Male reproductive characteristics and genetic polymorphism of feral raccoons (*Procyon lotor*) in Hokkaido

（　北海道に生息する外来種アライグマ(*Procyon lotor*)における
雄の繁殖特性および遺伝多型に関する研究　）

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CONTENTS

ABBREVIATIONS

3 βHSD	3 β-hydroxysteroid dehydrogenase
AR	Androgen receptor
BL	Body length
BW	Body weight
EP	The extrusibility of the penis
EP＋	An extrusible penis
EP－	An penis with the preputia remaining
FSH	Follicle-stimulating hormone
LH	Luteinizing hormone
MOE	Ministry of the Environment
mtDNA	Mitochondrial DNA
P450arom	Aromatase cytochrome P450
P450c17	17-α hydroxylase cytochrome P450
P450scc	Cholesterol side-chain cleavage cytochrome P450
Plasma T	Plasma testosterone concentration
SS	Spermatogenetic score
STD	Seminiferous tubule diameter
Tissue T	Testicular tissue testosterone concentration
TS	Testis size
TW	Testis weight

PREFACE

Raccoons in Hokkaido

The raccoon (*Procyon lotor*) (Fig. I) is a mammal indigenous to North America. It has become feral in Japan as a result of escaping from captivity and unscrupulous releases since the first report of raccoon naturalization in Inuyama, Aichi Prefecture in 1962 [Ando and Kajiura 1985]. In Hokkaido, escapes and unscrupulous releases of pet raccoons have been reported since the 1970s, and feral raccoons have been increasing in number and spreading throughout Hokkaido [Asano et al. 2003a, Ikeda 1999]. In the early 1990s, inhabitation of feral raccoons had been detected in only limited regions, however by 2013, their habitat has been distributed in 144 out of 179 municipalities (80.4%) in Hokkaido (Fig. II). There may be some reasons for this increase and wide-spreading, such as omnivorous behavior, adaption to cold weather, absence of predators and high reproductive potential [Ikeda 1999]. Until now, an eradication program has been conducted by Hokkaido government, Ministry of the Environment (MOE), each municipalities and agricultural cooperative society under the Invasive Alien Species Act, which came into force in 2005 by MOE as a aim to ensure biodiversity conservation in ecology, maintain biological safety of the human lives and health, and contribute the development of agriculture, forestry and fisheries industry. By 2009, the number of raccoons captured under eradication program reached more than 4,000 every year in Hokkaido (Fig. III); however, control has not been successful yet. Difficulty in controlling the population may be partly due to a lack of basic biological information about raccoons.

To decrease the raccoon population size efficiently, it is necessary to gather

basic biological information: Particularly to understand the reproductive characteristics in the habitat, is crucial [Kato et al. 2009]. And it is also important to infer dispersing process and to reveal population units with geographical or genetic barrier for effective management. This whole study was performed to elucidate one aspect of reproductive physiology and genetic background of feral raccoons in Hokkaido.

MALE REPRODUCTIVE CHARACTERISTICS

Chapter 1 Puberty and Seasonal breeding

Background

Raccoons are promiscuous seasonal breeders. In Hokkaido, the mating season for raccoons is from January to March, and considering an approximately 63-day gestation, litters are born between March and May [Asano et al. 2003b], which is similar to the timing in North America [Johnson 1970, Sanderson and Nalbandov 1973, Stuewer 1943].

Female reproductive physiology has been reported in North America, native habitat and also in Japan, introduced area. In west central Hokkaido, 66% of juveniles and 96% of yearlings and adult females get pregnant [Asano et al. 2003b], indicating that the majority of females reach puberty during the first mating season of their life. Age at the onset of puberty is critical for determining individual lifetime reproductive success, and ultimately, age at puberty influences population growth [Bronson and Rissman 1986]. Therefore, the timing of puberty is an essential factor affecting population growth [Oli and Dobson 2003]. In reproductive seasonality, late-born pups have been detected in Hokkaido [Asano et al. 2003b]. In North America, it has been reported that some females that failed to get pregnant or lost their pups can reproduce again after April, although they basically reproduce once a year during the mating season [Berard 1951, Gehrt and Fritzell 1996, Sanderson and Nalbandov 1973, Stuewer 1943]. These reports demonstrated seasonal flexibility in female raccoon reproduction.

Understanding female reproductive physiology, which can directly help to

explain number increasing and population growth, are considered to be an important element in wildlife management. On the other hand, male reproductive physiology is sometimes unregarded. In many species, however, male individual dispersal, home range or migrating pattern can be affected by sexual maturity or seasonal reproductive behavior [Edelman and Koprowski 2006, Fritzell 1978a, Greenwood 1980]. Thus, investigating male reproductive physiology is also essential to control invasive alien species whose habitat has been still dispersing. So far, few studies focusing on reproduction in male raccoons were done in Japan, therefore, in this Chapter, the author clarified the timing of puberty and seasonal reproduction in male raccoons in Hokkaido.

Sample collection

Animals

The author collected carcasses of feral male raccoons that were euthanized in the eradication program in west-central Hokkaido from 2008 through 2011 (Fig. 1-1). In total, 219 male raccoons were employed for this study. Body weights (BW) and body lengths (BL) were measured, and the extrusibility of the penis (EP) was checked. The left testis was weighed, and the long diameter, short diameter and thickness were measured; the testis and epididymis were then immediately fixed for about 12 h in Bouin's solution for histological observation. Blood was placed in a 5 ml heparin tube and spun at 1,050 g for 10 minutes. The plasma was removed and stored at −30°C until assay. The lower jaws were boiled, and then the lower canine teeth were removed for age determination.

The author also used a captive juvenile and a 5-year-old adult raccoon kept

at the Asahiyama Zoological Park and Wildlife Conservation Center, Asahikawa, Hokkaido (Fig. 1-1). The author collected samples once a month from December 2008 to March 2010 under anesthesia using the following injectable anesthetics administered intramuscularly: 0.3 mg/kg midazolam (Dormicum, Astellas, Tokyo, Japan), 80 μg/kg medetomidine hydrochloride (Domitor, Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan), and 3.0 mg/kg ketamine hydrochloride (Ketalar, Daiichi Sankyo Co., Ltd., Tokyo, Japan). At the time of collection, the raccoon was weighed, testis size including the preputium was measured, 10 ml blood was collected from the jugular vein in heparin tubes, and testes were biopsied for samples of approximately 4 mm^3. Biopsies alternated between the left and right testis each month, i.e., each testis was sampled bimonthly to minimize discomfort due to repetitive surgical trauma. Testicular samples and blood were treated in the same way as in the feral raccoons. All samplings were performed using methods approved by the Animal Care and Use Committee of Hokkaido University (approval No: JU9129).

Age determination

Feral raccoons were categorized into juveniles (0 years old), yearlings (1 year old), subadults (2 years old) and adults (over 3 years old) by examining their body size, tooth eruption and root foraminal closure of the canine teeth [Grau et al. 1970, Sanderson 1961]. In the yearlings, subadults and adults, age was determined by the number of cementum annuli of the canines [Grau et al. 1970, Hachiya and Ohtaishi 1994]. In Hokkaido, most litters are born between March and May [Asano et al. 2003b]; therefore, in this study, all raccoons were assumed to have been born on 1 April, and age was evaluated accordingly.

Section 1 Seasonal changes of spermatogenetic activity in adult males

Introduction

In many seasonally breeding mammals, males exhibit a seasonal cycle in reproduction, with spermatogenesis and testicular steroidogenesis limited to a specific period of the year [Tsubota et al. 1997]. For example, raccoon dogs (*Nyctereutes procynoides*) [Qiang et al. 2003] and Hokkaido brown bears (*Ursus arctos yesoensis*) [Tsubota and Kanagawa 1989] can produce mature spermatozoa around the mating season; however, testicular function significantly declines during other periods, and spermatozoa cannot be observed in the nonbreeding season. The reduction in testicular function in the nonbreeding season can prevent energy loss because producing spermatozoa in the absence of rutting females in the nonbreeding season has little meaning. Raccoons are also seasonally breeding mammals, so it could be thought that male testicular function declines during the nonbreeding period. However, the existence of seasonally late-born pups suggests that some raccoon males can produce spermatozoa after the mating season. Moreover, the conclusions concerning whether or not male raccoons have seasonal testicular changes have varied among studies [Kaneko et al. 2005, Sanderson and Nalbandov 1973, Stuewer 1943]. In North America, differing rates of spermatozoa presence in summer, the nonbreeding season, have been reported, which suggests that male raccoons may have different breeding cycles according to habitat area or surrounding environment. However, few details are known in Hokkaido raccoons.

The aim of this study was to clarify reproductive characteristics of male

raccoons in Hokkaido by examining the seasonal changes in spermatogenesis and peripheral testosterone concentration in both feral and captive raccoons.

Methods

Histology

Testicular tissues were dehydrated in an ethanol series, embedded in paraffin wax, sectioned at a thickness of 4 μm and stained with hematoxylin-eosin. Four fields were chosen from the entire testis under microscope using low (×100) and high (×400) magnification, and from each, ten seminiferous tubules were chosen randomly. The mean diameter of the seminiferous tubules was measured by micrometer and image analysis software (ImageJ, W.S. Rasband, U.S. National Institutes of Health, Bethesda, MD, USA). In the captive raccoon, 10 seminiferous tubules were chosen randomly from one or two fields.

Evaluation of spermatogenesis

Spermatogenetic Score (SS) was evaluated as the mean value of each seminiferous tubule chosen, as described above, according to a score based on the most advanced spermatogenetic cells present [Kaneko et al. 2005]: SS 1, spermatogonia; SS 2, no cells further than primary spermatocytes; SS 3, some cells further than secondary spermatocytes; SS 4, round spermatids; and SS 5, elongated spermatids and/or spermatozoa (Fig. 1-2).

In feral raccoons, the presence level of spermatozoa in the cauda epididymis was evaluated. It was calculated using image analysis software (ImageJ), from the

mean value of ten ducts of the cauda epididymis chosen randomly under low (×100) magnification with a microscope. The criteria were as follows: Level 0, no spermatozoa; Level 1, spermatozoa observed in less than 10% of the duct lumen; Level 2, spermatozoa observed in 10-50% of the duct lumen; and Level 3, spermatozoa observed in more than 50% of the duct lumen (Fig. 1-3).

Enzyme immunoassay

Each month, up to 5 raccoon samples were chosen randomly. Plasma testosterone concentrations (Plasma T) were measured by testosterone assay (enzyme immunoassay) [Iibuchi et al. 2010]. Testosterone-3-CMO-HRP (FKA101, Cosmo Bio, Tokyo, Japan) was diluted 600,000-fold with assay buffer. Standard testosterone (Cayman, Ann Arbor, MI, USA) was diluted in the 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 Co., Tokyo, Japan) was used as the secondary antibody. The minimum detectable level of testosterone was 4.9 pg/well, and the intra- and inter-assay coefficients of variation were 10.5% and 15.7%, respectively.

Statistical analysis

Because the number of feral raccoon samples from November to February was small, the data were compared among the four seasons as follows: spring (April–June), summer (July–September), autumn (October–December) and winter (January–March). In the feral raccoons, testis weight (TW), seminiferous tubule diameter (STD), SS, presence level of spermatozoa in the cauda epididymis and Plasma T were analyzed using a one-way ANOVA and Scheffe's F test for pairwise

comparison. All values are presented as the mean ± SEM and were calculated using Microsoft Excel 2003 for Windows (Microsoft, Redmond, WA, USA). Because the data under captivity were obtained from one individual continuously, statistical analysis was not performed.

Results

Feral raccoons

In the 68 adult raccoons over 3 years old, the monthly BW increased from September (6.2 ± 0.4 kg) to November (10.7 kg), decreased gradually from December (10.0 kg) to the following March (6.5 ± 0.6 kg) and did not change remarkably from March to September (Table 1-1). As for spermatogenetic evaluation, SW, STD, SS and presence levels of spermatozoa in the cauda epididymis changed among the seasons (Fig. 1-4). TW exhibited a maximum of 11.3 ± 0.4 g, in winter, and decreased to a minimum of 7.3 ± 0.4 g, in summer (Fig. 1-4A). STD increased beginning in summer, with a minimum diameter of 196.9 ± 4.5 μm, and exhibited a maximum diameter of 225.6 ± 7.2 μm in autumn (Fig. 1-4B). SS also increased beginning in summer, with a minimum score of 3.6 ± 0.2, and exhibited a maximum score of 4.8 ± 0.0 in winter (Fig. 1-4C). Presence level of spermatozoa in the cauda epididymis also increased beginning in summer, with a minimum level of 0.6 ± 0.2, and exhibited a maximum level of 2.8 ± 0.0 in winter (Fig. 1-4D). The values of TW, SS and presence level of spermatozoa in summer were significantly lower than in the other seasons ($P < 0.01$ or 0.05). STD in summer was significantly lower than in autumn ($P < 0.01$). In summer, from July to September, the mean values of presence

level of spermatozoa in the cauda epididymis were remarkably low, although individual values varied widely, and some individuals showed a high value even during this period. Among the total of 26 individuals in summer, 12 individuals had no sperm (value 0), while 14 individuals had sperm (value > 0) (Fig. 1-5). The mean value of Plasma T was low from spring to autumn and was significantly higher in winter ($P < 0.01$), with the highest concentration (2.69 ± 1.01 ng/ml) found in winter and the lowest concentration (0.15 ± 0.02 ng/ml) found in summer (Fig. 1-6).

Captive raccoon

The results for STD, SS, and Plasma T evaluated in one captive male continuously from April 2009 to March 2010 are shown in Fig. 1-7. The monthly STD decreased from April to July and increased gradually from July to March, with a minimum mean diameter of 198 μm, in July, and a maximum mean diameter of 259 μm, in April (Fig. 1-7A). The monthly SS remained relatively high from November to June, with a maximum mean score of 4.9, in February, and a minimum mean score of 2.7, in August (Fig. 1-7B). Spermatozoa could not be observed in seminiferous tubules from July to September. The monthly Plasma T remained low from June to August, with a minimum concentration of 0.47 ng/ml, in July, and varied erratically from September, reaching a maximum of 19.50 ng/ml, in April (Fig. 1-7C).

Discussion

In this study, TW, STD, SS and presence level of spermatozoa in the cauda

epididymis were used as indicators of seasonal morphological and physiological changes in the male gonad, in accordance with reports in other species [Howell-Skalla et al. 2002, Kawauchi et al. 2003, Qiang et al. 2003, Tsubota et al. 1997]. These values changed seasonally and were significantly higher around the winter mating season and lower in summer. Whether or not male raccoons have seasonal testicular changes has been controversial in North America. In Michigan, Stuewer [1943] concluded that males were capable of breeding at all times of the year because the testis size did not change remarkably throughout the year. In Illinois, on the other hand, Sanderson and Nalbandov [1973] observed that a majority of the adult males did not have sperm in the epididymides during summer. In Alabama, male raccoons seemed to show a definite seasonal cycle in testis size [Johnson 1970]. Therefore, there may be regional differences in seasonal changes of male reproduction. In Japan, one study on male raccoon reproduction was reported by Kaneko et al. [2005], who evaluated STD, SS and serum testosterone concentration from June to October in Hokkaido. They concluded that spermatogenesis did not become less active in summer (July to September) as compared with spring (June) and autumn (October). There were no remarkable differences among seasons in their study; however, differences among seasons were observed in the present study. This difference might be because they had only a limited number of samples during a limited period from June to October (n = 6 in June, 2 in July, 2 in August, 3 in September and 1 in October). In the present study, the author examined the remainder of the year, including the mating season from January to March.

In the present study, the highest values for TW, SS and presence level of spermatozoa in the cauda epididymis were seen during the winter mating season.

On the other hand, the peak of STD was in autumn. This difference may be due to individual variability given the remarkably high value in two individuals in autumn (261.6 and 250.0 μm). Spermatogenetic activity generally declined in summer, but there was great variation in the degree of spermatogenetic decline among individuals (Fig. 1-5). As a result, individuals with spermatozoa were observed in every month throughout the year. In many seasonal breeding mammals, spermatogenesis declines largely in the nonbreeding season [Qiang et al. 2003, Tsubota and Kanagawa 1989, Tsubota et al. 1997]. Also, in many domestic animals, high temperatures in summer reduce semen quality [Ulberg 1958]. In these animals, after spermatogenesis declines, some spermatozoa can be observed remaining in the cauda epididymis for some time. In this study, however, almost all individuals presenting with spermatozoa during summer exhibited higher values (> 4) in spermatogenetic score, suggesting active production of spermatozoa. Therefore, it can be inferred that some individuals produce spermatozoa even in summer. Furthermore, the captive male raccoon maintained active spermatogenesis and produced spermatozoa from October to June, while spermatozoa were not observed for the three months from July to September. These results suggest that the time and duration of aspermatogenesis varied among individuals. Therefore, raccoons producing spermatozoa can be observed in a group or local population throughout the year. In North America, the seasonal decline in testicular activity differs by region [Johnson 1970, Sanderson and Nalbandov 1973, Stuewer 1943]. From a study examining the epididymis in 28 captive males held in outdoor cages throughout the year, Sanderson and Nalbandov [1973] reported that individual males have periods averaging 3-4 months when they are incapable of breeding, but in a large group of raccoons, sperm production could be found in some animals at

any given time. Their report supports the present finding on the flexibility of reproductive decline in feral male raccoons.

In North America, the raccoon mating season is generally from January to March. However, a second estrus after mating season or late litters have been reported [Berard 1951, Gehrt and Fritzell 1996, Johnson 1970, Sanderson and Nalbandov 1973, Stuewer 1943]. The physiological details of the second estrus in female raccoons are not known, but Gehrt and Fritzell [1996] reported that females were consorting with males an average of 9 days (n = 6, range = 6-14 days) after the loss of their first litters. In Hokkaido, although mating peaks in February and litters are born between March and May as in North America, two females captured in July were reported as pregnant, which indicates that mating later in the season can occur [Asano et al. 2003b]. In the present study, it was revealed that male raccoons have indefinite seasonal reproductive changes, and individuals producing spermatozoa can be observed throughout the year. This means that some male raccoons have spermatozoa after the mating season and may mate with females in their second estrus.

Testicular function is affected by androgen, the dominant steroid hormones in males. In many male mammals, androgen produced from Leydig cells stimulates spermatogenesis, especially during and after meiosis, maturation of spermatozoa and to elongate spermatozoa existing period in cauda epididymis [Uemura 2008]. In many seasonal breeders, such as raccoon dogs [Yongjun et al. 1994], black bears (*Ursus americanus*) [Tsubota et al. 1997], brown bears [Tsubota and Kanagawa 1989] and martens (*Martes melampus melampus*) [Kawauchi et al. 2003], seasonal changes can be observed in spermatogenesis, morphology of the testis and peripheral androgen concentration. The present results showed that the Plasma T

increased from the premating season and declined at the end of the mating season, similar to reports in other species. In the present study, spermatogenesis was active, as during the mating season, after April when the testosterone concentration had declined. Spermatogenesis after testosterone concentration decline has also been reported in other mammals [Lee et al. 2001, Tsubota and Kanagawa 1989], which suggests that a high peripheral testosterone concentration is not necessary for maintaining spermatogenesis. It is thought that high peripheral testosterone levels affect aggressiveness and sexual behavior in male mammals [Lees 1965, Lincoln 1971]. Therefore, a relatively high testosterone concentration in winter may drive male raccoons to sexual behavior such as defending territories, searching for females and competing with other males. Compared with feral males, the concentration in the captive male remained generally higher and for a longer duration. These differences may be due to good nutritional condition and the presence of a mature female raccoon in the next cage.

In conclusion, in feral male raccoons in Hokkaido, spermatogenesis was found to be significantly less active in summer and more active in winter with a high peripheral testosterone concentration. However, even in summer, the aspermatogenetic duration varied among individuals. Therefore, some raccoons producing spermatozoa exist throughout the year. This great variation in seasonal reproductive physiology is unique among seasonal breeding mammals. This suggests that raccoons, as a group or local population, might be able to reproduce throughout the year. Most animals have difficulty surviving and spread out of their original range; however, this perennial reproductive potential may explain why raccoons have been increasing in number rapidly in Hokkaido since their introduction.

