

A skeletochronological study of growth, longevity, and age at sexual maturity in a population of *Rana latastei* (Amphibia, Anura)

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Longevity and age at sexual maturity in an Italian population of *Rana latastei* were studied by skeletochronology performed on the phalanges. Frogs collected in 1998 and 1999 by drift fences and pitfall traps were marked by toe-clipping. After marking, individuals were released and the cut phalanges were processed for skeletochronological analysis. The maximum age so far recorded was 3 years in males and 4 years in females. The smallest male and female that were sexually mature on the basis of histological analysis of the gonads were 36 and 35 mm snout vent length (SVL), respectively. In both sexes, most individuals were estimated to breed shortly after emergence from their first overwintering. Among the European Brown Frogs, *Rana latastei* appears to be one of the shortest-lived and one of the first to reach sexual maturity.

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1. Introduction

Rana latastei is an anuran amphibian belonging to the Brown Frogs group and is distributed in the Po river plain, the Ticino Canton and northeastern Istria (Grossenbacher 1997). Of the European amphibians, *R. latastei* is one of the most important in terms of conservation and protection because of its restricted range and fragmentation of its habitats (Boano and Sindaco 1992). The species is included both in Appendix II of the Bern Convention, adopted by the Member States of the Council of the Europe in the 1979 and in Annexes B and D of DPR No. 357 of September 8 1997 (rules for the enforcement of Directive 92/43/EEC). *R. latastei* is also listed as a vulnerable species in the IUCN Red List of Threatened Animals (IUCN 1996).

Despite its conservation importance, very little is known about the life history of *R. latastei* (Pozzi 1980; Dolce *et al* 1985). Our study analyses some demographic parameters of a population of *R. latastei* by skeletochronological method.

Skeletochronology can be a reliable tool to assess individual mean longevity, growth rates, and age at sexual maturity (Castanet *et al* 1993; Smirina 1994). In many amphibians and reptiles, skeletochronology can be performed on the phalanges, and hence, represents a powerful technique, mainly in the case of endangered species, since it allows individuals to be marked for field study and skeletal elements to be obtained for skeletochronology without sacrificing the individuals (Castanet and Smirina 1990; Denton and Beebee 1993; Guarino *et al* 1999). To test the reliability of the skeletochronological data obtained from the phalanges, we also examined a small sample of femurs.

2. Materials and methods

2.1 Field techniques

The population examined was located in the Brusa Marsh (province of Verona, northern Italy) at about 20 metres

Keywords. Amphibia; growth; longevity; *Rana latastei*; skeletochronology

Abbreviations used: EB, Endosteal bone; LAG, lines of arrested growth; OM, outer margin; PB, periosteal bone; RL, reversal line; SVL, snout vent length.

above sex level. Frogs were collected in 1998 and 1999 on a monthly basis from March to October, during the annual activity period of this species. Sampling was performed by means of pit-fall traps placed 5 m apart along fences surrounding the pools visited by frogs. The sampling site was visited each month for 8–10 consecutive days. From each specimen, sex, total snout vent length (SVL) to the nearest mm, body weight, and reproductive status were recorded. Before release, each specimen was marked by clipping the second phalanx of the second toe of the left forelimb. The cut toe was immediately fixed in 70° ethanol and the animal was released. All the animals found dead near the sampling sites and still in a good state of preservation were immediately fixed *in toto* in 70° ethanol. These specimens ($n = 5$) together with tissue samples from sacrificed adults (two males and five females), after anaesthesia in MS-222, were used to study the bone tissue organization, to compare the skeletochronological results obtained in femur and phalanx of the same individual, and to analyse the status of the gonads.

2.2 Skeletochronology

Skeletochronological analysis was carried out according to a protocol tested on other amphibian species (Guarino et al 1995a, 1998). Cryostat cross-sections (12 µm thick) were stained using Erlich's haematoxylin for 25 min. The lines of arrested growths (LAGs) number observed in the bone sections was independently calculated by two of us in a blind fashion. Whenever there was discrepancy the sections were reanalysed until a consensus was reached.

