Long-term effects of superoxide and DNA repair on lizard telomeres

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Abstract
Telomeres are the non-coding protein-nucleotide "caps" at chromosome ends that contribute to chromosomal stability by protecting the coding parts of the linear DNA from shortening at cell division, and from erosion by reactive molecules. Recently, there has been some controversy between molecular and cell biologists, on the one hand, and evolutionary ecologists on the other, regarding whether reactive molecules erode telomeres during oxidative stress. Many studies of biochemistry and medicine have verified these relationships in cell culture, but other researchers have failed to find such effects in free-living vertebrates. Here, we use a novel approach to measure free radicals (superoxide), mitochondrial "content" (a combined measure of mitochondrial number and size in cells), telomere length and DNA damage at two primary time points during the mating season of an annual lizard species (Ctenophorus pictus). Superoxide levels early in the mating season vary widely and elevated levels predict shorter telomeres both at that time as well as several months later. These effects are likely driven by mitochondrial content, which significantly impacts late season superoxide (cells with more mitochondria have more superoxide), but superoxide effects on telomeres are counteracted by DNA repair as revealed by 8-hydroxy-2′-deoxyguanosine assays. We conclude that reactive oxygen species and DNA repair are fundamental for both short- and long-term regulation of lizard telomere length with pronounced effects of early season cellular stress detectable on telomere length near lizard death.

KEYWORDS
Ctenophorus pictus, flow FISH, mitochondrial mass, qPCR, superoxide, telomeres

1 | INTRODUCTION

Telomeres are tandem nucleotide repeats found at the ends of chromosomes (TTAGGG in most Metazoans; Blackburn & Gall, 1978; Gomes, Shay, & Wright, 2010; Hug & Lingner, 2006). These nucleotide repeats are involved in several important functions, such as the triggering of cellular, and possibly, tissue and whole-organism senescence (von Zglinicki, 2002). Furthermore, telomeres ensure coding DNA is not lost when linear DNA is replicated (Hug & Lingner, 2006), prevent the accidental fusion of chromosomes by the repair machinery of the cell (through tight binding with proteins, Houben, Moonen, van Schooten, & Hageman, 2008), and are required for the correct
alignment and separation of chromosomes during cell division (Lin, Smith, & Blackburn, 2004). These processes are costly, and telomeres become shorter over the life of an organism (at least in homeotherms) due to repeated cellular fission and damage by reactive oxygen species (ROS) and other reactive molecules, especially during stress (Chen, Hales, & Ozanne, 2007; Ludlow, Spangenberg, Chin, Cheng, & Roth, 2014; Reichert & Stier, 2017). However, recent work in birds (Boonekamp, Bauch, Mulder, & Verhulst, 2017) suggests that six independent oxidative stress markers are uncorrelated with telomere attrition, calling into question the relative role and effect of reactive molecules for telomere dynamics. These are important observations and suggest incomplete understanding of the causal links between reactive molecules, oxidative stress, telomere attrition and life history evolution (see also Janssens & Stoks, 2018).

The telomeric sequence possesses unique features (e.g., long, tandem G-rich repeats) that predispose telomeres to a variety of DNA damage from intracellular metabolic processes such as hydrolysis, oxidative alkylation and DNA base mismatches, as well as by insult from exogenous sources, which will not be included in this study (e.g., UV light; Jia, Her, & Chai, 2015). To protect genomic stability, Metazoan cells have evolved highly conserved DNA repair mechanisms to remove and repair such DNA lesions (Ahmed & Lingner, 2018; Jia et al., 2015; Kansara & Gupta, 2018; Tan et al., 2017). One group of repair pathways for telomeric maintenance is excision and repair of bases (BER), nucleotides (NER) and DNA mismatches (MMR). BER is a cellular mechanism that corrects discrete, small DNA base lesions caused by oxidative deamination and, when defective, can elevate mutation rate and cancer risk (Jia et al., 2015).

Observations of telomere shortening in haematopoietic mouse cell cultures, in normoxia (20%) or in the presence of an oxidant (hydrogen peroxide), indicate that oxidative guanine damage affects telomere maintenance in diverse ways. The BER pathway is required for maintaining telomere integrity (at least in mammals), although the exact mechanism for removing telomeric 8-oxo-G residues is still unclear (Jia et al., 2015). The DNA repair assay in the current work detects 8-hydroxy-2′-deoxyguanosine (8-oxo-dG), the most abundant type of base lesion repaired in telomeric sequences (Jia et al., 2015). In the current work, we have therefore focused on BER as our target repair pathway.

