Skeletochronology and Geographic Variation in Age Structure in the Wood Frog, *Rana sylvatica*

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ABSTRACT—Age structure of a breeding population of *Rana sylvatica* from southwestern Québec, Canada, is described based on the enumeration and analysis of Lines of Arrested Growth (LAGs) in phalanges. Analyzed using skeletochronology, ages ranged from 1–4 yr among 98 males and from 2–4 yr among 33 females. Females were on average slightly older than males, but the difference was not significant. Mean snout-vent length was 43.6 mm ± 2.0 (SD) among 179 males and 48.8 mm ± 2.7 among 33 females; the difference between the sexes was significant. Endosteal resorption completely destroyed LAG 1 in 6.1% of frogs, about twice as frequently in males as in females. A minimum of three LAG enumeration readings by two observers differed for 24.4% of preparations and independent readings by the same observer differed for 15.3% of preparations, highlighting the importance of reducing subjectivity in skeletochronological analyses. This was accomplished, in part, by plotting LAG diameters on a histogram whereby outliers from an expected normal distribution indicated loss of inner LAGs via endosteal resorption. Consistent with predictions regarding environmental influences on anuran populations, southern Québec *R. sylvatica* matured later and were larger than more southerly conspecifics from a low-elevation area, but matured earlier and were smaller than southerly frogs from a high-elevation area.

Interpopulational variation in life history characters due to climatic effects has been shown to exist in several anuran species. Cold temperatures at high altitudes and latitudes delay both growth and development in larval amphibians (Berven 1982a, b; Berven and Gill, 1983; Hemelaar, 1988), producing cohorts which are larger and/or older upon attainment of normal developmental stages than conspecifics living under milder conditions. Berven (1982a) attributed delayed maturation among high elevation *Rana sylvatica* relative to a nearby low-elevation population primarily to climatic differences. Similar findings have been reported for *R. temporaria* (Ryser, 1996) and *Bufo bufo* (Hemelaar, 1988).

The annual periodicity of Lines of Arrested Growth (LAGs) in several temperate anuran species has been demonstrated (Smirina, 1972; Hemelaar and van Gelder 1980; Gibbons and McCarthy, 1983). Skeletochronology based on the enumeration of LAGs in anuran phalanges has been used to assess age, age at sexual maturity, and longevity. Coupled with body size data from a subsample of the population, skeletochronology even enables retrospective estimates of body size and growth rates (Hemelaar, 1985, 1988; Augert and Joly, 1993; Smirina, 1994). This non-lethal aging technique allows for the collection of demographic information on anuran populations much more rapidly than mark-recapture (Halliday and Verrell, 1988). However, due to endosteal bone resorption, skeletochronological evidence requires some analysis before ages can be assigned confidently. Where possible, age estimates based on skeletochronology should be compared with individuals of known age from the same population for confirmation (Halliday and Verrell, 1988).

LAGs in older individuals can be obscured by replacement of periosteal bone with endosteal bone, which originates at the perimeter of the marrow cavity and progresses toward the bone perimeter (Hemelaar, 1985). Since LAGs appear in periosteal bone, endosteal resorption has the potential to distort skeletochronological age estimations by destroying LAGs. In phalanges in which LAG 1 (deposited during the first postmetamorphic hibernation) has been completely destroyed by endosteal resorption, the innermost visible LAG was actually deposited during the second, rather than the first, post-metamorphic hibernation. Because complete LAG resorption cannot be identified by simple visual inspection, diameter values for the first (innermost) and second visible LAGs from each section should be calculated and analyzed. Based on this analysis, age estimates from visual LAG enumeration can be corrected to reflect the true age structure of the sampled population.

