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LIFE-HISTORY TRAITS AND THE FIRST DEMOGRAPHIC DATA OF IRANIAN POPULATION OF THE WEST ASIAN LEMON-YELLOW TREE FROG, *HYLA SAVIGNYI* (AUDOUIN, 1827)

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The life-history of an organism consists of its lifetime pattern of growth, development, storage, age, and reproduction. In this study, some life-history traits of *Hyla savignyi* were studied in populations from different parts of Iran. The microscopic and macroscopic analysis showed that testicular activity in *H. savignyi* is potentially continuous, reaching its peak level in April. Metamorphosis was completed in approximately 102 days after egg deposition, and body size at metamorphosis was 10 mm. Significant sexual size dimorphism was present in all populations, and a larger female asymptotic body size was observed (43.07 mm for females vs 41.16 mm for males). The adult survival rate (S) and life expectancy (ESP) were the same for both sexes (S = 0.73 and ESP = 4.2 years). Age and body size were positively correlated with each other for both females and males. Maximum longevity was recorded to be six years in both females and males, and ages of sexual maturity were estimated to be two or three years in breeding individuals. The adult sample age ranged from two to six years (mean age of females: 4.40±0.68 years; males: 3.63±0.13 years). Our data confirm the general patterns of body size variation and mean age in anurans and show that females are larger than males and live longer.

Key words: life-history, *Hyla savignyi*, metamorphosis, sexual size dimorphism (SSD), survival rate, life expectancy, tree-frog.

INTRODUCTION

The amphibian growth rate is influenced by temperature, rainfall and humidity, diet, competition, predation, and intrinsic characteristics, e.g., body length and egg size (Duellman & Trueb 1986, Berven 1982). A variation in these characters is correlated with altitude and latitude; they can potentially be used to assess amphibians' lifespan under different climatic conditions (Berven & Chadra 1988, Berven *et al*. 1979). Sexual maturity is an important phase in the life-history of any organism, and parameters such as age, size, and the formation of sex cells define this stage (Bernardo 1993, Adolph & Porter 1996). There are various hypotheses regarding how biotic and abiotic factors influence age, length, and body size at sexual maturity; the optimal timing of sexual maturity is ideally achieved when the costs and benefits of maturing at different ages and lengths are balanced around an equilibrium point or along with a reaction norm (Stearns & Koella 1986). Intraspecific phenotypic plasticity provides an important source of information for studying the evolution of life-history traits and adaptation to different environmental pressures (Berven 1982). The traits that constitute an organism's life-history can also be seen as "coadapted and designed by natural selection to solve a particular ecological problem" (Stearns 1976), which in turn makes it important whether diversity among populations and species is generally phenotypic or genotypic (Berven & Gill 1983).

Spermatogenesis is one of the basic aspects of the reproductive cycle in vertebrates (Konvalina *et al.* 2018). The annual spermatogenic cycles in anurans have been grouped into three main categories: continuous, discontinuous and potentially continuous (Lofts 1974, Kaptan & Murathanoğlu 2008). Tropical species occurring in environments that do not show major fluctuations in climatic conditions have evolved a continuous spermatogenic cycle (Kaptan & Murathanoğlu 2008) indicated by the continuous presence of spermatozoa in the testes throughout the year (Lofts 1974, Sasso-Cerri *et al.* 2004). Spermatogenesis in temperate anurans is completely seasonal and can take the form of either discontinuous or potentially continuous types (Kaptan & Murathanoğlu 2008). In the discontinuous type, males enter an inactive sexual phase after spawning; in the potentially continuous type, they show a partial cessation of spermatogenic activity during certain seasons of the year, though primary spermatogonia remain sensitive gonadotrophic stimulation (Huang *et al.* 1997).

In the case of inter-population variation, amphibians are often well suited for life-history studies. Many amphibian populations show marked fluctuations in size and age structure (Driscoll 1999). Knowledge of variation in age and body size of individuals is necessary to develop management plans and protect amphibian populations in their natural environment (Driscoll 1999).