At a cellular level, it is well accepted that oxidation compromises telomere length and attrition through a variety of mechanisms (Fouquerel, Parikh, & Opresko, 2016; von Zglinicki, 2002), a damage that is repaired in vivo but with varying success for a number of reasons. For example, contributing factors to this net attrition effect may be age- and sex-specific dynamics in the production of telomerase, endogenous antioxidants (e.g., superoxide dismutase, catalase), and how the costs for the production of these enzymes are traded off against other self-maintenance processes (Monaghan, 2010; Monaghan & Haussmann, 2006; Olsson, Wapstra, & Friesen, 2017, 2018). In practice, any process that elevates metabolic rate and increases the level of not-fully reduced oxygen, or fails to counter these effects with endogenous antioxidants, is a potential inducer of oxidative stress. For example, increased testosterone levels (or reduced levels of oestrogen) may increase telomere attrition and explain sex differences in longevity (males often have shorter lives, Barrett & Richardson, 2011; Brooks & Garratt, 2017), with telomere shortening triggering cellular senescence (Valko et al., 2007). Given the benefits and costs (sometimes substantial) of "long" telomeres (increased risk of cancer; Olsson et al., 2017, 2018), we therefore expect classic life history trade-offs to be mediated by, or reflected in, telomere dynamics that should vary across taxa or morphs with different reproductive tactics.

Our perspective on the current study is one of evolutionary biology using a short-lived lizard species with a wealth of information from prior work in the wild and in the lab, with considerable information on ROS and telomere biology, the Australian painted dragon lizard (Ctenophorus pictus; Healey & Olsson, 2009; Healey, Uller, & Olsson, 2007; Olsson, Wilson, Isaksson, Uller, & Mott, 2008; Olsson, Healey, Perrin, Wilson, & Tobler, 2012). Males have distinct morph-based strategies, differ in a number of reproductive parameters, and are short-lived, surviving for about 1 year in the wild (Friesen, Johansson, & Olsson, 2017; Olsson, Tobler, Healey, Perrin, & Wilson, 2012; Olsson, Healey, Wapstra et al., 2007). This makes them an outstanding vertebrate model for analysing components of ageing and life histories in a single reproductive season. Here, we use this model system to test the following hypotheses: (a) Does superoxide (SO) vary across the season, before and after reproduction, in terms of mean and variance? (b) Does superoxide covary negatively with telomere length early in life? (c) Does superoxide covary negatively with telomere length late in life, also when early-life telomere length is controlled for statistically? (d) Does DNA (erosion and) repair assays (8-oxo-dG) predict positive telomere length late in life? (e) Does mitochondrial cell content explain SO variance?

2 | METHODS

This work was performed under the Animal Ethics permit 2013/6050 at the University of Sydney. Mature male and female lizards (~9 months old) were caught by noose or hand at Yathong Nature Reserve, NSW, Australia (145°35′E; 32°35′S) during the Australian spring (ca. mid October, 2014) and taken to holding facilities at the University of Sydney where they were housed during the experiments. Adult males were housed individually in opaque plastic tubs (330 × 520 × 360 mm) with sandy substrate and exposed to a 12 hr light: 12 hr dark cycle. Males and females could observe each other through "windows" in the cage walls, cut out and covered with a wire mesh (else females will not go through a normal ovarian cycle and fertilization—they need male presence for this to happen; Uller & Olsson, 2005). The males and females were all participating in breeding experiments reported on elsewhere and had plenty of interactions with social/sexual partners. However, because males painted dragons are strongly territorial and highly aggressive, males were kept separately and with no visual interactions for ethical reasons. The lizards were fed mealworms and crickets, dusted with calcium and multivitamins, to satiation every day, and the cages were misted with water once a day. Heat lamps and ceramic hides were provided to allow the lizards to thermoregulate to their preferred body temperature (36°C; Mats Olsson, unpublished data obtained from cloacal temperature readings in the wild).
Our main methodological approach is flow cytometry, since this powerful technique makes it possible to measure, in tandem, levels of free radicals (here, superoxide) the content of mitochondria in cells (i.e., a measure influenced by both the size and number of mitochondria in cells; Cottet-Rousselle, Ronot, Leverve, & Mayol, 2011; Xiao, Deng, Zhou, & Tan, 2016) that can be argued to be main contributors to level of superoxide (since more mitochondria yield more electron transport chains leaking reactive oxygen species). Using Flow FISH on the same samples makes it possible to also assess telomere length and attrition. Molecular techniques for measuring telomere length all have their respective pros and cons. This is not the right forum for an extensive debate on methods (but see reviews by Aubret, Bonnet, Shine, & Lourdais, 2002; Baird, 2005; Gutierrez-Rodrigues, Santana-Lemos, Scheucher, Alves-Paiva, & Calado, 2014; Nakagawa, Gemmell, & Burke, 2004; Nussey et al., 2014), but to verify the robustness of our Flow FISH telomere analyses, we also ran our samples using Quantitative PCR (qPCR) and looked for congruence between Flow FISH and qPCR telomere estimates.

