FULL PAPER *Wildlife Science*

Reproductive traits of the Ryukyu long-furred rat (*Diplothrix legata*) on Okinawa-jima Island

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ABSTRACT. The Ryukyu long-furred rat, *Diplothrix legata*, is a large rodent distributed only on Amami-ohshima Island, Tokuno-shima Island and Okinawa-jima Island, Japan. This animal is endangered as a result of deforestation, predation by introduced carnivores and mortality caused by vehicles. We performed theriogenological examinations of 32 male and 25 female Ryukyu long-furred rats carcasses collected from wild populations on northern Okinawa-jima Island from December 2005 to September 2013. Adult males had remarkably large preputial glands. Seminiferous diameter of adult was significantly small (136 ± 28 μm, n=8) from April to August. Numerous spermatozoa were observed from September through February, and seminiferous diameter was significantly large (216 ± 27 μm, n=12) during this time in adults; testes length changed in a similar pattern. These findings indicate that the mating season may occur from September through February. Size (body length) at sexual maturity was estimated to be >560 mm in both sexes. From observation of corpora lutea and placental scars, litter size was estimated to range from 2 to 12 (average=6, n=4). These results provide fundamental knowledge that will be beneficial for *in situ* and *ex situ* conservation of this rare species.

KEY WORDS: *Diplothrix legata*, reproductive organ, Ryukyu long-furred rat, seasonal change, seminiferous tubule

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The Ryukyu long-furred rat (*Diplothrix legata*) is a large rodent distributed only on Amami-ohshima Island, Tokuno-shima Island and Okinawa-jima Island, Japan [8]. It lives in natural forests of chinquapins and oaks and is an omnivore that eats chinquapins seeds and insects [6, 8, 15].

This rare species is listed as endangered on the International Union for Conservation of Nature (IUCN) Red List [7] and the Japanese Red List [12]. The major threats to the Ryukyu long-furred rat are deforestation and predation by feral dogs, cats and introduced mongooses (*Herpestes auropunctatus*) [7]. Vehicle-caused mortality of a number of Ryukyu long-furred rats has recently been reported by the Ministry of the Environment, Japan. Because of its low population numbers, it is difficult to collect biological information about the Ryukyu long-furred rat.

Effective conservation and management of wild animals requires an understanding of their reproductive traits. However, no extensive surveys have been performed for the Ryukyu long-furred rat, and little is known about its ecology and physiology [7, 8]. Theriogenologic data would help in estimating population dynamics and in achieving *in situ* and *ex situ* conservation. The purpose of this study was to clarify reproductive characteristics in wild Ryukyu long-furred rats.

MATERIALS AND METHODS

Animals: We collected 32 male and 25 female Ryukyu long-furred rats. Those were thought to have been killed by vehicles from December 2005 to September 2013 in the Yambaru District, northern Okinawa-jima Island, Japan (26°00′–27°00′N, 127°30′–128°30′E), which is in the subtropical zone. Total body length (TBL) and body weight (BW) were measured to within 1 mm and 1 g, respectively. Coat appearance was examined for assessment of developmental stage. In females, thickness of lacteal glands and length of teats were examined macroscopically. Lacteal gland thickness over 1 mm and teat length over 3 mm were defined as “thick lacteal glands” and “elongated teat”, respectively, for descriptive purposes in this study. The animals were then autopsied, and reproductive organs were observed macroscopically. Testes (males) and ovaries and uteri (females) were surgically removed. The size of testes and preputial glands was measured at the point of greatest diameter and width, respectively, in 1 mm increments. Ovaries were weighed to the nearest 0.1 g. Ovary weight and the size of testes and preputial glands were presented as the average values of the bilateral organs. The presence of follicles and corpora lutea in ovaries, and fetuses and placental scars in uteri, was examined macroscopically. Female that had large antral follicles (>3 mm), large corpora lutea (>3 mm), fetus or thick lacteal glands was defined as reproductively active in this study. Testes were fixed in Bouin’s solution or 10% neutral buffered formalin for histological observation. If

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sampling or measurement was not possible because of damage or decay, data from that specimen were not used.

