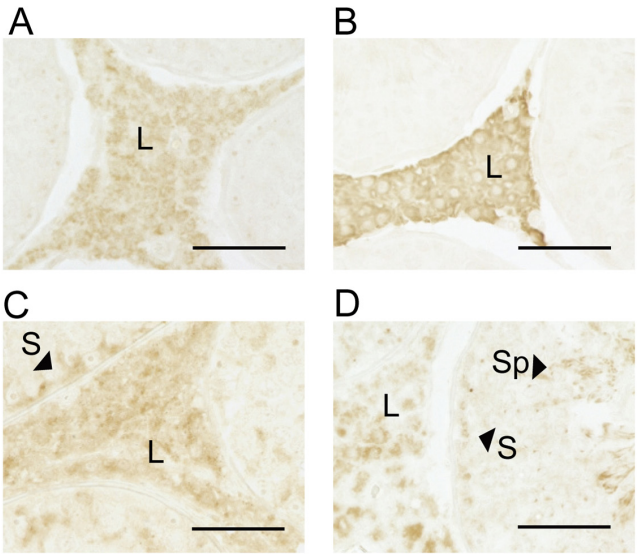

Fig. 2. Immunohistochemical localization of four steroidogenic enzymes (A, P450scc; B, P450c17; C, 3βHSD; D, P450arom) in raccoon testes in the winter mating season. L, Leydig cells; S, Sertoli cells; Sp, Spermatids. Bar=50 µm.