To evaluate the processes of bone remodelling, which might cause the complete destruction of LAGs and consequently lead to underestimation of individual age, osteometrical analysis was performed on cross-sections. The sections observed under the microscope were recorded by colour video camera ProgRES 3008 and processed by a personal computer with the image analysis software KS300. For each phalanx, we selected at least three cross-sections, at mid-diaphyseal level, with the smallest marrow cavity. The perimeters of medullary cavity (MC), reversal line (RL), each visible LAG, and periosteal outer margin (OM) were measured in adults and compared with the perimeter of OM measured in juvenile frogs close to the first overwintering, but which lacked LAGs in the phalanx sections. Based on Pozzi (1980), Boano and Sindaco (1992), and our personal observations, we considered the juveniles, sampled in September and October the largest body size (SVL ranging from 28 to 38 mm) as the individuals that had not yet overwintered since metamorphosis.

As in other skeletochronological studies (Ento and Matsui 2002), the growth pattern in SVL of both sexes of *R. latastei* was estimated by fitting the logistic func-

tion as: $L = a/(1 + b \cdot e^{-ct})$; where L is the average SVL at a given age t ; a is the asymptotic SVL; e is the exponent for natural logarithms, b is the growth rate, c is the constant determined by allowing t to be 0, and t is the time (age) in years. We used the software PAST (version 0.94) to compute the logistic function.

2.3 Experimental validation of skeletochronology

To verify the periodicity of LAG formation, we performed experiments with fluorescent vital labelling. Four adult frogs (1 male, 40.7 mm SVL, and 3 females, 41.9, 44.7, 44.8 mm SVL, respectively) were caught before their overwintering (10 October 2000) and taken to an aquaterrarium, where they were kept for five months (until mid February 2001) under outdoor conditions and fed *ad libitum*. As soon as the frogs were introduced into the aquaterrarium, they were toe-clipped (second toe of the left hand) and injected with calcein (0.1 mg/g body weight), a vital fluorochrome, according to Francillon-Vieillot and Castanet (1985). The phalanges were demineralized, cross-sectioned, and stained with Erlich's haematoxylin. All the frogs underwent normal overwintering (late November–January) and survived until to the end of the experiment. At emergence, frogs were toe-clipped again (second toe of the right hand) and then released into the natural environment, except one individual which was euthanized and stored in ethanol. The phalanges clipped after the overwintering period were split into two equal parts: one half was decalcified and stained with Erlich's haematoxylin. The other half was embedded in resin, smoothed by grinding up to 50–80 µm in thickness, and observed under fluorescent microscope. From the sacrificed specimen, histological preparations of the femur and phalanx were obtained.

2.4 Assessment of sexual maturity

Individual age at sexual maturity was assessed by noting the secondary sexual characters (nuptial thumbpads in males and vitellogenic eggs in females) in the sampled specimens, and by histological analysis of the gonads from the specimens found dead ($n = 5$) or euthanized ($n = 7$). Gonads were embedded in paraffin and the sections (7 µm) were stained with Galgano's trichrome (Mazzi 1977).

3. Results

3.1 Body size

Table 1 shows a comparison of the SVL (mean ± SD) of individuals sampled during the two years of our research. Data were subdivided into three principal periods of the

annual cycle of the species: (i) reproductive period (March–April); (ii) period of metamorphosis and immediate post-metamorphic growth (May–August); and (iii) pre-wintering growth period (September–early November). Juveniles were subdivided into size classes of individuals smaller than 28 mm SVL and between 28–37 mm SVL.

In both the sample years, the majority of sexually mature individuals were collected during the reproductive period; almost no adults (1998, $n = 1$; 1999, $n = 0$) were collected during the period of metamorphosis; few adults (1998, $n = 4$) were collected during the pre-wintering period. Numerous neometamorphs (on average 17 mm SVL) and several individuals (with SVL greater than 20 mm) were sampled from June to August (table 1). Hence, in this period the class of juveniles below 28 mm was predominant. During the pre-wintering period the percentage of juveniles with SVL less than 28 mm was very low, therefore several subadults between 28 and 37 mm SVL were collected (table 1). Among adults, there was a non-significant difference in SVL between males and females (unpaired t -test 1998: $t = 1.57$, $df = 24$, $P = 0.12$; 1999: $t = -1.93$, $df = 58$, $P = 0.056$) although females tended to be the largest (table 1).

