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# Estimation of Age Structure by Skeletochronology of a Population of *Hynobius nebulosus* in a Breeding Season (Amphibia, Urodela)

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**ABSTRACT**—Using skeletochronology, we determined the age structure of adult *Hynobius nebulosus* from Kyoto in the breeding season of 1998. From previously marked individuals, the lines of arrested growth proved to be formed once per year, indicating the number of winters each salamander experienced. The age at first reproduction was estimated to be 2.8–2.9 yrs of age in males and 3.8–3.9 yrs in females. The oldest males and females were 9.8–9.9 and 5.8–5.9 yrs of age, respectively, and, therefore the longevity in this species was estimated to be more than 9 yrs for males and 5 yrs for females. The growth curve of male's body size estimated indicated that the growth rate much decreases after males attained sexual maturity. Because body sizes of adults greatly vary even within an age class, it is dangerous to estimate individual age from the size frequency data at least in adults. We discussed age properties in *Hynobius* by comparing lentic and lotic breeders.

**Key words:** age estimation, age structure, *Hynobius nebulosus*, skeletochronology, growth curve

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## INTRODUCTION

Skeletochronology is one of the techniques of age determination and studies the presence of growth layers in the long bones by counting the lines of arrested growth (LAG). This technique is convenient and suitable for investigating the population age structure in nature without killing animals. In these days, there have been many studies of age determination for various lower tetrapods using this technique (e.g., Halliday and Verrell, 1988; Castanet *et al.*, 1988), because many important aspects of population life history can be elucidated by knowing exact age of each individual involved. Thus, the growth rate, longevity, and the age at first breeding have already been clarified in populations of various amphibian species (e.g., Hemelaar, 1988; Caetano and Castanet, 1993; Wake and Castanet, 1995). Using these data, Tilley (1980) studied the intraspecific life history variation of two populations from different environments, and Francillon-Vieillot *et al.* (1990) discussed evolutionary strategies of hybrids. In fact age determination has been a very important subject in the study of amphibian biology.

In Japan, only few skeletochronological studies have been conducted so far for amphibians (e.g., *Rana tagoi*: Kusano *et al.*, 1995a; *R. sakuraii*: Kusano *et al.*, 1995b), and in urodeles, only two studies for *H. kimurae* (Misawa and Matsui, 1999) and *Cynops pyrrhogaster* (Marunouchi *et al.*, 2000) are available. There are two groups in *Hynobius* that exhibit different modes of breeding (i.e., lentic and lotic), and *H. kimurae* is a member of the lotic breeding group. Between these two groups, not only the breeding site but also the size and number of eggs laid are different. Because these reproductive parameters are important life history traits, there may be difference in age properties between the two groups.

Although there have been several life history studies, including age and growth, of lentic breeding *Hynobius tokyoensis* (as *H. nebulosus tokyoensis*, Kusano, 1980, 1981, 1982), the individual age has never been estimated skeletochronologically. Therefore, the first purpose of this paper is to make a first attempt to examine the applicability of skeletochronology to lentic breeding *H. nebulosus*. On the basis of results obtained, we analyzed age structure, age at first reproduction, and growth pattern of this species whose local populations have been markedly declining and called for immediate conservation measure.

The second purpose is to generalize some life history differences between lentic and lotic breeding small salamanders. This species is a close relative of *H. tokyoensis*

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studied by Kusano (1980, 1981, 1982) and has a wide range of distribution in western Japan. On the other hand, *H. kimurae* mentioned above (Misawa and Matsui, 1999) is a lotic breeder and has also wide distribution range in central to western Japan. We, therefore, can compare age and growth characteristics between the two species that represent two different modes of life history.