Histological observations: Fixed testes were dehydrated and embedded in paraffin according to standard procedures. Tissue sections (4 μm thick) were stained with hematoxylin and eosin and observed using a light microscope (BZ-9000, Keyence, Osaka, Japan). The short diameter of 10 randomly selected round seminiferous tubules (long diameter / short diameter <1.3) was measured using the BZ-9000 analysis application (Keyence). The presence of spermatozoa was examined by observing the seminiferous tubule lumen. Male that had spermatozoa in seminiferous tubules was defined as reproductively active in this study.

Sexual maturity: Male sexual maturity was defined as presence of spermatozoa in seminiferous tubules in the estimated mating season. In females, presence of elongated teats, placental scars and corpora lutea were considered to be evidence of the sexual maturation. We defined the developmental stages as adult, subadult and juvenile from the coat appearance, body size and sexual maturity; adults had attained sexual maturity, subadults were similar to adults in external appearance, but had not attained sexual maturity, and juveniles had not attained sexual maturity and had smaller body size. Also, some animals were allocated as adult even though their sexual maturity or activity could not be observed, if they had similar external appearance and exceeded the minimum body size of other sexually matured or active animals.

Statistical analyses: The values are presented as means ± SD. The differences between estimated mating and non-mating seasons was tested by Student's *t*-test. Differences with $P<0.05$ were regarded as statistically significant.

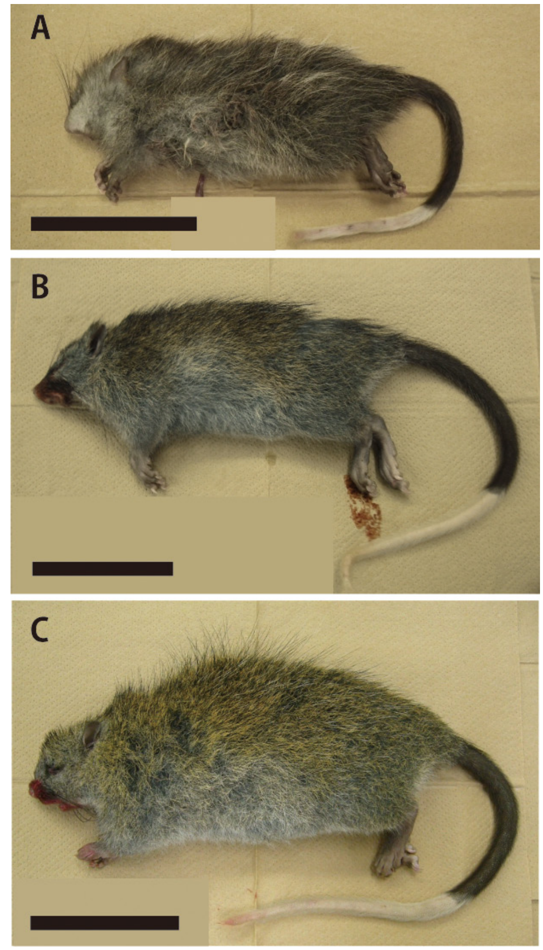

Fig. 1. Appearance of Ryukyu long-furred rats in juvenile (A), sub-adult (B) and adult (C). Scale bars=100 mm.

RESULTS

Coat appearance, body size and sexual maturity: The appearance of Ryukyu long-furred rats at each developmental stage is illustrated in Fig. 1. The relationship between TBL and sexual maturity is shown in Fig. 2 and Table 1. The number of animals allocated to each developmental stage and month for both sexes is shown in Table 2. Adults had yellowish-brown hair, some long (>50 mm) bristle hairs on their backs (Fig. 1C) and TBL >560 mm. Most animals (12 of 15 males and 6 of 9 females) with TBL >560 mm were sexually active or mature. The coats of subadults resembled those of adults except for silky gray hair on the lower back (Fig. 1B), and the TBL of subadults ranged from 500 to 560 mm. Juveniles had silky gray hair on the trunk (Fig. 1A) and TBL <500 mm. Coat color differences between males and females were not observed. Body weights of adult male and female were 587 ± 121 g (n=12) and 570 ± 108 g (n=6), respectively.

Morphology of the male reproductive organs: Male reproductive organs consisted of paired testes, urethra, penis and associated ducts and glands (i.e., seminal vesicles, prostate, bulbourethral gland and preputial gland) (Fig. 3A). The preputial glands of adults were significantly larger during September through February (23.7 ± 5.6 mm, n=13) than during March through August (13.5 ± 4.6 mm, n=9) (Fig. 4C).

