Telomere shortening rate predicts species life span

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Telomere shortening to a critical length can trigger aging and shorter life spans in mice and humans by a mechanism that involves induction of a persistent DNA damage response at chromosome ends and loss of cellular viability. However, whether telomere length is a universal determinant of species longevity is not known. To determine whether telomere shortening can be a single parameter to predict species longevities, here we measured in parallel the telomere length of a wide variety of species (birds and mammals) with very different life spans and body sizes, including mouse (*Mus musculus*), goat (*Capra hircus*), Audouin's gull (*Larus audouinii*), reindeer (*Rangifer tarandus*), griffon vulture (*Gyps fulvus*), bottlenose dolphin (*Tursiops truncatus*), American flamingo (*Phoenicopterus ruber*), and Sumatran elephant (*Elephas maximus sumatranus*). We found that the telomere shortening rate, but not the initial telomere length alone, is a powerful predictor of species life span. These results support the notion that critical telomere shortening and the consequent onset of telomeric DNA damage and cellular senescence are a general determinant of species life span.

telomere | life span | species

Humans have relatively short telomere lengths from 5 to 15 kb [1–3], and yet humans have much longer life spans than mice, which can start with telomere lengths around 50 kb [4, 5]. Previous studies have suggested that the telomere shortening rate rather than the initial telomere length is the critical variable that determines species life span [4, 6–10]. In particular, we have previously shown that human telomeres shorten at a rate of ~70 bp per y [1], which is in line with the rate published by other authors [3, 11–14], while mice telomeres shorten at a rate of 7,000 bp per y [4]. These different rates of telomere shortening between human and mice could explain the different longevities of mice and humans. However, the telomere shortening rate has been investigated to date in few species [4, 6–10, 15, 16], and using different techniques, which has prevented side-by-side comparisons of telomere shortening rates in phylogenetically distant species with different body sizes and life spans.

Here, to address whether telomere length and/or telomere shortening rates could explain species longevity, we measured telomere length in peripheral blood mononuclear cells from individuals of different species of birds and mammals at different ages in parallel, and calculated the telomere shortening rate per year in each species. A longitudinal study of telomere length was not considered here owing to the very different longevities of the species included in this study. Future studies warrant this type of analysis to understand telomere dynamics at the individual level. To measure telomere length, here we used a high-throughput quantitative fluorescence in situ hybridization (HT Q-FISH) technique, which allows to quantify individual telomere signals at a single-cell level [1] rendering data on both the average telomere length and individual telomere signals (*SI Appendix*, Table S1 and Fig. 1) [17, 18]. In particular, we measured, in parallel, the telomeres of laboratory mice (*Mus musculus*) (Fig. 1*A*), bottlenose dolphins (*Tursiops truncatus*) (Fig. 1*B*), goats (*Capra hircus)* (Fig. 1*C*), reindeer (*Rangifer tarandus*) (Fig. 1*D*), American flamingos (*Phoenicopterus ruber*) (Fig. 1*E*), griffon vultures (*Gyps fulvus*) (Fig. 1*F*), Audouin's gulls (*Larus audouinii*) (Fig. 1*G*), and Sumatran elephants (*Elephas maximus sumatranus*) (Fig. 1*H*). Laboratory mice were included as a control, as we had

