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One scute ring per year in *Testudo graeca*? A novel method to identify ring deposition patterns in tortoises

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Abstract. A reliable estimation of individuals' age is helpful to conduct demographic studies on wildlife populations. In tortoises, many studies have estimated individuals' age by counting growth rings on their scutes, assuming one ring per year (1:1 ratio). However, the accuracy of this method is controversial. The ring deposition pattern can vary depending on species, or even populations, and should be studied comprehensively. We studied the ring deposition pattern of *Testudo graeca* in southeastern Iberian Peninsula, using recaptures of 156 individuals between 2004 and 2010. We used a novel approach to explore the ring deposition pattern and to test possible differences between localities and individuals. Our results revealed that most analysed individuals (57.4%) showed a 1:1 ratio, in which rings were deposited during months of activity (spring to autumn). However, we found a trend to count less rings than years, which underestimated 1 year every 3 or 4 years. No differences in the deposition patterns were found among sites, sizes or sexes because the halt in growth during hibernation equally affects all tortoises in all sites. Our results support that the assumed 1:1 ratio in the assignment of individuals' age is too simplistic. Since ring deposition patterns are complex, the use of statistical approaches capable of handling deviations from the assumed deposition ratios can help to better depict population age structures.

Keywords. Growth rings, age estimation, spur-thighed tortoise, ring deposition pattern.

INTRODUCTION

An accurate estimation of individuals' age is key for the study of life history and population dynamics of species (Ricklefs, 1990). A population's age distribution is also a basic parameter to assist with management and conservation strategies (Slobodkin, 1963). In turtles and tortoises, there are three main approaches to estimate age: skelotochronology (Zug et al., 1986), growth models (Carr and Goodman, 1970) and growth ring counts (Agassiz, 1857; hereinafter GRC). Each one of the methods has its pros and cons (Wilson et al., 2003; Curtin et al., 2008; Armstrong and Brooks, 2014). Firstly, although skelotochronology can provide reliable data about age it is an invasive method that just can be used with death animals and also shows some biases (Curtin et al., 2008). On the other hand, growth data models allow to produce age estimates in big long-lived species (Armstrong and Brooks, 2014), but some papers exemplifies case studies where size is weakly related to age (e.g., Carr and Goodman, 1970; Congdon et al., 2001). In the present work, we focus into the GRC method, probably the most extensively used, but that it is not free of criticisms (Wilson et al., 2003).

GRC was first described by Agassiz (1857) and was used to estimate age in *Chrysemys picta*. Although several studies have supported it (Graham, 1979; Zug, 1991; Germano and Bury, 1998; Bertolero et al., 2005), others have questioned its use (Tracy and Tracy, 1995; Kennett, 1996; Brooks et al., 1997). In particular, Wilson et al. (2003)

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reviewed 143 papers that provided age data by counting growth rings. Most papers (67%) presented no data to support the use of GRC, and several researchers cited Sexton (1959) as a validation method. However, in that paper, the author built a theoretical model without testing the relationship between rings and age.

In accordance with Wilson et al. (2003), we identified four critical sources of mismatches that question the accuracy of GRC as an age estimator. These sources can be divided into two groups. Firstly, deposition of rings may not follow the usually assumed one ring per year (1:1 ratio) because: 1) some individuals may deposit two rings or more in the same season during periods or in habitats with good resources availability (Tracy and Tracy, 1995; Aresco and Guyer, 1998; Berry, 2002); 2) some individuals may stop growing, and may not even deposit any ring for years (Turner et al., 1987). Secondly, even if ring deposition follows a 1:1 ratio, other factors may negatively affect the accuracy of the technique: 3) scutes worn-out from friction may make it impossible to discern individual rings, thus age may be underestimated (Aresco and Guyer, 1998; Litzgus and Brooks, 1998; Berry, 2002); and 4) identification of rings requires an observer's arbitrary decision, and repeated measures may yield different results. This inherent subjectivity of ring counts may be exacerbated by those rings produced in years after reaching maturity as they might be too small to be accurately counted (Lambert, 1982; Galbraith and Brooks, 1987; Zug, 1991; Germano and Bury, 1998).