## MATERIAL AND METHODS

Study site was located in the campus of Kyoto University, Yoshida Nihonmatsu-cho, Sakyo-ku, Kyoto, Japan (35°08'N, 135°47'E, 98 m above sea level). This site was about 2430 m<sup>2</sup> in area and surrounded by dry asphalt streets that completely prohibited migration of salamanders. This area was covered with bushes, low bamboos *Sasa*, and broad-leaved trees, such as *Celtis*, *Salix*, and *Aucuba* that provide a lot of fallen leaves on the ground. The breeding site was a small artificial pond, about 36 m in circumference and 60 cm in depth with soft mud and fallen leaves on the bottom. The population of *H. nebulosus* inhabits the study site derived from eggs and larvae originally introduced artificially from other places in Kinki district from 1983 to 1991 by one of us (MM). From 1996 to the present, metamorphs have been captured, individually marked by toe clipping, measured for body size, and released periodically.

In the present study, data obtained for adults in the breeding season of 1998 was mainly used. Sampling of adults was made in the breeding season, between early January and early April. Here, breeding season means a period when adults in a reproductive condition were observed in the pond. The breeding adults, collected by hand net three times a week from 1900 hr to 2100 hr, were carried to the laboratory each time. After anesthetized by 1% MS-222 (Ethyl 3-aminobenzoate, methanesulfonic acid salt), the snout-vent length (SVL) from the tip of the snout to the anterior corner of the cloaca was measured to the nearest 0.1 mm with a caliper. The third toe of the left foot was amputated when individual marking was made by toe clipping, and preserved in 10% formalin for skeletal-

chronology. Some individuals have been recaptured over years.

For preparing bone sections, we followed Kusano *et al.* (1995a); toes were washed in the running water for 24 hr, decalcified in 5% nitric acid for 15–30 min, and again washed in the running water for 24 hr. We cross-sectioned the toes (20–22 μm thick) by a freezing microtome, stained with hematoxylin (Mayer's acid hemalum) for 30 min, examined under a light microscope (X 100–400), and checked the number of LAGs present in the bone.

The bone sections of juveniles collected before hibernation, in November 1998, and overwintered adults collected after breeding season, in late April 1998, were examined to elucidate formation process of LAGs. In addition, the sections of phalanges of several adults captured after one or two years were compared to examine the change in LAG numbers over years.

We estimated the growth pattern in the SVL of male salamanders by fitting the Logistic Model  $L = a / (1 + e^{c-bt})$ , where L is the SVL (mm) at time t (year), a is the asymptotic size, c is the constant determined by putting 0 to t, and b is the instantaneous growth rate (Misawa and Matsui, 1999). In this estimate, we used data of unsexed juveniles and estimates of the parameters provided by a software KaleidaGraph 3.0.4 following Zar (1996). We could not estimate a growth curve for females because of their small sample size.

We examined sexual difference in age structure of reproductive adults by  $\chi^2$ -test. Mann-Whitney U-test was applied for sexual differences in average age of reproductive adults. Difference in SVL was compared among the different ages by Kruskal-Wallis's test. The significance level was set at 0.05.

## RESULTS

In 1998, salamanders bred from 12 January to 3 April and we captured 425 male and 28 female adults in this period. The mean ( $\pm$ SD) SVL of breeding adults was 53.9 ( $\pm$ 2.54) mm in males and 46.7 ( $\pm$ 0.16) mm in females. The range of SVL was 41.9–70 mm in males and 43.5–61.4 mm in females.

