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A skeletochronological analysis of age, growth and longevity of the Indian Green Frog *Euphlyctis hexadactylus* (LESSON, 1834) (Anura: Ranidae)

Skelettochronologische Analyse von Alter, Wachstum und Lebenserwartung beim Indischen Wasserfrosch *Euphlyctis hexadactylus* (LESSON, 1834) (Anura: Ranidae)

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KURZFASSUNG

Die vorliegende Arbeit untersucht die Altersstruktur einer indischen Population des Sechszehenfrosches, *Euphlyctis hexadactylus* (LESSON, 1834), mit skelettochronologischer Methoden an Phalangen und Langknochen. Zweiundfünfzig Frösche aus drei (Alters)gruppen (frisch Metamorphosierte und Jungtiere, adulte Männchen, adulte Weibchen) wurden während dreier Jahre (2002-2004) aufgesammelt. An den mit Äther narkotisierten Tieren wurden Messung von Kopf-Rumpf-Länge und Masse durchgeführt und zur Individualmakierung in einer Wachstumsstudie für Fang- und Wiederfangvergleiche Zehen amputiert. Der bisher einzige Wiederfang, ein Frosch im zweiten Jahr nach erfolgter Markierung, zeigte eine Vermehrung der Wachstumsstillstandsringe (LAGs) um die Zahl zwei und belegte damit die jährliche Ring-Bildung. Keine Wachstumsstillstandsringe fanden sich auf Knochenquerschnitten von frisch Metamorphosierten und Jungtieren. Die Knochen adulter Frösche wiesen ein bis dreizehn Wachstumsstillstandsringe auf. Männliche Frösche waren älter / lebten länger (maximal 14 Jahre) als weibliche (maximal 6 Jahre). Beide Geschlechter erreichten die Geschlechtsreife im zweiten der Metamorphose folgenden Jahr. In allen Altersgruppen waren die Männchen verhältnismäßig kleiner als die Weibchen. Die Korrelation zwischen Kopf-Rumpf-Länge bzw. Körpermasse und der Anzahl der Wachstumsstillstandsringe war erwartungsgemäß positiv.

ABSTRACT

Age structure in a population of the Indian Green Frog, *Euphlyctis hexadactylus* (LESSON, 1834) was assessed by skeletochronology of phalanges and long bones. Fifty-two frogs forming three (age) groups (metamorphosed froglets and juveniles, adult males, adult females) were collected over a period of three years (2002–2004). All the specimens were anesthetized with ether prior to measurement of snout-vent length and weight. Toe clipping was applied in order to mark the frogs individually for recognition after subsequent recapture and study of their growth. The only frog recaptured was found in the second year of marking. Skeletochronology of the recaptured frog showed two more lines of arrested growth (LAGs) confirming the annual formation of LAGs or growth rings. No LAG was observed in the cross sections of the bones of froglets and juveniles. However, the adult frogs revealed one to thirteen lines of arrested growths (LAGs) in their bone histology. The study also showed the male frogs to be older / live longer (maximum age 14 years) than the females (maximum age 6 years). However, both the male and female frogs attained sexual maturity in the second year after metamorphosis. In all the age groups the male frogs were comparatively smaller in size than the females. The expected positive correlations between both body size (SVL) as well as body mass (WT) and number of LAGs was demonstrated.

KEY WORDS

Amphibia: Anura: Ranidae: *Euphlyctis hexadactylus*, skeletochronology, population ecology, age structure, lines of arrested growth (LAGs), mark-recapture, longevity, India

INTRODUCTION

Skeletochronology is known to be an effective and reliable method of estimation of age in amphibians (SMIRINA 1972; HEMELAAR 1981; GIBBONS & MC CARTHY 1983; HALLIDAY & VERRELL 1988; CASTANET & SMIRINA 1990, 1994; ESTEBAN et al. 1996; KUMBAR & PANCHARATNA 2002; PANCHARATNA & DESPANDE 2003; GUARINO et al.

