# Does oxidative stress shorten telomeres?

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# Does oxidative stress shorten telomeres?

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Oxidative stress shortens telomeres in cell culture, but whether oxidative stress explains variation in telomere shortening *in vivo* at physiological oxidative stress levels is not well known. We therefore tested for correlations between six oxidative stress markers and telomere attrition in nestling birds (jackdaws *Corvus monedula*) that show a high rate of telomere attrition in early life. Telomere attrition was measured between ages 5 and 30 days, and was highly variable (average telomere loss: 323 bp, CV = 45%). Oxidative stress markers were measured in blood at age 20 days and included markers of oxidative damage (TBARS, dROMs and GSSG) and markers of antioxidant protection (GSH, redox state, uric acid). Variation in telomere attrition was not significantly related to these oxidative stress markers ($|r| \leq 0.08$, $n = 87$). This finding raises the question whether oxidative stress accelerates telomere attrition *in vivo*. The accumulation of telomere attrition over time depends both on the number of cell divisions and on the number of base pairs lost per DNA replication and, based on our findings, we suggest that in a growing animal cell proliferation, dynamics may be more important for explaining variation in telomere attrition than oxidative stress.

## 1. Introduction

Telomeres are terminal DNA–protein complexes that act as 'protective caps' of linear chromosomes [1]. Telomeres shorten with age and telomere length has been found to predict remaining lifespan (e.g. [2,3]). Identifying the mechanisms causing telomere attrition is therefore of interest, because it may contribute to the identification of physiological processes underlying variation in health and lifespan. Mechanisms of telomere attrition have been well studied in cell culture, revealing that oxidative stress is a key factor that accelerates telomere attrition in a dose-dependent manner [4,5]. However, cell cultures are not organisms, and oxidative stress levels *in vitro* are difficult to scale to oxidative stress in whole organisms. This raises the question whether oxidative stress also shortens telomeres *in vivo*. We are aware of six recent studies of the link between oxidative stress and telomere attrition *in vivo*, with mixed results, but sample sizes were modest and they included few oxidative stress parameters that were measured after the telomere attrition had already occurred, i.e. not in the period between the baseline and follow-up telomere sample [6–11]. The role of oxidative stress in telomere attrition *in vivo* is therefore currently unclear.

We measured telomere attrition between ages 5 and 30 days in free-living jackdaw nestlings in which we previously demonstrated a high rate of telomere attrition over this period, making jackdaw nestlings a suitable study system [12]. We collected an additional blood sample at an intermediate age (day 20) to measure six commonly used oxidative stress markers that have been implicated to be important in growth and fitness, to investigate the extent to which oxidative stress and telomere dynamics were related. This panel included markers of oxidative damage (TBARS and dROMs, both markers of lipid

