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ASSESSMENT OF AGE AND INTERSEXUAL SIZE DIFFERENCES IN *BUFO BUFO*

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Abstract - Numerous studies have underlined the complex nature of relationship between age, size, and reproductive traits in anurans. One of the most intriguing problems for evolutionary biologists is intersexual difference in body size (SSD). For testing various hypotheses about SSD, we need reliable estimates of its extent (the important issue being the choice of trait for analysis), as well as the accurate determination of individual age. The measures of SSD may be subject to error if estimated from populations with unknown age distribution; amphibians continue to grow throughout their life and SSD is linked to sex differences in traits such as age at maturity and lifespan. In the present paper, we analyze problems involved in accurate determination of age structure and factors that may lead to under- or overestimation of individual age, as well as the problem of appropriate choice of traits, in the light of our experience and results of investigating populations of common toad (*Bufo bufo*) in the vicinity of Belgrade.

Key words: Onthogeny, phylogeny, sexual size dimorphism, age structure, maturity, skeletochronology, *Bufo bufo*

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INTRODUCTION

Numerous studies have underlined the complex nature of relationship between age, size, and reproductive traits in anurans. For evolutionary biologists, one of the most intriguing problems is intersexual difference in body size (SSD). It is a common phenomenon in anurans and various hypotheses have been proposed to explain its direction and extent (e.g., Shine, 1979; Woolbright, 1983; Halliday and Verrel, 1986; Arak, 1988). In order to test these hypotheses for a particular species and allow for comparison with other species, we need reliable and comparable estimates of SSD.

One of the important issues is the choice of traits for analysis (Cvetković, in press). In amphibians, snout-vent length (SVL) is commonly used as the indicator of overall body size. Though comparative studies of SSD are based mainly on this trait, it has been reported that in a number of species other characters (e.g., ones related to specific reproductive behavior or feeding) diverge more in this respect.

However, the measures of SSD may be subject to error if estimated from populations with unknown age distribution (Arak, 1988). Amphibians continue to grow throughout their lifetime, and SSD is linked to sex differences in traits such as age at maturity and lifespan, among others. In addition, some of the models concerning evolution of SSD focus on the role of intersexual differences in age structure of breeding populations (e.g., Monnet and Cherry, 2002). Hence, the accurate determination of individual age and age at maturation is essential.

Mark-recapture and skeletochronology are considered the only reliable methods for age estimation in amphibians (Halliday and Verrel, 1988), but skeletochronology allows for a much more rapid collecting of demographic data in natural populations. This method is based on the presence of growth marks or lines of arrested growth (LAG) in cross-sections of long bones and phalanges. On stained cross-sections, growth marks appear as concentric paler zones of periosteal bone, separated by narrow dark resting lines. The annual rhythm of the resting lines has been demonstrated in many species;

in temperate zones, LAGs are formed during hibernation.

Over the past decades, skeletochronology has been successfully used for age determination in a variety of amphibian species, temperate (e.g., Gibbons and McCarthy, 1983; Francillon-Vieillot and Castanet, 1985; Francillon-Vieillot *et al.* 1990; Cvetković *et al.* 1996; Miaud *et al*. 1999), as well as tropical and subtropical (Kumbar and Pancharatna, 2002; Lin and Hou, 2002; Morrison *et al*. 2004).

This method provides accurate estimations of individual age, age at sexual maturation, and longevity. However, in some cases problems can arise due to the resorption process or presence of double and false lines. Bone is a complex, dynamic tissue; continuous remodeling processes may alter or destroy the original patterns of growth marks (Enlow, 1969). The extent of this remodeling is related to various factors: metabolism, lifespan, and body size (de Ricqles, 1976); and size and character of the mechanical load on the bone (Klevezal and Kleinenberg, 1967). Remodeling involves the replacement of periosteal bone by endosteal bone, and may cause the resorption of initially formed year rings (Castanet, 1975). Resorption of periosteal bone starts from the inner surface of the bone and is marked by a hematoxylinophilic line - the "resorption line" (Klevezal and Kleinenberg, 1967). As a result of resorption of the initial rings, the age of some individuals may be underestimated.

Common toad, *Bufo bufo*, is a species with wide geographical and altitudinal distribution in Europe (Arnold, 2002); it prefers forests, but also occurs in fields, gardens, and human settlements (Radovanović, 1951). Like other widely distributed anuran species, it can be used as an indicator of environmental changes on a large scale, provided that patterns of variation of fitness components and age structure are known throughout its range. Intraspecific variation in age structure and body size along latitudinal and altitudinal gradients is a well-known phenomenon in anurans (Miaud *et al*. 1999), but the patterns are inconsistent. Although *B. bufo* has been the subject of numerous studies (e.g., Gittins *et al.* 1982; Hemelaar, 1983; Reading, 1988, 1991; Hoglund, 1989; Hoglund and Saterberg, 1989; Cvetković *et al.* 2003), data concerning life-history traits, and especially age structure of breeding populations, from parts of its range (including the Balkans) are still scarce.

