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**Temperature-independent telomere lengthening with age in the
long-lived human fish (*Proteus anguinus*)**

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ABSTRACT

Despite number of studies showing negative relationship between age and telomere length, the universality of this pattern has been recently challenged mainly in ectothermic animals exhibiting diverse effect of age on telomere shortening. However, data on ectotherms may be strongly affected by thermal history of the individuals. We thus investigated the age-related changes in relative telomere length in the skin of a small but long-lived amphibian living naturally in stable thermal environment over its entire life allowing the comparison with other homeotherms animals like birds and mammals. The present data showed a positive relation between telomere length and individual age, independent of sex and body size. A segmented analysis highlighted a breakpoint in the telomere length-age relationship, suggesting that telomere length reached a plateau at the age of 25 years. Further studies focusing on the biology of animals that live much longer than expected based on body mass will contribute to our better understanding of how ageing processes evolved and may also bring innovation for extend human health span.

INTRODUCTION

The DNA repairing machinery of the cell maintains genomic integrity notably *via* the recognition and reparation of DNA breaks (1). Eukaryotic telomeres, by their ability to form a t-loop structure with associated proteins, are the structure allowing to distinguish the ends of linear chromosomes from DNA breaks, and thus to avoid unnecessary emergency cell responses (2). In the absence of active enzymatic reset by telomerase (3), telomere ends inevitably erode over time in the adult soma (4), at a rate that can be accelerated by all kind of stressors (5), which ultimately lead to cell senescence (6).

The extensive effort in studying telomere dynamics in birds and mammals confirmed, except in rare cases, the age-related shortening of telomeres, and its negative link with individual lifespan (e.g. 7, 8, 9). Still, telomere changes with age are supposed to be completely different in ectotherms, which are animals that grow continuously and generally preserve their telomerase activity in somatic cells over life (e.g. 10). Surprisingly, change in telomere length with age in ectotherms was shown to be diverse, with either continuous shortening or no differences between young and old individuals (11, reviewed in 12) sometimes with marked sex-differences (11, 13). Such variability among ectotherm species may be attributed to intra-sex differential investment in reproduction (11) but also to environmental temperature that drive ectotherms' metabolism, growth and even ageing rates (14, 15). The thermal performance curves, which reflect both how environmental temperatures affected individual traits like early development and present performances, have been recently suggested as a key framework to understand ectotherm telomere dynamics within and between species (16). Up-to-date, telomere dynamics with age in ectotherm models maintained in a stable thermal environment during the entire life has never been studied.

The olm salamander *Proteus anguinus* is an outstanding animal model to address the telomere and ageing question. This animal is called “human fish” due to its extreme longevity (mean and maximum lifespan estimated to 68 and more than 100 years respectively) for its body mass (> 20 g) and neither the study of its basal metabolic rate or of its antioxidant capacities shed lights on the nature of the mechanisms sustaining such an exceptional long life (17, 18). In addition, the olm is the only obligate cave dwelling vertebrate in Europe (19), and thus live in a stable thermal environment over its entire life allowing the comparison with other homeotherms animals like birds and mammals without the potential confounding effect of temperature variation.

Taking advantage of a nearly 70 years breeding program of the olm, we investigated the age-related changes in relative telomere length (RTL, obtained via qPCR, see below) in a sub-population of 41 individuals. This breeding program conducted in a natural cave in Moulis (France) provides available living individuals kept in the dark in a controlled-temperature facility (12°C) and information on their development and life cycle in a natural environment.

MATERIALS AND METHODS

- Studied species and individuals

All animals (n = 41) came from the Moulis captive population that is the only successful programme allowing to study of the biology of this amphibian. The founder individuals of the population came from successive imports from the Piuka cave river near the town of Planina in Slovenia (20, 21). Reproductions has been observed since 1959 (21) with animals fed with chironomid larvae ('blood worms') once a week and the aquaria kept in the dark in a controlled-temperature facility (12°C). Individuals used in this study were found newly and naturally dead (with yet no sign of decomposition) from 2003 to 2011. Their snout-vent length (SVL) was measured (± 1 mm) for 29 individuals, which were then fixed by submersion in ethanol 70°. Our sampled population was composed of 41 individuals, 17 males, 12 females and 12 with undetermined sex, but all with known age at death (from 4 to 40 years-old). No ethical authorization was needed to obtain samples.

