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Anatomical characteristics of male reproductive organs in the Korean water deer (*Hydropotes inermis argyropus*)

한국 고라니 수컷 생식기관의 해부학적 특징

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이 논문을 수의학석사 학위논문으로 제출함

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2013 년 6 월

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Abstract

The Korean water deer which is endemic in China and Korea, is currently over-populated in some region in South Korea. In order to accumulate basic reproductive information for the conservation and the wildlife management of this species, the male reproductive organs from this species were macroscopically, histologically and immunohistochemically observed throughout the year. The location and shape of testes and accessory reproductive glands (ampulla, vesicular gland, prostate gland and bulbourethral gland) of the water deer showed a resemblance to other small ruminant species. The size of the testes were increased from October and decreased from February. The status of spermatogenesis was shown from October to January. It appeared that the breeding season of the male Korean water deer is from October to January. The immunolocalization of the cytoskeletal proteins in the testes and epididymis were investigated in the Korean water deer and Reeves' muntjac as the comparison among cervid species. The distribution pattern of cytoskeleton proteins in the Korean water deer and Reeves' muntjac was similar. From this results, it appears that these distribution

patterns of cytoskeletal proteins may be common in the cervids. The fundamental reproductive information obtained in this study may contribute to the conservation and wildlife management of the Korean water deer.

Keywords: anatomy, cytoskeletal protein, *Hydropotes inermis argyropus*, Korean water deer, reproductive organs, wild animals,

Student Number: 2010-21643

Contents

General Introduction

The Korean water deer, *Hydropotes inermis argyropus*, belongs to the Family cervidae which can be classified into three subfamilies: Hydropotinae, Odocoileinae and Cervinae. The water deer is the only species that belongs to Hydropotinae and is different from other subfamilies by the phylogenic evidences, including the lack of antlers and the existence of well-developed upper canine in males (Figs. 1&2) (Cap *et al*., 2002; Kuznetsova *et al*., 2005). This species is distributed all over South Korea (*H. i. argyropus*) and in limited areas in around East China (*H. i. inermis*) (Fig. 3(A)). The water deer has been introduced to Europe in the 1900's and resulting captive and feral populations remained viable but remarkably stable in number (Hofmann *et al.,* 1988; Kirkwood *et al*., 1988) (Fig. 3(B)). Although the population of this species in China is designated as the endangered situation (Fang and Wan, 2002; Zhu *et al*., 2004), the over-populated situation in some regions in South Korea has caused various conflicts to human life, including severe agricultural damages. The studies on this species had been ecologically (Dubost *et al*., 2008, 2011; Zhang, 2000) and genetically (Hu *et al*., 2006; Koh *et al*.,

2009) performed and the growth and reproduction of water deer have been investigated in the introduced population in Europe (Dubost *et al*., 2010; Kirkwood *et al*., 1988; Mauget *et al*., 2007). As the anatomical studies, the skull (Jung *et al*., 2004; Kim *et al*., 2013a; Kim *et al*., 2013b) and the branching pattern of aortic arch (Ahn *et al*., 2008) have been studied. Although many anatomical studies on the reproductive organs have been reported in some other cervidae species including Sika deer (*Cervus nippon*) (Hayakawa *et al*., 2009, 2010) and roe deer (*Capreolus capreolus*) (Goeritz *et al*., 2003), there have been no reports on the reproduction of the endemic population of this species in Korea or China. The accumulation of the basic information on reproduction is meaningful for both the conservation and management of water deer in Korea and China. In this study, therefore, anatomical characteristics of male reproductive organs of the Korean water deer were analyzed and described in the following two chapters. In chapter 1, the anatomical characteristics of male reproductive organs were observed and measured by macroscopically and histologically. In chapter 2, the localization of cytoskeletal proteins in testis were observed by immunohistochemistry. In this chapter, the testes of Reeves' muntjac were observed as the comparison.

Fig. 1. The male Korean water deer. No antlers and the existence of well-developed upper canine are their unique anatomical appearances (Photo taken by JoonHyuk Sohn).

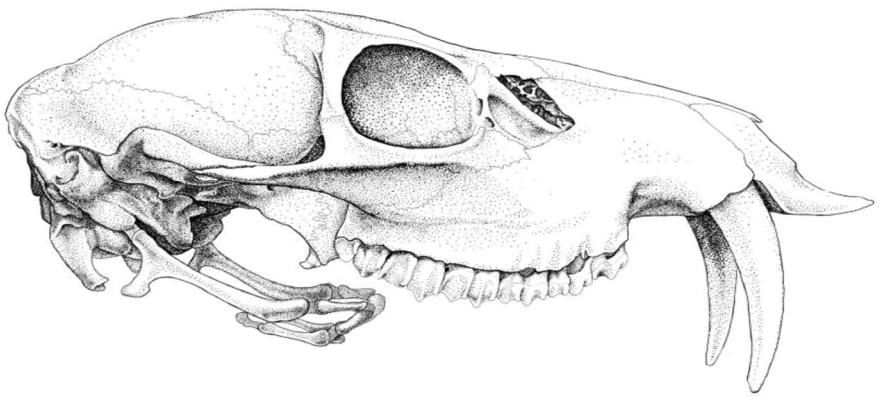

Fig. 2. The skull of male Korean water deer (Picture drawn by HeeJung Hwang).

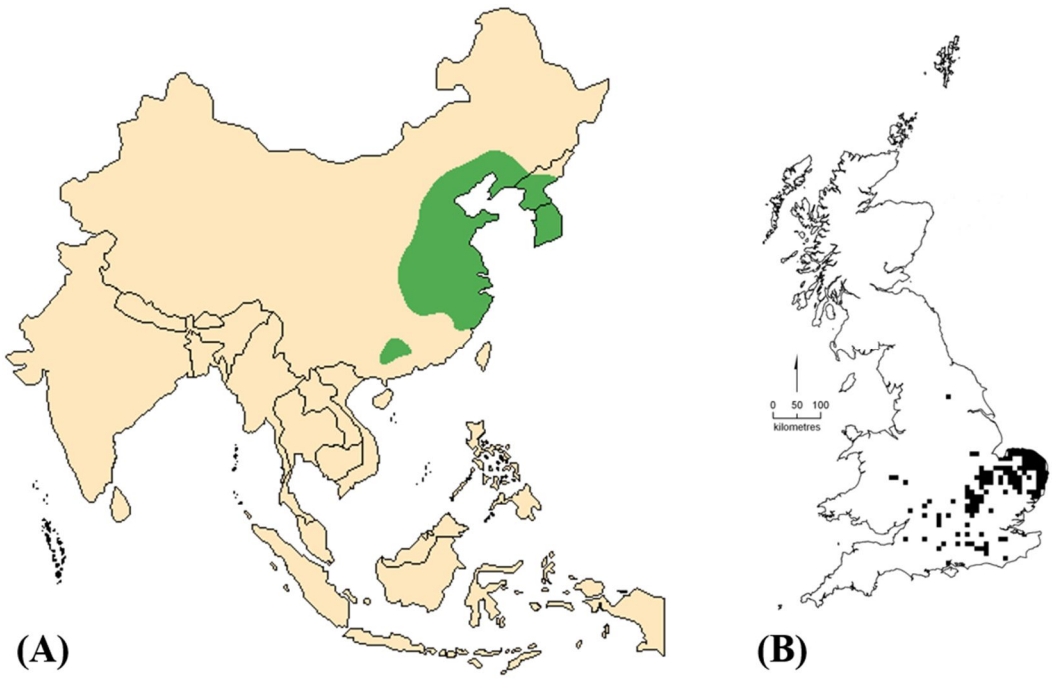

Fig. 3. Distribution of water deer in East Asia (A) (Sheng *et al*., 2000) and U.K (B) (figure from the British deer society).

Chapter 1.

Observation of male reproductive organs in Korean water deer (*Hydropotes inermis argyropus*)

Introduction

The Korean water deer, *Hydropotes inermis argyropus*, belongs to the family cervidae and genus Hydropotes. The studies on this species had been ecologically (Dubost *et al*. 2008, 2011; Zhang 2000) and genetically (Hu *et al*. 2006; Koh *et al*. 2009) performed. The growth and reproduction of water deer have been investigated in the introduced population in Europe (Dubost *et al*. 2010; Krikwood *et al*. 1988; Mauget *et al*. 2007). However, there have been no reports on the reproduction of the endemic population in China or Korea. Many types of papers have been reported in other deer species, including red deer (*Cervus elaphus*), fallow deer (*Dama dama*), white-tailed deer

(*Odocoileus virginianus*), moose (*Alces americanus*), elk (*Alces alces*), roe deer (*Capreolus capreolus*) and mule deer (*Odocoileus hemionus*). For example, in the red deer, the reproduction (Guiness *et al*., 1971; Kaji *et al*., 1988; Lincoln, 1971b), managements (Fuller, 1990; Kirkpatrick, 1980; Leopold, 1933), nutrition (Fennessy *et al*., 1991) and embryology (Krzywinsky, 1987) have been thoroughly studied. These basic information would contribute to the conservation medicine for this species.