previously shown a rate of telomere shortening of around 7,000 bp per y, which is 100-fold faster than that reported in humans [4]. The initial telomere length of the different species under study was estimated by linear regression (Fig. 1). Note that the initial telomere length value is only an estimate and telomere length dynamics may not be linear during the early stages of life [19]. First, we confirmed a very high rate of telomere shortening in our current mouse cohort of 6,420 bp per y (Fig. 1*A*), similar to that previously described by us [4]. Bottlenose dolphins showed a telomere shortening rate of 766 bp per y (*SI Appendix*, Table S1 and Fig. 1*B*) and an estimated initial telomere length around 90.7 kb (Fig. 1*B*). Goats showed a telomere shortening rate of 363 bp per y (Fig. 1*C*) and an estimated initial telomere length around 10.4 kb. Reindeer showed a telomere shortening rate of 531 bp per y (Fig. 1*D*) and an estimated initial telomere length of ~ 19.8 kb. American flamingos showed a telomere shortening rate of 105 bp per y (Fig. 1*E*) and an estimated initial telomere length around 21.0 kb. Griffon vultures had a telomere shortening rate of 209 bp per y (Fig. 1*F*) and an estimated initial telomere length around 19.8 kb. Audouin's gulls had a telomere shortening rate of 771 bp per y (Fig. 1*G*) and an estimated initial telomere length around 35 kb. Sumatran elephants have a telomere shortening rate of 109 bp per y (Fig. 1*H*) and an estimated initial telomere length around 36.3 kb. In the case of griffon vultures and Sumatran elephants, we were limited to the few available individuals at the Madrid Zoo; thus, in these cases the values obtained should be considered as a first approximation to the telomere shortening rates in these species.

We next investigated relationships between telomere length, telomere shortening rate, and species life span. For the species maximum life span, we used the AnAge database [20]. The average life spans were obtained from various sources (*SI Appendix*, Table S1). First, we did not find any correlation between the

Significance

The exact causes of aging are still not understood, and it is unclear why some species live less than 1 d, while others can live more than 400 y. Research suggests that telomeres are related to the aging process, but a clear relationship between the life span of a species and initial telomere length has not been observed. Here, we measure the telomere lengths of a variety of different species. We find that, in fact, there is no strong correlation between the life span of a species and initial telomere length. However, we find a strong correlation between the telomere shortening rate and the life span of a species.

**Fig. 1.** Telomere measurements for various species. The telomeres were measured by HT Q-FISH in individuals of different ages for (*A*) mice (*Mus musculus*), (*B*) bottlenose dolphins (*Tursiops truncatus*), (*C*) goats (*Capra hircus*), (*D*) reindeer (*Rangifer tarandus*), (*E*) American flamingos (*Phoenicopterus ruber*), (*F*) griffon vultures (*Gyps fulvus*), (*G*) Audouin's seagull (*Larus audouinii*), and (*H*) Sumatran elephants (*Elephas maximus sumatranus*). Each point represents the values for a different individual. The correlation coefficient (R^2), slope (rate of telomere shortening in kilobases per year), and *y* intercept (initial telomere length) are presented on the graphs.

estimated initial telomere length and species longevity (Fig. 2 *A–D*). In particular, a graph of the species maximum life span versus the estimated initial telomere length resulted in an R^2 value of 0.0190 with a linear regression curve (Fig. 2*A*), and an R^2 value of 0.0407 with a power law regression curve (Fig. 2*B*). A graph of the species' average life span versus the estimated initial telomere length resulted in an R^2 value of 0.125 with a linear regression curve (Fig. 2*C*), and an R^2 value of 0.145 with a power law regression curve (Fig. 2*D*). Note that there is even a trend for shorter life spans with longer initial telomere lengths with the low R^2 values just mentioned, and negative slopes in the regression line equations (Fig. 2 *A–D*). Also note that the inverse correlation between average life span and initial telomere length ($R^2 = 0.125$; Fig. 2*C*) was better than that between maximum life span and initial telomere length ($R^2 = 0.019$; Fig. 2*A*). These findings agree with a previous study that compared telomere length in more than 60 different species (21). Although the telomere shortening rate was not measured in that study, the authors concluded that the life span of a species could not be predicted from the initial telomere length and that there was a trend for short-lived species to have longer telomeres (21).

Interestingly, when we plotted maximum life span versus the rate of telomere shortening for the different species, we obtained a power law curve with an R^2 value of 0.829 (Fig. 2*E*). The equation from this curve can be used to predict the life span of a species when given the telomere shortening rate without using any information about the initial telomere length with an R^2 value of 0.782 (Fig. 2*F*). The same graphs can be made using the average life span instead of the maximum life span (Fig. 2 *G* and *H*), and in this case the power law curve R^2 value is 0.934. The observation that life span versus telomere shortening rate fits a power law curve is in agreement with many natural phenomena fitting either a power law or exponential curve such as population growth, temperature cooling/heating, city sizes, species extinction, body mass, individual incomes, and the number of connections to nodes in a scale-free network, among others (22–25).

