Comparison of the effectiveness of phalanges *vs.* humeri and femurs to estimate lizard age with skeletochronology

M. Comas, S. Reguera, F. J. Zamora–Camacho, H. Salvadó & G. Moreno–Rueda

Comas, M., Reguera, S., Zamora–Camacho, F. J., Salvadó, H. & Moreno–Rueda, G., 2016. Comparison of the effectiveness of phalanges *vs*. humeri and femurs to estimate lizard age with skeletochronology. *Animal Biodiversity and Conservation*, 39.2: 237–240, https://doi.org/10.32800/abc.2016.39.0237

Abstract

Comparison of the effectiveness of phalanges vs*. humeri and femurs to estimate lizard age with skeletochronology.*— Skeletochronology allows estimation of lizard age with a single capture (from a bone), making long–term monitoring unnecessary. Nevertheless, this method often involves the death of the animal to obtain the bone. We tested the reliability of skeletochronology of phalanges (which may be obtained without killing) by comparing the estimated age from femurs and humeri with the age estimated from phalanges. Our results show skeletochronology of phalanges is a reliable method to estimate age in lizards as cross–section readings from all bones studied presented a high correlation and repeatability regardless of the bone chosen. This approach provides an alternative to the killing of lizards for skeletochronology studies.

Key words: Conservation, Demography**,** Growth, Population structure

Resumen

Comparación de la eficacia de las falanges respecto húmeros y fémures para la estimación de la edad en lagartijas mediante esqueletocronología.— La esqueletocronología permite estimar la edad de las lagartijas a partir de una sola captura (tomando una muestra de hueso), lo que hace innecesario un seguimiento a largo plazo. No obstante, los estudios esqueletocronológicos suelen implicar la muerte del animal para obtener el hueso. Nosotros probamos la fiabilidad de la esqueletocronología a partir de falanges (que pueden obtenerse sin matar al animal), comparando la edad estimada a partir de fémures y húmeros con la estimada a partir de falanges. Nuestros resultados muestran que la esqueletocronología de falanges es un método fiable para estimar la edad en lagartijas, ya que las lecturas de las secciones de todos los huesos estudiados presentaron una alta correlación y repetibilidad, independientemente del hueso escogido. Este método es una alternativa a la muerte del animal en estudios de esqueletocronología.

Palabras clave: Conservación, Demografía, Crecimiento, Estructura poblacional

Received: 3 IV 16; Conditional acceptance: 4 V 16; Final acceptance: 11 V 16

Mar Comas, Ecología Integrativa, Estación Biológica de Doñana (EBD–CSIC), Avda. Américo Vespucio s/n., Sevilla 41092, Spain.– Senda Reguera, Francisco J. Zamora–Camacho & Gregorio Moreno–Rueda, Depto. de Zoología, Fac. de Ciencias, Univ. de Granada, 18071 Granada, Spain.– Francisco J. Zamora–Camacho, Dept. of Biological Sciences, Dartmouth College, Hanover, New Hampshire, USA.– Humbert Salvadó, Lab. of Protistology, Depto. de Biologia Animal, Fac. de Biologia, Univ. de Barcelona, Avda. Diagonal 643, 08028 Barcelona, Spain.

Corresponding author: Mar Comas. E–mail: mar.comas@ebd.csic.es

Introduction

Demography studies, which require determination of the age of the animals studied, are fundamental in population ecology, in conservation biology and in wildlife management. However, knowing the age of animals usually requires longitudinal studies, in which animals are captured and marked for long–term monitoring (Sutherland, 1997). Mark–recapture is a useful and precise method but it is time–consuming

ISSN: 1578–665 X
eISSN: 2014–928 X

© 2016 Museu de Ciències Naturals de Barcelona

and may be difficult in elusive species or those with high rates of movement. Moreover, marks may have negative consequences on individuals (Murray & Fuller, 2000). Alternative methods for mark–recapture are few. Nevertheless, some ectotherms with indeterminate growth may present a cyclic growth pattern in hard body structures, corresponding to alternate periods of growth and resting. In this way, age can be estimated by examining cyclic growth patterns in bones (Castanet, 1994).

