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Estimation of age and longevity of the Indian Bullfrog *Hoplobatrachus tigerinus* (DAUDIN, 1802): A skeletochronological study
(Anura: Ranidae)

Abschätzung von Alter und Lebenserwartung beim Indischen Ochsenfrosch *Hoplobatrachus tigerinus* (DAUDIN, 1802): Eine skelettochronologische Untersuchung
(Anura: Ranidae)

KATTI PANCHARATNA & SURESH M. KUMBAR

KURZFASSUNG

Die vorliegende skelettochronologische Studie untersucht Alter und Lebenserwartung beim Indischen Ochsenfrosch *Hoplobatrachus tigerinus* (DAUDIN, 1802). Achtundsechzig Frösche (Körpermasse: 25 - 600 g; Kopf-Rumpflänge: 6,5 - 17 cm) aus der Küstenregion von Karwar (14°50'N, 74°15'E) Südindien, standen zur Verfügung. Im Labor wurde die Masse und Größe jedes Frosches erhoben und die vierte (längste) Zehe der Hinterbeine unter schwacher Äthernarkose abgetrennt und histologisch untersucht. Die Schnitte von 10 µm Dicke wurden mit Harris Hämatoxylin gefärbt. Femur, Humerus, Tibiofibula und Radioulna von sechs großen Individuen wurden gleichfalls histologisch untersucht, um die Knochenresorptionsrate in diesen Knochen mit jener in Phalangen zu vergleichen. Schnitte durch die Diaphysenmitte von Phalangen- und Extremitätenknochen zeigten Wachstumsringe, die jeweils aus einer breiteren Wachstumszone und einer chromophilen Linie verzögerten Wachstums (LAG) bestanden. Von den Fröschen wiesen 7.4% keine LAGs auf, 30.9% jeweils eine, 27.9% zwei, 17.6% drei, 10.3% vier und 5.9% der Frösche hatten fünf LAGs in ihren Phalangenquerschnitten. Die Rückrechnung zeigte, daß bei größeren Tieren zumindest eine LAG aufgrund von Resorption verloren gegangen war. Die Ergebnisse legen nahe, daß diese Froschart im Freien über sieben Jahre alt werden kann.

ABSTRACT

An investigation was carried out to estimate the age and longevity of the Indian Bullfrog *Hoplobatrachus tigerinus* (DAUDIN, 1802). Sixty-eight frogs (body mass: 25 - 600 g; snout-vent-length: 6.5 - 17 cm) collected from the coastal region of Karwar (14°50'N, 74°15'E) southern India, were examined. In the laboratory, body mass and size of each frog were recorded, the fourth (longest) toe of both right and left hind limb were clipped under mild ether anesthesia and processed for histology. Sections of 10 µm thickness were stained with Harris hematoxylin. Femur, humerus, tibiofibula and radioulna of six large sized frogs were also processed for histology in order to compare the rate of bone resorption in these bones with that of phalanges. Mid-diaphyseal sections of phalanges and limb bones exhibited growth rings, each consisting of a broader growth zone, and a chromophilic line of arrested growth (LAG). Among the frogs studied, 7.4% showed no LAGs, 30.9% one LAG each, 27.9% two LAGs, 17.6% three LAGs, 10.3% four LAGs and 5.9% of frogs exhibited five LAGs in their phalangeal histology. Back calculation indicated that in larger-sized frogs, at least one LAG is lost due to resorption. The results suggest that this frog can attain an age of more than seven years in nature.