Age determination studies, in particular, are needed to understand the dynamics and life-history of populations of amphibians and reptiles. The skeletochronology of amphibians has been widely established for around three decades (Gibbons & McCarthy 1986, Morrison *et al*. 2004, McCreary *et al.* 2008). The effects of climate conditions on the ectotherm vertebrate metabolism are recorded in their growth by the formation of lines of arrested growth, LAGs, providing a suitable method for determining age in natural populations (Olgun *et al*. 2005). The cool season gives rise to narrow and dark rings in bone corresponding to reduced growth and higher concentrations of organic and inorganic materials; in contrast, the warm season leads to the formation of looser and wider rings (Gibbons & McCarthy 1986, Morrison *et al*. 2004).

The West Asian Lemon-yellow Tree Frog, *Hyla savignyi* (Audouin, 1827) is widely distributed in western Asia. In Iran, it can be found in the North, West and Southwest regions of the Iranian plateau at sites with permanent water and dense grass in the Zagros and Alborz Mountains, at altitudes ranging from 350 to 2000 m (Baloutch & Kami 1995, Cheatsazan *et al*. 2005). We collected samples of *H. savignyi* from some populations in Iran. The primary aim of our study was to determine testicular activity, reproduction season, and time and size at metamorphosis in *H. savignyi* of these populations. We also analyzed and compared the growth rate, age structure, age at sexual maturity, and longevity in the species.

MATERIALS AND METHODS

Study area and sampling – The study areas were located in two Iranian Provinces, Lorestan and Markazi. Frog samples were collected under permit 95/10168 granted by the Iranian Department of Environment. Surveys were conducted at night from March to October 2019 in various aquatic habitats, including ponds, unstable stagnant waters, rock and soil pools, wetlands, and creeks. A total of 72 specimens (67 males, 5 females) of *H. savignyi* were caught by hand. Snout-vent length (SVL) was measured with a digital calliper to the nearest 0.01mm. The second segment was cut from the longest toe (4th) of the right or left hindlimb and placed in formaldehyde (10%) for histological analysis from each specimen. Examined female frogs were subsequently released at the collection site of their capture, whereas males were transported to the Zoology Laboratory, Arak University, Arak, Iran, for use in the histological analysis of the gonads to estimate the reproductive season.

Reproductive season – Male frogs were euthanized with chloroform, and their gonads were removed and placed in formaldehyde (10%). All experimental procedures and animal care were performed according to the protocols approved by the Department of Biology of Arak University. Histological preparations were made according to Bancroft and Stevens (1990). Each testis was dehydrated in alcohol, cleared in xylene, embedded in paraffin wax at 56°C (melting point), then sectioned at 5–7 μm thickness. Each section was then stained using the Hemotoxylin and Eosin (H & E) staining method. Cross-sections prepared from each sample were examined to detect different types of spermatogenic cells, such as spermatogonia, primary and secondary spermatocytes, spermatids, and free spermatozoa. According to Behera (2012), during the spawning season, testis lobules of the banded gourami (*Trichogaster fasciata*), a seasonally breeding tropical freshwater fish, were completely packed with spermatozoa, the testicular and lobular walls became thinner, and the intralobular space decreased, whereas in the post-spawning season there was a remarkable decrease in spermatozoa. We used the above pattern to interpret the cross-sections of male gonads of *H. savignyi*, because there is the same reproductive pattern in both species (Rugh 1951). We compared mean testis size (testis length) and weight in different months of the year. Descriptive statistics, a one-way analysis of variance (ANOVA) and Scheffe's post hoc tests were used to analyze seasonal variation in histological and quantitative studies.

Age determination – Age estimation was performed using the standard method of skeletochronological analysis according to Castanet and Smirina (1990) by counting the lines of arrested growth (LAGs) in stained cross-sections of the phalangeal bones (Jin *et al*. 2017). Phalanges were decalcified in nitric acid (5%), and after dehydration, serial sections (18–20 μm) from the mid-diaphyseal region were cut using a freezing microtome and then stained with H & E. Cross-sections were explored under both a light microscope (Olympus BX51) and Scanning Electron Microscopy (SEM). The cross-sections were fixed under a light microscope (Olympus BX51) and were independently confirmed by two observers for age estimation. Longevity (maximum lifespan) was estimated according to Otero *et al*. (2017) as the maximum number of LAGs counted in adults. To estimate age at sexual maturity, we used a combination of three methods (average of all three methods): 1. the smallest distance between two subsequent LAG (Ryser 1998); 2. the minimum number of LAG counted in breeding individuals (Otero *et al*. 2017); and 3. lowest age recorded amongst the breeding individuals (Arisoy & Başkale 2019).

