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Age and Body Size of the Shangcheng Stout Salamander *Pachyhynobius shangchengensis* (Caudata: Hynobiidae) from Southeastern China

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Abstract Age and body size are critical for understanding life history evolution and ecology. In this study, the age and body size of the Shangcheng stout salamander, *Pachyhynobius shangchengensis*, from a population in Anhui Province, China, were studied by skeletochronology. The mean age was 8.8 ± 0.2 (mean ± SD) years in females and 9.6 ± 0.2 in males and ranged 5–13 years for both sexes. The mean age was significantly different between sexes. The mean body size and mass were (100.21 ± 0.91) mm and (31.76 ± 0.73) g in females, and (105.31 ± 1.23) mm and (37.14 ± 1.12) g in males, respectively. Males were significantly larger and heavier than females, indicating sexual size dimorphism. There was a significant positive correlation among body size, body mass, and age, suggesting that the oldest individuals are larger and heavier. The growth rate in males was significantly higher than in females. The present study provides preliminary data on life-history traits which can be helpful for future studies of this species and other hynobiid salamanders.

Keywords age structure, growth rate, life history, skeletochronology

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1. Introduction

Age and body size are two of the most important attributes of an animal's life history (Kusano *et al*., 2010), including for amphibians (Duellman and Trueb, 1994; Misawa and Matsui, 1999; Roff, 2002; McCreary *et al*., 2008). Skeletochronology is considered a reliable method for estimating the age of amphibians, based on counting lines of arrested growth (LAGs) in phalangeal bones (Castanet and Smirina, 1990; Castanet *et al*., 1993). For example, this method has been successfully used in urodeles (e.g., Caetano and Castanet, 1993; Caetano and Leclair, 1996; Bovero *et al*., 2006; Eden *et al*., 2007; Üzüm, 2009; Farasat and Sharifi, 2016; Altunişik, 2018). Compared with the other methods (body size correlation and mark-recapture) of estimating age in amphibians, skeletochronology has the advantages of being time-saving and individuals do not have to be sacrificed.

The family Hynobiidae is the third largest group within the amphibian order Caudata, and species are widely distributed, including from Kamchatka through Siberia to European Russia, Turkmenistan, Afghanistan, and Iran, as well as eastward to Korea, Japan, and China (Frost, 2020). Age of hynobiid salamanders has been the focus of the attention of biologists (e.g., Ento and Matsui, 2002; Kusano *et al*., 2006; Lee and Park, 2008; Matsuki and Matsui, 2009, 2011; Lee *et al*., 2010; Hasumi, 2010; Yamamoto *et al*., 2011; Hasumi and Borkin, 2012; Zivari and Kami, 2017), and these studies have mainly focused on the species in Japan, South Korea, Mongolia, and Iran. China has the highest diversity in the family Hynobiidae (Frost, 2020), but there is little biological information on the species in this area, including a lack of skeletochronology approaches.

The Shangcheng snout salamander, *Pachyhynobius shangchengensis* Fei, Qu and Wu, 1983 (Urodela: Hynobiidae), is an endemic salamander, which is only distributed in the Dabie Mountains, Southeastern China (Fei *et al.*, 2006). This salamander inhabits small hill streams with slow to moderate flow, feeds on small aquatic invertebrates (Chen, 1992), and has relatively low vagility. Xiong *et al.* (2019) examined sexual dimorphism, but there is no age information for this species. In this study, the age and body size of this salamander from the Yaoluoping population of Dabie Mountain, Yuexi County, Anhui Province, China, were studied. The main aims were to: (1) estimate age and body size of both sexes; (2) compare age, body size, and growth rate between sexes; and (3) describe the relationship between age and body size.

2. Material and methods

2.1 Sampling A total of 190 adult specimens (104 males and 86 females) were caught by hand at night from the Yaoluoping population of Dabie Mountain, Yuexi County, Anhui Province, China (30°58′N, 116°04′E; 1,135 m above sea level), June, 2015. As the breeding season may be in April and May (Pasmans *et al.*, 2012), these specimens may be individuals post-breeding or in the non-breeding season. Upon arrival to the laboratory, animals were euthanized via submergence in a buffered MS-222 solution and then stored in 10% formalin. Voucher specimens were deposited in the Museum of Anhui University. Specimens were sexed by inspection of the gonads through a small ventro-lateral incision, and sexual maturity was determined according to the development of gonads.

