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DIFFERENTIAL GROWTH AND LONGEVITY IN LOW AND HIGH ALTITUDE *RANA IBERICA* (ANURA, RANIDAE)

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Age, body size and histological bone growth were analysed in two populations of *Rana iberica* that were genetically similar and which represented the altitudinal extremes of the species range. In spite of having a much longer hibernation, mountain frogs were significantly bigger (snout-vent lengths) for all ages, with the exception of the pre-maturity period. Mountain frogs were longer-lived (oldest females 6 years, males 5 years, one male outlier 9 years) than lowland frogs, where at most only one individual of each sex attained the age of 4 years. The minimum age at sexual maturity was 2 years for both sexes and populations. For both sexes, and using different assumptions about the duration of the period of seasonal activity, the relative contribution of the growth rate component seems slightly higher (46-75 % for females, 60-82 % for males) than differences in life span (i.e. total days of activity: 25-54 % for females, 18-40 %) in accounting for the overall size differences found between populations.

Key words: age, altitude, Anura, growth, skeletochronology

INTRODUCTION

The Iberian brown frog *Rana iberica* Boulenger, 1879, is an endangered endemic species from the Iberian Peninsula. This species is one of the few non-Mediterranean Spanish anurans, distributed in the Atlantic Eurosiberian realm throughout north-western Spain, north and central Portugal and the Sistema Central, a mountain range which crosses the Iberian Peninsula from ENE to WSW (Crespo, 1997; Esteban, 1997; Fig. 1). A few relict populations of the species have also been found in the Basque Country (Esteban, 1997). The distribution of *Rana iberica* shows a wide altitudinal range (Pleguezuelos & Villafranca, 1997), although the habitat it uses across this range is similar. *Rana iberica* is found in the humid northern regions, even at sea level (Galán, 1982, 1989), whereas in drier areas, such as central Spain, this species is only a mountain form, found at over 2000 m (Esteban, 1997; Pleguezuelos & Villafranca, 1997). The very different winter climatic conditions under which these populations are found causes drastic variations in their seasonal period of activity (Esteban, 1990).

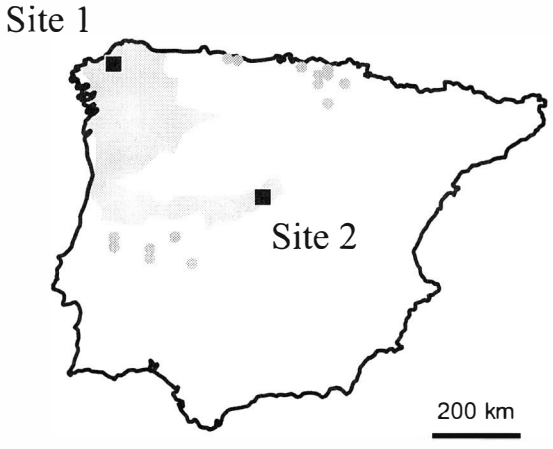

FIG. 1. Distribution range of *Rana iberica*, with indications of the sites studied. Range of *R. iberica* in grey. Site 1: lowland population of Monfero. Site 2: high mountain population of El Espinar.

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Some morphological studies show that a large variation in body size occurs among different populations of this species (Lizana, Pérez-Mellado & Ciudad, 1987; Galán, 1989). This variation is present despite the fact that this species shows very low genetic differentiation, as inferred from allozyme electrophoretic studies, throughout its range (Herrero, Arano & Esteban, 1990; Arano, Esteban & Herrero, 1993). In ectothermic animals with continuous growth, such as amphibians, differences in size among populations may be the result of differential growth rates, sexual maturity onset, yearly activity period (total days of annual activity), and longevity (total years lived). Thus, there are plastic phenotypic responses to local environmental conditions that alter individual growth time and/or energy budget constraints (Berven, 1982; Hemelaar, 1988).

