HERPETOZOA 19 (3/4): 111 - 119
Wien, 30. Jänner 2007

Age determination by skeletochronology in the Common Indian Toad *Bufo melanostictus* SCHNEIDER, 1799
(Anura: Bufonidae)

Skelettochronologische Altersbestimmung bei der Schwarznarbenkröte, *Bufo melanostictus* SCHNEIDER, 1799
(Anura: Bufonidae)

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KURZFASSUNG

Die Abschätzung von Alter, Lebenserwartung sowie Alter bei Erreichen der Geschlechtsreife und bei der Fortpflanzung anhand skelettochronologischer Befunde wird für Schwarznarbenkröten, *Bufo melanostictus* SCHNEIDER, 1799 aus dem östlichen Indien beschrieben. Bei achtundsechzig während des Jahres 2003 gesammelten Individuen aus drei Altersgruppen wurden nach Betäubung mit Äther die Kopf-Rumpflänge gemessen und die vierte Zehe des linken Hinterbeines abgetrennt. Die Geschlechtsbestimmung erfolgte nach äußeren Merkmalen. Zur Bestimmung des Gonadenstatus und zur histologischen Untersuchung von Oberarm-, Oberschenkel- und Zehenknochen diente die Sektion von acht Tieren aus verschiedenen Altersgruppen. Die Knochen wurden in 70%igem Alkohol konserviert und in 10%igem EDTA entkalkt und für die histologische Untersuchung aufbereitet. Die Kröten wurden nach Kopf-Rumpflänge und Gonadenstatus drei Altersgruppen zugeordnet (Gruppe I: frisch metamorphosierte Individuen; Gruppe II: Jungtiere; Gruppe III: geschlechtsreife Tiere).

Zehenknochen erwiesen sich als repräsentativ für die Altersbestimmung, da die Anzahl der LAGs bzw. Wachstumsringe innerhalb jedes der acht untersuchten Exemplare in Femur, Humerus und Phalangenknochen jeweils die gleiche war. Der erste Wachstumsring erschien im zweiten Lebensjahr; Knochenquerschnitte von Tieren der Gruppe I und II zeigten keine Wachstumsringe. Kröten der Gruppe III hatten 1 bis 11 Wachstumsringe. Daraus wird geschlossen, daß *B. melanostictus* in der Natur bis zu 12 Jahre alt werden kann. Männchen wie Weibchen erreichen die Geschlechtsreife während ihres zweiten Lebensjahres. Klammernde Männchen sind immer kleiner als ihre weiblichen Partner und sind entweder jünger oder gehören derselben Altersgruppe an wie ihre Weibchen.

ABSTRACT

Skeletochronological estimation of age, longevity, age at sexual maturity and breeding of east Indian specimens of the Common Indian Toad *Bufo melanostictus* SCHNEIDER, 1799 have been described in the present study. Sixty-eight toads of three different age groups collected throughout the year 2003 were anaesthetized with ether prior to measurement of snout-vent length and clipping of fourth toe of left hind limb. Sex determination was based on external features. In order to determine the gonadal status, eight toads of different age were sacrificed. The same animals were used to study histology of long (humerus, femur) and phalangeal bones. Bones were preserved in 70% alcohol, decalcified with 10% EDTA and processed for histology. Toads were assigned to three groups depending on the SVL and gonadal status (group I: recently metamorphosed; group II; juveniles; group III: mature males and females).

Phalangeal bones turned out to be representative for age determination because number of growth marks in femur, humerus and phalanx was identical within each of eight specimens studied in this respect. The first growth ring appeared in the second year of life. Cross sections of bones of group-I and group-II toads revealed no growth rings. Toads belonging to group III showed 1 to 11 growth rings. Thus it is concluded that in nature, the maximum age of *Bufo melanostictus* is approximately 12 years. Both males and females attain sexual maturity during their second year of life. The amplectant males are always smaller in size than their female partners. These males are either younger than the female partners or of the same age group.