2003). In cross sections of both the long bones and the phalanges growth marks correspond to broader zones formed during the periods of growth in phases of activity and narrower lines of arrested growth (LAGs) formed during hibernation. So, one LAG (and its adjoining growth ring) represents one year of growth, which corresponds to the age of the individual (HALLIDAY & VERRELL 1988; CASTANET & SMIRINA 1990, 1994). For larger amphibians, particularly the anurans, examination of the phalanges is the method usually applied for this purpose (GITTINS et al. 1982; GIBBONS & MC CARTHY 1983; KUMBAR & PANCHARATNA 2001a; GUARINO et al. 2003), precluding the need to sacrifice any animal and making the technique more compatible with mark-recapture or other ecological investigations. A few reports are available on age determination by phalangeal skeletochronology of marked and recaptured anurans, which confirm the reliable use of phalanges in the determination of age (HEMELAAR & VAN GELDER 1980; GIBBONS & MC CARTHY 1983; MARNELL 1997; TEJEDO et al. 1997; SULLIVAN & FERNANDEZ 1999; MARUNOUCHI et al. 2000; JACOB et al. 2002).

There are also reports of skeletochronological age estimation in some amphibians inhabiting south India (KULKARNI & PANCHARATNA 1996; KUMBAR & PANCHARATNA 2001a, 2001b, 2002; PANCHARATNA & DESPANDE 2003). But there is no data on skeletochronological age estimation of amphibians from the eastern part of India except for *Bufo melanostictus* (NAYAK et al. 2007). The present work aims at describing the age structure of a small east Indian population of the Indian Green Frog *Euphlyctis hexadactylus* (LESSON, 1834), based on skeletochronology data gained in a still ongoing mark recapture project.

MATERIALS AND METHODS

Fifty-two specimens of various size (10 metamorphosed froglets and immatures/juveniles, 16 mature/adult females 26 mature/adult males, Table 1) of the Indian Green Frog *Euphlyctis hexadactylus* were randomly collected in three consecutive monsoon periods (June to August 2002, 2003, 2004) from ponds in the nearby areas of Jagatsinghpur, Orissa (Eastern India, 20°10'N 86°12'E). The captured specimens were anesthetized with ether, weighed (to the nearest gram) by a single pan balance and snout-vent length (SVL) was measured to the nearest mm with a digital caliper. The adult frogs were sexed on the basis of secondary sexual characters such as vocal sacs and presence of thumb pads. Mature females were distinguished from immatures by their swollen abdomen. The frogs were individually marked by toe clipping (MARTOF 1953) and released at the capture sites. Thus, the technique of toe clipping was employed in both age determination and as a tool in the mark recapture project.

For comparative skeletochronological data, four adult frogs were autopsied and long bones along with toes were surgically removed and fixed in 70% alcohol. In the second year of capture, one marked frog was recaptured and examined for the increase in number of LAGs in long bones and phalanges.

All fixed bones were washed thoroughly in running tap water for one hour. The bones were decalcified in 10% EDTA solution for 24-48 h depending upon the size of the bones. After decalcification, the bones were washed in running tap water for 24 h and processed for paraffin block preparation. Serial sections were cut (8-10μ in thickness) by a rotary microtome and stained with Delafield hematoxylin and eosin. The mid-diaphyseal cross sections were examined under a compound microscope. Lines of arrested growth (LAGs) were counted and recorded for each animal. Photographs were taken from appropriate sections. The correlations between body mass (WT) and age, body size (SVL) and age as well as body size (SVL) and body mass (WT) were studied and assessed by scatter plots. The correlation coefficient (Pearson *r*) was calculated (STEEL & TORRIE 1980).

Table 1: Body size (SVL), body mass (BM) and number of Lines of Arrested Growth (LAGs) in immatures (postmetamorphic froglets included), mature females and mature males of *Euphlyctis hexadactylus* (LESSON, 1834). n - sample size; SD - Standard deviation.

Tab. 1: Körpergröße (SVL, mm), Körpermasse (BM, g) und Anzahl von Linien verminderten Wachstums (No. of LAGs) bei Juvenilen (einschließlich frisch verwandelter Jungtiere), reifen Weibchen und reifen Männchen von *Euphlyctis hexadactylus* (LESSON, 1834). n - Stichprobengröße; Mean±SD - Arithmetisches Mittel ± Standardabweichung; (Range) - Spannweite; Median/Mode - Median/Modalwert.

