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Morphological variability and age structure in a population of *Bufo verrucosissimus* (Anura: Bufonidae) from Artvin, Turkey

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Abstract

Morphological variability and age structure in a population of *Bufo verrucosissimus* (Anura: Bufonidae) from Artvin, Turkey. We investigated morphological diversity (external body traits, coloration patterns and skin structure) and age structure in a population of Caucasian toad (*Bufo verrucosissimus*) from Lake Borçka Karagöl, situated in the vicinity of Artvin on the northeastern border of Turkey and Georgia. Age was determined using phalangeal skeletochronology. The external morphological variability was assessed using the linear measurement of 27 body-related characters and the photographs of each specimen. According to the results, the means of head length and head width were found significantly lower in Karagöl population as opposed to Caucasian populations, but average body size did not differ among all populations. Coloration and pattern features of the specimens from Karagöl were identical to those of Caucasia. Most of the females have brown tones and males are usually olive green and brown. The indiscrete elongated dark bands on parotoids did not extend behind the gland. PCA analysis confirmed a clear separation between the sexes and a high degree of female-biased sexual size dimorphism was determined based on the body size (SDI index: +0.41). The constructed Von Bertalanffy growth curve models yielded similar profiles in both sexes. Body size and age were significantly correlated in both models, but growth coefficient value was higher in males. Accordingly, the mean age of the Karagöl population is greater in both sexes, but the lifespan and maximum age are greater in Caucasus populations.

Keywords: Body measurements, Caucasian toad, Life history, Skeletochronology.

Resumo

Variabilidade morfológica e estrutura etária em uma população de *Bufo verrucosissimus* (Anura: Bufonidae) de Artvin, Turquia. Investigamos a diversidade morfológica (características externas do corpo, padrões de coloração e estrutura da pele) e estrutura etária em uma população do sapo caucasiano *Bufo verrucosissimus* do Lago Borçka Karagöl, situado nas proximidades de Artvin, no nordeste da fronteira da Turquia com a Geórgia. A idade foi determinada utilizando

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esqueletocronologia falageal. A variabilidade morfológica externa foi avaliada utilizando a medida linear de 27 caracteres relacionados ao corpo e fotografias de cada espécime. De acordo com os resultados, as médias do comprimento e largura da cabeça foram significativamente menores na população de Karagöl do que nas populações caucasianas, mas o tamanho médio do corpo não foi diferente entre as populações. As características de coloração e padrão dos espécimes de Karagöl foram idênticas às do Cáucaso. A maioria das fêmeas apresenta tons castanhos, e os machos são geralmente verde oliva e castanhos. As faixas escuras alongadas não se estendem por alémde das glândulas paratóides. A análise PCA confirmou uma separação clara entre os sexos, e um elevado grau de dimorfismo sexual nas fêmeas foi determinado com base no tamanho do corpo (índice SDI: +0,41). Os modelos construídos de curva de crescimento de Von Bertalanffy produziram perfis semelhantes em ambos os sexos. O tamanho do corpo e a idade foram significativamente correlacionados em ambos os modelos, mas o valor do coeficiente de crescimento foi mais elevado nos machos. Consequentemente, a idade média da população de Karagöl é mais elevada em ambos os sexos, mas a expectativa de vida e a idade máxima são maiores nas populações do Cáucaso.

Palavras-chave: Esqueletocronologia, História de vida, Medidas corporais, Sapo-caucasiano.

Introduction

The Caucasian toad, *Bufo verrucosissimus* (Pallas, 1814), occurs throughout the Caucasian diversity hotspot classified as Transcaucasia covering the center of Caucasia including Artvin (on the northeastern of Turkey) and Ciscaucasia covering the southernwest of Russian Federation representing the northern part of the Caucasus (Orlova and Tuniyev 1989, Kidov 2009, Recuero *et al.* 2012, Tuniyev *et al.* 2014). In addition, the presence of the toad was also reported in the south of Turkey (Antalya, Mersin, Osmaniye, Hatay) (Özdemir *et al.* 2020) and from Lebanon (Moukhtara) (Jablonski and Sadek 2019). The species was previously delimited under four morphology based subspecies, namely *B. verrucosissimus verrucosissimus* (Pallas, 1814) spreading from the Black Sea coast of Turkey to the inner part of Georgia and extending to Great Caucasus mountains, *B. verrucosissimus turowi* (Krasovsky, 1933) from in a narrow zone at the north of Greater Caucasus, *B. verrucosissimus circassicus* (Orlova and Tuniyev, 1989) from the northern coastal side of Greater Caucasus Mountain Range, and *B. verrucosissimus tertyschnikovi* (Kidov, 2009) inhabiting Stavropol Krai of the Russian Federation. However, the latest genetic studies have indicated the presence of three different lineages (Pisanets *et al.* 2009, Garcia-Porta *et al.* 2012, Özdemir *et al.* 2020) rather than morphological units and the subspecies are no longer recognized (Frost 2021).

Bufo verrucosissimus is known from Lake Karagöl (41°23' N, 41°51' E; 1,450–1,480 m a.s.l.,) is situated in the district of Borçka (Artvin city) near the northeastern border of Turkey and Georgia (Figure 1). It is a 5-ha freshwater lake with a maximum depth of 25 m. Streams and rainfall are the sources feeding the lake (Kopar and Sever 2008). The lake and its surrounding have a rich fauna and flora with a mean air temperature of 2.0–6.1°C. Lake Karagöl and its surroundings are part of the Eastern Black Sea Mountains (Eastern Pontides) shaped by Alpine orogeny and volcanism events. The lithology in and around the lake consists of volcano-sedimentary sequences. The site has a rich potential in terms of streams and resources fed by rain and spring waters in addition to the continuously flowing rivers (Kopar and Sever 2008, Nergiz and Alar 2019).

