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Immunohistochemical Localization of Steroidogenic Enzymes in the Testis of the Sika Deer (*Cervus nippon*) During Developmental and Seasonal Changes

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Abstract. Testicular steroidogenesis and spermatogenesis during developmental and seasonal changes were investigated in male sika deer (*Cervus nippon*), a short-day seasonal breeder, to clarify the physiological mechanisms for reproductive function. The immunohistochemical localization of steroidogenic enzymes (P450scc, P450c17, 3βHSD and P450arom), spermatogenesis and cell proliferation were analyzed in the testes of fetal (164 to 218 days of fetal age), fawn (0 years old), yearling (1 year old) and adult (more than 2 years old) male sika deer. Three kinds of steroidogenic enzymes, P450scc, P450c17 and 3βHSD, essential for the synthesis of testosterone were located only in the Leydig cells of the testes from the fetal period, and these localizations did not change during developmental or seasonal stages. Immunoreactivity for P450arom, a key enzyme converting testosterone to estradiol, was also localized only in the Leydig cells of testes but was also further limited to the testes of yearlings and adults. Seminiferous tubules had already formed in the fetal testes examined in the present study. Spermatogenesis started in yearlings and was more active in the breeding season. In the adult sika deer testes, the Leydig cells, which displayed immunoreactivities for steroidogenic enzymes, changed to have more cytoplasm in the breeding season than in the non-breeding season. Cell proliferation of Leydig cells was hardly observed in adult testes during seasonal changes. The present results suggested that sika deer testes start to synthesize testosterone from the fetal period, that seasonal changes in testosterone and estradiol syntheses are dependent on the quantitative variation of steroidogenic enzymes synchronized with the size of Leydig cells and that estradiol synthesized in yearling and adult testes makes a contribution to the initiation and recrudescence of spermatogenesis and spermiogenesis in the sika deer.
Key words: Developmental change, Seasonal change, Sika deer, Steroidogenic enzyme, Testis

Many species of the Cervidae are short-day seasonal breeders. It has been reported that the red deer (*Cervus elaphus*) shows clear seasonal changes in the gonads, reproductive tracts, accessory genital glands and various other organs [1]. The sika deer (*Cervus nippon*) is the only Cervidae inhabiting Japan except for a feral foreign cervid, Reeves' muntjac (*Muntiacus reevesi*). This species is also a short-day seasonal breeder, and the rut is observed from October to December, at which point the testicular weight and diameter of the seminiferous tubules are increased in males [2].

Reproductive events are maintained through intricate regulatory mechanisms induced by regulatory factors, such as hormones and growth factors. Therefore, comprehensive studies on reproductive functions are necessary to understand the complicated interactions between these factors, and reproductive techniques based on the

elucidated functions will make conservation of endangered animals and wildlife management possible.

In males, sex steroid hormones, such as androgen and estrogen, are synthesized in the testes, of which steroidogenesis participates with several steroidogenic enzymes [3]. Androgen operates on not only the testes themselves but also accessory genital glands by way of its receptor (androgen receptor), regulating the differentiation and growth of these organs and spermatogenesis in the testes [4]. Estrogen is also concerned with development and spermatogenesis in the testes [5, 6]. Therefore, an understanding of the dynamics of sex steroid hormones is important to reveal testicular functions.

Analysis of serum concentrations of sex steroid hormones is a general approach to demonstrate an endocrinological strategy for reproduction. In several seasonal breeders, the serum concentrations of steroid hormones exhibit seasonal fluctuations [7–13]. In addition, the immunolocalization of steroidogenic enzymes in the testes has been reported to change seasonally in the black bear and northern fur seal [12, 13]. Moreover, it has been reported that the expression of steroidogenic enzymes in the testis is different among