Skeletochronology and bone histology have proved to be excellent tools in evaluating the physiological activity induced in amphibians by seasonal changes. Such changes lead to the formation of bone growth marks, such as 'zones', i.e. thicker layers of bone laid down during periods of fast osteogenesis, and 'lines of arrested growth' (LAGs) formed in periods when osteogenesis is very slow or non-existent (Castanet,

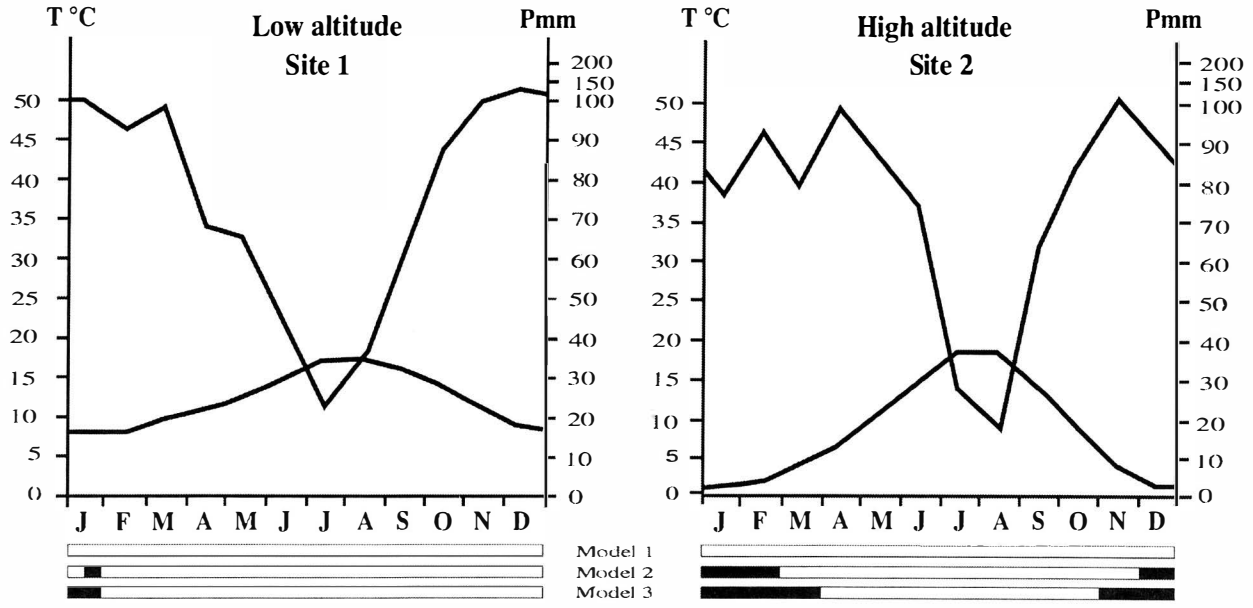

FIG. 2. Climatic diagrams and phenological assumptions for the populations of low altitude (Site 1) and high altitude (Site 2). T°C: temperature in degrees Celsius. Pmm: precipitation in cubic mm per m^2. For models, black lines indicate periods of hibernation (see Table 3).

Francillon-Vieillot, Meunier & de Ricqlès, 1993). A zone followed by a LAG corresponds to a single annual cycle of activity in cold or temperate regions. The number of growth marks and their histological structure have been used in a large number of amphibian species to estimate individual age (Smirina, 1994), and they also enable us to trace the history of growth year by year.

In this article we study the relationships between age, size and histological bone growth in two selected populations of *Rana iberica*. These populations represent the altitudinal extremes of the species range (Fig. 1). Taking advantage of the genetic similarity between the populations at the isozyme level (Nei genetic distance 0.019; Arano *et al.*, 1993), this study will analyse the degree to which body size is related to longevity, growth rates and duration of the annual activity period.

