

Timing of Puberty and Its Relationship with Body Growth and Season in Male Raccoons (*Procyon lotor*) in Hokkaido

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Abstract. The raccoon (*Procyon lotor*), indigenous to North America, has naturalized in Japan as an invasive alien species, having been introduced into the country in the 1970s. In Hokkaido, the northernmost island of Japan, feral raccoons have been increasing in number and spreading throughout the island. The age at the onset of puberty for raccoons is important for estimating individual lifetime reproductive success and population growth. The present study 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.

Key words: *Procyon lotor*, Puberty, Raccoon, Reproduction, Spermatogenesis

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The raccoon (*Procyon lotor*) is a mammal indigenous to North America that has naturalized in Japan as a result of escape from captivity and irresponsible releases since the 1970s [1]. In Hokkaido, the northernmost island of Japan, feral raccoons have been increasing in number and spreading throughout the island [1, 2]. An eradication program has been conducted under the Invasive Alien Species Act, which came into force in 2005 as a response to agricultural damage, adverse effects on biodiversity, and the possibility of zoonotic infection. However, controls have not yet been successful. For effective population control, gathering basic biological information, particularly concerning reproductive characteristics in the wild, is crucial.

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 [2], which is similar to the timing in North America [3–5]. In the central region of Hokkaido, 66% of juveniles and 96% of yearlings and adult females get pregnant [2], 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 [6]. Therefore, the timing of puberty is an essential factor affecting population growth [7].

Puberty in male animals is defined by Lincoln [8] 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 [4]. In Alabama [3], Michigan [5] and North Dakota [9], males reach puberty during the second mating season as yearlings. In Texas, on the other hand, only 20% of yearlings possess sperm [10]. Such differences might be caused by the surrounding environment, such as climate, temperature, light, and season, as well as food availability [11]; however, the timing of the onset of puberty is not known for male raccoons in Hokkaido.

We studied the timing of puberty and identified possible triggers of the onset of prepubertal development in male raccoons by examining changes in testicular developmental using both feral and captive raccoons in Hokkaido.

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Materials and Methods

Animals

We collected carcasses of feral male raccoons that were euthanized in the eradication program in west-central Hokkaido from May 2008 to March 2011 (Fig. 1). In total, 151 male raccoons under 2 years old were employed for this study (Table 1). Raccoon carcasses were weighed, body lengths were measured, and the extrusibility of the penis was checked. The left testis was weighed, and the long diameter, short diameter and thickness were measured; the testis was then immediately fixed for about 12 h in Bouin's solution for histological observation. The lower jaws were boiled, and then the lower canine teeth were removed for age determination.

We also used a captive juvenile raccoon kept at the Asahiyama Zoological Park and Wildlife Conservation Center, Asahikawa, Hokkaido (Fig. 1). We collected samples once a month from December 2008 to June 2009 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, Fukushima, Japan), and 3.0 mg/kg ketamine hydrochloride (Ketalar, Daiichi Sankyo, Tokyo, Japan). At the time of collection, the raccoon was weighed, testicular size including the preputium was measured, 10 ml blood was collected from the jugular vein, and testes were biopsied for samples of approximately 4 mm³. 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 were treated in the same way as in the feral raccoons. Blood was spun at 1,050 g for 10 min, and plasma was removed and stored at -30 C until the assay. 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) and adults (over 2 years old) by examining their body size, tooth eruption and root foraminal closure of the canine teeth [12, 13]. In the yearling and adult groups, age was determined by the number of cementum annuli of the canines [12, 14]. In Hokkaido, most litters are born between March and May [2]; therefore, in this study, all raccoons were assumed to have been born on 1 April, and age was evaluated accordingly.

Histology

Testicular tissues were dehydrated in an ethanol series, embed-

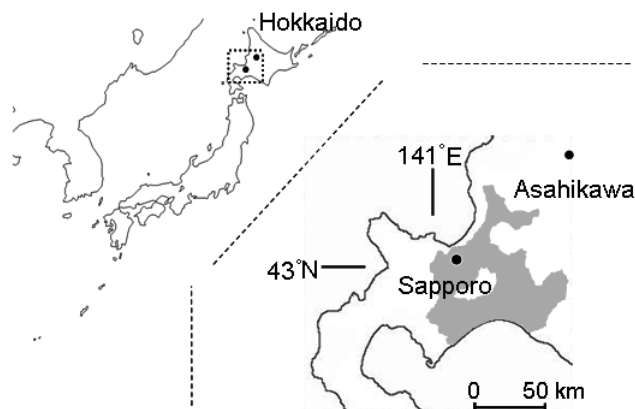


Fig. 1. The captive raccoon was located in Asahikawa, and the gray area represents the study area for feral raccoons in the west-central region of Hokkaido, Japan (2008–2011).