Table 1. Developmental stages and number of animals used in the present study

Developmental stage	Season	Month	Number
Fetus (164–218 days)			7
Fawn (0 years old)	Breeding season	October and November	3
	Non-breeding season	January	4
Yearling (1 year old)	Non-breeding season	July	4
	Breeding season	November	3
Adult (>2 years old)	Breeding season	October and November	10
	Non-breeding season	January	4
		March	4
		July	3
Total			42

the developmental stages [14–17]. In the testes of adult sika deer, it has been reported that the immunohistochemical localization of steroidogenic enzymes found in the breeding season [18]. In the sika deer, the testes change in size and structure dramatically with not only sexual maturity but also seasonal transitions [2]. Therefore, fluctuations in the localization of steroidogenic enzymes in the testes may be found during developmental and seasonal changes, and these fluctuations may lead to hints that are helpful in elucidation of testicular control mechanisms.

The present study analyzed the immunohistochemical localization of steroidogenic enzymes in the testes of sika deer during developmental and seasonal changes to clarify the functional mechanisms of reproduction of male sika deer.

Materials and Methods

Testes were obtained from a total of 42 male Hokkaido sika deer (*Cervus nippon yesoensis*), a subspecies of the sika deer living in Hokkaido, Japan, in the districts of Nishiokoppe, Okoppe, Ohmu, Ashoro, Nakasatsunai and Nakanoshima Island between November 2002 and July 2007. Thirty-two wild male deer were hunted in October and November (breeding season) and January, March and July (non-breeding season) with official permission. Three adult males kept at a deer farm were immobilized by an injection of a mixture of ketamine hydrochloride (1.5 mg/kg) and xylazine hydrochloride (1.2 mg/kg) [19, 20]. After chemical immobilization, a piece of a testis was biopsied from each animal. The developmental stages and numbers of animals are shown in Table 1. The testes of seven male fetuses (164 to 218 days of fetal age) were collected from pregnant females captured between March and June. The fetal age was estimated by body weight [21]. The age of each animal after birth was estimated by branching of antlers and dentition [22], and the animals were then categorized as fawns (0 years old), yearlings (1 year old) and adults (more than 2 years old). Because Hokkaido sika deer usually give birth in June [20, 23], the ages of the deer were classified into the next category beginning in June.

The removed testes were immediately fixed in Bouin's fluid or phosphate-buffered (pH 7.2) 10% formalin. After fixation, the tissue samples were dehydrated in a graded series of ethanol, cleared

in xylene and embedded in paraffin (Paraplast Plus®, Kendall, Mansfield, MA, USA). The samples were cut serially at 4 μm thickness and mounted on aminopropyl-triethoxy-silane-coated slides (S8226, Matsunami Glass, Osaka, Japan). After deparaffinization, the testicular tissue sections were stained with hematoxylin and eosin (HE) to investigate the general structure and spermatogenesis.

To detect the localization of steroidogenic enzymes, the sections were stained immunohistochemically using an avidin-biotin peroxidase complex (ABC) method [24] or the ImmPRESS™ (polymerized reporter enzyme staining system) method (Vector Laboratories, Burlingame, CA, USA). The primary antibodies used in this study are summarized in Table 2. The antibodies against P450scc, P450c17, 3βHSD and P450arom were used in previous studies on testicular steroidogenesis [12, 15, 18, 25, 26].

For the ABC method, the deparaffinized sections were incubated with methanol containing 0.3% H_2O_2 for 10 min at room temperature (RT) to block endogenous peroxidase activity and then with normal goat serum (1:50, S-1000, Vector Laboratories) for 30 min at RT to prevent nonspecific staining. The samples were incubated with each primary antibody overnight at 4 C. After an overnight incubation, the secondary antibody, biotinylated anti-rabbit IgG raised in the goat (1:200, BA-1000, Vector Laboratories), was applied for 30 min at RT, and then the sections were incubated with ABC for 30 min at RT (1:2, PK-6100, Vectastain *Elite*® ABC Kit, Vector Laboratories). The immunoreactive sites were visualized using Tris-HCl buffer (pH 7.4) containing 0.02% 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.006% H_2O_2. For the ImmPRESS™ method, the sections were incubated with ImmPRESS anti-rabbit IgG (MP-7401, ImmPRESS™ reagent, Vector Laboratories) for 30 min at RT after overnight incubation with the primary antibodies, and the binding sites were visualized using acetonitrile (SK-4700, Vector® SG Substrate Kit, Vector Laboratories).

