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ORIGINAL RESEARCH

Shorter telomeres are associated with shell anomalies in a long-lived tortoise

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Abstract

Age-related telomere length (TL) variation is relatively well-described for mammals, birds and other model organisms. Nevertheless, it remains largely unknown in ectotherms, especially turtles and tortoises, which are extremely long-lived species with slow or negligible senescence. In this study, we described TL dynamics in wild spur-thighed tortoises (*Testudo graeca*), one of the chelonian species with the lowest aging rates. By combining cross-sectional (single) and longitudinal (capture–recapture) samplings, we assessed the relationship between TL and individual characteristics (sex, age, individual growth rate, body condition index, presence of shell anomalies). We did not find any association between TL and sex, individual growth rate, or body condition. However, the relationship with age remains uncertain, likely due to the complex dynamics of TL over time. Interestingly, shorter telomeres correlated significantly with shell anomalies, which are usually assumed as a fitness proxy for reptiles. Overall, our results suggest TL as a potential indicator for ontogenetic studies on tortoises, while its utility as a marker of biological age appears limited.

Introduction

Telomeres are non-coding DNA sequences involved in several molecular and cellular functions, including chromosome protection against replication problems, avoidance of subtelomeric gene expression, or genome stability preservation (Blackburn, 2005; Monaghan, 2014). Although telomere studies in non-model species have become more common in the last decade, the majority of studies have been focused on endotherms (mammals and birds) (Wilbourn et al., 2018). Most of these studies have reported that individuals with shorter telomeres and/or experiencing faster telomere shortening rates have increased mortality, disease risk and reduced longevity (Olsson et al., 2018a, 2018b; Tobler et al., 2022; Tricola et al., 2018; Wilbourn et al., 2018).

Telomere length (TL) shortening occurs as a result of base pair loss during cell replication (Monaghan & Ozanne, 2018), resulting in telomere shortening as organisms age in many endotherms (Simide et al., 2016). However, there is no clear unique pattern described for TL dynamics in ectotherms (Fitzpatrick et al., 2021; Olsson et al., 2018a, 2021), which could even change across their life stages (e.g. amphibian metamorphosis, Burraco et al., 2023; Sánchez-Montes et al., 2020). For instance, different strains of zebrafish (*Danio rerio*) have been reported to exhibit both stable telomere length (TL) and age-related telomere shortening (Henriques et al., 2013; Lund et al., 2009). Conversely, in female sand lizards (*Lacerta agilis*), telomere elongation has been observed with age (Olsson et al., 2010). The fact is that post-natal TL dynamics in ectotherms do not necessarily match patterns of endotherms because of physiological responses, metabolic rates, or telomerase activity (enzyme responsible for telomere restoration) differ among organisms with these two thermoregulation strategies (Fitzpatrick et al., 2021).

Among ectotherms, reptiles are particularly interesting for studying telomere dynamics because of their phenotypic plasticity, autotomy and, in some cases longevity (Bateman et al., 2009; da Silva et al., 2022; Olsson et al., 2010, 2018a, 2018b; Reinke et al., 2022). In reptiles, the relation between TL and individual characteristics differs among clades, conspecific individuals, or even tissues and cell types of the same individual (Fitzpatrick et al., 2021; Olsson et al., 2018a, 2020, 2021; Rollings et al., 2019; Serén et al., 2023). Furthermore, studies on lizards, alligators and snakes have explored the effects of age, sex and life history traits, such as growth rates or reproductive investment, revealing diverse TL dynamics (Olsson et al., 2021). These dynamics range from TL elongation during juvenile phases to TL shortening with age or in response to increased growth and/or reproductive investment (see Olsson et al., 2018a for a review; Fitzpatrick et al., 2021; Rollings et al., 2019). It is worth mentioning that most of the studies conducted to date have been performed under laboratory conditions or using zoo animals (Hatase et al., 2008; Olsson et al., 2018b) which leaves telomere dynamics in wild populations poorly understood. In reptiles, telomere shortening or elongation can reflect environmental stress, parasitic pressure, self-maintenance or other life history investments like reproduction, which are key pressures in ecological terms (Brown et al., 2021; Sparks et al., 2022). Therefore, given the reflection on fitness of TL dynamics, studies need to focus more on non-model organisms in the wild.

In this study, we describe TL dynamics in wild spur-thighed tortoises (*Testudo graeca*). To the best of our knowledge, TL has never been studied before in terrestrial chelonians. Chelonians are extremely long-lived animals and, among them, *T. graeca* has been recently identified as having one of the lowest aging rates (i.e., the speed at which the mortality rate increases with age; da Silva et al., 2022). *Testudo graeca* has been described as a species with a slow pace of life due to high adult survival rates, long generation times and low and variable reproductive patterns (Rodríguez-Caro et al., 2023). In captivity, spur-thighed tortoises can live for up to 160 years (Bruce, 2004), and the maximum ages reported for wild individuals exceed 40–50 years (Andreu et al., 2000; Lambert, 1982). Moreover, annual survival rates increase with age from 20 to 41% in immatures (≤4 years) and up to 95 to 98% in adults (≥9 years) (Graciá et al., 2020; Rodríguez-Caro et al., 2019; Sanz-Aguilar et al., 2011).

In the present study, we explore age-related changes of TL in blood cells of *T. graeca* and how TL relates to different individual traits such as sex, growth rate, individual body condition and presence of shell anomalies. For doing so, we combined cross-sectional (single) and longitudinal (capture–recapture) samplings. Cross-sectional approaches allow TL variation among individuals of the same population with different characteristics (e.g., age) to be widely explored, but may be influenced by factors like cohort age range or survival effects (Chen et al., 2011). Longitudinal samplings in the wild are demanding for being based on recapture events, but better allow subtle TL dynamics to be explored over time. For example, longitudinal studies prevent from biases arising from individual variability in interstitial telomeric sequences (ITS), which might potentially affect cross-sectional studies (Foote et al., 2013). Although it is worth noting that ITS are infrequent among chelonians (Clemente et al., 2020).