As the accumulation of the basic information on reproduction is meaningful for both the conservation and management of water deer in Korea and China, the anatomical characteristics of male reproductive organs of the Korean water deer were analyzed both macroscopically and histologically in this chapter.

Materials and Methods

Water deer roadkills were collected from Gyeonggi, Chungbuk and Chungnam wild animal rescue centers and Cheorwon center of the Korean association for bird protection in Korea. Eighty–seven male individuals were obtained during from October 2010 to November 2012. Male reproductive organs, including testes and accessory reproductive organs, were excised and fixed in 10% formalin solution until observation. The size and weight of the testes and accessory reproductive organs were measured and observed. Afterwards, they were dissected and dehydrated in a graded series of ethanol, cleared in xylene and embedded in paraffin. These embedded organs were then sectioned at 4μm and placed on silane-coated glass slides. After deparaffinization, these sections were stained with Hematoxylin and Eosin (H&E). All images were taken by a BX51 light microscope (Olympus, Tokyo, Japan) equipped with a digital camera (DP71, Olympus) connected to a computer monitor. Eruption and abrasion of the molar teeth were examined in order to ensure that the animals used in this study were fully grown. The statistical differences of the size and weight of the accessory reproductive organs between

two periods (October to December of 2010 and April to June of 2011) were analyzed using student's *t*-test. We considered differences to be significant if $p<0.05$.

Result

Upon the observation of the molar teeth, all individuals used in this study were classified as adults.

Testis

The testis was found outside of the body, positioned vertically between the legs. Each testis had an oval shape and was attached to the epididymis within the scrotum. There were no significant differences regarding the location and shape of the testes. The volume and weight of the testes as well as the existence of spermatids in the convoluted seminiferous tubules are shown in Table 1. The volume and weight of the testis were varied throughout the year (Fig. 4). It was observed that testes become larger from September, largest on December and January and smaller from March. The spermatids in the convoluted seminiferous tubules were only observed in the specimens collected from October to January (Fig. 5).

Table 1. The volume and weight of testis and the existence of spermatid in the convoluted seminiferous tubules

Specimen number	Collected date	Volume of testis (cm^3)*	Weight of testis (g)		Spermatid
			R	L	
KJ0572	2010-10-30	10.45	6.4	5.9	+
KJ0573	2010-11-03	22.04	12.8	11.8	+
KJ0574	2010-11-05	10.35	5.2	7.4	+
KJ0580	2010-11-21	9.27	5.2	5.1	+
KJ0576	2010-11-29	16.93	8.6	8.6	+
KJ0577	2010-12-01	32.51	18.7	16.5	+
KJ0582	2010-12-03	12.46	7.9	8.0	+
KJ0583	2010-12-03	19.12	11.5	11.2	+
KJ0578	2010-12-05	19.28	9.9	11.3	+
KJ0585	2010-12-16	25.72	14.4	14.0	+
KJ0587	2010-12-19	19.96	10.1	11.0	+
KJ0589	2010-12-19	23.88	14.6	13.0	+
KJ0590	2010-12-19	26.49	14.9	12.2	+
KJ0591	2010-12-24	9.86	5.3	5.9	+
KJ0592	2010-12-24	17.89	10.2	10.7	+
KJ0594	2011-04-01	9.16	5.3	5.9	-
KJ0595	2011-05-18	5.76	3.4	3.4	-
KJ0597	2011-05-18	11.44	3.8	6.6	-
KJ0599	2011-06-18	7.51	4.3	5.5	-
KJ0608	2011-06-19	8.96	5.9	6.0	-
KJ0609	2011-06-21	9.65	5.8	6.0	-
KJ0610	2011-07-04	13.12	7.6	7.9	-
KJ0611	2011-07-04	10.20	6.7	6.3	-
KJ0612	2011-07-06	5.79	5.3	4.5	-
KJ0837	2011-07-28	3.45	6.0	4.5	-
KJ0839	2011-09-09	6.71	12.8	11.6	-
KJ0840	2011-09-09	5.73	7.1	7.9	-
KJ0841	2011-09-14	3.45	5.5	5.8	-
KJ0842	2011-09-15	6.85	13.5	12.7	-
KJ0843	2011-10-15	7.98	9.0	12.3	-
KJ0844	2011-10-26	11.31	15.2	14.4	-
KJ0850	2011-11-15	15.32	17.2	17.7	+
KJ0851	2011-11-25	9.44	14.8	15.2	+

Table 1. The volume and weight of testis and the existence of spermatid in the convoluted seminiferous tubules (continued)

Specimen number	Collected date	Volume of testis (cm^3)*	Weight of testis (g)		Spermatid
			R	L	
KJ0852	2011-11-28	19.17	23.1	22.4	+
KJ0581	2011-12-02	13.81	8.1	9.1	+
KJ0853	2011-12-06	15.55	17.9	19.0	+
KJ0854	2011-12-07	10.78	15.4	14.8	+
KJ0856	2011-12-22	8.80	9.5	8.6	+
KJ0857	2011-12-22	17.60	22.4	20.1	+
KJ0858	2011-12-22	14.57	18.6	19.0	+
KJ0859	2012-01-02	10.79	14.9	14.0	+
KJ0861	2012-01-08	9.00	11.9	12.8	+
KJ0863	2012-01-11	4.74	7.5	6.9	+
KJ0864	2012-01-11	11.37	15.2	15.4	+
KJ0865	2012-01-16	7.23	10.8	9.5	+
KJ0866	2012-02-15	5.08	6.6	6.8	-
KJ0867	2012-02-15	8.12	13.2	12.9	-
KJ0868	2012-03-01	8.02	11.0	12.6	-
KJ0869	2012-03-09	5.51	7.8	7.9	-
KJ0870	2012-03-09	7.92	11.4	9.4	-
KJ0871	2012-03-18	5.08	9.0	6.5	-
KJ0872	2012-04-10	4.01	7.4	6.1	-
KJ0873	2012-04-17	5.92	9.6	9.4	-
KJ0874	2012-04-17	5.43	8.4	8.1	-
KJ0875	2012-04-30	3.79	5.9	5.8	-
KJ0876	2012-05-01	3.45	5.2	5.5	-
KJ0877	2012-05-02	3.58	5.1	5.4	-
KJ0878	2012-05-07	5.30	7.8	7.6	-
KJ0879	2012-05-07	2.00	4.1	3.8	-
KJ0880	2012-05-08	4.85	7.7	7.4	-
KJ0881	2012-05-10	6.71	9.5	9.4	-
KJ0882	2012-05-11	3.55	5.5	5.2	-
KJ0883	2012-05-14	4.30	7.9	7.3	-
KJ0884	2012-05-16	3.05	4.5	4.1	-
KJ0885	2012-05-18	5.00	6.8	7.5	-
KJ0886	2012-05-21	8.68	11.6	10.0	-

Table 1. The volume and weight of testis and the existence of spermatid in the convoluted seminiferous tubules (continued)

Specimen number	Collected date	Volume of testis (cm^3)*	Weight of testis (g)		Spermatid
			R	L	
KJ0887	2012-05-23	4.22	5.5	5.4	-
KJ0888	2012-05-23	4.39	7.9	7.5	-
KJ0889	2012-05-25	6.54	9.3	8.9	-
KJ0890	2012-05-29	4.39	6.4	5.7	-
KJ0891	2012-06-01	3.94	5.0	5.2	-
KJ0892	2012-06-04	3.49	4.5	6.9	-
KJ0893	2012-06-05	4.56	6.5	8.0	-
KJ0894	2012-06-05	5.23	6.8	7.8	-
KJ0895	2012-06-05	6.26	8.0	9.0	-
KJ0897	2012-06-07	3.33	4.7	4.2	-
KJ0989	2012-06-20	4.46	6.1	7.0	-
KJ0898	2012-06-25	2.59	3.5	3.6	-
KJ0990	2012-06-30	3.27	5.0	5.1	-
KJ0991	2012-07-06	5.25	2.1	11.9	-
KJ0992	2012-07-21	4.62	8.3	7.5	-
KJ0904	2012-08-31	3.58	5.3	4.5	-
KJ0905	2012-09-21	8.47	12.0	14.4	-
KJ0907	2012-09-29	11.02	14.0	13.4	-
KJ0908	2012-11-03	6.32	10.0	9.4	-
KJ0909	2012-11-06	10.13	12.8	14.4	+
KJ0911	2012-11-13	12.66	13.4	14.8	+

* Volume of testis was calculate as follows: $V = 4/3\pi abc$ (a: long axis; b: short axis, c; width of testis). R: right, L: left. +: spermatid were discovered in the convoluted seminiferous tubules, -: spermatid were not discovered in the convoluted seminiferous tubules.