The lake has an ice cover (10–14 cm) in February and the active period for anurans was from March to October. A total of five species of anurans—*Rana macrocnemis* Boulenger, 1885,

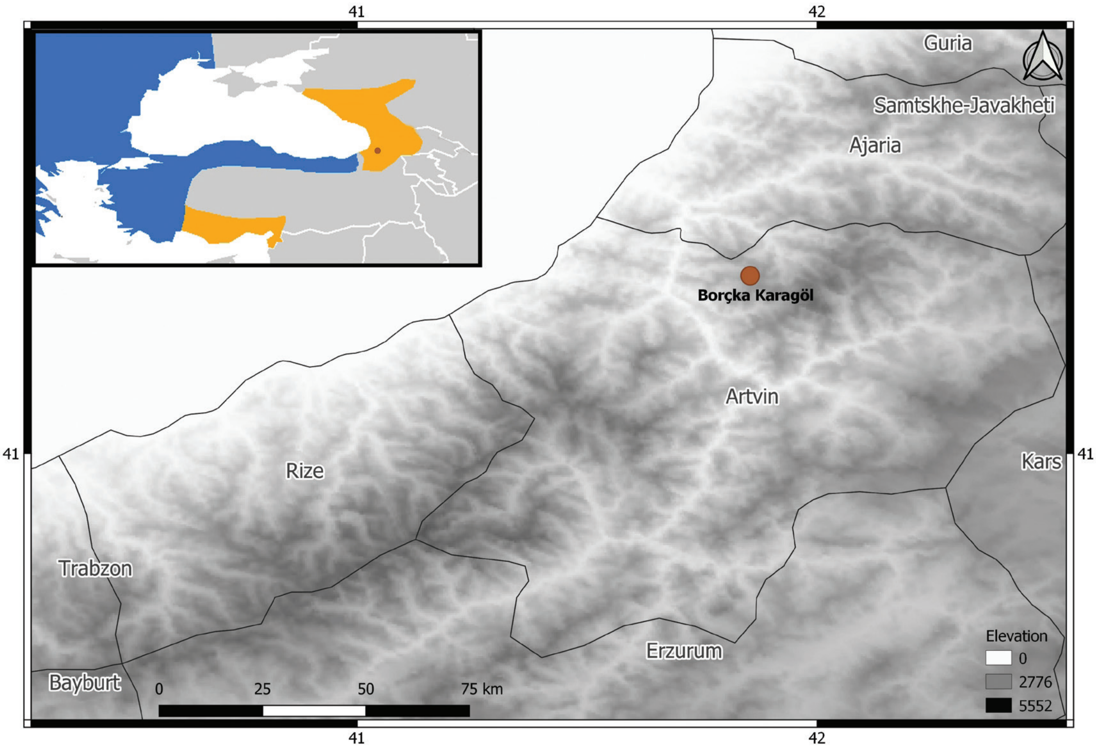

Figure 1. The location map of Lake Karagöl, Artvin, Turkey. The inset map was modified from Özdemir *et al.* (2020) and represents the distribution of *Bufo verrucosissimus* (yellow) and *B. bufo* in surrounding regions (blue).

Pelophylax ridibundus (Pallas, 1771), *Hyla orientalis* Bedriaga, 1890, *Pelodytes caucasicus* Boulenger, 1896, and *Bufo verrucosissimus*—and two newts—*Mertensiella caucasica* (Waga, 1876) and *Ommatotriton ophryticus* (Berthold, 1846)—were found in the Lake Karagöl and its vicinity (Afsar *et al.* 2018).

Age and body size are two important indicator of amphibian life history (Üzüm *et al.* 2020) and their relationship is critical when assessing evolutional-ecological processes. Although age structure of *B. verrucosissimus* was studied in a population at the border of Caucasia (Gokhelashvili and Tarkhnishvili 1994, Tarkhnishvili 1994, Zazanashvili and Mallon 2009), no data exist from Turkey. In this study, we describe comprehensive morphology and age structure of a local population of *B. verrucosissimus* from Lake Karagöl.

Materials and Methods

A total of 29 adult specimens (14 ♀♀, 15 ♂♂) were collected during the breeding season in 2019. Initially, the toads were anesthetized with 250 mg/L MS222. Thereafter, 27 external morphological characters (Özdemir *et al.* 2020) were measured for each of the toads: snout–vent length (SVL), length of the head (LHEAD), width of the head (WHEAD), minimum distance between the nostrils (INTNOS), distance between the nostril and the tip of snout (NOSTIP), minimum distance from the nostril to the anterior corner of the eye (NOSEYE), eye–

tympanum distance (EYETYM), horizontal diameter of the eye (DEYE), diameter of the tympanum (DTYM), length of the parotoid (LPAR), distance between the elbows with humerus kept perpendicular to the body axis (WGRASP), radio ulna length (RADUL), length of the hand (LHAND), length of the first finger (L1FING), length of the femur (LFEM), length of the tibia (LTIB), length of the tarsus (LTARS), length of the foot (LFOOT), minimum distance from the distal extremity of the inner metatarsal tubercle and the web between the third and fourth digit (WEB), length of the metatarsal tubercle (LMET), interorbital distance (IOD), anterior parotoid distance (PDA), posterior parotoid distance (PDP), left parotoid width (LPW), right parotoid width (RPW), length of the inner metatarsal tubercle (LIMT) and width of the inner metatarsal tubercle (WIMT).

All measurements were taken by the same researcher with using a digital calliper to the nearest 0.01 mm. The sex of adult specimens was determined in the field using secondary sexual characters. To examine qualitative traits, each individual was photographed. After sampling process, the individuals were released to the area where captured. This study was done with the permission of the local ethic committee (Republic of Turkey Recep Tayyip Erdogan University Local Ethics Committee for Animal Experiments, approval reference number: 2019/9).

Morphological Analyses

Captured specimens were analysed using photographs of their external body traits, coloration patterns and skin structure with reference to the former studies (Orlova and Tuniyev 1989, Sinsch *et al.* 2009). To make numerically explicit the morphological features of the Karagöl population, measurements were computerized using SPSS v22 (IBM 2013) and then transferred in R environment using *foreign* package (R Core Team 2021). Descriptive statistics of each character were calculated with the *psych* package (Revelle 2019). Normality assumptions were confirmed using the Shapiro-Wilks test. We used a One Sample t-test to compare our data with the average measurements of previous studies. To compare the means of characters between sexes, the Student t-test was carried out using the *stats* package (R Core Team 2021). The sexual dimorphism index (SDI) was calculated according to the formula introduced by Lovich and Gibbons (1992): the mean SVL of the bigger sex was divided by the mean SVL of the smaller sex and subtracted from one (SVLbig/SVLsmall-1) to achieve an approximate relationship of sexual size dimorphism (SDI > 0 when females are larger than males, SDI < 0 when males are larger than females). To visualize the separation between sexes, log10-transformed data were subjected to PCA analysis using *FactoMineR* (Le *et al.* 2008) and *factoextra* (Kassambara and Mundt 2020) packages. Data visualization was performed using *ggplot2* (Wickham 2016), *grid* and *gridExtra* (Auguie 2017) packages. All the analyses were run using R Programming Language (R Core Team 2021).