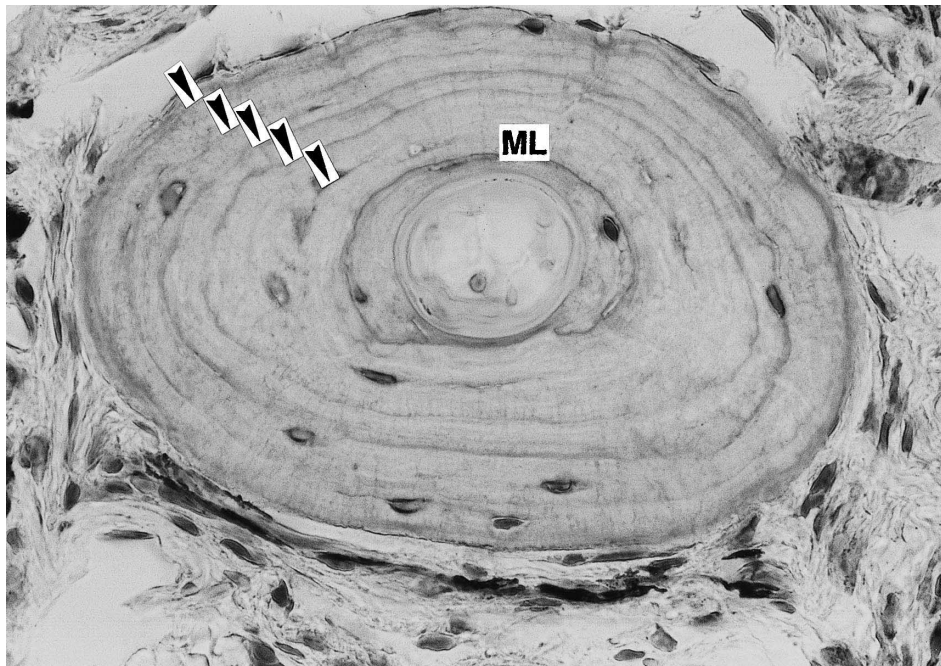
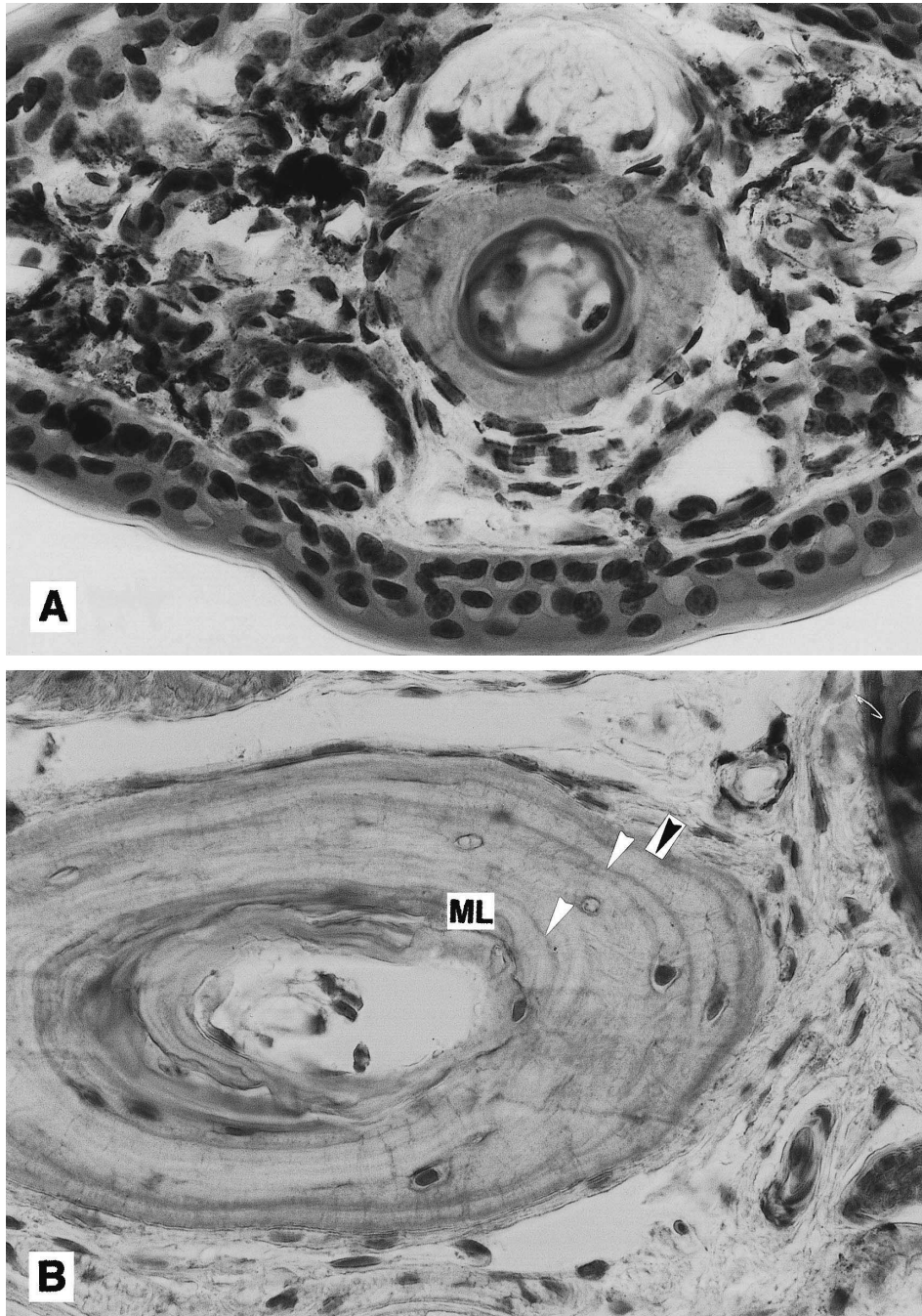


