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Review

How Old Is a Turtle? Challenges in Interpreting Age Information in Sea Turtles

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Abstract: Marine turtles are iconic, globally distributed large reptiles with a largely oceanic life history that makes it difficult to characterize population demography and dynamics. This is significant because all marine turtle species are currently considered vulnerable or endangered. Knowledge of the age of individuals is central to our understanding of the life history of a species and an important consideration in the design of management and conservation strategies. Herein, we review different methods used to estimate the absolute, relative and physiological age of marine turtles, as well as their underlying hypotheses and challenges in their interpretation. We conclude that, at present, there is no validated method that establishes the absolute age of an individual from field studies.

Keywords: aging; marine turtles; skeletochronology; sclerochronology; telomere; DNA methylation

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1. Introduction

Age is a fundamental variable in life-history theory and population modeling because it impacts a wide range of factors that are essential for understanding and predicting population dynamics and for making informed policy and planning decisions [1]. Age distribution affects the demographic structure of a population, which, in turn, influences birth rates, death rates and migration patterns [2]. Different age groups may have distinct mortality and fertility rates, and understanding these can help predict population growth or decline [3]. Marine turtles also exhibit late maturity, and there can be substantial time lags between impacts and noticeable effects on a marine turtle population. Given this, there are both fundamental and practical reasons for determining the age of individuals in a population.

Before discussing the aging of marine turtles, it is important to understand the periodic growth pattern of these species. When growth is periodic, growth marks are deposited at the periphery of hard tissues (bones and scutes), and if the deposits are annual, they provide the opportunity to age the individual [4]. The use of scutes is restricted to the hawksbill sea turtle, *Eretmochelys imbricata* (Linnaeus, 1766), due to its particular scute structure, but long bones of all marine turtle species can be used. Three types of skeletal growth marks are distinguished on the basis of their specific histological characteristics [5]: (1) the "zones", broad fast-growing layers, alternate with (2) "annuli", corresponding to relatively slow osseous depositions, and (3) lines of arrested growth show a temporary arrest of osteogenesis. These are generally more translucent and narrower than the fast-growing layers. It should be noted, however, that these lines of arrested growth visualized on histological sections are not exclusively the result of growth arrest but can also represent a metabolic change, with differing proteins associated with these lines of arrested growth [6].

From the perspective of life-history strategies, this growth periodicity has often been interpreted as a constraint in ectotherms such as marine turtles. However, this could

also be interpreted as an adaptive strategy, as it can be seen also in endotherms [7,8]: Growth is often interrupted during the unfavorable season at the same time as body temperature drops, metabolic rate slows and plasma levels of insulin-like growth factor-1 that promotes bone growth decrease, part of a plesiomorphic thermometabolic strategy for energy conservation. Conversely, episodes of intense tissue growth coincide with peak metabolic rates and hormonal changes correlated with the onset of the favorable season, indicating increased efficiency in the acquisition and utilization of seasonal resources. Growth modulation is therefore the result of an interaction between environmental factors and hormonal control of metabolism and not merely an effect of the environment [9].

Lines of arrested growth can also be observed in animals under thermal or environmental stress [10]. This phenomenon is well known in humans and corresponds to the Harris lines [11]. Variation in photoperiod can also be a proximate factor triggering variation in growth rate, which can lead to the appearance of lines of arrested growth [8]. In temperate species, aestivation lines are also observed, and sometimes several lines correspond to growth resumption during the winter period.

Marine turtles exhibit what is known as indeterminate growth, i.e., growth continues throughout the animal's life, and the relationship between the period when growth slows down and sexual maturity is not precisely defined [12]. Methods that use the age at which a fraction of the asymptote of a given growth model is reached (logistic, Gompertz or von Bertalanffy, for example) [13–15] or even indeterminate methods such as a Generalized Additive Mixed-Model (GAMM) [16] are based on false assumptions. There is no simple, general relationship between growth rate and sexual maturity, and the time lag between the two phenomena can be several years [17,18]. In addition, complex interactions between growth rate, age of sexual maturity, individual size and resource quantity have been observed in ectotherms [19]. Such a complex interaction has also been observed in sea turtles [20]. Age at sexual maturity is an individual characteristic, and, for species such as sea turtles that experience a very wide variety of environmental conditions, determining an average age is of little practical use. Instead, it would be more useful to discuss the probability of reaching sexual maturity as a function of the animal's size [21].

