Sex-Specific Associations between Telomere Dynamics and Oxidative Status in Adult and Nestling Pied Flycatchers

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ABSTRACT

Oxidative stress can contribute to an acceleration of telomere erosion, leading to cellular senescence and aging. Increased investment in reproduction is known to accelerate senescence, generally resulting in reduced future reproductive potential and survival. To better understand the role played by oxidative status and telomere dynamics in the conflict between maintenance and reproduction, it is important to determine how these factors are related in parents and their offspring. We investigated the relationship between oxidative status and telomere measurements in pied flycatchers (*Ficedula hypoleuca*). Total antioxidant status (TAS) in plasma, total levels of glutathione in red blood cells (RBCs), and oxidative damage in plasma lipids (malondialdehyde [MDA]) were assessed in both parents and nestlings. Telomeres were measured in RBCs in adults. Our results showed sex differences in oxidative variables in adults that are likely to be mediated by sex steroids, with testosterone and estrogens increasing and reducing, respectively, the production of reactive oxygen and nitrogen species. We found a negative association between telomere length (TL) and MDA in adults in the previous season. Moreover, TL was positively associated with TAS in females, while telomere shortening (ΔTL) correlated positively with MDA in males in the current year. These associations could be reflecting differences between sexes in reproductive physiology. We found a positive correlation between parental ΔTL and nestling MDA, an example of how parental physiological aging could affect offspring quality in terms of oxidative stress that highlights the constraints imposed by higher rates of ΔTL during reproduction and rearing.

Keywords: Ficedula hypoleuca, oxidative stress, telomere length, telomere shortening.

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Introduction

Oxidative stress is defined as the breakdown in the equilibrium between antioxidant defenses and the generation of prooxidants, which leads to oxidative damage to biomolecules (Finkel and Holbrook 2000; Halliwell and Gutteridge 2015). Oxidative stress may have important health-related implications (Ames et al. 1993) that affect several fitness-related traits and shape animal life-history evolution (Costantini 2008; Dowling and Simmons 2009; Monaghan et al. 2009; Costantini et al. 2010; Metcalfe and Alonso-Alvarez 2010). In addition to its effect on macromolecules, oxidative stress could increase senescence rates through its effect on telomere erosion, which ultimately leads to cellular senescence and aging (Von Zglinicki 2002; Monaghan and Haussmann 2006; Aubert and Lansdorp 2008).

Telomeres occur at the ends of the linear chromosomes of most eukaryotes. They are specialized nucleoprotein structures consisting of highly conserved noncoding sequence. Together with various associated proteins, telomeres cap the chromosome ends (Blackburn 1991; Armanios and Blackburn 2012), preventing chromosome degradation and maintaining genome stability during cell division. In addition to this protective role of coding DNA, telomeres are also thought to play a central role in the regulation of chromosome segregation during both mitosis and meiosis (Aubert and Lansdorp 2008). In the absence of restoration and repair processes, telomere length declines with each cell division; the amount of telomeric DNA lost per round of cell division can be increased by certain factors, most notably oxidative stress (Von Zglinicki 2002).

Telomeres of a critically short length have been associated with premature aging syndromes and reduced survival (Bauch et al. 2013). It has been reported previously in avian studies that birds show somatic telomere shortening that has been linked to longevity, as in the case of Seychelles warblers (*Acrocephalus*