KEY WORDS

Amphibia: Anura: Bufonidae, *Bufo melanostictus*, skeletochronology, age determination, growth marks, lines of arrested growth (LAG), longevity, sexual maturity, breeding, eastern India

INTRODUCTION

The use of bone histology and application of skeletochronological techniques in vertebrates has been recognized as the most meaningful and practicable method not only to assess individual age, but also to determine the rate of growth, age at sexual maturity and the longevity of various vertebrates (fishes - QUASIM & BHATT 1966; SEMAKULA & LARKIN 1968; lizards - PEABODY 1961; SMIRINA 1974; PATNAIK & BEHERA 1981; MAHAPATRO et al. 1989; turtles - CASTANET & CHEYLAN 1979; birds - KLEVEZAL et al. 1972; mammals - KLEVEZAL & KLEINENBERG 1967). The method is based on the presence of growth layers recorded in cross-sections of long bones including phalanges. In amphibians, such cyclic growth rings or lines of arrested growth (LAGs) are considered to mirror the annual growth periodicity of an individual (HALLIDAY & VERRELL 1988; CASTANET & SMIRINA 1990) in that cyclic changes in the metabolic activity are paralleled by cyclic changes in the density of the bone layers. Thus, positive correlation between snout-vent-length (SVL) and number of growth marks indicates that larger individuals have experienced a greater number of growth cycles and therefore are older. Such positive correlation has been reported for *Bufo melanostictus* SCHNEIDER, 1799 inhabiting southern India (KUMBAR & PANCHARATNA 2001) and several other anuran species (*Rana temporaria* LINNAEUS, 1758 - RYSER 1988; *Polypedates maculatus* (GRAY, 1834), *Microhyla ornata* (DUMÉRIL & BIBRON, 1841), *Hoplobatrachus tigerinus* (DAUDIN, 1802) - KUMBAR & PANCHARATNA 2001; *Bufo bufo* (LINNAEUS, 1758) - HEMELAAR 1983; *Bufo pentoni* ANDERSON, 1893 - FRANCILLON-VIEILLOT et al. 1984; *Bufo calamita* LAURENTI, 1768 - TEJEDO 1991; *Euphlyctis cyanophlyctis* (SCHNEIDER, 1799) - KULKARNI & PANCHARATNA 1996).

There are several reports on skeletochronology of a variety of temperate amphibians (HEMELAAR 1981, 1988; SMIRINA 1983, 1994; FRANCILLON et al. 1984; CASTANET & SMIRINA 1990; CHERRY & VIEILLOT 1992; WAKE & CASTANET 1995; ESTEBAN et al. 1996; TEJEDO et al. 1997; SULIVAN & FERNANDEZ 1999; JAKOB et al. 2002). Skeletochronology of some few tropical anurans has also been investigated (KULKARNI & PANCHARATNA 1996; GUARINO et al. 1998; PANCHARATNA et al. 2000; KUMBAR & PANCHARATNA 2001, 2002; PANCHARATNA 2002; PANCHARATNA & DESHPANDE 2003). However, the anurans inhabiting eastern India are not yet explored sufficiently.

The aim of the present investigation was to employ skeletochronological methods in the determination of the age of the Common Indian Toad, *Bufo melanostictus* SCHNEIDER, 1799 inhabiting Bhubaneswar, eastern India and to compare our results with those obtained from *B. melanostictus* inhabiting Dharward, southern India (KUMBAR & PANCHARATNA 2001).

Histology of long (humerus, femur) and phalangeal bones of animals of all available age groups have been investigated to correlate the number of growth rings with body size, age, longevity, age at breeding and sexual maturity.

