広島大学総合博物館研究報告 Bulletin of the Hiroshima University Museum 9: 41–47, December 25, 2017

論文 Article

Age determination by skeletochronology of the Japanese giant salamander *Andrias japonicus* (Amphibia, Urodela)

Hiromi YAMASAKI[1,2], Yuki TAGUCHI[3], Shinji MINAMI[3], Kazushi KUWABARA[4] and Norio SHIMIZU[5]

Abstract: In the past several years, a number of studies have been carried out on the behavioral and reproductive ecology of the Japanese giant salamander *Andrias japonicus*, but the age and longevity of *A. japonicus* has not yet been studied. In this study, we attempted to establish an age determination method using specimens (age: 1 to 11 years old) from Hiroshima City Asa Zoological Park that lived and died in captivity. The cross sections of phalangeal bones were nearly circular in shape, and hematoxylinophilic lines that were interpreted as lines of arrested growth (LAGs) were observed in the periosteal tissue; this suggests that this technique can be used to estimate the age of *A. japonicus*. The number of LAGs was one less than the number of winters that each individual experienced. We could observe LAGs in both frozen and 10% formalin specimens. LAGs could be confirmed even for specimens that had been fixed in formalin for up to 30 years. By using this method, it was suggested that the lifespan of this species could be determined from specimens existing in museums, zoos, and aquariums worldwide. It also showed potential for providing important conservation information, such as generation time and age structure of populations in the field.

Keywords: giant salamander, *Andrias japonicus*, age determination, skeletochronology, amphibians, natural monument

Ⅰ. Introduction

Information concerning age is very important in understanding life history traits of animals and evaluating the status of populations. Therefore, several methods have been developed and used for estimating age of animals. In the past, some researchers thought that age could be estimated from total length, but the fact that 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).

Skeletochronology is an effective tool and has been widely used for over 30 years to evaluate age and growth in amphibians (Castanet and Smirina, 1990; Smirina, 1994). This technique has proven to be an excellent tool for investigating population age structure without sacrificing specimens (e.g., Francillon-Vieillot et al., 1990; Rogers and Harvey, 1994; Kusano et al., 1995). The method is based on the presence of growth layers recorded in cross sections of long bones (Halliday and Verrell, 1988; Castanet and Smirina, 1990). Annual seasonal changes and the physiological response of amphibians cause the formation of bone growth marks, including zones of thicker layers of bone laid down during periods of fast osteogenesis; lines of arrested growth (LAGs) are formed during periods when osteogenesis is slow or inactive (Castanet and Smirina, 1990). A zone followed by a LAG typically corresponds to a one-year cycle of activity in cold or temperate regions and has been confirmed as an annual mark for several amphibians (Smirina, 1994; Misawa and Matsui, 1999; Ento and Matsui, 2002). At present, the validity of this method is confirmed; thus, there is no need to comment further on the validity and advantages of the method described above, particularly in comparison with studies using only morphometric data or body size data (Castanet and Smirina, 1990).

Japanese giant salamander, *Andrias japonicus*, is

---

1 Graduate student, Graduate School of Biosphere Science, Hiroshima University
2 Present Address: Yamaguchi Prefectural Hagi Fisheries Promotion Office
3 Hiroshima City Asa Zoological Park
4 Society of Japanese Giant Salamander
5 Hiroshima University Museum

one of the largest extant amphibians in the world, and it spends its whole life in water, with its eggs fertilized externally (Kobara, 1985). It is endemic to the three main islands of Japan: Honshu, Shikoku, and Kyushu (Matsui and Hayashi, 1992; Matsui, 2000). It is known that the maximum total length reaches 150.5 cm (Kuwabara, 2004; Kawakami et al., 2005), the lifespan is over 60 years, the distribution is extensive from upstream to downstream (Tochimoto et al., 2007), and it is thought to be at the top of the river food chain (Kobara, 1985). The breeding ecology has been elucidated by observation of a natural nest (Kawamichi and Ueda, 1998) and an artificial nest (Kuwabara and Nakagoshi, 2009). Paternal care behaviors have also been studied (Okada et al., 2015), and gradually the whole life history is being elucidated.