- DNA extraction and PCR procedures

Relative telomere length (RTL) was evaluated using the qPCR methodology (22), based on DNA extraction obtained from skin samples. The tissue samples (of approximately 20mg) were introduced into an Eppendorf tube and mixed into 200 μ l of 10% Chelex-100 resin solution. 10 μ l of proteinase K (Boehringer Mannheim) at 15 mg/ml is added to the solution which is then incubated at 55° C for an hour while being shaken. The mixture is then

87 incubated further at a temperature of 90°C for 15 minutes to increase the extent of protein
88 denaturation. The tubes containing the extracted DNA and the Chelex resin, together with the
89 degraded proteins, were stored at 4° C or at -20°C. Before its use, the mixture was shaken in a
90 vortex mixer and centrifuged at 10,000 rpm for ten seconds to deposit the mass formed by the
91 resin and the proteins, and then stored at -20°C. In order to purify the gDNA, NucleoSpin
92 gDNAclean –up kits were used (Macherey Nagel, Duren Germany) and DNA quantification
93 was determined by Fluorescence (QuantiFluor® ONE dsDNA System, Promega, Madison,
94 WI, USA). Two µl of supernatant are used for each 10 µl of final volume of the PCR reaction
95 mixture. DNA quality was checked for solvents presence using a NanoDrop 1000
96 (ThermoFisher Scientific, Waltham, MA, USA) spectrophotometer (absorbance ratio
97 A260/280; A260/230) and for DNA degradation using gel migration. Two different
98 sequences of genomic DNA were amplified to obtain both a control signal accounting for
99 variation in DNA quantity (Recombinase Activity Protein 1 gene (RAG1, 287 bp, NCBI
100 number EF107303.1) and of the telomeric sequences. Forward and reverse primers were 5'-
101 CCA GGC TAT CAC CCC TTT GA - 3' and 5'- TCG CTT ACA TCT CCC ATC CC - 3' for
102 the RAG1 gene and 5'- CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG -
103 3' and 5'- GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT - 3' for the
104 telomeric sequence. Amplifications were conducted in one plate, using one distinct control
105 gene and telomere run. qPCR conditions were 2 min at 95°C followed by 40 cycles of 15 s at
106 95°C, 30 s at 60°C and 30 sec at 72°C, or of 2 min at 95°C followed by 30 cycles of 15 s at
107 95°C, 30 s at 60°C and 30 sec at 72°C, for the control gene and telomere amplifications,
108 respectively. Reactions were done in a master mix prepared for each primer set, with 5 µL
109 GoTaq QPCR Mix (Promega, Madison, WI, USA). We used 2 ng of DNA and the telomere
110 primers at a concentration of 200 nM and the control gene primers at 7.5 nM (for a final
111 reaction volume of 10 µL in each well). All samples were run in duplicates and intra-

individual repeatability of RTL, evaluated using the Intra Class Coefficient (23), was of 0,973.

- Statistics

Statistics were done using R version 3.5.1. RTL were log transformed to obtain a normal distribution of our response variable and standardized (z scores). Normality was checked using the quantile – quantile function *qqnorm* both on the RTL values and model residuals distributions. We investigated if RTL was related to age at death, using a Linear model with a quadratic Age^2 term to control for non-linear age-related variation in ectotherms (24) and controlling for sex effects. Before calculating the Age^2 variable, Age was initially scaled to avoid collinearity (25), further checked using the *vif* function (“car” R package). Also, because the olm salamander is an indeterminate growing organism (see results), and that telomere length and growth are suspected to be interconnected (26), the individual SVL as body size measurement was initially added as a fixed factor. This reduced our sample size to 29 individuals, including 17 males and 12 females. Duration of sample storage (range: 9-17 years) was also included in our model as a fixed factor (and not random factor as it did not follow a Normal distribution). Our full model was therefore: $RTL \sim Age^2 + Age + Sex + SVL + storage\ duration$. Selection of the best model was done using the *Mumin* R function, based on AIC criterion. The selection was adapted to conserve the *Age* variable we were interested in, and no more than 4 final variables given our sample size, and consequently without including interaction terms. We choose the most complete model within a AIC difference of 2 (excluding Age^2 did not change the results, see ESM). Age breaking points in telomere length change over time was assessed using the Segmented R package, calculating breakpoints in the slope of the RTL - Age relationships (27). Since our data were cross-sectional (non-repeated individuals over age), we tested for selective disappearance of individuals with short telomeres at adult age in two ways. First by evaluating the effect size of

