

Accuracy Assessment of Skeletochronology in the Arizona Tiger Salamander (*Ambystoma tigrinum nebulosum*)

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Skeletochronology is the most commonly used tool for evaluating the age of amphibians and reptiles. However, the accuracy of skeletochronology is questionable because few studies have utilized individuals of known age to calibrate their results. In this study, the accuracy of skeletochronology was assessed using individual Arizona Tiger Salamanders (*Ambystoma tigrinum nebulosum*) that were of known ages that varied from 2–15 years. Cross-sections of salamander toes were analyzed to compare the number of LAG (lines of arrested growth) to the actual age of known cohorts. Our results suggest that skeletochronology was an inaccurate tool for estimating age in this population of *A. tigrinum nebulosum* because it grossly underestimated age. The high intensity of endosteal resorption coupled with rapprochement of the most peripheral LAG may have produced these results. Our research indicates that skeletochronological studies that do not use individuals of known age for calibration may underestimate age.

DATA on the age of individuals is often critical for understanding the ecology of populations, including the construction of life tables and evaluation of age structure (Krebs, 2001). The results of a life table, including survivorship, fecundity, and net reproductive rate, and the shape of age-structure diagrams, are dependent on the accuracy of age classes used in these analyses (Castanet and Smirina, 1990). Because life tables and age structure are vital tools for understanding population dynamics (Patón et al., 1991; Castanet et al., 1996; Krebs, 2001), the accuracy of age estimation is critical for population ecologists and conservation biologists. Age estimation is particularly critical for species in which age structure may provide a vital biomonitoring tool, such as in amphibians (Smirina, 1994).

Among amphibian populations, three main methods of age estimation have been used: age and body size correlation, mark and recapture, and skeletochronology. Age and body size correlation is thought to be the least accurate method of age estimation (Halliday and Verrell, 1988). Due to substantial overlap of body size distribution between age classes, the relationship between body size and age is usually quite weak (Hagström, 1977; Halliday and Verrell, 1988; Smirina, 1994). In contrast, mark and recapture data provide exact ages of individuals that were marked at a known time (during their first year), and provide the most accurate data for age analyses. However, because of the difficulty in tagging small, young amphibians, loss of individuals due to migration, high mortality, and the time cost of marking and recapturing hundreds or thousands of individuals, mark and recapture

is considered an inefficient method for aging (Halliday and Verrell, 1988; Smirina, 1994).

Skeletochronology is the most commonly used method for aging amphibians (Caetano, 1990; Castanet and Smirina, 1990; Francillon-Viellet et al., 1990). This technique utilizes stained cross-sections of long bone, which allow visualization of lines of arrested growth (LAG). Studies of a variety of amphibian taxa suggest that each LAG corresponds to an annual resting growth period and that the number of LAG represents the age of the animal (Gibbons and McCarthy, 1983; Caetano and Castanet, 1993). Because LAG are clearly visible in bones such as phalanges, the determination of age only requires the removal of a toe, allowing the animal to be released with minimal trauma (Patón et al., 1991; Miaud et al., 1993). Thus, skeletochronology can be a quick and efficient means of age estimation, requiring minimal field time without sacrificing the animal.

Factors such as double LAG, resorption, and rapprochement, however, could affect age determination via skeletochronology. For example, double LAG can occur as a result of a double cycle of annual activity (hibernation and aestivation; Caetano, 1990). During the process of bone development, new layers of bone are formed, and other layers, which were previously formed, are sometimes resorbed into the endosteum (Smirina, 1994). Endosteal resorption may occur as a result of calcium being extracted from the bone and utilized for important physiological functions of the amphibian such as blood coagulation and capillary integrity (Moore, 1964). As a result, resorption may destroy one or more of the inner growth rings, resulting in an underestimation of age (Forester and Lykens, 1991). Rapproche-

ment occurs in older individuals when LAG becomes more tightly compacted as a result of reduced growth rate, creating difficulty in accurately deducing the number of LAG (Castanet and Smirina, 1990; Francillon-Viellet et al., 1990).

