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## A Skeletochronological Study of Growth and Age in Relation to Adult Size in *Batrachoseps attenuatus*

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**ABSTRACT.** — The age of specimens of the plethodontid salamander *Batrachoseps attenuatus* was estimated using skeletochronology. This species differs from other urodeles in that it develops directly within the egg capsule, with no larval period, and it is active in the winter rather than in the summer. Although lines of arrested growth are somewhat irregular and more difficult to interpret than in other species of salamanders studied, age of most individuals was estimated with a high degree of confidence. Maximum adult size in the population studied was about 50 mm snout-vent length. Specimens larger than 40 mm in length ranged in age from four to eight years. Once sexual maturity is achieved, size is a poor estimator of age in this species.

In amphibians and reptiles in which annual growth is cyclical, with alternating periods of relatively rapid and relatively slow growth, skeletochronology can be used to estimate the age of individual organisms (Castanet et al., 1993).

Cyclical growth results in the production of growth marks in bones and some other hard tissues, and information provided by the annular pattern can be used to obtain accurate estimates of age in amphibians and reptiles (e.g.,

Castanet and Smirina, 1990). Periodicity in growth is revealed in the histology of the bone, with broad, less dense zones being associated with periods of rapid growth, and narrower, more dense zones called annuli (*sensu* Peabody, 1961) being associated with periods of decreased growth rates. These latter periods are manifest in distinctive layers of increased density known as lines of arrested growth (LAG). One broad layer plus one LAG generally constitute a complete cycle of growth, typically annual for amphibians that live in regions with predictable climatic cycles of warm and cold, or wet and dry seasons. However, under certain climatic conditions two LAG may be produced in a single year (Caetano et al., 1985). Studies of marked individual newts (*Triturus cristatus*) of known initial age that extended over several years demonstrated that each LAG corresponds to a winter period of inactivity, and that each broad zone corresponds to the preceding period of summer activity (e.g., Francillon, 1979, 1980; Caetano and Castanet, 1987). Because of these experimental demonstrations of the utility of the method, skeletochronology has become widely used by ecologists studying salamander populations (e.g., Smirina and Sofianidu, 1985; Verrell and Francillon, 1986; Houck and Francillon-Vieillot, 1988; Guyétant et al., 1992; Francillon-Vieillot et al., 1990; Montori, 1990; Flageole and Leclair, 1992; Miaud et al., 1993).

When conducting comparative studies of the relation of phylogeny and ontogeny, the relation between size and age is of special importance (Alberch et al., 1979). When age is not determined, size is sometimes substituted, because during the main growth phase there is a general correlation between size and age. However, following attainment of sexual maturity the correlation of size and age is suspect, and may be entirely absent. The present study was undertaken as a component of comparative studies of the ontogeny of salamanders that lack a larval stage and undergo encapsulated development, hatching from the egg as miniature adults (there is no metamorphosis). Prior skeletochronological studies have been biased toward members of the families Salamandridae and Ambystomatidae. The species studied have a biphasic life cycle with a larval period usually of one year or less and a discrete metamorphosis. We studied a population of the California slender salamander, *Batrachoseps attenuatus*, a miniaturized species that develops directly within the egg capsule. This species has an activity pattern very different than that of European newts and American ambystomatids that have been the subject of skeletochronological studies published to date. *Batrachoseps* is active only during the winter rainy period, a time of

relatively low temperature, and is inactive during the hot, dry summer. It is important to learn if standard skeletochronological techniques can be applied in such situations, because over 50% of the species of salamanders are direct developing and most of these occur in tropical environments which have patterns of seasonality (typically wet-dry) that differ from the north temperate (cold-warm) environments of salamanders studied to date.

#### MATERIALS AND METHODS

Salamanders were collected from the field in Berkeley, Alameda Co., California, in January, 1988, and were kept in the laboratory at seasonal light conditions and 14 C from two to eleven months as a component of a study of tooth replacement. When sacrificed, specimens were fixed in 10% buffered formalin and then transferred to 70% ethanol for storage. The specimens studied were measured (from snout to posterior end of vent, SVL) and sexed. In addition to 23 adults, two small specimens ca. 12 mm SVL and in their first year of life were examined. Specimens were prepared for skeletochronological study following standard techniques (Caetano et al., 1985). Frozen sections of demineralized bones (femur and humerus) were cut at 15 micrometers and stained in Ehrlich's hematoxylin. Sections from the midshaft of the diaphysis were mounted in an aquamounting medium, examined by normal micrography and photographed at a constant magnification for analysis of age and growth. The perimeter of each LAG was measured with a curvimeter. Initial counts of LAG were done separately ("blindly") by each author, and results were then compared and final ages assigned. There was high general agreement, but a few instances required consultation and re-evaluation. Finally, both authors agreed on all assignments.