Understanding the deposition pattern of rings in tortoises is a premise for using the GRC technique in age inferences (Germano and Bury; 1998, Wilson et al., 2003; Bertolero et al., 2005). For age estimates from GRC to be reliable, ring production has to be cyclical (i.e., regular) and at known deposition rates (Germano, 1988; Zug, 1991; Germano and Fritts, 1994; Wilson et al. 2003). However, deposition rates can vary among species, or even populations, if they withstand different climatic conditions (e.g., Moll and Legler, 1971; Berry, 2002). In the *Testudo* genus, the results of previous studies show that the reliability of GRC as an age estimator diminishes with the individual's age (Diaz-Paniagua et al., 2001 for *T. graeca*) and some authors consider the GRC technique is only reliable for juveniles (Bertolero et al., 2005, Attum et al., 2011; for *T. hermanni* and *T. kleinmanni*, respectively). These results suggest uncertainty in the accuracy of age-based demographic studies since species dynamics relies heavily on adult dynamics (Diaz-Paniagua et al., 2001). Therefore, comprehensive studies to address the accuracy of the GRC method are required.

The purpose of this work is to study the growth ring deposition pattern of the spur-thighed tortoise (*T. graeca*) population in southeastern Iberian Peninsula. In particular the specific goals of this study are twofold: 1) to test whether the deposition pattern follows a 1:1 ratio and understand the temporal context; and 2) to explore possible differences between sizes, sexes, stages and samples sites. In order to address these objectives, we start by analysing the mismatch between the number of deposited rings and the elapsed time between captures of individuals and develop a novel method to infer the deposition ring patterns of the species. Our results will provide valuable information to further demographic studies of this endangered population. Moreover, the undertaken approach can prove useful to study other chelonian populations as a first step to address the accuracy of the GRC technique.

MATERIALS AND METHODS

Study system and data collection

T. graeca is a long-lived species and individuals can live up to 30 years (Diaz Paniagua et al., 2001). In southeastern Iberian Peninsula the species displays strong seasonal activity patterns (Perez et al., 2002). The main activity season occurs in spring (March to June), when the species' principal breeding season (mating and laying of eggs) takes place, and a secondary activity season occurs in autumn (September to November) when most, but not all, individuals are active (Perez et al., 2002). These two activity periods are separated by two inactivity periods, summer and winter, due to scarce food resources and extreme temperatures (Perez et al., 2002).

Monitoring was carried out at 18 sampling sites over 6 years (2004-2010), which are representative of the species' entire range in southeastern areas of the Iberian Peninsula (Fig. 1), although one locality comprises more than half of the data (Galera, n = 99, 63% of the individuals analysed). Surveys were conducted during the active period walking though the habitat in line transects and capturing the tortoises in opportunistic encounters. Individuals were marked with an individual code by notching the marginal scutes. From each individual, carapace length (CL) and scute ring count was recorded. Rings were counted from the third costal scute to the right of the carapace (Fig. 2) because, as in other tortoises, the rear part of the carapace receives less wear than the front part or the plastron (Germano, 1988). Ring width was measured to the nearest millimetre using a digital calliper. Sex of individuals was identified by secondary characters (Lopez-Jurado et al., 1979). Unsexable immature individuals were classified as subadults. Tortoises were released in the same place that it was found. Measurements were taken by 19 different specialists. To minimize errors and bias, these specialists followed the same measurement protocol and were trained for standardization under supervision of experienced researchers with captive animals before fieldwork. In total we captured 156 tortoises at least twice (74 females, 70 males and 12 juveniles). Only one recapture per tortoises was used in order to maintain the independence of the data.

Fig. 1. Samples sites inside the distribution area in the southeast of Spain, between the provinces of Murcia and Almeria. The black points are the sampled sites and the black star is the site called "Galera", the place with the higher number of captured tortoises. The area in the all samples sites is 1 km^2.

Mismatches due to errors in measurements

In a first step, we excluded the individuals for whom ring counting was not feasible due to worn-out scutes. In order to ensure that the number of rings deposited between captures was not strongly affected by inaccurate observations, we first checked the reliability of our data. In particular, we attempted to identify the last rings of the previous capture during subsequent captures using ring width and relative ring position (Fig. 2). Those individuals where this identification was not possible were removed from further analyses. We compared the size distribution before and after remove the individuals using a chi-squared analysis to test whether the size frequencies are maintained after the individuals were discarded. We also compared the size of the discarded males and females with those non-discarded individuals by means of a t-statistic ($\alpha = 0.95$). In order to test out the errors made by the specialists, we used a chi-squared analysis to test the frequencies of the accurate and inaccurate counts that they carried out.