Skeletochronology

The 4^{th} toe of the hind limb was clipped for each toad and preserved in 95% ethanol for the skeletochronology. We applied standard skeletochronology procedure following Castanet and Smirina (1990) to elicit the age structure of the Lake Borçka Karagöl population. Phalanges were manually cleaned from soft tissues and stored in 70% ethanol. Samples were washed in the tap water approximately one hour, decalcified in 5% nitric acid around 1.5 hours. Cross sections of 18 μm were obtained from second phalanges using a freezing microtome (Thermo Shandon Cryostat, Germany). The cross sections were stained with Ehrlich’s haematoxylin around 15 min. Photos were taken using an Olympus BX51 microscope at 200× and 400× magnifications for each individual. Age estimation was done counting the lines of arrested growth (LAGs) that develop during hibernation, the only period of arrested activity in the year. The calculation was done by two independent researchers (N. Özdemir and C. Dursun) using an Olympus BX51 microscope (Figure 2). To avoid probable errors of the age estimation due to medullary resorption, we used diaphysis sections in which the periosteal bone size reaches its maximum and that of the medullar cavity is at its minimum (Oromi *et al.* 2016).

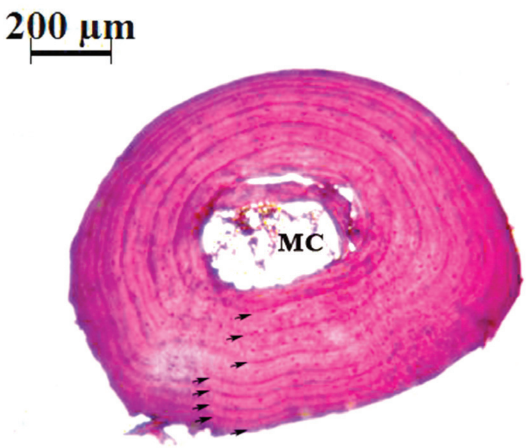

Figure 2. Cross-section image through phalanges of eight years old female specimen.

Since the age classes are discrete variables, we compared age differences between sexes using the Mann-Whitney U test by comparing median values in the *stats* package. We predicted growth curve models under the typical Von Bertalanffy's equation modified by Beverton and Holt (1957): $Lt = L_\infty\{1\text{-}\exp[-k\ (t\text{-}t_0)]\}$ where Lt is the expected or average length at the time (or age) t, L_∞ is the asymptotic average length, k is the so-called Brody growth rate coefficient and t_0 is a modelling artifact that is said to represent the time or age when the average length was zero. To obtain parameter values and execute the analyses *FSA* (Ogle *et al.* 2021), *FSAdata* (Ogle 2019), *FSAsim* (Ogle 2020) and *nlstools* (Baty *et al.* 2015) packages were used following the guide *fishR Vignette* (Ogle 2013). To visualize growth curve, we also added a hypothetical individual to the dataset using the reference of Afrin *et al.* (2019) under the given parameters: SVL at metamorphosis is fixed to mean 12.00 mm and age at metamorphosis is 0.3 year. Since we lacked the individuals between 0–4 years in females, the growth curve did not fit the data. Therefore, we estimated the body length of a female at age 2 years to a shape consistent with the curve of the male dataset and we applied the missing value analysis based on regression which is advisable method to fill the gaps in data for the constructed models (Raghunathan 2004, Zhang 2016, Johnson *et al.* 2021).

Results

Morphology

The head is not proportionately larger compared to the body size. Snout is wide and short, and rostrum is extensively rounded. The parallel positioned parotoid glands are apparent but narrow. Dorsal surface is covered with large, round, and smooth warts. Ventral side has small smooth knobs. The warts at the corners of mouth and flanks of the head bear thorny keratinized spines. Females are wartier and spinier. Males have relatively smooth skin with fewer spine intensity. Males mostly have a green dorsum, rarely brown. Females are generally brown but some have a dorsally brick-red and brown-greenish mixture. A clear majority of specimens had irregular dark spots locating on dorsal warts. Ventral side is off-white, cream colored and covered with generally visible brown patterns. The bands located on parotoids are indiscrete and never extending beyond parotoids. The laterally extended light bands are more observable in females but absent in a few individuals (Figure 3).

Descriptive statistics of the measurements are summarized in Table 1. The data indicated that females were larger than males in all cases. Also, significant differences were found in the

Figure 3. The specimens representing both sexes from the population of Lake Karagöl: (**A**) A male specimen; (**B**) A female specimen.

investigated characters between sexes ($p < 0.05$). The first principal component explained 83% of the variance; 91.09 % of the total variance was explained by four principal components. All variables showed positive loadings for PC1 which is representing the variation relevant to the size. The highest loads were observed for WHEAD, LHEAD and SVL, respectively. In the second principal component RADUL and PDP had the highest positive loads whereas NOSTIP and LIMT showed the lowest negative loads (Table 2). Males and females are clearly distinguished in the morphospace based on size, but they represented similar shape characteristic corresponding to the explained variance by PC2 (Figure 4). Sexual dimorphism index was calculated + 0.41 and this rate verified female-biased sexual size dimorphism, as well.

Skeletochronology and Intraspecific Phenotypic Variation

The median values of age were found 5 and 7 years for males and females, respectively. Mann-Whitney U test also pointed the significant difference between sexes in terms of age structure (W = 34.5; $p < 0.01$). The average age was found 5.13 and 6.64 years for males and females, respectively. Males aged between 2–7 years and females 5–8 years (Table 3).

The Von Bertalanffy's growth curves adequately fitted the relationship between age and SVL in the constructed models. The models represented identical growth trajectories in terms of curve shapes (Figure 5). The estimated asymptotic SVL of females was 101.72 ± 1.55 mm with 95% confidence interval between 98.36