the differences in RTL between young (<17 years) and adult individuals using the Cohen-d value (“lsr” R package) and, second, by testing differences in variance among young (4-10 years and 11-16 years) and adults (17-30 years and 31-40 years) groups (“DescTools” R package, Kruskal-Wallis test) [see (18) for data on age at sexual maturity]. $P \leq 0.05$ was considered significant and effect size interpreted following Cohen (28).

RESULTS

Table 1 presents the output of the generalized linear model testing for change of RTL with age at death in the olm. We found a significant and positive effect of age at death on RTL, independent of sex (even if males tended to have shorter RTL) and body size (also negative tendency). We also found a negative effect of storage duration on RTL values. There was no indication of a quadratic relationship of RTL with age at death. However, segmented analysis highlighted a breakpoint in the RTL-age relationship, suggesting that RTL reached a plateau at the age of 25 years (Figure 1). Body size (SVL, 134.2 ± 4.9 mm, mean \pm S.E.M.) was found to increase over age following a significant quadratic interaction with sex (GLM, $\text{Age}^2 \times \text{sex}$, $F_{1,24} = 14.4$, $P < 0.001$), males growing faster than females.

The significant increase in RTL with age at death could be due to the selective disappearance of individuals with short telomeres. However, Cohen’s d effect sizes between young (<17 years old) and adults was of 0.312 [by convention small to medium effect (28)] and even smaller when calculated from the residuals of RTL with storage duration ($d = 0.173$).

Variances among age at death categories were not found to be significantly different among young and adult age categories (Chi-square = 3, $P = 0.392$), despite a visual tendency to decrease from early-life young stage (4-10 years, var = 2.27) to the other life stages (young 10-16 years, 0.19; adults 17-30, 0.82; adults 31-40, 0.53).

161

162 **DISCUSSION**

163 Telomere length is commonly expected to decline with age. However, because initially most
164 studies focused on mammals and birds (29), it rapidly called into question the universality of
165 the observed patterns and proposed hypotheses. Evidences soon accumulated showed that
166 there is no single, universal pattern of telomere erosion even in endotherms (e.g. 30) but more
167 evidently in ectotherms (e.g. 31). Thus, in order to better understand ongoing selection and
168 evolution of telomeres and telomere-regulating processes, a larger panel of non-model
169 organisms presenting unusual telomere dynamics needed to be incorporated, as they lend
170 themselves better to research direct links between telomere traits and components of fitness.
171 According to the present data, the olm joins the few species exhibiting positive relationship
172 between RTL and age. Even if such positive correlation has been considered as an artefact of
173 measurement error (32), increasing arguments (both theoretical and empirical) plead for
174 considering it to be a real biological phenomenon (33, 34). Even if we cannot exclude a
175 simple TL elongation with no effect on mortality whatsoever, two hypotheses could explain
176 the present results: the "selection hypothesis" stating that the longest-lived individuals start
177 with the longest telomeres and variation in telomere length decreases with age due to the
178 selective disappearance of individuals with short telomeres. Alternatively, the "elongation
179 hypothesis" relies on the idea that the longest-lived individuals are those able to lengthen
180 telomeres with age, perhaps, due to the higher levels of telomeres maintenance pathway.
181 Since no age effect on variance has been detected, our data plead for the "elongation
182 hypothesis" but the assessment of both individual telomere dynamics and of the telomerase
183 activity would be necessary to confirm it.

