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A skeletochronological estimation of age structure in a population of the Guenther's frog, *Hylarana guentheri*, from western China

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Abstract. Age determination in amphibians is crucial to investigation of life-history traits. In this context, we studied age structure of the Guenther's frog, *Hylarana guentheri*, population from southwestern China located at an altitude of 300 m using skeletochronological method. Our results showed that age in adult males ranged from 1 to 4 years, and from 2 to 6 years in adult females. Average age in females was significantly older than males in 2008 and 2009. Also, females had significantly larger average body size than males in both years. A significant relationship between age and body size within each sex was found in this species for both years. The ANCOVA analysis revealed that there was significant difference in body size between sexes when the effect of age was removed. The von Bertalanffy's model showed that females had larger asymptotic body size than males, but growth rate of females was smaller than males.

Keywords. Skeletochronology, age structure, *Hylarana guentheri*, sexual size dimorphism.

INTRODUCTION

Mark-recapture studies to obtain data on age, growth and longevity of animals are very time-consuming. An alternative tool to get such data for amphibians is skeletrochronology which is considered the most reliable method of age estimation by counting the lines of arrested growth (LAGs) recorded in long bones (Hemelaar and Van Gelder, 1980; Castanet and Smirina, 1990). It has been successfully used on a variety of species from temperate zone (*Bufo bufo*, Hemelaar, 1988; *Rana temporaria*, Miaud et al., 1999; *R. latastei*, Guarino et al., 2003; *R. chensinensis,* Lu et al., 2006; *R. muscosa*, Matthews and Miaud, 2007; *R. holtzi*, Guarino and Erismis, 2008; *R. ridibunda*, Kyriakopoulou-Sklavounou et al., 2008) and tropical regions (*R. nigrovittata,* Khonsue et al., 2000; *Limnonectes limnocharis,* Pancharat-

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na and Deshpande, 2003). Recently, it also has applied to species from subtropical regions (*Mantidactylus microtympanum*, Guarino et al., 1998; *Boophis occidentalis*, Andreone et al., 2002; *R. swinhoana,* Lai et al., 2005; *Dyscophus antongilii*, Tessa et al., 2007; *B. andrewsi,* Liao, 2009; *Amolops mantzorum,* Liao and Lu, 2010a; *Hyla annectans chuanxiensis*; Liao and Lu, 2010b; *R. limnocharis*; Liao et al., 2010b). The accurately assessed individual age allowed us greater understanding of life histories in amphibians.

The Guenther's frog, *Hylarana guentheri*, is a species endemic to china that is widely distributed in paddyfields. It is commonly the dominant amphibian species even where the amphibian population is declining. This species is a medium-sized frog [adult snout–vent length (SVL) ranges from 62 to 80 mm], showing a female-biased size dimorphism. During the breeding period, males actively search for mates and attract them by advertisement calls (Fei and Ye, 2001). Egg-laying extends from March to May, and the Guenther's frog may be classified as a prolonged breeder (sensu Wells, 1977). Currently, despite breeding ecology, habitat use and morphological traits of the species has been reported (Zhao and Adler, 1993; Fei and Ye, 2001), information about demographic data on the species during the breeding period remains largely unknown.

In this study, we used phalangeal skeletochronological method to assess individual age, growth and longevity of *H. guentheri* in two successive years. Our aims were 1): to compare the age structure, body size and growth of a *H. guentheri* population living western China where these demographic parameters are unavailable; 2) to gain insight into factors that affect the differences in body size between males and females.

MATERIALS AND METHODS

The studied population was located in some rice fields of Shidong town in Anju County (30°23'N, 105°22'E), at an altitude of 300 m a.s.l., in western China. The rice fields are filled with water to a depth of 8-10 cm. The vegetation of the study site is typical eucalyptus (*Eucalyptus robusta*), oriental arborvitae (*Platycladus orientalis*), silvergrass (*Miscanthus floridulu*) and bulrush (*Phragmites australis*). As being a subtropical region, climate in the study area is strongly seasonal. Annual average temperature ranges between 16.9-17.2 °C and annual total precipitation is 908-993 mm. Monthly average temperature in summer and winter is 38 °C and -3 °C, respectively. Frogs hibernate in holes on land, and migrate in March to lay eggs in the rice fields (Fei and Ye, 2001). Adults and juveniles migrate back to the hole for hibernation in November at the end of the active season.