Growth estimation – We used Shapiro-Wilk's test to assess normality and the Mann-Whitney U-test test to compare the age structure between sexes. Sexual size dimorphism was computed according to the sexual dimorphism index, where SDI = (average length of larger sex/average length of smaller sex) ±1; the result is arbitrarily defined as positive (+1) if females are larger than males, and negative (-1) if males are the larger sex (Lovich & Gibbons 1992). Survivorship rate for adults of this species was calculated by the following formula: $S = T / (R + T - 1)$, where S is the finite annual adult survival rate estimate, $T = N1 + 2N2 + \ldots + nNn$, $R = \Sigma Ni$, and Ni described as the number of the adult in the age group i. Adult Life expectancy (ESP) was computed by the formula of Seber (1973) , where $ESP = [0.5 + 1 / (1 - S)$ and S = adult survival rate. As with the general patterns of ectotherms, amphibian growth follows an S-shaped curve (von Bertalanffy 1957). The growth rate was assessed according to the von Bertalanffy equation: $SVLt = SVLasym.max (1 e - k (t - t0))$, where age at metamorphosis is t0, SVL at the age of metamorphosis is SVLt0, and the growth coefficient called k (von Bertalanffy 1957). To determine the age and size at metamorphosis, one egg clutch of *H. savignyi* was collected from a natural and clean pond in the Makhmalkoh -Khorramabad region 48.30°N, 33.59°E and 1300 m above sea level (a.s.l.), on 21 January 2017. Clutches were stored in separate polyethylene tubs (600 × 400 × 250 mm) filled with water from the natural habitat and transported to the Biology Laboratory, Arak University, Arak, Iran. We used the NLS method in "stats" package on R software to fit the Bertalanffy model.

RESULTS

Reproductive season

We found that peak reproductive activities (spawning season) in *H. savignyi* occur from late March to late April (Fig. 1*a*), and the period of lowest sperm production (post-spawning season) was during the cool season from August–February (Fig. 1*b*). Microscopic examination of the histological sections of male testes indicated that the total length of the breeding season was somewhat longer, from late March to late July; after that, the number of cells in the final stages of spermatogenesis (spermatids and sperm) gradually declines. During this period, the germinal layer begins to rebuild, and only spermatogonia and spermatocytes can be observed in the seminiferous tubules. Besides, the interstitial tissue thickness increased during this period, and the lumen of seminiferous tubules contained large ducts almost devoid of sperm. This is a confirmation of our statistical analysis (Fig. 1).

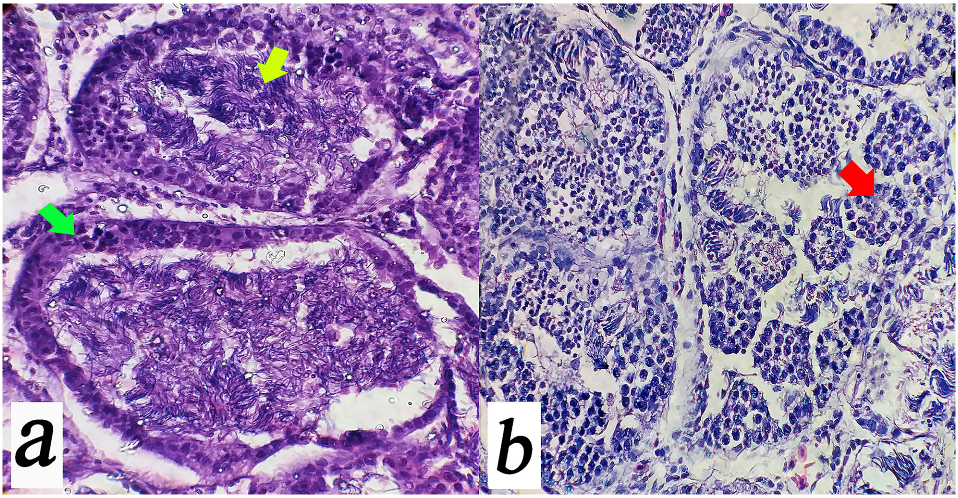

Fig. 1. Comparison of reproductive state in male *H. savignyi* during the: a) active period April, yellow arrow: spermatozoa, green arrow: spermatogonia; b. inactive period following the spawning season in October, red arrow: spermatocyte cyst

Our variance analysis showed that the testes differed significantly in mean weight and size across months ($p < 0.001$). Scheffe´s post hoc test showed that the testes differed significantly in April compared to other months. Specifically, male testes attained their greatest size and mass in April as compared to other months (Fig. 2). Consequently, these findings support the histological analysis.

