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Differentiation of bone tissue and long bone development in the Uludağ frog, *Rana macrocnemis* tadpoles

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Abstract. Long bone development and differentiation during metamorphosis (Gosner Stages 36 to 46) of *Rana macrocnemis* from high altitudes Uludağ (Bursa, Turkey, 2275 m a.s.l.) were investigated. We observed both longitudinal and radial growth of bones in the diaphysis and epiphyses. Longitudinal growth of long bones is a result of increasing thickness of periosteal bones. In tadpoles of *R. macrocnemis*, long bones are formed by perichondral ossification and differentiation of a cartilage responsible for enlargement of the medullar cavity. Ossification of the femur starts at Stage 39 and progresses at the latter Stages. We suggest that not only the epiphyseal cartilage, but also the periosteum play an important role in a longitudinal growth of long bones in amphibians.

Key words: *Rana macrocnemis*, metamorphosis, long bone development, Western Anatolia.

Introduction

Perichondral ossification is preceded or accompanied by an endochondral ossification in developing long bones of vertebrates (Carter 1998). Particularly, in mammals and birds, the developing long bones of the limbs is the result of endochondral ossification, in which cartilage serves as the initial skeletal element and is later replaced by bone tissue (Simsa & Monsonego Ornan 2007). In lizards, perichondral and endochondral ossification proceed synchronously (Carter 1998). In contrast to the endochondral ossification in mammals, birds and lizards, perichondral ossification dominates in early long bone development in basal tetrapods and dinosaurs, and extant bony fish (Haines 1942, Reid 1984, 1996, Barreto et al. 1993). In amphibians long bones are formed by perichondral or intramembranous ossification (Felisbino & Carvalho 2002). The perichondral ossification of amphibian long bones advances toward the bone ends faster than the endochondral ossification (Carter 1998). Histogenesis of long bones in higher vertebrates has been studied thoroughly (e.g. Bloom & Fawcett 1986, Olsen et al. 2000); however, this process is still poorly known in amphibians (Rozenblut & Ogielska 2005). Postembryonic development and ossification of fore- and hind limbs were described in some ranids, such as *Rana pipens* (Kemp & Hoyt 1969), *R. temporaria* (Zawadowska & Faber 2002) and *Pelophylax ridibundus* and *P. esculentus* (Rozenblut & Ogielska 2005). In larval anurans, ossification of chondral primordia of long bones starts at the end of premetamorphosis (Stage 34) and is extensive during prometamorphosis (Stages 36-41); the process is completed during metamorphic climax (Stages 42-45) (Rozenblut & Ogielska 2005).

The purpose of the present study was to complete our knowledge of ossification in amphibians by studying the development and differentiation of femora in *Rana macrocnemis* from larval Stages 36 to 46.

Materials and Methods

Rana macrocnemis tadpoles were collected from Lake Kilimli, Uludağ (40° 04′ 672′′ N, 29° 13′ 293′′ E, 2275 m a.s.l.) (Bursa, Turkey) between June and August 2007. Developmental Stages were determined using Gosner's (1960) developmental table. Studies of long bone growth were performed on femora of metamorphosing tadpoles at Stages 36 to 46. Data concerning body length (SVL) and total length (TL) used in this study are summarized in Table 1. Femora were dissected from tadpoles (5 specimens for each Stage) and soft tissues were removed from the bones before fixation in 10% formaldehyde for 24h. We used Rozenblut & Ogielska (2005)'s modified method for decalcification. After fixation, the bones were decalcified in a 1:1 mixture of 10% formic acid and 4% formaldehyde for 30 min. The bones were then washed with distilled water to remove decalcification medium and then embedded in paraffin, sectioned at 7-10 µm, and stained with Gill's Hematoxylin and trichrome of Goldner's. The slides were photographed with Olympus 51-Altra 20 Soft Imaging System and investigated with light microscope. The study protocol was approved with Decision No. 2007/4-1 by the Animal Ethical Council of the Faculty of Pharmacy at Ege University.

Results

The development and differentiation of femora were analyzed in metamorphosing tadpoles at Gosner Stages 36 – 46. Longitudinal and transversal growth of these bones was studied through the examination of the diaphyses and epiphyses. In the larvae of *Rana macrocnemis*, long bones are formed by perichondral ossification.

