

Annular bone growth in phalanges of five Neotropical Harlequin Frogs (Anura: Bufonidae: *Atelopus*)

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Abstract

Annular bone growth in phalanges of five Neotropical Harlequin Frogs (Anura: Bufonidae: *Atelopus*). Skeletochronological studies were conducted on museum specimens representing five species of the highly threatened Neotropical genus *Atelopus* (Bufonidae). We detected annular bone growth (expressed as lines of arrested growth [LAGs]) patterns in each species, and this might provide insight to understand demographic constituency in future studies. In four of the five species under consideration, LAG counts in fore and hind limb bone occurred in a 1:1 ratio, indicating that bone growth was consistent within each individual. The use of skeletochronology in understanding historic and existing populations of *Atelopus* might assist *in situ* and *ex situ* population managers in making informed strategic conservation plans.

Keywords: age estimation, demographic constituency, lines of arrested growth, skeletochronology.

Resumo

Crescimento do osso anular de falanges de cinco espécies de anuros neotropicais (Anura: Bufonidae: *Atelopus*). Estudos de esqueletocronologia foram realizados em amostras representativas de cinco espécies do gênero Neotropical altamente ameaçado *Atelopus* (Bufonidae). Detectamos crescimento ósseo anular (expresso em linhas de crescimento) em todas as espécies, o que pode oferecer subsídios para entender a estrutura demográfica em estudos futuros. Em quatro das cinco espécies consideradas, as contagens das linhas de crescimento nos ossos dos membros anteriores e posteriores ocorreram em uma proporção de 1:1, indicando que o crescimento do osso foi consistente dentro de cada indivíduo. O uso da esqueletocronologia na compreensão das populações históricas e atuais de *Atelopus* pode auxiliar os profissionais que trabalham com manejo *in situ* e *ex situ* no delineamento de planos de conservação estratégicos bem-embasados.

Palavras-chave: esqueletocronologia, estimativa de idade, estrutura demográfica, linhas de crescimento.

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Introduction

Studies on demography and longevity constitute a disproportionately small number of works on amphibians (Duellman and Trueb 1986), even of “common” species. Halliday and Verrell (1988) considered two techniques reliable for estimating age in amphibians and reptiles: capture-mark-recapture, and skeletochronology. Skeletochronology involves visualizing and counting annular lines of arrested growth, or “LAGs” (located between bone growth areas) from cross-sectioned diaphyses of long bones such as femora or phalanges (Halliday and Verrell 1988, Castanet and Smirina 1990). Skeletochronological studies could be important in understanding historic demographic constituencies from populations of currently endangered species. Museum collections may hold crucial age/longevity information in these species from when originally collected under prior healthy population statuses. This would appear to be the case in the genus *Atelopus*, where many museum collections hold scores of specimens taken from individual locations at a particular point in history (pers. obs. from multiple well-known and substantial collections in the United States).

A central, and now widely demonstrated and accepted, assumption of skeletochronology is that seasonal dormancy or inactivity leads to LAG formation. Thus, the technique is suitable for age estimation of individual amphibians and reptiles from populations occurring in seasonal climates (Halliday and Verrell 1988, Morrison *et al.* 2004, Matsuki and Matsui 2009). Skeletochronology was first applied widely to anurans occurring in seasonal climates in Europe (Hemelaar 1985, Castanet and Smirina 1990, Castanet *et al.* 1993, Smirina 1994) and North America (Bastien and LeClair 1992, Sagor *et al.* 1998, Redmer 2002). Skeletochronology may be used as an effective non-lethal technique to sample age of anuran populations at chosen or successive points in time, thus, demographic parameters (such as age of reproductive adults)

may be tracked over a period of years. Skeletochronology has been increasingly used to assess individual growth and age structure within populations of amphibians from tropical or subtropical areas (e.g., Guarino *et al.* 1998, Pancharatna *et al.* 2000, Khonsue *et al.* 2000a, b, 2001, Kumbar and Pancharatna 2001, Morrison *et al.* 2004, Lai *et al.* 2005). In these tropical studies it was sometimes assumed that dry/wet seasonality serves as the climatological cause for haematoxylinophilic lines of arrested growth. Recent researchers have found that in a seasonal habitat the LAGs formation may be under genetic control rather than seasonal (Marangoni *et al.* 2009). Others have found that in yearly stable environments, LAGs could be irregular or altogether absent (Kusrini and Alford 2006, Tessa *et al.* 2007).