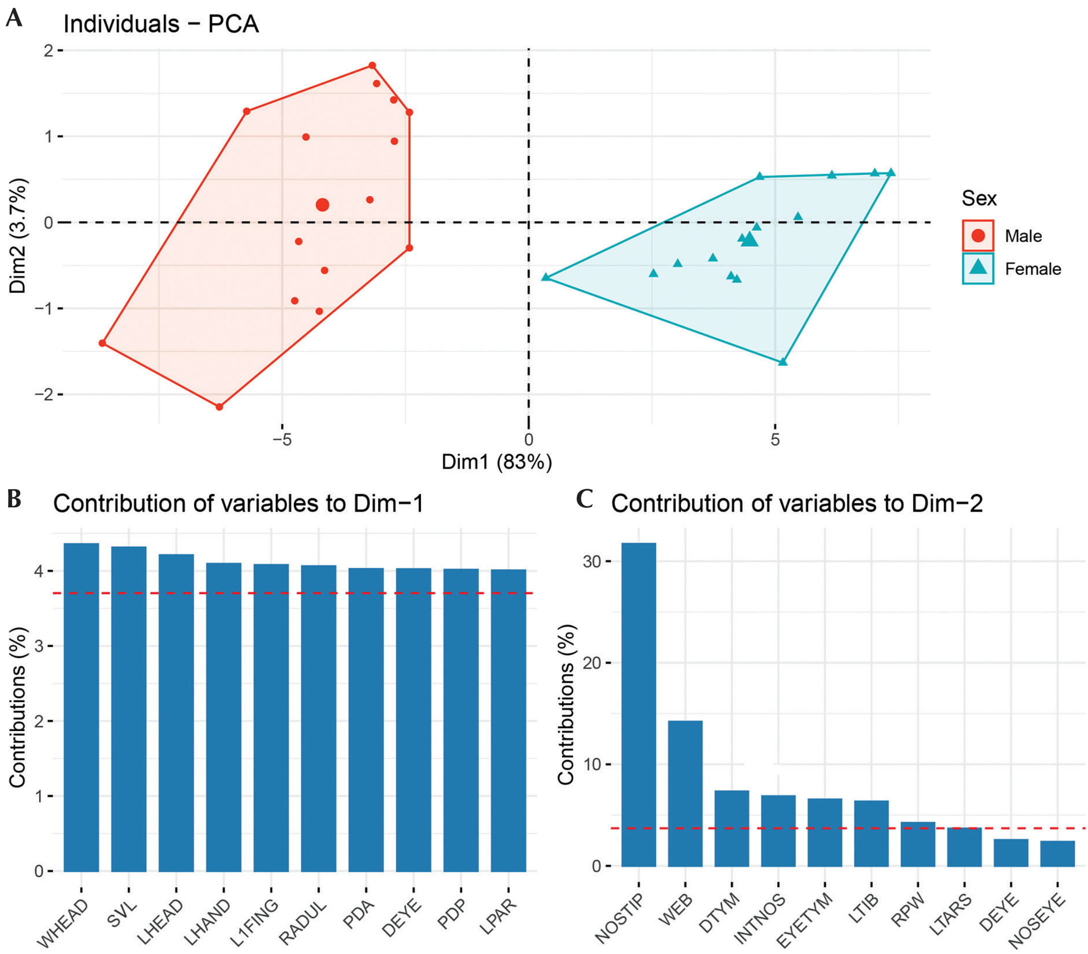

Figure 4. The scatter plot of PCA analysis based on the morphometric measurements (**A**), and the top ten variables contributing to PC1 (**B**) and PC2 (**C**).

and 105.07 mm and it is lower than the maximum SVL (112.59 mm). The growth coefficient (K) is 0.88 ± 0.16 (confidence interval: 0.51–1.24). On the other hand, the estimated asymptotic SVL of males (75.55 ± 0.91 mm) was lower than females with 95% confidence interval between 73.57–77.53 mm. However, K was calculated as 0.99 ± 0.14 (confidence interval: 0.67–1.31) which is higher than females. The estimated asymptotic SVL was not higher than maximum recorded SVL value (79.86 mm). The final models were found statistically significant for all parameters ($p < 0.05$). The correlation of age and SVL was higher in males (r = -0.60) than females (r = -0.44). The negative direction of the correlation values indicated that the growth is decreasing in higher ages.

The intraspecific comparison yielded statistically significant results between Karagöl and Caucasus populations. Caucasian populations

Table 1. Descriptive statistics of morphometric characters (SE: Standard error of mean; Min: Minimum value; Max: Maximum value). Test results and significance values of each character were given in the same line. Significance levels were flagged with asterisk (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Positive test values indicate female biased differences.

Characters	Mean ± SE (mm)	Min	Max	Mean ± SE (mm)	Min	Max	Test Value	Sig.
	Males			Females				
SVL	73.91 ± 1.05	65.74	79.86	102.67 ± 1.49	90.86	112.59	$t = 15.946$	***
LHEAD	17.15 ± 0.32	14.98	19.52	24.13 ± 0.47	21.12	27.89	$t = 12.511$	***
WHEAD	23.67 ± 0.34	20.23	25.29	33.12 ± 0.56	28.73	36.56	$t = 14.728$	***
INTNOS	4.83 ± 0.12	3.88	5.66	6.04 ± 0.10	5.34	6.99	$t = 7.637$	***
NOSTIP	3.01 ± 0.14	2.22	4.15	3.60 ± 0.11	2.92	4.32	$t = 3.154$	**
NOSEYE	3.68 ± 0.12	3.01	4.47	5.14 ± 0.14	4.27	5.83	$t = 7.942$	***
EYETYM	2.68 ± 0.11	2.00	3.27	4.25 ± 0.11	3.79	4.97	$t = 10.350$	***
DEYE	7.08 ± 0.14	5.84	7.90	8.94 ± 0.20	7.53	10.33	$t = 7.747$	***
DYTM	2.54 ± 0.13	1.91	3.49	3.17 ± 0.17	2.07	4.37	$t = 3.031$	***
LPAR	15.57 ± 0.36	12.38	17.72	22.33 ± 0.43	19.85	24.56	$t = 11.978$	***
WGRASP	61.57 ± 1.11	52.39	70.48	74.72 ± 1.67	64.70	88.33	$t = 6.655$	***
RADUL	24.57 ± 0.35	22.08	27.11	30.52 ± 0.56	26.07	33.51	$t = 9.102$	***
LHAND	19.40 ± 0.42	14.85	21.63	26.60 ± 0.60	22.67	30.10	$t = 9.918$	***
L1FING	7.74 ± 0.24	5.50	9.39	12.04 ± 0.35	9.36	13.90	$t = 10.376$	***
LFEM	31.27 ± 0.58	27.28	35.68	41.55 ± 0.77	37.15	46.68	$t = 10.231$	***
LTIB	19.79 ± 0.29	17.55	21.78	27.13 ± 0.52	23.48	30.28	$t = 12.558$	***
LTARS	18.08 ± 0.28	16.67	20.17	24.14 ± 0.50	21.49	27.30	$t = 10.773$	***
LFOOT	34.20 ± 0.62	29.44	38.16	40.28 ± 0.73	35.42	44.26	$t = 6.374$	***
WEB	20.40 ± 0.47	16.89	23.76	23.78 ± 0.59	19.86	27.07	$t = 4.504$	***
LMET	2.71 ± 0.11	1.94	3.41	3.90 ± 0.10	3.34	4.40	$t = 7.961$	***
IOD	8.67 ± 0.24	7.43	10.41	12.34 ± 0.33	9.79	14.62	$t = 9.111$	***
PDA	17.06 ± 0.31	14.77	19.20	23.13 ± 0.58	19.39	28.22	$t = 9.433$	***
PDP	24.95 ± 0.50	20.59	28.27	35.70 ± 0.92	30.85	42.03	$t = 10.483$	***
RPW	5.27 ± 0.07	4.61	5.66	8.17 ± 0.20	7.10	10.08	$t = 13.686$	***
LPW	5.17 ± 0.08	4.37	5.66	7.93 ± 0.30	5.08	10.08	$t = 9.117$	***
LIMT	4.42 ± 0.10	3.56	4.91	6.32 ± 0.14	5.08	7.47	$t = 10.854$	***
WIMT	2.52 ± 0.06	1.95	2.83	3.41 ± 0.10	2.72	4.13	$t = 7.791$	***

