

THE USING OF SKELETOCHRONOLOGY AS A SCREENING METHOD FOR AGE DETERMINATION OF ALPINE NEWTs (*MESOTRITON ALPESTRIS*): A TECHNICAL REPORT

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Abstract

MAKOVICKÝ PETER, KOPECKÝ OLDŘICH, MAKOVICKÝ PAVOL, MATLACH RADEK. 2015. The Using of Skeletochronology as a Screening Method for Age Determination of Alpine Newts (*Mesotriton Alpestris*): a Technical Report. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis*, 63(2): 439–446.

Skeletochronology is a widely used method for age determination in amphibians. This method is based mainly on the histological examination of the finger bones. However, the lengths of utilized severed fingers have not been specified in previous studies. The objective of this study was to analyse the structure of line arrested growth (LAGs) involving taking only the last two phalanges of a finger, and using the entire finger of Alpine newts (*Mesotriton alpestris*). Altogether 432 fingers were taken from four localities in the Czech Republic during the newt breeding period. The first group (group A) contained fingers that consisted of the last two phalanges (330 samples), and the second (group B) contained complete fingers with all phalanges (102 samples). All fingers were processed using standard histological methods and stained with hematoxylin-eosin. Phalange cross-section slides were made, and the ages of the individuals were determined by the number of LAGs. From two phalanges it is determine the age of 17.87% of newts; however, age determination was successful in 49.01% of newts when using whole fingers. Age determination success rate differences between groups were significant ($P < 0.001$). This is a histological study and it is recommended utilizing whole fingers in future *Caudata* amphibian screening age studies.

Keywords: age determination, Alpine newt, amphibian, bone histology, caudata, screening, line arrested growth, skeletochronology, zoology

INTRODUCTION

One of the fundamental parameters of population is age structure. Hence precise animal age determination is often crucial for an effective conservational approach. There has been a lot of discussion surrounding the recent global decline among amphibians (Gherghel *et al.*, 2008). Their age determination is technically demanding and cannot be carried out without invasive

intervention. For amphibian age determination, the skeletochronology method is the most widely used (Caetano and Leclair, 1996; Jakob *et al.*, 2002; Makovický *et al.*, 2011; Uzum, 2009). This technique utilizes stained cross sections of bone, which allow visualization of lines of arrested growth (LAGs). There were two types of growth lines described (Bruce *et al.*, 2002). It is annuli and LAGs. Annuli are composed by thin layers

of avascular bone with parallel-aligned bone fibers and LAGs are much thinner and have fewer bone fibers by volume (Francillon-Vieillot *et al.*, 1990). In studies where the age of the individual is only one of the observed variables and precise results are required, skeletochronology is based on large bones such as *humerus* or *femur* (Olgun *et al.*, 2001). However, a method based on the use of large bones is necessary to kill examined individuals. An alternative to the large bone method is to use only bones from digits, which allows amphibians to survive. Moreover, due to their regenerative capacity, consequences of this method are considered minimal, and will not greatly affect their existence. This time it is known, that skeletochronology as a method is used only for estimation of age and it is also well known, that this method is not accurate. In our meaning, there for the purposes of skeletochronology should be used only those parts of bones, which cause minimal trauma. The most suitable alternative is phalange of animals with the ability to complete regeneration. However, no literature has determined how many phalanges of digits should be severed. A smaller segment of a severed digit will mean fewer traumas for the animal, but also less material for age determination. All of these questions are a part of zoological research and they are in the context

with animal protection. This is a histological study and the objective of our study was to analyse the structure of LAGs involving taking only the last two phalanges of a longest finger, and using the whole longest finger. As a model organism, we used a common European amphibian, the Alpine newt (*Mesotriton alpestris*).

MATERIAL AND METHODS

Ethical Principles of the Study

This study was approved by the Ethics Committee of Landscape protected area – Orlické hory, number 00444/OH, Czech Republic.

Animals

Alpine newts were captured individually from the shore using dip nets or directly to the hand during the reproduction periods from 2005 to 2009 (Figs. 1A, 1B). The survey was carried out in four localities in the Czech Republic, all lies in Pardubický region: Zabitý (elevation: 500m; character: eleven unstable water-filled vehicle-track ruts in spruce forest with max depth 0.2m and max. surface 1.6 m²; localization: 49°57'11" N, 16°23'54" E), Hylváty (elevation: 355m; character: five unstable water-filled vehicle-track ruts in spruce



1: A: This is a view on the one little newt. B: Please look and note size comparison among adult male hands and newt. C: Please note size of the one little fixed sample in labelled tube. D: Several fixed samples in many tubes from one locality.

forest with max depth 0.2 m and max. surface 1.3 m²; localization: 49°57'29'' N, 16°23'53'' E), Horní Morava (elevation: 760 m; character: abandoned quarry with stable pool, max depth 0.7 and surface 55 m²; localization: 50°9'42'' N, 16°49'16'' E) and Zhoř (elevation: 480 m; character: small garden concrete pool with max depth 0.5 m and surface 3 m²; localization: 49°54'7'' N, 16°22'60'' E).