Our primary objective was to determine whether skeletochronology could be a useful method for estimating age in five species of *Atelopus* (*A. chiriquiensis*, *A. peruensis*, *A. zeteki*, *A. lozanoi*, and one species not yet described). In this genus, most species are threatened or endangered with extinction from habitat fragmentation and degradation, over-collection and especially from chytridiomycosis (La Marca *et al.* 2005). Many species like *A. senex* (Costa Rica), *A. chiriquiensis* (Panama and Costa Rica), *A. lozanoi* (Colombia), *A. peruensis* (Peru), and *A. ignescens* (Ecuador) are already presumed to be extinct (La Marca and Reinthaler 1991, La Marca *et al.* 2005, J. Lynch pers. com.). A few others (e.g., *A. zeteki*) are presumed extinct in the wild, but are sustained through ongoing *ex situ* conservation efforts, (e.g., captive breeding in zoological facilities) (Gagliardo *et al.* 2008, Mendelson 2011). Due to the critically imperiled status of much of this genus, skeletochronological analysis of amphibian tissues from museum collections may provide some insights into historic demographic constituency of populations, species, and could also assist in planning or management of captive populations, as well as founder wild populations if reintroduction is attempted in the future.

If the primary objective could be met, then the secondary objective of this study was to provide evidence in the literature to which researchers and museums could refer, to make the case for wider, yet minimally destructive sampling of *Atelopus*. This would help conservation and captive managers better assess current population status and constituency by comparing to historic ones.

We examined histologically prepared cross-sectioned and stained *Atelopus* phalanges in order to determine whether annular growth is detectable in this genus. We also attempted to determine if periodical growth rings and LAGs are present in a one-to-one ratio in penultimate phalanges from toes and fingers.

Materials and Methods

Species

Histological sectioning was conducted on preserved specimens of five Neotropical species of *Atelopus*: (1) *A. chiriquiensis* (2:7; $N = 9$) from the highland cloud forests (2,200 m asl) of western Panama, *A. peruensis* (6:8; $N = 14$) from the puna and sub-puna northern Peruvian Andes (2,800–4,200 m asl; study specimen elevation unknown), *A. zeteki* (2:2; $N = 4$) from the low to middle elevation moist forests (1,000 m) of central Panama, *A. lozanoi* (3:2; $N = 5$) from the high elevation páramo (3,000–3,300 m asl) of Parque Nacional Nacional Chingaza, Colombia, and *Atelopus* sp. (7:0; $N = 7$) (Nusagandi), from the lowland rain forest (600 m asl) of Parque Nacional Nusagandi, Kuna Yala, Panama. The SVL for each specimen was taken using a Fisher Scientific digital caliper (model # 14-648-17). Considering the highly threatened status of *Atelopus* species throughout their range, a very limited number of specimens were allowed for destructive sampling from the Museum of Biological Diversity at the Ohio State University (*A. peruensis*) in Columbus and the Field Museum of Natural History (*A. zeteki*) in Chicago. Specimens of *Atelopus* sp. (Nusagandi),

A. lozanoi, and *A. chiriquiensis* were obtained from prior studies under natural field conditions by EDL and Carlos Navas in 1994 and 1995 (see Lindquist and Swihart 1997, Lindquist *et al.* 1998). Unfortunately, only male specimens of *Atelopus* sp. from Parque Nacional Nusagandi were available. The *A. zeteki* specimens came from specimens of limited information from the Field Museum of Natural History. Specimens used for this study were legally obtained through field collection and museum collections.

Histology

One finger and one fourth toe were amputated from each specimen. Digits were fixed for a minimum of 24 hr in 10% formalin, rinsed 12 hr in tap water, and stored in 70% ethanol. The penultimate phalanx from each digit was decalcified for 24 hr in Kristensen's formic acid solution, and then rinsed for 24 hr in running tap water. Phalanges were automatically cleared and stained (V.I.P. Tissue-Tek® Machine), then embedded in small paraffin blocks. The tissues were sectioned to 10 μm with a rotary microtome. Mid-diaphyseal cross sections were mounted on microscope slides with adhesive gelatin. Tissues were dehydrated with a series of alcohol steps, stained for 12 minutes with Shandon® Instant Regressive Hematoxylin, and brought to water, at which time they were covered with Permount® mounting medium and glass coverslips.

