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An assessment of the reliability of growth rings counts for age determination in the Hermann's Tortoise *Testudo hermanni*

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Abstract. Growth ring counts on the shell have been widely used for age estimation in Chelonians. However, as stated by Wilson et al. (2003), most studies have applied this method without assessing its reliability by proving that 1 : 1 ratio between number of rings and real age exists. In the present study, the reliability of this method is analysed for a population of Hermann's Tortoise, *Testudo hermanni* introduced in the Ebro Delta (NE Spain). Age estimations were obtained from direct observations of tortoises in the field in 2000 ($n = 82$) together with those from photographs of the same and other individuals of the population taken between 1991 and 2001 ($n = 356$). A second photograph was taken at one or more years after the first one for 101 individuals. Results of Model II linear regression analysis indicated that the method was reliable only for tortoises between 0 and 7 years old, whereas tended to underestimate age for those between 8 and 11 years. Since, sexual maturity in this population is attained around 8 years (mean for both sexes), ring counts are only reliable for juveniles and subadults. Finally, it is noteworthy that the results coming for photographs were equivalent to those coming from direct observations in the field.

Introduction

Individual age determination allows us to study the life history strategies of species in a more reliable way than categorisation by age classes (i.e. juveniles and adults). When available, age-dependent estimations can be obtained for parameters such as survival, mortality, growth and reproductive traits (i.e. age of sexual maturity and fecundity). However, this approach cannot be applied in many long-term studies since it requires either the monitoring of individuals from birth or indirect ageing methods that do not affect survival (Germano and Bury, 1998). Growth ring counts on the shell of chelonians provide indirect evidence for age calculation in this group. This method has been widely used (see reviews by Graham, 1979; Dunham et al., 1988; Dunham and Gibbons, 1990; Zug, 1991; Germano and Bury, 1998), although some authors have questioned its validity (Tracy and Tracy, 1995; Kennett, 1996; Brooks et al., 1997; Wilson et al., 2003).

Growth rings on tortoise shell depend on the successive deposition of layers of epithelial tissue during periods of intensive growth alternated with grooves generated when growth stops (Castanet and Cheylan, 1979; Germano, 1988; Zug, 1991). To provide a reliable estimation of age, rings should be deposited cyclically and the rhythm of deposition should be determined (Graham, 1979; Germano, 1988; Zug, 1991; Germano and Fritts, 1994; Wilson et al., 2003). Thus, if the period when growth is slow or null occurs once a year (i.e. hibernation), there should be a 1 : 1 relation between the numbers of rings and periods of arrested growth. If this is the case, the number of cycles will coincide with age.

Several studies have used growth rings to determine age in Hermann's tortoise, *Testudo hermanni* Gmelin 1789 (Castanet and Cheylan, 1979; Stubbs et al., 1984; Stubbs and Swingland, 1985; Stubbs et al., 1985; Meek, 1989; Nougarède, 1998; Willemsen and Hailey, 1999), although only Castanet and Cheylan (1979) proved that ring production followed an annual rhythm. Nevertheless, this result may be not directly generalised to other populations or

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species. Wilson et al. (2003) proposed that the age determination method should be calibrated for each population and species since ring deposition may change with local environmental conditions and phylogeny. Thus, here we tested whether ring counting is a reliable method for age assessment in a population of *T. hermanni* from the Ebro Delta and, if so, to determine the age range in which satisfactory results can be obtained.

Furthermore, although direct ring count in the field was the method most used here, in other studies moulds or photographs were taken. The latter provide a permanent record of the specimens and can be analysed later in more detail (Galbraith and Brooks, 1987; Brooks et al., 1997). Since most of the information in this study comes from photographs, an additional objective was to assess the validity of this method for ring counts.

Material and methods

Here we analysed a population of Hermann's tortoise from a stock of adults that were introduced into the Ebro Delta Natural Park (Tarragona Province, Catalonia, NE, Spain) in 1987 in order to conserve this species in this area (Bertolero et al., 1995; Bertolero, 2002). Between 1991 and 2001, 448 tortoises, all of which belonged to the first and second generations that were born free, were marked (Bertolero, 2002).

Study site. This population inhabit several islets of one the reserves of the Natural Park. The space around the islets lacks vegetation and sea cover depends on season and weather. The islets are constituted by dunes with small slopes fixed by psammophile and halophile vegetation. A small forest of White Pine, *Pinus halepensis*, covers parts of the area and also contributes to fixing sand and retaining humidity. The climate is littoral Mediterranean, with a long dry summer (between 1987 and 2001: mean annual rainfall = 450.2 l/m^2; mean temperature = 17.2°C). More details on habitat, climate and the introduction project can be found in Bertolero (2002).