Alternatively, more linear life span predictions can be made using both the initial telomere length and the rate of telomere shortening. In this case, it seems unlikely that species die when their telomeres are completely eroded since the life spans predicted by complete telomere erosion are longer than the observed life spans for most species (*SI Appendix*, Table S1). Instead, we find here that
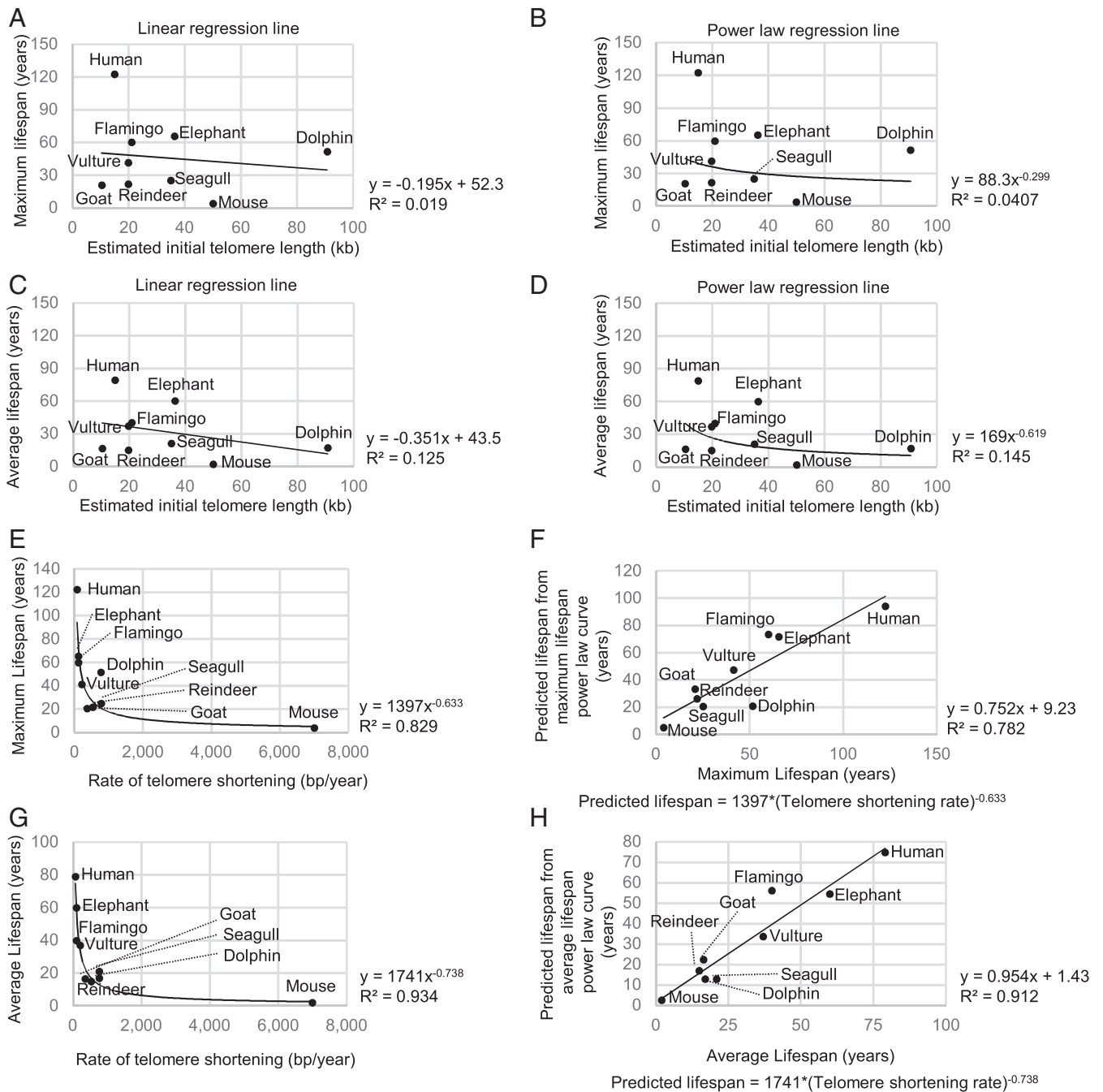
Fig. 2. Species life span predictions with telomere parameters I. (*A*) Maximum life span vs. estimated initial telomere length fit with a linear regression line. (*B*) Maximum life span vs. estimated initial telomere length fit with a power law regression line. (*C*) Average life span vs. estimated initial telomere length fit with a linear regression line. (*D*) Average life span vs. estimated initial telomere length fit with a power law regression line. (*E*) Maximum life span vs. rate of telomere shortening. (*F*) The predicted life span vs. the maximum life span. The predicted life span is calculated by using the telomere shortening rate in the power law regression equation from *E*. (*G*) Average life span vs. rate of telomere shortening. (*H*) The predicted life span vs. the average life span. The predicted life span is calculated by using the telomere shortening rate in the power law regression equation from *G*.

