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Source: Current Herpetology, 37(1) : 58-68

Published By: The Herpetological Society of Japan

URL: https://doi.org/10.5358/hsj.37.58

Current Herpetology 37(1): 58–68, February 2018
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doi 10.5358/hsj.37.58

Body Size and Age Structure in Two Populations of Tokyo Daruma Pond Frog, *Pelophylax porosus porosus*

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Abstract: Body size and age of two populations of the endangered species *Pelophylax porosus porosus* from the Kanto Plain, Japan (paddy fields in valley bottoms [Zushi] and open plains [Hayamajima]) were investigated. Age was estimated by skeletochronology of the phalanges. Frogs grew rapidly between 0 and 1 year of age in both populations. The asymptotic snout-vent length (SVL) of both sexes peaked at 2 years. Age at sexual maturity was estimated to be 0–1 years for males, but 1–2 years for females. SVL in adults ranged from 45.2 to 67.2 mm in males, and 60.2 to 88.8 mm in females. In both populations, females were significantly larger than males. However, the SVL was not significantly different between the populations or between age classes in both sexes. Lines of arrested growth (LAGs) were observed in the periosteal tissue. Longevity was estimated to be 3 and 4 years in males and females, respectively. *Pelophylax p. porosus* is suggested to be the most short-lived and the first to reach sexual maturity compared with the other *Pelophylax* species in Japan, although the age structure differed between the populations. In both sexes, when comparing the age of 1 year old or older frogs, the Zushi population was significantly older than the Hayamajima population, but the longevity of the Hayamajima population was shorter than that of the Zushi population. As the two study sites differed in agricultural management, it is possible that the resulting environmental conditions influenced the longevity of *P. p. porosus*.

Key words: Age; Endangered species; Lines of arrested growth; *Pelophylax porosus porosus*; Skeletochronology; Snout-vent length

INTRODUCTION

The Japanese Pond Frog, or Tokyo Daruma Pond Frog, *Pelophylax porosus porosus*, is

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an endemic subspecies distributed in eastern Honshu Island (Sendai Plain, Kanto Plain, central and southern Niigata Prefecture, and central and northern Nagano Prefecture) and part of Hokkaido Island, where it was artificially introduced (Maeda and Matsui, 1999; Saito and Hachiya, 2002). This species inhabits rice field environments (Maeda and Matsui, 1999) that have been maintained through the artificial disturbance caused by paddy cultivation. Therefore, changes to paddy field environments, including the modernization of cultivation technology, have caused declines in the frog's population. Thus, *P. p. porosus* is a suitable model species for clarifying how changes in rice field environments are linked to declines in frog numbers.

To protect and conserve a species effectively, it is important to understand its ecological characteristics, such as the life-history traits of populations. However, our knowledge of the ecology of *P. p. porosus* remains limited. Previous studies focused on the habitat use of this species (Osawa et al., 2003; Osawa and Katsuno, 2003), and the environment that it inhabits (Azuma and Takeuchi, 1999; Sato and Azuma, 2004). Other studies have focused on the biological characteristics of this species, including its breeding ecology (Shimoyama, 1989), reproductive traits (Serizawa and Serizawa, 1990), size distribution and growth (Togane et al., 2005), and size and age at sexual maturity of females (Togane et al., 2009). Matsui (2005) suggested that we could presume the age distribution, growth rate, and longevity of the populations of this species if we could estimate the age of individuals.

Methods used to determine the age of amphibians include mark-recapture of known individuals, extrapolation of body size frequency data, skeletochronology, and estimates from testes lobules (Kusano et al., 1995). In particular, many studies have reported the effectiveness of skeletochronology (e.g., Hemelaar and van Gelder, 1980; Gibbons and McCarthy, 1984; Hemelaar, 1985; Acker et al., 1986; Castanet et al., 1996). Some studies in Japan have previously used skeletochronology to determine the age of frogs (e.g., Kusano et al., 1995; Khonsue et al., 2000; Marunouchi et al., 2002). For example, Khonsue et al. (2002) used a section of the phalanx to report the age structure of a population of the other subspecies, *P. p. brevipodus*, which has been assigned an endangered status. By using phalanx for skeletochronology, damage to individuals in the population is avoided, which is particularly important when assessing endangered species.

