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Age, size and growth of the endemic Anatolian mountain frog *Rana holtzi* from Turkey

Claude Miaud[1], Nazan Üzüm[2], Aziz Avci[2] & Kurtuluş Olgun[2]

[1]*Laboratoire d'Ecologie Alpine, UMR 5553, Université de Savoie, CISM, Le Bourget du Lac, France*
[2]*Adnan Menderes Üniversitesi, Fen-Edebiyat Fakültesi, Biyoloji Bölümü, Aydýn, Turkey*

We estimated the age, size and growth patterns of a mountain ranid, *Rana holtzi*, from Central Anatolia, using skeletochronology. We used lines of arrested growth (LAGs) recorded in phalanges to estimate the age of juveniles and adults. Results showed that age at maturity in this population was 4–5 years (median age of juveniles was 3.5 yrs and minimum age for adults was 4.0 yrs for both sexes). Median ages of males and females were similar (6 yrs) and longevity reached 8.0 yrs for males and 10.5 yrs for females. Mean body and tibia length of males was significantly larger than that of females. Growth of the Anatolian mountain frog was well described by the von Bertalanffy model, and males and females did not differ in growth parameters. These demographic parameters are compared with those obtained with other amphibians, especially ranids. The sexual size dimorphism observed – larger males than females – has rarely been observed previously and should prompt observers to collect other biological information on this rare endemic species.

Key words: amphibian, life-history trait, sexual size dimorphism, skeletochronology

INTRODUCTION

The so-called brown frog (subgenus *Rana*) is a group of eleven species of Western Palearctic amphibians (Veith et al., 2003). Brown frogs occur throughout Europe and Asia Minor (Baran & Atatür, 1986; Gasc et al., 1997) and vary greatly in ecology and distribution: species can be generalists (e.g. *Rana temporaria* breeds in numerous aquatic places and occurs from lowland habitats up to 2745m a.s.l. in the Alps), or specialists (e.g. *Rana graeca* breeds in running mountain brooks of the southern Balkans and adjacent regions).

The Anatolian mountain frog *Rana holtzi* (Werner, 1898) is one of three minor Asia Minor and Caucasian endemics (with *R. camerani* Boulenger, 1886 and *R. macrocnemis* Boulenger, 1885). The taxonomic status of these species has been repeatedly questioned. The first described was *R. macrocnemis*, found at Uludağ by Boulenger (1885). The second was *R. camerani*, described from Mount Erciyes in Turkey (Werner, 1902), and the third was *R. holtzi* from Maden Lake in the Taurus mountains (Werner, 1898). Morphological (Baran, 1969) and osteological (Özeti, 1970) analysis argues for the existence of three distinct species, but the morphological closeness has led to on-going questions regarding their taxonomical status (e.g. Borkin, 1997; Kuzmin, 1996; Tarkhnishvilli, 1996; Tarkhnishvilli et al., 1999). Çevik et al. (2006) concluded from morphometrical and serological analyses that only two distinct mountain frog species, *R. holtzi* and *R. macrocnemis*, inhabit the three known localities of Anatolia.

The Anatolian mountain frog, *R. holtzi*, exhibits one of the smallest distribution ranges of all the Western Palearctic amphibians: it is currently found in only two lakes (Karagöl and Çinigöl, separated by a distance of 2 km) in the Bolkar mountains of Central Anatolia. Apart from some ecological data (Baran et al., 2001), the biology of the Anatolian mountain frog remains largely unknown. The aim of this paper is to present original data on age and growth in this endemic species. Skeletochronology is considered the most reliable method of age estimation in amphibians (Castanet & Smirina, 1990; Halliday & Verrell, 1988), and is now widely used (Castanet, 2002). It is based on the counting of lines of arrested growth (LAGs) recorded in long bones, and knowledge of the rhythm of deposition allows estimation of individual age. We hypothesized that the Anatolian mountain frog is exposed to climatic conditions leading to such deposition in winter; the use of phalanges prevented the sacrifice of individuals.