Although these three factors can impede age estimates, it has been suggested that their effect on the accuracy of skeletochronology can be reduced by using individuals of known age for calibration (Castanet and Smirina, 1990; Smirina, 1994). Although several studies have supported the assumption that one LAG equals one year by following individual amphibians in the field for 1–3 years (Gibbons and McCarthy, 1983; Francillon-Viellet et al., 1990; Buhlmann and Mitchell, 2000) or under laboratory conditions for 1–4 years (Francillon, 1979; Kumbar and Pancharatna, 2002, 2004), only one study (Tejedo et al., 1997), to the best of our knowledge, has assessed the accuracy of skeletochronology using individuals of known age. Previous studies that did not utilize known-age individuals thus can not assess the number of LAG lost to resorption or rapprochement during life history events, such as metamorphosis, maturation, or aging (Castanet et al., 1996; Forester and Lykens, 1991).

Comparing the accuracy of skeletochronological estimates to actual age is critical to evaluate its usefulness for age estimation, and because numerous studies have already assumed that it is an accurate method (Rebelo and Caetano, 1995; Wake and Castanet, 1995; Caetano and Leclair, 1996). Here we test the hypothesis that skeletochronology is an accurate, reliable method of age determination by sampling a population of Arizona Tiger Salamanders, *Ambystoma tigrinum nebulosum*, of known age. Sex, growth rate, and body condition could influence bone development and growth, which could subsequently impede the accuracy of skeletochronology. Thus, we tested the hypothesis that female salamanders would have less accurate estimates than males due to greater energy allocation to reproduction rather than development. Finally, we tested the hypothesis that salamanders with slower growth and poorer body condition would have less accurate estimates than those experiencing faster growth or that were in better body condition, because of increased available energy for bone development in the former group.

MATERIALS AND METHODS

The Tiger Salamander, *Ambystoma tigrinum*, is a geographically widespread, morphologically variable species (Collins et al., 1993). The

Arizona Tiger Salamander, *Ambystoma tigrinum nebulosum*, is native to the western United States, from western Colorado and Utah to south-central New Mexico and central Arizona (Behler and King, 1979). The population studied here is located within The Nature Conservancy's Mexican Cut Nature Preserve (3,640 m²) in south-central Colorado. The within-site variation in habitats that comprise this area, coupled with autumnal, semi-permanent, and permanent ponds, creates an ideal environment for studying salamander life history (Wissinger and Whiteman, 1992; Whiteman and Wissinger, in press). The Mexican Cut salamander population is facultatively paedomorphic; depending on environmental conditions, individual larvae either metamorphose into terrestrial adults or retain their aquatic morphology and become mature paedomorphic adults (Semlitsch, 1985; Whiteman, 1994). Both metamorphs and paedomorphs were utilized in this study.

Beginning in 1988, individuals from this population were captured by hand or using dip nets, marked by toe clipping (beginning in 1989), and measured for snout-vent length (SVL) and mass. Cohorts of known-age larvae (denoted by the year of hatching: 1988, 1989, 1990, 1999, 2000, 2001; the population experienced little recruitment in other years [Whiteman and Wissinger, in press]) were clipped once they had reached sufficient size and were followed through metamorphosis and/or maturity as a paedomorphic adult. Thirty-seven salamanders of known age (2–15 years) were captured during July and August 2003 for use in skeletochronology and were also utilized to compare age and body size.