#### RESULTS

*Histology.*—As in other salamanders (Caetano et al., 1985), the humerus of *B. attenuatus* had undergone less remodeling than the femur, and was more suitable for skeletochronological analysis. However, in two individuals the femur was more suitable than the humerus, which had been completely remodeled. Neither bone could be used for one individual, which was eliminated from the study. In one instance skeletochronological analysis was not possible because of difficulties in determining the growth marks late in life, but minimal estimates of age were obtained. In all individuals, histology of the midshaft of the humeral diaphysis was relatively uniform and was used for interindividual comparison.

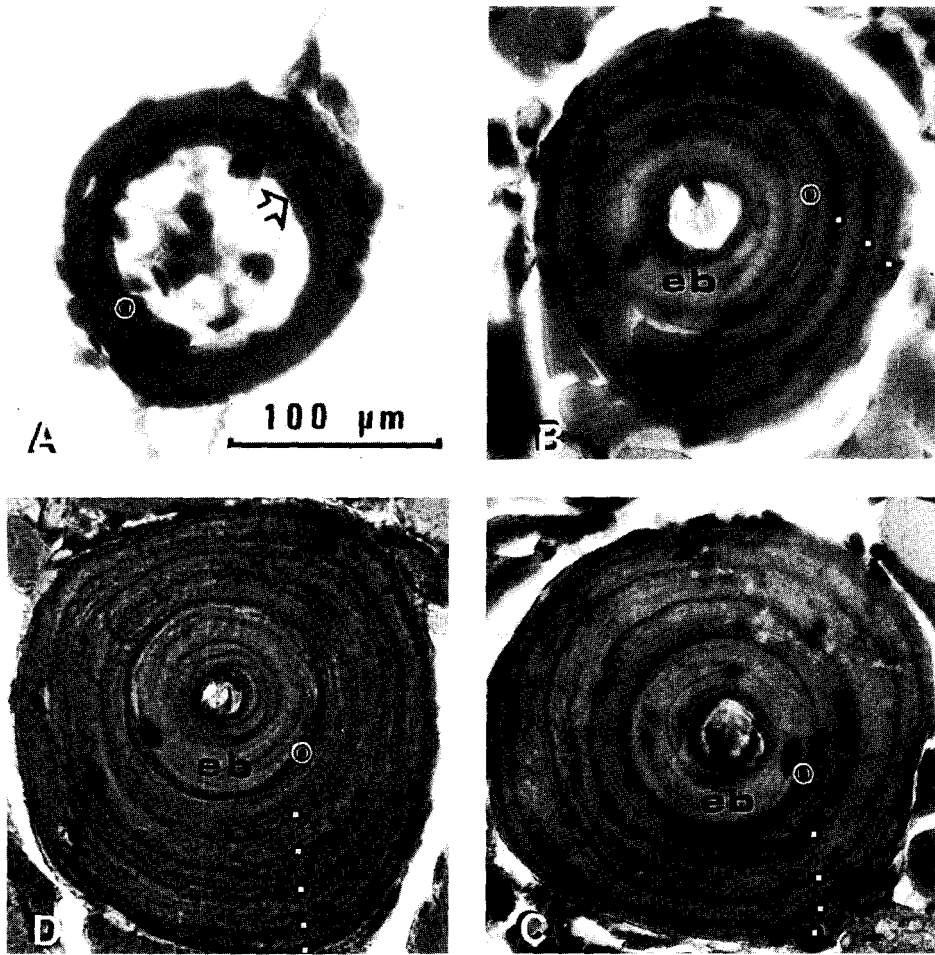


FIG. 1. Cross sections of the humerus of individuals of *Batrachoseps attenuatus*. (A.) An individual in its first year of life. Hollow circle = first LAG which has not yet been reached by endosteal resorption. Hollow arrow = remains of the embryonic bone. (B.) An individual in its fourth year of life (3 LAG following starting point). Hollow circle = starting point for counting growth marks, corresponding to the first LAG more or less merged with the reversal line and densely stained; solid circle = LAGs; eb = endosteal bone. (C.) An individual in its fifth year of life (4 LAG following starting point). (D.) An individual in its sixth year of life (5 LAG following starting point).

Patterns of growth marks at this level were similar (Fig. 1). The primary cortical bone, as typical of urodele periosteal bone, was made of a roughly parallel-fibered matrix (e.g., pseudolamellar bone), which sometimes was more strictly lamellar at the periphery, especially in old individuals. Osteocytes were large but scarce, and randomly distributed, as in most urodele bones. Lines of arrested growth (LAG) were evident in all individuals and these varied from one individual to the next in the degree of distinctness and regularity. In some individuals the LAG were locally split and sometimes merged with bony lamellae when lamellar bone was present.