2.1 | Blood sampling

Blood (~150 μl) was sampled at three time points (October, December and February) over 3 days using a syringe by rupturing vena angularis (in the corner of the mouth) with the tip of a capillary tube. Aliquots (10 μl) of each sample were immediately diluted into 1.5 ml of cryopreservation buffer for analysis with flow cytometry (see below), another aliquot of 10 μl whole blood was added to 90 μl PBS (1:10) and placed on ice for flow cytometric analysis of ROS and mitochondrial mass, and the remainder was diluted with 1 ml of RNAlater (Sigma Aldrich, Australia) for qPCR analysis and stored immediately at −80°C. For the Flow FISH analysis, the manufacturer’s recommendation of using standard cryopreservation methods for blood cells was followed (Nazarpour et al., 2012). A haemocytometer was used to determine that C. pictus whole blood contains approximately 1 × 10⁹ RBCs per ml; therefore, 10 μl of whole blood from C. pictus contains 6 × 10⁶ cells, which is 3x the number of cells required for the FISH analysis. Whole blood (10 μl) was added to 1.5 ml cryopreservation buffer kept on ice (30% foetal bovine serum, Sigma 12003C); 60% RPMI-1640 medium (Sigma R8758; 10% DMSO, Sigma D4540) to achieve the recommended cell mass of 2–4 × 10⁶ per ml of cryo-buffer. Samples were placed in a foam box in a −80°C freezer to slowly cool the cells to avoid rupture and where they were kept until analysis.

2.2 | Quantifying superoxide and cell mitochondrial content

Samples of peripheral blood (70 μl) were diluted with nine volumes of phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄, pH 7.4) and stored on ice prior to analyses, which were completed within 4 hr of sampling. Prior to staining, diluted blood was diluted a further 50-fold with PBS and then centrifuged (300 g for 5 min) to pellet cells; each cell pellet corresponded to 10 μl of whole blood. Cells were resuspended in 100 μl of PBS containing either no additions (unstained control), 5 μM MitoSOX Red (MR; Thermofisher), or a mixture of 0.1 mM dihydrotrihydroxamic acid 123 (DHR; Thermofisher) and 50 nM MitoTracker Deep Red FM (MTDR; Thermofisher). The fluorescence from these probes can be used to detect, respectively, superoxide ions (MR), any reactive oxygen species (DHR) and the content of mitochondria in a cell, largely independent of mitochondrial membrane potential (MTDR; Xiao et al., 2016). MR, DHR and MTDR were added from stock solutions in dimethylsulfoxide (DMSO); the final concentration of DMSO was 0.2% (v/v) or less. Cells were subsequently incubated at 37°C for 30 min, then washed with PBS by centrifugation as described above and held on ice until analysed by flow cytometry; 50,000 events were acquired for all samples. Flow cytometry was performed using a Becton Dickinson LSFortessa X20, with excitation at 488 nm (MR and DHR) and 633 nm (MTDR), and emitted fluorescence collected using band pass filters of 575 ± 13 nm (MR), 515 ± 10 nm (DHR), and 660 ± 20 nm (MTDR). Data were acquired and analysed using FACSDIVA version 4.0.1 (Becton Dickinson, Sydney, Australia) and Flio (v9.1; TreeStar Inc., USA) software, respectively. On the basis of forward angle laser scatter and side angle laser scatter, a number of blood cell populations were discerned; the results obtained were similar for all these populations. For each sample, the arithmetic mean fluorescence for all 50,000 cells acquired was determined using Flio software and used to compare between samples and treatments. The accuracy of flow cytometry results from samples from the same individuals has been measured in a separate experiment (see Olsson et al., 2008 for further details), involving 14 males with a correlation coefficient between samples of r = 0.97, p < 0.0001. Thus, our flow cytometry technique can be argued to be highly consistent.

2.3 | Quantifying telomere length—flow FISH

We used the Telomere PNA Kit/FITC for flow cytometry (Dako), which is recommended by the manufacturer as being suitable for use with nucleated cells from all vertebrates (http://www.dako.com.au/ar42/p107840/prod_products.htm).