The fourth toe on the right rear foot was excised from each individual and stored in 70% ethanol. Toes were transported to the laboratory at Murray State University, rinsed in de-ionized water, and then decalcified in 3% nitric acid. A cryostat (HM 505 E) was used to section the toes at 20 μ m. Sections were stained using Harris' haematoxylin to create visible LAG (Kumbar and Pancharatna, 2001), which were counted directly from slides using a phase-contrast microscope. At least 12 sections per toe were collected, but only the sections that illustrated the clearest LAG were used. Age was determined using two to three observers who counted LAG independently to prevent any bias, considering the outer perimeter of the bone as a LAG (Patón et al., 1991). Each LAG had to completely circumvent the section of bone to be counted. If there were discrepancies between the number of LAG counted between the first two observers (who counted all specimens), a third observer was

utilized to count the number of LAG. In all cases, observers were blind to the identification of the individual, its actual age, and the counts of other observers. To compare the accuracy of LAG in phalanges versus the humerus, we obtained samples from three individuals that died in this population, and found that in each case the number of LAG matched exactly.

Regression analysis was conducted to determine if there was a relationship between the number of LAG and actual age and between last known SVL and actual age. The potential for differences in these relationships between paedomorphic and metamorphic adults were tested using analysis of covariance. Percent error (difference between actual age and number of LAG divided by actual age) was calculated to determine if a relationship existed between increased age and accuracy.

The 1988 cohort was analyzed in more detail to assess factors that might influence LAG, such as differences in sex, growth rate, and change in body condition among individuals. This cohort was chosen because it had the largest sample size ($n = 20$) and was the oldest available (15 years). To determine if the number of LAG was sex dependent, a U-test was used because of non-normal distributions that could not be controlled with transformation. To assess growth rate, snout-vent length was divided by the age of each individual. The average change in body condition was calculated as:

$$\frac{(\text{Mass}_2/\text{Svl}_2) - (\text{Mass}_1/\text{Svl}_1)}{\text{number of years between measures}}$$

where Svl_1 and Mass_1 are the snout-vent length and mass at first capture and Svl_2 and Mass_2 are the snout-vent length and mass at the most recent capture. The effects of growth rate and change in body condition on the number of LAG were evaluated using simple regression. All statistics were conducted using StatView (SAS Institute, Cary, North Carolina, 1999). When multiple tests were conducted on the same variable, we reduced α based on the number of tests performed (Rice, 1989).

RESULTS

The relationships between the number of LAG and actual age were not statistically different between paedomorphic and metamorphic adults (slope: $F_{1,33} = 0.09$, $P = 0.77$, intercept: $F_{1,33} = 0.04$, $P = 0.84$); therefore, a single regression line was used. A significant positive relationship was evident between the number of LAG and actual age ($F_{1,35} = 98.2$, $P < 0.0001$, $R^2 = 0.74$; Fig. 1A)

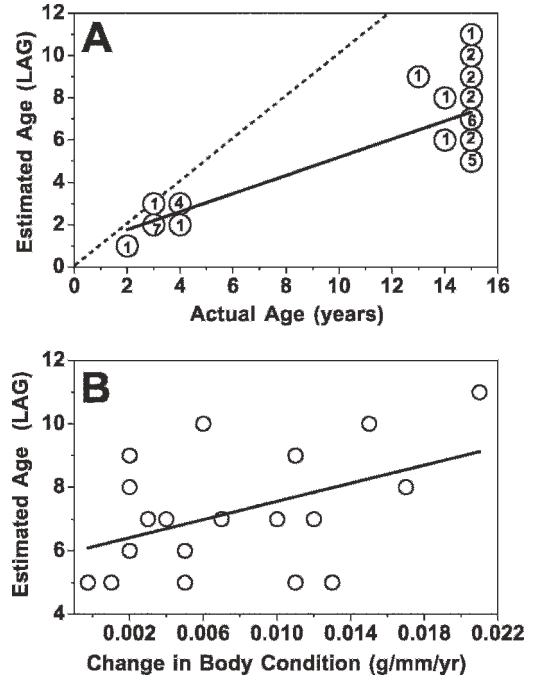


Fig. 1. (A) The relationship between estimated age and actual age of Arizona Tiger Salamanders (*Ambystoma tigrinum nebulosum*). The dashed line represents predicted age estimates assuming a 1:1 correspondence between LAG and age in years. The solid line represents age determined by skeletochronology. The numbers inside circles represent the number of individuals at each point. (B) The relationship between estimated age and change in body condition of 15-year-old individuals.

