AGE STRUCTURE IN A DECLINING POPULATION OF RANA TEMPORARIA FROM NORTHERN ITALY

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In recent years the population of *Rana temporaria* from Laghi Paradiso (northern Italy) declined without any appearing reason. We applied skeletochronology to study the age structure of this population during the breeding season 2005. Males were on average smaller in body length than females. The modal age was 6 yrs in males and 3 yrs in females. Maximum age ascertained (10 yrs) was the same in both sexes. Sexual maturity was attained after the second or third year from metamorphosis in males and after the third year in females. Based on skeletochronological patterns, we conclude that in the examined population of *R. temporaria* females are larger than males not because they are on average older but because they grow faster beginning their pre-adult stages of life (2 yrs), this fact contributing to sexual size dimorphism in adults. Comparisons with skeletochronological data obtained from other European populations of *R. temporaria* confirm that in this species body length, longevity and age at maturity are inversely correlated to the activity period. Finally, we aimed to investigate potential demographic factors responsible for decrease in the study population

Key words: *Rana temporaria,* age structure, skeletochronology, population decline

INTRODUCTION

Many amphibian populations show marked fluctuations in their size and structure. The knowledge of individual age and of population age structure might be very useful for understanding the causes of population instability and for developing appropriate management and conservation plans of populations in their natural environments (DRISCOLL 1999). Skeletochronology is one of more reliable methods to estimate individual age, enabling also growth rates and age at sexual maturity to be ascertained (CASTANET *et al*. 1993). Up to 2004 more than 110 amphibian species had been investigated by skeletochronological methods (ISHCHENKO 2004). Skeletochronology consists in the careful interpretation of lines of arrested growth (LAGs) recorded in bone tissue by which individual age can be assessed. For several years, this technique has been successfully applied on phalanges by many researchers worldwide, being useful particularly in demographic studies (KHONSUE *et al*. 2001, GUARINO *et al*. 2003, MORRISON *et al*. 2004). In fact, the removal of one or more phalanges enables a bone sample to be obtained

without sacrificing the animals and allows them to be marked for subsequent iden-
tification in field studies.

The purpose of this investigation was to study the age structure of a north-
western Italian alpine population of European common frog, *Rana temporaria.*
During the last decade, this population has shown marked numerical decrease
without a clear explanation (personal communication by the staff of the Park) but
its demographic parameters have never been studied. *R. temporaria* has an exten-
sive distribution throughout Europe (GROSSENBACHER 1997); although in Italy it
is found only in the Alps and in the northern Apennines (BERNINI & RAZZETTI
2006). Among European anurans this species is one of the most widely studied by
skeletochronology (GIBBONS & MCCARTHY 1984, GUYÉTANT *et al*. 1988, RYSER
1988, 1996, ESTEBAN 1990, AUGERT & JOLY 1993, MIAUD *et al*. 1999). Conse-
quently, there are thus copious data on longevity, age at sexual maturity and age
structure for different populations of *R. temporaria* inhabiting different climatic
areas. By comparing our skeletochronological data with those reported elsewhere
we finally aimed to investigate the potential demographic factors responsible for a
decrease in the population in question.

MATERIALS AND METHODS

Study area and sampling

The study population breeds in a small permanent lake locally called "*Il paradiso delle rane*"
in the comune of San Giorio di Susa (1250 m a.s.l.), within the Orsiera – Rocciavré Natural Park.
Close to this lake there is another small lake, in witch just a few frogs lay eggs. The study lake is iso-
lated from the other by more than 4 km and about 700 meters of altitude. Maximum dimension of the
study lake are 110 m × 70 m with a surface area averaging 4,000 m²; maximum depth is about 2 m.
The lake is surrounded mainly by mixed broadleaf and coniferous woods, with a few small herba-
ceous clearings.

One hundred frogs (79 males and 21 females) were collected during the breeding period 2005,
from the end of March to the end of April. Drift fences with pitfall traps were used to sample frogs
during their breeding migration and dipnets to sample frogs during their aquatic phase. We placed
drift fences just on the southern side of the pond, where frogs lay eggs, before reproductive activity.
We didn't put any trap on the northern side because no frog was ever observed there by the staff of the
Park. Furthermore, the northern side was frozen at the beginning of the study. Frogs were sexed by
external secondary characters and measured from snout to urostyle to the nearest 0.1 mm with a cali-
per. For age determination by skeletochronology, from each animal we removed the second phalanx
of the fourth toe from the right forelimb. The phalanges were fixed with 70% ethanol and stored until
processed for skeletochronological analysis. After registration and toe-clipping, the frogs were re-
leased in their original capture site.

