

# A Skeletochronological Study of Breeding Females in a Population of Japanese Clouded Salamanders (*Hynobius nebulosus*)

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The age structure of breeding females of *Hynobius nebulosus* has not been studied sufficiently. We estimated the ages of 76 individuals from a population in Kyoto by using skeletochronology. The mean age and snout-vent length (SVL) of this population were 4.6 years and 55.7 mm, respectively. It was estimated that the youngest females breed two years post hatching at a mean SVL of 46.5 mm, but a larger number of individuals begins breeding at three years and a mean SVL of 52.2 mm. Because most males also start to breed at three years, there seems to be no gender difference in the timing of sexual maturation. The age of the oldest female was estimated to be 11.8 years. It is possible that the life history of *H. nebulosus* is characterized by early maturation and arrested growth, and short longevity.

**Key words:** Life history traits, skeletochronology, age structure, age at first reproduction, longevity, *Hynobius nebulosus*

## INTRODUCTION

The Japanese clouded salamander, *Hynobius nebulosus*, is mainly distributed in the lowlands of western Japan and usually breeds in still waters, such as wetlands and small pools (Matsui et al., 2006). Between 1983 and 1991, eggs of *H. nebulosus* from some localities of the Kinki District were introduced to a tree-covered green space (2430 m<sup>2</sup>) with an artificial pond on the campus of Kyoto University, in the city of Kyoto, central Japan (35°08'N, 135°47'E, 98 m a.s.l.). They have become established and the population has stabilized (Ento and Matsui, 2002; Matsuki and Matsui, 2009). Since 1996, the life history of salamanders in this population has been studied in order to gain basic ecological information about *H. nebulosus* to aid in its conservation. During the initial phases of the study, only dip nets were used to collect adult salamanders (dip netting), but since 2003, drift fencing (Dodd Jr. and Scott, 1994; Halliday, 2006) has been used to catch animals.

In this population, Ento and Matsui (2002) studied a number of life history traits by elucidating ages of breeding individuals by means of skeletochronology, a technique used to estimate the age of vertebrates by counting layer structures formed periodically in long bone tissues (e.g., Smirina, 1994; Castanet et al., 2003). However, in the study of Ento and Matsui (2002), the number of females was insufficient to draw definitive conclusions, raising the need for additional investigations to clarify life history traits of female *H. nebulosus* by using a larger number of samples. This dif-

ficulty was overcome by introduction of the drift fence trap. The female capture rate increased dramatically and it became possible to obtain a sufficient number of females to apply skeletochronology.

The validity and high accuracy of skeletochronology have been confirmed in this population of *H. nebulosus* by the use of age-known individuals that had been given cohort markings immediately after metamorphosis (Matsuki and Matsui, 2009). In the present study, we conducted skeletochronology to elucidate age structures of female *H. nebulosus* collected in the breeding season, so as to contribute to the understanding of basic life history traits of this species.

## MATERIALS AND METHODS

### Sampling strategy

Breeding females were captured mainly by drift fencing, supplemented by dip netting, from the fall of 2007 to the spring of 2008 (2008 breeding season). For the drift fence trap, the breeding pond was enclosed with galvanized steel sheets (30 cm high and 10 cm deep), and 14 pitfalls made of plastic bottles were set at equal intervals on both the inner and outer sides of the fence. The cover lids of the pitfalls were kept open between late November 2007 and early May 2008, and inspection was made three times a week during this period. Dip netting was made once a week starting from the day of the first capture by pitfalls; two persons were engaged in this procedure over a period of two hours (1900–2100 h).

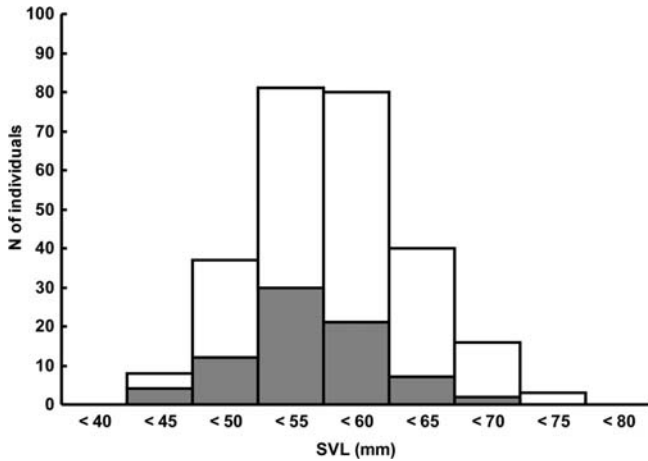
Between 24 December 2007 and 11 April 2008, a total of 265 breeding females were captured (63% by drift fencing, 27% by dip netting, and 10% by other methods). All individuals captured were carried to the laboratory at once, sexed, and recorded for SVL (snout-vent length, from the tip of the snout to the anterior end of the cloaca) to the nearest 0.1 mm by a slide caliper and body mass to the nearest 0.1 g by an electric balance. All individuals have been tagged by passive integrated transponder (PIT) tags since 1998, so we also checked for individual tag number, and in cases of first capture, the individual was inserted with a PIT tag. After these proce-