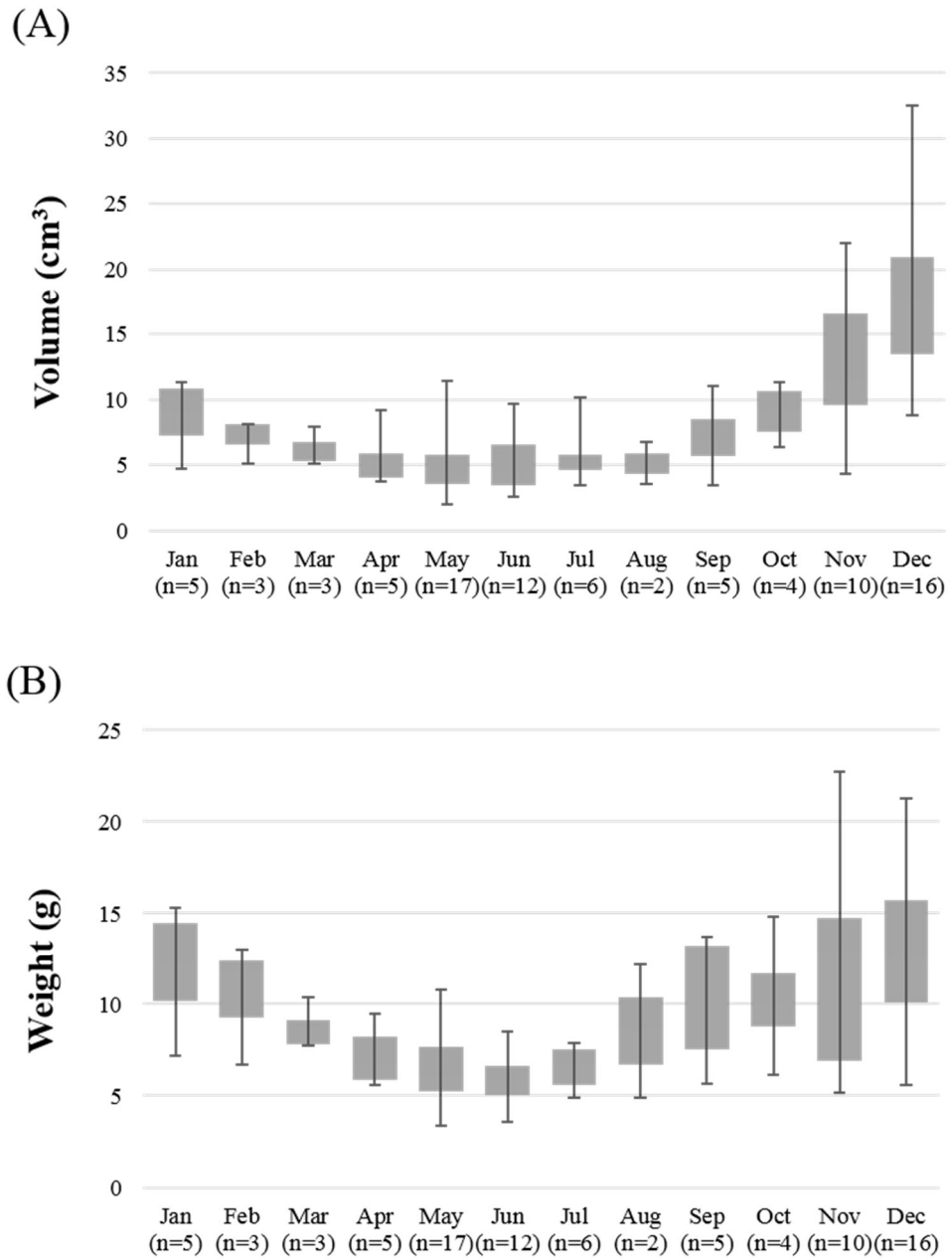

Fig. 4. The chronological change of volume (A) and weight (B) of testes. The testes becomes larger from September, largest on January and smaller from February. Boxes range the standard deviations and whiskers indicate the highest and lowest values.

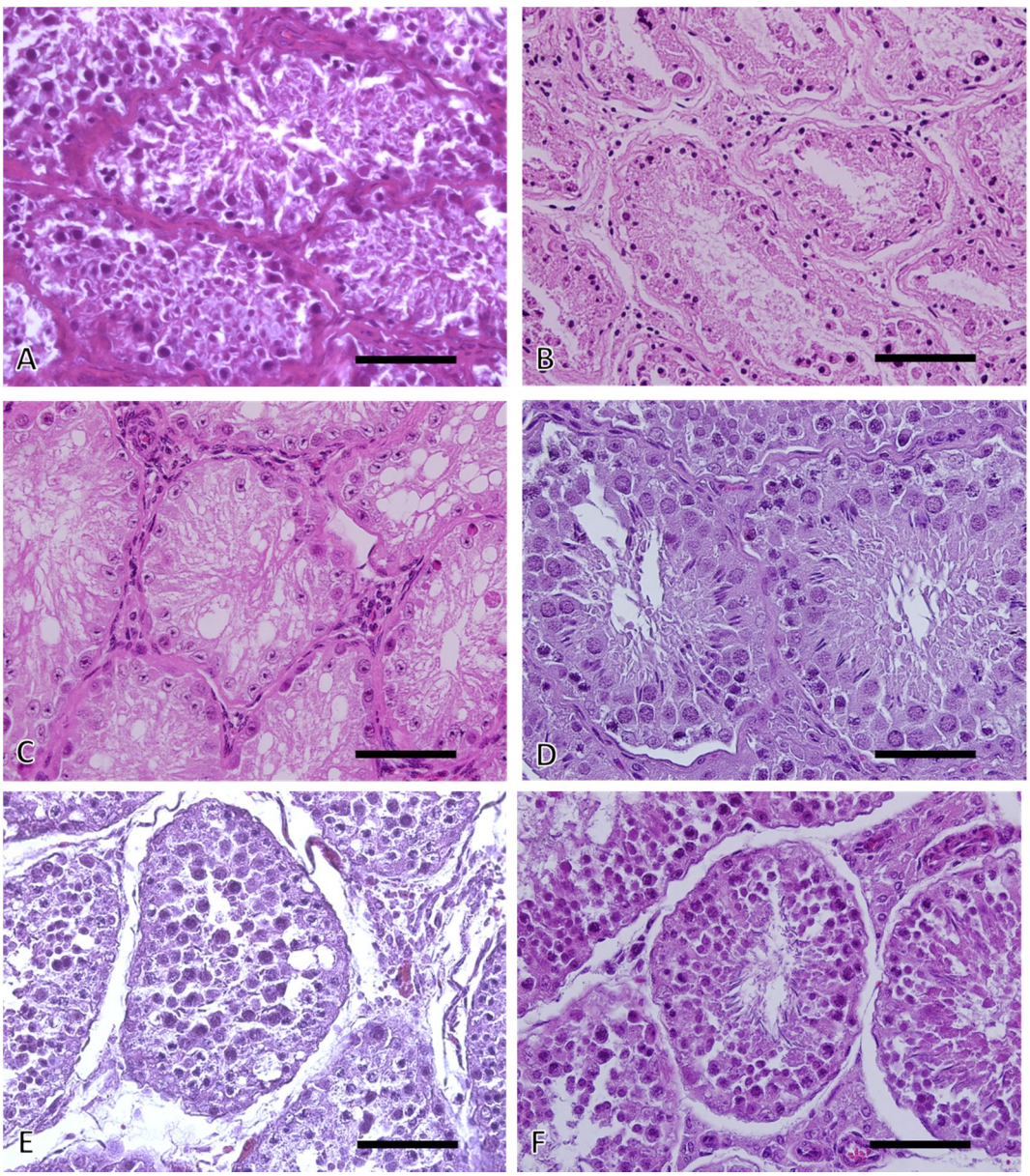

Fig. 5. The convoluted seminiferous tubules through the year. The spermatids were only observed in the specimens collected from late October to January in the Korean water deer. (A; January, B; February, C; July, D; October, E; November, F; December) (H&E staining) Scale bar = 25 μm

Accessory reproductive organs

Macroscopic observation

Four accessory reproductive organs (ampulla, vesicular glands, prostate glands and bulbourethral glands) were identified (Fig. 6). The ampulla is an enlarged part of the deferent duct before it reaches the genital fold. The duct narrows as it moves away from the ampulla and dips under the body of the prostate gland. The paired vesicular glands are the largest of the accessory genital glands in this species. It is a round-shaped, hard and compact organ with an uneven surface. The free ends of the gland are directed cranially and slightly laterally, and they lie in the genital fold, which is dorsal to the bladder. The terminal segments of the ureters and deferent ducts are positioned between the left and right glands. The body of prostate gland is bilobed and lies transversely on the dorsal surface of the urethra, just caudal to the vesicular gland.