Sampling

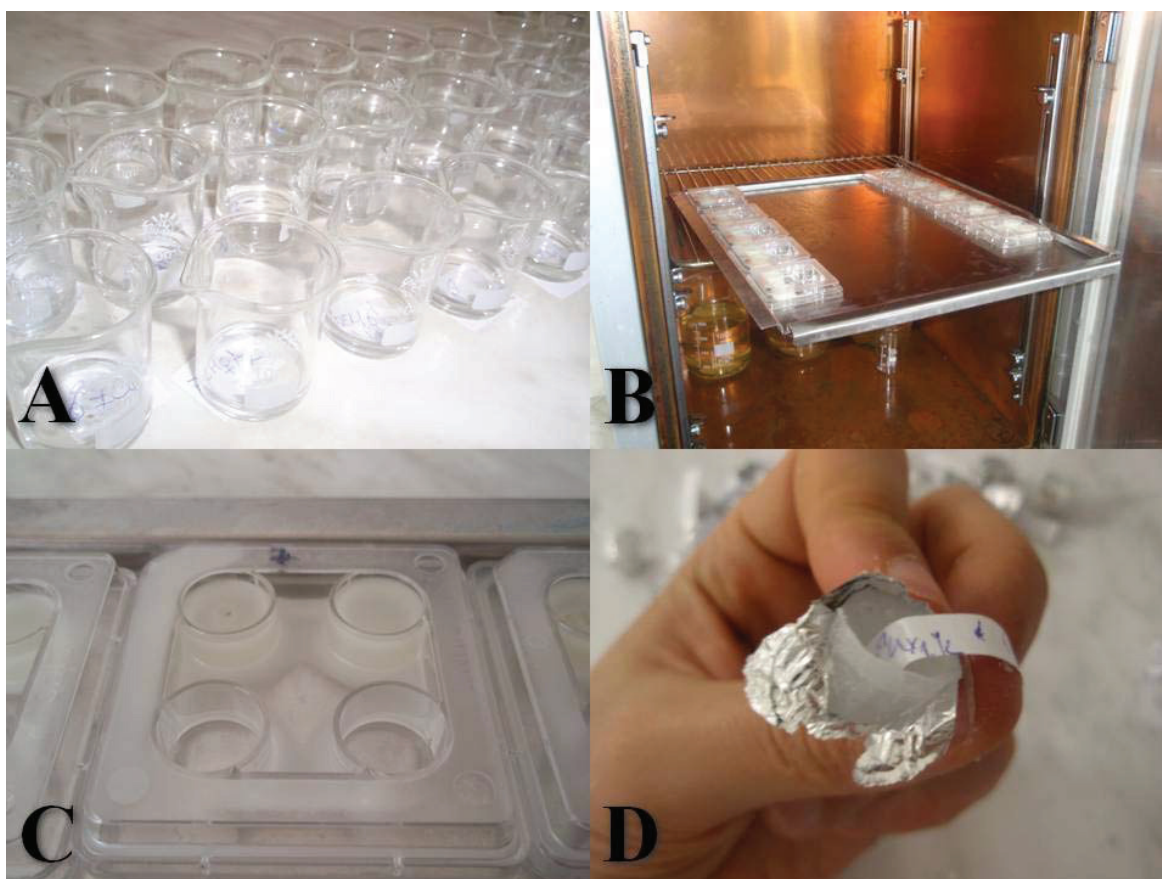
Altogether 432 samples were used for this study. Between 2005 and 2007, 330 individuals were captured on localities Hylváty, Zabitý, Horní Morava, and Zhoř, and it we took the last two phalanges from the longest finger of the left front limb (group A). Between 2008 and 2009, 102 newts were captured on localities Zabitý, Horní Morava, and Zhoř. We severed the whole longest finger from the left front limb of each of these individuals (group B). Sampling was done with nail scissors and without anesthetization. After each individual sampling, the scissors were disinfected in 70% alcohol. The removed digits were preserved in 70% alcohol and stored in labelled tubes (Figs. 1C, 1D).

