

Growth marks in natterjack toad (*Bufo calamita*) bones: histological correlates of hibernation and aestivation periods

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Skeletochronological assessment of the growth marks in the phalange bones of two natterjack populations (*Bufo calamita*) inhabiting northern Spain (Balaguer, Mas de Melons, Catalonia, 41°N) revealed that most individuals produced more than one line of arrested growth (LAG) per year, in contrast to conspecifics in other populations throughout the geographic range. Moreover, up to 19.2% of toads showed faint broad lines of reduced growth (annuli) in a given year. We developed a quantitative method based on the distinction between summer and winter growth periods in the surface area of periosteal bone produced to identify multiple LAG formation. Statistical evaluation of growth areas demonstrated that a precise distinction was feasible for individuals of a longevity up to five years. We related the number of LAGs per winter (one in 53.3% of 334 observations, two in 32.0%, three in 11.7% or four in 3.0%) to features of the local winter climate. The influence of climate (mainly temperature) accounted for 88.2% of total variation in the number of LAGs per year. Juveniles interrupted hibernation more frequently for winter growth periods than adults, indicating ontogenetic variation in hibernation behaviour. We also investigated the frequency of annuli generally produced during the summer growth period and thus representing histological traces of slowed growth during aestivation. There was no significant correlation between aestivation rates and local climate suggesting that neither ambient temperature nor moisture were key factors in inducing aestivation at the studied localities. In conclusion, the incidence and frequency of multiple growth marks per year represent informative histological correlates of local environmental impacts on life history.

Key words: annuli, environmental impact, growth pattern, life history, multiple LAGs, skeletochronology

INTRODUCTION

Since the pioneering studies on *Necturus maculosus* (Senning, 1940) and on *Rana catesbeiana* (Schroeder & Baskett, 1968) skeletochronology has become a standard procedure in estimating the age of amphibians (Castanet, 2002). Non-lethal age determination became available when Smirina (1972, 1974) demonstrated that phalanges obtained by toe-clipping provided the same information as larger tubular bones such as the femur and humerus. It is the annual alternation between the broad growth zone of periosteal bone and the narrow, densely stained line of arrested growth (LAG) that allows for a backward dating of bone samples. The precision of the age estimates, i.e. the correspondence of LAGs with the repetition of the annual cycle, has been tested for in capture–mark–recapture studies or by vital dye injections depositing time marks in the bone tissue (e.g. Smirina, 1972; Francillon, 1980; Hemelaar & van Gelder, 1980; Smirina & Makarov, 1987; Böll et al., 1997; Tejedo et al., 1997). These and other studies confirmed the formation of one LAG per year in most populations of palaeartic and subtropical species (reviews: Castanet et al., 1993; Smirina 1994; Morrison et al., 2004). Available evidence supports the hypothesis of Castanet et al. (1993) that LAG formation is ultimately caused by a genetically

based, circannual rhythm which under natural conditions becomes synchronized with, and reinforced by, the seasonal cycle.

However, exceptions to the “one LAG per year” rule have been observed occasionally. In the life history of some species, singular events such as metamorphosis represent a physiological stress strong enough to promote the formation of a supplementary line, as first observed in *Bufo bufo* (Hemelaar, 1985). The line of metamorphosis is usually easy to distinguish from regular LAGs due to its more or less diffuse nature between larval and terrestrial stage periosteal bone (e.g. *T. karelinii*, Olgun et al., 2005; *B. calamita*, *B. viridis*, Sinsch, unpubl. observations). Furthermore, abnormal climate impacts, diseases or individual starvation periods are thought to cause two instead of one LAG per year (double lines; Castanet et al., 1993) or broad annuli as a result of decreased osteogenesis (Peabody, 1958). The direct linkage of a supplementary growth mark to a specific environmental cause is mostly obscure, except for the lines or annuli in *T. marmoratus* (Caetano et al., 1985), *T. karelinii* (Olgun et al., 2005), *S. salamandra* (Alcobendas & Castanet, 2000) and *Pelobates cultripipes* (Leclair et al., 2005), which are related to aestivation. A further complication is the fact that most supplementary lines are histologically indistinguishable from LAGs with an an-

nual periodicity, and therefore they have usually been ignored (Castanet et al., 1993).

In this paper, we propose a quantitative method to identify non-annual LAGs and correlate their incidence with local climate features for an anuran amphibian. We chose the natterjack toad *Bufo calamita* because the skeletochronological features of 16 populations throughout its entire geographic range are well known and have been used for demographic life history analyses (Leskovar et al., 2006; Marangoni, 2006). The populations inhabiting central and northern Europe with cold winters and moderate summer temperatures exhibit the regular pattern of one LAG formed per year, though very rarely supplementary lines occur in a few individuals in some populations, including Germany (Schnäbele, 1987; Sinsch, 1998; Große, 1999; Leskovar et al., 2006), Belgium (Stevens et al., 2003), England (Denton & Beebee, 1993; Banks et al., 1993) and Ireland (Gibbons & McCarthy, 1983). The same seems to occur in southern Spain (Andalusia: Tejedo et al., 1997; Marangoni, 2006). In contrast, supplementary LAG formation is common in two populations inhabiting the northern part of the Iberian Peninsula with Mediterranean mild winters and hot summers (Catalonia: Leskovar et al., 2006). The skeletochronological features of 96 individuals of these peculiar populations form the database for this paper. Specific aims are 1) to provide a quantitative method of distinguishing histologically regular summer growth periods from occasional growth events during short warm winter periods, and 2) to identify the relationship between the presence and quantity of supplementary growth marks and local climate features.

MATERIALS AND METHODS

Study areas

Populations of the natterjack toad *Bufo calamita* were studied in the province of Lleida in Catalonia (Spain) at Balaguer and Mas de Melons. Climate is Mediterranean and moderately continental with average summer temperatures of 24.7 °C (July) and winter temperatures of 5.3 °C (January, meteorological station: Lleida – Observatorio 2, 192 m a.s.l., period 1971–2000; Instituto Nacional de Meteorología, pers. comm.). The average number of days with temperatures below 0 °C was 37 per year. Total annual precipitation averaged 369 mm.

