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ACTUAL AND OSTEOCHRONOLOGICAL ESTIMATED AGE OF NATTERJACK TOADS (*BUFO CALAMITA*)

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The estimation of individual ages in natural populations of amphibians is not a trivial undertaking (Halliday & Verrell, 1988). Intuitively, a mark-recapture method is the best approach to establish actual age by marking individuals at known ages, i.e. at metamorphosis, and recapturing them later when sexual maturity is attained and individuals migrate to ponds. However, this approach may involve two important costs. First, juveniles may exhibit very low survival rates (Smith, 1987; Semlitsch *et al.*, 1988; Berven, 1990; Reading *et al.*, 1991; Scott, 1994) and a large number of toadlets must therefore be marked. Second, if significant immigration rates occurred from unmarked populations, immigrants would be of indeterminate age compared with marked individuals of known age. An alternative method to estimate age in amphibians that overcomes these problems is the indirect assessment of age by studying the pattern of growth layers in the osseous tissues and counting the lines of arrested growth (LAGs) laid down in the midshaft diaphysis of long bones. This method is known as skeletochronology and in the past 15 years has become a widely used tool for ageing individual amphibians (see Smirina, 1994 for a review). This method, however, requires two conditions to be valid: (1) bone growth pattern must correlate with fairly constant chronological period; and (2) no significant variability in the genetic and/or environmental factors that control bone growth must exist within populations, thus allowing demographic comparisons both at intra- and interpopulational level. Only a few investigations have dealt with both problems in natural populations (Smirina, 1972; Hemelaar & van Gelder, 1980; Gibbons & McCarthy, 1983; Frétey & Le Garff, 1996). All of these were made with recaptured animals that had achieved sexual maturity. LAGs were annual and this pattern did not show variability across individuals in these populations.

The present study was conducted in a natterjack toad (*Bufo calamita*) population located in Sierra Morena (Córdoba province, southern Spain). As part of a long-term study, we marked around 2500 metamorphs by cutting the longest toe of the hind foot during the last two weeks of May 1992. Toadlets were seen around the ponds for nearly a month and then dispersed or aestivated because of the dry summer. In the first week of February 1995 rains filled the temporary ponds and a total of seven adult toads with clipped toes were recaptured. These animals allowed us to establish direct correspondence between an observed pattern of bone growth and the actual age of toads, which has never been reported in any amphibian species. We toe clipped the second outer toes of the right fore feet and these were preserved in 70% alcohol. We prepared bone sections for skeletochronological analysis following techniques published elsewhere (Hemelaar & van Gelder, 1980; Gibbons & McCarthy, 1983). From the toe selected, only the third phalange was used. This was washed in water for one hour, decalcified in 3% nitric acid for five hours and then rinsed in tap water overnight. Transverse sections of 15 µm were cut using a freezing microtome, stained with Ehrlich's haematoxylin for 15 min and rinsed in tap water for 30 min. Finally they were mounted in Aquamont and protected with a glass coverslip. The sections were examined with a Zeiss light microscope and photographed at a constant magnification. Age was determined by interpreting LAGs.

Five out of seven individuals clearly exhibited two distinct haematoxylinophilic lines of arrested growth. Individuals 7 and 16, however, exhibited an additional inner LAG (Fig. 1a). This line had probably been destroyed in the rest of toads due to bone remodelling at the periphery of the medullar cavity. Toads nos. 1 and 5 differed from this growth pattern showing two and one additional lines respectively (e.g. Fig. 1b).

These supplementary lines were more weakly expressed than the former and appeared alternated and more or less equidistant from the other, so it is improbable that they represented double-lines as reported in

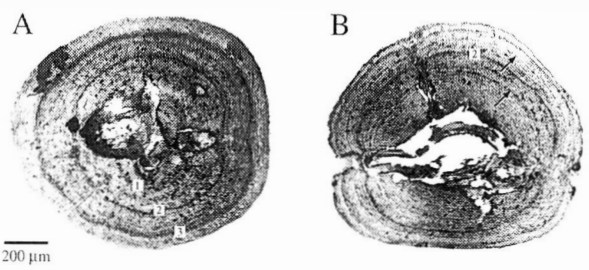

FIG. 1. Haematoxylin-stained cross-sections of phalangic dyaphises in adult *Bufo calamita* metamorphosed in May 1992 and recaptured in February 1995. Numbers are designated lines of arrested growth (LAG). (A) Toad no. 7 female, 78 mm snout-urostyle length; (B) Toad no. 1 male, 81 mm snout-urostyle length. This individual showed two additional LAGs marked with arrows. Inner line one was lost probably due to the process of bone remodelling at the periphery of the medullar cavity.

other amphibian species (Francillon, 1979; Caetano & Castanet, 1993). The periodical or occasional character of these lines, and therefore their chronological value, cannot be stated here, given the small sample, but they very probably reveal the potential for intrapopulational variation in the number of arrested growth periods that natterjacks belonging to the same population may exhibit.

The number of growth rings may be correlated with the pattern of activity and growth that natterjacks exhibit in southern Spain. Contrary to northern populations, activity is minimal during the hot and dry summer, and this is presumably the period of arrested growth when LAGs are formed. Toads emerge after the autumn rains, but breeding is delayed until winter. A fraction of yearly adult growth, estimated from recaptured individuals, was achieved during the period of breeding (January-April), with remaining growth presumably occurring during autumn (Tejedo, 1992, Fig. 4). Whether juveniles exhibit this same growth pattern remains unknown, but juveniles were frequently observed near the breeding area in autumn and winter, and it seems unlikely that they were active during the dry summer. Therefore, the three LAGs observed may correspond to the aestivation periods of 1992 (first LAGs not resorbed in toads nos. 7 and 16, Fig. 1a), 1993 and 1994.

This paper has reported the first evidence of the correspondence between an observed pattern of bone growth and the actual age of toads. The fact that some individuals, in spite of the small sample, exhibited variation in the bone growth pattern by expressing light but distinct additional LAGs, casts doubt on the reliability of skeletochronology in estimating age in natural populations of amphibians in warm temperate climates. Complementary information about actual activity and retreat periods of the animals in natural populations together with information based on recaptured individuals of known real age is necessary to check and standardize ageing estimates based on bone growth.

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