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Evidence for True Fall-mating in Japanese Newt *Cynops pyrrhogaster*

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The mating season of Japanese newt *Cynops pyrrhogaster* is generally thought to occur once a year in spring to early summer, during the months of April to June, as in many other Japanese amphibians. However, in fall, from September to October, we often observed breeding colored males demonstrating a mating behavior with females in the field. In this study, in order to identify their true mating season, we anatomically and histologically investigated the annual maturation cycle of gonads and reproductive organs, including cloacal spermathecae in females, and, using a molecular marker, identified the seasonal origins of sperm, which are released in spring to perform insemination. We found that, in fall, ovaries are somewhat immature, while the testes were mature and the sperm already stored in the deferent ducts. Females stored a significant amount of sperm in around 80% of the spermatechae examined in October and 100% in December. When artificially ovulated in March before contact with male partners after hibernation, the females spawned fertilized eggs and these developed normally. Finally, we identified heterozygous genotypes of the visual pigment gene for the two different population types in the embryos, which were derived from a female who established contact with males of the same population in fall and then switched to males from another population until oviposition in spring. We therefore, conclude that the true mating season of this species occurs from fall to early summer, interrupted only by winter, and lasts six months longer (from October to June) than generally believed.

Key words: fall mating, newt, spermathecae, sperm, maturation

INTRODUCTION

The Japanese fire belly newt, *Cynops pyrrhogaster*, is endemic to Japan and widely distributed in the main islands of Honshu, Shikoku, and Kyushu, as well as in the adjacent islands. These newts inhabit mainly ditches beside paddy fields; its habitat, however, is very wide-ranging, from seaside ponds to bodies of water located on mountains at altitudes as high as 2000 m. The mating season is widely thought to occur during the months of April to June. In mating, the male waves its breeding blue-colored tail and engages in a courtship ritual with a female, who, in a successful mating attempt, picks up and incorporates a spermatophore that the male releases by dropping it onto the bottom of a body of water. The sperm are stored in the spermathecae, which is a complex of tubular glands ("a simple type" according to the classification of Sever, 2002) in the dorsal roof of the cloaca, and are released on matured eggs passing through the cloaca of the female during oviposition. Since this newt has an internal fertilization system with the long-term sperm storing spermathecae of female, mating does not always coincide with spawning (egg laying). This is in contrast to other Japanese oviparous amphibians of salamanders and anurans that have an external fertilization system, in which the mating directly induces spawning.

Many Japanese amphibians mate from spring to summer, and some frogs and almost all *Hynobius* urodeles begin mating from winter. The mating season of Japanese amphibians generally occurs once (or twice in a few species) a year, and continues without a seasonal break. While being aware of such general background information regarding the reproductive behavior of Japanese amphibians, we often observed mating behaviors in *Cynops pyrrhogaster* in the months of September and October in the field; males waved the breeding blue colored tail and engaged in courtship with females, just as they do in spring, but egg laying was not observed. Fall-mating has also been observed in other newts, such as *Plethodon cinereus* and *Notophthalmus viridescense* from USA, but this is interpreted as an autumnal "false breeding season" (Gergits and Jaeger, 1990; Sever et al., 1996; Sever, 1997). In the Japanese fire belly newt, the breeding color of males and their mating behavior in fall have been observed by other researchers (Tsutusi, 1931; Iwawasa, 1996). Iwasawa and Ishii (1990) also examined annual cycle of testis maturation and found that the weight of testes reached its maximum during the months of September and October, and that androgen secretion activity reached a maximum twice a year, first during September

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to November and then from January to February. More recently, the increase in locomotor activity in male newts, for breeding in autumn, was shown by elevated synthesis of the neurosteroid, 7α-hydroxypregnenolone, and simultaneously elevated expression of Cyp7B, catalyzing its formation, in the brain (Haraguchi et al., 2010). These reports and our observations suggest that the Japanese fire belly newt males are reproductively ready to release mature sperm in fall, but no direct proof for the true fall mating has ever been attempted. We therefore, in the present study, examined the annual cycle of maturation in gonads and reproductive organs, including female spermathecae, and identified the seasonal origins of sperm used for insemination in spring using a molecular marker. Finally, we showed that females incorporate spermatophores in fall and use these for insemination in spring over the winter. This indicates that fall mating in this species is true, not a false-mating phenomenon, challenging us to change the commonly held view on the spring mating season of this species.