MATERIALS AND METHODS

Study area and species

The studied population was located in Lake Karagöl (37°24'N, 34°33'E), at 2560 m a.s.l., in the Bolkar mountains of Niğde province (Central Anatolia). Karagöl is a small tectonic lake of about 450 × 175 m. The maximum depth is 12 m. The lake is surrounded by an alpine grassland, and is filled with melting snow (and possibly with groundwater from Lake Çinigöl, 2580 m a.s.l., situated 2 km from Lake Karagöl). The lake is covered with ice and snow during some 5–7 months each year. Melting snow water feeds this crater lake between April and June via a canal located at the eastern side of the lake. The southern side of the lake abuts a steep slope, while the other sides are covered with meadows. These meadows attract animals (invertebrates and vertebrates) that introduce nutrients to the lake. However, the water is always particularly limpid, and the phyto- and zooplankton diversity and biomass are low (Baran et al., 2001).

Correspondence: Claude Miaud, Laboratoire d'Ecologie Alpine, UMR 5553, Université de Savoie, CISM, F 73376 Le Bourget du Lac, France. *E-mail:* claude.miaud@univ-savoie.fr

Table 1. Climatic conditions recorded at the meteorological station of Kars (1340 km from Çinigöl, 2500 m a.s.l., Central Anatolia).

Recorded in 2001	Annual	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sept.	Oct.	Nov.	Dec.
Average temperature (⁰C)	4.2	–11.5	–9.7	–3.8	4.8	10.1	13.5	17.3	17.2	13.3	6.9	0.3	–7.3
Relative air humidity (%)	70	73	74	75	70	69	68	64	62	61	68	76	76
Precipitation (mm)	501.2	23.2	26.6	28.2	45.4	83.8	78.7	54.0	42.8	29.0	39.0	28.0	22.4
Precipitation (days)	133.7	10.8	10.9	11.8	13.3	18.2	15.1	10.2	8.2	7.2	9.1	8.8	10.0
Highest temperature (⁰C)	35.4	8.4	11.0	18.8	24.2	27.0	31.4	35.4	35.4	32.6	26.8	21.2	11.4
Lowest temperature (⁰C)	–39.6	–39.6	–37.0	–33.3	–22.6	–7.0	–4.0	0.1	–1.9	–6.0	–17.5	–30.0	–35.0

The active period of *R. holtzi* starts in May and ends in October, a period when temperature allows breeding and feeding activities. Table 1 shows climatic conditions recorded at the meteorological station of Kars (2500 m a.s.l., 1340 km from Karagöl). While Kars is far from Karagöl, the meteorological station is at the same altitude as the study site, and the climate observed in Karagöl is similar to that recorded in Kars (K. Olgun, pers. obs.). Frogs hibernate on land, in the meadows, and migrate in May to lay eggs in the lake (Baran et al., 2001). Tadpoles develop until the end of September and transform into small, terrestrial froglets. The aquatic phase (egg and tadpole) is thus achieved in about 4–5 months. Froglets and adults migrate back to the meadows at the end of the active season.

Individual capture and body size measurements

Many frogs reached the lake for breeding and were easily caught by hand from the shore and from the meadow edge on 29 June 2003. Body length (snout–urostyle length) and tibia length were measured to the nearest mm with a calliper. Body length was recorded to infer frog growth, and tibia length because of its importance in previous Anatolian frog taxonomical studies (e.g. Baran, 1969). Sexual size dimorphism (SSD) was estimated with the Lovich and Gibbons (1992) sexual dimorphism index SDI: mean length of the larger sex divided by the mean length of the smaller sex with the result arbitrarily defined as positive (minus 1) when females are larger and negative (plus 1) in the converse case.

Males and females were identified by their general colour and secondary sexual characteristics (the presence of nuptial pads on the underside of the forelimb thumbs in males). Small individuals (<35 mm) were classified as juveniles. Three individuals (one of 38 and two of 40 mm body length) were also classed as juveniles because of the absence of nuptial pads or of female aspect.

Skeletochronological analysis

The two distal phalanges of the larger toe of the right hind limb were clipped and preserved in 90% alcohol. The standard skeletochronological procedure (e.g. Castanet et al., 1977; Miaud, 1991) was used to determine age. Cross sections (12 mm) from the diaphyseal region of the decalcified phalanx were obtained using a freezing microtome and were stained with Ehrlich's haematoxylin. Cross-sections were observed through a light microscope, and one bone section from each frog was photographed (at the same magnification) using a camera lucida, allowing for simultaneous comparison and facilitating the analysis of the bone growth pattern. Two different people (N.U. and C.M.) with previous experience of the technique performed the analysis of growth marks. The maximum difference for LAGs counted was one year and, in this case, the age was estimated at ±1 year.