The Balaguer study site (41°46′30″N, 0°46′50″E, 240 m a.s.l.) is located about 20 km north of the city of Lleida and included clay and gravel pits within arable land. Toads reproduced in many ephemeral water bodies that were distributed within the winter cereal fields. The behavioural ecology of natterjacks in this area has been described previously (Miaud et al., 2000; Miaud & Sanuy, 2005). The Mas de Melons study site (41°30′50″N, 0°43′30″E, 240 m a.s.l.) is located about 15 km south-east of the city of Lleida and included pasture areas and arable land (winter cereal fields). Toads reproduced in a permanent eutrophic cattle pond and in ephemeral water bodies in the vicinity of the pond.

Sampling procedure and skeletochronological analysis

A total of 96 adult toads were collected during the reproduction period at the breeding pond: 1) Balaguer: 34 males and 14 females (23 March–1 April 2004); 2) Mas de Melons: 44 males and 4 females (21–23 April 2004). The variation in sex ratio is due to the fact that collecting surveys were not exhaustive and thus samples represent a random sample of the local breeding assemblage. Toads were released again *in situ* following sex determination, measuring of snout–vent length (SVL, to the nearest mm), and toe-clipping (third or fourth toe of the right hindlimb). The toes were stored in 70% ethanol at room temperature.

Laboratory protocols followed the standard methods of skeletochronology (Smirina, 1972; Kuhn, 1994; Leskovar et al., 2006). Samples were embedded in Historesin™ (Jung) and stained with cresylviolet (details in Sinsch et al., 2001). The diaphysis was cross-sectioned at 10 µm using a Jung RM2055 rotation microtome. Cross-sections were examined using light microscopy at magnifications of 200× using an Olympus BX 50. A Sony Cybershot DSC-T1 was used to take digital images from those diaphysis sections in which the size of the medullar cavity was at its minimum and that of bone at its maximum.

One digital photo per individual was selected for further analysis. Images were optimized with respect to contrast and intensity using Corel Photo House, version 1.10.072, to facilitate the distinction of lines stained within the periosteal bone from the surrounding areas. Besides the line of metamorphosis, two categories of growth marks were distinguished (Fig. 1): 1) narrow, strongly stained lines which sometimes formed closely neighbouring groups, i.e. LAGs in *sensu stricto*; 2) broad, faintly stained, often interrupted annuli of low osteogenesis (slow bone growth) adopting the terminology proposed by Peabody (1958). Image files were transferred to UTHSCSA ImageTool, version 3.0, and calibrated using a standard micrometer. The area of periosteal bone between two neighbouring LAGs was measured to the nearest µm² to obtain a two-dimensional estimate for magnitude of three-dimensional bone growth. The size of bone produced during the first uninterrupted activity period of life (aquatic + terrestrial period until first hibernation) was quantified as the area delimited by the first LAG, i.e. including the medullar cavity. Growth areas were plotted successively from the innermost to the outermost one to distinguish those produced during summer from those produced during winter (the newly developed method is described in detail in the results). Based on this distinction, the data set obtained for each locality included the variables sex, age (as number of hibernations) and number of LAGs per hibernation in five winters (1999–2000, 2000–01, 2001–02, 2002–03, 2003–04) with 48 observations (= individual toads) each. The presence and number of annuli were recorded for the three outermost summerly growth periods, e.g. for 2001, 2002 and 2003.

Climate records

Local microclimate data (1999–2004) were obtained from the meteorological station Vallfogona de Balaguer

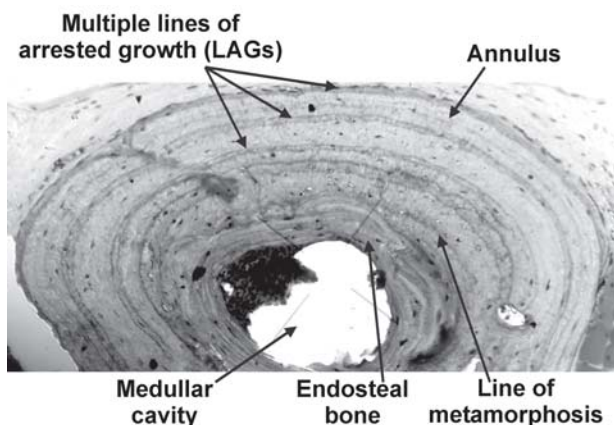


Fig. 1. Stained cross section of the phalange bone of male 94 from Mas de Melons. Arrows indicate the types of growth marks commonly observed in natterjack toads of populations from northern Spain.

(41.785°N, 0.829°E; 245 m a.s.l.) close to the Balaguer study site and from the station Castellidans (41.53°N, 0.754°E; 225 m a.s.l.) close to the Mas de Melons study site. As the activity of natterjack toads is influenced mainly by the ambient temperature and moisture, we focused analysis on seven variables: 1) daily average air temperature, 2) daily maximum air temperature, 3) daily minimum air temperature, 4) number of days per month with temperatures below zero, 5) number of days per month with precipitation, 6) monthly sum of precipitation (mm), and 7) evapotranspiration.

Based on these daily records we calculated local averages or sums for each yearly period that we considered as “winter” or “summer”. Inspection of the annual variation of temperature suggested that a period beginning on 1 October and ending on 31 March of the following year covered the total duration of each winter, while a period beginning on 1 May and ending on 30 August included all days with continuously high temperatures, i.e. summer. Consequently, we obtained a winter data set which included seven variables (the six-month averages of the three temperature variables, the total number of days with frost and precipitation, the sum of total winter precipitation and the total evapotranspiration during winter) with 10 observations (five winters at two localities) each. The summer data set included six variables (the four-month averages of the three temperature variables, the total number of days with precipitation, the sum of total precipitation and the total evapotranspiration during summer) with six observations (three summers at two localities) each.