Fig. 1. Five LAGs (arrows) and a metamorphosis line (ML) seen in phalangeal bone of an adult male.

The section of toe phalanges of breeding adults exhibited narrow, concentric, hematoxylinophilic rings (Fig. 1). These darkly stained rings were interpreted as LAGs, and one ring in the innermost side as metamorphosis line (ML), which was always found in juveniles just after metamorphosis. We could distinguish them easily from their different shapes. In almost all sections, this ML was found to be retained (Figs. 1–3) and the resorption of LAGs was rarely found. The section of newly metamorphosed juveniles captured in November before their first overwintering showed

ML but no LAG. In the section of overwintered adults collected in late April after the breeding season, the newest LAG was clearly separated from the cortex and indicated that the bone already started growth (Fig. 2). The comparisons of phalangeal sections taken from 11 individuals that were captured after one or two years (10 captured in the 1996 and 1998 breeding seasons, and one captured in the 1996 and 1997 breeding seasons) clearly indicated that LAGs were formed over corresponding winters (see Fig. 3).

From above results it is clear that in adults participated



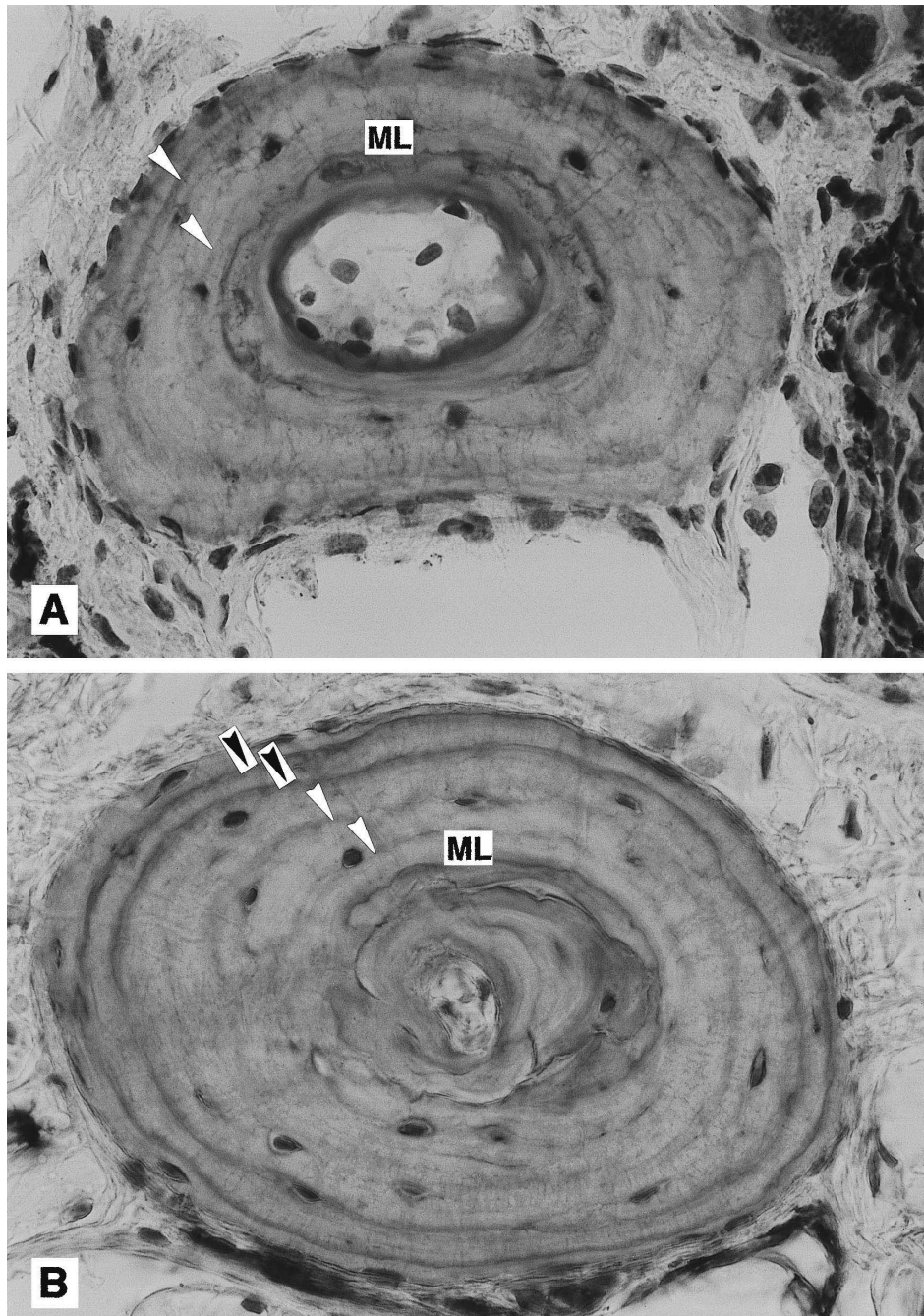