Growth was estimated using the von Bertalanffy (1938) model, taking into account only the juvenile and adult stages of the life cycle (Hemelaar, 1988; Arntzen, 2000; Olgun et al., 2001) as:

$$BL_t = BL_{max} - (BL_{max} - BL_{met})e^{-k(t-tmet)}$$

where t is the age in years, BL_t is the total length at age t and BL_{max} is the estimated average maximum total length that can be reached. BL_{met} is the fixed average total length at metamorphosis. The growth coefficient, k, is the rate at which BL_{max} is reached, and determines the shape of the curve. Because we did not collect froglets at metamorphosis, size at metamorphosis was estimated from the age/size linear regression estimated in older juveniles (1–4 years old): body size = 0.18 + 0.69 × age, r=0.73, $F_{1,8}$ =9.08, P=0.017, n=10. The parameters BL_{max}, k, and their asymptotic confidence intervals (CI) were estimated by non-linear least-square regression (Statistica 5.0/W, Statsoft Inc., U.S.A.). Two estimated parameters (BL_{max} and k) were considered significantly different (at the 0.95 level) when their respective confidence intervals did not overlap (El Mouden et al., 1999; Miaud et al., 2001).

Most distributions of variables (body length, tibia length, age) deviated from a normal distribution (Shapiro–Wilk test) and we used non-parametric tests (Mann–Whitney U-test) to compare variables between sexes and the Spearman correlation to infer a relationship between variables.

RESULTS

Lines of arrested growth in the phalanges

Lines of arrested growth were present in cross sections of juvenile and adult phalanges. They appeared as thin and approximately concentric layers, more intensely stained than the rest of the cross-section (Fig. 1A–D).

In juveniles, some cartilage remained in the marrow cavity, and the youngest individuals did not show endosteal resorption (Fig. 1A). In the oldest juveniles (Fig. 1B) and adults, endosteal resorption was always present (Fig. 1C–D). Because this resorption can erode the first deposited LAGs, we compared the diameter of the smallest juvenile cross-section (one year old without resorption) to the diameter of the resorption line in the oldest individuals. In all of them, the resorption eroded the first line,