Age group Altersgruppe	n	SVL (mm) Mean±SD (Range)	BM (g) Mean±SD (Range)	No. of LAGs Median/Mode (Range)
Immatures Jungtiere	10	52.3±19.43 (12.0-75.0)	32.2±12.69 (8.0-49.0)	0/0 (0)
Mature females Reife Weibchen	16	105.5±24.19 (75.0-145.0)	146.5±79.65 (58.0-300.0)	2/1 (1-5)
Mature males Reife Männchen	26	88.11±18.41 (57.0-140.0)	96.73±45.49 (40.0-250.0)	2/2 (1-13)

RESULTS

Histological sections of phalangeal bones of post-metamorphic froglets (Fig. 1A) revealed the central marrow cavity (mc) to be surrounded by the endosteal layer (e). The endosteal layer lined the middle broad cortex (c), which was externally bounded by the periosteal layer (p). The cortex (c) or cortical layer was made up of a homogenous matrix (m) with evenly scattered osteocytes (o). In the mature (adult) frogs, thin and darkly stained concentric chromophilic lines were observed in the cortex above the endosteal layer (Figs. 1B, 1C). The lines were distinct and separated by lighter zones. The darker lines were interpreted as lines of arrested growth (LAGs) and the broader lighter zones as growth rings. One growth ring along with its neighboring LAG was considered as an annual ring or growth mark (Figs. 1B-2C).

No LAGs were observed in the immature frogs (post-metamorphic froglets and immatures, SVL: 12.0-75.0 mm). Among the mature frogs (SVL: 57.0-145.0 mm), one to thirteen LAGs were observed in the cross sections of bones. Synoptic data on body mass (WT), body size (SVL) and number of LAGs in the immature, and mature male and female frogs studied are represented in Table 1. The number of LAGs in both the long bones and phalanges was found to be the same within each individual out of the four specimens sacrificed. In one sacrificed male frog measuring 96.0 mm in SVL and weighing 120.0 g, four LAGs were observed both in long and digit bones (Figs. 2A, 2B). Both the smallest mature female (SVL: 75.0 mm, WT: 58.0 g) and mature male (SVL: 57.0 mm, WT: 40.0 g) showed one LAG each. A larger female frog measuring 135.0 mm in SVL and 230.0 g in body mass was also sacrificed and revealed four LAGs (Fig. 1C) each in long bones and digits. Among the remaining frogs not sacrificed, the largest captured female frog (SVL: 145.0 mm, WT: 300.0 g) showed five LAGs and was considered the oldest female frog in the present study. The largest mature male frog (SVL: 140.0 mm, WT: 250.0 g) revealed four LAGs whereas seven LAGs were observed in a comparatively smaller (SVL: 100.0 mm, WT: 120.0 g) male (Figs. 3A, 3B). The highest number of LAGs was thirteen seen in the phalangeal cross section of a mature male measuring 97.0 mm in SVL and weighing 103.0 g (Figs. 2C, 3A). This frog was the oldest living frog in the study. The above finding indicates that, neither body size (SVL) nor weight (WT) should be considered as the sole criterion for estimation of age in *E. hexadactylus*.

As a part of a long term mark-recapture study a single marked frog was recaptured two years after the first capture. At first capture, the frog was immature and without any LAG (Fig. 1A), measuring 55 mm in body size (SVL) and weighing 30 g. At recapture after two years, it was mature and was identified as a male frog. It measured 70 mm in body size (SVL), weighed 90 g and showed two LAGs as per expectation (Fig. 1B).

DISCUSSION

In each of the four individuals studied, the number of LAGs in the long bones remained the same as in the phalangeal bones. Similar observations have been reported for several temperate climate anurans (HEMELAAR & VAN GELDER 1980; DENTON & BEEBEE 1993; GUARINO et al. 2003) as well as a few Indian frogs (PANCHARATNA & DESPANDE 2003). As a consequence, phalangeal bones alone (gained by toe-clipping) should serve for age determination without sacrificing the animal.

Skeletochronology based on phalangeal bone cross sections can be considered as one of the most reliable methods of estimation of age in *E. hexadactylus*. Bone histology of the marked and recaptured frog showing increase in the number of LAGs (two LAGs in two years) confirmed earlier findings of annual LAG formation reported for several anurans inhabiting temperate regions (*Rana temporaria* LINNAEUS, 1758 - SMIRINA 1972; *R. latastei* BOULENGER, 1879 - GUARINO et al. 2003; *Bufo bufo* (LIN-