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sechellensis; Barrett et al. 2013) and zebra finches (*Taeniopygia guttata*; Heidinger et al. 2012). However, telomere length has been suggested to reflect an individual's biological state rather than its chronological age (Bauch et al. 2013). Studies in the field and in the laboratory have shown that increased investment in reproduction can accelerate senescence and that telomeres may constitute a link between reproductive investment and its fitness consequences (Monaghan and Haussmann 2006; Kotrschal et al. 2007; Bauch et al. 2013). When reproductive investment exceeds what is sustainable for parents, the costs, in terms of decreased longevity, may become apparent through accelerated aging (Santos and Nakagawa 2012; Badás et al. 2015). Thus, studies increasing brood size in a range of organisms have shown that costly reproductive events may have a negative effect on telomere dynamics for breeding adults (Reichert et al. 2014) and developing offspring (Nettle et al. 2013; Boonekamp et al. 2014; Herborn et al. 2014). To better understand the role played by oxidative status and telomere dynamics in mediating the conflict between maintenance and reproduction, one important challenge is to determine how telomere shortening and oxidative stress are related to each other. Moreover, since telomeres are linked to aging processes, they should then also be associated with other variables that change with aging, especially foraging and reproductive behaviors (Young et al. 2015), and they could modulate the trade-offs between maintenance and reproductive investment (Reichert et al. 2014). Thus, intergenerational effects of these interlinked processes might influence fitness via effects on offspring health and longevity. In addition, it has been shown that males and females respond differently to reproduction and stressful conditions in terms of oxidative stress and telomere dynamics (Kotrschal et al. 2007; Young et al. 2013; López-Arrabé et al. 2016). Understanding these physiological sex-specific costs is essential to fully appraise these life-history trade-offs.

In this study, we investigated the relationship between oxidative status and telomere length in breeding adults of the two sexes in short-lived altricial birds and, using longitudinal data, estimate the rate of telomere shortening. In addition, we explored possible cross-generational associations between telomere length and telomere shortening measured in adults and oxidative status in offspring. For these purposes, during two reproductive seasons we collected data from adults of known age and their nestlings of a pied flycatcher (*Ficedula hypoleuca*) population breeding in central Spain.

To evaluate the oxidative status of individuals in this study, we obtained measures of both oxidative damage and antioxidant defenses. We measured levels of plasma malondialdehyde (MDA), a by-product of lipid peroxidation (Halliwell and Gutteridge 2015), as a proxy of oxidative damage (Mateos et al. 2005; Halliwell and Gutteridge 2015; Sepp et al. 2012). To monitor antioxidant defenses, we used two independent markers: total antioxidant status (TAS) in plasma and total glutathione (GSH) levels in red blood cells (RBCs). Telomere length was measured in RBCs. Uric acid in plasma is correlated with TAS values, so we used this biochemical parameter to control this antioxidant biomarker. We examined the relationship between telomere dynamics (length and shortening) and oxidative status. We predicted that adults with shorter telomeres or higher rates of telomere shortening would (i) show higher levels of oxidative stress and/or (ii) produce nestlings with increased levels of oxidative stress (higher levels of oxidative damage and/or reduced antioxidant defenses).

Methods

General Field Methods

The study was conducted during the springs of 2013 and 2014 in a montane forest of Pyrenean oak (*Quercus pyrenaica*) at 1,200 m asl in Valsaín, central Spain (40°54′N, 4°01′W), where long-term studies of cavity-nesting birds have been ongoing since 1991. In the study area there are 570 nest boxes (for dimensions, structure, and placement of nest boxes, see the appendix in Lambrechts et al. 2010) occupied by pied flycatchers, great tits (*Parus major*), nuthatches (*Sitta europaea*), and blue tits (*Cyanistes caeruleus*).