In Kanto District (Honshu Island, Japan) the distribution and population density of *P. p. porosus* is rapidly decreasing. This species is classified as near threatened by the Red Data Book of Japan (Fukuyama, 2014), and as a vulnerable (VU) species in the Red Data Book of Kanagawa Prefecture (Arai, 2006). In the Red Data Book of Tokyo Metropolis, this species is ranked as critical (CR) in 23 wards of the capital city, and as endangered (EN) or VU in the other cities and district (Fukuyama, 2013). This study reports the body sizes and age structures of two populations of *P. p. porosus* inhabiting paddy fields in valley bottoms and open plains in Kanto District.

MATERIALS AND METHODS

Study site

This study was carried out at two locations in Kanto District, Honshu Island, Japan, and included paddy fields: (1) in valley bottoms and (2) in open plains. Paddy fields situated in valley bottoms were located in Zushi-Onoji Historic Environmental Conservation Area, Zushi, Machida City, Tokyo. Open plains paddy fields were located in Hayamajima, Shiroyama Town, Sagamihara City, Kanagawa Prefecture (Shiroyama Town, Tsukui District in the same prefecture was merged into Sagamihara City in March 2007).

The "Act on Natural Conservation and Restoration in Tokyo" was enacted in Zushi (35°35'N, 139°25'E, altitude 65 m above sea level [a.s.l.]) by the Tokyo Metropolitan

Government. At this site, paddy cultivation is performed in an ill-drained paddy field, and farm ponds are present, as well as an artificially constructed earth canal. Many paddy fields, fallow fields, and earth canals are present in this area, where water remains even during periods of no cultivation. *Pelophylax p. porosus* is present in this area from mid-April. Hayamajima (35°30'N, 139°19'E, 56 m a.s.l.) is located in the Sagami River basin, and is a well-drained paddy field during the winter season. This area is farmed using large-sized machines. The irrigation canal is separated from drainage canals, and water is typically pumped into paddy fields via underground pipes, and is drained from fields to underground again. *Pelophylax p. porosus* is present in this area from early May, with large numbers being detected in late-May, when the paddy fields are covered by water.

Sample collection and age determination

We investigated the frog populations at Zushi from 2004 to 2006, and at Hayamajima in 2006. Froglets that had recently metamorphosed were collected at each site in July to August. Frogs were collected between September and November, when annual growth was almost over. Age was determined by counting the lines of arrested growth (LAGs), because diapause occurs only once a year in *P. p. porosus*. At both locations, frogs were captured by hand or using dip nets. Snout-vent length (SVL) was measured to the nearest 0.1 mm with a slide clipper. Body mass (BM) was measured to the nearest 0.1 g with a digital scale. Sex and stage of maturation of individuals were also recorded. We distinguished mature males based on the presence of secondary sexual characteristics, such as vocal sacs and nuptial pads. However, it is difficult to distinguish immature males from mature females based on morphological characteristics. At Zushi, the SVL of the smallest mature female was previously recorded as 58.3 mm (Togane et al., 2009). Thus, in this study, females were defined as individuals with an SVL of 60 mm or larger that did not exhibit the secondary sexual characteristics of males. After measuring and sexing individuals, the third toe of the left or right hind leg was clipped off and preserved in 10% formalin, to determine age by skeletochronology. Froglets that had recently metamorphosed were measured at each site in July to August. All examined frogs were immediately released at the point of capture.

Skeletochronology

Age was determined by investigating LAGs, which remain on the bone during diapause (Halliday and Verrell, 1988). The method used to determine the age of each frog followed that used by Kusano et al. (1995), with some improvements. The amphibian bone may be resorbed by the inside. Hemelaar (1985) reported that bone resorption is less likely in the phalanx of the fore limbs compared to that of the hind limbs. After confirming that the rings skeletochronology of phalanges from both the fore and hind limbs from the same mature frog produced the same results, we consistently used the hind limb for all subsequent frogs. In brief, the phalanges were washed in running water for 24 h. Subsequently, they were decalcified in 5% nitric acid for 60–180 min, or in 5% formic acid for 60–180 min, or in K-CX for 24 h at room temperature or low air temperature (4–10 C). After decalcification, the bones were washed again in running water for 24 h, and dehydrated in sucrose liquid. Decalcified bones were embedded in O.C.T. compounds, and were cross-sectioned (15–20 μm thick) with a freezing microtome (MICROM HM500). Cryostat sections were dried naturally for 24 h, dipped into phosphate-buffered saline for 20 min, and stained with hematoxylin (Mayer's acid hemalum) for 10 min. We examined the sections under a light microscope (OLYMPUS BX51; magnification: 200–600X), and counted the number of LAGs present on the periosteal bone. We photographed the stained sections using an Axio CammRc5. Age was defined as the number of rings present on a stained section.

