Determination of age, longevity and age at reproduction of the frog *Microhyla ornata* by skeletochronology

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Skeletochronological estimation of age, longevity, age at sexual maturity and breeding of *Microhyla ornata* was done. Frogs (*n* = 62) were collected locally in August (rainy season) 1997 and brought to the laboratory. Body mass and snout-vent-length (SVL) of each frog was recorded; the 4th toe of both the hind limbs was clipped under anaesthesia, fixed in 10% formalin, demineralized in 5% nitric acid and processed for histology. Limb bones (femur, humerus, tibiofibula and radioulna) of 6 large sized frogs were also processed for skeletochronology in order to study the rate of resorption. Gonads of 25 frogs (belonging to different body size ranges) were processed for histology in order to ascertain the gametogenic status of individual frogs. One to four growth rings consisting of growth zones and lines of arrested growth (LAGs) were noticed in frogs of different body sizes; the number of LAGs remained identical in all the limb bones and phalanges in 5 out of 6 frogs. Back calculation indicated that the resorption rate is very low in this frog. Male frogs possessed sperm bundles in seminiferous tubules in the 1st year, while females showed yolky follicles in the ovary in the 2nd year. Frogs found in amplexus were 3–5 years old. The results suggest that this frog may live for a maximum of 5 years in the natural population.

1. Introduction

Amphibians and reptiles reflect growth marks corresponding to broader zones linked to fast growing periods, together with lines of arrested growth (LAGs) which correspond to resting periods in their hard tissues (e.g. Hemelaar 1981, 1983; Castanet and Smirina 1990; Smirina 1994). Since these LAGs represent the number of bone growth cycles that the animal has experienced (which are by and large annual in many amphibian species studied) they are used as indices of aging, and as such, skeletochronology has emerged as the most reliable and powerful tool to estimate the age and longevity of amphibians (Castanet and Smirina 1990; Smirina 1994). Almost all the studies on the determination of age and longevity of amphibians originate from temperate areas (references in Hemelaar 1981, 1983; Castanet and Smirina 1990; Smirina 1994; Esteban *et al* 1996). Similar studies on species inhabiting warmer areas or the tropics are scanty (Esteban *et al* 1996).

The present work is an attempt to study the age structure of the burrowing frog *Microhyla ornata* inhabiting Dharwad (15°17′N, 75°3′E), southern India. The occurrence of growth marks in the cross section of the femur of this frog has been recently reported (Kumbar and Pancharatna 2001). This study emphasizes the skeletochronological estimation of (i) age at attainment of sexual maturity, (ii) age at breeding, and (iii) longevity of frogs in the natural population.

2. Materials and methods

Sixty-two frogs [*M. ornata*, body weight: 0·60–1·65 g, snout-vent-length (SVL): 2–2·8 cm] collected from the Karnatak University Campus, Dharwad, in the month of

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August (rainy season) 1997 were used in the present study. After capture, frogs were transported to the laboratory and housed in glass aquaria. Of the 62 frogs, 10 (5 pairs) were found in amplexus; they were transported separately in plastic bags and placed in 5 different aquaria. Each frog was anaesthetized (using anaesthetic ether), the body mass and SVL were recorded; the 4th toe of each of the hind limbs was clipped, fixed in 10% formalin and numbered serially. Six large sized frogs were used for skeletochronology of the long bones of the limbs (femur, humerus, tibiofibula and radioulna) in order to compare the rate of resorption in the phalanges and in these bones (Esteban *et al* 1996; Marnell 1997). Twenty-five frogs belonging to different body size ranges were autopsied and their gonads were processed for histology to study the reproductive status of the individual frogs (the femurs of all these frogs were also processed for skeletochronology). The remaining frogs were released.

