

Usefulness of postpygal caudal vertebrae and osteoderms for skeletochronology in the limbless lizard *Anguis veronensis* POLLINI, 1818

(Squamata: Sauria: Anguidae)

Die Brauchbarkeit postpygaler Schwanzwirbel und Osteoderme in der Skeletochronologie der extremitätenlosen Echse *Anguis veronensis* POLLINI, 1818
(Squamata: Sauria: Anguidae)

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KURZFASSUNG

In der vorliegenden Studie untersuchten die Autoren die Brauchbarkeit von postpygalen Schwanzwirbeln und Osteodermen zur skeletochronologischen Altersbestimmung bei Individuen der fußlosen Echse *Anguis veronensis* POLLINI, 1818. Dabei erwies sich die Altersbestimmung anhand von Osteodermen als unsicher, da in den untersuchten Tieren die Anzahl der Linien verlangsamen Wachstums (LAGs) sowohl zwischen einzelnen Osteodermen als auch innerhalb derselben variierte. Im Gegensatz dazu waren Neuralbögen der Schwanzwirbel in der Skeletochronologie insofern verwertbar als die Anzahl der LAGs in diesen Strukturen innerhalb jedes der untersuchten Tiere relativ konstant war. Unter der Annahme, daß alljährlich eine LAG gebildet wird, war das älteste Individuum der Untersuchung neun Jahre alt. Nicht ausgeschlossen werden kann allerdings eine zu niedrige Einschätzung des Alters, bedingt durch Knochenumbau unter völliger Zerstörung von LAGs. In der skeletochronologischen Altersbestimmung von *Anguis* ist deshalb bei Verwendung von Schwanzwirbeln eine vorsichtige Interpretation angezeigt.

ABSTRACT

In this study, the authors tested the usefulness of postpygal caudal vertebrae and osteoderms for the assessment of individual age in the limbless lizard *Anguis veronensis* POLLINI, 1818, employing the skeletochronological method. The results showed that osteoderm-based skeletochronology was unreliable, since within any specimen examined the number of lines of arrested growth (LAG) varied among and even within osteoderms. On the contrary, skeletochronology was to some degree applicable to the neural arch of the caudal vertebrae, as the LAG number was roughly stable in all neural arches per animal studied. Assuming that one LAG is formed per year, the oldest individual in the present study was nine years old. However, age underestimation due to bone remodeling that completely destroyed the LAGs cannot be ruled out. Therefore, cautious interpretation is required in the skeletochronological age estimation of *Anguis* if caudal vertebrae are analyzed.

KEY WORDS

Reptilia: Squamata: Sauria: Anguidae, *Anguis veronensis*, skeletochronology, age determination, caudal vertebrae, osteoderms, morphology, ecology

INTRODUCTION

Skeletochronology is a widely used method for estimating individual age and growth rates of vertebrates, using incremental growth marks in the bone (CASTANET et al. 1993; CASTANET 2006). Concerning reptiles, this technique was successfully applied to numerous taxa inhabiting temperate (e.g., CASTANET & ROCHE 1981; GUARINO et

al. 2010; GÜL et al. 2014) subtropical and tropical (PATNAIK & BEHERA 1981; CASTANET & GASC 1986; PANCHARATNA & KUMBAR 2005) areas. Previous studies showed that diaphyseal cross sections of limb long bones were the most informative elements for skeletochronological analysis. Therefore, tetrapods with largely reduced limbs or

limbless taxa pose specific problems to the skeletochronological approach. For these vertebrates, alternative methods were developed, such as the use of growth marks in certain skull bones (e.g., the wall of the *fossa mandibularis* of the temporal bone and the ectopterygoid bone) or caudal vertebrae, entire or sectioned. The former method, applied in different species of snakes (CASTANET 1974; HAYASHI & TANAKA 1981; COLLINS & RODDA 1992) requires sacrifice of the studied individuals whereas, the use of caudal vertebrae minimizes injuries, especially when natural urotomy, that characterizes many lizards, is exploited (GUARINO 2010). Also the study of osteoderms was proposed as potentially useful for skeletochronology and harmless to the animals. So far, this non-lethal skeletochronological procedure was tested for a few species only (TUCKER 1997). Osteoderms are bony plates present within the dermis, under epidermal horny scales on the head or the whole body of crocodilians and

numerous saurian taxa, including Anguidae. The use of osteoderms in skeletochronology was recently questioned by BOCHATON et al. (2015) in diploglossine lizards, which are placed within the family Anguidae (PYRON et al. 2013).