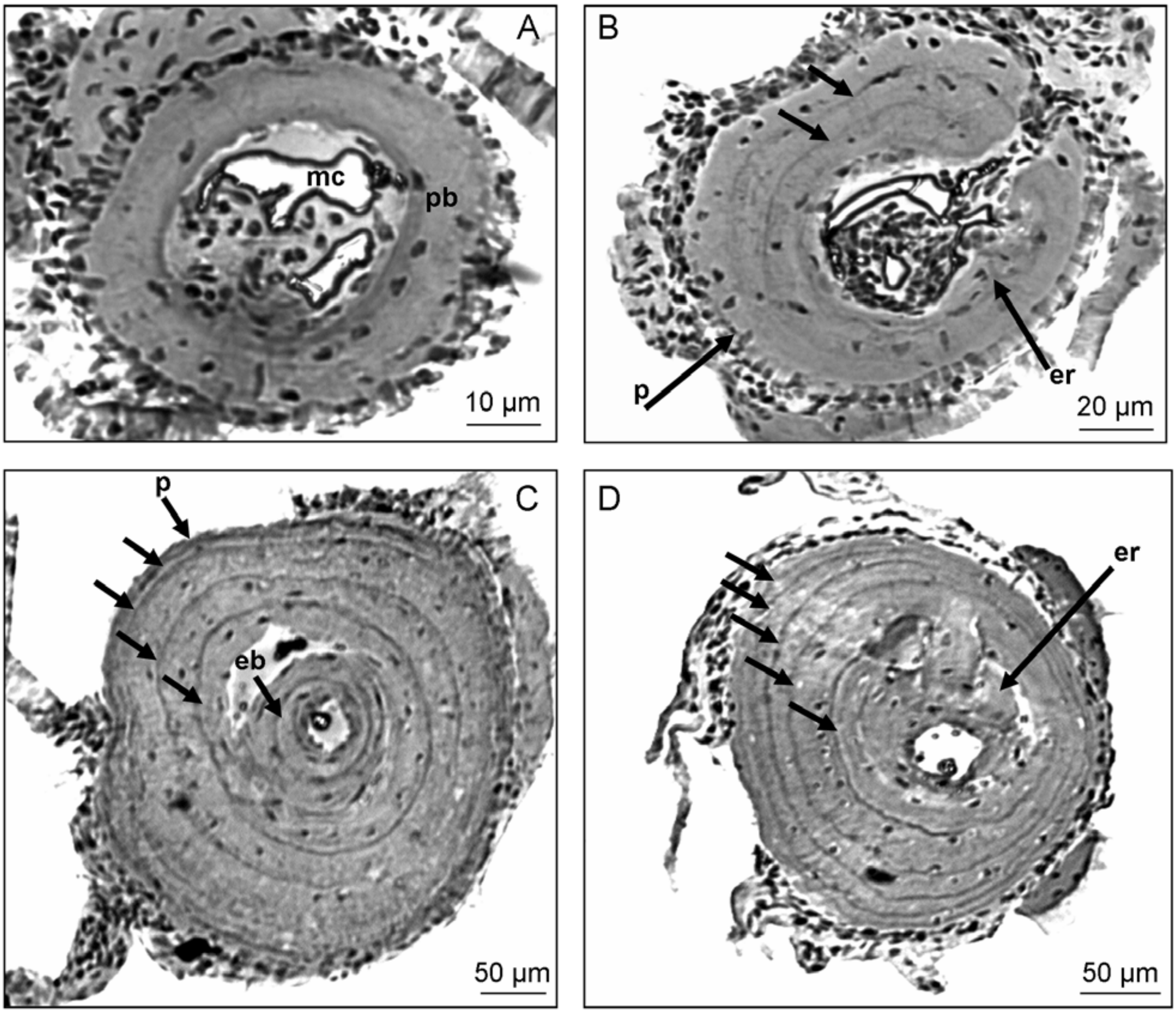

Fig. 1. Cross-sections (12 µm thick, Ehrlich's haematoxylin) at the diaphysis level of a phalange of juvenile, male and female Anatolian mountain frogs *Rana holtzi* from Lake Karagöl in Central Anatolia (Turkey). A) 1-year-old juvenile, 22 mm body length (×400). No Line of Arrested Growth (LAG) was observed in the periosteal bone. B) 3-year-old juvenile, 39 mm body length (× 400). Two LAGs were observed in the periosteal bone (arrows). Endosteal resorption was present. C) 6-year-old male, 48 mm body length (× 100). Four LAGs were observed in the periosteal bone (arrows). The innermost LAG was eroded by endosteal resorption. Note that LAGs are also present in the endosteal bone. D) 7-year-old female, 52 mm body length (× 100). Five LAGs were observed in the periosteal bone (arrows). The innermost LAG was eroded by endosteal resorption. Abbreviations: eb = endosteal bone, er = endosteal resorption, mc = marrow cavity, p = periphery, pb = periosteal bone.

but did not eliminate the second one. Because tadpoles metamorphose at the end of the active season, LAGs observed in phalanges were deposited only during the juvenile and adult stages. At more than 2500 m a.s.l. in the Bolkar mountains, this population is exposed to strong, yearly, cyclical climatic variation (Table 1). We thus assumed that the number of LAGs is equivalent to the number of winters experienced by each individual (Fig. 1A–C), thus giving a direct estimate of individual age.

Fifty-five bones (representing 13 juveniles, 22 males and 20 females) were analysed, 89% of which (n=49) allowed for an estimation of the individual's age. The age was estimated at ±1 year in 8% (n=4) of the usable phalanges. Unusable phalanges resulted from histological procedure problems such as decalcification, staining or sectioning outside the diaphyseal region.