The disseminated part surrounds the urethra completely. The bulbourethral gland consists of two round-shaped, independent lobes. They lie on the dorsal surface of the urethra opposite to the ischiatic arch and are covered by the proximal part of the thick

bulbospongiosus muscle. Volumes of these four accessory reproductive organs are recorded in Table 2. The difference in the volumes of accessory reproductive organs between October to December and April to June was not significant without bulbourethral gland (Fig. 7). In the bulbourethral glands, the difference in volume was shown difference significantly between these two periods.

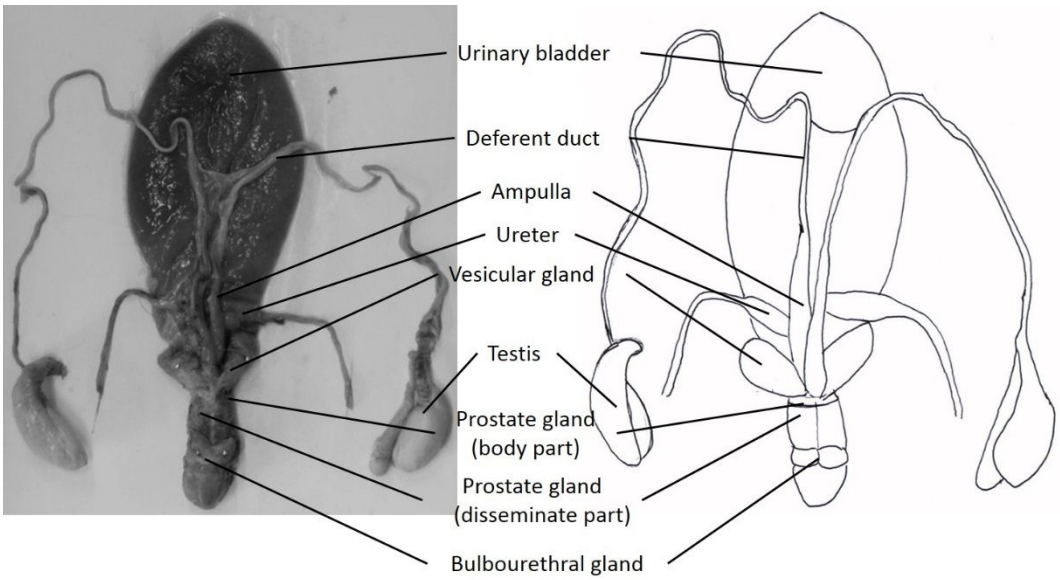

Fig. 6. Photograph (left) and schematic drawing (right) of the male reproductive organs in the Korean water deer.

Table 2. The volume of accessory reproductive organs in the Korean water deer

Specimen number	Collected date	Volume of ampulla gl. (cm^3)	Volume of vesicular gl. (cm^3)	Volume of prostate gl.-1 (cm^3)	Volume of prostate gl.-2 (cm^3)	Volume of bulbourethral gl. (cm^3)
KJ0572	2010-10-30	1.52	3.25	0.92	8.81	1.38
KJ0573	2010-11-03	2.02	2.02	2.04	11.99	0.56
KJ0574	2010-11-05	4.17	1.74	0.34	18.17	1.01
KJ0576	2010-11-29	4.99	4.39	0.54	12.36	1.76
KJ0577	2010-12-01	2.15	6.51	0.74	13.67	1.50
KJ0578	2010-12-05	2.03	4.79	0.89	9.89	1.52
KJ0582	2010-12-03	3.66	3.72	0.73	19.40	2.24
KJ0583	2010-12-03	2.44	4.92	0.45	10.50	1.92
KJ0585	2010-12-16	2.49	4.84	0.82	17.74	2.54
KJ0589	2010-12-19	2.02	2.09	0.46	17.87	1.41
KJ0590	2010-12-19	2.86	3.48	0.58	18.47	1.48
KJ0592	2010-12-24	4.03	4.52	1.42	15.45	1.39
KJ0594	2011-04-01	1.12	4.15	0.24	10.91	1.17
KJ0595	2011-05-18	2.12	3.33	0.53	14.93	0.83
KJ0597	2011-05-18	1.75	5.45	0.71	11.72	1.08
KJ0599	2011-06-18	2.01	5.73	1.02	16.23	0.49

* Volume of accessory reproductive organs (ampulla, vesicular, prostate (body, disseminate part, bulbourethral) was calculate as follows: $V = 4/3\pi abc$ (a: long axis/2; b: short axis/2, c; width/2 of accessory reproductive organs). Prostate gl.-1; body part, prostate gl.-2; disseminate part.

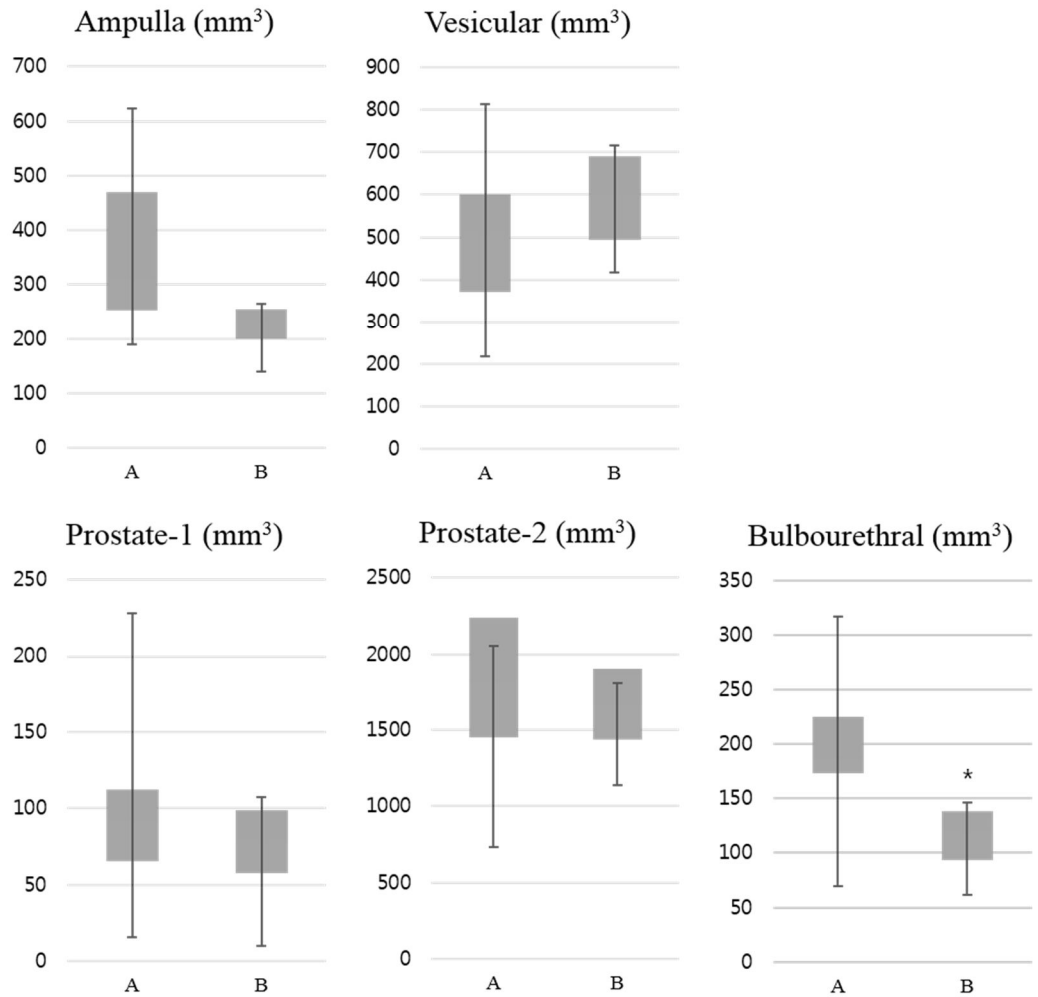

Fig. 7. The comparison of the volume (mm^3) of accessory reproductive organs (ampulla, vesicular gland, prostate gland-1 and 2, bulbourethral gland) in the two periods (A; October to December, B; April to July). *$p<0.05$, Boxes range the standard deviations and whiskers indicate the highest and lowest values. (prostate gland-1; body part, prostate gland-2; disseminate part)

Histological observation

In the period of October to December, the luminal secretions from secretory epithelia of all accessory reproductive organs were observed, but not in the periods of April to July. The epithelia of the secretory alveoli were lined by single columnar cells in the ampulla and by single cuboidal cells in the whole parts of prostate glands in the both periods of October to December and April to July. In the bulbourethral and vesicular glands, the epithelia of the secretory alveoli in the period of October to December were lined by single cuboidal cells and in the period of April to July by single columnar cells. The luminal secretion were observed in the all accessory glands in the period of October to December (Fig. 8).

