Preliminary data on size and age of Oreolalax sterlingae (Nguyen et al., 2013)

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Preliminary data on size and age of *Oreolalax sterlingae* (Nguyen et al., 2013)

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Amphibians are currently considered the most threatened vertebrate class, with 41% of assessed species threatened with extinction (IUCN, 2022). Many amphibians remain poorly known and understudied and this paucity of knowledge may impede conservation actions targeting threatened amphibians (Rowley et al., 2009; Nori et al., 2018). Age structure and longevity are important to determine, particularly for highly threatened species, as this information can be vital in extinction risk assessment and in informing conservation management strategies.

Species living at higher elevation and or colder environments usually live longer (see review in Stark and Meiri, 2018). Although body mass and length may be indicative of age, they cannot be used to accurately estimate the age of an individual amphibian (Halliday and Verrell, 1988) and skeletochronology is considered a more reliable inference of age (Castanet and Smirina, 1990). Skeletochronology has been successfully used to estimate the age of frogs, especially those inhabiting areas with distinct seasonality as this seasonality results in discontinuous growth (Morrison et al., 2004; Liao and Lu, 2010a). During periods of reduced activity, lines of arrested growth (LAGs) form as bone tissue apposition stops or is greatly reduced. Studies have shown that a zone of bone growth followed by a LAG usually corresponds to an annual activity cycle in temperate amphibians (Smirina, 1994; Morrison et al., 2004; Liao and Lu, 2010a).

Sterling's Toothed Toad, (*Oreolalax sterlingae* Nguyen et al., 2013; Fig. 1A), is an Endangered species that is thought to be endemic to the Hoang Lien Range in northern Vietnam (Nguyen et al., 2013; Tapley et al., 2020; IUCN, 2021). This megophryid frog is only known to occur above 2345 m elevation in bamboo forest associated with rocky rivulets and rocky streams (Nguyen et al., 2013; Tapley et al., 2020; IUCN, 2021) and elfin moss forest (Tapley et al., 2017). Tadpoles develop in large pools in rocky streams (Rowley et al., 2017) and have been observed year-round on Mount Fansipan (Tapley et al., 2020). Adult male specimens are reported to reach 36.8 mm in size and females 44.6 mm in size (Nguyen et al., 2013). These frogs are likely to be seasonally active; over several field seasons active adult frogs have been observed in March (2018), April (2019), May (2022), June (2012, 2016; 2018, 2019, 2021, 2022), September (2015; 2017, 2018, 2020). During a two-day field survey undertaken in December 2017 we did not encounter any active *O. sterlingae* (L. Nguyen unpubl. data).

The most immediate threats to *O. sterlingae* include habitat degradation associated with tourism (Rowley et al., 2013; IUCN, 2021). It is also expected that the forest habitat of *O. sterlingae* has been greatly reduced in extent and quality due to historic burning and subsequent ecosystem conversion (Nguyen and Harder, 1996). The restriction of this species to high altitude forest also makes it inherently vulnerable as tropical montane forests are expected to be particularly prone to alteration by climate change (Foster, 2001).

We used skeletochronolgy to estimate ages of *O. sterlingae* to better understand the life history of this Endangered species.

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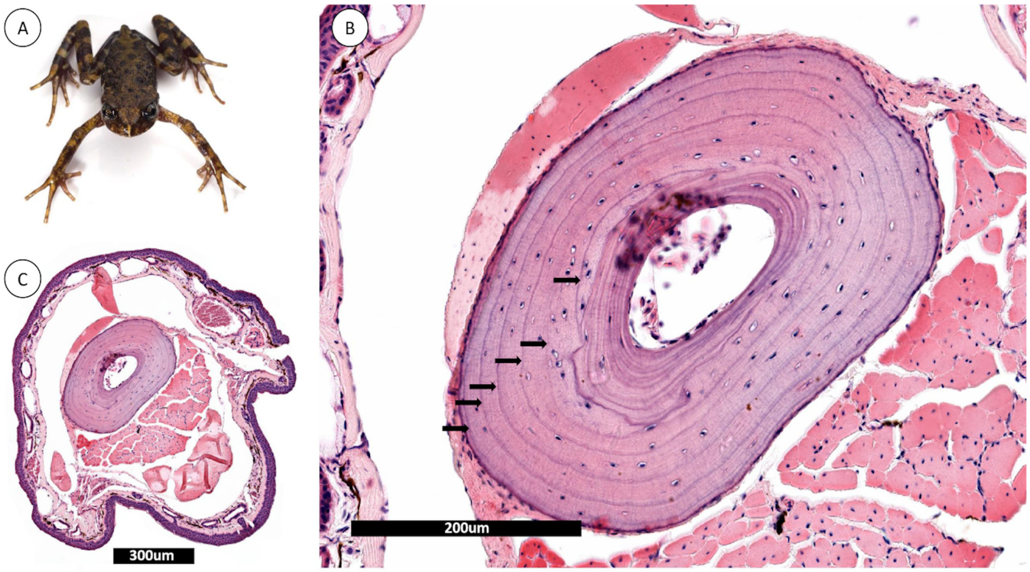