MATERIALS AND METHODS

Newts

Newts of the species *Cynops pyrrhogaster* were collected from paddy fields or areas by the side of the Yoshii River in Kamisaibara of Okayama Prefecture in Japan, in every month from September of 1999 to September of 2000. The animals were kept at 4°C in a refrigerator, and were subjected to investigation within the next four days. Newts that had engaged ovulation were maintained outside (not in a refrigerator) under a roof to avoid exposure to direct sunlight, and were fed solid food manufactured for trout farming twice a week. Oviposition was induced by several injections of 40 units of human chorionic gonadotrophin (hCG, "Gonatropin") per female. After anesthetization with a 0.1% ethyl-m-aminobenzoate methanesulfonate and dissection, the whole body, ovary, oviducts, testes and vas deferens (deferent ducts) were weighed. Some sperm ducts were cut into small pieces to facilitate the counting of the number of sperm in deferent ducts, and the sperm were suspended in DeBoer's solution for counting by a hemocytometer. Newts of the Oita population used for identification of the seasonal origin of sperm were collected from Usuki city of Oita Prefecture, Japan, in 2009.

Histological analysis

A part of cloaca was removed by cutting the body diagonally from the posterior of hind-limbs to the end of cloaca, and then fixed with Bouin's solution for 6–12 hr. Ovaries were fixed with Smith's solution overnight, or with Bouin's solution for 6–12 hr. Testis, deferent duct, and oviduct were fixed with Bouin fixation for eight hours, replaced with 70% ethanol including two drops of $LiCO_3$ solution for bleaching. These tissues were dehydrated through a series of ethanol immersions, preserved in isoamyl acetate, and finally embedded in paraffin including Lemosol, and sectioned at 11 μm thickness, followed by staining with Schiff and Fast Green for observation under a microscope.

DNA analysis

DNA of the larvae was extracted from the tail using DNeasy kit (Qiagen) following the manufacturer's instructions. An approximately 600 bp fragment of a visual pigment gene was amplified by PCR in a reaction solution including 2.5 μl of 10 × reaction buffer, 2 μl of 2.5 mM dNTP, 0.1 μl of Ex Taq (Takara, Japan) with 0.5 μl of each of primers: forward-5′GAT ATA AGA TGC CCA AAA CAC TTC3′ and reverse-5′GGA AAA ATG CGT CTT GTC CAG TG3′, at 35 cycles of 94°C for 30 s, 64°C for 30 s, 72°C for 30 s, ending at 72°C for 2 min. Ten μl of the amplified solution was digested with 0.3 μl of restriction enzyme *Hinc*II for 2 hr at 37°C, and was electrophoresed in 6% polyacrylamide gel, followed by staining with ethidium bromide.

RESULTS

Annual cycle of maturation of gonads and reproductive organs

Ovary and oviduct

Annual changes in weights of ovary and oviduct from females, calculated as a ratio to the total body weight (including ovary and oviduct), are shown in Fig. 1A, B. Weights of ovary and oviduct, respectively, were the lowest in the months of July and August in summer after the mating