MATERIALS AND METHODS

Histological samples of 113 specimens of *Rana iberica*, collected in different years from two areas in Spain (Fig. 1) were studied. One sample (Site 1) comes from the north-western Iberian region, in the neighbourhood of Monfero (43° 20' N, 8° 20' W; province of Coruña), and is composed of 49 adults (27 males and 22 females). This area is situated at an altitude of between 100 and 200 m. The relevant climatic conditions at the site have been inferred from the meteorological station in the nearby town of Betanzos, where an annual rainfall of 900-1000 mm and a mean air temperature range of 13-14 °C was recorded over 37 years (Fig. 2; León-Llamazares, 1988). The second sample (Site 2) is composed of 64 adults (26 males and 38 females) from the Sistema Central mountains at altitudes from 1600 to 1700 m, the majority collected in the area between El Espinar and La Granja de San Ildefonso (40° 45' N, 4° 10' W; province of Segovia). Our meteorological data for this region comes from the station at La Granja de San Ildefonso. The climate here is characterized by a cold winter, with an annual precipitation of around 1200 mm, and an average yearly temperature between 7 and 9 °C over 41 years. The climatic diagram for site 2 is presented in Fig. 2, based on León-Llamazares (1987).

Esteban (1990) presents general data on the seasonal phenology of the populations analysed here. Nevertheless, on account of the high interannual phenological variation expected, we have used three working assumptions that will cover all the relevant possibilities about the duration of the activity period. The first, admittedly unrealistic, assumes that frogs are active all year in both populations. The second projects a hibernation period of 15 days (350 active per year) for the lowland population. This is a minimum estimation, deduced from the fact that lines of arrested growth (LAGs) are present in the population, and each of these marks require from 15 days to 5 weeks to develop (Smirina, Klevezal & Berger, 1986). Therefore, we think that LAGs are formed in Monfero during the coldest month, which has a mean temperature of 7.8 °C. The corresponding situation in the high mountains would be 275 days of activity, a figure that assumes that the majority of the frog population is not active in months with a mean temperature below 5 °C. This result is precisely equivalent for Site 2 to a hibernating period during the months in which the mean of the minimal temperatures is below 0°. The third assumption assigns a hibernation period of one month to the

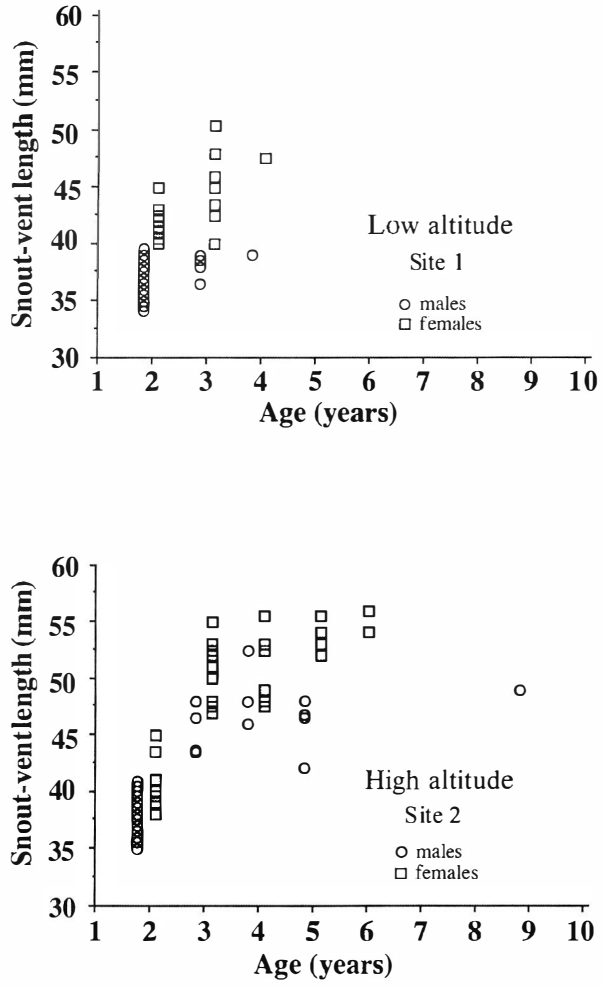

FIG. 3. Plot of age (LAG number) versus SVL for low (Site 1) and high (Site 2) altitude populations of *Rana iberica.*

lowland region and five months to the mountain region, with a monthly mean temperature of 8 °C for activity as the common threshold. Nevertheless, a mountain hibernation of five months is a non-realistic maximum, because we have frequently observed active frogs during most of both November and March.