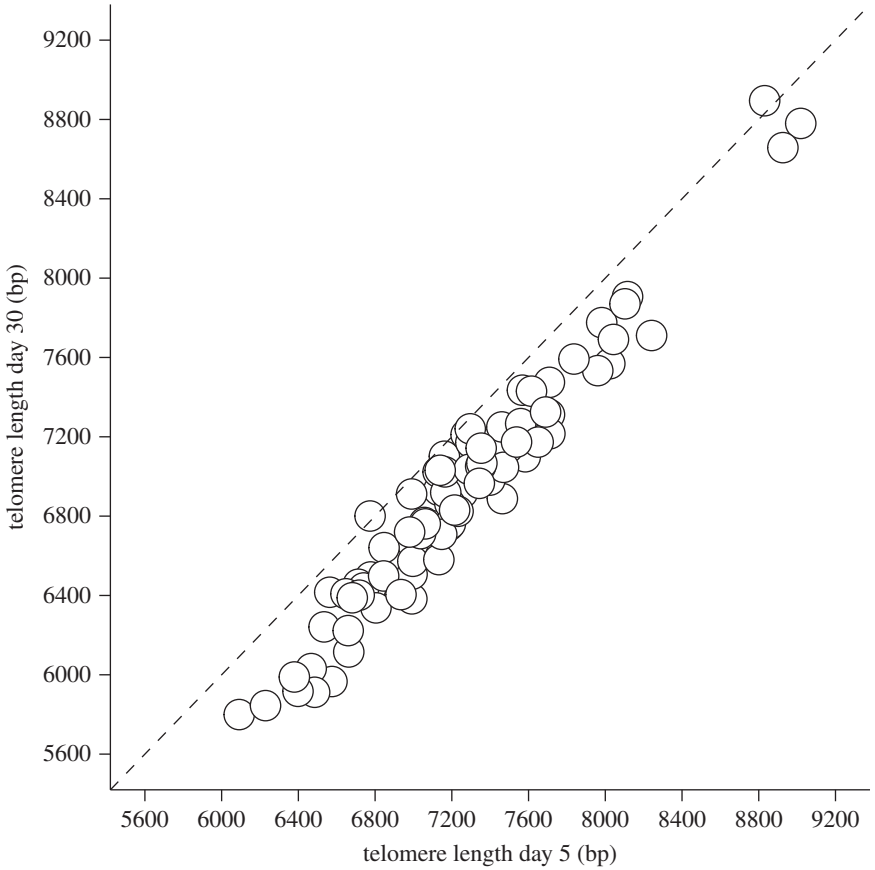


**Figure 1.** Telomere length at age 30 days plotted against telomere length at age 5 days in jackdaw nestlings ($r = 0.97$, $n = 87$). The dashed line represents equal values of day 5 and 30 telomere lengths and hence the distance below this line reflects the telomere shortening.

peroxidation [13,14]; GSSG, oxidized glutathione [13,15]) and markers of antioxidant capacity (GSH, reduced glutathione [13,15,16]; uric acid, a compound with antioxidant capacity [17]; redox state, the ratio of oxidized over total glutathione [13,15]). We measured oxidative stress in blood, while telomere shortening occurs in the haematopoietic stem cells, but blood and tissue oxidative stress variables are highly correlated across the body [18].

## 2. Material and methods

We studied a natural population of jackdaws in 2014 in five nest-box colonies in the vicinity of Groningen, The Netherlands (53.1708° N, 6.6064° E). General field procedures were as previously described [19]. We collected ±70 µl blood samples during early (day 5) and late development (day 30, just before fledging; hatching of the oldest chick = day 1) to measure telomere dynamics in the nestling period ($n = 87$ nestlings from 40 broods). In addition, we collected a larger blood sample at day 20 (±1 ml) for oxidative stress measurements. These larger blood samples were stored at −80°C after centrifugation.

### (a) Telomere measurements

Telomere length was measured in erythrocytes with pulsed-field gel electrophoresis [20]. DNA was extracted from erythrocyte nuclei using the CHEF Genomic DNA Plug kit (Bio-Rad, Hercules, CA, USA). DNA was digested overnight using proteinase K (50°C) and then subsequently digested with a combination of *Hind*III (60 U), *Hinf*I (30 U) and *Msp*I (60 U) for 18 h at 37°C in NEB2 buffer. Digested DNA was separated using pulsed-field gel electrophoresis at 14°C for 24 h (3 V cm$^{-1}$, switch times: 0.5–7.0 s). Dried gels were hybridized overnight at 37°C with $^{32}$P-end-labelled oligo (5′-CCCTAA-3′)$_4$ that binds to the 3′ end-cap telomere overhang. Radioactive signal was quantified with the PerkinElmer cyclone storage phosphor system. Individual telomere length size distributions were quantified with densitometry using grey intensity values in ImageJ v. 1.38x, obtaining the mean telomere length of the sample [20]. The within-individual repeatability of telomere length, estimated using the day 5 and 30 samples with birdID as random effect, was 84.5%. This value underestimates the true repeatability of the measurements because telomeres had shortened in the 25-day period and shortening rate varied among individuals. When correcting day 30 telomere length for the average shortening between days 5 and 30, we estimate the repeatability to be 97.3%, corresponding to the measurement repeatability of previous telomere projects in our laboratory.