Statistical analyses

Means are always given with the corresponding 99% confidence interval following Bonferroni correction and compared using a one-way ANOVA and a complementary multiple range test. The potential effects of sex, age, locality and year on the number of lines of arrested growth per winter were estimated using a 4-factor MANOVA. A multiple linear regression model (procedure: backward

selection; F-to-remove = 4) was used to delimit a prediction model for the number of LAGs per winter based on meteorological variables (see section on climate record). The single factor accounting for most of the variance explained by meteorological variables was determined using single linear regression and Pearson’s product-moment correlation analysis. As the scarcity of data of the summer data set did not permit a multiple regression analysis, we tested for correlations between the frequency of individuals with annuli and the meteorological variables in simple regression analyses. Significance level was set at $\alpha=0.05$. All calculations were based on the procedures of the program package Statgraphics Plus for Windows, version 5.0.

RESULTS

Patterns of LAG, annuli and growth zone formation

The number of narrow hematoxylinic lines per toad, i.e. LAGs, varied between two and 10 at Balaguer, and between three and 10 at Mas de Melons; the number of faint and often broad growth marks (annuli) between two LAGs was 0–2 (Fig. 1). The line of metamorphosis was visible in several individuals (e.g. Fig. 1). In those specimens in which it had been replaced during the formation of endosteal bone, at least 80% of the first LAG remained unaffected. Whereas the larger size of innermost growth zone (i.e. the bone produced during the larval development and terrestrial life during the first hibernation) is a general pattern in all studied individuals, there is a large variation in the area of subsequent growth zones (see Fig. 2 for a sample of four individuals). The latter growth pattern is the spatial equivalent to the linear grouping of LAGs to units of several lines with very short distances among each other and larger distances to the next line or group of lines (e.g. Fig. 1).

Quantitative identification method of summer and winter growth periods

Until presently, the linear proximity of lines has been used as the only criterion to distinguish multiple lines deposited within one year from successive annual LAGs representing different years (e.g. Francillon-Vieillot et al., 1990; Castanet et al., 1993; Smirina, 1994). However, distances between LAGs as a linear measure may constitute misleading estimators of the three-dimensional annual bone growth intensity because a short distance between lines in the peripheral section corresponds to a larger volume of bone than the same distance measured close to the medullar cavity. Consequently, we propose an improved method to identify multiple LAG formation in a single year by comparing the size of successive growth areas (= two-dimensional estimate) instead of distances between LAGs.

In amphibians growth may occur anytime, provided that body temperature remains within a suitable range and sufficient nutrients are available. Thus, mild winter periods as well as the continuum of spring, summer and fall may provide suitable growth conditions for natterjacks. The criterion for the assignment of bone growth periods

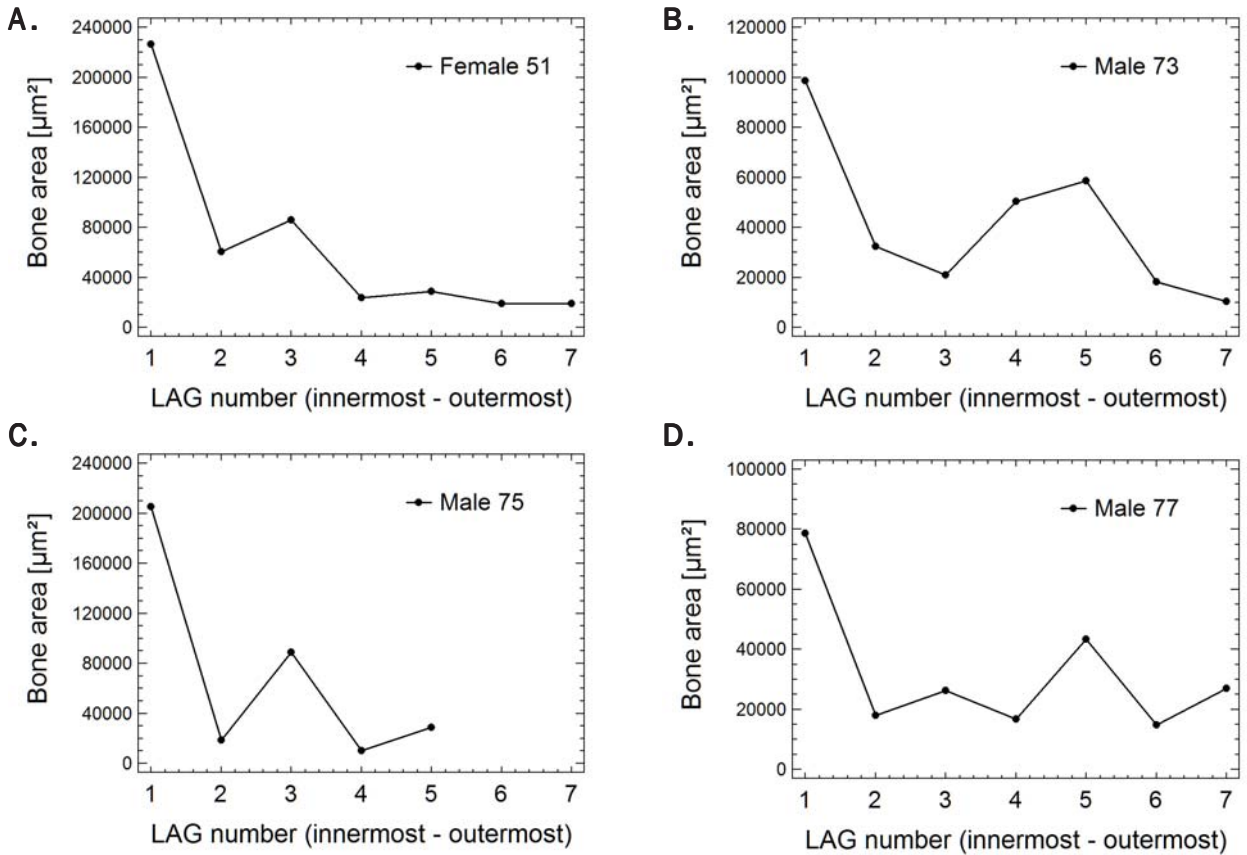


Fig. 2. Growth pattern (area of periosteal bone) of four individuals of the Mas de Melons population. Note that the area delimited by LAG 1 also includes the medullar cavity.

to either summer continuum or winter is size (= surface area visible in cross sections) in comparison to that of later growth periods. Specifically, if the area of any later growth period was at least twice as large, the smaller one was considered to be the result of a winter growth period. The rationale for this criterion is the fact that even considering the age-dependent decrease of growth rate and year-to-year variations in climate and food availability, the area of periosteal bone produced during summer (6 months) should considerably exceed that deposited during warm winter periods (2–3 weeks at most at the two studied localities).