Histological Analysis

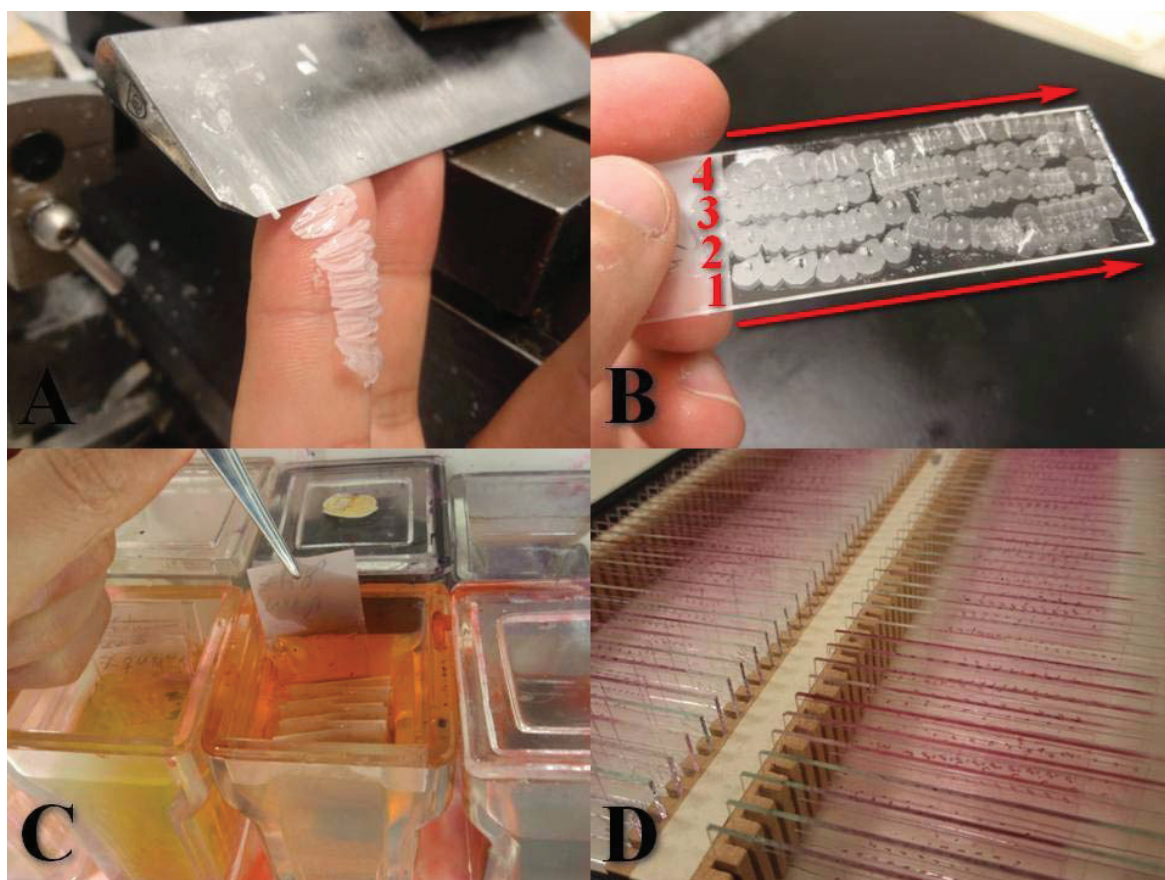
Samples were decalcified, further processed by standard histological methods and embedded in paraffin blocks (Figs. 2A, 2B, 2C, 2D). Three to five µm-thick slices were cut from each sample to the standard slides (Bamed, Czech Republic) (Figs. 3A, 3B). The slices were stained with haematoxylin-eosin (HE) (DiaPath, Srl., Italy) (Figs. 3C, 3D). The samples were described and LAGs in each of the samples were evaluated using a light-microscopic picture using an Olympus AX70 Provis microscope (Japan). All the samples, where were LAGs clearly visible were evaluated. So successfully examined slide was those one, where LAGs were visible. In other samples, which were any LAGs, or there were difficult to see them, or these were merged with each other, age has been undetected.

Statistical Analysis

Differences in age determination success rates between groups A and B were compared by Statistica 9.0 (Stasoft, Inc. 2009) using a 2 × 2 contingency table.



2: A: It is photo showing individual manual processing of some samples. B: Individual samples in paraffin bath in thermostat. C: Detailed photo showing some samples in paraffin bath. D: It is a view on a little paraffin block with one sample.



3: A: It is view on cutting of sample with some paraffin slices using microtome. B: More serial paraffin slices were cut from each sample to one slide. Please note, that there are 1–4 continuous lines with serial paraffin slices from one sample in succession in the direction of arrows. C: It is a view on staining of the slices. D: The view on the stained and mounted microscope glasses in the boxes.

RESULTS

Objective Results

In group A from total 330 samples were able to evaluate 59 samples, what is 17.87% success rate. In the group B from total 102 samples were able to evaluate 50 samples, what is 49.51% success rate. Total of 432 samples were able to evaluate 109 samples, what is 25.23% success rate. The difference in age determination success rates between groups A and B was statistically significant ($P < 0.001$) (Tab. 1).