As TL has been described as a marker of aging in other reptiles (Burraco et al., 2020), we could expect to find an association between TL and age in the cross-sectional study, as well as significant TL shortening over time in the longitudinal study. On the other hand, sex-specific differences in TL should arise if differences in reproductive investment and/or trade-offs exist (Rollings, Friesen, et al., 2017; Rollings, Uhrig, et al., 2017). In the case of *T. graeca*, females are typically larger than males (Benelkadi et al., 2022). In relation to this, we also expect the animals that grow more (or more quickly) to show shorter TL (Monaghan & Ozanne, 2018; Rollings, Friesen, et al., 2017; Rollings, Uhrig, et al., 2017). Growth involves an important energy investment in all organisms by increasing oxidative stress and DNA damage, which are closely related to telomere shortening (Monaghan & Ozanne, 2018). We also hypothesize that body condition may be related to TL because body condition reflects the immediate size-to-weight ratio, which is used as a proxy for both health status and self-maintenance (Barrett & Richardson, 2011; Burraco et al., 2020; Olsson et al., 2021; Rollings, Uhrig, et al., 2017). Additionally, this assessment of body condition has been linked to physiological costs of reproduction and to sex-specific life strategies (Friesen et al., 2021; Rollings, Uhrig, et al., 2017). Finally, we expect a relationship between TL and the alteration of shell scute pattern. In chelonians, the number of shell scutes is a highly conserved trait at the species level (Zimm et al., 2017). At the individual level, the occurrence of shell anomalies is related to stress during egg incubation, environmental factors, inbreeding and epigenetic-related problems (Velo-Antón et al., 2011; Zimm et al., 2017). Thus, we may expect that tortoises with such anomalous numbers of scutes will have shorter telomeres. Altogether, our results will contribute to a better understanding of how TL is influenced by individual factors and with the passing of time in long-lived species in the wild.

Materials and methods

Study system, sampling and data collection

Testudo graeca is the most widely distributed tortoise species in the Western Palearctic, with different lineages ranging over North Africa, the Near and Middle East and southern Europe (Fritz et al., 2007, 2009; Graciá et al., 2017; Javanbakht et al., 2017; Mikulíček et al., 2013). Here we sampled a population from arid environments in SE Spain (Figure S1).

We conducted a cross-sectional study between 2005 and 2019 in spring to sample 90 tortoises that represented the SE Spanish distribution range (39 males, 38 females and 13 subadults with ages ranging from 2 to 27 years; Table S1, Figure S1). We collect blood samples and they were conserved in 70% ethanol (stored at −4°C until analysed). Each tortoise was permanently marked by carapace notching and the following individual traits were recorded: sex (according to external secondary characters; Lopez-Jurado et al., 1979); age (estimated from carapace ring counting, Rodríguez-Caro et al., 2015); body weight (g); plastron and carapace length (mm); shell anomalies (occurrence of any deviation from the typical number and arrangement of shell scutes, Benelkadi et al., 2022) (see *Fieldwork Information* on Methods in Appendix S1 for more details). The frequency of such anomalies in the SE Spanish *T. graeca* population has been estimated at 17% by Moreno (2017). Our cross-sectional sampling included 20% of the tortoises with anomalous scute counts (Table S1).

In addition to the information recorded during fieldwork, two more descriptors were estimated. Firstly, a growth rate score per individual (mm/year, *k*) was calculated separately for males and females using carapace length and age in the von Bertalanffy model (Fabens, 1965; Rodríguez-Caro et al., 2013) (Equation S2). This model is typically used to describe chelonians growth (e.g., Benítez-Malvido et al., 2019; Zivkov et al., 2007) and allows estimations of each individual's growth rate since its birth to the current measurement time. This approach compares individual growth to the growth pattern of the whole population. Secondly, individual body condition was estimated using the residuals of the linear regression analysis between weight and plastron length (Rollings et al., 2019). It is necessary to notice that these two variables show in our dataset a robust linear correlation coefficient of 0.93, as indicated by a Pearson test ($P < 2.2e{-}16$).

For the longitudinal study, we twice sampled 14 adult females, which were captured and recaptured with time intervals from 1 to 14 years (Table S2, Figure S1). We focused on females because they are much easier to recapture after long time intervals due to their smaller home ranges and higher site fidelity (Graciá et al., 2020).

Laboratory analyses and relative telomere length calculation

DNA was extracted from blood samples using the DNeasy Blood and Tissue kit (Qiagen) following the manufacturer's protocol. Telomere length was measured using the real-time quantitative PCR (qPCR) method described in Cawthon (2002, 2009), adapted for tortoise and turtle samples by Plot et al. (2012). We used the highly conserved subunit of ribosomal DNA 18S as a single-copy gene (commonly employed in phylogenetics, intra- and interspecific comparisons and telomere studies in reptiles and other taxa to calculate relative TL) (Plot et al., 2012; Yang et al., 2018). DNA samples were assayed using the Maxima SYBR Green qPCR Master Mix (Thermo Scientific) in a StepOnePlus system (Applied Biosystems). The efficiency of each amplicon was estimated from the slopes of the amplification curves for each qPCR reaction using LinReg PCR software and it varies from an average of 1.900 for telomere plates and of 1.768 for 18S plates (Ruijter et al., 2009). The individual plates included the samples for telomeres or 18S amplification (run on different plates), a reference sample (Gold Sample) and a negative control (water). To ensure the robustness of our measurements, all samples, including the negative control and golden sample, were run in triplicate. The relative telomere length (T/S ratio) was calculated using the formula described by Raschenberger et al., 2016, which takes into account variations in telomere and 18S amplification efficiencies, as well as the amplification efficiencies of the golden sample (Equation S1). For this formula, we used a mean of the efficiency calculated with LinReg PCR software for each of the three replicates.

Statistical analysis

Cross-sectional study (single sampling)

In case of cross-sectional study, we made the statistical analysis in two steps. To explore the relation between T/S ratio (dependent variable) and individual characteristics (age, sex, *k*, body condition scores and the presence or absence of shell anomalies; independent variable), we used univariate models in linear regression. We made a logarithm transformation of T/S to standardize the variable from a normal distribution before construct all models (log T/S ratio). The log T/S ratio and sex relation was tested only for adults by a one-way analysis of variance (ANOVA). Age was analysed using a both linear and quadratic regressions that allow to explore nonlinear TL dynamics. To avoid sex-specific effects above body condition index, we employed sex as a covariable in the linear regression models. Finally, we built a multivariate model to assess the combined effect of the variables that resulted significant in the univariate models. We employed a stepwise backward analysis using the Akaike Information Criterion (AIC) for model selection to identify the most relevant variables influencing TL. All analyses were performed using basics *stats* functions of R software environment (R Core Team, 2022).