Testes: Seasonal changes in seminiferous tubule diameter and testes length in adult males are shown in Fig. 4. The seminiferous tubule diameter in adult was significantly small (136 ± 28 μm, n=8) from April to August and significantly large (216 ± 27 μm, n=12) from September to February (Fig. 4A). From April to August, three out of eight adult rats had no spermatozoa in the seminiferous tubules, and their seminiferous tubules contained Sertoli cells and germ cells from spermatogonia to degenerating round spermatids (Fig. 5A and 5B). From September to February, the seminiferous tubules contained Sertoli cells and germ cells from spermatogonia to spermatozoa (Fig. 5C and 5D). The length of testes changed in a similar manner; testes were significantly short (16.2 ± 2.0 mm, n=7) from April to August and long (23.4 ± 5.2 mm, n=11) from September to February in adults. The diameter of seminiferous tubules and the length of testes in immature animals (subadults and juveniles) were 106 ± 38 μm (n=5) and 11.7 ± 5.7 mm (n=6), respectively.

Morphology of the female reproductive organs: Female reproductive organs consisted of paired ovaries and ovi-

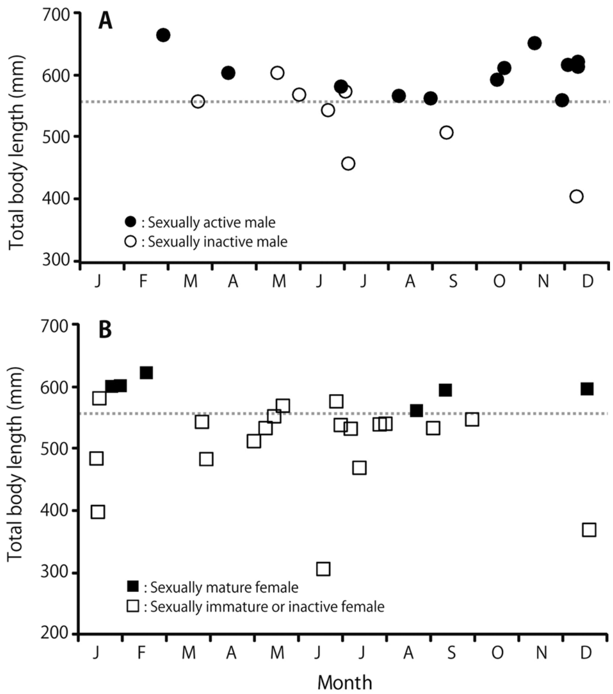

Fig. 2. Relationships between total body length and sexual maturation in males (A) and females (B). Solid circles indicate males with spermatozoa in the seminiferous tubules. Open circles indicate males with no spermatozoa in the seminiferous tubules. Solid squares indicate females with corpora lutea, elongated teats or placental scars, which are indicative of the sexual maturation. Open squares indicate females with no corpus luteum, short teats or no placental scar. In this study, indicators of sexually activity (large corpora lutea (>3 mm), large antral follicles (>3 mm), thick lacteal glands or fetuses) were not observed in females. Dotted line indicates 560 mm.

ducts, uterus, cervix (Fig. 3B), vagina and paired preputial (clitoral) glands. Seasonal changes in adult female preputial glands were small (13.6 ± 5.1 mm, n=9).

Ovary, uterus and mammaries: We did not detect large antral follicles, fetuses, thick lacteal glands or large corpora lutea. Because of the small corpora lutea and follicles, ovary weight was low throughout the year (0.09 ± 0.05 g, n=7) with one exception. In that case, the ovaries weighed 0.20 g, and many small follicles (approximately 2 mm diameter) were observed.

On the other hand, several females had experienced ovulation, pregnancy or lactation. Of the seven adult females collected throughout the year, three (43%) had small corpora lutea (approximately 1–2 mm diameter). The number of corpora lutea ranged from 2 to 12 (average=5.6). Six (60%) out of the ten adult females collected throughout the year had elongated teats, and four (50%) out of eight adult females collected throughout the year had placental scars. The number of placental scars ranged from 2 to 12 (average=6.0).