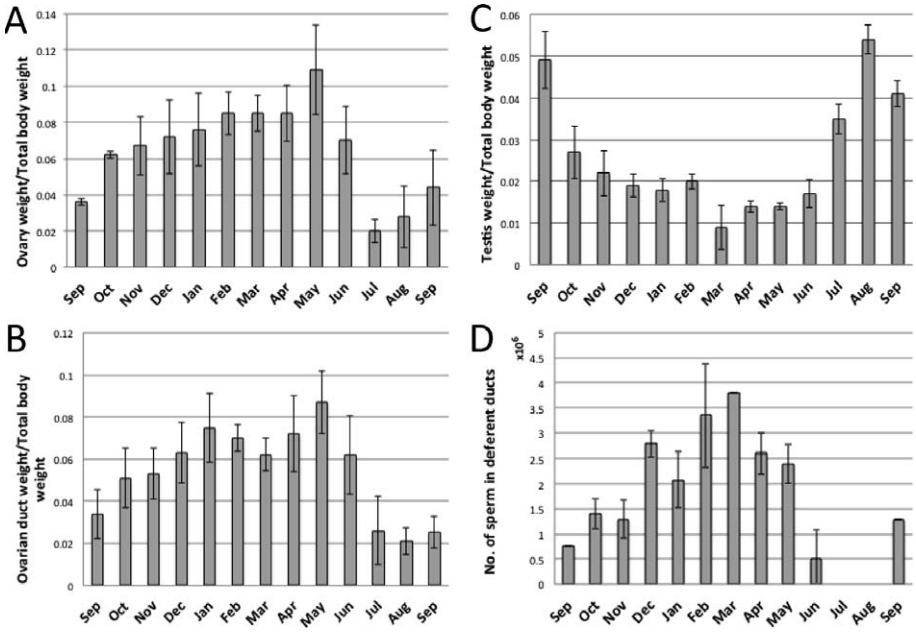

Fig. 1. Annual changes in the weights of ovary and oviducts of female newts and weight of testis and number of sperm in the deferent ducts of male newts. **(A)** The weight of the ovary of each female was calculated as a proportion of the total weight of the body. After the mating season in July and August, the ovary weight is the lowest, which gradually increases and then decreases again in the following June. **(B)** The weight of the oviduct of each female was calculated as a proportion of the total weight of the body. The annual change in the weight of the oviduct is very similar to that of ovary. **(C)** The weight of the testis of each male was calculated as a proportion of the total weight of the body. The pattern of change is different from those of ovary and oviduct. The weight of the testis is lowest in March, which gradually increases during spring and summer, and then turns to decrease in September. **(D)** The annual pattern of change in the number of sperm in deferent ducts is similar to those of ovary and oviduct, the lowest being in July and August after the mating season, which gradually increases in September and then decreases in the following April. Bars indicate standard deviations of the mean values.

season, and these gradually increased thereafter until the following May. Histologically, the ovary included proliferating oogonia in the follicles and many prematured small oocytes at the previtellogenic stage, which measured around 30 μm in diameter in July (Fig. 2A). In the same month, oocytes at the early vitellogenic stage measured between 90 and 350 μm in diameter were observed as well (Fig. 2B). In September, some nearly mature oocytes at the mid-vitellogenic stage, containing a rather brownish cytoplasm and having diameters between 200 and 750 μm, were also observed (Fig. 2C). During the mating season in the month of May, many completely matured vitellogenic oocytes, measuring between 1500 and 1900 μm in diameter, were observed, whose germinal vesicles, located in the animal hemisphere, were surrounded with yolk granules (Fig. 2D).

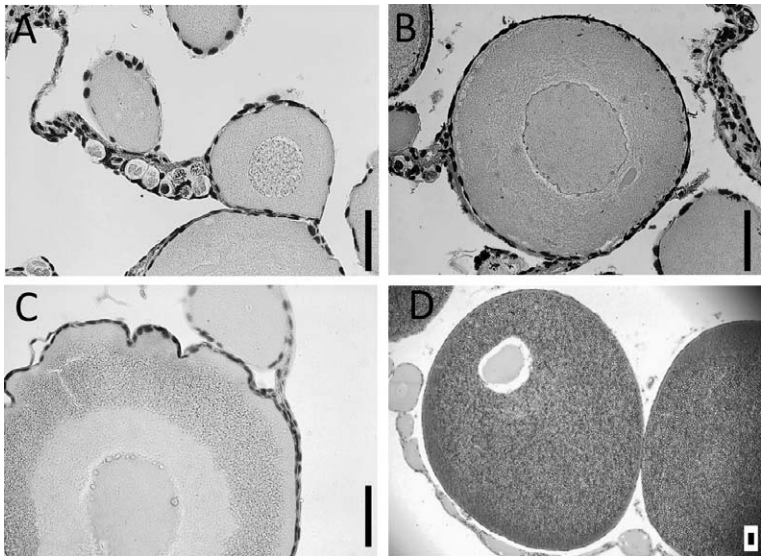

Fig. 2. Histological cross sections of ovaries. Ovaries of females examined in July **(A, B)**, September **(C)**, and the following March **(D)**. Bar, 100 μm.

