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Skeletochronological Assessment of Age in the Himalayan Crocodile Newt, *Tylototriton verrucosus* (Anderson, 1871) from Thailand

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ABSTRACT.– Lines of arrested growth (LAGs) were assessed in Thai population of the protected and rare Himalayan crocodile newt, *Tylototriton verrucosus* (Anderson, 1871) from Northern Thailand using skeletochronology. LAGs were clearly discernable in phalangeal bone cross-sections and could be interpreted as age. The number of LAGs in the sampled breeding population was found to be 4-8 in males (n=12) and 4 and 6 in females (n=2), consistent with previous observations that sexual maturity is reached at four years of age in both sexes. Although limited in sample size, the data suggest that males with ages over eight years are rare or absent in the breeding population studied. This non-lethal technique thus appears of potential use in ecological studies requiring non-lethal sampling of tropical urodela, including rare and or protected species.

KEY WORDS: Skeletochronology, LAGs, Tropical newt, *Tylototriton verrucosus,* Thailand

INTRODUCTION

The Himalayan crocodile newt, *Tylototriton verrucosus* (Anderson, 1871) (Urodela: Salamandridae), is a species of urodelan amphibian that is distributed mainly in the high altitudes and cold climate of the eastern Himalaya region within northeast India (Sikkim and Darjeering), Bhutan, eastern Nepal, northern Vietnam and southern China (Stuart et al., 2008), where it is in Red List Category and Criteria as least concern ver 3.1 (Stuart et. al., 2008). However, small populations exist outside this region including within the highlands of Northern Thailand, which represent the southernmost limit of its known range and where it is even rare, patchily distributed, and protected by Thai law (Nabhitabhata et al., 2000). Although some information on the habitat, breeding behavior, larval stages and parental care has been published, largely from the eastern Himalayan populations, there is both scant information on this species in its isolated habitat in Northern Thailand as well as, in particular, on its longevity, demography, survival rate, reproductive mode and breeding behavior throughout its entire range. These traits, through local selection and adaptation, are likely to vary across the species' range yet are critical to understand for implementing, for example, effective conservation measures. However, previous studies on this species have largely only reported their specific location (Smith, 1924; Taylor, 1962; Beaver, 1982) and some information on morphology and ecology (Wongratana, 1984).

Skeletochronology, especially the use of lines of arrested growth (LAGs) is widely used to determine the age in amphibians and reptiles (Castanet and Smirina, 1990; Castanet et al., 1993, 1996). However, although skeletochronology is established as a reliable method to assess age in

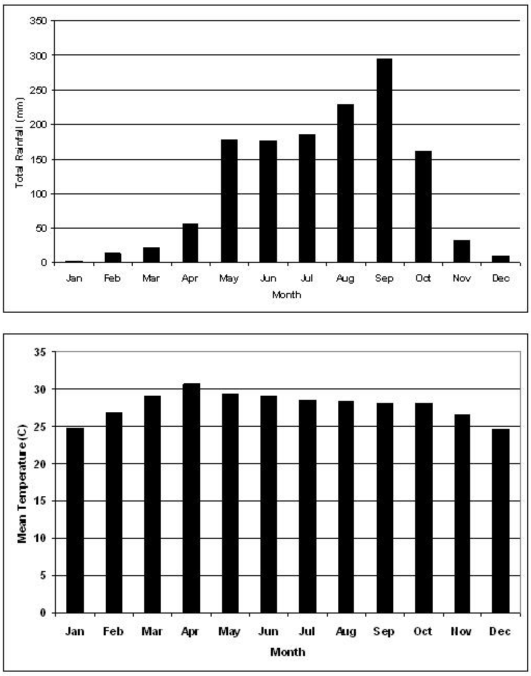

FIGURE 1. The mean total rainfall (mm) and temperature (^{o}C) of Pitsanulok Province during 1999-2009.

amphibians, it has been applied mainly to temperate species (Halliday and Verrell 1988), because hibernation or prolonged dormancy in that region. Its use in temperate amphibians for age determination has revealed how different life-history traits in populations from different altitudes or environmental contexts may be marked by differences in longevity, in age and size at sexual maturity and in the relationships between body size and growth rate (Berven, 1982; Hemelaar, 1985; Caetano and Castanet, 1993; Diaz-Paniagua and Mateo, 1999; Kutrup et al., 2005). However, LAGs are not only laid down in the long bone during hibernation in temperate regions, but also in the hash period, such as the summer season or unconditioned temperature or rainfall, in tropical or desert areas (Esteban et al., 1996; Khonsue et al., 2002; Kumbar and Pancharatna, 2004). This later point is of both interest and concern, however. Since, in tropical and subtropical regions, food availability caused by seasonal climatic change will also affect the formation of lines of arrested growth (LAGs) (Guarino et al., 1998; Kumbar and Pancharatna, 2001a,b, 2004; Lin and Hou, 2002), an annual seasonality could be expected to lead to LAGs as annual rings as in hibernating temperate amphibians and thus the general applicability of LAGs.