At this locality, all frogs were searched with sampling line of 2500 m length and 2 m width in April 2008 and 2009, when they were active and in breeding condition. All observed frogs on the bank of rice fields along the line were caught using a battery flashlight for illumination. Each adult specimen was sexed by their secondary sexual characteristics (the nuptial pads on the first finger for male, eggs readily visible by the skin of the abdomen for female). The individuals were classed as juveniles owning to the absence of nuptial pads or of female aspect. Body sizes (the snout–vent length: SVL) were measured to the nearest 0.02 mm with a caliper. The last two phalanges of the right hind limb were clipped, for a mark-recapture study, and stored in 10% neutral buffered formalin for skeletochronological analysis. All frogs examined were released at the points of capture. A total of 78 adult males, 61 adult females and 35 juveniles were collected during this study. Using the mark-recapture data and the Lincoln-Petersen index (Caughley, 1977), we estimate the population size to be about 558 individuals.

The standard skeletochronological procedure (e.g., Lu et al., 2006; Liao, 2009; Liao and Lu, 2010a) was used to determine age. For each individual digit, we cleaned of surrounding tissues of the phalanges, and then put them in 5% nitric acid to decalcify for 48 h. These decalcified digits were stained for 75 min in Ehrlich's haematoxylin. Subsequently, these stained bones were dehydrated through successive ethanol stages. Phalanges were then processed for paraffin embedding in small blocks. Cross-sections (13 μm) thick were obtained by means of rotary microtome, and the phalanx with the smallest medullar cavity were selected and mounted in aqueous synthetic resin. The sections were observed though a light microscope and the best of them were photographed at selected magnifications using a camera lucida. The analysis of lines of arrested growth (LAGs) was performed by two different persons (W.B. Liao and T.L. Yu) with previous experience of the technique. As suggested by Castanet and Smirina (1990), a thin layer of chondroid tissue situated between periosteal and endosteal bones probably are the remnant of the larval cartilage precursor. It is originally described as "Wandständiger Knorpel" by Kastschenko (1881) and has been indentified to define accurately the endosteal resorption in amphibians (Rozenblut and Ogielska, 2005).

Sexual size dimorphism was described by SDI index (Lovich and Gibbons, 1992). The equation form was: SDI = (mean length of the larger sex/mean length of the smaller sex) ± 1 (SDI = 0 when both sexes are of similar size, SDI > 0 when females are larger than males). The relationship between body length and age using inferred data was examined. Growth was estimated using von Bertalanffy's (1957) function, a model demonstrated to be suitable for anurans (Lu et al., 2006; Ma et al., 2009). The equation form was: $S_t = S_{max} \times (1 - e^{-kt+b})$, where S_t is body size at age t, S_{max} is the estimated asymptotic body size, k is a growth coefficient relating to rate of decline in growth as frogs attain the maximum body size, b is a constant. Growth rate can be calculated with the following equation: $R = dS/dt = k \times (S_{max} - S_t)$, which is maximal when S_t is minimal.

We assessed interaction between age and sex using general linear models (GLMs) treating SVL as a dependent variable. We used non-parametric tests (Mann-Whitney U tests) to compare inter-sex differences in body size and age and the Spearman correlation to infer a relationship between variables. We also conducted ANCOVAs with age as covariate to see significance of differences in body size between sexes after removing the effects of age. All probabilities were two-tailed, and the level of significance was $P < 0.05$. The values given are shown as mean ± SD.

RESULTS

Lines of arrested growth (LAGs) were present in cross sections of juvenile and adult phalanges (Fig. 1). In juveniles, endosteal resorption was not observed in the samplings (Fig. 1A). In the adults, we found that endosteal resorption incompletely eroded the first (innermost) periosteal LAGs in twelve males and eight females. However, the first LAGs of three males and two females were completely eroded by endosteal resorption due to the absence of KL, and we added one year as their true age. False lines would result in serious difficulty in interpreting age of individuals, but they did not affect LAGs counted in the sample. Two very close hematoxynophilic lines that were considered as double LAGs were observed in five males and four females (about 5.8% of the sample). As a rule these lines must be counted as a single true LAG in the samplings (Fig. 1D).

We captured 139 adult frogs (78 males and 61 females) in successive two years resulting in a male-biased sex ratio of 1.28:1. The sex ratio did not differ from 1:1 ($\chi^2 = 2.08$, df = 1, P = 0.15). A total of 104 individuals (35 juveniles, 45 males and 33 females) were captured and marked by toe-clipping in 2008. We captured 70 individuals (9 juveniles, 41 males and 20 females) and recaptured 2 juveniles, 4 males and 6 females in 2009. Frequency of recapture (as an indictor of survival) in males was smaller than females. Frequency of recapture of males and females was 8.9% and 18.2%, respectively.