The kit is based upon the hybridization of a synthetic DNA/RNA analogue, conjugated with FITC, capable of binding to telomeres in a sequence-specific manner obeying the Watson–Crick base pairing rules. The kit was used primarily for two reasons: (a) unlike methods such as Telomere Restriction Fragment analysis using Southern Blotting (TRF), this kit does not suffer from the interaction of sub-telomeric sequences, and (b) the probe hybridizes with telomere repeat sequences (TTAGGG) typical of vertebrates including lizards, and the resulting fluorescence intensity of the cells is directly correlated with the length of the telomeres. This method therefore provides a relative indication of telomere length between blood cells from different individuals. Tests of reproducibility of the Telomere PNA kit/FITC were performed at Dako’s laboratories using human blood. Relative Telomere Length values showed a coefficient of 8%–13% of single determinations and 6%–9% for duplicate determinations.
Cryopreserved blood was gently brought to room temperature and washed in PBS by centrifugation (as described above), before subsequent processing within 4 hr of sampling. Relative telomere length was compared between blood cells of individual lizards using the Dako kit, following the manufacturer’s instructions. Cells were counter-stained with propidium iodide (PI) to assess total nucleic acid content. Flow cytometry was performed using a Becton Dickinson Fortessa X-20, with excitation at 488 nm and emitted fluorescence collected using band pass filters of 515 ± 10 nm (fluorescein) and 695 ± 10 nm (PI). More than 90% of the cells had a similar level of PI staining, and this major population was electronically gated to select them for analysis of the level of telomere probe hybridization. Between 5% and 9% of blood cells had higher levels of PI staining, and these were electronically excluded from the analyses (the excluded lizard blood cells may be undergoing cell division and therefore contain inherently higher levels of nucleic acids). Following the manufacturer’s instructions, cultured CCRF-CEM cells (human acute lymphoblastic leukaemia cell line; Sigma) were used as control cells, and were mixed with each blood sample before processing and analysis. Thus for each individual sample, the telomere-associated FITC fluorescence of lizard blood cells was reported relative to that of an internal standard (co-processed and co-analysed control CCRF-CEM cells). Data were acquired and analysed as described above.

2.4 | Quantifying DNA erosion (OXO)

8-hydroxy-2′-deoxyguanosine (8-oxo-dG) is a modified nucleoside base, which is the most commonly studied and detected by-product of DNA damage that is excreted upon DNA repair (Wu, Chiu, Chang, & Wu, 2004; Zhang, Xu, Kamendulis, & Klaunig, 2000). We used the HT 8-oxo-dG ELISA Kit (Trevigen, Catalog No. 4370-096-K), which identifies repaired DNA by assaying the excised, damaged DNA, which hence is a frequently used biomarker of oxidative DNA damage and oxidative stress. The kit is based upon a competitive binding ELISA, and features an 8-oxo-dG-specific monoclonal antibody, an enzyme-labelled secondary antibody (HRP conjugate) and detection substrate (tetramethylbenzidine, TMB). The anti-8-oxo-dG monoclonal antibody binds in a competitive manner to 8-oxo-dG in the sample, standard or pre-bound wells on the plate. Anti-8-oxo-dG bound to 8-oxo-dG in the sample or standard is washed away while those captured by the immobilized 8-oxo-dG are detected with the HRP conjugate and TMB and the absorbance measured in a microplate reader at 450 nm. The analysis was performed as outlined by the manufacturer on plasma diluted 10x with Assay Diluent in duplicates. The samples were run on five separate plates with an interplate coefficient of variation of 5.9. There was no effect of plate number on assay score in the beginning or late in the season (p = 0.27 and 0.67, respectively; Proc GLM, SAS).