A. japonicus became a federally protected species under the "special natural monument" designation in 1952. It has been also listed under Appendix I of the Convention on International Trade of Endangered Species (CITES) since 1975 (Matsui and Hayashi, 1992), and was designated as vulnerable in the Japanese Ministry of Environment's Red Data Book (Ministry of the Environment Government of Japan, 2017). Although collecting this species is prohibited throughout its geographic range (Matsui and Hayashi, 1992), except for a few reserves, most of its habitat remains unprotected (Kobara, 1985). Dams and bank protection walls constructed for flood and erosion control, agriculture, hydraulic power generation, and road construction severely affect a large portion of *A. japonicus*'s riverine habitat (Matsui, 2000; Tochimoto, 2005).

Although *A. japonicus* moves along the river before and after the breeding season (Taguchi 2009), it was confirmed that weirs inhibited its upstream migration, suggesting the possibility of a negative effect on breeding (Taguchi and Natuhara, 2009). In recent years, global amphibian population declines have been reported (Houlahan et al., 2000; Stuart et al., 2004), and habitat loss, fragmentation, and degradation are among the largest threats to amphibian populations (Stuart et al., 2004; Cushman, 2006). In this species, it is also suggested that artificial river environments may have a negative influence on population structure by preventing recruitment of young individuals (Okada et al., 2008). In long-lived hellbenders of the same Cryptobranchidae family, a remarkable concentration of elderly individuals and a significant population decline have been seen (Wheeler et. al., 2003). In recent survey, an unusual percentage of large individuals has been confirmed (Yamasaki et al., 2013), and this species is considered to be in a similarly large-scale decline. Age is one of the most fundamental pieces of information for understanding life history, and it provides important information for conservation, such as total lifespan, age of sexual maturity, and age structure of populations in the field. However, research on age has not been conducted in this species. It is known from a breeding experiment in a tank that the age of this species is difficult to estimate by simply using total length and body weight (Kobara, 1985); hence, it is necessary to establish a reliable age assessment method, in order to obtain information on age.

Thus, this paper is the first attempt to examine the applicability of skeletochronology to *A. japonicus*.

Ⅱ. Materials and Methods

The specimens that we examined were 6 individuals (age: 1 to 11 years old) of *A. japonicus*, that hatched and died in a captive breeding program at Hiroshima City Asa Zoological Park (Table 1). Asa Zoo has succeeded

Table 1 Sample No., total length (TL), body weight (BW), birth year, date of death, age, and preservation method. In Hiroshima City Asa Zoological Park, *Andrias japonicus* hatched in mid to late October, thus the age was calculated based on a birth date of October 15th.

Sample No.	TL(mm)	BW(g)	Birth year	Date of Death date/month/year	Age	Preservation method
1	362	340	2004	5/6/2012	7	Frozen
2	78	2.43	1983	4/9/1985	1	10% formalin
3	185	50	2007	2/8/2013	5	Frozen
4	320	183	2004	22/10/2012	8	Frozen
5	345	460	2004	6/8/2013	8	Frozen
6	550	1180	1990	6/3/2002	11	10% formalin

in captive breeding this species since 1979 (Kobara et al., 1980; Kuwabara et al., 1989) and has accumulated specimens. At the conservation breeding center of the Japanese giant salamander, which is an outside facility, *A. japonicus* hatched in mid to late October, thus the age was calculated based on a birth date of October 15th. Both well and stream water were used for breeding; only well water was used for rearing salamanders, at a temperature of 10.5 to 21.3°C (all individuals experienced low water temperature in the winter). As appropriate, feed was given to all animals; thus, we assumed that animals had not been starved. After death, the specimens were immediately frozen or stored in 10% formalin. The standard skeletochronological procedure was used to determine age (Leclair and Castanet, 1987; Castanet and Smirina, 1990). The bone section used for age determination was from the 2nd or 3rd phalange of the hind limb (i.e., "toe clipping"). Frozen specimens were thawed and fixed with 10% formalin before the experiment.