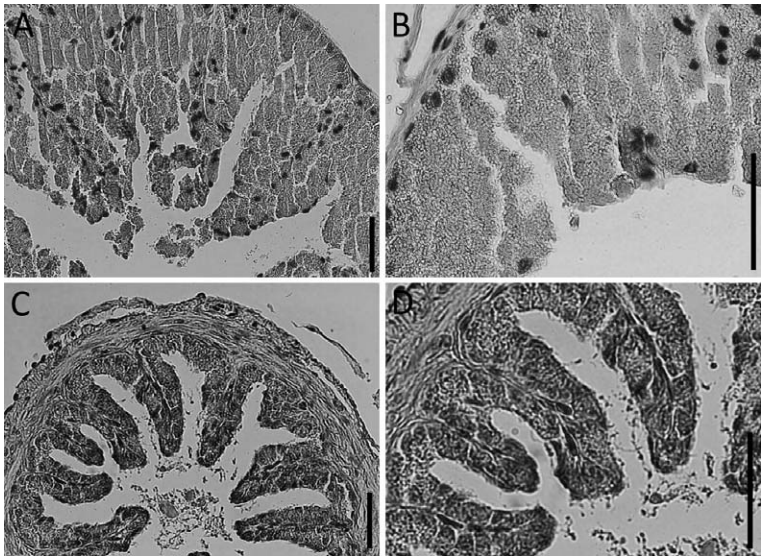

Fig. 3. Histological cross sections of oviducts. Oviducts of females examined in May **(A, B)** and September **(C, D)**. The jelly-like substances are accumulated in the epithelium of oviduct in May during mating season, but not in September after the mating season. Bar, 100 μm.

The oviducts were 200 to 300 μm in width during the mating season in May, and histologically, a large amount of jelly was accumulated in the epithelial cells (Fig. 3A, B). After completion of the mating season, in the month of September, the oviducts shrunk to 150 to 200 μm in width and the oviductal epithelial cells were rearranged into a single layer, and did not contain any jelly material (Fig. 3C, D).

Testis and deferent duct

The testis consists of a number of lobes (3.9 ± 1.4 lobes a male, n = 85), and the weight of the testis was counted as the total weight of all lobes. The weight of testis relative to total body weight (including testis and deferent duct) was lowest during the mating season in the months of March to June, which gradually increased from July and then quickly decreased in October, followed by the maintenance of a steady weight over the winter months from November to February (Fig. 1C). In contrast, the number of sperm stored in the deferent ducts was lowest in the summer months of July and August after the mating season; this gradually increased from September in fall and then decreased until the following June (Fig. 1D). The inner structure of the testis in this newt is characterized by two apparently different parts: one in which well-developed mature sperm are abundant, and the other, in which spermatogenesis is ongoing, showing germ cells at various stages of development from spermatogonia to spermatids (Fig. 4). The region showing spermtogenesis was small in the month of April, which is the mating season, when the testis weight is the lowest (Figs. 1C, 4A), while this very part occupied more than half of the testis in September after the mating season, when the testis weight was close to its maximum for the year (Figs. 1C, 4B). Therefore, the weight of testis is in proportion to the size of the part in testis showing spermatogenesis, whereas the proportion is inverse to the number of sperm present in the deferent ducts. Sperm were abundant in the deferent duct in April, whereas their content was poor in the months of August and September, which then quickly increased thereafter (Fig. 1D). Histologically, the deferent ducts, when observed under microscope, were observed to be filled with sperm in the month of April (Fig. 4C), whereas none appeared in August (Fig. 4D).

Spermathecae

The female newt has spermathecae, which are a compound tubular gland in the dorsal roof of the cloaca. A female receives a spermatophore from a male at mating, and the sperm are stored in the spermathecae. We examined the annual cycle of sperm number in the spermathecae. Although a total count of the number of sperm in all spermathecae was difficult to obtain, due to their complex structure, we made serial histological sections of

spermathecae and observed the localization of sperm in the spermathecal tubules. We classified the state of sperm storage in the tubules into three stages, as follows.