To this end, Kumbar and Pancharatna (2001a) confirmed that the numbers of LAGs that appear in bone cross-sections of tropical amphibians are a fair representation of the individual's age. This technique was applied to *T. verrucosus*, but only on a population from the subtropical region in Sikkim, India (Kuzmin et al., 1994), leaving open the two potential caveats of the effects of (i) different habitat and climates and associated physiological adaptations, and (ii) differences in population genetics, when considering applying this technique to the remote northern Thailand populations of *T. verrucosus*. Recently, a study on the age of *Bufo melanostictus* confirmed that LAGs were formed annually in this tropical toad (Khonsue et al., 2000, Kumbar and Pancharatna, 2004), perhaps suggesting that indeed LAG formation and correlation to subject age may be general to tropical as well as temperate amphibians. The aim of the present study was to evaluate if the skeletochronological technique could be applied to tropical urodeles without lethal dissection, using the Himalayan crocodile newt, *T. verrucosus*, population from Northern Thailand as the model system. The age and longevity of breeding population were also investigated and discussed.

FIGURE 2. Adult *Tylototriton verrucosus* (SVL about 9-10 cm).

MATERIALS AND METHODS

Study site and field method

Small ponds, surrounded by grass and high trees in a hill evergreen forest at Pitsanulok Province, Thailand (16° 59’ 17” N, 101° 00’ 05” E, Alt. 1,296 m), were observed during the 15^{th} – 16^{th} May and the 16^{th} of July 2004. The mean total rainfall (mm) and temperature (^{o}C) during 1999-2009 were shown in Figure 1. Breeding populations of *T. verrucosus* were collected from these ponds (Fig. 2) and anesthetized in 1% (v/v) solution of MS-222 (methane tricaine sulfonate). The amplexus newt and egg mass were observed in the ponds. The snout-anterior part of vent length (SVL); was measured to the nearest 0.1 mm, with a digital caliper and the 3^{rd} finger was chopped off and preserved in 10% (w/w) formalin for later analysis. The newts were then allowed to recover and kept under observation until their release the next day at the site of collection.

Skeletochronological method

The skeletochronological procedure followed Khonsue et al. (2000, 2001 and 2002). The removed fingers were washed in running water for 24 hrs, decalcified in 5% (v/v) nitric acid for 30 - 60 min, and then washed in running water for 24 hrs. The resulting phalangeal bones were then cross-sectioned (20-22 µm thick) on a freezing microtome, typically yielding 20-30 sections per bone, and the sections stained with hematoxylin (Mayer’s acid hemalum) for 30-45 min. Sections from the central region (typically ~5 sections) of the diaphysis were selected and mounted in glycerin after rinsing with tap water. Using light microscopy at 400-x magnification, the cross-sectioned phalangeal bones were screened and scored for the number of

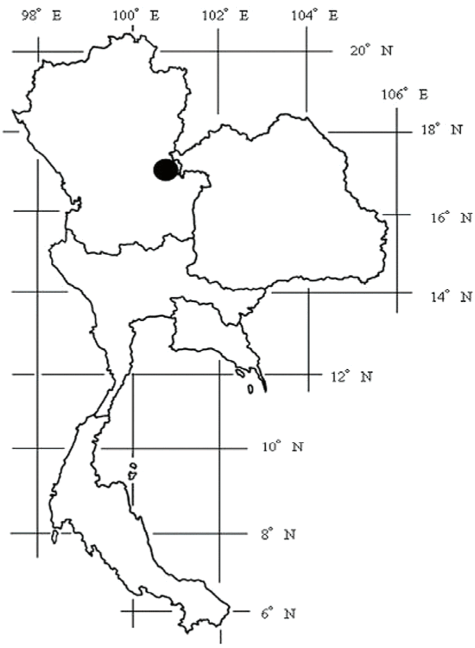

FIGURE 3. Map of Thailand. Black circle indicates Phitsanulok Province, study area.

LAGs by WK and TC. For each individual, at least 5 cross sections were scored to check for consistent reproducibility and reduce the risk of errors due to localized breakage of LAGs by endosteal resorption. The thickness of each LAG band and the distance between bands were measured using ocular micrometer taking at least 5 measurements per section. Photomicrographs were taken of representative sections.