The Italian Slow-worm, *Anguis veronensis* POLLINI, 1818, is a legless lizard of the Family Anguidae, spread throughout the Italian Peninsula and south-eastern France. Until a few years ago, the Italian Slow-worm populations were ascribed to the species *Anguis fragilis* LINNAEUS, 1758, widespread in most of Europe, and only recently were elevated to the rank of a separate species according to molecular and cytogenetic evidence (GVOŽDÍK et al. 2013; MEZZASALMA et al. 2014). Very little is known about the life span of *Anguis* species and the age structure within their populations. The present study aims to test the usefulness both of subterminal caudal vertebrae and osteoderms in skeletochronological studies of *A. veronensis*.

MATERIALS & METHODS

Eight individuals (1 newborn, 3 males, 3 females, 1 unsexed adult) of *A. veronensis* collected at Ruvo del Monte (Potenza, Southern Italy) and Valle Agricola (Caserta, southern Italy) were used for the study. Tail fragments were taken from individuals previously studied (MEZZASALMA et al. 2013) with the approval of institutional committees.

All had intact (non-regenerated) tails. Snout-vent length (SVL) was measured to the nearest 1 mm with a ruler, and the sex was determined by the presence or absence of hemipenes. Before their release in the capture site, from each individual a small piece (about 4 mm) of the tail tip was clipped, fixed and stored in 70 % ethanol for laboratory analysis.

To identify, in a pilot test, the most suitable section planes for skeletochronological analyses (i.e., those parts of the caudal vertebrae or osteoderms that are characterized by strong development of bone and little remodeling activity), two small pieces of tail were taken from the newborn, one

male and one female individual, each. After embedding in paraffin according to standard technique, the tail fragments were sectioned transversely or longitudinally using a rotary microtome. The sections, 7 µm thick, were stained with Mayer's hemalum and eosin (Carlo Erba, Milano, Italy), and observed under the microscope using both ordinary and polarized light.

Skeletochronological analyses were performed according to standard protocols (GUARINO et al. 2004; GUARINO 2010). After removing the scales, the remaining piece of tail was decalcified with 5 % nitric acid for about three hours, rinsed with tap water (overnight), and cross-sectioned at 12 µm thickness, using a cryostat (Leica Reichert Jung 2800N Frigocut). The sections were stained with Mayer's hemalum (25 min) and mounted in aqueous resin (Carlo Erba, Milano, Italy). Lines of arrested growth (LAGs) were independently counted by two researchers (FMG and MM) using a light microscope equipped with an image analyzer.



Fig. 1: Parasagittal section of the postpygal portion of the subterminal part of the tail of *Anguis veronensis* POLLINI, 1818, stained with Mayer's hemalum-eosin. Vertical lines (a – f) indicate the levels of the cross-sections shown in Figs. 2 (A – F). Scale bar: 330 µm.
 Abb. 1: Parasagittaler Schnitt im postpygalen Schwanzbereich von *Anguis veronensis* POLLINI, 1818. Färbung: Mayers Hämalaun-Eosin. Die vertikalen Linien (a – f) bezeichnen die Lage der Querschnitte in Abb. 2 (A – F). Maßstab: 330 µm.