Clipped toes and limb bones were cleaned, demineralized in 5% nitric acid and processed for histology. Paraffin sections (8 µm) of the distal phalanx were stained with Harris haematoxylin (Kulkarni and Pancharatna 1996; Rossell and Sheehan 1998). Mid-diaphyseal sections were selected and observed under a compound microscope. Growth zones and LAGs were clearly visible in the cross sections of limb bones and phalanges. Since the sections of the femur were relatively larger compared to other bones and LAGs were distinct, the perimeters of the central marrow cavity (MC), endosteal bone (EB), LAGs and the periosteal bone outer margin (PBM) of this bone of each frog, were measured using an ocular micrometer. The rate of endosteal resorption during bone remodelling was estimated by back calculation, i.e. by comparing the perimeter of the first LAG or PBM of the youngest frog with that of the MC of older frogs (Castanet *et al* 1996).

The meteorological data used in the present study were obtained from the University of Agricultural Sciences, Dharwad.

3. Results

Monthly variation in ambient temperature, relative humidity and rainfall in the year 1997–1998 is shown in figure 1. Although the mean monthly temperature varied between 21·7°–28·7°C, the relative humidity ranged from 61%–93% and that of rainfall from 0–202 mm (figure 1).

The cross sections of the phalanges of *M. ornata* showed growth rings, each ring composed of a faintly stained broader growth zone, and a darkly stained chromophilic line, the LAG (figure 2). The presence of either double lines or partly resorbed LAGs was not noticed. In all 62 frogs used for phalangeal skeletochronology, the LAGs were clear and distinct. One to four

LAGs were observed in the phalangeal cross sections of frogs of different body sizes. In two small sized (SVL: 2 cm) frogs (3·23%), LAGs were completely absent (table 1, figure 2A). Eighteen frogs (29·03%) showed 1 LAG each (table 1, figure 2B), 15 frogs (24·19%) 2 LAGs, another 15 frogs (24·19%) 3 LAGs (figure 2C) and 12 frogs (19·36%) exhibited 4 LAGs in the phalangeal cross sections (table 1).

The measurements of the MC, EB, LAGs and PBM of the femur of 31 frogs indicated that the perimeter of MC or EB in no case exceeded that of the PBM of the smallest sized frogs (figure 3).

In the six large sized frogs, in which all the limb bones were processed for skeletochronology, the number of LAGs remained identical in all the limb bones and phalanges in five frogs (table 2; figure 2C, D). In only one frog, the femur and humerus showed an extra ring compared to the phalanx, radioulna and tibiofibula (table 2).