Figure 1. (A) *Oreolalax sterlingae* in life, (B) Cross section of the third toe of the right foot of an adult female *O. sterlingae* AMS R 177532, (C) Magnified toe cross section, each black arrow pointing towards a line of arrested growth. Photos by Jodi J.L. Rowley and Elaine Chew.

Methods

Study area. Mount Fansipan (Lao Cai Province, Vietnam) is the highest point in Indochina, at an elevation of 3143 m. The vegetation on Mount Fansipan between 2200 to 2900 m elevation consists of elfin moss forest (Frontier Vietnam, 2007). Annual temperatures range from -3–20 °C with an average of 15 °C, meanwhile frosts are frequent in the coldest months and there are typically 2–3 days of snow per year (Nguyen and Harder, 1996). Annual rainfall is 3500 mm and there are no months without rainfall (Nguyen and Harder, 1996; Nguyen et al., 2000).

Sampling and body measurements. We examined 17 *O. sterlingae* specimens (seven male, ten female) at the Australian Museum (voucher numbers AMS R177528, AMS R177530–42 and AMS177544–46) and toe samples taken from these specimens. These specimens were collected from stream habitats on Mount Fansipan (22.3146°N, 103.7657°E, elevation 2783 m, Hoang Lien National Park, Sa Pa District, Lao Cai Province, Vietnam) in June 2012. Specimens were fixed in 10% formalin and then stored in 70% ethanol. At the time the specimens were collected, body mass in life was recorded to the nearest 0.1 g using Pesola® spring scales (Pesola AG, Schindellegi, Switzerland). The snout to vent length (SVL) of preserved specimens was measured to the nearest 0.1 mm with digital callipers. Sex and maturity were determined by the presence or absence of nuptial pads and spines on the chest (secondary sexual characteristics of male *O. sterlingae*; Nguyen et al., 2013), and/or the presence of eggs (visible through the ventral surface of the females) and inspection of gonads via dissection.

Skeletochronological analysis. We followed a standard skeletochronology protocol to assess individual age through the count of lines of arrested growth (LAGs). The method was developed in the Histopathology Lab Veterinary Pathology Diagnostic Services, School of Veterinary Science, University of Sydney. We used the third toe of the right foot of each specimen. Toes were placed into processing cassettes and immersed in a decalcifying solution consisting of 10% formic acid in 10% buffered formalin (100 mL concentrated formic acid and 900 mL of 10% buffered formalin). Toes were kept overnight in this solution and up to 48 hours. Toes were then washed in running water to remove surface calcium and then processed in an automatic tissue processor (Medite TPC Trio) over 12 hours. Toes were then vertically embedded in Paraplast Tissue embedding wax. Blocks were trimmed of excess wax and cut at 8 µm onto Histobond®+ Slides, then dried overnight at 58 °C.

Staining method. Slides were dewaxed through changes of xylol, absolute ethanol, 95% and 70% ethanol prior to being washed in water. Sections were then stained in Whitlock's haematoxylin for eight minutes prior to being washed in running tap water, they were then stained in Scott's blueing solution for two minutes and then washed in running tap water two minutes before being contrasted with Eosin for 30 seconds. Sections were then dehydrated through two changes each of 70%, 95% and 100% ethanol. Slides were then placed in a 50% mixture of absolute ethanol and xylol. Sections were mounted with a 60 mm coverslip using D.P.X mounting fluid to create a permanent mount.

Age assessment. Age was assessed by four of the authors (BT, JJLR, CP and CM) who read digital images of the toe sections and counted the LAGs, each LAG was interpreted as an annual cycle of growth and indicative of age (Fig. 1B–C). Where there were discrepancies between authors regarding the number of LAGs, the sections were read again until a final consensus was reached.