Ring deposition pattern

We analysed the temporal deposition patterns of rings, by means of comparing the increment of the number of rings (dR) and the increment of time between captures according to different hypotheses of deposition patterns (dT_{DP}). The difference between these two values is the bias between time and rings, under the five different hypothesised deposition patterns (BTR_{DP}).

$$BTR_{DP} = dR - dT_{DP}$$

Deposition pattern hypotheses (DP, Table 1) were formulated considering that the detection of a new ring requires more

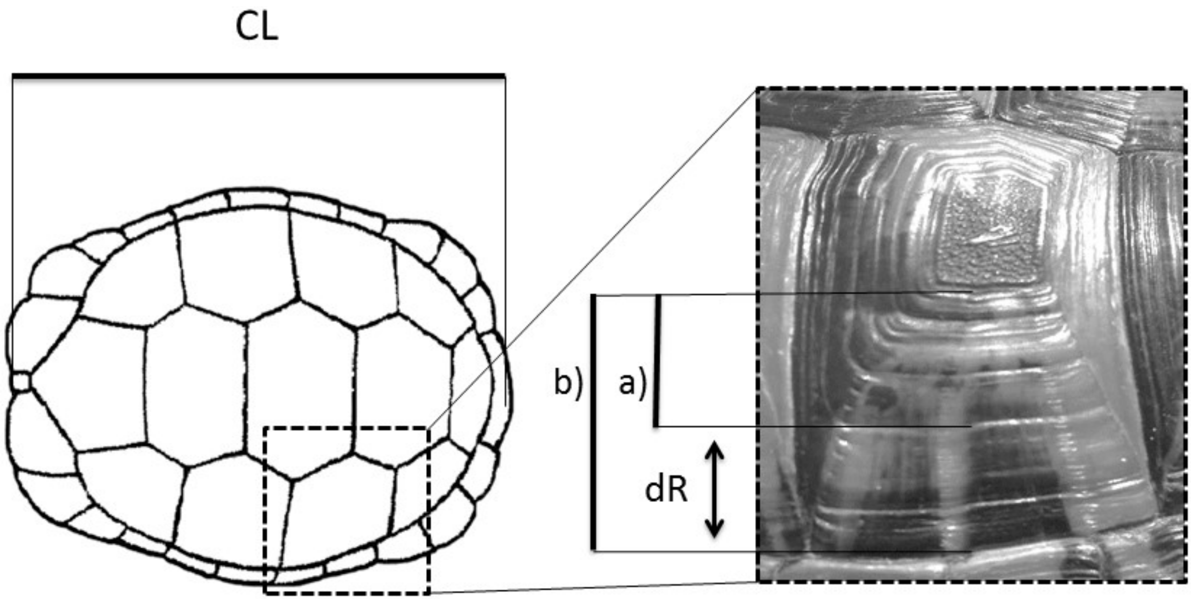

Fig. 2. Tortoise carapace showing the third scute and the carapace length (CL) measure. a) The rings counted in the first capture and b) the rings counted in the recapture, where dR is the difference between a) and b).

Table 1. Abbreviations for the bias between time and rings (BTR_{DP}) and time elapsed between captures at different hypotheses of deposition pattern (dT_{DP}).

Deposition Pattern (DP) Hypothesis	Bias Variables (BTR_{DP})	Time Variables (dT_{DP})**	Equation*
1 ring per year	BTRy	dTy	dTy=no. months/12
1 ring per spring	BTRs	dTs	dTs=no. months in spring/4
1 ring per autumn	BTRa	dTa	dTa=no. months in autumn/3
1 ring per active season, spring and autumn	BTRsa	dTsa	dTsa=no. months in spring or autumn/7
1 ring per spring and another ring per autumn	BTRs+a	dTs+a	dTs+a=no. months in spring/4 + no. months in autumn/3

*Springs include four months (March, April, May and June) and autumn three (September, October and November).
**We rounded off dT_{DP} to create a discrete variable.

than half the time contemplated in the hypothesis, because we consider that we can detect the ring "in growth" before the complete ring is deposited. For example, under the first hypothesis of one ring per year (dTy), we assumed that we would be able to identify a new ring after the sixth month. Under the second and third hypotheses of one ring per spring (dTs) and one ring per autumn (dTa), we could be able to see the ring after two spring months, and after one and a half autumn month, respectively. Under the fourth hypothesis of one ring per active season, including both spring and autumn (dTsa), we should detect a new ring after 3.5 months of spring or autumn. Finally, our fifth hypothesis states that one ring is deposited in spring and another in autumn (dTs+a), thus one ring should be detected in at least two spring months and another ring in, at least, one and a half autumn months (Table 1).

