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Life History Traits in a Turkish Population of the Agile Frog *Rana dalmatina* Fitzinger in Bonaparte, 1839 (Anura: Ranidae)

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Abstract: Using skeletochronology, we assessed the relationships of age and body size (SVL) in individuals from a population of the agile frog *Rana dalmatina* near the village of Özyurt, North-western Türkiye. The mean age and SVL of adult frogs were 4.50 ± 0.38 years and 61.54 ± 1.31 mm (6.46 ± 0.33 years and 67.86 ± 1.00 mm in females and 3.00 ± 2.71 years and 56.70 ± 1.26 mm in males, respectively). Intersexual differences in body size were female-biased (SSD = 0.196) while the age ranged between 4-8 years in females and 2-6 years in males. There was a positive correlation between body size and age (i.e., larger frogs were older) in both female and male individuals of this species.

Key words: mean age; body size; age at maturity; longevity; skeletochronology

Introduction

Determination of age, sexual dimorphism and relationships between age and body size in amphibians are important to reach knowledge on life-history traits (i.e. longevity, growth, population dynamics and the effects of environmental conditions on age and size amphibians) of these animals.

Skeletochronology, one of the age determination methods, is a very important method to examine the age structure of populations and to examine the life span of various species (Castanet 1994, Miaud et al. 2001, Cogălniceanu & Miaud 2003, Guarino et al. 2008, Patrelle et al. 2012). The number of studies addressing age determination to learn about the life-history traits of frog species has been increasing in recent years (Čavlović et al. 2018, Togane et al. 2018, Caldart et al. 2019, Guarino et al. 2019, Zhang et al. 2020). There is also an increase in the studies using skeletochronology and determining the age structure of Turkish frogs (Ismail & Çiçek 2017, Erismis 2018, Başkale et al. 2018, Arisoy & Başkale 2019).

The agile frog *Rana dalmatina* Fitzinger in Bonaparte, 1839 is a medium-sized frog with a body length of up to 9 cm (Baran et al. 2021). It is distributed over a wide area in West and Central Europe (Austria, Belgium, Czech Republic, Denmark, France, Germany, Hungary, Italy, Luxembourg, Slovakia, Slovenia, Spain, Sweden, Switzerland and the United Kingdom) and on the Balkans (Albania, Bosnia and Herzegovina, Bulgaria, Croatia, Greece, Montenegro, North Macedonia, Romania, Serbia and Thrace part of Türkiye) and Black Sea coasts (Türkiye and Ukraine); it generally prefers forests, grasslands and wetlands (Kaya et al. 2009, Baran et al. 2021). It has been classified as LC (Least Concern) in the IUCN Red List of Threatened Animals (Kaya et al. 2009). *Rana dalmatina* has terrestrial habits and occurs in various environments, mainly

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deciduous forests but also open habitats like grasslands within forested areas, from sea level up to 1500 m a.s.l. (Speybroeck et al. 2016). Studies examining large numbers of individuals of this species and focusing on their population biology are scarce, as they hide well when they are on land except during the breeding period when they enter the water. Although there are studies on the life history traits (age, size and growth) of the agile frog populations from Italy and the Iberian Peninsula (Guarino et al. 1995, Sarasola-Puente et al. 2011), the knowledge about the age structure of the population of the species in Türkiye is limited to the preliminary study of Genç & Tok (2021), who used skeletochronology on samples collected from different populations but not the relationships between the age and body length in a population.

The aim of the present study is to obtain knowledge on the mean age, longevity, age at sexual maturity, sexual size difference and age-length relationship in a Turkish population of *R. dalmatina*.

Materials and Methods

A total of 30 adult individuals of *Rana dalmatina* (17 ♂♂ and 13 ♀♀) were caught from a population (Özyurt population located in Devrek District, Zonguldak Province, Türkiye) on 10-15 April 2019. This population (41°04ˈ51.3ˈˈN, 31°48ˈ34.1ˈˈE) was located in a lowland area at altitude of 626 m a.s.l. The habitat of frogs consisted of a reed bed located in the middle of a forest of pine trees. Other amphibians sharing this living area: *Hyla orientalis* (Bedriaga, 1890), *Pelophylax ridibundus* (Pallas, 1771), *Lissotriton kosswigi* (Freytag, 1955) and *Ommatotriton nesterovi* (Litvinchuk, Zuiderwijk, Borkin & Rosanov, 2005).