The Metamorphosis Line (ML), which is similar in appearance to a LAG, is deposited during metamorphosis, before any LAGs are deposited. The positive identification of the ML in phalangeal sections from adult frogs thus indicates

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that complete LAG resorption has not occurred, allowing the calculation of the distribution of known LAG 1 diameters. Based on this distribution, the innermost visible LAG in sections in which the ML has been resorbed can be positively identified as either LAG 1 where complete LAG resorption has not occurred (if the diameter falls within the range of known LAG 1 values) or LAG 2 where it has (if the diameter is >2 SD above the mean of known LAG 1 diameters). However, if the ML cannot be positively identified in a large proportion of the sample, it is still possible to identify complete LAG resorption. The diameters of the first (innermost) and second visible LAGs can be plotted in histogram form. If the sample size is sufficiently large, the distributions of each should be approximately normal. A normal curve can be fitted to the distributions, and cases of complete LAG resorption should be evident as positive outliers in the distribution of the diameters of the first visible LAG. This method should be particularly valid where sample size is large or complete LAG resorption is rare. With a small sample, the distributions of LAG diameters may not reflect the normal distribution clearly enough for a curve to be fitted to the data. If complete LAG resorption occurs with nearly 100% frequency, which may be a concern in long-lived frogs, use of the histogram technique could lead to the underestimation of all ages; thus identification of the ML would be the preferred technique.

Bastien and Leclair (1992) used skeletochronology to compare demographic characteristics of *R. sylvatica* from Trois-Rivières, Québec, Canada with conspecific low- and high-elevation populations from Maryland and Virginia described by Berven (1982a). Since their sample size was relatively small, the LAG diameter data do not reflect a normal distribution, and the authors were forced to arbitrarily estimate the threshold diameter for the innermost LAG, above which they concluded LAG resorption had occurred. Because this threshold directly affects age estimates, there must be minimal uncertainty about it in order for age data to be accurate. Our aim was to collect sufficiently large a sample so that LAG resorption could be identified with confidence, and thereby to improve on Bastien and Leclair's (1992) study. Therefore, we intensively collected age structure data for a single breeding Québec *R. sylvatica* population and compared them with the more southerly population data of Berven (1982a). In so doing, we also considered the problem of LAG enumeration subjectivity and the interpretation of bone histological marks. Based on differences in the length of the growing season between the sites, it was expected that age at first
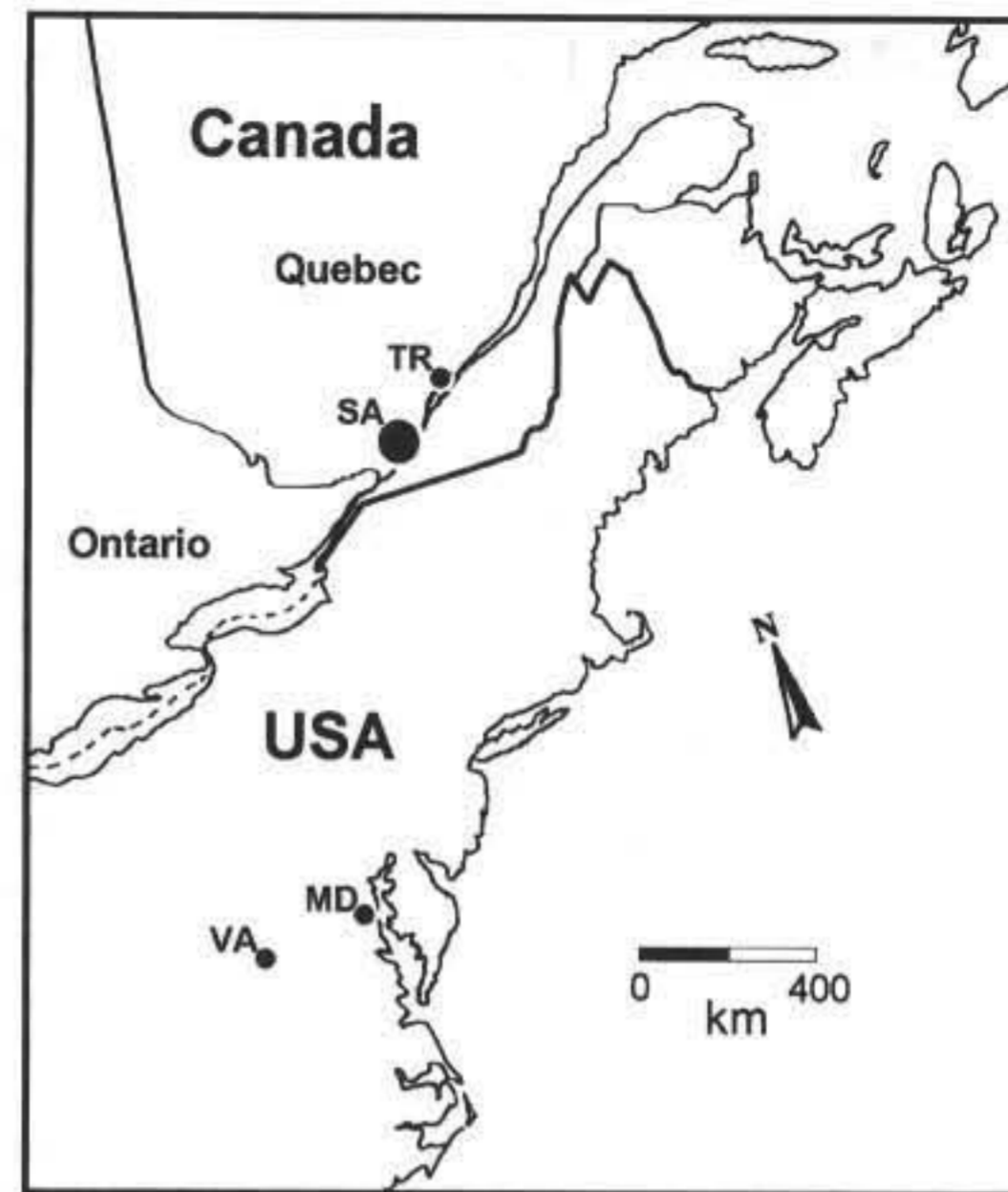