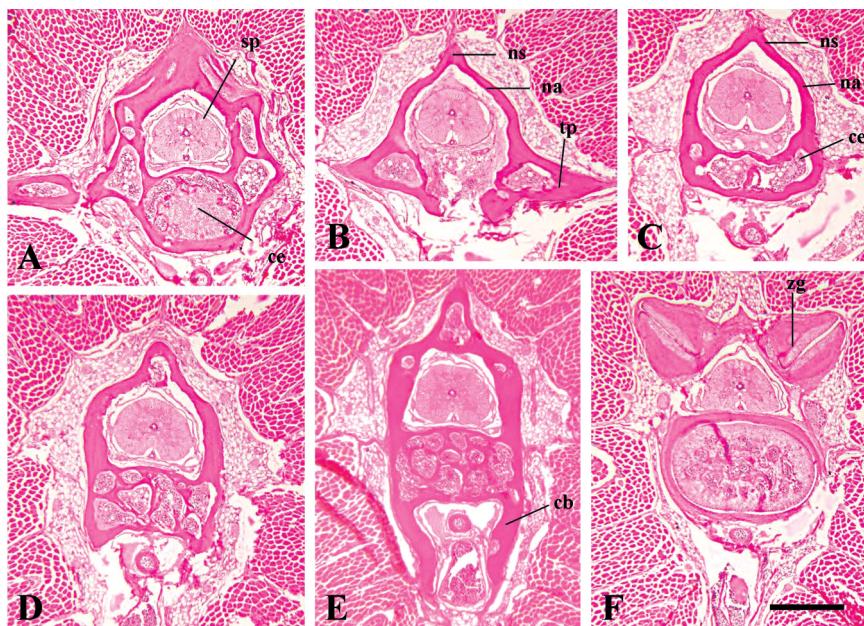


Fig. 2: For the legend see opposite page. / Abb. 2: Legende siehe gegenüberliegende Seite.

RESULTS

Table 1: Snout-vent length (SVL, in mm) and number of visible Lines of Arrested Growth (LAG) in subterminal caudal vertebrae of eight individuals of *Anguis veronensis* POLLINI, 1818, examined in this study. F – Female, M – Male; NB – newborn; ND – not determined.

Tab. 1: Kopf-Rumpf-Länge (SVL, mm) und Anzahl der erkennbaren Linien verlangsamten Wachstums (LAG) in subterminalen Schwanzwirbeln der acht untersuchten Individuen von *Anguis veronensis* POLLINI, 1818. F – Weibchen, M – Männchen; NB – Neugeborenes; ND – unbestimmt.

Code	Sex	SVL	LAG
Afra NB	ND	61	0
Afra ND	ND	125	4
Afra 2	F	155	7
Afra 3	F	197	7
Afra 4	M	143	5
Afra 5	M	166	9
Afra 7	M	127	3
Afra 10	F	112	7

The observation of serial histological cross-sections revealed that in *A. veronensis* the portion of the caudal vertebra most suitable for skeletochronological interpretation was the neural arch, inasmuch as it is quite developed and less affected by remodeling phenomena than other parts of the vertebra

(Figs. 1, 2). Consequently, the present skeletochronological analysis is focused on the neural arch. The latter was mainly composed of parallel-fibered bone with distinct hematoxylinophilic lines interpretable as LAGs (Fig. 3), even if they were not always clearly expressed throughout the neural arch extension. The maximum number of counted LAGs was 9; the newborn individual displayed no LAG (Table 1).

Osteoderm cross sections showed two layers of bone, an outer, superficial layer and a basal plate (terminology according to ZYLBERBERG & CASTANET 1985). The superficial layer was composed mainly of woven bone and appeared completely dark (or extinct) when observed in polarized light, under crossed Nichols (Figs. 4A, 4B). This layer was characterized by tubercles in the posterior portion of the osteoderm. The basal plate was composed mainly of lamellar bone and revealed alternate dark and light strata in polarized light (Figs. 4A, 4B). Both layers showed hematoxylinophilic lines but the number of these lines, both in the superficial and the basal stratum, was often different within the same individual, depending on the osteoderm or its portion examined (Figs. 4C, 4D).

DISCUSSION

This study shows that in *A. veronensis* the individual age assessment based on osteoderm skeletochronology is inaccurate since the LAG number varies between different osteoderms and even within the same element. On the contrary, the skeletochronology is potentially applicable to the neural arch of the caudal vertebrae as the LAG number was roughly stable within each animal tested. Yet, in the case of the caudal vertebrae, caution is needed because some problems, such as the rapprochement of the LAGs and the formation of supplementary bone marks (*sensu* CASTANET et al. 1993) can complicate LAG counting.

To reliably estimate individual age, each skeletochronological study requires to be validated as regards both the periodicity of formation of the LAGs and the possible

effects of bone remodeling that can provoke complete destruction of one or more LAGs, resulting in an underestimation of the animal's real age. Concerning the periodicity of LAG formation, it is reasonable to assume that in *A. veronensis* one LAG is formed per year during the wintering period of inactivity, as was demonstrated for other seasonally active lizard species from temperate areas (PILORGE & CASTANET 1981; CASTANET 1985).