At the earlier Stages of metamorphosis, the femur has a fully cartilagous structure. Mesenchymal cells differentiate and transform into chondroblasts at Stage 36. At this Stage, initial cartilage differentiation occurs and the femur primordium resembles an articular cartilage tissue. The forming bone is encircled by perichondrium in the outermost part. At Stage 38, chondroblasts are surrounded by matrix, chondrocytes appear and differentiation of cartilage becomes rapid (Fig. 1A). At Stage 39 the amount of cartilage matrix decreases and the chondrocytes begin to differentiate (Fig. 1B). The cells increase in volume and their growth (hypertrophy) becomes more evident. The process proceeds from proximal to distal direction. As cells undergo hypertrophy, thinning of the cartilage matrix occurs. At Stages 40 and 41, the periosteal bone is shaped as a thin cylinder along the femur and is seen on transverse sections as a ring of compact tissue (Fig. 2A, B). As a result of the inclusion of a thin periosteal bone into the epiphysis, the inner and outer part of the epiphyseal cartilage start to separate from each other and form the lateral articular cartilage (Fig. 3A). The metaphyseal cartilage

Table 1. Descriptive statistics of body (SVL) and total length (TL) of *Rana macrocnemis* tadpoles in Uludağ (Bursa/Turkey).

Stages	36		37		38		39		40		41	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
SVL	15.5	1.2	16.9	1.1	17.6	0.8	17.5	1.3	17.3	1.0	17.3	1.0
TL	38.2	2.3	42.2	2.2	46.3	2.3	48.3	2.3	46.1	3.1	47.7	2.6
Stages	42		43		44		45		46			
SVL	18.4	3.9	16.1	0.5	17.4	0.8	17.2	0.6	19.7	0.8		
TL	42.9	4.2	27.4	0.6	19.5	1.7	18.7	0.9	-	-		

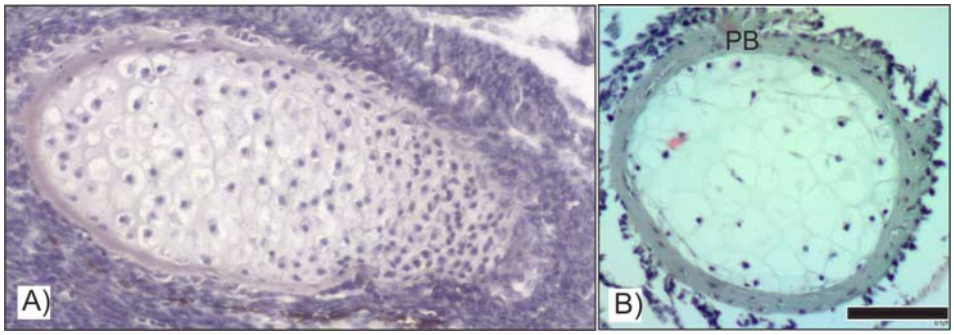

Figure 1. Transverse sections from diaphyseal part of femora of tadpoles at Stage 38 (**A**) and 39 (**B**) in *Rana macrocnemis*. PB: periosteal bone. (Gill's Hematoxylin). Scale Bar, 100 µm.

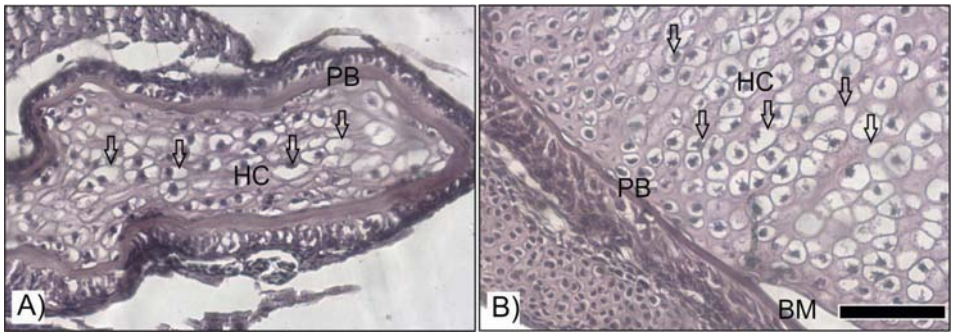

Figure 2. *Rana macrocnemis* tadpole at Stage 40 (**A**) and 41 (**B**). At this Stages cartilage cells continue differentiation and arrows show hypertrophy of cells, HC: hypertropic chondrocytes; PB: periosteal bone, P: periosteum, BM: medullar cavity. (Gill's Hematoxylin) Scale Bar, 100 µm.