and was considerably lower than a predicted line of 1 LAG = 1 year. For the oldest age class (15 years), LAG varied from 4–11, although most ranged from 4–6. The percent error increased with age, from 16.5% in 2–4 year olds to 46.7% in 13–15 year olds. There was no significant difference in the number of LAG between males and females from the 1988 cohort ($U = 29.0$, $P = 0.92$). Regression indicated that the number of LAG was not significantly related to growth rate ($F_{1,19} = 0.38$, $P = 0.54$, $R^2 = 0.02$), but there was a weak but significant relationship between number of LAG and change in body condition ($F_{1,19} = 4.83$, $P < 0.0001$, $R^2 = 0.21$, $\alpha = 0.025$; Fig. 1B) among animals from this year class.

Endosteal resorption of the innermost LAG was observed in all individuals, with the most extensive resorption occurring in older individuals greater than 12 years (Fig. 2). Also, rapprochement occurred in the periphery of the periosteal region of some older individuals.

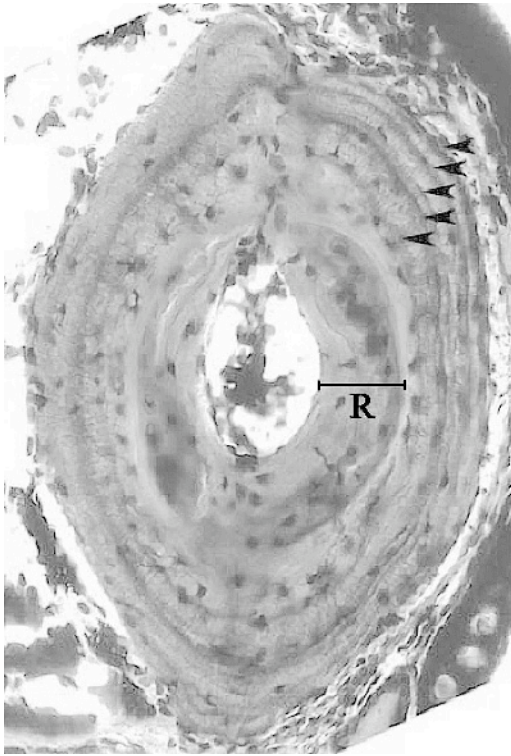


Fig. 2. Cross-section of a 15-year-old individual exhibiting five LAG, indicated by black arrows. R = endosteal resorption of LAG.

DISCUSSION

Skeletochronology is arguably the most common tool used to decipher the age of various amphibian taxa (Halliday and Verrell, 1988; Castanet and Smirina, 1990; Smirina, 1994). Many studies assume that LAG are equivalent to age (Patón et al., 1991; Esteban et al., 1996; Parham et al., 1996), yet without accuracy assessment and subsequent calibration in order to adjust for confounding factors such as resorption and rapprochement, age estimation via skeletochronology could be highly inaccurate (Castanet and Smirina, 1990; Smirina, 1994; Sullivan and Fernandez, 1999).

Our accuracy assessment agrees with this hypothesis; the relationship between the actual regression line and the expected line revealed that the number of LAG is a highly conservative and inaccurate estimate of age for this population (Fig. 1A). The number of LAG in relation to actual age varied by only 0–2 LAG in the younger age classes, but only one of 14 individuals had LAG which equaled its actual age. In the older age classes, all skeletochronological estimates

were inaccurate and varied by 5–11 LAG when compared to actual age (Fig. 1A).