ded in paraffin wax, sectioned at a thickness of 4 µm, and stained with hematoxylin-eosin. Four fields of view were chosen from the entire testis under a microscope using low (×100) and high (×400) magnification. From each field, 10 seminiferous tubules were chosen randomly, and the mean diameter of the seminiferous tubules was measured using a micrometer and image analysis software (ImageJ, W.S. Rasband, US 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

Spermatogenesis was evaluated as the mean value of each seminiferous tubule chosen, as described above, according to a score based on the most advanced spermatogenic cells present [15, 16]: score of 1, spermatogonia; score of 2, no cells further along than primary spermatocytes; score of 3, some cells further along than secondary spermatocytes; score of 4, round spermatids; and score of 5, elongated spermatids and/or spermatozoa.

The existence of spermatozoa in the cauda epididymis was also checked for each individual.

Enzyme immunoassay

Plasma testosterone concentrations were measured using a testosterone assay (enzyme immunoassay) [16]. Testosterone-3-CMO-HRP (FKA101, Cosmo Bio, Tokyo, Japan) was diluted 600,000-fold with assay buffer. Standard testosterone (Cayman, Ann Arbor, MI,

Table 1. Monthly number of captured feral male raccoons

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 with spermatozoa in the cauda epididymis are shown in parentheses. Newborn juveniles were never captured before June.

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, Tokyo, Japan) was used as the secondary antibody. The minimum detectable level of testosterone was 4.9 pg/well, and the intra- and interassay coefficients of variation were 10.5% and 15.7%, respectively.

Statistical analysis

Spermatogenic scores were compared between groups of individuals with an extrusible penis and groups with the preputia remaining using Mann-Whitney U tests in Microsoft Excel 2003 for Windows.

Results and Discussion

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). In this study, testicular weight (TW), spermatogenic score (SS) and seminiferous tubule diameter (STD) were used as indicators of seasonal morphological and physiological changes in the male gonad in accordance with reports for other species. These values increase remarkably during prepubertal development in several species such as red deer stags (*Cervus elaphus L.*) [8], rhesus monkeys (*Macaca mulatta*) [17] and Japanese monkeys (*Macaca fuscata*) [18].

Body length (BL) greatly increased during the juvenile period (Fig. 2A). In almost all individuals, TW and SS remained low until around April of the yearling stage and then greatly increased from May to the following November (Fig. 2B and C). 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. 2D). 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 2-year-old individuals, TW, SS and STD declined from June to September (Fig. 2B–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). In adult males older than 3 years, spermatogenesis was significantly less active in summer than during other seasons [16]. Two-year-old individuals 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, in some juveniles, TW, SS, and STD were detected at levels as high as in yearlings captured after October (Fig. 2B–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). To determine the difference between these early maturing juveniles and other juveniles, the relationship between each of these three values and raccoon body condition was examined using BL (Fig. 3). In Hokkaido, the body weight 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 [16]. Therefore, BL is considered to be a

more suitable parameter than body weight for estimating raccoon body development. In juveniles, TW and STD increased slightly based on BL development, and after reaching a BL of about 55 cm, these two values increased remarkably (Fig. 3A and C). Similarly, SS remained at a score of 1 until a BL of about 55 cm and subsequently increased remarkably (Fig. 3B). The age at the onset of puberty is strongly influenced by the plane of nutrition availability [8], 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 [6]. 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 [19]. These body weights were remarkably heavier than the average weight of 6-month-old juveniles (females, 4.62 kg; males, 5.42 kg [20]) and relatively heavier than even adults. Dorney [19] 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 our 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 two 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 [8] 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 our study, 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. 4A and B). In addition, STD and plasma testosterone concentration began to increase at 8 months old (in January; Fig. 4B) based on body weight gain. Furthermore, elongated spermatozoa were observed to be released within the lumen of seminiferous tubules in April, only 11 months after birth. 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 [16]. Generally, the timing of reaching puberty is influenced by the surrounding environment [11], and puberty appears to represent the start of the seasonal cycle of testicular activity [8, 18]. 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 [8] 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 [21]. 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.

