

# Skeletochronology of phalanges underestimates the true age of long-lived Alpine newts (*Ichthyosaura alpestris*)

Alexander Wagner<sup>1</sup>, Robert Schabetsberger<sup>2</sup>, Marc Sztatecsny<sup>1</sup> & Roland Kaiser<sup>2</sup>

<sup>1</sup>Department of Evolutionary Biology, University of Vienna, Austria

<sup>2</sup>Institute of Zoology, University of Salzburg, Austria

Skeletochronology has become the most widely used method of ageing amphibians. However, bone remodelling, double lines and rapprochement of peripheral lines all hamper exact skeletochronological age determination. In a long-term field study, the age of long-lived Alpine newts (*Ichthyosaura alpestris*) from a high-altitude population was estimated from cross-sections of phalanges at the time of tagging with PIT tags and again 10–16 years later. The skeletochronologically assessed age at recapture underestimated the time since first capture by 2–19 years, and the observed deviance increased with increasing age estimates at first capture.

*Key words:* age, amphibians, longevity, mark–recapture, urodeles

High-altitude or -latitude habitats are characterized by low temperatures, low food availability and a short activity period (Nagy & Grabherr, 2009). Amphibians from such habitats show a number of specific characteristics in their life history, such as slow growth after metamorphosis, increased age at maturity and increased longevity (reviewed in Miaud & Merilä, 2000). To study interspecific variation in such age-associated traits and their change over time (e.g. linked to global warming), reliable and accurate methods of determining an amphibian's age are needed.

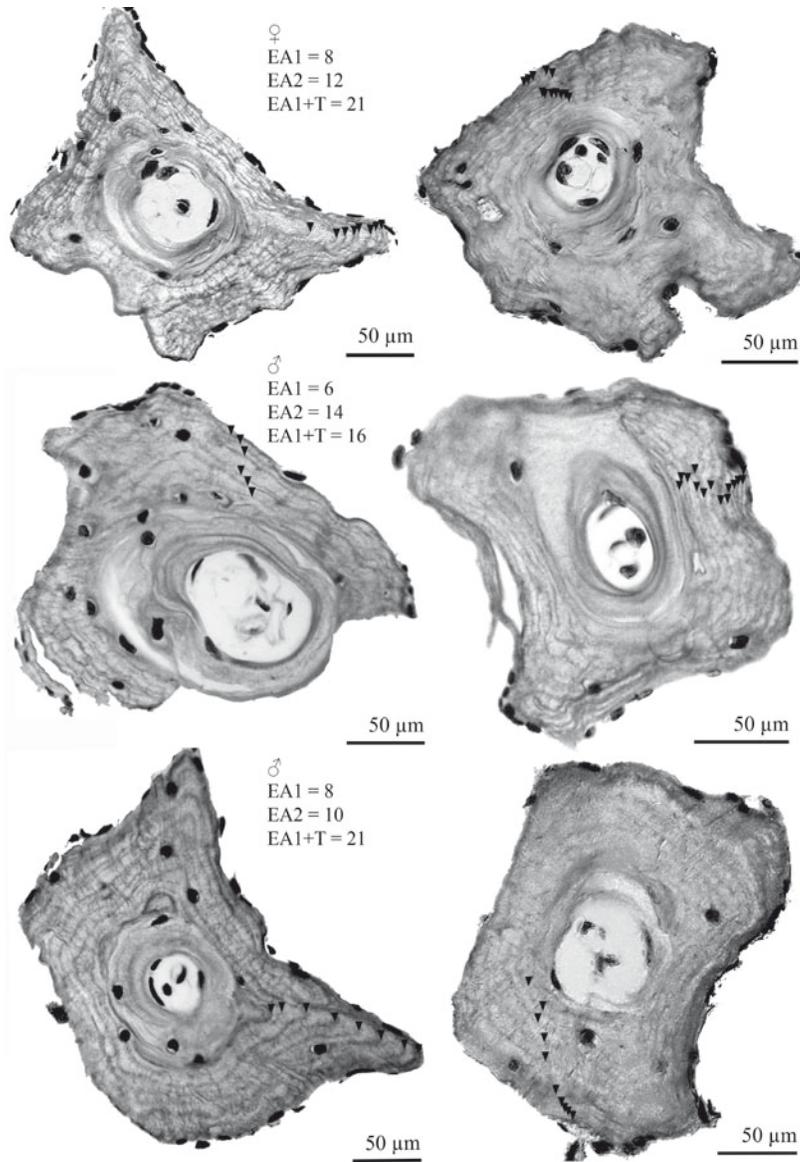
There are two methods for determining the age of amphibians, skeletochronology and mark–recapture investigations. The latter provides the most reliable data, but when mark–recapture data are unavailable, skeletochronology has turned out to be most adapted for ageing amphibians (Halliday & Verrell, 1988). Concentric rings resulting from rapid summer growth bands shifting with winter lines of arrested growth (LAGs) can be counted on bone cross-sections analogous to tree rings. The usage of long bones (e.g. humeri or femora) gives the most reliable LAG count data, but requires killing the animal. To avoid sacrificing amphibians, phalanges are now used for skeletochronology in anurans and larger urodeles (e.g.