Skeletochronological analysis

Skeletochronological analysis were performed according to the standard protocol (GUARINO *et al*. 1995, 1998). After removing the surrounding tissue, the phalanx was decalcified with 5% nitric acid for about 1 h 15 min, rinsed with tap water for at least 4 h and mounted in cryocompound OCT-tissue tek (Sigma). Cross sections at mid-diaphyseal level, 12 µm thick, were obtained by cryostat and stained with Mayer's acid hemalum. For each sample, about twenty sections were observed under Leica light microscope equipped with a Leica DC 100 digital colour video camera. Of these sections, we counted the number of lines of arrested growth (LAGs) in the periosteal bone. Subsequently, in order to estimate the number of LAGs potentially lost during growth owing to endosteal resorption, three representative sections with the smallest medullar cavities were acquired and transformed as digital photos. On these sections, we performed osteometrical analysis using NHI-image software. The perimeters of reversal line (RL), that is the boundary between periosteal and endosteal bone, of the first innermost (towards medullar cavity) visible LAGs, and of the periosteal bone outer margin (PBM) of the youngest (with low LAG number) frogs were measured and compared with the RL and first LAG perimeters of the largest frogs (GUARINO *et al*. 2004). The measures were taken on all frogs.

In agreement with other authors (SMIRINA 1994, MIAUD *et al*. 1999, ROZENBLUT & OGIELSKA 2005) and taking into account the microclimatic parameters of the sampling area where frogs regularly overwinter during the cold months (November to March), we assumed that each LAG corresponds to an annual arrest of individual growth. Consequently, it was possible to assess the individual age and age structure of the population by counting the number of total LAGs (number of visible LAGs plus number of totally resorbed LAGs).

In order to estimate age at sexual maturity, we followed the criterion of LAG rapprochement as proposed by KLEINEBERG and SMIRINA (1969) and widely used by other authors (FRANCILLON-VIEILLOT *et al*. 1990, LECLAIR *et al*. 2005, TSIORA & KYRIAKOUPOLOU-SKLAVOUNOU 2002). Therefore, the first decreasing interval between LAGs, which is supposed to indicate the attainment of sexual maturity, was identified for each section.

All statistical analyses specified in the text were performed with the program Systat version 5. A 5% significance level was selected.

RESULTS

*Body size and sexual dimorphism*

In the breeding season examined, the sex ratio was 3.8: 1 in favour of males. All males were judged sexually mature by nuptial pads except for one small individual (50.1 mm), codified as RT 111. Mean body length ± SD was 79.5±7.8 mm (N = 78, range 61.1–85.2) in males and 86.1±13.8 (N = 21, range 65.8–120) in females. The larger body size of females than males was significant (unpaired Student's t-test: t = 3.04, df = 97, P = 0.03). Distribution of body length of the collected frogs, separated by sexes and grouped into 2 mm classes according to RYSER (1988) is shown in Figure 1. Male length exhibited a unimodal distribution where the most represented class was that with body length between 80 and 81.9 mm. As regards females, we observed an irregular distribution of body size.

