

Body Size and Age Structure of Breeding Populations of the Salamander, *Hynobius tokyoensis* (Caudata: Hynobiidae)

TAMOTSU KUSANO^{1*}, TAKEHITO UEDA², AND HIKARU NAKAGAWA¹

¹ Department of Biology, Faculty of Science, Tokyo Metropolitan University, 1–1 Minami-ohsawa, Hachioji, Tokyo 192–0397, JAPAN

² Northern Biology Laboratory, 7–13–18 Minamikojiwa, Edogawa-ku, Tokyo 133–0056, JAPAN

Abstract: Body size and age structure were studied for two breeding populations of the salamander, *Hynobius tokyoensis*, in the southern Kanto plain of Honshu, Japan. The mean snout-vent lengths (SVLs; measured from the tip of snout to the posterior end of cloaca) of males and females were 64.1 and 66.5 mm at Habu in Tokyo, and 63.1 and 65.3 mm at Isumi in Chiba prefecture, respectively. The differences in SVL were not significant between sexes or populations. The breeding adults, successfully aged by skeletochronology using phalanges, were 4–21 years old. The median age was significantly older in the Habu population (eight years) than in the Isumi population (five years), and the age at first reproduction estimated from the age at which the growth of individuals was greatly retarded was two to six years. The modal age at first reproduction was four years for both sexes in both populations except for males at Isumi (three years). In the latter, age at first reproduction was significantly younger than in the others. We discuss the ecological significance of interpopulational variation in age structure clarified by skeletochronology.

Key words: Age at first reproduction; Age structure; Body size; *Hynobius tokyoensis*; Skeletochronology

INTRODUCTION

Body size and age are two of the most important variables in population studies of long-living species, such as salamanders and newts. Usually body size can be measured easily. In contrast, it is difficult to determine ages of individual animals; hence, there is, as yet, insufficient information on the age structures for amphibian populations.

Four methods have been used for aging

amphibians: recapture of known individuals, extrapolation from size-frequency data, skeletochronology, and testis lobation. However, only mark-recapture and skeletochronology are considered to be reliable (see Halliday and Verrell, 1988). Mark-release-recapture potentially provides the most reliable data on age, but this method is very labor-intensive and takes a long time to yield results. On the other hand, skeletochronology using phalanges has proved to be an excellent tool for investigating population age structure without sacrificing specimens, and in Europe and North America many studies have been conducted successfully for amphibians by this method (e.g., Smirina,

* Corresponding author. Tel: +81-42-677-1111 ext. 3761; Fax: +81-42-677-2559;
E-mail address: tamo@comp.metro-u.ac.jp

1972; Hemelaar and van Gelder, 1980; Gibbons and McCarthy, 1983; Verrell and Francillon, 1986; Forester and Lykens, 1991). Although this technique has also been applied to many Japanese anuran species (e.g., Kusano et al., 1995a, b; Khonsue et al., 2001; Marunouchi et al., 2002), there have been only a few skeletochronological studies on Japanese urodeles to date (Misawa and Matsui, 1999; Marunouchi et al., 2000; Ento and Matsui, 2002).

In this paper, we report the body size of breeding adults of the salamander *Hynobius tokyoensis* in Tokyo and Chiba Prefecture. Also, we document the age structure and age at first reproduction as determined by skeletochronology for these populations. We then compare the body size and age structure between them, and discuss the ecological significances of inter-population differences in these variables.

MATERIALS AND METHODS

Study site

Hynobius tokyoensis is a typical lentic breeding salamander distributed in the Kanto district and part of Fukushima Prefecture, Japan. Some ecological aspects of *H. tokyoensis*, such as population dynamics and life histories, have already been documented (Kusano, 1980, 1981, 1982; Kusano and Miyashita, 1984).