We followed breeding activities from nest construction to fledging in nest boxes occupied by pied flycatchers. Egg laying in our population of pied flycatchers begins in late May, as previously described (Sanz and Moreno 1995). Females lay on average six eggs, and chicks usually fledge at the age of 17 d. Adult males and females were captured in their nest boxes with traps while provisioning nestlings of 7–8 d, ringed if necessary or identified, and weighed to the nearest 0.01 g with a digital balance. From every adult bird we obtained a blood sample, collected in heparinized microcapillaries, of about 120 μL from the brachial vein ($N = 62$ samples from 31 individuals [19 females and 12 males] captured in 21 nest boxes in 2013 and recaptured in 25 nest boxes in 2014). The whole procedure took less than 5 min after capture. Blood samples were stored in Eppendorf tubes in an icebox until they were processed in the laboratory on the same day. Plasma was separated from blood by centrifugation (10 min at 12,000 rpm), and both fractions were stored at −80°C until analyzed for MDA, TAS, and uric acid in plasma and total GSH and telomere length in RBCs (see below). Because the total volume of some plasma samples was not sufficient to carry out all biochemical analyses, we prioritized the assays as follows: MDA, TAS, and uric acid levels. This explains why sample sizes for assays differ. Since hemolysis can induce an efflux of intracellular prooxidants and antioxidant molecules into plasma, it can confound levels of oxidative markers measured in plasma samples. One observer (J. López-Arrabé) therefore assessed the degree of hemolysis by visual inspection of plasma redness in a continuous gradient from 0 (no hemolysis) to 2 (high degree of hemolysis).

In both years, on day 13 (hatching date = day 1) nestlings from studied adults were ringed, weighed, and measured, and a blood sample was collected following the same protocol as for adults. In 2013 we collected blood from two randomly selected nestlings of each nest ($N = 40$ nestlings from 21 nest boxes), whereas in 2014 we collected blood samples from all chicks in the nest ($N = 74$ nestlings from 14 nest boxes).

Adults ranged from 1 to 5 yr old. The exact age of most adults was known, as they had been ringed as nestlings in the

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study area. To estimate the age on first capture of those individuals that were not raised in the study area, we assumed a conservative age of 2 yr on the basis of patterns of age at first reproduction observed in birds of exactly known age (Potti and Montalvo 1991).

Lipid Peroxidation Assays

Plasma concentrations of MDA were calculated as described in López-Arrabé et al. (2014*b*). In brief, a standard curve was prepared for calibration using a 1,1,3,3-tetraethoxypropane stock solution serially diluted in 40% ethanol. Butylated hydroxytoluene, phosphoric acid, and thiobarbituric acid (TBA) solutions were added to each plasma sample and standard. Samples were then incubated on a dry bath to allow formation of MDA-TBA adducts. After that, pure *n*-butanol was added to each sample and standard. Tubes were vortexed and centrifuged, and the upper phase was collected and transferred into a high-performance liquid chromatography (HPLC) vial for analysis. Samples were injected into an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA). Data were collected using a fluorescence detector (G1321A; Agilent Technologies). Repeatability (following Lessells and Boag 1987), calculated on a set of samples assayed in duplicate, was high ($r = 0.722$, $N = 66$, $P < 0.001$). Interassay coefficient of variation (CV) was 8.08%.

Total Antioxidant Status

TAS measures the capacity of plasma samples to counteract a redox reaction induced by free radicals (Miller et al. 1993; Cohen et al. 2007) and is primarily the result of the pooled effect of all extracellular antioxidant compounds of the blood (Costantini 2011). TAS was analyzed as described in López-Arrabé et al. (2014*b*). As standard for the assays we used Trolox (a water-soluble α-tocopherol derivative), and TAS levels are expressed in Trolox-equivalent units. The assays were run on a Synergy HT multimode microplate reader (BioTek Instruments, Winooski, VT). To accurately control the reaction time, only one column of the plate was used at a time. To standards and samples were sequentially added metmyoglobin (a mix of equal volumes of myoglobin [M0630-250MG; Sigma-Aldrich, St. Louis, MO] and potassium ferricyanate), ABTS (the chromogen 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid), and H_2O_2, which started the reaction. Kinetic measurements were immediately started, recording absorbance at 660 nm every 5 s. The temperature was maintained at 37°C during assays. All samples were assayed in duplicate, and results showed high repeatability ($r = 0.981$, $N = 234$, $P < 0.001$) and an interassay CV of 3.63%.