3.2 Bone histology and skeletochronological analysis

Figure 1 represents phalangeal cross-sections of *R. latastei*. In the metamorphs, smaller than 30 mm SVL, the phalanges at mid-diaphyseal level were formed by a layer enclosing a broad central cavity of bone marrow. In the subadults and the adults, the diaphyses of the phalanges and femurs were composed of two compact and concentric bone layers: an outer and broader layer of periosteal bone (PB), and an inner and narrow layer of endosteal bone (EB). In six individuals, the two layers were separated by irregular and strongly haematoxylinophilic lines interpreted as RL. EB and PB did not always differ in histological organization. Hence it was difficult to distinguish them. In fact in both the bone layers flattened and randomly distributed osteocytes were often observed, although the cells were more abundant in the outer bone. In the phalanges and femurs of most adults, strongly haematoxylinophilic, circular and more or less complete periosteal lines (table 2; figure 1) were interpreted as LAGs. Double lines or false lines (*sensu* Castanet and Smirina 1990) were rarely observed. In individuals, in which both femur and phalanx were examined, the number and optical appearance of LAGs were the same. LAGs were absent in all the juveniles and in the 10 adults (seven males and three females). Of those adults, six individuals (five males and one female), ranging 37–41 mm SVL, were sampled after emergence (March); the remaining four frogs, measuring 35–41 mm SVL, were collected in September and October.

In most adults, bone remodelling along the inner periosteal layer was documented. Osteometrical measurements showed that the perimeters of RL in a randomly selected sample of adult males ($n = 15$) and females ($n = 17$), did not exceed that of OM of the juveniles close to first overwintering (figures 2 and 3). The perimeter of the first visible LAG was only smaller than that of juvenile OM in seven individuals (figure 4). A comparison between the RL perimeter of adults and the perimeters of the first visible LAG showed that, out of a total of 32, in 11 individuals, including four collected after emergence and without LAG, the perimeter of the RL exceeded that of the first visible LAG.

3.3 Experiments of fluorescent labelling of the bone

At the beginning of the experiment, none of the four frogs which were labelled showed LAGs in phalangeal sections.

Table 1. Number of sampled individuals (n), SVL (expressed as mean \pm SD) and range, during the two years of research.

	Males		Females		Juveniles					
	n	Mean \pm SD range	n	Mean \pm SD range	< 28 mm		28–37 mm			
					n	Mean \pm SD range	n	Mean \pm SD range		
1998										
repr	11	42.8 \pm 4.5 36–49	15	40.7 \pm 2.2 37–44	0	–	–	0	–	–
met	1	40	0	–	80	17.9 \pm 4.3 11–27	2	30.5 \pm 2.2 29–32		
prewin	4	38.7 \pm 2.5 36–42	2	36.4 \pm 1.4 35–37	4	24.5 \pm 2.5 21–27	8	33.6 \pm 2.6 28–37		
1999										
repr	34	43.5 \pm 4.7 36–47	26	46.0 \pm 5.3 39–58	0	–	–	0	–	–
met	0	–	0	–	122	17.5 \pm 3.1 14–27	3	29.7 \pm 1.9 28–32		
prewin	4	37.2 \pm 0.9 36–38	0	–	1	25	–	2	34.0 \pm 2.9 32–36	

Repr, reproductive period; met, metamorphosis and immediate post metamorphosis period; prewin, pre-wintering period.

At the end of the experiment, two frogs exhibited a faint haematoxylinophilic line on the periosteal outer margin of the phalanx. In the ground sections of the phalanges a fluorescent mark was observed both in the outer part of the periosteal bone and in the endosteal bone along the margin surrounding the marrow cavity (figure 5). At the end of the experiment, from the comparison between the phalangeal ground sections and phalangeal stained section, it appeared that the fluorescent periosteal mark was close to the peripherally deposited LAG.

