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*

**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 (Cvetkovic, 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; Francellon-Vieillot and Castanet, 1985; Francellon-Vieillot et al., 1990; Cvetković et al., 1996; Miaud et al., 1999), as well as tropical and subtropical (Kunbar 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; Høglund, 1989; Høglund 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-
tunguished 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 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.
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; Tomasević 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 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-
ruption (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 Plath and Lathrop (1993).

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REFERENCES


Castanet, J. (1975). Quelques observations sur la presence et la structure (Grant No. 1725).


Hemelaar, A. (1985). An improved method to estimate the number of year rings resorbed in phalanges of Bufo bufo (L.) and its application to populations from different latitudes and altitudes. Amphibia-Reptilia 6, 323-341.


ОДРЕЂИВАЊЕ СТАРОСТИ И ИНТЕРСЕКСУАЛНИХ РАЗЛИКА У ВЕЛИЧИНИ ТЕЛА КОД BUFO BUFO

ДРАГАНА ЦВЕТКОВИЋ1, НАТАША ТОМАШЕВИЋ2, И АЛЕКСИЋ И ЈЕЛКА ЦРНОБРЊА-ИСАИЛОВИЋ2

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Бројне студије истакле су сложену природу односа између старости, величине тела и репродуктивних особина код бестрехих водоземаца. За еволуционе биологе, један од најинтригантнијих проблема је интересексуална 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 узрсна структура дате популације; водоземци настављају да расту током живота, а SSD је повезана са интересексуалним разликама у особинама као што су време полног сазревања и дужина живота. У овом раду анализирамо проблеме везане за тачну процену узрсне структуре и чиноце који могу да доведе до прецењених или потцењених вредности, као и проблеме адекватног избора особина за анализу, с обзиром на наша искуства и резултате прочувања популација Bufo bufo из околине Београда.