We defined just metamorphosed juveniles, which appeared after the proximate breeding season, as 0 years. The LAG numbers were counted independently by at least two collaborators, to improve the precision of age determination.

Analysis

The data accumulated to determine the SVL and age of frogs between September and November were separated with respect to sex at the two locations for analysis. Metamorphic size was analyzed between the two locations, regardless of the date of metamorphosis. At Zushi, many mature frogs were observed in June, while newly metamorphosed froglets appear from July (Togane et al., 2005). Thus, we set the starting point arbitrarily as June 1, to specify the age of each individual. We used a logistic model to estimate the growth pattern. We set the newly metamorphosed body size to an initial value when we estimated the growth curve. We used juveniles' data collected from September to November for both males and females. We estimated the model parameters by applying non-linear regression using nls() functions in R version 3.2.3 (Crawley, 2007; R Core Team, 2015). The following logistic model was used: $L=a/(1+e^{c-bt})$, where L is the SVL (mm) at time t (yr); 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; Khonsue et al., 2001; Khonsue et al., 2002). Estimates of asymptotic size and instantaneous growth rate were given with the corresponding 95% confidence interval. Differences in SVL and age between samples were tested for statistical significance using the Mann-Whitney U-test and Welch's t-test. The significance level was set at 0.05 (two-sided). Growth parameters were statistically compared based on a 95% confidence interval.

RESULTS

Body size

We analyzed a total of 569 frogs in the two populations. The frequency distribution for body size of all individuals captured and measured at the two study sites is shown in Fig. 1. A total of 365 frogs were captured at Zushi. The mean SVL for males, females, and juveniles was 57.5 mm (n=107, SD=5.51 mm), 67.6 mm (n=77, SD=6.14), and 47.4 mm (n=181, SD=7.46), respectively. Males (SVL 45.2–69.3 mm) were significantly smaller than females (SVL 60.2–88.8 mm) (Mann-Whitney U-test: Z=9.13, P<0.01).

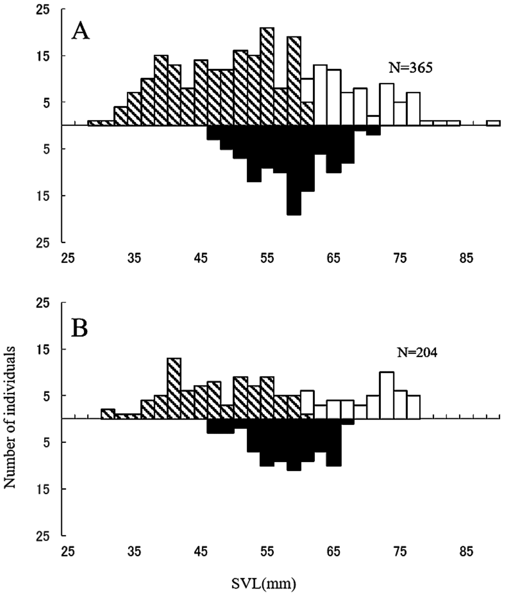

FIG. 1. Frequency distribution of SVL for *Pelophylax porosus porosus* at Zushi (A) and Hayamajima (B). Solid squares, open squares, and hatched squares indicate adult males, adult females, and juveniles, respectively.