Skeletochronological Evaluation

Cross sections were visualized with a Nikon Eclipse E600 stereoscopic microscope. For each sample, we individually attempted to count periosteal and endosteal LAGs, and noted if there appeared to be bone resorption or remodeling. When resorption and/or remodeling were noted, a count of visible LAGs were provided and would present a conservative estimate of age if prior LAGs were obliterated (see Wagner *et al.* 2011). Because *Atelopus* phalanges are very small, and endosteal LAGs

often were compact, all age estimates were based only on counted periosteal LAGs. We compared our findings after evaluating each sample, and if there was disagreement we re-evaluated the samples a second time. If after the second evaluation disagreement still occurred over the evaluation, the sample would have been eliminated from the study, but this did not happen.

Results

Snout-vent length ranges for each species/sex are as follows: *Atelopus chiriquiensis* ♂ = 31.6–33.3 mm ($N = 2$) and ♀ = 37.3–46.1 mm ($N = 7$), *A. peruensis* ♂ = 33.6–45.5 mm ($N = 6$) and ♀ = 42.1–44.71 mm ($N = 8$), *Atelopus lozanoi* ♂ = 28.0–29.3 mm ($N = 3$) and ♀ = 38.7–46.2 mm ($N = 2$), *Atelopus* sp. (Nusagandi) ♂ = 25.1–30.3 mm ($N = 7$), and *Atelopus zeteki* ♂ = 35.6–37.6 mm ($N = 2$) and ♀ = 47.0 mm ($N = 2$; one specimen missing head and hence, no SVL). All of these specimens fall in adult size ranges for each species (Gray and Canatella 1985, Lötters 1996, Osorno-Muñoz *et al.* 2001, Savage 2002, pers. obs.). As in all known *Atelopus* species, we witnessed sexual dimorphism in size with females being larger (Gray and Canatella 1985, Lötters 1996, Osorno-Muñoz *et al.* 2001, Savage 2002, pers. obs.). Females in *A. peruensis* and *A. zeteki* were all gravid upon inspection, but lack specific collection data (i.e. date, temperature, etc.).

We found that each of five species of *Atelopus* in this study possessed LAGs in periosteal and endosteal phalangeal bone (see Figure 1). However, *A. lozanoi* exhibited the least defined LAGs of those observed. Periosteal bone exhibited the best defined LAGs in all specimens observed. Likewise the number of periosteal LAGs was in agreement between fore and hind limb phalanges. Table 1 provides minimum, maximum and average number of periosteal LAGs for each species. In 15 specimens among the five species studied in this genus (38.5%), we observed outward endosteal

bone growth extensive enough that it likely obliterated innermost LAGs in periosteal bone. For two species, *Atelopus* sp. (Nusagandi) and *A. zeteki*, no remodeling nor resorption was observed. In other species (*A. peruensis* and *A. chiriquiensis*), this problem was much more significant (see Table 1). In these latter cases skeletochronology would lead to underestimation of the number of countable LAGs, and hence would not be an appropriate aging tool. This problem has also been reported by other skeletochronological studies of amphibians (Eden *et al.* 2007, Wagner *et al.* 2011). The number of LAGs detected in phalanges correlated on a 1:1 ratio between fingers and toes for four of the five species used in this study, and is an indication that the influences on annular growth and resorption/remodeling rates are approximately the same in long bones (see Table 1). A correlation analysis seeking SVL-LAG agreement was not calculated due to the low statistical power afforded by the low sample sizes used in this study.

Discussion

Presence of periosteal LAGs and annular bone growth indicates that age could be estimated in some species of *Atelopus*. The finding of LAGs in phalanges of *Atelopus* is consistent with those of other skeletochronology studies in tropical zones (Guarino *et al.* 1998, Khonsue *et al.* 2000a, b, Pancharatna *et al.* 2000, Kumbhar and Pancharatna 2001, 2002). However, while seasonality in food resources, temperature and rainfall can be demonstrated in tropical or subtropical areas, especially along elevation and moisture gradients (Karr and Freemark 1983, Guarino *et al.* 1998), the processes that lead to LAG formation in anurans is yet unresolved. Kumbhar and Pancharatna (2002) unsuccessfully attempted to correlate LAG formation with the passage of one year in frogs maintained under laboratory conditions. Morrison *et al.* (2004) demonstrated that in *Litoria chloris* and *L. pearsoniana* (Hylidae) and *Mixophyes fleayi* (Myobatrachidae),