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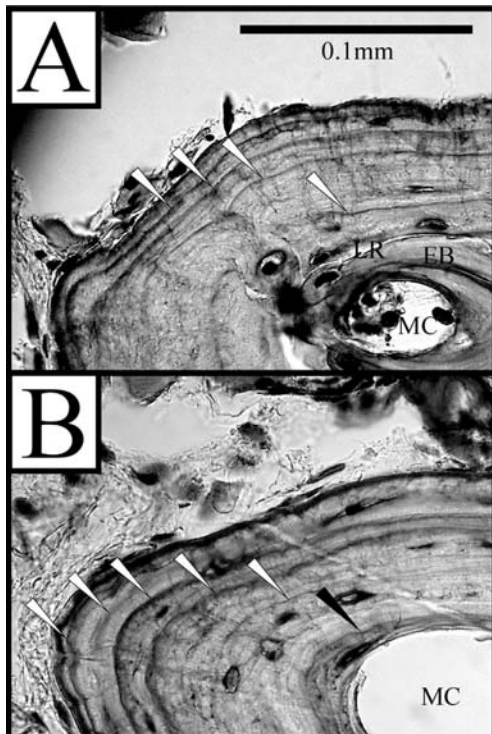
dures, females, mostly gravid ones, collected by traps outside the pond were released inside of the drift fence, as they were thought to be ready for breeding. Spent females collected by traps inside of the fence were released.

**Skeletochronology**

Toe samples for skeletochronological analysis were obtained from females captured mainly between 28 January and 11 April



**Fig. 1.** SVL distribution of female *Hynobius nebulosus* caught in the breeding season from the end of 2007 to spring of 2008 (N = 265). Shaded area represents the number of individuals analyzed by skeletochronology (N = 76).



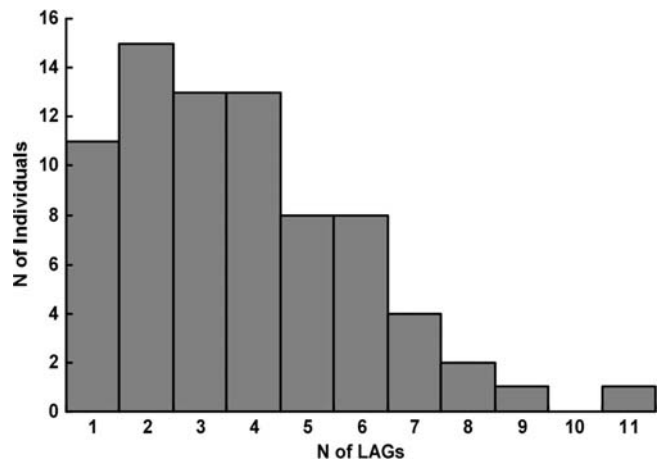
**Fig. 2.** Cross-sections of digits in (A) four-year-old and (B) five-year-old individuals of *Hynobius nebulosus*. White arrows and a black arrow represent LAGs and a ML, respectively; MC = marrow cavity; EB = endosteal bone; LR = line of resorption. Terminology for bony tissues followed Smirina (1994).

2008. Because of time limitations, we could not analyze all the clipped toes. We selected individuals of a specific SVL, so as to reflect the SVL distribution of all the individuals captured. As a result, we used 69 female *H. nebulosus* for skeletochronology. For analyzing age structure of the breeding population, we combined the previously published data for 11 females to confirm the validity of skeletochronology. The exact ages of these individuals were revealed by cohort markings and skeletochronology (Matsuki and Matsui, 2009).

The skeletochronology procedure (e.g., collection and storage of toe samples, preparation and observation of cross-sections, and counting of LAGs) was identical to that reported by Matsuki and Matsui (2009). Ages of individuals were estimated on the basis of an assumption that all larvae hatch on 1 April every year.