**Fig. 2.** Phalanges of a young before its first winter showing the absence of LAG (A), and of a 3 yrs adult just after overwintering showing a new LAG (dark arrow) at the edge of the phalange (B). White arrows show old LAGs.

in the breeding between January and April, a new LAG had not yet been formed. On the other hand, larvae usually hatched after April and metamorphose by autumn. Thus, the adult collected in the breeding season and with  $X$  LAGs (i.e., overwintered  $X$  times) corresponds to the one which experienced  $X$  years and 10–12 months from its hatching.

Out of a total of 453 individuals captured, we examined 443 (415 males and 28 females) and failed to determine age of only six (=1.4%) in which one had LAGs resorbed and the other five had no LAGs in their regenerated toes. The age

structure of these adults is shown in Fig. 4. As is clear in this figure, sexes differed in age structure ( $\chi^2$ -test,  $P < 0.01$ ). The median (and mean) age was 3.90 (4.28) yrs in males and 4.80 (4.84) in females, and females were significantly older than males ( $U$ -test,  $P < 0.01$ ). The oldest individual was 9.8–9.9 yrs old in males and 5.8–5.9 in females, while the minimum age was 2.8–2.9 yrs in males and 3.8–3.9 in females.

In the breeding males, individuals of the age class 2 (2.8–2.9 yrs of age) were most numerous and the number of individuals decreased with the increase of age. By con-



**Fig. 3.** Phalanges of an individual first captured in 1996 (A) and recaptured in 1998 (B). White arrows indicate old LAGs and dark arrows additional two new LAGs.