The active period of frogs extends between early April and early October in the Özyurt population. According to climate data provided by the 9th Ankara Meteorology Regional Directorate, the mean annual temperature and precipitation over the last decade at the Özyurt site were 14°C and 103.21 mm, respectively. During the active period of frogs, the mean temperature and precipitation humidity were 18.6°C and 79.46 mm, respectively.

The frogs were caught by hand and sexed by direct examination of sexual characters (presence of grey-coloured nuptial pads on the first fingers of the male individuals during the breeding season). Snout-vent length (SVL) was measured to the nearest 0.1 mm using a digital calliper and was presented as Min-Max (Mean ± SD). We quantified Sexual Size Dimorphism (SSD) with the index of Lovich & Gibbons (1992) according to the following formula:

SDI = (mean length of the larger sex / mean length of the smaller sex) ± 1

For each frog, the second phalange of the longest finger of the hind limb was clipped and preserved in 10 % formalin solution for subsequent histological analyses. After registration and toe-clipping, the frogs were released back into their natural habitats. The animals were treated in accordance with the guidelines of the Ethics Committee of the Karadeniz Technical University.

The procedure of skeletochronology was based on a calculation of the lines of arrested growth (LAGs) in transverse sections of the middle part of the phalangeal diaphyses (in that case a portion of the second phalanx of the longest toe) (Bülbül et al. 2018). In the cross-sections of the present study, it was observed that the resorption zone did not reach the first LAG in all specimens. We evaluated the occurrence of endosteal resorption by direct observation of the cross-sections under the microscope.

We followed the experimental procedure used in the study of Bülbül et al. (2018). The clipped toes of the frogs were first preserved in a 10 % solution of formaldehyde, followed by peeling to facilitate the cross-sectioning process. Decalcification of bone tissue was performed by keeping the tissues for 2.5 hours at 5 % nitric acid solution. After decalcification, all samples of the toes were loaded into a tissue processing system (Leica TP1020 tissue processor). The skeletochronology protocol lasted 16 hours with 80-minute periods (change time of the solution) using solutions of alcohol (eight times), xylene (two times) and paraffin (two times), respectively. Later, all tissue samples were embedded in paraffin with a tissue embedding device (Thermo). The cross-sections (14 μm) were obtained from embedded phalanges with a rotary microtome. Using haematoxylin (non-acidified type, Thermo Scientific™ Shandon™ Harris Haematoxylin), the cross-sections were stained for 2 minutes. Entellan (Merck brand "Entellan® new", rapid mounting medium for microscopy) was employed for mounting the stained cross-sections on microscope slides. Finally, the cross-sections were studied under a light microscope and photographed with a ZEISS Axio Cam ERc 5s camera.

In order to minimize the error rate during counting the number of LAGs, the cross-sections were independently evaluated by two observers (M. Albayrak & U. Bülbül) and the results were compared. Not always the double lines represented real two LAGs due to the species having two arrested growth periods in the same year. However, most of