General Histological Description

In the centre of the samples, there is a layer with predominantly hyaline cartilage, which is surrounded by a thin layer of elongated cells forming the helical core of perichondrium. The cartilage merges smoothly with the peripheral part of the bone. In some samples, centrally located bone tissues are clearly visible. LAGs in different groups were visible in cross-section of bone tissue. In some samples, there were no LAGs, but only thin, dense and often featureless circular lines that overlap each other; this was evident in samples taken from only part of the finger. Sparse cells, fibrocytes

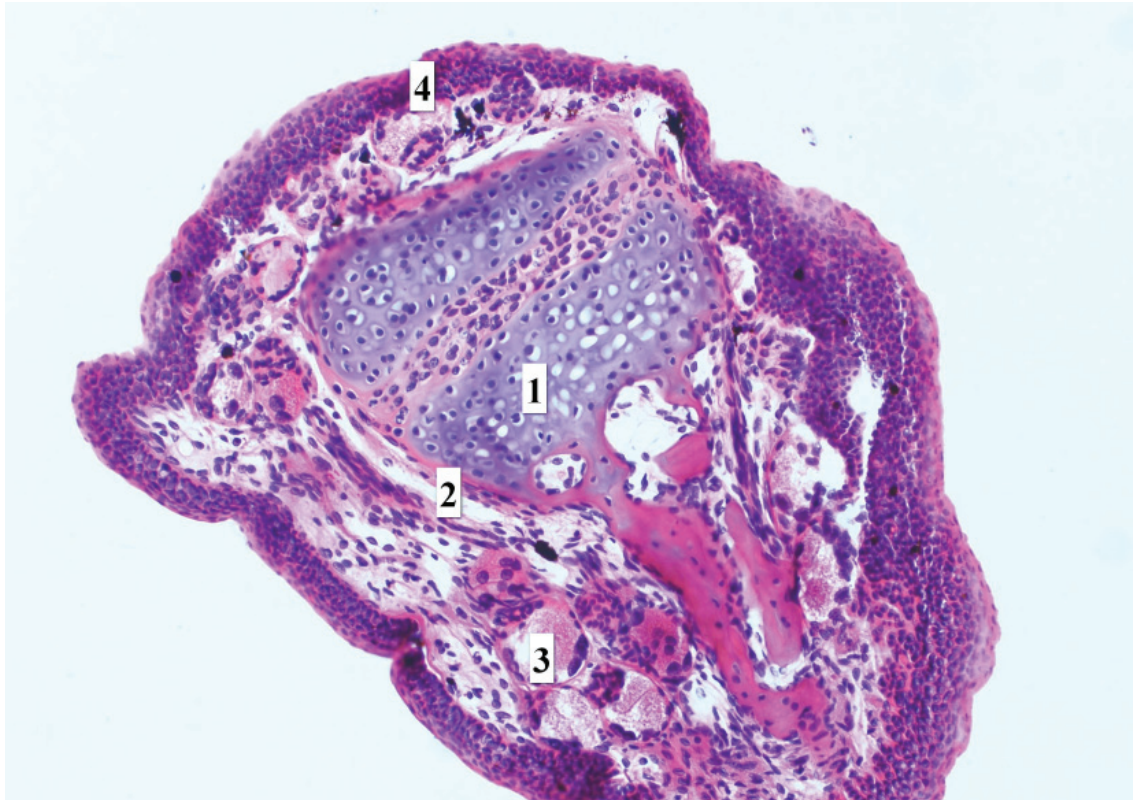
and unevenly distributed pigment cells can be seen on the periphery of bone tissue. The other part of the interstitial tissue consists of numerous thin-walled blood vessels of a predominantly larger calibre. The surface is covered with a multilayer of keratinized epithelia and numerical pigment epithelium cells.

Histological Description of Group A

From histological point of view appears as small ovoid formation with centrally localized layer of hyaline cartilage that is bind by thin layer of elongated cells. These cells have spiral cores which forms perichondrium (Fig. 4). In some sections, both bone and compact tissue with prominent Havers system is visible as same as well separated and noticeable LAGs. Surface is composed of multilayer keratinized epithelium (Fig. 5).