However, without the accuracy assessment based on individuals of known age it is impossible to verify the reliability of this method in *A. veronensis*. Furthermore, one cannot exclude the possibility that the age of the individuals examined here was underestimated, owing to bone remodeling. The probability that one or more LAGs are com-

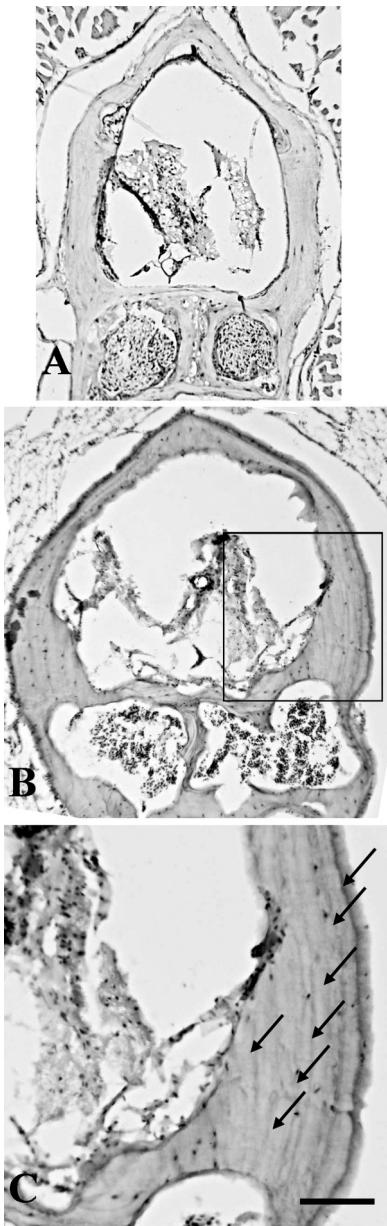


Fig. 2 (opposite page, bottom): Cross sections of a postpygal caudal vertebra of *Anguis veronensis* POLLINI, 1818, at the levels shown in Fig. 1. Staining with Mayer's hemalum-eosin. cb – chevron bone; ce – centrum; na – neural arch; ns – neural spine; tp – transverse process; zg – pre- and postzygapophyses.

All the figures are at the same magnification. Scale bar: 320 µm.

Abb. 2 (gegenüberliegende Seite, unten): Querschnitte eines postpygalen Schwanzwirbels von *Anguis veronensis* POLLINI, 1818 an den in Abb. 1 gezeigten Positionen. Färbung: Mayers Hämalaun-Eosin. cb – Hämabogen; ce – Zentrum; na – Neuralbogen; ns – Dorsalfortsatz; tp – Querfortsatz; zg – Prä- und Postzygapophysen. Balkenlänge entspricht 320 µm, Teilbilder im selben Maßstab.

pletely destroyed by bone remodeling is generally evaluated through the back-calculation method (CASTANET et al. 1993; GUARINO et al. 2004; GUARINO 2010). This method is applicable only to structures of more or less regular geometric shape (for example diaphysis of the femora or phalanges) but not irregular structures, such as vertebrae. In this study, the possibility of complete removal of one or more LAGs appears supported by the fact that individuals with SVL very different (Afra 3 and Afra 10) have the same number of LAGs.

So far, there is no data on the individual age of wild Eurasian Slow-worms; ecological studies on *Anguis* species report only data on SVL (STUMPEL 1985; CAPULA et al. 1992; LUISELLI et al. 1994; SOS & HERZEG 2009). Even taking into account the small size of the sample examined and the possible underestimation of LAG number, the present findings indicate individual ages much lower than those reported for animals in captivity, which may attain more than 50 years (LUISELLI et al. 2011). The increased longevity of individuals in captivity relative to that observed in individuals from natural populations is found in many other species and it is argued to be the consequence of a series of artificial conditions such as the absence of predators and the constant availability of food (GUARINO 2010).

Fig. 3 (left): Cross sections of postpygal caudal vertebrae of *Anguis veronensis* POLLINI, 1818, stained with Mayer's hemalum. A – newborn, 61 mm SVL, no Lines of Arrested Growth present; B – Female, 112 mm SVL; C – Boxed area of B in higher magnification, seven LAGs (arrows) are visible. Scale bar represents 175 µm in A and B, 80 µm in C.