The osteological material and the data about sex and snout-vent lengths (SVL) for the specimens come from the herpetological collection of the Museo Nacional de Ciencias Naturales (Madrid, Spain). This material was previously used in other studies about the genetic variability (Herrero *et al.*, 1990; Arano *et al.*, 1993) and morphology (Galán, 1982, 1989) of this threatened endemic species, and no additional specimens have been collected for our study.

Skeletochronological preparations were made, with slight modifications, according to the Smirina (1972) protocol. A tibiofibula of each specimen was decalcified in nitric acid 3% for 5 hrs and washed overnight in running tap water. Sections of the diaphysis of frozen bones were cut using a cryostat microtome. Sections were 20 mm thick, stained for 15 minutes at room temperature with Ehrlich hamatoxylin and mounted in aqueous synthetic resin (Aquamount) after being rinsed for at least 5 minutes in tap water. The analysis and interpretation of growth marks was done under an ordinary light microscope, followed by photomicrography. On each photograph, a curvimeter was used to measure the perimeters of the marrow cavity, the outer margin of the tibiofibula, and each line of arrested growth (LAG). In all tibiofibular cross-sections, one or more stained LAGs were clearly distinct and easy to count. We accept the assumption that each LAG represents the end of a one year cycle, as demonstrated for several ranids in cold and temperate climates (Smirina, 1972; Gibbons & McCarthy, 1983; Patón, Juarranz, Sequeros, Pérez-Campo, López-Torres & Barja de Quiroga, 1991).

The software packages Mathematica 3.0 (Wolfram, 1996) and Statview IV were used for the calculations. Interpolation in the developmental trajectories were obtained by fitting low-order polynomial curves between the data points. We have used step-wise interpolation functions generated by the Mathematica standard interpolation facilities. These curves used are not intended to have any biological meaning, and their equations are not given; they only provide the best possible accuracy for interpolation and further estimation of the two relative components (growth rate and total days of activity) of final size.

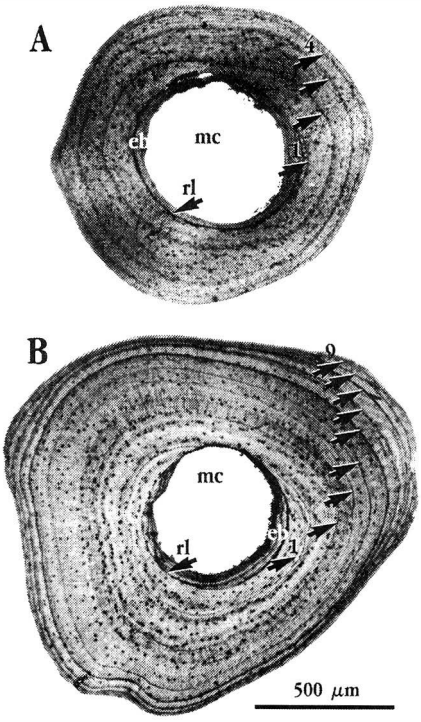

FIG. 4. Cross sections taken at the middle of the tibiofibular diaphysis of *Rana iberica.* A: female from Site 1 (SVL= 49 mm), showing four LAGs. B: male from Site 2 (SVL= 50 mm), with nine LAGs. eb: endosteal bone; mc: medullar cavity; rl: resorption line.