Reading, 1991; Flageole & Leclair, 1992; Semlitsch et al., 1993; Guarino et al., 1995; Miaud et al., 1999). There are several challenges to skeletochronology, however, that may be more pronounced with phalanges due to their small diameter: additional LAGs or double LAGs as the result of a double annual growth cycle may occur (Sinsch et al., 2007), endosteal resorption can erode LAGs, and rapprochement of lines deposited late in life can render exact age determination impossible (Castanet & Smirina, 1990). To avoid the misinterpretation of skeletochronology data, in particular for aged individuals, calibration (i.e. analysing bones from animals of known age) is required. Until now, however, calibration of skeletochronology on amphibians of higher age has only been performed for captive animals, except by Eden et al. (2007) and Tejedo et al. (1997). Therefore little is known about the reliability of ageing data from free-ranging long-lived amphibians.

In our study, we made use of a long-term mark–recapture investigation in a high-altitude population of Alpine newts (*Ichthyosaura alpestris*; Faber, 1997), to investigate the deviance of the age at first capture plus the time that had passed between captures and estimated age at recapture. The animals had been individually marked with passive integrated transponders (PITs) and were toe clipped at the same time. Knowing each individual's estimated age when first marked (EA1), the time passed since marking (T) and the estimated age at the time of recapture (EA2) allowed us to test the reliability of skeletochronology using toes for the first time in a natural high-altitude population of Alpine newts.

The Alpine newt occurs in Europe from sea level up to 2500 m a.s.l. in the Swiss Alps (Gasc et al., 1997), and extensive data on age, body length and growth are available (Miaud et al., 2000; Schabetsberger & Goldschmid, 2001). Our study site is located in the Northern Limestone Alps of Austria at 1900 m a.s.l. and is characterized by a plateau containing a few hundred pools (see Faber, 1997, for details). Between 1993 and 1999 Alpine newts from 17 ponds were marked with PITs and the middle toe of a hind limb was removed for age determination (Faber, pers. comm.). Between May and September 2009 30 ponds were resampled for four days each month by dipnetting. All newts caught were scanned for PITs with a portable reader (Trovan LID-560). From tagged newts the second toe (pointing towards the belly) of the left hind limb was clipped and immediately stored in 70% alcohol.

In the laboratory, skin and muscles were removed from the phalanges and the largest proximal digit of each toe was decalcified in Bouin's solution. After ten days it was embedded into paraffin, sectioned with a Leica Super Cut microtome at 10 µm, mounted on slides and stained with Mayer's haematoxylin (Hemelaar, 1985). While focusing through a section under a Nikon Eclipse E800 microscope (10 × 10 magnification), seven to ten pictures were taken and overlaid with the software CombineZ (<http://www.micropics.org.uk>). The LAGs of six mid-diaphyseal sections from one phalanx were counted and the mean was calculated.



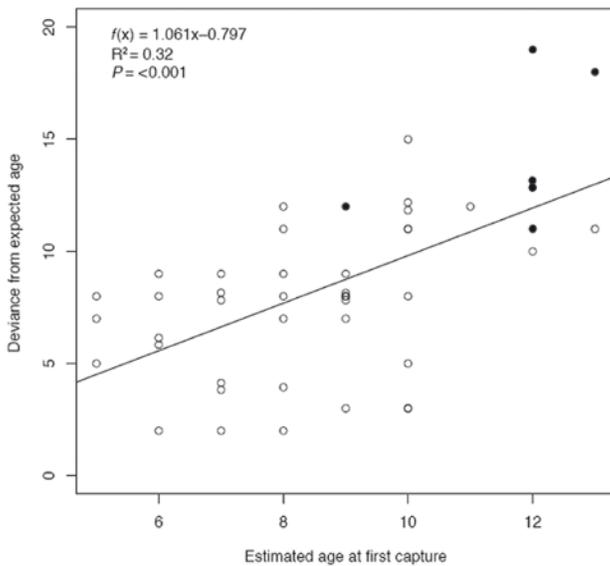
**Fig. 1.** Comparison of cross-sections of phalanges of Alpine newts removed in 1996 and 1999 (left) and 10–13 years later (right). Estimated age at first capture (EA1), and recapture event (EA2) and elapsed time (T) between these events are shown for each pair.