Longitudinal study (capture–recapture sampling)

To investigate individual telomere dynamics, we estimated changes in the logarithm of T/S ratio for 14 captured–recaptured females in different time intervals ($\Delta\log(\text{T/S ratio}) = 2^{\text{nd}}$ capture $\log(\text{T/S ratio}) - 1^{\text{st}}$ capture $\log(\text{T/S ratio})$). For each female, significant differences in TL between the capture and recapture times were assessed by *t*-tests using sample triplicates (Table S3). Subsequently, we conducted a linear regression between $\Delta\log(\text{T/S ratio})$ (dependent variable) and capture intervals (independent variable). All the analyses were done in R project (R Core Team, 2022).

Results

Cross-sectional study (single sampling)

The cross-sectional data analysis revealed that TL was not influenced by sex (Table S4), individual growth rate (*k*) or body condition (Table S5). Nevertheless, we found that shell anomalies were a significant predictor of TL ($P = 0.04$, Estimate $= -0.14 \pm 0.06$, $R^2 = 0.06$, d.f. = 53; Table S5). Tortoises with shell anomalies are more likely to have shorter telomeres (Fig. 1). Regarding the influence of age on TL, significant effects were observed only in accordance with the quadratic regression model. The results indicated that the telomeric length is greater in individuals of intermediate ages (linear term: $P = 0.02$, quadratic term: $P = 0.01$, $R^2 = 0.05$; see Table S5). However, in the multivariate stepwise analysis, the best model describing TL variation only retained the presence of shell anomalies (starting model including age with linear and quadratic terms and shell anomalies: AIC = −165.85; final model including only shell anomalies: AIC = −161.66), being therefore identical to the univariate model explored before (Table S5).

Longitudinal study (capture–recapture sampling)

The capture–recapture data revealed diverse individual TL dynamics over time: out of the fourteen analysed tortoises, eight showed significantly shorter telomeres when recaptured, five displayed no significant differences and one presented significant telomere elongation (this last one with a 1-year capture interval). The linear regression conducted to assess the relation between Δ(log T/S) ratio and the time interval between recaptures resulted in a non-significant relation ($P = 0.421$, Estimate = -0.07 ± 0.08, d.f. = 13, $R^2 = -0.023$, Fig. 2) (Table S6).

Discussion

In this study, we explored how TL changes with age and relates to important individual traits during the postnatal life in wild spur-thighed tortoises. Our univariant exploration revealed a negative relation between TL and the presence of shell anomalies and a quadratic relationship between TL and age. However, this latter relationship was not retained in the best multivariate analysis built with a stepwise procedure, and the capture–recapture experiment unveiled diverse individual TL dynamics over time. Furthermore, we found no relationship between TL and sex, individual growth rate or tortoise body condition. The lack of relation between some variables and TL may arise from limited statistical power owing to a relatively modest sample size, or because we studied individuals inhabiting homogeneous conditions (a single population). However, overall, our results around TL and aging align with previous studies and support the notion that telomeres shorten more slowly—or have more diverse dynamics—in long-lived species than in short-lived ones (Dantzer & Fletcher, 2015; Haussmann et al., 2003).

Our study is pioneering for establishing the relation between occurrence of morphological anomalies and TL in a wild-living ectotherm. In reptiles, scalation asymmetries and anomalies are strongly influenced by environmental factors and nest conditions during embryo incubation (Peet-Paré & Blouin-Demers, 2012; Zimm et al., 2017), and also by inbreeding or genetics (Brown et al., 2017; Velo-Antón et al., 2011). Subtle deviations from bilateral symmetry are frequently used as a fitness proxy (Cordero Rivera et al., 2008; Leary et al., 1984; Velo-Antón et al., 2011) which is, however, not always justified (Brown et al., 2017; Lens et al., 2002; Löwenborg et al., 2011). Our results suggest a link between developmental

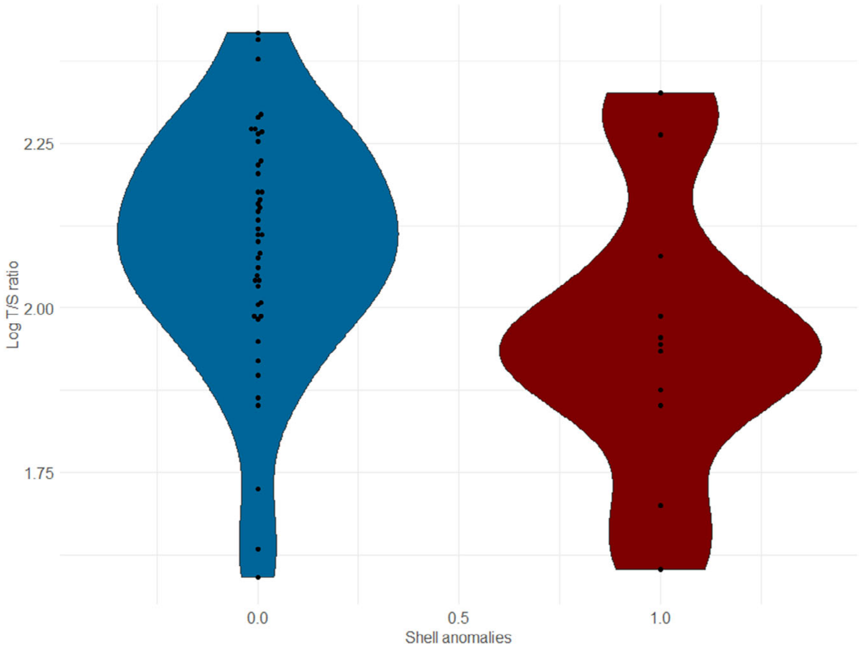

Figure 1 Violin plot representing the distribution of telomere length (logarithm of T/S ratio) in relation to the presence or absence of shell anomalies in *Testudo graeca* at the cross-sectional study (Sample size = 55, $P = 0.04$, Estimate = -0.14 ± 0.06, $R^2 = 0.06$, d.f. = 53; Table S5). Dots and white bars represent the median and first to third quartiles, respectively, while the density curves depict the probability distribution of the dependent variable.