2.2 Data collection Body size (snout-vent length) was measured to the nearest 0.1 mm using a digital caliper. Size dimorphism was calculated using the size dimorphism index (SDI) of Lovich and Gibbons (1992), in which SDI = (size of larger sex/size of the smaller sex) ± 1. The value + 1 is used if males are larger than females and – 1 if the opposite is true. The result is arbitrarily defined as positive if the females are larger and negative in the converse situation. Body-weight (g) was recorded to the nearest 0.1 g using an electronic balance. For each salamander, the second phalange of the longest finger of the left hindlimb was clipped and preserved in 10% formalin solution for subsequent histological analyses.

The histological analysis followed standard skeletochronological methods (Castanet and Smirina, 1990), with slight modifications as follows: phalanges were washed with tap water for 24 h; decalcified in Plank decalcifying solution for 14 h; dehydrated in 70%, 80%, 85%, 90%, 95%, and 100% alcohol for 2 h, 2 h, 1 h, 1 h, 1 h, and 20 min, respectively; treated using n-butyl alcohol; and then embedded in paraffin. Cross-sections (10 μm) of the diaphyseal part of each phalanx were obtained from embedded phalanges using a Leica RM 2135 type microtome (Leica Microsystems, Wetzlar, Germany), and then were stained with Haematoxylin-Eosin (HE) (10 min in Haematoxylin solution and 5 min in Eosin solution). Age was determined by counting the number of lines of arrested growth (LAG) in the periosteal bone of the phalanges under an OLYMPUS CX31 light microscope, and photographs were taken with an OLYMPUS DP26 digital camera connected to the microscope. LAGs in bone sections were independently counted by two observers blindly. Whenever there was a discrepancy, sections were reanalyzed until consensus was reached.

Endosteal resorption may affect the accuracy of LAG counting (Hemelaar and Van Gelder, 1980), and can be confirmed by identifying the Kastschenko Line (KL; the interface between the endosteal and periosteal zones; Rozenblut and Ogielska, 2005). Complete resorption of the innermost LAG was also confirmed based on the difference in diameter between LAG and KL (Liao and Lu, 2010). The double lines in the cross-sections were not incorporated into age estimation.

2.3 Data analysis The Kolmogorov-Smirnov test was used to compare age structure between the sexes, and the Mann-Whitney U test was used to compare average age (because of non-normality). Student's t-tests were utilized to compare body size and body mass between sexes. Pearson's correlation coefficient was used to examine relationships among body size, body mass, and age. Univariate analysis of covariance (ANCOVA) was conducted to explore the patterns of body size and body mass between sexes, with age as the covariate. Growth was assessed using non-linear regressions in SPSS 22.0 using Von Bertalanffy's (1957) equation, $S_t = S_{max}(1 - e^{-kt+b})$, where S_t is SVL (mm) at age t, S_{max} is the estimated asymptotic maximum size, k is a growth coefficient and b is a constant. The growth rate was calculated as $R = dS/dt = k(S_{max} - S_t)$. Survival rate (S) for adult individuals was calculated from age structure based on the formula of Robson & Chapman (1961): $S = T/(R+T-1)$, where S is the finite annual survival rate estimate, $T = N_1+2N_2+3N_3+4N_4+\ldots nN_n$, $R = \Sigma N_i$, and N_i is the number of individuals in age group i. Adult life expectancy (ESP), the expected life span of individuals that have reached maturity, was calculated according to the formula of Seber (1973): $ESP = 0.5 + 1/(1 - S)$, where S represents survival rate. All statistical analysis was carried out with SPSS software, version 22.0 (SPSS Inc., Chicago, IL, USA). Values are presented as mean ± standard error of the mean, and the significance level used in all tests was $P < 0.05$.