KEY WORDS

Amphibia: Anura: Ranidae: *Hoplobatrachus tigerinus*, age, longevity, skeletochronology, growth mark

INTRODUCTION

In vertebrates, especially amphibians and reptiles, annual cyclicity in osteogenesis and its cessation, leave marks in the structure of bones that indicate the number of growth cycles experienced by the animal and, therefore, the age of the individual (PEABODY 1961; MINA 1974; HEMELAAR 1981; FRAZIER 1985; CASTANET et al. 1993; GUARINO et al. 2003). The validity and reliability of skeletochronology in the determination of age and longevity were thoroughly reviewed (HALLIDAY & VERRELL 1988; CASTANET & SMIRINA 1990; SMIRINA 1994). It is evident from these reviews that a majority of studies on the age determination of amphibians originate from temperate zones, where drastic fluctuations in the ambient temperature become a limiting factor for feeding activity and enforce seasonal bone growth. Comparative studies on tropical amphibian representatives are scanty (HALLIDAY & VERRELL 1988; SMIRINA 1994; ESTEBAN et al. 1996). The present investigation is an attempt to estimate the age and longevity of the Indian Bullfrog *Hoplobatrachus tigerinus* (DAUDIN, 1802), in a population inhabiting the coastal region of Karwar, southern India, using skeletochronology.

MATERIALS AND METHODS

Specimens of *Hoplobatrachus tigerinus* (body mass: 25 - 600 g; body size [snout-vent-length, SVL]: 6.5 - 17 cm; n = 68) were collected randomly from the coastal area of Karwar, southwest India (14°50'N, 74°15'E) between August and October 1997. In the laboratory the body mass (to the nearest gram) and snout-vent length (SVL, measured to the nearest mm using a thread) of each frog were recorded under mild ether anesthesia. The 4th (longest) toe of both right and left hind limb of each frog were clipped and fixed in 10% formalin and numbered serially. All the long bones of limbs (femur, humerus, tibiofibula and radioulna) of six large sized (SVL: 9 - 17 cm) frogs were also processed for skeletochronology in order to compare the number of growth marks and the rate of endosteal bone (Eb) resorption in these bones with those occurring in phalanges. The remaining frogs were released. Clipped toes and limb bones were cleaned and demineralized in 5% nitric acid and processed for histology. Paraffin sections of 10 µm thickness were cut on a microtome and stained with Harris hematoxylin (KULKARNI & PANCHARATNA 1996; ROSSELL & SHEEHAN 1998). Mid-diaphyseal sections of the limb bones and the distal phalanx were chosen for observation under a compound microscope. Growth zones and lines of arrested growth (LAGs) were clearly visible in the cross-section of phalanges and limb bones.

The diameters of marrow cavity (Mc), LAGs and periosteal bone margin (Pbm) were measured from phalangeal cross sections of all frogs using an ocular micrometer. The loss of LAGs if any, owing to endosteal resorption was determined using the 'back calculation method', i.e., by comparing the diameters of Pbm of smaller frogs with those of the marrow cavity of larger frogs (CASTANET et al. 1996; KUSANO et al. 1995a, 1995b; KUMBAR & PANCHARATNA 2001b; KUMBAR & PANCHARATNA 2003). The relationship between the number of growth marks versus body mass and body size were assessed by drawing scatter plots and calculating correlation coefficients (r) (STEEL & TORRIE 1980).

The meteorological data used in the present study were obtained from the Government of India Meteorological Center, Bangalore, India.

RESULTS

Monthly variations in the ambient temperature, rainfall and relative humidity at Karwar during the year 1997 (January 1998 included) are shown in figure 1. Although, the mean temperature varied from 24.3°C to 30.5°C, the changes in mean relative humidity were between 69% to 88%, and that of the rainfall between 0 to 1258 mm (fig. 1).

Cross-sections of phalanges and long limb bones of *H. tigerinus* showed growth rings, each ring composed of a faintly stained broader growth zone, and a darkly stained condensed chromophilic line, the LAG (fig. 2). Expression of LAGs as double and triple lines was also noticed. In 42 frogs (61.8%), LAGs were represented exclusively as single lines while, in 23 specimens (33.8%) both single and double lines were observed; in three frogs (4.4%) there were triple lines along with single and double lines.

Table 1: Number of LAGs (lines of arrested growth) in bones, body size (SVL – Snout-vent-length) and probable age in 68 individuals from a natural population of *Hoplobatrachus tigerinus* (DAUDIN, 1802).