Skeletochronological interpretation

Hematoxynophilic lines with typical features of LAGs were observed in pha-
langeal sections of all frogs except the individual RT 111 (Fig. 2*a*). As a rule, LAGs
were strongly hematoxynophilic, circular and complete, with low variability in
their optical sharpness. In the frogs with more than five LAGs (Figs 2*c, e*; 3*d, e*) the
outermost lines were closely adjacent and often hard to count. In many of these
cases, careful observation at higher magnification (400×) of several serial sections
near to mid-diaphyseal level, preferably at the periphery of periosteal bone where
it forms a crest on which the phalangeal ligament was inserted (Figs 2*d, f*; 3*f*), al-
lowed us to clearly discern the peripheral LAGs and to reliably count them. Double
LAGs (two very closely adjacent growth marks) were rarely observed and were
counted as a single LAG in accordance with other authors (FRANCILLON *et al*.
1990, LECLAIR *et al*. 2005). False LAGs (incomplete and faint hematoxynophilic
lines) were also rare (Fig. 3*a*) and were not considered as true LAGs. Apart from
RT 111, twelve medium-sized frogs (8 males with body length ranging from 61 to
77 mm; 4 females with body length from 69.5 to 90 mm) showed no LAG on the
outer margin of periosteal bone (Fig 2*a, b*).
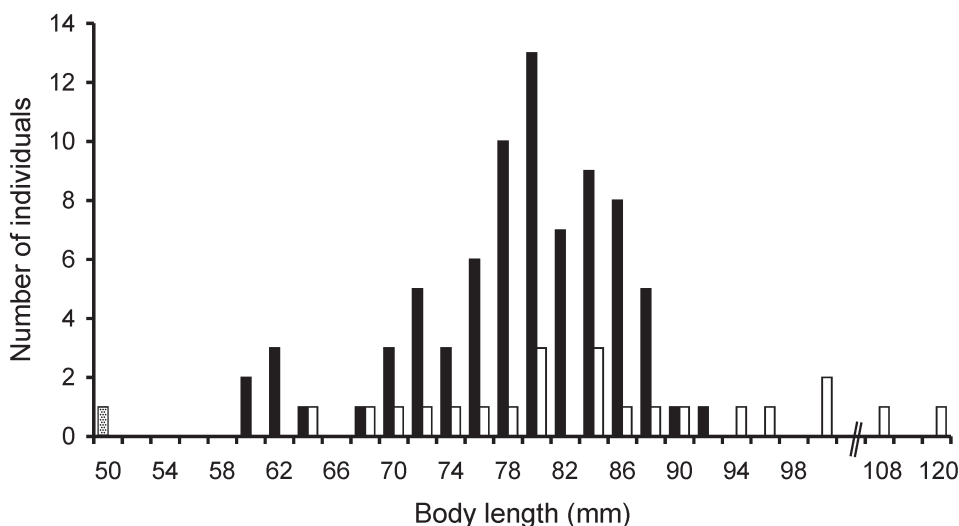
Fig. 1. Body length distribution (2 mm classes) of male (filled bars) and female (empty bars) *Rana
temporaria*. The dotted bar represents a juvenile male
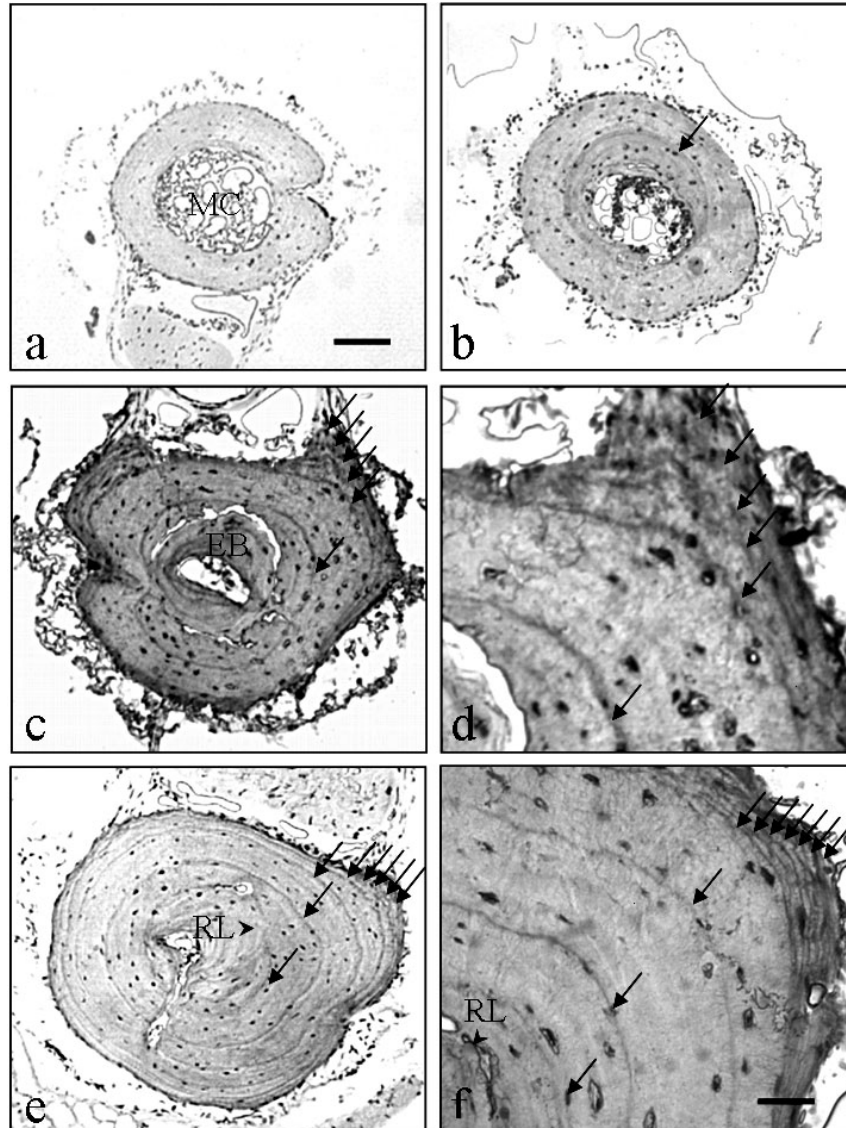
Fig. 2. Diaphyseal cross-sections of phalanges of *Rana temporaria* males. (a) Juvenile, 50.1 mm in body length, without LAGs. (b) Individual, 63 mm in body length, with 1 visible LAG plus one non-visible probably because it is confluent with the outer margin of periosteal bone. (c) Individual, 74.7 mm in body length, with 6 LAGs. (d) Same individual as in Fig. 2c but at higher magnification. 5 LAGs can be more easily counted in the ridge at the periphery of periosteal bone. (e) Individual, 85.2 mm in body length, with 8 LAGs. (f) Individual, 89 mm in body length, with 10 LAGs, of which the peripheral are very close to each other. Abbreviations: EB = endosteal bone; MC = medullar cavity; RL = reversal line. Arrows indicate lines of arrested growth (LAGs). Scale bar, 100 μm in Figs 2a–e; 35 μm in Figs 2d,f