MATERIALS AND METHODS

Bufo melanostictus specimens of different size groups (SVL: 10.5 - 104.0 mm) were collected from the vicinity of Utkal University campus and the city of Bhubaneswar (20°18'N, 85°50'E) during various months of the year 2003. Following capture, the toads were anaesthetized with ether. SVL was recorded and the fourth toe of the left hind limb of each specimen was clipped. Eight toads belonging to different age groups were sacrificed to study the gonadal status (Table 1). The same animals were used for histology of long (humerus, femur) and phalangeal bones. Bones were fixed in 70% ethanol. Depending on body size and gonadal status, toads were assigned to three groups as follows (Table 2): group I (10 post-metamorphic toadlets, sexually undifferentiated, SVL: 10.0-27.2 mm), group II (20 juvenile males and fe-

Table 1: Sex, gonadal status, snout-vent-length (SVL, mm) and number of LAGs in femur, humerus and phalanx of eight *Bufo melanostictus* SCHNEIDER, 1799. J - juvenile, M - male, F - female, IMG - immature gonads, MG - mature gonads.

Tab. 1: Geschlecht, Gonaden-Entwicklungsstatus, Körpergröße (SVL, mm) und Anzahl der Linien verzögerten Wachstums (LAGs) in Femur, Humerus und Phalanx bei acht *Bufo melanostictus* SCHNEIDER, 1799. J - Jungtier, M - Männchen, F - Weibchen, IMG - unreife Gonade, MG - reife Gonade.

ID No lfd. Nr.	Sex	Gonad. Status	SVL	Nr. of LAGs Anzahl LAGs
1	J	IMG	29.5	0
2	J	IMG	35.0	0
3	M	MG	41.5	1
4	M	MG	67.4	2
5	M	MG	77.0	5
6	F	MG	46.5	1
7	F	MG	71.0	3
8	F	MG	99.5	6

Table 2: Body size (SVL, mm) and number of LAGs in different age groups of *Bufo melanostictus* SCHNEIDER, 1799. n - number, SD – standard deviation, SU - sexually undifferentiated, JF - juvenile female, JM - juvenile male, MF - matured females, MM - matured males.

Tab. 2: Körpergröße (SVL, mm) und Zahl der Linien verzögerten Wachstums (LAGs) in verschiedenen Altersgruppen von *Bufo melanostictus* SCHNEIDER, 1799. n - Anzahl, SD – Standardabweichung, SU - Geschlechtsunterschied nicht ausgeprägt, JF - juvenile Weibchen, JM - juvenile Männchen, MF - reife Weibchen, MM - reife Männchen.

Group Gruppe	(n)	Mean SVL ± SD Mittlere SVL ± SD	No. of LAGs Anzahl LAGs
I (SU)	(10)	17.44±6.77	0
II (JF)	(10)	37.36±5.32	0
II (JM)	(10)	36.37±3.19	0
III (MF)	(20)	79.07±14.95	1-11
III (MM)	(18)	67.24±10.69	1-5

males, SVL: 30.2 - 42.5 mm), group III (38 mature males and females, SVL: 41.5 - 104 mm). The features body size (SVL), expression of secondary sexual charactes, gonadal status and number of LAGs as observed in eight specimens was made available as a criterion for sexual maturity in animals which were not dissected (Table 1). Besides, six amplectant pairs were also investigated (Table 3).

Table 3: Body size (SVL, mm), number of LAGs and age in twelve individuals of six amplectant pairs of *B. melanostictus* SCHNEIDER, 1799. F - mature female, M - mature male.

Tab. 3: Körpergröße (SVL, mm), Anzahl Linien verzögerten Wachstums (LAGs) und Alter bei zwölf (sechs Pärchen) *B. melanostictus* SCHNEIDER, 1799. F - reifes Weibchen, M - reifes Männchen.

Amplectant pair No. Pärchen Nr.	SVL	Sex	No. of LAGs Anzahl LAGs
1	77.0	F	3
	67.0	M	2
2	93.0	F	7
	74.0	M	3
3	85.0	F	5
	70.0	M	3
4	87.0	F	3
	77.0	M	3
5	85.0	F	3
	66.0	M	3
6	75.0	F	2
	69.5	M	2

The bones were decalcified at room temperature in 10% EDTA (ethylene diamine tetra acetic acid). The period of decalcification ranged from 24 to 48 h depending on the size of the bones. After decalcification, the bones were washed thoroughly in running tap water for 24 h. Following washing, the bones were dehydrated and processed for paraffin block preparation. Serial sections (8-10 µm) were cut using a rotary microtome. The sections were stained with Delafield hematoxylin and eosin and examined under a compound microscope. Photographs were obtained from appropriate sections.