Tab. 1: Anzahl LAGs (Wachstumsstillstands-Zonen) in den Knochen, Kopf-Rumpflänge (SVL) und vermutliches Lebensalter bei 68 Individuen aus einer natürlichen Population von *Hoplobatrachus tigerinus* (DAUDIN, 1802).

Number of LAGs Anzahl LAGs	SVL (cm)	Age (year of life) Alter (Lebensjahr)	Number of frogs Anzahl Frösche	%
0	09.3 ± 0.8	1st	5	07.4
1	10.5 ± 0.2	2nd	21	30.9
2	12.9 ± 0.3	3rd	19	27.9
3	13.6 ± 0.5	5th	12	17.6
4	14.6 ± 0.4	6th	7	10.3
5	14.1 ± 0.4	7th	4	05.9

Histology of the distal phalanx of the smallest (body mass: 25 g, SVL: 6.5 cm) frog of our capture showed a large marrow cavity in the center, circumferenced by a thin periosteal bone (fig. 2a), the outer margin of which measured 504 µm in diameter (fig. 3). Four frogs (7.4%, SVL: 9.3 ± 0.8 cm) showed no LAGs in the periosteal bone (table 1) but, in these frogs bone remodeling was initiated and the formation of an endosteal layer from the inner part of periosteal bone was noticed. Twenty-one frogs (30.9%, SVL: 10.5 ± 0.2 cm) showed one LAG each in the periosteal layer (table 1). Nineteen frogs (27.9%, SVL: 12.9 ± 0.3 cm) exhibited two LAGs (table 1); 12 frogs (17.6%, mean SVL: 13.6 ± 0.5 cm) showed three periosteal LAGs (fig. 2b). Four LAGs were present in the cross-section of phalanges of seven frogs (10.3%, SVL: 14.6 ± 0.4 cm) (table 1). And, another four frogs (5.9%, SVL: 14.1 ± 0.4 cm) showed five periosteal LAGs in phalangeal histology (table 1, fig. 2c).

In four out of six large-sized frogs (in which all limb bones were processed for skeletochronology), the number of LAGs was identical in all long bones and phalanges (table 2). In one of the frogs, the phalanx was short of one LAG compared to limb bones and in another, femur and humerus exhibited an extra ring compared to phalanx, radioulna and tibiofibula (table 2).

The measurements of the diameters of marrow cavity, endosteal bone, LAGs and periosteal bone margin of frogs representing all the age groups are shown in figure 3. When 'back calculation' was made in order to estimate the loss of LAGs, if any, due to endosteal resorption, it was found that in frogs exhibiting more than four LAGs, the first LAG had undergone resorption which was also confirmed by phalangeal histology (figs. 2c and 3).

There was a positive correlation between the number of growth marks and both body mass ($r = 0.71$, fig. 4) and body size ($r = 0.72$, fig. 5).

Table 2: The number of lines of arrested growth (LAGs) as found in different bones of six individuals of *Hoplobatrachus tigerinus* (DAUDIN, 1802).

Tab. 2: Anzahl der Wachstumsstillstands-Zonen (LAGs) in verschiedenen Knochen von sechs Individuen von *Hoplobatrachus tigerinus* (DAUDIN, 1802).

Frog #	Femur	Humerus	Radioulna	Tibiofibula	Phalanx
17	1	1	1	1	1
22	3	3	2	2	2
25	2	2	2	2	2
73	3	3	3	3	3
75	1	1	1	1	1
26	4	4	4	4	3