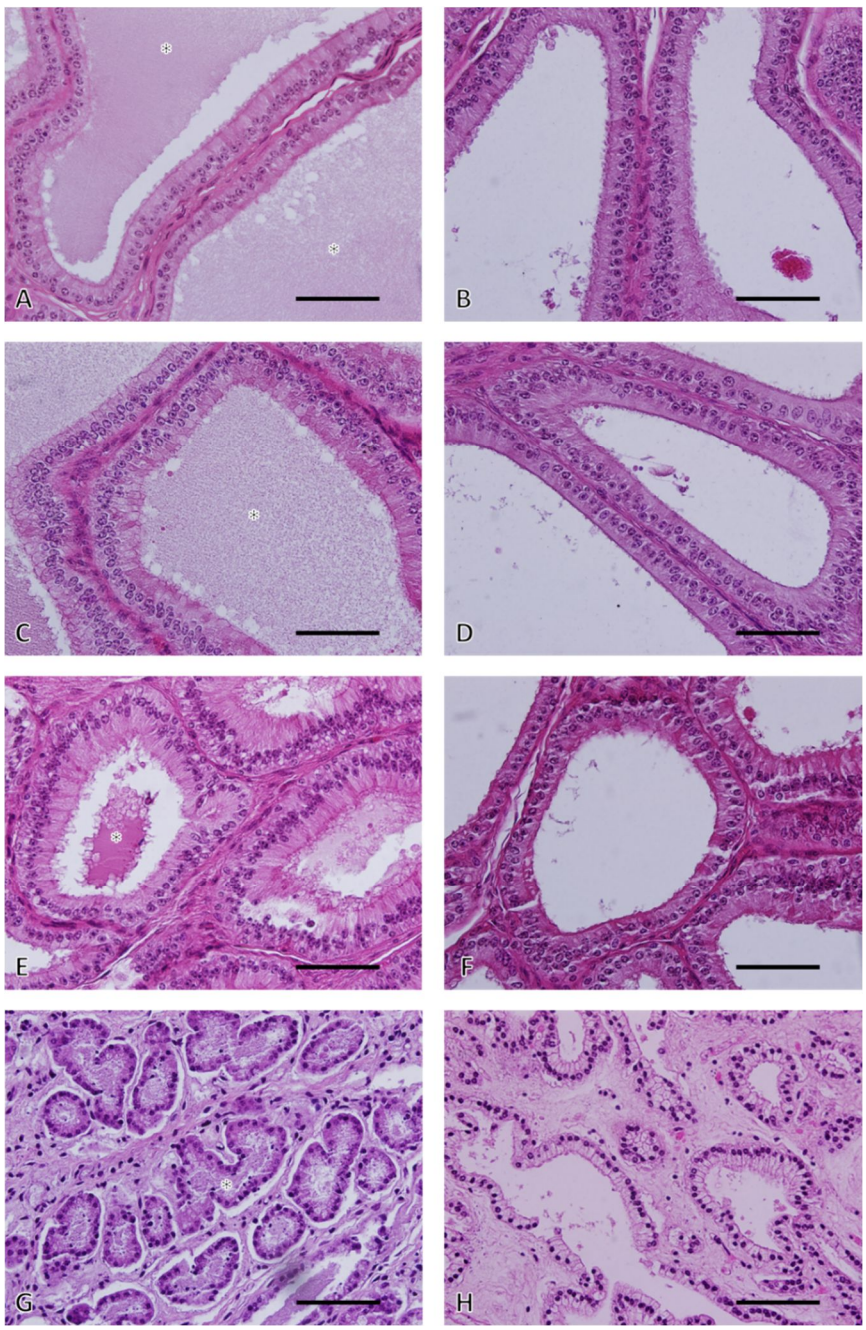

Fig. 8. Histological views of the accessory reproductive organs of the water deer. (A, B: ampulla, C, D: vesicular gland, E, F: prostate gland, G, H: bulbourethral gland) (A, C, E, G: October to December, B, D, F, H: April to July) (H&E stain) Note the luminal secretion in these four glands on October to December (*) Scale bar = 25μm

Discussion

There have been very few anatomical papers on the Korean water deer only describing the skull structure (Jung *et al*., 2004; Kim *et al*., 2013a; Kim *et al*., 2013b) and the branching pattern of aortic arch (Ahn *et al*., 2008). This is the first report in the anatomy of the reproductive organ in Korean water deer. The Korean water deer has the same four typical accessory reproductive glands as other ruminant species (Dyce *et al*., 2010). Their shapes and histological structures also resemble other small ruminant species, including goats and sheep (Bacha and Bacha, 2000; Banks, 1993; Eurell *et al*., 2006). Many cervid species of Northern temperate origin, such as the roe deer (*Capreolus capreolus*), show seasonal production of spermatids (Lincoln, 1992). In the case of Sika deer (*Cervus nippon*) which inhabits in Japan, the spermatogenic process occurs in July or August and its activity reaches its peak in late October. In February or March, termination of the spermatogenesis was observed (Suzuki *et al*., 1992). According to the results obtained from this study, the spermatogenesis of the water deer also occurs from October to January.

Seasonal changes in size and weight of the accessory genital organs were only noted in the bulbourethral glands. The histological study showed differences in secretory activities between October to December and April to July based on the existence of luminal secretions. It has been known that the functional activity of the most of male accessory glands are testosterone dependent. Because of the reduction of the size and weight of the testis during April to July, the secretory activities of the bulbourethral glands may decline.

These finding suggests that the breeding season of this animal is from October to December, when the male reproductive functions are most active. The histological characteristics of testis including the classification of the seminiferous epithelial cycle (Hayakawa *et al*., 2009) and immunohistochemical localization of steroidogenic enzymes (Hayakawa *et al*., 2010) have been clearly demonstrated in Sika deer which inhabit in Japan. Further histological and/or immunohistological studies are needed to elucidate the mechanism of the reproductive seasonality of the Korean water deer. The localization of cytoskeletal proteins in testes and epididymis will be clarified in the following chapter.

Chapter 2.

Immunolocalization of cytoskeletal proteins in the testes of two Asian cervids: Korean water deer (*Hydropotes inermis argyropus*) and Reeves' muntjac (*Muntiacus reevesi*)

Introduction

Macroscopic and histological observations of the male reproductive organs of the Korean water deer have been performed and demonstrate that the Korean water deer possesses the same four typical accessory reproductive glands as other ruminant species. Seasonal changes in size and weight of the testis were also clearly noted and the breeding season of the animal has been shown to be from October to December, when the male reproductive functions are most active (chapter 1 of this thesis, Sohn and Kimura, 2012).

The cytoskeleton proteins are comprised of microfilaments (actins), intermediate filaments (desmin and vimentin), microtubules (tubulin), neuro-filament proteins, glial fibrillary acidic proteins and cytokeratin. Their roles in cellular structure and function include maintaining cell shape and polarity, positioning of intracellular organelles, forming of cytoplasmic extensions, and anchoring of organelles to the plasma membrane. The localization of testicular cytoskeletal proteins in several species has been immunohistochemically examined including pig (van Vorstenbosch *et al*., 1984), sheep (Steger and Wrobel, 1994), buffalo (Cruzana *et al*., 2006), cow (Devkota *et al*., 2006), lesser mouse deer (Sasaki *et al*., 2010), camel (Rodriguez *et al*., 1999), Japanese black bear (Komatsu *et al*., 1998) and rat (Zhu *et al*., 1997) to elucidate the functional roles of the cytoskeletal proteins. These studies revealed that the testicular localization of each cytoskeletal protein was different among species and the distribution pattern changed with testicular development. However, studies on the testicular cytoskeletal proteins in Cervids have not been reported. To understand the general characteristics in the cervid, the Reeves' muntjac was also studied as the comparison.