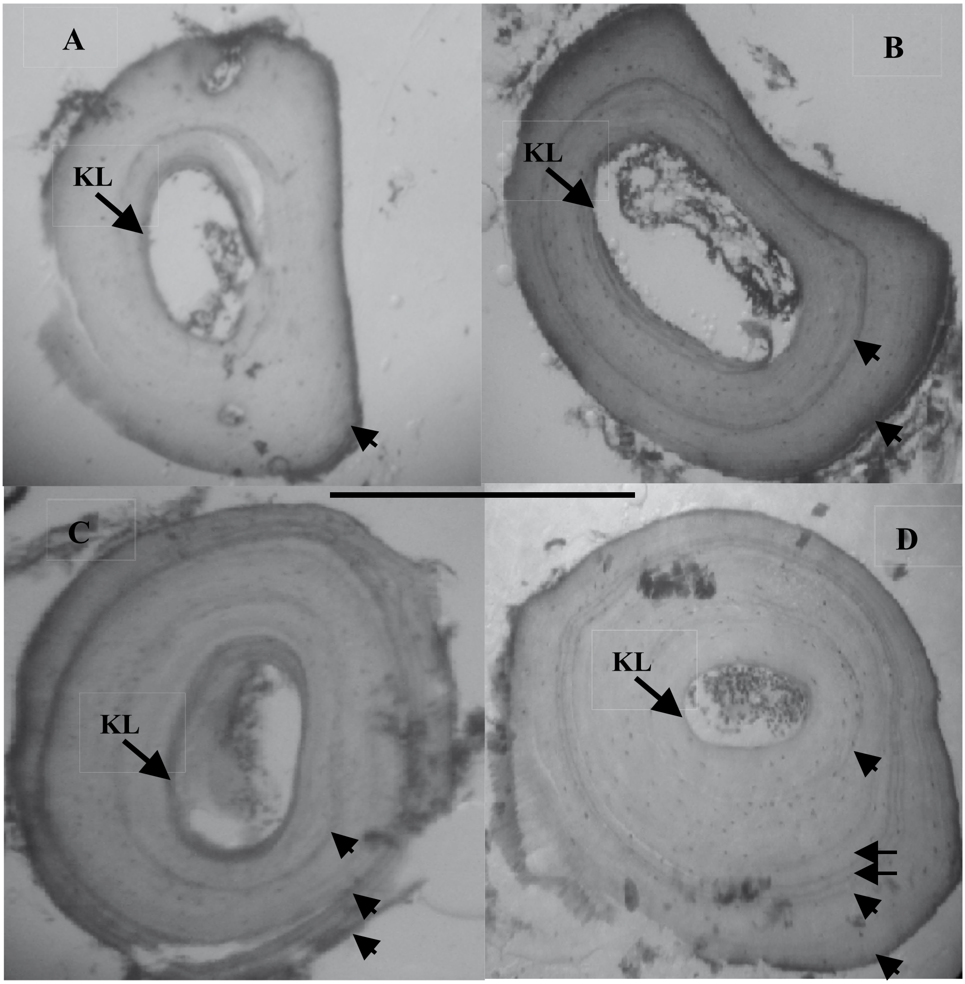

Fig. 1. Four selected examples of hematoxylin-stained cross-sections of the longest phalange of *Hylarana guentheri* for two successive years in a subtropical population, western China (A: 1-yr old male; B: 2-yr old female; C: 3-yr old male and D: 4-yr old female, the second LAGs is double). Arrows indicate the lines of arrested growth (LAG). KL represents Kastschenko Line, the interface between the endosteal and periosteal zones. Scale bar: 300 μm.

Adult age ranged from 1 to 4 years in males, and from 2 to 6 years in females (Fig. 2). Age distributions between males and females did not differ markedly in 2008 and 2009 (Kolmogorov-Smirnov test: 2008: $D = 0.58$, $P = 0.89$; 2009: $D = 0.85$, $P = 0.45$). Females had significantly older average age than males in two years (Table 1; Mann–Whitney U-test: 2008, $Z = 3.21$, $P = 0.003$; 2009, $Z = 2.22$, $P = 0.03$).

