Decline in telomere length with increasing age across nonhuman vertebrates

Decline in telomere length with increasing age across non-human vertebrates: a meta-analysis

Short title: Telomere attrition across non-human vertebrates

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Abstract:

The prediction that telomere length (TL) shortens with increasing age is a major element in considering the role of telomeres as a key player in evolution. While telomere attrition is found in humans both *in vitro* and *in vivo*, the increasing number of studies reporting diverse age-specific patterns of TL challenges the hypothesis of a universal decline of TL with increasing age. Here we performed a meta-analysis to estimate the relationship between TL and age across 175 estimates encompassing 98 species of vertebrates. We found that, on average, TL does decline with increasing age during adulthood. However, this decline was weak and variable across vertebrate classes, and we also found evidence for a publication bias that might weaken our current evidence of decreasing TL with increasing age. We found no evidence for a faster decline in TL with increasing age when considering the juvenile stage (from birth to age at first reproduction) compared to the adult stage. Heterogeneity in TL ageing rates was explained by the method used to measure telomeres: detectable TL declines with increasing age were found only among studies using TRF with in-gel hybridisation and qFISH methods, but not in studies using qPCR and Southern blot-based TRF methods. While we confirmed that TL declines with increasing age in most adult vertebrates, our results identify an influence of telomere measurement methodology, which highlights the need to examine more thoroughly the effect of the method of measurement on TL estimates.

Keywords: Ageing, Telomere attrition, Life-history traits, Systematic review, Telomere Restriction Fragment, qPCR

Introduction:

Telomeres are composed of non-coding DNA sequences repeated in tandem (TTAGGG in most animals) at the extremity of chromosomes in eukaryotes (Blackburn, 1991). Telomeres prevent chromosomal fusions during mitosis and play an essential role in chromosomal segregation during cell division (Aubert & Lansdorp, 2008). However, each time a cell replicates, DNA polymerase is unable to fully replicate the 5'-3' strand (the so-called "end-replication problem") leading to a loss of nucleotide sequences (Levy et al., 1992). This telomere attrition has attracted a lot of attention because short telomeres can be responsible for chromosomal fusions, and ultimately lead to replicative cell senescence and apoptosis. Although telomere length (TL) declines with each mitotic cell cycle due to the end-replication

problem, oxidative damage on telomeric DNA is another mechanism involved in accelerated telomere attrition (von Zglinicki et al., 2001; Reichert & Stier, 2017). Accumulation of oxidative damage, known as oxidative stress, is favoured when the generation rate of reactive oxygen species (ROS) exceeds the antioxidant machinery. ROS are highly reactive and cause damage to nucleic acids, proteins and lipids. Because of their guanine rich structure, telomeres are highly sensitive to oxidative damage (Kawanishi & Oikawa, 2006). Telomere erosion patterns can be modulated by the activity of telomerase. This enzyme is a reverse transcriptase that adds telomeric repeats and can potentially compensate telomere shortening (Sherratt et al., 2004). In mammals, the activity of telomerase in most somatic cells is negatively linked to the species body mass and is mostly expressed (at least in fibroblasts) in species weighing less than 2kg (Gomes et al., 2011) and in very proliferative tissue (*e.g.* antlers in cervids; Li, 2012). In birds, high telomerase activity was found in bone marrow in two long-lived species but not in two short-lived species (Haussmann et al., 2007). Telomerase activity was found in somatic tissue in the limited number of fish, reptiles and amphibians studied to date (Gomes et al., 2010), which suggests different telomere dynamics compared to birds and mammals. However, available data remain scarce and little is known about telomerase activity in vertebrates and the effect of this activity on age-related telomere dynamics.

Early research on telomere dynamics in humans found that TL consistently declines with increasing age in cultured fibroblasts (Harley et al., 1990) and the research that has been done on human's telomere dynamics since (Aubert et al., 2012; Lapham et al., 2015) has led to the same conclusion. The shortening of telomere repeats is now considered as a marker of ageing (López-Otín et al., 2013) and telomere length is known to predict subsequent morbidity (Herrmann et al., 2018). In addition, shorter telomeres are linked to an increased mortality risk in both humans and non-human vertebrates (Wang et al., 2018; Wilbourn et al., 2018). Telomere length and dynamics have also been linked to growth (Monaghan & Ozanne, 2018) and reproduction (Sudyka 2019), conferring them a possible key role in mediating life-history trade-offs. Since the early 2000s, we have witnessed a burst of studies measuring TL in laboratory animals and wild populations (Monaghan et al., 2018). Similarly to the patterns observed in humans, most of these studies reported a decline in TL with increasing age (*e.g.* Pauliny et al., 2006 in two species of birds, the dunlin (*Calidris alpina*) and the sand martin (*Riparia riparia*), Beirne et al. 2014, in the badger (*Meles meles*)). However, other studies reported no decline (*e.g.* Horn et al., 2011 in the kakapo, *Strigops habroptilus*; Lewin et al., 2015 in the spotted hyena, *Crocuta crocuta*) or even an increase in TL with age (Hoelzl et al.,