184 Previous studies found that slightly negative or even positive telomere rates of change
185 (TROC) were positively linked with maximum lifespan (9) and also associated with the

species pace of life (*i.e.* slow-living species loose less (or even gain) telomeres per unit of time than fast-living species) (35, 36, 37). Our TROC estimate is not directly comparable with previously published values (ours is obtained using qPCR measurements, not in bp of telomere DNA lost per year). Still, our results are in congruence with these findings since we found that the very slow pace of life of *P. anguinus* is associated with a slightly positive TROC estimate (see Table 1). While being not obviously hypometabolic (38), the olm can dramatically reduce movements and metabolic rate during food scarcity (39), and could survive years without eating (40). The free radical generation is probably at low enough levels (17), enabling this species to accumulate very little oxidative damage over its life span. A longitudinal study of individuals is needed to confirm our interpretation of cross-sectional data, and would allow for an exploration of how the complex telomere maintenance process (41, 42) explain the presently described positive telomere dynamics in this species.

Interestingly, *P. anguinus* seem to belong to those long-lived organisms that live much longer than expected based on body mass (18) and exhibit telomere lengthening or no change with age (43, 44). The extreme longevity of those types of organisms is partly explained by their ecological niche or by their physical aptitude which reduces the predation risk and avoid adverse conditions (45, 46, 47). In parallel, increased or unchanged RTL with age could be a signature of small and long-lived species. In fact, if long telomeres protect from cell senescence and a stable somatic telomerase activity help to sustain high metabolic costs, the overuse of telomere maintenance processes may be paid as increased cell immortalization risks. Consequently, small and long-lived rodents have evolved specific anti-cancer mechanisms (48). Whether this is the case of the olm remains an open question.

With a significant increase of TL length with age in a stable thermal environment, the present data confirm more complex telomere dynamics than a simple decline with age throughout life in vertebrates. Studying the biology of long-lived animals staying healthy and capable of

reproduction until death will contribute to our better understanding of how ageing processes evolved in response to environmental factors (49). It may also lead to novel interventions that extend human health span. Further studies focused on others small and long-lived ectotherms like some rockfishes' species or the red sea urchin are of prime importance to fully decipher interaction between body size, longevity and telomere dynamic.

Data accessibility

The data for this study have been deposited on Dryad under the following DOI: doi:10.5061/dryad.1jwstqk0c (50) and are directly accessible via the following weblink: <https://datadryad.org/stash/share/EcPEv3jwqMg0sHyo726uASDIZhlpSPtjoBWESdkqgHs>.

Author contribution

YV established the study design, AD conducted DNA extraction, SZ established and conducted the qPCR measurements on *Proteus* samples, FC analyzed the data. YV, GO & FC co-wrote the manuscript, with input from AD and SZ.