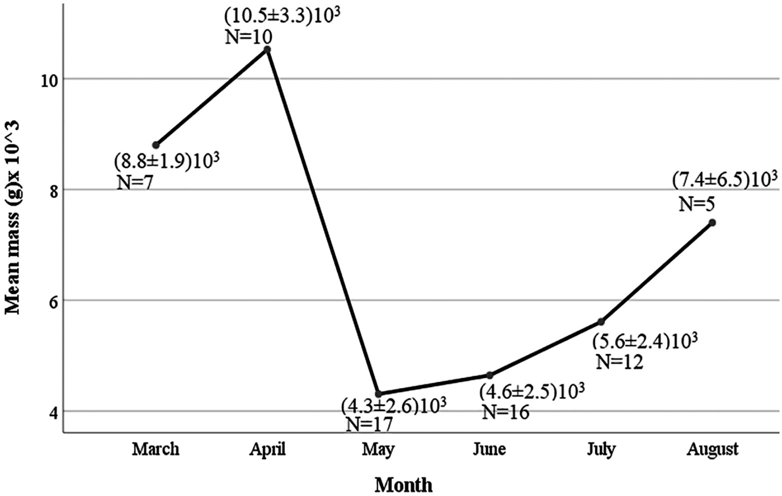

Fig. 2. Change in mean testis mass ± standard deviation in *Hyla savignyi* in different months of the year

Table 1. Comparison of life-history traits of *H. savignyi* in Iran. S: survival rate; ESP: life expectancy.

Sex	n	Average age (years)	Range age	Average SVL (mm)	Range SVL (mm)	S	ESP (years)
Female	5	4.40±0.68	3–6	40.92±2.08	36.04–47.06		
Male	67	3.63±0.13	2–6	38.47±0.45	29.34–44.03	0.73	4.2

Growth estimation

According to our results, the first larvae completed metamorphosis (t0) about 0.3 years after egg deposition. At that time, metamorphosed larvae had total body lengths of 10 mm (Lt0). The growth coefficient was 0.77 in males and 0.73 in females. Both sexes had a higher maximum SVL (47.06 mm for females and 44.03 mm for males) than asymptotic SVL (SVLasym was 43.07 mm for females and 41.16 mm for males). We found that females were slightly larger than males (Table 1). Although the SDI was 0.06, indicating an intersexual difference in body length (female-biased SSD), SVL did not differ significantly among females and males according to the Mann-Whitney test; P = 0.207.