3.4 Age structure, growth and age at sexual maturity

The age distribution of *R. latastei* is reported in figure 6. The data were pooled from two years of sampling. We considered individual age to be equivalent to the number of visible LAGs plus the possible LAGs completely resorbed, as estimated by osteometry. In particular, one LAG was assumed to be lost owing to endosteal resorption

in the 11 individuals showing the RL perimeter greater than that of the first visible LAG, including the six adults showing no LAGs, collected shortly after emergence. The modal age of both sexes was 1 year. The maximum age recorded was three years in males and four in females. SVL and age were positively correlated both in males (Pearson's correlation, $r = 0.80$, $df = 24$, $P < 0.001$) and females ($r = 0.92$, $df = 22$, $P < 0.001$).

Table 2. Number of individuals of *R. latastei* for each LAG class.

No. of LAGs	Males	Females
0	7 (27%)	3 (12.5%)
1	12 (46%)	11 (46%)
2	6 (23%)	6 (25%)
3	1 (4%)	3 (12.5%)
4	0	1 (4%)

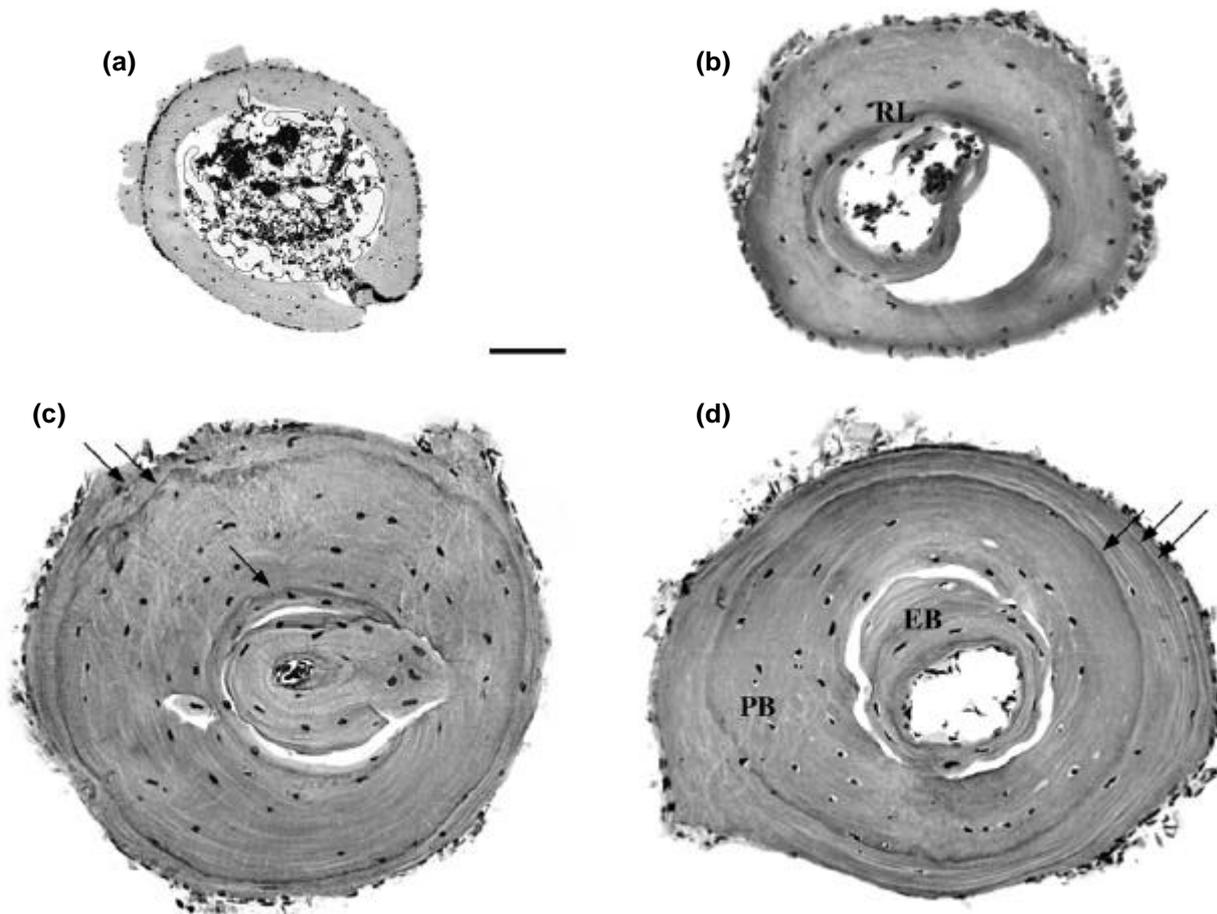


Figure 1. Phalangeal cross sections of *R. latastei*. (a) Juveniles, 18 mm in SVL, showing absence of LAG. (b) Subadult, 38 mm in SVL, showing no LAG. (c) Male, 48 mm in SVL, showing three LAGs (arrows). (d) Female, 54 mm in SVL, showing three LAGs (arrows) to which, by osteometry, one LAG totally resorbed was added. EB, endosteal bone; PB, periosteal bone; RL, reversal line; Scale bar = 60 μm .

Our data on mean SVL at a given age were used to determine growth curves with a logistic model for both sexes (figure 7). Up to one year of age, the growth curve appeared similar in males and females. Beyond this age, growth curves differed between the sexes. The growth rate was higher for females ($b = 0.48$) than for males ($b = 0.27$).

