Effectiveness of phalanx skeletochronology to estimate age in living reptiles

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Effectiveness of phalanx skeletochronology to estimate age in living reptiles

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Abstract

Demographic studies are fundamental in population ecology, as well as in conservation biology and wildlife management. However, few methods are available to determine the exact age of animals. Mark-recapture is usually the main method to study demography, but this approach is highly time-consuming and needs long-term monitoring. For species for which recapture is not feasible, this method is not valid. However, in vertebrates with indeterminate growth, such as fish, amphibians, and reptiles, skeletochronology is a method that allows age to be estimated from a bone. Nevertheless, studies of skeletochronology frequently involve the death of the animal to obtain the bone. In the present study, we test the reliability of phalanx skeletochronology, comparing the readings from the most commonly used bones in reptile skeletochronology (femur and humerus) with the age estimated from phalanges. Our results show phalanx skeletochronology to be a reliable method for estimating age in lizards without killing them. Cross-section readings from all bones studied presented a high correlation and repeatability, regardless of the phalanx chosen. These findings imply that, to apply skeletochronology, phalanges must be used instead of other bones that mean the death of the animal, and the killing of lizards for skeletochronology studies is no longer justified. This alternative is especially relevant for endangered species, considering that obtaining a representative sample usually requires a considerable number of individuals.

Keywords: conservation, demography, growth, population structure.

Introduction

Determining the age of animals under study is necessary to understand several evolutionary and ecological processes, such as terminal investment, senescence, life-time reproductive success, longevity, and fitness (Roff, 2002). Demography studies -which require knowing the age of the animals studied- are fundamental in population ecology, as well as in conservation biology and wildlife management (Beiswenger, 1986; Eaton et al., 2005).

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, although it presents a number of limitations. For example, it is highly time-consuming, especially in long-living species. Furthermore, recapture may be difficult in species which have high rates of movement or dispersion, or are elusive. Moreover, marks frequently have negative consequences on individuals, and therefore, this method has an undesirable impact on the populations studied (Murray & Fuller, 2000). If marks alter animal behaviour, physiology, or risk of being depredated or parasitized, conclusions should be drawn with caution (e.g. review in Fair et al., 2010). Moreover, mark-recapture does not solve the problem of the aging of unmarked individuals with unknown growth histories (Leskovar et al., 2006; Sinsch, 2015).

Mark-recapture has few alternative methods. Nevertheless, some ectotherms with indeterminate growth (i.e. which grow throughout their lifespan) may present a cyclic growth pattern in some hard body structures, corresponding to alternate periods of growth and resting, which may be used for determining the age of the individual (e.g. Marschal et al., 2004). For example, the number of layers (growing periods) in fish otoliths and scales or in tortoise scutes are used for determining age (Polat et al., 2001, Rouag et al., 2007).

Similarly, the age of vertebrates with indeterminate growth may be estimated by examining cyclic growth patterns in their bones, by means of skeletochronology (Castanet & Smirina, 1990; Castanet, 1994). Ectotherm vertebrates with indeterminate growth that have resting periods show chromophilic lines in their bones: lines of arrested growth (hereafter, LAGs), which correspond to resting periods, together with broader zones of osteogenesis generated during growing periods (Castanet & Smirina, 1990). When LAGs identify years, age can be estimated, making skeletochronology a useful method for determining age (Castanet, 1994).

The femur and humerus are the most commonly used bones in lizard skeletochronology studies (Castanet & Smirina, 1990; Piantoni et al. 2006; Guarino, 2010, Arakelyan et al., 2013). However, the use of the humerus and femur has the disadvantage that individual must be dead or even killed specifically 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. Sinsch et al., 2002; Grafe et al., 2011; Dubey et al., 2013). Clipping of one or two toes does not significantly reduce survival (Mccarthy & Parris, 2004, Grafe et al. 2011; Guimarães et al., 2014). Moreover, cutting phalanges has no significant effects on key traits of animal behaviour, such as sprint speed (Huey et al., 1990; 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 examine how well the use of phalanges works to estimate age in reptiles in comparison with the use of the femur and humerus. For this, we consulted a collection of preserved individuals of the lizard *Psammodromus algirus*, at the University of Granada (Spain). We estimated the age of these lizards by using phalanges, humerus, and femur, and compared the estimates made by the three types of bones.