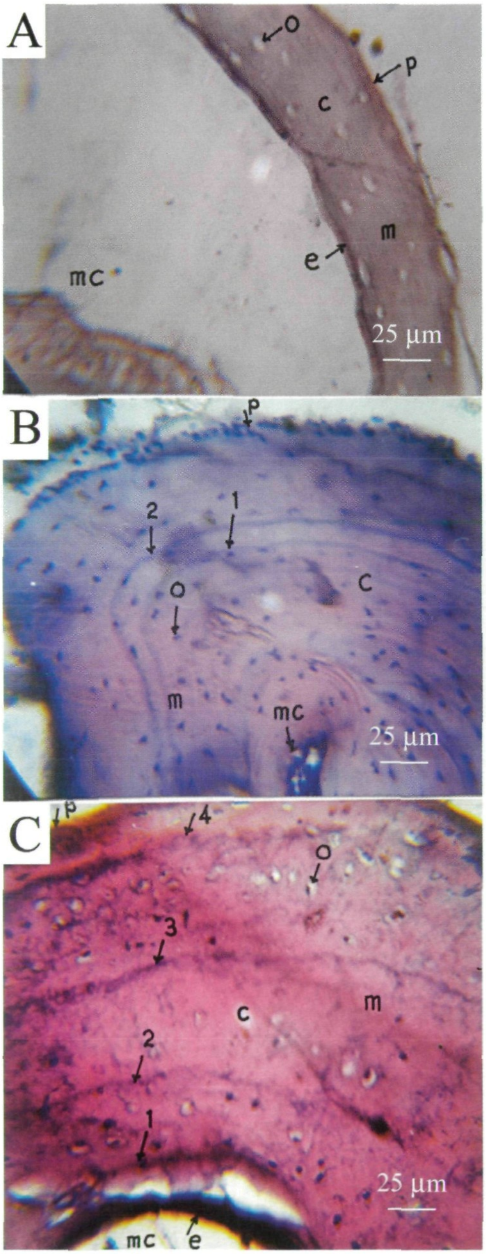

Fig. 1 (left): Cross sections through phalangeal bones of *Euphlyctis hexadactylus* (LESSON, 1834). c - cortex; e - endosteal layer; m - matrix; mc - marrow cavity; o - osteocytes; p - periosteal layer; 1-4 - Lines of Arrested Growth (LAGs) 1-4.

A - 4th phalanx of an immature male at first capture (SVL: 55.0 mm), showing no LAGs.

B - 3rd phalanx of the recaptured male (SVL: 70.0 mm), showing two LAGs (1-2).

C - 4th phalanx of a mature female (SVL: 135.0 mm), showing four LAGs (1-4).

Abb. 1 (links): Querschnitte durch Phalangenknochen von *Euphlyctis hexadactylus* (LESSON, 1834). c - Kortex; e - Endost; m - Matrix; mc - Markhöhle; o - Osteozyten; p - Periost; 1-4 - Linien verminderten Wachstums (LAGs) 1-4.

A - Die vierte Phalange eines unreifen Männchens beim Erstfang (KRL: 55,0 mm) zeigt keine LAGs.

B - Die dritte Phalange des wiedergefangenen Männchens (KRL: 70,0 mm) zeigt zwei LAGs (1-2).

C - Die vierte Phalange eines reifen Weibchens (KRL: 135,0 mm) zeigt vier LAGs (1-4).