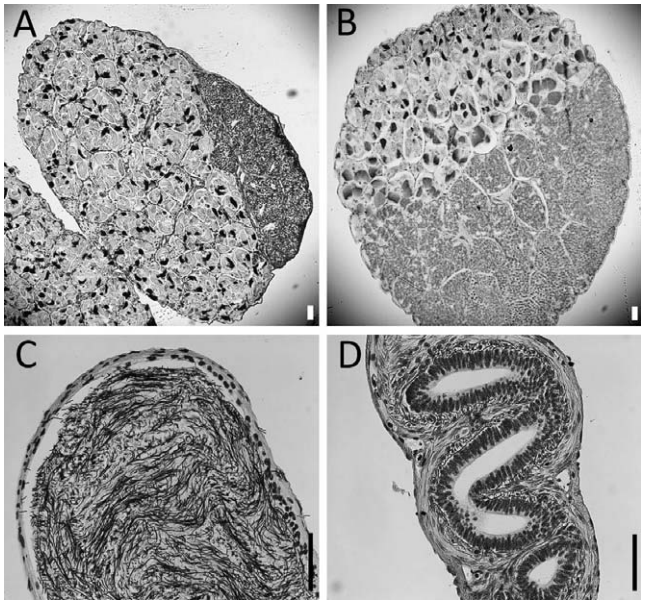

Fig. 4. Histological cross sections of testes and deferent ducts. In April, when the testis weight is the lowest, the part of testis (right part in A) where spermatogenesis is intense is smaller, whereas the other part that contains only the complete sperm is larger **(A)**. In September, when the weight of testes is close to maximum, the part where spermatogenesis occurs is larger, occupying more than half of the testis **(B)**. Sperm are abundant in the deferent duct in April **(C)** during the mating season, whereas none exist in August after the mating season **(D)**. Bar, 100 μm.

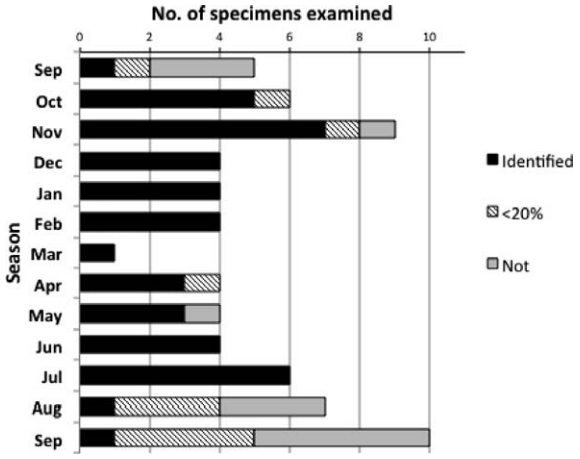

Fig. 5. Annual change in the appearance of sperm in the spermathecae in females. Sperm were observed in the spermathecae of around 80% of females examined in October and the percentages were even higher during the period of October to July. Particularly, spermathecae of all females examined during winter and early spring from December to March contained much sperm. Solid bar indicates the percentage of females whose spermathecae filled with sperm were more than 20%, shaded bar indicates less than 20%, and light gray bar indicates spermathecae with no sperm.

(I) "Identified" stage, in which large amounts of sperm are stored, and sperm were observed in more than 20% of the spermathecal tubules.
(II) "20%" stage, in which small amounts of sperm are stored, and sperm were observed in less than 20% of the tubules.
(III) "Not identified" stage, in which no sperm was observed.

The results are shown in Fig. 5. Sperm stored in the spermathecae were scarce in the months of August and September, and then they increased and became abundant from the month of October till July. During the period from December to March, sperm were always observed in nearly all spermathecae of females examined (Figs. 5, 6).

Artificial ovulation and spawning

Three females were caught in December of 2009 from the field, and were kept in the laboratory without any contact with males until the 23rd of March. On March 24, the females were ovulated by injections of hCG into the abdomen. The females spawned 10, 90 and 22 eggs, respectively, of which seven (70%), 66 (73.3%) and 5 (22.7%) developed normally. The result clearly indicates that the females received spermatophores in fall and retained them over the winter, and that the sperm inseminated the matured eggs in spring. In addition, we ovulated three females with hCG in December, which once spawned fertilized eggs in May of the year and were separated from males and kept over the summer at laboratory, but none of them spawned fertilized