Table 2. Principal component loadings of PC1–PC4 extracted from correlation matrix.

Characters	PC1	PC2	PC3	PC4
SVL	0.2076	-0.0897	0.0043	-0.0408
LHEAD	0.2051	-0.1096	0.0769	0.0834
WHEAD	0.2087	-0.0697	0.0345	0.1113
INTNOS	0.1932	0.2619	-0.0683	-0.0502
NOSTIP	0.1325	0.5630	-0.4834	0.0614
NOSEYE	0.1858	0.1541	0.0841	0.5773
EYETYM	0.1812	-0.2557	-0.0478	0.3107
DEYE	0.2005	0.1596	-0.0902	-0.0046
DTYM	0.1295	0.2707	0.7908	-0.0076
LPAR	0.2001	0.0413	-0.0501	-0.0732
WGRASP	0.1972	0.1181	0.0885	0.0348
RADUL	0.2015	0.0034	0.0489	-0.0579
LHAND	0.2023	0.0209	-0.0445	-0.1145
L1FING	0.2019	-0.0315	-0.0245	0.0877
LFEM	0.1991	0.0124	-0.1035	0.0422
LTIB	0.1929	-0.2518	0.0007	-0.2471
LTARS	0.1930	-0.1917	0.1133	-0.2867
LFOOT	0.1931	0.1495	0.1188	-0.1504
WEB	0.1767	0.3768	0.0337	-0.3127
LMET	0.1831	-0.1027	0.0033	0.3826
IOD	0.1993	0.0259	0.0503	0.1225
PDA	0.2006	-0.1089	-0.0619	0.0732
PDP	0.2003	-0.0387	-0.0807	-0.1574
RPW	0.1991	-0.2057	-0.1379	-0.1016
LPW	0.1984	-0.1186	0.0437	0.1702
LIMT	0.2000	-0.1274	-0.0343	0.0001
WIMT	0.1875	-0.1498	-0.1492	-0.2082
Eigenvalue	22.40	1.00	0.72	0.40
Variance (%)	82.99	3.71	2.90	1.48
Cumulative variance (%)	82.99	86.70	89.61	91.09

Table 3. Descriptive statistics of age groups in both sexes of *B. verrucosissimus* from Lake Karagöl (SVL: Snout–vent length, SE: Standard error of mean).

Male Age Groups			
Age	***N***	**Mean SVL ± SE (mm)**	**Range (mm)**
2	1	66.58 ± 0.00	66.58
4	4	71.82 ± 1.15	69.45–73.89
5	3	73.48 ± 1.46	70.61–75.41
6	5	76.62 ± 0.98	74.81–79.86
7	2	77.60 ± 0.89	76.7–78.49
Female Age Groups			
Age	***N***	**Mean SVL ± SE (mm)**	**Range (mm)**
5	1	102.83 ± 0.00	102.83
6	4	99.02 ± 2.88	90.86–104.23
7	8	101.34 ± 1.85	93.3–107.7
8	1	108.45 ± 0.00	108.45

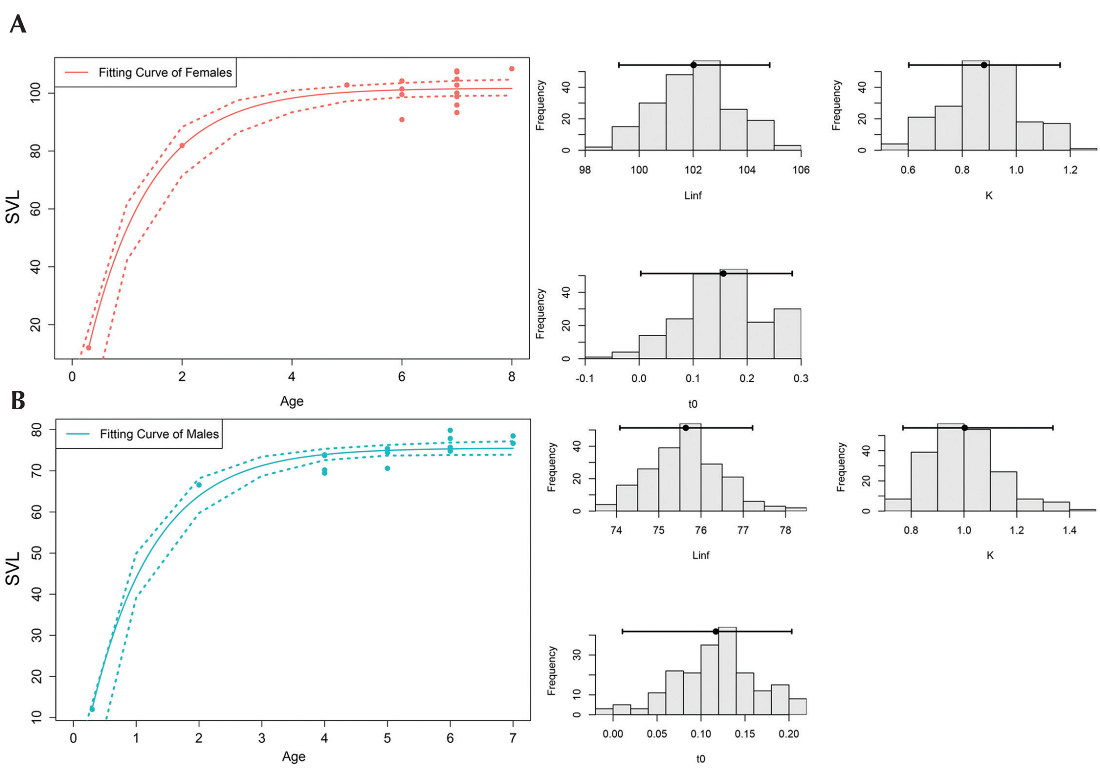

Figure 5. The Von Bertalanffy growth models and bar graphics of the relevant parameters for females (**A**) and males (**B**).

have larger body proportion in comparison with Karagöl population. Even though the size of body segments is larger in the other populations, the body length was not significantly different between Lake Karagöl and the population which was previously described as *B. verrucosissimus turowi* subspecies (Table 4).