A total of 204 frogs were captured at Hayamajima. The mean SVL of males, females, and juveniles was 57.2 mm (n=72, SD=4.90), 65.9 mm (n=46, SD=5.00), and 46.3 mm (n=86, SD=7.38). Males (SVL 46.2–65.4 mm) were significantly smaller than females (SVL 60.6–75.8 mm) (Mann-Whitney U-test: Z=8.23, P<0.01). The SVL was not significantly different between the Zushi and Hayamajima populations (Mann-Whitney U-test: males, Z=0.34, P=0.73; females, Z=1.43, P=0.15;

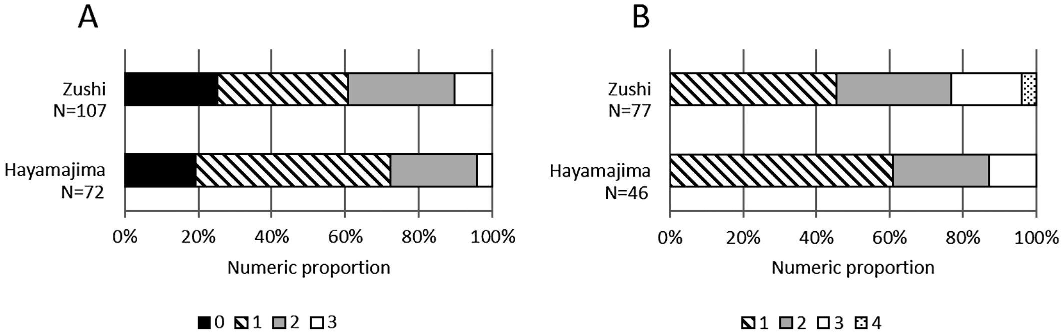

FIG. 2. Age composition of adult males (A) and adult females (B) of *Pelophylax porosus porosus* in the Zushi and Hayamajima populations.

juveniles, Z=0.74, P=0.46). Sexual size dimorphism was present in both populations, with females being larger than males.

Age composition

Out of 635 samples that were processed, 569 samples showed recognizable bone structures. Destruction and resorption of LAGs occurred in approximately 10% of samples. The age composition of mature frogs in the Zushi and Hayamajima populations is shown in Fig. 2. Males of 0 to 3 years old were present at both sites. Males of 4 years old or older were not found at either study site. In comparison, females of 1 to 4 years old were present at Zushi, while females of 1 to 3 years old were present at Hayamajima. Mature females aged 0 years were not present at either site. Overall, 60.7% of mature males and 45.5% of mature females belonged to the 0–1 year age class at Zushi. In comparison, 72.2% of mature males and 60.9% of mature females belonged to the 0–1 year age class at Hayamajima. Thus, mature males were relatively younger than mature females in both populations.

Younger individuals (0 and 1 year age class) of mature males account for 72.2% of individuals at Hayamajima (Fig. 2) and 60.7% at Zushi (Fig. 2). Older males (3 years age class) accounted for 4.2% and 10.3% of all individuals at Hayamajima and Zushi, respectively. When comparing the age of 1 year males or older males, the Zushi population was significantly older than the Hayamajima population (Welch's t-test: df=133.41, t=2.40, $P<0.05$).

More of the younger mature females (1 year age class) were present in the Hayamajima population compared to the Zushi population. More 2 and 3 year old females were present in the Zushi population compared to the Hayamajima population. Only Zushi contained females that were 4 years old. Consequently, the Zushi population was significantly older than the Hayamajima population with respect to females (Welch's t-test: df=109.64, t=2.02, $P<0.05$).

Overall, the Hayamajima population contained relatively younger mature frogs than the Zushi population.

Body size and age structure

The SVLs in each age class of the Zushi and Hayamajima populations are shown in Table 1. No significant difference was detected in SVL for males of the same age class in each population (Mann-Whitney U-test: 0 year, Z=0.28, P=0.78; 1 year, Z=0.33, P=0.74; 2 years, Z=0.81, P=0.42; 3 years, Z=0.86, P=0.39). The SVL of 1 year old females from the Zushi population was significantly smaller than those from the Hayamajima population (Mann-Whitney U-test: Z=2.12, $P<0.05$). In contrast, no significant difference in SVL was detected for 2 and 3 year old females in the two populations (Mann-Whitney U-test: 2 years, Z=1.90, P=0.06; 3 years, Z=0.12,

TABLE 1. Mean±SD of snout-vent-length (SVL in mm) of the *P. p. porosus* in each age class of Zushi and Hayamajima populations. Range are shown in parenthesis.