Applying this quantitative method to the cross-sections of the 96 individuals studied, we found that the individual differences in total bone size and growth rates accounted for a wide intrapopulation variability in the size of the innermost area delimited by the first distinguishable LAG (Fig. 2). In order to reduce this scatter, we standardized data by calculating the size of successive bone areas relative to area of the first growth period. For example, a relative growth area size of less than one means that its absolute area was smaller than that of the innermost one. In a first step, we applied this analysis exploratively to the central region of the bone cross-sections because growth rates during the first two years of life are maximum and winter growth events should be easiest to detect. In fact, we found two types of growth pattern which differed significantly amongst each other (ANOVA; $F_{2,85}=440.8$, $P<<0.00001$): the relative bone size produced during the second growth period of life was ei-

ther $48\pm 4\%$ of that of the first one (summer growth period), or $14\pm 4\%$ (winter growth period). The number of successive short growth periods varied between one and three. The areas of these growth zones (1a–c in Fig. 3A) did not differ significantly (ANOVA; $F_{2,27}=0.14$, $P>0.05$), neither did the area of a long growth periods either following immediately the first one or one or more previous short growth periods (2 and 2' in Fig. 3A; ANOVA; $F_{1,27}=1.01$, $P>0.05$). Consequently, in the second step of analysis we pooled successive short growth periods between two long growth periods and equivalent long growth periods (Fig. 3B). During the first five years of life the bone area produced during winter growth periods was significantly smaller than the previous and following areas resulting from summer growth (ANOVA, multiple range test; $P<0.01$). All later growth periods added too little bone area to distinguish among summer and winter periods. Thus, multiple lines formed during the first four winters of a toad's life can be detected precisely by applying the quantitative, area-based method presented here.

Prior to the quantitative analysis of growth patterns, two of the authors visually inspected all cross-sections and independently assigned LAGs to either annual growth interruptions or to multiple lines. This qualitative assignment yielded a 95% correspondence to the quantitative one. However, the quantitative method was more sensitive in older individuals with multiple lines in the outer perimeter of the bone where the distance between LAGs becomes small. Consequently, we conclude that despite the larger number of LAGs the toads' age varied

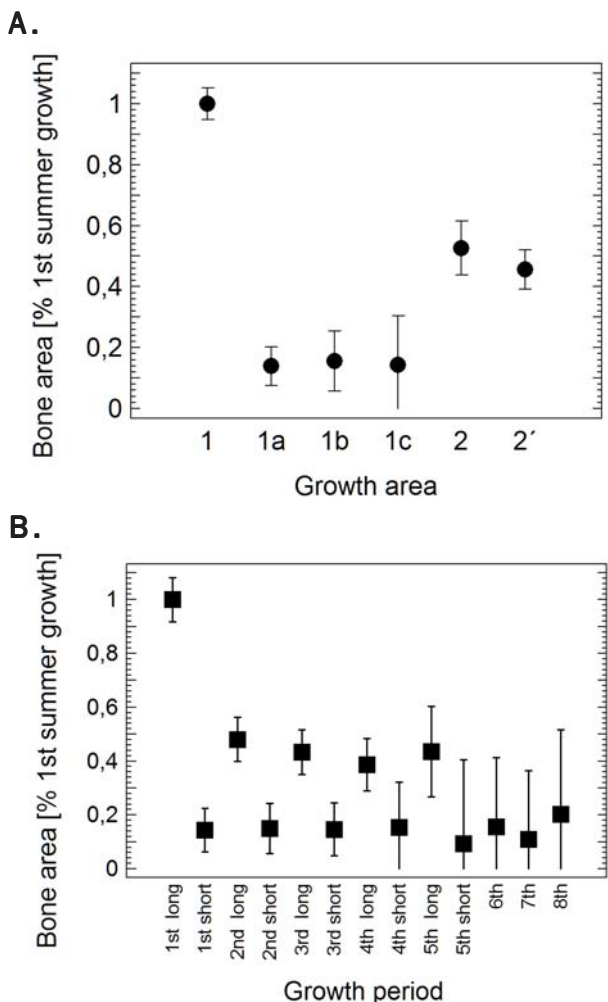


Fig. 3. Standardized growth pattern (size of growth areas is given relative to the area of periosteal bone and medullar cavity enclosed by the the first LAG) of all studied natterjack toads. A) Size of the innermost successive growth areas: 1 = 1st uninterrupted growth period; 1a, 1b, 1c = 1st, 2nd or 3rd short growth period following 1; 2 = long growth period directly following 1; 2' = long growth period following 1 and 1-3 small growth areas. B) Size of all successive growth areas: short growth periods between two long growth periods are pooled. Data are presented as means and corresponding 99% confidence interval.

Table 1. Multifactorial analysis on the sources of variation in the number of lines produced per hibernation. Age = number of hibernations before the winter in study.

Source	Sum of squares	df	Mean sum of squares	F	P
Main effects					
Sex	0.001	1	0.001	0.00	>0.05
Age	8.193	5	1.639	3.17	0.0083
Locality	5.609	1	5.609	10.85	0.0011
Year	30.771	4	7.693	14.87	<0.0001
Residual	164.457	318	0.518		
Total (corrected)	211.518	329			