**RESULTS**

Of 69 individuals for which skeletochronology was applied, 65 had aged successfully, while the remaining four showed abnormal patterns in bone histology and were excluded from the analysis. Accordingly, coupled with 11 individuals of known age (see above), the ages of 76 individuals were determined. The SVL distribution of these 76 individuals is shown in Fig. 1. Their mean SVL ( $\pm 2SE$ ),  $53.9 \pm 1.2$  mm, was slightly smaller than that of total 265 females captured (mean =  $55.7 \pm 0.7$  mm, range = 40.7–74.2 mm; Fig. 1).



**Fig. 3.** Age distribution of female *Hynobius nebulosus* estimated by skeletochronology (N = 76).

**Table 1.** Mean and range of SVL of female *Hynobius nebulosus* at specific number of LAGs.

N of LAGs	N of individuals	Mean of SVL ( $\pm 2SE$ )	Range of SVL
1	11	$46.5 \pm 2.2$	40.7–54.0
2	15	$52.2 \pm 1.5$	47.6–57.5
3	13	$54.3 \pm 1.8$	50.0–60.8
4	13	$53.4 \pm 1.9$	46.9–58.6
5	8	$55.4 \pm 2.2$	51.7–60.5
6	8	$57.8 \pm 2.1$	52.3–62.3
7	4	$59.3 \pm 2.2$	56.5–61.8
8	2	65.0	65.0–65.0
9	1	62.8	–
11	1	61.1	–
Total	76	$53.9 \pm 1.2$	40.7–65.0

The number of LAGs observed ranged from 1–11 (Fig. 2), with a mean of  $3.7 \pm 0.5$  (2SE). Metamorphosis lines (ML; Fig. 2B) were observed in the periosteal bone in 27.6% (21/76) of the total number of individuals. Individuals with two LAGs were most numerous, accounting for 19.7% (15/76) of the total (Fig. 3). The mean and range of SVL at specific number of LAGs are shown in Table 1. The mean SVLs ( $\pm$  2SE) of the individuals with one and two LAGs were  $46.5 \pm 2.2$  mm and  $52.2 \pm 1.5$  mm, respectively.

The mean age estimated as shown above was  $4.6 \pm 0.5$  (2SE) years old, and the youngest and oldest individuals, respectively, were estimated to be 1.9 and 11.8 years old from the time of hatching.

## DISCUSSION

In the study of small salamanders of the genus *Hynobius*, biological traits of females have been much less clear compared with males because the number of females captured is usually much smaller than males (e.g., Nishikawa et al., 2007; Matsui et al., 2009). In our survey during the breeding season in 1998 at the present study site, only dip netting was employed as a sampling method. As a result, only 28 females were captured, in contrast to 425 males collected (Ento and Matsui, 2002). In fact, 310 pairs of egg masses were confirmed in the season, meaning that more than 90% of females that participated in breeding must have gone unnoticed. This difference in detectability is attributable to the behavioral difference between the sexes; males stay in the breeding pond for a long time during the breeding season, whereas females immediately leave it after egg-deposition (Sato, 1943; Matsuki and Matsui, unpublished data).

The efficiency of collecting females increased greatly with the adoption of drift fencing, with approximately 400 females captured every breeding season between the time the fences were introduced (end of 2002) and spring of 2007 (Matsuki and Matsui, unpublished data). In this way, the efficiency of capturing individuals can vary considerably depending on the method employed, and it is particularly important in the ecological study of small salamanders to adopt effective sampling methods. Although a smaller number of females than males has been captured in spite of the use of drift fences in several ambystomatid salamanders (e.g., Husting, 1965; Douglas, 1979), such a disproportion in sex ratio proved to be not the case in our species.

We estimated ages of only about one-third of breeding females captured, but the findings from these limited individuals are considered to reflect the age structure of the breeding female population, because we chose individuals of various body size evenly sampled over the breeding season from January to April.

In our results, at least a partial metamorphosis line (ML) was recognized in 27.6% of individuals examined, and this value is larger than the 17.5% reported by Matsuki and Matsui (2009). These two values also differ from Ento and Matsui (2002) who found ML in almost all individuals they studied. This inconsistency could be caused by confusing lines of resorption with MLs in earlier studies (e.g., Fig. 2B in Ento and Matsui, 2002), but it does not affect estimating ages because MLs have not been counted as LAGs in all the studies.