The breeding populations of *H. tokyoensis* were studied at two main sites: one in Habu, Hinode-machi, Tokyo and the other in Isumi-shi, Chiba prefecture (Fig. 1). The study site in Habu (35°44'N, 139°14'E) is a small pond, 200 m asl, located in a hilly region covered with forests of planted coniferous trees, *Cryptomeria japonica* and *Chamaecyparis obtuse*, and with secondary deciduous forest. The pond was about 10 m² in area and 15–20 cm in maximum depth. Detailed descriptions of this site and the breeding population are given in previous papers (Kusano, 1980, 1981, 1982). In Isumi-shi, we chose three sites: Mangi, Sakuta, and Fukaya. These sites are located in a lowland rural area consisting of paddy fields, and forests of planted coniferous trees *C. japonica* and evergreen broadleaf

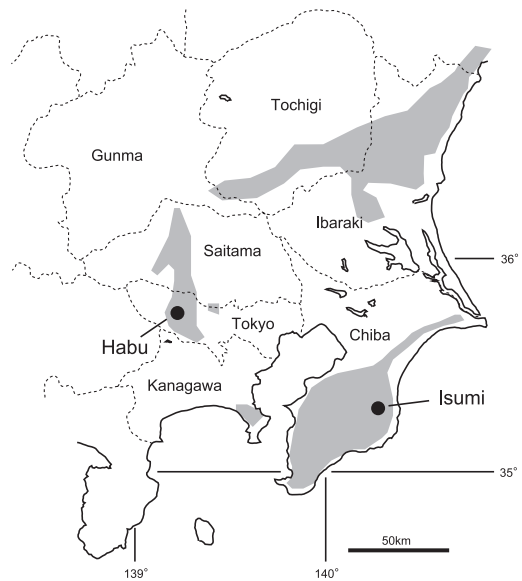


FIG. 1. Map of the Kanto district, Japan, showing the range of distribution of *Hynobius tokyoensis* (gray area) and the study sites (solid circles).

trees *Castanopsis sieboldii*, *Quercus myrsinaefolia* and *Q. glauca*. The study site at Mangi (35°18'N, 140°20'E, 20 m asl) is a small, roundish, spring-fed pond measuring about 2 m in diameter. The study site at Sakuta (35°17'N, 140°17'E, 20 m asl) is an irrigation ditch, 50 cm in width and 15 m in length, bordering a paddy field. The site at Fukaya (35°18'N, 140°17'E, 40 m asl) is a small swamp bordered by a road. Since these three sites in Isumi are located within a distance of 4 km, data from them were pooled as Isumi under an assumption that salamanders from these localities belong to the same population, or at least, metapopulation.

Body size and age determination

At Habu, we collected breeding adults by hand during the day from February to April in 2004 and 2005. These adults were sexed on the basis of the status of secondary sexual characters, such as morphology of the cloacal region (Sato, 1943), and their snout-vent length was measured (SVL; from the tip of the snout to the posterior end of the cloacal

opening) to the nearest 0.5 mm with a sliding caliper after anesthetization with MS-222 solution. In some of the adults collected, the third or fourth toe of a hindleg was clipped off, and stored in a separate vial containing 10% buffered formalin for skeletochronological analysis. The adults were returned to the collection sites soon after these procedures. We also collected some additional samples in the breeding seasons of 2000 and 2006.

At Isumi, skeletochronological samples were collected in the breeding season of 2002 (February 1 to April 2). Since the standard body length of adults at Isumi was measured differently from the way adopted for the adults at Habu (i.e., measured to the anterior, not posterior, end of cloacal opening; henceforth referred to as SVL_{ant}), their values were later converted using the relationship between SVL_{ant} and SVL in the same sample as below, so as to be comparable with SVL values of the latter. First of all, both SVL_{ant} and SVL were measured for adults in the breeding seasons of 2005 and 2006 at Isumi. Using the resultant data set, a highly significant correlation between these two variables was confirmed ($n=12$, $R^2=0.96$, $P=3.76 \times 10^{-8}$) and a regression equation was obtained ($SVL=1.113SVL_{ant}-0.414$). Then, SVL_{ant} s from all Isumi adults were converted to SVLs with this equation.

The method used to determine the age of each adult was essentially the same as that used by Kusano et al. (1995a, b). The phalanges were decalcified in 5% nitric acid for 0.5–1 h, cross-sectioned (20 μ m thick) using a freezing microtome, and stained with hematoxylin (Mayer's acid hemalum) for 30 min. Sections with smaller medullary cavities were selected for observation from the central region of the diaphysis, because resting lines were absent or incomplete in the epiphysis, and were examined under a light microscope using an ocular micrometer at 600 \times magnification.