The reduced accuracy of skeletochronology with age found in this study may be the result of extensive resorption that occurred throughout the salamander's life, resulting in a gross underestimate of the age of older individuals (Forester and Lykens, 1991; Sullivan and Fernandez, 1999). Resorption has been observed to be more intense in populations of high altitude (Caetano and Castanet, 1993; Caetano and Leclair, 1999), and our studied population is at the elevational extreme for salamanders (3640 m; Behler and King, 1979). High-elevation wetlands tend to be oligotrophic and may lack important minerals essential for normal growth (Turner et al., 1994). For example, the extensive bone resorption exhibited by this population might be correlated to low levels of calcium in the environment. Calcium, essential for the maintenance of homeostasis, can be extracted from bone tissue and allocated for important metabolic functions (Stiffler, 1993; Wright and Whitaker, 2000). However, calcium levels measured at Mexican Cut (Wissinger and Whiteman, 1992) were within the range of a host of other wetlands that varied in elevation and geographic location (Corn et al., 1989; Schalles et al., 1989; Rowe and Dunson, 1993), suggesting that resorption due to calcium deficiency is no more likely at Mexican Cut than at other localities.

Rapprochement was also apparent in older individuals, resulting in very thin LAG around the peripheral area of the bone section, creating great difficulty in counting the number of LAG. Since aestivation does not occur in this population, the variation in the number of LAG is unlikely a result of double LAG (Caetano and Castanet, 1993), but could be the result of individual differences in rapprochement in relation to changes in body condition (Fig. 1B). Such differences might occur such that those salamanders with the best body condition have the most visible LAG because they are able to allocate more energy into bone growth, which would explain the large variation in LAG among 15 year olds (Fig. 1A). For example, during certain years competition between paedomorphic individuals led to negative growth rates (Whiteman et al., 1996), possibly increasing LAG rapprochement.

The increased percent error in our skeletochronology estimates with age suggests that the factors that influence the accuracy of skeletochronology in this population (resorption, rapprochement) compound with age. As individuals age, there is more time for these factors to affect the number of LAG, leading to less

accurate age estimates. This result suggests that short-term studies using young individuals (Gibbons and McCarthy, 1983; Francillon-Viellet et al., 1990; Buhlmann and Mitchell, 2000) may not be sufficient to properly calibrate skeletochronology, particularly in long-lived species.

Skeletochronology proved to be an unreliable tool for the estimation of age in this particular population of *A. tigrinum nebulosum*. The inaccuracy of skeletochronology in the study population may be the result of a localized effect and may not reflect studies conducted elsewhere. However, this and other studies (Castanet and Smirina, 1990; Sullivan and Fernandez, 1999) suggest that without accuracy assessment it is impossible to ascertain the credibility of this methodology. Some short-term (1–3 year) studies have illustrated that one LAG is equivalent to one year (Rebelo and Caetano, 1995; Buhlmann and Mitchell, 2000; Kumbar and Pancharatna, 2004). These studies, however, did not compare individuals of known age. The only study to use known-age individuals, besides the present study, is Tejedo et al. (1997), which followed seven *Bufo calamita* individuals in the field over three years. Tejedo et al. (1997) found substantial variation in LAG within this sample (2–5 LAG; expected = 3) and concluded that skeletochronology may not be reliable for temperate amphibians. Hemelaar (1985) observed extensive variation in the degree of resorption among *Bufo bufo* populations, even from the same climatological conditions, indicating that age determination requires resorption to be estimated for each population separately. Therefore, age estimates should be reevaluated for those studies that utilize calibration from one population to evaluate the ages of a different study population (Caetano and Castanet, 1993; Caetano and LeClair, 1996; Parham et al., 1996), and future studies should conduct accuracy assessment, calibration, and age estimation using the same population. Our results also suggest that the utilization of extant species in order to calibrate age for extinct species such as tyrannosaurids (Erickson et al., 2004) should also be reevaluated. Thus, skeletochronological studies that do not assess the accuracy of age estimation and utilize calibration as a means of increasing accuracy are of questionable value for understanding demographic and population trends.

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