This research complies with the laws and ethical standards of China. All animal procedures were approved by the Animal

Care and Use Committee of the College of Animal Science and Technology, Henan University of Science and Technology (CAST2015040010). All field work with the animals was conducted according to relevant national and international guideline

3. Results

A total of 185 of 190 individuals were aged successfully by skeletochronology, as five (four males and one female) showed abnormal patterns in bone histology and were excluded from the analysis. In all phalangeal cross-sections, stained growth lines were discernable and easy to count (Figure 1). Ages ranged from 5–13 years in both sexes; the age structure is shown in Figure 2. Individuals of eight- and nine-year-olds were predominant in females, whereas ten years was the most common age in males. Age structure differed significantly between males and females (Kolmogorov-Smirnov test: D = 1.89, P < 0.05). The mean age was 8.8 ± 0.2 in females and 9.6 ± 0.2 in males (Table 1); males were significantly older than females (Mann-Whitney U-test, P < 0.05).

Figure 1 Transverse sections of phalanx bones of adult *Pachyhynobius shangchengensis* with six LAGs (black arrows). KL, the Kastschenko line (white arrow); EB, endosteal bone; PB, periosteal bone; MC, marrow cavity.

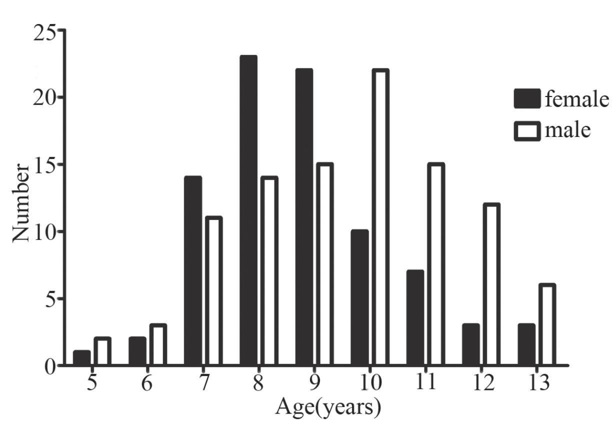

Figure 2 Age structure of *Pachyhynobius shangchengensis*.

Means of body size and body mass were (102.97 ± 0.81) mm and (34.67 ± 0.72) g for all individuals ((100.21 ± 0.91) mm, (31.76 ± 0.73) g in females and (105.31 ± 1.23) mm, (37.14 ± 1.12) g in males, respectively) (Table 1). Males were significantly larger and heavier than females (body size: t = –3.333, df = 174.650, P < 0.05; body mass: t = –4.017, df = 166.059, P < 0.05). The sexual dimorphism index was –2.05. A significant positive correlation was found between body size and mass (females, r = 0.823, P < 0.05; males: r = 0.920, P < 0.05), body size and age (females, r = 0.974, P < 0.05; males: r = 0.975, P < 0.05), body mass and age (females, r = 0.594, P < 0.05; males: r = 0.761, P < 0.05); the oldest individuals were the largest and heaviest. When the effect of age was controlled for, the intersexual difference was only found in body mass ($F_{1, 182}$ = 6.110, P < 0.05), and not in body size ($F_{1, 182}$ = 2.109, P = 0.148). Mean body size and mass of females was larger than males in age classes five, six, and seven, and smaller than males in the other age classes. A significant difference in body size was only found in age eleven and twelve classes, and body mass in age eleven classes (P < 0.05, Table 2).

Growth parameters estimated by von Bertalanffy's growth model are shown in Table 3. The maximum body size recorded in males and females was smaller than estimated asymptotic size. Males had larger asymptotic size and growth coefficients than that of females. The growth rate in males (5.30 ± 0.12) was significantly higher than in females (3.79 ± 0.08; Mann-Whitney U-test, P < 0.05). Since age structure differed between sexes, S and ESP calculations were carried out separately. S and ESP of adult males were 0.906 and 11.1 years, respectively, and those of adult females were 0.899 and 10.4 years.