The fit between the hypothesized deposition patterns and the observed data was tested by a t-test. According to the hypotheses put forward, if the BTR_{DP} did not significantly differ from zero ($\alpha = 0.95$), we assumed that the number of rings represents the time interval. Differences between sexes, between stage classes (adults and juveniles), and among samples sites (comparing "Galera" with the other sample sites) were tested by means of a Wilcoxon test. We used linear regression to assess how the best fitted BTR_{DP} varied with the time that elapsed between capture and recapture (dT_{DP}) and with a body measure (CL, Fig. 2).

RESULTS

Of the 156 recaptured individuals, 41 (26.3%) were ruled out from further analyses given the possible mismatches due to errors in measurements in their observa-

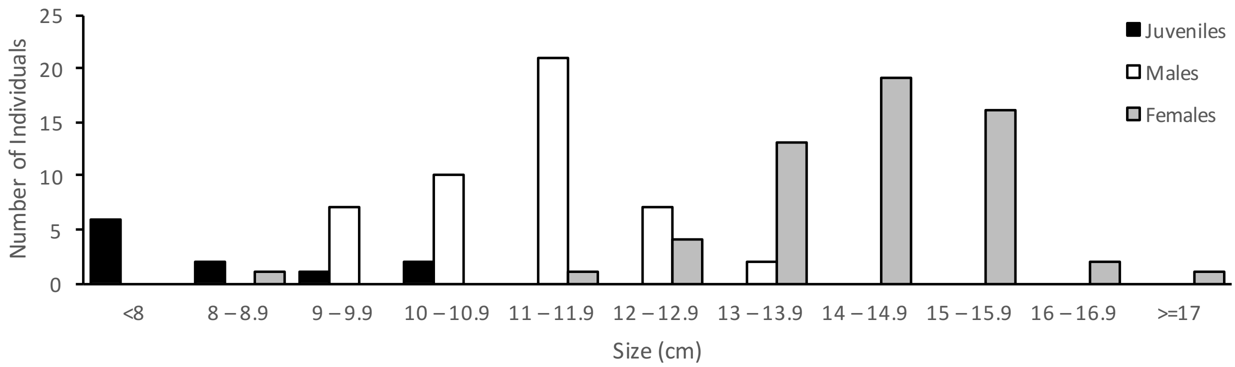

Fig. 3. Size distribution patterns in the population after some individuals were ruled out for error measures (n=115).

Table 2. Descriptive statistical data calculated for the bias variables (After some tortoises were ruled out for errors measures, n=115).

Bias variables	Mean	SD	T-test (x=0), df =114	p.value
BTRy	-0.27	1.119	-2.58	0.011
BTRs	-0.29	1.114	-2.76	0.007
BTRa	-0.26	1.093	-2.56	0.012
BTRsa	-0.16	1.048	-1.60	0.112
BTRs+a	-1.36	2.053	-7.09	0.000

BTR is the bias variable obtained with different temporal hypothesis (More information in Table 1).

tions. In seven tortoises, scutes were very worn-out (4.5% of the total sample) and, in 34 tortoises the pattern of ring measures between recaptures vastly differed and did not allow an accurate estimation of the number of rings deposited (21.8%). The size class distribution was no different before and after ruling them out (χ^2 = 2.35, df = 10, P = 0.99; Fig. 3). We found no differences in size between discarded and non-discarded female tortoises (147.7 ± 14.9 mm and 143.8 ± 12.9 mm, respectively; P > 0.05). Nevertheless, discarded males (116.9 ± 8.9 mm) were significantly larger than non-discarded ones (112.5 ± 10.1 mm; P = 0.034). Juveniles not were analysed because only one juvenile was discarded.

We detected no differences among all the specialists when comparing their number of accurate and inaccurate observations (χ^2 = 14.53, df = 13, P = 0.34), nor even between those 10 observers who, together, achieved more than 80% of observations (χ^2 = 10.77, df = 9, P = 0.29).

