Application of Skeletochronology in Aging Larvae of the Salamanders *Gyrinophilus porphyriticus* and *Pseudotriton ruber*

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**ABSTRACT.**—We evaluated ages of larvae in southern Appalachian populations of the salamanders *Gyrinophilus porphyriticus* and *Pseudotriton ruber* by the method of skeletochronology applied to femurs. In *P. ruber*, the method yielded a strong correlation between snout–vent length and age and provided estimates in agreement with earlier studies of larval growth and age that were based on size-frequency data. However, in *G. porphyriticus*, the correlation between snout–vent length and age was weak and nonsignificant. This result was in accordance with previous studies that showed that larvae of this species do not sort by size into discrete age classes. The failure of skeletochronology in *G. porphyriticus* was attributed to either extreme variation in growth rate or to some deficiency of the methodology itself when applied to this species. We suggest that larvae of *G. porphyriticus* may not develop well-defined annual bone layers because of their adaptation to relatively aseasonal, subterranean microhabitats in headwater springs. In contrast, larvae of *P. ruber*, although overlapping in macrohabitat with *G. porphyriticus*, are essentially surface-dwellers that are exposed to the seasonal climatic fluctuations of the region.

The genera *Gyrinophilus* and *Pseudotriton* are allied members of the basal clade of plethodontid salamanders (Chippindale et al., 2004), and share numerous morphological, reproductive, and behavioral characters (Lombard and Wake, 1986; Collazo and Marks, 1994; Houck and Sever, 1994; Sever, 1994; Beachy, 1997). Metamorphosing species of both genera attain relatively large sizes prior to metamorphosis and may have larval periods that extend for two years or more (Ryan and Bruce, 2000). Two of the species, *Gyrinophilus porphyriticus* and *Pseudotriton ruber*, have broadly overlapping geographic ranges in eastern United States (Petranka, 1998) and often occur syntopically in springs and headwater streams (Bruce, 2003). In the southern Blue Ridge Mountains, there are microhabitat differences associated with larval morphology: larvae of *P. ruber* tend to occur under surface debris on fine substrates of the stream bed, whereas larvae of *G. porphyriticus* are more likely to be found below the surface in the saturated beds of coarser substrate materials (Bruce, 2003).

In *P. ruber*, body size measurements in large samples of larvae tend to resolve into bi- or polymodal distributions. Series of such distributions in samples taken over the annual cycle reveal age structure of the larval component of the population. Based on this approach, the typical larval phase of *P. ruber* in the southeastern United States has been estimated to vary from 18–23 months in the upper Coastal Plain (Semlitsch, 1983), to 27–32 months in the southern Blue Ridge Mountains and upper Piedmont (Bruce, 1972a, 1974). Because of overlap of successive size classes, Bruce suggested that some larvae in the populations he studied may metamorphose early, at 15–20 months, whereas others may extend the larval period an additional year, to 39–44 months. Semlitsch (1983) indicated that Coastal Plain larvae may delay metamorphosis an additional year, to metamorphose at 30–35 months.

In contrast, the larval phase of *G. porphyriticus* is poorly understood. Bruce (1980), again using analysis of body-size distributions to identify age classes in samples from different months, estimated larval periods of 36–60 months in a single population in the southern Blue Ridge Mountains. However, the resolution of age classes by size was poor. Two possible contributing factors are an extended oviposition season and highly variable individual growth rates.

In the several decades since these studies were conducted, skeletochronology has come into wide use as a method of individually aging ectothermic vertebrates in temperate climates (Castanet and Smirina, 1990; Castanet et al., 1993; our terminology follows that of the latter authors). The technique involves identification of growth marks (GM), consisting of bone layers or zones laid down during phases of active osteogenesis, separated by narrower layers that reflect reduced osteogenesis. In urodeles, the latter may take the form of annuli, representing layers of slower bone growth, or of resting lines, representing phases of arrested growth. Resting lines are referred to as lines of arrested growth or LAGs. Annuli and LAGs form in the limb bones when osteogenesis slows during seasons of the year when overall growth declines. This is often an effect of cold temperatures in the winter months in northern temperate species.