the length of the telomeres when species die at the age of the maximum life span appears to be ∼50% of the original telomere length for that particular species, when considering the average of all of the species measured (*SI Appendix*, Table S2). Interestingly, when considering the timepoint of the average life span, the telomere length appears to be ∼75% of the original length (*SI Appendix*, Table S2). Therefore, we can calculate the life span of a species if we assume that telomeres shorten with a constant linear rate and that the time of death will occur once the telomeres have

shortened to 50% or 75% of the original telomere length. The equation of the estimated life span if telomeres shortened to 50% of the original length is as follows: ((Initial telomere length) − (Initial telomere length) × 0.5)/Telomere shortening rate. A plot of the estimated life span at 50% original telomere length vs. the maximum life span yields an R^2 of 0.565 (Fig. 3*A*). The estimated life span at 50% original telomere length vs. the average life span yields an R^2 of 0.694 (Fig. 3*B*). Similar graphs are presented for 75% original telomere length (Fig. 3 *C* and *D*). With this dataset, the
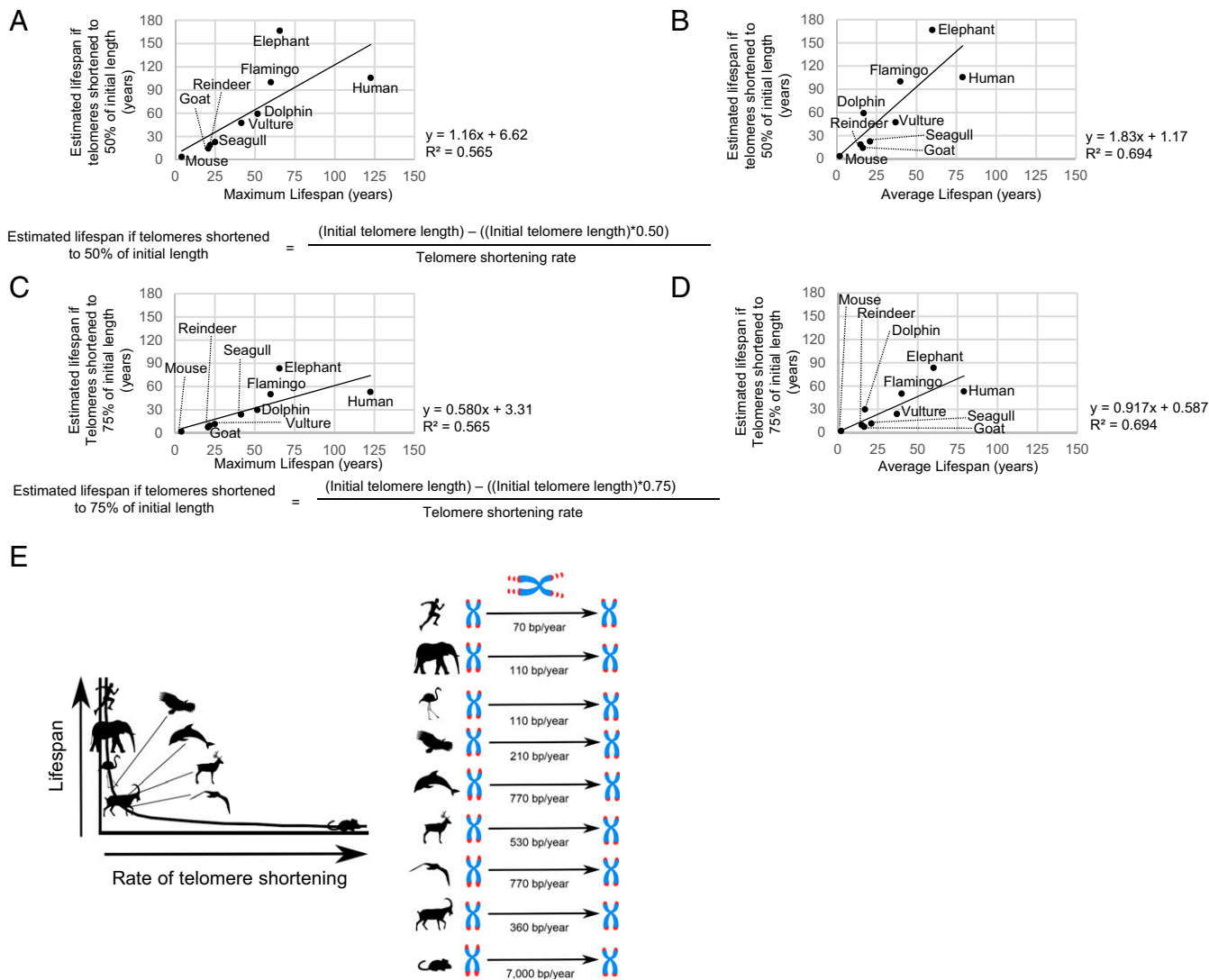
graph with 75% original telomere length vs. the average life span provides the most accurate results with an R^2 of 0.694. Although the R^2 value is the same as the value in Fig. 3B, this graph also has a slope that is closer to a value of 1, indicating a smaller shift between the actual and estimated life spans. Note that better correlation coefficients are obtained with the power law regression curves using the telomere shortening rate without taking the initial telomere length into consideration (Fig. 2 E–H).

Another trait that correlates with life span is body mass (26). In general, larger species such as elephants and whales have longer life spans than small species such as mice and rabbits. One investigation compared the mass and life span of 1,456 different species, and found a trend for longer life span with larger mass ($R^2 = 0.397$) (26). With the species in our dataset, we also observed a correlation between mass and life span (*SI Appendix*, Table S3). The species telomere shortening rate also correlated with body weight with an R^2 of 0.413 (*SI Appendix*, Fig. S1). Species with

higher body weights tend to have lower telomere shortening rates and longer life spans.

Some authors have shown an inverse correlation between life span and heart rate, a variable related to organismal metabolism (27, 28), although more extensive studies do not seem to support this notion (29). Here, we set to address a potential correlation between heart rate and telomere length. First, we observed a correlation between life span and heart rate with our dataset (*SI Appendix*, Table S3). We also found a linear correlation between the telomere shortening rate and the heart rate with an R^2 of 0.974 (*SI Appendix*, Fig. S2 *A* and *B*).

