NOTE *Anatomy*

Immunohistochemical Localization of Steroidogenic Enzymes in the Testis of Hokkaido Sika Deer (*Cervus nippon yesoensis*)

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ABSTRACT. The testes from 15 adult male Hokkaido Sika deer (*Cervus nippon yesoensis*) were collected during the rutting season (October and November). We investigated the localization of 4 kinds of steroidogenic enzymes (P450scc, 3βHSD, P450c17 and P450arom) immunohistochemically in these testicular samples. The specific immunoreactivities to these enzymes were detected only in the cytoplasm of Leydig cells. This differs to the enzyme distributions reported previously in Japanese black bear, Japanese raccoon dog, Hokkaido brown bear and American black bear, in which the same immunoreactivities were detected in Leydig cells, Sertoli cells and/or spermatogenic cells. The current study suggests that in the testes of the Hokkaido Sika deer, testosterone and estradiol-17 β may be synthesized in the Leydig cells only.

KEY WORDS: Hokkaido Sika deer (*Cervus nippon yesoensis*), immunohistochemistry, steroidogenic enzyme.

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The Sika deer (*Cervus nippon*) is one of Japan's largest terrestrial mammals and its management, together with that of its principal predator the Japanese brown bear (*Ursus arctos yesonensis*), is extremely important. Of the Sika deer, the sub-species living on Hokkaido island, the Hokkaido Sika deer (*Cervus nippon yesoensis*), is the largest. They are seasonal breeders with their rut occurring from October to December [22]. The histology of the postnatal testicular development and seasonal changes of the testes of Honsyu Sika deer (*Cervus nippon centralis*) in Nara Park (34°40' N, 135°50' E; Nara, Japan) were reported [26]. In a later study of the annual changes in the testis size, normal histology of seminiferous tubules and changes in plasma testosterone levels of wild Hokkaido Sika deer were reported by Suzuki *et al.* [22].

Seasonal changes in wild of testicular steroidogenesis and spermatogenesis have been reported in the following species; American black bear [23], Northern fur seal [24], Japanese raccoon dog [21] and Japanese black bear [14]. In these wild animals, the immunohistochemical studies on the testis demonstrated different distributions of immunoreactivities for steroidogenic enzymes in cellular elements in and around the seminiferous tubules associated with seasons and the species examined.

For their long-term conservation and management a better understanding of reproductive strategies of male Sika deer, in particular knowledge of testicular steroidogenesis is critically important. However no immunohistochemical studies on the testicular steroidogenic enzymes have been conducted to date on Cervidae. Consequently when the opportunity arose to obtain appropriate samples we undertook the present study to identify the sites of steroidogenic enzymes in the testes of male Hokkaido Sika deer.

To do this a total of 15 adult males of Hokkaido Sika deer were sacrificed in the autumn (October and November) of 2003. Five deer (more than 4-year-old) which were kept at a deer farm (Ashoro; 43°15'N, 143°33'E; Eastern Hokkaido, Japan), were obtained in early October with the official permission from Ashoro Town. Two wild deer (more than 3-year-old) were sacrificed in late October for scientific research with the government permission in Nishi-Okoppe (44°20'N, 142°55'E; Northern Hokkaido, Japan) and 8 wild deer (more than 3-year-old) were sacrificed in November by hunting in Nishi-Okoppe and Ohmu (44°35'N, 142°56'E; Northern Hokkaido). The estimated age of each animal was determined by assessing the branching of it's antler, as well as it's dentition [18]. The present study was approved by the Animal Research Committee of Obihiro University of Agriculture and Veterinary Medicine (Obihiro, Japan).

Immediately following the death of each animal its testes were removed and fixed in Bouin's fluid or phosphate buffered (pH 7.4) 10% formalin. Tissue samples were embedded in paraffin using routine techniques and cut serially at 4 μm. After deparaffinization, testicular tissue sections were stained with hematoxylin and eosin (HE) for the investigations of general structure and spermatogenesis. To detect the localization of steroidogenic enzymes, a separate series of sections were stained immunohistochemically using the avidin-biotin peroxidase complex (ABC) method [10]. This entailed using biotinylated anti-rabbit IgG (1:200 dilution, BA-1000, Vector Laboratories, Burlingame, CA, U.S.A.) and Vectastain *Elite* ABC Kit (1:2 dilution, PK-6100, Vec-

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tor Laboratories) according to the manufacture's protocols. First layer specific antibodies were raised in rabbits against bovine adrenal cholesterol side-chain cleavage cytochrome P450 (P450scc) [1], human placental 3β-hydroxysteroid dehydrogenase (3βHSD) [4], porcine testicular 17α-hydroxylase cytochrome P450 (P450c17) [7] and human placental aromatase cytochrome P450 (P450arom) [13]. These antibodies were the same as those used in the previous study [16, 21, 23, 25]. The specific first layer antibodies were used at a 1:500 dilution. Controls were treated with normal rabbit serum instead of the primary antisera.