FIG. 1. Eastern North America showing the Ste-Anne-de-Bellevue study site (SA) in southern Québec and other localities mentioned in the text: Trois-Rivières (TR), Virginia (VA), and Maryland (MD).

reproduction among southern Québec wood frogs would be intermediate between those of the high-elevation and the low-elevation southern populations.

MATERIALS AND METHODS

Breeding adult *Rana sylvatica* were sampled between 15 and 20 April, 1996 from a small, undisturbed pond located in the Morgan Arboretum ecological reserve in Ste-Anne-de-Bellevue, Québec, Canada (45°25'N, 73°57'W, altitude 150 m; Fig. 1), on the island of Montréal. The pond is 10 m in diameter and is surrounded by mature deciduous forest. Based on annual number of frost free days, this site has an average growing season of approximately 137 d, extending from mid-May through early October.

We captured 179 males and 33 females by hand and dip-net. Most frogs were captured either calling or in amplexus on 19 and 20 April and sexed by secondary sex characteristics and the production of sperm or ova. Snout–vent length (SVL) was measured for all frogs by a single observer. Taking care to minimize trauma to the frog, the fourth toe from the right hind limb of 98 males and all females was clipped at the articulation proximal to the third phalange, and all frogs were then released at the site of capture.

Clipped toes were stored in 10% neutral buffered formalin for a minimum of five days be-

TABLE 1. Age structure (percent frequency) of a breeding population of *Rana sylvatica* from Ste-Anne-de-Bellevue, in southern Québec, Canada (present study) compared to ages of first time breeders from lowland (Maryland) and high-elevation (Virginia) sites in the southern part of the species' range (from Berven, 1982a). Data from Maryland and Virginia were based on mark-recapture of frogs from several ponds in each locality over a period of three years.

	S. Québec		Maryland		Virginia	
Age	Males	Females	Males	Females	Males	Females
1	4.1	0	86.0	1.1	0	0
2	49.0	45.5	14.0	98.9	13.6	0
3	40.8	33.3	0	0	83.7	44.2
4	6.1	21.2	0	0	2.7	55.8

TABLE 2. LAG diameter values for breeding adult *Rana sylvatica* at Ste-Anne-de-Bellevue, Québec.