Average body size of females was significantly larger than that of males (Table 1; Mann-Whitney U-test: 2008, $Z = 6.22$, $P < 0.001$; 2009, $Z = 4.93$, $P < 0.001$). There was a significant difference in SVL between females and males within each age group (Table

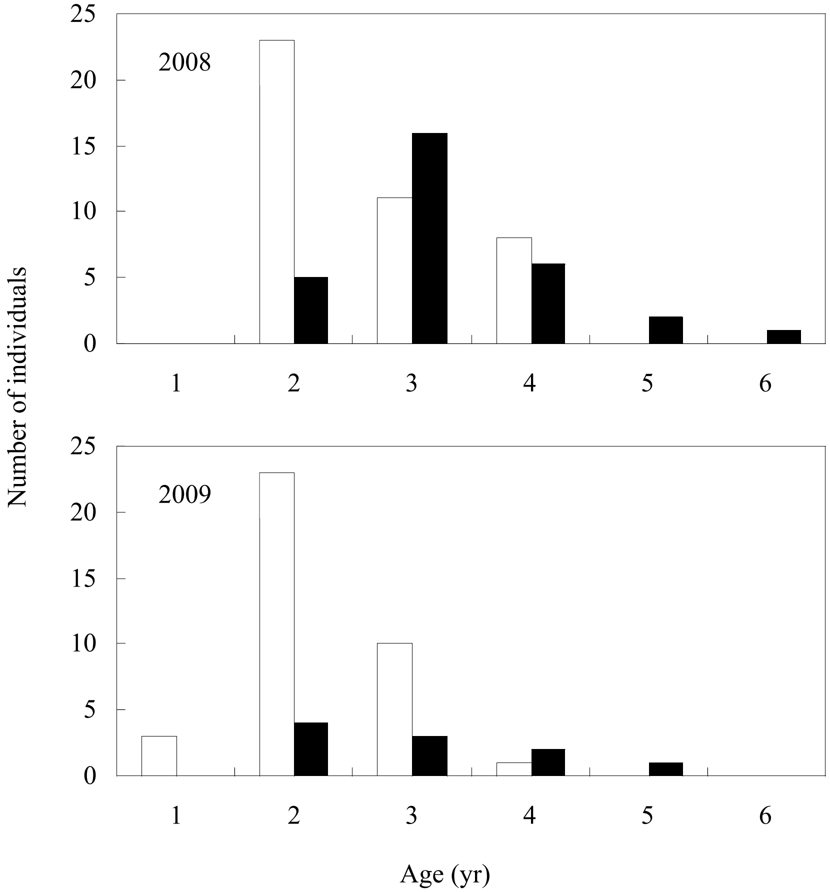

Fig. 2. Relationship between body size and age classes of *Hylarana guentheri* (male, open bars; female, close bars) for two successive years in a subtropical population, western China. Growth curves follow the von Bertalanffy model. Body size in mm is shown as mean ± SD.

2; all $P < 0.05$). A non-significant interaction between sex and age ($F_{1,121} = 0.21$, $P = 0.81$) revealed that relationship between age and size did no differ apparently among the sexes. The ANCOVAs analysis showed that the difference in body size between sexes remained significant (2008, $F_{1,74} = 49.92$, $P < 0.001$; 2009, $F_{1,47} = 11.34$, $P < 0.002$) when the significant effect of age was controlled in both years (2008, $F_{1,74} = 28.21$, $P < 0.001$; 2009, $F_{1,47} = 26.54$, $P < 0.001$).

The Spearman correlation showed that significant relationships between age and SVL in both years were found within each sex (2008: male, $r_s = 0.57$, $n = 42$, $P < 0.001$; female, $r_s = 0.56$, $n = 32$, $P = 0.001$; 2009: male, $r_s = 0.55$, $n = 37$, $P < 0.001$; female, $r_s = 0.72$, $n = 10$, $P = 0.019$). The sexual dimorphism index (SDI) with body size in 2008 and 2009 was -0.102 and -0.112, respectively.

The von Bertalanffy's model provided a good description for the relationship between body size and age for the populations in either sex [2008, male, $S_t = 73.5 \times (1 - e^{-0.72t-0.99})$,

Table 1. Body size and age of *Hylarana guentheri* for two successive years in a subtropical population from western China. Values in descending order are mean ± SD and sample size.