Summary

In Chapter 1 - Section 1, the author investigated seasonal changes in spermatogenesis and peripheral testosterone concentration of raccoons in Hokkaido. In the present study, external characteristics and histology of the testis and epididymis and the plasma testosterone concentration were investigated in 68 feral male raccoons and once a month in one live, captive male. The feral males exhibited seasonal changes in spermatogenesis, showing active spermatogenesis in autumn, winter and spring (from October to June) with noted spermatogenesis and inactive spermatogenesis in summer (from July to September) with lower percentage of spermatozoa existence in the cauda epididymis. Even in the inactive period, spermatozoa were observed in about half of the individuals (14/26); therefore, individuals producing spermatozoa existed every month throughout the year. Plasma testosterone concentrations were significantly high in the winter mating season. In the captive male, plasma testosterone concentrations were low from June to August, and spermatozoa could not be observed from July to September. These results suggest that raccoons exhibit seasonality of reproduction, but the time and duration of spermatogenetic decline varies widely among individuals. This individual variation in the inactive period is a feature of male raccoon reproduction and is unique among seasonally breeding mammals.

Section 2 Timing of puberty of males

Introduction

Puberty in male animals is defined by Lincoln [1971] as the period when accessory organs and secondary sexual characters develop under the influence of the testes and the animal first becomes fertile. The timing of puberty in male raccoons varies among populations in North America. In Illinois, more than half of juveniles have the ability to produce spermatozoa during the first mating season [Sanderson and Nalbandov 1973]. In Alabama [Jornson 1970], Michigan [Stuewer 1943] and North Dakota [Fritzell 1978b], males reach puberty during the second mating season as yearlings. In Texas, on the other hand, only 20% of yearlings possess sperm [Wood 1955]. Such differences might be caused by the surrounding environment, such as climate, temperature, light, and season, as well as food availability [Donovan and Ten Bosch 1965]; however, the timing of the onset of puberty is not known for male raccoons in Hokkaido.

In this Section 2, the author studied the timing of puberty and identified possible triggers of the onset of prepubertal development in male raccoons by examining changes in testicular development using both feral and captive raccoons in Hokkaido.

Methods

Histology

Testicular tissues were treated as the same method which was described in Section 1.

Evaluation of spermatogenesis

Spermatogenetic score (SS) was evaluated as the same method which was described in Section 1. The existence of spermatozoa in the cauda epididymis was also checked for each individual.

Enzyme immunoassay

Plasma testosterone concentrations (Plasma T) of one captive male were measured by the same method which was described in Section 1.

Statistical analysis

In all individuals, the extrusibility of the penis (EP) was checked. SS were compared between groups of individuals with an extrusible penis (EP+) and groups of whose penis with the preputia remaining (EP–) using Mann-Whitney *U* tests in Microsoft Excel 2003 for Windows (Microsoft, Redmond, WA, USA).

Results

A total of 151 male raccoons were classified as juveniles (n = 39), yearlings (n = 85) and 2 years olds (n = 27) by age determination (Table 1-2). Body length (BL) greatly increased during the juvenile period (Fig. 1-8A). In almost all individuals, testis weight (TW) and SS remained low until around April of the yearling stage and then greatly increased from May to the following November (Fig. 1-8B and C). Seminiferous tubule diameter (STD), although relatively variable, also remained low until around April of the yearling stage and then increased greatly from May to

the following November (Fig. 1-8D). Values of all three indicators increased remarkably beginning in May of the yearling stage, and after the following October, spermatozoa were observed in the cauda epididymis of all individuals until May of the 2-year-old stage. In some juveniles, TW, SS, and STD were detected at levels as high as in yearlings captured after October (Fig. 1-8B, C and D), and spermatozoa were observed in the cauda epididymis in one individual in November, one individual in February and two individuals in March (Table 1-2). In 2-year-old individuals, TW, SS and STD declined from June to September (Fig. 1-8B, C and D), and spermatozoa were only observed in 16.6% (3/18) of individuals during this period. Subsequently, after October, spermatozoa were again observed in all individuals (Table 1-2).

The relationships between each three values (TW, SS and STD) and BL in juveniles were shown in Fig. 1-9. TW and STD increased slightly based on BL development, and after reaching a BL of about 55 cm, these two values increased remarkably (Fig. 1-9A and C). Similarly, SS remained at a score of 1 until a BL of about 55 cm and subsequently increased remarkably (Fig. 1-9B).

In the captive juvenile that was fed during winter, testis size (TS) and SS began to increase rapidly beginning at 9 months old (in February; Fig. 1-10). In addition, STD and Plasma T began to increase at 8 months old (in January; Fig. 1-10B) along with body weight gain. Furthermore, elongated spermatozoa were observed to be released within the lumen of seminiferous tubules in April, only 11 months passed after birth.

When EP was compared with spermatogenetic activity, all individuals which had spermatozoa in the cauda epididymis until September of the yearling stage exhibited EP+. In the other immature individuals, the penis was not

extrusible until April of the yearling stage (EP+ = 0%; 0/42). The monthly percentage of EP+ appeared to increase gradually from May to August, although the percentage in August was low because of the small number of individuals captured (EP+ = 18%, 3/17 in May; 34%, 14/41 in June; 70%, 7/10 in July; and 33%, 1/3 in August). And then, almost all males had attained EP (EP+ = 90%, 27/30) after October of the yearling stage. The values of SS were compared between groups based on the extrusibility of the penis (EP+ or EP−) during the periods of May to September and October to April using Mann-Whitney *U* tests. SS for EP+ raccoons was significantly higher than for EP− raccoons during both periods ($P < 0.001$). However from May to September, the values for the two groups overlapped, ranging from 1.3 to 3.6 and many individuals without spermatozoa production were also EP+ (Fig. 1-11A). In October to April, on the other hand, no SS values overlapped between the two groups. All EP+ individuals had spermatozoa, and all EP− individuals lacked spermatozoa (Fig. 1-11B).

Discussion

TW, SS and STD, which were used as indicators of seasonal morphological and physiological changes in the male gonad, had been applied to see sexual development of males in other species. These values increase remarkably during prepubertal development in such as red deer stags (*Cervus elaphus*) [Lincoln 1971], rhesus monkeys (*Macaca mulatta*) [Van Wagenen and Simpson 1954] and Japanese monkeys (*Macaca fuscata*) [Nigi et al. 1980]. In the majority of male raccoons in this study, these three values started to increase in May of the yearling stage, and active

spermatogenesis was observed in following October (Fig. 1-8). In adult males older than 3 years, spermatogenesis was significantly less active in summer than during other seasons in Section 1. Two-year-old individuals in this study showed the same seasonal changes as adult males. Therefore, 2-year-old males were considered to have reached puberty, and they exhibited seasonal testicular activity. These results indicate that for the majority of male raccoons in Hokkaido, prepubertal development begins in May of the yearling stage, and spermatozoa production is complete before the second mating season of the yearling stage.

However, active spermatogenesis with producing spermatozoa was observed in some juveniles. To determine the difference between these early maturing juveniles and other juveniles, the relationship between each of these three values; TW, SS and STD, and raccoon body condition was examined using BL (Fig. 1-9). In Hokkaido, the body weight (BW) of raccoons exhibits seasonal changes, with increases from April to November and decreases from December to March due to the lack of food in a severe winter (shown in Section 1). Therefore, BL is considered to be a more suitable parameter than BW for estimating raccoon body development. As the results, testicular development occurred after BL reached about 55 cm. The age at the onset of puberty is strongly influenced by the plane of nutrition availability [Lincoln 1971], likely because all facets of mammal development ultimately depend on available calories and nutrients, and the reaction of peripubertal mammals to all other environmental factors depends in part on normal growth [Bronson and Rissman 1986]. Unusual body growth gain in juveniles has been reported in the wild in Wisconsin: a female and male juvenile with weights of 7.38 and 7.94 kg, respectively, were recaptured 6 months after birth [Dorney 1953]. These BWs were much heavier than the average weight of

6-month-old juveniles (females, 4.62 kg; males, 5.42 kg [Asano et al. 2003a]) and relatively heavier than even adults. Dorney [1953] suggested that nutrient supply from an abundance of crippled ducks in the habitat of these juveniles may have accounted for their large weights. The early-maturing feral individuals in the present study were captured in or close to barns in agricultural fields; thus, they likely had access to ample food even in winter. Therefore, if male raccoons can achieve adequate body growth, the onset of puberty development is likely to begin, and spermatozoa production will begin during the juvenile stage, which is earlier than in other individuals. Four yearlings in April and 2 in July with spermatozoa production were thought to be early-maturing males that had attained puberty during the first mating season and continued to show active spermatogenesis until being captured.

Lincoln [1971] found that when provided supplementary feed during the winter months, stag calves developed pedicles, an external aspect of secondary sexual development in this species, several months earlier than animals in the wild. In this study, the captive juvenile was observed producing spermatozoa only 11 months after birth (Fig. 1-10). These results can support the possible relationship between body growth gain and early pubertal development in the feral juveniles described above. Although the age of attaining puberty varied in juveniles and yearlings in this study, spermatozoa production was only observed after autumn for both stages. This timing coincided with the recrudescence of seasonal active spermatogenesis in adult males in Section 1. Generally, the timing of reaching puberty is influenced by the surrounding environment [Donovan and Ten Bosch 1965], and puberty appears to represent the start of the seasonal cycle of testicular activity [Lincoln 1971, Nigi et al. 1980]. The year in which the onset of puberty

occurs may be controlled by nutritional conditions, and the specific time of year may be controlled by other factors [Lincoln 1971] such as day length and ambient temperature. In female raccoons, stimulation from increasing day length drives them to prepare for reproductive conditions at the end of winter [Bissonnette and Csech 1938]. The influence of seasonal factors on the onset of puberty in male raccoons is not known; however, attaining puberty might require both adequate body nutrient development and some seasonal stimulation.

After attaining puberty, the extent of the reduction in testicular activity during the summer varied among individuals. Although 2-year-old males continued to produce spermatozoa until May, the values of TW, SS and STD in some individuals declined after June to levels comparable to those in yearlings from June to September (Fig. 1-8). Thus, yearlings captured from June to September likely included early-maturing raccoons that exhibited low values of TW, SS, and STD during this period due to seasonal testicular changes. Considering that 18.6% (8/43) of individuals attained puberty early during the period from November as juveniles until May of the yearling stage, it is possible that about 20% of yearlings captured from June to September were individuals that had already attained puberty.

In some regions, EP has been used as an external characteristic for estimating age or the onset of puberty. In Florida, adults can be distinguished from juveniles and yearlings by the penis extrusible [Kramer et al. 1999]. In Illinois, the majority of males achieve EP+ during the juvenile period [Sanderson and Nalbandov 1973]. In the present study, the percentage of EP+ increases gradually from May to September. This period corresponded to that of substantial testicular development, suggesting that attaining EP+ appears to occur during prepubertal development. To evaluate whether EP+ or EP– can serve as an indicator of

spermatogenesis in Hokkaido, values of SS were compared based on the extrusibility of the penis. As the result, during the period of October to April, all males which attained puberty exhibited EP+ and all immature males exhibited EP– (Fig. 1-11). Therefore, EP can be used as an external indicator of attaining puberty for individuals captured only from October to April in Hokkaido.

Sexual maturity, which is the status of an animal that assumes an effective role in reproduction of the population, is distinct from puberty, which is the status of an animal that first becomes capable of reproduction [Asdell 1946]. In North America, yearling males are thought to be rarely capable of reproducing because they are socially immature, even after puberty is attained [Bissonnette and Csech 1938, Kramer et al. 1999, Jornson 1970, Sanderson and Nalbandov 1973, Stuewer 1943]. In high-density areas where competition between adult males is intense, yearling males might not be able to reproduce. However, in Hokkaido, which maintains many areas with low densities of raccoons, only about 40 years have passed since the introduction of raccoons, and yearlings may be able to participate in reproduction. Yearling males disperse from their natal habitat area during the spring-summer before their second mating season [Fritzell 1978a]. Therefore, attaining puberty before the second mating season may be important in that they can reproduce in the new dispersal area. Moreover, the participation of these young males in reproduction may be one cause of the rapid increase in the abundance and dispersal of raccoons throughout Hokkaido. To reveal such relationship between sexual maturation of young males and population dynamics, further detailed study is required on the reproductive activity of young males in the wild.

In conclusion, for the majority of male raccoons in Hokkaido, prepubertal development began in May of the yearling stage, and puberty was attained in

October prior to the second mating season during the yearling stage. However, if male raccoons were able to attain enough body growth before the first mating season, the onset of pubertal development occurred and spermatozoa production was achieved during the juvenile stage, which was earlier than in other individuals. In both juveniles and yearlings, spermatozoa production was only observed after autumn, and this timing coincided with the recrudescence of seasonally active spermatogenesis in adult males. Therefore, attaining puberty appears to require both enough body nutrient development and several environmental factors that control seasonal testicular changes in male raccoons.

Summary

In Chapter 1 - Section 2, the author investigated the timing of and potential factors affecting the onset of puberty in male raccoons in Hokkaido. External characteristics and histology of testes were studied in 151 male feral raccoons and in 1 captive juvenile. For the majority of feral yearling raccoons, prepubertal development began in May, and spermatozoa production began in October prior to their second mating season. However, some larger juveniles attained puberty during the juvenile period. The captive juvenile, which was fed throughout the winter, attained puberty only 11 months after birth. These results suggest that if male raccoons can achieve enough body growth before the first mating season, puberty can be attained early. In both juveniles and yearlings, spermatozoa production was only observed after autumn. This timing coincided with the recrudescence of seasonally active spermatogenesis in adult males.

Therefore, attaining puberty in male raccoons appears to require both adequate body nutrient development and several environmental factors that control seasonal testicular changes.

Chapter 2 Changes in the immunolocalization of steroidogenic enzymes and the androgen receptor in testicular tissue

Introduction

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 were detected in the mating season, and, in contrast, inactive spermatogenesis along with low testosterone concentrations were detected in the non-breeding season in many seasonal breeding males, such as Hokkaido sika deer (*Cervus nippon yesoensis*) [Suzuki et al. 1992], American black bears (*Ursus americanus*) [Tsubota et al. 1997] and Hokkaido brown bears (*Ursus arctos yesoensis*) [Tsubota and Kanagawa 1989].

The raccoon is a long-day seasonal breeder, with mating in the winter, from January to March, in Hokkaido [Asano et al. 2003b]. In Chapter 1 - Section 1, the author found that adult male raccoons exhibited active spermatogenesis with high plasma testosterone concentrations (Plasma T) in the winter mating season, as in other seasonal breeding mammals. 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 procynoides*) [Qiang et al. 2003], Japanese black bears (*Ursus thibetanus japonicus*) [Okano et al. 2003], and ground squirrels (*Citellus dauricus Brandt*) [Zhang et al. 2010]. The immunolocalization and the seasonal changes varied 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 Chapter 1 - Section 1, some male raccoons were found to produce spermatozoa actively despite low Plasma T in the summer non-breeding season. 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 factors. First, testosterone concentrations in local sites in testicular tissue may be independent of peripheral testosterone concentrations and be relatively high in testes with active spermatogenesis. Second, metabolism and reactivity against testosterone in testicular tissue 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*) [Tahka et al. 1997] and big fruit-eating bats

(*Artibeus lituratus*) [Oliveira et al. 2009]. Thus, clarifying the localization and expression changes in steroidogenic enzymes and the AR can help in understanding the mechanisms that regulates spermatogenesis and testicular steroidogenesis in the raccoon testes.

In this Chapter, to assess changes in the biosynthesis, metabolism, and reactivity of testosterone, the localization and immunohistostaining intensity of steroidogenic enzymes and AR were investigated according to season and spermatogenetic activity in feral raccoons. The goals of this Chapter were to identify factors related to seasonal changes in testosterone production and differences in spermatogenetic activity in the summer non-breeding season.

Materials and Methods

Sample collection

The author collected carcasses of adult male raccoons that were euthanized for eradication control in west-central Hokkaido from 2010 to 2013. In total, 15 raccoons were used in this study. Five were collected from February through early April and 10 were from July. The 15 male adult raccoons were classified into 3 groups according to the season and level of spermatogenetic activity. Group WIN contained samples from the winter (n = 5). Samples from the summer in which the author observed spermatozoa in the cauda epididymis with active spermatogenesis (Spermatogenetic Score; SS > 4) in the seminiferous tubules, were classified into group SUM+ (n = 5). Samples from the summer in which the author observed inactive spermatogenesis (SS ≤ 4) in the seminiferous tubes without spermatozoa in

the cauda epididymis, were classified into group SUM− (n = 5).

Raccoon carcasses were weighed and measured for body length (BL), blood was obtained by cardiac puncture, and the testes were removed. Blood was placed in a 5 ml heparin tube and centrifuged at 1,050 g for 10 min. The plasma was removed and stored at −30°C until assayed. The testes were brought on ice to the laboratory within 1 h after euthanasia. The left testes were weighed and 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 treated as the same method which was described in Chapter 1 - Section 1. In histological sections which were stained with hematoxylin and eosin, spermatogenesis was evaluated as the same method which was described in Chapter 1 - Section 1. 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), were immunostained. The primary antibody references are summarized in Table 2-1. The sections were activated in immunostimulatory with using DakoCytomation Target Retrieval Solution (S3307, DakoCytomation, 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 the 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 T was measured by the same method which was described in Chapter 1 - Section 1.

HPLC/ESI-MS/MS

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, then after centrifuging at 1,100 g for 10 min under 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 at 15,000 g for 15 min under 4°C.

Testisticular tissue testosterone concentration (Tissue T) was measured using high-performance liquid chromatography-electrospray-ionization tandem mass spectrometry (HPLC: Shimadzu LC-20 series, ESI-MS/MS: LCMS-8030, Shimadzu Corporation, Kyoto, Japan). Extracted samples (50 μl) were injected on to the Hypersil GOLD column (Thermo Scientific, MA, USA) with the 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 0-2 min: B conc. 50%, 2-5 min: B conc. 50-95%, 5-6.5 min: B conc. 95%, and 6.5-8 min: B conc. 50%. 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 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 nebulizing gas flow at 3 l/min, dry gas flow at 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 two of the three groups with Microsoft Excel 2003 for Windows (Microsoft, Redmond, WA, USA). SS was not subjected to a statistical analysis, because samples in Summer were allocated to two groups based on SS. 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 in WIN (n = 5), SUM+ (n = 5) and SUM− (n = 5). There was no significant difference in BL or body weight (BW) among the three groups (P = 0.64 and 0.95, respectively; Fig. 2-1). Testis weight (TW) in WIN

was significantly heavier than that in SUM− ($P < 0.01$; Fig. 2-1).

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 (7.6 ± 3.4 ng/ml) was higher than the other two groups (0.8 ± 0.1 ng/ml in SUM+ and 0.6 ± 0.1 ng/ml in SUM−; Fig. 2-1). Regarding Tissue T, although only 3 fresh testes samples were collected in the winter, the WIN value (16.8 ± 6.17 ng/100 mg tissue) was significantly higher than those of the other 2 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. 2-1). There was no significant difference between the SUM+ and SUM− values.