		LAG Diameter (μm)		
LAG #	N	(mean $\pm$ SD)	Min	Max
1	121	324 $\pm$ 41	249	448
2	120	456 $\pm$ 32	358	536
3	58	479 $\pm$ 35	381	564
4	12	483 $\pm$ 34	442	563

fore the third phalange was isolated, cleaned of surrounding tissues, and prepared for sectioning following the methods of Leclair and Castanet (1987). Phalanges were decalcified for 3 h in 3% nitric acid, then soaked overnight in distilled water. The diaphyseal portion of each phalange was cut into 16 to 20 μm thick transverse sections at -25 C using an Ames Lab-Tek Cryostat Freezing Microtome. Sections were immediately stained for 22 min in Ehrlich's hematoxylin and rinsed with distilled water. The smallest diameter diaphyseal sections (Hemelaar, 1985) were mounted in a drop of glycerol on glass microscope slides for skeletochronological analysis. Sections were examined at least three times independently by two observers. Where LAG counts differed, sections were re-examined together by both observers until consensus was reached.

Because frogs were captured shortly after emergence from hibernation, LAGs deposited during the previous winter's hibernation were not discernible from the outer edge of the bone. The outer edge was therefore counted in all cases as a LAG. The diameters of the Resorption Line (the outer extent of endosteal resorption, delineated by the boundary between endosteal and periosteal bone), as well as all visible LAGs including the outer edge of the bone section, were digitized and measured using SigmaScan (version 3.92, Jandel Scientific, Corde Madera, CA). The longest and shortest perpendicular axes of each of four diaphyseal sections per phalange were measured, following the methods of Hemelaar (1985). Axis measurements were multiplied together and the square root of the product calculated. This value, the average diameter of the line being measured, was averaged across the four sections measured per phalange. To identify cases of LAG resorption, the diameters of the innermost and second visible LAGs were

plotted on a frequency distribution. Diameters for the innermost visible LAG which were >2 SD greater than the group mean were interpreted as cases of LAG 1 resorption, where the innermost visible LAG was actually LAG 2, not LAG 1.

RESULTS

Mean age ($\pm$ SD) of breeding males from the surveyed population was 2.49 $\pm$ 0.68 yr (N = 98), while females averaged 2.76 $\pm$ 0.79 yr (N = 33). The difference in age was not significant (Student's t-test: $P = 0.121$). Although a few male yearling breeders were identified, the two and three yr age classes were best represented in both sexes. Four yr olds were also identified in both sexes (Table 1).

Mean SVL was 43.6 $\pm$ 2.0 mm (N = 179) for males and 48.8 $\pm$ 2.7 mm (N = 33) for females. The difference in body size between the sexes was significant (Student's t-test: $P < 0.001$). SVL was positively correlated with age (r = 0.415; $P < 0.001$) but size ranges of different age classes overlapped extensively, making age estimation from size data alone impossible for this population.

LAG diameters increased with the age at which they were deposited (Table 2). Diameter values for the first (innermost) and second visible LAGs both exhibited approximately normal distributions. Based on these distributions, we concluded that complete resorption of the innermost LAG occurred in 6.1% (8 of 131) of frogs (Figs. 2 and 3). Resorption occurred in 7.1% (7 of 98) of males and in 3.0% (1 of 33) of females. Instances of LAG resorption were identified as sections in which the innermost visible LAG had a diameter greater than 2 SD above the mean value for the group. Because the innermost visible LAG was never more than 2 SD above the LAG 2 mean, we concluded that complete resorption of both LAG 1 and LAG 2 did not occur (Fig. 3). No phalange could be described as fully intact, having sustained no endosteal resorption.

Differences in LAG counts among independent readings occurred in 24.4% (32 of 131) of cases. For 15.3% (20 of 131) of phalanges ex-

amined, two readings conducted by the same observer differed.