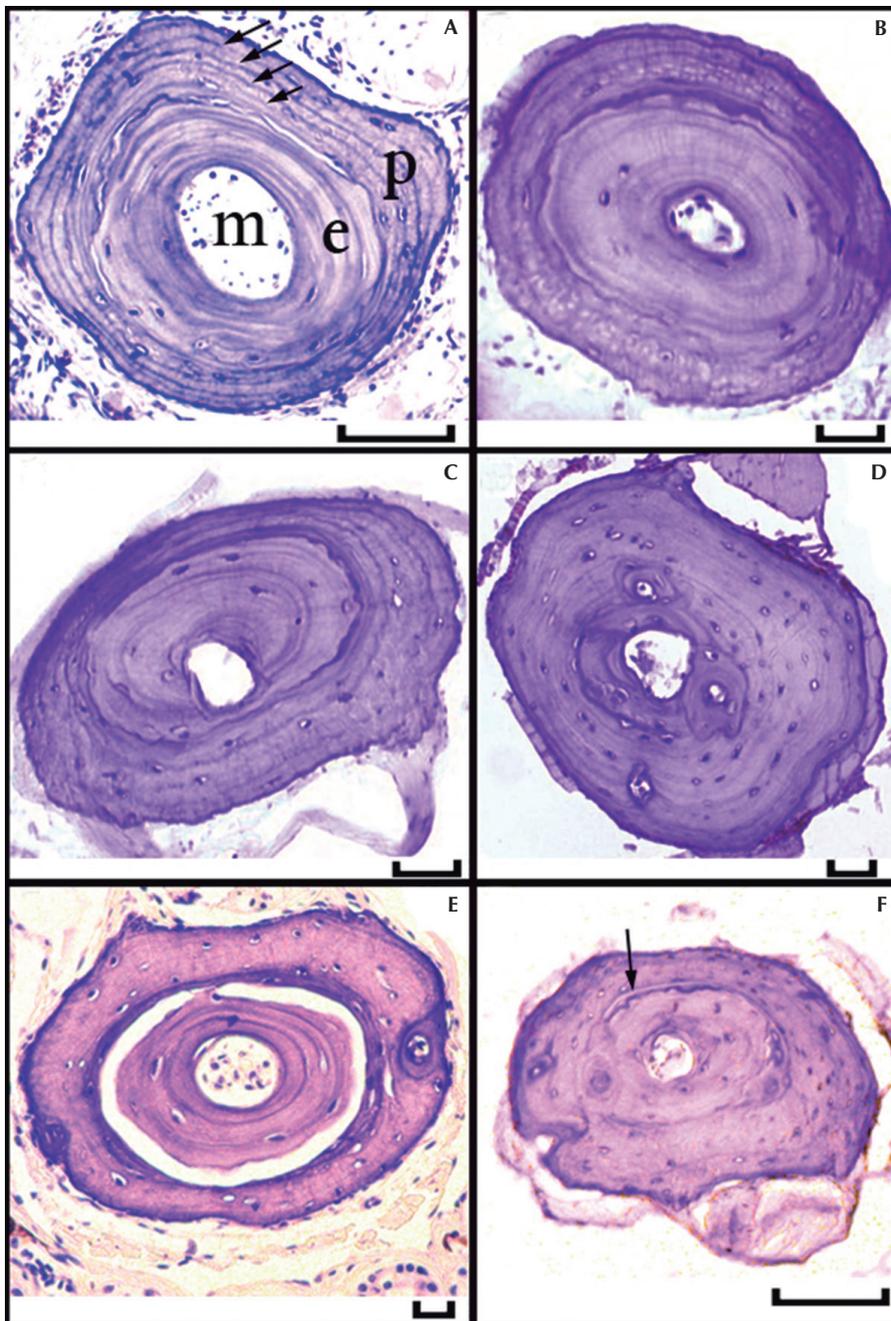


Figure 1. Photomicrographs showing periosteal and endosteal lines of arrested growth in phalanges of (A) *Atelopus peruvensis*, indicating LAGs (arrows), endosteal (e) and periosteal (p) bone, and the medullary cavity (m); (B) *Atelopus* sp. (Nusagandi, Panama); (C) *A. lozanoi*; (D) *A. chiriquiensis*; and (E) *A. zeteki*; (F) an example of remodeling in *A. chiriquiensis* (arrow). Photomicrographs were taken using a Nikon Coolpix 990 coupled to a Nikon Eclipse E600 binocular microscope. Bars in the lower right of each micrograph represent 0.1 mm.