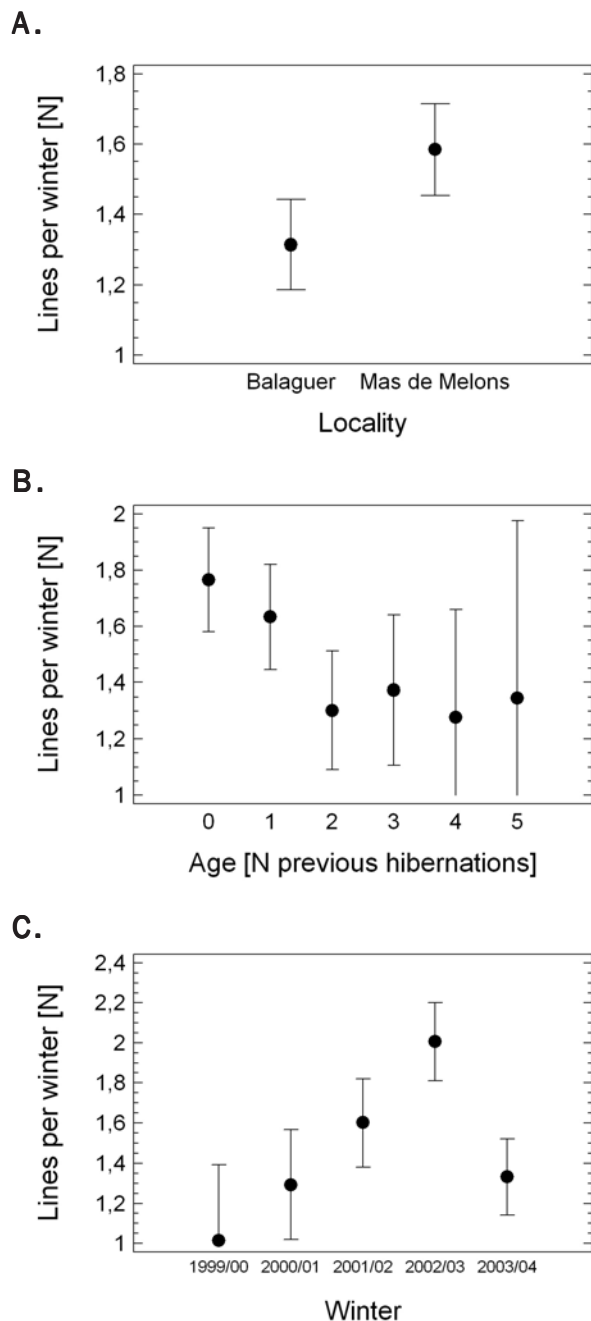


Fig. 4. Multifactorial ANOVA of the frequency of multiple line production in natterjack toads. Statistical details are given in Table 1. A) Local variation; B) variation attributed to the age at hibernation; C) variation among five successive winters. Data are presented as means and corresponding 99% confidence intervals following Bonferroni correction.

between two and seven years at Balaguer, and between two and eight years at Mas de Melons (Leskovar et al., 2006).

Factors influencing the number of lines per winter

The number of hematoxylinic lines per annual hibernation period varied between one and four at both localities, i.e. hibernation was interrupted by three short growth periods at most. A multifactorial ANOVA with the

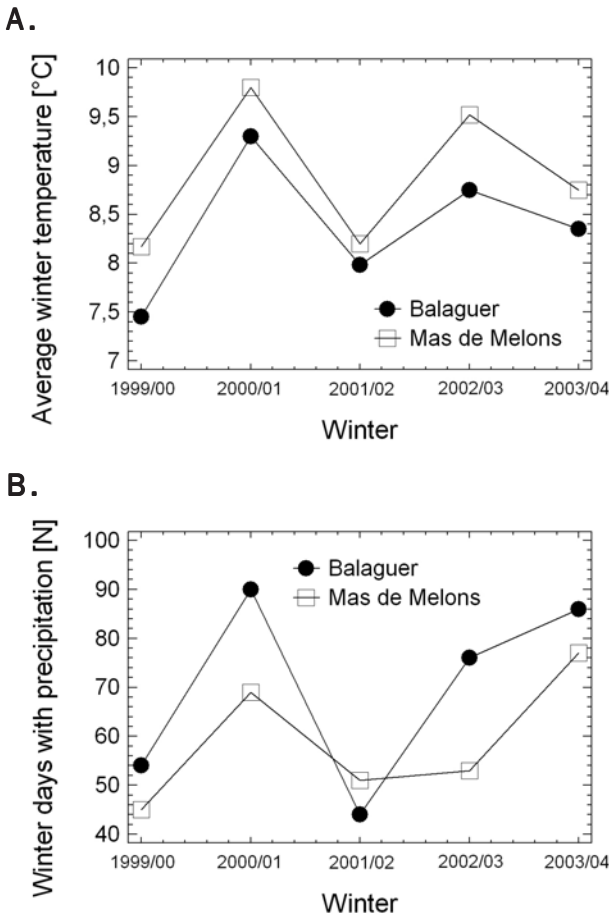


Fig. 5. Local meteorological features during winter (defined as the period October to March of the following year). A) Average air temperature at height of 2 m; B) number of days with precipitation.

Table 2. Prediction model for the number of lines produced per hibernation obtained using multiple regression analysis (procedure backward selection). Age = number of hibernations before the winter in study. a) Regression model, R^2 (adjusted for df) = 88.2%; b) ANOVA.

a)

Parameter	Estimate±SE	T statistic	P
CONSTANT	-4.01±1.21	-3.32	0.0295
Frost days (N)	0.02±0.01	2.50	0.0671
Temperature (max)	1.69±0.34	5.06	0.0072
Temperature (min)	2.85±0.54	5.24	0.0063
Average temperature	-3.36±0.71	-4.76	0.0090
Precipitation (mm)	-0.005±0.001	-4.78	0.0088

b)

Source	Sum of squares	df	Mean sum of squares	F	P
Model	0.948	5	0.190	14.45	0.0114
Residual	0.053	4	0.013		
Total (corr.)	1.001	9			