Discussion

In this study, we described the morphological characteristics of Lake Karagöl populations of *Bufo verrucosissimus.* At first, we tested the body size difference between sexes and the results confirmed a high degree of female-biased sexual size dimorphism which is common phenomenon in anurans (Kupfer 2007) regarded as an outcome of sex-specific patterns of sexual and natural selection. Numerous studies have reported female-biased sexual dimorphism in the genus *Bufo* (Gittins *et al.* 1980, Reading 1991, Tarkhnishvili 1994, Kutrup 2001, Cvetković *et al.* 2005, 2007, Litvinchuk *et al.* 2008, Mozaffari and Moghari 2012, Cadjenović *et al.* 2013, Arntzen *et al.* 2013, Ergül-Kalayci *et al.* 2019, Dursun *et al.* 2022). Female biased sexual size dimorphism is a common phenomenon in 90% of anuran species (Shine 1979). The larger body of females is a fecundity advantage in explosive breeder species due to faster growth rate and delayed reproduction to allocate energy (Hoglund and Robertson 1987, Kuhn 1994, Tomašević *et al.* 2008) besides different age structure of breeding population between sexes (Kusano *et al.* 2010).

We examined the population with regard to smooth and slightly spined warts. Females have more keratinized spines on the warts than males. The well-developed (thorny) spines were found on the chest and legs, and the lateral side of the head. Cadjenović *et al.* (2013) implied that *Bufo bufo* (Linnaeus, 1758) males were commonly characterized with poorly expressed warts with thorn-like ends, whereas most females have visible warts with very developed thorn-like ends on the head region. Arntzen *et al.* (2013) described similar observations between the sexes of *B. bufo* and *Bufo spinosus* Daudin, 1803 species. Therefore, the density of the keratinized spines may be a sexual dimorphic feature as noted in Lake Karagöl population. Dry warty skin is presented as main characteristic of bufonid species (Pough 2015) with the spines originating from epidermis (Regueira *et al.* 2016). The density of spines on the head was used to discriminate *Bufo* taxa (De Lange 1973, Lüscher *et al.* 2001, Arntzen *et al.* 2013). A high density of keratinized spines on the head was observed in *B. spinosus* and some *B. bufo* populations. Therefore, we assume that the lower density of spines is specific of *B. verrucosissimus.*

In this study, we revealed the age structure of the Lake Karagöl population for the first time. The range of ages was between 2–8 years in the population, but median differences indicated that females are significantly older than males. Growth models showed similar curves that described the correlation between age and SVL. The models indicated that slower growth was observed after 2–3 years for males and 3–4 years for females and was supported by the higher growth coefficient of males. In amphibians, males generally reach sexual maturity one year earlier than females (Miaud *et al.* 1999, Leclair *et al.* 2005, Liao *et al.* 2010). Reading (1991) implied that the minority of *B. bufo* males and females reached sexual maturity at ages of two and four years in the United Kingdom. Ergül-Kalayci *et al.* (2019) reported a significant decrease in the growth at the age of maturity ranging from 2–3 years in three different *B. bufo* populations in Turkey. Matushkina *et al.* (2015) mentioned that *Bufo eichwaldi* Litvinchuk, Borkin, Skorinov, and Rosanov, 2008 males and females reach sexual maturity after 2 and 3 winters, respectively. Therefore, our results are consistent with the previous studies in terms of growth patterns. The main reason behind delayed sexual maturity of females is related to allocation of energy to reproduction like in majority of amphibian species (Gibbons 1984, Pizzatto and Marques 2006, Yu *et al.* 2022).

Table 4. The results of One Sample t test among Karagöl population and Caucasian populations. Analyses were run with the mean values of previous studies against to our data. Positive t values are representing Karagöl biased differentiation, whereas that of negative for the other populations. Significance levels were flagged with asterisk (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Non-significant differences were presented as "n.s".

Study-Subspecies	Males								Females						
	Characters	***N***	**Mean**	**Min**	**Max**	**SD**	**Test Value**	**Sig.**	***N***	**Mean**	**Min**	**Max**	**SD**	**Test Value**	**Sig.**
Tarknishvili and Gokhelashvili (1999)	SVL	27	77.08	69.60	85.60	4.29	*t* = -3.018	**	8	115.1	104.00	124.20	6.37	*t* = -8.339	***
	LHEAD	27	19.41	18.00	21.80	0.86	*t* = -7.164	***	8	26.49	23.30	28.80	1.80	*t* = -5.025	***
	WHEAD	27	25.03	22.80	30.00	1.51	*t* = -3.993	**	8	39.90	37.00	44.70	2.85	*t* = -12.219	***
	INTNOS	27	8.37	7.20	10.10	0.72	*t* = -28.79	***	8	12.11	11.20	13.70	0.74	*t* = -62.395	***
	LPAR	27	15.30	13.5	18.00	2.09	*t* = 0.732	n.s	8	23.50	20.30	26.80	2.12	*t* = -2.6951	*
	LFEM	27	26.29	24.00	30.90	2.23	*t* = 9.003	***	8	36.44	33.70	39.30	1.68	*t* = 6.611	***
	LTIB	27	28.52	25.10	31.80	2.22	*t* = -29.785	***	8	38.45	36.00	41.50	1.94	*t* = -21.834	***
	Characters	***N***	**Mean**	**Min**	**Max**	**SD**	**Test Value**	**Sig.**	***N***	**Mean**	**Min**	**Max**	**SD**	**Test Value**	**Sig.**
Orlova and Tuniyev (1989)	SVL	13	85.88	78.00	97.00	4.65	*t* = -11.396	***	10	119.98	113.00	124.00	4.00	*t* = -11.613	***
	LHEAD	13	22.46	20.00	25.00	1.41	*t* = -16.840	***	10	29.55	27.00	32.50	1.64	*t* = -11.550	***
	WHEAD	13	27.96	25.00	30.00	1.30	*t* = -12.594	***	10	41.17	38.50	43.00	1.45	*t* = -14.507	***
	INTNOS	13	4.28	3.70	4.80	0.29	*t* = 4.479	***	10	6.08	5.00	6.70	0.51	*t* = -0.418	n.s
	LPAR	13	19.04	16.00	23.00	2.34	*t* = -9.512	***	10	24.94	23.00	27.00	1.20	*t* = -6.010	***
	LFEM	13	37.38	32.00	43.00	3.21	*t* = -9.685	***	10	48.00	43.00	53.00	3.13	*t* = -8.340	***
	LTIB	13	34.42	30.00	40.00	2.52	*t* = -49.908	***	10	43.30	41.00	47.00	1.71	*t* = -31.193	***
	Characters	***N***	**Mean**	**Min**	**Max**	**SD**	**Test Value**	**Sig.**	***N***	**Mean**	**Min**	**Max**	**SD**	**Test Value**	**Sig.**
Orlova and Tuniyev (1989)	SVL	1	73.50	-	-	-	*t* = 0.389	n.s	2	102.75	91.50	114.00	-	*t* = -0.053	n.s
	LHEAD	1	19.50	-	-	-	*t* = -7.450	***	2	25.00	23.60	26.40	-	*t* = -1.847	n.s
	WHEAD	1	25.40	-	-	-	*t* = -5.079	***	2	38.15	36.30	40.00	-	*t* = -9.006	***
	INTNOS	1	7.00	-	-	-	*t* = -17.646	***	2	6.15	5.30	7.00	-	*t* = -1.137	n.s
	LPAR	1	17.20	-	-	-	*t* = -4.473	***	2	23.10	22.40	23.80	-	*t* = -1.774	n.s
	LFEM	1	35.50	-	-	-	*t* = -6.466	***	2	46.15	45.00	47.30	-	*t* = -5.947	***
	LTIB	1	32.00	-	-	-	*t* = -41.654	***	2	41.50	41.10	41.90	-	*t* = -27.719	***