Accordingly, in this species counting LAG is

neither straight-forward nor easy. After hatching, the medullary cavity is wide and the thickness of the bone crown is thin (i.e., the cortico-diaphyseal index is low). The remnant of the resorbed embryonic cartilage at the border of the medullary cavity persists; it corresponds to what has become known as "Kastshenko's line" in species which undergo metamorphosis (Francillon, 1980). Secondly the medullary cavity becomes filled progressively with endosteal bone, and is separated from the zone of periosteal bone by a reversal line; only near the center of the bone of adult salamanders is any hollow region found. The first internal hematolymphatic line observed on the bone sections is often thicker and more densely stained

(Fig. 1C, D) than are other LAG. This line probably corresponds to the first LAG which either lies close to the reversal line or even to Kastshenko's line, depending on the situation in different individuals and at different levels of the long bone. The first dense structure encountered in individuals in their first year of life (Fig. 1A) and which persists in older individuals (Fig. 1B-D), was taken as the starting point for counting growth marks and estimating age. LAG also are found in the endosteal bone, but they are not used to estimate age.

*Age and Size.*—We were able to study patterns of growth and to estimate age for 22 of the 23 adult individuals examined. Figure 2 presents the relationship between age, as determined in this study, and size for these individuals. There was no significant correlation between size and age ( $r^2 = 0.119$ ,  $P > 0.05$ ).

We estimate that all of the adult specimens examined were at least in their fourth year of posthatching life, and two of them were in their eighth year. The smallest adult specimen analyzed (35.8 mm SVL) was in the youngest age class (4). The largest specimen analyzed (46.9 mm SVL) was in the oldest age class (8). However, there was extensive overlap of ages and sizes, and a specimen between 40 and 45 mm SVL could be anywhere between 3+ and 7+ yr of age. The largest animal in each age class fell between 44.9 and 46.9 mm SVL. The age class with the largest membership in our relatively small sample was year 6, with eight individuals.

*Growth.*—Because animals were maintained in the laboratory for up to eleven months, field and laboratory growth, both variable among individuals, were confounded in the last year of life. Accordingly, incremental growth during the period from the last LAG cannot be compared with field growth. We measured growth in terms of area in standardized but arbitrary units in order to study the pattern of growth prior to capture (i.e., prior to the last LAG). Because the period of surface activity for this species is from roughly October to May, and the animals were captured in January, 1988, we interpret the area between the outermost LAG and the immediate internal LAG as growth during the Fall, 1987–Spring, 1988 active period.

Although growth rate varied, the Fall, 1986–Spring, 1987 active period was especially favorable for most specimens studied. It was the period of maximal growth for 13 specimens. Furthermore, in 15 cases the period from Fall, 1986–Spring, 1987 resulted in more growth than in either the preceding or succeeding year. In two other cases this period witnessed more growth than the preceding or the following year, but in four cases growth was poorer than either the preceding or succeeding year. However,

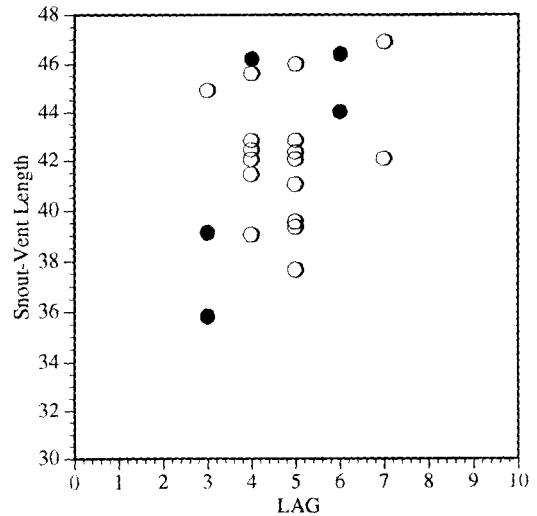


FIG. 2. Plot of LAG number and adult body size (SVL) for 22 specimens of *Batrachoseps attenuatus*, showing that there is no statistical relationship. The value of  $r^2$  for the regression is 0.119 ( $P > 0.05$ ). Females, hollow circles; males, solid circles.

each of the seven years covered by the study was a year of maximal growth for at least one individual, so presumably environmental heterogeneity in conditions favorable for growth existed in the small area of the study (less than 100 m<sup>2</sup>).

We were unable to detect any differences in male or female patterns in our small sample.