The smallest sexually mature male, which had sperms in its seminiferous tubules, was 36 mm SVL. The smallest sexually mature female showing yolky follicles in the

ovary was 35 mm SVL. Both individuals were captured in the autumn of 1998; neither presented LAGs.

4. Discussion

Our study shows that skeletochronology can be successfully applied to *R. latastei* using the second phalanx of the forelimb. In fact, most phalangeal sections exhibited distinct LAGs. Further, when the phalanx and femur sections from the same individual were compared, we found that the number and pattern of LAGs were identical.

It has been generally hypothesized that the growth marks visible in bones are the result of a genetically controlled growth rhythm which is synchronised and reinforced by particular environmental conditions, such as seasonality (Castanet *et al* 1993). The annual rhythm of the LAG has been demonstrated in temperate (Francillon-Vieillot and Castanet 1985) as well as tropical anurans (Kumbar and Pancharatna 2002). Results of the labelling of bone suggest that in *R. latastei* the formation of LAGs is annual. It is likely that in this species one LAG is produced when the individual overwinters.

In *R. latastei*, however, individual age cannot be assessed only by a simple enumeration of the visible periosteal LAGs in phalangeal cross-sections. Indeed, we found the absence of LAGs in some individuals ($n = 6$) phenotypically recognisable as adults. Bone remodelling, particularly endosteal resorption, represents one of the serious difficulties in skeletochronological interpretation because

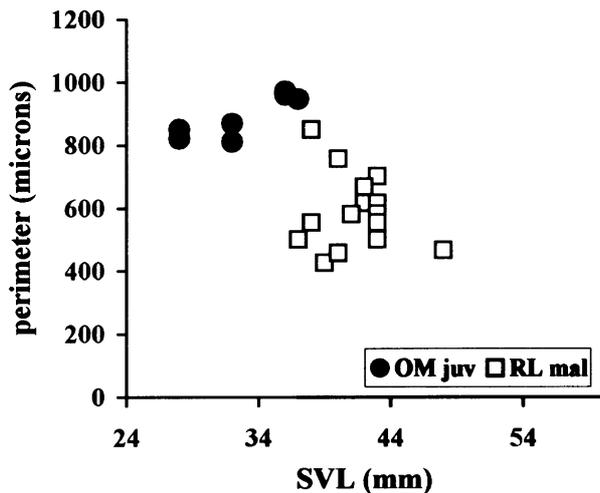


Figure 2. Comparison between the perimeters of periosteal outer margin (OM) of the juveniles close to the first overwintering with the reversal line (RL) perimeter of males. Values are the mean obtained from three measurements, at mid-diaphyseal level, on three different phalangeal sections.