Age determination

Lines of Arrested Growth, visualized and scored as above, were interpreted as yearly age bands of the *T. verrucosus* specimens. The rational is based on the observation of Kumbar and Pancharatna (2004) who suggested that LAGs are formed annually in the phalanges of tropical anurans, or at least in *Bufo melanostictus*.

Sex identification

The gender of each individual was determined from their breeding behavior and cloacal morphology. Males have an elongate cloaca whilst that for females is swollen and round.

RESULTS

A total of 14 specimens comprised of 12 males and 2 females were captured from the study site (Fig. 3). Their size (SVL) varied from 58.0 - 70.2 mm for males (N=12), with an average (± 1 S.D.) of 66.14 ± 4.30 mm, and from 69.4 and 80.1 mm for females (N=2).

In all examined sections, the haematoxylinophilic lines, interpreted as LAG, were observed clearly in the cross-sections (Fig. 4). LAGs were closer together near the margin of the bone opposite from the bone center and so the distance between LAG-1 and LAG-3 was much larger than that for LAG-3 to LAG-8 in both sexes (Fig. 4A, B).

The number of LAGs was found to vary from 4 - 8 for the 12 male samples, and a 4 and 6 for the two females samples (Fig. 5).

Although the sample size is very small, our data indicated that the growth rate in mature adult is very small in both sexes (Fig. 5).

DISCUSSION

In accordance with the previous reports for other tropical species (Khonsue et al., 2000; Kumbar and Pancharatna, 2004), which have suggested that the number of LAGs in tropical anurans are representative of the age of the animal. We have followed this idea in this study, but note that we have not formally established it to be true for tropical *T. verrucosus*. Rather, the ages of

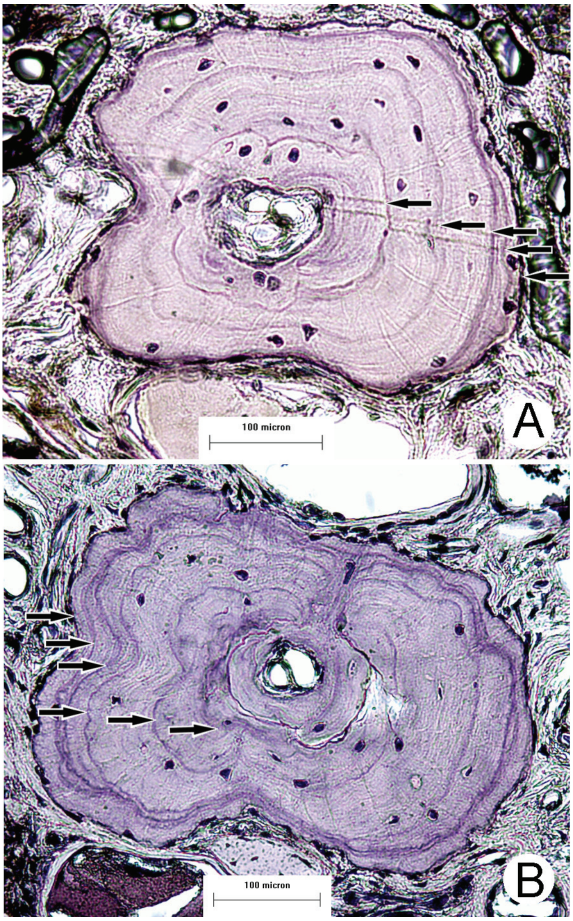

FIGURE 4. Representative phalangeal bone cross-sections from the newt, *T. verrucosus*. **A.** Adult male, 69.2 mm SVL, with five LAGs. **B.** Adult female, 80.1 mm SVL, with six LAGs. Arrows indicate the LAGs.

the newts determined by this methodology merely correlate with some known broadly age-dependent behaviors.

As reviewed in Khonsue et al. (2002), the skeletochronological method, if valid, is useful because important ecological data, such as evaluating age structures and breeding demographics at different altitudes or habitats, delayed sexual maturity, differences in longevity etc., in addition to estimating population sizes, can be obtained using only a finger or toe bone with minimum damage to the animal, and an expected absence of mortality or impairment to reproductive success. Thus, this method has been applied for several threatened and or protected anuran species, where lethal dissections to allow examination of internal morphologies would be unethical and illegal. Moreover, this method appears to be valid in not only anuran species, but in other amphibians and in both temperate and tropical habitats (Caetano and Castanet, 1993; Castanet et. al., 1996).