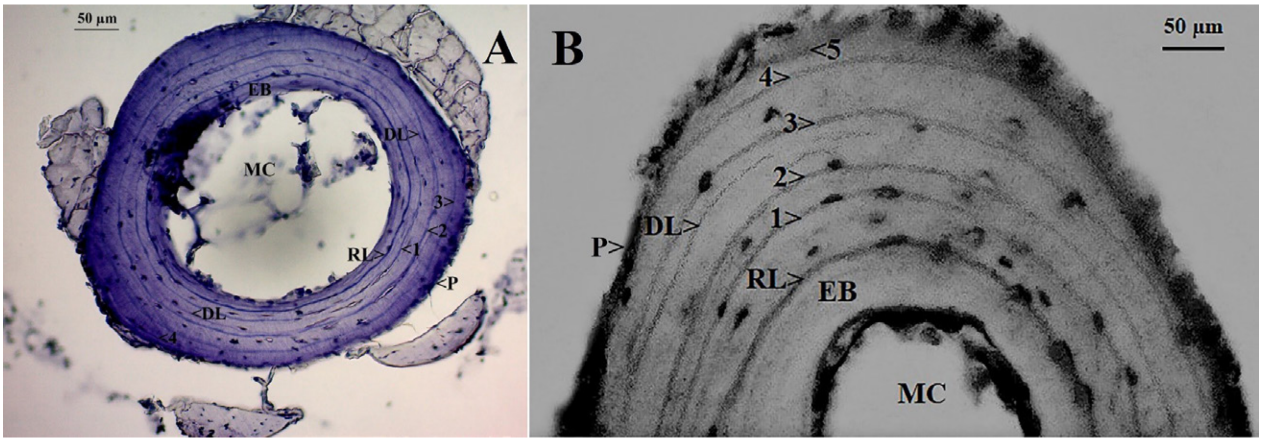

Fig. 1. A. Cross-section (20 µm thick) of a toe bone of a four-year-old male (60.11 mm SVL) of *Rana dalmatina* from the Özyurt population in Türkiye. The age was derived from the presence of five lines of arrested growth (LAGs 1-4) surrounding the resorption line. **B.** Cross-section (20 µm thick) of a toe bone of a five-year-old female (63.61 mm SVL) of *Rana dalmatina* from the Özyurt population in Türkiye. The age was derived from the presence of five lines of arrested growth (LAGs 1-5) surrounding the resorption line. MC – marrow cavity; EB – endosteal bone; RL – resorption line; DL – double line; P – periphery.

the environmental influences affecting the growth of a LAG occur within one year and the observed double lines were considered as one LAG in the age determination as suggested by Aragón & Fitze (2014), Guarino & Erismis (2008) and Bülbül et al. (2018). The distance between two adjacent LAGs has been accepted as a marker of individual growth in a given year (Kleinenberg & Smirina 1969, Özdemir et al. 2012). The point where an obvious decrease in spacing between two subsequent observed LAGs was taken to mark the age when sexual maturity was achieved (Ryser 1988, Özdemir et al. 2012, Bülbül et al. 2016). Besides LAGs, one ring in the innermost side was interpreted as metamorphosis line (ML). LAGs and ML can be easily distinguished from their different shapes as described by Ento & Matsui (2002).

As indicated in the study of Leskovar et al. (2006), we described the age-related parameters estimated variables: (1) age at maturity: the minimum number of LAGs counted in reproductive individuals; (2) longevity: the maximum number of LAGs counted in reproductive individuals; (3) potential reproductive lifespan: the difference between longevity and age at maturity.

All statistic tests were processed with IBM SPSS 22.0 for Windows. Since age classes and SVL measurements were normally distributed (One-Sample Kolmogorov-Smirnov Test, P> 0.05) within the sample, the parametric Independent Samples T-Test was used for comparison of means and Pearson's Rank Correlation Test ($P < 0.01$) to analyse correlations.

Results

A growth zone and thin haematoxylinophilic outer line corresponding to a winter line of arrested growth were present in cross sections of the phalanges in 100 % (n = 30) of adult, individuals (Fig. 1). The resorption zone did not reach the first LAG in all specimens. The resorption zone clearly seemed out of the endosteal bone in all preparations and never created difficulty for age determination. In most of the younger individuals, ML was found to be present while it was present in few older individuals only. The double lines were observed in 12 (40 %) adult individuals of *Rana dalmatina* from the Özyurt population and endosteal resorption was observed for only one (3.33%) adult individual. It was observed that male individuals reached sexual maturity at the age of 2 years while the age at maturity of females was 4 years.

SVL was 60.28-73.12 (67.86 ± 3.61) for females and 43.18-64.08 (56.70 ± 5.20) for males; age was 4-8 (6.46 ± 1.19) and 2-6 (3 ± 1.11), respectively. The mean SVL and age of all individuals (male and female) were 61.54 ± 7.20 mm and 4.50 ± 2.80 years. Age ranged from 4-8 years in adult females and 2-6 years in adult males (Fig. 2). The mean age of the specimens was different significantly between the sexes (Independent Sample T-Test; t = 8.148, df = 28, P = 0.000). The potential reproductive lifespan was estimated to 4 years in both sexes.

Intersexual differences in body size (length) were female-biased (SSD = 0.196). The mean SVL (t = 6.594, df = 28, P = 0.000) was significantly dif-

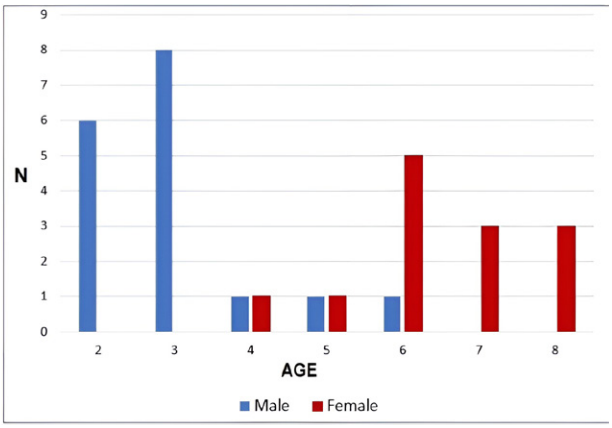

Fig. 2. Age frequency of male and female individuals of *Rana dalmatina* in the Özyurt population.

ferent between the sexes. There was a strong correlation between SVL and age for females (Pearson's correlation coefficient (r = 0.895, P = 0.000035) and there was a correlation in male individuals of the species (r = 0.525, P = 0.0302) (Fig. 3).