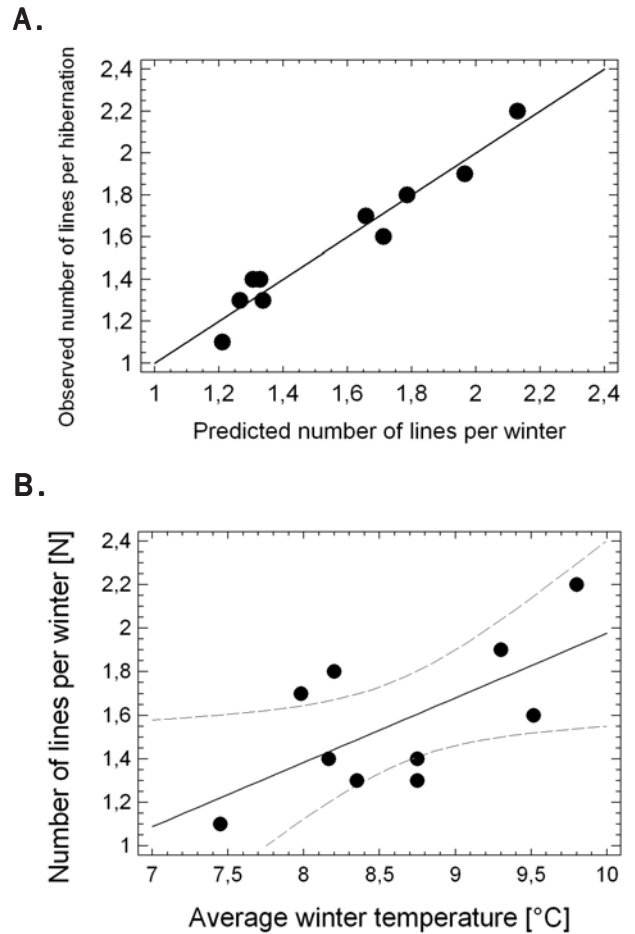


Fig. 6. Multiple regression analysis of the covariation of meteorological variables and the number of multiple lines. Statistical details are given in Table 2. (A) Fit of the multiple regression model; (B) Simple linear regression model of the most influential single factor temperature (outer lines represent the 95% confidence interval of the regression line).

independent factors sex, age, locality and winter showed that only sex did not affect the quantity of multiple lines (Table 1). The average number of lines was significantly greater at Mas de Melons (1.6) than at Balaguer (1.3, Fig. 4A). The average number of lines per hibernation was significantly larger in younger stages (i.e. less than two years old, juvenile and subadult toads) than in adult stages (1.6–1.8 vs approximately 1.3, Fig. 4B). The average number of lines corresponding to the five hibernation periods considered in this analysis varied between 1 (1999/2000) and 2 (2002/03, Fig. 4C).

Local climate during winter varied among the years with respect to temperature and to precipitation (Fig. 5). At the Balaguer locality winter was always colder and mostly more humid than at the Mas de Melons site. The best subset of the seven meteorological variables used for a multiple regression analysis included four temperature variables and total precipitation during winter (Table 2). The variability of these variables accounted for 88.2% of total variance in the average number of multiple lines, i.e. model prediction and observed values agreed widely (Fig. 6A). Average air temperature accounted for most of

Table 3. Frequency of LRG deposition per year and population and local microclimate during summer (May–August). Temperatures are given as arithmetic means for the summer period. BA = Balaguer, MM = Mas de Melons.

Parameter	2001		2002		2003	
	MM	BA	MM	BA	MM	BA
Frequency (%) of individuals with:						
1 annulus	0	0.108	0.149	0.1042	0.043	0.125
2 annuli	0	0	0.043	0	0.023	0.063
Rain days (N)	23	27	23	27	22	24
Precipitation (mm)	99.4	103	112	82.8	148.1	149.7
Evapotranspiration	676.3	542.5	474.9	912.1	556.6	593.3
Temperature (max)	29.9	29.7	28.7	28.6	31.9	31.8
Temperature (min)	14.2	15.2	14.0	14.5	15.6	16.4
Average temperature	21.9	22.3	21.2	21.4	23.6	23.9

the variation in LAG number and explained 43.2% of total variation (linear regression model: $n \text{ LAGs} = 0.986 + 0.296 \cdot \text{temperature}$; Fig. 6B).

Periods of reduced growth during summer

Occasionally, one or two annuli of reduced growth were visible within the summer growth period (Fig. 1). Annuli were easily distinguishable from LAGs because they always stained more faintly and were often broader (similar to those in *Pelobates cultripedis*; Leclair et al., 2005). The frequencies of individuals producing annuli ranged between 0 and 19.2% per population and year (Table 3). In the toads of the Balaguer population, annulus deposition was more frequent than in those of the Mas de Melons population. In neither population was a significant correlation between the frequency of annuli and any of the local climate variables detected (regression analyses, $P > 0.05$).

DISCUSSION

During the past decades of skeletochronological age assessment in amphibians, the presence of “double lines”, or more precisely “multiple lines”, has mostly been considered an occasional, enigmatic and disturbing deviation from the rule that the equivalent of an annual growth period is a single broad radial area of periosteal bone plus a line of arrested growth. The rule describes correctly the situation in populations of species living in temperate climates with a markedly seasonal annual cycle with either harsh winters or hot and dry summers (cause: genetically based rhythm plus environmental reinforcement; Castanet et al., 1993; Smirina, 1994), or in subtropical climates in which growth is not constrained by environmental conditions (cause: genetic base; Morrison et al., 2004). In this paper we present the first quantitative area-based method to distinguish between multiple lines per year and annual LAGs and explore their ecological significance for the life history cycle.

Validation of method to distinguish summer and winter growth periods

The validation of the identification method requires the assessment of the growth pattern in the tubular bone of

anurans that follow the “one LAG per year” rule as the baseline condition. It is generally agreed that 1) bone formation does not cease completely in wild-ranging amphibians that die long before reaching senescence (e.g. *Bufo calamita*, Leskovar et al., 2006, Sinsch et al., 2006), 2) the magnitude of bone formation decreases with age and 3) sexual maturity leads to a sharp decrease in bone formation (“rapprochement”; Kleinenberg & Smirina, 1969; Hemelaar & van Gelder, 1980; Castanet et al., 1993; Smirina, 1994). Numerous studies used the diameters of LAGs as a surrogate measure of bone growth (e.g. Alcobendas & Castanet, 2000; Olgun et al., 2005). The result of these measurements is a growth curve showing a linear to asymptotical relationship between diameter and age. However, bone growth is three-dimensional, from which the diameter method captures merely one axis. As the effort needed to estimate spatial bone growth would be disproportionate to the information obtained, we refined the linear method to the area measurements presented here, which cover at least two of three dimensions of growth. These measurements require a single cross-section of the diaphysis as before, but PC-based image analysis programs permit an easy assessment of the area of periosteal bone deposited each year. So, we can identify the baseline condition as an (often exponentially) decreasing relationship between bone area produced in successive years and age (e.g. Fig. 2A). Deviations from this condition are considered manifestations of environmental impacts that, instead of reinforcing, are superimposed on the annual periodicity. Of course, there are detection limits. In the case of the natterjack populations studied here, deviations from the basic pattern were possible with a reasonable precision in those individuals whose longevity was five years at most.