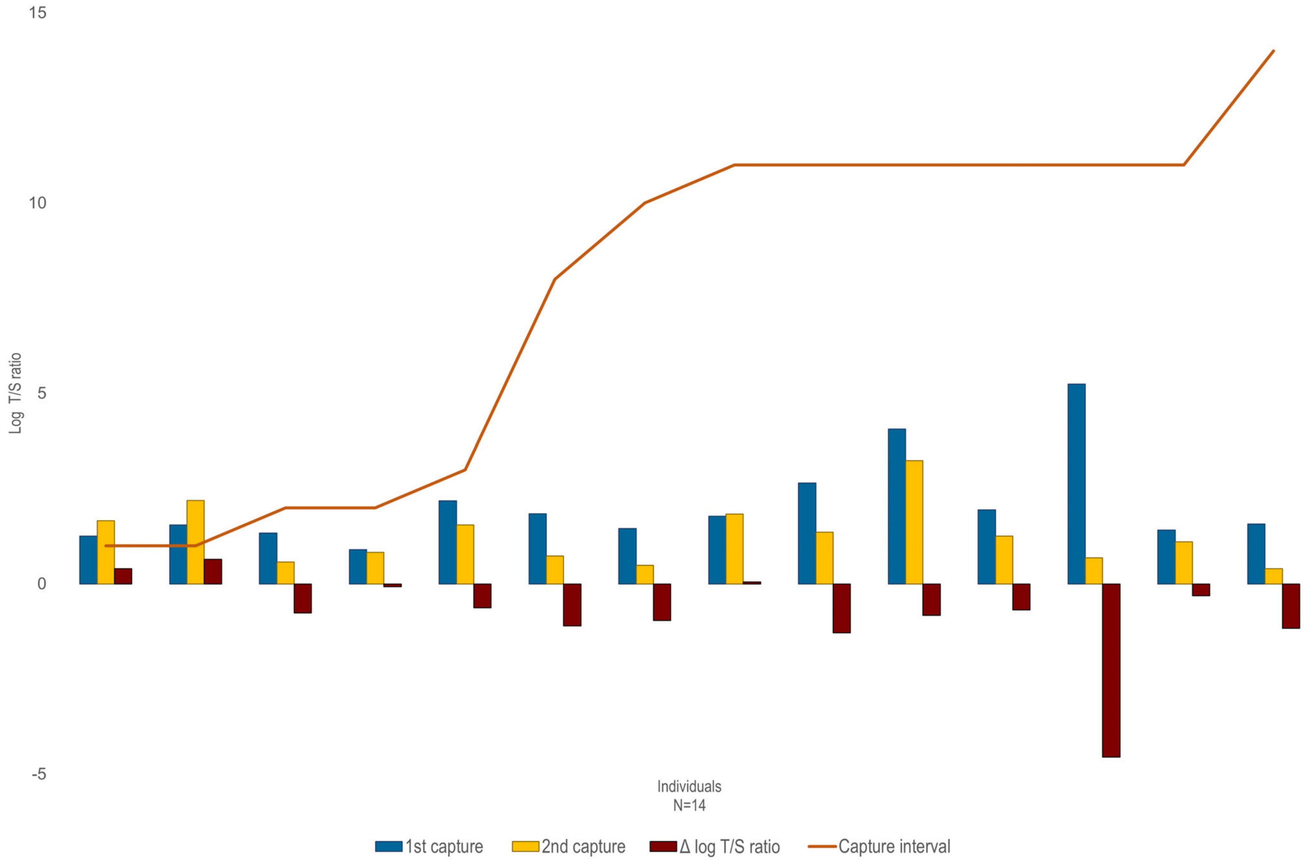

Figure 2 Telomere length variation at the longitudinal study. For each individual *Testudo graeca* ($N = 14$), bars represent the logarithm of T/S ratio (1^{st} capture in blue, 2^{nd} capture in yellow) and logarithm of ΔlogT/S ratio (calculated as the 2nd capture logT/S ratio minus the 1st capture logT/S ratio). Therefore, negative values of ΔlogT/S ratio represent telomere shortening over time, while positive values represent telomere elongation. Significant differences between capture and recapture logT/S ratios are denoted by an asterisk (see *t*-test results in Table S3). Individuals are sorted based on the capture interval, which is represented by the orange line.

instability and TL in blood cells of adult *T. graeca*. Taking into account that the incubation of eggs and the development of tortoises to their adult stage occur at different times and in very different environments, we would venture to propose that the observed relationship is attributable to endogenous factors (i.e., genetic and epigenetic factors), rather than exogenous (developmental environments). Further research (including experimental designs) may elucidate causality and potential sources for this relation, and also the consequences of shell anomalies on *T. graeca* fitness.

Regarding sex, previous studies tended to report that the heterogametic sex, or the sex growing faster or which needs to maintain a larger body mass presents shorter telomeres (Barrett & Richardson, 2011). However, Remot et al. (2020) tested these expectations in 51 vertebrate species (including mammal, bird, fish and reptile species), and did not find any of them to be generally supported. Alternative mechanisms behind sexual dimorphism may modulate or mitigate sex-specific differences in TL (Rollings et al., 2019; Rollings, Friesen, et al., 2017; Rollings, Uhrig, et al., 2017). *Testudo graeca* lacks sex chromosomes (Pieau, 1975), but the larger size and body mass of females led us to expect sex-related differences in TL. Telomere length is positively related to survival in many taxa (Wilbourn et al., 2018) and sex-biased survival rates have been found in species with different TL between sexes (Rollings et al., 2019). The SE Spanish population of *T. graeca* shows a balanced sex ratio and a high adult survival rate for both sexes (98.4% for males and 94.7% for females) (Graciá et al., 2017; Rodríguez-Caro et al., 2013), which is compatible with our results. In fact, it has been reported that *T. graeca* females buffer disturbances by constant-through-time survival rates of adults at the expense of wide variability in reproductive rates (Rodríguez-Caro et al., 2021). This results in the question whereas tortoises adjust their reproductive investment in the long term to avoid TL damage or not.