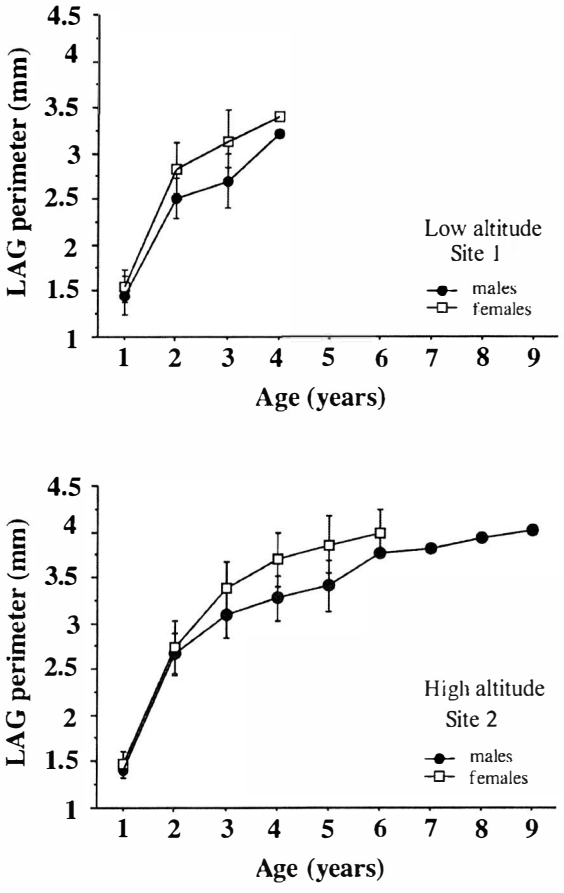

FIG. 5. Mean tibiofibular annual growth in low (Site 1) and high (Site 2) altitude populations of *Rana iberica*. Male perimeters at 5-8 years (Site 2) are based on one 9 year-old individual. Age (in years) is not biologically equivalent in both sites because their annual activity periods are different (see text). Black dots: males. Open squares: females. Vertical bars represent standard deviation.

TABLE 1. Number of individuals (*N*), mean size in mm (SVL), standard deviation and range, for each sex in both populations. *U*-test: probabilities in the Mann-Whitney *U*-test for differences between sexes and sites.

	Site 1	Site 2	*U*-test (sites)
Males			
N	27	26	
Mean±SD	36.93±1.62	41.33±4.66	$P<0.001$
Range	34.0-39.5	35.0-49.0	
Females			
N	19	38	
Mean±SD	43.53±2.99	48.75±5.28	$P<0.001$
Range	40.0-50.5	38.0-56.0	
U-test (sexes)	$P<0.001$	$P<0.001$	

RESULTS

Osseous remodelling was detected in the centre of the cross section of 24.5% and 28% of the individuals in the populations of Site 1 and Site 2 respectively. In most cases this endosteal resorption was slightly asymmetrical and did not completely destroy the first LAG. However, we suspect that LAG 1 was removed in 4 and 3 specimens from Site 1 and Site 2 respectively, because the mean perimeter of the medullar cavity for these individuals (Site 1: 1.66 mm, SD=0.10; Site 2: 1.76 mm, SD=0.24) was significantly larger ($P<0.01$ for both sites in Mann-Whitney *U*-tests) than the LAG 1 perimeters in the subsample that did not show complete resorption (Site 1: 1.48 mm, SD=0.20; Site 2: 1.44 mm, SD=0.12). Likewise, the innermost LAG of the individuals sus-

TABLE 2. Mean and standard deviation of LAG perimeter for males and females in both populations. *U*-test: Mann-Whitney *U*-test for differences between sites.

	Site 1		Site 2		
LAG	*N*	Perimeter (mm)	*N*	Perimeter (mm)	*U*-test
Males					
1	18	1.43±0.21	21	1.41±0.09	$P=0.370$
2	27	2.49±0.22	26	2.68±0.22	$P=0.003$
3	6	2.67±0.30	11	3.11±0.23	$P=0.010$
4	1	3.02	7	3.27±0.25	–
5	–	–	5	3.41±0.28	–
6	–	–	1	3.78	–
7	–	–	1	3.80	–
8	–	–	1	3.83	–
9	–	–	1	3.87	–
Females					
1	16	1.53±0.18	25	1.47±0.14	$P=0.522$
2	19	2.81±0.28	38	2.73±0.29	$P=0.441$
3	9	3.21±0.40	28	3.41±0.29	$P=0.041$
4	1	3.45	15	3.70±0.29	–
5	–	–	7	3.87±0.32	–
6	–	–	2	3.98±0.26	–