Of the 25 frogs used to study the gametogenic status of the gonads, 6 were females and 19 were males. The histology of the ovaries revealed that all the six females contained yolky or vitellogenic follicles; these frogs exhibited 1–4 LAGs in the phalangeal cross section (table 3). Sperm bundles were observed in the cross sections of the
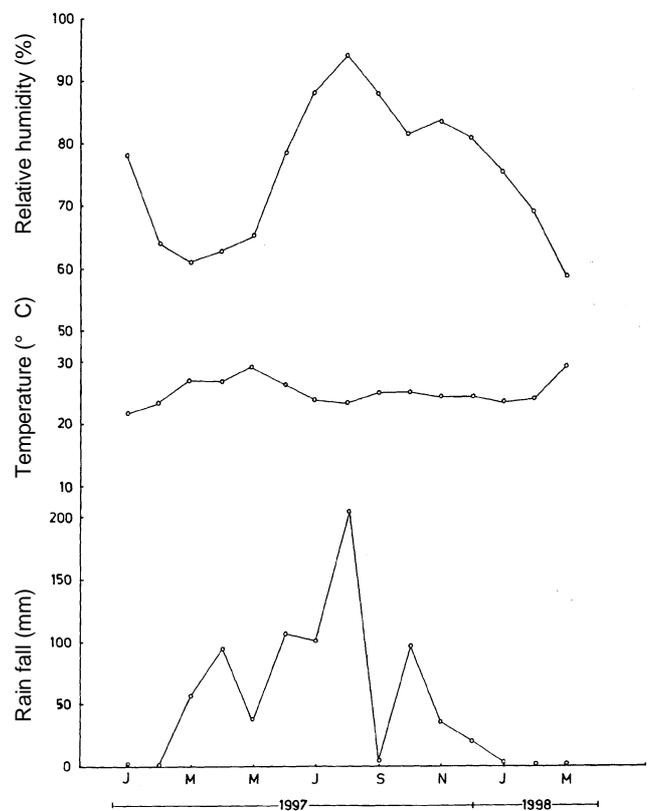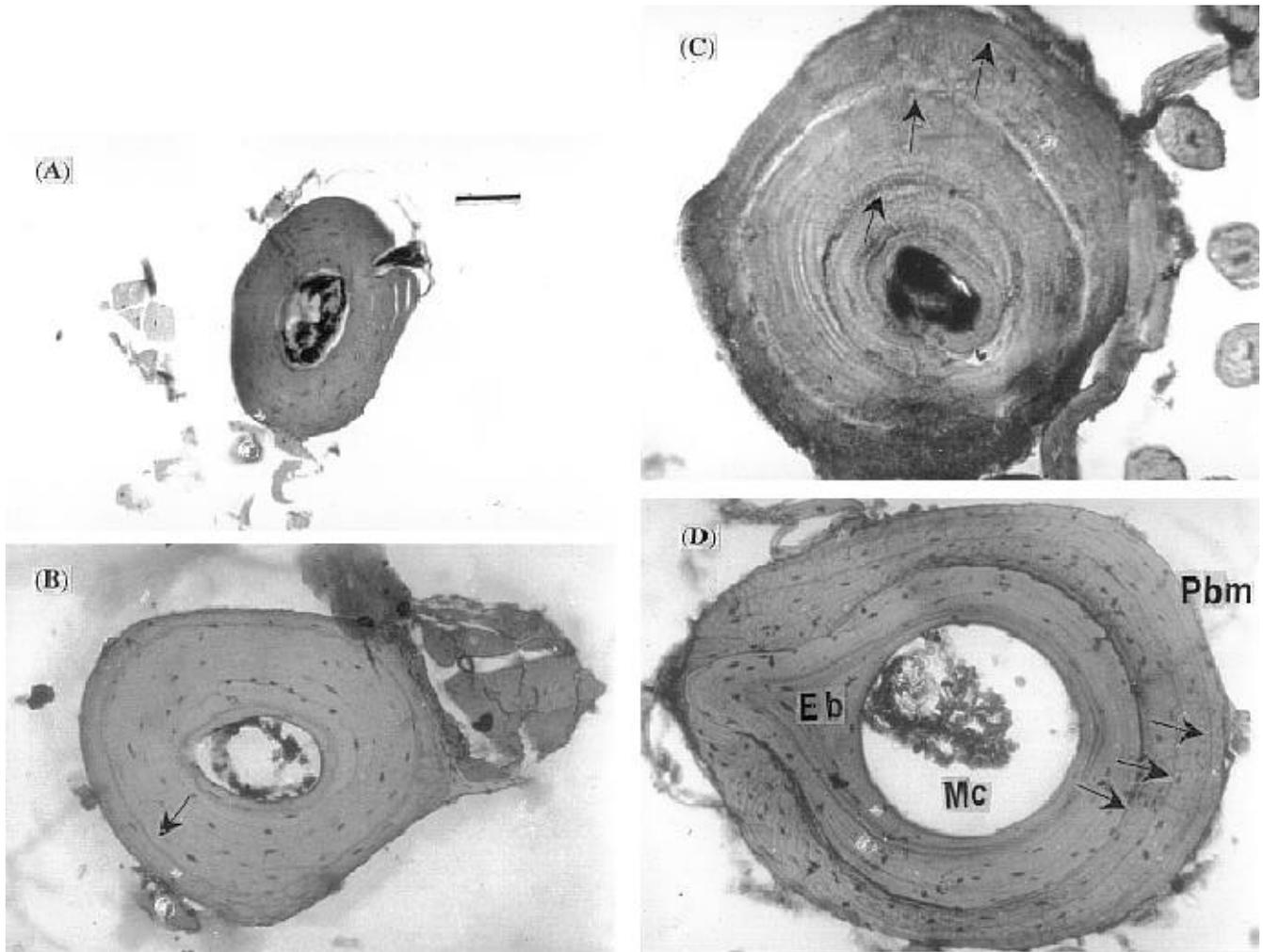

Figure 1.   Monthly rainfall (mm), temperature (°C) and relative humidity (%) in Dharwad.

