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Skeletochronological data on age, body size and mass in the Indian Cricket Frog *Limnonectes limnocharis* (BOIE, 1835) (Anura: Ranidae)

Skelettochronologische Daten zu Alter, Körpergröße und -masse bei *Limnonectes limnocharis* (BOIE, 1835) (Anura: Ranidae)

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KURZFASSUNG

Beim Reisfrosch *Limnonectes limnocharis* (BOIE, 1835) aus den südindischen Tropen wurden die Parameter Alter, Alter zur Zeit der Fortpflanzung und Resorptionsrate des Knochens mit Hilfe skelettochronologischer Methoden untersucht und die Beziehungen zwischen Körpergröße, -masse und Alter dargestellt. Die Individuen waren unterschiedlich groß und zeigten in Phalangenquerschnitten ein bis vier Wachstumsmarken (0 - 3 Zonen verlangsamten Wachstums). Bei zwei von fünf Fröschen war die Anzahl der Wachstumsmarken in der untersuchten Phalange nicht gleich jener Anzahl, die im Oberarm-, Unterarm-, Oberschenkel- oder Unterschenkelknochen festgestellt wurde. Diese intra-individuellen Unterschiede traten allerdings nur bei 3 von 25 untersuchten Querschnitten (= 12 %) auf, sodaß die Skelettochronologie anhand der Phalangen bei dieser Froschart ziemlich ähnliche Ergebnisse liefert wie anhand der langen Extremitätenknochen. Der Vergleich von Querschnitten jüngerer und älterer Individuen wies darauf hin, daß die Resorptionsrate vernachlässigbar gering ist, sodaß die Zahl der Wachstumsmarken unmittelbar das Alter widerspiegelt. Bei drei von vier Pärchen in Amplexus waren die Weibchen größer und hatten mehr Wachstumsmarken (waren älter) als ihre männlichen Partner. Die Korpergröße war kaum (r = 0,21), die Körpermasse nicht (r = -0,04) mit der Anzahl der Wachstumsmarken korreliert. Die Untersuchungsergebnisse legen nahe, daß der Frosch in der Natur ein Alter von 4 Jahren erreicht und vom zweiten bis zum vierten Lebensjahr fortpflanzungsfähig ist.

ABSTRACT

In specimens of the Indian Cricket Frog *Limnonectes limnocharis* (BOIE, 1835), originating from the tropics of southern India, age, age at reproduction and rate of bone resorption was studied by means of skeletochronological methods; the relationship between body size, body mass and age was analyzed. One to four growth marks (0-3 lines of arrested growth) were observed in the cross sections of phalanges in specimens of different body size. In two out of five frogs, the number of growth marks in the phalanges was not paralleled by the number of growth marks in all of their limb long bones i. e. femur, humerus, radio-ulna, and tibio-fibula. However, this deviation from intra-individual homogeneity was observed only in 3 out of 25 cross-sections (= 12 %) suggesting that phalangeal skeletochronology leads to similar results as that of other limb long bones in this species. Back-calculation (comparison of cross-sections of younger and older individuals) indicated that the rate of bone resorption is negligible meaning that the number of growth marks directly depicts the age of the individuals. In three out of four amplectant couples studied, the females were larger in size and possessed a greater number of growth marks (i.e., were older) compared to their male partners. Body size showed poor (r = 0.21) and body mass no (r = -0.04) correlation with the number of growth marks. The results suggest that in the natural population this frog reaches an age of 4 years. Reproductive age of the individuals are the 2nd, 3rd and 4th years.

KEY WORDS

Amphibia: Anura: Ranidae; *Limnonectes limnocharis*, skeletochronology, age, body size, sexual maturity, population ecology

INTRODUCTION

Recent reviews dealing with the age and longevity of amphibians reveal that there is a large body of literature accumulated on the skeletochronological age estimation of Urodela and Anura inhabiting the cold temperate area (SMIRINA 1994; ESTEBAN et al. 1996; PANCHARATNA 2002). Although the tropics are rich in diverse amphibian species, information on the age and longevity of these animals is scanty owing to the paucity of studies and necessitates investigations on the determination of age of amphibians inhabiting this region.