Reeves' muntjac is one of the muntjac species and can be found in southeastern

China and Taiwan as their habitat. The males have antlers and their upper canines are apparent and protrude from the side of their mouths. This species was introduced into Japan as an exhibition animal and naturalized in the southern part of Chiba prefecture between the 1960s and 1980s (Asada *et al*., 2000), and onto Izu-oshima island in 1970 (Ohdachi *et al*., 2009). In the genus Muntjac, some studies on their reproduction and growth have been performed (Chaplin, 1976; Chapman *et al*., 1984; Chapman *et al*., 1985; Chapman and Harris, 1991).

The male breeding season in the Japanese population of Reeves' muntjac has not been clearly reported, however, the parturition has been observed throughout the year in Chiba prefecture, Japan (Ohdichi *et al*., 2009).

In this study, therefore, the distribution of cytoskeletal proteins in the testes of the Korean water deer and Reeves' muntjac were examined immunohistochemically to gather basic reproductive information for wildlife management and conservation for these species.

Materials and Methods

Testes and epididymides collected from five adult Korean water deer and three adult Reeves' muntjac were used in this study. Samples from road-killed Korean water deer were obtained within the period of late September to December, 2011, in Korea. Three Reeves' muntjacs were culled for the purpose of population control in Chiba prefecture, Japan, in March, 2005. The testes and epididymides of these animals were fixed in 10% formalin. The samples were transferred to 70% ethanol, dehydrated in graded series of ethanol, cleared in xylene, and embedded in paraffin.

Tissue samples were cut serially at 4 μm thickness and placed on silane-coated slides (5116-20F, Muto Pure Chemicals Co., Ltd., Tokyo, Japan). Deparaffinized sections were used for hematoxylin and eosin (H&E) and stained immunohistochemically using the avidin-biotin peroxidase complex (ABC) methods (Hsu *et al*., 1981). In this study, each process was treated in a microwave processor (MI-77, AZUMAYA, Tokyo, Japan). For the antigen retrieval, the slides were placed horizontally on a turntable, and incubated for 15 min at 97 ℃ in the retrieval buffered solution (Target retrieval solution high pH,

S3307, DAKO, Glostrup, Denmark). The slides were then immersed in methanol containing 0.3% H_2O_2 for 10 min at room temperature to block the endogenous peroxidase activity. To prevent nonspecific staining, normal goat serum (1:50, S-1000, Vector Laboratories., Burlingame, CA, USA) were applied to the specimens. The sections were then irradiated for 20 min with each primary antibody in the microwave followed by the instruction manual of the microwave processor. The primary antibodies used in this study were the following: monoclonal anti-porcine stomach desmin in mouse (1:50, code M724, DAKO, Glostrup, Denmark), polyclonal anti-calf lens vimentin in rabbit (1:100, code VIP, MEDAC, Hamburg, Germany), monoclonal anti-human α-smooth muscle actin (α-SMA) in mouse (1:1,000, Clone 1A4, Sigma Chemical Co., St. Louis, MO, USA) (Devkota *et al*., 2006; Harikae *et al*., 2012). For secondary antibodies, the sections with biotinylated anti-rabbit IgG in goat (1:200, BA-1000, Vector Laboratories Inc.) or biotinylated anti-mouse IgG in goat (1:200, BA-9200, Vector Laboratories Inc.) were irradiated for 5 min, and then the sections were incubated with ABC-kit (1:2, PK-6100, Vectastain Elite ABC kit, Vector laboratories) for 5 min. The binding sites were visualized with tris-HCl buffer (pH 7.4) containing 0.02% 3, 3'-diaminobenzidine

hydrochloride (DAB) and 0.006% H_2O_2. After incubation, the sections were washed with 0.01 M phosphate buffered saline (PBS, pH7.4), dehydrated in graded series of ethanol, cleared in xylene and coverslipped. All images were taken by a BX51 light microscope (Olympus, Tokyo, Japan) equipped with a digital camera (DP71, Olympus) connected to a computer monitor.

Result

The testes and epididymides of both Korean water deer and Reeves' muntjac possess general histological structures that are common in most mammals. The elongated spermatids in the clearly opened lumen of the convoluted seminiferous tubules and the sperm atozoa in the deferent ducts were recognized in all specimens used in this study. In addition, the polynuclear spermatids and the exfoliation of spermatocytes into the lumen were not observed (Fig. 9). These observations obviously indicated that the active spermatogenesis was performed in these specimens. Desmin was immunohistochemically detected in the myoid cells of the seminiferous tubules (peritubular myoid cells) and walls of blood vessels in the testis of Korean water deer and Reeves' muntjac. The sub-epithelial myoid cells of epididymal ducts also showed the immunoreactivity for desmin (Table 3, Fig. 10C, 10D). The immunoreactivity of vimentin in the Korean water deer and Reeves' muntjac was shown in the peritubular myoid cells and perinuclear region of the Sertoli cells (Table 3, Fig. 11A, 11B). In the Leydig cells, the immunoreactivity of vimentin was detected in both species (Table 3, Fig. 11A, 11B). Vimentin

immunoreactivities were also shown in the sub-epithelial myoid cells of the epididymal ducts and the stromal cells among the ducts (Table 3, Fig. 11C, 11D). An intense immunoreactin for α-SMA in two species was restricted to the smooth vascular muscle cells and peritubular myoid cells which surrounded the seminiferous tubules, in the testes (Table 3, Fig. 12A, 12B). The sub-epithelial myoid cells of the epididymal ducts also showed immunoreactivity for α-SMA.

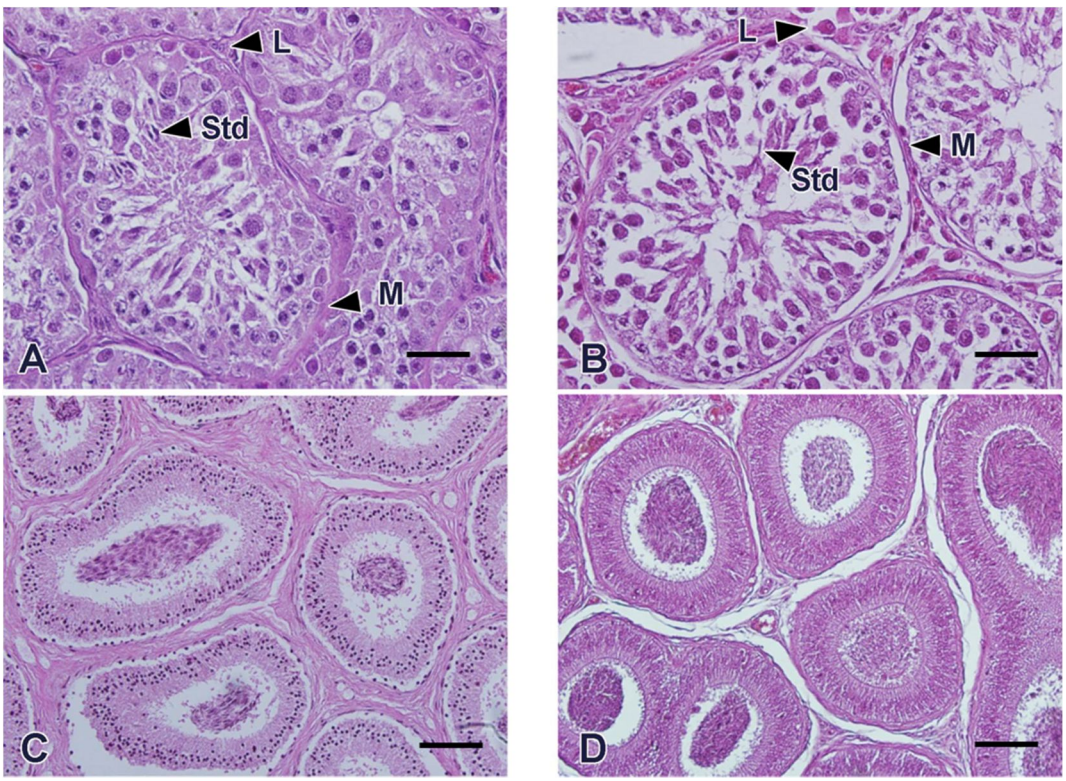

Fig. 9. Histological view of the testis and epididymis of the Korean water deer (A, C) and Reeves' muntjac (B, D).