Table 4. *Continued.*

Study-Subspecies	Characters	Males N	Males Mean	Males Min	Males Max	Males SD	Males Test Value	Males Sig.	Females N	Females Mean	Females Min	Females Max	Females SD	Females Test Value	Females Sig.
Orlova and Tuniyev (1989)	SVL	10	81.01	75.60	86.90	3.51	*t* = -6.759	***	1	123.20	-	-	-	*t* = -13.774	***
	LHEAD	10	20.20	17.40	26.20	2.31	*t* = -9.670	***	1	28.40	-	-	-	*t* = -9.098	***
	WHEAD	10	26.11	23.40	28.00	1.52	*t* = -7.163	***	1	42.40	-	-	-	*t* = -16.723	***
	INTNOS	10	4.60	4.00	5.30	0.44	*t* = 1.863	n.s	1	6.00	-	-	-	*t* = 0.403	n.s
	LPAR	10	16.80	14.70	19.80	1.64	*t* = -3.375	**	1	26.40	-	-	-	*t* = -9.371	***
	LFEM	10	30.32	24.00	36.70	4.24	*t* = 2.401	*	1	47.00	-	-	-	*t* = -7.047	***
	LTIB	10	30.41	22.40	33.40	3.35	*t* = -36.231	***	1	42.00	-	-	-	*t* = -28.684	***
	Characters	**N**	**Mean**	**Min**	**Max**	**SD**	**Test Value**	**Sig.**	**N**	**Mean**	**Min**	**Max**	**SD**	**Test Value**	**Sig.**
Kidov (2009)	SVL	6	69.75	64.00	75.50	4.41	*t* = 3.957	***	2	101.6	99.70	103.50	-	*t* = 0.717	n.s
	WHEAD	6	24.32	23.00	25.50	0.92	*t* = -1.909	n.s	2	38.60	37.00	40.20	-	*t* = -9.877	***
	INTNOS	6	3.93	3.40	4.20	0.30	*t* = 7.362	***	2	6.35	6.30	6.40	-	*t* = -3.193	***
	LPAR	6	15.67	14.10	17.40	1.29	*t* = - 0.281	n.s	2	22.2	22.10	22.30	-	*t* = 0.297	n.s
	LFEM	6	31.85	30.20	33.60	1.31	*t* = -0.218	n.s	2	46.05	45.20	46.90	-	*t* = -5.818	***
	LTIB	6	28.17	27.20	28.80	0.61	*t* = -28.591	***	2	38.30	38.20	38.40	-	*t* = -21.545	***

Although we presented the first report on the Turkish populations, the age structure of *B. verrrucosissimus* was studied in Caucasia. Gokhelashvili and Tarkhnishvili (1994) extensively investigated life-history traits of six anuran species during two consecutive years at the border of central Georgia, which has similar ecological conditions with the area of Lake Karagöl. The vegetation is mainly composed of mixed forest [*Abies nordmanniana* (Steven) Spach, *Picea orientalis* (L.) Peterm., *Carpinus caucasica* Grossh., *Fagus orientalis* Lipsky], the elevation is between 900–1200 m and the annual precipitation is 1000 mm (Tarkhnishvili 1993). In this context, they aged 105 adult *B. verrucosissimus* specimens (49 males, 56 females), and the age varied between 2–9 years (mean: 4.04 years) in males and 3–10 years in females (mean: 5.96). Tarkhnishvili (1994) indicated that the maximum age is reached around 9–10 years for both sexes of the species. Accordingly, the lifespan and maximum age are higher in Georgian populations. Iskanderov (2009) noted that the sampled Caucasian toads from Azerbaijan were aged 5–8 years and 75–80% of the population ranged between 8–12 cm in body size. Recently, Afrin *et al.* (2022) showed that the age varied between 3–7 years in males, and 5–12 years in females on the Stavropol uplands in Russia. A shorter lifespan is generally explained by early maturity and high reproductive investment while delayed maturity is observed in long-lived amphibian species (Guarino *et al.* 2010, Oromi *et al.* 2012, Stănescu *et al.* 2016; Székely *et al.* 2018). However, these patterns can also be observed between different populations of the species under severe environmental pressures or stressors. For instance, Zamora-Camacho and Comas (2017) reported a lower mean age of *Epidalea calamita* (Laurenti, 1768) populations from agrosystems than natural habitats, and they implied that agrosystem toads compensate for lesser reproductive event with more breeding attempt. Marangoni *et al.* (2018) observed life history traits in *Chacophrys pierottii* (Vellard, 1948) due to aridity effect. Moreover, forest destruction, drying of wetlands, intentionally killing and road mortality are destructive effects on hygrophilous forest species. Zazanashvili and Mallon (2009) noted that degradation of natural sites and the effect of tourism are serious threats to *B. verrucosissimus* since the species can not hide from potential enemies. According to the Afsar *et al.* (2018), the main threats to amphibians in Lake Karagöl and surroundings are hotel wastes, camping and picnicking activities, and noise pollution. Moreover, the introduction of carp fish by resident people may cause the decline of the Lake Karagöl population due to the invasion of breeding sites, egg and tadpole mortality, and avoidance of breeding habitats. Therefore, we assume that the difference between the longevity of Georgia and Karagöl populations may be related to these factors.