Bone growth is known to be a cyclical phenomenon in many amphibians and reptiles, which is generally attributed to the seasonal feeding activity that is enforced by environmental factors, particularly the temperature in temperate areas (HEMELAAR 1981; CASTANET & SMIRINA 1990; SMIRINA 1994; WAKE & CASTANET 1995; ESTEBAN et al. 1996). This cyclicity in osteogenesis results in the formation of growth marks in bones (growth zones formed during warm summer months and lines of arrested growth during cold winter months) which are used as indices for the process of aging (SMIRINA 1994). The validity and the reliability of the use of bone growth marks in estimating the age and longevity of amphibians have been reviewed thoroughly (CASTANET & SMIRINA 1990; SMIRINA 1994; CASTANET et al. 1996; ESTEBAN et al. 1996). Experimental studies have confirmed that the formation of these growth marks is annual in temperate amphibians and, therefore, they may be regarded as 'year rings' for the estimation of the age of the individual animal (HEMELAAR & VAN-GELDER 1980; HEMELAAR 1981; TEJEDO et al. 1997; KUMBAR & PANCHARATNA 2002a, 2002b). By enumerating bone growth marks in either the phalanges or long bones of limbs, age has been estimated for a number of temperate zone amphibians (references in CASTANET & SMIRINA 1990; SMIRINA 1994; CASTANET et al. 1996; ESTEBAN et al. 1996). Information on age and longevity of amphibians inhabiting areas with little annual temperature fluctuations such as the tropics is comparatively scanty (ESTEBAN et al. 1996).

A few available reports on the skeletochronological age determination of tropical amphibians indicate that these animals also express growth marks in the hard tissue such as bones as clearly as reported in temperate species (FRANCILLON et al. 1984; KULKARNI & PANCHARATNA 1996; GUARINO et al. 1998; PANCHARATNA et al. 2000; KUMBAR 2001; KUMBAR & PANCHARATNA 2001a, 2001b; PANCHARATNA 2002; KUMBAR & PANCHARATNA 2002a, 2002b). However, the pattern of distribution and intensity of expression of growth marks in the osseous tissue is known to vary from one species to the other depending on habitat type, altitude of the place of inhabitance, and extent of fluctuations in surrounding environmental factors (CASTANET & SMIRINA 1990; SMIRINA 1994). Further, the rate of endosteal bone resorption and periodicity in the formation of growth marks which play a decisive role in the applicability and reliability of skeletochronology to estimate the age may also vary with the species (SMIRINA 1994; PANCHARATNA 2002). Therefore, careful studies on individual species are necessary before concluding and generalizing any aspect on aging and longevity of a group of amphibians inhabiting a geographical area.

The present study is an attempt to understand age structure in the Cricket Frog, *Limnonectes limnocharis* (BOIE, 1835), inhabiting the tropics of southern India. The skeletochronological aspect of the specimens was related to body size and reproductive age.

MATERIALS AND METHODS

Forty-four individuals of the frog *Limnonectes limnocharis* weighing between 1 and 7 g in body mass and measuring 16 to 45 mm in snout-vent-length (SVL) were collected from the areas around Dharwad (15° 17'N 75°03'E), in the first week of November 2001. All specimens (including 4 amplectant couples) were transported to the laboratory (the amplectant couples were transported separately in polyethylene bags and each couple was placed in a separate aquarium) where body mass was determined to the nearest gram by weighing on a single pan balance and body size (SVL) was measured to the nearest millimeter using a scale. The 4th (longest) toe was clipped (under mild ether anesthesia) from the right hind limb of each frog including those of amplectant couples, fixed in 10% formalin and numbered serially. Five large-sized frogs (from this sample) were autopsied and the long bones of the limbs (femur, humerus, radio-ulna and tibio-fibula) were excised and fixed in 10% formalin. The other specimens were maintained in the laboratory for a week until the wound on the right hind limb was healed completely and then released at the site of capture. Twenty tadpoles (just prior to metamorphosis) were collected from the same area in the last week of June 2002. They were allowed to metamorphose in the laboratory and young froglets were obtained. In 10 pre-metamorphic tadpoles and 10 post-metamorphic young froglets the 4th toe of the right hind limb was excised, fixed in 10% formalin and numbered serially.