DISCUSSION

Age Structure and the Environment.—Based on length of the growing season in each locality (Maryland: 177 d, Virginia: 121 d [Berven, 1982a], Ste-Anne-de-Bellevue: 137 d), we expected both age at first reproduction and average size of southern Québec frogs to be intermediate between those of frogs from the low- and high-elevation southern populations. Frogs from the 1996 breeding group in Ste-Anne-de-Bellevue matured later than frogs from the low-elevation Maryland population, and earlier than frogs from the high-elevation Virginia population (Berven, 1982a). Both sexes from the 1996 Ste-Anne-de-Bellevue breeding group and from Trois-Rivières (Bastien and Leclair, 1992) were slightly larger than breeding adult frogs from lowland Maryland (Table 3). Yet they were smaller than frogs from the high-elevation Virginia habitat. The colder temperatures and shorter growing seasons in habitats at both higher altitude and higher latitude may constrain anuran growth and development both directly by reducing the metabolic rate and indirectly through a reduced food supply (Berven, 1982a). Coupled, these constraints on growth and development impose delayed maturation upon the population. Because there is only one breeding event per season in *R. sylvatica*, frogs that fail to reach maturity during their first spring after metamorphosis grow for a second full season before reproduction. Consequently, frogs which mature during their second spring after metamorphosis are typically larger than frogs from the same cohort which reach maturity during the previous year. A longer term study would provide valuable confirmation of this result by accounting more effectively for normal annual fluctuations in recruitment and population structure.

Skeletochronology.—Because the ML was not identifiable in frogs from Ste-Anne-de-Bellevue, we used the distributions of the diameters of the first and second visible LAGs to identify cases of complete LAG resorption. Since the distribution of the diameters of the first visible LAG so nearly approximate the normal distribution (Fig. 3), cases of complete LAG resorption are easily identifiable as positive outliers (>2 SD above the LAG 1 mean) in the distribution of the diameters of the first LAG. Complete LAG resorption was rare in this population, and because it was easily identifiable, did not complicate age estimation. Although the histogram method provided a reliable estimate of the frequency of complete LAG resorption in *R. sylvatica* from southern Québec, it might not provide
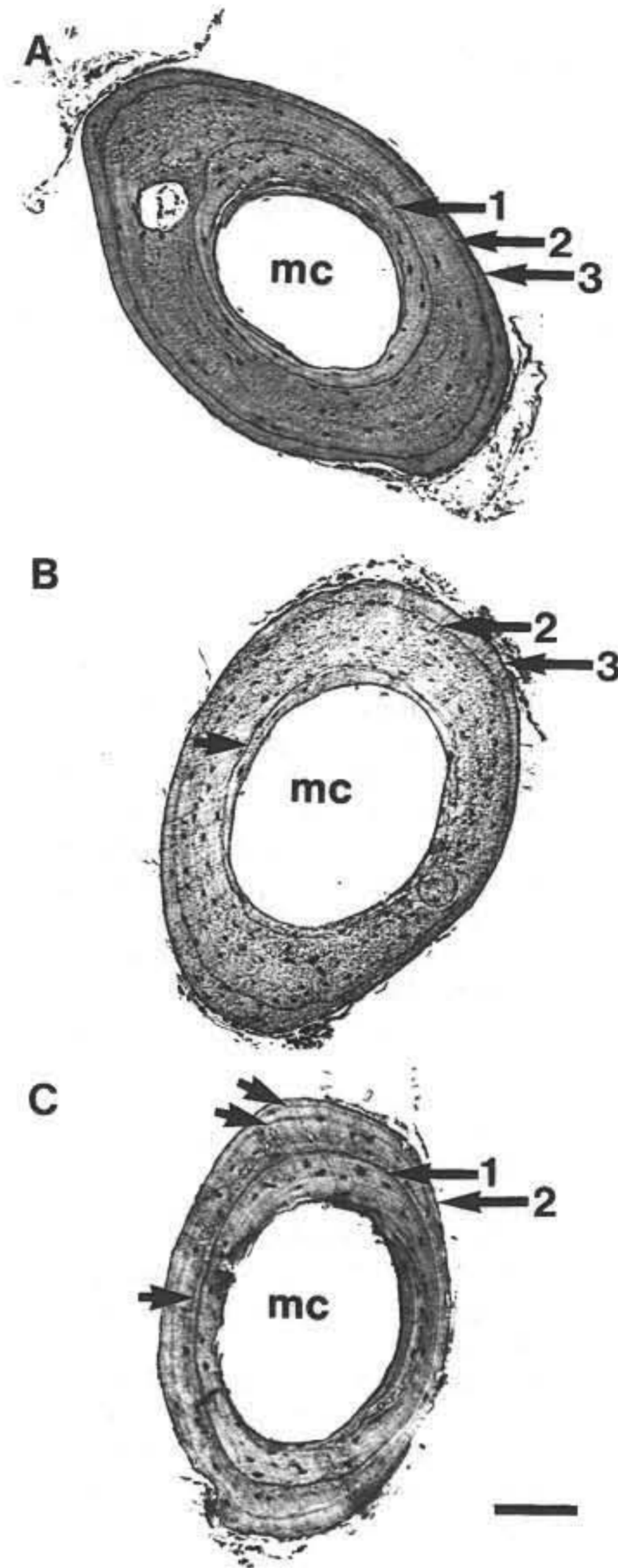