2016 in the edible dormouse, *Glis glis*; Ujvari & Madsen, 2009 in the water python, *Liasis fuscus*). Patterns of telomere attrition also vary within species, which suggest that environmental conditions might to some extent also modulate telomere dynamics (Salmón et al., 2016; Wilbourn et al., 2017). Various patterns (*i.e.* an increase, a decrease or no relationship of TL with age) that has been reported across all classes of vertebrates calls into questions the hypothesis that TL is a universal marker of ageing. Here, we use meta-analyses to test whether or not the decline in TL with increasing age constitutes a general pattern in vertebrates. We also aim to identify factors that could modulate the strength of the decline in TL with increasing age, and determine whether these are ecological, biological or methodological.

One factor that might explain variation in the relationships reported between TL and age among vertebrates is phylogeny. Indeed, telomerase is differentially expressed throughout life across taxa and across species within taxa (Gomes et al., 2010; Olsson et al., 2018). Besides telomerase expression, fish, reptiles and amphibians are indeterminate growers (in contrast with birds and mammals), which might influence the relationship with age. Indeed, somatic growth is linked to an increase in cell division and oxidative stress that will impair TL (Monaghan & Ozanne, 2018). It is then expected that organisms that grow throughout their life should display different age-specific patterns of TL than determinate growers.

Related to phylogeny, the decline in TL with increasing age might vary with the species-specific life history. So far, studies have highlighted that telomere attrition rate negatively correlates with maximum lifespan in birds (Dantzer & Fletcher, 2015; Tricola et al., 2018) and more generally that telomere attrition rate is linked to the species position along the slow-fast continuum of life histories (Dantzer & Fletcher, 2015), with fast-living species (*i.e.* short-lived species that start to reproduce early and produce several offspring per year) displaying steeper rates of telomere attrition than slow-living species (*i.e.* long-lived species that start to reproduce late and produce one offspring or less per year). The difference in telomere attrition rate between short- and long-lived species might be due to a difference in telomerase expression between those species or a lower level of oxidative stress in long-lived species than in short-lived ones (Tricola et al., 2018; Vágási et al., 2019). However, this relationship has not yet been investigated in non-avian taxa, due to an evident lack of studies available. Besides the slow-fast continuum, we should observe different patterns of telomere dynamics, at least in mammals, in different-sized species because the activity of telomerase is higher in mammal species weighing less than 1kg (Gomes et al., 2011).

In addition, because TL shortens with each mitotic cycle and cellular division may occur at a faster rate during the growth period, this may lead to a steeper decline in TL during the juvenile stage than during the adult stage (Monaghan & Ozanne, 2018). Substantial telomere loss has been found during the embryonic and the nestling stages in three species of birds (Boonekamp et al., 2014; Stier et al., 2020; Vedder et al., 2017) compared to adults. In addition, greater telomere shortening throughout the juvenile stage compared to the adult stage has been observed in some bird species (Hall et al., 2004; Pauliny et al., 2006) as well as in mammals (Aubert et al., 2012; Fairlie et al., 2016; Frenck et al., 1998; Seeker et al., 2018). A human-based mathematical model further suggests that the rapid telomere loss during the juvenile stage (a four-fold difference between juvenile and adult stages, (Zeichner et al., 1999)) could be explained by a higher turnover rate of peripheral mononuclear cells in children than in adults (Sidorov et al., 2004). Hence, depending on the life stage analysed, the relationship between TL and age may not be consistent.

The type of data collected might also influence the reported patterns. Indeed, because short telomeres might be associated with a higher mortality risk (e.g. Bichet et al., 2020; Bize et al., 2009; Wilbourn et al., 2018), the selective disappearance of individuals carrying short telomeres could lead to underestimation of the age-specific decline in TL with increasing age (see also Nussey et al., 2008 for a broader discussion of the importance of considering selective disappearance in the context of senescence). Therefore, the analysis of age-specific TL data collected from longitudinal studies, where known-aged individuals are regularly sampled throughout their life, will provide a much more accurate pattern of the changes in TL with increasing age.