Intracellular Total GSH Level

GSH is a tripeptide thiol functioning in the protection of cells against free radicals and is often considered the most important endogenous antioxidant (Meister 1991; Wu et al. 2004). Total GSH levels in RBCs were determined as described in López-Arrabé et al. (2014*a*) and Romero-Haro and Alonso-Alvarez (2015). In brief, RBC samples were diluted and homogenized in a stock buffer and mixed with trichloroacetic acid. The mixture was vortexed and centrifuged, and the supernatant was separated. The next steps were performed on a Synergy HT multimode microplate reader (BioTek Instruments). To samples (supernatant) we added a mixture of NADPH (nicotinamide adenine dinucleotide phosphate) and DTNB (5,5-dithio-bis-(2-nitrobenzoic acid)). Then a GSH reductase solution was added after 15 s, and the absorbance at 405 nm was monitored after 15 and 45 s. The change in absorbance was used to determine the intracellular total GSH concentration by comparing the output with the results from a standard curve generated by serial dilution of GSH. The assays were performed at 37°C, and only one column of the plate was used at a time in order to control reaction times accurately. A set of samples assayed in duplicate showed high repeatability ($r = 0.983$, $N = 106$, $P < 0.001$) and an interassay CV of 3.89%.

Measurement of Uric Acid Levels

TAS levels can be affected by the concentration of uric acid, which may confound the interpretation of this antioxidant biomarker (Cohen et al. 2007; Costantini 2011). Uric acid is the main form of nitrogen excretion in birds and its levels increase quickly after feeding and during fasting (Alonso-Alvarez and Ferrer 2001), but it is also a powerful antioxidant that is frequently positively related to TAS values (Cohen et al. 2007; Hõrak et al. 2007; Pérez-Rodríguez et al. 2008). Nevertheless, whether uric acid is actively regulated in response to oxidative stress is still unclear (Romero-Haro and Alonso-Alvarez 2014) because its variability is often explained by amino acid catabolism or inflammation (Sautin and Johnson 2008). Therefore, we also quantified this plasma metabolite to statistically control for its effect in the analyses. Following previous studies in birds (e.g., Pérez-Rodríguez et al. 2008; Romero-Haro and Alonso-Alvarez 2015), plasma levels of uric acid were measured using commercial kits (11522; Biosystems, Barcelona, Spain) based on the uricase/peroxidase method (Fossati et al. 1980). Analyses were run in 96-well plates using the same microplate reader mentioned before. A total of 150 μL of the chromogen was added to 5 μL of each plasma sample or the standard (a 6 mg/dL uric acid solution). Plates were incubated for 5 min at 37°C, and absorbance was subsequently measured at 520 nm. A subset of samples assayed in duplicate showed high repeatability ($r = 0.99$, $N = 45$, $P < 0.001$). Interassay CVs were 2.79%.

Telomere Analysis

Blood is a good tissue for measuring avian telomere lengths because erythrocytes are nucleated and thus allow for large amounts of DNA to be obtained from small samples (Barrett et al. 2013). Genomic DNA was extracted from RBCs using the Macherey-Nagel Nucleospin Blood Kit (Macherey-Nagel, Düren, Germany) by resuspending 15 μL of RBCs in 185 μL of