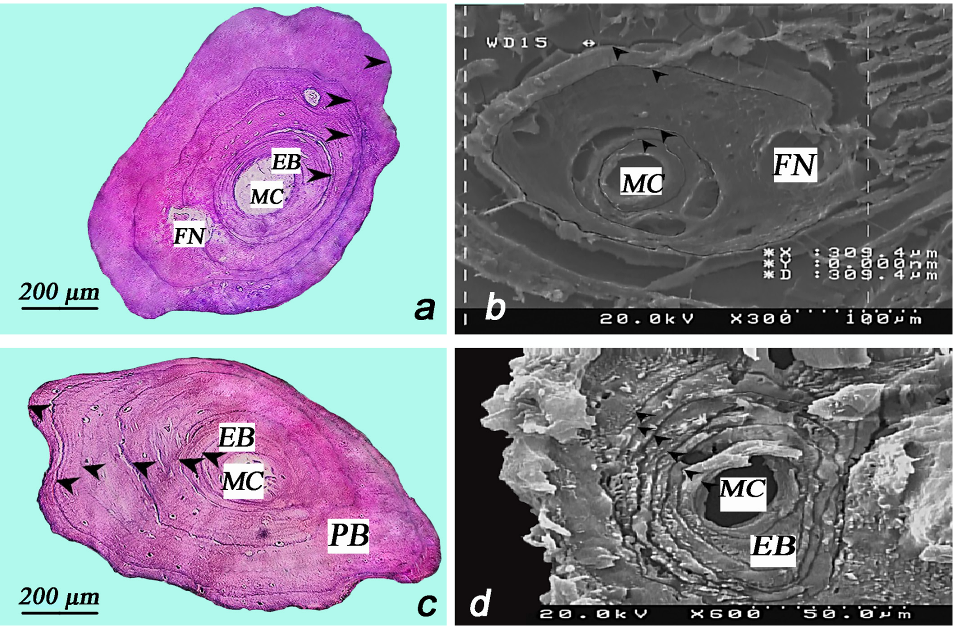

Fig. 3. Light-microscope photographs (a and c) and images from low-magnification SEM (b and d) of lines of arrested growth (LAGs) of second phalangeal bones in *H. savignyi*. a: 4-years old male (SVL = 42.25); b: 4-year old male (SVL = 40.29); c and d: 6-years old female (SVL = 47.06); MC = marrow cavity, EB = endosteal bone, PB = periosteal bone, FN = nutrient foramen

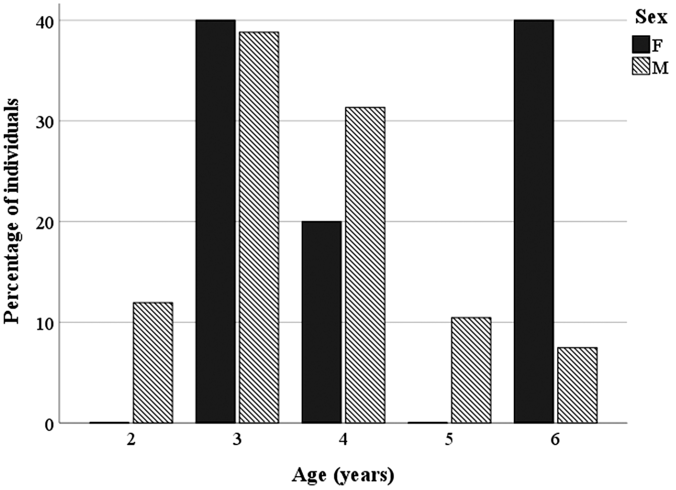

Fig. 4. Age distributions of specimens according to sex in *Hyla savignyi*

Bone histology and skeletochronological analysis
(age structure, age at sexual maturity, longevity, and growth)

In all cross-sections, hematoxynophilic lines were marked and estimated age obtained by both direct observation in the light microscope (Fig. 3*a, c*) and visualization using low-magnification SEM (Fig. 3*b, d*). The mean age of females was 4.40±0.68 years (range 3–6 years), while the average age in males was 3.63±0.13 years (range 2–6 years; Table 1). There was no significant difference in age structure between females and males (Mann-Whitney test; W = 215.5, P = 0.27); therefore, survival rate (S) and life expectancy (ESP) calculations were combined (S = 0.73, ESP = 4.2 years) for both sexes (Table 1). Ages of sexual maturity were 2 or 3 years in breeding individuals, and longevity (maximum lifespan) was found to be 6 years for both sexes (Table 1 & Fig. 4).

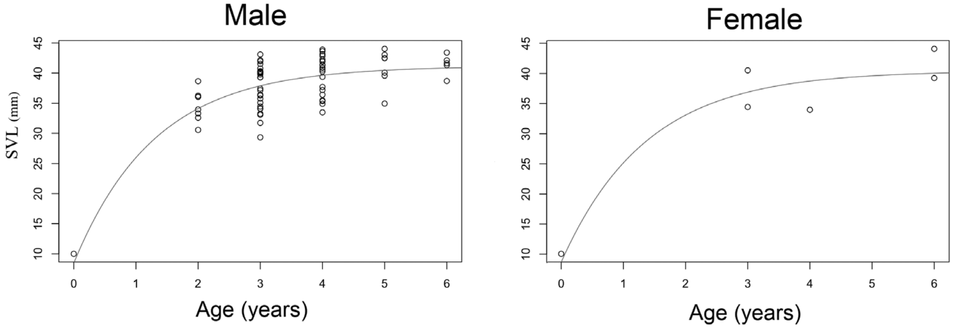

Fig. 5. Relationship between snout-vent length (SVL) and age structure of breeding female and male *Hyla. savignyi*

A significant positive correlation was detected between age and body size (SVL) in both sexes (Spearman's correlation; $\varrho = 0.51$, $P < 0.001$). The growth curves represented a good fit for a correlation between body size and age (Fig. 5).