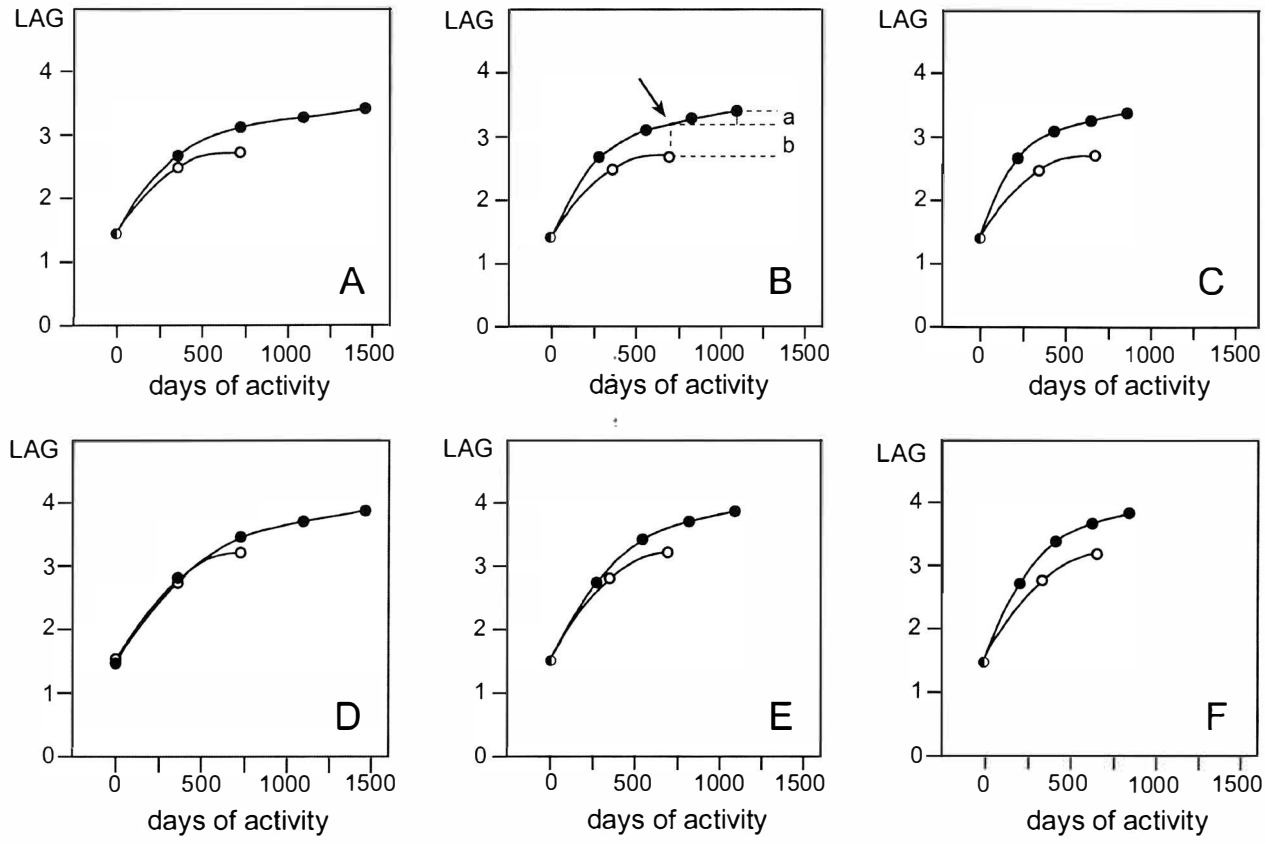

FIG. 6. Growth of the central tibiofibular perimeter (LAG, in mm) for different phenological assumptions. The history of growth has been inferred directly from maximum LAG perimeters in samples >5 (see text). A-C: males. D-F: females. A,D: 365 days of activity. B, E: 350 (Site 1) and 275 (Site 2) days of activity. C, F: 335 (Site 1) and 215 (Site 2) days of activity. Upper curves (black dots) correspond to the high altitude Site 2. Arrow: interpolation point on Site 2 of the maximum age on Site 1. Final size on Site 2 caused by a differential life span component (a) or by differences in growth rate (b).