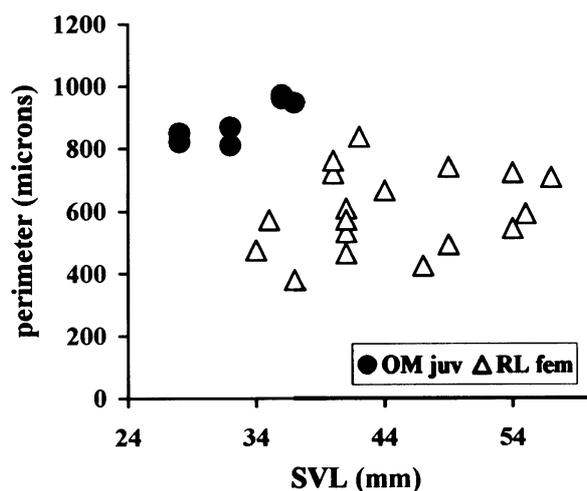


Figure 3. Comparison between the perimeters of OM of the juveniles close to the first overwintering with the RL perimeter of females. Values are the mean obtained from three measurements, at mid diaphyseal level, on three different phalangeal sections.

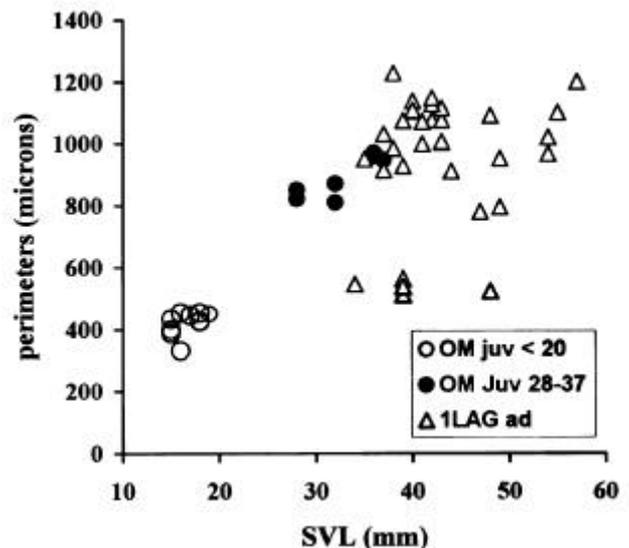


Figure 4. Comparison between the OM perimeters of juveniles and the perimeters of the first visible LAG of adult males and females. Values are the mean obtained from three measurements, at mid-diaphyseal level, on three different phalangeal sections.

it can cause the complete loss of one or more periosteal LAG and, therefore, result in underestimation of age (Castanet *et al* 1993). Therefore, as others have already demonstrated (Hemelaar 1985; Leclair and Castanet 1987; Guarino *et al* 1999), it is crucial to perform osteometrical analysis to ascertain whether bone resorption might have completely removed one or more LAG. Another important approach is to correlate skeletochronological interpretation to the life history of the species.

In our study, osteometrical analysis indicated that one LAG was completely resorbed in 11 frogs, which included four out of six individuals showing no LAG and sampled shortly after overwintering. The absence of LAGs in two individuals sampled post-emergence and showing poor endosteal remodelling is hard to explain. In this regard, it is interesting to note that in our sample several ($n = 5$) phalangeal sections of individuals captured after emergence showed no LAG on the outer margin. Some authors (Rogers and Harvey 1994; Sagor *et al* 1999) consider that in the individuals sampled immediately after the emergence from overwintering, the outer edge of the bone might not be distinguishable from the last LAG, i.e. the LAG formed during the previous overwintering period. Hence, they always count the outer periosteal margin as a LAG. Alternatively, it may be supposed that peripheral remodelling causes the destruction of the outer LAG. Both these assumptions seem to be partially possible for *R. latastei*, given that many individuals sampled after overwintering showed a clearly discernible LAG on the outer bone edge. In addition, peripheral bone remodelling

was not observed. Therefore, our findings suggest that some individuals might have continued to grow during the winter period which usually corresponds to a quiescent period for this species. During mild winter days, individuals of *R. latastei* as well as *Bufo bufo* have been observed near their reproductive sites (Pozzi 1980; S Mazzotti, unpublished results). Furthermore, our results obtained on the individuals injected with calcein corroborate this theory. Indeed, at the end of the experiment the calceinized frogs showed a thin and poorly discernible peripheral LAG.