Materials and methods

Fourteen *Psammodromus algirus* from the scientific collection of 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 evaluated for age estimation by means skeletochronology (Castanet & Smirina, 1990).

We ran several tests to estimate the necessary time for decalcification. Finally, the samples were decalcified in 3% nitric acid for at least 3 hours and 30 minutes. Although we used only one phalange per lizard, the phalanx number was assigned at random in order to examine whether different phalanges are more or less suitable for estimating age. The basal and middle phalanges of each finger provide better resolution than does the most distal phalange. Decalcified samples were conserved in PBS (phosphate-buffered saline) solution with sucrose (for cryoprotection) for at least 48h at 4ºC, until 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 is used to improve the placing of the cross-sections

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on glass-slides. Chromium (III) potassium sulphate is used to improve sample conservation before applying the staining and fixation protocol. Glass slides were submerged for at least 5 min in glycerol-chromium (III) potassium sulphate solution and then oven dried for 24 h. Finally the treated slides were refrigerated until used.

For cross-sections, samples were embedded in gel O.C.T. (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 min and then the excess stain was rinsed by washing the slides in tap water for 5 min. Later, stained sections were dehydrated with an alcohol series (70%, 96%, 100%; 5 min each), washed in xylol for 15 min, and were finally fixed with DPX (mounting medium for histology) and mounted on slides.

Thereafter, cross-sections were 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 is known -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. Different LAG pattern depending on age may be explained because juvenile lizards usually are more active and show activity periods more intermittent than adults (Rose, 1981).

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 the 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 with the formula $r_i = B/(B+W)$, where B is the variance between individuals and W is the variance within individuals, estimated from an one-way ANOVA (Senar, 1999).

Results

In all lizards the number of LAGs remained almost identical for all limb bones analysed and among the three independent readings of the sections, independently of the phalanx number used (for phalanx: $r_j = 0.982$, $F_{(13, 28)} = 112.8$, $p < 0.001$; humerus: $r_j = 0.982$, $F_{(13, 27)} = 108.7$, $p < 0.001$; femur: $r_j = 0.984$, $F_{(9, 18)} = 123.1$, $p < 0.001$; 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). A lizard (ID number 10113) showed one extra ring in two of the readings, one of the phalanx and other of the humerus (Table 1). In another lizard (ID number 10112), the readings did not completely coincide for one year (Table 1).

Discussion

We found that age -estimated from the number of LAGs in all bones studied- was identical in the 85.7% of the lizards studied, confirming that phalanx skeletochronology is a reliable method to estimate age in living reptiles, as found in amphibians (Kumbar & Pancharatna, 2001). Our results show that section readings from different bones presented high correlation and repeatability. However, sections from humeri and phalanges were better than those from femurs, and even in some individuals it was not possible to obtain good sections from femurs because were more difficult to cut.

In the case of phalanges, the results were equally reliable irrespective of the phalanx used. These results imply that phalanx skeletochronology should be used instead of skeletochronology with other bones that require the death of the animal, especially in the case of endangered species. Moreover, it should be noted that toe clipping with proper disinfection does not decrease survival (Mccarthy & Parris, 2004; Grafe et al., 2011; Guimarães et al., 2014). The fact that age was equally well estimated with any phalanx number implies that the toe used is irrelevant. Nonetheless, we suggest avoiding clipping toes with special importance for animal movements, such as the longest toe.

The applications of phalanx skeletochronology in ecology and conservation biology are numerous. It 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. For example, we can estimate the conservation status of lizards in isolated zones difficult to access with regular visits in which we can collect a sample of phalanges. Changes in the demographic pyramid may indicate lack of turnover in the population, and therefore, the decline of that population (Skalski et al., 2010). In this way, phalanx skeletochronology allows an easy, economic, and ethical way to monitor herpetofauna.

In evolutionary ecology, the study of life history is a central issue (Roff, 2002). Studies on senescence, for example, need to know the age of animals. Therefore, different techniques have been developed to estimate the age of animals when mark-recapture is not available (Guerin, 2004). Despite this, studies on senescence in reptiles are scarce (Patnaik, 1994). For example, Nussey et al., (2013) found only 7 studies showing senescence in reptiles (vs. 149 in birds and 165 in mammals): 2 turtles, 1 snake, 1 skink, and 1 lizard (*Zootoca vivipara*, with 3 studies). Skinks and lizards are appropriate for phalanx skeletochronology, but the four studies on skinks and lizards were based on mark-recapture (Ronce et al., 1998; Richard et al., 2005; Isaksson et al., 2011; Massot et al., 2011), with the consequent expenditure of time and money, as well as the disturbance caused to the animals studied. Therefore, the application of phalanx skeletochronology could aid studies on age-related physiology, reproduction, survival, etc. in reptiles with a reduction in costs and disturbance to animals, thereby providing an efficient and cheap alternative to the mark-recapture approach, in addition to having less impact on animals.