FIG. 2. Variation in hematoxylin-stained transverse sections from the diaphyseal portion of phalangeal bones of breeding adult *Rana sylvatica*. mc = marrow cavity. (A) Male from the 3 yr age class for which LAGs are easily visible and no LAG resorption has occurred. (B) Male from the 3 yr age class. Endosteal resorption (short arrow) has completely destroyed LAG 1. (C) Male from the 2 yr age class. LAG enumeration in this section is complicated by the presence of numerous false lines (short arrows).

such an unambiguous result when applied to more long-lived species in which complete LAG resorption is more common or where there are not enough measurements of intact LAG 1 for the normal distribution to be apparent. Further,
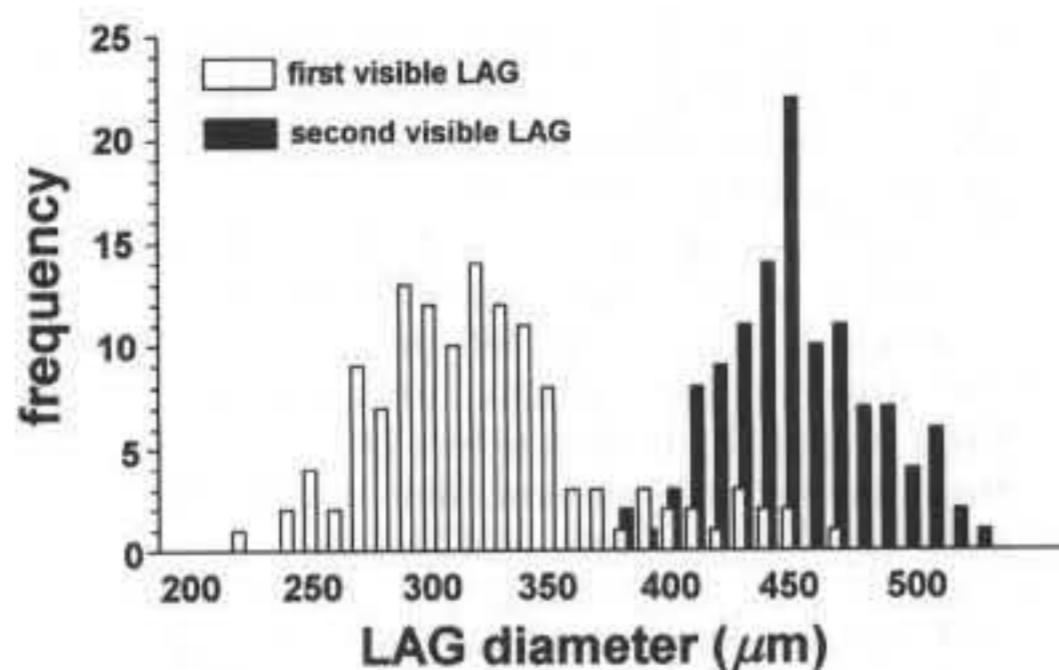
FIG. 3. The distributions of diameters of the first (innermost) and second visible LAGs in phalangeal sections from a southern Québec population of *Rana sylvatica*. The first deposited LAG has been resorbed in sections in which the diameter of the first visible LAG is greater than 425 μm.