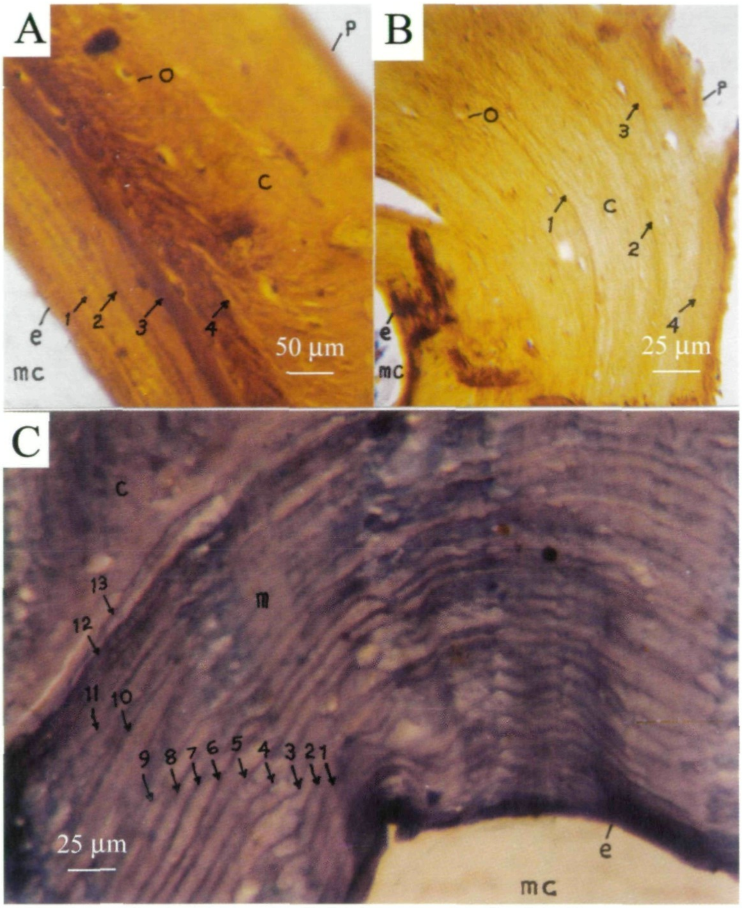

Fig. 2: Cross sections through the femur and phalangeal bones of *Euphlyctis hexadactylus* (LESSON, 1834).
c - cortex; e - endosteal layer; m - matrix; mc - marrow cavity; o - osteocytes; p - periosteal layer; 1-13 - LAGs 1-13.
A - Cross section through the femur bone of a mature male (SVL: 100.0 mm) showing four LAGs (1-4).
B - Cross section through the 4th phalanx of the same frog as in A showing four LAGs (1-4).
C - Cross section through the 4th phalanx of the oldest frog (SVL: 97.0 mm) showing 13 LAGs (1-13).

Abb. 2: Querschnitte durch Femur und Phalangenknochen von *Euphlyctis hexadactylus* (LESSON, 1834).
c - Kortex; e - Endost; m - Matrix; mc - Markhöhle; o - Osteozyten; p - Periost;
1-13 - Linien verminderten Wachstums (LAGs) 1-13.
A - Der Querschnitt durch das Femur eines reifen Männchens (KRL: 100,0 mm) zeigt vier LAGs (1-4).
B - Der Querschnitt durch die vierte Phalange desselben Frosches wie in A zeigt vier LAGs (1-4).
C - Der Querschnitt durch die vierte Phalange des ältesten Frosches (KRL: 97,0 mm) zeigt 13 LAGs (1-13).

NAEUS, 1758) - HEMELAAR & VAN GELDER 1980 and *B. calamita* LAURENTI, 1768 - TEJEDO et al. 1997).

Lack of LAGs in immature frogs indicated that the animals had not yet gone through the dormant stage in winter, when usually a LAG is laid down in the bone. The sections showed a thin cortical matrix with a small number of osteocytes distributed in it. However, in mature frogs the bones had become comparatively thicker.

The smallest mature male (SVL: 57.0 mm) as well as female (SVL 75.0 mm) showed one LAG each. No frog without