pected of complete resorption (Site 1: 2.67 mm, SD=0.26; Site 2: 2.54 mm, SD=0.22) was within the range of the LAG 2 for the specimens which preserved LAG 1 (Site 1: 2.27-3.32 mm; Site 2: 2.30-3.25 mm). We conclude that LAG have been completely lost in the above mentioned specimen, and we have therefore added one to the number of counted LAGs. This remodelling process is mainly produced when the growth rate is highest, before the onset of sexual maturity (Leclair, 1990), and has been reported in other *Rana* species (Leclair & Castanet, 1987; Esteban, 1990; Esteban, García-París & Castanet, 1996; Sagor, Ouellet, Barten & Green, 1998).

As indicated in Table 1, females were on average larger than males in both populations. For both sexes, the population of Site 2 was significantly larger (Table 1). Galán (1982, 1989) finds similar sizes to our Site 1 in populations from La Coruña (SVL males 29.2-38.6 mm; females 36.6-51.0 mm), although in our sample we found a slightly smaller minimum size.

The age structure of the populations, determined by LAG counts, is shown in Fig. 3 in relation to SVL. In Site 1, males had a mean age of 2.26 years (SD=0.53), while the mean age for females was 2.53 (SD=0.61). The male subsample from Site 2 had a mean age of 3.04 (SD=11.66) and the female value was 3.37 yrs (SD=1.17). Significant sexual differences occur in age in both populations (Mann-Whitney *U*-test $P>0.05$), but both sexes from Site 2 were older than their counterparts from Site 1 ($P<0.01$). The modal values for ages were 2 yrs in the males of both populations and in the females at Site 1, and 3 yrs for females at Site 2.

The skeletochronological pattern allows us to infer the age at which sexual maturity is attained (Castanet *et al.*, 1993). The minimum age for maturity observed here was 2 yrs in both sexes and populations, and no significant differences in body size at maturity was found between populations ($P>0.05$).

The oldest individuals were found in the highland population, where the oldest females were 6 yrs old and the males 5 yrs old, with the exception of a single male (SVL 50 mm) which had lived for 9 yrs (Fig. 4B). In comparison, the members of the lowland population were younger, and only one individual of each sex attained the age of 4 yrs (Fig. 4A).

An analysis of growth patterns in terms of age and size throughout each individual's life, as preserved in the histological bone marks, has been carried out. LAG perimeters, which represent the bone perimeter at the end of each growth season, are well known measures which can be adopted as reliable indicators of past size and growth (Hemelaar, 1988). Correlation between

TABLE 3. Comparison of tibiofibular growth for each sex and population. Annual percentage (%) and daily (μm) increase in mean perimeter for each age. M1: model of 365 days of annual activity in both populations. M2: model of 350 days of activity for Site 1 and 275 days for Site 2. M3: model of 335 days of activity for Site 1 population and 215 days for Site 2.

Years of life	Site 1				Site 2			
	%	M1	M2	M3	%	M1	M2	M3
Males								
1st	100				100			
2nd	174	203	211	221	190	247	327	419
3rd	186	33	34	36	221	85	113	144
4th	–	–	–	–	232	30	40	51
5th	–	–	–	–	242	27	36	47
Females								
1st	100				100			
2nd	183	227	237	248	186	235	312	399
3rd	209	71	74	78	232	127	168	215
4th	–	–	–	–	252	54	72	92
5th	–	–	–	–	263	232	42	54

SVL and tibiofibula perimeter, for each population and sex, was found to be significant in our study (Site 1: males r=0.56, females r=0.81, Site 2: males r=0.89, females r=0.85; P<0.05). Fig. 5 shows the tibiofibular growth for both populations. The regression analysis of tibiofibular perimeter on age showed a highly significant correlation for both sexes and populations (P<0.01), although the tibiofibular perimeters vary substantially within most age groups, and the largest individuals were not always the oldest ones. As expected, immature individuals grew much faster in both populations (Table 3). Growth rates were higher for females than for males (P<0.05).