Eleven individuals with one LAG are estimated to have started breeding at 1.9–2.0 years (mean SVL = 46.5 mm) from the time of hatching. This implies that the time at sexual maturity is earlier than this age. In addition, the percentage of these young individuals among the breeding females is not definitely small (Fig. 3). Such an age structure has never been reported in the breeding population of any other Japanese *Hynobius* species (see below), and the possibility of overlooking some factors like endosteal resorption cannot be ruled out. Results of our previous study from mark-recapture and bone histology actually indicated age underestimation in some individuals (Matsuki and Matsui, 2009). However the ratio of such individuals was small and the present result is thought to reflect actual age composition of the population. Our previous study (Matsuki and Matsui, 2009) also indicated that some males similarly mature at the same young stage. In contrast, such young individuals were not observed in the 1998 breeding season (Ento and Matsui, 2002). The reason for this discordance is unknown. However, there is no appreciable difference in the body size between the two breeding seasons: The mean  $\pm$  2SE [range] of SVL in 28 females in 1998 was  $53.9 \pm 1.6$  [43.5–61.4] mm (recalculated from the original data), which is not much different from that in 265 females in 2008 ( $55.7 \pm 0.7$  [40.7–74.2] mm). Thus, females may begin breeding at a certain minimal size rather than a certain minimal age.

Because the individuals with two LAGs predominated in number, a larger number of females are thought to breed at three years (mean SVL = 52.2 mm) after hatching. This period agrees with the case in the majority of males (Ento and Matsui, 2002). Ento and Matsui (2002) speculated that females mature later than males and start to breed at the age of four to five years. However, this may be an overestimation of the age at first reproduction in females, based on the small number of females captured in 1998. Although many studies suggest that female urodeles tend to become sexually mature later than males (e.g., Houck, 1982; Misawa and Matsui, 1999; Bruce et al., 2002), the results of the present study do not support such a tendency in our *H. nebulosus* population. In order to confirm if this is specific to the *H. nebulosus* population studied, it is necessary to reassess other species using effective sampling methods and to ascertain the age of each individual.

The age of the oldest female was estimated to exceed 11.8 years in our study. However, individuals older than eight years accounted for only about 5% of the total females examined (Fig. 3), and extremely old females are not common in our study site. The estimated maximum longevity ranges from 12 to 23 years in some Japanese urodeles (Misawa and Matsui, 1999; Marunouchi et al., 2000; Kusano et al., 2006; Sakamoto, 2008), and a female *H. nebulosus* is reported have lived over 15 years in captivity (Tanabe and Matsui, 1997). Besides, maximum longevity of more than 12 years is known in 57.5% out of 40 species of urodeles throughout the world (review by Wells, 2007). Therefore, *H. nebulosus* seems to be not an essentially short-lived species, but actual longevity in the wild would surely be much shorter.

Of the five *Hynobius* species studied to date, the minimal ages of breeding females are estimated to be five to seven years in lotic breeders, *H. kimurae* (Misawa and Matsui,

1999), *H. naevius* (as the large type of *H. naevius sensu lato*: Tominaga and Matsui, 2008), and *H. yatsui* (as the small type of *H. naevius sensu lato*: Sakamoto, 2008). By contrast, minimal ages of two to five years are reported for lentic breeding species, *H. tokyoensis* (Kusano, 1982; Kusano et al., 2006) and *H. nebulosus* (this study). Consequently, the age at first reproduction seems to differ between lotic and lentic breeders, with the lentic species tending to breed earlier in their life history than do the lotic breeding species.

The unique life history of the *H. nebulosus* population we studied would be more clearly characterized by comparing some traits such as mean age and mean body size (SVL) with other *Hynobius* species hitherto studied: populations of *H. kimurae* from Tokyo and Kyoto (Misawa and Matsui, 1997, 1999); populations of *H. tokyoensis* from Tokyo and Chiba (Kusano et al., 2006); a population of *H. nebulosus* from Kyoto (Ento and Matsui, 2002; this study). Because SVL measured by Kusano et al. (2006:  $SVL_{\text{post}}$  = from the tip of the snout to the posterior end of the cloaca) differs from the length in other studies ( $SVL_{\text{ant}}$  = tip of the snout to the anterior end of the cloaca), we converted  $SVL_{\text{post}}$  to  $SVL_{\text{ant}}$  by using the following formula:  $SVL_{\text{post}} = 1.113SVL_{\text{ant}} - 0.414$  (Kusano et al., 2006).

