FULL PAPER *Theriogenology*

Reproductive Cycle Observation of the Okinawa Rail (*Gallirallus okinawae*) in the Wild

Manabu ONUMA[1)]*, Takashi NAGAMINE[2)], Yumiko NAKAYA[2)] and Yasuko NEAGARI[3,4)]

[1)]*Ecological Genetics Analysis Section, Center for Environmental Biology and Ecosystem, National Institute for Environmental Studies, 16–2 Onogawa, Tsukuba, Ibaraki 305–8506,* [2)]*Conservation & Animal Welfare Trust, #308–7–205 Maehara, Uruma, Okinawa 904–2235,* [3)]*Laboratory for Intellectual Fundamentals for Environmental Studies, National Institute for Environmental Studies, 16–2 Onogawa, Tsukuba, Ibaraki 305–8506 and* [4)]*Office of Wildlife Management, Wildlife Division, Nature Conservation Bureau, Ministry of the Environment, 1–2–2 Kasumigaseki, Chiyoda-ku, Tokyo 100–8975, Japan*

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ABSTRACT. The captive breeding program of the Okinawa rail started in 2008. For successful captive breeding, information related to reproduction, such as age at sexual maturity, testicular cycles and ovulatory cycles, is essential to predict when reproduction is possible and when certain reproductive behaviors are most likely to occur. We made gross and histological observations of the reproductive organs of Okinawa rails to gain understanding of sexual maturity, the testicular cycle and the ovulatory cycle. We found that the weight of the testis was smallest in December and largest in March. Changes in the diameter of the seminiferous tubule showed the same pattern. Mature sperm were observed from March to June. The heaviest ovary was observed in April. A single peak of reproduction, from March to April, was observed in males and females. Our observations suggested that the Okinawa rail is a seasonal breeder. Establishing suitable breeding pairs will be critical to ensure success of the Okinawa rail captive breeding program. Our results suggested that pairing must be started before March. If supportive breeding is used, semen should be collected from March to June and artificial insemination conducted in April.

KEY WORDS: captive breeding, *Gallirallus okinawae*, okinawa rail, ovulatory cycle, testicular cycle.

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Okinawa rail (*Gallirallus okinawae*) is an almost flightless bird, endemic to the northern part of Okinawa Island [13]. Wild populations of Okinawa rail are in decline because of habitat loss and predation by feral cats, dogs and also potentially Javan mongooses (*Herpestes javanicus*) [1, 9]. There was an estimated 1,500–2,100 birds in 1985, which was reduced to 820–1,300 birds in 2006 [9]. Because of significant habitat loss and the decline in population size, Okinawa rail has been protected as a National Endangered Species since 1993. In 2006, it was categorized as Critical and currently it represents one of the most endangered species in Japan. The Japanese Ministry of Environment initiated a captive breeding project for the species in 2008 as a strategy to potentially prevent their extinction. A captive breeding program is one of a range of potential conservation strategies, and there have been successful captive breeding programs for birds including the California condor (*Gymnogyps californianus*), the Guam rail (*Gallirallus owstoni*) and the Pink pigeon (*Nesoenas mayeri*). Captive breeding programs have also been conducted in Japan for some endangered avian species such as the Japanese crested ibis (*Nipponia Nippon*), the Oriental stork (*Ciconia boyciana*) and the Red headed wood pigeon (*Columba janthina nitens*).

Information related to reproduction, such as age at sexual maturity, the testicular cycle and the ovulatory cycle of the target species, is essential for successful captive breeding because it can help predict when reproduction is possible and when certain reproductive behaviors are most likely to occur. This information can be used to effectively establish breeding pairs, collect semen and perform artificial inseminations. However, for the Okinawa rail, there is still a lack of information regarding sexual maturity, and the testicular and ovulatory cycle. Thus, we made gross and histological observations of the reproductive organs of the Okinawa rail to gain understanding of sexual maturity, and the testicular and ovulatory cycle.

* CORRESPONDENCE TO: ONUMA, M., Ecological Genetics Analysis Section, Center for Environmental Biology and Ecosystem, National Institute for Environmental Studies, 16–2 Onogawa, Tsukuba, Ibaraki 305–8506, Japan.
e-mail: monuma@nies.go.jp

MATERIALS AND METHODS

Animals: 30 males and 20 females wild Okinawa rails were used for the present study. These birds had died from traffic accidents between May 2005 and November 2010 in the Yambaru area of Okinawa prefecture, Japan. The age of the birds was categorized into four groups, juveniles, subadults, adults born a year before and full adults, based on their body weight, and the color of their feathers and beak (Fig. 1).