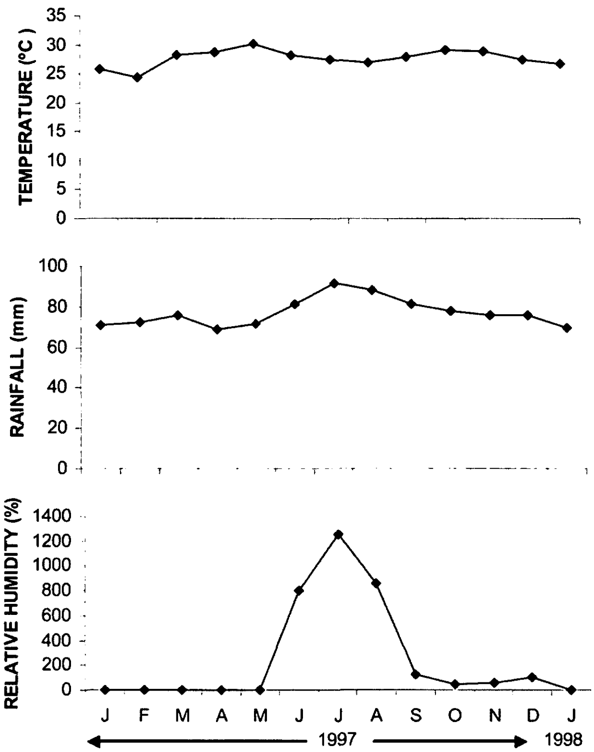


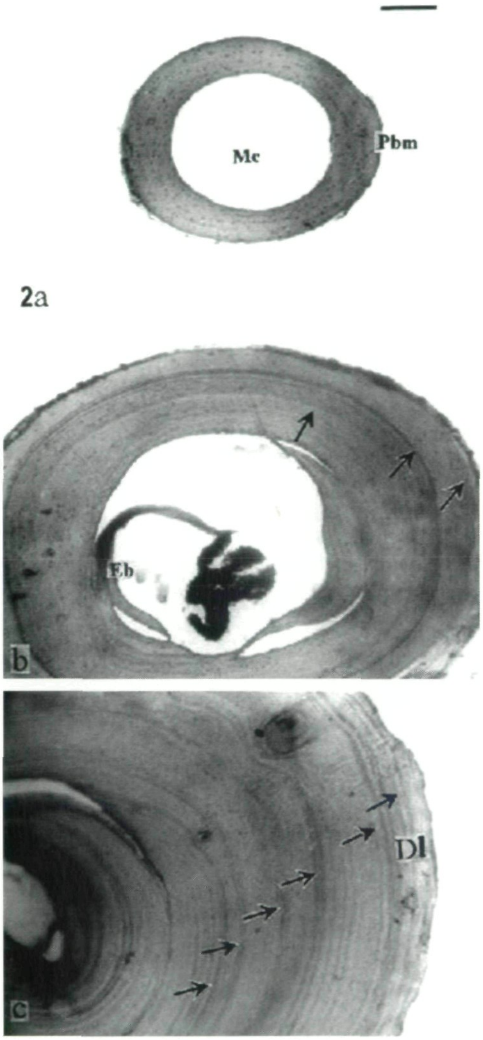
Fig. 1. Monthly averages of temperature (°C), rainfall (mm), and relative humidity (%) for Karwar, southwestern India, indicated for the period of January 1997 to January 1998.

Abb. 1: Die Monatsmittelwerte von Temperatur (°C), Niederschlagsmenge (mm), und relativer Luftfeuchte (%) von Karwar, Südwest-Indien, angegeben für die Zeit Jänner 1997 bis Jänner 1998.

DISCUSSION

The Indian Bullfrog *H. tigerinus* is widely used as a model for teaching and research in physiology and reproduction because of its large size and availability. Although, a lot of work has been carried out on the various aspects of reproduction, the aging and longevity of this frog are poorly understood and no skeletochronological study has been carried out previously on this species.