Immunohistochemical localization and expression differences in enzymes and AR

Immunolocalization of the 4 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 of part of Sertoli cells. Immunostaining for P450arom was detected in the cytoplasm of Leydig cells, the cytoplasm of part of Sertoli cells, and spermatids (Fig. 2-2). AR was detected in nuclei of Leydig cells and Sertoli cells. No immunostaining was detected in control sections (NC in Fig. 2-3). Immunostaining for some of the enzymes became scarce or was absent in summer samples compared with WIN. Differences in immunohistochemical localization among groups are shown in Fig. 2-3, and immunostaining intensity of the enzymes in Leydig cells and of AR in Leydig cells and Sertoli cells is shown in Table 2-2. Immunostaining of 3βHSD in

Sertoli cells and immunostaining of P450arom in Sertoli cells and spermatids were observed only in part of cells even in one individual sample, therefore comparison of immunopositive intensity was not performed in these cells. 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 intensity in SUM+ was weaker. In SUM−, 3βHSD was detected in 2 out of 5 samples and no immunohistochemical reaction was observed in the other 3 samples. P450arom was detected in WIN and SUM+, but was weak in SUM−. Immunostaining of AR in Leydig cells and Sertoli cells was strong in WIN and SUM+ samples and relatively weak in SUM− samples, while intensities varied in 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-breeding season, some raccoons exhibited active spermatogenesis, producing spermatozoa, despite low testosterone concentrations, at the same level as other summer samples, when spermatogenesis was inactive. These results are similar to the result in Chapter 1 - Section 1. Changes in Tissue T correlated with peripheral changes. Thus, the first possibility would seem to be eliminated, that the local testosterone concentration in testicular tissue may be a proximate factor for maintaining spermatogenesis over the summer. Between the two groups in the summer, according to spermatogenetic activity, there was no

difference in BL or BW. 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 [Payne and Hales 2004]. Sex steroid hormones, such as testosterone and estradiol, are synthesized from cholesterol, which is metabolized into pregnenolone, primarily by P450scc. In the next step, pregnenolone is converted into androstenedione by P450c17 and 3βHSD through two pathways. Then, 17β-hydroxysteroid dehydrogenase type 3 converts androstenedione into testosterone, and finally P450arom converts testosterone into estradiol. The presence of these steroidogenic enzymes in testicular tissues has been reported in mammals, and in this present study, immunohistochemically, P450scc, P450c17, 3βHSD, and P450arom were detected in raccoon testes. All 4 enzymes were located in the cytoplasm of Leydig cells. Androgen synthesis has been detected generally in Leydig cells in many mammalian species [Payne and Youngblood 1995, Wang et al. 2003]; thus, Leydig cells are likely to also be a major site of steroidogenesis in raccoon testes. 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 [Hall et al. 1969], some evidences indicate that Sertoli cells possess 3βHSD and can metabolize steroids [Welsh and Wiebe 1978]. Immunoreactivity for 3βHSD in Sertoli cells was also reported in cynomolgus monkey (*Macaca fascicularis*) testes [Liang et al. 1998]. In the present study, though not all Sertoli cells exhibited immunopostive against 3βHSD even in WIN samples, the results suggest that Sertoli cells, as well as Leydig cells, may play a role in producing androgens in raccoon testes. Estradiol is thought to be also essential for male reproduction, for stimulating sperm

maturation [Carreau et al. 2007] and absorbing rete testis fluid to concentrate sperm in semen [Carreau et al. 2011]. P450arom has been detected in Leydig cells, Sertoli cells, and germ cells in some animals, such as Japanese black bears [Komatsu et al. 1997, Okano et al. 2003], Hokkaido brown bears [Tsubota et al. 1993], raccoon dogs [Qiang et al. 2003], laboratory mice [Nitta et al. 1993], and laboratory rats [Levallet et al.1998]. Also, in raccoon testes, various cells, including Leydig cells, Sertoli cells, and spermatids, are considered to be sources of estradiol.

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 [Tahka et al. 1982] and American grey squirrels (*Sciurus carolinensis*) [Pudney and Lacy 1977], and P450scc and P450c17 in DLS rams [Price et al. 2000]. Seasonal changes in 3βHSD expression were demonstrated in raccoon dogs [Qiang et al. 2003], Japanese black bears [Komatsu et al. 1997] and Colorado mule deer (*Odocoileus hemionus*) [Markwald et al. 1971] by immunohistological evaluation, and raccoon dogs [Weng et al. 2012] 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 raccoon testes, as in the other mammals mentioned. Decreasing testosterone over the summer might be related to decreases in number or size changes of Leydig cells [Hayakawa et al. 2010] and decreased TW (Fig. 2-1), in addition to 3βHSD expression changes. Also, changes in luteinizing hormone (LH) secretion, which controls these factors [Keeney et al. 1988], are thought to be part of another regulatory system.

When SUM− samples were compared with SUM+, testosterone concentrations in both plasma and tissue were not significantly different. However, immunoreactivity of P450arom was scarce in Leydig cells in SUM−. Estradiol is also considered to be important for testicular development and spermatogenesis [Hess et al. 1997]. Synthesis of estradiol by P450arom occurs in various tissues and cells in mammals, such as adipose tissue, bone, ovary, placenta, and the testis [Payne and Hales 2004]. 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 in Leydig cells, along with spermatogenetic activity in the summer season. Thus, P450arom expression in raccoon testicular tissue might contribute to keeping spermatogenetic activity during the summer non-breeding season.

AR was detected strongly in Leydig cells and 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 [Blok et al. 1989, Verhoeven and Cailleau 1998]. In further research, such upstream regulatory factors should be investigated.

In conclusion, the present study showed that morphological, histological, and endocrinological differences in testes of feral male raccoons occurred with the seasons and spermatogenetic activity. Four steroidogenic enzymes and AR were immunolocalized in raccoon testicular tissue and changes in immunohistostaining 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, but not to 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.

Summary

The raccoon is a seasonal breeder, with a mating season in the winter. In Chapter 1 - Section 1, adult male raccoons exhibited active spermatogenesis, with high plasma testosterone concentrations, in the winter mating season. Maintenance of spermatogenesis generally requires high testosterone, produced by steroidogenic enzymes. However, even in the summer non-breeding season, some males were producing 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-breeding season, morphological, histological and endocrinological changes in the testes of feral male raccoons should be known. In this Chapter 2, to assess changes in the biosynthesis, metabolism and reactivity of testosterone, the localization and immunohistostaining intensity of four steroidogenic enzymes; cholesterol side-chain cleavage cytochrome P450 (P450scc), 17-αhydroxylase cytochrome P450 (P450c17), 3 β-hydroxysteroid dehydrogenase (3βHSD) and aromatase cytochrome P450

(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 they were scarce in aspermatogenic testes. This suggests that spermatogenesis in raccoon testis might be maintained by some mechanism that regulates P450arom expression, in synthesizing estradiol, and AR expression, in controlling reactivity to testosterone.

GENETIC POLYMORPHISM

Chapter 3 A genetic method for sex identification of raccoons

Introduction

Sex is one of the most important pieces of information about an animal, as it is related to physiology, behavior, and reproduction. Thus, developing methods for sex identification is essential in many fields of study, including zoology and ecology.

In some mammalian species, the sex of adult individuals can be determined relatively easily based on differences in body size or sexually dimorphic characters, including the larger body weight in male Primates, Proboscidea, Pinnipedia, Ruminnantia, and Mustelidae [Ralls 1977], antlers in male Cervidae [Geist and Bayer 1988], and manes of male Suidae, Bovidae, and Ottariidae [Kunz et al. 1996]. However, even in such mammals, sex determination before sexual maturation can be difficult. The raccoon is a middle-sized carnivore in which both sexes have the same appearance and an almost identical adult body size (asymptotic body weight in Hokkaido: 6.26 kg for males and 5.36 kg for females; asymptotic body length: 59.0 cm for males and 55.7 cm for females [Asano et al. 2003a]). Male raccoons have an *os penis*, which can be used for sex determination. However, it is difficult to identify the sex of a raccoon based on its appearance alone at any stage of life. Thus far, no genetic method using non-invasive materials has been established to determine the sex of raccoons. The development of such a genetic method would be useful for conservation genetics studies or biological analyses of raccoons.

Genetic sex determination in mammals is based mostly on the specification of the Y chromosome in males. Easy and rapid PCR-based amplification methods have been developed for many mammalian species based on differences in the genes of the X and Y chromosomes, including the amelogenin genes *AMELX* and *AMELY* [Ennis and Gallagher 1994], the sex-determining region Y (*SRY*) gene [Griffiths and Tiwari 1993], and the zinc finger protein genes *ZFX* and *ZFY* [Aasen and Medrano 1990]. *ZFX/ZFY* have been used in rapid amplification methods for sex identification in many mammals, including forest musk deer (*Moschus berezovskii*) [Qiao et al. 2007], Hokkaido sika deer (*Cervus nippon yesoensis*) [Yamazaki et al. 2011], American minks (*Neovison vison*) [Shimatani et al. 2010], and dogs (*Canis familialis*) [Murakami et al. 2001]. The aim of this study was to identify differences between *ZFX* and *ZFY* in raccoons and to establish a genetic method for sex determination of raccoons.

Materials and Methods

Sample collection and DNA extraction

Hair or whisker samples were collected from the carcasses of feral raccoons (20 males and 20 females) that were euthanized for eradication control in Hokkaido. The sex of the animals was determined at the time of sampling by checking for an *os penis*. The samples were kept at −20°C with silica gel until DNA extraction. DNA was extracted from root parts of hair (8 to 10 strands) or whisker (3 to 4 strands) to final volume 30-50 μl using an ISOHAIR™ kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol.

PCR amplification

In 4 females and 4 males which were chosen randomly, amplification of *ZFX/ZFY* was performed using the universal primers described by Aasen and Medrano [1990], P1-5EZ (5′ - ATAATCACATGGAGAGCCACAAGCT - 3′) and P2-3EZ (5′ - GCACTTCTTTGGTATCTGAGAAAGT - 3′), in a 25 μl reaction containing 1 μl DNA extract, 0.625 U of KAPA*Taq* EXtra DNA polymerase (NIPPON Genetics Co. Ltd., Tokyo, Japan), 5 μl of 5× KAPA*Taq* EXtra buffer (NIPPON Genetics Co. Ltd.), 5 μl of 25 mM $MgCl_2$ (NIPPON Genetics Co. Ltd.), 0.75 μl of dNTP Mix (10 μM each; NIPPON Genetics Co. Ltd.), and 1.25 μl of each of the primers described above. The reaction conditions were: 1 cycle of 95°C for 10 min; 35 cycles of denaturation at 95°C for 30 sec, annealing at 53°C for 30 sec, and extension at 72°C for 30 sec; and 1 cycle of 72°C for 10 min. To confirm amplification, 5 μl of the products were electrophoresed on a 1.5% agarose gel. The products were purified using a NucleoSpin Gel and PCR Clean-up kit (Takara Bio Inc., Shiga, Japan). The purified male fragment was cloned into pGEM-T easy vector (Promega Co., Madison, WI, USA) and transformed into competent DH5α *Escherichia coli* cells.

Sequence analysis

The cloned products of *ZFX* and *ZFY* were sequenced using a Big Dye Terminator version 1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) in both directions with the primers described above. The sequences were analyzed using an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, Foster-City, CA, USA). Nucleotide sequence data reported are available in the DNA Data Bank of Japan databases under accession numbers *ZFX*: AB856034 and *ZFY*: AB856035.

Design of primers for sex determination

Specific primers for sex determination were designed according to differences between the sequences of *ZFX* and *ZFY* based on positions 323 and 325. ZFX-IPfw (5′-AGCCGTACCAGTGCCAGTA-3′) and ZFX-IPrv (5′- GCGGACCTATAC TCGCAGTAC-3′) were complementary to *ZFX*. ZFY-IPrv (5′-TGCAGACCTATACTC GCAGAAT-3′) and ZFY-IPfw (5′-AGCCGTACCAGTGCCAATT-3′) were complementary to *ZFY* (Fig. 3-1).

PCR test for sex determination

For sex identification, 2 sets of four primers (set I: P1-5EZ, P2-3EZ, ZFX-IPfw, and ZFY-IPrv; set II: P1-5EZ, P2-3EZ, ZFX-IPrv, and ZFY-IPfw) were applied. The sizes of the expected products in set I were 141 and 447 bp for females and 141, 346, and 447 bp for males. On the other hand, these in set II were 345 and 447 bp for females and 141, 345, and 447 bp for males. To confirm the amplification pattern, 5 μl of the products were electrophoresed on a 1.5% agarose gel with 20 samples of each sex.

Comparison with other species

These primer sets were applied in three native carnivores in Hokkaido, raccoon dogs (*Nyctereutes procyonides albus*), Hokkaido brown bears (*Ursus arctos yesoensis*) and red foxes (*Vulpes vulpes*). DNA from 4 females and 4 males in each animal were extracted as the same method described above, and PCR amplification was conducted to be compared with electrophoresis image of raccoons.

Results

The fragment lengths were 447 bp for *ZFX* and *ZFY* amplified using the universal primers, P1-5EZ and P2-3EZ. The sequences of the *ZFX* fragment from 4 females and 4 males were identical, while those of the *ZFY* fragment from 4 males were also identical. Between the sequences of *ZFX* and *ZFY*, 14 base differences were identified (96.9% homology) (Fig. 3-1).

Using the 2 sets of primers, a 141-bp fragment from *ZFX* and a 346-bp fragment from *ZFY* were successfully amplified with primer set I, and a 345-bp fragment from *ZFX* and a 141-bp fragment from *ZFY* were successfully amplified with primer set II. In electrophoretic profiles, results of PCR tests were obtained as expected. In primer set I, 2 bands of 141 and 447-bp fragment was detected for females and 3 bands of 141, 346, and 447 bp for males, and in set II, 2 bands of 345 and 447 bp for females and 3 bands of 141, 345, and 447 bp for males (Fig. 3-2). The author confirmed that the same electrophoretic profile in total 20 individuals of each sex.

Amplification pattern in 3 native species was shown in Fig. 3-3. In all species, electrophoretic profiles in both sexes were the same in either primer set. With primer set I, the smallest molecular weight band (about 141 bp) and the largest molecular weight band (around 447 bp) were detected in raccoon dogs and bears, and only the smallest molecular weight band was detected in foxes. With primer set II, only the largest molecular weight band (around 447 bp) were detected in raccoon dogs and bears, and only the smallest molecular weight band was detected in foxes.

Discussion

In the present study, a PCR test for sex determination in raccoons using universal primers and newly designed primers was developed. Sequence analysis after PCR with universal primers revealed 14 nucleotides differences between *ZFX* and *ZFY*. The new primer ZFX-IPfw and ZFY-IPfw were designed based on 2 different sites while ZFY-IPrv and ZFX-IPrv were designed based on 3 sites; of these, ZFY-IPrv was considered to be highly specific for ZFY due to the absence of a 346-bp fragment in the female samples with primer set I. And ZFY-IPfw was also considered to be highly specific for ZFY due to the absence of a 141-bp fragment in the female samples with primer set II. Therefore, the sex of raccoons could be determined rapidly by agarose gel electrophoresis based on the appearance of band position using primer set I or II. No amplification or a single band was taken to indicate a technical failure.

Both primer sets could not distinguish sex of other 3 native species (Fig. 1-3). With primer set I, foxes showed only the smallest molecular weight band, however, raccoon dogs and brown bears showed the same band pattern of raccoon females. On the other hand, with primer set II, foxes showed only the smallest molecular weight band, and raccoon dogs and brown bears showed only the largest molecular weight band (Fig. 3-3). From these results, applying primer set II could determine sex of raccoons specifically, and moreover could distinguish raccoon's electrophoresis pattern from other 3 native species in Hokkaido.

In Japan, the raccoon has been considered a feral animal since the 1970s [Ikeda, 1999], and an eradication program was started following passage of the Invasive Alien Species Act in 2005. For effective control, basic biological information,

such as on population dynamics and reproductive biology, is required. Therefore, sex determination methods are essential. Male raccoons have an *os penis*, so sex can be identified by close observation of the lower abdominal region or checking for the existence of the *os penis*. The present genetic method enables sex determination in the laboratory using a small amount of DNA samples, such as road-kill carcasses, hair-trap samples or feces whose sex are unclear. Furthermore, PCR amplification with primer set II can distinguish raccoons from 3 native carnivores of raccoon dogs, Hokkaido brown bears and red foxes, even if it is uncertain whether the owner of a sample is a raccoon or not. In a captive situation, human handling of newborn cubs for sex identification can be stressful for both cubs and parents. The present genetic method can be used in such cases to avoid excessive human interference.

In conclusion, a genetic method for sex determination in raccoons was developed based on the zinc finger protein genes, *ZFX* and *ZFY*. In a sequence analysis of partial fragments of the genes (447 bp), 14 nucleotide differences were detected between *ZFX* and *ZFY* (96.9% homology). Four novel internal primers specific for *ZFX* or *ZFY* were designed. PCR amplification using 2 primer sets followed by agarose gel electrophoresis enabled sex determination. The bands of 141 bp, and 447 bp were in both sex and 346-bp band was specific only in male with primer set I. The bands of 345 bp and 447 bp were in both sex, and 141-bp band was specific only in male with primer set II, which could distinguish raccoon's electrophoresis pattern from 3 native carnivores in Hokkaido. Genetic sex determination methods for raccoons have not been previously developed. Further development of genetic methods targeting other genes such as the amelogenin genes or *SRY* gene can support accuracy of sex determination by using multiple genetic methods.

Summary

Sex identification of mammals is essential in many fields of study, including zoology and ecology. In some species, the sex of adult individuals can be determined relatively easily based on appearance. However, in raccoons, both sexes have the same appearance and almost identical body size. Although male raccoons have an *os penis*, which can be used in sex determination, it is difficult to identify the sex of a raccoon without a physical examination (i.e., by appearance alone). In such animals, a genetic method for sex determination can be useful; however, genetic-material-based techniques have not been developed for raccoons. In this Chapter 3, a genetic method for sex determination in raccoons was developed based on nucleotide differences of the zinc finger protein genes, *ZFX* and *ZFY*. Four novel internal primers specific for *ZFX* or *ZFY* were designed. PCR amplification using 2 primer sets followed by agarose gel electrophoresis enabled sex determination. The bands of 141 bp and 447 bp were in both sex and 346-bp band was specific only in male with primer set I. The bands of 345 bp and 447 bp were in both sex and 141-bp band was specific only in male with primer set II, which could distinguish raccoon's electrophoresis pattern from 3 native carnivores in Hokkaido; raccoon dogs, Hokkaido brown bear and red foxes. This method will be useful for biological analyses of raccoons.

Chapter 4 Mitochondrial DNA polymorphism of raccoon population in Hokkaido

Introduction

Phylogeographic studies can find the relationships between population genetic structures of organisms and geographical tracts [Avise et al. 1987]. In this field, molecular genetic approaches play a important role to investigate genetic variability [Randi et al. 2000] and geographical genetic barrier [Thulin and Tegelstrom 2002] in native species. Moreover, in cases of non-native species, these approaches can be powerful tools to detect the ecological and evolutionary impact in introduced area [Miura 2007]; for example to reconstruct the histories of the biological invasions, to find dispersal processes, and to detect evidence of population bottlenecks and founder events. Until now, various phylogeographic studies have been conducted in numerous invasive species from plants to mammals for finding origin of the population, spread pattern and invasion pathway, and genetic structure and level of genetic variation following invasion [Puillandre et al. 2008]. In many studies, mitochondrial DNA (mtDNA), which has high rate of evolution [Brown et al. 1979], was used as a genetic marker for determining relationships of genetic divergence in recent times among/within species. In particular, control region (D-loop), which is one of most variable regions in mtDNA with high substitution rates, has been applied in studies of various invasive species [Le Roux and Wieczorek 2009].

Phylogenetic diversity of raccoons was reported in native habitat, North

America. Pons et al. [1999] first analyzed 294-bp partial D-loop sequence, and detected 9 haplotypes in Virginia, Maryland, Illinois, Arizona and Quebec. Subsequently, Helgen et al. [2008] found other 9 haplotypes in the same 294-bp fragment from some regions in USA and West Indian islands, where raccoons were introduced from mainland of USA. In the same year, Cullingham et al. [2008] conducted a phylogenetic study straddle wide range of Eastern North America, and reported 76 haplotypes of 467-bp D-loop fragment, which was longer in 3' end and partially overlapped with Pons' fragment. In very recent years, some studies of invasive raccoons in Europe detected the same haplotypes described by Cullingham et al. [2008] in USA and concluded that multiple introduction had happened in Europe [Alda et al. 2013, Biedrzycka et al. 2013, Frantz et al. 2013].

In Japan, Takada [2011] found 18 haplotypes of 264-bp D-loop fragment from Honshu, Kyushu and Hokkaido. Takada-Matsuzaki et al. [2004] and Takada [2011] investigated phylogenetic distribution in municipalities in central Hokkaido and found 6 haplotypes. These haplotypes were also found in other prefectures in Honshu and Kyushu, indicating that multiple haplotypes occurred sympatrically in many municipalities. With these results, Takada-Matsuzaki et al. [2004] indicated that multiple maternal populations were related at founder events, including re-introduction between Honshu and Hokkaido and frequent immigration among municipalities. However, sampling field in their studies was limited only around Sapporo and there is no report of the genetic distribution of raccoons throughout Hokkaido, despites sighting of raccoons has been recorded in almost all municipalities (Fig. II). Moreover, dispersal processes in Hokkaido and comparison of polymorphism with native reports in North America have never been discussed before. Thus, the aim of this present research is to understand the founder invasive

events of raccoons in wider regions in Hokkaido with identifying geographical differentiation of D-loop lineages in mtDNA.

Materials and Methods

Sample collection and DNA extraction

Hair, whisker or tissue samples were collected from 526 carcasses of feral raccoons that were euthanized for eradication control in 44 municipalities in Hokkaido from 2010 to 2013 (Table 4-1, Fig. 4-1). The sampling area was chosen where raccoons were captured in 2009 (based on the data from Hokkaido government). The sex of some of the animals was determined at the time of sampling by checking for an *os penis*. In other samples whose sex information was lacked, sex was identified with the method developed in Chapter 3. The hair and whisker samples were kept at −20°C with silica gel until DNA extraction. DNA was extracted to final volume 30-50 μl using an ISOHAIR™ kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol.