General methods. In the first capture, the carapace and the plastron of each tortoise were photographed to make a graphic data-base of the population. This procedure was carried out between 1991 and 1992 and, later, between 1995 and 2001. From 1995 onwards, some of the individuals recaptured within a minimum of one year, were photographed. From the 448 tortoises localised, 356 (79.5%) were photographed. Of these, 101 were photographed a second time in the following years (mean time between photos $\pm$ SD: 2.9 $\pm$ 1.5 years, range: 1-10 years).

Photographs were examined under a magnifying glass for ring recounts. Scutes were mainly abdominal and humeral but, in some cases, the third vertebral scute was also used. In the recounts, annual rings were considered as the spaces between two well-marked lines of arrested growth (Germano, 1988; Zug, 1991). Other secondary lines ("false rings") could, however, appear within the main ring, indicating minor growth arrests throughout the annual cycle (Dunham et al., 1988).

September 1 was fixed as the birth date for each year, since most hatchlings appear in this month (Cheylan, 1981). Thus, an individual with one ring was considered to belong to the age class 0 if observed before September 1 but age class 1 if observed after this date but before hibernation, in spite of showing the same ring number.

Two photographic recounts of rings were carried out independently and by the same observer. In the first (recount A), in January 2000, 311 individuals were analysed whereas in the second (recount B), November 2001, 356 tortoises were observed (tortoises from 2000 and 2001). Additionally, the rings of another 82 tortoises were counted by direct observation during intensive field monitoring in 200l (recount C).

Statistical treatment. The intra-class correlation coefficient r_I (Krebs, 1989; Sokal and Rohlf, 1995) was calculated to determine the repeatability of the ring counts from the photographs. It is usually accepted that when $r_I > 0.70$ repeatability in the measures occurs, although values over 0.90 are more reliable (Senar, 1999). The difference between ages obtained from recounts A and B was calculated to determine the proportion of bias.

Model II linear regression analysis was used to test the 1 : 1 relation between rings counted and the theoretical age, because the two variables were not controlled by the researchers (Legendre and Legendre, 1998; Legendre, 2001). If the correlation obtained was significant and the slope of the regression did not differ from 1.00 (within the 95% confidence interval of slope), the number of rings counted corresponds to the age of the individual (Germano, 1988). Three regression analyses were carried out (tables 1 and 2). In the first, data from only the individuals photographed twice were analysed. Since rings were counted in all photos only in recount B, this constituted the input entered in the analysis. The number of rings in the first photo was assumed to correspond to the true age (A1PB) and the number of years elapsed until the second photo (YEARS) was added. Therefore, regression was performed between the theoretical age when the second photo was taken (TA2PB) and the ring number observed (A2PB). The second regression was carried out between the number of rings counted by direct observation in 2000 (A00) and the theoretical age of the tortoises that year, calculated from the first photo of recount A (A1PA), adding the years elapsed until 2000 (TAA; table 2). Finally, the third regression was similar to the second but calculated the theoretical age from the first photo of recount B (A1PB).

Results

The ages obtained from recount A varied between 0 and 8 years while in recount B they were between 0 and 11 (table 3). Repeatability for the whole sample was high ($r_I = 0.914$; $n = 311$). The age values of these two recounts coincided for 74.6% of cases. A lack of coincidence was mostly due to an overestimation of 1 year in the second recount (21.9% of cases) and less to an underestimation of 1 year (2.9%). The maximal discrepancy between the two counts was in one specimen whose age was overestimated by 2 years and in other that was underestimated by 2 years (0.6% of the sample).

Age range, either rings in the second photo (A2PB) or values calculated when second photo from the time passed from the first (TA2PB), varied between 0 and 11 years. Correlation between these two variables was significant ($r = 0.95$; $P < 0.001$) and the slope was 0.90, significantly different from 1.00 (table 4, fig. 1).

However, when we studied only those individuals under than 8 years (sexual maturity in this population; Bertolero, 2002), according to the second photo, the slope was not significantly different from 1.00 (table 4). The age calculated from rings in the second photo was greater than the theoretical age in 20.8% of cases and lower in 28.7% (table 5).

Correlation between the number of rings counted in 2000 and the theoretical age in that year (TAA and TA1PB) was significant (recount A: $r = 0.94$, $P < 0.001$; recount B: $r = 0.98$, $P < 0.001$). In both cases, regression slopes were significantly different from 1.00 (table 4). As in the first regression, when only tortoises classified as younger than 8 were considered, slopes did not differ from 1.00 (table 4). Nevertheless, in both cases regressions remained significantly different from 0.00 (recount A: $r = 0.93$, $P < 0.001$; recount B: $r = 0.96$, $P < 0.001$). In 2000 and in the two

Table 1. Definition of variables used.