Skeletochronological observations on this frog reveal the presence of growth marks consisting of broader growth zones and chromophilic LAGs in the cross-sections of phalanges and limb bones (KUMBAR & PANCHARATNA 2001a, fig. 2). This frog shows a clear-cut seasonality in body mass and fat-body mass (PRAMODA & SAIDAPUR 1984; HOQUE & SAIDAPUR 1994) suggesting indirectly that bone growth is a cyclical phenomenon. Both body and fat-body mass reach their minimal values between June - August, which coincides with the onset of monsoon rains and breeding activity of the frog; from September onwards there is an increase in body and fat-body mass, and they attain their maximal values between December and April (PRAMODA & SAIDAPUR 1984; HOQUE & SAIDAPUR 1994). Therefore, in this frog, the LAG(s) may be laid down between June - August when the body growth and food reserves almost ceased



Figs. 2a-2c. Cross-sections of distal phalanges of *Hoplobatrachus tigerinus* (DAUDIN, 1802) (Hematoxylin). 2a - Phalangeal histology showing no LAG (smallest sized frog in the sample), 2b - with three LAGs, and 2c - five LAGs. Scale line = 100 µm. SVL – Snout-vent-length; Mc - Marrow cavity; Eb - Endosteal bone; Pbm - Periosteal bone margin; LAGs - Lines of arrested growth; Dl - Double lines.

Abb. 2a-2c. Querschnitte durch distale Phalangen von *Hoplobatrachus tigerinus* (DAUDIN, 1802) (Hämatoxylin). 2a – Querschnitt ohne LAG (kleinster Frosch in der Untersuchung), 2b – mit drei LAGs und 2c - fünf LAGs. Maßstab = 100 µm. Mc - Markhöhle; Eb - Endostaler Knochen; Pbm – Rand des Periostalen Knochens; LAGs - Wachstumsstillstands-Zonen; Dl - Doppellinien.

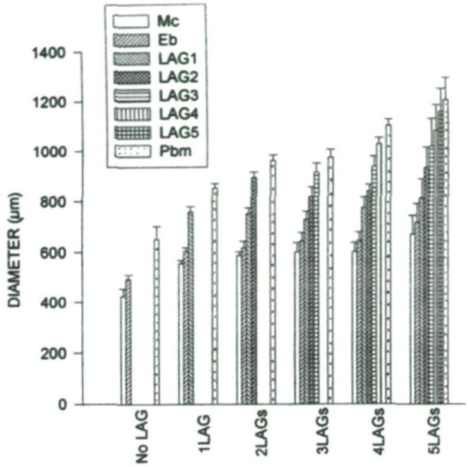


Fig. 3: Diameters (mm) of marrow cavity (Mc), endosteal bone (Eb), lines of arrested growth (LAGs) and periosteal bone margin (Pbm) of phalange bones of *Hoplobatrachus tigerinus* (DAUDIN, 1802). Values are mean ± standard error, n = 68.

Abb. 3: Die Durchmesser (µm) von Markhöhle (Mc), endostalem Knochen (Eb), Wachstumsstillstands-Zonen (LAGs) und periostalem Knochen (Pbm) bei Fingerknochen von *Hoplobatrachus tigerinus* (DAUDIN, 1802). Angegeben sind der Mittelwert ± Standardfehler, n = 68.

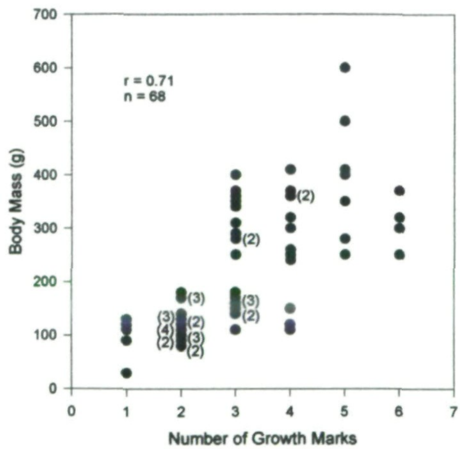


Fig. 4: Relationship between body mass (g) and number of growth marks in bones of 68 specimens of *Hoplobatrachus tigerinus* (DAUDIN, 1802). In parentheses: number of dots in identical position.

Abb. 4: Beziehung zwischen Körpermasse (g) und Anzahl der Wachstumszonen in den Knochen bei 68 Exemplaren von *Hoplobatrachus tigerinus* (DAUDIN, 1802). In Klammern die Anzahl von Punkten in identer Position.