Stained sections showed a series of narrow, concentric, hematoxylinophilic rings separated by wider zones of paler background material. These dark-stained rings were interpreted as

the result and products of arrested growth during winter, and were thus regarded as resting lines (RL; see Hemelaar and van Gelder, 1980). The age of each individual was determined by counting the number of RLs in the periosteal bone. Since specimens were collected during the breeding season, i.e., the period during which no growth should have occurred in the current year, the outer margin of the bone was regarded as an additional RL (Smirina, 1972). Because these RLs were nearly circular or elliptic, the longest and shortest axes of each RL were measured using an ocular micrometer, and the geometric mean was taken as the diameter of the RL. It is known that resorption of the periosteal bone occurs from the inner surface of the bone, and that the process of resorption is marked by a resorption line: a narrow irregular hematoxylinophilic line (see Hemelaar and van Gelder, 1980). We also measured the diameter of the resorption line.

Statistical analysis

We used the software R 2.3.1 (R Development Core Team, 2006) for statistical analyses. To test differences in body size distributions between sexes and populations, pairwise comparisons were conducted using Welch's test, because of the heterogeneity of variances (Zar, 1996). The differences in age distributions were also tested between sexes and populations using the Mann-Whitney test because of non-normality. Acceptance levels for simultaneous statistical tests were adjusted by the sequential Bonferroni procedure (Rice, 1989). Results where the adjusted P-values were less than 0.05 were judged statistically significant.

RESULTS AND DISCUSSION

Body size and sexual dimorphism

A total of 90 and 153 breeding adults were measured at Habu and Isumi, respectively. Since the yearly variation of SVL at Habu was small in both sexes and the sample size of each year was insufficient, the data were pooled for

all years. The SVLs of breeding adults ranged from 50.5–73.0 mm (\bar{x} =64.1 mm) for males and from 60.0–75.5 mm (\bar{x} =66.5 mm) for females at Habu (Fig. 2). At Isumi, SVLs ranged from 48.5–80.0 mm (\bar{x} =63.1 mm) for males and from 54.0–83.0 mm (\bar{x} =65.3 mm) for females. We could not detect any significant differences in SVL between sexes or populations.

At both sites, we failed to detect any significant sexual size dimorphism, although mean SVL was larger in females than in males (Fig. 2). The absence of statistical significance may be due to the small sample size, especially for females at Habu ($n=17$). A large data set for the body size of the breeding population at Habu has already been reported by Kusano

(1982) and Inoue (1988). They surveyed the population in nine breeding seasons between 1976 and 1987, and measured a total of 583 males and 273 females. In their data, the SVL ranged from 48.5–79.5 mm (\bar{x} =61.9 mm, $SD=4.9$) for males and from 55.5–75.5 mm for females (\bar{x} =65.2 mm, $SD=4.0$), and the difference was highly significant between the sexes (Welch's test, $t=10.54$, $df=632.05$, $P < 0.001$). Therefore, it seems obvious that a significant sexual size dimorphism actually exists in the Habu population, but the magnitude of the dimorphism is so small as to obscure its statistical significance with a relatively small data set.

Age structure and age at first reproduction

A total of 81 adults from Habu and 84 adults from Isumi were aged successfully by skeletochronology. In all phalangeal cross sections, stained RLs were distinct and easy to count. We could not find any metamorphosis lines (Misawa and Matsui, 1999; but see Ento and Matsui, 2002). The cross sections had 4–21 RLs, and the donors' ages were thus estimated to be 4–21 years.

Because all specimens examined were adults, the process of resorption may have destroyed the older RLs especially at one year of age in our sections (Hemelaar and van Gelder, 1980). Although this may present a serious problem for age determination, complete resorption of the first RLs in the breeding adults was reported to be rare in Japanese hynobiid salamanders (Misawa and Matsui, 1999; Ento and Matsui, 2002). This suggests that age determination by skeletochronology may be only slightly noised by the resorption of RLs. Even so however, since we could not make enough attempts to compensate effects of resorption in our sample, ages of some adults may have been underestimated in reality.