Population	Sex	Age (yr)	N	SVL (mm)
Zushi	Male	0	27	50.6±2.50 (45.2–54.8)
		1	38	57.4±3.61 (48.3–65.3)
		2	31	61.1±3.40 (55.9–69.3)
		3	11	64.2±2.12 (60.2–67.2)
		4	0	
	Female	0	0	
		1	35	64.7±4.85 (60.2–79.2)
		2	24	67.1±4.28 (61.3–76.4)
		3	15	72.4±5.17 (60.7–82.7)
		4	3	80.1±7.62 (74.6–88.8)
	Juvenile	0	121	43.5±7.01 (27.6–58.2)
		1	58	54.2±3.67 (46.6–59.9)
		2	2	58.9±1.27 (58.0–59.8)
Hayamajima	Male	0	14	50.8±3.80 (46.2–57.9)
		1	38	57.1±3.12 (50.9–63.3)
		2	17	61.3±3.32 (54.4–65.4)
		3	3	63.4±0.70 (62.7–64.1)
		4	0	
	Female	0	0	
		1	28	67.5±5.26 (60.6–75.7)
		2	12	70.0±4.11 (62.6–75.6)
		3	6	72.2±3.33 (67.4–75.8)
		4	0	
	Juvenile	0	80	45.5±6.96 (30.4–58.9)
		1	6	57.3±2.12 (54.8–59.6)

$P=0.91$). Juveniles were significantly larger in the Hayamajima population compared with the Zushi population for both age classes (Mann-Whitney U-test: 0 year, $Z=2.60$, $P<0.05$; 1 year, $Z=2.22$, $P<0.05$). However, no significant difference in SVL was detected at the metamorphosis stage between the two populations (Zushi: 21.6±2.3 mm, Hayamajima: 21.5±2.5 mm, Mann-Whitney U-test: $Z=0.04$, $P=0.97$). Thus, the SVL of mature frogs was not significantly different between the two populations, with the exception of 1 year old females. The SVL of juveniles (0 and 1 year old) was significantly different in the two populations, whereas no difference in SVL at metamorphosis was found.

Growth patterns

Growth parameters were calculated from a logistic model, to produce a growth curve (Fig. 3). Frogs grew rapidly from 0 year to 1 year in all populations. The asymptotic SVL of males and females peaked at 2 years. The asymptotic SVL (mean±SE) of males and females was 63.00±2.19 mm and 72.17±2.21 mm, respectively, in the Zushi population, and 62.07±3.12 mm and 71.00±2.97 mm in the Hayamajima population, respectively. There

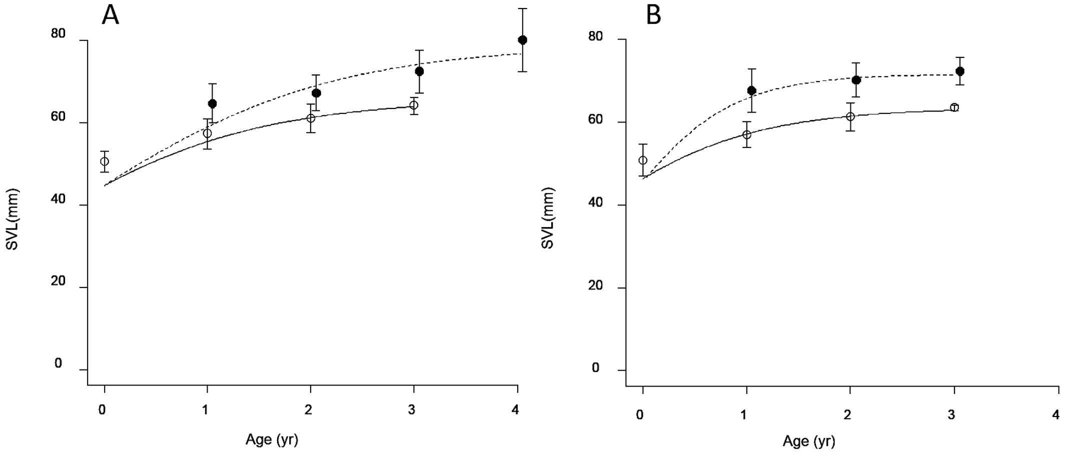

FIG. 3. Growth pattern of *Pelophylax porosus porosus* in the Zushi (A) and Hayamajima populations (B). Closed circles and open circles indicate adult males and adult females, respectively. Solid and dashed lines are adult males and adult females, respectively. Error bars indicate standard deviation.