Table 1. Perimeters (μm) of reversal line (RL), 1st and 2nd visible lines of arrested growth (LAG), and periosteal bone margin (PBM) in males and females *R. temporaria* showing up to 3 LAGs. For classes of 2 and 3 LAGs, values are expressed by means ± SD

n LAG	males					females				
	n	RL	1th LAG	2th LAG	PBM	n	RL	1th LAG	2th LAG	PBM
1	1	709	–	–	1170	1	–	–	–	–
2	6	744.7±135.9	1008.3±130.1	1380.2±99.1	1466.3± 88.2	2	789.1±164.1	1055.5±119.5	1437.5±95.5	1502.1± 3.5
3	11	770.5±148.1	950.9±253.8	1303.2±135	1520.9±119.2	7	848.5±172.7	1103.8± 87.1	1495.3±86.6	1626.8±98.2

Osteometric analysis and bone growth

For both sexes, bone outer perimeter was significantly correlated to body size (Pearson's correlation, males: r = 0.63, t = 6.25, df = 59, $p < 0.01$; females: r = 0.74, t = 4.10, df = 14, $p < 0.01$). Consequently, data for the growth rate of the phalanx thickness can serve as predictors of the body growth rate. In most of the phalanges we observed bone remodelling (endosteal resorption) in the innermost portion of periosteal bone concomitantly with endosteal bone formation (Figs 2*c, e, f*; 3*c, d, e*). Endosteal resorption varied greatly among individuals but was similar for males and females. Indeed, perimeter of RL (mean ± SD, males: 830±147 μm, N = 71, range = 445–1130; females: 851±133 μm, N = 17, range: 571–1031) did not differ between the sexes by unpaired Student's test (t = 0.53, df = 86, $p > 0.05$). In males with 2 or 3 visible LAGs the perimeter of RL and of the first LAG did not exceed that of PBM of the juvenile RT 111 except for two males (79.8 and 72.3 mm, respectively). Consequently, it seemed reasonable to conclude that in males with 2 or 3 LAGs no LAG was destroyed due to endosteal resorption except for two males for which one LAG was assumed to be lost. In females with 2 or 3 visible LAGs, the same type of analysis showed that in only one individual (81.4 mm) one LAG was very probably lost. Based on osteometric parameters from frogs with 2 and 3 visible LAGs we estimated the loss of LAGs, if any, in the frogs with more than 3 visible LAGs. Thus, we found only two males (84.6 and 86.2 mm, respectively) with resorption of the first LAG. Based on osteometric analysis we also extrapolated the rate of bone growth in the sexes. In frogs with 2 and 3 LAGs, the perimeter of the first LAG did not significantly differ between the sexes by unpaired Student's test (Table 1). By contrast, in the frogs with 3 LAGs a difference at the significance limit between sexes was observed in the perimeter of the second LAG (t = 3.33, df = 16, $p = 0.04$) and PBM (t = 1.96, df = 16, $p = 0.06$)
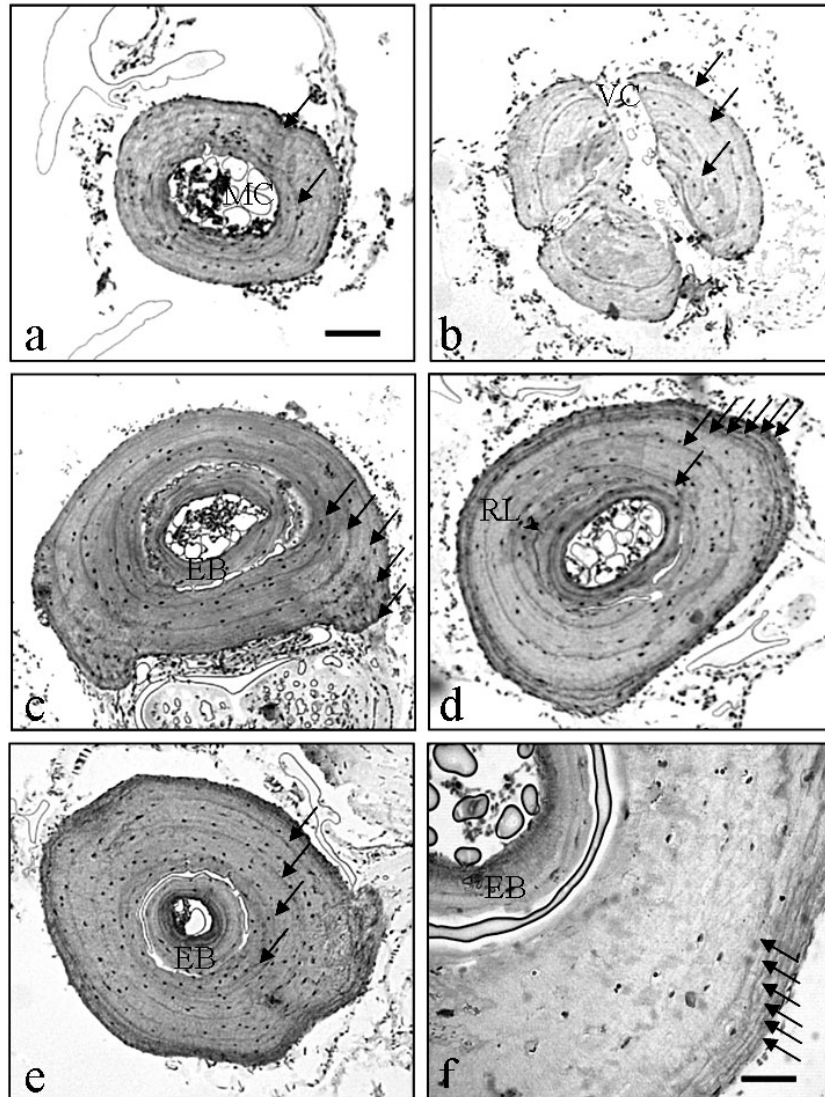
Fig. 3. Diaphyseal cross-sections of phalanges of *Rana temporaria* females. (a) Individual, 70 mm in body length, with 1 visible LAG plus one confluent with the outer margin of periosteal bone. Some false lines are also present. (b) Individual, 73.2 mm in body length, with 2 visible LAGs plus one confluent with the outer margin of periosteal bone. (c) Individual, 85.7 mm in body length, with 5 LAGs. (d) Individual, 101 mm in body length, with 7 LAGs. (e) Individual, 120 mm in body length, with the first 4 but not the peripheral LAGs clearly distinguishable. (f) Same individual as in the previous figure but adjacent section at higher magnification showing 6 distinct LAGs at the periphery of periosteal bone. Based on these observations it is concluded that this frog had 10 LAGs. Abbreviations: EB = endosteal bone; MC = medullar cavity; RL = reversal line; VC = vascular canal. Arrows indicate lines of arrested growth (LAGs). Scale bar, 100 μm in Figs 3a–e; 38 μm in Fig. 3f