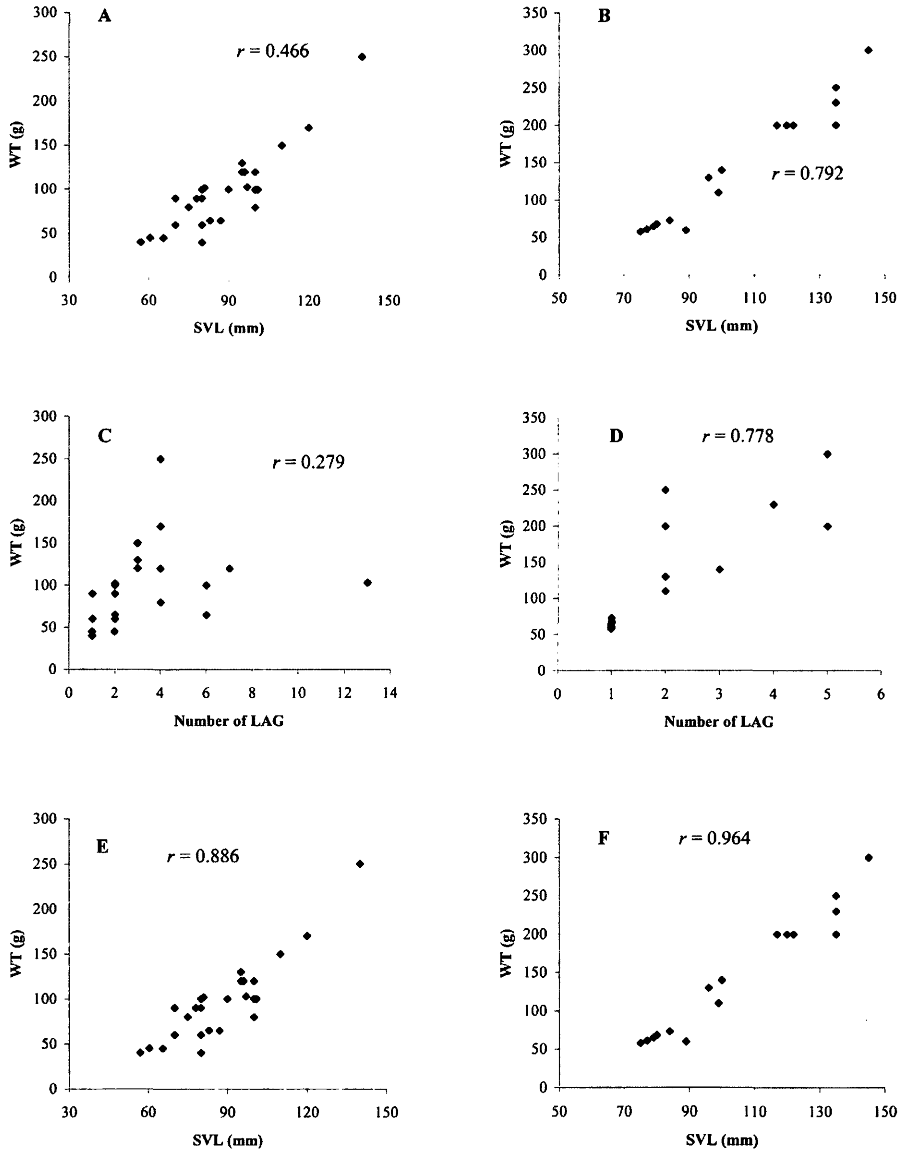

Fig. 3: Correlation between snout-vent length (SVL, mm) and number of LAGs (A, B) and between body mass (WT, g) and number of LAGs (C, D) as well as between body mass (WT, g) and snout-vent length (SVL, mm) (E, F) in 42 mature *Euphlyctis hexadactylus* (LESSON, 1834). A, C, E – males, B, D, F – females.

Abb. 3: Die Beziehung zwischen Kopf-Rumpf-Länge (SVL, mm) und der Anzahl der Linien verminderten Wachstums (LAGs) (A, B) sowie zwischen Körpermasse (WT, g) und Anzahl der LAGs (C, D) und zwischen Körpermasse (WT, g) und Kopf-Rumpf-Länge (SVL, mm) (E, F) bei 42 reifen *Euphlyctis hexadactylus* (LESSON, 1834). A, C, E – Männchen, B, D, F – Weibchen.

LAG was found to be mature. From this we concluded that sexual maturity in this species is attained after the formation of the first LAG. The smallest mature male and female frog showing one LAG each must have completed one year and the LAG was laid down in winter. With the following spring and summer, these individuals entered into a new growth phase in which the formation of the second growth ring occurred. During this growth phase the frogs became sexually mature and ready for breeding in the monsoons. Therefore, the age at sexual maturity in these frogs can be considered as more than one year, i.e., 1+ year as described by MARNELL (1997). In the present study 1+ old year frogs are described as two years old. Similar attainment of sexual maturity has been reported for two Indian anurans, i.e., *Microhyla ornata* (DUMÉRIL & BIBRON, 1841) by KUMBAR & PANCHARATNA (2001b) and *Euphlyctis cyanophlyctis* (SCHNEIDER, 1799) by KULKARINI & PANCHARATNA (1996). The oldest male frog (SVL: 97.0 mm, WT: 103.0 gm) in the present study showed 13 LAGs indicating the age of the frog to be 13+ years or 14 years (Fig. 2C). But the oldest female frog revealed only 5 LAGs indicating its age to be 5+ years or 6 years. It has been reported that the longevity of temperate anurans inhabiting colder regions is usually higher than of tropical anurans inhabiting warmer regions (SMIRINA 1994). The temperate frog species, *R. ridibunda* PALLAS, 1771, *R. esculenta* (LINNAEUS, 1758) and *R. temporaria* survive for 12, 12 and 14 years, respectively. Longevity of *B. bufo*, *B. pentoni* ANDERSON, 1893 and *B. arenarum* (HENSEL, 1867) inhabiting temperate regions has been described to be 12, 6 and 8 years, respectively (SMIRINA 1994). Longevity reports of some tropical anurans of south India described the maximum age of *Euphlyctis cyanophlyctis* to be 7 years; (KULKARNI & PANCHARATNA 1996), of *Microhyla ornata* to be 5 years (KUMBAR & PANCHARATNA 2001b) and of *Limnonectes limnocharis* (BOIE, 1835) to be 4 years (PANCHARATNA & DESPANDE 2003). The present finding showed that east Indian *E. hexadactylus* became older than anurans inhabiting south