In conclusion, our findings imply that killing lizards to do skeletochronology is no longer justified. Phalanx skeletochronology allows the age estimation of lizards, with numerous useful applications in demographic studies.

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

ID number	Phalanx			Femur			Humerus		
	1st reading	2nd reading	3th reading	1st reading	2nd reading	3th reading	1st reading	2nd reading	3th reading
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
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

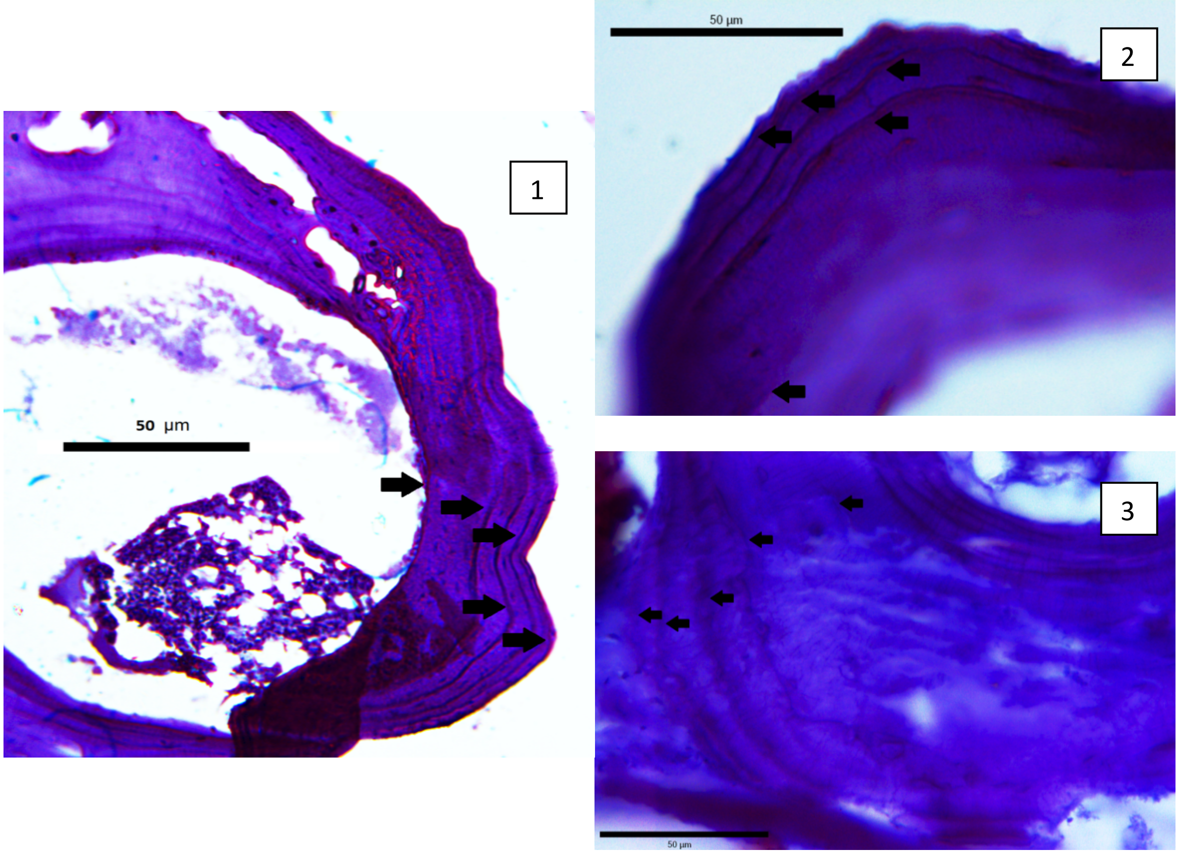

Figure 1. The figures show cross-sections of different long bones of the same individual (femur [1], humerus [2], and phalanx [3]), where 5 LAGs can be observed (ID number 10055). Photo credit: Mar Comas