We did not find any relation between TL and individual growth rate (*k*) or body condition descriptors. Growth patterns in *T. graeca* have been used in ecological studies to describe the long-term effect of habitat disturbances (like fires) on survival rates (Rodríguez-Caro et al., 2013). Body condition is used as a proxy of immediate health (e.g., to reveal the effects of virus infections) (Garcia-Morante et al., 2016). Not finding a relation of these estimates with TL could be the consequence of relatively homogeneous growth conditions and the good health of the analysed animals (we did not find any signs of disease or anorexia symptoms). Further studies may explore the potential impacts on TL of habitat disturbances like habitat loss and fragmentation, fire or drought.

According to the relation between TL and age, some previous cross-sectional studies for other extremely long-lived species found TL maintenance and elongations in wild populations (Dantzer & Fletcher, 2015). For example: (i) TL shortening between chick stage and adulthood, but TL maintenance in an adult wandering albatross (*Diomedea exulans*; Hall et al., 2004); (ii) no correlation with age in the ocean quahog (*Artica islandica*; Gruber et al., 2014); and (iii) apparent TL lengthening with age in Leach's storm petrel (*Oceanodroma leucorhoa*; Haussmann & Mauck, 2008). By explaining the absence of shortening patterns at the population level, it has been suggested that some individuals are able to elongate telomeres ('elongation hypothesis') and/or, alternatively, that selection acts against individuals with short telomeres ('selection hypothesis') (Haussmann & Mauck, 2008). In our study, we found unclear cross-sectional TL and age relation in *T. graeca*, and diverse individual TL dynamics over time when analysing females' capture–recapture data. Further research involving larger sample sizes, fitness traits and telomerase activity could definitely elucidate the contribution of each hypothesis to the cellular effects on *T. graeca* with the passing of time. For the moment, our results suggest that TL may have limited utility as a marker of biological age.

Conclusions

Despite the increasing number of studies on telomere dynamics in ectotherms, it remains essential to further investigate genetics, physiology and evolutionary ecology in relation to telomeres in reptiles (Olsson et al., 2021). Our study contributes to a better understanding of TL dynamics in long-lived species and reports for the first time the relationship between TL and the presence of shell anomalies. Specifically, we found shorter TL in animals with shell anomalies, but further research is necessary to elucidate the causality and potential sources of this link. Moreover, we did not find a clear relation between TL and age, but noted diverse individual TL dynamics over time. In the same way, we found no relation to link TL with sex, individual growth or body conditions. Altogether, these results suggest TL as a potential indicator for ontogenetic studies on tortoises, while its utility as a marker of biological age and the other studied traits appears limited.

Acknowledgments

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Author contributions

EG, JCNA and AG conceived this study. AG; AMJ; EG; MIGF and RCRC collected samples and recorded the information from tortoises during fieldwork. AMJ, CK, EG, JCNA, and MIGF processed blood samples and performed laboratory analyses. All the authors participated in the data analysis. AMJ led the writing. All the authors discussed the results, collaborated during writing and reviewed the paper.

Conflict of interest

All authors declare no conflict of interest regarding this paper or any information generated or extracted from it.

References

Andreu, A. C., Díaz-Paniagua, C., & Keller, C. (2000). *La tortuga mora en Doñana*. Asociación Herpetologica Española (Monografías de Herpetología, Vol. 5).

Barrett, E. L. B., & Richardson, D. S. (2011). Sex differences in telomeres and lifespan. *Aging Cell*, **10**, 913–921.

Bateman, P. W., Fleming, P. A., Bateman, P. W., & Bennett, N. (2009). To cut a long tail short: A review of lizard caudal autotomy studies carried out over the last 20 years. *Journal of Zoology*, **277**, 1–14.

Benelkadi, A. H., Rodríguez-Caro, R. C., Amroun, M., & Graciá, E. (2022). The shell measurements that best describe sexual dimorphism in the spur-thighed tortoise *Testudo graeca* from Algeria. *Basic and Applied Herpetology*, **36**, 19–30.

Benítez-Malvido, J., Giménez, A., Graciá, E., Rodríguez-Caro, R. C., de Ybáñez, R. R., Siliceo-Cantero, H. H., & Traveset, A. (2019). Impact of habitat loss on the diversity and structure of ecological networks between oxyurid nematodes and spur-thighed tortoises (*Testudo graeca L.). PeerJ*, **2019**, 1–26.

Blackburn, E. H. (2005). Telomeres and telomerase: Their mechanisms of action and the effects of altering their functions. *FEBS Letters*, **579**, 859–862.

Brown, G. P., Madsen, T., Dubey, S., & Shine, R. (2017). The causes and ecological correlates of head scale asymmetry and fragmentation in a tropical snake. *Scientific Reports*, **7**, 11363.

Brown, T. J., Spurgin, L. G., Dugdale, H. L., Komdeur, J., Burke, T., & Richardson, D. S. (2021). Causes and consequences of telomere lengthening in a wild vertebrate population. *Molecular Ecology*, **31**, 5933–5945.

Bruce, R. K. (2004). *Timothy the tortoise: The remarkable story of the Nation's oldest pet*. Orion Books.

Burraco, P., Comas, M., Reguera, S., Zamora-Camacho, F. J., & Moreno-Rueda, G. (2020). Telomere length mirrors age structure along a 2200-m altitudinal gradient in a Mediterranean lizard. *Comparative Biochemistry and Physiology Part A: Molecular Integrative Physiology*, **247**, 110741.

Burraco, P., Hernandez-Gonzalez, M., Metcalfe, N. B., & Monaghan, P. (2023). Ageing across the great divide: Tissue transformation, organismal growth and temperature shape telomere dynamics through the metamorphic transition. *Proceedings of the Royal Society A. Biological Science*, **290**, 1992.

Cawthon, R. M. (2002). Telomere measurement by quantitative PCR. *Nucleic Acids Research*, **30**, 1–6.