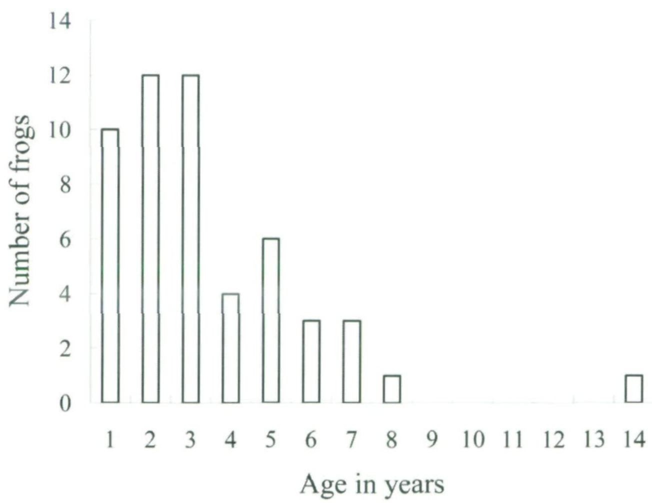

Fig. 4: Frequency distribution of the number of LAGs found in the east Indian population of *Euphlyctis hexadactylus* (LESSON, 1834) studied. The graph also shows the distribution of the 52 randomly sampled individuals according to different age groups.

Abb. 4: Häufigkeitsverteilung der Anzahl der Linien verminderten Wachstums (LAGs) bei den Individuen der untersuchten ostindischen Population von *Euphlyctis hexadactylus* (LESSON, 1834). Die Darstellung zeigt gleichzeitig die Verteilung der 52 zufällig ausgewählten Individuen auf Altersgruppen.

India. A similar difference in maximum age has also been reported for *Bufo melanostictus* (SCHNEIDER, 1799). The difference is described as being related to the geographical position of the two regions (NAYAK et al. 2007). Based on the present study, longevity of *E. hexadactylus* can be compared with that of temperate anurans.

Females were comparatively larger in size (SVL) and weighed more than the males (Table 1). A positive correlation was found to exist between the SVL and the number of LAGs for both the males and the females (Figs. 3A, 3B). The correlation coefficient (r) was 0.792 for females and 0.466 for the males. A similar positive correlation was observed between body mass (WT) and number of LAGs. The r values were 0.279 and 0.778 for the males and the females, respectively (Figs. 3C, 3D). As expected the correlation between body mass (WT) and SVL was positive. The r values were 0.886 and 0.964 for the males and females, respectively (Figs. 3E, 3F). Thus, the result indicates that in general the larger frogs (in size and weight) have experienced a greater number of growth cycles and are older. But, size and weight should not be considered as the sole criteria for age estimation, because the oldest frog with thirteen LAGs was 97.0 mm in SVL and 103.0 g in weight whereas the largest frog measuring 140.0 mm in SVL and 250.0 g in weight showed only four LAGs.

Out of the randomly collected fifty-two specimens studied, ten frogs (19.23%) were younger than one year and twelve frogs (23.07%) were in the second year of life. Another twelve frogs (23.07%) belonged to the age group 'third year', four frogs (7.69%) belonged to the fourth year, six (11.53%) to the fifth year, three (5.76%) to the sixth year, three (5.76%) to the seventh year, one frog (1.92%) to the eighth year and one frog (1.92%) belonged to the fourteenth year age group. As must be expected for the age structure of a sustainable natural population, younger frogs were more in number than older frogs (Fig. 4).

From the present skeletochronological study, it is concluded that (i) the maximum age of *E. hexadactylus* in nature is at least 14 years; (ii) maturity both in males and females is attained at the age of two years; (iii) males live longer than the females; (iv) the females are larger in size and body mass than the males and (v) (as expected) a positive correlation exits between 'SVL and age', 'body mass and age' as well as " SVL and body mass".

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