DISCUSSION

Coat appearance and body size are basic growth indicators that enable estimation of developmental stage by observation of mammals in the wild [4]. Although coat characteristics in adult Ryukyu long-furred rats have been reported previously [8], relationships between coat appearance, TBL and sexual maturity have been clarified in the present study. The degree of sexual maturity can be estimated by coat appearance or morphometric characteristics in many species. For example, in eastern gray squirrels (*Sciurus carolinensis*), fur on the rumps of adults has a distinct yellowish streak near the base that is absent in juveniles [18]. In the large Japanese field mouse (*Apodemus speciosus*), most males with BW >30 g and most females with BW >25 g have attained sexual maturity [14].

Table 1. Characteristics of each developmental stage in Ryukyu long-furred rats

Characteristic	Juvenile	Sub-adult	Adult
Coat appearance	Silky gray hairs on trunk	The coat resembles that of adult in appearance; however, silky gray hairs remain on lower back	Yellowish brown hairs and partly long (more than 50 mm) bristle-hairs on back
Total body length (mm)	Less than 500	500–560	More than 560
Mean ± SD	—	—	Male: 621 ± 30 (n=18) Female: 594 ± 16 (n=8)
Reproduction	Sexually immature	Sexually immature	Sexually mature

Table 2. The number of animals allocated to each developmental stage and month for both sexes (male/female) in this study

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total
Adult	0/3	1/1	0/0	1/0	2/1	1/1	1/0	2/1	0/1	2/0	2/0	3/1	15/9
Subadult	0/0	0/0	1/0	0/0	0/3	1/1	0/3	0/0	1/2	0/0	0/0	0/0	3/9
Juvenile	0/2	0/0	0/1	0/0	0/0	0/1	1/1	0/0	0/0	0/0	0/0	1/1	2/6
Total	0/5	1/1	1/1	1/0	2/4	2/3	2/4	2/1	1/3	2/0	2/0	4/2	20/24

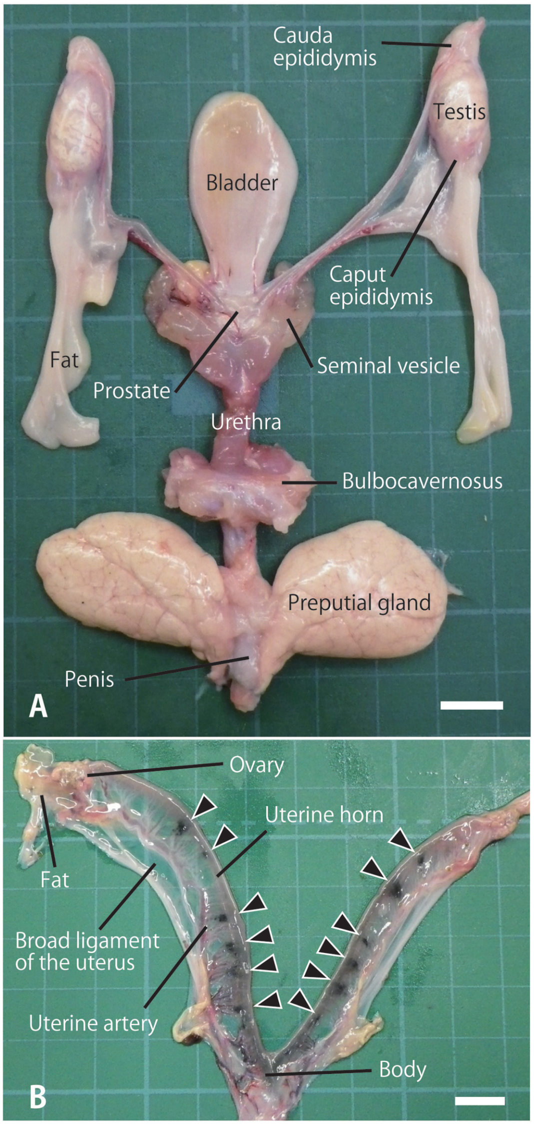

Fig. 3. Reproductive organs in a sexually mature male (A) and female (B) in the estimated breeding period. Arrowheads indicate placental scars. Scale bar=10 mm.