Femur and humerus are the most commonly used bones in reptile skeletochronology studies (Castanet, 1994). Their use, however, has the disadvantage that individuals must be dead or even specifically killed to obtain the bones, which, besides ethical concerns, precludes future studies or experiments with these specimens for which age has been estimated. Alternatively, researchers could use phalanges (easily obtained by toe clipping) to estimate age (*e.g.*, Dubey et al., 2013). Clipping of one or two toes does not significantly reduce survival (Perry et al., 2011) and has no significant effects on key traits of animal behaviour, such as sprint speed (Husak, 2006). Therefore, estimating individual age with skeletochronology of phalanges would allow experimentation or future studies with animals of known age.

In the present study, we examined the usefulness of phalanges to estimate age in reptiles in comparison with the use of the femurs and humeri. We used a collection of preserved individuals of the lizard *Psammodromus algirus* at the University of Granada (Spain). We estimated the age of these lizards using phalanges, humeri, and femurs, and compared the estimates made by the three types of bones.

Material and methods

Fourteen *Psammodromus algirus* from the scientific collection at the University of Granada were used for the skeletochronological analysis. No lizard was killed for this study. These lizards had died from natural causes while in captivity or by accident while handling during a longstanding study on this species (less than 1% of the lizards handled during the study died). Bodies were preserved in 70% ethanol. Later, long bones (femurs, humeri, and phalanges) were removed and age was estimated by means skeletochronology (Castanet & Smirina, 1990).

We performed several trials to estimate the time needed for decalcification. Finally, the samples were decalcified in 3% nitric acid for at least three hours and 30 minutes. Although we used only one phalanx per lizard, the phalanx number was assigned at random in order to examine whether different phalanges are more or less suitable to estimate age. The basal and middle phalanges of each finger provide better resolution than does the most distal phalanx (Castanet & Smirina, 1990). Decalcified samples were conserved in PBS (phosphate–buffered saline) solution with sucrose (for cryoprotection) for at least 48 h at 4ºC, after which they were sectioned with the freezing microtome.

Glass–slides were treated (prior to use) with a solution of glycerol (5 gr/L) and chromium (III) potassium sulphate (0.5 gr/L). Glycerol was used to improve the placing of the cross–sections on glass–slides. Chromium (III) potassium sulphate was used to improve sample conservation before applying the staining and fixation protocol. Glass slides were submerged for at least 5 minutes in glycerol–chromium (III) potassium sulphate solution and then oven dried for 24 h. The treated slides were then refrigerated until use.

For cross–sections, samples were embedded in gel OCT (optimum cutting temperature) and then sectioned at 10–12 µm for phalanges and 14–30 µm for the longer bones, using a freezing microtome (CM1850 Leica) at the Centre of Scientific Instrumentation of the University of Granada. Cross–sections were stained with Harris hematoxylin for 20 minutes. The excess stain was then rinsed by washing the slides in tap water for 5 minutes. Later, stained sections were dehydrated with an alcohol series (70%, 96%, 100%; 5 min each), washed in xylol for 15 min, fixed with DPX (mounting medium for histology), and mounted on slides.

Cross–sections were made and examined for the presence of LAGs using a light microscope (Leitz Dialux20) at magnifications from 50 to 125x. With a ProgresC3 camera, we took several photographs (a mean of 33.67 per individual) of various representative cross–sections, discarding those in which cuts were unsuitable for examining the LAGs. We selected diaphysis sections in which the size of the medullar cavity was at its minimum and that of the periosteal bone at its maximum (Castanet & Smirina, 1990).

Because inferring age from the number of LAGs requires knowing the annual number of periods of arrested growth for each year, we compared our age estimates with juveniles, whose age was known to be less than a year. Multiple LAGs were found in juveniles in their first period of growth —which were counted as a single year—, while adults usually showed a single additional LAG per year. When various LAGs were found closely together, they were considered as a single LAG in order to avoid overestimation of age. A different LAG pattern depending on age may be explained by juvenile lizards usually being more active and showing more intermittent activity periods than adults (Carretero & Llorente, 1995).

The number of LAGs detected in the periosteal bone was independently counted three times by the same person but on different occasions, always blindly regarding specimen identification (Sagor et al., 1998). Lizards were collected in summer. Therefore, LAGs deposited during previous winter hibernation were discernible from the outer edge of the bone. Consequently, the outer edge of the bone was not counted as a LAG.