After removing phalanges from preserved specimens, we washed the bones in the tap water for about one hour, decalcified in 5% nitric acid for between 30 minutes to 5 hours, and then washed them in running water overnight. These decalcified phalanges were subsequently dehydrated through successive ethanol stages. Phalanges were then processed for paraffin embedding in small blocks. Cross-sections (6 μm thick) were obtained by means of rotary microtome (PR-50;Yamato Koki, Saitama, Japan). The sections were mounted onto microscope slides and stained with hematoxylin for 60 minutes in Mayer's hematoxylin at room temperature, and then washed for 20 minutes in tap water. The slides were coverslipped using a toluene-based resin, creating a permanent mount. We chose sections from the central regions of the diaphysis and counted the number of LAGs under a light microscope at 100-400× magnification.

Each sample was handled according to the laws and regulations of the Ministry of the Environment.

Ⅲ. Results

The cross sections of phalangeal bones were nearly circular in shape, and hematoxylinophilic lines interpreted as LAGs were observed in the periosteal tissue (Fig. 1). There was no difference in the number of LAGs in the second and third phalanges (Fig. 1a, b). Although LAGs were not uniformly clear, we could count their number

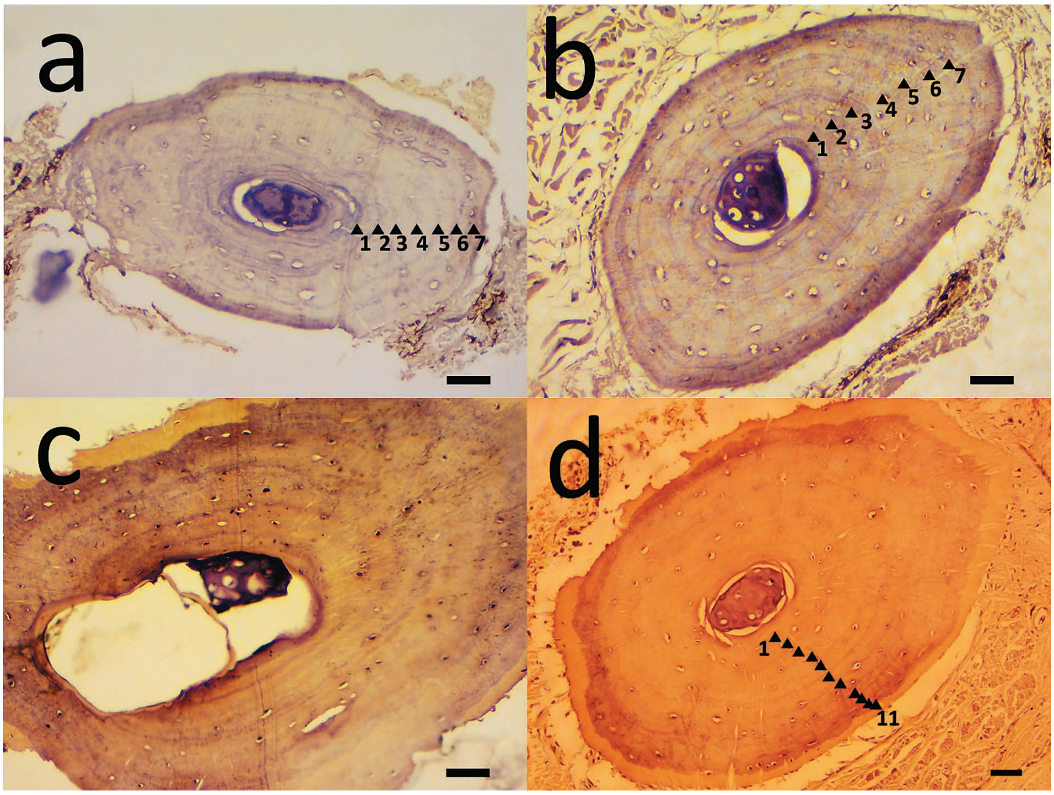

Fig. 1 LAGs observed in the toe bones of *Andrias japonicus*. (a) 2nd bone of Sample No.1. (b) 3rd bone of No. 1. (c) Hollow bone tissue of No. 6. (d) No. 6 (10% formalin specimen). Scale bars = 100 μm.