A, B: Elongated spermatids in the lumen of the convoluted seminiferous tubules in the testis (H&E staining). Scale bar = 25 μm

C, D: Spermatozoa in the lumen of the epididymis ducts (tail) (H&E staining). Scale bar = 50 μm

Std: Spermatid, L: Leydig cells, M: peritubular myoid cells.

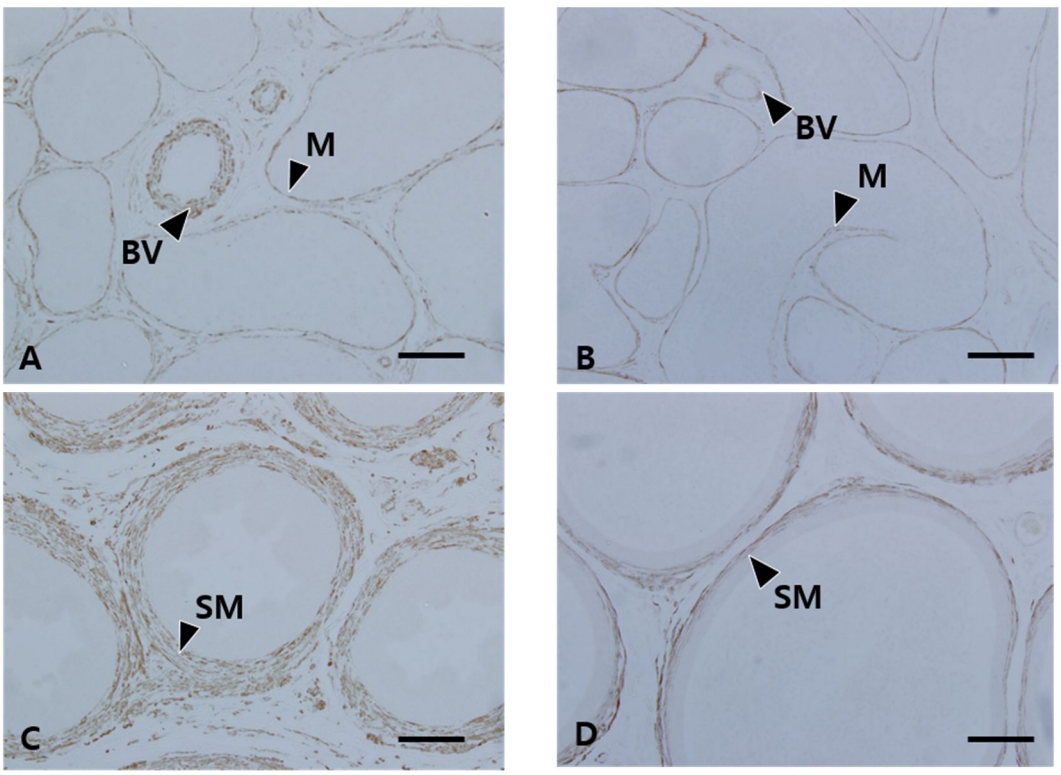

Fig. 10. Immunolocalization of desmin in the testis and epididymis of the water deer (A, C) and muntjac (B, D).

A, B: In the testis, desmin was found in the peritubular myoid cells (M) and the walls of blood vessels (BV). Scale bar = 50 μm.

C, D: immunoreactivity for desmin was detected in the sub-epithelial myoid cells (SM) of epididymal ducts. Scale bar = 25 μm.

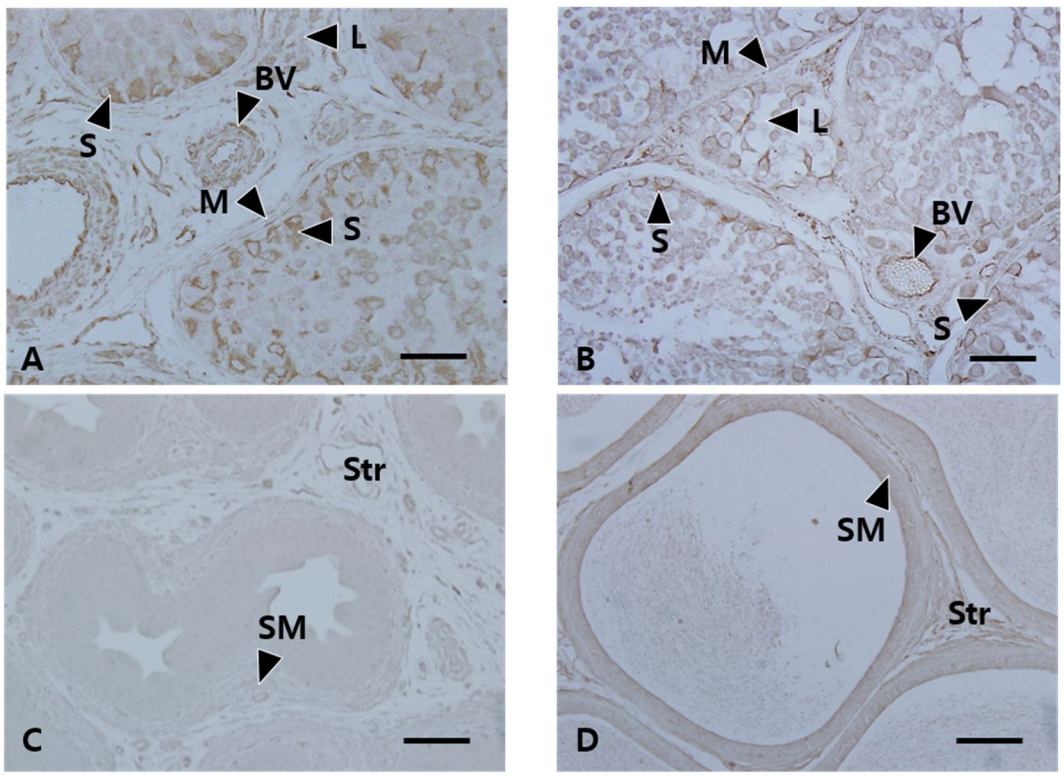

Fig. 11. Immunolocalization of vimentin in the testis and epididymis of the water deer (A, C) and muntjac (B, D).

A, B: Vimentin was found in the Sertoli cells (S), Leydig cells (L), peritubular myoid cells (M), and the walls of blood vessels (BV). Scale bar = 25 μm.

C, D: Vimentin was expressed in the sub-epithelial myoid cells (SM) of epididymal ducts and the stromal cells (Str) among the ducts. Scale bar = 50 μm.

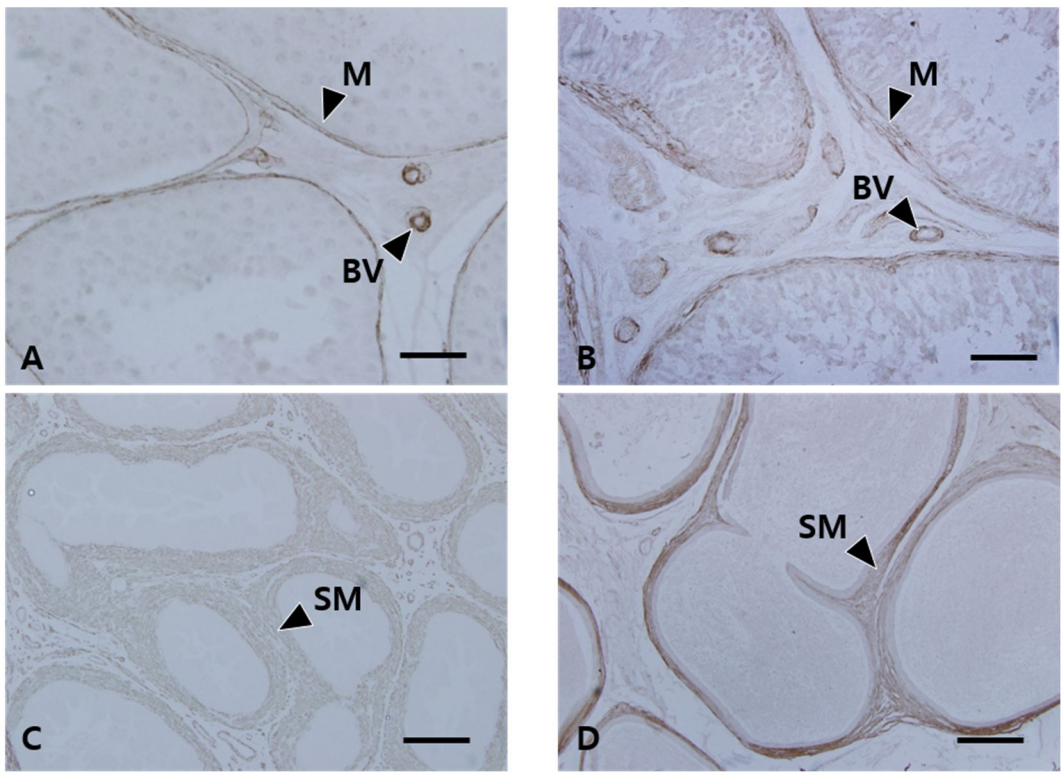

Fig. 12. Immunolocalization of α-smooth muscle actin (α-SMA) in the testis of the water deer (A, C) and muntjac (B, D).