A curious by-product of the development of the quantitative method is the confirmation that an experienced observer can identify about 95% of winter growth periods by simple visual inspection of the cross section, i.e. without any quantitative measurement. However, the mismatches revealed two weaknesses of qualitative visual inspections: 1) because of the fast growth during the first two years of life, distances between LAGs delimit-

iting winter growth events were sometimes relatively large and multiple lines were confounded with annual LAGs; 2) in some older individuals (more than three years old), the distances between LAGs become so small that even significant differences in bone area were not recognizable. Thus, here we provide a useful tool for interpreting growth patterns that works without previous experience in skeletochronology.

Ecological significance of supplementary growth marks

The populations studied here and a further one in the same region (Monegros; Sanuy & Oromi, unpubl. observations) differ from all other natterjack populations in the unusual incidence of multiple growth periods delimited by LAGs within a single year (Leskovar et al., 2006). Multiple LAGs with up to four discernable lines have also been observed in a high-altitude population of the newt *Triturus marmoratus* (Fig. 4A,B in Caetano et al., 1985). The number of LAGs per year and their frequency decreased from high to low altitude and from north to south in the Portuguese populations of this newt (Caetano & Castanet, 1993). Regular formation of two lines per year occurred also in Turkey in the newts *Mertensiella luschani* (Olgun et al., 2001) and *Triturus karelinii* (Olgun et al., 2005). If the frequent observation of multiple line formation at Mediterranean localities (northern Portugal and Spain, western Turkey) is not merely by chance, specific climate features may be the common explanation. Our multifactorial analysis indeed suggests that mild Mediterranean winters often permit natterjacks to interrupt hibernation for short growth periods. Future experimental manipulation and subsequent placing of time marks (dye injections) will prove whether or not the correlation between average winter temperature and multiple line formation is based on a causal relationship, as proposed. If our interpretation of the natterjack growth pattern at the Catalonian localities is correct, the main environmental modification of growth pattern occurs during winter, unlike the situation in *T. marmoratus* inhabiting high altitudes in which summer growth seems to be affected (Caetano et al., 1985).

In natterjacks, we also found a weak histological correlate of aestivation during summer; however, unlike in *T. marmoratus*, faint lines of reduced growth (LRGs) were occasionally formed. The histological appearance of natterjack annuli is obviously more similar to that reported in *T. karelinii* for aestivation growth marks (Olgun et al., 2005) than in *T. marmoratus* (Caetano et al., 1985). While multiple LAG formation was closely related to winter climate features, no clear association between annulus formation and hot summer periods was detectable. This may have multiple causes: local differences in food availability, land use and/or differential thermal qualities of shelter sites such as rock fissures, rabbit burrows and shallow self-dug holes. Thus, the available data on summer climate may not be representative of the microclimate at toad refugia.

Correlation analysis indicates that the hibernation behaviour of the natterjacks at the two localities studied undergoes an ontogenetic change. The frequency of win-

ter growth periods was considerably greater in juveniles than in reproductive adults, which rarely interrupted hibernation, suggesting that they prefer different winter refugia. Juveniles seem to hibernate at sites that are more exposed to local temperature variations and may respond with little delay to warmer winter periods permitting food uptake and growth. Thus, the annual period of growth and, consequently, size at maturity can be increased. The large size of Iberian natterjacks may be a result of this peculiar hibernation strategy, and as size is directly related to fecundity in females, fitness appears to be optimized as well (Sinsch, 1998; Leskovar et al., 2006).

In conclusion, the formation of multiple growth marks in a single year offers valuable insights into local modifications of the life history strategies of ecologically plastic amphibians such as the natterjack toad (Leskovar et al., 2006; this study). The incidence and frequency of multiple growth marks should no longer be considered as a rare aberration of growth pattern, but as an informative histological correlate of local environmental impacts.

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REFERENCES

- Alcobendas, M. & Castanet, J. (2000). Bone growth plasticity among populations of *Salamandra salamandra*: interactions between internal and external factors. *Herpetologica* 56, 14–26.
- Banks, B., Beebee, T.J.C. & Denton, J. S. (1993). Longterm management of a natterjack toad (*Bufo calamita*) population in southern Britain. *Amphibia-Reptilia* 14, 155–168.
- Böll, S., Erben, R.G. & Linsenmair, K.E. (1997). Wie zuverlässig ist die skeletochronologische Altersbestimmung bei der Geburtshelferkröte *Alytes obstetricans*? *Mertensiella* 7, 315–327.
- Caetano, M.H. & Castanet, J. (1993). Variability and microevolutionary patterns in *Triturus marmoratus* from Portugal: age, size, longevity and individual growth. *Amphibia-Reptilia* 14, 117–129.
- Caetano, M.H., Castanet, J. & Francillon, H. (1985). Détermination de l'âge de *Triturus marmoratus marmoratus* (Latreille 1800) du Parc National de Peneda Gerês (Portugal) par squelettechronologie. *Amphibia-Reptilia* 6, 117–132.
- Castanet, J. (2002). Amphibiens et reptiles non aviens: un matériel de choix en squelettechronologie. *Bulletin de la Société Herpétologique de France* 103, 21–40.
- Castanet, J., Francillon-Vieillot, H., Meunier, F.J. & Ricqlès, A. (1993). Bone and individual aging. *Bone, Vol. 7. Bone Growth*, 245–283. Hall, B.K. (ed.). Boca Raton: CRC Press.
- Denton, J.S. & Beebee, T.J.C. (1993). Densityrelated features of natterjack toad (*Bufo calamita*) populations in Britain. *Journal of Zoology* 229, 105–119.