Variable	Definition
YEARS	Number of years between 1st and 2nd photos
A1PA	Age obtained from 1st Photo in recount A (January 2000)
TAA	Theoretical Age of a tortoise of recount A in 2000
A1PB	Age obtained from 1st Photo in recount B (November 2001)
TA1PB	Theoretical Age of a tortoise of recount B in 2000, obtained from its 1st Photo
A2PB	Age obtained from 2nd Photo in recount B (November 2001)
TA2PB	Theoretical Age of a tortoise of recount B in the year of the 2nd Photo but obtained from the 1st photo
A00	Age determined from direct observation of the tortoises during 2000

Table 3. Number of tortoises classified by age in the two recounts: A (November 2000) and B (January 2001).

Age	Recount A		Recount B	
	N	%	*n*	%
0	153	49.20	159	44.66
1	78	25.08	88	24.72
2	28	9.00	52	14.61
3	13	4.18	13	3.65
4	12	3.86	10	2.81
5	10	3.22	13	3.65
6	11	3.54	11	3.09
7	5	1.61	7	1.97
8	1	0.32	1	0.28
10	–	–	1	0.28
11	–	–	1	0.28
Total	311		356	

Table 2. Summary of the relationships between variables. Rec. = recount.

Rec.	Date	Method	"Real" age variable	Derived variable: "real" age + time (years)	Regression
A	Jan. 2000	Photos	A1PA	A1PA + no. years until 2000 = TAA	2: A00 *vs.* TAA
B	Nov. 2001	Photos	A1PB	A1PB + no. years until 2000 = TA1PB	3: A00 *vs.* TA1PB
		Photos	A2PB	A1PB + YEARS = TA2PB	1: A2PB *vs.* TA2PB
C	2000	Direct obs.	A00		

Table 4. Regressions between the ages determined by ring counts and the theoretical age. Age column indicates the range of ages analysed in each regression. When 1 falls within the confidence interval (CI95%) of slope (b_1) then regression do not differ significantly from 1 and the relation between years and rings can be consider as 1 : 1. Similarly, when 0 falls within the confidence interval (CI95%) of the intercept (b_0) then the intercept do not differ significantly from 0.

Regression	Age	Equation	b_1 *CI* 95%	b_0 *CI* 95%	*n*
A2PB *vs.* TA2PB	0-11	A2PB = 0.437 + 0.900*TA2PB	0.846-0.958	0.115-0.741	101
	0-7	A2PB = 0.096 + 1.012*TA2PB	0.913-1.122	−0.323-0.474	70
A00 vs. TAA	0-11	A00 = 0.408 + 0.885*TAA	0.795-0.984	−0.249-1.007	51
	0-7	A00 = −0.200 + 1.113*TAA	0.948-1.311	−0.886-0.373	26
A00 vs. TA1PB	0-11	A00 = 0.108 + 0.914*TA1PB	0.871-0.957	−0.117-0.324	82
	0-7	A00 = 0.042 + 0.977*TA1PB	0.902-1.058	−0.105-0.177	50

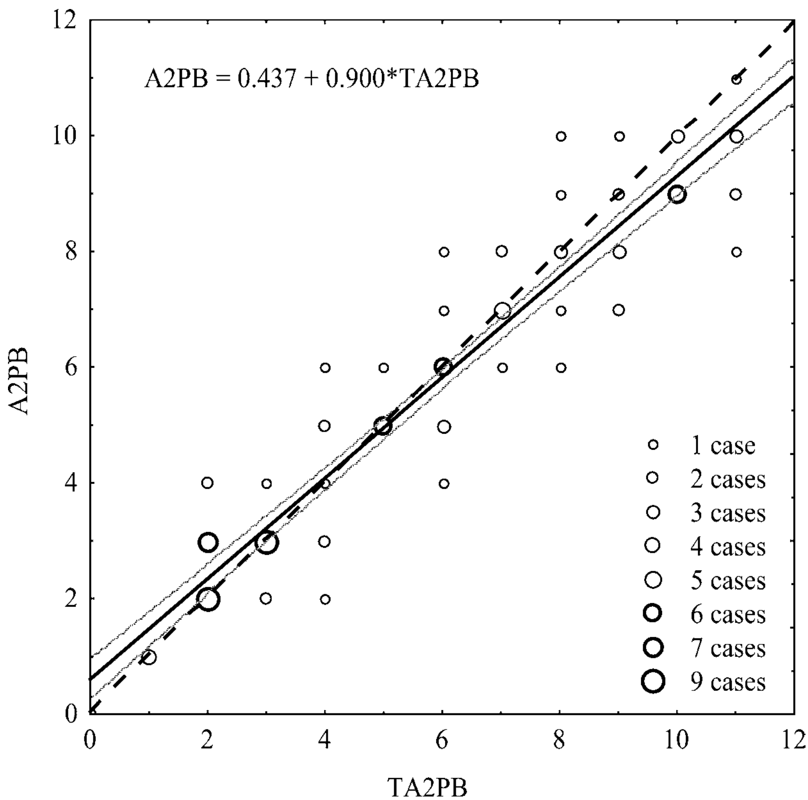

Figure 1. Model II regression line between age and ring number (continuous) and confidence intervals 95% (dotted) in the *Testudo hermanni* population in the Ebro Delta. Dashed line indicates the theoretical relation 1 : 1.