Testis and ovary: Testes and ovaries were isolated during postmortem examinations performed at the National Institute for Environmental Studies. The left testis was used the present study and right testis was cryopreserved with freezing medium (10% DMSO/FBS) for the environmental specimen banking program. Left testicular weight was measured using scales and cut in the median line along their

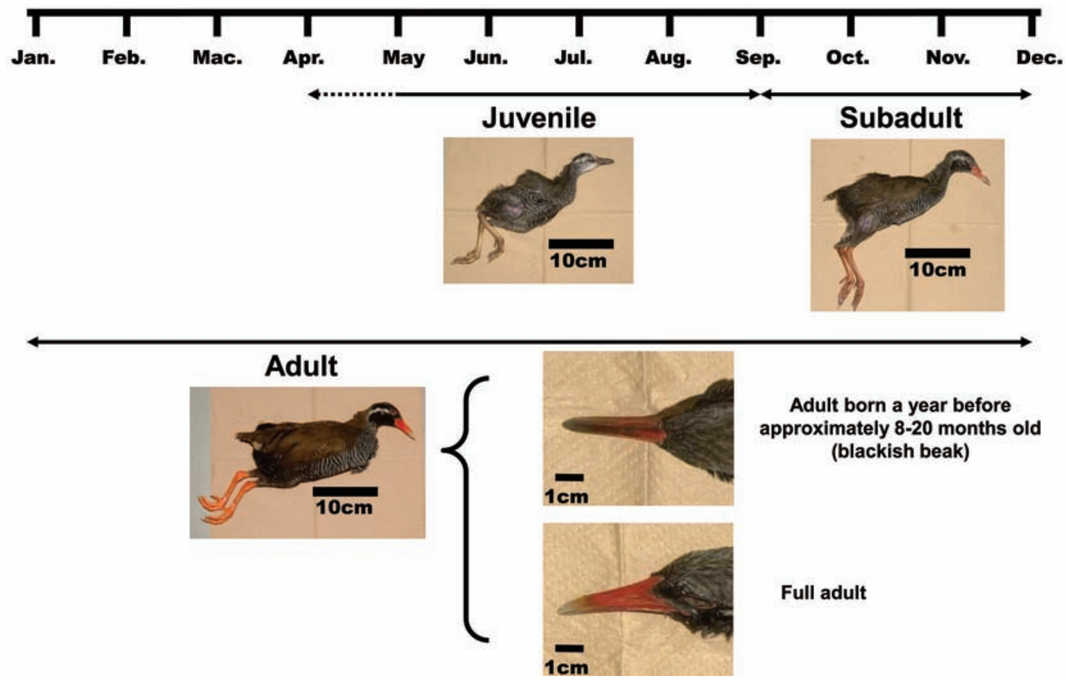

Fig. 1. Age determination criteria of the Okinawa rail.

long axis. The tissues were then fixed by 10% buffered formalin for histological observation. Ovaries were also weighed and cryopreserved with freezing medium (10% DMSO/FBS) for the environmental specimen banking program.

Histological observation of testes: The fixed testes were processed through graded ethanol for dehydration and embedded in paraffin. Sections (4 μm) of paraffin-embedded testes were taken from long axis direction of the organ and were mounted on gelatin coated slides. The sectioned tissues were stained with hematoxylin and eosin. Seminiferous tubules and spermatogenesis were observed using an Olympus microscope (BX41) with a ×100 magnification. The diameters of 20 randomly selected round-shaped seminiferous tubules were measured using WinRoof (mitani corporation, Fukui, Japan). Then, the mean value of the diameter in each testes was calculated by a statistical computing function of WinRoof .