RESULTS

In general, histology of humerus, femur and phalanges revealed a central marrow cavity and an outer cortical layer (Figs. 1A, 1B). The cortical layer was lined by the endosteal layer on its inner side and periosteal layer towards the outer side. In between these two layers, a distinct matrix was present in which the osteocytes were distributed evenly. Thin and darkly stained chromophilous lines known as lines of ar-

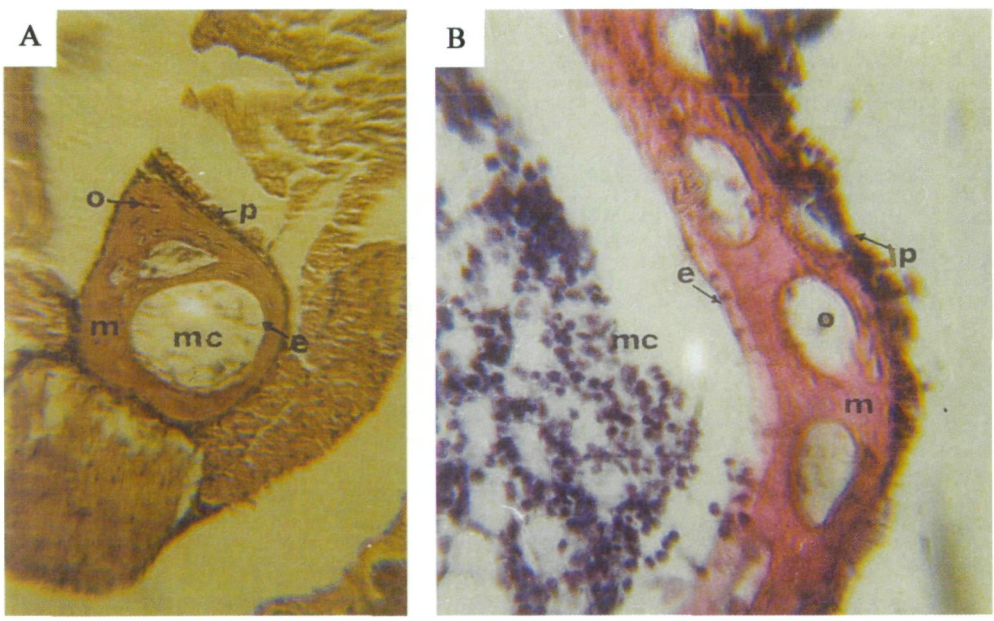
rested growth (LAGs) were observed (Figs. 2A – 2D) in the matrix of the matured toads. The intermediate zones between two LAGs were lighter in color and referred to as the annual growth rings. The LAGs were devoid of cellular elements. SVL and number of LAGs, as found in the individuals of three age groups analyzed are represented in Table 2 and Figs. 3 and 4.

Within each of eight specimens studied (Table 1), the number of growth marks was the same in femur, humerus and phalangeal bone. Thus, phalangeal LAG counts paralleled accurately humeral and femoral LAG counts in this toad and could be used as the only source of skeletochronological data.

A positive correlation was established between the number of LAGs and SVL for male (r = 0.664) and female (r = 0.782) toads and both sexes collectively (r = 0.714) (Figs. 3, 4).