Ring deposition pattern

We analysed the deposition pattern in 115 individuals (57 females, 47 males and 11 juveniles). The mean interval between captures was 1.0 ± 1.2 years. In all the different hypothetical temporal deposition patterns tested, the mean of the time that had elapsed between captures was longer than the mean of the number of deposited rings

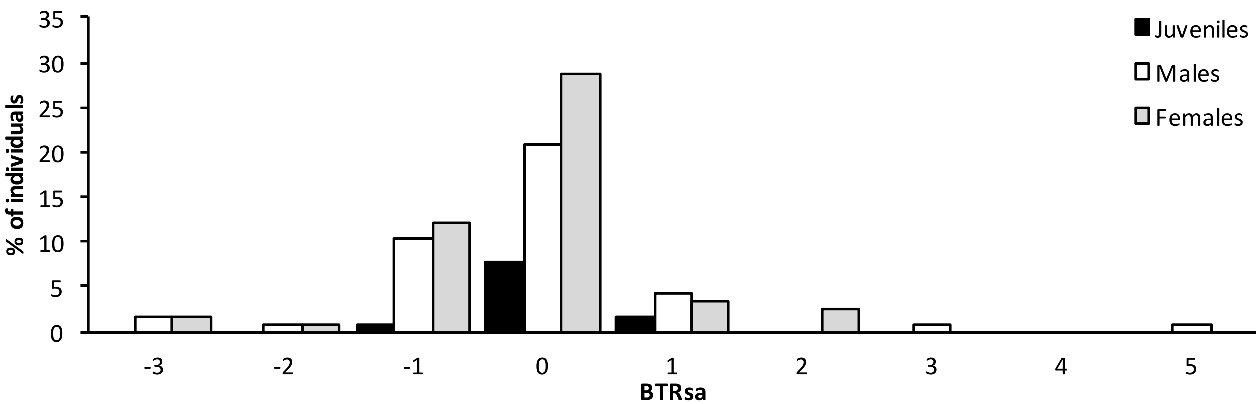

Fig. 4. Frequency of individuals with a bias according to BTRsa (bias variable based on the hypothesis that one ring is deposited per active season – spring and autumn). When BTRsa is 0, there is a 1:1 ratio (one ring per year)

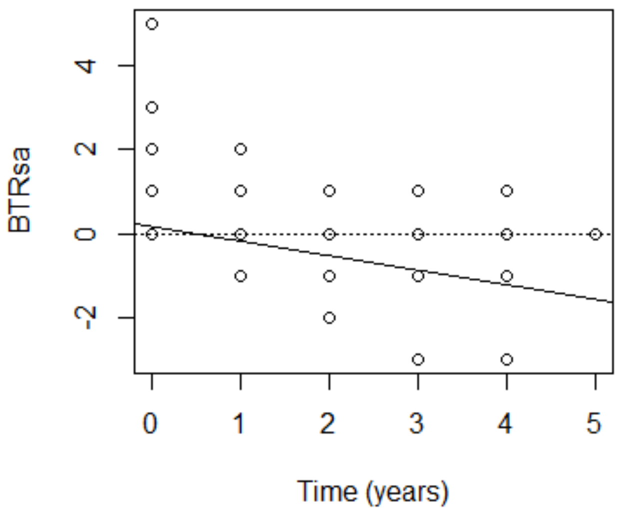

Fig. 5. Regression between BTRsa (bias variable based on the hypothesis that one ring is deposited per active season – spring and autumn) and the time elapsed between recaptures (dTsa). Bold line is the result of the regression.

(Table 2). The temporal deposition pattern BTRsa (one ring jointly developed in spring and autumn) was the only pattern that statistically matched the observed data (Table 2). Based on this assumption, 57.4% of the analysed tortoises exactly followed a 1:1 deposition pattern (i.e., BTRsa = 0, Fig. 4). Nonetheless, the BTRsa variable correlated negatively with the time that had elapsed between captures ($R^2 = 0.0153$; $P < 0.001$). Namely, the trend was to count fewer rings than expected (Fig. 5).

Finally, no differences were found in the temporal deposition patterns between males and females, between adults and juveniles or between the Galera location and the remaining sites (W = 1287.5; W = 519; W = 1011; respectively, $P > 0.05$), and BTRsa also had no relation with individual sizes ($P > 0.05$).