The fact that in about 13% of the adults the perimeter of the inner visible LAG was smaller than that of the outer periosteal margin of the juveniles close to first overwintering, strongly suggested that in these adults the inner LAG was a metamorphosis line. Hemelaar (1985) and Castanet and Smirina (1990) have described this metamorphosis line in a few species of amphibians as a chromophilic perimedullar line which may form in correspondence with metamorphosis. As a rule, the metamorphosis line is associated with the presence of woven fibered embryonic bone. Our study does not support this supposition because perimedullar lines were never found in association with embryonic bone. In addition, we never observed metamorphosis lines in newly metamorphosed individuals. The variation in the perimeter of the first visible LAG suggests that there is plasticity in the body size at which the individuals of *R. latastei* overwinter the first time.

Among the Brown Frogs thus far analysed by skeletochronology, our results for *R. latastei* in the population

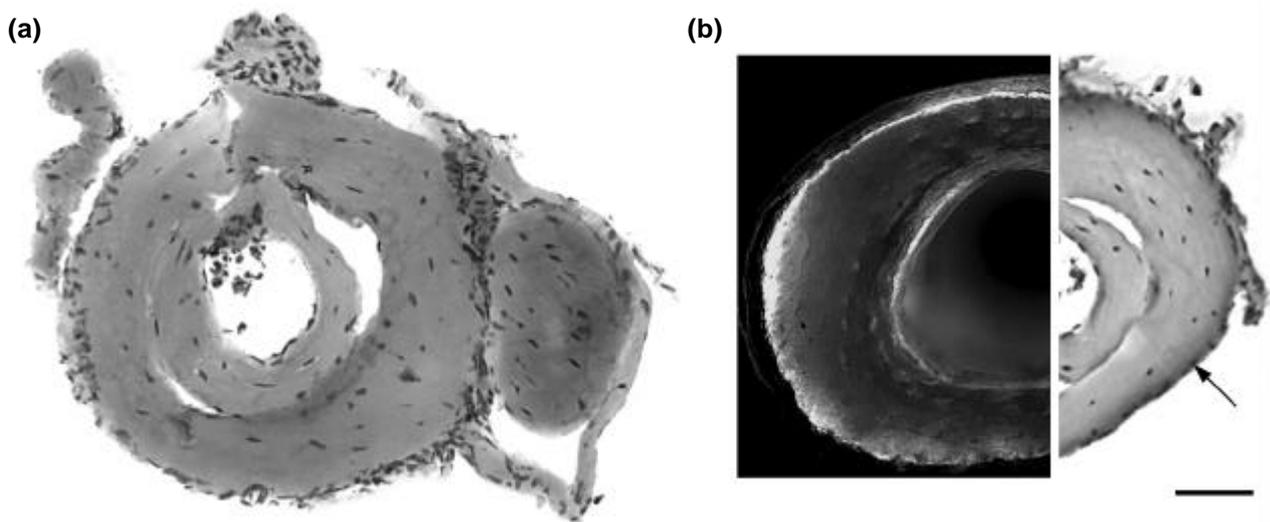


Figure 5. Phalangeal cross-sections of an individual, 40 mm in SVL, before (a) and after (b) injection with calcein. (b) Graphically composed from two photos from serial sections (observed under ultraviolet microscope and light microscope, respectively) in order to show the correspondence between fluorescent label and LAG. Phalanx clipped pre-injection does not show LAG. Phalanx clipped at the end of experiment shows, under ultraviolet microscope, a fluorescent mark at the periphery of periosteal bone and a faint chromophilic annulus on the outer periosteal edge, under light microscope. Scale bar = 65 μ m

we studied show that it is one of most short-lived and one of the first to reach sexual maturity. The maximum age recorded was three years in males, and four in females; in both the sexes, the one year age class was best represented. Similar life spans have been reported in a few Brown Frog species, such as *R. japonica* (Marunouchi *et al* 2002) and *R. sylvatica* (Sagor *et al* 1999). However, in most European Brown Frog species, such as *R. temporaria* (Gibbons and McCarthy 1983; Augert and Joly 1993; Ryser 1996), *R. dalmatina* (Guarino *et al* 1995a; Cavallotto and Giacoma 2000), *R. italica* (Guarino *et al* 1995a, b), and *R. arvalis* (Lyapkov 1999), life span is greater than five years. However, it must be taken into account that there are often inter-population differences in longevity and age at maturity