Table 2 Age, number of winters survived, and number of LAGs.

No.	Age	Number of winters survived	Number of LAGs
1	7	8	7
2	1	2	1
3	5	6	5
4	8	8	7
5	8	9	8
6	11	12	11

by closely examining several sections for each individual. As a result, we were able to count LAGs for all six samples that underwent the assessment. The result clearly shows that the number of LAGs was one less than the number of winters each individual had experienced (Table 2). However, we observed a hollow structure in the periosteal tissue in some phalangeal bones, and it was not possible to accurately count LAGs in such cases (Fig. 1c).

We could observe LAGs in both frozen and 10% formalin specimens (Fig. 1d). LAGs could be confirmed even for specimen No. 2, that had been fixed in formalin for up to 30 years.

Ⅳ. Discussion

Although the technique of skeletochronology has been reported to be relatively difficult to apply for some urodele (Wake and Castanet, 1995) and anuran (Hemelaar, 1988) species, it has been used in many other amphibian species (Francillon-Vieillot et al., 1990; Castanet et al., 1996). This is the first study using skeletochronology to estimate the age of Cryptobranchidae. In this study, we confirmed that age determination by skeletochronology is also applicable for *A. japonicus*.

From our results, it was suggested that LAGs were formed in winter, which is consistent with that observed in *Hynobius kimurae* (Misawa and Matsui, 1999) and *Hynobius nebulosus* (Ento and Matsui, 2002) of the same suborder (Cryptobranchoidea) in Japan. The number of LAGs is one less than the number of winters survived, because of the fact that limbs are generated while absorbing egg yolk, which occurs during the first winter season after hatching, and phalanges are not yet fully formed. From specimens No. 4 and No. 6 it was estimated that the formation time of LAG was from October 22 to March 6; it is considered that the precise formation time of LAGs can be confirmed by examination of samples before and after the winter season.

Also, since the periosteal bone tissue generally shows the history of the growth rate, it is possible to estimate how much it grew at each age, not only in captivity but also in the wild, by observation of LAG. However, in the wild, the external environmental conditions, including food availability, are very different from captive rearing conditions. In addition, compared to the current captive conditions, if the environment differs depending on the facility, it is possible that repeated studies may not obtain the same results as presented here. Therefore, it is desirable to improve our general understanding by increasing the number of samples from different locations, to eventually assess the longevity of *A. japonicus* for each endemic river population and captive facility.

It has been confirmed that bone resorption occurs in some amphibian species (Hemelaar, 1985; Leclair and Castanet, 1987). We also observed a hollow structure (with the possibility that resorption had occurred) in the periosteal tissue in some phalangeal bones in this species. Since some LAGs are not uniformly and clearly visible, it is thus desirable to create consecutive slices; doing so would allow a more accurate estimation of LAGs through comparison with other cross sections. Hemelaar (1985) shows the age assessment method for LAGs in resorbed bone. In larger and longer living individuals, it is considered that resorption may have occurred, and it becomes difficult to count LAG. A method for assessing LAGs in resorbed bone for *A. japonicus* now needs to be established.

Specimens stored either frozen or in formalin were used in this study; the preservation method had few negative effects on the LAGs. LAGs could be identified even in specimens preserved in formalin for up to 30 years. About 20 years ago, follow-up surveillance with Passive Integrated Transponder (PIT) tags first began to improve the accuracy of identification for individuals of this species. It takes a longer time to estimate the life span of long-lived *A. japonicus* by recapture, which can reportedly live more than 60 years. However, if the LAG assessment method is used, it may become possible to drastically shorten the time required to determine the life span.