The Lake Karagöl population differs from the toad populations inhabiting Caucasia in terms of body size, with Caucasus populations being significantly larger (see Tables 2 and 4). The maximum length of the species measured in Caucasia is 154 mm (Gumilevsky 1939). Moreover, the findings obtained in previous studies also validated the size difference between Lake Karagöl and Caucasian populations (Tarkhnishvili 1993, Litvinchuk *et al.* 2008, Iskanderov 2009, Sinsch *et al.* 2009). In amphibians, the geographic distributions of morphological traits can be influenced by different environmental factors such as precipitation, temperature, water balance and altitude (Bidau *et al.* 2011, Green 2015, Boaratti and Silva 2015, Guo *et al.* 2019) as well as life-history traits (Miaud *et al.* 1999, Morrison and Hero 2003, Womack and Bell 2020). The ranges of the populations compared in this study are characterized by different floristic districts of the Caucasus (Oganesian 1995) with different altitudes. Ergül-Kalayci *et al.* (2019) studied body size variation of three *B. bufo* populations from different altitudes (Trabzon, 1090 m a.s.l.; Kastamonu, 925 m a.s.l.; Yalova, 65 m a.s.l.) and they reported that Trabzon population was smaller than the others. We also revealed similar patterns in terms of the body size variation between *B. verrucosissimus* populations and the Lake Karagöl population. Those at the highest altitude had smaller body sizes and proportions. However, the previous studies reported no difference (Cvetković *et al.* 2009) or smaller body size in the lowlands (Hemelaar 1988) for *B. bufo*. Ergül-Kalayci *et al.* (2019) suggested that body condition can show negative correlation with the amount of snowfall, and it can reduce the body size due to short annual activity periods due to shortening the feeding period between the metamorphosis and first hibernation. Moreover, Womack and Bell (2020) investigated evolution of anuran body size using 2434 species and they indicated that the effect of altitude and microhabitats caused size differences. Therefore, we propose that the intraspecific body size variation of *B. verrucosissimus* populations is caused from the environmental characteristics affecting phenotypic plasticity. Lastly, the presence of carp fish in Lake Karagöl can be taken into consideration, because Lardner (2000) noted that *B. bufo* tadpoles tend to respond to predator presence by reducing their growth rate to tolerate weak swimming ability. This behavioral mechanism may be a defensive behavior in *B. verrucosissimus*.

The means of head length and head width were significantly lower in Karagöl specimens as opposed to Caucasian populations. The members of the Karagöl population have narrower and shorter heads. The highest head values were recorded in the populations from Agurchik and Krepostnaya (Orlova and Tuniyev 1989). In addition, we revealed similar differences between populations in the following characters: femur, tibia, and parotoid length (see Table 2). Therefore, the population inhabiting Lake Karagöl is smaller than Caucasian representatives in terms of head proportion and body segments. Most of the variation in head size is generally driven by body size representing allometry for amphibians. However, these differences might be due to the diet preferences and local environmental conditions. Crnobrnja-Isailović *et al.* (2012) described the head size and width differences between the sexes of *B. bufo* based on the prey size, and they suggested that the prey items of females have higher proportions opposed to male diets. Hudson *et al.* (2018) studied the geographical divergence in head width of *Rhinella marina* (Linnaeus, 1758) populations. They noted that the head width of samples from native ranges was higher than the translocated populations. Moreover, the head width was correlated with maximal ingestible prey size and prey-handling ability of the toads. Lastly, the authors proposed that the geographical divergence in relative head widths is a result of rapid evolution and the adaptation to the local conditions causing plasticity. These character differences were also reported for different species such as *Boana albopunctata* (Spix, 1824) (as *Hypsiboas albopunctatus;* Guimarães *et al.* 2011), *Bufotes boulengeri* (Lataste, 1879) (as *Bufo boulengeri;* Nabil and Sarra 2011), and *Pelophylax saharicus* (Boulenger, 1913) (as *Rana saharica;* Amor *et al.* 2009). Concerning the head measurements, the observed differences between *B. verrucosissimus* populations yield similar patterns with the other amphibian species, a result of plasticity as found in body size variation. Even though INTNOS was evaluated as a distinguishing character between populations, it was not totally supported by our measurements except for the round rostrum shape in the Karagöl population (Orlova and Tuniyev 1989, Kidov 2009).

Most females demonstrated brown shades whereas males were usually olive green-brown, and dark brown ventral sides occurred in both sexes. Color and pattern features of the specimens from Lake Karagöl are identical to Murgul-Artvin (Kutrup 2001) and Georgia populations from The Black Sea coast (Orlova and Tuniyev 1989, Tarkhnishvili and Gokhelashvili 1999, Sinsch *et al.* 2009). Although reddish brown males were rarely reported from Georgia, they were not observed in Karagöl. The remaining *B. verrucosissimus* populations were characterized by various shades of brown (Orlova and Tuniyev 1989). The elongated dark bands extending posteriorly along parotoid glands were previously noted as a specific character in the populations which were formerly assessed as *B. verrucosissimus verrucosissimus* (Sinsch *et al.* 2009). However, the bands were not discrete in the Karagöl samples. Zhelev *et al.* (2020) studied color polymorphism in two distinct *Bufotes viridis* (Laurenti, 1768) populations in Bulgaria and they reported that one of the populations mostly had morphs with dark backgrounds, whereas the other population was dominated by light colored individuals. They explained the observed variation by environmental differences due to the habitat characteristics relevant to vegetation type which can play a role in thermoregulation as well as defensive adaptations as camouflage seen in numerous anuran species (Hoffman and Blouin 2000, Zakharova *et al.* 2012, Zhelev *et al.* 2014, Wassef *et al.* 2019). Regarding our results, we also suggest that eco-geographical conditions and habitat characteristics may explain the observed polymorphism in *B. verrucosissimus.*

In sum, our study described general morphological traits and the lifespan and age distribution of the Karagöl population for the first time. Further studies are needed on the life-history traits of this population using ecological parameters.

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