Individuals	2008		2009	
	Body size (mm)	Age (yrs)	Body size (mm)	Age (yrs)
Juvenile	51.8 ± 6.9 (n = 26).		48.9 ± 6.9 (n = 9)	
Male	68.7 ± 3.7 (n = 45)	2.6 ± 0.8 (n = 42)	67.0 ± 3.7 (n = 41)	2.2 ± 0.6 (n = 37)
Female	75.7 ± 3.8 (n = 33)	3.4 ±1.0 (n = 32)	74.5 ± 4.9 (n = 20)	3.0 ± 1.1 (n = 10)

Table 2. Difference in body size between males and females within each age class group *Hylarana guentheri* in a subtropical population western China. Values in descending order are mean ± SD and sample size.

Age	2008				2009			
	Male	Female	Z	P	Male	Female	Z	P
1					63.2 ± 1.7 (n = 3)			
2	67.0 ± 3.7 (n = 23)	72.3 ± 3.0 (n = 5)	2.61	< 0.01	66.1 ± 3.2 (n = 23)	70.2 ± 2.7 (n = 4)	1.95	< 0.05
3	70.4 ± 2.6 (n = 11)	76.0 ± 3.5 (n = 16)	3.65	< 0.001	69.9 ± 3.3 (n = 10)	74.4 ± 1.5 (n = 3)	2.12	< 0.05
4	70.0 ± 1.9 (n = 8)	75.8 ± 2.1 (n = 6)	2.45	< 0.05	72.6 (n = 1)	77.5 ± 7.2 (n = 2)		
5		81.1 ± 0.1 (n = 2)				77.2 (n = 1)		
6		80.3 (n = 1)						

$r^2 = 0.32$; female, $S_t = 80.4 \times (1 - e^{-0.51t-1.34})$, $r^2 = 029$; 2009, male, $S_t = 71.9 \times (1 - e^{-1.21t-0.11})$, $r^2 = 0.64$; female, $S_t = 78.6 \times (1 - e^{-0.86t-0.40})$, $r^2 = 0.59$; Fig. 3]. The results showed that females were generally larger than males throughout the life cycle, but growth rate of males was greater than females in the population (2008, male, $R = 3.35$; female, $R = 2.11$; 2009, male, $R = 8.31$; female, $R = 4.31$).

DISCUSSION

This is the first study using skeletochronology to estimate the age structure of a *H. guentheri* population in western China. Stained LAGs were clearly visible between zones of thicker layers of bone deposited in growth periods (Fig. 1). The pattern of LAG deposition is considered to be genetically controlled with reinforcement by seasonality such as