Clipped toes and limb bones were cleaned, washed in water for 1-2 hours; they were de-mineralized in 5% nitric acid and processed for paraffin embedding. Sections of 8 μm thickness of the second phalanx of the 4th toe and of limb bones were cut on a rotary microtome, stained with Harris hematoxylin and mounted in glycerine (KULKARNI & PANCHARATNA 1996). Mid-diaphyseal sections (12 per bone) were chosen for observation under a compound microscope for the presence of growth marks, which were enumerated when present.

In order to account for the number of growth marks potentially lost in the process of aging owing to endosteal resorption during bone remodeling, "back calculation" was performed by comparing the diameters of the marrow cavity (MC) of the larger frogs with that of the first line of arrested growth (LAG, when present) or the periosteal bone margin (PBM) of the laboratory metamorphosed froglets (CASTANET et al. 1996).

In all animals, the diameters of MC, LAGs and PBM of the second phalanx of the 4th toe were measured to the nearest 1.0 μm using an ocular micrometer. In total, 788 cross sections (528 of phalanges and 20 of long bones from adults + 240 of phalanges of pre and post metamorphic froglets) were analyzed in the present study. The diameters of all above structures measured were represented by the mean values of their longest and shortest axes each. Means and standard errors were calculated, correlation coefficients (Pearson *r*) (STEEL & TORRIE 1980) were used to show the relationship between body mass, body size, and number of LAGs present.

RESULTS

Cross-sections of phalanges and limb bones of post-metamorphic *Limnonectes limnocharis* showed a central bone marrow cavity (Mc) surrounded by an inner narrow endosteal bone layer (Eb) and an outer broader periosteal bone layer (fig. 1). Series of thin darkly stained chromophilous lines were observed both in the endosteal (not further analyzed, see SMIRINA 1994: 135) and periosteal layers. The lines in the periosteal layer were distinct, separated by wider light purple rings and used for age estimation because of the following reason: The darker thin lines were interpreted as lines of arrested growth (LAGs) and the broader light purple colored zones with sparsely distributed osteocytes as growth rings. Each growth ring along with its LAG was considered as an 'annual ring' or growth mark (figs. 1A-1D). Between zero and three LAGs (between 1 and 4 growth rings) were observed in the specimens studied (fig. 1). As can be concluded from earlier studies on South Indian frogs (see discussion), the first LAG appears in the rainy season following metamorphosis i.e., approximately one year after the froglets emerge. Hence, the presence of three LAGs (and four growth rings) means that the frog is four years old (fig. 2D). The LAGs were found spaced rather equidistantly and expressed exclusively as a single line. Double or triple or partly resorbed lines were not taken into account in any frog. Such false lines are represented in fig. 1B in the form of two thin poorly developed lines distally from the LAG.

For body mass (g), SVL (mm) and number of LAGs in the individuals studied see table 1. Twenty-three out of 44 frogs (52.3%), showed no LAGs, 10 frogs (22.7%) possessed 1 LAG each, 9 frogs (20.5%) exhibited 2 LAGs and 2 frogs (4.6%) expressed 3 LAGs each (table 1 and figs. 1A-1D).