#### DISCUSSION

We found no significant correlation between size and age in *Batrachoseps attenuatus*. Our largest animals fall into all age classes detected. Furthermore, relatively small animals can be several years older than much larger animals (a 37.6 mm SVL specimen is in its sixth year of life, while a 44.9 mm SVL specimen is in its fourth year, i.e., has 3 LAG). Our results can be compared with those of Hendrickson (1954), who studied growth of marked individuals in a population of *B. attenuatus* approximately 15 km distant from the site of capture of our animals. Hendrickson reported only a fraction of his data. He noted a slowdown in growth at sizes greater than 35 mm, and extrapolated growth from his full (but unreported) data set to suggest that some individuals in his population might have been as old as ten years. We are unable to evaluate this extrapolation, but our results suggest that while his maximal age estimate appears reasonable, it is risky to make extrapolations of growth rate to age in adults of this species. Maiorana (1976) also summarized an unpublished data set for this species

from the vicinity of Berkeley, California. She found that sexual maturity was attained after 3.5 yr (4 dry seasons), and while she presented no data on adult life span, she predicted that it fell within the range of 2 to 6 years. Accordingly, a mature individual would have experienced a minimum of four periods of inactivity (should show 4 LAG) and could be expected to have a maximum of between 6 and 10 LAG. While our maximal numbers are within the expected range, we found adults with only 3 LAG and accordingly suspect that individuals may mature earlier than Maiorana thought.

The only other published skeletochronological study of plethodontid salamanders is for *Desmognathus ochrophaeus*, a species with a larval stage (Houck and Francillon-Vieillot, 1988). These authors found that body size was significantly correlated with age in their sample of 29 males, although much variation in age was found among individuals of the same body size. They found one eight and one seven year old animal, but most animals were between four and six years of age. Their largest animal was four years old.

Not only is *Batrachoseps attenuatus* a smaller species than *Desmognathus ochrophaeus*, but it also is a direct-developing form that lacks a free-living larval stage. Nevertheless, skeletochronological analysis proved feasible. Based on our analysis, *B. attenuatus*, although small, is relatively long-lived (maximal size in our sample is 46.9 mm SVL, an animal in its eighth year). This species also differs from *D. ochrophaeus* in having an active period in the cool but wet winter and an inactive period in the warm and dry California summer. Our observations indicate that LAG formation takes place in *Batrachoseps* during the summer dry period, when the species is subterranean. Individuals may feed only minimally, if at all, during this period. However, we had difficulty in interpreting some LAG; some very close lines or some inexact separation of lines may indicate either episodes of feeding during the dry season, or the effects of mid-winter dry spells of several weeks, during which activity is restricted. Because over one-half of all species of salamanders are direct-developing forms, our findings for *Batrachoseps* suggest that skeletochronology should be attempted with the tropical plethodontids, all of which develop directly, and many of which experience at least short times of inactivity during seasonal dry periods.

The two plethodontids studied by skeletochronological methods do not live as long as members of the families Salamandridae or Ambystomatidae. Montori (1990) reported that *Euproctus asper* can live to be at least 26 yr old. Larger species of the genus *Triturus* can live at

least 18 yr, but the smaller species rarely exceed 10 yr (reviewed in Miaud, 1992). Flageole and Leclair (1992) used skeletochronology to show that *Ambystoma maculatum* reaches 32 yr of age under natural conditions.

Halliday and Verrell (1988) stressed the need for empirical studies of the relationship of size and age in amphibians and reptiles, and cautioned against assuming that larger animals are necessarily older. The use of an age-size correlation even within a single species is risky unless one has some knowledge of growth rate differences among populations. Halliday and Verrell (1988) appropriately remark that "this method assumes what it sets out to demonstrate." There are several studies of salamandrids and ambystomatids that showed a positive but weak association between size and age (cf. Flageole and Leclair, 1992; Castanet et al., in prep.). In fact, the size-frequency distribution generally is able to predict age until sexual maturity, but once sexual maturity is achieved reliable age groups no longer can be recognized. Some of the best data relevant to this issue have been presented for desmognathine plethodontid salamanders by Bruce (1988, 1989, 1990, 1993). However, even in these species, age groupings are unreliable after sexual maturity is achieved. Estimation of age in adults requires skeletochronological analysis. Our data for *Batrachoseps attenuatus* agree with these findings, for we find no correlation between size and age for adult specimens. Maiorana (1976) predicted that age structure could not be deduced from size-frequency distributions for this species, because of complex allocations of energy to growth, maintenance, and reproduction associated with the unpredictability of the climate in central California and the small adult body size characteristic of this species. Our data are in accord with her predictions.

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