The aim of the present study was to analyze problems involved in accurate determination of age structure and factors that may lead to under- or overestimation of individual age, as well as the problem of appropriate choice of traits for studying intersexual size differences, in the light of our experience and results of investigating populations of common toad (*Bufo bufo*) in the vicinity of Belgrade.

MATERIAL AND METHODS

This work is part of a larger ongoing project involving assessment of the body size-age relationship and life history variation in amphibian populations in the vicinity of Belgrade. Analyses were conducted on samples collected from two localities: Trešnja and Zuce. The study sites are near Belgrade, in an agricultural area (and thus under the impact of human activities). Trešnja is a small artificial lake, known as an important breeding site for several amphibian species, while the other site is a medium-sized pond near the village of Zuce.

Adults of *B. bufo* were collected during three successive years (2001-2003), from March to November. Here we review our results concerning the problem of choice of variables for SSD analysis. A total of 20 morphometric characters were analyzed. Details of measurement procedures, statistical analyses performed, and statistical packages are given elsewhere (Cvetković *et al*. 2003, 2005, in press).

The skeletochronology procedure followed that of Miaud (1992). The longest toe of the right forelimb was cut off and stored in 75% alcohol. Muscle and skin were removed and the phalanx was washed in distilled water. The bones were then decalcified in 5% nitric acid and washed in running water. Cross sections (14 μm) from the diaphyseal region of the proximal phalanx were obtained using a freezing microtome, stained with Ehrlich's hematoxylin, and observed under a light microscope. Lines of arrested growth were counted and checked independently by the authors.

RESULTS AND DISCUSSION

Skeletochronological age determination was successful in 100 out of the 114 individuals. Cross-sections of phalanges showed sharp LAGs, which were easily dis-

tinguished from the rings of periosteal bone deposited during the activity period. In 14 individuals (12.3%), the LAGs were not distinct enough to allow precise counting due to poor affinity of the resting lines for hematoxylin or to inadequate quality of the cross section.

In this sample, all bones showed endosteal resorption. In all sections of the phalanges, the resorption was moderate to high, i.e., the first LAG was eroded partially or completely (Fig. 1). The loss of the first and sometimes the second LAG as the result of resorption has been

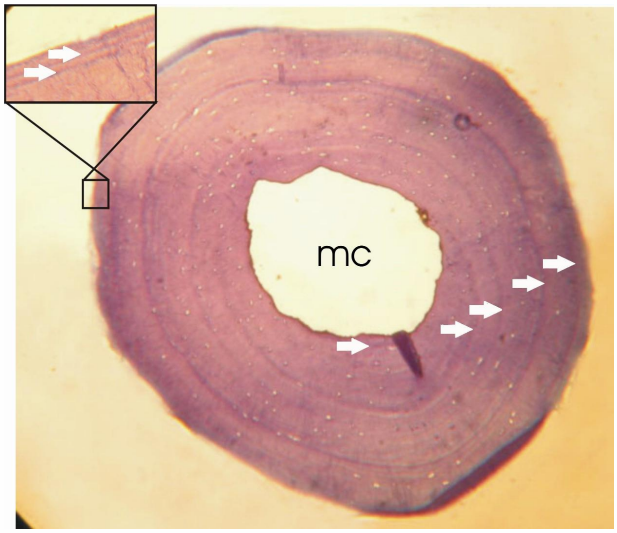

Fig. 1. Phalangeal cross section of female *Bufo bufo*: Seven LAGs (LAGs appear as thin dark lines), the first LAG is almost completely eroded; arrows - LAGs, mc - marrow cavity.

previously reported in *B. bufo* (Hemelaar, 1985; Fretey and Le Garff, 1996) and other species as well. Thus, Smirina (1972) found that in *Rana temporaria* from a continental climate zone the first year ring had always been resorbed completely and only remnants of the second year ring remained. Similar results were reported for the desert species *Bufo pentoni* (Barbault *et al.* 1979). Individual variation in the degree of resorption - no resorption, resorption of one or two LAGs - was found in *B. bufo* populations from the Netherlands and Poland (Hemelaar, 1981; Smirina, 1983).