Nondestructive research is common for this species, a special natural monument. However, by using and improving the LAG assessment method, using accumulated specimens from museums, zoos, and aquariums worldwide, it is expected that a big discovery such as the determination of lifespan will be made. This will strengthen the support that is necessary for future field applications. It will also increase the importance of the role that these facilities (museums, zoos, and aquariums) play in the conservation of creatures and protection of cultural property.

To obtain information concerning age of amphibian populations, age assessment using bone has been an indispensable method in the wild for many years. Information on age in the wild gives a timescale to the life history of an organism, and it provides important information such as total lifespan, age of sexual maturity, and population age structure. Long-lived creatures have slow growth rates and long generation times; this characteristic means that their adaptability to environmental change is weak (Congdon et al., 1993, 1994). In hellbenders, a higher proportion of larger individuals and a remarkable decline in population numbers were observed simultaneously (Wheeler et al., 2003). *A. japonicus* has also been confirmed to possess these vulnerable characteristics in response to environmental change under captive breeding (Kobara, 1985), and the unusual proportion of large individuals has also been confirmed in the field (Yamasaki et al., 2013). It is difficult to prove the decline of long-lived animals, but the evidence that *A. japonicus* is in a crisis situation is becoming evident. In order to evaluate the health of populations and carry out effective conservation, life expectancy in the field, age of maturity, and population age structure are important information.

This paper is the first attempt to examine the applicability of skeletochronology to *A. japonicus*, and it proposes a method to clarify this information which has not been elucidated so far. Information on age may become a tool that will more strongly appeal to the urgency of conservation. However, we suggest that the introduction of this method to the field should be considered carefully, because there are reports that toe removal has a potentially negative effect on migration (Clarke, 1972; Mccarthy and Parris, 2004), although other research did not find this (Ott and Scott, 1999), and there are various arguments about ethical issues (May, 2004; Funk et al., 2005). Regarding the conservation of this species, which is a special natural monument, this method should be seriously considered not only by biologists but from all perspectives.

【Acknowledgements】

We would like to thank Professor Yoichi Sakai and Associate Professor Takeshi Tomiyama from Biology of Aquatic Resources Laboratory of the Graduate School of Biosphere Science, Hiroshima University for allowing us to use the experimental facility. We are also grateful to Tetsuo Takamatsu, Katsunori Sato, and Kenta Tsuchioka for their considerable support to us during the fieldwork. We would like to thank Editage (www.editage.jp) for English language editing. This work supported by JSPS KAKENHI Grant Numbers JP 26870400, JP 17K 01209.

References

Caetano M. H. and Castanet J. (1993): Variability and microevolutionary patterns in *Triturus marmoratus* from Portugal: age, size, longevity and individual growth. *Amphibia-Reptilia*, 14, 117-129.

Castanet J. and Smirina E. M. (1990): Introduction to the skeletochronological method in amphibians and reptiles. *Annales des Sciences Naturelles, Zoologia*, 11, 191-196.

Castanet J., Vieillot H. F. and Bruce R. C. (1996): Age estimation in Desmognathine salamanders assessed by skeletochronology. *Herpetologica,* 52, 160-171.

Clarke R. D. (1972): The effect of toe clipping on survival in Fowler’s Toad (*Bufo woodhousei fowleri*). *Copeia*, 1, 182-185.

Congdon J. D., Dunham A. E. and van Loben Sels R. C. (1993): Delayed sexual maturity and demographics of Blanding’s turtles (*Emydoidea blandingii*): implications for conservation and management of long-lived organisms. *Conservation Biology*, 7, 826-833.

Congdon J. D., Dunham A. E. and van Loben Sels R. C. (1994): Demographics of common snapping turtles (*Chelydra Serpentina*): implications for conservation and management of long-lived organisms. *American Zoologist*, 34, 397-408.

Cushman S. A. (2006): Effect of habitat loss and fragmentation on amphibians: a review and prospectus. *Biological Conservation*, 128, 231-240.

Ento K. and Matsui M. (2002): Estimation of age structure by skeletochronology of a population of *Hynobius nebulosus* in a breeding season (Amphibia, Urodela). *Zoological Science*, 19, 241-247.