- Francillon, H. (1980). Mise en évidence expérimentale du caractère annuel des lignes d'arrêt de croissance (LAC) chez le triton crêté, *Triturus cristatus* (LAUR.). *Bulletin de la Société Zoologique de France* 105, 343–347.
- Francillon-Vieillot, H., Arntzen, J.W. & Geraudie, J. (1990). Age, growth and longevity of sympatric *Triturus cristatus*, *T. marmoratus* and their hybrids (Amphibia, Urodela): a skeletochronological comparison. *Journal of Herpetology* 24, 13–22.
- Gibbons, M.M. & McCarthy, T.K. (1983). Age determination of frogs and toads (Amphibia, Anura) from northwestern Europe. *Zoologica Scripta* 12, 145–151.
- Große, W.R. (1999). Altersbestimmung bei mitteleuropäischen Amphibien mittels Skeletochronologie am Beispiel der Kreuz-, Erd- und Wechselkröte (Anura, Bufonidae). *Elaphe* 7, 73–76.
- Hemelaar, A.S.M. (1985). An improved method to estimate the number of year rings resorbed in phalanges of *Bufo bufo* (L.) and its application to populations from different latitudes and altitudes. *Amphibia-Reptilia* 6, 323–342.
- Hemelaar, A.S.M. & Gelder, J.J.V. (1980). Annual growth rings in phalanges of *Bufo bufo* (Anura, Amphibia) from the Netherlands and their use for age determination. *Netherlands Journal of Zoology* 30, 129–135.
- Kleinenberg, S.E. & Smirina, E.W. (1969). A contribution to the method of age determination in amphibians. *Zoologicheskij Zhurnal* 48, 1090–1094.
- Kuhn, J. (1994). Lebensgeschichte und Demographie von Erdkrötenweibchen *Bufo bufo bufo* (L.). *Zeitschrift für Feldherpetologie* 1, 3–87.
- Leclair, M.H., Leclair, R. & Gallant, J. (2005). Application of skeletochronology to a population of *Pelobates cultripes* (Anura: Pelobatidae) from Portugal. *Journal of Herpetology* 39, 199–2007.
- Leskovar, C., Oromi, N., Sanuy, D. & Sinsch, U. (2006). Demographic life history traits of reproductive natterjack toads (*Bufo calamita*) vary between northern and southern latitudes. *Amphibia-Reptilia* 27: 365–375.
- Marangoni, F. (2006). Variación clinal en el tamaño del cuerpo a escala microgeográfica en dos especies de anuros (*Pelobates cultripes* y *Bufo calamita*). PhD thesis, Seville, Spain: University of Seville.
- Miaud, C., Sanuy, D. & Avrillier, J.N. (2000). Terrestrial movements of the natterjack toad *Bufo calamita* (Amphibia, Anura) in a semi-arid, agricultural landscape. *Amphibia-Reptilia* 21, 357–369.
- Miaud, C. & Sanuy, D. (2005). Terrestrial habitat preferences of the natterjack toad in a landscape of intensive agricultural activity. *Amphibia-Reptilia* 26, 359–366.
- Morrison, C., Hero, J.M. & Browning, J. (2004). Altitudinal variation in the age at maturity, longevity, and reproductive lifespan of anurans in subtropical queensland. *Herpetologica* 60, 34–44.
- Olgun, K., Miaud, C. & Gautier, P. (2001). Age, size and growth of the terrestrial salamander *Mertensiella luschani* in an arid environment. *Canadian Journal of Zoology* 79, 1559–1567.
- Olgun, K., Uzum, N., Avci, A. & Miaud, C. (2005). Age, size and growth of the southern crested newt *Triturus karelinii* (Strauch 1870) in a population from Bozdag (western Turkey). *Amphibia-Reptilia* 26, 223–230.
- Peabody, C.E. (1958). A Kansas drought recorded in growth zones of a bullsnake. *Copeia* 1958, 91–94.
- Schnäbele, H. (1987). Grundlagen der Altersbestimmung durch Skeletochronologie bei Kreuzkröten (*Bufo calamita*) und Wechselkröten (*Bufo viridis*). Ein Beitrag zum Schutz bedrohter Tierarten. *Beihefte Veröffentlichungen Naturschutz Landschaftspflege Baden-Württemberg* (Karlsruhe) 41, 485–497.
- Schroeder, E.E. & Baskett, T.E. (1968). Age estimation, growth rates and population structure in Missouri bullfrogs. *Copeia* 1968, 538–592.
- Senning, W.C. (1940). A study of age determination and growth of *Necturus maculosus* based on parasphenoid bone. *American Journal of Anatomy* 66, 483–495.
- Sinsch, U. (1998). Biologie und Ökologie der Kreuzkröte. Bochum, Laurenti Verlag.
- Sinsch, U., di Tada, I.E. & Martino, A. (2001). Longevity, demography and sex-specific growth of the Pampa de Achala toad, *Bufo achalensis* CEI, 1972. *Studies on Neotropical Fauna and Environment* 36, 95–104.
- Sinsch, U., Lehmann, H. & Geiger, A. (2006). Zur potenziellen und realisierten Lebenserwartung von Kreuzkröten (*Bufo calamita*). *Zeitschrift für Feldherpetologie* 13: 101–112.
- Smirina, E.M. (1972). Annual layers in bones of *Rana temporaria*. *Zoologicheskij Zhurnal* 51, 1529–1534.
- Smirina, E.M. (1974). Prospects of age determination by bone layers in Reptilia. *Zoologicheskij Zhurnal* 53, 111–117.
- Smirina, E.M. & Makarov, A.N. (1987). On ascertainment of accordance between the number of layers in tubular bones of amphibians and the age of individuals. *Zoologicheskij Zhurnal* 66, 599–604.
- Smirina, M.E. (1994). Age determination and longevity in amphibians. *Gerontology* 40, 133–146.
- Stevens, V.M., Wesselingh, R.A. & Baguette, M. (2003). Demographic processes in a small, isolated population of natterjack toads (*Bufo calamita*) in southern Belgium. *Herpetological Journal* 12, 59–67.
- Tejedo, M., Reques, R. & Esteban, M. (1997). Actual and osteochronological estimated age of natterjack toads (*Bufo calamita*). *Herpetological Journal* 7, 81–82.

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