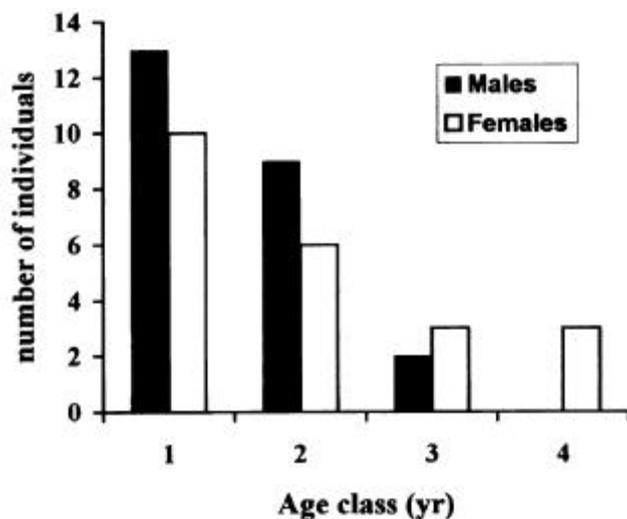


Figure 6. Age class distribution of adult males and females of *R. latastei*.

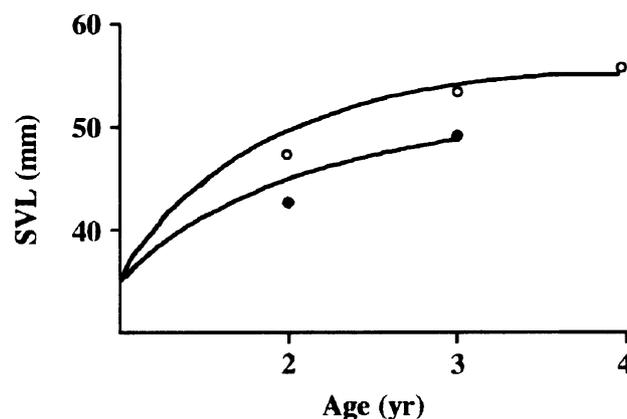


Figure 7. Growth curves for males (black circles) and females (white circles) of *R. latastei* by Logistic model. Data from 1998 and 1999 were pooled. The Logistic functions are: $L = 48/(1 + 0.27 * e^{-1.49t})$ for males; $L = 56/(1 + 0.48 * e^{-1.22t})$ for females.

depending on latitude and/or altitude. Many amphibians, including Brown Frogs, are thus more longer lived with increasing latitude and/or altitude (Guarino *et al* 1995b; Esteban and Sanchiz 2000; Leclair *et al* 2000). Therefore, comparative studies of populations living in climatologically different regions are necessary to determine whether our report on the short life span of *R. latastei* represents an intrinsic (genetic) characteristic of the species and/or is the result of one or more environmental factors.

The results show that both the sexes are potentially able to reproduce in the first breeding season after the first metamorphosis. Males possessed sperm bundles in their seminiferous tubules, while some females had yolky follicles in the ovary at about 5 months from metamorphosis. Many other Brown Frog species, like *R. temporaria* (Gibbons and McCarthy 1983; Ryser 1988), *R. italica* (Guarino *et al* 1995a), *R. dalmatina* (Cavallotto and Giacoma 2000), and *R. iberica* (Esteban and Sanchiz 2000) attain sexual maturity after the second year from metamorphosis. Moreover, in *R. latastei*, variation in size between froglets beginning overwintering for the first time may cause not only the difference in their size at emergence but also the difference in the size of their phalanges and, hence, of the perimeter of the LAGs.

As in many amphibian species, in *R. latastei* male and female growth rates are high until sexual maturity, and decrease thereafter. For both sexes of *R. latastei*, the growth curve is quite similar to that of other amphibians, such as *R. temporaria* (Ryser 1988) and *R. subaquavocalis* (Platz *et al* 1997). The growth curves indicates that after the attainment of sexual maturity, growth rate appears to be higher in females than in males.

In conclusion, phalangeal skeletochronology is a valuable method for age assessment of *R. latastei*, but, in accordance with Sagor *et al* (1999), we recommend that care be exercised to avoid conditions which can lead to underestimation of age. Further, research is required to confirm whether the low life span values we obtained are the general rule for *R. latastei*.

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