2.5 | Quantifying telomere length—qPCR

To assess the reliability of the Flow FISH analysis, we also performed qPCR analyses of telomere length on our early season samples. Our qPCR methods on this system have been described in detail elsewhere (Rollings et al., 2017) so here we only give a summary. In brief, we purified DNA from 50 μl blood using a DNeasy Blood and Tissue Kit (Qiagen, Australia), according to the manufacturer’s instructions. RNase A (Qiagen) was added at the recommended concentration. The DNA concentration (ng/μl) of each sample was measured in duplicate using a Pherastar FS (BMG, Labtech, Germany) and aliquots diluted to 10 ng/μl using the AE buffer provided in the DNA extraction kit. Samples were then stored at −30°C. Telomere length was measured using real-time quantitative PCR (qPCR) using SensiMix SYBR No-ROX Kit (Bioline, Sydney, Australia). The telomere primers used were Telb1 (5′-CGGTTTGGGTGCGGGTTGGTTGGTTGGTT-3′) and Telb2 (5′-GGCCTGCTGACACCCCTACGCTACGCT-3′; Criscuolo et al., 2009). The 18S ribosomal RNA (18S) gene (92 bp ampli-con in Anolis) was selected as the reference gene as it had previously been validated in a reptile (Plot, Criscuolo, Zahn, & Georges, 2012). The primer sequences used were 18S-F (5′-GAGTTGAAATTCTTGGACCGG-3′) and 18S-R (5′-CGAAACCTCCGACTTTCCGTTC-3′). The melt curves produced for both telomere and 18S after amplification by qPCR displayed a single peak, indicating specific amplification of the DNA sequence. The qPCR was performed in a final volume of 20 μl for both telomeres and 18S. Ten nanogram of DNA was used per reaction, and the primers were used at a concentration of 250 nM. 11.25 μl SensiMix SYBR No-ROX Master Mix (Bioline) was added per reaction, and MgCl₂ was added for a reaction concentration of 1.7 mM. Reactions were run in triplicate for each sample. Amplifications were carried out in a Rotor-Gene 6000 thermocycler (Qiagen) using an initial Taq activation step at 95°C for 10 min, and a total of 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 15 s. A melt curve was created after each run over the temperature range of 60–95°C to ensure no non-specific product amplification. No-template control reactions were run in triplicate for each primer set during every qPCR run to ensure no contamination. Standard curves were created, using the blood of a randomly selected lizard, for both telomeres and 18S to ensure consistent rates of amplification over a wide range of DNA concentrations. The reaction was considered consistent when a straight line with an R² exceeding 0.985 could be fitted to the values obtained. The efficiency of the telomere amplification was 1.05, and the efficiency of the 18S amplification was 0.96. LinRegPCR 2015.2 (Ruijter et al., 2009; Tuomi, Voorbraak, Jones, & Ruijter, 2010) was used to analyse the qPCR data.

2.6 | Statistical analysis

We use GLMs in SAS 9.4 for all analyses. For all analyses, our main time points of comparison are early in the Austral spring at the onset of the mating season (October: “early season” sample), and during the Autumn, or late in the mating season (February: “late season” sample). For superoxide and mitochondrial content, we also report on a sampling event in mid-December. Preliminary analyses showed that superoxide level varied considerably early in the mating season, and our previous analyses have shown differences among morphs in a variety of reproductive traits (Olsson, Healey, Wapstra et al., 2007; Olsson, Tobler et al., 2012; Olsson, Wilson et al., 2008; Rollings et al., 2017). We therefore first analysed any direct effects
of “morph” (head colour and bib presence/absence) on levels of superoxide. These variables did not have a significant direct effect on superoxide \((p > 0.17)\) in this data set and were therefore not considered further. This agrees in detail with previous work (Olsson et al., 2008; but for subtler interactive effects see Friesen, Johansson et al., 2017; Friesen, Wilson et al., 2017). A corresponding analysis of morph on mitochondrial content in October revealed a strong effect of head colour morph (Model \(F_{4,66} = 4.41, p = 0.0032\)) with orange males having much higher mitochondrial content than other morphs (parameter estimate = 954.6, ±256, SE), \(t = 3.7, p = 0.0004\), but with no effect of presence/absence of a bib (\(p = 0.23\)). This morph difference in mitochondrial content disappeared towards the end of the mating season (December, \(p = 0.90\)), and stayed non-significant late in the season (February, \(p = 0.84\)). Given the significant effects of morph early in the season, and our previous discoveries with respect to morph differences, we ran all analyses with and without morph and bib in the models and report the effects in table legends within the Results section (none were statistically significant). For logistical reasons, early season sampling took place over 3 days. Since flow cytometry analysis can show among-batch variance, we therefore also ran a separate analysis with telomere length and early superoxide standardized for repeated sampling over these 3 days (mean set to zero and standard deviation to one). The significant relationship between standardized superoxide and telomere length reported in Table 1 was not significant (\(p > 0.52\)). These relationships are plotted in Figures 5 and 6.

When parameter distributions did not meet model assumptions, parametric analyses were supported by non-parametric analyses (Spearman rank-order correlations between target traits). DNA repair was analysed under the prediction that increased DNA repair predicts longer telomeres. We therefore specifically tested the directional prediction in more identified sequence repeats by Flow FISH (i.e., longer telomeres—DNA repair, it is expected to regain the ability to hybridize, resulting in more successful hybridization at compromised excision-repair will elongate telomeres. Given that the kit we used for Flow FISH analysis is based upon the hybridization of a synthetic DNA/RNA binding to telomeres in a sequence-specific manner, less successful hybridization is expected at compromised (unrepaired) telomeric sequence. Once the telomeric sequence is repaired, it is expected to regain the ability to hybridize, resulting in more identified sequence repeats by Flow FISH (i.e., longer telomeres). We therefore specifically tested the directional prediction that more DNA repair makes telomeres longer (and thus report one-tailed \(p\)-values).