The specimens from Site 2 attained significantly greater sizes in tibiofibular perimeter than those from Site 1, at the age of 2 yrs and 3 yrs (males) and 3 yrs (females), in Mann-Whitney U-tests (P<0.005). This size difference is indicative of a faster bone growth in highland individuals after maturity, for all possible assumptions about periods of hibernation (Table 3, Fig. 6).

Fig. 6 shows the overall histological growth trajectories for each population, sex and annual activity assumption. These diagrams give a general view of the relative contributions of growth rates and longevity in accounting for the final size differences between populations. The difference in ordinates (LAG perimeter) in Fig. 6 between the final points of both trajectories indicates the total size difference. The difference in ordinates between the final point of the shortest lived trajectory (Site 1) and their corresponding interpolation (i.e., with the same numbers of days of activity) in the trajectory of Site 2, indicates the contribution of differential growth rates to the overall size comparison. Any growth increase after this point in Site 2 is exclusively caused by a differential longevity factor (Fig. 6B). The results were slightly different for males and females, but in both sexes the growth rate played a more important role than the age, with percentages around 60%, 79%, and 82% for males, and 46%, 70%, and 75% for females under hibernating assumptions A to C respectively.

DISCUSSION

Several of the results that we have found agree with similar studies done on other populations of brown frogs (Sagor, Ouellet, Barten & Green, 1998). The positive relationship between increasing body size and altitude is a frequent observation. Our samples from almost the lowest and highest elevations can be compared with the intermediate sizes (maximal SVL 42.0 mm for males and 50.7 for females) present in another *Rana iberica* population at medium altitude (around 850 m) from the western Sistema Central (Lizana *et al.*, 1987). The increase in longevity in amphibian populations living in mountain conditions has already been cited in other European brown frogs (Guarino, Angelini, Giacoma & Cavallotto, 1995; Ryser, 1996) and in numerous other amphibians (see review in Smirina, 1994). This phenomenon has been related to a delay in the onset of sexual maturity for populations living at lower temperatures (Stearns, 1989; Charnov, 1990; Charnov & Berrigan, 1990), and to different rates of predation derived from the shorter duration of the annual activity period (Licht, 1976; Ryser, 1996). Nevertheless, no delayed maturity exists in any of our populations, and Guarino *et al.* (1995) have found similar ages for maturity in two populations of *Rana italica*, a similar European brown frog species.

Our results should be taken as preliminary until more detailed ecological and life cycle studies are available for the populations examined. Nevertheless, the results do not completely conform with current life-history theory for ectotherms, as summarized by the

'developmental temperature-size rule' (Atkinson & Sibly, 1997). Size differences are not found at the end of the juvenile period - they increase during the adult growth period. According to the most realistic phenological assumption, we have estimated that growth rates account for no less than 46-75% (females), and up to 60-82% (males), of the total size differences between populations (Fig. 6). These facts can be explained in several ways. On the one hand, we could suppose that growth rate, as a complex life history trait, has been subject to selection, and that this genetic differentiation is not reflected in the neutral loci sampled on allozymes (Latta, 1998). On the other hand, if genetic differentiation is negligible at all levels, as both environments (and presumably food supplies) are quite similar during the common activity period of frogs, then an environmentally induced factor (temperature) would account for the size differences recorded (Partridge & French, 1996; Atkinson & Sibly, 1997). Both explanations cannot fully explain the lack of size differences at the time of the first LAG, unless growth metabolic inductors were drastically different, or some biased compensatory growth occurred in late autumn. Therefore, it seems possible that resource allocation differences may be involved. The metabolic adult rate in the highland population, as observed in the male skeletochronology sections, may perhaps not necessarily be associated with first reproduction, which may have occured during the third year in a fraction of the highland population. In this way, a considerable energetic cost would have been avoided by the two year-old males. Furthermore, undetected differences may exist in tadpole size and time of metamorphosis.

The histological data sets here compiled show that growth rate plays a more important role than longevity (i.e. total years lived) or activity period (total days of activity lived) in explaining the size differences observed between lowland and highland populations. Further research is necessary for a more detailed quantitative description of the differential ontogenetic size trends and their causal basis.

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