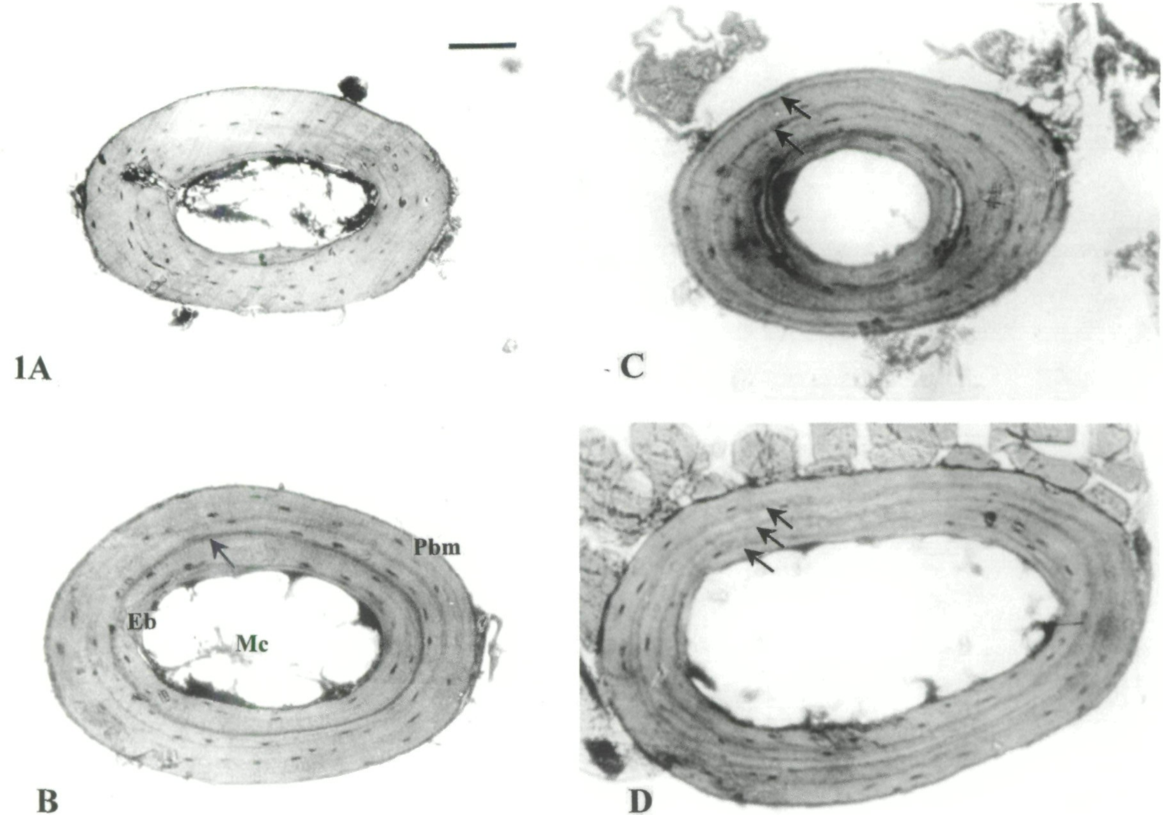
Table 1: Body mass (g), snout vent-length (SVL, mm) and number of bone growth marks (lines of arrested growth, LAGs) in a sample of 44 specimens of *Limnonectes limnocharis* (BOIE, 1835). Specimen numbers followed by a, b, c, or d indicate the four amplectant couples studied.

Tab. 1: Körpermasse (g), Kopf-Rumpf-Länge (SVL, mm) und Anzahl von Knochen-Wachstumsmarken (Zonen verlangsamten Wachstums, LAGs) in einer Stichprobe von 44 Exemplaren von *Limnonectes limnocharis* (BOIE, 1835). Die Exemplare mit dem Nummernzusatz a, b, c oder d bezeichnen die vier untersuchten Pärchen.

Specimen Exemplar	Body mass (g) Körpermasse (g)	Body size (SVL, mm) Kopf-Rumpf-Länge (mm)	Number of LAGs Anzahl LAGs
1	6	45	0
2	4	30	1
3 c	2	30 (M)	2
4	5	30	0
5	3	30	1
6	7	30	0
7	3	27	1
8	2	30	1
9	3	30	3
10	4	30	0
11 a	2	28 (F)	3
12	2	27	2
13	4	32	2
14 c	4	30 (F)	2
15 b	2	28 (M)	2
16 a	2	27 (M)	2
17	3	28	0
18	2	28	1
19	3	32	1
20	4	29	1
21 d	2	31 (F)	2
22	3	30	0
23	3	29	0
24	2	23	0
25	2	24	2
26	3	22	0
27	1	16	0
28	1	17	0
29 d	1	23 (M)	1
30	1	16	0
31	2	31	0
32	3	30	1
33 b	3	30 (F)	2
34	2	27	1
35	1	20	0
36	2	20	0
37	2	25	0
38	4	34	0
39	3	30	0
40	3	27	0
41	2	25	0
42	2	26	0
43	1	25	0
44	1	26	0