Discussion

Among the adult individuals (17 males and 13 females) of the Özyurt population, the oldest male individual was six years old and the oldest female individual was eight years old. Contrary to our findings, the longevity of *Rana dalmatina* from the Iberian Peninsula (in the samples of 63 males and 30 females) was found to be eight years in males and six years in females by Sarasola-Puente et al. (2011). In addition, Guarino et al. (1995) reported the maximum age of the species in an Italian population (18 males and six females) as five years in both sexes. Similarly, Genç & Tok (2021) when studying four males and four females of *R. dalmatina* from Çanakkale Province and seven males and one female of the species from the Kırklareli Province in Türkiye, found the longevity as five years in females and four years in males. The mean age, longevity and age at maturity can be influenced by genetic differentiation among different species or environmental factors when comparing among populations of the same species (Dubey et al. 2013, Gül et al. 2014, Bülbül et al. 2016, Sinsch & Dehling 2017). The individuals of *R. dalmatina* from the Özyurt Population live under stable and moderate climate conditions. Therefore, it can be considered normal for frogs to have a relatively high longevity (eight years) in the Özyurt population.

The males of *R. dalmatina* in the Özyurt population reached sexual maturity at two years of age, while in the females of the population sexual maturity age was four years. Similarly, Sarasola-Puente et al. (2011) reported that males reached sexual maturity at two years of age, although sometimes they can reach it at only one year of age. The females reached sexual maturity at two years of age, while a subset of the female population reached sexual maturity at three years of age. In addition, Genç & Tok (2021) determined the sexual maturity ages in both males and females of the species as two-three years. The age of sexual maturity may vary in different populations of the same species as well as in females and males within the same population (Bülbül et al. 2020). Amphibians at high altitudes or high latitudes grow more slowly and take longer to reach sexual maturity than do populations of the same species in warmer climates (Wells 2007). The altitude and latitude of the populations of *R. dalmatina* in the studies of Sarasola-Puente et al. (2011) and Guarino et al. (1995) are not strongly different from the results for the Özyurt population.

The body length of females of *R. dalmatina* was bigger than males (Guarino et al. 1995, Sarasola-Puente et al. 2011). Similarly, the mean SVL of females in the Özyurt population was found to be significantly higher than of males. According to the findings of Sarasola-Puente et al. (2011), the maximum SVL of *R. dalmatina* was 73 mm for females and 59.5 mm for males. In addition, Guarino et al. (1995) reported that the highest SVL was close to 64 mm in females and 57 mm in males. Similar to these findings, the maximum SVL was found to be 73.12 mm in female individuals and 64.08 mm in male individuals of the Özyurt population. The SVL may differ among populations from different geographical regions. In anurans, body size is expected to increase in colder areas, being inversely related to climatic factors (i.e., heat and sunlight) directly conditioning individual thermoregulation (Hutchinson & Dupre 1992). There is not much temperature difference among Türkiye, Italy and the Iberian Peninsula.

SSD in anurans is positively correlated with sex differences in age at maturity (Zhang & Lu 2013) and sexual differences in growth rates (John-Alder & Cox 2007, Tomašević-Kolarov et al. 2010). In accordance with this, we found that females reached sexual maturity later than males and the female-biased SSD for the Özyurt population was statistically significant. The female-biased SSD was also reported by Petrović et al. (2017) in adult individuals of *R. dalmatina* from Serbia and Montenegro. Another important factor in SSD is longevity (Liao & Lu 2010, Liapkov et al. 2010). Congru-