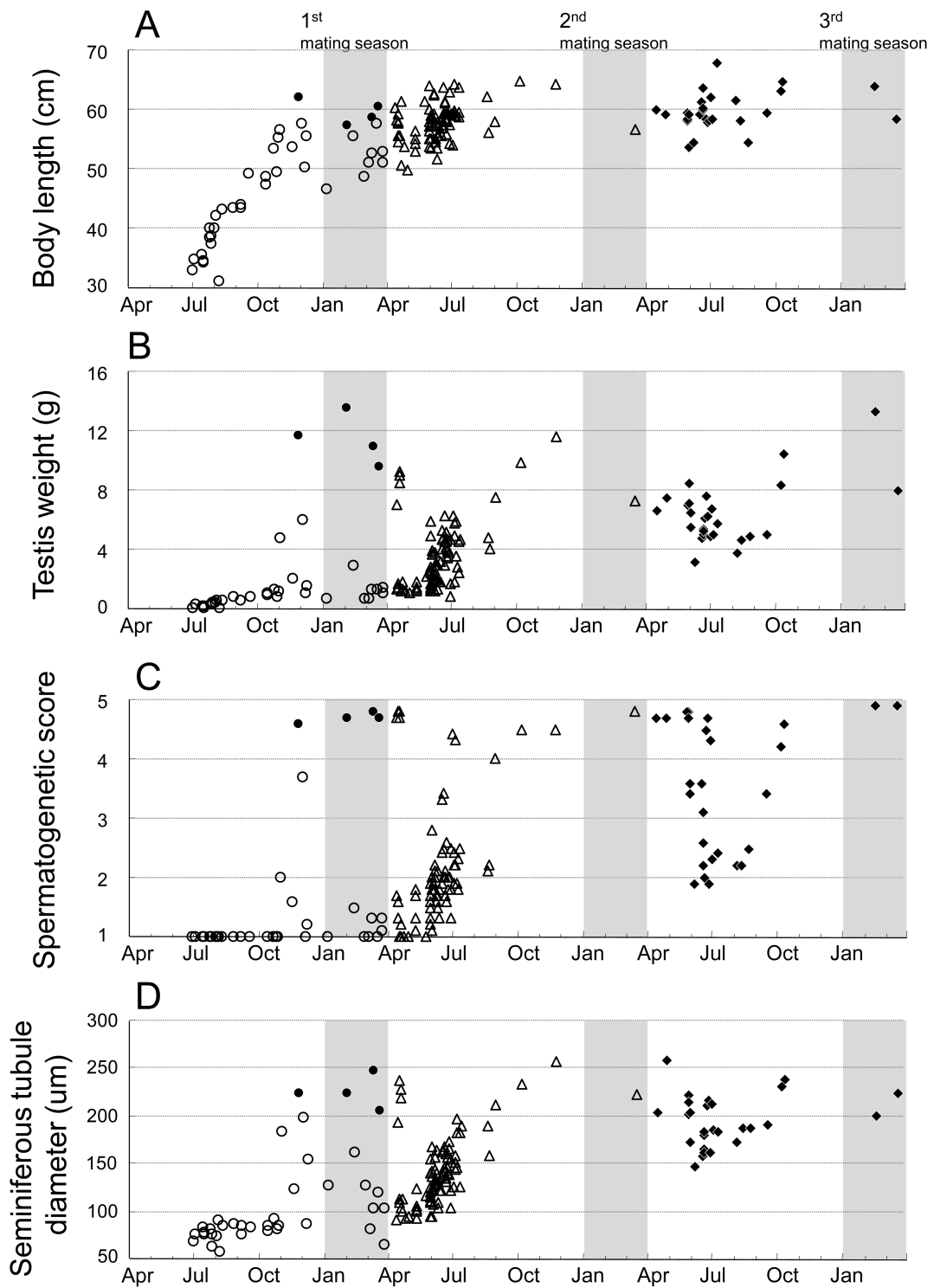


Fig. 2. Individual values of body length (A), testis weight (B), spermatogenic score (C) and seminiferous tubule diameter (D) in fetal male raccoons by month. The shaded area represents the mating season, from January through March. The filled circles (●) represent juveniles for which spermatozoa were observed in the cauda epididymis, the unfilled circles (○) represent the other juveniles, the unfilled triangles (△) represent all yearlings and the filled diamonds (◆) represent all 2-year-old individuals.