Breeding adults were estimated to be 4–21 years old at Habu, and the mean and median ages were 9.0 ($SD=3.8$, $n=65$) and 8 years in males, and 8.7 ($SD=2.2$, $n=16$) and 8 years in females, respectively (Fig. 3). The age of breeding adults was estimated to be 4–13 years

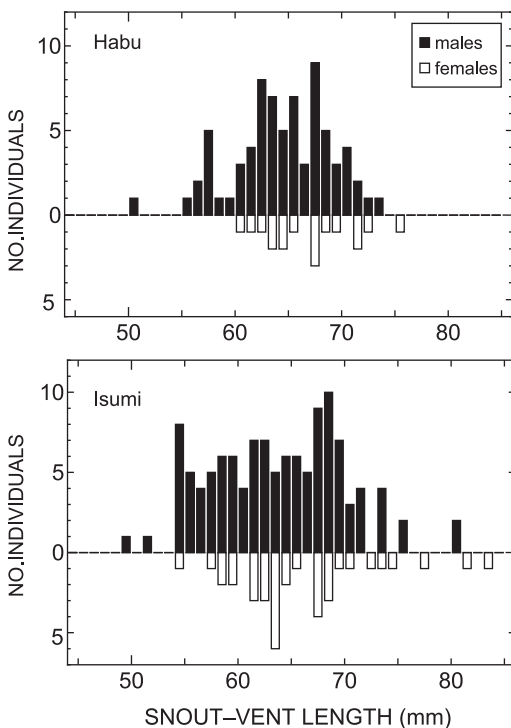


FIG. 2. Snout-vent lengths of breeding adults of the two *Hynobius tokyoensis* populations. Means \pm SDs were 64.1 \pm 4.5 mm ($n=73$) for males and 66.5 \pm 4.3 mm ($n=17$) for females in the Habu population, and 63.1 \pm 6.1 mm ($n=117$) for males and 65.3 \pm 6.4 mm ($n=36$) for females in the Isumi population.

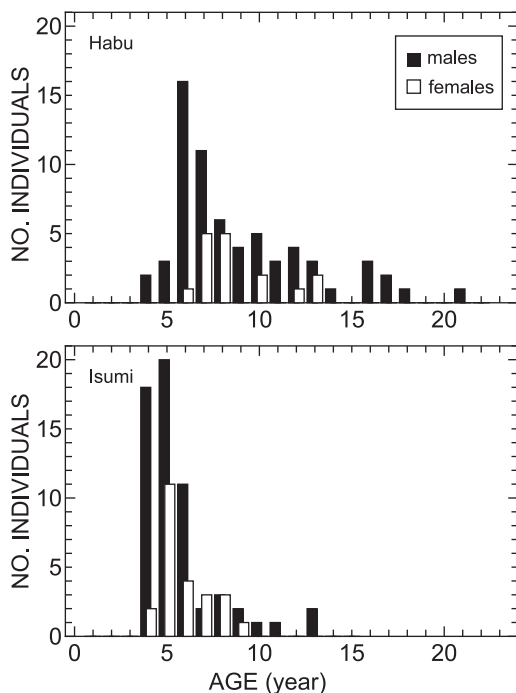


FIG. 3. Age structures of breeding populations of *Hynobius tokyoensis* determined by skeletochronology. Means \pm SDs were 9.0 ± 3.8 years ($n=65$) for males and 8.7 ± 2.2 years ($n=16$) for females in the Habu population, and 5.7 ± 2.1 years ($n=60$) for males and 5.9 ± 1.4 years ($n=24$) for females in the Isumi population.

at Isumi, and the mean and median ages were 5.7 ($SD=2.1$, $n=60$) and 5 years in males, and 5.9 ($SD=1.4$, $n=24$) and 5 years in females. Breeding adults were significantly older at Habu than at Isumi ($P < 0.001$), but the sexual differences were not significant in either population ($P > 0.3$; Fig. 3).

The youngest breeding individuals were four years old in males and six years old in females at Habu, and four years old for both sexes at Isumi. This fact suggests that *H. tokyoensis* needs at least four to six years to begin breeding activities. Kusano (1982), on the basis of growth rates of juveniles under laboratory conditions, mean SVL at metamorphosis, and the minimum SVL of breeding adults in the field, estimated that age at first reproduc-