Postovulatory follicles observation: Postovulatory follicles (POFs) can be distinguished by gross observation in some avian species, such as the ring-necked pheasant (*Phasianus colchicus*) [4, 6, 7]. The POFs of the pheasant persist for several months as 1–2 mm pigmented (reddish-brown) structures. Similar structures were observed by gross observation in Okinawa rails (Fig. 2a). The results of histological observation on the structure using an Olympus microscope (BX41) with a ×100 magnification and KEYENCE microscope (BZ-9000) with a ×20 magnification showed that there was no oocyte in the structure (Fig. 2b). The results suggested that the POFs of the Okinawa rail showed the same appearance of the ring-necked pheasant POFs and it can be confirmed by gross observation as pigmented (reddish-brown) structures. Thus the presence of POFs was recorded during postmortem examination.

RESULTS

Testicular shape and weight: The comparative results of testicular shape by gross observation among in March (Fig. 3a), August (Fig. 3b) and December (Fig. 3c) showed that the testis in March was enlarged. Temporal changes of testicular weight in Okinawa rail are shown in Fig. 4. The heaviest left testis in an adult was observed in March (1.34 g). The observed value of weight decreased to 0.12 g in August. The lightest left testis in an adult was observed in October and December (0.08 g).

Seminiferous tubule shape, diameter and spermatogenesis: The comparative results of seminiferous tubule shape by microscopic observation among in March (Fig. 3d), August (Fig. 3e) and December (Fig. 3f) showed that the seminiferous tubule in March was enlarged. Temporal changes of seminiferous tubule diameter are shown in Fig. 5. The largest diameter in an adult was observed in March (323.7 μm). The observed value of diameter decreased to 108.9 μm in August. The smallest diameter in an adult was observed in December (95.5 μm) (Fig. 5). Mature sperm were observed in 5 adult males between March and June (Figs. 4 and 5). Importantly, spermatogenesis was observed in an individual that was born a year before (Fig. 6a, b). This male had the second heaviest testicular weight (1.09 g) and the largest seminiferous tubule diameter (323.7 μm).

Ovary shape and weight: The comparative results of

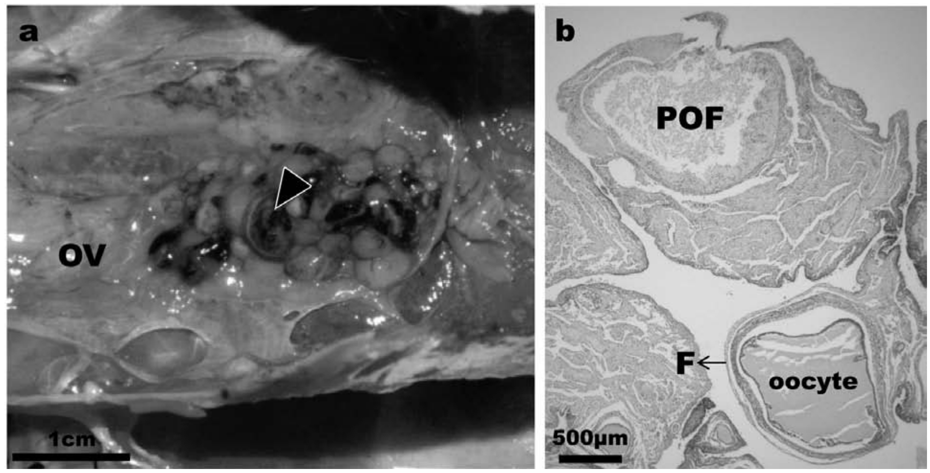

Fig. 2. Gross and histological view of an ovary from the adult Okinawa rails in March. Reddish-brown pigmented structures were observed on the surface (a). The result of histological observation on the structure showed that no oocyte was observed in the structure (b). Thus reddish-brown pigmented structures are postovulatory follicles. ▲, the region for histological observation. OV, oviduct. F, non-ovulated follicle. POF, post ovulatory follicles.