Given the backdrop of Bruce’s (1980) equivocal analysis of larval size-frequency data, our original goal in this study was simply to age larvae of *G. porphyriticus* by skeletochronology, to determine variation in the duration of the larval period. However, when we encountered difficulties in interpreting the preliminary results, we expanded the study to include larvae of
**P. ruber.** This allowed a comparison of the efficacy of skeletochronology between a species in which the larval period is relatively well understood (*P. ruber*) and a close relative in which the larval period remains unresolved (*G. porphyriticus*). Based on documented differences between the species in behavior and microhabitat selection in the southern Blue Ridge, we hypothesized that skeletochronology would provide a more reliable estimation of the larval period in *P. ruber* than in *G. porphyriticus*.

**MATERIALS AND METHODS**

As part of a study on the larval ecology of *G. porphyriticus* and *P. ruber* (Bruce, 2003), larvae of both species were collected from several populations in the Chattooga River watershed, near the common boundary of North Carolina, South Carolina, and Georgia, in the southern Blue Ridge Mountains. Habitats were described in the 2003 paper; however, it can be noted herein that larvae of *G. porphyriticus* were most often found in rheocrenes and helocrenes in subsurface microhabitats in the interstices among the gravel, pebble, and cobble of the streambed. In contrast, larvae of *P. ruber* were usually located in limnocrenes and helocrenes, on the surface of the mineral substrate, hidden in slitted mats of decaying leaves. There was no obvious variation in the habitat affinities of larvae of either species between the two study periods.

Although the 2003 study did not include temperature data, annual temperature profiles demonstrating thermal stability of springs having populations of larval *G. porphyriticus* and *P. ruber* in the Chattooga and an adjacent watershed were provided in an earlier report (Bruce, 1968).

Larvae were killed by immersion in MS-222, fixed in 10% formalin, and transferred to 70% ethyl alcohol. Snout–vent lengths (SVL), taken to the posterior edge of the cloacal slit, were measured after preservation. One of us (RCB) estimated ages by comparing the SVL measurements to the histograms in Bruce (1972a, 1980); these estimates were not provided to the second author (JC) until he had completed the skeletochronological evaluation of growth marks. This procedure was followed to minimize bias in relating size, growth marks, and age estimates.

One femur of each larva was removed and demineralized for 6 h in 5% nitric acid. Cross-sections were made with a frozen microtome (Leitz Kryomat 1700) midway along the diaphysis of the bone. The sections were stained with Ehrlich’s hematoxylin for 5 min and preserved in aquamounting resin. For each larva, microscopic GM analysis of the femur sections was performed by the second author two or three times, with several weeks delay between observations. Photographs were taken at the same magnification with a numeric Olympus camera.

We assumed that growth marks in larvae of these species were deposited annually, based on similar patterns of bone histology in other urodèles, including both larvae (Lima et al., 2001; Miaud et al., 2001; Bruce et al., 2002) and adults (Castanet et al., 1996; Alcobendas and Castanet, 2000; Bruce et al., 2002; Homan et al., 2003). Difficulties in the reading and interpretation of growth marks in plethodontid salamanders have been discussed by us in a recent paper (Ash et al., 2003), and these precautions were followed in the present study.

However, two issues require further explication. First, in larvae of both *G. porphyriticus* and *P. ruber*, including the smallest individuals, a dark-staining line was usually observed adjacent to the medullar cavity. This line apparently develops at or near the time of hatching, and is referred to as a hatching line (hl) rather than an annulus or LAG. The hatching line forms the inner boundary of the bone layer deposited during the first season of active osteogenesis. Second, in other species of plethodontids, the innermost bone layers of the diaphysis may break down as individuals age. Such endosteal resorption results in loss of one or more LAGs. This condition was encountered in one larva of *P. ruber* but in no other specimens.

**Age Calculation.**—Females of *P. ruber* oviposit in the autumn, with hatching following in late autumn and winter (Bishop, 1925, 1941; Fowler, 1962; Bruce, 1972a, 1974). For populations in the southern Blue Ridge close to those of the Chattooga watershed, Bruce estimated a mid-December to mid-February hatching period. In assigning ages based on LAG counts, our criterion was that a LAG separated from the hatching line forms in January as the modal month of hatching. On this basis, the first visible LAG would not be expected to form until the following winter (i.e., at an age of 12 months after hatching). Thus, larvae in their first year would be expected to show a hatching line but no LAG, with the first LAG becoming apparent during the second growing season as new bone is deposited around the LAG.