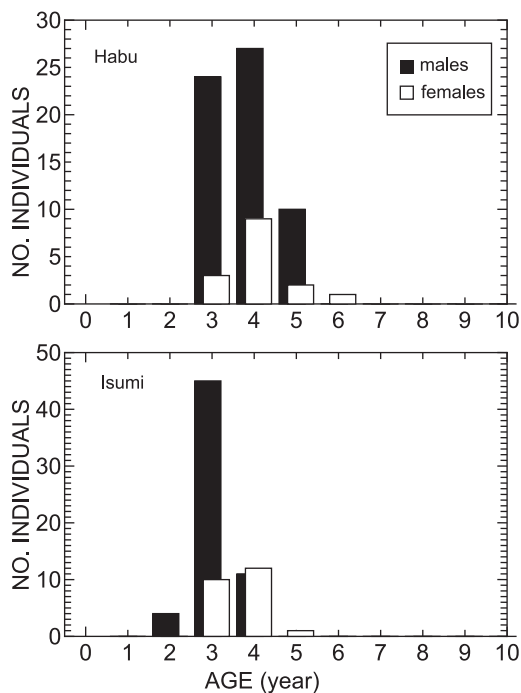


FIG. 4. Frequency distribution of age at first reproduction in *Hynobius tokyoensis* estimated from the age at which the growth of individuals was greatly retarded. Means \pm SDs were 3.8 ± 0.7 years ($n=61$) for males and 4.1 ± 0.8 years ($n=15$) for females in the Habu population, and 3.3 ± 0.6 years ($n=60$) for males and 4.1 ± 0.8 years ($n=24$) for females in the Isumi population.

tion is four to five years in the Habu population of *H. tokyoensis*. This range of estimated ages at first reproduction agrees roughly with the estimation in the present study, but the estimate from the age structure may be biased and overestimated, because age at first reproduction may differ greatly among individuals, and the sample was not large enough for both sexes, especially females.

We tried to estimate age at first reproduction in another way. In general, animals allocate a large amount of energy to reproduction, and therefore, their growth is usually greatly retarded after reaching sexual maturity (e.g., Kusano, 1982; Ryser, 1989). A smaller interval between two RLs indicates retarded growth and the first one of such smaller intervals may

correspond to the age at sexual maturity (but see Augert and Joly, 1993). Age at first reproduction was thus estimated for both sexes in both populations (Fig. 4).

By this method, *H. tokyoensis* at Habu was estimated to start breeding from three to six years of age, with a mean of 3.8 years for males and 4.1 years for females, and a median of four years for both sexes. At Isumi, age at first reproduction was estimated to be two to five years, with a mean of 3.3 years for males and 4.1 years for females (Fig. 4), and median of three and four years, respectively. The modal age at first reproduction was four years for both sexes in both populations except for males at Isumi, in which the modal age was three years. Age at first reproduction was significantly younger for males at Isumi than females at Isumi and both sexes at Habu (all P 's < 0.003).

Ages at first reproduction estimated by this method tended to be younger than the minimum ages of breeding adults (Figs. 3 and 4). For instance, many males were estimated to start breeding at three years of age, but there were no three-year-old males in either of the two breeding populations. We are not sure of the cause of discrepancy. Presumably this reflects inaccuracy in estimating age at first reproduction from the growth pattern of RLs, underestimating the age in *H. tokyoensis* (see Augert and Joly, 1993). Or, this may reflect heterogeneity in catchability among breeding adults of different ages (Van Gelder and Rijdsdijk, 1987), because younger and smaller adults tend to appear at the breeding site later in the season (Kusano, 1980). Young adults that breed for the first time may not have been captured at the breeding site in the present study. The minimum estimated age of breeding adults is reliable only when samples of the breeding populations are large enough.

Relationship between body size and age

Amphibians are generally considered to show indeterminate growth, leading to strong correlation between the body size and age even in adults (Duellman and Trueb, 1986), although

Halliday and Verrell (1988) questioned this assumption. In *H. tokyoensis*, significant correlations were recognized between body size and age in both sexes in both populations ($r > 0.47$, $p < 0.05$; Fig. 5) except for females at Isumi ($r = 0.32$, $n = 24$, $P = 0.13$). Comparisons by analysis of covariance (ANCOVA) of regressions of SVL on age between males of the two populations revealed a significant difference in slope (0.66 mm/year [SE=0.15] at Habu and 1.4 mm/year [SE=0.33] at Isumi: $F_{1,121} = 4.882$, $P = 0.029$), indicating that the annual growth rate of breeding males was greater at Isumi than at Habu. Sexual difference in