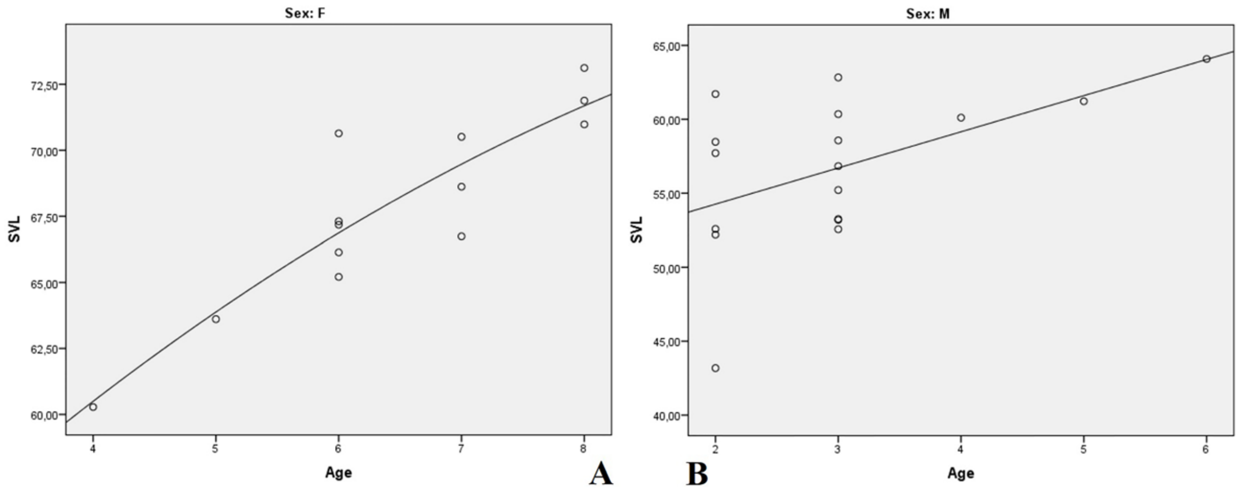

Fig. 3. Age-SVL relationships of both female and male individuals of *Rana dalmatina* from the Özyurt population. **A.** Females. **B.** Males.

ently, we found that longevity was higher in females than males. These results show that age at maturity and longevity may be the main determinants of SSD in the Özyurt population of *R. dalmatina*. However, we did not analyse the growth curves in each sex. On the other hand, differences in age at maturity between sexes could be observed if the sample size had been increased.

There was a correlation between SVL and age for both females and males of *R. dalmatina* in the Özyurt population. This allows in this species to use body size as a predictor of age. Similarly, Sarasola-Puente et al. (2011) that the correlation between age and SVL is statistically significant in both sexes of the species in an Iberian Peninsula population. However, Guarino et al. (1995) observed a positive correlation between SVL and age only in females of *R. dalmatina* from an Italian population. The limited sample size of the females (only six individuals) may be the reason for this result.

The environmental factors may have some effect on endosteal resorption and altitude in the living area may cause differences in endosteal resorption (Smirina 1972). In addition, Caetano & Castanet (1993) reported that individuals living in low-altitude populations have a lower rate of resorption compared to those living in high-altitude regions. Correspondingly, a low rate (3.3%) of endosteal resorption was found in the Özyurt population with low altitude (626 m a.s.l.).

The formation of a double line depends on conditions that are not exactly specified (Curtin et al. 2005). An environmental factor that can adversely affect the adequate nutrient intake in the habitat where the animals are present or the development of the creature may make it possible to form a double line or triple line (Yakin & Tok 2015). In the Özyurt population, a considerable amount (40%) of double-ring formation was observed. In this population, the adult individuals of the agile frog have many rivals (the adult individuals of *H. orientalis*, *P. ridibundus*, *L. kosswigi* and *O. nesterovi*) for limited food sources. These competitors may be preventing the individuals of *R. dalmatina* from utilising ample food resources and this feeding situation may have led to a high rate of double line formation. On the other hand, Guarino & Erismis (2008) reported that two instead of one LAG per year (double lines; Castanet & Smirina 1990, Castanet et al. 1993), related to a double cycle of annual activity (hibernation and aestivation), have been reported (Caetano et al. 1985, Caetano 1990, Caetano & Leclair Jr. 1999, Olgun et al. 2005, Iturra-Cid et al. 2010).

In conclusion, we evaluated some of the life history traits in a Turkish population of *R. dalmatina* and compared our findings with the results of other studies in the literature. There are limited studies revealing the life history characteristics of *R. dalmatina*. Studies to be carried out in the next years in other populations of *R. dalmatina* living under different ecological conditions may provide a better interpretation of the data obtained in the present study.

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