develops in the internal portion of the periosteal bone (Fig. 3B). This structure consists of two parts, namely resting (1), proliferation (2) and hypertrophic (3) zones. At Stage 42, the hypertrophied cartilage cells start degenerating. At Stage 43, an increase is seen in the amount of matrix, with parallel to the amount of hypertrophy (Fig. 4A). At Stage 44, the diaphyseal cartilage cells begin to disappear as a result of the degeneration and the area of the bone matrix increases (Fig. 4B). At Stages 45 and 46, some clustering called osteogenic bud that consists of perichondrial osteoprogenitor and blood cells is observed in the gaps resulting from cartilage degeneration (Fig. 5A, B). At Stage 46, degeneration of chondrocytes in the diaphysis stops when marrow cavity reaches the epiphyses. As a result, the cartilage is restricted only to the epiphyses and the marrow cavity is successively filled with marrow cells. The differentiations of chondrocytes are responsible for enlargement of bone marrow, so transversal growth of entire bone is completed by differentiation of chondrocyte cells.

Longitudinal growths of bones take place in the osteochondral ligament (OCL) that is situated inside of the perichondrium and is visible in the transversal sections of epiphyses of femora (Fig. 3B). This structure attaches the periosteal bone to the articular cartilage. The longitudinal growth of a bone is a result of new osteocytes that are generated by OCL and thus an increase in thickness of bone is completed by apposition of new bone tissue.

Discussion

In mammals the growth cartilage is situated inside the periosteal bone and plays an important role during the endochondral ossification (Hunziker 1994, Olsen et al. 2000). In amphibians this structure was referred to metaphyseal cartilage (Dell-Orbo et al. 1992). We also use this term for this portion of epiphysis. Chondrocyte proliferation, hypertrophy and death within the metaphyseal cartilage are subjects of complex regulation and enlargement of bone marrow cavity (Olsen et al. 2000). The metaphyseal cartilage of the epiphysis is composed of cellular zones named resting, proliferation and the hypertrophy zones, by other authors (Katschenko 1881, Dickson 1982, Felisbino & Carvalho 1999, Rozenblut & Ogielska 2005, Olsen et al. 2000).

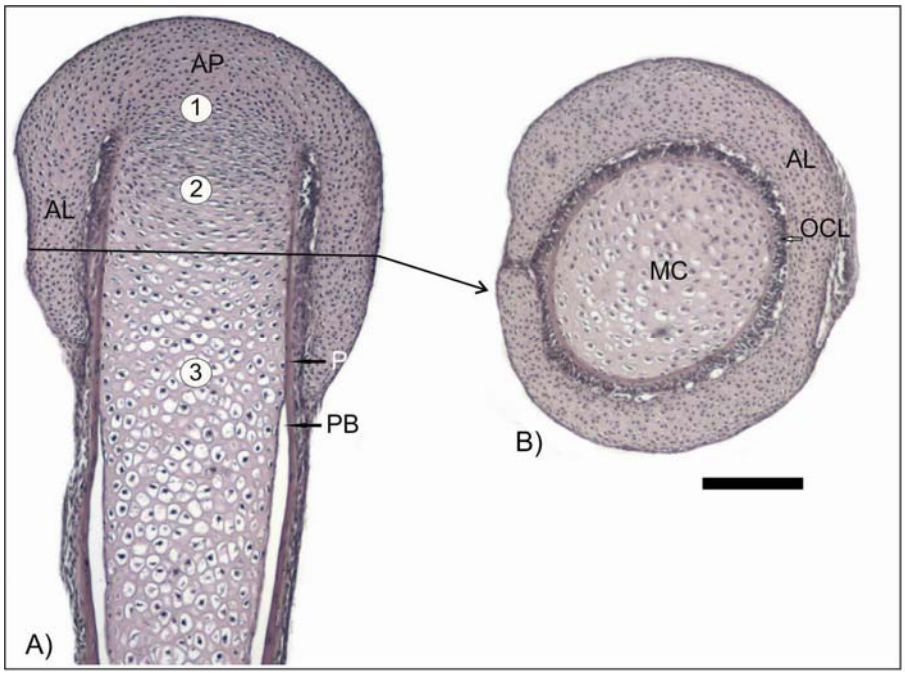

Figure 3. *Rana macrocnemis* tadpole at Stage 42. **A**: Longitudinal sections of distal part of the femur, **B**: Transverse sections of proximal epiphyses of the femur. 1: resting zone, 2: proliferating zone, 3: hypertrophic zone, AL: lateral articular cartilage, AP: articular cartilage proper, MC: metaphyseal cartilage, OCL: osteochondral ligament. (Gill's Hematoxylin) Scale Bar, 200 µm.