### (b) Oxidative stress measurements

We measured TBARS, reactive oxygen metabolites (ROMs), protein carbonyl, uric acid and triglyceride concentrations in blood plasma, and reduced and oxidized glutathione concentrations in whole blood. For details of the methods, see the electronic supplementary material S1.

Oxidative stress measurements were done using 96-well plates and between-plate-variation was normalized by subtracting the respective plate mean values from the individual oxidative stress values prior to statistical analyses. All variables were subsequently log-transformed to normalize the distribution, and subsequently transformed to a standard normal distribution to enable a direct comparison of model coefficients between oxidative stress variables. To allow a log transformation, distributions were made numerically positive by adding a fixed number per variable to make the lowest value in that variable

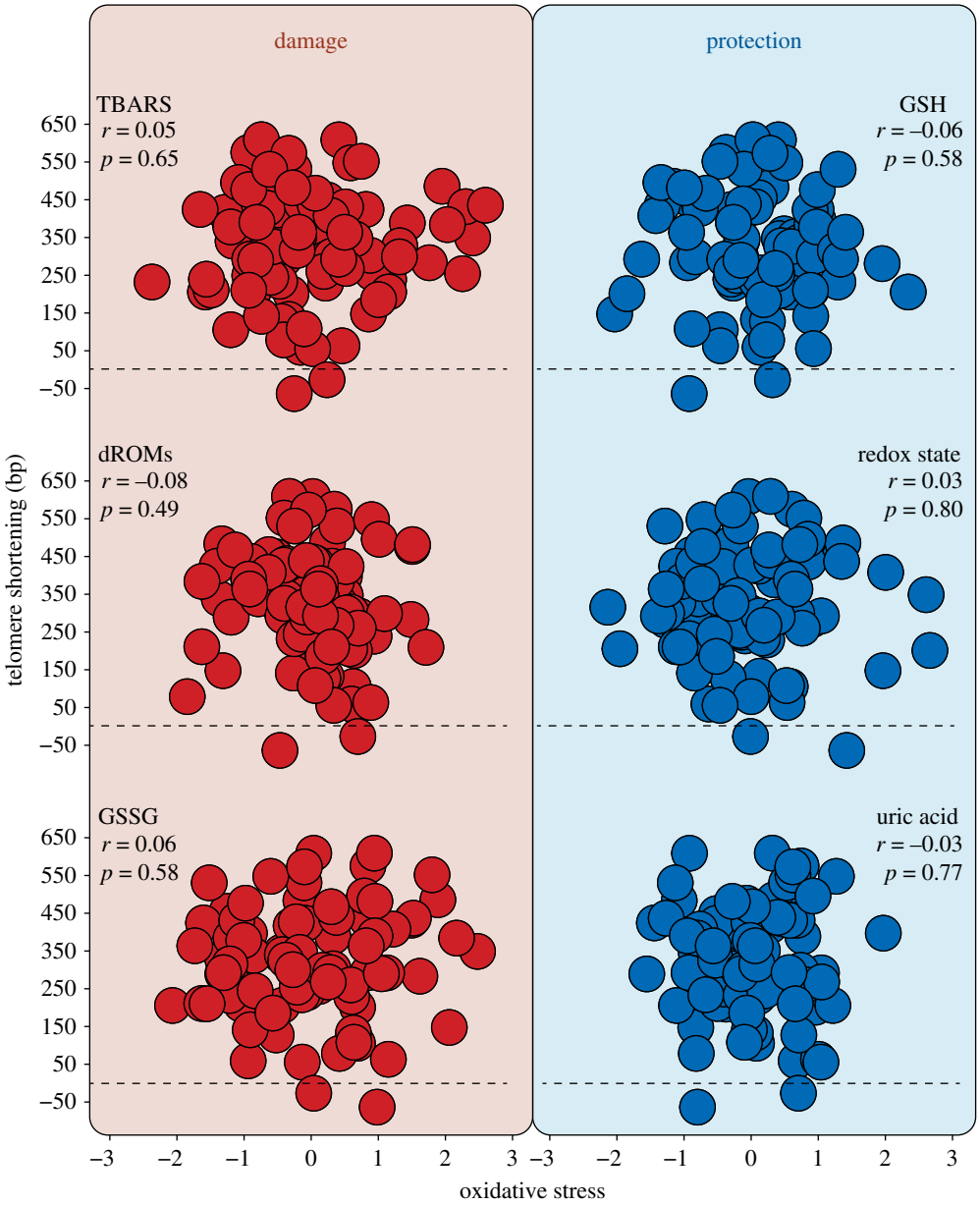


**Figure 2.** Association between oxidative stress variables and telomere shortening ($n = 87$). The oxidative stress variables were transformed to a standard normal distribution and units on the *x*-axis therefore represent standard deviations. d-ROMS and uric acid were corrected for plasma triglycerides and handling time, respectively (see the electronic supplementary material SI for details). The Pearson correlation coefficients ($r$) are based on the data as shown, but *p*-values are from the mixed models. (Online version in colour.)

greater than zero. We verified that the raw data produced highly similar results.

### (c) Statistical analyses

We used mixed-effects models with restricted maximum log-likelihood estimation in R-lme4 [21] and included birth nest as random factor to account for the within-brood dependence of siblings due to shared early environmental and genetic effects. When testing oxidative stress effects on telomere attrition, each model included only the oxidative stress variable as fixed effect in addition to the random effect.

## 3. Results

Nestling telomere lengths at ages 5 and 30 days were strongly correlated ($r = 0.97$). Nestlings lost on average 323 bp (s.d. = 146) over this period (figure 1), but telomere attrition varied strongly between nestlings (CV = 45%). We tested the associations between telomere attrition and our panel of oxidative stress variables, but none of the oxidative stress variables were associated with telomere attrition (figure 2). The strongest correlation was close to zero and in the opposite direction from what we expected (dROMs, $r = -0.08$, $p = 0.49$). We further tested whether the inclusion of sex, brood size and nestling mass on day 20 and their interactions affected the relationship between oxidative stress and telomere attrition, but this did not change the results. Given our sample size, we would have been able to detect an association of $R^2 > 0.078$ with power 80%, suggesting that an existing relationship that we did not detect is likely to be weaker than this limit.