Finally, the method used to measure TL is known to be a source of heterogeneity in TL that potentially influences age-specific patterns. Currently, three methods are generally employed (Nussey et al., 2014), which provide absolute or relative measures. The first technique used is the telomere restriction fragment method (TRF), which involves the use of restriction enzyme to digest the genomic DNA without cutting within the telomeric fragment. Telomeric fragments are then separated by gel electrophoresis and probed with telomeric sequences providing an absolute measure of the mean TL in a sample of cells (Harley et al., 1990). Two different types of TRF have been developed: TRF with in-gel hybridization (hereafter TRFI) and TRF followed by a southern blot (hereafter TRFS). In TRFI the probe binds to the single-stranded overhang of the telomere and therefore only telomeres at chromosome ends are measured. In contrast, DNA is denatured in TRFS and the probe binds

not only to terminal telomeres but also to interstitial telomeric sequences, which are telomere repeats located all along chromosomes, that can vary in length both between and within species (Foote et al., 2013). The second technique is the quantitative real time PCR method (qPCR) and gives a relative measure of TL by amplifying telomeric fragments and a non-telomeric reference gene that does not vary in copy number (Cawthon, 2002). As for TRFS, qPCR amplifies both telomeres and interstitial telomeric sequences. Finally, the quantitative fluorescent in situ hybridisation (qFISH) technique uses a specific fluorescent-conjugated telomere probe to quantify average TL from tissues or isolated cells (Lansdorp et al., 1996). It is important to mention that methodologies are not equally distributed across the phylogeny. Indeed, while qFISH methodology is barely used in ecology, TRFI is mostly used in birds. In a previous meta-analysis looking at sex differences in TL in humans (Gardner et al., 2014), TL was found to be longer in women only in studies using the TRF (TRFS and TRFI pooled) method. In a meta-analysis linking TL and mortality in vertebrates (Wilbourn et al., 2018), the negative relationship between TL and mortality was stronger in studies using qPCR.

We performed a phylogenetically-controlled meta-analysis to examine the relationship between TL and age in non-human vertebrate species. We predicted that telomere dynamics with age should vary depending on the class of vertebrates due to differences in telomerase expression. More precisely, we expected a stronger decline in species where telomerase expression seems to be repressed (e.g. large mammals and short-lived birds). We also predicted that TL declines on average with increasing age across vertebrates but with a steeper decline during the juvenile than the adult stage. Based on both theoretical aspects and previous empirical studies, we also predicted that the decline in TL should be more pronounced in short-lived species, since the TL decline is fastest in short-lived species of birds. We also predicted that the decline in TL with increasing age is more likely to be recorded in longitudinal studies due to selective disappearance in transversal studies. All analyses were corrected for the method used to measure telomeres and associated heterogeneity was calculated to assess whether the method used influenced the detected association between telomere length and age.

Material and methods:

1) Literature search

The literature search was performed using the ISI Web of Science Database in December 2019 and updated in May 2021. Key words used in the Topic window for the search were

186 telom* not (clinic OR hospital). The keyword telom* was used instead of telomere to ensure
187 studies on telomerase were included in the results. By using "not (Clinic OR hospital)" we also
188 avoided a lot of articles from medical literature. We restricted our search to the following
189 categories: "*Evolutionary biology*", "*Marine Freshwater Biology*", "*Multidisciplinary*
190 *sciences*", "*Geriatrics gerontology*", "*Physiology*", "*Zoology*", "*Environmental sciences*",
191 "*Fisheries*", "*Ecology*", "*Agriculture dairy animal sciences*", "*Veterinary sciences*". We
192 limited our search to articles providing TL estimates for known age individuals of vertebrate
193 species. Since the main goal of our analysis focused on the non-manipulated decline of TL with
194 increasing age we excluded studies of laboratory strains of mouse and rat as well as
195 experimental studies (*e.g.* pathogen infection, diet experiment etc.). We found very few studies
196 (i.e. 4 studies only in birds) that measured TL at different time point during the first month of
197 life. Hence, we decided to include only studies that covered more than the first month of life in
198 the juvenile stage to avoid this very limited number of studies that focus solely on the first
199 month of life due to a manifest lack of data. When data were not available within the main text
200 or supplementary materials, we requested the dataset directly from the authors. Thirty-two (out
201 of 43) authors kindly provided their dataset. At the end of the literature survey, we ended up
202 with 87 articles (See **Figure 1**).

203

204 2) Effect size calculation

205 To assess the relationship between TL and age, we calculated Fisher's Z transformation of
206 the correlation coefficient using the following procedure. We first fitted either a linear model
207 between TL and age for transversal studies or a linear mixed-effects model (package *lme4*
208 (Bates et al., 2020)) with individual identity as a random factor for longitudinal studies. We
209 then computed the correlation coefficient:

210 $$r = \frac{t * \left(1 + \frac{Ni}{No} * R\right) * \sqrt{1 - R}}{\sqrt{t^2 * \left(1 + \frac{Ni}{No} * R\right)^2 * (1 - R) + No - k}}$$

211 with t being the t-value of the linear regression, Ni being the number of individuals, No the
212 number of observations, k the number of parameters including the intercept and R the intra-
213 class correlation (Nakagawa & Cuthill, 2007). In the case of transversal studies (i.e. studies
214 without repeated measurements), the formula become:

$$r = \frac{t}{\sqrt{t^2 + df}}$$

because in this case R = 0, Ni/No = 1 and the number of degree of freedom df is No – k.