Three out of the five large-sized frogs (which were used for skeletochronological comparison of limb bones) showed identical numbers of LAGs in the phalanges and other limb bones (table 2). In the remaining two there was some variation found in that in one frog, there were two LAGs in the phalanx, femur and tibio-fibula and one in the humerus and radio-ulna while in the other, the femur showed an extra LAG compared to other bones (table 2). Collectively seen, three counts out of 25 (= 12%) showed some deviation from intra-individual homogeneity.

The diameters of the marrow cavity of larger frogs in no case exceeded that of the periosteal bone margin (PBM) of laboratory



Figs. 1A - 1D: Hematoxylin stained cross sections of the second phalanx of 4th toe in four individuals of *L. limnocharis* (BOIE, 1835) showing no LAG = line of arrested growth (A), 1 LAG (B), 2 LAGs (C) and 3 LAGs (D). Scale line represents 100 mm. Mc - Marrow cavity, Eb - Endosteal bone, Pbm - Periosteal bone margin. Arrows indicate LAGs.

Abb. 1A - 1D: Die Hämatoxylin-gefärbten Querschnitte der zweiten Phalange der vierten Zehe von vier Individuen von *L. limnocharis* (BOIE, 1835) zeigen keine (A), eine (B), zwei (C) und 3 (D) Zonen verlangsamten Wachstums (LAGs). Maßstabsbalken entspricht 100 mm. Mc - Markhöhle, Eb - endostaler Knochen, Pbm -periostaler Knochenrand. Pfeile zeigen auf die Zonen verlangsamten Wachstums (LAGs).

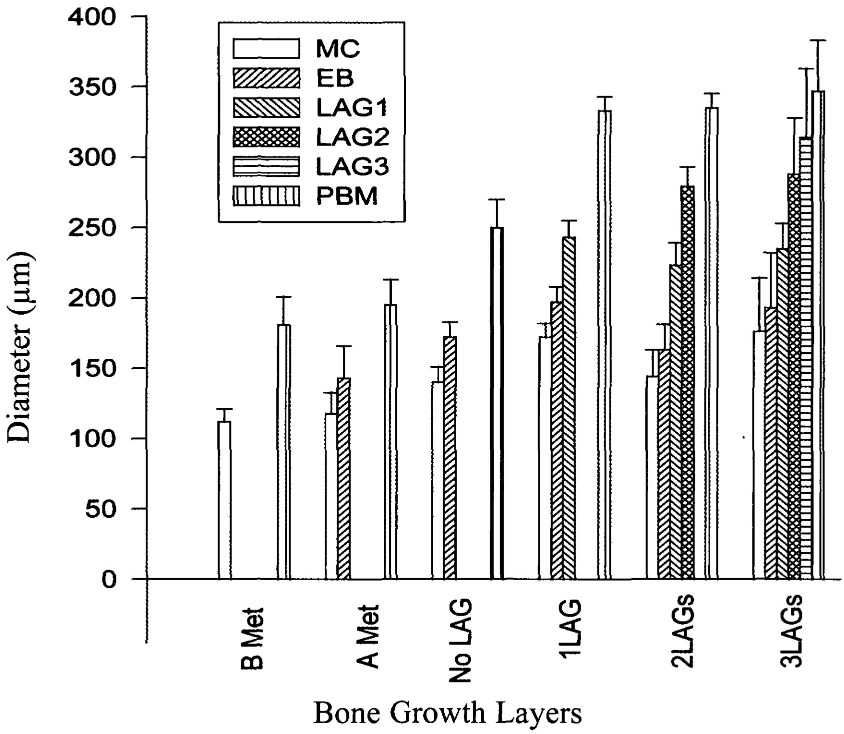


Fig. 2: Diameters (µm) of marrow cavity (MC), endosteal bone (EB), LAGs (1 - 3) and periosteal bone margin (PBM) of the second phalanx of the 4th toe in *L. limnocharis* (BOIE, 1835) indicated for tadpoles (B Met - Before Metamorphosis, n = 10), young froglets (A Met - After Metamorphosis, n = 10), and frogs displaying zero (n =23), one (n = 10), two (n = 9) or three (n = 2) lines of arrested growth (LAGs). Error bars represent standard error.