We used principal components analysis (singular value decomposition) to assess whether covariation among the six oxidative stress variables could predict telomere attrition. The first two principal components predicted a substantial amount of variation (51 and 20%), but neither predicted telomere attrition ($p > 0.52$).

## 4. Discussion

We tested the hypothesis that oxidative stress accelerates telomere attrition *in vivo*, but we detected no significant associations between telomere attrition and a panel of six oxidative stress variables despite (i) large variation in telomere attrition, (ii) a sample size that allowed us to identify modest effect sizes with reasonable power, and (iii) using a panel of six oxidative stress markers. The most parsimonious view emerging from our data is therefore that oxidative stress has little effect on telomere attrition *in vivo*, at least in growing nestlings. At the same time, it is a truism that the absence of a process cannot be proven, and there are of course limitations to our study that potentially obscured oxidative stress effects on telomere attrition. For example, oxidative stress is challenging to assess reliably [22] and we cannot rule out that other oxidative stress markers or tissues would have yielded a different result. Furthermore, oxidative stress levels could have fluctuated over time and it remains unclear whether a snapshot at day 20 is representative of the total oxidative stress in the period over which telomere attrition was determined. Finally, we did not measure telomerase activity and hence it would be informative to verify the extent to which oxidative stress and telomerase activity are correlated.

The difficulty in quantifying or manipulating oxidative stress hampers convincing refutation of any oxidative stress–related hypothesis, which in turn makes it more difficult to publish negative results [23]. For example, among the four published studies relating telomere attrition to oxidative stress, the non-confirmative results were not presented as a main research finding [8–11], which contrasted with papers with confirmative results [6,7], which also contained non-confirmative results that could be given equal weight. This difference creates a bias in the perception of the role of oxidative stress in telomere attrition. Clearly, more studies on the link between oxidative stress and telomere attrition *in vivo* are needed and we should in particular welcome the unbiased publication of confirmative and non-confirmative findings.