We finally transformed the correlation coefficient to Fisher's Z:

$$Zr = \frac{1}{2}\log(\frac{1+r}{1-r})$$

And we calculated the sampling variance associated:

$$Vz = \frac{1}{Ni - 3}$$

Zr was used over the correlation coefficient because Zr is normally distributed and thus not bounded between –1 and 1 unlike the correlation coefficient. A negative effect means a decrease of TL with increasing age, whereas a positive effect means an increase of TL with age. Following the Cohen's rule of thumb (Cohen, 1977), absolute values of Zr of 0.25, 0.5 and 0.75 represent low, moderate and strong relationship, respectively

Fisher' z was calculated separately in juveniles and adults (*i.e.* before and after the species-specific age at first reproduction, defined as the earliest age at which females give birth). We also calculated Zr on data combining juveniles and adults ('all age-classes' dataset), which leads to three distinct datasets.

3) Moderators included

To investigate the various factors that could explain the direction and the magnitude of the relationship between TL and age, we included the following moderators as covariates in the meta-analytic models:

- The age at first reproduction (hereafter AFR) of the species was used as a proxy of the slow-fast continuum of life histories (Gaillard et al., 2005).
- The mean body mass of adult females for each species was included since body mass is negatively correlated with telomerase expression in mammals (Gomes et al., 2011).
- The method of telomere measurement was included as a four-level factor: TRFI, TRFS, qPCR and FISH.
- The type of study was included as a two-level factor ("Transversal" vs. "Longitudinal").

- The ratio of the age range covered by the data (hereafter lifespan coverage) corresponds to the age range covered by the data divided by the maximum lifespan of the species. With a wider lifespan coverage, we expected a more accurate estimate and so a higher probability to detect a change in TL with age. However, this moderator was not tested on the juvenile dataset because the age range was inevitably too low in this specific case.

All species information (*i.e.* AFR, mean adult female body mass and maximum lifespan) was retrieved in the literature (**See Supplementary material**).

4) Meta-analysis

The meta-analysis was performed using R (version 4.0.2) (R Core Development Team, 2020). Phylogenetically-corrected mixed effect models were run using the package *metafor* (Viechtbauer, 2010). To assess phylogenetic relatedness, since there is no single pan-vertebrate phylogeny, a phylogenetic tree was built using the website http://www.timetree.org/ (Kumar et al., 2017). A correlation matrix of phylogenetic relatedness among the species was then extracted from the tree.

Random-effects meta-analytic models were performed with the effect sizes entered as the dependent variable along with the sampling variance associated with each effect size included in the model. The phylogeny (using the phylogenetic distance matrix), the population and the species independently of the phylogeny were included as random factors. The same model was used for each age class (juvenile, adult and 'all age classes' datasets) (See **Table 1** for a summary of the data analysed). To quantify heterogeneity in our data accounting for random factors, we estimated I^2 for each random factor (i.e. the percentage of the total variance explained by each random factor) as well as I^2 total (i.e. the percentage of the total variance explained by the between-study variance) following Nakagawa & Santos (2012).

First the overall association between TL and age was quantified using the intercept model (i.e. null model). Next, we added the taxonomic class as a fixed effect to analyse the heterogeneity between vertebrate classes. Finally, to explain the variability in the association, we built a full model that included the moderators listed above as fixed effects for the dataset on adults and on 'all age classes'. For the dataset on juveniles, we tested the same moderators but we removed the lifespan coverage. For all models, AFR and body mass were log-transformed to improve normality. Since results from the 'all age classes' dataset were

qualitatively the same as results from the adult dataset, we decided, for the sake of clarity, to present only the results on juveniles and on adults in the main text. Results on the 'all age classes' dataset are therefore presented in **Supplementary** (**Table S1-S4 and Figure S1-S4**). To avoid the use of multiple correlated proxies of life-history (See **Supplementary, Table S11**), AFR and body mass were included separately in the full model. Since results did not change qualitatively whether we used AFR or body mass, we decided to present results using only the AFR. In addition, the use of phylogenetically-controlled mixed models allowed us to test the effect of different life history variables while accounting for the phylogenetic relatedness among species. The sex of individuals might be a source of heterogeneity in telomere dynamics as males and females of the same species might have a different relationship between TL and age (Barrett & Richardson, 2011; but see Remot et al., 2020). However, due to a high number of studies that did not report information regarding the sex of the individuals, we analysed the effect of sex separately and these results are presented in supplementary section (**See Supplementary, Table S5-S7**). For this complementary analysis, we built a new dataset by calculating Zr for each sex separately.

Models with every possible combination of fixed effect were then compared using the corrected Akaike Information Criterion (AICc) using the *MuMIn* package (Bartoń, 2019), selecting the model with the lowest AIC value. When models were within two AICc units, the simpler model was retained (Burnham & Anderson, 2002).