4. Discussion

The age of sexual maturity of a species is affected by environmental conditions and influenced its fitness (Kusano, 1982). Most hynobiid salamanders have relatively early age at sexual maturity, such as *H. yangi* (females at three years and males at two years, Lee *et al*., 2010), *H. quelpaertensis* (both sexes at three years, Lee *et al*., 2010), *Salamandrella keyserlingii* (females at 3–4 years and males at 2–3 years, Hasumi, 2010), and *H. lichenatus* (both sexes at four years, Yamamoto *et al*., 2011). The observed minimum age of *P. shangchengensis* was 5 years in both sexes, suggesting that both sexes take at least 5 years to attain sexual maturity. Maximum longevity of more than 12 years is known in 57.5% of 40 species of urodeles throughout the world

Table 1 Body size (SVL), body mass and age of *Pachyhynobius shangchengensis* between sexes. Values are mean ± SE.

sex	n	Body size (mm)	Body mass (g)	Age (yrs)
female	85	100.21±0.91	31.76±0.73	8.81±0.18
male	100	105.31±1.23	37.14±1.12	9.59±0.19

Table 2 Comparison of body size (SVL) and body mass between sexes for each class of *Pachyhynobius shangchengensis*. Mean ± SE are given with samples sizes and ranges of body size and body mass of each age class in parentheses.

years	Body size (mm)				Body mass (g)			
	female	male	t	P	female	male	z	P
5	93 (93.0, n=1)	80.2±3.6 (76.7–83.8, n=2)			28.2 (28.2, n=1)	15.7±0.6 (15.1–16.2, n=2)		
6	81.4±3.4 (78.0–84.8, n=2)	81.7±1.0 (80.0–83.5, n=3)	−0.072	0.935	24.2±4.3 (19.9–28.5, n=2)	19.3±1.4 (17.8–22.1, n=3)	1.083	0.275
7	92.8±1.7 (85.1–103.0, n=14)	89.3±2.2 (82.3–103.4, n=11)	1.26	0.22	26.8±1.3 (19.6–35.7, n=14)	24.0±2.5 (14.3–38.8, n=11)	1.072	0.295
8	98.0±1.3 (88.7–111.5, n=23)	99.2±2.9 (87.8–128.0, n=14)	−0.436	0.666	29.3±1.3 (17.1–41.5, n=23)	30.6±2.2 (21.0–52.8, n=14)	−0.524	0.604
9	101.6±1.2 (92.3–112.7, n=22)	102.0±1.8 (92.0–114.3, n=15)	−0.222	0.825	32.7±1.2 (24.2–48.7, n=22)	35.0±2.5 (20.1–60.6, n=15)	−0.934	0.357
10	103.8±2.3 (92.5–116.3, n=10)	108.1±1.3 (92.4–120.3, n=22)	−1.747	0.091	35.4±2.0 (25.6–46.0, n=10)	39.7±1.3 (28.2–54.3, n=22)	−1.8	0.082
11	110.0±1.1 (105.5–114.2, n=7)	114.6±1.2 (107.8–129.4, n=15)	−2.415	0.025	37.5±1.3 (32.6–41.8, n=7)	46.9±1.5 (37.0–57.0, n=15)	−3.945	0.001
12	108.2±2.4 (103.6–112.0, n=3)	117.2±1.3 (109.4–126.2, n=12)	−3.145	0.008	36.6±3.8 (29.3–42.0, n=3)	44.2±1.7 (35.2–54.3, n=12)	−1.992	0.068
13	114.2±3.2 (109.4–120.2, n=3)	120.1±2.2 (112.4–126.9, n=6)	−1.556	0.164	43.0±0.1 (42.8–43.1, n=3)	50.1±3.2 (37.5–58.8, n=6)	−1.512	0.174

Table 3 Growth parameters of *Pachyhynobius shangchengensis* estimated by von Bertalanffy's growth model ($S_t = S_{max}(1-e^{-kt+b})$).

Character	Female	Male
The number of samples	85	100
Asymptotic size S_{max} (mm)	143.29	159.925
Growth coefficients k	0.088	0.097
Constant b	−0.436	−0.163
Growth rate R	3.79	5.3

(Wells, 2007), which also has been shown for most hynobiid salamanders (e.g., *H. kimurae*, Misawa and Matsui, 1999; *H. tokyoensis*, Kusano *et al.*, 2006; *H. lichenatus*, Yamamoto *et al.*, 2011; *P. gorganensis*, Zivari and Kami, 2017). Maximum longevity of *P. shangchengensis* was 13 years in this study, suggesting it has a typical lifespan for this group of species.