Figure 2. Cross section of phalanges of *M. ornata* showing no LAG (**A**), 1 LAG (**B**) and 3 LAGs (**C**). Cross section of femur of the frog with 3 phalangeal LAGs (**D**) (haematoxylin). Scale line = 50 μm. Mc, marrow cavity; Eb, endosteal bone; Pbm, periosteal bone margin; arrows, LAGs.

Table 1. Age frequencies of *M. ornata* in the natural population.

Number of LAGs	Age	Number of frogs	Per cent
0	1st year	2	3·23
1	2nd year	18	29·03
2	3rd year	15	24·19
3	4th year	15	24·19
4	5th year	12	19·36

Table 2. A comparison of the number of LAGs recorded from different limb bones and phalanx in six frogs (*M. ornata*).

Frog No.	Femur	Humerus	Radioulna	Tibiofibula	Phalanx
1	3	3	3	3	3
2	3	3	3	3	3
3	4	4	3	3	3
4	1	1	1	1	1
5	3	3	3	3	3
6	3	3	3	3	3

seminiferous tubule of the testis of all the 19 male frogs including the two which did not show any LAGs (table 3). Among the 5 pairs of frogs found in amplexus, in the 1st pair, female showed 2 and male 3 LAGs; 2nd pair, female 3 and male 3; 3rd pair, female 4 and male 4; 4th pair, female 3 and male 4; and 5th pair, female 3 and male 4

LAGs each in the cross sections of both phalanges and femur (table 3).

4. Discussion

The available literature on the skeletochronological age estimation of amphibians reveals that the layered structure

and cyclic pattern of growth of bones is common for amphibians which is generally attributed to enforced seasonal feeding activity owing to marked variations in surrounding climatic factors, e.g. the extreme seasonal shifts in temperature of temperate areas, and the typical wet and dry conditions of the humidity cycle in the tropics (Smirina 1994; Wake and Castanet 1995; Esteban *et al* 1996). *Microhyla ornata*, found around Dharwad, leads a fossorial mode of life during most of the year and comes out for breeding with the onset of the monsoon. This frog exhibits a clear seasonality in body mass, testicular weight and spermatogenic activity (Kanamadi and Hiremath 1993). Skeletochronological observations show the presence of growth marks consisting of growth zones (generally formed due to the faster growth of bone) and LAGs (that reflect slower or arrested bone growth) in the cross sections of the phalanges and femur (Kumbar and Pancharatna 2001 and the present study). Furthermore, lack of double lines (which indicate a double annual growth cycle

including an instance of aestivation and a hibernation period) or partly resorbed LAGs, suggests that the frog may experience an uninterrupted and regular annual bone growth cycle. The meteorological data indicate that, although the annual temperature variation of Dharwad is within 7°C, the rainfall and relative humidity exhibited considerable annual variation (figure 1). Therefore, the cyclic pattern in the bone growth of *M. ornata* may be due to the annual rainfall pattern which in turn may affect regular feeding activity by diverting the frogs towards reproductive activity. Furthermore, there was no drastic difference in the body size of males and females of this species unlike that reported for many amphibians in which the female is usually larger than the male (references in Cherry and Francillon-Vieillot 1992).