In addition, to investigate the cellular proliferation of adult deer testes during developmental and seasonal changes, the sections were stained immunohistochemically with monoclonal antibody against rat proliferating cell nuclear antigen (PCNA; Table 2) and visualized using an ABC kit. For the negative controls, the sections were treated with each normal serum or 0.01 M PBS instead of the

Table 2. Characteristics and dilutions of primary antibodies

Antigen	Host	Type	Dilution	Catalog No. or Source
Rat cholesterol side-chain cleavage cytochrome P450 (P450scc)	Rabbit	Polyclonal	1:200	AB-1244 Chemicon International, Temecula, CA, USA
Porcine 17α-hydroxylase cytochrome P450 (P450c17)	Rabbit	Polyclonal	1:500	[49]
Human 3β-hydroxysteroid dehydrogenase (3βHSD)	Rabbit	Polyclonal	1:500	[50]
Human aromatase cytochrome P450 (P450arom)	Rabbit	Polyclonal	1:500	[51]
Rat proliferating cell nuclear antigen (PCNA)	Mouse	Monoclonal	1:100	11434 ICN Pharmaceuticals, Cleveland, OH, USA

primary antibody.

Results

General structure of testis

The testes of male sika deer showed remarkable histological changes during development. In the earliest fetal testis examined in this study (164 days of fetal age), the seminiferous tubules had already been formed. However, in the fetuses and fawns, the germ cells had not yet differentiated into spermatogenic cells, and thus spermatogenesis was not observed (Fig. 1A and B). In the fetal testis at 164 days, the interstitial tissue containing the Leydig cells was loosely dispersed among the seminiferous tubules (Fig. 1A). In the fawns, the Leydig cells were gathered, and the cytoplasm was more extensively developed (Fig. 1B).

In the yearlings, spermatozoa were not detected in July, although germ cell differentiation into spermatogenic cells had already started (Fig. 1C). On the other hand, in November, yearling deer completed spermatogenesis and began producing spermatozoa (Fig. 1D). In the adult testes, histological changes in the seminiferous epithelium were clearly shown during seasonal changes. In October and November, the seminiferous tubules were well developed and showed active spermiogenesis (Fig. 1E). In January, spermiogenesis was inactive, but a few spermatozoa were still found in the seminiferous tubules (Fig. 1F). However, in March, the seminiferous epithelium was regressed, and degenerated spermatocytes and spermatids were left out of the Sertoli cells (Fig. 1G). In July, spermatogenesis recrudesced, and the seminiferous tubules were found to contain many spermatocytes and occasionally spermatids, but no spermatozoa (Fig. 1H).

In the breeding season, the Leydig cells, which occupy the interstitial tissue surrounding the seminiferous tubules, contained extensive cytoplasm (Fig. 1E). In the non-breeding season, especially March, the Leydig cells were regressed, and their cytoplasm was less abundant (Fig. 1F–H). Therefore, the interstitial area became smaller in the non-breeding season than in the breeding season (Fig. 1E–H).