Preliminary analyses indicated considerable mismatch between EA1+T and EA2, exclusively underestimating the age at recapture (Fig. 1). The calculated deviance ((EA1+T)–EA2) ( $n=45$ , mean =  $8.46 \pm 3.99$  SD) fitted a normal distribution (Shapiro–Wilk normality test,  $W=0.9626$ ,  $P=0.1538$ ). Least squares linear regression was conducted to address the question of whether the deviance is related to the individual's initial age. Analyses were performed in R with base packages (R Development Core Team, 2010).

A total of 28 female and 17 male adult Alpine newts were resampled after 10–16 years. The mean EA1±SD for females was  $10 \pm 2.1$  (range 5–13) and for males  $7 \pm 1.7$  (range 5–12) years. The mean EA2±SD of recaptured

females was  $13 \pm 3.5$  years (range 8–22), that of males  $11 \pm 2.5$  (range 7–16), and the mean deviance between EA2 and EA1+T in males and females was  $7 \pm 3$  (range 2–11) and  $9 \pm 4.4$  (range 2–19) years, respectively. An individual's age at first capture positively influenced the deviance ( $R^2=0.32$ ,  $P \leq 0.001$ , Fig. 2).

Our results suggest that skeletochronology of phalanges grossly underestimates the true age of long-lived Alpine newts at high altitudes. The deviance increased with increasing age, showing that remodelling processes in the bones and rapprochement of peripheral LAGs increasingly reduce the reliability of age estimates in long-lived individuals. In this respect the study confirms results obtained for tiger salamanders in the Rocky Mountains (Eden et



**Fig. 2.** The relationship between skeletochronologically estimated age at first capture and the deviance of this age plus the time that had passed between captures and estimated age at recapture. Filled dots symbolize individuals where EA2 was lower than EA1.

al., 2007). It is very likely that most skeletochronological studies at high altitude or latitude conducted so far have underestimated the true age of amphibians.

Endosteal resorption probably accounts for EA1 sometimes exceeding EA2 by up to five years (Fig. 2). However, a maximum deviance of up to 19 years suggests that rapprochement of peripheral LAGs is the main reason for underestimating the true age. Cross-sections of humeri of a nearby population showed up to 12 narrow LAGs after the newts had reached sexual maturity with an estimated age of 10 years (Schabetsberger & Goldschmid, 1994). In a consecutive study the bones were embedded into cold-polymerizing resin and the thickness of cross sections was reduced to 7  $\mu\text{m}$  (Schabetsberger et al., 2001). This method allowed a higher resolution of LAGs deposited late in life and yielded a maximum age of 29 years, which was almost reached in this study (28 years estimated in a female individual for EA1+T). Assuming that such old animals do not grow anymore, we believe that Alpine newts may live well beyond the age of 30 years. Hence the only reliable method of obtaining exact age estimates for long-lived amphibians is to permanently mark them after metamorphosis and recapture them later in life. However, to our knowledge no method exists for applying time-specific permanent marks on large numbers of small metamorphs that can be recognized up to three decades later. Although the reliability of skeletochronological age estimates is higher in short-lived amphibian species, there is a need to develop new techniques to permanently mark young metamorphs.

**Acknowledgements.** We want to thank Helmut Faber for providing toes and PIT data from marked newts, as well as Julia Felling, Walter Hödl and Stefan Kummer for their support in the field and helpful comments on the manuscript. MS received funding from FWF grant P22069 during the writing of the manuscript. Field work was conducted under permit number FA13C-53S-7/2009-71.