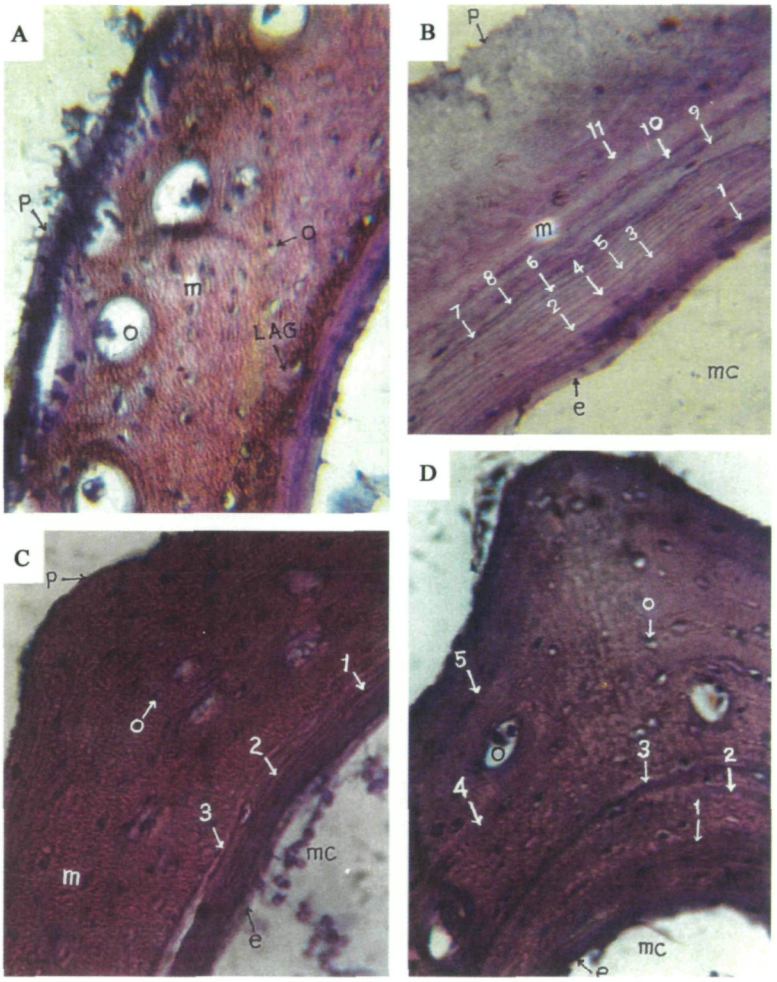
Group I (post-metamorphic toadlets, sexually undifferentiated) [n = 10, SVL: 10.0-27.2 mm, captured from July to September] (Table 2). The matrix of the femur bone was devoid of any line of arrested growth. Smaller osteocytes were scattered throughout the matrix (Fig. 1A).

Group II (juvenile males and females) [n = 20, SVL: 30.2-42.5 mm in 10 females, 33.0-40.5 mm in 10 males, collected from August to November] (Table 2). Histology of bones of both males and females revealed no LAGs. Cross section of femur showed the cortical layer to be comparatively thicker than in group I toads. Both inner endosteal and outer periosteal layers were distinct; large osteocytes were present (Fig. 1B).



Figs. 1A and 1B: Cross sections (C. S.) of femur bones of *Bufo melanostictus* SCHNEIDER, 1799.
1A - C. S. (20x) through femur of metamorphosed toad from group I (SVL = 10.0 mm) shows central marrow cavity (mc), inner endosteal layer (e), outer periosteal layer (p), few osteocytes (o) in the matrix (m) and no LAGs.
1B - C. S. (80x) through femur of juvenile male toad from group II (SVL = 37.0 mm) shows a thicker cortical layer without LAG and larger osteocytes (o) in the matrix (m).