Immunohistochemistry

In all the testes examined in this study, the immunoreactivities for P450scc, P450c17 and 3βHSD were localized only in the Ley-
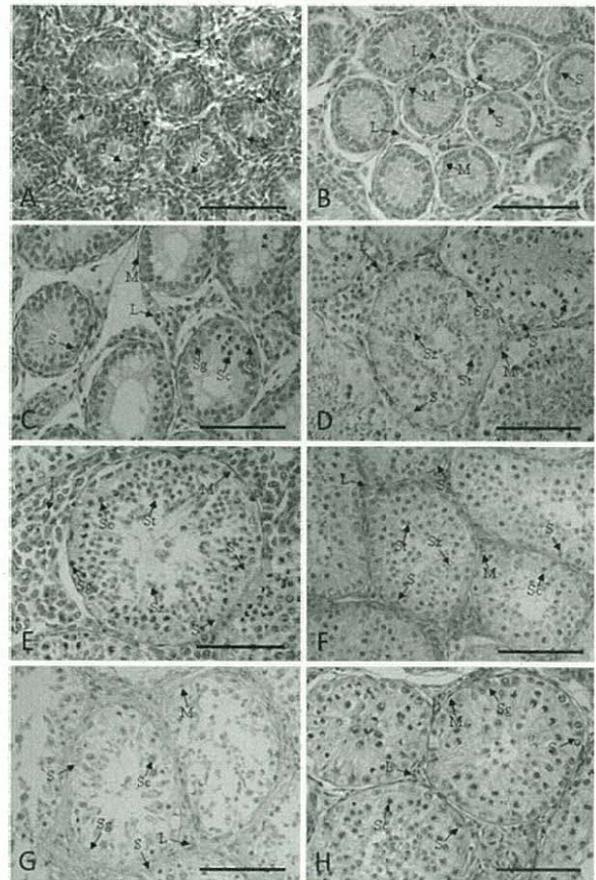

Fig. 1. General structure of the testes in the sika deer. (A) The earliest fetus, 164 days of fetal age, (B) fawn, (C, D) yearling (C, July; D, November), (E–H) adult (E, November; F, January; G, March; H, July). G: Germ cells L: Leydig cells M: Myoid cells Sg: Spermatogonia Sc: Spermatocytes St: Spermatids Sz: Spermatozoa S: Sertoli cells. Bar=100 μm.

dig cells (Figs. 2 and 3). Furthermore, Leydig cells also showed immunoreactivity for P450arom, but this was limited to the testes of yearlings and adults (Fig. 4). The immunointensities of all steroidogenic enzymes, except for P450arom, did not show clear

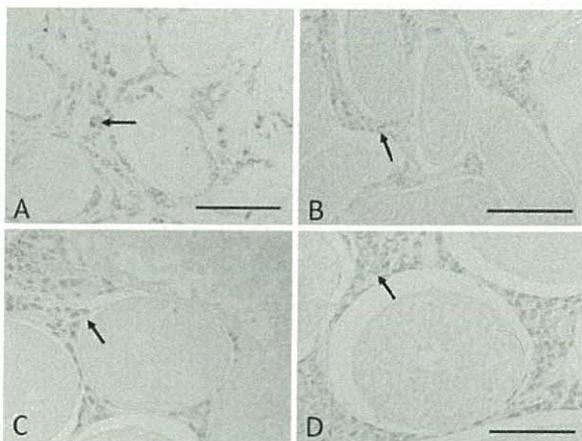**Fig. 2.** Immunohistochemical localization of P450scc in the testes of the sika deer during development. Immunoreactivity for P450scc is localized only in Leydig cells (arrows) during all developmental stages. (A) Fetus, (B) fawn, (C) yearling and (D) adult in the breeding season. Bar = 100 μm.
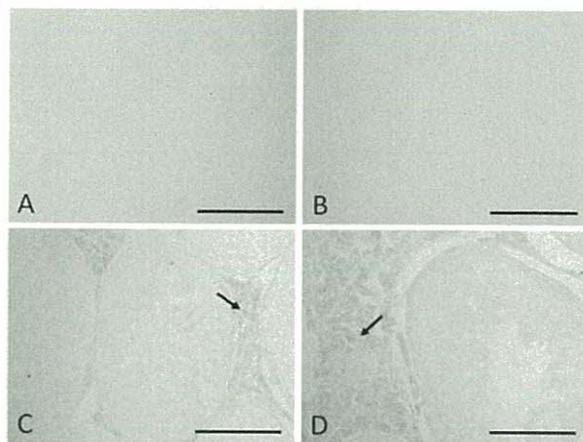