Orchard plots were used to visualize the results from both meta-analyses and meta-regressions (Nakagawa et al., 2019). The orchard plot displays the estimate of the model and its confidence interval. In addition, it also displays the prediction interval and the effect size of each study allowing us to visualize precisely the heterogeneity in the data. Orchard plots were drawn using the package *orchaRd* (Nakagawa et al., 2019).

5) Publication bias:

To test for possible publication bias, we used contour-enhanced funnel plots to represent the precision of each study (*i.e.* the inverse of the standard error) against the estimate of the study. When the data points are symmetrically distributed around the mean, this indicates an absence of publication bias. In addition to funnel plots, we performed an Egger's regression (Egger et al., 1997), which is a linear regression of the mean of each study against their precision. However, because means are not independent, we fitted the linear regression on the residuals of the meta-analysis (which are independent) against its precision. In absence of

publication bias, the intercept of the regression should not differ from zero. To confirm and to assess the influence of the publication bias, the trim and fill method was used (Viechtbauer, 2010). This method estimates the number of missing studies on one side of the funnel plot and provides an adjustment of the meta-analysis mean according to those missing studies.

Results:

1) Meta-analyses in juveniles and in adults

In our meta-analyses, the relationship between TL and age was weak and negative. The negative relationship was statistically significant in adults (estimate and 95% CI: -0.16, [-0.26:-0.06]) but not in juveniles (estimate and 95% CI: -0.13, [-0.50:0.24]). However, estimates in adults and in juveniles were not detectably different, as their confidence intervals overlapped; **Table 2, Figure 2 and Figure 3**).

2) Heterogeneity analysis

The total heterogeneity was high and similar for juveniles (I^2 total = 84.06%) and adults (I^2 total = 83.94%). In juveniles, the total heterogeneity was mainly due to the phylogeny (I^2 phylogeny = 54.51%) and the heterogeneity within populations (I^2 population = 29.55%). However, the species independently of the phylogeny and the population explained the I^2 Total in adults, and the phylogeny had little contribution to total heterogeneity (**Table 2**).

3) Meta-regression

We found a negative (but weak) association between TL and age in juvenile and adult stages of all taxa (except for juvenile reptiles, likely due to the low number of effect sizes). In mammals, estimates were similar between juvenile and adult stages but their 95% CI overlapped zero (estimate and 95% CI = -0.04, [-0.24:0.17] and -0.09, [-0.26:0.08] for juveniles and adults, respectively). In fish, estimates were moderate and statistically significant for juveniles (estimate and 95% CI = -0.47, [-0.69:-0.26]) but low and not statistically significant for adults (estimate and 95% CI = -0.14, [-0.42:0.15]). Finally, in birds, estimates were negative and the 95% CI overlapped zero in juveniles but not in adults (estimate and 95% CI = -0.17, [-0.36:0.01] and -0.22, [-0.36:-0.08]). For adult reptiles, estimates were very close to zero (estimate and 95% CI = -0.08, [-0.38:0.22]) (**Figure 4**)

In the juvenile dataset, the model selected was the null model, meaning that none of our moderators, independently of phylogeny, explained the heterogeneity in the relationships between telomere length and age during this stage (**Table S8**). The selected model for adults included the type of study and the method of TL measurement (**Table S9**). When tested separately, the relationship was weak and negative in both Transversal and Longitudinal studies but not statistically different (estimate ± 95% CI = -0.20, [-0.31:-0.08] and -0.11, [-0.26:0.03] for Transversal and Longitudinal studies, respectively). Regarding the effect of the method, the association between TL and age was negative whatever the method considered. However, the estimate was strongly negative for FISH (estimate and 95% CI = -0.73, [-1.18:-0.28]) and small to moderate for TRFI (estimate and 95% CI = -0.30, [-0.49:-0.12]). The estimates for studies using TRFS and qPCR were small and their 95% CI overlapped zero (estimate and 95% CI = -0.23, [-0.49:0.03] and -0.07, [-0.16:0.03]; **Table 3, Figure 5**).

In the adult dataset, we performed the same meta-regression as above but this time using data restricted to the methods that provided evidence for a decline in telomere length with increasing age (i.e. TRFI and FISH). The model selected was the null model, meaning that none of our variables could explain the decline of telomere length with increasing age **(Table S10)**.

4) Publication bias analysis

There was no detectable publication bias for the juvenile dataset because the intercept of the Egger's regression was not different from zero (Estimate ± SE: 0.06 ± 0.09) and the trim and fill method did not detect any missing studies (**Figure 6**). However, we found a funnel plot asymmetry in the adult dataset. The intercept of the Egger's regression was different from zero (Estimate ± SE: -0.39 ± 0.07) and the trim and fill method detected 56 missing studies on the right-hand side of the funnel plot (**Figure 6**). Those missing studies might change the estimate of the meta-analysis mean (change in the meta-analysis mean = 0.13, SE = 0.04) leading to a weaker relationship between TL and age (estimate after the trim and fill = -0.03).