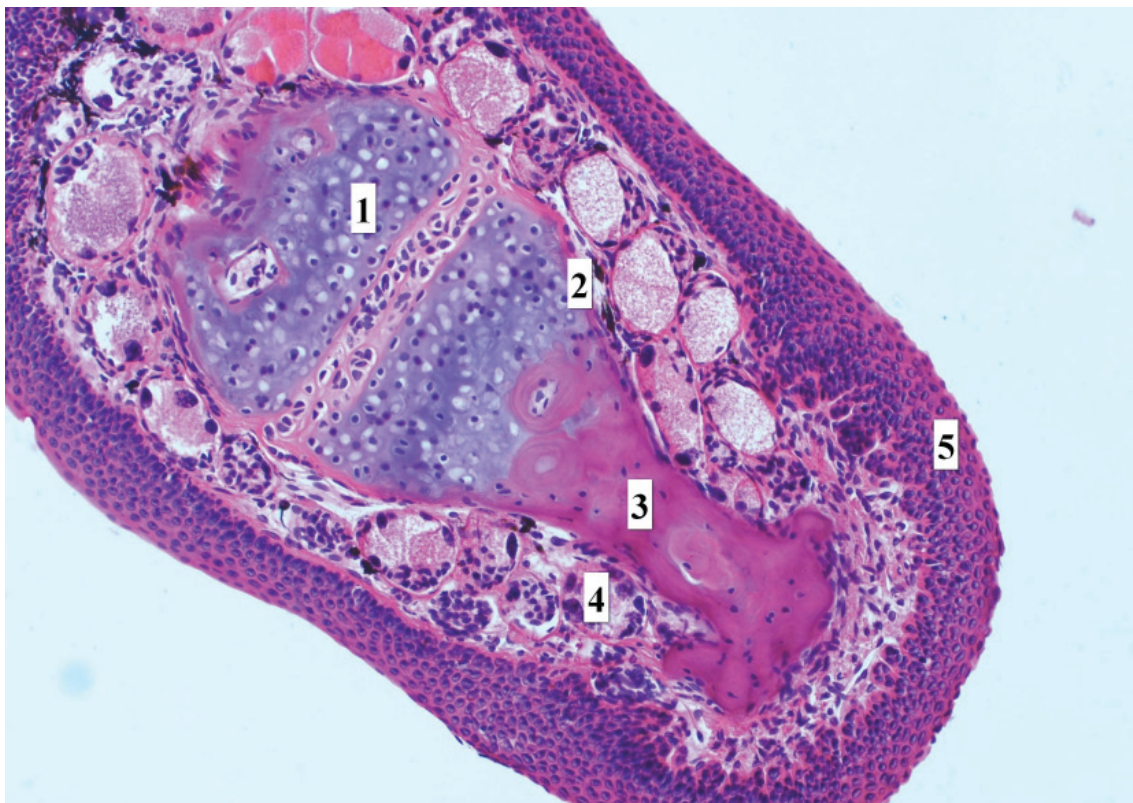
Histological Description of Group B

It is a cross section of the phalange with clearly visible bone tissue. Serial sections further extend into hyaline cartilage, eventually severing epiphysis plate (Fig. 6). In compact bone tissue is visible Havers system with particular LAGs (Fig. 7). The surface is covered by multilayer epithelium mixed with high number of pigment cells.



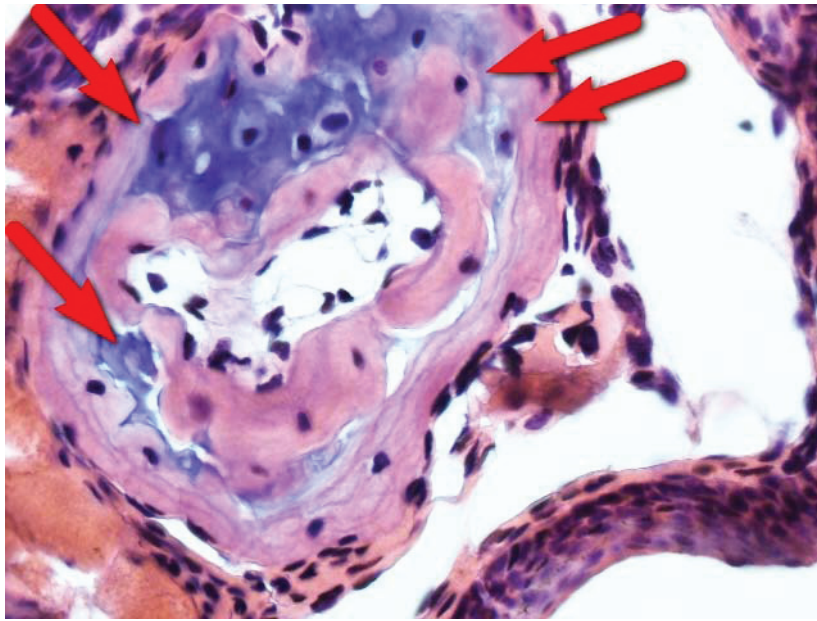
4: It is a cross section of the apical part of the finger with cartilaginous tissue and the adjacent layer of connective tissue, which is covered with multilayer epithelium

Legends: HE, 100x. A group. 1: hyaline cartilage, 2: perichondrium, 3: blood vessel, 4: epidermis.