One of the problems generally associated with skeleto-chronological age estimation is the phenomenon of bone resorption (Castanet and Smirina 1990; Smirina 1994; Esteban *et al* 1996). At the early period of growth when
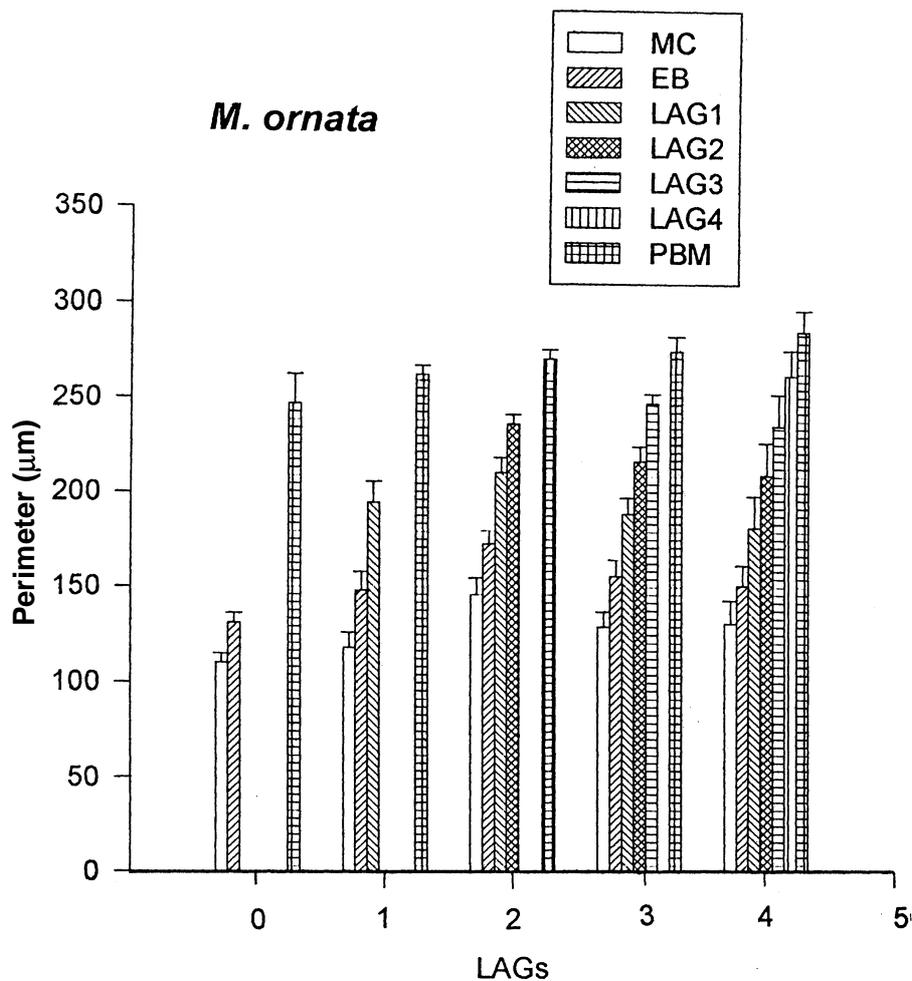
Figure 3. Perimeter of MC, EB, LAGs and PBM of femur of *M. ornata*. Values are mean ± standard errors, *n* = 31.

structural remodelling of the bone occurs, the endosteal layer is formed which often replaces the inner part of the periosteal bone; this may cause the resorption of a few earlier formed LAGs and consequently, the under estimation of age (Hemelaar 1981; Castanet and Smirina 1990; Smirina 1994; Esteban *et al* 1996). Since phalanges are small sized bones, the resorption effects may be greater. Recent studies emphasize the usage of the long bones of the limbs such as the femur or humerus or radioulna and tibiofibula for skeletochronology (Plytycz *et al* 1995; Wake and Castanet 1995; Esteban *et al* 1996; Pharam *et al* 1996; Marnell 1997). In particular, the tibiofibula of ranids is known to show slow rate of resorption and is therefore ideal for skeletochronology (Esteban *et al* 1996). In the present study, when the skeletochronological comparison of different limb bones and phalanges was made, in 5 out of 6 frogs the number of LAGs was identical in all the bones indicating that phalangeal skeletochronology is reliable in > 90% of frogs. The back calculation method further indicated there may not be loss of any LAG due to resorption since the perimeter of MC of larger frogs never exceeded that of the 1st LAG or PBM of younger frogs (figure 3), confirming the reliability of phalangeal skeletochronology.