Cawthon, R. M. (2009). Telomere length measurement by a novel monochrome multiplex quantitative PCR method. *Nucleic Acids Research*, **37**, 1–7.

Chen, W., Kimura, M., Kim, S., Cao, X., Srinivasan, S. R., Berenson, G. S., Kark, J. D., & Aviv, A. (2011). Longitudinal versus cross-sectional evaluations of leukocyte telomere length dynamics: Age-dependent telomere shortening is the rule. *The Journals of Gerontology Gerontology: Series A*, **66**, 312–319.

Clemente, L., Mazzoleni, S., Pensabene Bellavia, E., Augstenová, B., Auer, M., Praschag, P., Protiva, T., Velenský, P., Wagner, P., Fritz, U., Kratochvíl, L., & Rovatsos, M. (2020). Interstitial Telomeric repeats are rare in turtles. *Genes (Basel)*, **11**(6), 657.

Cordero Rivera, A., Ayres Fernández, C., & Velo-Antón, G. (2008). High prevalence of accessory scutes and anomalies in Iberian populations of *Emys orbicularis*. *Revista Española Herpetología*, **22**, 5–14.

da Silva, R., Conde, D. A., Baudisch, A., & Colchero, F. (2022). Slow and negligible senescence among testudines challenges evolutionary theories of senescence. *Science*, **376**, 1466–1470.

Dantzer, B., & Fletcher, Q. E. (2015). Telomeres shorten more slowly in slow-aging wild animals than in fast-aging ones. *Experimental Gerontoly*, **71**, 38–47.

Fabens, A. J. (1965). Properties and fitting of the von Bertalanffy growth curve. *Growth*, **29**, 265–289.

Fitzpatrick, L. J., Olsson, M., Pauliny, A., While, G. M., & Wapstra, E. (2021). Individual telomere dynamics and their links to life history in a viviparous lizard. *Proceedings of the Royal Society B: Biological Sciences*, **288**, 20210271.

Foote, C. G., Vleck, D., & Vleck, C. M. (2013). Extent and variability of interstitial telomeric sequences and their effects on estimates of telomere length. *Molecular Ecology Resources*, **13**, 417–428.

Friesen, C. R., Wilson, M., Rollings, N., Sudyka, J., Giraudeau, M., Whittington, C. M., & Olsson, M. (2021). Exercise training has morph-specific effects on telomere, body condition and growth dynamics in a color-polymorphic lizard. *The Journal of Experimental Biology*, **224**(9), jeb242164.

Fritz, U., Harris, D. J., Fahd, S., Rouag, R., Graciá Martínez, E., Giménez Casalduero, A., Široký, P., Kalboussi, M., Jdeidi, T. B., & Hundsdörfer, A. K. (2009). Mitochondrial phylogeography of Testudo graeca in the Western Mediterranean: Old complex divergence in North Africa and recent arrival in Europe. *Amphibia-Reptilia*, **30**(1), 63–80. https://doi.org/10.1163/156853809787392702

Fritz, U., Hundsdörfer, A., Široký, P., Auer, M., Kami, H., Lehmann, J., Mazanaeva, L., Türkozan, O., & Wink, M. (2007). Phenotypic plasticity leads to incongruence between morphology-based taxonomy and genetic differentiation in western Palaearctic tortoises (*Testudo graeca* complex; Testudines, Testudinidae). *Amphibia-Reptilia*, **28**(1), 97–121.

Garcia-Morante, B., Pénzes, J. J., Costa, T., Martorell, J., & Martínez, J. (2016). Hyperplastic stomatitis and esophagitis in a tortoise (*Testudo graeca*) associated with an adenovirus infection. *Journal of Veterinary Diagnostic Investigation*, **28**, 579–583.

Graciá, E., Rodríguez-Caro, R. C., Sanz-Aguilar, A., Anadón, J. D., Botella, F., García-García, A. L., Wiegand, T., & Giménez, A. (2020). Assessment of the key evolutionary traits that prevent extinctions in human-altered habitats using a spatially explicit individual-based model. *Ecological Modelling*, **415**, 108823.

Graciá, E., Vargas-Ramírez, M., Delfino, M., Anadón, J. D., Giménez, A., Fahd, S., Corti, C., Jdeidi, T. B., & Fritz, U. (2017). Expansion after expansion: Dissecting the phylogeography of the widely distributed spur-thighed tortoise, *Testudo graeca* (Testudines: Testudinidae). *Biological Journal of the Linnean Society*, **121**, 641–654.

Gruber, H., Schaible, R., Ridgway, I. D., Chow, T. T., Held, C., & Philipp, E. E. R. (2014). Telomere-independent ageing in the longest-lived non-colonial animal, *Arctica islandica*. *Experimental Gerontoly*, **51**, 38–45.

Hall, M. E., Nasir, L., Daunt, F., Gault, E. A., Croxall, J. P., Wanless, S., & Monaghan, P. (2004). Telomere loss in relation to age and early environment in long-lived birds. *Proceedings of the Royal Society of London B*, **271**, 1571–1576.

Hatase, H., Sudo, R., Watanabe, K. K., Kasugai, T., Saito, T., Okamoto, H., Uchida, I., & Tsukamoto, K. (2008). Shorter telomere length with age in the loggerhead turtle: A new hope for live sea turtle age estimation. *Genes & Genetic Systems*, **83**, 423–426.

Haussmann, M. F., & Mauck, R. A. (2008). Telomeres and longevity: Testing an evolutionary hypothesis. *Molecular Biology and Evolution*, **25**, 220–228.

Haussmann, M. F., Winkler, D. W., O'Reilly, K. M., Huntington, C. E., Nisbet, I. C. T., & Vleck, C. M. (2003). Telomeres shorten more slowly in long-lived birds and mammals than in short-lived ones. *Proceedings of the Royal Society B: Biological Sciences*, **270**, 1387–1392.

Henriques, C. M., Carneiro, M. C., Tenente, I. M., Jacinto, A., & Ferreira, M. G. (2013). Telomerase is required for zebrafish lifespan. *PLoS Genetics*, **9**(1), e1003214.