Discussion:

Our meta-analysis based on 175 effect sizes and 98 species, encompassing birds, mammals, fish and reptiles, revealed a negative relationship between TL and age in adults but not in juveniles. However, the relationship was both weak and variable across taxonomic classes, which suggests more complex telomere dynamics than a simple decline with increasing

366 age throughout life in vertebrates. Despite the high number and diversity of species analysed,
367 we were not able to include any amphibian species. Our dataset was also biased in favour of
368 birds that were over-represented (45 bird species and 81 effect sizes out of 98 species and 175
369 effect sizes) relative to the other taxonomic classes. Along with the taxonomic class effect, we
370 also found a strong effect of the method used to measure telomeres in adults. Indeed, the
371 relationship was statistically significant only when using TRFI and qFISH methodologies.

372 Contrary to our hypothesis, we found a similar decline in age-specific TL during the
373 whole juvenile and adult stage. Our results suggest that juveniles and adults do not differ in TL
374 attrition rates on average across our set of species. Two non-mutually exclusive hypotheses
375 could be made to explain this lack of difference in telomere dynamics among life stages. First,
376 we used the age at first reproduction as the cut-off age between juveniles and adults. However,
377 the period of most rapid growth (which corresponds to the period of highest cell division and
378 thus decline in TL) might not cover the entire period before the age at first reproduction in all
379 taxa (Monaghan & Ozanne, 2018). In birds, for instance, final body size is reached by fledgling
380 or shortly after while sexual maturation could be achieved several years later for long-lived
381 birds. This is well illustrated in the wandering albatross where chicks reached adult body size
382 at around 10 months old (Mabille, et al., 2004) but individuals only start to reproduce around 7
383 years old (Froy et al., 2013). While there is compelling evidence that telomeres shorten at a
384 faster rate during embryonic development (Stier et al., 2020) and nestling stage in some bird
385 species (Boonekamp et al., 2014; Vedder et al., 2017), we excluded those articles from our
386 analyses due to the lack of data available covering the first month of life. The lack of available
387 data on TL change over the earliest developmental stages could lead us to underestimate the
388 decline of telomere length in juveniles. Repeated sampling in very young individuals, especially
389 in taxa other than birds, is clearly needed to better understand early-life telomere dynamics and
390 the link between growth and telomere shortening. However, repeated sampling of very young
391 individuals in wild populations may be both ethically and logistically challenging. A second
392 explanation is that variation exists in the form of the relationship between TL and age, being
393 curvilinear in some species (Rollings et al., 2017; Ujvari et al., 2017) and bi-phasic (Aubert et
394 al., 2012; Hall et al., 2004) in others, so that different relationships cancel into noise and we
395 cannot pick up the expected difference between juveniles and adults. To perform our meta-
396 analyses, we made strong simplifying assumption (*i.e.* linearity of the relationship between TL
397 and age and a cut-off between juvenile and adult stages using the AFR) that researchers working

on single species do not have to make. In that respect, some relationships we estimated might be at odds with what is reported in the literature.

Among the different moderators included in our analyses, we found that the method used to measure telomeres influenced the mean effect size in adults. More precisely, we found stronger negative relationships in studies using TRFI and qFISH methodologies, but weaker and not statistically significant relationships in studies using TRFS and qPCR. Conversely, in a previous meta-analysis linking TL and mortality risk in non-human vertebrates (Wilbourn et al., 2018), the relationship was stronger in studies using qPCR. In another meta-analysis looking for the association between stress exposure and TL (Chatelain et al., 2020), the association was 34% stronger when telomeres were measured with qPCR than TRF. The stronger association in qPCR was explained by a possible publication bias in qPCR studies, which is supposed to be higher than in TRF since qPCR is a cheaper and faster method than TRF. Chatelain et al. (2020) also explained such discrepancies by the fact that mean TL might be more accurately estimated using qPCR since TL distribution can be highly skewed and thus poorly reflected by average value obtained using TRF. On the other hand, a meta-analysis of human studies found sex differences only in articles using TRF (Gardner et al., 2014). The authors suggested that this difference could arise from higher measurement error in qPCR than in TRF. To date, only one published study compared estimates of TL using qPCR and TRFS on the same sample in relation to age (Aviv et al., 2011). While the authors found a higher measurement error in qPCR than in TRFS (6.45% against 1.74%), age accounted for 17.2% of the inter-individual variation of TL measured with qPCR against 29% using TRFS. While the number of studies measuring telomeres in wild vertebrates has increased dramatically in recent years, TRF, considered as a 'gold standard' (Nussey et al., 2014), is being used less and less in favour of qPCR because qPCR requires less DNA and is easier to perform than TRF (Lai et al., 2018). However, qPCR assay is known to have higher measurement error than TRF in humans (Aviv et al., 2011; Elbers et al., 2014) and is subject to well-position effects in thermocyclers, which, when not controlled, increase the overall measurement error (Eisenberg et al., 2015). In overall, qPCR require to be highly optimized in order to increase the precision and the accuracy of telomere length measurement (Eastwood et al, 2018; Lin et al., 2019). However, a higher measurement error in qPCR than in TRF cannot explain the pattern we observe since in our analysis we detected an effect of age using TRFI but not using qPCR nor TRFS. Another explanation for the discrepancy between the methods used is that TRFI uses non-denatured DNA, contrary to TRFS and qPCR. By denaturing the DNA, TRFS and qPCR measure, in addition to telomeres,