Abb. 1A und 1B: Querschnitte von Oberschenkelknochen von *Bufo melanostictus* SCHNEIDER, 1799.
1A - Der Schnitt (20x) durch den Oberschenkelknochen metamorphosierter Kröten der Gruppe I (Kopf-Rumpf-Länge = 10,0 mm) zeigt die zentrale Markhöhle (mc), die innere endostale Schicht (e), die äußere periostale Schicht (p), wenige Knochenbildnerzellen (o) in der Grundsubstanz (m) und keine Linien verzögerten Wachstums.
1B - Der Schnitt (80x) durch den Oberschenkelknochen einer jungen männlichen Kröte der Gruppe II (Kopf-Rumpf-Länge = 37.0 mm) zeigt eine dickere Rindenschicht ohne Wachstumsstillstandslinie und größere Knochenbildnerzellen (o) in der Grundsubstanz (m).



Figs. 2A – 2D: Cross sections (C. S., 200x) of different bones of matured (Group III) *Bufo melanostictus* SCHNEIDER, 1799.
2A - C. S. through femur of a female toad (SVL = 46.5 mm) shows a single LAG, peripheral larger osteocytes (o) and smaller osteocytes scattered throughout the matrix (m).
2B - C. S. through 4th phalanx of female toad (SVL = 104.0 mm) shows eleven LAGs (1-11).
2C - C. S. through 4th phalanx of male toad (SVL = 71.0 mm) shows three LAGs (1-3), larger osteocytes (o) are present in the central part and smaller osteocytes are scattered in the matrix (m).
2D - C. S. through 4th phalanx of a male toad (SVL = 78.0 mm) shows five LAGs (1-5), few larger osteocytes (o) are confined to the central part and smaller osteocytes are scattered throughout the matrix (m).

Abb. 2A – 2D: Querschnitte (200x) durch verschiedene Knochen reifer Individuen (Gruppe III) von *Bufo melanostictus* SCHNEIDER, 1799.
2A - Der Oberschenkelknochen einer reifen weiblichen Kröte (Kopf-Rumpf-Länge = 46.5 mm) zeigt eine einzige Linie verzögerten Wachstums, randlich größere (o), in der Grundsubstanz verteilt kleinere Knochenbildnerzellen (m).
2B - Ein Knochen der 4. Zehe einer weiblichen Kröte (Kopf-Rumpf-Länge = 104.0 mm) zeigt elf Linien verzögerten Wachstums (1-11).
2C - Ein Knochen der 4. Zehe einer männlichen Kröte (Kopf-Rumpf-Länge = 71.0 mm) zeigt drei Linien verzögerten Wachstums (1-3), größere Knochenbildnerzellen in der Mitte (o), kleinere in der Grundsubstanz verteilt (m).
2D - Ein Knochen der 4. Zehe einer männlichen Kröte (Kopf-Rumpf-Länge = 78.0 mm) zeigt fünf Linien verzögerten Wachstums (1-5), wenige größere, auf die Mitte beschränkte (o) und kleinere über die Grundsubstanz verteilte Knochenbildnerzellen (m).

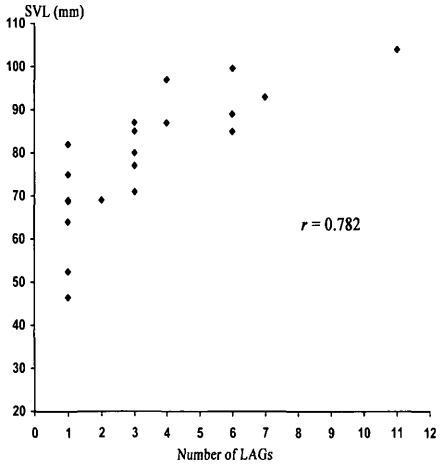


Fig. 3: Correlation ($r = 0.782$) between body size (SVL, mm) and number of lines of arrested growth (LAGs) in female *Bufo melanostictus* SCHNEIDER, 1799.

Abb. 3: Korrelation ($r = 0,782$) zwischen Körpergröße (Kopf-Rumpf-Lämge, SVL, mm) und der Zahl der Linien verzögerten Wachstums (LAGs) bei Weibchen von *Bufo melanostictus* SCHNEIDER, 1799.