Javanbakht, H., Ihlow, F., Jablonski, D., Široký, P., Fritz, U., Rödder, D., Sharifi, M., & Mikulíček, P. (2017). Genetic diversity and quaternary range dynamics in Iranian and transcaucasian tortoises. *Biological Journal of the Linnean Society*, **121**, 627–640.

Lambert, M. R. K. (1982). Studies on the growth, structure and abundance of the Mediterranean spur-thighed tortoise, *Testudo graeca* in field populations. *Journal of Zoology*, **196**, 165–189.

Leary, R. F., Allendorf, F. W., & Knudsen, K. L. (1984). Superior developmental stability of heterozygotes at enzyme loci in salmonid fishes. *American Naturalist*, **124**, 540–551.

Lens, L., van Dongen, S., Kark, S., & Matthysen, E. (2002). Fluctuating asymmetry as an indicator of fitness: Can we bridge the gap between studies? *Biological Reviews of the Cambridge Philosophycal Society*, **77**, 27–38.

Lopez-Jurado, L. F., Talavera-Torralba, P. A., Ibañez-González, J. M., MacIvor, J. A., & García-Alcazar, A. (1979). Las tortugas terrestres *Testudo graeca* y *Testudo hermanni* in España. *Naturalia Hispanica*, **17**, 1–61.

Löwenborg, K., Shine, R., & Hagman, M. (2011). Fitness disadvantages to disrupted embryogenesis impose selection against suboptimal nest-site choice by female grass snakes, *Natrix natrix* (Colubridae). *Journal of Evolutionary Biology*, **24**, 177–183.

Lund, T. C., Glass, T. J., Tolar, J., & Blazar, B. R. (2009). Expression of telomerase and telomere length are unaffected by either age or limb regeneration in Danio rerio. *PLoS One*, **4**, e7688.

Mikulíček, P., Jandzik, D., Fritz, U., Schneider, C., & Široký, P. (2013). AFLP analysis shows high incongruence between genetic differentiation and morphology-based taxonomy in a widely distributed tortoise. *Biological Journal of the Linnean Society*, **108**, 151–160.

Monaghan, P. (2014). Organismal stress, telomeres and life histories. *Journal of Experimental Biology*, **217**, 57–66.

Monaghan, P., & Ozanne, S. E. (2018). Somatic growth and telomere dynamics in vertebrates: Relationships, mechanisms and consequences. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, **373**, 20160446.

Moreno, M. (2017). *Factores ambientales y genéticos que influyen en la presencia de anomalías morfológicas en Testudo graeca*. Trabajo Final de Grado. Facultad de Ciencias Experimentales. Universitat Miguel Herández.

Olsson, M., Geraghty, N. J., Wapstra, E., & Wilson, M. (2020). Telomere length varies substantially between blood cell types in a reptile: Telomere length in different blood cells. *Royal Society Open Science*, **7**, 192136.

Olsson, M., Pauliny, A., Wapstra, E., & Blomqvist, D. (2010). Proximate determinants of telomere length in sand lizards (*Lacerta agilis*). *Biology Letters*, **6**, 651–653.

Olsson, M., Wapstra, E., & Friesen, C. (2018a). Ectothermic telomeres: It's time they came in from the cold. *Philosophical Transactions of the Royal Society. Series B: Biological Sciences*, **373**, 20160449.

Olsson, M., Wapstra, E., & Friesen, C. (2021). Ectothermic telomeres: It's time they came in from the cold. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, **373**(1741), 20160449.

Olsson, M., Wapstra, E., & Friesen, C. R. (2018b). Evolutionary ecology of telomeres: A review. *Annals of the New York Academy of Sciences*, **1422**, 5–28.

Peet-Paré, C. A., & Blouin-Demers, G. (2012). Female eastern hog-nosed snakes (*Heterodon platirhinos*) choose nest sites that produce offspring with phenotypes likely to improve fitness. *Canadian Journal of Zoology*, **90**, 1215–1220.

Pieau, C. (1975). Temperature and sex differentiation in embryos of two chelonians, *Emys orbicularis L* and *Testudo graeca L*. In R. Reinboth (Ed.), *Intersexuality in the animal kingdom* (pp. 332–339). Springer.

Plot, V., Criscuolo, F., Zahn, S., & Georges, J. Y. (2012). Telomeres, age and reproduction in a long-lived reptile. *PLoS One*, **7**, 1–6.

R Core Team. (2022). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. https://www.R-project.org/

Raschenberger, J., Lamina, C., Haun, M., Kollerits, B., Coassin, S., Boes, E., Kedenko, L., Köttgen, A., & Kronenberg, F. (2016). Influence of DNA extraction methods on relative telomere length measurements and its impact on epidemiological studies. *Scientific Reports*, **6**, 1–10.

Reinke, B. A., Cayuela, H., Janzen, F. J., Lemaître, J., Gaillard, J., Lawing, A. M., Iverson, J. B., & Christiansen, D. G. (2022). Diverse aging rates in ectothermic tetrapods provide insights for the evolution of aging and longevity. *Science*, **1466**, 1459–1466.

Remot, F., Ronget, V., Froy, H., Rey, B., Gaillard, J.-M., Nussey, D. H., & Lemaître, J.-F. (2020). No sex differences in adult telomere length across vertebrates: A meta-analysis. *Royal Society Open Science*, **7**, 200548.

Rodríguez-Caro, R. C., Capdevila, P., Graciá, E., Barbosa, J. M., Giménez, A., & Salguero-Gómez, R. (2021). The limits of demographic buffering in coping with environmental variation. *Oikos*, **130**, 1346–1358.

Rodríguez-Caro, R. C., Graciá, E., Anadón, J. D., & Gimenez, A. (2013). Maintained effects of fire on individual growth and survival rates in a spur-thighed tortoise population. *European Journal of Wildlife Research*, **59**, 911–913.

Rodríguez-Caro, R. C., Graciá, E., Blomberg, S. P., Cayuela, H., Grace, M., Carmona, P. C., Pérez-Mendoza, H. A., Giménez, A., & Salguero-Gómez, R. (2023). Anthropogenic impacts on threatened species erode functional diversity in chelonians and crocodilians. *Nature Communications*, **14**, 1542.