Next, to investigate the effect of the multiple variables on life span when combined into the same model, we performed a multivariate linear regression. The input variables of telomere shortening rate, initial telomere length, body mass, and heart rate were fit to either the average life span or the maximum life span. The data used for the regression are presented in *SI Appendix*, Table S4. The log value of all of the data points was used for the regression

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$$\text{Estimated lifespan if telomeres shortened to 50\% of initial length} = \frac{(\text{Initial telomere length}) - ((\text{Initial telomere length})*0.50)}{\text{Telomere shortening rate}}$$

$$\text{Estimated lifespan if telomeres shortened to 75\% of initial length} = \frac{(\text{Initial telomere length}) - ((\text{Initial telomere length})*0.75)}{\text{Telomere shortening rate}}$$

Fig. 3. Species life span predictions with telomere parameters II. (*A*) The estimated life span if telomeres shortened to 50% of the original length vs. the maximum life span. (*B*) The estimated life span if telomeres shortened to 50% of the original length vs. the average life span. (*C*) The estimated life span if telomeres shortened to 75% of the original length vs. the maximum life span. (*D*) The estimated life span if telomeres shortened to 75% of the original length vs. the average life span. The estimated life span is calculated using the following equation: ("Telomere length original" − "Telomere length original" × "percent of original length")/"Telomere shortening rate." (*E*) Graphical illustration which shows the main finding from this paper, which is that faster telomere shortening rates result in shorter species life spans.

instead of the original values. Each variable vs. the average life span or maximum life span had either a higher linear R^2 correlation coefficient when using log-transformed data, or there was no noticeable change in the correlation coefficient in the case of the initial telomere length variable. The model fit to the average life span resulted in an R^2 value of 0.997 and an adjusted R^2 value of 0.992 (*SI Appendix*, Table S5), demonstrating that these variables can predict the average life span. The *P* values (listed in the Pr(>|t|) column) were statistically significant for all variables. The telomere shortening rate was the most statistically significant variable (*P* = 0.000422). The model fit to the maximum life span resulted in an R^2 value of 0.950 and an adjusted R^2 value of 0.884 (*SI Appendix*, Table S6), demonstrating that the variables can also predict the maximum life span. In this case, only the telomere shortening rate variable was statistically significant (*P* = 0.0218). Again, we found an inverse relation between average life span and initial telomere length with a *P* value of *P* = 0.0302, with short-lived species having initial longer telomeres (*SI Appendix*, Table S5). Also, in the multivariate analysis, the relationship between the initial telomere length and the maximum life span was not significant in agreement with a weaker inverse correlation between initial telomere length and maximum life span compared with average life span (Fig. 2 *A* and *C*). Thus, these findings confirm that the telomere shortening rate (negative correlation), initial telomere length (negative correlation), body weight (positive correlation), and heart rate (negative correlation) can predict species life span and that, among these variables, the variable with the greatest power to predict the life span is the telomere shortening rate.

Finally, one caveat of studies with animals of different ages is that an effect can occur in which old animals with short telomeres selectively disappear due to death, and these telomeres are consequently not measured at older ages. Therefore, the telomere length could be artificially high at the older ages since only the animals with longer telomeres continue to survive at these ages. However, the fact that telomere shortening with age fits a linear regression in the majority of the species studied indicated that this phenomenon is not very distorting in our current study. Also, such disappearance of animals would only be expected to occur at very late ages, and the majority of the animals in this study were not extremely old (*Methods*).

Conclusions

Although a number of previous studies measured telomere length in different species (30–35), few of them determined the telomere shortening rates (4, 6–10, 15, 16). In this regard, some studies found a correlation between telomere shortening rates and species' life spans, including previous work from our group in mice and humans (1, 4, 6–10); however, these studies did not compare side-by-side telomere shortening rates in phylogenetically distant species by using a single technique to measure telomeres.

In our current study, telomere length and the rate of telomere shortening from multiple species with very different life spans, including birds and mammals, was acquired in the same laboratory by using the sensitive HT Q-FISH technique which allows to determine absolute telomere length values in units of base pairs as well as individual telomere signals. A limitation of the current study is, however, the few available individuals for some species.

The results shown here indicate that the telomere shortening rate of a species can be used to predict the life span of that species, at least with the current dataset (Fig. 3*E*). We observed that mean telomere length at birth does not correlate with species life span since many short-lived species had very long telomeres, and long-lived species had very short telomeres. Future studies warrant determination of telomere shortening rate in species such as the naked-mole rat or the bat, which do not match their predicted life span well according to their body size (26, 36).