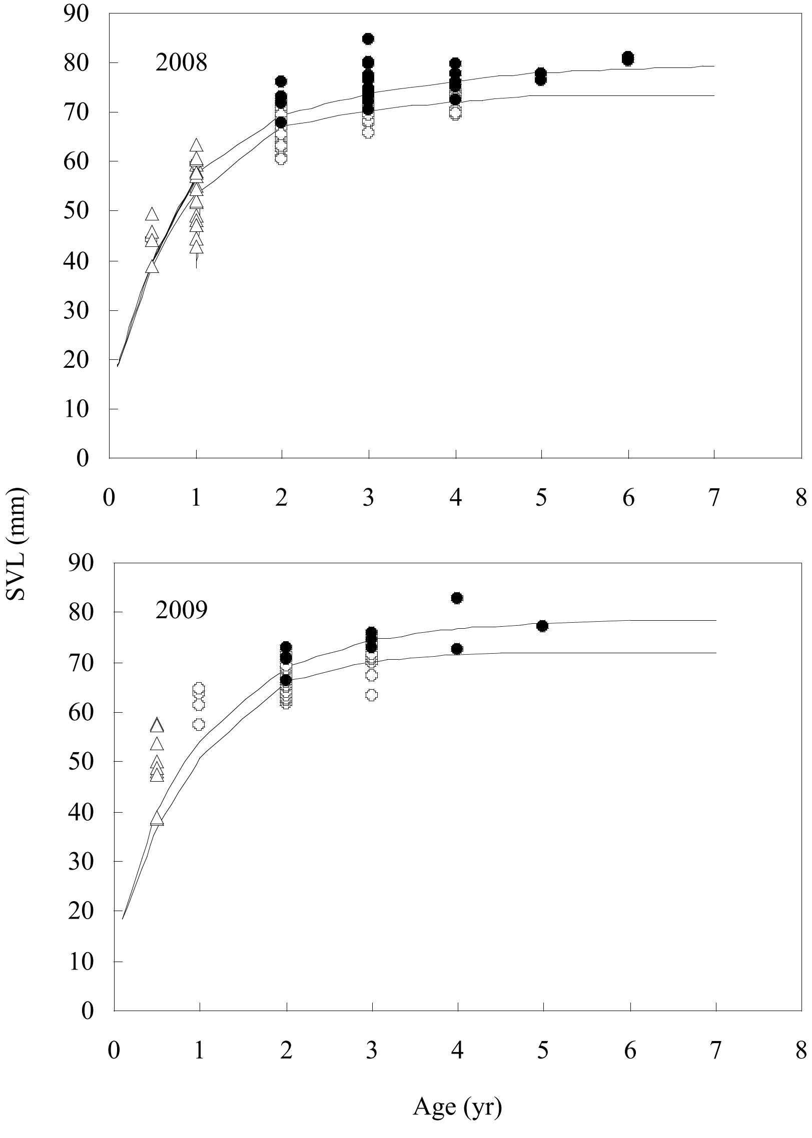

Fig. 3. Growth curve of the breeding population of *Hylarana guentheri* from western China (Juvenile, open triangles; male, open circles; female, close circles) following the von Bertalanffy model for two successive years.

temperature fluctuations (Alcobendas and Castanet, 2000). In our study site, the active period is limited to March-November and growth mark deposit annually in winter. Winter growth marks can be significantly expressed in *H. guentheri*, as previously observed in other species in subtropical regions (Guarino et al., 1998; Morrison et al., 2004; Lai et al., 2005; Liao and Lu, 2010a). Completive LAG resorption based on the occurrence of KL was easily identifiable in this species, and it did not complicate age estimation. In some temperate amphibians, double LAGs are the expression of growth interruption twice a year and show a periodic pattern (Caetano and Castanet, 1993). In our study, *Hylarana guentheri* experienced growth interruption twice a year; the occasionally occurring double lines were easily identified because the annual growth zones were by far broader than the distance between the double lines and thus, did not deteriorate precision of age estimates. In these individuals, false lines differed markedly from LAGs because they were incomplete and faint hematoxynophilic lines.

For many amphibian species it is reported that males reach sexual maturity one year earlier than females (Miaud et al., 1999; Kyriakopoulou-Sklavounou and Grumiro, 2002; Eaton et al., 2005; Liao et al., 2010a). We also found that *H. guentheri* males reached sexual maturity earlier than females. In the *H. guentheri* population, longevity of males was shorter than that of females, as observed previously in most anurans (Khonsue et al., 2001; Ento and Matsui, 2002; Matthews and Miaud, 2007; Liao, 2009). The fact that males tended to shorter lifespan than females resulted from the lower survival of males.

Significant correlation was found between age and SVL for both years, as reported in other anurans (Hemelarr, 1983; Ryser, 1996; Lu et al., 2006). There were significant overlaps of the body size distributions between age classes in mature individuals, therefore, body size by *H. guentheri* was not considered as a reliable predictor of age. This tendency has been pointed out by many previous authors in other amphibians (Halliday and Verrell, 1988; Platz and Lathrop, 1992; Kutrup et al., 2005).

Sexual size dimorphism is observed in many amphibians, and females are larger than males (Shine, 1979; Monnet and Cherry, 2002). In *H. guentheri*, adult males had significantly smaller size than females in two years. Larger body size in females has been discussed in relation to the fact that females need more stored energy to allocate much more to gonad and embryo development (Shine, 1979; Halliday and Verrell, 1988). By contrary, males need relatively low investment of energy for reproduction in that spermatogenesis is less costly than vitellogenetic growth of oocytes (Jørgensen, 1992).

The difference in body size between sexes is also often explained by post-maturation growth rate and growth duration (or age). For *H. guentheri,* growth rate of males was larger than females, and also grew more quickly towards the asymptotic body size than females. Difference in growth rate between sexes would contribute marginally to the observed sexual size dimorphism. Amphibians have indeterminate growth (Halliday and Tejedo, 1995). Thus, the earlier age at sexual mature of males in this study resulted in the differences in SVL between sexes because males took more energy in reproduction rather than growth. A larger size in females can also be attained thanks to a longer life span and larger mean age in this species, a pattern common among many amphibians (Monnet and Cherry, 2002; Matthews and Miaud, 2007), including subtropical species (Morrison et al., 2004; Lai et al., 2005). Moreover, genetic factor may result in difference in body size inter-sex in inter- and intra-population (Berven, 1982; Miaud et al., 1999). Future studies need to solve the views.

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