Statistical analyses. All analyses were conducted in R 4.2.0 in RStudio (2022.02.2 Build 485) using the stats package (R Core Team, 2022). Nearly all male frogs had the same number of LAGs and so meaningful analysis of LAGs against SVL could not be undertaken due to insufficient LAG variation. A Kruskal-Wallis test was used to compare SVL, mass and LAG number between sexes. The relationship between SVL and LAG number for female frogs was explored by constructing a Linear Model (LM) with the structure SVL ~ LAG number, and then conducting likelihood ratio tests (LRT) against null models. SVL was chosen as the dependent variable as under the alternative hypothesis, age (with LAGS acting as a proxy) should drive increased body size. We did not analyse mass against LAGs as mass and SVL are tightly correlated for any given species, and mass is much more subject to short-term variation due to hydration and nutritional state than is SVL (Dodd, 2010). Alongside primary test outcomes, we also report r^2 for the linear model; this statistic represents the proportion of the variance for a dependent variable that is explained by an independent variable in a regression model, and is a common measure of model fit.

Literature review. To compare the maximum age of *O. sterlingae* with that of other frogs from montane systems in subtropical Asia with similar climate and season patterns to Mount Fansipan we reviewed the literature to compare the maximum reported age of frogs (Ma et al., 2009; Chen et al., 2012; Li et al., 2013; Liao and Lu, 2010a,b, 2011, 2012; Lou et al., 2012; Huang et al., 2013; Hsu et al., 2014; Meng et al., 2014; Yuan et al., 2021); for the purpose of our literature review we defined montane as above 1500 m above sea level.

Results

LAGs were present in all sampled specimens (Fig. 1B–C; Table 1). The innermost lines were closer together than the outer lines and staining of lines was equal across the toe cross sections. The mean number of LAGs in male *O. sterlingae* (n = 7) was 2.86 (SD ± 0.35) with a range of 2–3 LAGs. The mean SVL in male *O. sterlingae* (n = 7) was 35.7 mm (SD ± 3.81) with a range in SVL from 28.6–41.3 mm. The mean mass in life in male *O. sterlingae* (n = 7) was 3.7 g (SD ± 0.98) with a range of 1.7–4.7 g. The mean number of LAGs in female *O. sterlingae* (n = 10) was 4.0 (SD ± 1.10) with a range of LAGs from 2–6. The mean SVL in female *O. sterlingae* (n = 10) was 42.3 mm (SD ± 1.98) with a range in SVL from 39.5–46.3 mm. The mean mass in life in female *O. sterlingae* (n = 7) was 6.0 g (SD ± 1.27) with a range of 4.7–8.9 g. Four of the female frogs we examined were gravid, these had 3, 5, 5 and 6 LAGs respectively (see Table 1 for details). AMSR 177528 and AMSR 177536 (age estimated at two and three years respectively) lacked secondary sexual characters; we inspected gonads and confirmed that these specimens were male.

Female frogs were significantly longer (X^2_1 = 9.15, p = 0.002), heavier (X^2_1 = 11.12, p < 0.001) and had more LAGs (X^2_1 = 5.46, p = 0.019) than male frogs.

LAG and SVL data for female frogs were Normally distributed (Shapiro-Wilk test; W_9 = 0.95, p = 0.7; W_9 = 0.96, p = 0.8, respectively). A Breusch-Pagan test confirmed homoskedasticity (BP_1 = 0.16, p = 0.69). There was a significant effect of LAG number on SVL for female frogs (F_1 = 5.72, p = 0.044), with a slope estimate of 1.11 (95% Confidence Interval 0.04–2.18), y-intercept estimate of 37.91 mm (95% Confidence Interval 33.48–42.34) and adjusted r^2 = 0.34; see Figure 1.

The maximum age of 6 for *O. sterlingae* was four years less than the longest reported living frogs from the region (see references in literature review). A maximum age of 9 years was reported for female *Amolops mantzorum* (David, 1872) at 1700 m elevation in China (Liao and Lu, 2010a). There have only been three other studies assessing the LAGs in megophryid frogs (Meng et al., 2014; Peng et al., 2021). A mean age of nine years is reported for *Scutiger ningshanensis* Fang, 1985.

Table 1. The number of lines of arrested growth, snout-vent length and mass of the *Oreolalax sterlingae* in this study. *Gravid female.