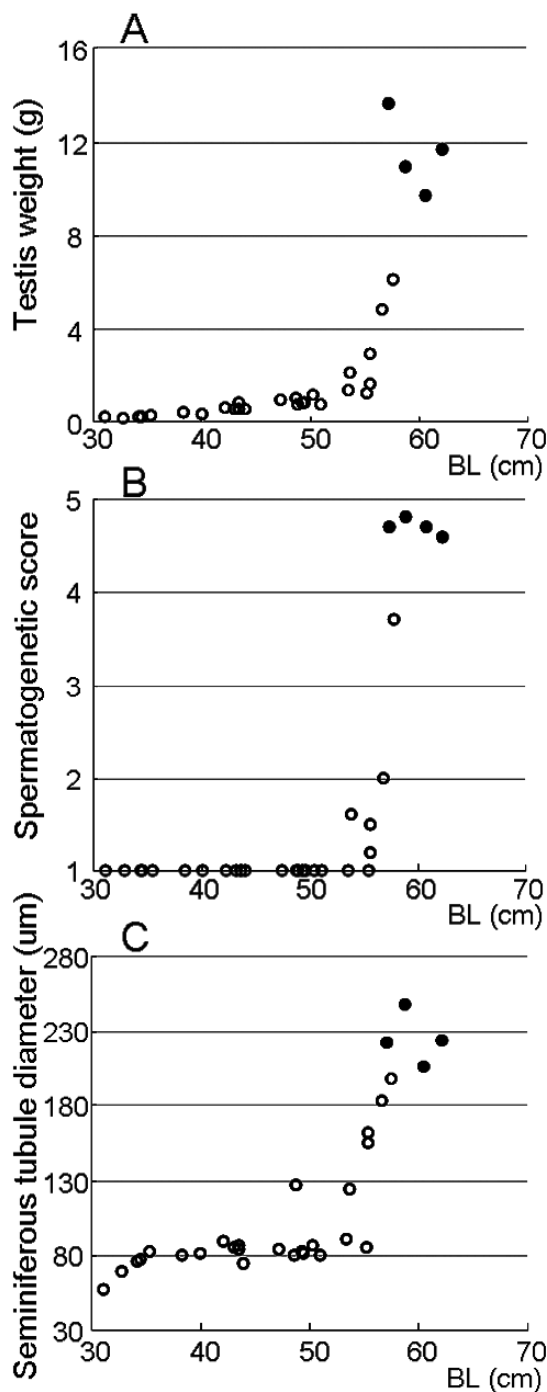


Fig. 3. Individual values of testis weight (A), spermatogenic score (B) and seminiferous tubule diameter (C) according to body length (BL) in juveniles. The filled circles (●) represent individuals for which spermatozoa were observed in the cauda epididymis, and the unfilled circles (○) represent individuals for which no spermatozoa were observed.

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. 2). 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, the extrusibility of the penis (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 [22]. In Illinois, the majority of males achieve EP during the juvenile period [4]. In the present study, all early-maturing individuals with spermatozoa observed 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). This period corresponded to that of substantial testicular development, suggesting that attaining EP appears to occur during prepubertal development. After this period, almost all males had attained EP (EP = 90%, 27/30 after October of the yearling stage). To evaluate whether EP can serve as an indicator of spermatogenesis, values of SS were compared between groups based on the extrusibility of the penis (EP+ or EP-) during the periods of May–September and October–April using Mann-Whitney U tests. SS for EP+ raccoons was significantly higher than for EP- raccoons during both periods ($P < 0.001$). However in May–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. 5A). In October–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. 5B). Therefore, EP can only be used as an external indicator of attaining puberty for individuals captured 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 [23]. In the US, yearling males are thought to be rarely capable of reproducing because they are socially immature, even after puberty is attained [3–5, 21, 22]. 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 [24]. 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