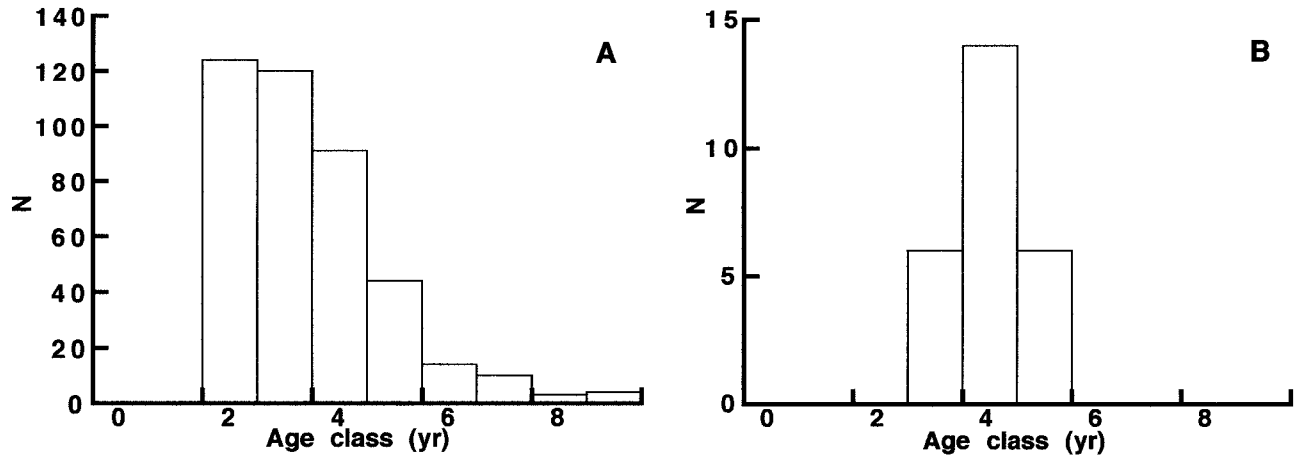


Fig. 4. Age structure of breeding males (A) and females (B) in 1998.

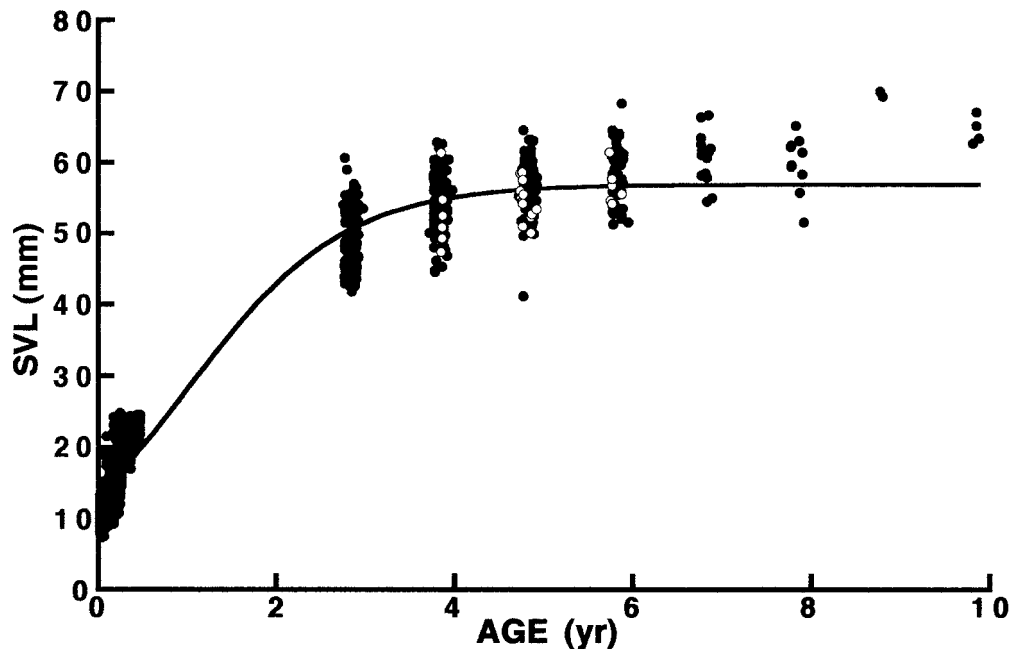


Fig. 5. SVL growth in males (closed circles) and females (open circles). In estimating the growth curve for males by Logistic Model ( $L=56.93/(1+e^{1.18-1.15x})$ ), data for unsexed juveniles just after metamorphosis were used.