Rodríguez-Caro, R. C., Graciá, E., dos Santos, R. M., Anadón, J. D., & Gimenez, A. (2015). One scute ring per year in *Testudo graeca*? A novel method to identify ring deposition patterns in tortoises. *Acta Herpetologica*, **10**, 77–84.

Rodríguez-Caro, R. C., Wiegand, T., White, E. R., Sanz-Aguilar, A., Giménez, A., Graciá, E., van Benthem, K. J., & Anadón, J. D. (2019). A low cost approach to estimate demographic rates using inverse modeling. *Biological Conservation*, **237**, 358–365.

Rollings, N., Friesen, C. R., Sudyka, J., Whittington, C., Giraudeau, M., Wilson, M., & Olsson, M. (2017). Telomere dynamics in a lizard with morph-specific reproductive investment and self-maintenance. *Ecology and Evolution*, **7**, 5163–5169.

Rollings, N., Friesen, C. R., Whittington, C. M., Johansson, R., Shine, R., & Olsson, M. (2019). Sex- and tissue-specific differences in telomere length in a reptile. *Ecology and Evolution*, **9**, 6211–6219.

Rollings, N., Uhrig, E. J., Krohmer, R. W., Waye, H. L., Mason, R. T., Olsson, M., Whittington, C. M., & Friesen, C. R. (2017). Age-related sex differences in body condition and telomere dynamics of red-sided garter snakes. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, **284**, 20162146.

Ruijter, J. M., Ramakers, C., Hoogaars, W. M. H., Karlen, Y., Bakker, O., van den Hoff, M. J. B., & Moorman, A. F. M. (2009). Amplification efficiency: Linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Research*, **37**, e45.

Sánchez-Montes, G., Martínez-Solano, Í., Díaz-Paniagua, C., Vilches, A., Ariño, A. H., & Gomez-Mestre, I. (2020). Telomere attrition with age in a wild amphibian population. *Biology Letters*, **16**(7), 20200168.

Sanz-Aguilar, A., Anadón, J. D., Giménez, A., Ballestar, R., Graciá, E., & Oro, D. (2011). Coexisting with fire: The case of the terrestrial tortoise *Testudo graeca* in mediterranean shrublands. *Biological Conservation*, **144**, 1040–1049.

Serén, N., Megía-Palma, R., Simčič, T., Krofel, M., Guarino, F. M., Pinho, C., Žagar, A., & Carretero, M. A. (2023). Functional responses in a lizard along a 3.5-km altitudinal gradient. *Journal of Biogeography*, **50**, 1–15.

Simide, R., Angelier, F., Gaillard, S., & Stier, A. (2016). Age and heat stress as determinants of telomere length in a long-lived fish, the Siberian sturgeon. *Physiological and Biochemical Zoology*, **89**(5), 441–447.

Sparks, A. M., Spurgin, L. G., van der Velde, M., Fairfield, E. A., Komdeur, J., Burke, T., Richardson, D. S., & Dugdale, H. L. (2022). Telomere heritability and parental age at conception effects in a wild avian population. *Molecular Ecology*, **31**, 6324–6338.

Tobler, M., Gómez-Blanco, D., Hegemann, A., Lapa, M., Neto, J. M., Tarka, M., Xiong, Y., & Hasselquist, D. (2022). Telomeres in ecology and evolution: A review and classification of hypotheses. *Molecular Ecology*, **31**, 5946–5965.

Tricola, G. M., Simons, M. J. P., Atema, E., Boughton, R. K., Brown, J. L., Dearborn, D. C., Divoky, G., Eimes, J. A., Huntington, C. E., Kitaysky, A. S., Juola, F. A., Lank, D. B., Litwa, H. P., Mulder, E. G. A., Nisbet, I. C. T., Okanoya, K., Safran, R. J., Schoech, S. J., Schreiber, E. A., … Haussmann, M. F. (2018). The rate of telomere loss is related to maximum lifespan in birds. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, **373**, 20160445.

Velo-Antón, G., Becker, C. G., & Cordero-Rivera, A. (2011). Turtle carapace anomalies: The roles of genetic diversity and environment. *PLoS One*, **6**, e18714.

Wilbourn, R. V., Moatt, J. P., Froy, H., Walling, C. A., Nussey, D. H., & Boonekamp, J. J. (2018). The relationship between telomere length and mortality risk in non-model vertebrate systems: A meta-analysis. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, **373**, 20160447.

Yang, M., Kong, X., Shi, W., Gong, L., Luo, H., & Wu, B. (2018). Remarkable sequence polymorphisms in 18S rDNA of Pleuronichthys cornutus (Pleuronectiformes: Pleuronectidae). *Gene*, **677**, 251–258.

Zimm, R., Bentley, B. P., Wyneken, J., & Moustakas-Verho, J. E. (2017). Environmental causation of turtle scute anomalies in ovo and in silico. *Integrative and Comparative Biology*, **57**, 1303–1311.

Zivkov, M., Ivanchev, I., Raikova-Petrova, G., & Trichkova, T. (2007). First data on the population structure, growth rate and ontogenetic allometry of the tortoise *Testudo hermanni* in eastern Stara Planina (Bulgaria). *Comptes Rendus de l'Académie Bulgare des Sciences*, **60**, 1015–1022.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Sampling sites of *Testudo graeca* in south-eastern Spain.

Table S1. Individual information used for the cross-sectional study.

Table S2. Individual information used for the longitudinal study.

Table S3. Variance testing and *t*-test analysis between captures.

Table S4. Statistical results from the ANOVA analysis to test the relationship between TL and sex, for the cross-sectional study.

Table S5. Statistical results from univariate linear regressions models and generalized linear models to describe the relationship between TL as dependent variable and age, *k*, individual body condition and the presence or absence of shell anomalies as independent variables.

Table S6. Statistical results from the linear model to assess the effect of capture interval above logarithm of TL variation between capture events (ΔlogT/S ratio).

Equation S1. Equation described on Raschenberger et al. (2015) for T/S ratio estimation above the single copy gen and GS.

Equation S2. Growth rate equations for *T. graeca* based on von Bertlanffy model (Rodríguez-Caro et al., 2013).