Francillon-Vieillot H., Arntzen J. W. and Geraudie J. (1990): Age, growth and longevity of sympatric *Triturus cristatus*, *T. marmoratus* and their hybrids (Amphibia, Urodela): A skeletochronological comparison. *Journal of Herpetology*, 24, 13-22.

Funk W. C., Donnelly M. A. and Lips K. R. (2005): Alternative views of amphibian toe-clipping. *Nature*, 433, 193.

Halliday T. R. and Verrell P. A. (1988): Body size and age in amphibian and reptiles. *Journal of Herpetology*, 22, 255-265.

Hemelaar A. (1985): An improved method to estimate the number of year rings resorbed in phalanges of *Bufo bufo* and its application to populations from different latitudes and altitudes. *Amphibia-Reptilia,* 6, 323-341.

Hemelaar A. (1988): Age, growth, and other characteristics of *Bufo bufo* from different latitudes and altitudes. *Amphibia-Reptilia*, 22, 369-388.

Houlahan J. E., Findlay C. S., Schmidt B. R., Meyer A. H. and Kuzmin S. L. (2000): Quantitative evidence for global amphibian population declines. *Nature*, 404, 752-755.

Kawakami Y., Hirao K. and Okada S. (2005): Note on maximum size of the Japanese giant salamander, *Andrias japonicus* from Tottori Prefecture, Japan. *Bulletin of the Tottori Prefectural Museum*, 42, 1-2. (in Japanese with English summary)

Kawamichi T. and Ueda H. (1998): Spawning at nests of extra-large males in the giant salamander *Andrias japonicus*. *Journal of Herpetology*, 32, 133-136.

Khonsue W., Matsui M. and Misawa Y. (2000): Age determination by skeletochronology of *Rana nigrovittata*, a frog from tropical forest of Thailand. *Zoological Science*, 17, 253-257.

Kobara J., Ashikaga K., Inoue T., Wakabayashi F., Kuwabara K. and Suzuki N. (1980): The study on the protection of Japanese giant salamander *Megalobatrachus japonicus,* in Hiroshima Prefecture. 5. The egg-laying in aquarium. *Journal of Japanese Association of Zoological Gardens and Aquariums*, 22, 67-71. (in Japanese)

Kobara J. (1985): *O-sansyo-uo [The Japanese Giant Salamander]*. Dobutsu-sha, Tokyo. (in Japanese)

Kusano T., Fukuyama K. and Miyashita N. (1995): Body size and age determination by skeletochronology of the brown frog, *Rana tagoi tagoi* in southwestern Kanto. *Japanese Journal of Herpetology*, 16, 29-34.

Kuwabara K., Suzuki N., Wakabayashi F., Ashikaga H., Inoue T. and Kobara J. (1989): Breeding the Japanese giant salamander *Andrias japonicus* at Asa Zoological Park. *International Zoo Yearbook*, 28, 22-31.

Kuwabara K. (2004): The mystery of the largest giant salamander in Japan. Matsui M. and Ikeda A. (ed.): *Hiroshima Prefecture's Wonder Dictionary*, Shinjinbutsuohraisya, Tokyo, Japan, pp. 216-218. (in Japanese)

Kuwabara K. and Nakagoshi N. (2009): Analysis on reproductive behavior of Japanese giant salamander, *Andrias japonicus* − observations on the breeding behavior and notes on the video imagery. *Natural history of Nishi-Chugoku Mountains,* 14, 11-50. (in Japanese with English summary)

Leclair R. Jr. and Castanet J. (1987): A skeletocronological assessment of age and growth in the frog *Rana pipiens schreber* (Amphibia, Anura) from southwestern Quebec. *Copeia*, 2, 361-369.

Matsui M. and Hayashi T. (1992): Genetic uniformity in the Japanese Giant Salamander, *Andrias japonicus*. *Copeia*, 1, 232-235.

Matsui M. (2000): Japanese Giant Salamander. Environment Agency (ed.): *Threatened Wildlife of Japan, Red Data Book. 2nd Edition*, Wildlife Research Center, Tokyo, Japan. pp.101. (in Japanese)

May R. M. (2004): Ethics and amphibians. *Nature*, 431, 403.