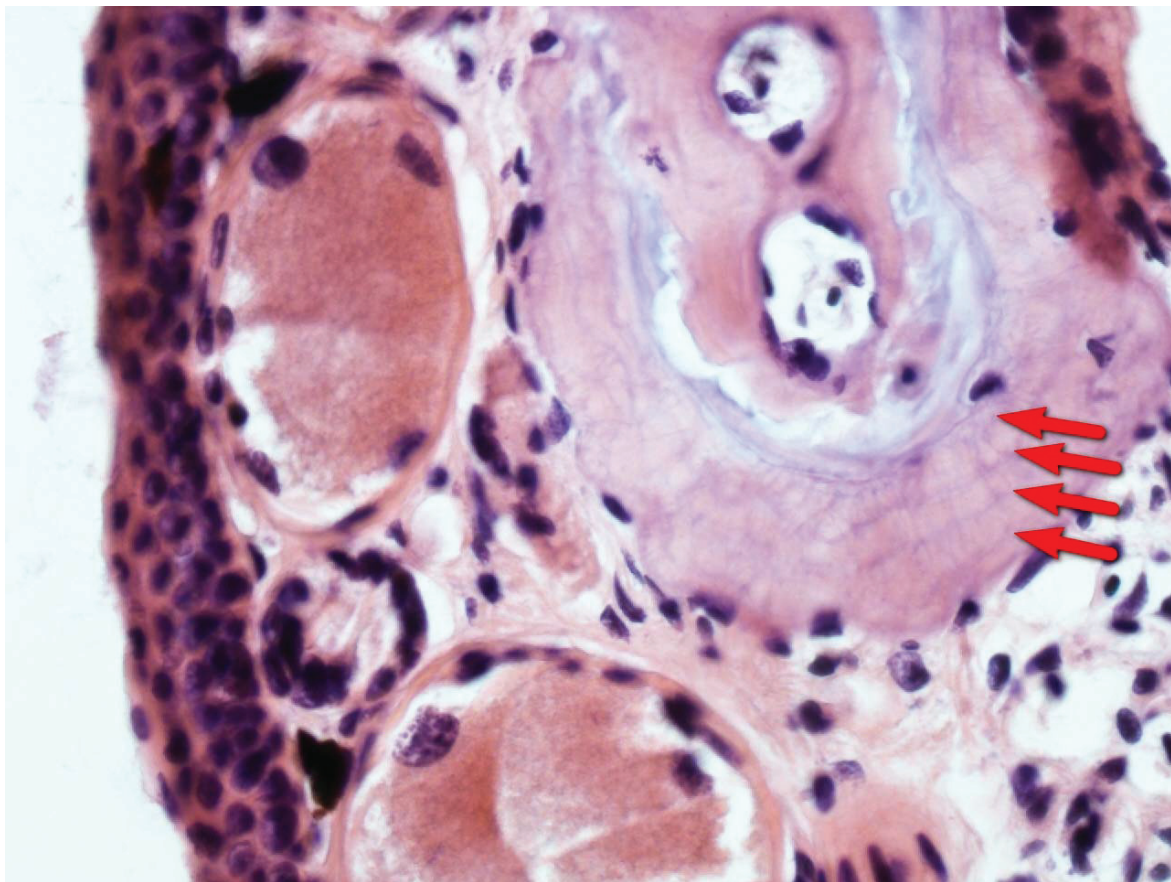


5: It is a part of finger on the cross-section with a central hyaline cartilage, bone tissue with adjacent interstitial tissue and epidermis

Legends: HE, 100x. A group. 1: hyaline cartilage, 2: perichondrium, 3: compact bone, 4: blood vessel, 5: epidermis.



6: This is a cross section of the finger with the marginal zone of bone to cartilage tissue (arrows). This view is from the contact of diaphysis and epiphysis of bone.
Legends: HE, 400x. B group



7: This is a view to the centre of the sample with bone tissue with four well visible lines as LAGs (arrows)
Legends: HE, 400x. B group

I: Comparison of two different skeletochronological approaches

Groups	NS	SES	PE
A	330	59	17.87%
B	102	50	49.01%
Suma	432	109	25.23 %

Legends: A: samples consist from last two phalanges of digit, B: samples consist of whole fingers, NS: Number of samples, SES: Successfully examined samples, PE: Percentage expression. Significance: $P < 0.001$.

DISCUSSION

The Havers system is a long cylinder which is consistent with the bone shaft. It is formed by a central canal surrounded by concentrically arranged lacunae and lamellae (Martiniaková *et al.*, 2006). This is why long bones used for amphibian age determination produce better results than bones, such as digits, from distal parts of the body do. The ages of 100% of individuals were often successfully determined when long bones were used. However, this approach requires the killing of animals. In ecological or ethological studies where there is a demand for live specimens, this approach is useless. Most studies utilize only digits for age determination; however, their results are less accurate than those from studies which utilize entire bones (Miaud *et al.*, 2000; Olgun *et al.*, 2005). Age determination accuracy rates in our experiment are (mainly in group B) comparable with those of previous studies. Conversely, in group A, only the apical parts of the bone along with dense, barely visible LAGs were observed in many slices. Furthermore, in group A, it were often observed only cartilaginous tissue in the investigated samples.