Table 1. Descriptive statistics on lines of arrested growth, estimated ages, finger-toe agreement, resorption/remodeling observed by *Atelopus* species. Periosteal LAGs (reported here) proved more reliable than endosteal LAGs, which often were resorbed or remodeled.

Species	Specimens (N)	Periosteal LAGs (mean \pm sd)	Estimated Age Range (min–max)	Finger/Toe Agreement (by specimen)	Resorption (%) / Remodeling Evident
<i>A. chiriquiensis</i>	9	3.33 \pm 0.71	2–4	88.88% (8/9)	66.67 (6/9)
<i>A. peruensis</i>	14	4.54 \pm 0.97	3–6	85.71% (12/14)	57.14 (8/14)
<i>A. lozanoi</i>	5	3.60 \pm 0.55	3–4	20% (1/5)	20.00 (1/5)
<i>A. sp.</i> (Nusagandi)	7	2.71 \pm 0.49	2–3	100% (7/7)	0 (0/7)
<i>A. zeteki</i>	4	3 \pm 0.0	3	100% (4/4)	0 (0/4)

all subtropical species, LAGs in individuals could be correlated to the passage of a year, making skeletochronology a successful and useful tool. However, these authors found that this technique proved unsuccessful in the *Litoria lesueuri*, a low elevation, subtropical species where the activity season was prolonged. A similar trend was also observed by Tessa *et al.* (2007) for *Dyscophus antongilii* found at sea level. Considering the numerous differences in environmental factors (elevation, seasonality, rainfall, prey availability, etc.) in the five species of *Atelopus* studied here, no clear causation for the presence of LAGs can be inferred at this time. However, in species such as *Atelopus sp.* (Nusagandi) and *A. zeteki* where 100% finger-toe LAG count correlation and 0% resorption and remodeling was observed, skeletochronology would seem to provide some promise for successful use in age estimation and population studies and management. At sites where *Atelopus sp.* (Nusagandi) and *A. zeteki* occur (or occurred), there is substantial seasonal variation in rainfall and temperature, and this could provide a basis for differences in the activity cycles, arthropod prey availability, and perhaps likewise, bone growth.

Comparison of LAGs in different years should be done in a greater number of taxa, and in particular, additional work (mark-recapture studies) would be helpful on the genus *Atelopus*

to calibrate the formation of LAGs to verify a 1:1 correspondence of LAG to seasonal or annual cycles. Assuming it is still possible to conduct mark-recapture studies on any of the remaining *Atelopus* species, these could provide a more direct calibration of annular bone growth and LAG formation, rendering skeletochronology to become an important method for estimating age in at least some *Atelopus* species. Considering a 1:1 LAG to annual cycle in *Atelopus sp.* and *A. zeteki*, this would indicate a surprisingly low longevity in these species, adding to the complexity and urgency of informed management strategies and conservation efforts (see Table 1). The majority of congeneric species are critically endangered, so an aging technique that is invasive in nature would not be applicable or advisable in demographic studies on these species. Due to the presence and prominence of intraspecific semaphoring (visual signaling) behavior in many *Atelopus* species (e.g., *A. zeteki*, *A. chiriquiensis*, and *A. varius*), caution should be used so as not to compromise individuals' ability to successfully communicate (Lindquist and Hetherington 1996, 1998, pers. obs.).

However, since there are many *Atelopus* specimens in museum collections, studies could be conducted using these instead of wild-caught specimens. The same case was made for many Madagascan species, such as *Scaphiophryne*

gottlebei and several of *Mantella* (Guarino *et al.* 2010, Jovanovic and Vences 2010). Likewise, studies on bone growth and age calibration among successful captive breeding programs at various zoos and research facilities could be informative, especially if they are paired with studies on specimens currently held in museums. Inferences based on historic demography on some species that are well represented in museum collections could be useful for planning and management (e.g., modeling or determining age/sex ratios) of populations held *ex situ* for conservation purposes, or for assessing population health for *in situ* management for extant species. Information on longevity also could be useful in determining when search efforts might be economically infeasible for species that are thought to have disappeared completely.

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