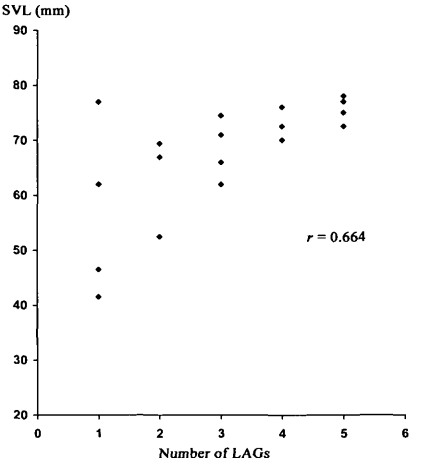


Fig. 4: Correlation ($r = 0.664$) between body size (SVL, mm) and number of lines of arrested growth (LAGs) in male *Bufo melanostictus* SCHNEIDER, 1799.

Abb. 4: Korrelation ($r = 0,664$) zwischen Körpergröße (Kopf-Rumpf-Lämge, SVL, mm) und der Zahl der Linien verzögerten Wachstums (LAGs) bei Männchen von *Bufo melanostictus* SCHNEIDER, 1799.

Group III (mature males and females) [n = 38, SVL: 46.5-104.0 mm in 20 females, 41.5-78.0 mm in 18 males] (Table 2). In the females, the number of LAGs ranged from one to eleven. The smallest mature female (SVL 46.5 mm) showed one LAG (Fig. 2A). In between endosteal layer and LAG a distinct lighter growth ring was present which was devoid of any cellular elements. Larger osteocytes were confined to the periphery whereas smaller osteocytes were distributed throughout the matrix. The longest female (SVL 104.0 mm) showed eleven growth rings (Fig. 2B). Similar growth rings were also observed in male toads. A smaller male measuring 66.0 mm in SVL showed three LAGs (Fig. 2C). Smaller osteocytes were scattered throughout the matrix while a few larger osteocytes were present in the central area of the matrix. Maximum number of LAGs observed in a male toad (SVL 75.0 mm) was five (Fig. 2D).

Six amplectant pairs were collected during the monsoon period (May - July). In all the pairs the females were larger than the males (Table 3). Histology of phalangeal bones of these pairs showed three to seven growth rings in females and two to three in males. In three pairs, the females had more growth rings than the males and in three pairs the numbers were equal.

DISCUSSION

Phalangeal bones turned out to be representative for age determination because the number of growth marks in femur, humerus and phalangeal bone was identical within each of the eight specimens studied. Similar observations have been reported from other amphibians also [*Limnonectes limnocharis* (BOIE, 1835), *Microhyla ornata* and *Euphlyctis cyanophlyctis*; PANCHARATNA & DESHPANDE 2003).

Both group I and group II toads showed no growth rings as expected because of their young age; they were captured within three to five months after mass breeding

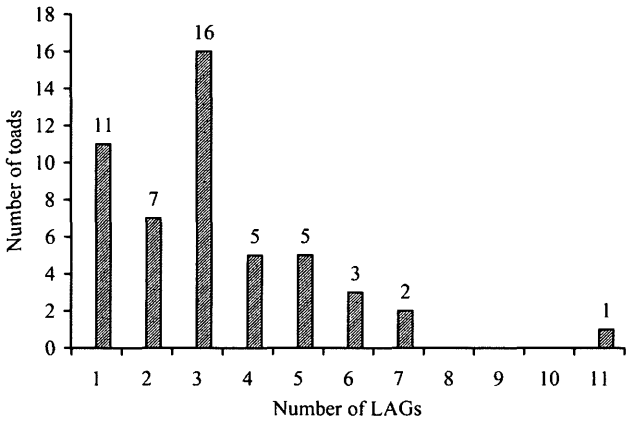


Fig. 5: Number of LAGs found in 50 randomly sampled mature individuals of *Bufo melanostictus* SCHNEIDER, 1799. The graph also shows the distribution of the toads according to different age groups.