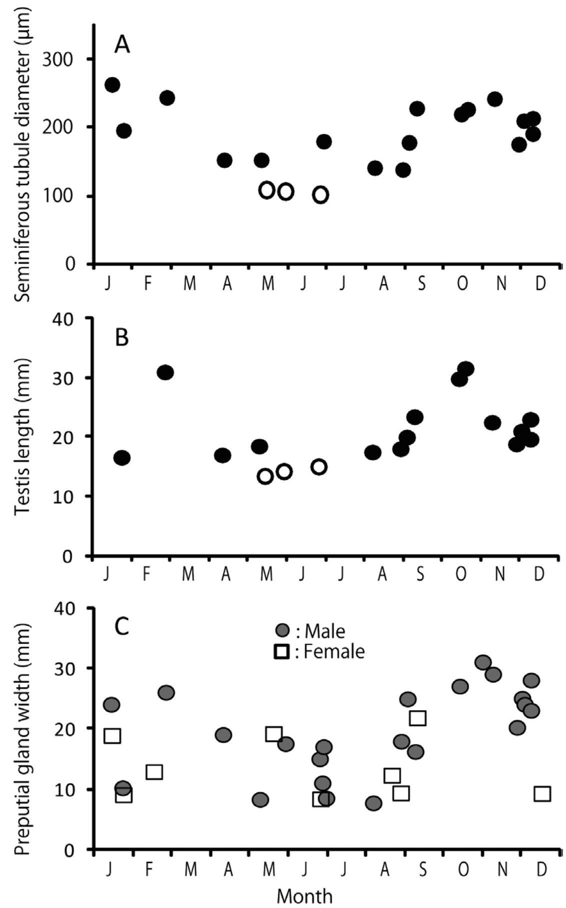

Fig. 4. Seasonal changes in seminiferous tubule diameter (A) and testis length (B) in sexually active and inactive adult males, and preputial gland width (C) in adult males and females. Solid circles indicate sexually active males with spermatozoa in the seminiferous tubules (A, B). Open circles indicate sexually inactive males with no spermatozoa in the seminiferous tubules (A, B).

Mammalian reproductive organs show species-specific morphology [9]. The morphology of Ryukyu long-furred rat reproductive organs, except for the preputial glands, resembled that of other rodents, such as laboratory rats [10]. Preputial glands of the Ryukyu long-furred rat were very large in males, and the size of these glands was synchronized with reproductive activity. Preputial glands are small in rodent species commonly studied in the laboratory [10]. Some rodents, such as the South American water rat (*Nectomys squamipes*), have fairly large preputial glands [19], which produce sex pheromones in males and females [16]. In male laboratory mice, preputial gland secretions attract females and stimulate attack behavior in other males [2]. Sex pheromones secreted from preputial glands may provide an important form of communication in the wild for the Ryukyu long-furred rat.

Environmental factors including food availability, ambient temperature, rainfall and the day/night cycle influence seasonal reproductive changes in wild mammals [1]. The seasonal changes in seminiferous tubule diameter and testes length, and the lack of spermatozoa in early summer in adult males indicate that the Ryukyu-furred rat would be a seasonal breeder. The potential mating season in this species is relatively long (September to February), as indicated by the large reproductive organs in males during this period. Small mammals generally have a longer reproductively active season than large or long-lived mammals [1], which represents an opportunistic breeding strategy for short-lived (usually <1 year) species because maximum reproductive success can be obtained by breeding at every available opportunity [3]. In this study, spermatozoa were even observed in some animals during the estimated non-mating season. This physiological