There is a consensus that skeletochronology is not a reliable method in the case of long-living amphibians as e.g. Alpine newt is, because growth is stopping after a given age. Wagner *et al.* (2011) proved that skeletochronology is not a suitable method for age determination of the Alpine newts (*Mesotriton alpestris*). It was also found that differences between real and estimated ages increased with age; the older an individual the bigger the difference is. The only suitable method is if marked animals are followed for years. In other study by Altunisik *et al.* (2014) was documented, that age was not correlated with body size for the individuals. Also research study of Eden *et al.* (2007) indicates that skeletochronological studies that do not use individuals of known age for calibration may underestimate age. On the other hand for example study of Khonsue *et al.* (2010) concludes that skeletochronology can be applied for ecological studies on protected species as newt. The similar conclusions were stated also previously study conducted by Bovero *et al.* (2006). Our study is without real control group, but the aim of our study was elsewhere. This is a histological study, which analyses the structure of LAGs in whole longest fingers and last two phalanges of the whole fingers of newt. It seems like, that for good visibility of individual LAGs it is necessary to cut the bone in the middle of diaphysis. Toward to epiphysis, there is starting overlap of individual LAGs. Some LAGs can also mutually fuse together through resorption or bone tissue rebuilding (Lima *et al.*, 2001). This resorption rates may differ depending on bone thickness. Also resorption or bone remodelling may affect the presence of LAGs. Contrarily, double-LAGs (Caetano and Castanet, 1993) or adjacent lines are sometimes observed in close proximity to LAGs (Miaud *et al.*, 1993). The distribution of osteons through bone tissue can also be modulated by mechanical factors, and digits are often damaged by newts; however, they were not visible in our experiment.

Our results show that age determination in populations of *Caudata* amphibians should be based on examination of whole digits, but 100% success is not guaranteed. Reading LAGs produce far better accuracy rates than does the examination of the last two phalanges. Regeneration of a digit is faster when only the last two phalanges of an individual are taken. In our experiment, no significant impact on health or behaviour was observed in newts with severed digits therefore, we do not expect any significant differences between results from group A and those of group B. The regenerative capacity of *Caudata* amphibians ensures the healing of wounds and tissue replacement within one year of sampling. Therefore, we recommend sampling whole fingers in future *Caudata* amphibian age determination screening studies.

REFERENCES

- ALTUNISIK, A., ERGUL, T., GÜL, C. *et al.* 2014. A skeletochronological study of the smooth newt *Lissotriton vulgaris* (Amphibia: Urodela) from island and mainland population in Turkey. *Ital. J. Zool.*, 81(3): 381–388.
- BOVERO, S., ANGELINI, C. and UTZERI, C. 2006. Aging *Salamandrina perspicillata* (Savi, 1821) by skeletochronology. *Acta Herpetol.*, 1(2): 153–158.
- BRUCE, R. C., CASTANET, J. and FRANCILLON-VIEILLOT, H. 2002. Skeletochronological analysis of variation in age structure, body size, and