Several methods have been applied to marine turtles to determine the age of individuals, which we have broadly divided into three categories: (a) *absolute age* as the age since the hatching of the individual, (b) *relative age* as the time between two events (observation, tagging, capture, or recapture) and (c) *physiological age* as the approximate age of an individual based on physiological characteristics. Herein, we review each age category, how it can be estimated and the limitations of each method.

2. Aging Methods and Limits

2.1. Absolute Age

2.1.1. Captivity Rearing

When an egg is incubated under artificial conditions or a hatchling is taken from a natural nest and the individual is reared in artificial conditions, the absolute age of this individual is obviously known. This approach has been used in different parts of the world. A large variation in age at maturity was observed in a 34-year study of captive green turtles, *Chelonia mydas* (Linnaeus, 1758), which followed individuals from hatching to beyond maturity [20]. The wide variation observed in captive turtles under similar conditions provided important insights into the variability that could be exhibited by wild populations experiencing stochastic conditions [20]. However, it is often difficult to compare growth under the rearing conditions in captivity with what would be experienced by turtles in the wild. For example, the growth rate of leatherback turtles, *Dermochelys coriacea* (Vandelli, 1761), in the wild was only 60% of the growth of conspecifics in captivity where individuals were fed ad libitum [12]. In addition, the logistics, cost and permit requirements of captive rearing of long-lived species mean that this is an impractical method to establish growth rates, size at age and age at maturity in marine turtles.

2.1.2. Tagging at Hatching

While some experimentation has been performed with passive internal transponder (PIT) tags [22,23], the most reliable and practical method of identifying hatchlings from the time of emergence involves mutilation tagging, which typically notches marginal scutes and codes turtles by year cohorts. Extensive notching (mutilation tagging) of South African loggerhead turtles, *Caretta caretta* (Linnaeus, 1758), was used to determine age and size at reproduction (putative first nesting season) and thus to identify the age and size for sexual maturation. A total of 332,811 juveniles were notched and then released as they emerged from nests between 1972 and 2002. The notching patterns on the marginal scutes encoded the year of emergence. From these, 137 clearly identified notched adult females were encountered at the nesting beach, and these were then assigned a known absolute age [24]. Two key findings emerged from this experiment: (1) size was a more important threshold for the initiation of the maturation process than age, and (2) the onset of sexual maturity was dependent on both intrinsic and extrinsic factors. However, the dynamic of growth was not accessible with these data, as turtles were only sighted at hatching and at (putative) first nesting. A similar experiment was conducted in Australia, during which 129,921 loggerhead hatchlings were marked as soon as they hatched. One immature female was recaptured after 15 years measuring 75.6 cm in CCL [25], and only one report of these reaching maturity after 29 years has been published from this experiment [26]. This method is clearly labor-intensive and unlikely to become a mainstream approach to understanding absolute age due to ethical considerations of the mutilation process and the low return rate in subsequent recaptures.

2.1.3. Skeletochronology-Based Age Determination

Skeletochronology is a tool used to determine the absolute age of individuals in populations with periodic growth [27]. This type of growth is observed in ectotherms in general and therefore in reptiles, including marine turtles [28]. Periodic growth is also found in leatherback turtles, which exhibit a particular form of endothermy known as gigantothermy [29], but it can also be found also in true endotherms [7,8]. The use of this technique requires special equipment for cutting, staining and visualizing bone sections, as well as experience in reading and interpreting histological sections. At first glance, the application of this technology to real-life cases seems straightforward: long bones are cut transversely and stained with Ehrlich's hematoxylin, and growth striations are visualized on the sections and counted. It is then hypothesized that one stria corresponds to one year, and by counting the number of striae, one can know the number of years since hatching. This theory works relatively well for short-lived ectotherms in temperate regions [27,28].

The authors who developed this technique for sea turtles were very cautious about the validity of the method on an individual scale: "The technique is not advocated as a method for the age determination of individual sea turtles" (in [30]). However, this caution has since been overlooked, and the method is often presented as reliable and accurate for estimating the absolute age of individuals (age since hatching); for example, "A robust approach to estimate critical age-specific demographic rates and population-level variation of these parameters is to use skeletochronology" (in [31]). A thorough analysis of the literature suggests that the initial caution regarding reliance on this method for determining absolute age remains valid.