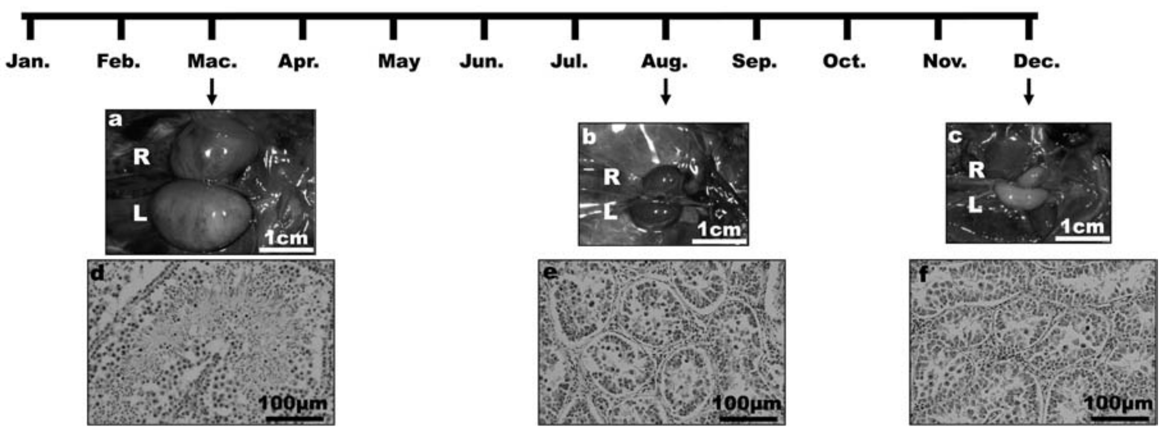

Fig. 3. Gross and histological observations of testes on adult Okinawa rails in March (a, d), in August (b, e) and in December (c, f). Testicular shape and seminiferous tubule shape were relatively enlarged in March comparing with the shapes in August and in December.

ovary shape by gross observation among in March (Fig.7a), August (Fig. 7b) and December (Fig. 7c) showed that preovulatory follicles were observed only in April. Temporal changes of ovary weight in wild Okinawa rail are shown in Fig. 8. The heaviest ovary of an adult was observed in April (15.14 g). The observed value of weight decreased to 0.50 g in August. The lightest ovary of an adult was observed in October (0.10 g).

Postovulatory follicles: POFs were observed in nine adult Okinawa rails between March and August (Fig. 8). In June, no POFs were observed on the ovary of an individual that was born a year before (Fig. 9a). On the other hand, postovulatory follicles were found in other four full adults in June (Figs. 8 and 9b).

DISCUSSION

The main purpose of the present study was to gain information regarding sexual maturity, the testicular cycle and the ovulatory cycle of wild Okinawa rail. The lightest left testes weight in adult was observed in October and December (0.08 g) and the heaviest weight was observed in March (1.34 g). Changes in the seminiferous tubule diameter showed the same temporal pattern. Mature sperm were observed during March to June. The heaviest ovary was observed in April. These data are consistent with a single peak of reproduction, during March to April, in males and females. The breeding season of the species was previously reported to be May to July. According to field observations, egg laying occurs from the beginning of April to the begin-