Again, this low rate of resorption may be possibly due to the habitat of this frog because amphibian species which are active throughout the year are known to show higher rate of resorptions in comparison with those which experience seasonally active and inactive periods (Esteban *et al* 1996). There seems to be species variation in the rate of resorption, as for example in *Rana sakurii* (Kusano *et al* 1995a), *Rana tagoi tagoi* (Kusano *et al* 1995b), *Bufo bufo* (Hemelaar 1981), *Triturus vulgaris* (Marnell 1997), and in *Rana perezi* (Plytycz *et al* 1995) wherein the innermost LAG is known to be invariably resorbed, while in desmognathine salamanders, Castanet *et al* (1996) report that at least 1–3 LAGs are lost due to endosteal resorption. In contrast, in the red hill salamander no loss of LAGs is reported since the endosteal growth is confined to the marrow cavity (Pharam *et al* 1996).

The histology of the gonads indicated that the frogs without LAGs (therefore in the first year of growth) also possessed spermatozoa in the seminiferous tubules suggesting that sexual maturity is attained in the first year itself in males while the females showed yolky follicles in the ovary in the second year (table 3). This is in general agreement with that reported for other tropical anurans (Barbault and Rodriques 1978; Kulkarni and Pancharatna

Table 3. Age related changes in the body mass, body length and gonadal status of *M. ornata*.

S. No.	Body mass (g)	Body length (cm)	LAGs	Sex	Gonadal status*
1	0·8	2·0	No	Male	62%
2	0·8	2·0	No	Male	56%
3	1·0	2·3	1	Male	48%
4	0·8	2·1	1	Female	Yolky follicles
5	0·6	2·5	1	Male	43%
6	1·0	2·1	1	Male	78%
7	0·9	2·0	1	Male	68%
8[a]	1·4	2·4	2	Female	Yolky follicles
9	0·8	2·0	2	Male	63%
10	1·0	2·5	2	Male	48%
11	1·0	2·1	2	Male	60%
12	1·0	2·2	2	Male	57%
13[b]	1·0	2·3	3	Male	73%
14	1·2	2·2	3	Male	65%
15[a]	1·0	2·4	3	Male	72%
16[e]	1·2	2·2	3	Female	Yolky follicles
17[b]	1·5	2·5	3	Female	Yolky follicles
18	1·0	2·5	3	Male	59%
19	1·0	2·5	3	Male	47%
20[d]	1·7	2·8	3	Female	Yolky follicles
21	1·0	2·2	3	Male	58%
22[c]	1·6	2·5	4	Female	Yolky follicles
23[e]	1·1	2·1	4	Male	72%
24[c]	1·3	2·3	4	Male	65%
25[d]	1·1	2·2	4	Male	80%

*Per cent tubules containing sperm bundles per testicular cross section in males.
[abcde]Each paired superscript indicates frogs found in amplexus.

1996; Pancharatna *et al* 2000). In the present study our sample consisted of 5 pairs found in amplexus; their skeletochronology indicated that these frogs possessed 2–4 LAGs. Therefore, the reproductive age in this species may vary between 3–5 years. Many amphibians inhabiting temperate areas are also known to be actively involved in reproduction between 2–6 years of age (references in Cherry and Francillon-Vieillot 1992).

In order to study the longevity of amphibians in natural populations, a random collection and large (> 60) sample sizes (consisting of all the possible age groups) are advised for skeletochronology (Smirina 1994). In the present study the age determination of 62 randomly collected animals indicated that 3% were in the first year, 29% in second year, 24% in third year, 24% in fourth year and 19% frogs in the 5th year of growth. Therefore, in the natural population, the frogs may live for a maximum of 5 years. The existing data on the longevity of anurans in nature reveal that *Bufo americanus*, *Bufo pentoni*, *Rana perezi* and *Rana pipiens* also live for 5–6 years while other anurans such as *Ascaphus truei*, *Rana temporaria*, *Bufo bufo*, *Rana ridibunda*, *Rana esculenta* are reported to live longer, i.e. 9–14 years (Hemelaar 1983; Cherry and Francillon-Vieillot 1992; Smirina 1994).

In conclusion, the present study reveals that skeletochronology is applicable to the tropical anuran *M. ornata*. In natural populations, this frog lives for a maximum of 5 years; the reproductive age ranges between 3–5 years. Sexual maturity is attained in the first and second year in male and females respectively.

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