- life history in three species of desmognathine salamanders. *Herpetologica*, 58(2): 181–193.
- CAETANO, M. H. and CASTANET, J. 1993. Variability and microevolutionary patterns in *Triturus marmoratus* from Portugal: age, size, longevity and individual growth. *Amphibia-Reptilia*, 14(2): 117–129.
- CAETANO, M. H. and LECLAIR, R. 1996. Growth and population structure of red-spotted newts (*Notophthalmus viridescens*) in permanent lakes of the Laurentian shield, Quebec. *Copeia*, 1996(4): 866–874.
- EDEN, C. J., WHITEMAN, H. H., DUOBINIS-GRAY, L. et al. 2007. Accuracy assessment of skeletochronology in the Arizona Tiger salamander (*Ambystoma tigrinum nebulosum*). *Copeia*, 2007(2): 471–477.
- FRANCILLON-VIEILLOT, H., DE BUFFRÉNIL, V., CASTANET, J. et al. 1990. Microstructure and mineralization of vertebrate skeletal tissues. 471–530. In: CARTER, J. G., *Skeletal biomineralization*. New York: Patterns, Processed, and Evolutionary Trends.
- GHERGHEL, I., STRUGARIU, A., GHIURCA, D. et al. 2008. The herpetofauna from the Bistrita river basin (Romania): geographical distribution. *North-West. J. Zool.*, 4(S1): S71–S103.
- JAKOB, C., SEITZ, A., CRIVELLII, A. J. et al. 2002. Growth cycle of the marbled newt (*Triturus marmoratus*) in the Mediterranean region assessed by skeletochronology. *Amphibia-Reptilia*, 23(4): 407–418.
- KHONSUE, W., CHAIANANPORN, T. and POMCHOTE, P. 2010. Skeletochronological assessment of age in the Himalayan Crocodile newt, *Tylotriton verrucosus* (Anderson, 1871) from Thailand. *Tropical Natural History*, 10(2): 181–188.
- LIMA, V., ARNTZEN, J. W. and FERRAND, H. M. 2001. Age structure and growth pattern in two populations of the golden-striped salamander *Chioglossa lusitanica* (Caudata, Salamandridae). *Amphibia-Reptilia*, 22(1): 55–68.
- MAKOVICKÝ, P., KOPECKÝ, O. a RAJMON, R. 2011. Naše zkušenosti s určováním věku čolka horského pomocí skeletochronologie. *Infovet*, 18(4): 183–184.
- MARTINIÁKOVÁ, M., GROSSKOPF, B., OMELKA, R. et al. 2006. Differences among species in compact bone tissue microstructure of mammalian skeleton: use of a discriminant function analysis for species identification. *J. Forensic Sci.*, 51(6): 1235–1239.
- MIAUD, C., JOLY, P. and CASTANET, J. 1993. Variation in age structures in a subdivided population of *Triturus cristatus*. *Can. J. Zool.*, 71(9): 1874–1879.
- MIAUD, C., GUYETANT, R. and FABER, H. 2000. Age, size, and growth of the alpine newt, *Triturus alpestris* (Urodela: Salamandridae), at high altitude and a review of life-history trait variation throughout its range. *Herpetologica*, 56(2): 135–144.
- OLGUN, K., MIAUD, C. and GAUTIER, P. 2001. Age, growth, and survivorship in the viviparous salamander *Mertensiella luschni* from southwestern Turkey. *Can. J. Zool.*, 79(9): 1559–1567.
- OLGUN, K., UZUM, N., AVCI, A. et al. 2005. Age, size, growth of the southern crested newt *Triturus karelinii* (Strauch 1870) in a population from Bozdag (Western Turkey). *Amphibia-Reptilia*, 26(4): 223–230.
- STASOFT, Inc – STATISTICA. 2009. Statistica 9.0, USA.
- UZUM, N. 2009. A skeletochronological study of age, growth and longevity in a population of the Caucasian Salamander, *Mertensiella caucasica* (Waga 1876) (Caudata: Salamandridae) from Turkey. *North-West. J. Zool.*, 5(1): 74–78.
- WAGNER, A., SCHABETSBERGER, R., SZTATECSNY, M. et al. 2011. Skeletochronology of phalanges underestimates the true age of long-lived Alpine newts (*Ichthyosaura alpestris*). *Herpetol. J.*, 21(2): 145–148.

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ERRATA

Article first published 30 April 2015 in:

MAKOVICKÝ PETER, KOPECKÝ OLDŘICH, MAKOVICKÝ PAVOL, MATLACH RADEK. 2015. The Using of Skeletochronology as a Screening Method for Age Determination of Alpine Newts (*Mesotriton Alpestris*): a Technical Report. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis*, 63(2): 439–446.

<http://dx.doi.org/10.11118/actaun201563020439>

Dear readers,
after the printing of the aforementioned issue we have discovered some errors that have occurred in this publication.

The corrections are as follows:

The latin name of the Alpine newt should read correctly *Ichthyosaura alpestris*.

In chapter **Ethical Principles of the Study** (p. 440) the text should read correctly:

The study was carried out under research permit number 00444/OH/2009.

Figures 1A and 1B (p. 440) should be correctly:



1: Alpine newt male during the mating season

In chapter **Objective Results** (p. 442) the last sentence should read correctly:

The difference in age determination success rates between groups A and B was statistically significant (2×2 contingency table, $P < 0.001$).

In **Tab. I** (p. 445) the legend should read correctly:

Legend: A: samples consist of last two phalanges of digit, B: samples consist of whole fingers, NS: Number of samples, SES: Successfully examined samples, PE: Percentage expression.

In chapter **Discussion** (p. 445) the last paragraph should read correctly:

Our results show that age determination in populations of newt amphibians should be based on examination of whole digits, but 100% success is not guaranteed. Reading LAGs produce far better accuracy rates than does the examination of the last two phalanges. Regeneration of a digit is faster when only the last two phalanges of an individual are taken. In our experiment, no significant impact on health or behaviour was observed in newts with severed digits, therefore, we do not expect any significant differences between results from group A and those of group B. The regenerative capacity of newt amphibians ensures the healing of wounds and tissue replacement within one year of sampling. Therefore, we recommend sampling whole fingers in future newt amphibian age determination screening studies.