Fig. 4. Immunohistochemical localization of P450arom in the testes of the sika deer during different developmental stages. Immunoreactivity for P450arom is localized only in the Leydig cells (arrows) of yearling (C) and adult (D) deer testes in the breeding season and not in the fetus (A) or fawn (B) testes. Bar=100 μm.
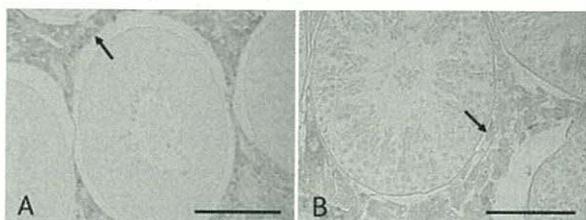

Fig. 3. Immunohistochemical localization of P450c17 and 3βHSD in the testes of adult sika deer in the breeding season. These enzymes are localized only in Leydig cells (arrows). (A) P450c17, (B) 3βHSD. Bar = 100 μm.
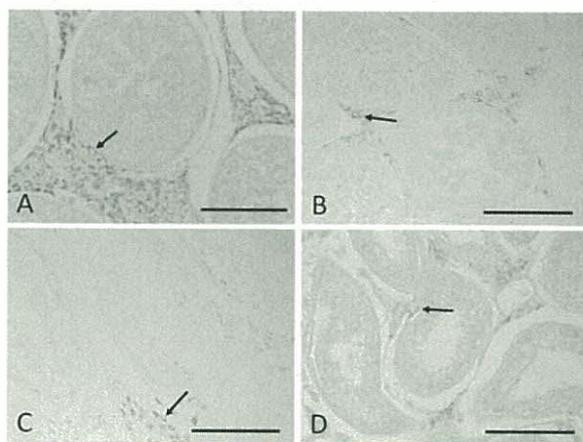

Fig. 5. Immunohistochemical localization of P450scc in the testes of adult sika deer during different seasonal periods. Immunoreactivity for P450scc is localized only in Leydig cells (arrows) and is strongest in the breeding season. (A) October, (B) January, (C) March, (D) July. Bar=100 μm.

developmental changes. However, all steroidogenic enzymes indicated strong immunoreactivities in the breeding season (Fig. 5). Moreover, in the breeding season, the Leydig cells with steroidogenic enzyme immunoreactivities became larger in size than those in the non-breeding season (Fig. 5).

In the fetal and fawn testes, the immunoreactivity for PCNA was localized in the Sertoli cells, Leydig cells, germ cells and myoid cells (Fig. 6A). However, in the yearlings and adults, the PCNA-immunoreactive Sertoli cells disappeared completely (Fig. 6B and C). In these two developmental stages, PCNA-immunoreactive Leydig cells and myoid cells were only few in number (Fig. 6B and C). On the other hand, spermatogonia and spermatocytes showed remarkable immunoreactivities for PCNA (Fig. 6B and C). In the yearlings and adults, the immunolocalization of PCNA did not differ throughout the seasonal changes (Fig. 6C and D).

Discussion

Sex steroid hormones such as testosterone and estradiol produced by steroidogenic enzymes play an essential role in reproductive functions. It is necessary to investigate the localization of steroidogenesis in the testis of each mammalian species because the localization is different among animal species and among developmental and seasonal changes. The present study is the first histological report on the testicular steroidogenesis in Cervidae during developmental and seasonal changes.