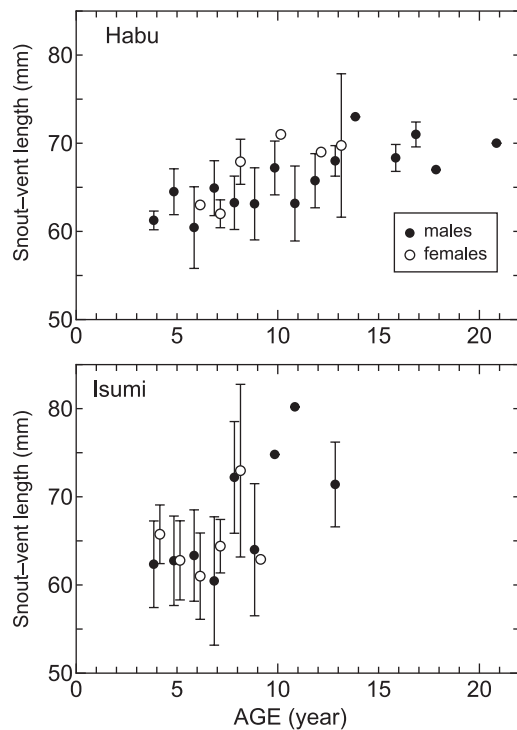


FIG. 5. The relationship between the body size (snout-vent length) and age in two populations of *Hynobius tokyoensis*. Circles and vertical bars indicate means and the ranges of \pm SDs, respectively. The size of each sample ranged from 1–20. Correlation coefficients (r) were 0.56 ($n = 65$, $P < < 0.001$) and 0.61 ($n = 16$, $P = 0.013$) for males and females at Habu, and 0.47 ($n = 60$, $P < 0.001$) and 0.32 ($n = 24$, $P = 0.13$) for males and females at Isumi, respectively.

relationship between SVL and age was not evident in either population (Fig. 5), probably because of the small sizes of the female samples.

Interpopulational differences

The SVL of breeding adults did not differ significantly between sexes or populations, although females tended to be slightly larger than males in both populations (Fig. 2). By contrast, the age structure was distinctly different between the two populations. The median of age was three years older at Habu than at Isumi, and this difference was mainly due to the difference in longevity and timing of sexual maturity between the two populations. The maximum age of males reached 21 years at Habu, while it was only 13 years at Isumi (Fig. 3). Similar high longevity has already been reported for a few Japanese urodeles: *H. kimurae* (20 years; Misawa and Matsui, 1999) and *Cynops pyrrhogaster* (23 years; Marunouchi et al., 2002). Males older than 10 years accounted for 28% of breeding males at Habu, but only 5% at Isumi (Fig. 4).

Males reached sexual maturity earlier at Isumi than at Habu, and some of the males at Isumi required only two years to start breeding (Fig. 4). Since the minimum SVL of breeding males was nearly 50 mm in both populations (Fig. 3; also see Kusano, 1982), earlier maturity suggests that juvenile growth is faster at Isumi than at Habu. In addition, interpopulational comparison of the relationship between SVL and age of breeding males showed faster growth after sexual maturity at Isumi (Fig. 5). However, because the sample of females was not large enough, it is difficult to draw definite conclusions on interpopulational variations for females and sexual differences solely on the basis of our present data.

ACKNOWLEDGEMENTS

We thank Norio Habu and his family for permission to use the study site at Habu and for their assistance in the field survey. We also thank the members of the Laboratory of

Animal Ecology, Tokyo Metropolitan University, for their assistance in the laboratory and two anonymous reviewers for giving helpful comments to improve the manuscript.