Tissue samples (muscles or testes) were cut into small pieces, and kept in 99% ethanol at −20°C until DNA extraction. DNA was extracted to final volume 50 μl using a Wizard® Genomic DNA purification Kit (Promega, Madison, WI, USA) according to the manufacturer's protocol.

PCR amplification and Sequence analysis

Amplification was performed using the universal primers described by Helgen et al. [2008], Proc L (5′-TCATCGAAAATAATCTGTTAAAATGAA-3′) and

Proc H (5′-CGGAGCGAGAAGAGGTACAC-3′), which amplify a 392-bp fragment of the mitochondrial D-loop region of raccoons. A 25 μl reaction volume were containing 1 μl DNA extract, 0.625 U of KAPA*Taq* EXtra DNA polymerase (NIPPON Genetics Co. Ltd., Tokyo, Japan), 5 μl of 5× KAPA*Taq* EXtra buffer (NIPPON Genetics Co. Ltd.), 5 μl of 25 mM $MgCl_2$ (NIPPON Genetics Co. Ltd.), 0.75 μl of dNTP Mix (10 μM each; NIPPON Genetics Co. Ltd.), and 1.25 μl of each of the primers (final concentration; 0.5 μM) described above. The reaction conditions were: 1 cycle of 95°C for 10 min; 35 cycles of denaturation at 95°C for 30 sec, annealing at 53°C for 30 sec, and extension at 72°C for 30 sec; and 1 cycle of 72°C for 10 min. To confirm amplification, 5 μl of the products were electrophoresed on a 1.5% agarose gel. The products were purified using a FastGene™ Gel/PCR Extraction kit (NIPPON Genetics Co., Ltd.).

The products were sequenced using a Big Dye Terminator version 1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). The sequences were analyzed using an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems) and differentiated into 7 haplotypes.

For amplification of longer fragments, another reverse primer, ProcR-add (5′-CCATGAATTAAACTGCACCA-3′), was designed based on the sequence reported by Frantz et al. [2013]. In 46 samples, which were picked up from 7 haplotype groups with covering all detected subprefectures, 709-bp longer fragments were amplified with primers Proc L and ProcR-add and sequenced in the same method described above.

Molecular data analysis

Seven haplotypes of D-loop fragment sequence were aligned and a

neighbor-joining phylogenic tree was constructed with using MEGA5 software (http://www.megasoftware.net/) [Tamura et al. 2011]. The genetic distances based on alignment gaps among the haplotypes were estimated from all substitutions and by using the 2-parameters distances method [Kimura 1981].

Results

A 345-bp fragment of D-loop (excluding primer length) in mtDNA was examined in 526 raccoons collected from 44 municipalities in Hokkaido (Table 4-1). As the results, 7 haplotypes were detected with 17 polymorphic sites (Fig. 4-2a); *north/all* (n = 234), *hdk/tka* (n = 117), *kmo/kmk* (n = 92), *asb* (n = 14), *asb/bie* (n = 62), *ash* (n = 5) and *iwa* (n = 2) (Table 4-1). These 345-bp sequences covered fragments in other studies conducted previously; 199 bp in Japan [Takada-Matsuzaki et al. 2004] and 294 bp in North America and West Indian islands [Helgen et al. 2008, Pons et al. 1999], and also partially covered 160 bp out of 653 bp (covering variable sites 186-345) in Eastern North America [Cullingham et al. 2008] (Fig. 4-3). To compare with report by Cullingham et al. [2008], a longer 682-bp fragment was analyzed in 46 samples; *north/all* (n = 16), *hdk/tka* (n = 13), *kmo/kmk* (n = 10), *asb* (n = 2), *asb/bie* (n = 2), *ash* (n = 1) and *iwa* (n = 2). These samples were chosen to cover all subprefectures. As the results, every sample from the same haplotype showed an identical sequence. In variable sites 346-682, there were another 9 polymorphic sites among 7 haplotypes (Fig. 4-2b).

Haplotype *north/all* were found in almost all subprefectures in sampling area (Table 4-1, Fig. 4-4), and detected in about half of samples (44.5%) in this study.

Especially, raccoons in Northern Hokkaido had only this haplotype. *north/all* matched haplotype RMT03 reported by Takada-Matsuzaki et al. [2004], which was detected in Ishikari and Iburi subprefectures in Hokkaido, and also Wakayama and Fukui prefecture in Honshu and Saga prefecture in Kyushu [Takada et al 2007, Takada 2011]. Haplotype *north/all* was identified with haplotypes PLO2 in USA [Cullingham et al. 2008].

Haplotype *hdk/tka* was found in about half of municipalities, but mainly in southern central Hokkaido (Table 4-1, Fig. 4-4). Especially, 95% of individuals in Tokachi subprefecture were this haplotype. The nucleotide sequence matched haplotype RMT05 reported in central Hokkaido by Takada-Matsuzaki et al. [2004], and also detected in Chiba prefecture in Honshu (AB462045 in GenBank by Tokunaga) and Saga and Nagasaki prefectures in Kyushu [Takada 2011]. Haplotype *hdk/tka* was identified with haplotype PLO 32 in USA [Cullingham et al. 2008].

Haplotype *kmo/kmk* was found mainly in west central Hokkaido (Fig. 4-4). This haplotype matched haplotype RMT02 reported in west central Hokkaido by Takada-Matsuzaki et al. [2004], and also detected in Nagano, Kanagawa, Wakayama, Osaka, Hyogo and Fukui prefectures in Honshu, and also Fukuoka, Saga and Nagasaki prefectures in Kyushu [Takada et al 2007, Takada 2011]. Haplotype *kmo/kmk* was identified with haplotype PLO 32 in USA [Cullingham et al. 2008].

Haplotype *asb* was found only in Sorachi and Ishikari subprefecture in this research (Table 4-1, Fig. 4-4). This haplotype matched haplotype RMT04 reported in Hokkaido, which was also found outside of Sorachi and Ishikari (Iwamisawa, Mikasa, Kuriyama, Naganuma, Hobetsu, Yoichi and Niki) in the report of

Takada-Matsuzaki [2004], and also in Kanagawa prefecture [Takada et al 2007]. There was no report in other countries.

Haplotype *iwa* was found only in one individual in Iwamisawa and one in Kitahiroshima (Fig. 4-1). The number of individuals detected in this study was small. However, 91.9% of raccoons captured in Tanabe city in Wakayama prefecture were identified as this haplotype (RMT07; Takada-Matsuzaki et al. 2004). Haplotype *iwa* was also detected in Hyogo, Tottori and Fukui prefectures in Honshu [Takada et al 2007]. Haplotype *iwa* was identified with haplotype PLO 32 in USA [Cullingham et al. 2008].

Haplotype *asb/bie* was found only in Kamikawa and Sorachi subprefecture (Fig. 4-1). There was no report so far in other place.

Haplotype *ash* was found only in 5 individuals in Asahikawa (Fig. 4-1). This haplotype differed one nucleotide of haplotype *asb/bie* (position 227: base A changed into base G, Fig. 4-2). There was no report so far in other place.

Discussion

In this study, 7 haplotypes from 526 samples were detected. Five of them were reported previously in central Hokkaido (RMT02, 03, 04, 05 and 07) by Takada-Matsuzaki et al. [2004] (Table 4-2). When add RMT06, which had been found only in Iwamisawa by Takada-Matsuzaki et al. [2004]. but not in the present study, founder event in Hokkaido was established from at least 8 female haplotypes. It might be underestimation, because founder phylogenetic diversity can diminish during long demographic changing according to the number of founder females and

survivorship. Compare with the reports in the native habitat in Eastern North America, where 76 haplotypes were detected from 311 samples [Cullingham et al. 2008], the number of haplotypes in Hokkaido is very small. Such low mitochondrial diversity was also reported in other introduced areas in Europe; only 2 haplotypes from 58 samples in Spain [Alda et al. 2013], 6 haplotypes from 193 samples in Germany [Frantz et al. 2013], and 4 haplotypes from 72 samples in Poland, Czech Republic and Germany [Biedrzycka et al. 2013]. Rapid expansion from small populations can involve serial bottlenecking with progressive loss of allelic diversity, so that populations in more recently colonized places should contain less genetic diversity [Santucci et al. 1998].

When the author compared 682-bp partial sequences in this study with reports in native habitat, there was no match between the present data and 18 haplotypes reported in the North America and West Indian islands [Pons et al. 1999, Helgen et al. 2008]. Compared with 653-bp fragment sites reported in Eastern North America [Cullingham et al. 2008], haplotypes *hdk/tka*, *kmo/kmk* and *iwa* were identified with PLO32 (Table 4-2). Base differences among these 3 haplotypes were detected only in variable site 58 and 160 (Fig. 4-2a) where Cullingham et al. [2008] did not analyze (Fig. 4-3). Thus, 1 of these 3 might be the same haplotype with PLO32. From the result of phylogenetic tree (Fig. 4-5), these 3 haplotypes were affiliated genetically each other, so haplotypes *hdk/tka*, *kmo/kmk* and *iwa* seemed to be related closely with haplotype PLO32. These 3 haplotypes were also detected in Honshu and Kyushu [Takada et al. 2007], indicating that raccoons from the same origin group in North America were introduced at several remote places in Japan at founder event. Another possibility is that re-introduction as second release over prefectures, from first introduced regions into the non-habitat area, happened after

first founder event as Takada-Matsuzaki et al. [2004] had mentioned. Haplotype *north/all* matched haplotype PLO2 in North America [Cullingham et al. 2008]. PLO2 is the most common haplotype widespreading in North America [Cullingham et al. 2008] and reported in non-native area; Germany, Spain, Poland and Czech Republic [Alda et al. 2013, Biedrzycka et al. 2013, Frantz et al. 2013]. This haplotype was also detected in Honshu and Kyushu [Takada et al. 2007]. Haplotype *north/all* was detected in introduced area widely across countries, which strongly suggested that a same genetic population of this haplotype was managed under captive from wild in North America, exported to the world as a pet or a fur-bearing animal, and was introduced in non-native regions.

From the aspect of genetic diversity among municipalities in Hokkaido, haplotype *north/all*, *hdk/tka* and *kmo/kmk* were widely detected throughout Hokkaido (Table 4-1, Fig. 4-4). This result suggests that introduction of females which had an identical gene pool happened at several places in Hokkaido, and these 3 haplotypes were related with early stage of founder events and have dispersed into wide range during long time. In Hokkaido, up to 4 different haplotypes were detected in 1 municipality (ASH and KUR, Table 4-1). In Kamikawa, Sorachi, Ishikari and Iburi subprefectures, more than three haplotypes were mix-detected, suggesting that in these regions, several founder females with different haplotypes were introduced sympatrically, and/or cross-municipalities migrating have resulted in genetic diversity in close regions. On the other hand, in Northern Hokkaido area of Soya and Rumoi subprefecture, only haplotype *north/all* was detected. Inhabitation in this area had reported relatively recently (Fig. II, Table 4-1, Fig. 4-1), indicating that very limited number of founder females which had the same genetic background had contributed to dispersal in this area.

In Tokachi subprefecture, 95% of samples were identified as haplotype *hdk/tka*. Though sighting of raccoons in Tokachi had been recorded in several municipalities in relatively early stage of founder era (Fig. II, Table 4-1, Fig. 4-1), however stable habitation had not confirmed until recent years, and capturing number has been recently increasing from 2009 in reports by Hokkaido government. This population growth in Tokachi, therefore, might come out following lag period of number expansion. And also another possibility was suspected that population growth in Tokachi has been attributed by immigration from neighboring Hidaka or Kamikawa subprefectures. In this study, showing 94% of samples in Hidaka were also haplotype *hdk/tka*, diversity and distribution of haplotypes between Tokachi and Hidaka was much more alike than in Kamikawa, which might indicate possibility that raccoons in Tokachi were originated from Hidaka, or vice versa.

Haplotypes *asb/bie*, *asb* and *iwa* were detected over several municipalities, however these distribution were limited in close regions (Fig. 4-4). Therefore, it can be thought that these 3 haplotypes were introduced in only small area and dispersed around the original area. Haplotype *ash* was detected in only 5 individuals in Asahikawa. In the sequence of this haplotype, only 1 base was displaced from haplotype *asb/bie* and there was no other report so far. Thus, haplotype *ash* might be a newly mutated haplotype from haplotype *asb/bie* and 5 samples can be relatives from the same mutated mother.

Invasion of raccoons have been reported so far in almost all municipalities throughout Hokkaido (Fig. II), though sampling in this study was limited in some of them (Fig. 4-1). The author started to collect genetic samples where raccoons were captured in 2009, and succeeded from 38 municipalities out of 72 captured area (52.7 % coverage) and 7 municipalities where no capturing in 2009 (KSH, SKA,

OBH, UHR, ABS, SHR and RUS in Fig. 4-1). Consequently, the author could not get samples from Eastern Hokkaido, Southern Hokkaido, and north regions between Soya and Kamikawa. In these regions, the evidence of raccoon inhabitation had reported at least once in the past. Although, any nuisance control has not been taken place, because population has not established until present or economical damage from raccoons is still little according to small number of inhabitation. To reveal genetic distribution and linkage among regions more deeply, sampling in such regions are required. And for efficient eradication program against invasive alien species, detailed habitat survey and countermeasure should be conducted immediately after presence of raccoons was confirmed.

Genetic analysis of mtDNA can provide information about polymorphic diversity of the introduced population, though it's not enough to estimate precise number of founder individuals. Moreover, genetic admixture of different haplotypes cannot be examined by only using maternal inheritance mtDNA. With using microsatellite analysis, more detailed information can be obtained such as population structure and within-population diversity, genetic intercross, and estimation of number of founder individuals as reported in invasive species; raccoons in Spain [Alda et al. 2013], Indian mongoose (*Herpestes auropunctatus*) in Okinawa [Thulin et al. 2006], sika deer (*Cervus nippon*) in Scotland [Senn and Pemberton 2009]. A microsatellite-based analysis can help to further understand invasive introduction event and founder background of raccoons.

In conclusion, a 345-bp fragment of D-loop (excluding primer length) in mtDNA was examined in 526 raccoons collected from 44 municipalities in Hokkaido. Including with the results of longer 682-bp fragments in 46 samples, 7 haplotypes were detected with 26 polymorphic sites, and geographical polymorphic distribution

was observed. Compared with reports in Europe as nonnative-range and in North America as native-range, the author detected one example of raccoon dispersing from North America to the world. From the results of distribution in Hokkaido and other regions in Japan, it is suggested that raccoons were introduced at several remote places in Japan at founder event, and also re-introduction as second release over prefectures, from first introduced regions into the non-habitat area, happened after first founder event. Further study to reveal invasive introduction event and founder background of raccoons are required for efficient eradication program against this invasive alien species.

Summary

Phylogeographic studies can find the relationships between population genetic structures of organisms and geographical tracts. In cases of non-native species, these approaches can be powerful tools to reconstruct the histories of the biological invasions, to find dispersal processes, and to detect evidence of population bottlenecks and founder events. In this Chapter 4, a 345-bp fragment of D-loop in mtDNA was examined in 526 raccoons collected from 44 municipalities in Hokkaido. Including with the results of longer 682-bp fragments in 46 samples, 7 haplotypes were detected with 26 polymorphic sites, and geographical polymorphic distribution was observed. Haplotype *north/all*, the most widely distributing haplotype in Hokkaido, was reported in other places in Japan, non-native area in Europe and also in native-range North America. This haplotype was strongly supposed to be introduced across countries by export from native habitat. Haplotypes *hdk/tka*,

kmo/kmk, *iwa* and *asb* were also detected in other prefectures in Japan, indicating that raccoons were introduced at several remote places in Japan at founder event. Second possibility is that re-introduction as second release over prefectures, from first introduced regions into the non-habitat area, happened after first founder event. The other haplotypes *asb/bie* and *ash* were detected only in limited close regions in Hokkaido. Therefore, these haplotypes were thought to be introduced in only small area and dispersed around the original area. Further study to reveal invasive introduction event and founder background of raccoons are required for efficient eradication program against this invasive alien species.

TABLES AND FIGURES

Fig. I　Raccoon (*Procyon lotor*)

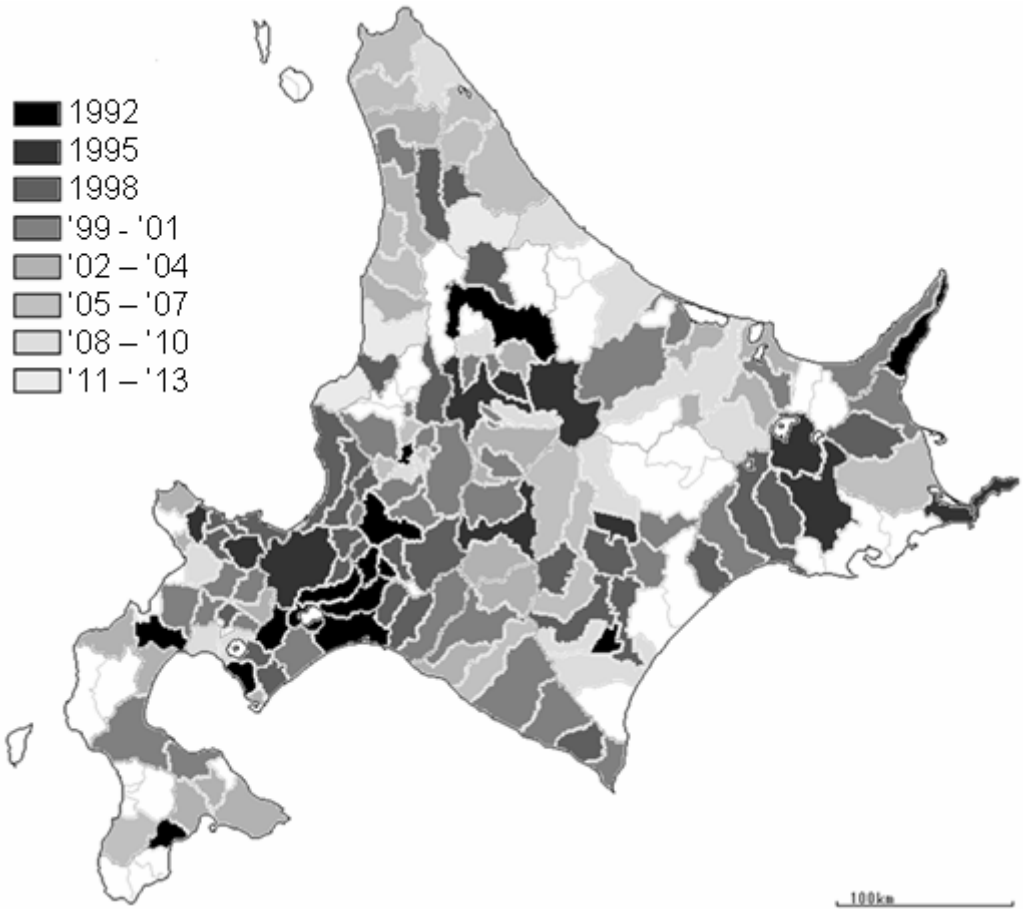

Fig. II
Municipalities where raccoon inhabitation was reported first time.
(Modified from Hokkaido government's report)

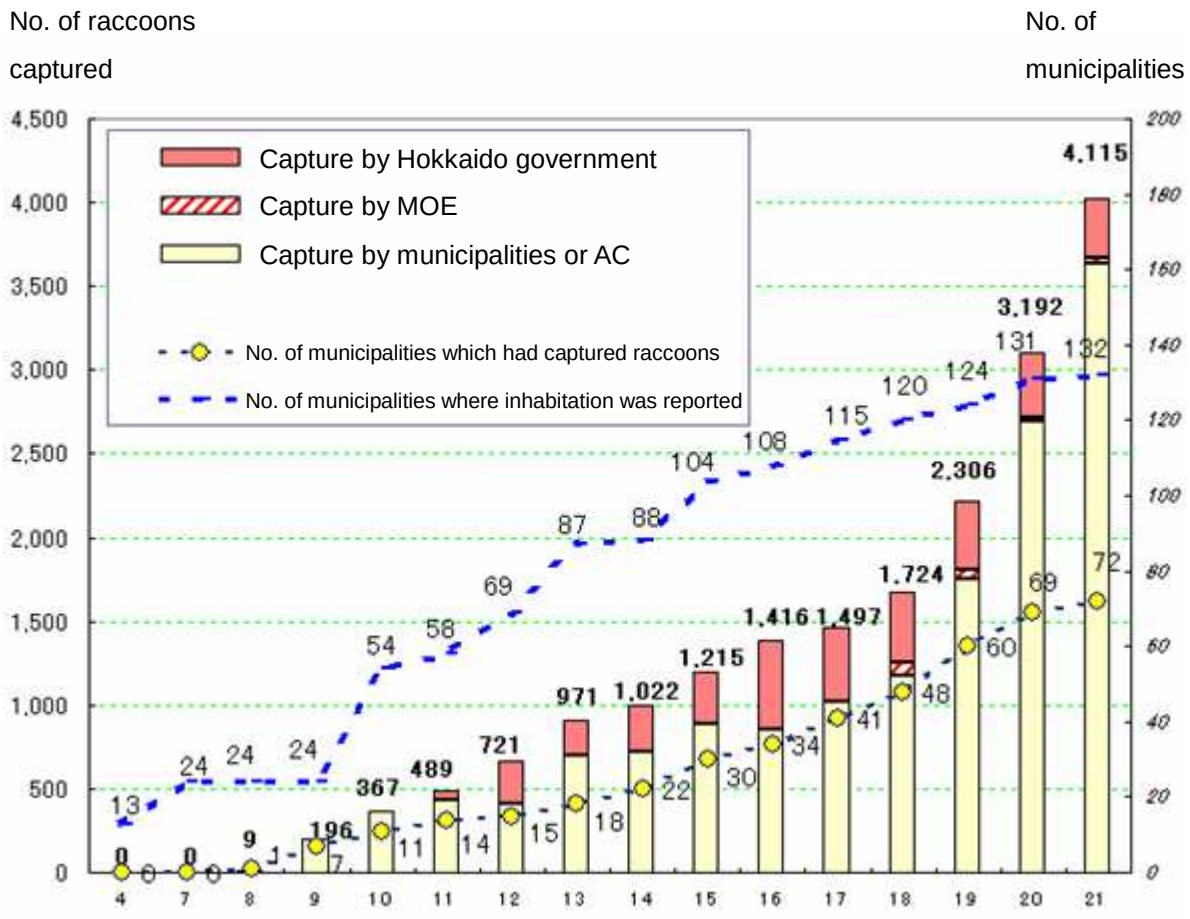

Fig. III

Number of municipalities first reported Raccoon inhabitation and number of raccoons captured in Hokkaido (modified from Hokkaido government's report).