Testes of adult Sika deer in this study had active spermatogenesis with all types of germ cells from spermatogonia to spermatozoa being present. The interstitium contained numerous Leydig cells (Fig. 1). The interstitial area was wider and contained more numerous large Leydig cells in samples taken in early October than in the late October and November (Fig. 1). Immunoreactivities for the steroidogenic enzymes, P450scc, P450c17, 3βHSD and P450arom were detected only in the cytoplasm of Leydig cells of all Sika deer (Fig. 2). Immunoreactivities were not detected in other testicular cells. The intensity levels of immunoreactivities for P450scc and P450c17 were high, but those of 3βHSD and P450arom were low. There was no detectable difference in the immunohistochemical characteristics of Leydig cells for each enzyme in the testes sampled in October or in November. All controls were negative.

An earlier study by Suzuki *et al.* [22] reported that in Hokkaido Sika deer on Nakanosima Island of Lake Toya (42°36'N, 140°51'E; Hokkaido, Japan) the onset of spermatogenesis occurred in July and/or August, and spermatogenic activity had already reached its peak in late October, at the beginning of the rutting season. According to the farm keepers in Ashoro (personal communication), rutting had not begun on the deer farm when we took the samples in early October of 2003. Consequently the testes used in this study should be representative of the early (early October) and full (late October and November) rutting period. Kameyama *et al.* [12] examined serum testosterone concentration in captured male Hokkaido Sika deer kept at Abashiri (44°01'N, 144°16'E; Northeastern Hokkaido, Japan), and reported that there was a peak of testosterone concentration between September and October, and that it had decreased significantly in November. In the present study the interstitial area occupied by Leydig cells was wider in testes collected in early October than in those from November. This could be reflected in changing testosterone concentrations of the deer.

Studies of steroidogenesis in seasonal breeding wild Japanese black bear [14], Hokkaido brown bear [25], American black bear [23], Northern fur seal [24] and Japanese raccoon dog [21] reported that the same enzymes P450scc, P450c17, 3βHSD and P450arom, were localized not only in the Leydig cells but also in the Sertoli cells and/or spermatogenic cells. Moreover, in Japanese black bear [14], American black bear [23] and Japanese raccoon dog [21], there were seasonal changes in the localization of these enzymes. In the present study on the testes of Hokkaido Sika deer taken over the rutting period, immunoreactivities for these same enzymes were detected only in the Leydig cells. Because the same antisera were used in all of these studies the differences of immunohistochemical localization are valid. The present results also suggest wide cross reactivity among the mammal species studied so far.

The essential enzymes in synthesis of androgen, P450scc, 3βHSD and P450c17, are recognized in not only the Leydig cells but also other testicular cells in various mammals [8]. In rat [20], however, these enzymes were detected only in the Leydig cells, and this is compatible with the present result.

Differing roles of estrogen in the testis are necessary for male reproduction and in fertility. In some mammals,

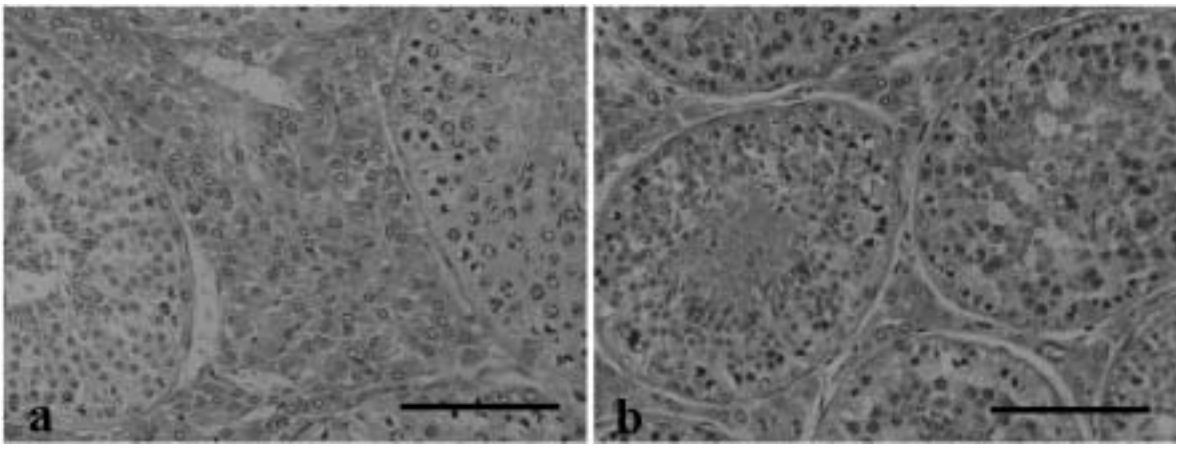

Fig. 1. a: Tissue section of testes sampled in early October was stained with HE. Spermatogenesis is very active. All types of spermatogenic cells and Sertoli cells are visible in the seminiferous tubules. The wide interstitial space is occupied with numerous and large Leydig cells. b: In testes sampled in November, spermatogenesis is very active, but the inter-tubular area is not so wide as that of testes sampled at early October (a). Bar: 100 μm.