3 | RESULTS

3.1 | Seasonal shifts in superoxide variation

Superoxide showed significantly higher variance early in the season (equality of variance test between the two sampling events, Folded F, Num DF = 75, DenDF = 77, \(F = 613.98, p < 0.0001\)). Thus, as individuals dropped in SO levels early (Spearman’s rank-order correlation, \(r_s = -0.40, p < 0.0003, N = 74\); Figure 1) but not late in the season (between December and February, \(r_s = -0.006, p = 0.957, N = 73\)), variation in superoxide levelled out during the latter half of the mating season.

### Table 1 Superoxide and DNA repair as predictors of early and late season telomere length

| (a) Response variable: telomere length, October | Estimate | SE  | t   | p   |
|-----------------------------------------------|----------|-----|-----|-----|
| Model \(F_{2,41} = 2.72, p = 0.08, R^2 = 0.12\) | Superoxide, October | -0.0004 | 0.0002 | -2.23 | 0.0310 |
| DNA repair, October | 0.0014 | 0.0016 | 0.89 | 0.1891 |

| (b) Response variable: telomere length, February | Estimate | SE  | t   | p   |
|-----------------------------------------------|----------|-----|-----|-----|
| Model \(F_{2,41} = 7.4, p = 0.002, R^2 = 0.27\) | Superoxide, October | -0.0006 | 0.0002 | -3.56 | 0.0010 |
| DNA repair, October | 0.0025 | 0.0014 | 1.80 | 0.0398 |

| (c) Response variable: telomere length, February | Estimate | SE  | t   | p   |
|-----------------------------------------------|----------|-----|-----|-----|
| Model \(F_{2,39} = 19.76, p < 0.0001, R^2 = 0.67\) | Superoxide, October | -0.0003 | 0.0001 | -2.63 | 0.0122 |
| Superoxide, February | -0.00002 | 0.00002 | -0.78 | 0.4385 |
| Telomere length, October | 0.6552 | 0.0979 | 6.69 | <0.0001 |
| DNA repair, October | 0.0017 | 0.00098 | 1.77 | 0.0423 |

Notes. Model (a): higher superoxide predicts shorter early season telomeres, but the effect of DNA repair this early in the season is not significant. Morph and bib effects were not significant (\(p > 0.40\)). Model (b): higher superoxide predicts shorter late season telomeres while elevated DNA repair predicts longer telomeres. Morph and bib effects were not significant (\(p > 0.52\)). Model (c): early season superoxide significantly, negatively predicts late season telomere length, independently of the (non-significant) effects of late season superoxide. The model explains 67% of variation in late season telomere length. Morph and bib effects were not significant (\(p > 0.59\)). These relationships are plotted in Figures 5 and 6.

3.2 | Early season superoxide and DNA repair effects on early season telomere length: simultaneous sampling of ROS and telomeres

Early season higher levels of SO were associated with shorter telomeres. However, this early in the season, DNA damage repair was not significantly affecting telomere length (Table 1a).

3.3 | Early season superoxide and DNA repair as predictors of late season telomere length: ROS and telomeres sampled with a time lag

Early season measures of superoxide were significantly associated with shorter telomeres late in the season (February). DNA repair showed positive effects on telomere length (\(p = 0.040\), one-tailed test; Table 1b).
FIGURE 1  Relationship between an individual’s superoxide level in October and December (Spearman’s rank-order correlation, $r = -0.40, p < 0.0003, N = 74$). This result disappeared in the corresponding correlation analysis between December and February ($r = -0.006, p = 0.957, N = 73$).

3.4  Early and late season superoxide and DNA repair as predictors of late season telomere length: early and late season superoxide contrasted

In this analysis, we also controlled for telomere length early in the season (thus, in reality this measures attrition, if this is not done, early season superoxide effects on late season telomere length is even stronger, $p = 0.0015$). Quite remarkably, variation in early season superoxide had a much more pronounced effect on late season telomere length than did late season superoxide, with the model explaining 67% of the variation in February telomere length (Table 1c).