recounts for the theoretical age, the number of rings oscillated between 0 and 11.

Discussion

Since Agassiz started using corneal ring counts to estimate tortoise age in the middle 19th century (in Germano and Bury, 1998), this method has been widely used (reviews by Graham, 1979; Dunham et al., 1988; Zug, 1991; Germano and Bury, 1998; Wilson et al., 2003). However, few studies have been performed with photographic material, which allows permanent records for further analysis (Galbraith and Brooks, 1987; Brooks et al., 1997), and few

Table 5. Difference between the rings counted in the second photo (recapture) and the theoretical age of each individual, considering the number of years since the first photo (capture). Positive values indicate overestimation, negative indicate underestimation.

Difference	*N*	%
−2	5	5.0
−1	16	15.8
0	51	50.5
1	21	20.8
2	7	6.9
3	1	1.0

have evaluated the effectiveness of this system (review by Wilson et al., 2003). In the case of *Testudo hermanni* from the Ebro Delta, our results show that ring counts from photographs is a feasible and highly repeatable method. Moreover, the degree of discrepancy between recounts from the same photographs was only ±1 year, which can be assumed as a reasonable error.

The maximal age determined in the tortoises observed and photographed in 2000 was 11 years. This age corresponds to the maximum value possible before September 2000 since the first adults were introduced in September 1987 (Bertolero, 2002) and, hence, the first hatching should have taken place at the end of summer 1988. Thus, it is possible to determine the maximal age possible in each year and the maximal age attained by a tortoise born free in this population.

As stated previously, annual periodicity in the deposition of main rings is necessary to validate this method (Graham, 1979; Germano, 1988, 1998; Litzgus and Brooks, 1998; Wilson et al., 2003). In the present study, the results of the three regressions did not corroborate this hypothesis since slopes differed from 1. This was due to a tendency to count fewer rings in the field (A00) and in the second photos (A2PB) compared with the theoretical age obtained from the first photo (TAA and TA1PB). In accordance with Brooks et al. (1997), ring recounts from photos tended to produce higher estimations than direct observations on the shell, although these authors also found that both types of counts underestimated the true age. In our case, underestimation was detected for theoretical ages higher than 8 both in the field and using photos (figure 1). On the other hand, between 0 and 7 years of age there was a 1 : 1 correspondence between years and rings. This result is similar to that obtained by Castanet and Cheylan (1979), who also studied *Testudo hermanni*. These authors demonstrated that ring formation during the first 7 years was annual (using specimens of known ages kept in open air terraria). They consider the method valid until 12 years in males and 15 in females, and that it is possible, in some cases, to reach estimations of 17-18 years.

The growth rate in tortoises generally decreases on reaching sexual maturity (Andrews, 1982) and, hence, the new rings deposited are thinner and difficult or impossible to count (Castanet and Cheylan, 1979; Germano, 1988, 1992). In the population studied, males are sexually mature at 6-8 and females at 8-10 (Bertolero, 2002). In fact, part of the individuals whose age was determined were already mature and showed reduced growth rings; consequently, their age may have been underestimated. This decrease in precision in sexually mature specimens has been reported for other tortoise species (Castanet and Cheylan, 1979; Germano, 1988; Galbraith and Brooks, 1989). In general, ring counts are reliable for immature or near mature tortoises (Germano and Bury, 1998).

In most studies it is impossible to calculate the age of the whole population; however, the determination of the age of a section provides the opportunity to analyse several aspects of the biology of a species. Therefore, given the limitations of ring counts (Wilson et al., 2003), we recommend establishing the age limit that ensures reliable age estimation in the chelonian population.

Conclusions

For the population of *Testudo hermanni* in the Ebro Delta, age determination on the basis of ring recounts, either in the field or from photographs, is reliable only for individuals under 7 years. This population segment corresponds to juveniles and sub-adults. At sexual maturity (around 8 years in this population) recounts may still be possible but tend to underestimate the true age and precision is lost because the new growth rings are very thin and difficult to observe either directly or through photos.

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