DISCUSSION

Activity periods and rings deposition in Testudo graeca

It has been proposed that the number of rings per year in the scutes of tortoises depends on the number of inactive periods experienced by the individuals, during which growth diminishes or stops (Wilson et al., 2003). The physiological process underlying ring deposition is based on the growth of the epidermis, when outer cells die and keratinise forms the scute layer. Our results fit this biological process and indicate that the seasonal activity pattern of *T. graeca* in southeastern Iberian Peninsula strongly influences its ring deposition pattern. The majority of individuals deposited one ring when one spring and one autumn had elapsed. We can, thus, infer that growth occurs during these activity seasons, and even in summer, when individuals grow, but probably more slowly. We were unable to detect differences in the number of deposited rings among males, females and juveniles, indicating that the growth halt during hibernation affects the entire population.

Do deposition patterns follow a one–ring-per-year ratio?

According to our results, the deposition of a complete ring requires both activity periods (spring and autumn) and a growth halt during the hibernation, what is the equivalent to 1 year. More than half of the analysed individuals followed a one-ring-per-year pattern, 57.4% of the analysed tortoises showed exactly a 1:1 deposition pattern (i.e., BTRsa = 0), and 90.4% revealed biases less than or equal to 1 year (Fig. 4). Overall our results support the one–ring-per-year ratio reported for other species (Germano, 1988; Iverson, 1988; Germano and Bury, 1998). However, slight individual deviations in the assumed pattern can result in inadequate age estimations, something which is especially problematic since chelonians are long-lived species. We identified a significant trend to visualise fewer rings than years, according to which 1 year is underestimated every 3 or 4 years (Fig. 5). The cumulative effect of this underestimation would specially affect accurate age assignments, whereas the consideration of departure in more complex statistical approaches could help better depict population age structures (i.e., a probabilistic assignation of individuals to age class intervals according to the inferred number of rings).

May other factors negatively affect the accuracy of the technique?

This work demonstrates that there is a relation between number of rings and tortoises' age, but possible sources of error may lead to inaccurate estimates. It is worth mentioning that 26.3% of recaptured individuals were ruled out to avoid inaccurate estimations of the number of rings deposited between recaptures. Although the majority of discards were due to inconsistencies in the data obtained by the specialists, no differences were found in accurateness between them, probably because they received the same training. Previous works that have reporting significant differences among observers found that discrepancies were related mainly with their ability to distinguish when a ring starts and finishes (Legler, 1960; Galbraith and Brooks, 1989; Germano and Bury, 1998).

The technique can be improved repeating counts by different specialist and training them according to possible ambiguities. These recommendations could be essential to guarantee data soundness, especially in long-term studies.

The other cause that led us to rule out some individuals was that tortoises showed worn-out or damaged scutes, especially the largest males of the population, in whom it was impossible to accurately infer the number of deposited rings among recaptures. We hypothesised that the higher rates of male movements lead to greater scute wear than in females, and rings are also more easily inferred in females due to their larger sizes (Anadon et al., 2012; Rodríguez-Caro et al., 2013). For spur-thighed tortoises in the Doñana National Park in southwestern Spain, Díaz-Paniagua et al., (2001) pointed out that the GRC method is reliable only for those individuals aged up to 20 years, older tortoises show too worn-out scutes to be counted

CONCLUSIONS

We assessed that GRC technique can be used to infer the approximate age of *Testudo graeca* in southeastern Iberian Peninsula and we develop a novel method to infer the deposition ring patterns in tortoises. We conclude that this population generally follows a one-ring-per-year ratio. Thus the present work support, with some cautions, the use of GRC to estimate age in further studies of this population, and also encourage the use of this approach to test the deposition patterns of other chelonians populations.

Despite the general trend (1:1 ratio), we also found notable variability associated with the individual's heterogeneity in ring deposition, and also due to mismatches among observations. Individual's heterogeneity could be assumed using statistical approaches capable of handling the deviations observed from deposition ratios, such as probabilistic assignations of individuals to age intervals instead of using specific age estimations. Regarding ring counting, we share the usual recommendation of training observers or increase the number of captured individuals, but also it would be convenient duplicate counts by different observers in order to avoid possible mistakes. In our study, we validated our data by at least two observations using recaptures at different times, but data could be easily improved using independent counts carried out by two specialist at the same time.

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