Age structure, age and body length, age at sexual maturity

Mean age ± SD was 6±2.14 (N = 79, range: 1–10 yrs) in males and 4.76±2.11 (N = 21, range: 2–10 yrs) in females and did significantly differ between the sexes (unpaired Student's-test: t = 2.36, df = 98, *p* = 0.02). For both sexes, age distribution is shown in Figure 4. Modal age was 6 years in males (about 24% of the sample) and 3 years in females (28.6%). For males, the 7-year age class was also well represented (about 21.5%). Linear regression analysis showed a significant correlation between age and body length in both sexes (Fig. 5). Regression equations were: y = 2.53x + 55 (r^2 = 0.48, N = 77, *p* < 0.01) for males, y = 5.36x + 60.6 (r^2 = 0.67, N = 19, *p* < 0.01) for females. However, although the largest individuals were almost always the oldest in both sexes, considerable overlap of the body lengths among the different age classes was observed. Comparing the age-specific mean size of both sexes, females always exhibited larger body size than males (Table 2) except the 5-year age class but this was probably due to the limited number of females available. We found statistical differences in the 3-year (t = 1.20, df = 5 15, *p* = 0.05), 6-year (t = 4.58, df = 21, *p* < 0.001) and 7-year age class (t = 3.26, df = 17, *p* < 0.01).

Males with two LAGs (n = 7) showed nuptial pads; therefore, they were considered sexually mature. Among males with 3 or more LAGs about 46% exhibited a decline in bone growth after the 2nd LAG (Fig. 2*c*), 39% after the third LAG (Fig. 2*f*) and 8% showed no clear decrease between two successive LAGs except
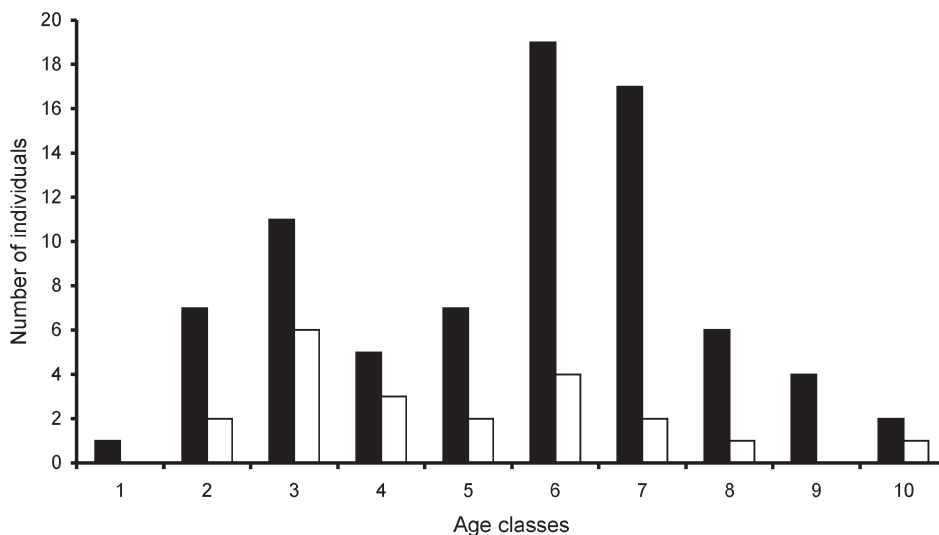
Fig. 4. Age distribution of male (filled bars) and female (empty bars) *Rana temporaria*

Table 2. Body length (in mm) for each age class in both sexes of *Rana temporaria*

| Age class | \| Males | | | | \| Females | | | |
	n	mean	SD	range	n	mean	SD	range
1	1	50.10	–	–	0	–	–	–
2	7	64.96	5.15	61.1–74.4	2	67.90	2.97	65.8–70
3	11	73.72	4.36	64.5–79.1	6	79.86	8.53	69.5–90.5
4	5	80.43	3.91	72.1–86.7	3	81.83	2.99	79–85
5	7	80.71	3.07	74.7–84.0	2	77.10	4.38	74–80.2
6	19	82.98	4.35	78–90.2	4	96.80	9.84	85.7–109.6
7	17	81.35	4.37	70.6–84.4	2	93.60	11.03	85.8–101.4
8	6	83.35	6.65	71.5–92.0	1	100.20	–	–
9	4	85.80	4.00	80,1–88.8	0	–	–	–
10	2	84.05	6.86	79.1–89	1	120.00	–	–
Total	79				21			