MOE: Ministry of the Environment, AC: Agricultural cooperative society.

Table 1-1

Monthly numbers of captured feral male raccoons over 3 years old and mean (± SEM) body weight in west-central regions of Hokkaido, 2008-2011.

Month	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Total
No. of animals	12	7	5	14	5	7	6	2	2	0	1	7	68
Body weight (kg)	6.5 ±0.2	6.9 ±0.3	6.5 ±0.3	6.8 ±0.3	7.0 ±0.4	6.2 ±0.4	8.5 ±0.6	10.7	10.0	-	7.0	6.5 ±0.6	

Table 1-2

Monthly numbers of captured feral male raccoons from juvenile to 2 years old in west-central regions of Hokkaido, 2008-2011.

Month	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Total
Juvenile	-	-	1	9	4	3	7	3 (1)	1	1	3 (1)	7 (2)	39
Yearling	11(4)	17	41	10 (2)	3	0	1 (1)	1 (1)	0	0	0	1 (1)	85
2 years old	2 (2)	3 (3)	11 (2)	3 (1)	3	1	2 (2)	0	0	0	1 (1)	1 (1)	27
Total													151

The numbers of individuals which have spermatozoa in the cauda epididymis were shown in parentheses.

New born juveniles were never captured before June.

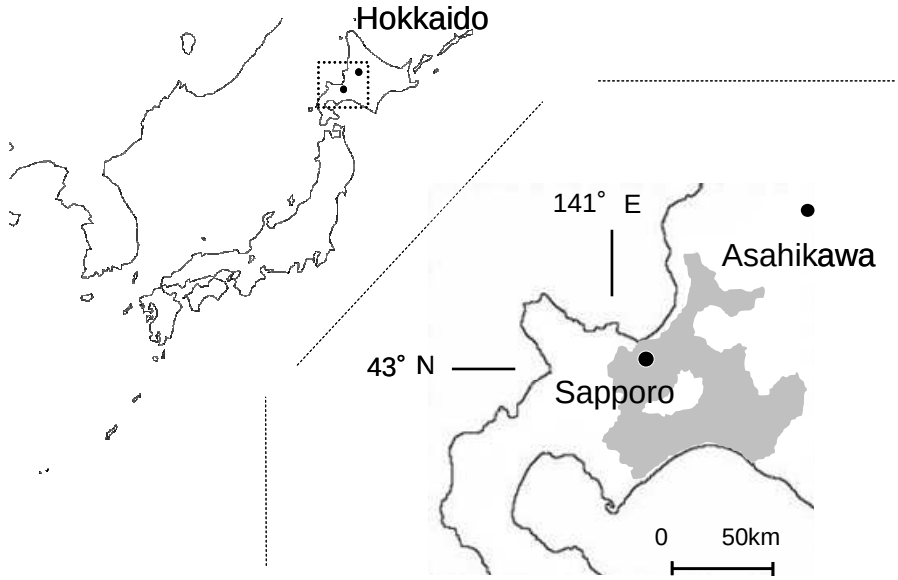

Fig. 1-1
The captive raccoons were located in Asahikawa, and the gray area represents the study area for feral raccoons in the west-central region of Hokkaido, 2008-2011.

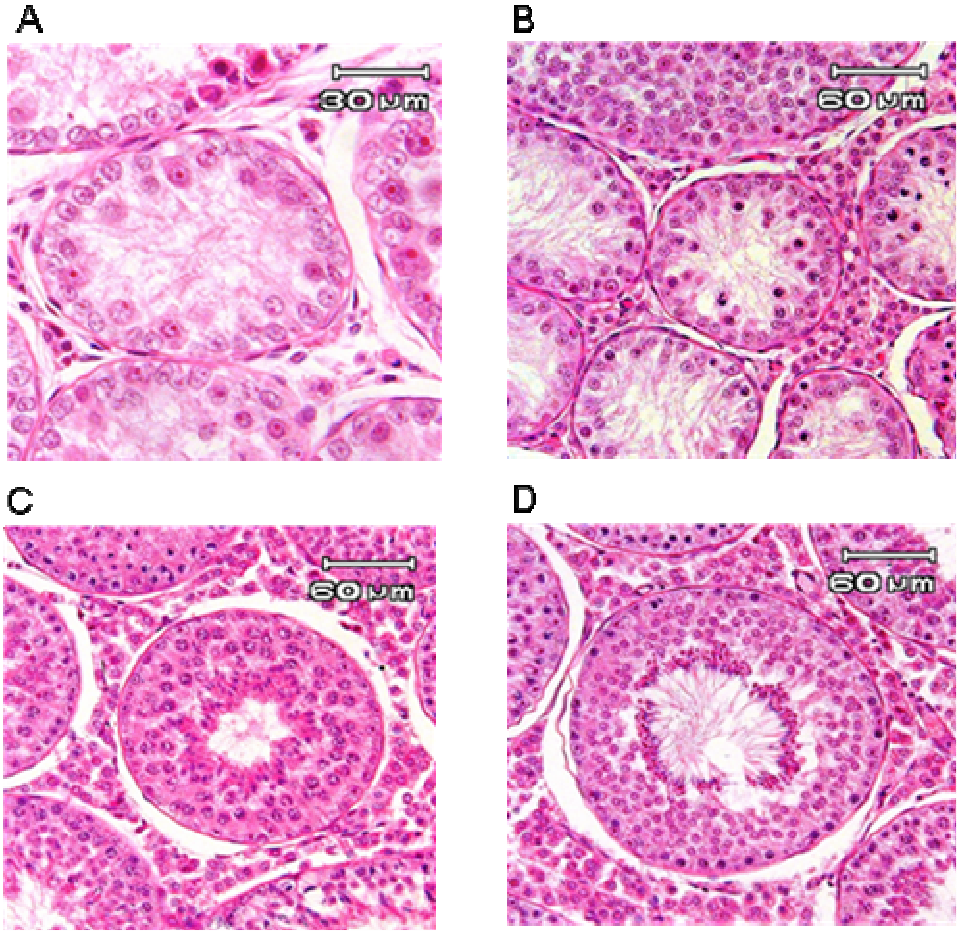

Fig. 1-2

Examples of seminiferous tubules showing each spermatogenetic score (SS) in hematoxylin-eosin sections.

A: SS 1, B: SS 2, C: SS 4 and D: SS 5.

Few seminiferous tubules showing SS 3 were observed.

SS was evaluated based on the most advanced spermatogenetic cells present as following;

SS 1: spermatogonia,

SS 2: no cells further than primary spermatocytes,

SS 3: some cells further than secondary spermatocytes,

SS 4: round spermatids,

SS 5: elongated spermatids and/or spermatozoa.

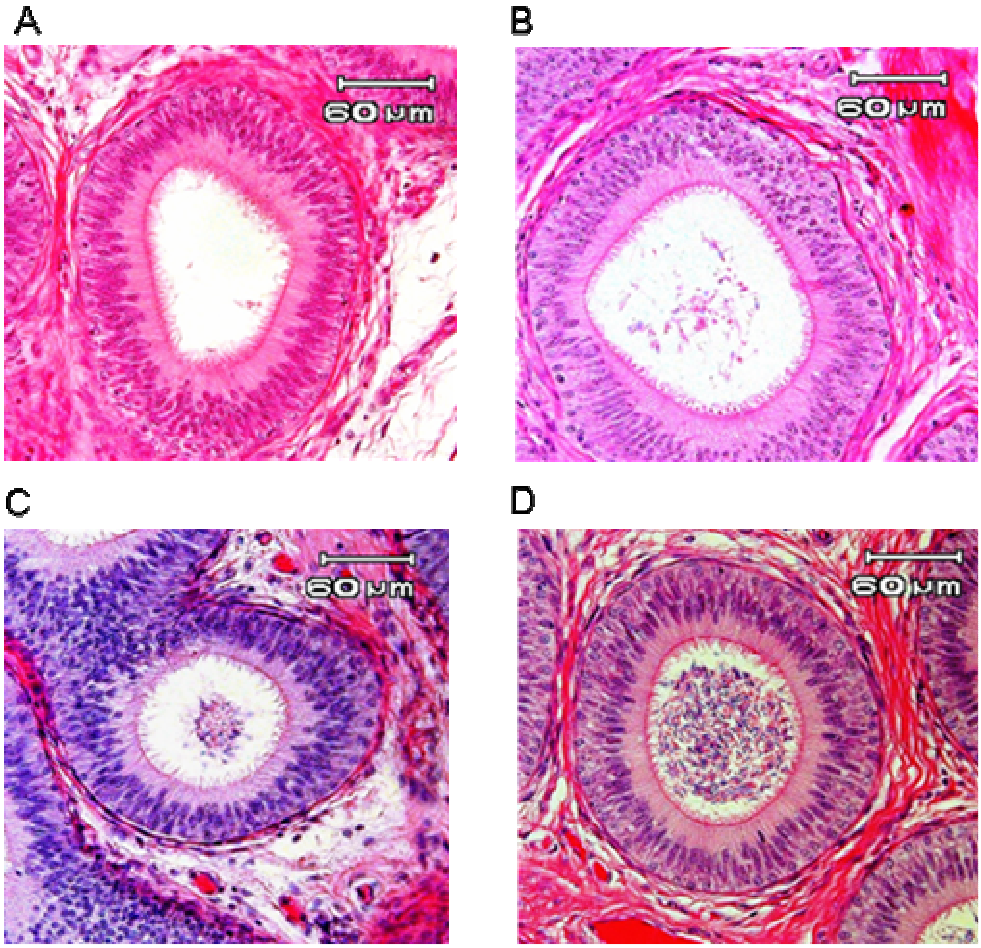

Fig. 1-3
Examples of cauda epididymis tubules showing each presence level of spermatozoa in hematoxylin-eosin sections.
A: Level 0, B: Level 1, C: Level 2 and D: Level 3.

The criteria were as following;
Level 0: no spermatozoa,
Level 1: spermatozoa observed in less than 10% of the duct lumen,
Level 2: spermatozoa observed in 10-50% of the duct lumen,
Level 3: spermatozoa observed in more than 50% of the duct lumen.

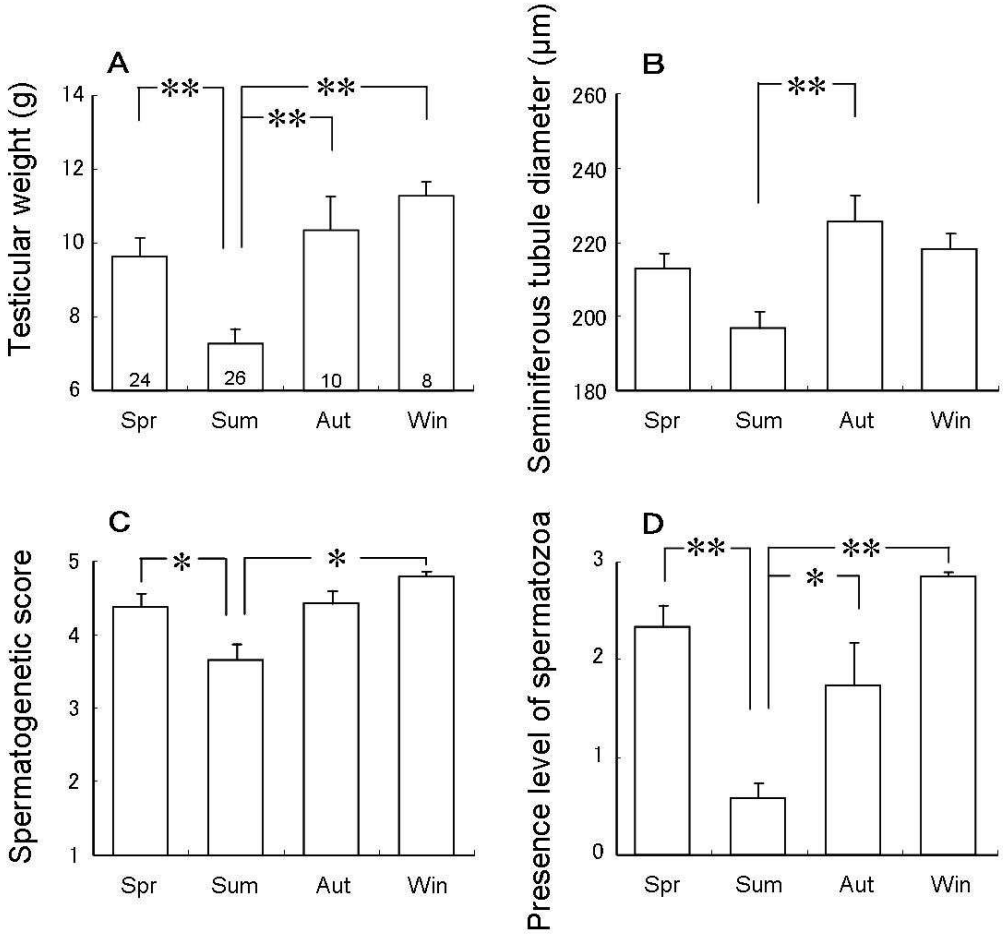

Fig. 1-4

Seasonal changes in testis weight (A), seminiferous tubule diameter (B), spermatogenetic score (C) and presence level of spermatozoa in the cauda epididymis (D) in feral male raccoons.

The number of samples used in each season is shown in each value bar. The sample numbers of B, C and D are the same as in A.

Values shown are means and error bars mean SEM.

Asterisks indicate significant differences between seasons (*P< 0.05, **P< 0.01) by Sheffe's F test.

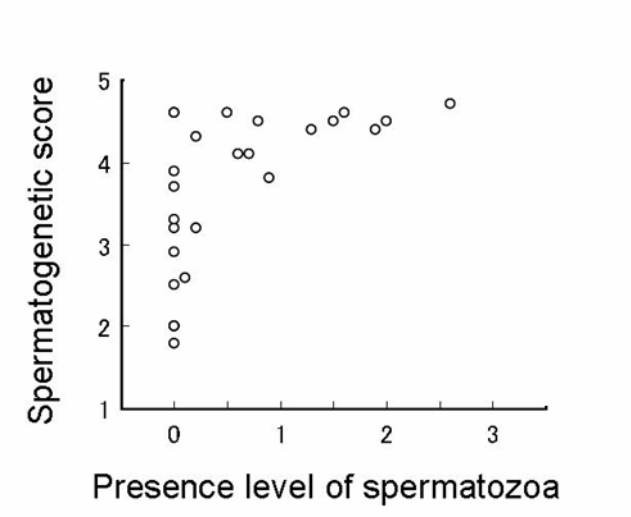

Fig. 1-5

Individual values correlating spermatogenetic score with presence level of spermatozoa in the cauda epididymis of adult males in summer from July to September (n = 26).

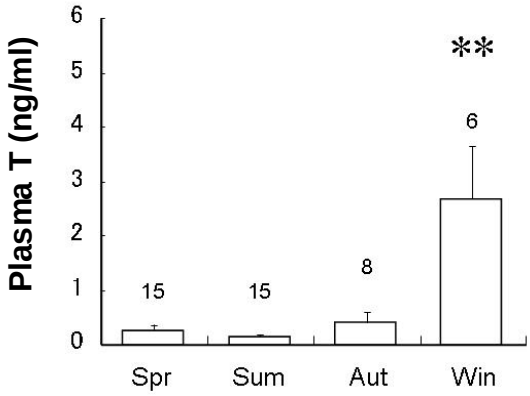

Fig. 1-6

Seasonal changes in the plasma testosterone concentration (Plasma T) in feral male raccoons. The numbers of samples used in each season are shown above each value. Values shown are means and error bars mean SEM.

Asterisks indicate significant differences between seasons (**P< 0.01) by Sheffe's F test.

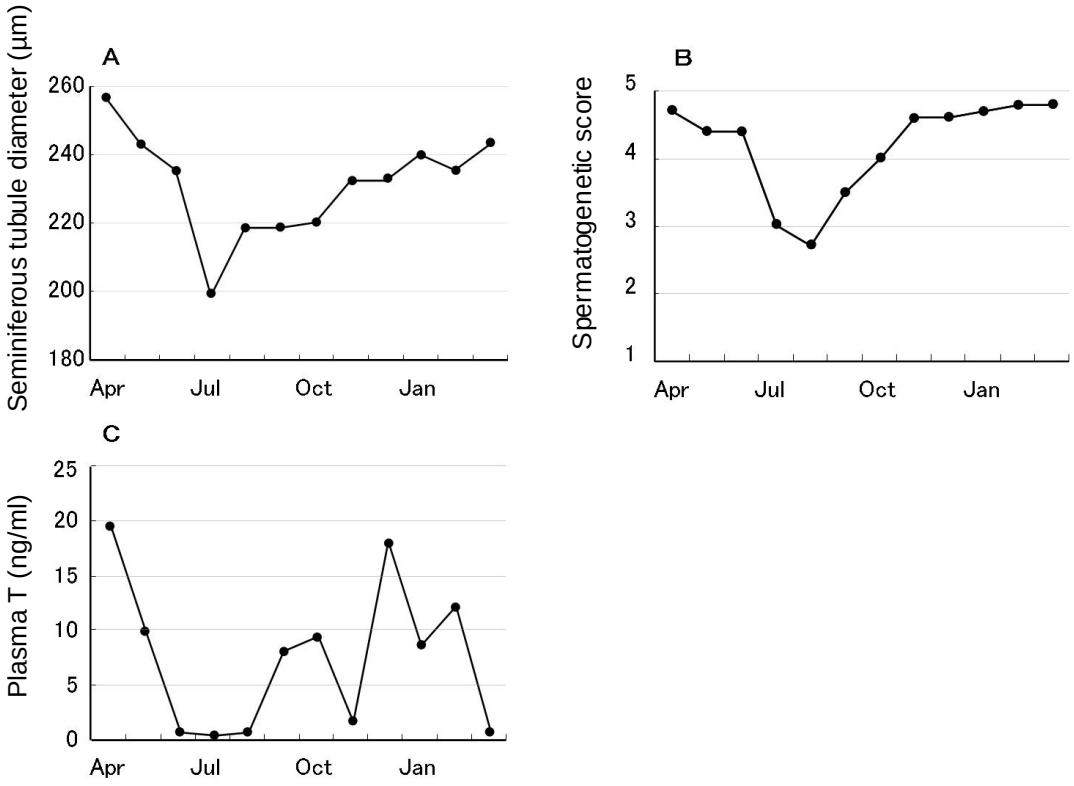

Fig. 1-7

Monthly changes in seminiferous tubule diameter (A), spermatogenetic score (B) and plasma testosterone concentration (Plasma T) (C) in one captive male raccoon from April 2009 to March 2010. Values shown are means in one individual.