Competing interest statement

The authors declare having no competing interests

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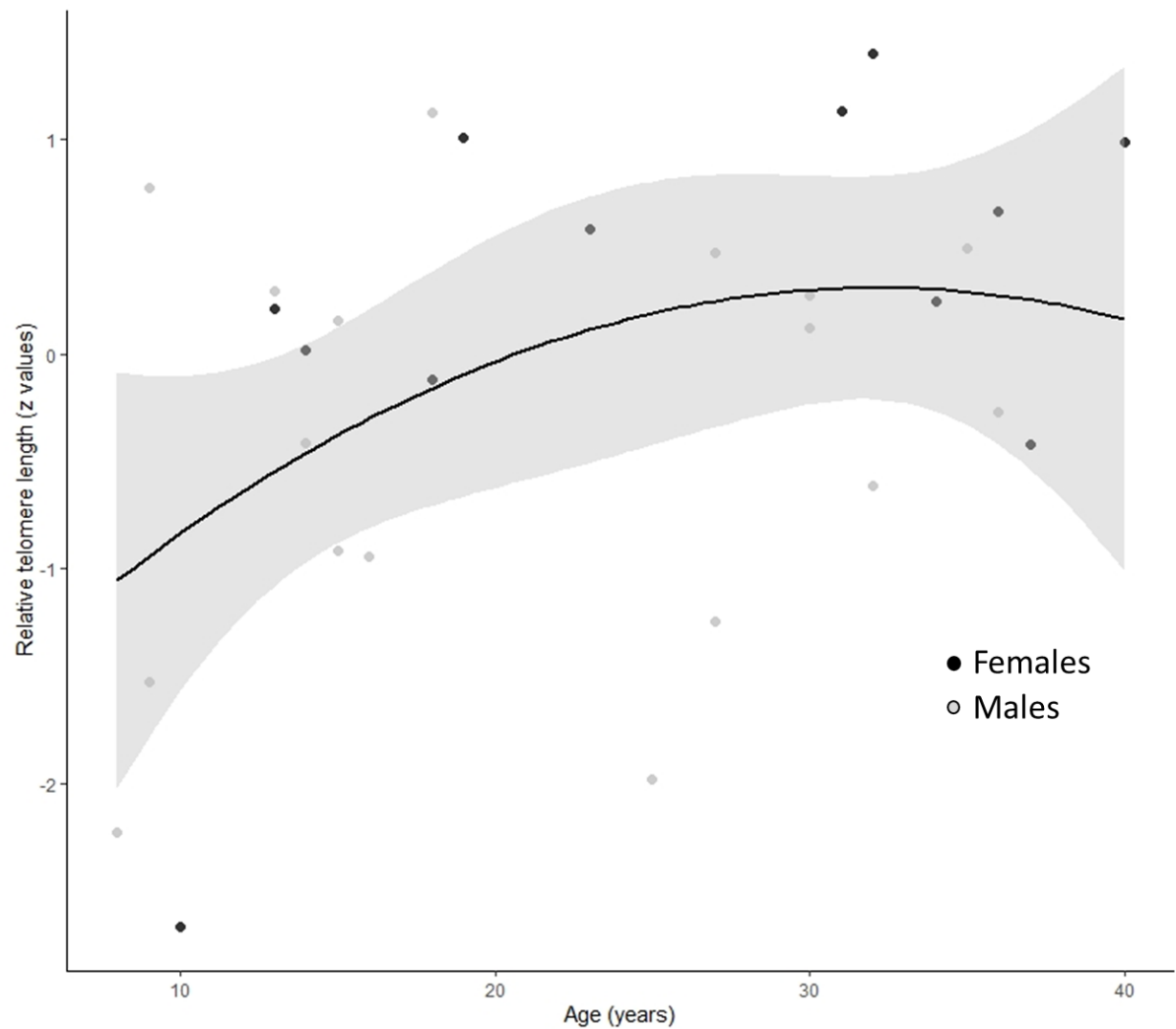
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Figure 1

Relative telomere length (z-transformed T/S ratio) of blind cave salamander in relation to age in 41 individuals. Confidence interval (95%) is indicated in grey. Values plotted are the predicted values obtained from the Linear Mixed Model using sample storage duration as a random factor, and sex and body size as cofactors.



383 Table 1

384 Relationship between relative telomere length (RTL) of individual olms and age (scaled
 385 square terms for a quadratic relationship), sex and body size (as the snout-vent length) tested
 386 using a Linear Mixed, with Relative telomere length (RTL) as response variable, and Age²,
 387 Age and storage duration as fixed factors. RTL is expressed as z-transformed log(T/S ratio).
 388 See Methods for the description of the model selection process. Vif values were of 1.05
 389 (Age²), 1.02 (Age), 1.06 (storage duration), suggesting no particular collinearity issues (all <
 390 5). Observations, 29; R² = 0,347.

Predictors	Estimates	Std Error	CI	F value	P
Intercept	2.32	0.92	0.42 – 4.23	2.51	0.019
Age²	-0.42	0.27	-0.97 – 0.14	1.439	0.242
Age	0.38	0.18	0.02 – 0.76	5.684	0.025
Storage duration	-0.16	0.07	-0.30 – -0.03	6.136	0.020
<i>Observations</i>	<i>29</i>				
<i>R²</i>	<i>0.347</i>				
<i>Adjusted R²</i>	<i>0.268</i>				

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