Mccarthy M. A. and Parris K. M. (2004): Clarifying the effect of toe clipping on frogs with Bayesian statistics. *Journal of Applied Ecology*, 41, 780-786.

Ministry of the Environment Government of Japan (2017): The 3rd Version of the Japanese Red List on Birds, Reptiles, Amphibians, and Invertebrates. http://www.env.go.jp/nature/kisho/hozen/redlist/MOEredlist 2017.pdf [Accessed 19 August 2017].

Misawa M. and Matsui M. (1999): Age Determination by Skeletochronology of the Japanese Salamander *Hynobius kimurae* (Amphibia, Urodela). *Zoological Science*, 16, 845-851.

Okada S., Utsunomiya T., Okada T., Zachary I. F. and Ito F. (2008): Characteristics of Japanese giant salamander (*Andrias japonicus*) populations in two small tributary streams in Hiroshima Prefecture, Western Honshu, Japan. *Herpetological Conservation and Biology*, 3, 192-202.

Okada S., Fukuda Y. and Takahashi M. (2015): Paternal care behaviors of Japanese giant salamander *Andrias japonicus* in natural populations. *Journal of Ethology*, 33, 1-7.

Ott J.A. and Scott D. E. (1999): Effect of toe-clipping and PIT-tagging on growth and survival in metamorphic *Ambystoma opacum*. *Journal of Herpetology*, 33, 344-348.

Rogers K. L. and Harvey L. (1994): A skeletochronological assessment of fossil and recent *Bufo cognatus* from South-central Colorado. *Journal of Herpetology*, 28, 133-140.

Smirina E. M. (1994): Age determination and longevity in amphibians. *Gerontology*, 40, 133-146.

Stuart S. N., Chanson J. S., Cox N. A., Young B. E., Rodrigues A. S. L., Fischman D. L. and Waller R. D. (2004): Status and trends of amphibian declines and extinctions worldwide. *Science*, 306, 1783-1786.

Taguchi Y. (2009): Seasonal movement of the Japanese giant salamander (*Andrias japonicus*) −Evidence for possible breeding migration by this stream-dwelling amphibian. *Japanese Journal of Ecology*, 59, 117-128. (in Japanese with English summary)

Taguchi Y. and Natuhara Y. (2009): Requirements for small agriculture dam to allow the Japanese giant salamander (*Andrias japonicus*) to move upstream. *Japanese Journal of Conservation Ecology*, 14, 165-172. (in Japanese with English summary)

Tochimoto T. (2005): Conservation of Japanese Giant Salamander, Matsui M. (ed.): *Directions in Batrachology*, Shokabo, Tokyo, Japan, pp.204-215. (in Japanese)

Tochimoto T., Taguchi Y., Onuma H., Kawakami N., Shimizu K., Doi T., Kakinoki S., Natuhara Y. and Mitsuhashi H. (2007): Distribution of Japanese giant salamander in Hyogo Prefecture, Western Japan. *Humans and Nature*, 18, 51-65. (in Japanese with English summary)

Wake D. B. and Castanet J. (1995): A skeletochronological study of growth and age in relation to adult size in *Batrachoseps attenuatus*. *Journal of Herpetology*, 29, 60-65.

Wheeler B. A., E. Prosen, A. Mathis and R.F. Wilkinson. (2003): Population declines of a long-lived salamander: a 20+-year study of hellbenders, *Cryptobranchus alleganiensis*. *Biological Conservation*, 109, 151-156.

Yamasaki H., Shimizu N., Tsuchioka K., Ueda S., Takamatsu T., Sato K. and Kuwabara K. (2013): Practical study for conservation of giant salamander *Andrias japonicus* in Toyosaka, Higashi-Hiroshima, Japan. *Bulletin of the Hiroshima University Museum*, 5, 29-38. (in Japanese with English summary)

(Received: 31 August 2017)
(Accepted: 6 December 2017)