Three steroidogenic enzymes, P450scc, P450c17 and 3βHSD, which are essential for the synthesis of sex steroid hormones, are localized not only in the Leydig cells but also in other testicular cells, such as Sertoli cells and spermatogenic cells in the monkey [27], American black bear [12], Japanese black bear [28], Hok-
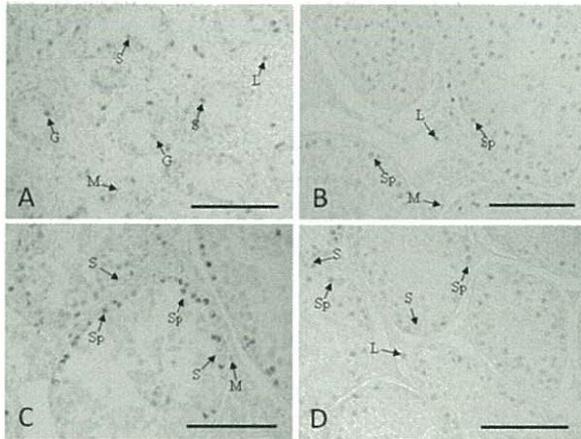
Fig. 6.   Immunohistochemical localization of PCNA in the testes of the sika deer. Immunoreactivity for PCNA is detected in the Leydig cells, germ or spermatogenic cells and myoid cells during all developmental stages. However, in Sertoli cells, immunolocalization of PCNA is only found in the fetus and fawn stages. Differences in localization were not found during the different seasonal periods. (A) Fetus, (B) yearling, (C, D) adult (C, November; D, March). L: Leydig cells G: Germ cells M: Myoid cells S: Sertoli cells Sp: Spermatogenic cells. Bar=100 μm.

kaido brown bear [26] and Japanese raccoon dog [25]. However, in the rat [29] and goat [15], these enzymes are detected only in the Leydig cells, in accordance with the present results. On the other hand, P450arom, the enzyme that converts testosterone to estradiol, is localized in various testicular cell types, such as Leydig cells, Sertoli cells and spermatogenic cells, of many mammals [6] including the American black bear [12], Japanese black bear [28, 30], Hokkaido brown bear [16], northern fur seal [13], Japanese raccoon dog [25], mouse [31] and rat [32]. However, in the sika deer, P450arom was localized only in the Leydig cells. This result was in accordance with the human [33], stallion [34, 35], ram [14], boar [17] and goat [15]. In the present study on sika deer testes, all of the steroidogenic enzymes examined were found only in the Leydig cells, suggesting that the Leydig cells may be the main testicular cells for production of sex steroid hormones, such as testosterone and estradiol.

In terms of the relationship between developmental changes and the syntheses of sex steroid hormones, the present results for the immunolocalization of P450scc, P450c17 and 3βHSD suggest that testosterone synthesis is already occurring in the fetal period in the Leydig cells of sika deer testes. Fetal testosterone synthesis has been demonstrated in the rat [29, 36], sheep [37], pig [38] and goat [15]. Testosterone is essential for male sexual differentiation and growth of male genital organs in the fetal period. In neonatal mouse testes, O'Shaughnessy et al. [16] reported that 17βHSD, which directly converts androstenedione to testosterone, is expressed at 10 days of age, disappears at 20 days, and then reappears with increased expression at 30 days. In the bull, the testosterone concentrations reach a minimum at 1 month of postnatal age and were restored again at 2 months [39]. In further studies

of the sika deer, it would be valuable to examine the changes in steroidogenesis during neonatal testicular development.