In *G. porphyriticus* in the southern Blue Ridge, egg-laying is apparently concentrated in the summer months, with hatching following in late summer and autumn (Bishop, 1941; Organ, 1961; Bruce, 1978). In populations near those in the Chattooga watershed, clutches and hatchlings were found in July and August (Bruce, 1978). Therefore, we set August as the modal hatching month in calculating ages from LAGs. Because there is apparently some growth in late summer and autumn of the first year (Bruce, 1980), we have assumed that a LAG separated from the hatching line forms in the first winter of life. This means, for example, that larvae taken in the warmer months between their first and second winters are expected to show a single LAG.

**RESULTS**

As is typical in urodèles, including plethodontids (Castanet et al., 1996; Bruce et al., 2002), histological organization of the diaphysis of long bones is simple in larval *G. porphyriticus* and *P. ruber*. The bone matrix, which is always avascularized, showed a gradation from a parallel-fibered structure, predominant in *P. ruber*, to a sublamellar organization, predominant in *G. porphyriticus*, with scarce but wide and somewhat flattened osteocytes. Growth zones appeared as broad bone layers that arise from sustained osteogenesis, but they were lightly stained in contrast to the alternating LAGs. In many individuals LAGs were replaced by wider annuli related to a temporary decrease in osteogenesis but without complete arrest. In several individuals of both species, annuli or LAGs were weakly expressed. Moreover, in sublamellar bone, mainly in *G. porphyriticus*, LAGs were often difficult to distinguish from bone lamellae, which made the counting of LAGs uncertain. The individuals considered too unreliable for aging were excluded from the analysis. Representative patterns of bone histology...
and growth marks in *P. ruber* and *G. porphyriticus* are shown in Figures 1 and 2.

In *P. ruber*, of the 24 larvae examined, four were considered unreliable. Of the remaining 20, nine showed no LAGs (Fig. 1A). All of the latter fell within the expected size range (20–31 mm) of first-year larvae (see histograms in Bruce, 1972a) and were estimated to range from six to 13 months, depending on the month of collection. Among the other 11, one or two LAGs were recorded (Fig. 1B,C). These were larger individuals (>30 mm), and, according to the dates of collection, fell within the size range of second- and third-year larvae.

A scatterplot of SVL versus age (Fig. 3A) in *P. ruber* showed the expected increase in SVL with age. The correlation is highly significant ($r^2 = 0.746$, $df = 18$, $P < 0.001$). Because the largest larvae (>35 mm) had attained metamorphic size, we calculated probable age at metamorphosis by adding the number of months between the date of collection and the usual season of metamorphosis (June and July), based on Bruce (1972a). This yielded estimates of metamorphic age of either 29–30 or 41–42 months in these populations. Although Bruce (1972a) concluded that the usual larval period in the southern Blue Ridge is 27–31 months, he allowed that some larvae may extend the larval period an additional year.

In *G. porphyriticus*, we could not as effectively compare our GM-based age estimates with those derived from SVL distributions because the latter are poor indicators of age structure (Bruce, 1980). In our sample of this species, seven of 24 larvae showed no clear pattern of LAGs and were considered unreliable. Even among the 17 in which LAG counts were obtained, several were difficult to resolve and the counts may be questionable. LAG numbers varied from one to four (Fig. 2A,B,C), yielding estimates of age from 12 to 51 months. No very small larvae from late summer through autumn samples were available, which probably accounts for the absence of null LAG counts. The upper limit of age falls within the 36–60 months larval period estimated by Bruce (1980) for a nearby population. However, in our sample, there was considerable variation in age among larger larvae, which suggests either a high level of variation in larval growth or a deficiency in the method. The skeletochronological data yielded estimates of attainment of metamorphic size (>55 mm, see Bruce, 1972b, 1980) at ages ranging from <2 to ≥5 years, which seems unlikely. The scatterplot of SVL against age (Fig. 3B) showed no obvious trend, and the correlation was nonsignificant ($r^2 = 0.146$, $df = 15$, $P = 0.130$).

**DISCUSSION**

We conclude that skeletochronology is a more reliable method of aging larvae in *P. ruber* than in *G. porphyriticus*. This specimen illustrates the difficulties in counting LAGs in larvae of both species. The larva was collected on 3 December and was most likely 35 months of age, which would give a prediction of metamorphosis at 41–42 months.
porphyriticus, at least in the southern Blue Ridge, and may have limited usefulness for determining population age structure in the latter species.