interstitial telomere sequences (ITS), which are telomeric repeats located inside the chromosomes (Foote et al., 2013; Meyne et al., 1990). ITS are known to be shorter than telomeres but should not shorten with increasing age (Foote et al., 2013). Accordingly, methodologies that include ITS may underestimate the average TL in a sample of cells. In addition, because ITS may also vary among individuals within a same species (Foote et al., 2013), including ITS could also decrease the power to detect differences in TL over time. However, this hypothesis could not explain the negative result we found in studies using qFISH, since qFISH also include ITS in the estimation of TL. Overall, we cannot explain the effect of TL measurement method we found in our meta-analyses. At present, only one meta-analysis in the field of telomere dynamics makes the distinction between TRFI and TRFS (Remot et al., 2020) and it is now necessary that forthcoming comparative and meta-analyses also make this distinction. In addition, reasons given to explain such discrepancies between methodologies remain hypothetical, and more research should focus on understanding how and why telomere measurement methods differ in the results they produce. To address this question, we recommend that further studies should focus on measuring TL in the same sample of known-age individuals using different methodologies. Finally, while the qFISH is rarely used in non-medical research (only 9 effect sizes in our dataset), the TRFI is almost exclusively used in birds (32 effect sizes in birds, 1 in mammals and 2 in reptiles, **see Supplementary, Table S12**). Hence, the effect of the method we found in our analyses and the fact that the methods used are not balanced across taxonomic classes could explain why we detected a decline in TL with advancing age in birds but not in mammals. In addition, because of the bias in methodologies, we may have underestimated the overall decline in telomere length with increasing age. To tease apart the effect of the phylogeny from the effect of the methodology, we recommend the use of methodologies such as TRFI and qFISH in taxa other than birds.

We found no evidence for an effect of the age at first reproduction nor a difference between the type of study (transversal vs. longitudinal) on the association between TL and age, contrary to what we expected. Previous studies found that telomere rate of change was positively linked with maximum lifespan in 19 species of bird (Tricola et al., 2018), and also associated with the species pace of life (*i.e.* fast-living species lose more telomeres per unit of time than slow-living species) in 14 species of birds (Dantzer & Fletcher, 2015). One potential explanation for this discrepancy with past studies of birds is that our analysis was performed in four different classes of vertebrates, encompassing 98 different species, with four different methods of TL measurement, while the two previous findings were made on birds only, whose

telomeres were measured using TRF only. Thus, the heterogeneity due to the high number of species and the various methodologies in our dataset could prevent us from finding a detectable effect. We also did not find any effect of the type of study on the association between TL and age. A possible source of heterogeneity that could potentially explain the weak relationship we observed in adults is the tissue sampled. While some studies did not find any effect of the interaction between tissue and age on TL, a recent study made on humans found different telomere attrition rates depending on the tissue sampled (Demanelis et al., 2020). At the within-tissue level, tissues are composed of various cell types that can have their own telomere dynamics. In the Australian Painted Dragon (*Ctenophorus pictus*), white blood cell (WBC) populations (lymphocytes and azurophils) have different TL, and TL is also much longer in WBC than in red blood cells (RBC) (Olsson et al., 2020). Given the lack of a nucleus in their erythrocytes, telomeres in mammals are mostly measured in WBC, which equates to measuring the average TL in a heterogeneous population of cells, each with potentially different sized telomeres. More problematically, it is known that the composition of WBC varies considerably with the age of individuals (Cheynel et al., 2017; Watson et al., 2017), which might cloud the pattern of telomere dynamics with age. From the few studies that measured cell-specific telomere dynamics, contrasting results occur. In the Soay sheep, while the composition of WBC varies with the age of individuals, it seems that those changes do not impact age-specific telomere dynamics (Watson et al., 2017). However, telomere attrition rates vary between subpopulations of leukocytes in humans (Lin et al, 2016), suggesting that average leukocytes TL might also vary following the composition of the cell population being measured. In our meta-analysis it was not possible to take into account the tissue sampled nor the cell type within tissue since most of the studies included (76%) measured TL in blood (whether RBC in birds, fish and reptiles or WBC in mammals) and because WBC are only measured in mammals, the tissue is confounded with the taxa. In addition, most of the other tissues present in our dataset were sampled once or twice (15 out of 21 tissues) meaning that we would not have enough repeated measures per tissue to detect tissue-specific telomere dynamics with age. We then need more studies that compare TL change with age over different tissues.