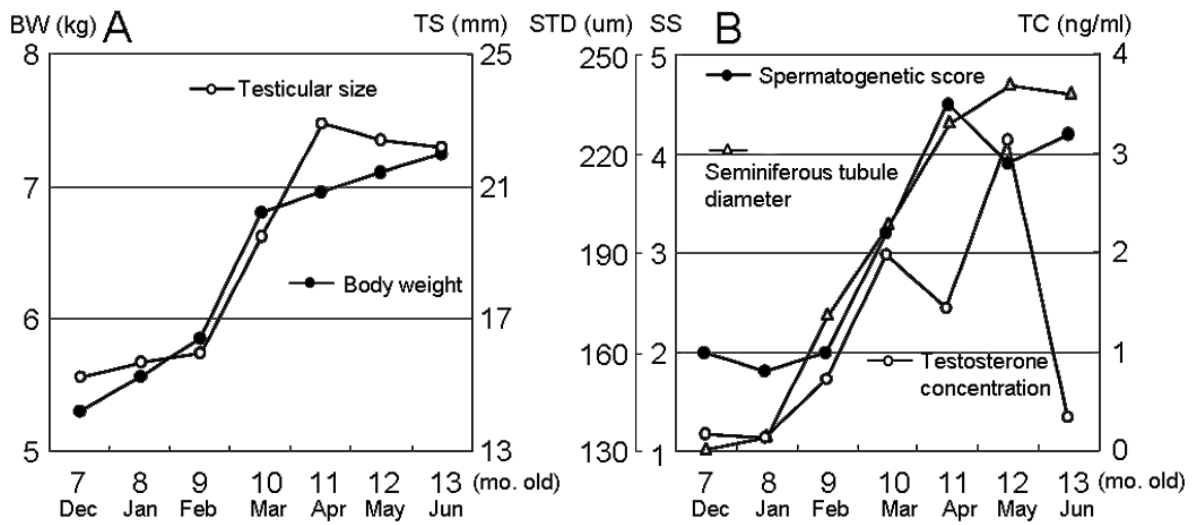


Fig. 4. Changes in body weight and testicular size (A), spermatogenic score, seminiferous tubule diameter and plasma testosterone concentration (B) in the captive juvenile according to age in months. Testicular size was calculated as (long diameter × short diameter × thickness)^{1/3} including the scrotum. BW = body weight, TS = testicular size, STD = seminiferous tubule diameter, SS = spermatogenic score and TC = plasma testosterone concentration.

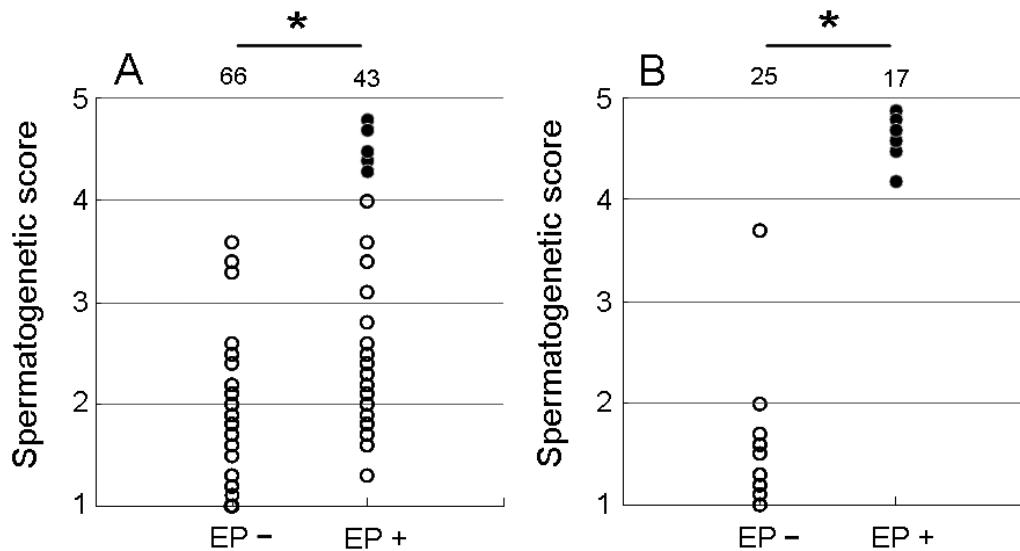


Fig. 5. Individual values of spermatogenic score for all male raccoons from May to September (A) and from October to April (B) according to whether the penis was extrusible (EP+) or not (EP-). The filled circles (●) represent individuals for which spermatozoa were observed in the cauda epididymis, and the unfilled circles (○) represent 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).

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.

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