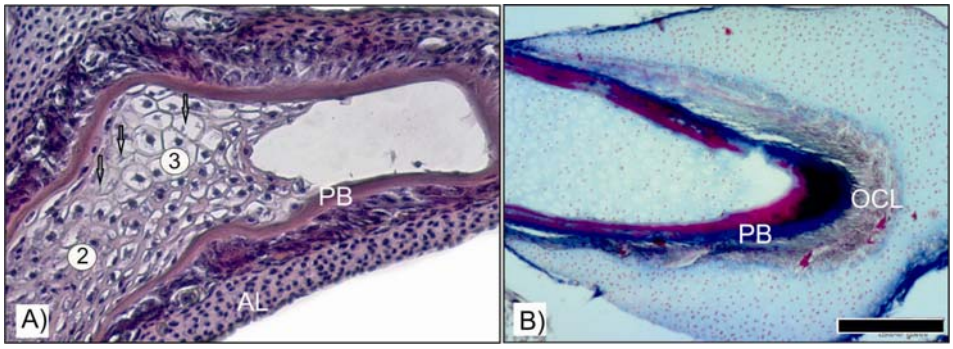

Figure 4. Longitudinal and transverse sections of femora of tadpoles at Stages 43 (**A**) (Gill's Hematoxylin) and 44 (**B**)., 2: proliferating zone, 3: hypertrophic zone. Arrows show degenerating chondrocytes in the hypertrophic zone. (trichrome of Goldner's) Scale Bar, 100 µm.

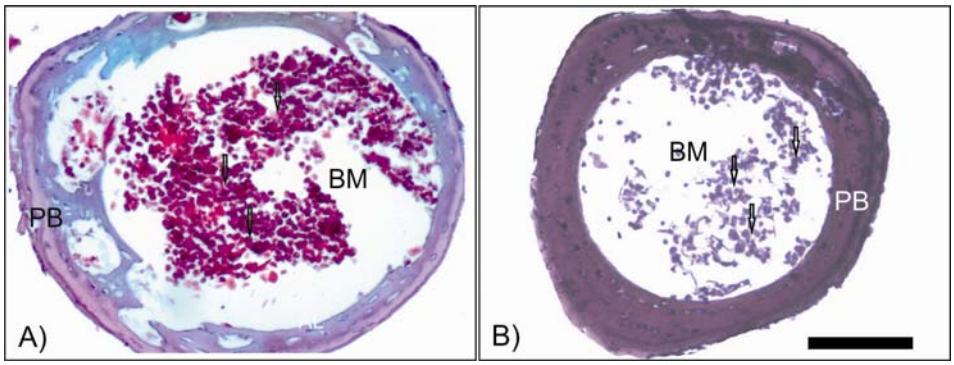

Figure 5. Transverse section of femora of tadpoles at Stages 45 (**A**) (trichrome of Goldner's) and 46 (**B**) (Gill's Hematoxylin) in *R. macrocnemis*. Arrows show osteoprogenitor cells in bone marrow cavity. Scale Bar, 100 µm.

Felisbino & Carvalho (2000) revealed that osteochondral ligament (OCL) is responsible for longitudinal growth of periosteal bone. This structure attaches the periosteal bone to articular cartilages. Our results related to the structure of OCL are similar to the findings of Felisbino & Carvalho (2000) and Rozenblut & Ogielska (2005).

Ossification of femur has been reported for a number of ranid species. These processes start at different Stages. For example, femur starts to ossify after Stage 34 in *R. temporaria* (Zawadowska & Faber 2002); Stage 35 in *R. pipiens* (Kemp & Hoyt 1969); Stage 36 (Haas 1999); Stage 40 in *P. adspersus* (Sheil 1999) and in European water frogs *P. lessonae*, *P. ridibundus* and *P. esculantus* (Rozenblut & Ogielska 2005). In our study, we observed that femur in *R. macrocnemis* started to ossify at Stage 39.

In conclusion, longitudinal growth and elongation of entire femora is a result of OCL activity, whereas radial growth is a result of the metaphyseal cartilage activity. The latter structure does not play a role in bone development, it is only assigned enlargement of the bone marrow. Long bones of *R. macrocnemis* tadpoles are formed by perichondral ossification and differentiation of a cartilage responsible for enlargement of the medullar cavity. Ossification begins at Stage 39 and progresses at the latter stages. Our findings also imply that not only the epiphyseal cartilage, but also the periosteum play an important role in the longitudinal growth of long bones in amphibians.

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