In marine turtles, several difficulties arise. The first is establishing whether a line of arrested growth corresponds to a calendar year or to a cycle that could correspond to migration or displacement, or, in adults, corresponds to reproductive cycles. Several studies have attempted to demonstrate that skeletal growth marks deposited in humeri are annual:

- Juvenile loggerhead turtles were injected intramuscularly with oxytetracycline to establish a chronology for the deposition of periosteal bone growth layers. Eight recaptures, covering intervals of 1 to 3 years, were used to demonstrate that these

growth layers were typically deposited on an annual basis [32]. Based on the published information, it is not possible to ascertain the intended meaning of "typically".

- A similar experiment was carried out on another individual that was found dead eight years after being tagged and injected intramuscularly with oxytetracycline. The authors reported good agreement between the number of lines of arrested growth and the time since tagging, although this was not visible in the sole published image (see Figure 1A in [33]).
- Another study used a 29.4 year-old loggerhead turtle that had remained permanently in captivity and an eight year-old juvenile released from captivity when it was two years old. Thirteen Kemp's ridley turtles, *Lepidochelys kempii* (Garman, 1880), of known age were also used. These had been reared for one year in captivity after emerging from a nest, tagged with passive integrated transponders and released [34]. Lines of arrested growth on images of the humeri of the young Kemp's ridleys were consistent with the known ages of the individuals. However, the lines of arrested growth on the older loggerheads were particularly difficult to interpret, in the authors' opinion, since they described double lines of arrested growth or blunt lines and endosteal resorption.
- Oxytetracycline was intramuscularly injected into 25 wild green turtles from a tropical environment (southern Bahamas) where temperature variations were low, and no growth marks were visible in biopsies of the recaptured individuals' humeri after periods of 1.3 or 2.4 years [35].
- In a study on green turtles in Hawaii, where temperature variations were more significant, 14 humeri were recovered from oxytetracycline-marked individuals. Of these, a fluorescent trace was visible in 10 individuals and interpretable in six. The number of lines of arrested growth was as expected in five individuals, the sixth one having double lines [36]. It is noteworthy that the determination of whether a line of arrested growth must be interpreted as double or not is impossible without knowing the animal's actual age, and this creates circularity in interpretation. Based on these results, the conclusion stated by [36] of "providing strong validation that growth marks are annual" appears very optimistic.

A second difficulty arises from bone resorption in the medullary cavity. Bone is deposited in the periosteum, i.e., on the periphery of the bone [37]. As the diameter increases, the bone in the internal cavity is either resorbed or remodeled. Bone remodeling is a normal physiological process used by vertebrates to maintain constant bone mass [38] or to mobilize calcium for reproduction [39]. As a result of this process, we lose information on the number of lines of arrested growth in this central zone, i.e., the oldest lines. For species with a short lifespan, it can be quite easy to reconstruct the lost individual history [40]. In long-lived species, the number of lines of arrested growth that are lost can be significant, and various methods have been proposed to reconstruct the number of lines of arrested growth lost through this medullary resorption. It should be noted, however, that depending on the method, the estimate of the total number of lines of arrested growth can vary by as much as a factor of two, whereas the basic assumptions of the different methods, such as the "ranking protocol" and "correction factor protocol" [41], are similar. For example, the estimated age of a 57.5 cm carapace length individual was 34 years using the ranking protocol and 17.7 years using the correction factor protocol (and this was not the most extreme difference) [41]. The choice of one method or another appears arbitrary: "We consider the correction factor protocol age estimates as biologically more plausible because of the disassociation of age and size" (in [41]), but the justification for the importance of the dissociation between age and size is unclear, since there exists a clear relationship between age and size, whereby size only increases during growth. Such an arbitrary choice of methodology makes comparison between studies very difficult, and it is not possible to determine whether biological or methodological differences are being measured.

A third problem with skeletochronology concerns the difficulty in reading histological sections and the variability of these sections in bones. Histological sections presented in the literature are often very typical, but in practice, many sections are very complex to

interpret. The number of lines of arrested growth counted by different researchers can vary considerably, and different sections of the same bone do not necessarily tell the same story. Zug, Chaloupka and Balazs [41] noted that the lines of arrested growth were absent on one side of the humerus in six out of 12 olive ridley turtles, *Lepidochelys olivacea* (Eschscholtz, 1829). The same was true of the loggerhead, where the growth marks were not equally distinct along the entire length of the humerus [33]. This point is of particular note with regard to sclerotic ossicles used in leatherback skeletochronology [13,42,43] since, depending on the orientation of the ossicle during cutting, the number of visualized lines of arrested growth can vary by more than a factor of two (M.G., pers. obs.). Depending on the choices made by the researcher, completely different results can be obtained, and what is presented in the publications is just one of many possible outcomes. This difficulty is classically encountered with skeletochronology [44] but is unfortunately discreetly ignored when it comes to justifying the use of the method to determine a marine turtle's absolute age.