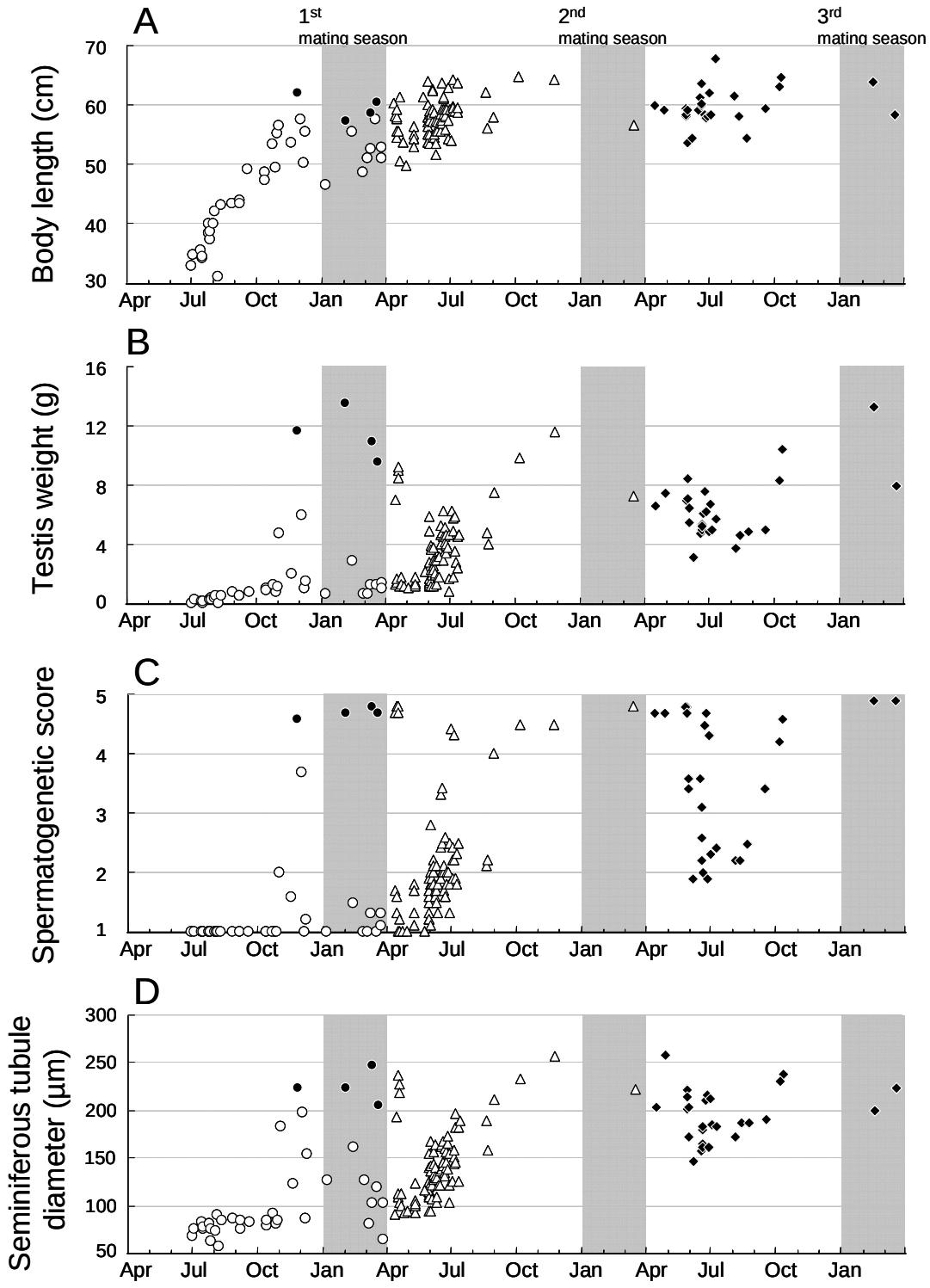

Fig. 1-8

Individual values of body length (A), testis weight (B), spermatogenetic score (C) and seminiferous tubule diameter (D) in feral, male raccoons by the month. The shaded area represents the mating season, from January through March. The symbol ●(n = 4) represents juveniles for which spermatozoa were observed in the cauda epididymis and ○(n = 35) represents the other juveniles. △(n = 85) represents all yearlings and ◆(n = 27) represents all 2-year-old individuals.

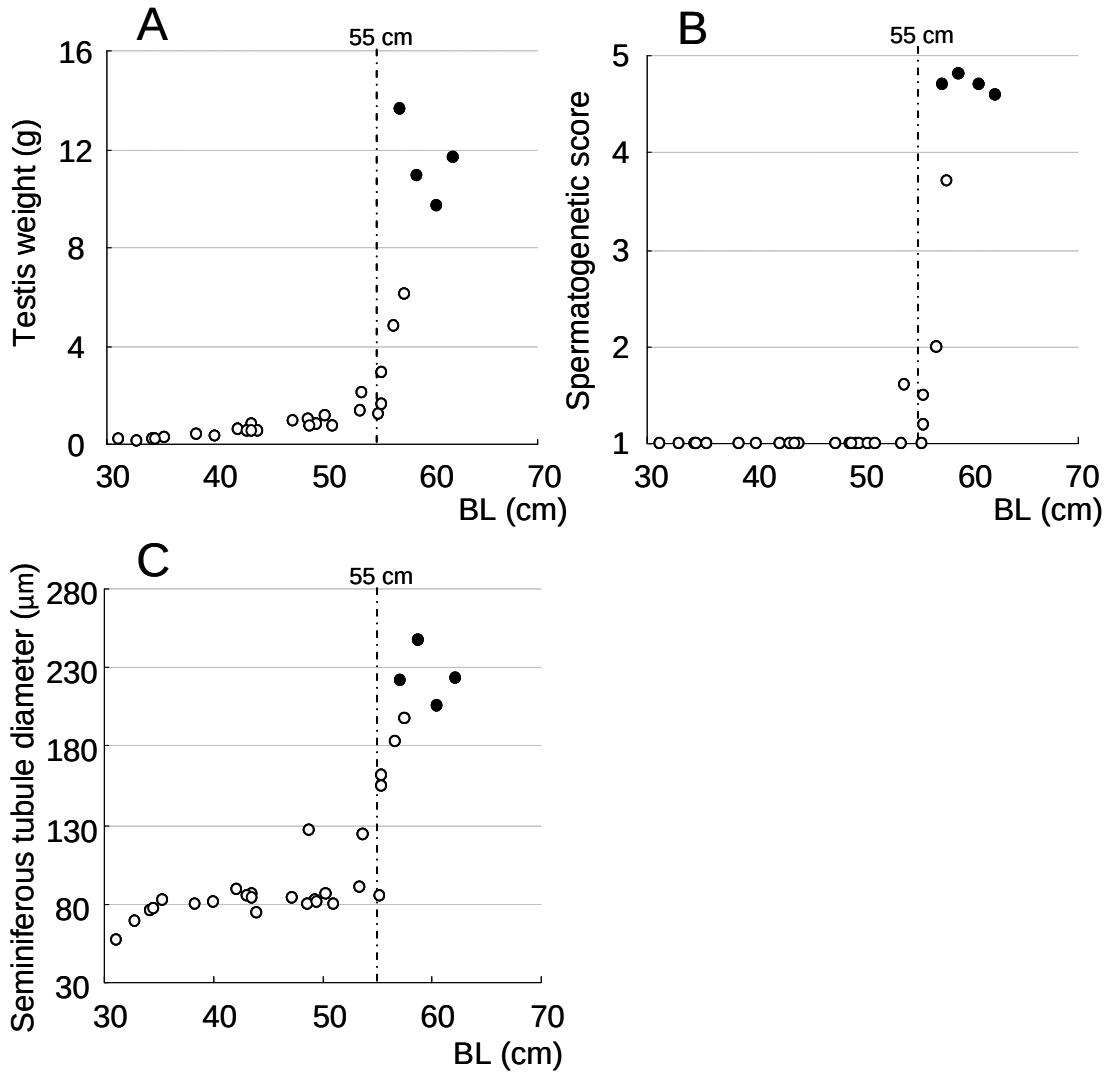

Fig. 1-9

Individual values of testis weight (A), spermatogenetic score (B) and seminiferous tubule diameter (C) according to body length (BL) in juveniles. The symbol ●(n = 4) represents individuals for which spermatozoa were observed in the cauda epididymis and ○(n = 35) represents individuals for which no spermatozoa observed.

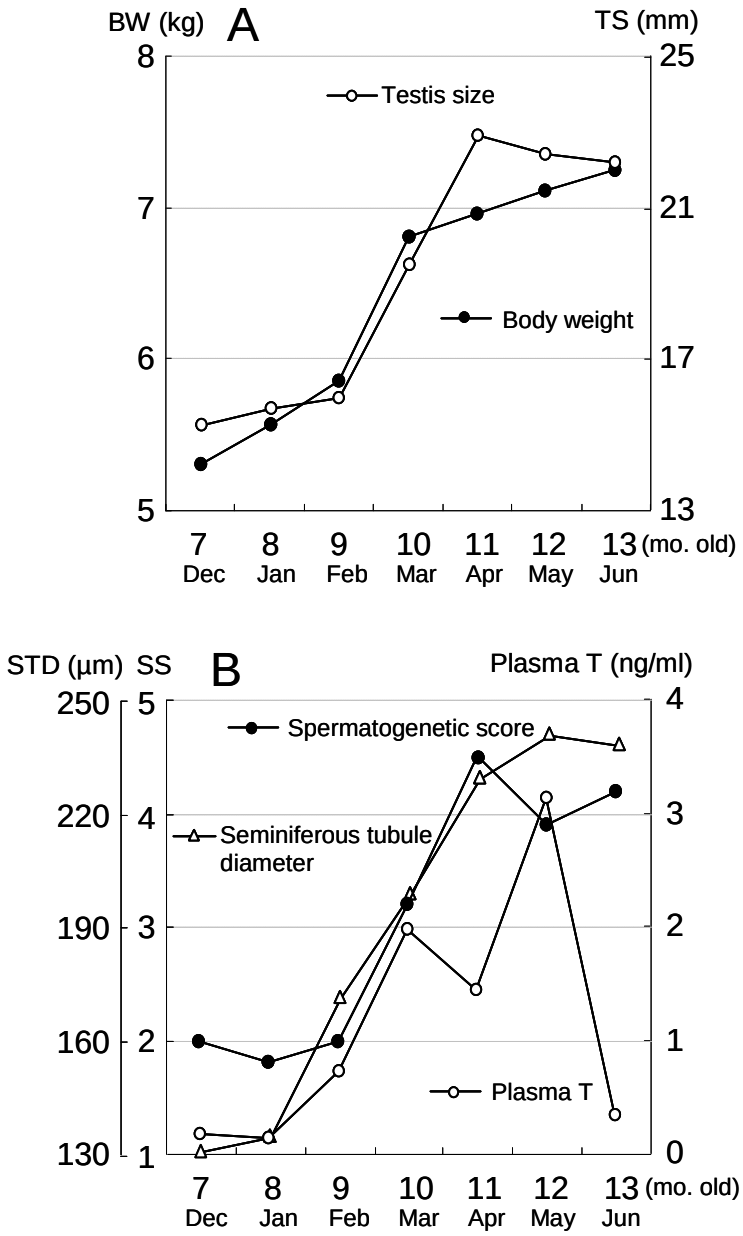

Fig. 1-10

Changes in body weight and testis size (A), spermatogenetic score, seminiferous tubule diameter and plasma testosterone concentration (B) in the captive juvenile according by age in months. Testis size was calculated as (long diameter ×short diameter × thickness)$^{1/3}$ including the scrotum. BW = body weight, TS = testis size, STD = seminiferous tubule diameter, SS = spermatogenetic score, and Plasma T = plasma testosterone concentration.

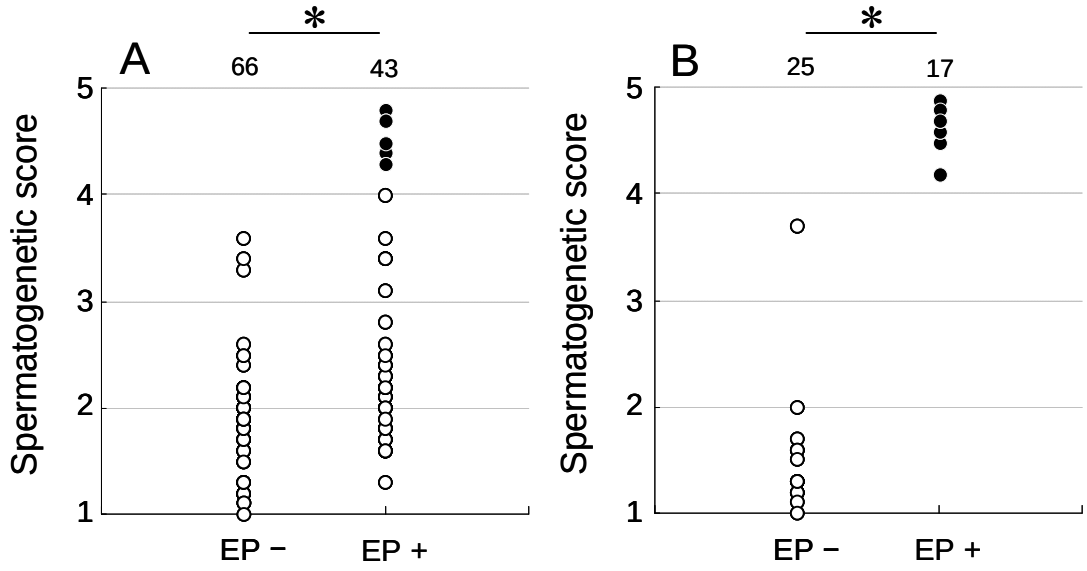

Fig. 1-11

Individual values of spermatogenetic score with all male raccoons from May to September (A) and from October to April (B) by the penis extrusible (EP+) or not (EP−). The symbol ● represents individuals for which spermatozoa were observed in the cauda epididymis and ○ represents individuals for which no spermatozoa were observed. The numbers of individuals are shown above each group. Asterisks indicate significant differences between groups (*$P < 0.001$ by Mann-Whitney U test).

Table 2-1
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	Anakwe and Payne 1987
Anti- 3β -hydroxysteroid dehydrogenase (3β HSD)	Human	Mouse	Monoclonal	1:100	sc-100466 Santa Cruz Biotechnology, Inc, CA, USA
Anti- 17α -hydroxylase cytochrome P450 (P450c17)	Porcine	Rabbit	Polyclonal	1:1000	Hales et al 1987
Anti- aromatase cytochrome P450 (P450arom)	Human	Rabbit	Polyclonal	1:100	Kitawaki et al. 1989
Anti- andorogen receptor (AR)	Human	Rabbit	Monoclonal	1:100	A9385 SIGMA-ALDEICH, St. Luis, USA

Table 2-2

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

Sample groups	Immunostaining intensity					
	P450scc	P450c17	3βHSD	P450arom	AR	
	L	L	L	L	L	S
WIN	++	++	++	+	++	++
SUM+	++	++	+	+	++	++
SUM−	++	+	$-^{3}/+^{2}$	-	$-^{3}/+^{2}$	$-^{2}/+^{3}$

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).

WIN: samples from winter (n = 5),

SUM+: samples from summer with active spermatogenesis (n = 5),

SUM−: samples from summer with inactive spermatogenesis (n = 5).

L: Leydig cell, S: Sertoli cell.

The intensity was evaluated when immunostaining detected (+), detected strongly (++), scarce or not (−).

Small numbers in the table indicate the number of samples (e.g., $-^{3}/+^{2}$ means that 3 samples were classified as − and 2 samples were classified as +).

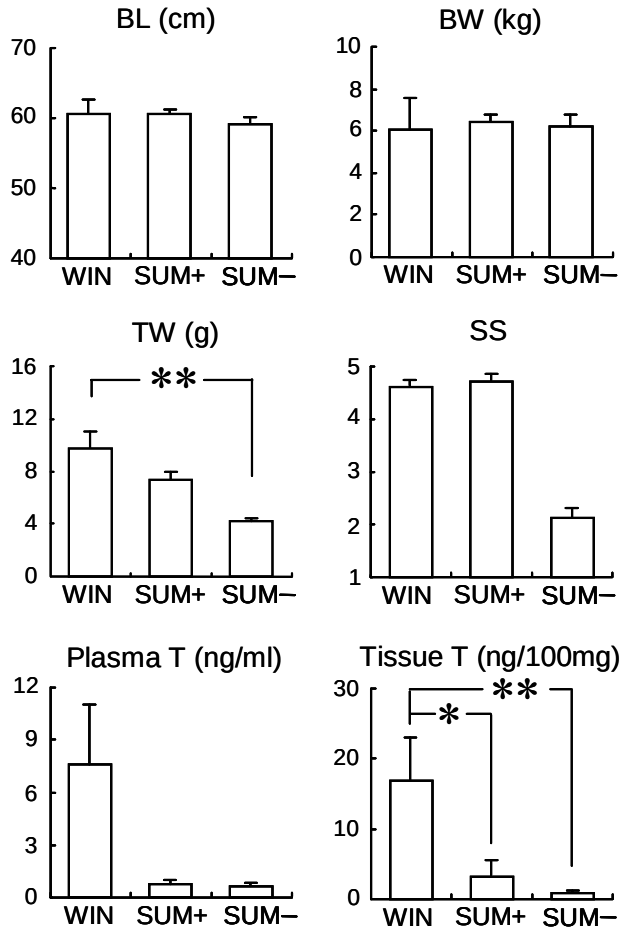

Fig. 2-1

Differences in body length (BL), body weight (BW), testis weight (TW), spermatogenetic score (SS), plasma testosterone concentration (Plasma T), and testicular tissue testosterone concentration (Tissue T; ng/100 mg testicular tissue) among three groups of WIN, SUM+ and SUM−.

WIN: samples from winter (n = 3 in Tissue T / n = 5 in other figures),

SUM+: samples from summer with active spermatogenesis (n = 5),

SUM−: samples from summer with inactive spermatogenesis (n = 5).

Values shown are means and error bars mean SEM.

Asterisks indicate significant differences between seasons (*P< 0.05, **P< 0.01) by Sheffe's F test. In SS, statistical analysis was not performed.