The number of LAGs resorbed in adults does not increase with increasing age - resorption of periosteal bone decreases sharply after the onset of sexual maturity. This was reported in previous studies on *Rana temporaria* and *B. bufo* (Smirina, 1972, 1983; Hemelaar, 1981; Fretey and Le Garff, 1996). Consequently, the observed differences among individuals (and among populations) with respect to resorption must have arisen before they attained maturity. In addition, we observed differences in resorption related to sex, confirming some previous findings (Smirina, 1983; Hemelaar, 1985; Fretey and Le Garff, 1996). Female *B. bufo* generally seem to be more affected than males. Endosteal resorption decreases after sexual maturation, and males mature earlier than females (1-2 years); therefore, delayed maturation of female toads is related to a higher degree of resorption.

Apart from resorption, the presence of lines which are not the result of hibernation affects accurate age estimation. These lines, observed in many amphibian species (e.g., Hemelaar and Van Gelder, 1980; Sagor *et al.* 1998; Guarino *et al.* 2003), are called double and false lines. False lines are generally fainter than LAGs, and do not form a complete ring around the bone section. They resemble LAGs in appearance and, like them, indicate temporary reduction in growth rate. However, LAGs result from hibernation, whereas false lines result from injury or reduced food supply, which compels the animal to focus available resources on sustenance rather than growth (Hemelaar, 1985; Sagor *et al.* 1998). The presence of double lines - pairs of very closely spaced concentric rings - reflects two periods of arrested growth in one year (hibernation and aestivation) or may be related to atypical climatic conditions (Klevezal and Kleinenberg, 1967; Hemelaar and Van Gelder, 1980) leading to interruption of hibernation. Therefore, double lines are equivalent to one year (i.e., counted as one LAG).

In our study, double and false lines were observed, though rarely (Fig. 2). But since caution was still needed, cross-sections were independently checked in order to avoid possible mistakes - for example, a double line may occasionally be mistaken for two LAGs (resulting in overestimation of age) or a LAG near the outer edge of the bone may be indistinct and overlooked (resulting in underestimation of age). The outer perimeter of the bone proved especially problematical for skeletochronological analysis. Outer LAGs were frequently very close to one another and to the edge of the bone, making the counting difficult (Fig. 3). More central LAGs are generally more widely spaced than those deposited later in life. Decreasing intervals between outer LAGs indicate that shift in resource allocation from growth to reproductive investment happened after the onset of sexual maturation

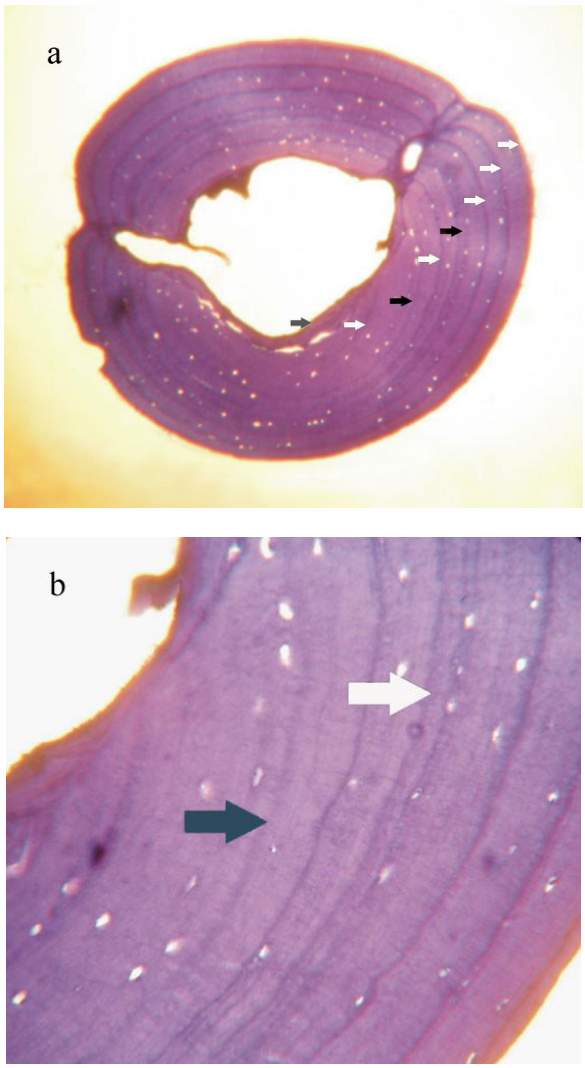

Fig. 2. Phalangeal cross section of female *Bufo bufo*: a) Six LAGs (the outermost LAG is confluent with the periphery of the bone), resorption is very high, a false line is present between the first and the second LAG, the third LAG shows a double line; gray arrow - endosteal bone, black and white arrows - false and double lines, respectively; b) close-up of false (black arrow) and double (white arrow) lines.