Another problem with this method concerns the compaction of lines of arrested growth from a period around sexual maturity, when the animal invests less in growth and more in reproduction. For old individuals, the compaction can be so extreme that separation of different lines of arrested growth is such that only a minimal age can be estimated with certainty. Furthermore, it should be emphasized that determination of age at maturity based on the age of slowdown growth and rapprochement of lines of arrested growth is not possible. It is possible only to define a period spanning several years of growth slowdown, not a specific age at maturity.

Finally, we have noted that the same individual may appear several times in different publications without this being explicitly noted. When meta-analyses are carried out, the fact that the same individual is used several times in separate analyses may impose a significant methodological and statistical bias. For this reason, we propose that each individual analyzed by skeletochronology should be associated with a unique digital identifier and that the list of identifiers be published in a Supplementary Material to each publication.

2.1.4. Sclerochronology (Scute)-Based Age Determination

The thick scutes of hawksbill sea turtles have rings of keratinized tissue with alternating dark or yellowish color patterns. Initial reports based on few individuals suggest that this alternation of color could be laid down on an annual basis and be used for ageing [45,46]. However, a lack of correlation between pigment bands and growth marks [47] suggests this approach may be less straightforward than originally thought. Abrasion of the epidermal surface results in growth mark loss in older individuals and could hinder the ability to age older individuals [47]. Complementing this, in another study where marginal scutes from 36 individual hawksbills representing all life stages and several Pacific populations and spanning eight decades were analyzed [48], the findings similarly did not provide clear annual growth records. This dataset demonstrated that, at least in some populations, the growth lines were not deposited on annual basis; for example, Hawaiian hawksbills deposited an average of eight growth lines annually (range 5–14) [48].

2.2. Relative Age

In the development of marine turtle growth models, the relative age of individuals (number of years since last capture or between two events) is often a reliable and useful metric. For example, such a method has enabled confirmation that marine turtles exhibit indeterminate growth [12,49]. If the sample size is large enough and includes turtles of various sizes, the entire lifespan of the animals can be documented, and reliable growth models can be developed without knowing absolute age. However, this method does not provide the exact age of individual turtles. It can provide a range of possible ages based on the size at first capture and recapture size, but not more.

2.3. Physiological Age

Various mechanisms lead to physiological changes during aging. If these changes accumulate at a constant rate, they may serve as potential age markers. Telomere shortening, which occurs during cell replication, was initially considered a predictor of age and age-related outcomes [50]. However, recent evidence suggests that the influence of telomere shortening on age and its associated effects is relatively modest in individuals [50]. Consequently, additional biomarkers are required to accurately predict age-related outcomes. The aging process also triggers multiple changes at the cellular and molecular levels of an organism, with research indicating that epigenetic alterations play a significant role in aging [51]. Epigenetic modifications involve changes in gene expression without alterations to the genome sequence. Prominent examples include histone modifications, DNA methylation and non-coding RNA, with dynamic changes in DNA methylation being the most widely documented in the aging process [52]. To date, only telomere length reduction and DNA methylation have been examined in marine turtles.