Age, body and tibia length in juveniles, males and females

Descriptive statistics for age, body length and tibia length are shown in Table 2. The median age of juveniles was 3.5 yrs, significantly less than the median age of males (Mann–Whitney U-test, U=9.5, P<0.0001, n_1=13, n_2=22) and females (Mann–Whitney U-test, U=9.5, P<0.0001, n_1=13, n_2=21). The minimum age for adults was 4.0 yrs for both sexes, while the oldest age was 5.0 yrs in juveniles. The higher proportion of five-year-old individuals (both males and females) in the age distribution

Table 2. Descriptive statistics for age, body length and tibia length in the Anatolian mountain frog *Rana holtzi* from Lake Karagöl, Central Anatolia (Turkey). *n* = sample size, SD = standard deviation, CV = coefficient of variation.

	Juveniles	Males	Females
Age (years)			
n	10	21	18
Mean	2.9	6.1	6.2
SD	1.7	1.1	1.5
Median	3.5	6.0	6.0
Minimum	1.0	4.0	4.0
Maximum	5.0	8.0	10.5
CV (%)	59.31	18.29	24.23
Body length (mm)			
n	13	22	20
Mean	28.4	46.0	44.2
SD	6.1	3.6	6.9
Median	30.9	46.8	43.7
Minimum	20.4	37.1	35.7
Maximum	39.3	51.1	68.2
CV (%)	21.62	7.82	15.61
Tibia length (mm)			
n	13	22	20
Mean	16.4	26.4	24.8
SD	4.6	2.2	3.8
Median	17.4	26.9	24.8
Minimum	10.1	21.4	20.7
Maximum	22.7	30.6	38.6
CV (%)	28.39	8.32	15.28

(Fig. 2A) confirmed that age at maturity in this population of Anatolian mountain frog was 4–5 years.

The median age of adults did not differ significantly (Mann–Whitney U-test, U=187.5, P=0.96, n_1=22, n_2=21). The maximum age recorded was 8.0 yrs for males and 10.5 yrs for females (Table 2 and Fig. 2).

The median body length of juveniles was 30.9 mm (Table 2). The minimum body length was 37.1 mm and 35.7 mm in males and females, respectively, and males were significantly larger than females (Mann–Whitney U-test, U=123.0, P=0.04, n_1=22, n_2=21).

The median tibia length of juveniles was 17.4 mm (Table 2), significantly smaller than the median tibia length of males (Mann–Whitney U-test, U=2.0, P<0.0001, n_1=13, n_2=22), and females (Mann–Whitney U-test, U=9.5, P<0.0001, n_1=13, n_2=21). The minimum tibia length was 21.4 mm and 20.7 mm in males and females, respectively, and male tibias were significantly larger than female tibias (Mann–Whitney U-test, U=105.5, P=0.0039, n_1=22, n_2=21).

Male body and tibia length were also less variable than those of the female (CV smaller in males than in females, Table 2). The sexual dimorphism index (SDI) was –0.04 and –0.07 with body and tibia length, respectively.

Table 3 summarizes relationships between variables. The length (body or tibia) of the Anatolian mountain frog was poorly or not correlated with age. On the other hand, the two morphologic measurements (body and tibia length) were strongly positively correlated in both sexes.