Abb. 2: Durchmesser (µm) von Markhöhle (MC), endostalem Knochen (EB), Zonen verlangsamten Wachstums (LAGs, 1 - 3) und periostalem Knochenrand (PBM) der zweiten Phalange der vierten Zehe bei *L. limnocharis* (BOIE, 1835). Die Werte und ihre Standardfehler sind angegeben für Kaulquappen (B Met, n = 10), frisch metamorphosierte Individuen (A Met, n = 10) und Frösche, bei denen keine (n = 23), eine (n = 10), zwei (n = 9) oder drei (n = 2) Zonen verlangsamten Wachstums (LAGs) festgestellt wurden.

metamorphosed froglets (fig. 2). This is also true for the apparently spacious marrow cavity depicted in figure 1D. Pearson's correlation coefficient (r) indicated poor correlation ($r = 0.21$) between the number of LAGs and the body size (fig. 3) and no correlation ($r = -0.04$) between the number of LAGs and the body mass (fig. 4).

In amplectant couples (i. e. sexually mature, reproductively active animals), snout-vent length ranged from 23 to 30 mm in males and 28 to 31 mm in females (table 1). Phalangeal histology of these frogs revealed the presence of 1 - 3 LAGs (table 3). In three out of four amplectant couples studied, female individuals were larger or heavier or possessed a greater number of LAGs compared to their male counterparts (table 3).

The cross sections of the second phalanx of the 4th toe of pre-metamorphic tadpoles showed a central marrow cavity and periosteal bone, while an endosteal layer was completely absent (fig. 2). In metamorphosed froglets an endosteal layer was noticed in between the marrow cavity and the periosteal layer (fig. 2).

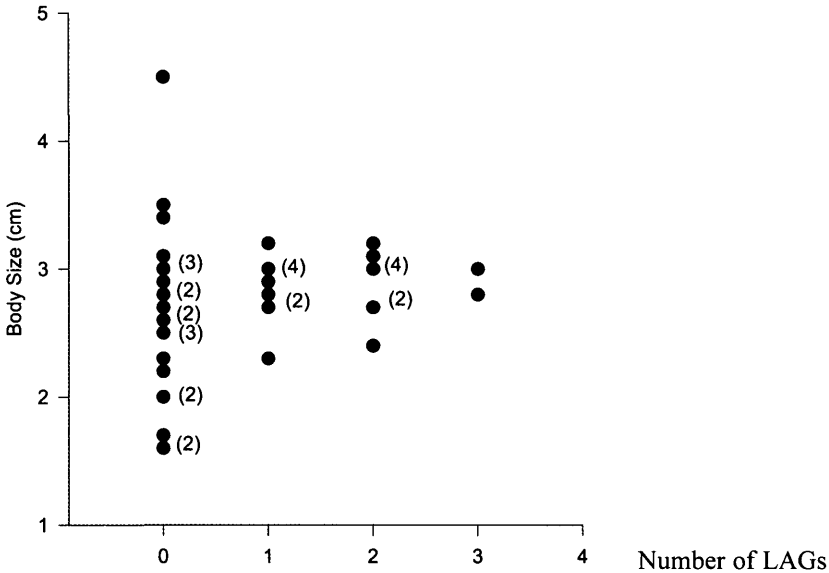


Fig. 3: Relationship (Pearson r = -0.04) between body size (SVL, cm) and number of lines of arrested growth (LAGs) in 44 specimens of *L. limnocharis* (BOIE, 1835). In parentheses: number of dots in identical position.

Abb 3: Beziehung (Pearson r = -0,04) zwischen Kopf-Rumpflänge (cm) und der Anzahl der Zonen verlangsamten Wachstums (LAGs) bei 44 Exemplaren von *L. limnocharis* (BOIE, 1835). Mehrfachbelegung in Klammern.