3.5  Mitochondrial content as a driver of superoxide

Late, but not early, season mitochondrial content covaried with late season superoxide (Table 2a,b, Figure 2a,b). Partial correlation analyses reconfirmed that only late season values correlated significantly when an outlier observation was removed (see figure legend; $r_{\text{partial}} = 0.477, p < 0.0001, N = 73$, the corresponding partial correlation between early mitochondrial content and late season superoxide did not reach significance when late season superoxide was held constant, $r_{\text{partial}} = 0.20, p = 0.093, N = 73$; Figure 2a,b).

3.6  Shaping telomeres: the combined effects of superoxide and DNA repair

To more comprehensively visualize the combined effects of superoxide (negative) and DNA repair (positive), we plotted their effects on telomere length at the end of the mating season close to the death of these annual lizards (Figure 3: response surface plot based on Proc g3grid and Proc g3d with a spline function in SAS 9.4). The spline function “smooths” the plot to help reveal the complex relationship between early season variation in superoxide, an estimate of DNA repair following several months exposure to superoxide, and end of season telomere length (near end of life). The plot is descriptive and not directly associated with a statistical analysis. The separate data plots for superoxide and DNA repair effects on February telomere lengths can be found in Figure 4 and 5 (Table 1c analysis).

3.7  Congruence between qPCR and flow FISH

Finally, we confirmed congruence between estimates of telomere length early in the season using Flow FISH and qPCR, an analysis that validates the similarity and robustness of the two techniques ($r = 0.521, p < 0.0001, N = 71$; Figure 6).

4  DISCUSSION

Recent research has shown complex interactions between reactive molecules, oxidative stress and telomere dynamics (Angelier, Costantini, Blévin, & Chastel, 2018; Haussmann & Marchetto, 2010). One contributing factor to telomere shortening under oxidative stress is an associated rapid and sustained decrease in telomerase activity, the telomere restoring enzyme. Thus, repair of the telomere sequence is compromised under physiological stress and telomere shortening is expected in stressed organisms (Kurz et al., 2004). We show significant positive (but modest) effects of DNA repair on telomere length, which supports the importance of DNA repair systems for restoring telomere length (our test is, logically, directional and one-tailed, whereas a two-tailed test incorporating an
unexpected DNA repair “erosion” effect on telomere length would not be significant). Once the telomeric sequence is repaired, a regained ability to hybridize is expected, and, as a consequence, more sequence repeats identified by Flow FISH (i.e., longer telomeres). This appears to be what is observed.

DNA damage repair response is repressed at intact telomeres, which slows down the cell cycle and further reduces repair efficiency (Ahmed & Lingner, 2018). In vitro experiments suggest that telomeric DNA is particularly reactive with ROS; double-stranded telomeric DNA is more susceptible to cleavage by ROS than non-telomeric DNA (Oikawa & Kawanishi, 1999). Similarly, production of 8-oxo-G (as measured in this study) was more prevalent in telomeric than in non-telomeric DNA with the same nucleotide content
from in vitro studies, that link ROS and oxidative stress to telomere shortening, these relationships are likely to fundamentally vary in vivo. Differences are, for example, expected between the sexes, across age categories, or between alternative morphs depending on variation in life history strategies and other potentially group-specific physiological factors, such as innate antioxidant production (Olsson, Tobler et al., 2012; Stauffer, Panda, & Ilmonen, 2017).

Another important aspect is to what extent (near) simultaneous sampling of ROS and telomere length (and, thus, oxidative stress) is informative with regards to telomere erosion and long-term life history consequences. Boonekamp et al.’s (2017) showed that telomere length was highly repeatable between days five and 30 in Jackdaws (Aves, Corvus monedula) and unrelated to six oxidative stress markers sampled at the same time. At first, this result seems intuitive, given that a ROS molecule is quickly neutralized by antioxidants (Halliwell & Gutteridge, 2015). However, the role of free radicals in the context of oxidative stress and disease, such as cancer, paints a very different picture with regards to long-term effects of ROS exposure. Our data corroborate this work, showing long-term implications of ROS on a short-lived lizard species, with superoxide having predictive effects on telomere length through at least some 70% of life span. These effects could mechanistically have a considerable role in life history traits and trade-offs, and be set early in life with predictive power for life (in agreement with e.g., results by Heidinger et al., 2012). Our data suggest that when mitochondrial content is higher, so, too, is superoxide. However, the strongest relationship between superoxide and telomere length is via high superoxide early in life, an effect that is carried through late into the season. Late in life, simultaneously sampled superoxide was more weakly related to telomere length, as indicated by a higher parameter estimate, that is, late season superoxide predicted a less steep decline in late season telomere length than did early season superoxide. Clearly, the time difference in sampling between superoxide and telomere length has only strengthened the ability to detect effects over a life time.