Accession #	Sex	Gravid	SVL (mm)	Mass (g)	LAGs	Log10LAGs
AMSR177528	Male	N/A	28.6	1.7	3	0.477121
AMSR177530	Male	N/A	36.9	4.7	3	0.477121
AMSR177531	Male	N/A	36.7	4.2	3	0.477121
AMSR177535	Male	N/A	35.2	3.6	3	0.477121
AMSR177536	Male	N/A	41.3	4.2	2	0.30103
AMSR177545	Male	N/A	34.3	3.5	3	0.477121
AMSR177546	Male	N/A	36.6	4.2	3	0.477121
AMSR177532	Female*	Yes	46.3	7	6	0.778151
AMSR177534	Female*	Yes	41.2	6	3	0.477121
AMSR177537	Female*	Yes	42.3	5.9	5	0.69897
AMSR177538	Female	No	43.5	6.2	4	0.60206
AMSR177539	Female	No	40.5	4.7	2	0.30103
AMSR177540	Female*	Yes	43.8	8.9	5	0.69897
AMSR177541	Female	No	39.5	4.7	4	0.60206
AMSR177542	Female	No	40.7	5.6	4	0.60206
AMSR177544	Female	No	43.2	5.5	3	0.477121
AMSR177533	Female	No	42.4	4.9	4	0.60206

occurring at elevations between 1590–2047 m elevation in China (Meng et al., 2014).

Discussion

Whilst our sample size was small and could be subject to sampling bias, our data indicate that *Oreolalax sterlingae* may not be exceptionally long lived relative to other anurans occurring in similar habitats in subtropical Asia. Longevity could be influenced by phylogeny (e.g., Stark and Meiri, 2018) but there are no comparative data for other megophryid frogs. The mean age of nine years reported for *Scutiger ningshanensis* suggests that other megophryid frogs may be longer lived. Unfortunately, the authors of this study did not report maximum age or provide details of sex.

Our sample size was small and limited to a single population and so there is some uncertainty of whether our sample is indicative of the overall age structure in the species. Male *O. sterlingae* in our sample were significantly smaller and younger than females (Fig. 2; Table 1) and there was very little overlap in size or age between the sexes (Fig 3). Males were significantly younger than females overall. In many anurans, there is asymmetric mortality between males and females (Lemckert and Shine, 1993; Alho et al., 2008) and it is possible that there is a sex-dependent differential mortality with males dying younger in *O. sterlingae*. Our sample did not include frogs with <2 LAGs; young frogs may move away from breeding sites after metamorphoses and only returning once they are sexually mature. Furthermore, there may have been seasonality effects biasing sampling; older males may exist in the population, but dwell close to breeding sites at different periods, or have different activity patterns that affected encounter rates. Our sample did not include any male frog with more than 3 LAGs, there is the possibility that LAGs may not be truly indicative of age.

Previous research has shown that in female frogs, there is a trade-off between time to sexual maturity and body size; selection favours females with large body sizes and increased fecundity (Gibbons and McCarthy, 1983; Gillespie, 2011). Our results indicate that female *O. sterlingae* are only ready for breeding at three years old. The same is likely in male frogs; male *O. sterlingae* in this study exhibited obvious secondary sexual characteristics.

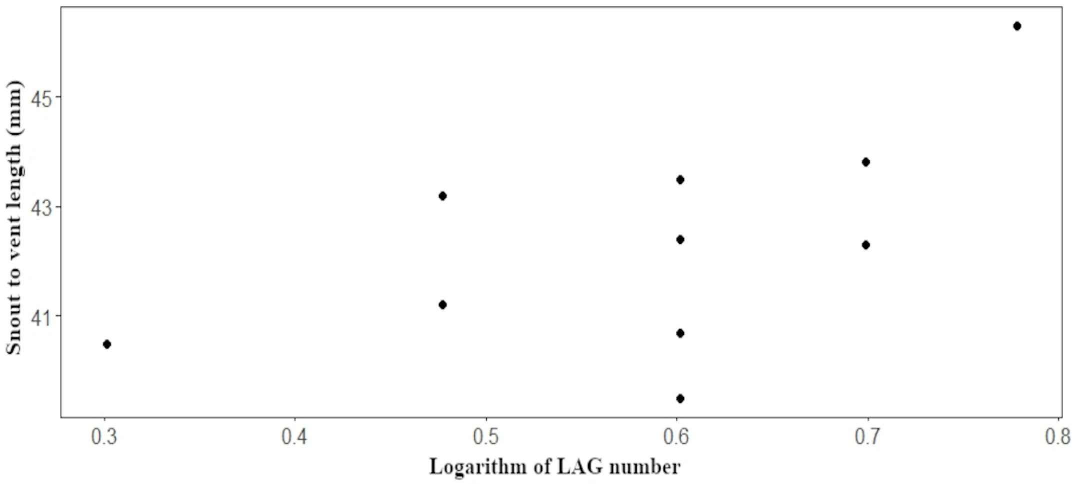

Figure 2. Scatter plot of snout-vent length and LogLAGs in female *Oreolalax sterlingae*.