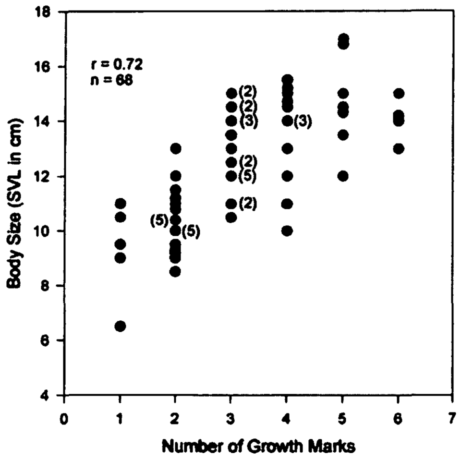


Fig. 5: Relationship between body size (SVL, cm) and number of growth marks in bones of 68 specimens of *Hoplobatrachus tigerinus* (DAUDIN, 1802). In parentheses: number of dots in identical position.

Abb. 5: Beziehung zwischen Kpf-Rumpflänge (SVL, cm) und Anzahl der Wachstumszonen in den Knochen bei 68 Exemplaren von *Hoplobatrachus tigerinus* (DAUDIN, 1802). In Klammern die Anzahl von Punkten in identer Position.

coinciding with the wet season of the year. From September onwards when body and fat-body masses begin to restore, the next osteogenic cycle may set in. Further, the thermal sensitivity of these frogs may enforce them to undergo aestivation and hibernation which is evident by the presence of double and triple LAGs (indicating resting periods within osteogenic phases) in more than 40% of the frogs in the natural population.

It is known that structural remodeling in bones and the rate of endosteal resorption severely influence skeletochronological interpretations in determining the age of amphibians (HALLIDAY & VERRELL 1988; CASTANET & SMIRINA 1990; SMIRINA 1994). In the present study, the skeletochronological comparison of limb bones and phalanges indicate that in four out of six frogs the number of LAGs was identical in all bones. In one frog, femur and humerus showed an extra LAG and in another, the phalanx was short of a LAG compared to other bones. Therefore, usage of long tubular bones such as femur or humerus may be ideal for skeletochronological age estimation of this frog, however, the results also suggest that phalangeal skeletochronology (which is a nonlethal method, therefore, ideal for assessing live samples) is reliable in 90% of frogs. Back calculation revealed that in all larger-sized frogs with more than four LAGs in phalangeal histology, the first LAG had undergone resorption. Therefore, frogs with four LAGs may be in their 6th year and those with five LAGs in the 7th year. Our previous studies on the age and longevity of southwest Indian species of anurans suggest that *Euphlyctis cyanophlyctis* (SCHNEIDER, 1799) [SVL = 3 - 7 cm], *Bufo melanostictus* (SCHNEIDER, 1799) [SVL = 5 - 11 cm], *Microhyla ornata* (DUMÉRIL & BIBRON, 1841) [SVL = 1.2 - 2.5 cm], and *Limnonectes limnocharis* (BOIE, 1835) [SVL = 1 - 4 cm], which have smaller body size, live for a maximum of five to six years (PANCHARATNA et al. 2000; KUMBAR & PANCHARATNA 2001a; PANCHARATNA 2002; PANCHARATNA & DESHPANDE 2003). While the results of the present study reveal that the larger (SVL > 17 cm) species *H. tigerinus* may live more than seven years in the natural population. In tropical anurans, as body size and mass show a positive correlation with the number of growth marks, whether the body size influences age or vice-versa, needs further elucidation.

In conclusion, the results of the present study reveal that growth marks are expressed as growth zones and LAGs in *H. tigerinus*. In larger-sized frogs at least one LAG is lost due to endosteal resorption. Double and triple LAGs indicating the prevalence of resting periods in between osteogenic cycles are common in this frog. In the natural population this frog may live for more than seven years.

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AUTHORS: Katti PANCHARATNA, Suresh M. KUMBAR, Department of Zoology, Karnatak University, Dharwad - 580 003, India < pancharatnak@yahoo.com >.

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