DISCUSSION

Generally, three patterns have been proposed for the annual spermatogenetic cycles in anurans: discontinuous, potentially continuous, and continuous (Lofts 1974). Frogs and toads in temperate regions have evolved a discontinuous or potentially continuous spermatogenic cycle, whereas those inhabiting tropical areas often have evolved a continuous type of spermatogenic cycle (for more information, refer to Huang *et al*. 1996, Kaptan & Murarhanoglu 2008). Huang *et al*. (1996) reported that the spermatogenetic cycle of the Taiwan endemic toad (*Bufo bankorensis*) is continuous. In another study, Lofts (1974) reported that the annual distribution of germ cells in *Pelophylax ridibundus* indicates that spermatogenetic activity appears to be continuous; dissimilar results were reported by Loumbourdis and Kyriakopoulou-Sklavonou (1991) and Kaptan and Murarhanoglu (2008) on populations from the northern part of Greece and plains of Geyve (East Marmara) respectively, where spermatogenetic activity is potentially continuous. Pesarakloo and Najibzadeh (2019) reported that *Pelophylax bedriagae* spawns in early April in breeding ponds in the Khorramabad region of western Iran. The sympatric amphibian species, *Bufotes variabilis* (Pallas) and *Hyla savignyi* are not active during this season (Baloutch & Kami 1995). Field observations of *H. savignyi* microscopic examination of histological sections of the testes and macroscopic analysis (testis weight and size) showed that a high level of breeding behaviour occurred in March–July, and the highest level of sperm production occurred in April. Thus, March–July was considered to be the breeding period. According to our results, accounting for the observed pattern of spermatogenetic activity and change in mass and size of the testes, the reproductive cycle of *H. savignyi* is the potentially continuous type.

According to our field observations, the metamorphosis was completed approximately 102 days after egg deposition, and body size at metamorphosis measured 10 mm. The embryonic and larval periods in anurans vary across between species and geographically within species in the region: Toxopeus *et al*. (1993) describe a study of *Pelodytes punctatus* from France (i.e., not Iran or elsewhere in Central Asia), so it doesn't make sense to cite it here in the context of "the region" (i.e., Central Asia). For example, the metamorphosis was completed in 73 days for *Rana pseudodalmatina* in Golestan Province, Iran (Pesarakloo *et al*. 2015), and 43–90 days for *P. ridibundus* in Anzali Lagoon, Iran (Mirzajani *et al*. 2006), but 80–120 days for another population of the latter species in France (Momeni & Zamatkesh 2005). In contrast, *P. bedriagae* had finished their development and commenced metamorphosis after 111 days (Pesarakloo & Najibzadeh 2019).

Generally, three patterns have been proposed for sexual dimorphism in amphibians, according to body size (SSD): Female-biased (females larger than males), male- biased (males larger than females), and unbiased (males equal to females) (Zhang *et al.* 2016). Bülbül *et al*. (2018) reported a slight female-biased SSD in the Enez population of *Bombina variegata*. According to our results, a female-biased SSD was observed in *H. savignyi*; SVLasym was 43.07 mm for females and 41.16 mm for males. Although no significant difference was observed between females and males concerning mean SVL and mean SVLasym, data obtained from the current study confirm the general patterns of body size variation in anurans and suggest that females are larger than males (Shine 1979, Serra-Cobo *et al*. 2000, Kupfer 2007, Reinhard *et al*. 2015, Zhang *et al*. 2016). According to previous studies, there are at least two reasons that could explain female-biased SSD in anurans. In one study, most males did not defend territories, offer resources to females, or care for offspring, and all males, small or large, were able to fertilize all eggs of any female (Vargas-Salinas 2006). Consequently, intersexual selection is not expected to favour the reproductive success of larger males (Vargas-Salinas 2006). Alternatively, age differences (Monnet & Cherry 2002, Young 2005, Liao *et al*. 2011) and survival (Vargas-Salinas 2006) between males and females can also explain female-biased SSD. Females are larger than males in some frogs because there are higher levels of mortality in males (Meshaka 2001). Indeed, lower survival in males means that females have more time to grow than males (Vargas-Salinas 2006).