What explains variation in telomere attrition when it is not oxidative stress? Cumulative telomere attrition is the product of the number of cell divisions and the number of base pairs that is lost per cell division. While cell culture studies express telomere attrition per cell population doubling time, and hence control for the rate of cell proliferation, *in vivo* studies investigate the cumulative telomere attrition. We hypothesize that the variation in telomere attrition we observed was largely caused by variation in cell proliferation rate, with only a modulating effect of, for example, oxidative stress. The among-individual variation in the rate of cell division could be of particular importance when studying telomere attrition during development, when cells proliferate most. To determine the relative importance of variation in cell proliferation rate versus oxidative stress as sources of telomere attrition *in vivo*, studies are required that measure both quantities simultaneously. Few studies have reported a positive relationship between nestling growth and telomere attrition (e.g. [12,24], but see [8]) and it would be informative to investigate the extent to which this pattern reflects a relationship between cell proliferation rate and telomere attrition.

Ethics. This work was approved by the University of Groningen animal ethics committee under licence number '6832 A' before conducting the research.

Data accessibility. The dataset is included as the electronic supplementary material (Boonekamp_data_ESM).

Authors' contributions. J.J.B. and S.V. designed the study, conducted the fieldwork, analysed the data and wrote the first draft of the manuscript. Measurements and analyses were done by C.B. (telomere length) and E.M. together with J.J.B. (oxidative stress). All authors contributed to editing the manuscript and gave final approval for publication.

Competing interests. We declare we have no competing interests.

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# Electronic supporting information of "Does oxidative stress shorten telomeres?"

## ESM-I (methods) p 1-2

*Blood oxidative stress chemistry measurements:*

*TBARS:* We used the QuantiChrom TBARS assay kit (DTBA-100 from BioAssay Systems) to quantify the concentration of thiobarbituric acid (TBA) reactive substances (TBARS) in the plasma. TBARS primarily consist of malondialdehydes (MDA), an established marker of lipid peroxidation. The reaction of TBARS with TBA form a pink coloured product, which intensity is proportional to the TBARS concentration in a sample and can be quantified by measuring the optical density at 535nm. To accomplish this 100ul plasma samples were thawed and de-proteinated prior to the assay with 200 ul ice cold TCA (10%), followed by 5 minutes of incubation on ice. Sample-TCA mixtures were subsequently centrifuged at 14,000 rpm in a micro-centrifuge and 200ul of clear supernatant was used for subsequent TBARS quantification. Standard curves were prepared in duplo by diluting the standard MDA that was supplied by the kit. Samples and standards were then treated with 200ul TBA reagent and incubated at 100 °C for 60 minutes. Finally, samples were loaded in duplicate (2 x 100ul) on 96-well plates and read at 535 nm using a micro-plate reader.

*ROMs:* We used the d-ROMs kit (MC 002) from Diacron, Italy , to quantify the concentration of plasma hydroperoxides that are formed during the process of lipid peroxidation. The d-ROMs test measures a sample's reactivity to a chromogen, which is quantified by measuring the optical density at 505/546 nm [1]. To achieve this, reactionmix was prepared according to the kit description. An alliquot of 200ul reactionmix was added to 20ul of thawed plasma sample and incubated for 90 minutes at 37 $^{o}$C. After incubation, samples were shortly centrifuged to precipitate any formed sediment. 200ul of supernatant was transferred to a 96-well plate and read at 505/546 nm using a micro-plate reader.

*Carbonyl:* We quantified the optical density at 375 nm of protein-carbonyl groups after tagging of plasma carbonyls with 2,4-dinitrophenylhydrazine (DNPH). We used two different protein carbonyl colorimetric assay kits (10005020 from Cayman Chemical and #K830-100 from BioVision) to assess the concentration of carbonyl in the plasma, and found that DNP hydrozone levels were below the detection limits of both kits. We therefore do not show results on plasma carbonyl.