Finally, the fact that the rate of telomere shortening can be used to predict life span suggests that the cellular effects induced by short telomeres, such as cellular senescence, may be the critical factor determining species longevity. In this regard, some studies correlate DNA repair ability to species longevity (37–39). In particular, the ability to repair UV-induced damage positively correlates with life span in different species, including primates (37, 38). Also, DNA repair rates are higher in longer living rodent species compared with rodent species with a shorter life span (39). It is interesting to note that short telomeres induce DNA damage, and in turn certain types of DNA damage, such as UV irradiation or oxidative stress, can also lead to telomere shortening (40–42).

Methods

Mice. The mouse strain was >95% C57BL/6 background. All mice were produced and housed in the specific-pathogen–free barrier of the Centro Nacional de Investigaciones Oncológicas (CNIO) institution in Madrid, Spain. After weaning, 5 mice were housed per cage and fed ad libitum with a nonpurified sterilizable Teklad 2018 18% protein rodent diet (Harlan; TD.2018S). All animal procedures were approved by the CNIO–Instituto de Salud Carlos III Ethics Committee for Research and Animal Welfare and conducted in accordance to the recommendations of the Federation of European Laboratory Animal Science Associations.

Blood Samples. Blood samples were obtained from the Madrid Zoo, with the exception of the mouse samples, which were obtained from the animal facility of CNIO, and the Audouin's gulls. Only 1 timepoint was measured for each individual, so this is a cross-sectional study. For mice (*Mus musculus*), blood was sampled from 7 individuals with an age range from 1.4 to 2.6 y. For dolphins (*Tursiops truncatus*), blood was sampled from 9 individuals with an age range from 8.6 to 50.1 y. For goats (*Capra hircus*), blood was sampled from 15 individuals with an age range from 0.85 to 10.1 y. For reindeer (*Rangifer tarandus*), blood was sampled from 8 individuals with an age range from 1.44 to 10.5 y. For American flamingos (*Phoenicopterus ruber*), blood was sampled from 17 individuals with an age range from 0.79 to 38.8 y. For the griffon vulture species (*Gyps fulvus*), blood was sampled from 6 individuals with an age range from 8.06 to 21.4 y. For the Sumatran elephant species (*Elephas maximus sumatranus*), blood was sampled from 4 individuals with an age range from 6.14 to 24.7 y. The Audouin's gulls were sampled at the breeding colony of the Ebro Delta (northeast Spain). For this Audouin's gull species, blood was sampled from 21 individuals (ranging from a few months old to 21.9 y old) were selected after determining age from polyvinyl chloride ring tags. The blood samples were processed with erythrocyte lysis buffer (Qiagen; catalog no. 79217) according to the manufacturer's protocol. Therefore, for all species, the telomeres were measured in the leukocyte cells. The samples were then frozen at −80 °C slowly in a Nalgene Cryo Freezing Container (Nalgene; catalog no. 5100-0001).

HT Q-FISH. The process for HT Q-FISH has been described previously (1). Briefly, frozen erythrocyte lysis buffer processed blood samples were first thawed quickly and resuspended in complete RPMI media. Cells were attached to the wells (30,000 to 150,000 cells/well) of clear-bottom black-walled 96-well plates (Greiner Bio-One, Inc.; catalog no. 655087), which had been precoated with a 0.001% (wt/vol) poly-L-lysine solution (Sigma; P8920-100 mL) for 30 min at 37 °C. The wells on the outer edge of the plate were not used. The cells were incubated for 37 °C for no more than 4 h before fixation. The cells were fixed by adding 200 μL of fixative solution (3:1 [vol/vol] methanol/acetic acid) slowly to the cells in a chemical hood and incubated for 10 to 15 min. The solution was removed, and this was repeated 3 more times. The plate was then fixed overnight at −20 °C with the fixative solution in the wells.