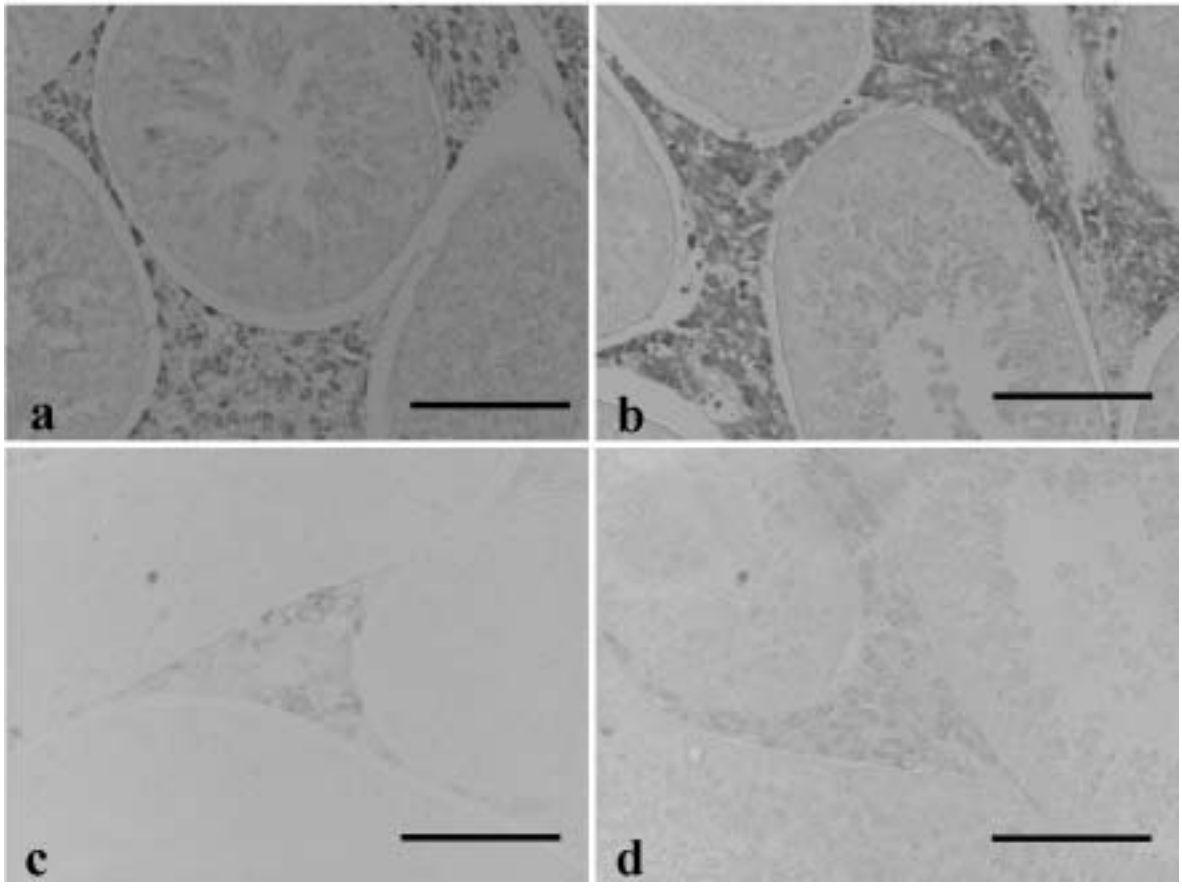

Fig. 2. Immunohistochemical staining for P450scc, P450c17, 3βHSD and P450arom (a, b, c and d, respectively) (early October). Immunoreactivities for these steroidogenic enzymes are detected in Leydig cells, but not in any cells in the seminiferous tubule. Staining intensity levels for P450scc and P450c17 are high, while those of 3βHSD and P450arom are low. Bar: 100 μm.

P450arom which converts testosterone to estradiol-17β, was localized in the Leydig cells, the Sertoli cells [14, 17, 19, 21] and spermatogenic cells [14, 15, 17, 19, 21, 23–25]. However, in ram [2], stallion [5, 9], human [3, 11], and boar [6], P450arom was localized only in the Leydig cells. The present study adds the Hokkaido Sika deer (*Cervus Nippon yesoensis*) to this list. In the present study, aromatization of testosterone in the testis of Hokkaido Sika deer was confirmed by immunohistochemical localization of P450arom.

The present results suggest that synthesis of testosterone and estradiol-17β in the testes of Hokkaido Sika deer might occur only in the Leydig cells. However, the staining intensity for 3βHSD and P450arom immunoreactivities was week, so further detailed studies of localization for these enzymes are needed. Since this study used only samples from rutting period, annual seasonal changes of steroidogenesis coupled with simultaneous hormonal investigations in wild deer need to be undertaken.

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