Abb. 5: Zahl der Linien verzögerten Wachstums (LAGs) in einer Zufallsstichprobe von 50 geschlechtsreifen *Bufo melanostictus* SCHNEIDER, 1799, bzw. die Verteilung der Kröten auf die verschiedenen Altersgruppen.

in May. However, in group II toads the bone cortex was thicker than in group I toads and there was an increase in number as well as size of the osteocytes. One to eleven LAGs were observed in group III toads. The smallest mature female (SVL 46.5 mm) showed one LAG (Fig. 2A) indicating its age to be more than one year and that females attain sexual maturity in the second year of their life. The highest number of LAGs recorded in the present study for a female toad (SVL 104.0 mm) was eleven. This toad is considered to be the oldest and twelve years old.

Skeletochronology of adult *B. melanostictus* inhabiting Dharward (15°17' N, 75°3' E) of southern India has been described earlier (KUMBAR & PANCHARATNA 2001) and shows the oldest toad to be five years old in contrast to the present investigation where the oldest toad is found to be twelve years old. This difference in longevity may be related to the geographical position of the respective regions because in the south, the annual variation in mean temperature normally does not exceed 10°C (KUMBAR & PANCHARATNA 2001) whereas the variation in temperature is around 25°C in the eastern part of India. Age of amplectant pairs has been described for the first time in this species.

The smallest mature male (SVL 41.5 mm) showed one LAG indicating the minimum age at sexual maturity to be two years. The longest male captured in the present study (SVL 78.0 mm) showed five LAGs (Fig. 2D) suggesting the age of the toad to be six years. Based on the present study it can be concluded that the longevity of female toads (twelve years) is higher than that of the males (six) in nature and maximum age is twelve years in contrast to the earlier report of five years (KUMBAR & PANCHARATNA 2001). The present finding is comparable to observations on the maximum age of *Bufo bufo* in nature (SMIRINA 1994) whose longevity is estimated to be twelve years. Longevity of other temperate species of *Bufo* (*B. americanus* HOLBROOK, 1836 - five years; *B. arenarum* HENSEL, 1867 - eight years and *B. pentoni* - six years; SMIRINA 1994) reported so far is less than in *B. melanostictus*. Comparison of longevity of various temperate (*Rana ridibunda* PALLAS, 1771, twelve years; *R. esculenta* LINNAEUS, 1758, twelve years; *R. temporaria*, fourteen years: SMIRINA 1994) as well as tropical anurans (*Euphlyctis cyanophlyctis*, seven years; KULKARNI & PANCHARATNA 1996: *Microhyla ornata*, five years; KUMBAR & PANCHARATNA 2001; *Limnonectes*

limnocharis, four years; PANCHARATNA & DESHPANDE 2003) shows that the temperate species usually live longer than the tropical species.

The age of 12 years (11 LAGs) should be considered exceptional as shown by the considerable gap in Figs. 3 through 5 while longevity of five (males) to eight (females) years is the rule in the *B. melanostictus* population studied.

Among 50 randomly collected mature specimens, the proportion of toads with three LAGs was highest (32%) followed by 22% which had one and 14% which had two LAGs. There was gradual decrease in number of toads with increase in number of LAGs (10% with four LAGs, 10% with five, 6% with six, 4% with seven 2% with eleven LAGs) indicating that in the population studied, the younger toads are more in number than the older individuals which is typical to steady populations (Fig. 5).

In conclusion, the present skeletochronological study reveals that in *B. melanostictus* (1) maximum age observed in nature was twelve years (2) females live longer than males (3) in amplectant pairs, males are smaller in size than their female partners and either younger than the females or of the same age group, (4) a positive correlation exists between SVL and age (r = 0.714), (5) both males and females attain sexual maturity in the second year after metamorphosis.

ACKNOWLEDGMENTS

Financial assistance from Department Research Support II - University Grants Commission, Government of India to Post Graduate Department of Zoology, Utkal University is gratefully acknowledged.

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DATE OF SUBMISSION: February 28, 2005 Corresponding editor: Heinz Grillitsch

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