A Pearson's correlation matrix was applied for the three age estimates and for each bone type. Repeatability (r_i) was estimated using the formula $r_i = B / (B+W)$, where B is the variance between individuals and W is the variance within individuals, estimated from a one–way ANOVA (Senar, 1999).

Table 1. Number of LAGs (age estimates) recorded from three readings (1, 2, and 3) of different limb bones: phalanx, femur, and humerus, of 14 individuals of *Psammodromus algirus:* ID. Identification code of each lizard.

Tabla 1. Número de LAGs (líneas de detención del crecimiento; indicador de la edad estimada) a partir de tres lecturas (1, 2 y 3) de diferentes huesos de las extremidades: falange, fémur y húmero de 14 individuos de Psammodromus algirus: *ID. Código de identificación de cada lagartija.*

	Phalanx			Femur			Humerus		
ID	1	2	3	1	2	3	1	2	3
10041	4	4	4	4	4	4	4	4	4
10032	3	3	3	3	3	3	3	3	3
10112	4	4	3	4	4	3	4	3	4
10113	3	3	4	3	3	3	3	3	4
10144	3	3	3	3	3	3	3	3	3
10055	5	5	5	5	5	5	5	5	5
10051	5	5	5	5	5	5	5	5	5

	Phalanx			Femur			Humerus		
ID	1	2	3	1	2	3	1	2	3
13104	5	5	5	–	–	–	5	5	5
13151	3	3	3	–	–	–	3	3	3
13155	1	1	1	1	1	1	1	1	1
13156	1	1	1	–	–	–	1	1	1
13158	2	2	2	2	2	2	2	2	2
13119	2	2	2	–	–	–	2	2	2
12132	3	3	3	–	–	–	3	3	3

Results

In all lizards the number of LAGs remained almost identical for all limb bones analysed and between the three independent readings of the sections, independently of the phalanx number used (for phalanx: $r_i = 0.982$, $F_{13, 28} = 112.8$, $P < 0.001$; humerus: $r_i = 0.982$, $F_{13, 27} = 108.7$, $P < 0.001$; femur: $r_i = 0.984$, $F_{9, 18} = 123.1$, $P < 0.001$; all Pearson's $r > 0.93$; table 1). In 12 lizards, age estimations were identical for all three readings and all bones studied (table 1; fig. 1).

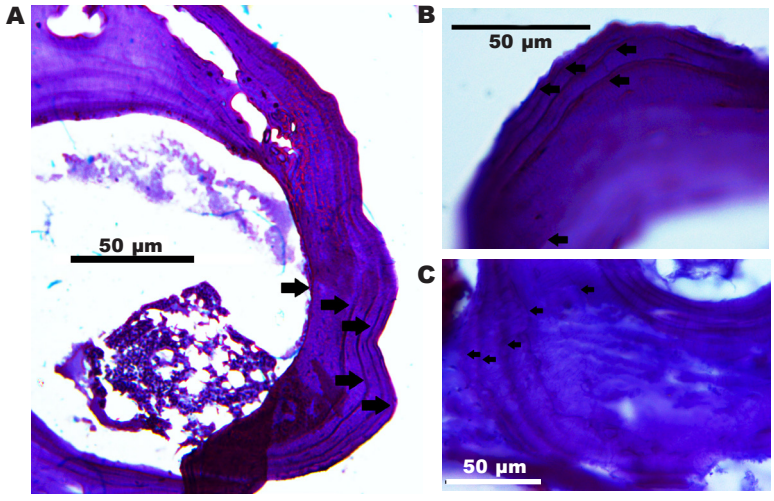

Fig. 1. Cross–sections of the three long bones of the same individual: A. Femur; B. Humerus; and C. Phalanx, where five LAGs can be observed (ID number 10055). First LAGs near the marrow cavity correspond to first year of growth. (Photos: Mar Comas.)

Fig. 1. Secciones transversales de los tres huesos largos del mismo individuo: A. Fémur; B. Húmero; C. Falange, donde pueden observarse cinco LAG (código de identificación: 10055). Las primeras LAG cerca de la cavidad de la médula ósea corresponden al primer año de crecimiento. (Fotografías: Mar Comas.)