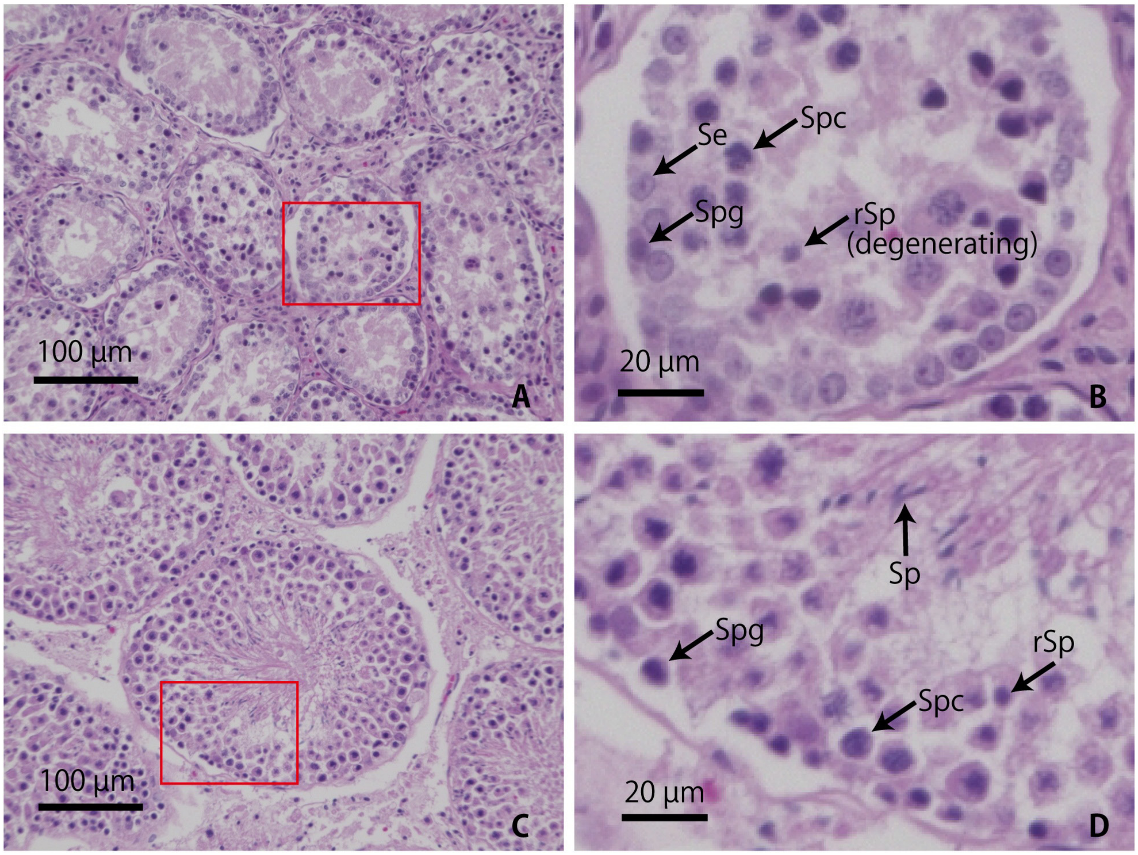

Fig. 5. Seminiferous tubules of adult Ryukyu long-furred rats in the estimated non-mating (May; A, B) and mating (October; C, D) seasons. (B) and (D) are high maginification figures of (A) and (C), respectively. Se, Sertoli cells; Spg, spermatogonia; Spc, spermatocytes; rSp, round spermatids; Sp, elongate spermatids. Hematoxylin and eosin staining.

state is common among seasonal breeders. For example, although the mating season of the Japanese black bear (*Ursus thibetanus*) is during June-August, spermatozoa are observed even in April, during the non-mating season [11]. The natural forests of Yambaru, our study area, are dominated by the evergreen oak, Japanese chinquapin (*Castanopsis sieboldii*) [6], which produces large quantities of nuts in autumn. Nut production by *C. sieboldii* was reported to have a positive effect on reproduction of the non-native black rat (*Rattus rattus*) on Amami-ohshima Island [5]. These acorns form an important part of the Ryukyu long-furred rat diet [15], and the availability of this food source in winter may help to enable winter breeding.

None of the collected Ryukyu long-furred rat females was reproductively active. An estimate of the gestation period is required to determine the delivery period from the known mating season. The mean gestation period in small rodents is approximately 20–30 days, while that of some large rodents is >2 months [13]. Embryonic diapause or delayed implantation, both of which extend the gestation period, is widespread in rodents [17]. Therefore, the delivery season cannot be estimated for the Ryukyu-furred rat from our data; additional data from pregnant or mothering females are needed to clarify this point.

In females, corpora lutea, placental scars and elongated teats indicate ovulation, pregnancy and lactation, respectively. From our observations of corpora lutea and placental scars, we estimate that the litter size of the Ryukyu-furred rat ranges from 2 to 12 with an average of 6. To our knowledge, pregnant Ryukyu long-furred rats have not been collected or captured to date, and further investigations of litter size are needed.

In conclusion, reproductive characteristics including seasonal changes, sexual maturity and morphology of reproductive organs in the Ryukyu long-furred rat were clarified in this study. The results presented here provide fundamental knowledge that will be useful for *in situ* and *ex situ* conservation of this rare species.

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