Table 3. Body lengths (range in mm), age at sexual maturity, maximum longevity from different populations of *Rana temporaria* studied by skeletochronology. In the case of studies performed on more consecutive years (e.g. RYSER 1988, AUGERT & JOLY 1993) data are pooled. Data reported in brackets are less frequent. Legend: NS= in the reference paper, it is not specified if the data refer to males or females; ND= data not reported

Locality (altitude)	Sex	Length (mm)	Age at maturity	Longevity	References
West Ireland (50 m)	M	46–78	2(3)	7	GIBBONS & MCCARTHY (1984)
	F	56–74	3(2)	7	
Spain (< 200 m)	M	ND	2 (NS)	8 (NS)	ESTEBAN (1990)
	F	ND			
East France (250 m)	M	43–70	1–2	6	AUGERT & JOLY (1993)
	F	47–80	2–3	6	
Swiss Alps (600 m)	M	59–91	2–3 (4)	8	RYSER (1988)
	F	62–98	3 (2–4)	8	
Italian Alps (1250 m)	M	61–90	3 (2)	10	Present work
	F	65–120	4 (5)	10	
North Spain (1700 m)	M	ND	3 (NS)	ND	ESTEBAN (1990)
	F	ND		11	
Swiss Alps (1930 m)	M	63–85	4	12	RYSER (1996)
	F	65–88	4	13	
French Alps (2000 m)	M	64.9–78	4	10	GUYETANT *et al.* (1988)
	F	72.7–87	5	8	
French Alps (2300 m)	M	70.4–82.6	4	12	MIAUD *et al.* (1999)
	F	82.3–90.2	4–5	15	

after the fifth or sixth LAG. By contrast, all females exhibited rapprochement after the third LAG, apart from two youngest females (65.1 and 70 mm, respectively). Accordingly, assuming the first LAG rapprochement to be an indicator of the onset of sexual maturity, males attained sexual maturity after the second or, to a lesser extent, third year, whereas all females matured after the third year.

DISCUSSION

In the present study the use of phalangeal growth marks allowed us to assess individual age and growth in most of the sampled frogs (about 89% of males and 70% of females) and only in a few individuals was age estimated with an error rate of one year. According to ROGERS and HARVEY (1994), the bone outer margin was counted in all cases as a LAG because the frogs were captured in the period immediately after hibernation.

Data on body size, age at sexual maturity and longevity of the *Rana temporaria* populations studied so far using skeletochronology are reported in Table 3. MIAUD *et al.* (1999) suggested that body size, longevity and age at sexual maturity seem to be inversely correlated to the activity period and show similar trends in both sexes. Our findings concur with this prediction and lend further contributions to the knowledge of demographic traits in this species. As regards age at sexual
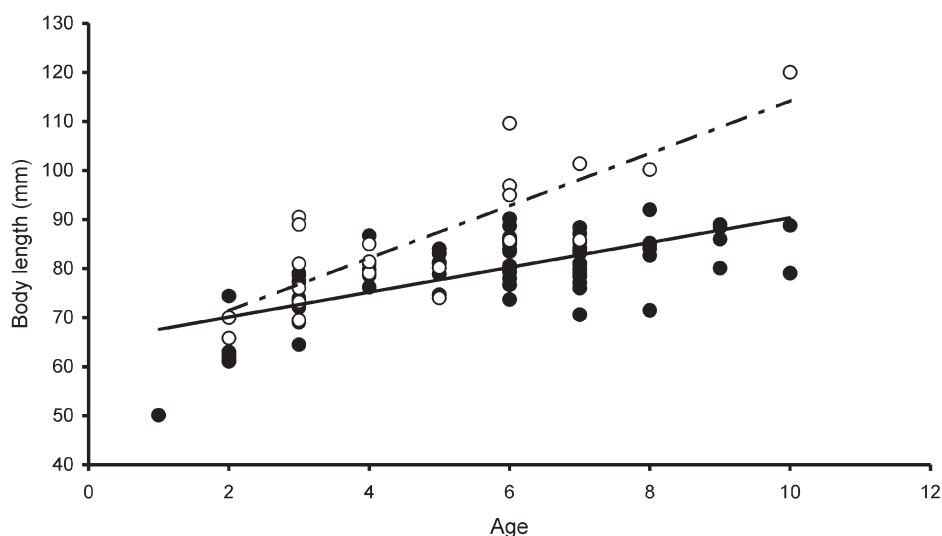
Fig. 5. Relationship between age and body length in male (filled circles) and female (empty circles) *Rana temporaria*

maturity, our results confirm for *R. temporaria* the general tendency to increased age of first reproduction in northern and upland frogs in comparison with southern and lowland frogs. In addition, males tend to breed for the first time earlier than females, the former reaching maturity chiefly after the 2nd year, the latter after the 3rd.