In this study only a small number of newts were collected (n=14), which restricts the analysis to being only preliminary for males (n=12) and excludes females (n=2). This is due to the facts that the species is both rare, limiting the number of specimens that could be found in Thailand (Nabhitabhata et al., 2000), preventing, for example, internal morphological examinations and destructive trapping and catching methods. Rather, here we just establish the likely validity of the skeletochronological analysis as a potentially non-lethal technique suitable for diverse ecological studies on rare and protected species, such as *T. verrucosus* in Thailand, which otherwise have almost no reported data on reproductive mode, breeding behavior, age structure and demography.

The appearance of LAGs in the pharangeal bone cross-sections were clear and the distance between each LAG was different. Kuzmin et al. (1994) reported that the growth rate of an animal decreases after attaining sexual maturity, which results in a decrease in the width of the LAGs in the bone. After three years of age the width of subsequent LAGS (4+ years) was decreased, in agreement with Kuzmin et al. (1994) they also reported that male and female *T. verrucosus* reach sexual maturity at around four years. Likewise, in this study samples

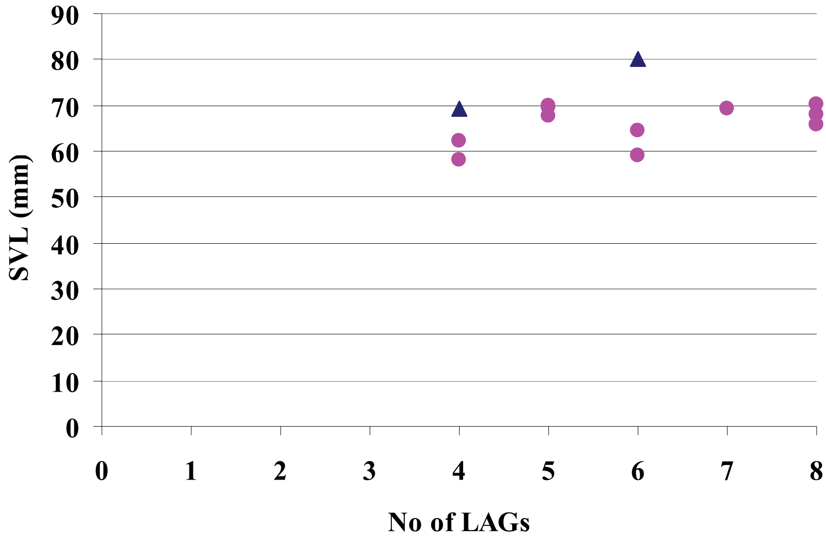

FIGURE 5. Tendency of the growth pattern of the newt, *T. verrucosus*. Circles and triangles indicate males and females, respectively.

were taken from visually observed breeding populations, and all were found to have at least four LAGs, consistent with years of age, and thus ageing ability, if this population also reaches sexual maturity at four years of age (Kuzmin et al., 1994). Thus, although not conclusive, the skeletochronology (LAGs) results are consistent with the breeding behavior observed in the field and that LAGs are laid down yearly. Indeed, after collection and overnight storage in plastic boxes prior to release the next day, they displayed clear breeding behavior and one of the two females laid eggs. However, the small sample size of this data set is insufficient to exclude, for example, a slight delayed sexual maturity in females thus accounting for their larger size, or a larger post sexual maturity growth rate than males.

Previously, the age of *T. verrucosus* has been reported to be successfully estimated by the skeletochronological technique in samples from the subtropical region in India (Kuzmin et al., 1994) whilst in the tropics the technique was reported to work on frogs such as *Rana tigrina*, *Polypedates maculatus*, *Microhyla ornata* and *Bufo melanostictus* (Kumbar and Pancharatna, 2001b, 2004). These studies confirmed that LAGs are formed in the periosteal bone and, therefore, could potentially be regarded as annual rings for estimating the age of these amphibians. Our result is the first to show the potential age estimation of newts from a tropical region by skeletochronology, using *T. verrucosus* at the lowest (warmest) range of its known distribution, at Pitsanulok Province, Thailand.

The longevity of 11 years reported by Kuzmin et al. (1994) was not observed in this study and it remains to be clarified by further sampling if this and other Thai populations do indeed have a shorter longevity, and if so why, as opposed to a stochastic artifact of a small and biased (breeding populations only) sample size and geographical variation. In addition, since we could observe only adult *T. verrucosus* at the breeding sites, it is plausible immature and older individual stay away from the breeding ponds.

We tentatively conclude that skeleton-chronology can be applied for ecological studies on protected species, or at least *T. verrucosus* from Thailand, although this requires further work for confirmation. Moreover, details on the growth pattern and both population and breeding population age structures are needed.

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