trast, individuals of the age class 4 predominated in females, being three times as many as the age class 3, and the number decreased in age class 5. SVL of adults collected in the breeding season greatly varied even within a same age class, and overlapped greatly among different age classes, resulting in no significant differences between different age classes in either sex (Kruskal-Wallis's test,  $P>0.05$  in both sexes; Fig. 5). In each corresponding age classes (3–5), females ( $n=26$ ) were significantly smaller than males ( $n=246$ ) ( $U$ -test,  $P<0.01$ )

As shown in the growth curve estimated by a logistic model (Fig. 5), the growth rate ( $b\pm 2SE=1.1482\pm 0.0574$ ) of males rapidly slowed down after they matured sexually.

## DISCUSSION

Our study site was an artificially constructed pool and the population of *H. nebulosus* studied was originally introduced there from the nearby localities. We, however, think the life history traits elucidated here are not much different from those of original populations. This is because *H. nebulosus* is known to have been distributed in regions including the study site some 50 to 70 years ago, and the study site, being isolated in an old campus of a university, has been affected by minimal disturbance in vegetations. The original sites of reproduction and habitats of *H. nebulosus* are ditches along paddy fields and hills around there, that could be judged even more affected by human activities

compared with our study site. Although we have no comparable data, the density of our population seems to be much higher than in the original populations. However, at least the body size of the salamander is similar to that of the populations from surrounding regions including the original populations (Tanabe and Matsui, 1997), and the seemingly high population density is not considered to have affected body size in the population studied.

Although the technique of skeletochronology has been reported to be relatively difficult to apply for some urodelan (Wake and Castanet, 1995) and anuran (Hemelaar, 1985) species, it has been used in many other amphibian species (e.g., Francillon-Vieillot *et al.*, 1990; Castanet *et al.*, 1996). In this study, we confirmed that age determination by skeletochronology is applicable also for *H. nebulosus*. Phalangeal sections of this species had clear hematoxylinophilic lines and unlike cases reported by Hemelaar (1985), there were few resorptions; in almost all sections we examined, we could find ML consistently. We, therefore, think the possibility of underestimation in determining the age of *H. nebulosus* was minimal.

The minimum age at first reproduction was estimated to be 2.8–2.9 yrs in males and 3.8–3.9 yrs in females. However, the number of 3.8–3.9 yrs females was much smaller than that of 4.8–4.9 yrs females. Therefore, most of the females were considered to mature in 4.8–4.9, and not 3.8–3.9 yrs, in contrast to males of which the majority begins breeding at the age of 2.8–2.9 yrs.

In the allied species *H. tokyoensis*, the age at first reproduction was estimated to be older than in *H. nebulosus*, being 4 yrs in males and 5 in females (Kusano, 1982). In Kusano's (1982) study, however, the age of individuals was expressed by the number of winters they experienced. According to his way, the age at first reproduction in our *H. nebulosus* corresponds to 3 yrs in males and 4–5 yrs in females, and the difference in the two species is approximately one year. This difference might have derived from the difference in the method adopted to estimate ages; Kusano (1982) estimated the age of *H. tokyoensis* from the combined data of growth rate of captive juveniles, size at metamorphosis in the field, and size of breeding adults.

Also, the difference of Kusano's (1982) estimation from our data might be derived from locally differential environmental conditions between the two species as Tilley (1980) reported. In order to solve this problem, skeletochronology should be applied to the age estimation of *H. tokyoensis*.