## REFERENCES

- Castanet, J. & Smirina, E.M. (1990). Introduction to the skeletochronological method in amphibians and reptiles. *Annales des Sciences Naturelles (Zoologie)* 11, 191–196.
- Eden, C.S., Whiteman, H.H., Duobinis-Gray, L. & Wissinger, S.A. (2007). Accuracy assessment of skeletochronology in the Arizona tiger salamander (*Ambystoma tigrinum nebulosum*). *Copeia* 2007, 471–477.
- Faber, H. (1997). Der Einsatz von passiven integrierten Transpondern zur individuellen Markierung von Bergmolchen (*Triturus alpestris*) im Freiland. Naturschutzrelevante Methoden der Feldherpetologie. *Mertensiella* 7, 121–132.
- Flageole, S. & Leclair, R. Jr. (1992). Étude démographique d'une population de salamanders (*Ambystoma maculatum*) à l'aide de la méthode squelette-chronologique. *Canadian Journal of Zoology* 70, 740–749.
- Gasc, J., Cabela, A., Crnobrnja-Isailovi, J., Dolmen, D., Grossenbacher, K., Haffner, P., Lescure, J., Martens, H., Martinez Rica, J., Maurin, H., Oliveira, M., Sofianidou, T., Veith, M. & Zuiderwijk, A. (1997). *Atlas of Amphibians and Reptiles in Europe*. Paris: Societas Europaea Herpetologica & Museum National d'Histoire Naturelle (IEGB/SPN).
- Guarino, F.M., Angelini, F. & Cammarota, M. (1995). A skeletochronological analysis of the syntopic amphibian species from southern Italy. *Amphibia-Reptilia* 16, 297–302.
- Halliday, T.R. & Verrell, P.A. (1988). Body size and age in amphibians and reptiles. *Journal of Herpetology* 22, 253–265.
- Hemelaar, A. (1985). An improved method to estimate the number of year rings resorbed in phalanges of *Bufo bufo* (L.) and its application to populations from different latitudes. *Amphibia-Reptilia* 6, 323–341.
- Miaud, C., Guyétant, R. & Elmberg, J. (1999). Variations in life-history traits in the common frog *Rana temporaria* (Amphibia: Anura): a literature review and new data from the French Alps. *Journal of Zoology* 249, 61–73.
- Miaud, C., Guyétant, R. & Faber, H. (2000). Age, size and growth of the Alpine newt, *Triturus alpestris* (Urodela: Salamandridae), at high altitude and a review of life-history trait variation throughout its range. *Herpetologica* 56, 135–144.
- Miaud, C. & Merilä, J. (2000). Local adaptation or environmental induction? Causes of population differentiation in alpine amphibians. *Biota* 2, 31–50.
- Nagy, L. & Grabherr, H. (2009). *The Biology of Alpine Habitats*. Oxford: Oxford University Press.
- R Development Core Team (2010). *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing.
- Reading, C.J. (1991). The relationship between body length,

- age and sexual maturity in the common toad, *Bufo bufo*. *Holarctic Ecology* 14, 245–249.
- Schabetsberger, R. & Goldschmid, A. (1994). Age structure and survival rate in alpine newts (*Triturus alpestris*) at high altitude. *Alytes* 12, 41–47.
- Schabetsberger, R., Jersabek, C.D. & Goldschmid, A. (2001). Sex reversal cannot explain female-biased sex ratios in high altitude populations of the alpine newt (*Triturus alpestris*). *Biota* 2, 75–87.
- Schabetsberger, R., Langer, H., Jersabek, C.D. & Goldschmid, A. (2000). On age structure and longevity in two populations of *Bufo bufo* at high altitude breeding sites in Austria. *Herpetozoa* 13, 187–191.
- Semlitsch, R.D., Scott, D.E., Pechman, J.H.K. & Gibbons, J.W. (1993). Phenotypic variation in the arrival time of breeding salamanders: individual repeatability and environmental influences. [Journal of Animal Ecology](#) 62, 334–40.
- Senning, W.C. (1940). A study of age determination and growth of *Necturus maculosus* based on the parasphenoid bone. [American Journal of Anatomy](#) 66, 483–494.
- Sinsch, U., Oromi, N. & Sanuy, D. (2007). Growth marks in natterjack toad (*Bufo calamita*) bones: histological correlates of hibernation and aestivation periods. [Herpetological Journal](#) 17, 129–137.
- Tejedo, M., Reques, R. & Esteban, M. (1997). Actual and osteochronological estimated age of natterjack toads (*Bufo calamita*). *Herpetological Journal* 7, 81–82.

Accepted: 2 February 2011