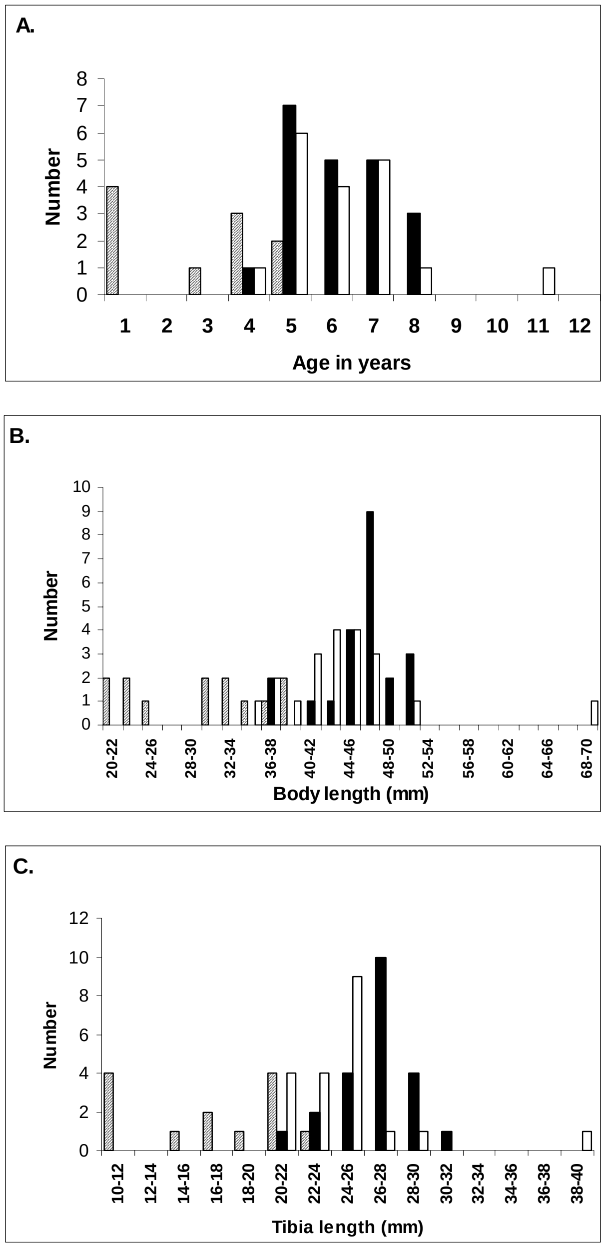

Fig. 2. A) Age distribution, B) body length and C) tibia length in Anatolian mountain frogs *Rana holtzi* from Lake Karagöl in Central Anatolia (Turkey). Hatched bars = juveniles, open bars = females, closed bars = males.

Growth in males and females

With r=0.701 and percentage of variance explained of 83.9% in males and r=0.515 and VE=71.7% in females, the von Bertalanffy growth model correctly fitted the age/body-length relationship of the sampled individuals (Fig. 3). Males and females did not significantly differ in growth parameters: the average maximum length (BL_{max}) was 53.4±1.36 mm for males and 57.6±2.44 mm for females. The growth coefficient k was 0.256±0.021 for males and 0.184±0.019 for females.

Table 3. Correlations among variables recorded in the Anatolian mountain frog *Rana holtzi* from Lake Karagöl, Central Anatolia (Turkey). R_s = Spearman correlation coefficient, *n* = sample size, *P* = significance.

	n	R_s	*P*
Males			
Age – total length	21	0.49	0.023
Age – tibia length	21	0.16	0.47
Total length – tibia length	22	0.80	<0.0001
Females			
Age – total length	18	0.37	0.126
Age – tibia length	18	0.43	0.073
Total length – tibia length	20	0.94	<0.0001