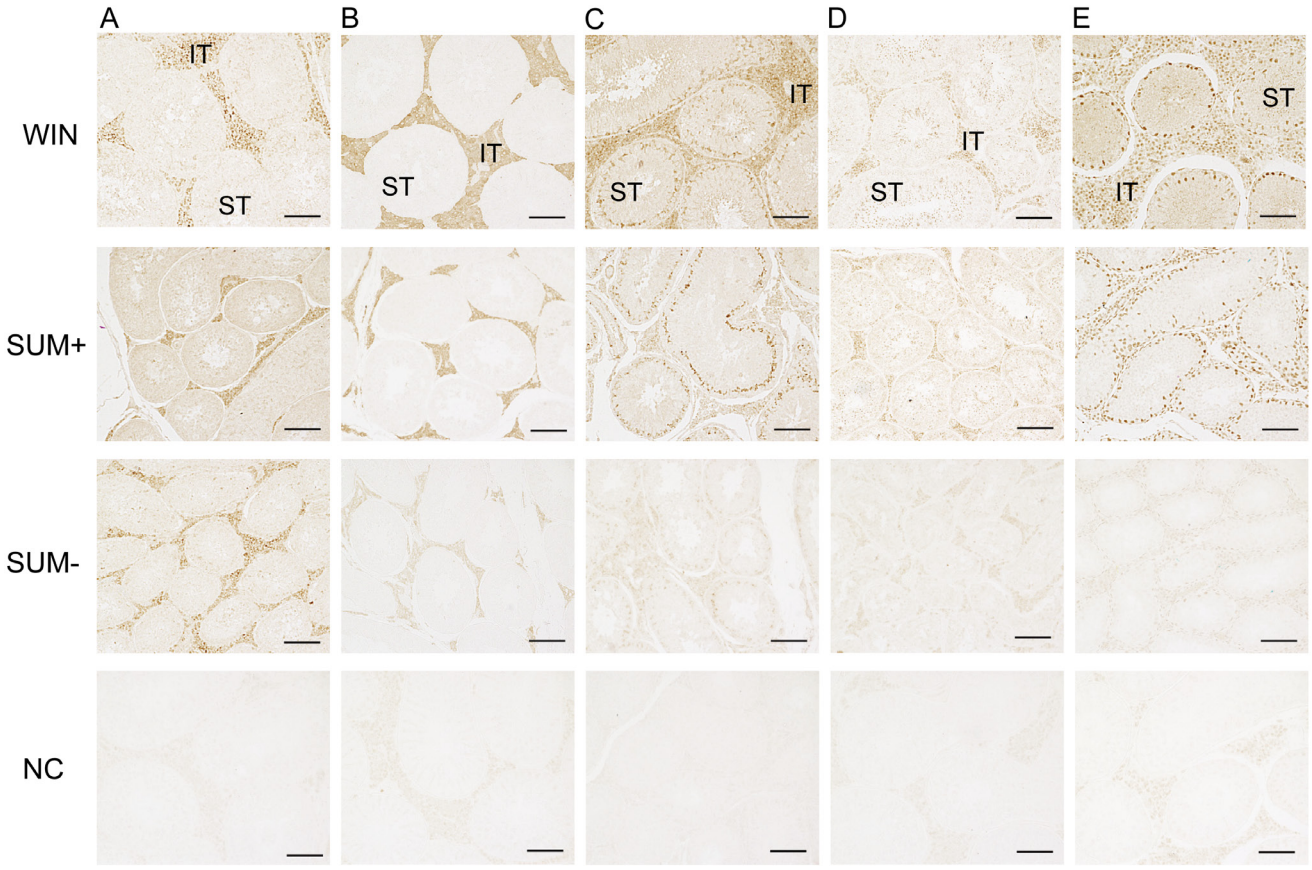

Fig. 3. Immunohistochemical staining of four steroidogenic enzymes (A, P450scc; B, P450c17; C, 3βHSD; D, P450arom) and (E): androgen receptor in representative samples of raccoon testes among the WIN, SUM+ and SUM– groups. NC, negative control; IT, interstitial tissue; ST, seminiferous tubules. Bar=100 µm.

Table 2. Immunostaining intensity of four steroidogenic enzymes and androgen receptor (AR) in raccoon testes among three groups

Sample group	Immunostaining intensity				
	P450scc	P450c17	3βHSD	P450arom	AR
WIN	++	++	++	+	++
SUM+	++	++	+	+	++
SUM–	++	+	– / + *1	–	– / + *1

Four steroidogenic enzymes were cholesterol side-chain cleavage cytochrome P450 (P450scc), 3β-hydroxysteroid dehydrogenase (3βHSD), 17α-hydroxylase cytochrome P450 (P450c17) and aromatase cytochrome P450 (P450arom). The intensity was evaluated when immunostaining detected (+), detected strongly (++), scarce or not (–). *1: Samples were classified as + and 3 samples were classified as –.

season, some raccoons exhibited active spermatogenesis, producing spermatozoa, despite low testosterone concentrations, the levels of which were the same as in other summer samples showing inactive spermatogenesis. These results are similar to our previous reports [6]. Changes in testosterone concentration in testicular tissue correlated with peripheral changes. Thus, the first possibility, that the local testosterone concentration in testicular tissue may be a proximate factor for maintaining spermatogenesis over the summer, would seem to be eliminated. Between the two groups in the summer, which were divided according to spermatogenetic activity, there was no difference in body length or body mass. Thus, the level of spermatogenetic activity was apparently independent from the raccoon's nutritional condition or body development.

Spermatogenesis is generally regulated by sex steroid hormones, and steroidogenesis is the result of the actions of steroidogenic enzymes [14]. The presence of these steroidogenic enzymes in testicular tissues has been reported in mammals, and in this present study, P450scc, P450c17, 3βHSD and P450arom were detected immunohistochemically in raccoon testes. All four enzymes were located in the cytoplasm of Leydig cells. Androgen synthesis has been detected generally in Leydig cells in many mammalian species [15, 16]; thus, Leydig cells are likely to also be a major site of steroidogenesis in raccoon testes. However, 3βHSD was also located in Sertoli cells in this study. Although the biosynthesis of androgens in the testes had been considered to occur only in Leydig cells [17], some evidence indicates that Sertoli cells possess 3βHSD and can metabolize steroids [18]. Immunoreactivity for 3βHSD in Sertoli cells was also reported in cynomolgus monkey (*Macaca fascicularis*) testes [19]. The results of the present study suggest that Sertoli cells, as well as Leydig cells, play a role in producing androgens in raccoon testes. Estradiol is thought to be also essential for male reproduction, for stimulating sperm maturation [20] and for absorbing rete testis fluid to concentrate sperm in semen [21]. P450arom has been detected in Leydig cells, Sertoli cells and germ cells in some animals, such as Japanese black bears [8, 22], Hokkaido brown bears [23], raccoon dogs [7], laboratory mice [24] and laboratory rats [25]. Also, in raccoon testes, various cells, including Leydig cells, Sertoli cells and spermatids, are considered to be sources of estradiols.