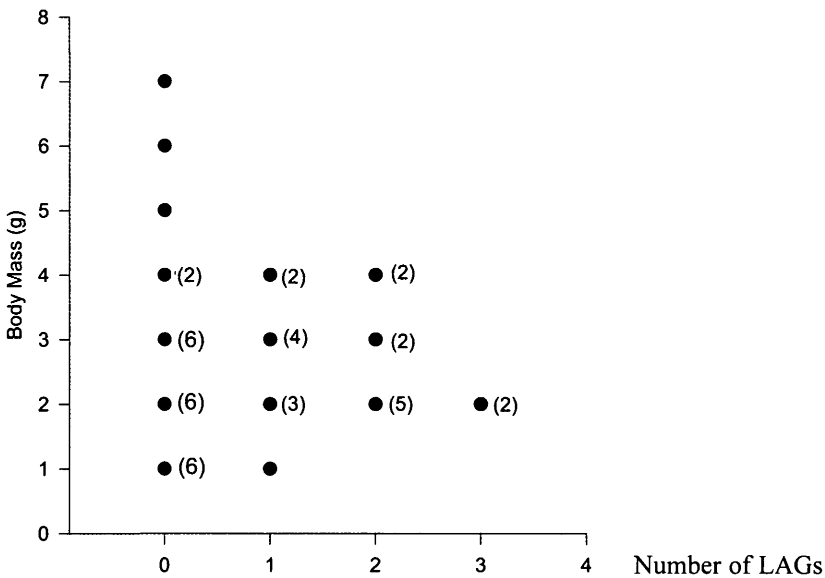


Fig. 4: Relationship (Pearson r = 0.21) between body mass (g) and number of lines of arrested growth (LAGs) in 44 specimens of *L. limnocharis* (BOIE, 1835). In parentheses: number of dots in identical position.

Abb 4: Beziehung (Pearson r = 0,21) zwischen Körpermasse (g) und der Anzahl der Zonen verlangsamten Wachstums (LAGs) bei 44 Exemplaren von *L. limnocharis* (BOIE, 1835). Mehrfachbelegung in Klammern.

Table 2: Number of lines of arrested growth (LAGs) in different limb bones of an individual as found in five specimens of *L. limnocharis* (BOIE, 1835).

Tab. 2: Die Anzahl der Zonen verlangsamten Wachstums (LAGs) in verschiedenen Extremitätenknochen eines Individuums wie sie bei fünf Exemplaren von *L. limnocharis* (BOIE, 1835) festgestellt wurden.

Specimen / Exemplar	Femur	Humerus	Tibio-fibula	Radio-ulna	Phalanx
1	0	0	0	0	0
2	2	1	2	1	2
3	2	2	2	2	2
4	2	2	2	2	2
5	3	2	2	2	2

DISCUSSION

Although annual formation of bone growth marks is well established for species of temperate regions such as *Rana temporaria* LINNAEUS, 1758 (SMIRINA 1972), *Rana esculenta* LINNAEUS, 1758 (FRANCILLON & CASTANET 1985), *Bufo bufo* (LINNAEUS, 1758) (HEMELAAR & VAN-GELDER 1980), *Triturus cristatus* (LAURENTI, 1768) (FRANCILLON 1980), and *Bufo calamita* LAURENTI, 1768 (TEJEDO et al. 1997), very recently this has also been reported in the tropical anuran species *Euphlyctis cyanophlyctis* (SCHNEIDER, 1799) and *Bufo melanostictus* SCHNEIDER, 1799 from southern India (KUMBAR 2001; KUMBAR & PANCHARATNA 2002a). As shown by previous studies on South Indian anuran species, LAGs are laid down in the wet months March through Septmber (KUMBAR 2001; PANCHARATNA 2002; KUMBAR & PANCHARATNA 2001a, 2001b; KUMBAR & PANCHARATNA 2002a, 2002b), which also coincides with the breeding activity of frogs. The tropical anurans have an extended breeding activity during which the feeding activity decreases and fat from fat bodies is mobilized; as a consequence the latter become totally depleted. This is most likely the time when the LAGs are laid down. After the breeding season the feeding activity is restored and fat bodies get repleted. In various Indian anurans, seasonal variation in the whole body as well as fat body weight was recorded. Therefore, the cyclic pattern in the bone growth of tropical (South Indian) anurans is attributed to the annual rainfall pattern which in turn affects the regular feeding activity by diverting the frogs towards reproductive activity (KUMBAR & PANCHARATNA 2001b).