For G. porphyriticus, the results indicate that growth rates of larvae are extremely variable, so that individuals of the same age vary widely in size. This is in accord with the failure of larval samples to sort by size into age classes (Bruce, 1980). Alternatively, our LAG counts may be inaccurate estimators of age in this species, because LAGs are weakly expressed in some individuals, may not form regularly, and may often be indistinguishable from bone lamellae. Low rates of osteogenesis associated with slow, year-round growth in cool, thermally stable microhabitats may be responsible. The formation of sublamellar bone in salamanders like G. porphyriticus is correlated with low rates of osteogenesis (Ricqlès et al., 1991).

The lack of well-defined age/size classes in large samples of larval G. porphyriticus may also be an outcome of a more prolonged, less seasonal period of

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**Fig. 2.** *Gyrinophilus porphyriticus*. Frozen cross-sections stained with Ehrlich’s hematoxylin of the femoral diaphyses of three larvae. hl = hatching line, numbers = LAGs or annuli. (A) 25.6 mm SVL larva having a hatching line and one wide LAG which appears double (= annulus). This larva was collected on 8 August and was estimated to be 12 months, based on an August hatching date (see text). Note position of LAG in middle of the diaphysis. (B) 38.7 mm larva collected 7 November showing 2 LAGs. Outside of the second LAG, growth during the second full growing season is represented by the bone between the LAG and the periphery. Age is estimated as 27 months. (C) 46.4 mm larva also collected on 7 November and having four LAGs. The inner two are wide and are considered annuli. Age estimate is 51 months.

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**Fig. 3.** Scatterplot of snout–vent length versus age in (A) *Pseudotriton ruber* (N = 20) and (B) *Gyrinophilus porphyriticus* (N = 17).
oviposition/hatching than in *P. ruber*, although this has not been documented. But such a difference would give rise to greater overlaps in body size between successive age classes, as observed (Bruce, 1980). In contrast, in larva of *P. ruber*, bone is mainly parallel fibered, which is indicative of more rapid growth (Ricqles et al., 1991). The age estimates based on LAG counts in *P. ruber* correlated much better with body size than in *G. porphyriticus* and agreed well with patterns of population age structure determined from size-frequency distributions (Bruce, 1972a).

In the southern Blue Ridge, larva of *G. porphyriticus* may undergo no pronounced reduction or cessation of growth in the winter months because of their affinity for subsurface microhabitats in springs wherein temperature is relatively constant year round (Bruce, 1968, 2003). Their adaptation to such microhabitats is expressed in their morphology (Brandon, 1966; Birchfield and Bruce, 2000). Although both *G. porphyriticus* and *P. ruber* co-occur in spring habitats, larvae of the latter tend to occur in surface microhabitats of limnocrenes and helocrenes, in contrast to the burrowing tendencies of larval *G. porphyriticus* in helocrenes and rheocrenes (Bruce, 2003). Thus, larvae of *P. ruber*, on average, may be exposed to more seasonal temperature regimes than those of *G. porphyriticus*. Larvae of the former species have a generalized stream-type larval morphology (Valentine and Dennis, 1964) and lack the adaptations of *G. porphyriticus* for subsurface life (Birchfield and Bruce, 2000). Therefore, it is likely that the larval growth pattern of *G. porphyriticus* reflects greater adaptation to cool, constant annual temperature regimes than that of *P. ruber*, with the difference expressed in the different modes of osteogenesis reported herein.

To estimate such parameters as variation in larval growth, larval age structure, and age at metamorphosis in *G. porphyriticus* probably it will be necessary to employ mark-recapture methods, in concert with seminatural cage experiments. In experimental studies of interspecific interactions among stream salamanders, several investigators have measured short-term growth of larval *G. porphyriticus* (in summer) using cage experiments but did not attempt to extrapolate these results to the longer term of the larval period (Beachy, 1994; Gustafson, 1993, 1994; Resetarits, 1991). Among the several species involved in these stream assemblages, the larval period of *G. porphyriticus* remains the least known. The latter species shows considerable variability in growth responses to the presence/absence of predators (Resetarits, 1991) and conspecifics (Gustafson, 1994) and to variation in prey density (Beachy, 1994). It remains a challenge to determine the influence of these factors over the time frame of the entire larval period of *G. porphyriticus*.

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