Abb. 3 (links): Querschnitte postpygaler Schwanzwirbel von *Anguis veronensis* POLLINI, 1818. Färbung: Mayers Hämalaun. A – Neugeborenes, 61 mm Kopfrumpf-Länge (KRL), keine Linien verlangsamer Wachstums (LAG) vorhanden; B – Weibchen, 112 mm KRL; C – Das in Abb B eingerahmte Feld vergrößert; sieben LAGs (Pfeile) sind erkennbar. Die Balkenlänge entspricht in A und B 175 µm, in C 80 µm.

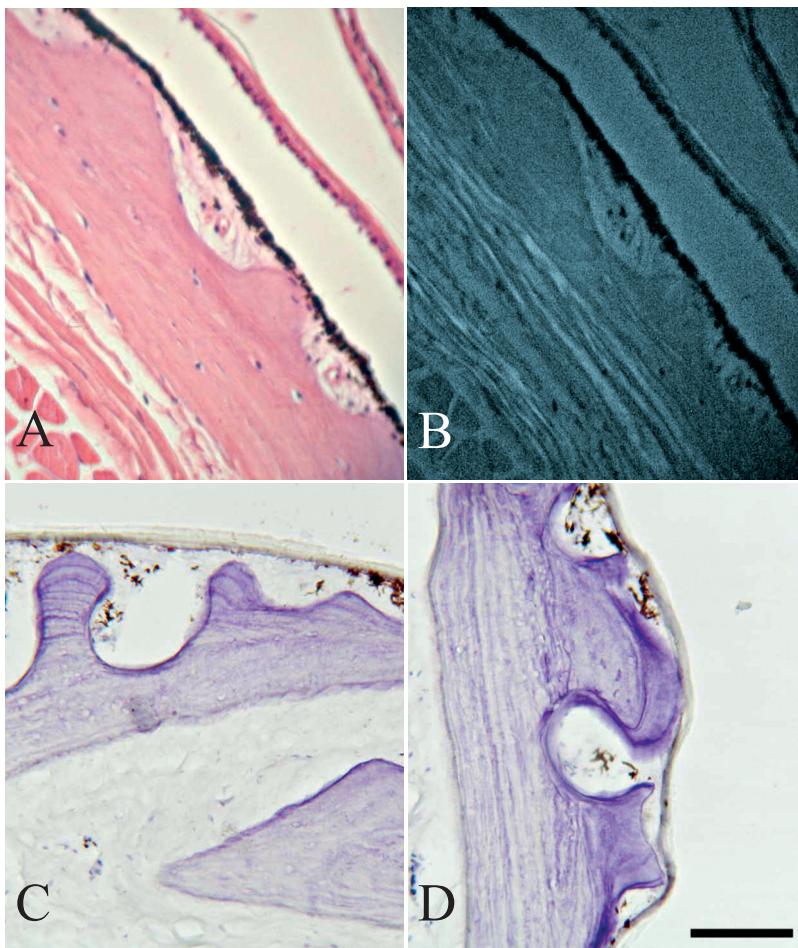


Fig. 4: Cross sections of osteoderms from the subterminal tail region of *Anguis veronensis* POLLINI, 1818. A – Female, 112 mm SVL, section stained with Mayer's hemalum-eosin and observed under transmitted ordinary light. B – Same section as in A, observed under polarized light. C and D – Male, 166 mm SVL; two different portions of caudal osteoderms, section stained with Mayer's hemalum. Several LAGs are visible. Scale bar represents 170 µm in A and B, 160 µm in C and D.

Abb. 4: Querschnitte von Osteodermen aus der subterminalen Schwanzregion von *Anguis veronensis* POLLINI, 1818. A – Weibchen, 112 mm Kopf-Rumpf-Länge (KRL); Färbung: Mayers Hämalaun-Eosin, Ansicht im Durchlicht; B – gleicher Schnitt wie in A, Ansicht im polarisierten Licht; C und D – Männchen, 166 mm KRL, zwei unterschiedliche Stellen; Färbung: Mayers Hämalaun. Mehrere Linien verlangsamen Wachstums sind erkennbar. Die Balkenlänge entspricht 170 µm in Abb. A und B, 160 µm in Abb. C und D.

ACKNOWLEDGMENTS

The authors are grateful to Stella DOLZATELLI for her assistance in the preparation of histological sec-

tions and Prof. Rakesh RASTOGI (both Università di Napoli “Federico II”) for language revision.

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DATE OF SUBMISSION: November 9, 2015

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