was a significant difference in the asymptotic size of males and females in the Zushi population (Males, $CI_{95\%}$: 58.63–67.38; Females, $CI_{95\%}$: 67.75–76.60; $P<0.05$), with females having a larger asymptotic size than males. In comparison, the asymptotic size of males and females was similar in the Hayamajima population. For the same sex, asymptotic size was similar in both populations. The instantaneous growth rate (mean±SE) of males and females was 1.85±0.30 mm and 1.63±0.16 mm, respectively, in Zushi, and 2.42±0.70 mm and 2.81±0.65 mm, respectively, in Hayamajima. Although the instantaneous growth rates of the two sexes were lower in Zushi compared to Hayamajima, there was no statistical difference between the two populations.

DISCUSSION

Skeletochronology

LAGs are formed when bone growth decreases during diapause. In the Honshu area of Japan, amphibians form one LAG per year in relation to hibernation (e.g., Kusano et al., 1995; Misawa and Matsui, 1999). Khonsue et al. (2002) validated the use of skeletochronological data in *P. p. brevipodus* by using skeletochronology in combination with capture-mark-recapture. They reported one LAG increase after one hibernation in the recaptured frogs, although the possibility of resorption of lines cannot be completely rejected. In other studies (e.g., Liao, 2011; Liu et al., 2012), an endosteal resorption of LAG was rarely observed in all individuals. In this study, resorption of LAGs and partially destroyed LAGs were observed in a part of samples (approximately 10%), although we did not use these unclear samples in our analyses. Generally, the process of resorption occurs in the innermost LAGs (e.g., Hemelaar and van Gelder, 1980), and the resorption will occur more frequently in older frogs than younger ones. So, exclusion of samples with resorption could affect the estimation of age structure, and it is possible that the age structure shown in this study is underestimated even slightly. The phalanx is used for the skeletochronology of many ranid species (Leclair and Castanet, 1987; Patón et al., 1991; Kusano et al., 1995; Sagor et al., 1998; Marunouchi et al., 2000; Khonsue et al., 2000, 2001, 2002). This current study also indicates that differences in the decalcification rate of different media did not affect the decalcification of each section. In particular, we adjusted the decalcification time based on the ability to detect LAGs clearly. Our results support the idea that the number of LAGs in *P. p. porosus* reflects the true age of individuals. Therefore, skeletochronology represents a suitable method to elucidate the age of individuals belonging to this subspecies.

Sexual size dimorphism and age at maturity

Sexual dimorphism was evident in our study, with *P. p. porosus* females being larger than males. This characteristic was also detected in the subspecies *P. p. brevipodus* (Khonsue et al., 2002) and many other anuran species (Monnet and Cherry, 2002). Previous studies suggest that sexual size dimorphism results from differences in age at sexual maturity between males and females, as well as differences in the rate of growth before sexual maturity (Monnet and Cherry, 2002). Males of *P. p. porosus* exhibited secondary sexual characteristics in the fall of the same year as metamorphosis (Serizawa and Serizawa, 1990; Maeda and Matsui, 1999). Our results supported these observations. Specifically, we estimated that the age at sexual maturity was as 0–1 years for male *P. p. porosus*. We also found that the age of sexual maturity was 1–2 years for females. Serizawa and Serizawa (1990) reported that female *P. p. porosus* became sexually mature when their SVLs exceeded 56 mm in the September after the first overwintering period. Consequently, females reach sexual maturity one year later than males. Therefore, the difference in age at sexual maturity between the sexes likely causes the detected sexual size dimorphism in this species.