Table 3: Body mass (g), snout vent-length (SVL, mm) and number of LAGs in the second phalangeal bones of the fourth toe in the eight individuals of four amplectant couples of *L. limnocharis* (BOIE, 1835). (F) - female, (M) - male.

Tab. 3: Körpermasse (g), Kopf-Rumpf-Länge (SVL, mm) und Anzahl der Zonen verlangsamten Wachstums (LAGs) in den zweiten Gliedern der vierten Zehe bei den acht Individuen von vier in Amplexus befindlichen Pärchen von *L. limnocharis* (BOIE, 1835). (F) - Weibchen, (M) - Männchen.

Couple Paar	Body mass (g) Körpermasse (g)	SVL (mm)	Number of LAGs Anzahl LAGs
1	2 (F)	28 (F)	3
1	2 (M)	27 (M)	2
2	3 (F)	30 (F)	2
2	2 (M)	28 (M)	1
3	4 (F)	30 (F)	2
3	2 (M)	30 (M)	2
4	2 (F)	31 (F)	2
4	1 (M)	23 (M)	1

In the present study phalangeal skeletochronology of *Limnonectes limnocharis* indicated that this frog lives at least four years after metamorphosis since the number of bone growth marks varied from zero to four in specimens of different body size (figs. 1, 3). Furthermore, the expression pattern of growth marks was largely congruent in phalanges and other limb long bones suggesting that phalangeal skeletochronology leads to similar results as that of any limb long bone in this frog species. This phenomenon is already known from other sympatric species (KUMBAR & PANCHARATNA 2001a, 2001b). That no periosteal LAGs are lost due to endosteal bone resorption was evident from back calculation, as the diameter of the marrow cavity of larger frogs in no case exceeded that of the PBM diameter of laboratory metamorphosed froglets (fig. 2). This condition is comparable to that of the burrowing frog *Microhyla ornata* (DUMÉRIL & BIBRON, 1841) and differs from other neighboring species of frogs such as, *E. cyanophlyctis*, *Hoplobatrachus tigerinus* (DAUDIN, 1802) or *B. melanostictus* in which one to three LAGs are known to be lost owing to endosteal resorption (KUMBAR & PANCHARATNA 2001a, 2001b).

The number of LAGs present in the phalangeal cross sections of amplectant couples indicated that the reproductive age of this frog species ranges from one to three years in males and two to four years in females (table 3). From our data it is furthermore clear, that, in the majority of amplectant couples, the females are larger and older than their male counterpart which is in agreement with observations made in other anuran species studied (CHERRY & FRANCILLON-VIEILLOT 1992; RYSER 1988; TEJEDO et al. 1992; PANCHARATNA et al. 2000) and seems to be true in all tropical Indian anurans studied so far (KUMBAR 2001; PANCHARATNA 2002; KUMBAR & PANCHARATNA 2001a, 2001b, 2002a, 2002b). Body mass or body size did not show any correlation with the number of growth marks. This suggests that body mass and size may not be a reliable criterion for age in *Limnonectes limnocharis* unlike in other sympatric species such as *H. tigerinus*, *E. cyanophlyctis, Polypedates maculatus* (GRAY, 1834) in which body size correlated positively with the number of LAGs (KUMBAR & PANCHARATNA 2001a).

In conclusion, the present skeletochronological study reveals that in the Indian Cricket Frog *L. limnocharis* (i) the maximum post-metamorphic age observed is 4 years; (ii) the qualification of phalangeal skeletochronology for estimating the age largely equals that of any other limb long bone; (iii) since the rate of endosteal resorption is negligible, the number of growth rings present directly depicts the age of the individuals (iv) reproductive age of these frogs ranges from the 2nd to the 4th year; (v) in amplectant couples, males are usually smaller and younger than their female counterparts.

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