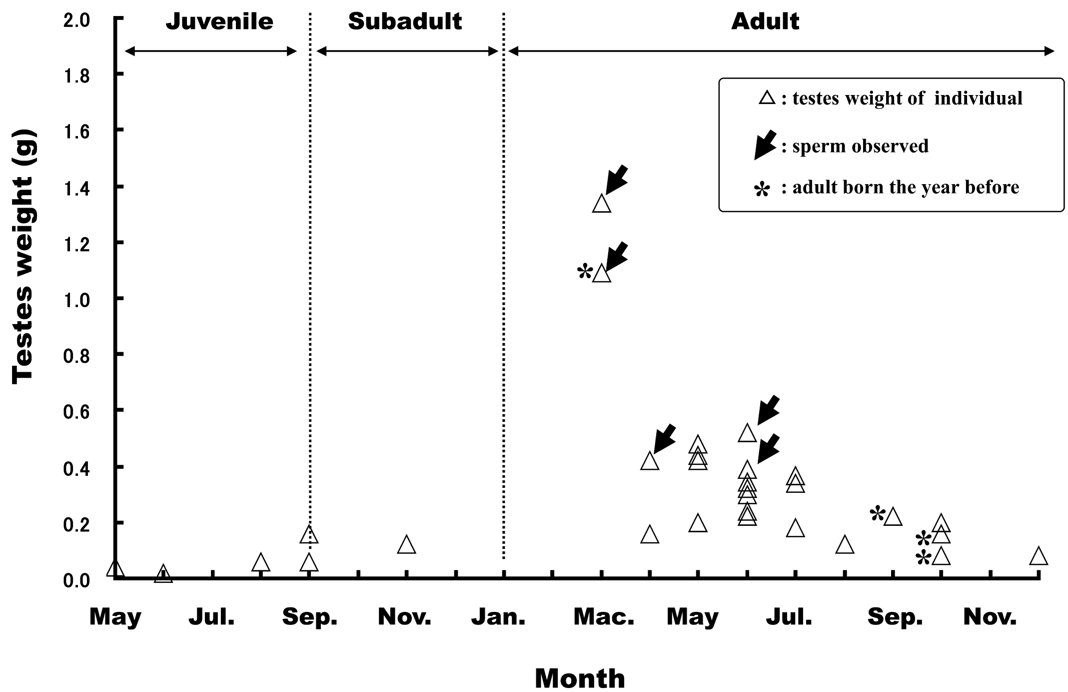

Fig. 4. Temporal changes in testis weight of the Okinawa rail. △, testes weight of individual. ⬋, sperm observed. *, adult born a year before.

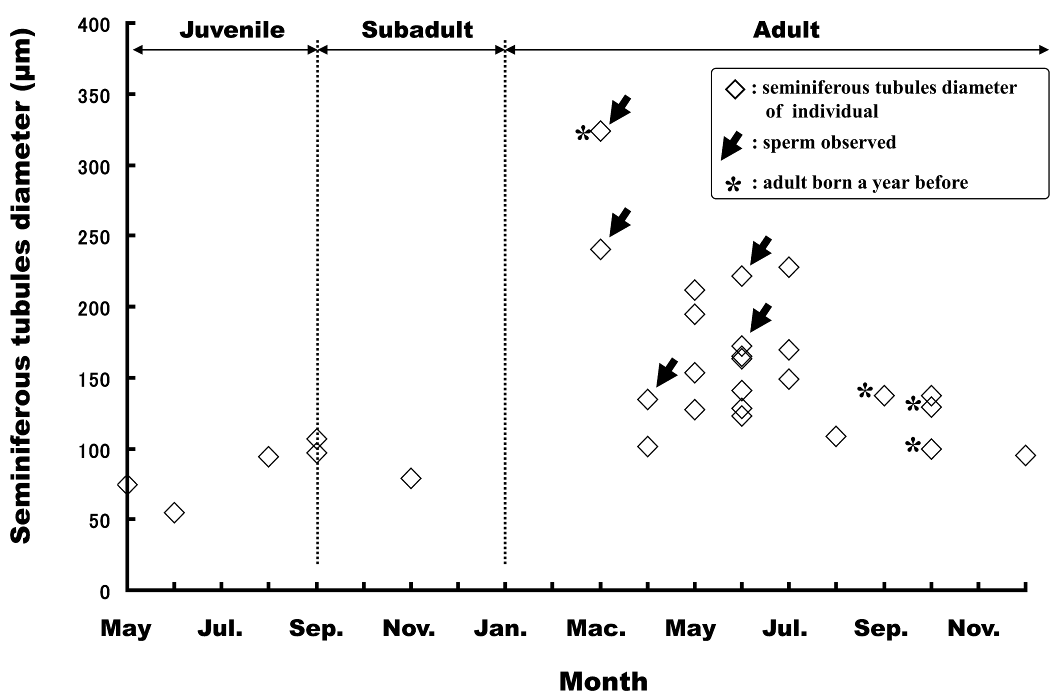

Fig. 5. Temporal changes in seminiferous tubules diameter of the Okinawa rail. ◇, seminiferous tubules diameter of individual. ⬋, sperm observed. *, adult born a year before.