We also found evidence for asymmetry in the funnel plot of the adult dataset. The trim and fill method estimated 51 missing effect sizes on the right-hand side of the funnel plot. Once these studies were accounted for, there was no evidence for a relationship between TL and age. However, the trim and fill method has shortcomings (especially when between-study heterogeneity exists (Peters et al., 2007)) and should be interpreted cautiously. Two hypotheses

could explain the funnel plot asymmetry. First, positive associations between TL and age might be less likely to be published without strong statistical support (i.e. high precision) since TL is commonly expected to decline with increasing age. Alternatively, such positive relationships could be truly rarely observed. Indeed, due to the 'end-replication problem' and oxidative damages, it is biologically less likely that TL declines than increases with age, meaning that, in this case, funnel plot asymmetry might be due to biological constraint rather than publication bias. Either way, we highly recommend that researchers measuring TL in known age individuals publish their data, no matter the effect found nor the statistical significance because only a higher number of studies will allow us to clarify whether this asymmetry is due to publication bias or biological constraint.

Our heterogeneity analysis reveals that the part of the total variance explained by the between-study variance in the meta-analysis performed on adults was mostly due to the heterogeneity among species (independently of the phylogeny) and populations (for species that were sampled in different populations), highlighting the role that environmental conditions can have on telomere dynamics. For example, in great tits (*Parus major*), individuals living in urban environments have shorter telomeres than individuals living in forest regardless of nestling origin (Salmón et al., 2016). In roe deer (*Capreolus capreolus*), shorter telomeres were observed in old individuals from a population facing strong resource limitation compared to those from a population experiencing better environmental conditions (Wilbourn et al., 2017). However, due to the lack of multiple sampling of the same species in contrasting environments, it was not possible to thoroughly examine the role played by environmental conditions on the relationship between telomere length and age.

Available data indicate that telomere dynamics are complex both within and among species. While we found an overall decline of TL with increasing age among adult vertebrates, the publication bias we found might weaken the strength of the association. In addition, our results mainly point out the variability of the relationship between TL and age and, because none of the biological moderators we tested explained this variability, suggesting that there is much we still do not know about telomere dynamics. In addition, as highlighted in previous meta-analyses (Chatelain et al., 2020; Wilbourn et al., 2018), the methodology used to measure telomeres remains an important source of heterogeneity, which is worrying since at least four different methods are currently used, potentially giving different results. It is important that future research aims to better understand how methodologies and phylogeny influence patterns of variation in TL in relation to age.

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Data accessibility: Data are available via the Dryad Digital Repository https://doi.org/10.5061/dryad.000000048

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Tables and Figures legends:

Table 1: Number of species, studies and effect sizes for the two meta-analyses performed on juveniles and adults.

Table 2: Effect size of the relationship between TL and age estimated from the meta-analyses we performed and I^2 associated with each random factor.

Table 3: Estimate and 95% confidence interval for the effect of the method used to measure TL in adults on the effect size of the relationship between TL and age.

Figure 1: PRISMA statement (adapted from Liberati et al., 2009).

Figure 2: The relationship between telomere length and age across adult non-human vertebrates. Species-specific estimates were calculated using the factor 'Species' as a moderator in the meta-regression model to calculate a mean relationship for each species.

Figure 3: Orchard plots for juveniles (A, in blue) and adults (B, in yellow). The point on the x-axis represents the estimate of the model, the black line in bold the confidence interval, the grey line in bold the prediction interval and coloured points represent the effect size of each study (the size of the points is proportional to the precision, calculated as the inverse of the standard error, of the study). Individual effect sizes are distributed on the y-axis to make them all visible. For the ease of interpretation, all effect sizes were back-transformed into correlation coefficients ($r = \tanh Zr$). The I^2 total is also reported.

Figure 4: Orchard plot for juveniles (A) and adults (B) split among taxonomic groups. The point on the x-axis represents the estimate of the model (back-transformed into correlation coefficients), the black line in bold the confidence interval, the grey line in bold the prediction

interval and coloured points represent the effect size of each study (the size of the points is proportional to the precision, calculated as the inverse of the standard error, of the study). k is the number of effect sizes for each level.

Figure 5: Orchard plot displaying the effect of the method of TL measurement in adults. The point on the x-axis represents the estimate of the model (back-transformed into correlation coefficients), the black line in bold the confidence interval, the grey line in bold the prediction interval and coloured points represent the effect size of each study (the size of the points is proportional to the precision, calculated as the inverse of the standard error, of the study). k is the number of effect sizes for each level.

Figure 6: Contour-enhanced funnel plot for the meta-analyses on juveniles (A) and adults (B). The precision (inverse of the standard error) is plotted against meta-analytic residuals. Areas of statistical significance are displayed in grey. While there was no detectable publication bias in juveniles, we found an important publication bias in adults with 56 missing studies on the right side of the funnel plot (white dots), estimated by the trim and fill method.