Growth coefficients were slightly higher in males (0.77) than in females (0.73). Our results are in contrast to those seen in most amphibians, which usually show higher growth coefficients in females than in males (Ryser 1988, Erişmiş & Chinsamy 2010, Çiçek *et al*. 2011, Patrelle *et al*. 2012, Erişmiş 2018, Başkale *et al*. 2018). Large body size in females may be affected by survival (Vargas-Salinas 2006) and age differences (Monnet & Cherry 2002, Young 2005, Liao *et al*. 2011). This is in agreement with our results, where the mean age of females was 4.40 years, while the average age in males was 3.63 years (Table 1). Indeed, lower survival in males means that females have more time to grow than males (Vargas-Salinas 2006). Wells (2001) argued that acoustic competition during the breeding season in frogs might explain lower local survival in males, a conclusion consistent with the literature (Tsiora & Kyriakopoulou-Sklavounou 2002, Socha & Ogielska 2010, Çiçek *et al*. 2011, Gül *et al*. 2011, Erişmiş 2018, Arisoy & Başkale 2019). The life expectancy of females and males of *Pelophylax caralitanus* was estimated to be 6.61 and 6.15 years, respectively (Arisoy & Başkale 2019), and the mean survival rates of females and males were calculated as 0.84 and 0.83, respectively. Similarly, the survival rate in adult *Rana temporaria* was 0.82 in males and 0.80 in females, and the expected total longevity of frogs that reached sexual maturity was 6.1 years in males and 5.5 years in females (Miaud *et al*. 1999). In the present study, the survival rate (S) was calculated to be 0.73 for both sexes, with a life expectancy (ESP) of 4.2 years.

Numerous researchers (e.g. Hemelaar 1985, Ryser 1988, Kutrup *et al*. 2005, Altunışık 2018) have found that the age at maturity may vary between different species and populations of anurans (Miaud *et al*. 2000, Altunışık 2018). In several studies, it was reported that male anurans mature earlier than corresponding females (Monnet & Chery 2002, Matthews & Miaud 2007, Kutrup *et al*. 2011). Intraspecific variation in age at maturity and adult body length along geographic (altitudinal and latitudinal) gradients is well-documented in anurans. However, patterns are often inconsistent (Miaud *et al*. 1999). Arisoy and Başkale (2019) showed that maturity ages were 2 or 3 years in both sexes of *P. caralitanus*, whereas the age at maturity of *R. dalmatina* was determined to be 2 years (Sarasola-Pue *et al*. 2011). In another study, Hemelaar (1988) estimated that age at maturity was 1.77 years for males and 2.49 years for females of *Bufo bufo*. The maximum age for *R. latastei* recorded to date was 3 years in males and 4 years in females (Guarino *et al*. 2003). Our data in the present study showed that maturity ages were about 2 or 3 years in breeding individuals, and longevity (maximum lifespan) was found to be 6 years for both females and males, consistent with the results of the study of *H. savignyi* in Turkey (Kalayci *et al*. 2015). Reznick (1990) proposed a model that implies that the correlation between age and body length at maturity remains constant over different environmental conditions. Halliday and Verrell (1988) stated that there are positive correlations between age and body size in amphibians. A strong positive correlation between SVL and age was reported for males and females of *B. variegata* (Bülbül *et al*. 2018) and *B. bombina* (Cogâlniceanu & Miaud 2003). In the present study, a significant positive correlation was detected between age and body size in both sexes of *H. savignyi*. Our results indicate that the largest individuals are almost always the oldest in both sexes and males were usually smaller and younger than females. Similar results have been reported in *H. savignyi* in Kermanshah province, Iran (Rastegar-Pouyani *et al*. 2015) and Nasiriyah province, Iraq (Aljaberi & Al Moussawi 2020), and in *H. arborea* in Turkey (Özdemir *et al*. 2012).

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