In the sika deer, estradiol is considered to be synthesized in the Leydig cells of yearling and adult testes because of the existence of P450arom. In the present histological observations, spermatogenesis started in yearlings. Therefore, in sika deer testes, we suggest that estrogen, mainly estradiol, is synthesized by the Leydig cells from at the least the yearling stage and has an influence on spermatogenesis and spermiogenesis. In previous reports, it has been mentioned that estrogen might have an effect on germ cell development via the estrogen receptor (ER) [40, 41]. On the other hand, in the goat, immunoreactivity for P450arom has also been found in fetal and neonatal testes [15]. Moreover, aromatase is expressed in the Leydig cells and Sertoli cells of fetal rodent testes, [40] and in the gonocytes and Leydig cells of fetal pig testes [42]. In fetal rat testes, estradiol promotes the proliferation of gonocytes [43] and has an effect on steroidogenic factor-1 (SF-1), which is important for gonadal differentiation and fetal Leydig cell functions [36]. Therefore, in the fetal period, estradiol is considered to be essential for fetal testicular development. However, in the fetal sika deer testes, immunoreactivity for P450arom was not detected. There are two possible explanations for these findings. One is that fetal sika deer depend on estradiol from maternal sources such as the placenta or ovary. The other is that estradiol synthesis and secretion may occur at an earlier fetal age than examined and then discontinues from the late fetal stage until the yearling stage.

In the present study on testicular seasonal changes, it was found that the interstitial area occupied by the Leydig cells was wider in the breeding season (October and November) than in the non-breeding season (January, March, and July). Furthermore, the seminiferous tubules were largely developed with increasing numbers of the spermatogenic cells and spermatozoa in the breeding season. This result corresponds with a previous report that the diameter of the seminiferous tubules of sika deer testes is significantly increased in the breeding season [2]. These seasonal histological changes of sika deer testes in the breeding season occur simultaneously with the increase in the serum testosterone concentration [10].

In immunohistochemical examinations, the localization of steroidogenic enzymes in the sika deer has been restricted to the Leydig cells throughout the seasonal changes, whereas the localization of enzymes in the bear [28] and Japanese raccoon dog [25] changed seasonally. In the sika deer, the intensity of immunoreactivity for each steroidogenic enzyme shows seasonal changes like other seasonal breeders, of which the intensity is increased when the testes are fully active [12, 44].

With regard to the seasonal testicular cell proliferation of the Leydig cells in yearlings and adults, there were only small number of PCNA-immunoreactive Leydig cells in each season. In adult roe deer testes, Leydig cells do not proliferate, while the size of the testes increases in the breeding season [45]. These results corresponded with the present observations in sika deer testes. Therefore, we suggest that enlarged Leydig cells have increased testosterone synthesis per active cell with increased levels of steroidogenic enzymes, which increase the whole quantity of testicular testosterone in the breeding season. Sertoli cells showed

PCNA immunoreactivity in fetuses and fawns, but not in yearlings or adults. In rat testes, it has been reported that the proliferation of the Sertoli cells is active during the perinatal period but not in the adult [46-48]. These previous results concur with those of the present research.

In the reindeer [8] and white-tailed deer [7], the male estradiol levels reach a maximum at the peak of the rut. The change in the estradiol concentration synchronized with the rut is similar to the case with testosterone. It is well-known that estradiol is directly converted from testosterone by P450arom. In the present study, almost all the enlarged Leydig cells in the breeding season showed P450arom immunoreactivity. This result suggests that sika deer testes might exhibit increased estradiol synthesis for spermatogenesis in the breeding season by conversion of the increased levels of testosterone. Tsubota *et al.* [12] reported a close relationship between the recrudescence of spermatogenesis and estradiol synthesis in the black bear. In male sika deer, the changes in serum estradiol levels and ER expression in testicular cells, including the spermatogenic cells, have not yet been examined. It is essential for understanding the role of estradiol in the recrudescence of spermatogenesis to clarify the relationship between the seasonal changes in serum estradiol and the ER testicular expressions in male sika deer.

In conclusion, the results of the present study indicate that testicular steroidogenesis of the sika deer occurs in the Leydig cells, which increases the levels of steroidogenic enzymes within the enlarged cytoplasm in the breeding season. Furthermore, they suggest that estradiol synthesized in yearling and adult testes makes a contribution to the initiation and recrudescence of spermatogenesis and spermiogenesis in the sika deer.

Acknowledgments

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