As in other populations *of Rana temporaria* (RYSER 1988, MIAUD *et al*. 1999), in the population from Laghi Paradiso there is a marked sexual dimorphism in body size, with females larger than males. Numerous proximate factors have been reported in literature to explain sexual dimorphism in anuran body size. They include growth rates different between the sexes before attainment of maturity, different age and/or body size between sexes at maturity, and different age structure between sexes in breeding populations (RYSER 1988, KHONSUE *et al*. 2001, MONNET & CHERRY 2002). In the case of *Rana temporaria* living at 2300 m a.s.l. (MIAUD *et al*. 1999), sexual dimorphism is due to the fact that females grow faster than males from metamorphosis to the 2nd or 3rd year whereas afterwards the growth rates are similar in the sexes. In addition, females tend to be older than males. In the case of two different Swiss populations of *Rana temporaria,* sexual dimorphism is largely the result of differential growth in a population living at 600 m. a.s.l. (RYSER 1988), whereas it is caused by both differential growth and differences in age structures in the population living at 1600 m 26 a.s.l. (RYSER 1996). In both Swiss populations, longevity is similar between the sexes. In our study, females are larger than males not because they are on average older but because they grow faster from their pre-adult stages (2 yrs). Comparisons with other populations seem indicate that sexual size dimorphism observed in *Rana temporaria* is not greater in upland or northern populations. However, in the majority of anuran species studied so far females are known to be larger than males (SHINE 1979) which is probably related to natural selection (MONNET & CHERRY 2002). In fact, previous studies on *Rana temporaria* showed that the largest females lay more numerous and larger eggs than smaller females (KOZLOWSKA 1971, CUMMINS 1986, GIBBONS & MCCARTHY 1986). Larger eggs develop faster than smaller eggs also because they have more nutrients. In unfavourable climates, such as in upland areas or in northern latitudes where the season for individual growth is short, faster development would prevent the risk that tadpoles do not complete metamorphosis before the onset of cold months. In addition, froglets can also have more time to forage and build up energy reserves before their first hibernation (MIAUD *et al*. 1999).

In this study, males are more numerous than females as in other populations of *Rana temporaria* (ELMBERG 1987, RYSER 1996). Interestingly, the modal age of females indicated a predominance of young breeders individuals (3 years) whereas there were few females older than 4 years, although they can live up to 10 years. By contrast, males were more abundant than females amongst the oldest age

classes (6 and 7 years). At present, we have no experimental data to explain the peculiar age structure of the *Rana temporaria* population studied herein. The general assumption that in a stable population the age distribution decreases with age (HEMELAAR 1988) seems consistent with the age structure of females, although the sample of females is restricted. It might be hypothesized that after the first reproduction only a small fraction of females survives for breeding in subsequent years owing to the strong reproductive effort and/or to other factors such as a higher rate of predation. In this regard it is worth remembering that about 50% of females examined by us were sampled by means of drift fences during migration towards the breeding site against 15% of the males. This suggests that most females, unlike males, overwinter away from the breeding site and consequently they are more exposed to the potential risks related to reproductive migration. Concerning the males, the paucity of 3–5 year-old individuals in comparison with 6–7 year-olds is 4 rather unexpected. However, age distribution of the anuran breeding population can change annually depending on recruitment rates, as observed for *Rana temporaria* as well (GIBBONS & MCCARTHY 1984, AUGERT & JOLY 1993). During the spring of 2005 we observed a total disappearance of tadpoles before their metamorphosis occurred, although it was not possible to identify the causes. If repeated in time, this observation could explain the scarcity of young adults with respect to older frogs, contrary to what is expected in the age composition of an unthreatened population. These aspects (different mortality between the sexes and possible failed recruitment of juveniles) currently being investigated by a capture-mark-release study.

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