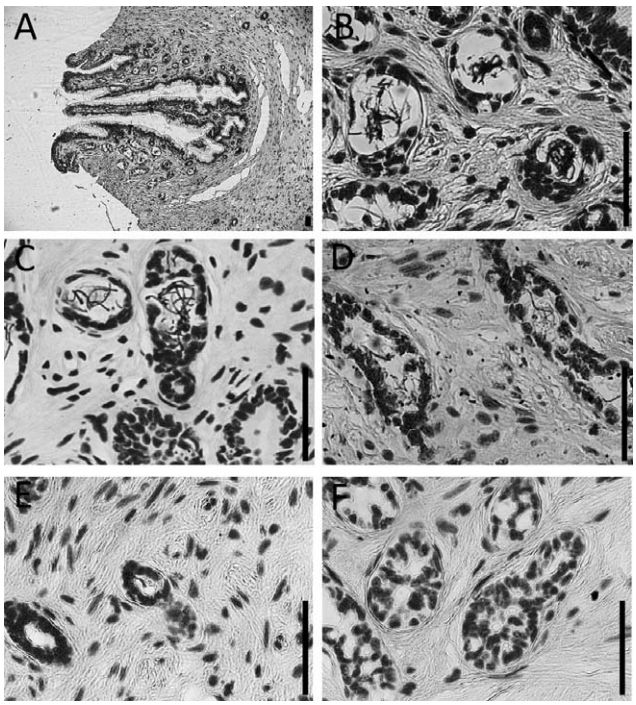

Fig. 6. Longitudinal and horizontal sections of the cloacal spermathecae in females. The spermathecae of newt is a complex of tubules on the dorsal roof of cloaca **(A)**. The horizontal sections of spermathecae of females examined in February **(B)**, June **(C)**, July **(D)**, August **(E)** and September **(F)**. Sperm are seen in the spermathecae in February **(B)**, July **(C)** and July **(D)**, but not in August **(E)** or September **(F)**. Bar, 100 μm.

eggs. This indicates that the sperm incorporated into females in spring could not survive over the summer. In contrast, when two and four females that were caught in September and November, respectively, were ovulated in October and December, they spawned six, three, ten, eight, 12 and six eggs, all of which developed normally. This again supports the notion that females mate and incorporate sperm from males in fall and are ready to spawn fertilized eggs.

Identification of the seasonal origins of sperm

In order to confirm the fall genesis of sperm that inseminated in spring, we examined genotypes of embryos using a molecular marker. Then, two geographically separated population, Okayama and Oita, were investigated for determination and comparison of nucleotide sequences of ten kinds of genes that were chosen from gene data bank. One of these, a visual pigment gene, was found to show sequence differences in a restriction enzyme site enabling us to distinguish the two populations: *Hin*cII cuts one site of Okayama fragment but none of the Oita fragment (Fig. 7A). All newts, 19 from Okayama and 10 from Oita, demonstrated their specific genotypes without exception. Using this restriction enzyme fragment length polymorphism (RFLP), we examined embryos that were naturally spawned from an Okayama female, which was caught in March from the field covered with snow and was then kept with Oita males until oviposition in the month of May at the laboratory. Of eight developed embryos naturally spawned from one female, two were homozygous for the Okayama type, whereas the remaining six were heterozygous for the Okayama and Oita (Fig. 7B). This provides direct evidence that the female spawned eggs that were inseminated with two varieties of sperm had been incorporated by the female before and after the month of March.

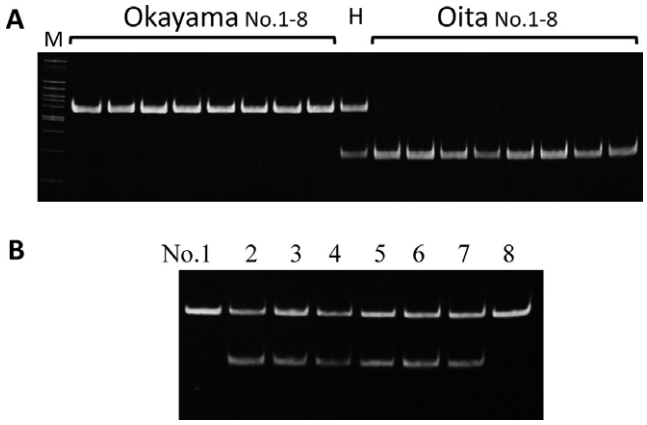

Fig. 7. *Hin*cII digestion of the amplified fragment of visual pigment gene. Samples of eight newts each from the two populations, Okayama and Oita, respectively, are arranged on the left and right, sandwiching the hybrid between the two populations **(A)**. The digested fragments of eight larvae are shown in **(B)**. Larvae were spawned from one female, which was caught in March from Okayama population and contacted to males from Oita population until oviposition. Tadpoles Nos.1 and 8 are homozygous for the Okayama, whereas the remaining Nos. 2–7 are heterozygous. M, DNA marker.