An interesting aspect of these discoveries is the significant morph difference in mitochondrial content early in the mating season, paired with the much larger variance in superoxide early in the mating season compared to when matings have ceased. This speaks of differences in reproductive strategies. As expected, superoxide covaries with mitochondrial content, but how this is related to among-morph differences in mitochondrial content and superoxide is incompletely understood. We know that: (a) red males are more aggressive and have higher testosterone levels; (b) yellow males are more subdued but have higher success in sperm competition; (c) there are confirmed differences in metabolic rate among morphs, and (d) the orange morph has been described as some intermediate between the red and yellow (Friesen, Wilson et al., 2017; McDiarmid, Friesen, Ballen, & Olsson, 2017; Olsson, Healey, & Astheimer, 2007; Olsson, Healey et al., 2012; Olsson, Healey, Wapstra et al., 2007; Olsson, Schwartz, Uller, & Healey, 2009; Olsson, Schwartz, Uller, & Healey, 2009; Olsson, Wilson et al., 2008). Lastly, recently a “blueish” morph was described that is behaviourally uncharacterized and does not quite fit into the “biochemical continuum” along the yellow-red morphs, perhaps due to lack of carotenoids or other “red/yellow” integumental pigments (Friesen, Wilson et al., 2017). In short, there is currently not a good explanation as to why orange males have the highest mitochondrial content early in the season. But—importantly—mitochondrial content is not the only determinant of ROS production. Other factors, such as production of innate antioxidants (like superoxide dismutase, catalase, urea; Halliwell & Gutteridge, 2015; Olsson, Healey et al., 2012), immunological ROS burst activities (to counter microbial insult; Tobler, Healey, Wilson, & Olsson, 2011), morph-, age-, and dominance-dependent stress and even neighbour morphotype (Olsson, Healey, Wapstra et al., 2007) may all contribute to the dynamics of superoxide, other reactive molecules and overall physiological stress (Costantini, 2014).

In previous work, we were only able to address some of these aspects, and logistic constraints (such as available plasma
volumes in small animals) make it impossible to simultaneously sample all desired parameters. In the current work, it would have been particularly interesting to also assess morph-specific levels of superoxide dismutase, since we know these levels at least differ markedly (4-fold) between the sexes, and perhaps also between morphs (Olsson, Healey et al., 2012). We hypothesize that further studies will detect balancing effects between mitochondrial content, superoxide and superoxide dismutase production, net telomere dynamics, and—perhaps—ultimately ageing and lifespan.

To make things even more complex, an evolutionary perspective calls for an assessment of regulatory effects of phenotypic traits of interest at a level of ongoing selection and evolution. Ideally, we would base these assessments on variation in individuals’ lifetime reproductive success, that is, the product of life span and reproduction. This would be greatly facilitated by working on a short-lived animal, like painted dragons (Olsson, Healey, Wapstra et al., 2007). However, sometimes not even exact knowledge of time of death will tell a complete telomere-based tale of age-related selection; many organisms, such as painted dragons, store sperm for substantial time and through multiple ovarian cycles, and hence males can sire offspring posthumously (Olsson, Schwartz et al., 2009). This clouds any association between life span and fitness: male telomere length at the end of life, some 6 months after copulation, would be under rudimentary ongoing selection if his sperm successfully keeps siring offspring in his absence (Olsson, Healey, Wapstra et al., 2007).

In our previous work (Olsson, Healey, Wapstra et al., 2007), we demonstrate that estimates of reproductive success (using molecularly assigned paternity) are approximately the same for all morphs in the wild (which may explain maintenance of polymorphism through time). The current study adds further complexity to this scenario by showing morph-independent, mechanistic effects of superoxide, which are predicting shorter telomeres, to some degree countered by DNA repair.

To conclude, future studies would benefit from continued work on short-lived organisms, in which ageing, life span and reproduction, can be estimated, but with animals still being large enough to permit plasma sampling sufficient to quantify “all” pieces in the “oxidative stress-repro-life-span” puzzle. We wish our colleagues the best of luck with finding these next generation Darwinian demons.

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**DATA ACCESSIBILITY**

Dryad: doi:10.5061/dryad.538044t.

**CONFLICT OF INTERESTS**

The authors have no conflict of interests to declare.

**AUTHOR CONTRIBUTIONS**

The study was conceived by M.O., C.R.F. and M.W. The molecular analysis was conducted by N.R., J.S. and C.M.W. The work was supervised by N.R., J.S. and C.M.W. The statistical analyses were performed by M.O. The literature was surveyed by M.O. and W.R.L. The manuscript was written M.O. and W.R.L. All authors read, reviewed and commented on the manuscript before submission.

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