The fixative solution was then removed, and the plate was dried on a hot plate at 37 °C 1 h in a chemical hood. The wells were rehydrated with 200 μL of PBS. The cells were fixed with 200 μL of 4% formaldehyde in PBS for 2 min at room temperature (RT). The plate was washed 3 × 5 min with PBS. The cell walls were degraded with prewarmed pepsin solution (100 mL of H_2O, 100 μL of 37% HCl [10.1 M HCl], and 100 mg of pepsin [Sigma-Aldrich; catalog no. P7000-25G]) for 15 min at 37 °C. The plate was washed 2 × 5 min with 200 μL of PBS, and then dehydrated with a series of 5-min 70%, 90%, and 100% ethanol washes. The plate was dried 1 h at 37 °C or overnight at RT.

Next, 50 μL of the hybridization solution containing the Tel-Cy3 PNA probe was added to the plate (95 μL of 1 M Tris, pH 7.0, 812 μL of $MgCl_2$ solution [25 mM $MgCl_2$, 9 mM citric acid, 82 mM Na_2HPO_4], 6.65 mL of deionized formamide, 475 μL of blocking reagent [10 g of blocking reagent (Boehringer; catalog no. 1093 657) dissolved with heat in 100 mL of maleic acid buffer, pH 7.5 (100 mM maleic acid, 150 mM NaCl)], 1.28 mL of H_2O, and 190 μL of Tel-Cy3 PNA probe solution [5 μg lyophilized Cy3-labeled (CCCTAA) 3 probe (Applied Biosystems) diluted in 200 μL of H_2O]). The DNA was denatured by heating the plate on a hot plate at 85 °C for 5 min. Then the plate was incubated 2 h at RT in the dark, washed with a plate shaker for 15 min with a solution containing 1 mL of 1 M Tris, pH 7, 1 mL of 10% BSA, 28 mL of H_2O, and 70 mL of formamide, and then washed 2 × 5 min with a

plate shaker with TBST (TBS [Tris-buffered saline, pH 7.0] with 0.08% Tween 20). Next, the plate was washed 1 × 5 min with a plate shaker with TBST containing 1 μg/mL DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride; Life Technologies; catalog no. D-1306) to stain the nuclei. Then the plate was washed 1 × 5 min PBS and 50 μL of Mowiol solution (10 g of Mowiol [polyvinyl alcohol; Calbiochem; catalog no. 475904], 25 mL of 85% glycerol, 25 mL of H_2O, 12 mL of 0.2 M Tris HCl, pH 8.5, and 2.5% [wt/vol] DABCO [1,4-diaza[2.2.2]bicyclooctane; Sigma-Aldrich; catalog no. D27802-25G]) was added. Plates were then sealed with aluminum foil lids (Beckman Coulter; catalog no. 538619) and stored at 4 °C in the dark. The plates were then processed by HT microscopy, as described in *HT Microscopy*, within 48 h.

HT Microscopy. Images were acquired on an Opera High Content Screening System (PerkinElmer) equipped with a UV lamp, 561-nm laser, and a 40×/0.9 N.A. water-immersion objective. Images were analyzed with Acapella Image analysis software (PerkinElmer). Data were analyzed with Microsoft Excel (Microsoft). Telomere fluorescence values were converted into kilobases by external calibration with the CCRF-CEM (7.5 kb), L5178Y-S (10.2 kb), and L5178Y-R (79.7 kb) cell lines (43, 44).

Abundance of Very Old Individuals in Different Species. We defined very old as the age above the value of 70% of the maximum life span for each species. For humans, this would correspond to an age of 122.5 × 0.7 = 73.5 y old. In our study, the number of old individuals (age greater than 70% of the maximum life span) sampled for each species is as follows: 0/7 (0%) for mice, 3/8 (37.5%) for dolphin, 0/15 (0%) for goat, 0/8 (0%) for reindeer, 0/16 (0%) for American flamingo, 0/6 (0%) for griffon vulture, 3/21 (14.3%) for Audouin's seagull, and 0/4 (0%) for Sumatran elephant.

Data Analysis. Graphs were created and data analysis was performed in Microsoft Excel. Multivariate linear regression was performed in the R statistics software (45).

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