LITERATURE CITED

- AUGERT, D. AND P. JOLY. 1993. Plasticity of age at maturity between two neighboring populations of the common frog (*Rana temporaria* L.). *Can. J. Zool.* 71: 26–33.
- DUELLMAN, W. E. AND L. TRUEB. 1986. *Biology of Amphibians*. McGraw-Hill, New York.
- ENTO, K. AND M. MATSUI. 2002. Estimation of age structure by skeletochronology of a population of *Hynobius nebulosus* in a breeding season (Amphibia, Urodela). *Zool. Sci.* 19: 241–247.
- FORESTER, D. C. AND D. V. LYKENS. 1991. Age structure in a population of red-spotted newts from Allegheny plateau of Maryland. *J. Herpetol.* 25: 373–376.
- GIBBONS, M. M. AND T. K. MCCARTHY. 1983. Age determination of frogs and toads (Amphibia, Anura) from north western Europe. *Zool. Scr.* 12: 145–151.
- HALLIDAY, T. R. AND P. A. VERRELL. 1988. Body size and age in amphibians and reptiles. *J. Herpetol.* 22: 253–265.
- HEMELAAAR, A. S. M. AND J. J. VAN GELDER. 1980. Annual growth rings in phalanges of *Bufo bufo* (Anura, Amphibia) from the Netherlands and their use for age determination. *Neth. J. Zool.* 30: 129–135.
- INOUE, M. 1988. Reproductive ecology of the salamander, *Hynobius tokyoensis*. Unpublished master's thesis. Tokyo University of Agriculture and Technology, Tokyo. (in Japanese)
- KHONSUE, W., M. MATSUI, AND Y. MISAWA. 2001. A comparison of age structures in two Populations of a pond frog *Rana nigromaculata* (Amphibia: Anura). *Zool. Sci.* 18: 597–603.
- KUSANO, T. 1980. Breeding and egg survival of a salamander, *Hynobius nebulosus tokyoensis* Tago. *Res. Popul. Ecol.* 21: 181–196.
- KUSANO, T. 1981. Growth and survival rate of the larvae of *Hynobius nebulosus tokyoensis* Tago (Amphibia, Hynobiidae). *Res. Popul. Ecol.* 23: 360–378.

- KUSANO, T. 1982. Postmetamorphic growth, survival, and age at first reproduction of the salamander, *Hynobius nebulosus tokyoensis* Tago, in relation to a consideration on the optimal timing of first reproduction. Res. Popul. Ecol. 24: 329–344.
- KUSANO, T., K. FUKUYAMA, AND N. MIYASHITA. 1995a. Age determination of the stream frog, *Rana sakuraii*, by skeletochronology. J. Herpetol. 29: 625–628.
- KUSANO, T., K. FUKUYAMA, AND N. MIYASHITA. 1995b. Body size and age determination by skeletochronology of the brown frog, *Rana tagoi tagoi* in Southwestern Kanto. Jpn. J. Herpetol. 16: 29–34.
- KUSANO, T. AND K. MIYASHITA. 1984. Dispersal of the salamander, *Hynobius nebulosus tokyoensis*. J. Herpetol. 18: 349–353.
- MARUNOUCHI, J., T. KUSANO, AND H. UEDA. 2002. Fluctuation in abundance and age structure of a breeding population of the Japanese brown frog, *Rana japonica* Guenther (Amphibia, Anura). Zool. Sci. 19: 343–350.
- MARUNOUCHI, J., H. UEDA, AND O. OCHI. 2000. Variation in age and size among breeding populations at different altitudes in the Japanese newts, *Cynops pyrrhogaster*. Amphibia-Reptilia 21: 381–396.
- MISAWA, Y. AND M. MATSUI. 1999. Age determination by skeletochronology of the Japanese salamander *Hynobius kimurae* (Amphibia, Urodela). Zool. Sci. 16: 845–851.
- R DEVELOPMENT CORE TEAM. 2006. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <http://www.R-project.org>.
- RICE, W. R. 1989. Analysing tables of statistical tests. Evolution 43: 223–225.
- RYSER, J. 1989. Weight loss, reproductive output, and the cost of reproduction in the common frog, *Rana temporaria*. Oecologia 78: 264–268.
- SATO, I. 1943. A Monograph of the Tailed Batrachians of Japan. Nippon Shuppan-sha, Osaka.
- SMIRINA, E. M. 1972. Annual layers in bones of *Rana temporaria*. Zool. Zh. 51: 1529–1534. (in Russian, with English abstract)
- VAN GELDER, J. J. AND G. RIJSDIJK. 1987. Unequal catchability of male *Bufo bufo* within breeding populations. Holarct. Ecol. 10: 90–94.
- VERRELL, P. A. AND H. FRANCILLON. 1986. Body size, age and reproduction in the smooth newt, *Triturus vulgaris*. J. Zool., Lond. 210: 89–100.
- ZAR, J. H. 1996. Biostatistical Analysis, 3rd ed. Prentice-Hall, Upper Saddle River.