Discussion

Age estimated from the number of LAGs in all bones was identical in 85.7% of the lizards. Section readings from different bones presented a high correlation and repeatability, similar to that found in a previous study in *Lacerta schreiberi* (Luís et al., 2003). These findings confirm that skeletochronology of phalanges is a reliable method to estimate age in reptiles. Sections from humeri and phalanges were better than those from femurs; furthermore, in some individuals we were unable to obtain good sections from femurs because they were more difficult to cut. The fact that age was equally well estimated with any phalanx implies that the toe used is irrelevant. Nonetheless, we suggest avoiding clipping toes with special importance for animal movements, such as the longest toe. These results imply that killing lizards is unnecessary to perform skeletochronology, and support the use of phalanges for skeletochronology rather than bones that require the death of the animal, especially in the case of endangered species.

The applications of this non–lethal approach in skeletochronology of phalanges in ecology and conservation biology are numerous and exceed those from skeletochronology implying the death of the specimen. For example, this method allows demographic studies with only one visit to the study area, making long–term studies unnecessary. This may fuel research programmes in areas of difficult access, where mark–recapture method would be ineffective. Skeletochronology of phalanges using this approach is a simple, economical, and ethical way to monitor herpetofauna. The application of skeletochronology of phalanges could also aid studies on age–related physiology, reproduction, and survival in reptiles, reducing disturbance to animals and providing an efficient and cheaper alternative to the mark–recapture approach with less impact on animals (Langkilde & Shine, 2006).

Acknowledgements

We are grateful to Concepción Hernández, from the Centre of Scientific Instrumentation of the University of Granada, for her help with the freezing microtome. David Nesbitt improved the English. Comments by Rodrigo Megía and two anonymous referees improved the manuscript.

References

Carretero, M. A. & Llorente, G. A., 1995. Thermal and temporal patterns of two Mediterranean Lacertidae. In: *Scientia herpetologica* (*Proceedings of the Seventh Ordinary General Meeting of the Societas Europaea Herpetologica*): 213–223 (G. A. LLorente, A. Montori, X. Santos & M. A. Carretero, Eds.). Asociación Herpetológica Española, Barcelona.

Castanet, J., 1994. Age estimation and longevity in reptiles. *Gerontology,* 40: 174–192.

Castanet, J. & Smirina, E. M., 1990. Introduction to the skeletochronological method in amphibians and reptiles. *Annales des Sciences Naturelles – Zoologie et Biologie Animale,* 11: 191–196.

Dubey, S., Sinsch, U., Dehling, M. J., Chevalley, M. & Shine, R., 2013. Population demography of an endangered lizard, the Blue Mountains Water Skink. *BMC Ecology,* 13: 4.

Husak, J. F., 2006. Does survival depend on how fast you can run or how fast you do run? *Functional Ecology,* 20: 1080–1086.

Langkilde, T. & Shine, R., 2006. How much stress do researchers inflict on their study animals? A case study using a scincid lizard, *Eulamprus heatwolei*. *Journal of Experimental Biology*, 209: 1035–1043.

Luís, C., Rebelo, R., Brito, J. C., Godinho, R., Paulo, O. S. & Crespo, E. G., 2003. Age structure in *Lacerta schreiberi* from Portugal. *Amphibia–Reptilia*, 25: 336–343.

Murray, D. L. & Fuller, R., 2000. A critical review of the effects of marking on the biology of vertebrates. In: *Research Techniques in Animal Ecology:* 15–64 (L. Boitani & T. K. Fuller, Eds.). Columbia University Press, New York.

Perry, G., Wallace, M. C., Perry, D., Curzer, H. & Muhlberger, P., 2011. Toe clipping of amphibians and reptiles: science, ethics and the law. *Journal of Herpetology* 45: 547–555.

Sagor, E. S., Ouellet, M., Barten, E. & Green, D. M., 1998. Skeletochronology and geographic variation in age structure in the wood frog, *Rana sylvatica. Journal of Herpetology,* 32: 469–474.

Senar, J. C., 1999. La medición de la repetibilidad y el error de medida. *Etologuía,* 17: 53–64.

Sutherland, W. J., 1997. *Ecological Census Techniques.* Cambridge University Press, Cambridge.