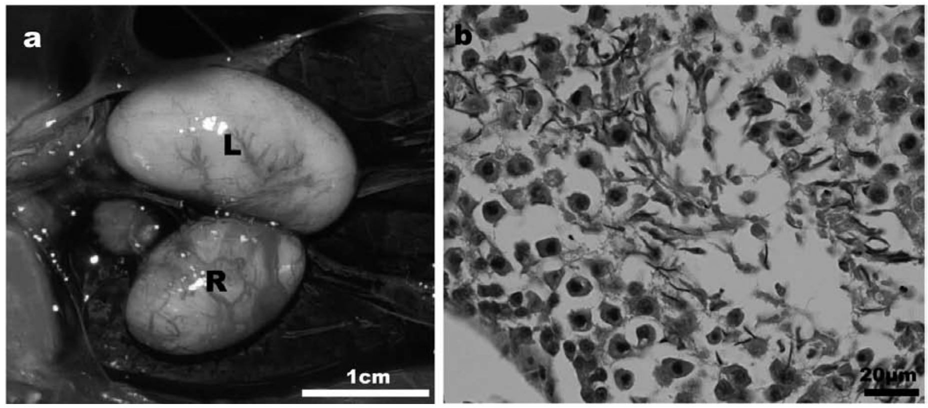

Fig. 6. Gross (a) and histological (b) observations on the testis of Okinawa rail that was born a year before in March (approximately 11 months old). The testis was the second heaviest testicular weight (1.09 g) and the largest seminiferous tubule diameter (323.7 μm). And matured sperms were observed in the lumen of seminiferous tubules (b). L, left testis. R, right testis.

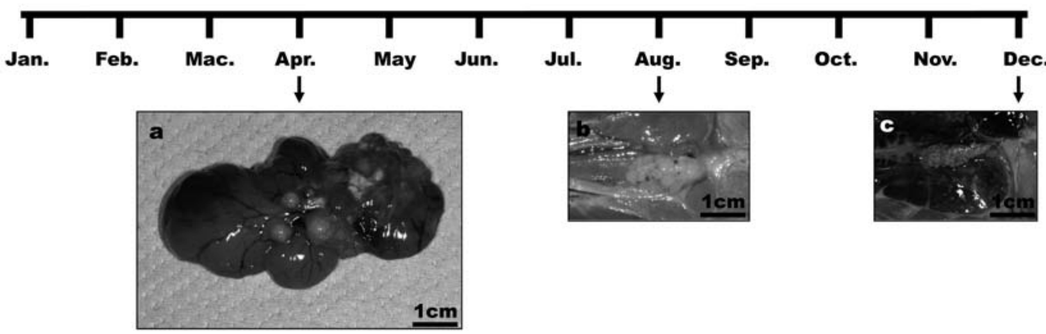

Fig. 7. Gross observations of ovaries on adult Okinawa rails in April (a), August (b) and December (c). Preovulatory follicles were observed only in April (a).

ning of May. Our results are consistent with these field observations and confirm that the Okinawa rail is a seasonal breeder. From a captive breeding management perspective, this information can be used to determine the optimal time to establish breeding pairs. Okinawa rails defend a territory and are monogamous, with a strong pair-bond [1]. Thus establishing suitable breeding pairs will be an important part of the captive breeding program. Our results suggested that pairing must be started before March. If supportive breeding is used, semen can be collected from March to June and artificial insemination must be conducted in April.

Testis condition in the first breeding season was observed in one Okinawa rail individual. This male had the largest seminiferous tubule diameter as well as mature sperm, which suggests that male Okinawa rails can mature in the first breeding season. Ovary condition in the first breeding season was also observed in one individual. No POFs were observed on the ovary. This suggested that female Okinawa rails do not reach sexual maturity in the first breeding season. The sample size of the present study is small. However, in many wild birds, juveniles do not usually attain sexual maturity in the first breeding season [5]. Therefore, female Okinawa rails may have this same reproductive characteristic. To make effective captive breeding pairs, our observation indicate that it is possible to use females in and after the second breeding season and adult males of any age.

Further research is required to ensure success of the captive breeding program of the Okinawa rail. First, a semen collection technique for the Okinawa rail must be established. Abdominal massage is applied to chicken and possibly can be applied to this species [2]. Research on the semen cryopreservation method is also important. Many different diluents and cryoprotectants have been used in chicken and wild birds [12]. The semen cryopreservation technique applied to Sandhill crane (*Grus Canadensis*) semen, from the same taxonomic order (Gruiformes), could be applied to Okinawa rail semen [3]. In addition, use of other reproductive cells must be considered. Cryopreservation of primordial germ cells (PGCs) must be considered to effectively use abandoned fertilized eggs. Chicken PGCs can be cultured and cryopreserved [8, 10, 11]. Reproductive chimeras in chickens have now been created by the transfer of PGCs, and eggs derived from transferred PGCs can be laid by a different breed of chicken [8, 10]. This technique should be considered as one of the options to conserve the