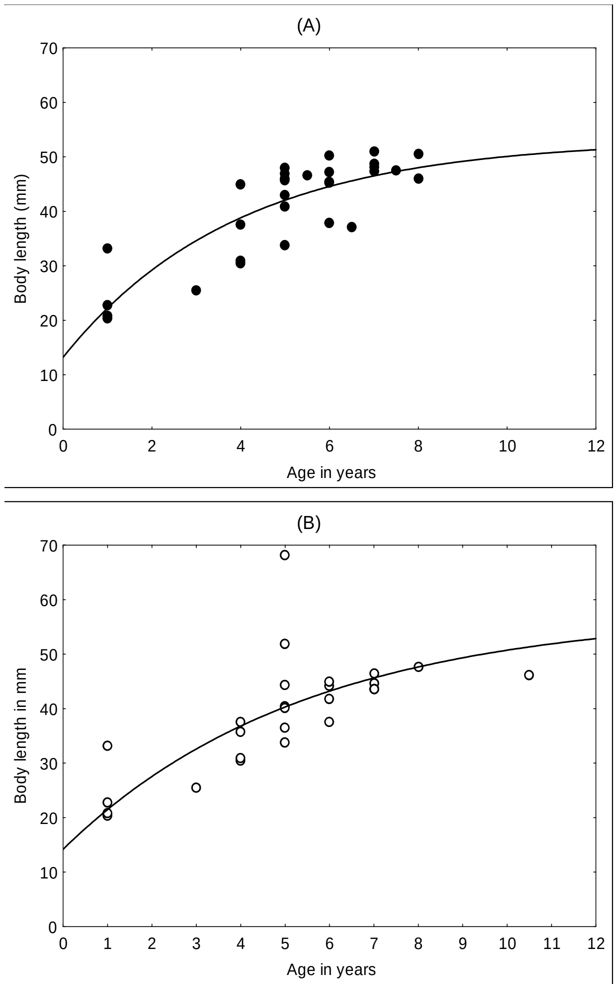

Fig. 3. Growth curves in A) male and B) female Anatolian mountain frogs *Rana holtzi* from Lake Karagöl in Central Anatolia (Turkey). Note that juveniles aged 1–3 years were the same individuals in A and B.

DISCUSSION

In the Anatolian mountain frog, the histo-morphological structure of the diaphysis of the phalange of the larger toe of the right hind limb was relatively uniform, with a bone matrix parallel-fibered. Stained LAGs were clearly visible (Fig. 1A–D) between zones of thicker layers of bone deposited during growth periods. The pattern of LAG deposition is considered to be genetically controlled with synchronization and reinforcement by seasonality (Alcobendas & Castanet, 2000). At the high altitude of Lake Karagöl in the Bolkar mountains, the active period is limited to May–October and growth stops during the remaining six months. The LAGS refer to this annual period of growth slow-down, and the age of the Anatolian mountain frog can be determined by counting these LAGs directly. Skeletochronology is widely used to estimate age in amphibians and reptiles (Castanet, 2002). Winter growth marks can be weakly expressed in warm, temperate climate regions (e.g. in *R. perezi* from southwest Spain [Esteban et al., 1996]). The LAGs in the Anatolian mountain frog were clearly stained, as previously observed in species exposed to harsh environmental conditions of high altitude or latitude (Esteban & Sanchiz, 2000; Lai et al., 2005; Leclair & Laurin,1996; Leclair et al., 2000; Miaud et al., 1999).

R. holtzi reached maturity at an age of 4–5 years, the median age of adults (6.0 years) was similar in the two sexes and longevity reached 8–10 years. These values are typical of species or populations living at high altitude, for example *Rana temporaria* in the Alps (Miaud et al., 1999) and *R. iberica* in the Pyrenees (Esteban & Sanchiz, 2000), and high latitude (e.g. *R. septentrionalis* in Quebec; Leclair & Laurin, 1996).

Sexual size dimorphism is observed in many amphibians, and ranid females are larger than males in *Rana ridibunda* (Yýlmaz et al., 2005), *R. porosa* (Khonsue et al., 2002), *R. epeirotica* (Tsiora & Kyriakopoulou-Sklavounou, 2002), *R. nigromaculata* (Khonsue et al., 2001a), *R. rugosa* (Khonsue et al., 2001b), *R. sylvatica* (Sagor et al., 1998; Leclair et al., 2000), *R. luteiventris* (Reaser, 2000), *R. temporaria* (Miaud et al., 1999), *R. perezi* (Esteban et al., 1996) and *R. septentrionalis* (Leclair & Laurin, 1996). In *R. holtzi* (this study), males were larger than females (male tibias were also larger than female tibias) as observed in *Rana nigrovittata* (Khonsue et al., 2000). It is difficult to conclude that males are larger than females in *R. holtzi* because Baran (1969) found that females were larger than males (range 41–61 mm for males and 41–74.5 for females, no mean value available), and Özeti (1970) described only osteological aspects without body size measurements.

The smaller size of one sex is often explained by a lower growth parameter, such as a smaller growth between metamorphosis and maturation as in *Rana temporaria* (Miaud et al., 1999) or as an adult in *R. iberica* (Esteban & Sanchiz, 2000). Growth parameters were, however, close between the sexes in the Anatolian mountain frog (k_{males}= 0.256±0.021 and $k_{females}$=0.184±0.019) and would contribute only marginally to the observed sexual size dimorphism. A larger size can also be attained thanks to a longer life span, but mean ages of *R. holtzi* were similar (and the oldest individual observed was a female). It would thus be very interesting to verify if this particular SSD is a constant characteristic of this population or species, and to look into, for example, behavioural ecology constraints that could lead to this bias.

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