It seems better not to use the size frequency data for age determination, because, as revealed in the present study, body size at least in adults highly varies within a same age group and largely overlaps among different age groups. The fact that the body size is a poor indicator for estimating the age of adults was reported in some studies (e.g., Halliday and Verrell, 1988; Caetano and Castanet, 1993; Khonsue *et al.*, 2000).

The growth rate in male *H. nebulosus* was high until sexual maturity but it slowed down thereafter. This must be

because individuals utilize energy mainly for growth before sexual maturation, but they turn to use most energy for reproduction once they matured (Hemelaar, 1988). In many urodelan and anuran species, females are reported to require longer time for sexual maturity than males (Tilley, 1980; Kusano, 1982), and this was the case in *H. nebulosus* we studied. This sexually different maturation time is generally considered to be resulted from a higher cost for reproduction in females than males (Misawa and Matsui, 1999), and this idea also seems to be applicable to *H. nebulosus* because the female of this species bears a large number of eggs (over 50 in the smallest female: Ento, unpublished data).

Misawa and Matsui (1999) reported that reproductive individuals are much older in lotic breeding *H. kimurae* compared with lentic breeding *H. nebulosus* here studied. Mean age in two populations of *H. kimurae* was found to be 8.8–9.1 yrs in reproductive males and 9.4–9.9 yrs in females (Misawa and Matsui, 1999), and more than twice that of *H. nebulosus* (4.34 yrs in males and 4.84 yrs in females).

Because there are many other *Hynobius* species in east Asia (Frost, 1985; Duellman, 1993), the different age properties seen between *H. nebulosus* and *H. kimurae* may not be generalized as that of lentic and lotic breeders, respectively, but no comparable and detailed data are available at present for any species of *Hynobius* other than these two species. Unlike lowland-living *H. nebulosus*, *H. kimurae* inhabits montane areas and breeds in small streams (Misawa and Matsui, 1997). Compared with *H. nebulosus*, females of *H. kimurae* are much larger [mean SVL=73–77 mm in two populations (Misawa and Matsui, 1997) v.s. mean SVL=47 mm in *H. nebulosus* (Ento, unpublished data)], lay much larger (mean ovum diameter=4.3–4.5 mm v.s. 2.2 mm in *H. nebulosus*) and less numerous eggs (mean=27–32 in two populations v.s. mean=120 in *H. nebulosus*), and hatched larvae first utilize yolk reserves, begin feeding later, and overwinter in some populations (Misawa and Matsui, 1997).

These reproductive properties are typical of lotic breeding salamanders (Sato, 1943). In *H. kimurae*, Misawa and Matsui (1999) found that clutch size did not correlate to female age, and that older and hence larger females tended to lay larger eggs that would increase larval survival (Kusano, 1982; Caetano and Leclair, 1996). From these observations Misawa and Matsui (1999) considered that the female fitness increased with age, not through an increase in the number of offspring per clutch, but through an elevation of survivorship per offspring.

On the other hand, minimum maturation age in female *H. kimurae* is reported to be 7 yrs (Misawa and Matsui, 1999), and nearly twice later than in *H. nebulosus* (3.8–3.9 yrs). Later maturation with larger eggs would be more advantageous since larval survival from larger eggs would be higher. This strategy could be elaborated only under the conditions of high postmetamorphic survival, and actually, minimum longevity in female *H. kimurae* is reported to be as

long as 12–17 yrs (Misawa and Matsui, 1999).

On the contrary, when the postmetamorphic survival rate is low, early maturation and participation in breeding, although with smaller body size (Diaz-Paniagua *et al.*, 1996) and hence with smaller eggs, would be more advantageous because, due to high mortality, females that mature later would have less chance to experience reproduction.

Because females of *H. nebulosus* we studied matured earlier at smaller size and started breeding at younger stage, the survival rate may be lower than that of *H. kimurae*. Much shorter minimum longevity observed in *H. nebulosus* in the present study (9 yrs) seems to support this hypothesis, but, unfortunately, we have at present no actual data of the survival rate to ascertain its validity, and detailed investigation of problems of postmetamorphic survival should be included in future studies.

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