DISCUSSION

The results of the present study indicate that the female of the Japanese fire red belly newt incorporates a spermatophore released from a male in fall, stores it in the cloacal spermatheca during the winter, and uses it for insemination to produce eggs, together with sperm newly incorporated in spring. This in turn indicates that this fall-mating is not false, but true. We therefore, need to recognize that the mating season is six months longer, from October to June, interrupted by hibernation in winter, than the one that has ever been believed generally, from April to June. In other newt species from the USA, some reproductive phenomena suggesting a fall mating have been documented, such as possession of sperm in the deferent ducts of males and in spermathecae of females, and mating or courtship behavior in fall. In *Plethodon cinereus*, some or all males examined have sperm in the vasa deferens, while females may or may not contain sperm in their spermathecae in October (Hood, 1934; Sayler, 1966; Werner, 1967; Sever, 1978). Gergits and Jaeger (1990) observed instances of courtship behavior in October in this species. A similar situation was found in *Eurycea cirrigera* (Sever, 1991) and in *Notophthalmus viridescens* (reviewed by Sever et al., 1996). In spite of these reports, the evidence to prove the mating in fall in these species lacked credibility (Sever, 1978; Sever, 1997). A major difference concerning reproductive situation in fall between the American newts and Japanese *Cynops pyrrhogaster* is the storage state of sperm in the spermathecae of females. Sperm were present in some, but not in all American newt females examined, whereas they were abundant in Japanese newts in the month of October and completely full in all females examined in December. This means that most females of the Japanese newt normally incorporate spermatophore from males in fall. This is the very first finding to show fall mating among newt species, and may well lend credibility to the possibility of true fall mating in other newt species.

The next question that arises is, how could the Japanese fire belly newt have established fall mating associated with no oviposition of females? We may find a hint by observing the geographic distribution and reproductive characteristics of *Cynops* group in Asia. The genus *Cynops* comprises eight species in Asia, and they are as follows: *C. ensiauda* in Nansei shoto (a southwestern group of islands) of Japan, six Chinese species of *C. cyanurus, C. chenggongenesis, C. orphicus, C. orientalis, C. wolterstorffi* and *C. fudingensis* in the southern regions (Zhao and Adler, 1993; Fei, 1999; Wu et al., 2010) and the present species *C. pyrrhogaster* in the main lands of Japan. The Chinese species, *C. orientalis*, lays eggs from March to July (Yang and Shen, 1993), and the spawning season is exceptionally extended until fall (October) on mountainous area at high altitude of 2100–2400 m in *C. c. chuxiongensis* (Fei and Ye, 1988). Of these eight species, *C. pyrrhogaster* is distributed in the northernmost region at the highest latitude. In addition, the mating season of the closely related species *C. ensicauda*, living in the southwestern group of islands of Japan, continues for a period of six months ranging from January to June (Sengoku and Nagasaka, 1999), which is much longer than *C. pyrrhogaster* was ever believed to be.

These facts have led us to develop the following hypothesis. *Cynops* group has a phyletic origin at south of China and thus originally had a continuous mating season of around five months, from March to July as seen in the present Chinese species. One group that invaded the Japanese Islands from the south is differentiated to *C. ensidauda*, and then the mating season was extended back toward winter. Unfortunately, we do not have any data and references to speculate on the reason of the seasonal extension, and thus at present it cannot be ruled out that the ancestral Chinese species already showed an extended mating season. The species, *C. ensidauda*, further evolved to *C. pyrrhogaster*, which extended its distribution toward north of Honshu mainland and happened to be the northern most end of distribution in *Cynops* group. Although the species still retained its inherited range of mating season in the main lands and possibly kept extending the mating season further toward fall, the cold winter did not allow the females to oviposit because of the extremely low ambient temperature, which prevents the development of embryos. Finally, this species established the present reproductive system, in which females began to mate and incorporate spermatophore in fall without oviposition. They then stored it in the cloacal spermathecae during the winter, and oviposed fertilized eggs in spring. This system is quite exceptional among amphibians living in Japanese islands, where many of these have a short and restricted mating season. *C. pyrrhogaster* probably evolved the present reproductive system as it possesses the unique reproductive mechanisms of internal fertilization, storage of sperm for a long period of time of around nine months in the spermathecae, as well as multiple mating.

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