Comparison between the two populations

We found that the two populations had different age structures. Even though there was no difference in the age at sexual maturity between the two populations, the Hayamajima population contained mature frogs that were relatively younger than the Zushi population. Thus, the longevity of the Hayamajima population seemed to be shorter than that of the Zushi population. However, no significant difference in the SVL of mature frogs was detected in the two populations. Previous studies suggested that differences in body size and age structure between populations often occur due to differences in altitude or latitude (e.g., Sagor et al., 1998; Hsu et al., 2014). For example, age at sexual maturity, longevity, and body size tend to be greater at higher altitudes compared to lower altitudes in many amphibians (Morrison and Hero, 2003). However, there was only a 9 m difference in altitude between our two study sites (Zushi and Hayamajima have altitudes of 65 m and 56 m a.s.l., respectively).

Our results might also be explained by differences in environmental conditions between the two study sites. For instance, Augert and Joly (1993) suggested that age structure, size at maturity, and body size varied between two neighboring populations of *Rana temporaria* due to differences in local environmental conditions. In our study, the two *P. p. porosus* populations were collected from paddy fields subject to different types of agricultural management. The paddy fields at Zushi are located in valley bottoms and are managed traditionally (Tagoku, 2010). In comparison, the paddy fields at Hayamajima are situated along a river basin, are surrounded by concrete irrigation canals, and are cultivated by modern practices. Therefore, the environmental conditions, such as water availability and food abundance, created by the different agricultural management practices in our two study sites might influence the longevity of *P. p. porosus*. Moreover, population density is an important factor affecting age structure and longevity of amphibian populations. More detailed studies are needed that collect quantitative data and investigate the population density of both study sites. This will be the subject of further study.

Comparison with other Pelophylax species

Two *Pelophylax* species have been documented in Japan. In addition, *P. porosus* include nominotypical subspecies *P. p. porosus*, and another subspecies *P. p. brevipoda.*

Because all *Pelophylax* species are threatened and listed in the Red Data Book of Japan (Fukuyama, 2014; Matsui, 2014a, b), comparison of their life history traits could help improve our understanding of the population dynamics and conservation requirements of this group of species. In our study, the age composition of *P. p. porosus* was 0–3 years for males and 0–3 and 0–4 years in females (depending on population). Previous studies reported the age composition of *P. p. brevipodus* and *P. nigromaculatus* as 0–4 years and 0–6 years in both sexes, respectively (Khonsue et al., 2001; Khonsue et al., 2002). The age composition of *P. p. porosus* is similar to that of *P. p. brevipodus*, whereas the mean age of mature *P. p. porosus* was older than that of *P. p. brevipodus*. On the other hand, the longevity of *P. p. porosus* was shorter than that of *P. nigromaculatus*. In comparison, Khonsue et al. (2002) reported that sexual maturity was attained by *P. p. brevipodus* in the fall of the year of metamorphosis for males and in the breeding season of the next year for females. This pattern was also recorded for *P. p. porosus* in the current study. Thus, *P. porosus* has shorter longevity after reaching maturity than *P. nigromaculatus*.

The instantaneous growth rate of *P. p. porosus* estimated by our study was higher than that estimated for both sexes of *P. nigromaculatus* (Khonsue et al., 2001) and *P. p. brevipodus* (Khonsue et al., 2002). In general, competition between syntopic species that occupy similar niches tends to influence certain ecological traits, including survival, growth, and fecundity (Berven, 1990). Khonsue et al. (2001) recorded higher growth rates in a *P. nigromaculatus* population without syntopic *P. p. brevipodus* than in a population with *P. p. brevipodus*. In our two study sites, no anuran species occupied similar niches to *P. p. porosus*. This absence of competitors might allow *P. p. porosus* to exhibit higher growth rates at our study sites. However, to confirm this hypothesis, comparative studies of populations inhabiting other areas are required.

In conclusion, our study demonstrates the utility of skeletochronology to determine the age structure and size at maturity of *P. p. porosus*. As a result, we were able to distinguish important differences between two populations inhabiting different environmental conditions and exposed to different intensities of anthropogenic activity. Based on our results, skeletochronology could be used to determine how different parameters drive population trends in different locations, which could ultimately help to improve the conservation and management of this species in Japan.

ACKNOWLEDGMENTS

We thank Koichi Tagoku (Director) and other members of the Machida Historic Environment Conservation Management Organization, and members of the Tama Environment Office attached to the Bureau of Environment for providing assistance with fieldwork in the conservation area. We also thank Tamotsu Kusano for providing statistical advice.

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Accepted: 15 January 2018