whereas the histogram method nevertheless appears to be reliable, a test using known-age individuals would provide valuable confirmation of the technique.

We have found that accurate demographic data are not as easy to obtain using skeletochronology as some authors suggest. Ages can not be estimated only on the basis of a simple count of visible LAGs in a phalangeal cross-section. LAGs should be measured to confirm the age at which they were deposited. Precise LAG diameter measurements are easy to obtain and greatly increase the confidence with which age estimations are reported. LAG measurements are recorded in the majority of studies on anuran populations using skeletochronology (Gibbons and McCarthy, 1983; Hemelaar, 1985; Leclair and Castanet, 1987; Bastien and Leclair, 1992; Kellner and Green, 1995; Kusano et al., 1995a, b), and the confirmation of age estimation that they provide is essential to the precision of the reported results. Measurements should be taken only from diaphyseal sections, and the same phalangeal bone should be examined for all individuals.

LAGs are sometimes incomplete, faint, or otherwise difficult to identify, which further complicates age determination. In our study, LAGs near the outer perimeter of the bone may have distorted some age estimates. Outer LAGs were frequently very close to one another and to the edge of the bone, making their enumeration, and the associated age estimation, difficult. In rare cases, a double line may be mistaken for two LAGs, resulting in an overestimation of the age of the sample being examined, or a LAG near the outer edge of the bone may simply be indistinct and overlooked. More central LAGs do not pose this problem because they are generally more widely spaced than those deposited later in life.

The difficulty of accurate LAG identification and interpretation of bone histological marks is evident in the high frequency with which different age estimations were recorded in our independent LAG readings. Differences in age estimations stemmed mostly from the difficulty of locating and identifying LAGs. In many phalanges, LAGs were close together or close to the bone perimeter and thus were difficult to identify. In cases in which different ages were reported for the same frog, the difference was most often the result of an oversight of a LAG by one observer. Interpretations of bone histological marks (genuine vs. false LAGs) also caused differences in age estimations for some individuals. False lines resemble LAGs in appearance and, like them, indicate temporary reduction in growth rate. LAGs, however, result from hibernation whereas false lines are thought to result from injury or reduced food supply (Hemelaar, 1985) which compel the animal to focus available resources on sustenance rather than growth. False lines generally are fainter than LAGs and do not form a complete ring around the bone section. Using multiple independent examinations of bone sections seems to be a simple and effective way to reduce the frequency of subjective error in age determination. We recommend this method in studies involving skeletochronology and caution against hasty acceptance of under-analyzed results in skeletochronology-based studies.

TABLE 3. Demographic parameters of *Rana sylvatica* populations from various localities. All values are means ± SD. [1] From Bastien and Leclair (1992). Data are pooled from two different breeding sites over a period of two years. [2] From Berven (1982a). Mark-recapture data pooled from numerous sites over a period of three years.

	Males		Females	
Location	Age (yr)	SVL (mm)	Age (yr)	SVL (mm)
Ste-Anne-de-Bellevue, Québec	2.49 ± 0.68	43.6 ± 2.0	2.76 ± 0.79	48.8 ± 2.7
Trois-Rivières, Québec[1]	—	43.6 ± 2.6	—	49.3 ± 2.5
High altitude Virginia[2]	2.89 ± 0.39	55.3 ± 3.1	3.56 ± 0.50	64.4 ± 3.5
Lowland Maryland[2]	1.16 ± 0.36	41.7 ± 3.7	1.99 ± 0.11	47.7 ± 3.7

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