A, B: In the testis, α-SMA was found in the peritubular myoid cells (M), and the walls of blood vessels (BV). Scale bar = 25 μm.

C, D: Immunoreactivity for α-SMA was detected in the sub-epithelial myoid cells (SM) of epididymal ducts. Scale bar = 50 μm.

Table 3. Summary of the immunohistochemical localization of the testis and epididymis in the Korean water deer and Reeves' muntjac.

	Testis				**Epididymis**	
	Peritubular myoid cells	Blood vessels	Sertoli cells	Leydig cells	Sub-epithelial myoid cells	Stromal cells
Desmin	++ / ++	+ / +	- / -	- / -	+ / +	- / -
Vimentin	+ / +	++ / ++	++ / ++	+ / +	++ / ++	+ / +
α-SMA	++ / ++	+ / +	- / -	- / -	+ / +	- / -

Korean water deer / Reeves' muntjac, -: negative, +: positive, ++: vividly positive reactions

Discussion

There have been very few papers on anatomical observation of the Korean water deer and Reeves' muntjac. The male genital organs (Sohn and Kimura, 2012), the skull structure (Jung *et al*., 2004; Kim *et al*., 2013a; Kim *et al*., 2013b), the rumen structure (Hofmann *et al*., 1988) and the branching pattern of the aortic arch (Ahn *et al*., 2008) have been anatomically studied in the Korean water deer. In the genus Muntjac, the parotid and mandibular gland (Adnyane *et al*., 2010), lingual papillae (Adnyane *et al*., 2011a; Zheng and Kobayashi, 2006), endocrine cells in the gastrointestinal tract (Adnyane *et al*., 2011b), Harderian and nictitans glands (Rehorek *et al*., 2007) and facial scent gland (Barrette, 1976) have been anatomically and histologically observed. However, this is the first report on the immunohistochemical localization of the cytoskeletal proteins in the testes of the Korean water deer and Reeves' muntjac.

The present immunohistochemical study demonstrates a pattern of localization of cytoskeletal proteins, including desmin, vimentin and α-SMA in the testis and epididymis of the Korean water deer and Reeves' muntjac. This localization pattern

suggests that the contents of the cytoskeletal proteins in the Korean water deer and Reeves' muntjac have a similar structure in the testis and the epididymis. Desmin was not expressed in the testicular cells, such as the Sertoli cells, spermatogenic cells and Leydig cells. In several mammals, desmin was found in the peritubular myoid cells; for example in the sheep (Steger and Wrobel, 1994), water buffalo (Cruzana *et al*., 2006), Japanese black bear (Komatsu *et al*., 1998) and rat (Frojdman *et al*., 1992; Virtanen *et al*., 1986). In the lesser mouse deer, however, desmin was detected only in the wall of blood vessels (Sasaki *et al*., 2010). In this study, the immunoreactivity for desmin was detected in the peritubular myoid cells, blood vessels and the sub-epithelial myoid cells of the epididymal ducts. From this result, the pattern of the desmin was shown to be different between two Asian cervids (Korean water deer and Reeves' muntjac) and mouse deer (*Tragulus javanicus*). The desmin localization pattern in the mouse deer belonging to infraorder Tragulinae may be characteristic among ruminants. In the testes of the Korean water deer and Reeves' muntjac, the immunoreactivity for vimentin was detected in the Sertoli cells, peritubular myoid cells and Leydig cells. This finding was also reported in the rhesus monkey (Zhang *et al*., 2004), rat (Aumuller *et al*., 1992; Wang *et*

al., 2002; Zhu *et al*., 1997), human (de Miguel *et al*., 1997; Maymon *et al*., 2000; Zhu *et al*., 1997), South American camelids (Amlani and Vogl, 1988), sheep (Steger and Wrobel, 1994), bull (Wrobel *et al*., 1995) and lesser mouse deer (Sasaki *et al*., 2010). Therefore, this localization pattern might be common in mammals.

Alpha-SMA, in the Korean water deer and Reeves' muntjac, was shown in the peritubular myoid cells and the wall of blood vessels of the testis and in the sub-epithelial myoid cells of the epididymal ducts. The peritubular myoid cells of the testis appeared in desmin, vimentin and α-SMA. The peritubular myoid cells play an important role in contractions of the seminiferous tubules to transport spermatozoa and testicular fluid (Maekawa *et al*., 1996; Miyake *et al*., 1986). Therefore, desmin, vimentin and α-SMA may be related to the myoid cell contraction. Likewise, cytoskeletal proteins were detected in the sub-epitherial myoid cells of the epididymal ducts. Therefore, these three cytoskeletal proteins might play collaborative roles in the epididymis. In the present study, the localization of the cytoskeletal proteins in the testis and epididymis of the Korean water deer and Reeves' muntjac was first identified.

These results suggest that the immunolocalization of cytoskeletal proteins in both animals have similar patterns in the testis and epididymis. This localization pattern might be common in the cervid species. These results will contribute to the conservation of management of these two Asian cervid species. Comparison of immunolocalization of cytoskeletal proteins to that in other cervidae species including the Sika deer (*Cervus nippon*) will be necessary in future studies.

The contents of this chapter has been published in Sohn *et al.*, 2013.

General conclusion

According to the results obtained from this study, the spermatogenesis of the water deer occurs from October to January. Although the histological study in the accessory genital organs showed differences in the secretory activities as evidence of luminal secretions between the two seasons. Seasonal changes in size and weight were only noted in the bulbourethral gland. There finding suggests that the breeding season of this animal is from October to January, when the male reproductive functions are most active.

In the immunohistochemistry, desmin, vimentin and α-SMA were appeared in the peritubular myoid cells of testis. This means desmin, vimentin and α-SMA might play a role in the myoid cell by their contraction for the seminiferous tubules for the transportation of spermatozoa and testicular fluids. In epididymis, cytoskeletal proteins were detected in the sub-epididymal myoid cells of epididymal ducts. These proteins might be play an important role in the epididymis. The location of cytoskeletal proteins were similar between Korean water deer and Reeves' muntjac. However, the existence

of desmin in peritubular myoid cells in the testis is varied among species.

The information obtained in this study regarding the reproductive morphology in the Korean water deer is the first report and there evidences may contribute to the conservation and management of this species.

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국문초록

고라니(*Hydropotes inermis*)는 우리나라와 중국의 고유종인 사슴과(Cervidae)의 동물로 우리나라의 일부 지역에서는 그 수가 과잉상태이다. 이들의 보존과 관리를 통한 개체 수 조절을 위해, 3년에 걸쳐 수컷 생식기관을 대상으로 고라니의 기본적인 생식에 관한 데이터를 육안해부학적, 조직학적, 그리고 면역조직학적 관찰을 실시하였다. 고라니 수컷은 고환과 덧생식샘(정관팽대, 정낭샘, 전립샘, 망울요도샘)의 위치와 모양이 다른 소형 반추류와 비슷하였다. 고환의 크기와 무게는 10월부터 증가하였으며, 2월부터는 감소됨을 관찰하였다. 정자형성은 10월부터 1월까지 이루어졌다. 이를 통해 고라니의 번식기간이 10월부터 1월까지임을 확인 할 수 있었다. 고라니와 같은 사슴과 동물인 Reeves' muntjac을 비교 종으로 이용하여 고환과 부고환의 세포골격 단백질을 면역조직학적 방법으로 확인하였다. 세포골격단백질의 분포양상은 두 종에서 비슷하였다. 이 결과로부터 세포 골격 단백질의 분포는 다른 사슴

과 동물들과 공동된 것임을 확인할 수 있었다. 본 연구를 통한 결과들은 한국고라니의 보존과 관리에 있어 기본적인 데이터가 될 것이다.

Keywords: 한국고라니, *Hydropotes inermis argyropus,* 해부학, 생식기관, 야생동물, 세포골격 단백질

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