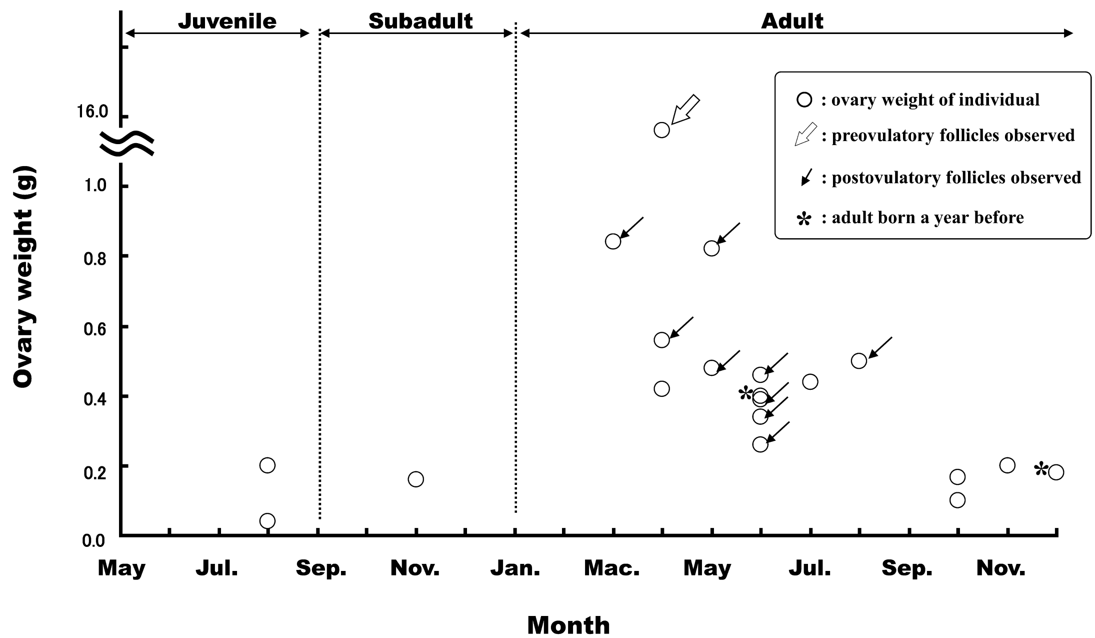

Fig. 8. Temporal changes in ovary weight of the Okinawa rail. ○,ovary weight of individual. preovulatory follicles observed. , postovulatory follicles observed. *, adult born a year before.

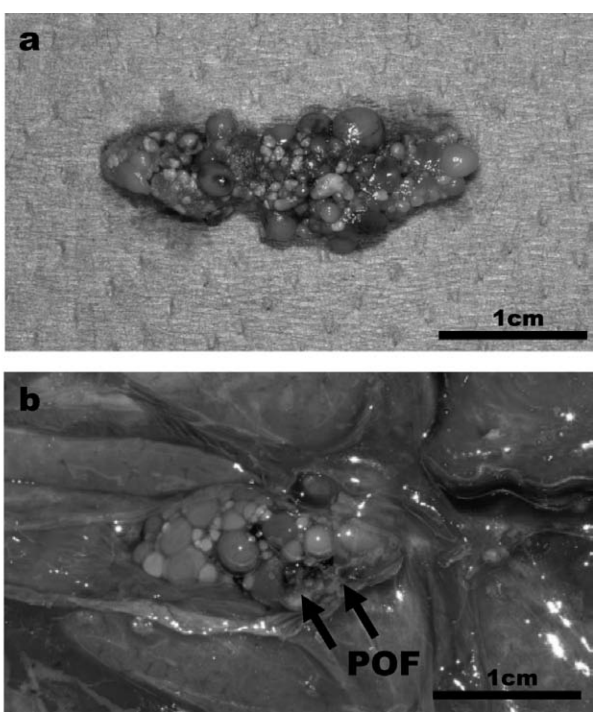

Fig. 9. Gross observation on the ovary of an individual Okinawa rail that was born a year before in June (approximately 14 months old) (a). No postovulatory follicles were observed on the ovary. On the other hand, postovulatory follicles (reddish-brown structures) were found in other four full adults in June (b). POF, postovulatory follicle.

Okinawa rail. Captive breeding programs usually start from a small number of founder individuals. Efficient use of reproductive cells could help prevent inbreeding depression. Our study provides important information on the Okinawa rail, however, it is essential to collect additional information, such as reproductive behavior, reproductive endocrinology, nutrition and genetics, to ensure success of the captive breeding program of the Okinawa rail.

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