Seasonal changes in steroidogenic enzyme expression in testicular tissue have been reported in some seasonal breeding animals, and the key enzyme associated with changes in the seasonal secretion of testosterone varies by species: P450c17 in bank voles [26] and American grey squirrels (*Sciurus carolinensis*) [27] and P450scc and P450c17 in DLS rams [28]. Seasonal changes in 3βHSD expression were demonstrated in raccoon dogs [7], Japanese black bears [22] and Colorado mule deer (*Odocoileus hemionus*) [29] by immunohistological evaluation and in raccoon dogs [30] by gene expression analyses. In the present study, P450scc and P450c17 were detected in all individuals throughout the seasons. Between WIN and SUM+, an intensity difference was observed only in 3βHSD. These results suggest that 3βHSD may play a key role in regulating testosterone production in Leydig cells in raccoon testes, as in the other mammals mentioned. Decreasing testosterone over the summer might be related to decreases in Leydig cell number or size changes [22, 31] and decreased testes weight, in addition to 3βHSD expression changes. Also, changes in luteinizing hormone (LH) secretion, which controls these factors [32], are thought to be part of another regulatory system.

When SUM– samples were compared with SUM+ samples, the testosterone concentrations in both plasma and tissue were not significantly different. However, P450arom expression decreased markedly and immunoreactivity was scarce in the SUM– group. Estradiol is also considered to be important for testicular development and spermatogenesis [33]. Synthesis of estradiol by P450arom occurs in various tissues and cells in mammals, such as adipose tissue, bone, the ovary, the placenta and the testis [14]. In raccoons, expression of P450arom in other tissues has not been noted, but in local testicular tissue, differences in the intensity of expression were observed, along with spermatogenetic activity, in the summer season. Thus, P450arom expression in raccoon testicular tissue might contribute to keeping spermatogenesis active during the summer non-mating season.

AR was detected strongly in Leydig cells and parts of Sertoli cells in SUM+, whereas it was detected weakly in both cells in inactive testes in SUM–. AR expression in Sertoli cells, which play a central role in nursing germ cells, decreased along with aspermatogenesis in summer. Thus, spermatogenesis in the raccoon testis may be maintained by reactivity to testosterone in Sertoli cells. AR expression in Sertoli cells has been reported to increase with stimulation of follicle-stimulating hormone (FSH) secretion [34, 35]. In further research, such upstream regulatory factors should be investigated.

The results in this study suggested that P450arom and AR were involved in maintenance of spermatogenesis in summer. However, there is a possibility that spermatogenetic activity was reduced promptly after the winter mating season and then was restarted by P450arom and AR expression in SUM+. In our previous study [6], we conducted histological observation of a testis repeatedly using one captive male and found that it took almost 4 months to restart spermatogenesis after the SS decline in summer. In addition, considering that all wild males showed active spermatogenesis until May in spring [6], spermatogenesis in SUM+ in the present study was thought to be maintained from the winter mating season, not reactivated after the aspermatogenetic period. The results for Plasma T and Tissue T suggested that a high testosterone concentration was not necessarily required for active spermatogenesis. In summer, even if the testosterone level was lower than in winter, spermatogenetic activity may be kept with the actions of testosterone on AR and of

estradiol synthesized by P450arom.

In summary, the present study showed that morphological, histological and endocrinological differences in testes of wild male raccoons occurred with the seasons and spermatogenetic activity. Four steroidogenic enzymes and the AR were immunolocalized in raccoon testicular tissue, and changes in immunohistochemical staining intensity were evaluated according to season and spermatogenesis. Seasonal changes in testosterone concentration correlated with 3βHSD expression, and spermatogenetic activity correlated with P450arom and AR expression. Maintaining spermatogenetic activity in the summer was related to metabolism and reactivity against testosterone, not with local testosterone concentrations in testicular tissue. Further investigations should examine how the differential effects of pituitary gonadotropins, such as FSH and LH, their receptors and estradiol expression affect spermatogenesis to clarify the details of the mechanism of the regulation of spermatogenesis in the raccoon testis.

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