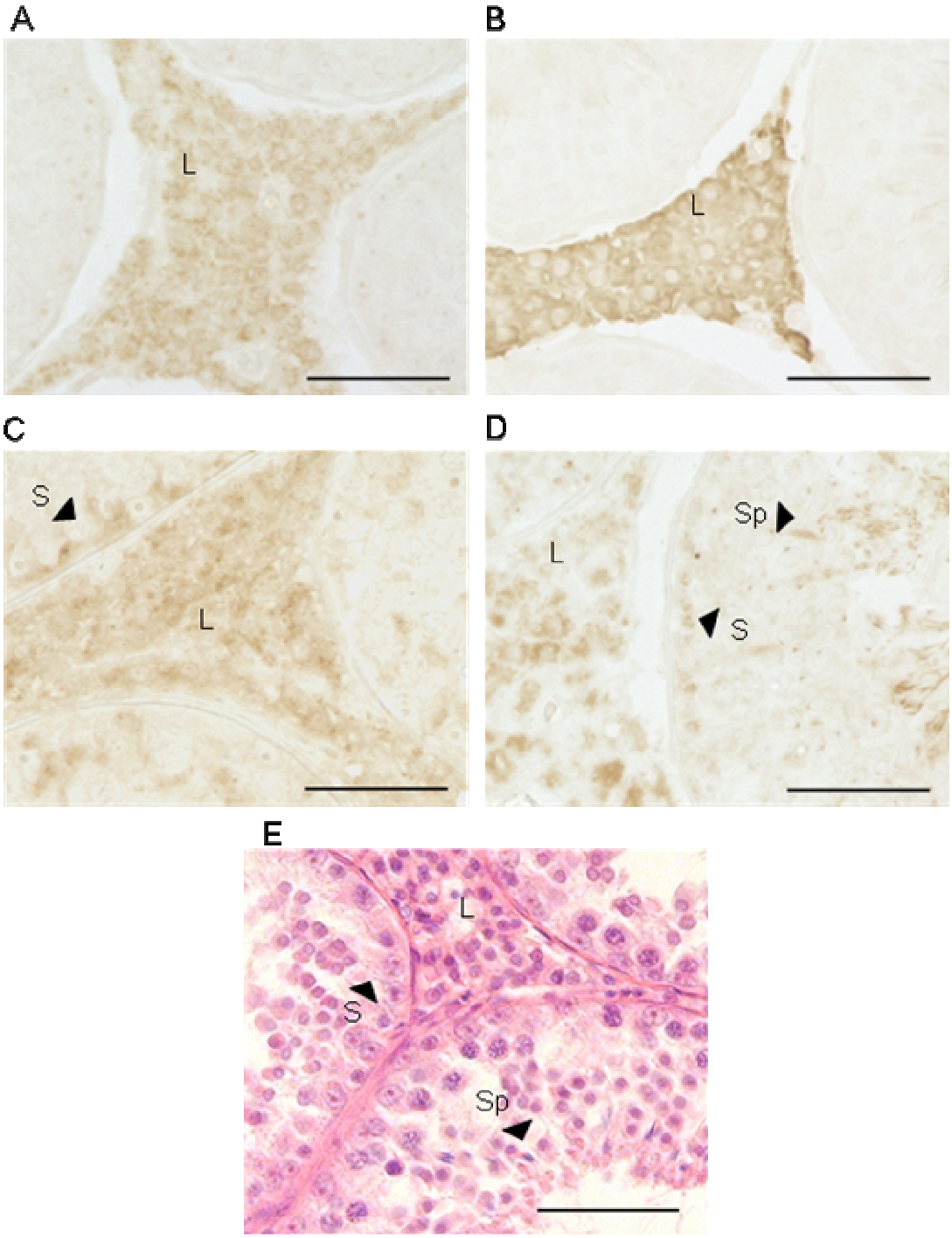

Fig. 2-2

Immunohistochemical localization of four steroidogenic enzymes (A: P450scc, B: P450c17, C: 3βHSD, D: P450arom) and E: hematoxylin-eosin staining section in raccoon testes in winter mating season.

L: Leydig cells, S: Sertoli cells, Sp: Spermatids.

Bar = 50 μm.

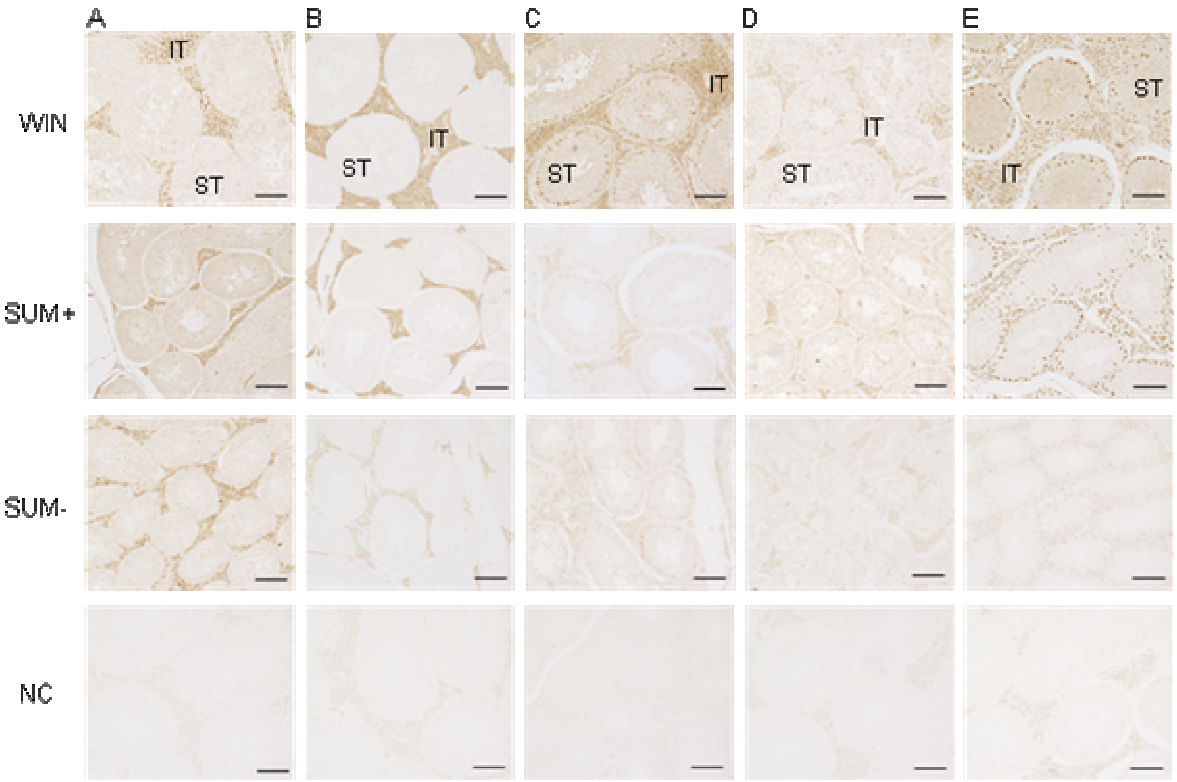

Fig. 2-3

Immunohistostaining of steroidogenic enzymes (A: P450scc, B: P450c17, C: 3βHSD, D: P450arom) and androgen receptor (E) in the representative examples of raccoon testes among three groups of WIN, SUM+ and SUM−. NC: negative control, IT: interstitial tissue, ST: seminiferous tubules.

WIN: samples from winter,

SUM+: samples from summer with active spermatogenesis,

SUM−: samples from summer with inactive spermatogenesis.

Bar = 100 μm.

Universal primers (⟶)

P1-5EZ: 5'- ATAATCACATGGAGAGCACAAGCT -3'
P2-3EZ: 5'- GCACTTCTTTGGTATCTGAGAAAGT -3'

Internal primers in set Ⅰ (- -▸)

ZFX-IPfw: 5'- AGCCGTACCAGTGCCAGTA -3'
ZFY-IPrv: 5'- TGCAGACCTATACTCGCAGAAT -3'

Internal primers in set Ⅱ (·····▸)

ZFX-IPrv: 5'- GCGGACCTATACTCGCAGTAC -3'
ZFY-IPfw: 5'- AGCCGTACCAGTGCCAATT -3'

Fig. 3-1
Partial sequence of the *ZFX* and *ZFY* genes of raccoons and the position of 2 universal primers and 4 internal primers

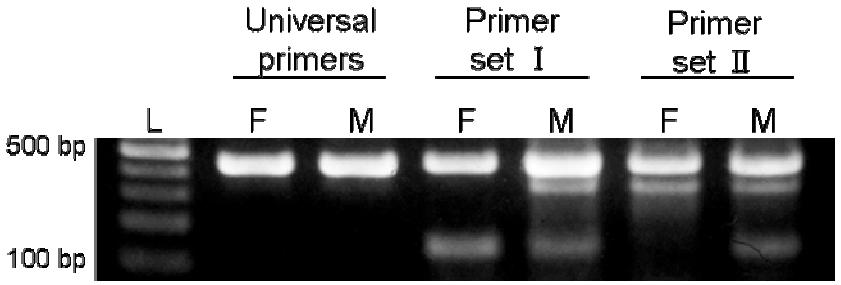

Fig. 3-2

PCR amplification results of fragments of ZFX and ZFY genes of raccoons in 1.5% agarose gel.
L : 500 bp ladder marker. F: females, M: males.
The samples from the same individuals were shown with three primer sets.

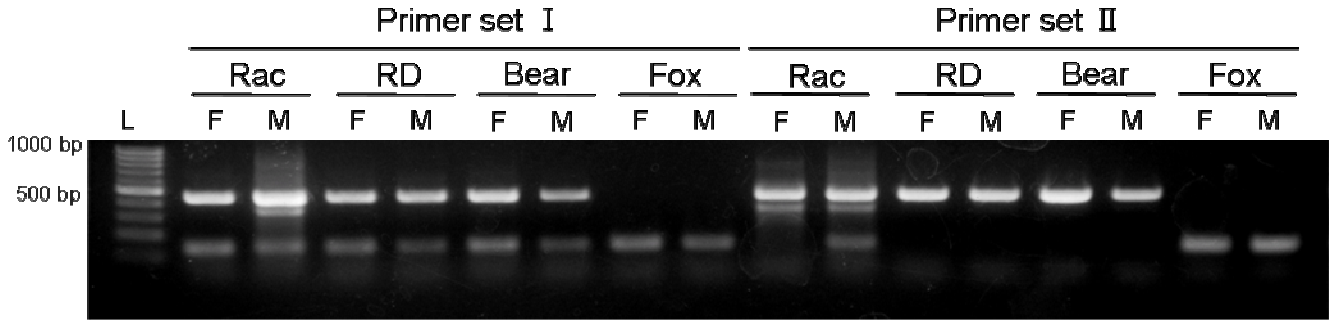

Fig. 3-3

PCR amplification results of fragments of ZFX and ZFY genes of four carnivore species in 1.5% agarose gel.
L : 1000 bp ladder marker. F: females, M: males.
Rac: Raccoons. RD: Raccoon dogs. Bear: Hokkaido brown bears. Fox: Red foxes
The samples from the same individuals were shown with both primer sets.

Table 4-1

Numbers and percentages of each haplotype in sampling municipalities.

Haplotypes				*north/all*		*hdk/tka*		*kmo/kmk*		*asb*		*asb/bie*		*ash*		*iwa*	
Sub-prefecture	Muni-cipality	ID	N	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Soya	Wakkanai	WKA	7	7	100												
	Toyotomi	TYO	19	19	100												
	Nakatonbetsu	NTB	20	20	100												
	Esashi	ESA	20	20	100												
Rumoi	Horonobe	HRN	20	20	100												
	Teshio	TSO	20	20	100												
	Enbetsu	ENB	3	3	100												
Kamikawa	Aibetsu	AIB	9					7	77.8			2	22.2				
	Toma	TMA	22	1	4.5			12	54.5			9	40.9				
	Asahikawa	ASH	24			1	4.2	2	8.3			16	66.7	5	20.8		
	Biei	BIE	19									19	100				
Sorachi	Iwamisawa	IWA	16					13	81.3	2	12.5					1	6.3
	Kuriyama	KUR	20	1	5.0	2	10	12	60	5	25						
	Ashibetsu	ASB	20							4	20	16	80				
	Tsukigata	TKI	5			3	60	2	40								
Hidaka	Hidaka	HDK	21	1	4.8	20	95.2										
	Biratori	BRA	3	2	66.7	1	33.3										
	Sinhidaka	NHD	20			20	100										
	Urakawa	URA	4			4	100										
Iburi	Abira	ABR	10	5	50	5	50										
	Atsuma	ATM	10	6	60	4	40										
	Mukawa	MK	5	1	20	4	80										
	Tomakomai	TOM	19	11	57.9	6	31.6	2	10.5								
	Date	DTE	20	14	70			6	30								
	Siraoi	SRA	1					1	100								
	Sobetsu	SOB	15	4	26.7			11	73.3								
Shiribeshi	Kimobetsu	KMO	19					19	100								
	Yoichi	YOI	20	20	100												
	Furubira	FRB	20	20	100												
	Kyowa	KYW	20	20	100												
Ishikari	Sapporo	SP	1	1	100												
	Kitahiroshima	KI	8	5	62.5			2	25							1	12.5
	Chitose	CTS	9	9	100												
	Tobetsu	TOB	13			8	61.5	2	15.4	3	23.1						
Abashiri	Abashiri	ABS	1			1	100										
	Shari	SHR	3	2	66.7			1	33.3								
Nemuro	Rausu	RUS	1			1	100										
Tokachi	Sikaoi	SKA	1			1	100										
	Shintoku	STK	13	1	7.7	12	92.3										
	Shimizu	SMZ	8			8	100										
	Memuro	MR	10	1	10	9	90										
	Kamishihoro	KSH	1			1	100										
	Urahoro	UHR	1			1	100										
	Obihiro	OBH	5			5	100										
Total			526	234	44.5	117	22.2	92	17.5	14	2.66	62	11.8	5	0.95	2	0.38

Table 4-2

Corresponding haplotype IDs which were identified in D-loop fragment among this study, reports in Japan by Takada-Matsuzaki et al. [2004] and Takada [2011],and a report in North America by Cullingham et al. [2008].

	This study	Takada-Matsuzaki et al. 2004 Takada 2011	Cullingham et al. 2008
Haplotype ID	*north/all*	RMT03	PLO 02
	hdk/tka	RMT05	PLO 32
	kmo/kmk	RMT02	PLO 32
	asb	RMT04	No report
	asb/bie	No report	
	ash	No report	
	iwa	RMT07	PLO 32

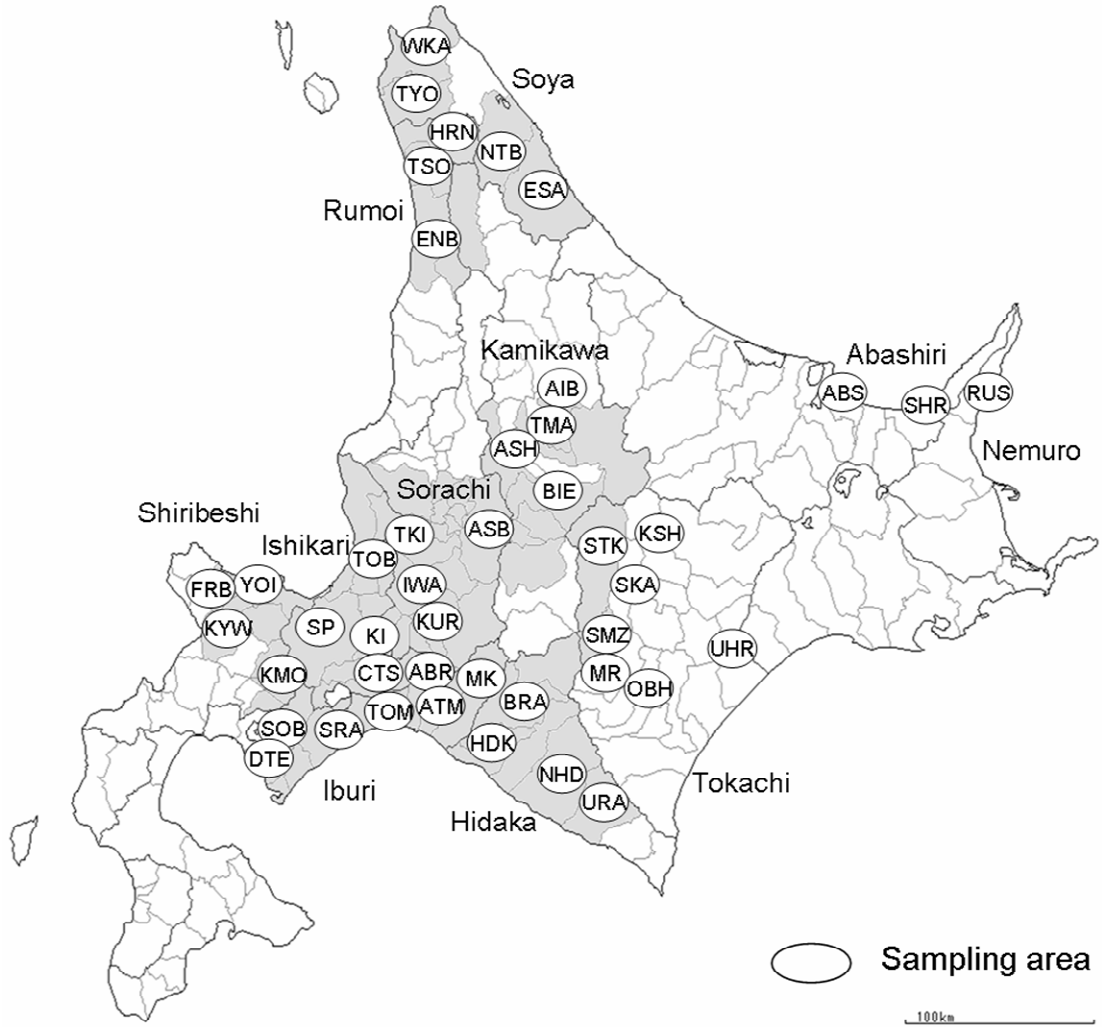

Fig. 4-1

DNA samples of raccoons from 44 municipalities were collected from 2010 to 2013 in this map.

Alphabets in each circle are comparable with municipality ID in Table 4-1.

Also subprefecture names in this map are comparable in Table 4-1.

Gray areas were municipalities where raccoons were captured in 2009.

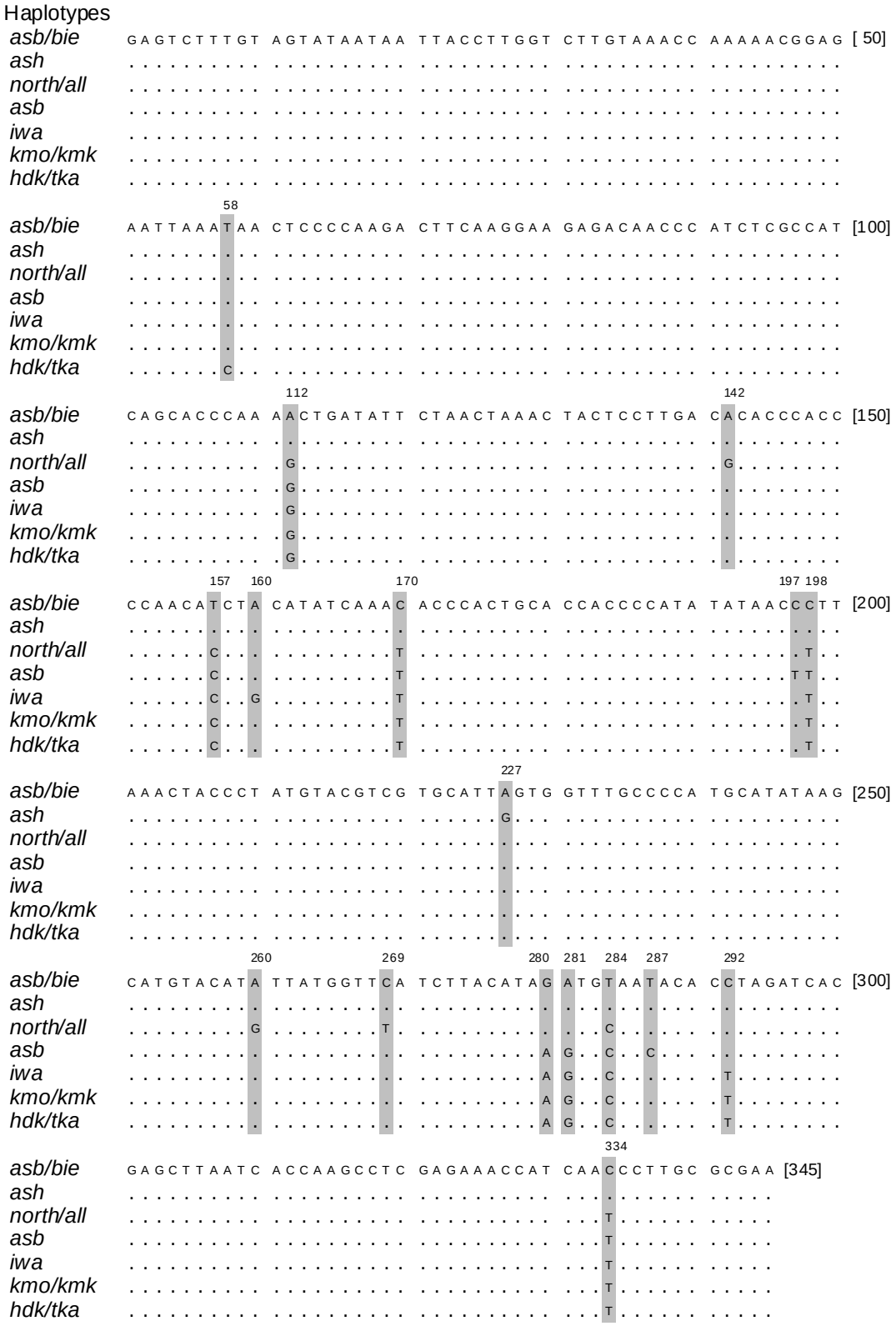

Fig. 4-2a

Sequence results of 7 haplotypes of base position from 1 to 345.