2.3.1. Telomere Length Reduction

Telomere reduction is both a marker of aging [53] and a cause of senescence [54]. Telomeres are reduced by a mechanical process at each cell division when telomerase activity is absent or insufficient in cells [55]. Most telomere sequences are located in the telomeric region in turtles [56] and a measure of telomere repeat sequences can be considered as a pertinent measure of telomeres length. Initial attempts to use the telomere length as an age criterion in turtles were made with the European freshwater turtle, *Emys orbicularis* (Linnaeus, 1758), using Southern blots and a telomere-sequence-specific probe [57]. In this species, no difference in average telomere length was observed between 15 adults (>20 years old) and 15 hatchlings. Moreover, the telomeres were particularly long (>20 kb) [57]. Great length of telomeres was also found in another freshwater turtle, *Trachemys scripta* (Schoepff, 1792), (≈50 kb) [58]. Blood cell telomere length similarly did not differ between hatchlings and adults of leatherback turtles [59]. No significant correlation between age and relative T/S ratios in the blood or epidermis (T/S is a measure of telomere length) was observed in loggerhead sea turtles [60]. The conclusion by [60] that "it was thus demonstrated that telomere length in epidermis could be a useful age estimator for sea turtles" is based on only one individual out of 20 that was the second oldest in the samples and had the smallest T/S ratio. Overall, however, there was no correlation between age and telomere length. Telomere length and telomerase activity were also measured in green turtle fibroblast skin cell culture. Telomere length and telomerase activity in cell subculture after 14 cycles of replication was greater than in subculture after five cycles. That is, older fibroblast skin cells had longer telomeres length than younger ones. However, based on morphology, skin cells showed senescence. It is possible the aging mechanism that the green turtle fibroblast skin cell culture underwent did not go through both telomere shortening and reduced telomerase activity [61]. These results reaffirm the original observations in European freshwater turtles [57] and leatherbacks [59], and we conclude that telomere length cannot be considered a reliable marker of age in turtles.

2.3.2. Methylation of DNA

The proportion of DNA methylation has been used to predict age in a wide range of mammals, fish and birds [62]. An epigenetic clock that predicts the age of marine turtles from skin biopsies has been developed [63]. The model was tested using DNA from known-age green turtles from two captive populations (12 individuals from La Réunion Kelonia center in Indian Ocean and 51 from Cayman Turtle Centre, Cayman Islands, Caribbean Sea) and two marked and recaptured wild turtles with known time intervals between captures (2.3 and 3 years). A total of 1,261,168 CpG (cytosine and guanine nucleosides separated by only one phosphate group in DNA) sites with DNA methylation levels were available, and global methylation did not significantly correlate with age (Pearson correlation = 0.10, p-value = 0.67). This result is not consistent with studies in other species [64,65]. Among

a subtotal of 844 CpG sites common to all turtle species, 51 were significantly correlated with age (51/844 = 0.06). This is approximately the expected proportion for a random set of methylation with a false discovery rate of 0.05. A selection of CpG sites among these 844 results was performed to best represent known age, resulting in 29 CpG selected sites, some of which were less methylated and others more methylated than had they been randomly chosen. Using an age prediction model with these 29 CpG sites, a high correlation between the true and predicted ages was found, but it should be noted that the use of Pearson correlation after a selection procedure is highly debatable. The results are promising but are close to what would be expected if the relationship between a large amount of CpG methylation information and age was purely random. Furthermore, a significant effect of rearing center in CpG methylation was noticed (Supplementary Figure S2C in [63]) which would be unexpected if CpG methylation had been related to the age of individuals. This effect was less visible when only 18 age-associated CpG sites selected for multiplex PCR were used (Figure 1 in [63]), but it could be due simply to an effect of lower sample size.

The relationship between age and DNA methylation is probably mediated by the presence of reactive oxygen species (ROS) [66]. ROS are derived from molecular oxygen and include a number of free radicals and reactive molecules, which can modify DNA, RNA, proteins and lipids. ROS are produced by metabolism as byproducts of several enzyme reactions for example in the Krebs cycle [67]. In such a context, it would be normal for a relationship between age and ROS to be observed, and DNA methylation could be a consequence of this relationship. This might also explain the strong effect of rearing centers (above) because it could reflect differences in rearing and feeding conditions. The relationship between CpG methylation and age of individuals is promising, but currently, the epigenetic clock in green turtles remains unvalidated in the field. Indeed, methylation status could reflect a cumulative physiological status rather than age, which could be tested by using two groups of individuals reared in captivity, with one group fed ad libitum and one group starved.

3. Conclusions

The purpose of this note is to alert researchers and species managers using age data that there are large levels of uncertainty involved in knowing the true absolute age of individual sea turtles. We demonstrate that estimating the absolute age of individuals of long-lived species using skeletochronology presents a very high level of uncertainty, that sclerochronology does not provide an age in years, that telomere length is not a proxy of age in turtles and that DNA methylation is a promising method to obtain a physiological age but not an absolute age. We believe there would be merit in developing a non-invasive method of marking cohorts of hatchlings at emergence to document absolute age at first nesting and develop credible size–age relationships. At present, only relative age seems reliable using the methodology available thus far for marine turtles, but this does not tell us as much as could be learned with absolute age.

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