Among these populations, *H. nebulosus* is the youngest and smallest in terms of mean values (Fig. 4). Even the next youngest and smallest, *H. tokyoensis*, from Chiba, is approximately one year older and slightly larger than *H. nebulosus* in both sexes. Another population of *H. tokyoensis* from Tokyo is two times older than *H. nebulosus* in both sexes. Furthermore, in *H. kimurae* from Tokyo and Kyoto, mean age is twice as old, and mean SVL is much greater than in *H. nebulosus*. It is thought that the younger age and smaller body size of the *H. nebulosus* population reflect earlier maturation, earlier arrested growth, and shorter longevity than in other species. Consequently, as far as the population in this site is concerned, the life history of *H. nebulosus* is characterized by such life history traits.

Intra- and interspecific life history variation, such as age at first reproduction and body size, is thought to be affected

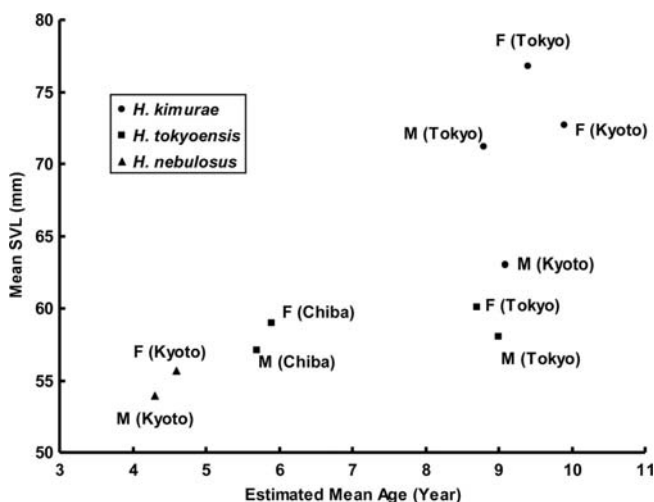


Fig. 4. Interspecific comparison among five populations of three Japanese *Hynobius* species. M = Male; F = Female.

by latitude and/or altitude-based environmental constraints on growth and development rates (Morrison and Hero, 2003), or by evolutionary responses to different predation pressures (Caetano and Leclair Jr., 1996). The study sites of *H. nebulosus* in Kyoto and *H. tokyoensis* in Chiba are located at relatively low altitudes of 98 m a.s.l. (Ento and Matsui, 2002) and 20–40 m a.s.l. (Kusano et al., 2006), respectively. By contrast, the study site of *H. tokyoensis* in Tokyo is located at 200 m a.s.l. (Kusano et al., 2006), and of *H. kimurae* in Tokyo and Kyoto at 450–550 m a.s.l. (Misawa and Matsui, 1999). These altitudinal differences in habitat may have caused differences in life history.

As discussed by Ento and Matsui (2002), early maturation and participation in breeding at a smaller body sizes would be more advantageous when the post-metamorphic survival rate is low. This is because, due to high mortality, females that mature later would have less chance to reproduce. Such life history traits are thought to be selected under environments that induce a low survival in adult animals (Horn and Rubenstein, 1984; Reznick et al., 1990). The necessity of detailed investigation of problems of post-metamorphic survival has already been stressed (Ento and Matsui, 2002), but, unfortunately, we have no such data in our population. However, metamorphosed *H. nebulosus* usually inhabits the forest floor near wetlands and paddy fields, adjacent to lentic waters (Sato, 1943; Tanabe and Matsui, 1997), and under such habitats, the rate of adult mortality may be higher than in the montane environments inhabited by such species as *H. kimurae*. Heavy predation, lack of shelters for hiding, and environment instability (e.g., drought, high temperatures) are thought to cause high mortality, it is therefore highly desirable to accumulate environmental data in future studies.

The genus *Hynobius* is remarkably diversified in Japan (Sato, 1943; Matsui et al., 2009), with very limited distribution ranges in each species (e.g., Matsui et al., 2004). This results in their endangered status (Environment Agency of Japan, 2006), but few ecological studies have been made on them to the present. Basic knowledge of life history traits, such as age and body size at first reproduction, and longevity, is not only indispensable for understanding life history evolution (e.g., Tilley and Bernardo, 1993), but is also very important for establishing appropriate conservation measures. Amid increasing demand for clarifying life history traits of salamanders, the non-lethal technique of skeletochronology will undoubtedly become a more and more a powerful tool.

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