*Uric acid:* We determined plasma uric acid concentrations using the QuantiChrom kit (DIUA-250) from BioAssay Systems. The kit utilizes the oxidation of a chromogen by uric acid, which is proportional to the optical density at 590nm. Samples were thawed to room temperature and gently mixed prior to the assay. Reaction mix was prepared according the kit descriptions. Blanks, standards, and samples (5ul) were set up using 96-well plates. 200ul of reaction mix was added to each sample and standard followed by incubation for 30 minutes at 20 °C. Plates were then read at 590nm using a micro-plate reader.

*Glutathione:* We measured both total glutathione (tGSH = GSH + 2*GSSG) and its oxidized form (GSSG) in whole blood samples using the EnzyChrom GSH/GSSG kit (EGTT-100) from BioAssay Systems. Glutathione acts as anti-oxidant and the ratio of oxidized versus total glutathione is considered to be an indicator of oxidative stress. The kit uses an enzymatic method utilising Ellman's reagent (DTNB) and glutathione reductase (GR). DNTB reacts with glutathione, forming a yellow product of which the rate of change in the optical density at 412nm is proportional to the concentration of glutathione. Before centrifuging the whole blood samples for harvesting plasma, 50ul whole blood was added into 2 tubes and one of the two tubes was treated with 1-methyl-2-vinylpyridinium triflate scavenger prior to storage at -80 °C. This scavenger eliminates all reduced glutathione (GSH) rendering the assay to quantify GSSG. Samples were thawed, mixed, and

incubated at room temperature for 10 minutes prior to the assay. Subsequently, samples were deproteinated using freshly prepared 5% metaphosphoric acid according the kit protocol. Working reagent (per reaction: 1ul GR enzyme, 0.25ul NADPH, 0.5ul DTNB, and 105ul assay buffer) was prepared and 100ul of working reagent was added to each sample and standard on 96-well plates. Optical densities were measured at 412nm immediately after mixing the plates and after 10 minutes of incubation at room temperature. GSH was determined by subtracting the amount of 2*GSSG from the total glutathione concentration.

*Plasma lipids:* We measured two indicators of plasma lipid mobilization – triglycerides and cholesterol – as follows: Standards were made in duplo by dissolving a calibrator (Cobas C.f.a.s. cat nr: 10759350) in demineralized water. Three 10ul plasma samples (one for triglyceride, one for cholesterol determination and one for blank) were dissolved in 90ul demi-water each and 500ul of Roche/Hitachi GRO-PAP reagents was added to each sample (for triglycerides: cat nr: 11730711216, for cholesterol: cat nr: 11489232216). Samples were mixed and incubated for 30 minutes at 37 °C and subsequently optical densities were determined at 473nm and 490nm with a spectrophotometer for the quantification of triglyceride and cholesterol concentrations, respectively. A blank dissolved sample was mixed with 500ul demi-water and optical densities were determined to control for initial plasma color.

*Confounding effects of plasma lipids and handling time*

Plasma lipid peroxidation markers have been found to co-vary with circulating lipid levels and as such high lipid peroxidation could in part be caused by high levels of circulating lipids [2]. We tested for such confounding effects by investigating the association of the log- and standard normal transformed TBARS and dROMs variables with plasma triglyceride levels using linear mixed effects models, including birth nest random effect. dROMs and triglycerides were positively associated (estimate ± s.e. = 0.420 ± 0.080, $p < 0.001$), and we used the residuals of this regression as corrected dROMs values in subsequent analyses. TBARS did not depend on triglycerides and hence we used uncorrected values in the data analyses.

Handling birds during sampling and measurement of the nestlings potentially causes stress and this may have induced confounding effects on the detected oxidative stress levels. We therefore included handling time in our analyses to check for such effects, and only found a significant negative relationship between handling time and uric acid (-0.029 ± 0.010, $p = 0.006$). To control for the effect of handling time we used the residual uric acid values in our analyses obtained from a model with handling time as single predictor and birth nest as random effect.

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