Among these haplotypes, 17 base differences were found.

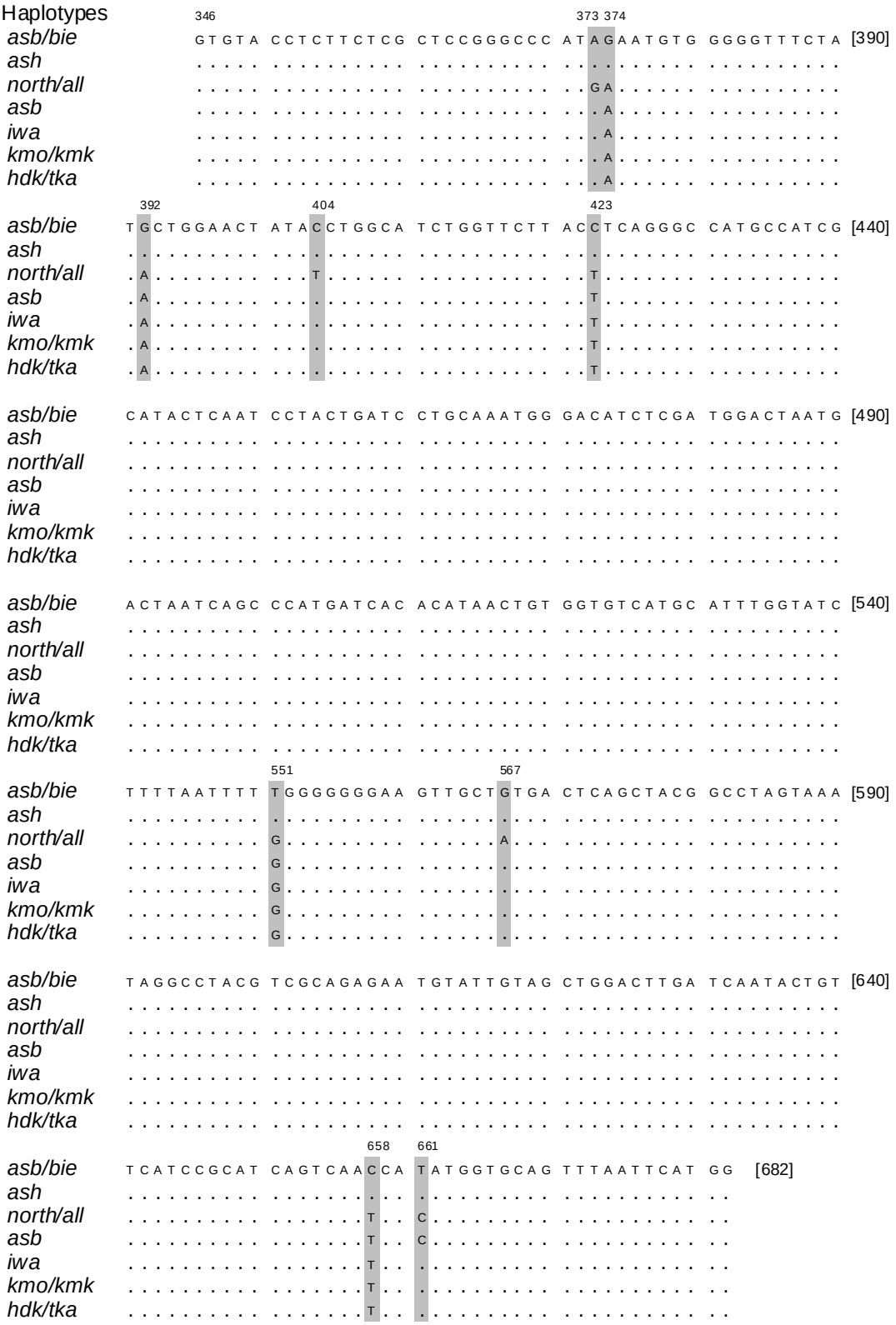

Fig. 4-2b

Sequence results of 7 haplotypes of base position from 346 to 682.

Among these haplotypes, 9 base differences were found.

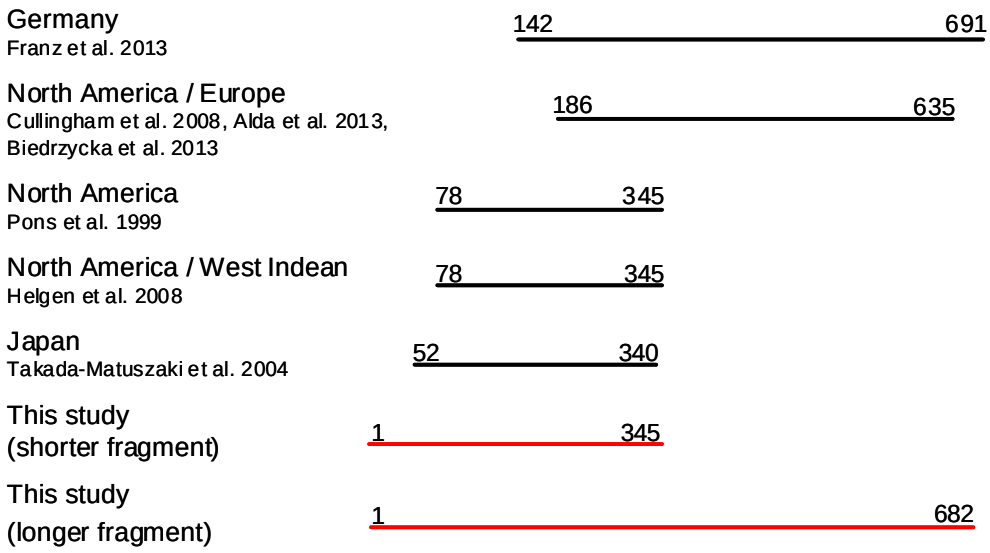

Fig. 4-3

Corresponding mtDNA D-loop fragments which was investigated in each study of raccoons.

The numbers were corresponding positions of start/end base compared with 682-bp fragment in this study.

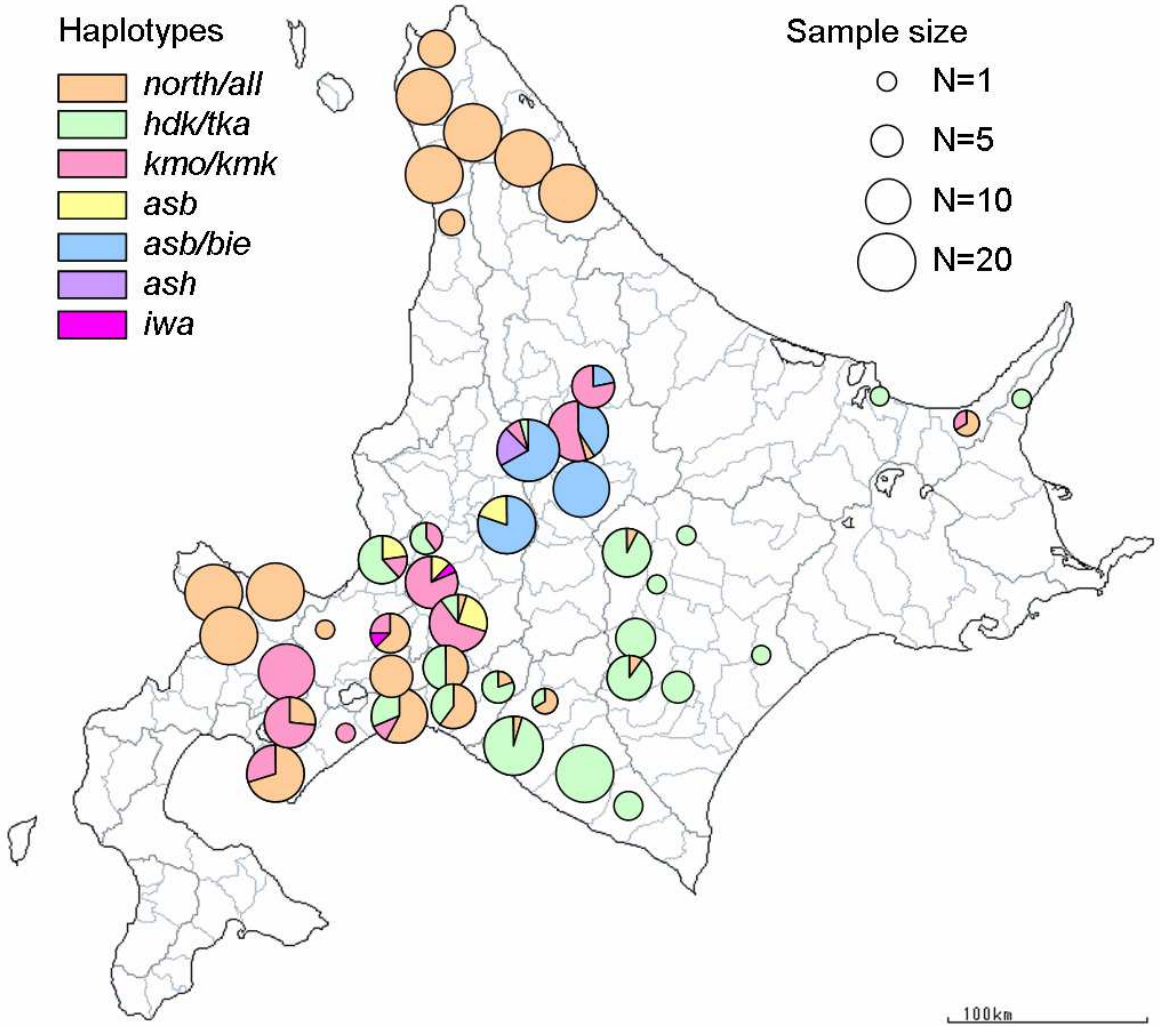

Fig. 4-4

Distribution of 7 haplotypes of raccoons in Hokkaido.

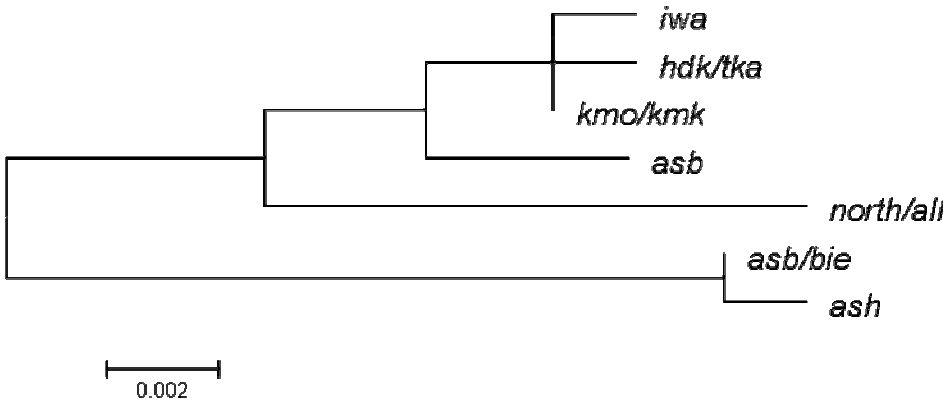

Fig. 4-5

Molecular phylogenetic analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [Tamura and Nei 1993]. The tree with the highest log likelihood (-1098.5094) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 7 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 682 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [Tamura et al. 2011].

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SYNOPSIS

Male reproductive characteristics and genetic polymorphism of feral raccoons (*Procyon lotor*) in Hokkaido

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The raccoon (*Procyon lotor*) is a mammal indigenous to North America, and has become feral since 1979 in Hokkaido as an invasive alien species. Until now, an eradication program has been conducted under the Invasive Alien Species Act; however control has not been successful yet. To decrease the raccoon population size efficiently, it is necessary to gather basic biological information. Particularly to understand the reproductive characteristics in the habitat is crucial. And it is also important to infer dispersing process and to reveal population units with geographical or genetic barrier for effective management. Thus this whole study was performed to elucidate one aspect of reproductive characteristics and genetic background of feral raccoons in Hokkaido.

First, timing of puberty and seasonal reproductive physiology were clarified in male raccoons with using histological and endocrinological method in total 219 male raccoons. For the majority of raccoons, which were born in spring, prepubertal development began in May in yearling stage, and spermatozoa production began in October prior to their second mating season in winter. On the other hand, some larger juveniles, which could achieve enough body growth, attained puberty during the juvenile period earlier than other many individuals. After reaching puberty, adult males exhibited active spermatogenesis with high peripheral testosterone concentration in winter mating season. In many seasonal breeding mammals, males generally exhibited inactive spermatogenesis

along with low testosterone concentration in non-breeding season. However in this study, some raccoon adult males were found to produce spermatozoa actively despite low plasma testosterone concentrations in the summer non-breeding season. Such an indefinite seasonal reproductive change in gonad is unique to raccoon males. Besides, to reveal the factors that regulate testosterone production and contribute to differences in spermatogenetic activity in summer, changes in the biosynthesis, metabolism and reactivity of testosterone were investigated with evaluating intensity of steroidogenic enzymes and the androgen receptor (AR) in 15 adult males. As a result, 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 testis. Immunostaining of P450arom and AR was detected in testicular tissues that exhibited active spermatogenesis in summer, while they were scarce in aspermatogenic testes. This suggests that spermatogenesis might be maintained by some mechanism that regulates P450arom expression, in synthesizing estradiol, and AR expression, in controlling reactivity to testosterone.

Second, mitochondrial DNA (mtDNA) polymorphism was clarified with using 526 DNA samples from 44 municipalities throughout Hokkaido. In most samples, sex was determined with *os penis* at the time of captured. In the other sex undetermined samples, a genetic method for sex identification was applied, which was developed based on nucleotides differences between *ZFX* and *ZFY* genes. With sequence analysis of 682-bp fragment in D-loop of mtDNA, 7 haplotypes with 26 polymorphic sites were detected and their geographical distribution in Hokkaido was observed. Including one haplotype which had been found previously in other report but not in the present study, founder event in Hokkaido was established from at least 8 female haplotypes. In 7 haplotypes in this study, 3 of them were distributed widely over subprefectures in Hokkaido and 5 haplotypes including these 3 were detected also in Honshu and Kyushu, indicating that raccoons were introduced at several remote places in Japan, or re-introduction as second release over prefectures happened after first foundation. The other 2 haplotypes were detected only in limited close regions in

Hokkaido, thus, these haplotypes were thought to be introduced in only small area and dispersed around there. Besides, a haplotype found in this study was identified as a report in non-native area in Europe and also native-range North America. An introduction of raccoons across countries by export from native habitat was revealed genetically in this study, which is the first report in Japan. Further study with using a microsatellite DNA can help to clarify genetic variability among/within population, geographical genetic barrier, and evidence of population bottlenecks at founder events.

Reproductive characteristics detected in this study, which showed a possibility of early-maturation and a perennial reproduction in male raccoons, are explaining one aspect of high reproductive potential of raccoons. And detecting geographical genetic distribution could partly infer a background of founder introduction. Further studies which investigate how and why raccoons could have been increasing and dispersing throughout Hokkaido are required for efficient eradication control against this invasive alien species.

SYNOPSIS (in Japanese)

北海道に生息する外来種アライグマ(*Procyon lotor*)における雄の繁殖特性および遺伝多型に関する研究

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アライグマ(*Procyon lotor*)は、北アメリカ原産の哺乳類であり、北海道では 1979 年以降野生化が確認され外来種として定着している。これまでアライグマによる農業被害が増加し、さらに生態系への影響および人獣共通感染症媒介の可能性などが危惧されることから、北海道では外来生物法のもと緊急対策としての捕獲・防除を行っている。しかし、現在分布拡大および個体数増加に歯止めがかかっているとはいえない状況である。外来生物の効果的な防除には、その生息地における基礎生物学的な情報の収集が不可欠であり、特に個体数増加に関わる繁殖生理学的情報、および分布拡大に伴う個体群の遺伝情報を明らかにする必要がある。その観点から、本研究においては雄の繁殖特性および北海道に分布する遺伝多型を明らかにすることを目的とした。

１）雄の繁殖特性

219 個体の雄アライグマの個体計測および生殖器の組織観察によって、性成熟時期を特定し、成雄の季節繁殖性を明らかにした。春に生まれた雄個体の多くは 1 歳で迎える翌 5 月から生殖腺の発達が始まり、冬の交尾期を前にした 10 月には精子形成が完了することが明らかになった。一方、十分に体が成長した個体については 0 歳のうちに精子形成を行っている早期性成熟個体が確認された。性成熟に至った雄は、冬の交尾期に高い血中テストステロン濃度を示し、活発に精子形成を行っていることが確認された。季節繁殖性を示す雄動物

の多くは、非交尾期には低血中テストステロン濃度に伴う精子形成機能の停止が認められるが、アライグマにおいては、非交尾期である夏に血中テストステロン濃度が低いにも拘わらず活発に精子形成を行っている個体が確認された。このことは雄アライグマの繁殖生理学的特徴であるといえる。さらに、夏の精子形成維持機構の詳細を明らかにするため、精巣組織における 4 種のステロイド代謝酵素およびアンドロジェン受容体(AR)の発現を、15 個体より得た精巣組織において免疫化学組織的手法を用いて評価した。その結果、季節的な血中テストステロン濃度の変化は 3βHSD の発現強度の変化と同調しており、テストステロン産生は 3βHSD 発現がキーファクターとなり季節的な変化を示していることが示唆された。また、非交尾期である夏に精子形成を行っていた精巣組織においては、P450arom および AR の発現が強く観察され、夏に精子形成を行っていなかった精巣組織ではそれらの発現が見られなかった。このことから、夏に精子形成機能を維持する機構には、P450arom によって産生されるエストラジオールの働きと、精巣組織における AR によるテストステロン感受性が維持される必要があることが示唆された。

２）北海道に分布する遺伝多型

北海道全域から 526 個体分の遺伝子材料を収集し、ミトコンドリア DNA の D-loop における遺伝多型を明らかにした。サンプルの多くは、その収集時に性別が確認できたが、一部性別不明のサンプルについては、*ZFX/ZFY* 遺伝子の塩基配列差異に基づく性判別手法を確立し、その手法を用いることで雌雄判別を行った。ミトコンドリア DNA の D-loop をターゲットとした PCR を行い、増幅された 682 bp の DNA 断片の塩基配列を解析した結果、北海道のアライグマにおいて 7 つのハプロタイプの遺伝型を確認した。先行研究の結果を鑑みると、北海道において導入された母集団は、少なくても 8 つの異なるハプロタイプを持つ個体から構成されていたことが示唆された。本研究で確認された 7 つのハプロタイプのうち、3 つは北海道に広く分布しており、それらを含む 5 つのハプロタイプは本州や九州でも存在が確認されていることから、アライグマの初期導入が原産地から日本の複数の地域に導入された、もしくは日本国内での移動による再導入が起こったことが示唆された。

その他 2 つのハプロタイプは北海道内の限局した地域にのみ認められた遺伝型であり、限られた地点で野生化し、その周辺のみで個体数を増加させていることが確認された。また、同様にアライグマが外来種として問題になっているヨーロッパで報告された北米原産のハプロタイプが北海道を含む日本の複数箇所で確認されたことから、本研究によってはじめて、原産国である北アメリカからペットや毛皮動物として世界中に輸出導入されたアライグマが日本の複数個所にも導入されたことが明らかになった。今後さらに調査地を広げ、マイクロサテライト DNA を対象とした解析を行うことで、個体群間および個体群内の遺伝的差異、地域間での遺伝的交流の有無、さらには導入初期の個体数推定などの解明が求められる。

本研究によって得られた繁殖生理学的知見は、アライグマの早期性成熟や非交尾期における生殖能力の維持を示すものであり、北海道での個体数増加の一因である繁殖力の強さの一端を解明することができた。また、アライグマの生息域拡大に伴う遺伝型の分布を明らかにしたことで、初期導入がどのような経緯で起こったのかを一部推察することができた。アライグマが北海道において個体数を増やすことができた要因と生息域を拡大させてきた経緯を明らかにすることで、今後のアライグマ防除計画等に活かしていくことが期待される。

Dedicated to my family.