(e.g., Miaud *et al.* 1999).

Sexual size dimorphism in species of the genus *Bufo*, may be pronounced or, on the contrary, absent (Halliday and Verrell, 1986; Sinsch *et al.* 2001; Monnet and Cherry, 2002). For example, in *B. calamita* males and females of the same age do not differ in size, but in *B. achalensis* females are markedly smaller than males (Sinsch *et al.* 2001). The case of *B. bufo* differs from these two examples: common toad is known for pronounced sexual dimorphism in body size, females being significantly larger than males.

In our study, differences between females and males in all analyzed morphometric traits were highly significant, confirming a very high level of SSD, though a much smaller subset of traits proved sufficient to describe relevant size differences (Cvetković *et al.* 2003; Tomašević *et al.*, in prep.). We found that snout-vent length (SVL), which is easy to measure and reflects overall size well, is most appropriate for the study of direction and extent of SSD in this particular species. This solves the problem of comparable estimates of size differences, since most comparative studies of SSD are based on this trait. It should also be mentioned that studies on selection

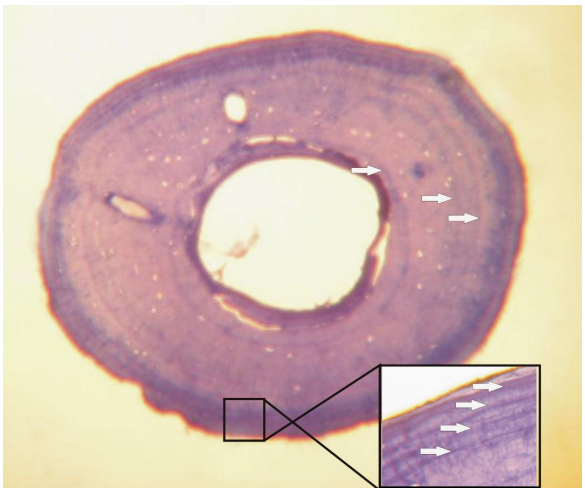

Fig. 3 Phalangeal cross section of male *Bufo bufo*: Seven LAGs, the first LAG is partially eroded, outer LAGs are very close to one another (indicating a shift in a resource allocation after sexual maturation).

of correlated characters, such as forearm length (Hoglund and Saterberg, 1989), did not reveal a significant indirect effect on SVL differences.

In conclusion, the present study has shown that the approach using snout-vent length as the main indicator of size is most appropriate for the purpose of studying the direction and extent of SSD in this species, and that skeletochronology is a reliable, non-lethal method for collecting data on age-structure. We had a high percent of successful age determination - most cross-sections had sharply resolved and easily distinguishable LAGs. However, care should be taken to avoid possible under- or overestimation of individual age.

The most common problems we encountered were: a relatively high, though variable, degree of endosteal reso-

rption (the first LAG was resorbed partially or completely); and very closely spaced outer LAGs in some individuals. Though false or double lines were not frequently observed, we conclude that they deserve special attention especially in view of their connection with atypical climatic conditions and unfavorable environmental influences - the pattern of growth marks (as well as substantial overlap in SVL of age classes) may indicate an experienced "bad year" in the past, i.e., conditions unfavorable for growth, as suggested by P l a t z and L a t h r o p (1993).

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ОДРЕЂИВАЊЕ СТАРОСТИ И ИНТЕРСЕКСУАЛНИХ РАЗЛИКА У ВЕЛИЧИНИ ТЕЛА КОД *BUFO BUFO*

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Бројне студије истакле су сложену природу односа између старости, величине тела и репродуктивних особина код безрепих водоземаца. За еволуционе биологе, један од најинтригантнијих проблема је интерсексуална разлика у величини тела (SSD). За тестирање хипотеза које се тичу SSD, неопходне су тачне процене њене величине (изузетно је важно питање избора особина које се користе у тој анализи), као и прецизно одређивање старости. Процена SSD може бити погрешна ако није позната узрасна структура дате популације; водоземци настављају да расту током живота, а SSD је повезана са интерсексуалним разликама у особинама као што су време полног сазревања и дужина живота. У овом раду анализирамо проблеме везане за тачну процену узрасне структуре и чиниоце који могу да доведеу до прецењених или потцењених вредности, као и проблеме адекватног избора особина за анализу, с обзиром на наша искуства и резулатате проучавања популација *Bufo bufo* из околине Београда.