Age structure is an important component of understanding the biology of an animal population (McCreary *et al.*, 2008). The observed minimum and maximum ages of *P. shangchengensis* were same for both sexes (5–13 years), but a significant difference was found in age structure between sexes; individuals of females were predominant in eight- and nine-year–old classes, whereas males were most common in the ten-year-old, males had a shift to older age classes (Figure 2). The age structure of *P. shangchengensis* is different from other hynobiid salamanders. In *H. nebulosus*, individuals at 2–4 years and 4 years were predominant in males and females, respectively (Ento and Matsui, 2002). In *H. yangi* and *H. quelpaertensis*, individuals at 3–5 and 4–6 ages were predominant in males and females, respectively (Lee *et al.*, 2010). In *S. keyserlingii*, individuals were concentrated in 3–5 and 3–6 ages in males and females, respectively (Hasumi, 2010). As shown in Figure 2, individuals at middle ages were relatively more abundant than those of younger and older age classes, and this suggested that this population of *P. shangchengensis* is stable. The survival rate and adult life expectancy of adult males were higher than that of females, as based on the difference in age structure. Males were significantly older than females in average age; this is also different from other hynobiid salamanders (e.g., *H. nebulosus*, Ento and Matsui, 2002; *H. leechii*, Lee and Park, 2008; *H. yangi*, Lee *et al.*, 2010; *S. keyserlingii*, Hasumi, 2010).

As an indeterminate growth animal, amphibians show a strong positive correlation between body size and age (Halliday and Verrell, 1988; Duellman and Trueb, 1994). Predicted asymptotic sizes (S_{max}) of both sexes were larger than measured body size, and there were positive correlations among body size, body mass, and age; this suggested that *P. shangchengensis* also is an indeterminate growth animal. The significant correlation between body size and age has also been found in other hynobiid salamanders, such as *H. tokyoensis* (Kusano *et* *al.*, 2006), *H. leechii* (Lee and Park, 2008), *P. gorganensis* (Zivari and Kami, 2017) but not for *H. lichenatus* (Yamamoto *et al.*, 2011). Asymptotic size and growth rate of males were higher than those of females, which can be explained by a difference in resource allocation between growth and reproduction (Marzona *et al.*, 2004; Mi, 2015). If animals allocate a large amount of energy to reproduction, their growth is usually reduced after reaching sexual maturity (Ryser, 1989). Females need more stored energy, as they need to allocate more energy to gonad and embryo development than males. Thus, the growth rate in females decreases after maturation, whereas males continue to grow.

Sexual size dimorphism (SSD) is a widespread phenomenon (Shine, 1979; Andersson, 1994; Kupfer, 2007). Females are larger in 60.8% of 79 urodele species (Shine, 1979). Interestingly, *P. shangchengensis* did not follow this tendency but instead exhibited male-biased dimorphism. This is consistent with the results of Xiong *et al.* (2019), which used a fewer number of individuals from the same population as used in this study. Male-biased dimorphism also has been reported in other hynobiid salamanders, e.g., *Liua shihi* (Zhang *et al.*, 2014) and *Onychodactylus zhangyapingi* (Xiong *et al.*, 2016). Though sexual selection has been promoted as the cause of male-biased SSD in *P. shangchengensis* (Xiong *et al.*, 2019), life-history traits (e.g., age structure, growth rate, and longevity) are also thought to be key factors influencing SSD (Morrison and Hero, 2003; Marzona *et al.*, 2004; Lu *et al.*, 2006; Altunisik *et al.*, 2014; Liao *et al.*, 2015; Mi, 2015). In the present study, a significant difference was found in age structure between sexes, but SSD was not obvious after correcting for age, suggesting that age structure mediates SSD. Maximum longevities of the two sexes are similar, but growth rate, growth coefficients, and survivorship rate of males is higher than those of females. Thus, differences in age structure, growth, and survivorship are responsible for male-biased SSD of *P. shangchengensis*.

This study was the first to determine age, growth rate, survival rate, and expected life span of *P. shangchengensis*. These data provide information on life-history traits which can be helpful for future studies of this and other hynobiid salamanders, and the study provides background information that can be used for the management of this species in China.

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