Our analyses showed that there is a significant relationship between age (or at least LAGs) and SVL in the sampled females of this species, with SVL increasing by approximately 1 mm for each LAG within the range of LAGs reported here. Although LAG data may be reasonably expected to follow a Poisson, rather than a Normal, distribution, in this case normality tests demonstrated that this assumption was upheld. Despite this, there was substantial spread around the regression line with a relatively low r^2 value, and relatively broad confidence intervals, indicating a large amount of error around any predictions of age based on SVL. SVL is therefore not a useful field measurement to accurately estimate age of animals, or to infer changes in the demographic structure of populations over time. A larger data set may help to create a model with better fit. If the same relationship between SVL and age exists for males as it did in females, then the difference in size between sexes could be due to age or actual dimorphism. Our dataset was limited but further studies could compare animals at the same age, or control for age.

Recently metamorphosed individuals and juveniles were not encountered at the time the animals were collected (J. Rowley. Pers. Obs.). Growth rates in amphibians are indeterminate and strongly dependent on numerous factors including genetics (Laugen et al., 2003), maternal effects (Dziminski and Roberts, 2006; Kaplan and Philips, 2006), temperature (Harkey and Semlitsch, 1988; Kaplan and Philips, 2006), resource availability (Alvarez and Nicieza, 2002; Pasmans et al., 2012), health status (Parris and Cornelius, 2004; Kelehear et al., 2011), stress levels (Denver, 2009; Michaels and Preziosi, 2015), exposure to environmental contaminants (Diana et al., 2000), reproductive costs (Ryser, 1989) and competition (Griffiths and Foster, 1998; Richter-Boix et al., 2013) all of which are likely to decouple the relationship between age and size. Growth rates in amphibians are also known to be asymptotic (e.g., Ryser, 1998; Kupfer et al., 2004; Cadeddu et al., 2012) and this could also be the case in *O. sterlingae*. The data in this study did form a linear relationship but if our sample size had been larger, including more

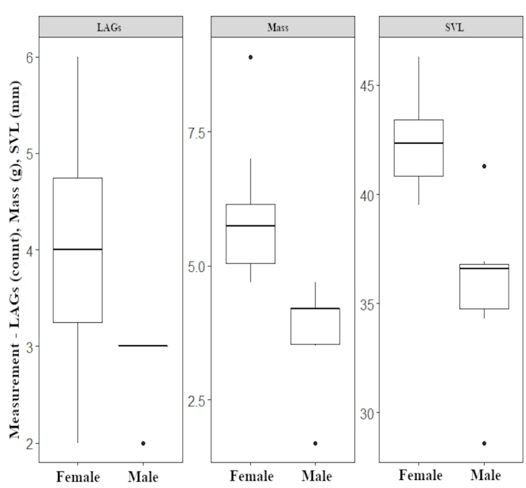

Figure 3. Box plots of male and female *Oreolalax sterlingae* lines of arrested growth, mass and snout-vent length.

extreme ages or sized, this linear relationship may have fit within a wider curvilinear relationship which could preclude linear modelling.

Four of the female frogs we examined were gravid, and had 3, 5, 5 and 6 LAGs respectively. It is possible that *O. sterlingae* may have a reproductive lifespan in excess of 3 years, assuming individuals grow and mature at similar rates (i.e., all can produce viable eggs from 3 years of age onwards). However, a greater sample size would be needed to confidently predict minimum age to reproduction.

Finally, some of the specimens in this study had a larger body size than those in the type series and a male and female in our study represent the maximum recorded size for both sexes in this species: male (AMSR 177536, 41.3 mm) and female (AMSR 177532, 46.3 mm).

The life history information presented here could contribute to the development of conservation strategies focusing on this Endangered amphibian. The data on age structure, longevity and reproductive lifespan could be used in combination with other data such as population size, fecundity, and distribution to undertake population viability analyses under different conservation management scenarios or incorporated into assessment of population extinction risk considering projected habitat loss and/or climate change. Unfortunately, we still lack important life history and population data for *O. sterlingae,* the population size and clutch size has not yet been reported. Future studies should aim to address these knowledge gaps.

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