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phosphate-buffered saline and following the manufacturer's protocol for DNA purification from whole blood. The concentration and purity of DNA samples were assessed using a NanoDrop 8000 spectrophotometer (Thermo Scientific), with all samples having $A_{260/280}$ and $A_{260/230}$ ratios >1.7 and 1.9, respectively. DNA samples were stored at −20°C. Relative telomere measurements were made using the quantitative polymerase chain reaction (qPCR) methods described by Criscuolo et al. (2009) with some modifications. All PCRs were carried out on a Mx3500 qPCR system (Agilent Technologies). Telomere length was measured as the ratio (T/S) of telomere repeat copy number (T) to control, single gene copy number (S) relative to a reference sample. The control, single copy number gene needs to have the same copy number among individuals in the population and within individuals over time. Here we used 18S as a nonvariable copy number gene (Voillemot et al. 2012). Forward and reverse telomere primers were 5′-CGGTTTGTTTGGGTTTGGGTTTGGGTTT GGGTTTGGGTT-3′ (Tel-1b) and 5′-GGCTTGCCTTACCCTT ACCCTTACCCTTACCCTTACCCT-3′ (Tel-2b), respectively, and forward and reverse 18S primers were 5′-GAGGTGAAATTCT TGGACCGG-3′ and 5′-CGAACCTCCGACTTTCGTTCT-3′, respectively. Telomere primers (Tel-1b and Tel-2b) were used at a final concentration of 500 nM, and 18S primers (forward and reverse) were used at a final concentration of 50 nM. DNA samples (10 ng) were assayed using the ABsolute Blue qPCR SYBR Green Low ROX master mix (Thermo Scientific). The telomere thermal profile was 15 min at 95°C followed by 27 cycles of 15 s at 95°C, 30 s at 58°C, and 30 s at 72°C. The 18S thermal profile was 15 min at 95°C followed by 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C. Both assays were followed by melt curve analysis of 58°–95°C 1°C/5-s ramp. A reference sample was serially diluted (from 40 to 2.5 ng/well) to produce a standard curve for each plate. This was used to calculate plate efficiencies, all of which fell within the acceptable range (mean ± SE: telomere, 106.56% ± 2.10%; 18S, 99.35% ± 9.76%), and only samples that fell within the bounds of the standard curve were included. One individual repeatedly fell outside the standard curve and was excluded from the analysis. Each sample was assayed in triplicate, and the mean of the three replicates was used. The telomere and 18S reactions were carried out on separate plates, and in both reactions the number of PCR cycles (C_t) required for the products to accumulate enough fluorescent signal to cross a threshold was determined. Individuals with relatively short telomeres are characterized by high C_t values, whereas those with relatively long telomeres are characterized by low C_t values (Cawthon 2002). The average intraplate variation of the C_t value was 2.05% for the telomere assays and 0.60% for the 18S assays, and the average interplate variation of the ΔC_t was 2.24% for the telomere assays and 0.91% for the 18S assays. R^2 values calculated from the reference curves of the qPCR runs were >0.99 for both the telomere assays and the GADPH assays. The mean CV for the T/S ratios was 20%. From these analyses we obtained three measures: initial relative telomere length measured in 2013 ($TL_{initial}$), telomere length in 2014 (TL_{2014}), and telomere shortening (ΔTL, the difference between TL_{2014} and $TL_{initial}$).

Statistical Analyses

To statistically control for potentially confounding effects, we explored the effect of the degree of hemolysis on the oxidative stress parameters of both nestlings and adults. In all cases hemolysis affected MDA levels (all $P \leq 0.05$). In these cases we controlled MDA levels, entered as an independent variable, by the degree of hemolysis in all analyses. Neither TAS nor GSH levels were affected by the degree of hemolysis (all $P > 0.05$). However, since uric acid has been shown to affect plasma TAS measures (Cohen et al. 2007; Pérez-Rodríguez et al. 2008), this marker of antioxidant capacity was always controlled for uric acid levels when entered into the models. In addition, we analyzed the relationships of telomere measures and oxidative variables with sex, age, and $TL_{initial}$ of adult individuals.

We ran independent models for two telomere measures (TL_{2014} and ΔTL of adult individuals) as dependent variables and oxidative stress parameters (MDA, TAS, and GSH levels of adults and nestlings) measured in 2013 and 2014 as independent variables. Analyses were run separately for the two study years. In all cases we controlled for laying date, clutch size, and age of adults by including them as covariates. Sex of adults and the interaction of sex with oxidative variables were included as fixed factors. The rate of telomere shortening was calculated as the difference in relative telomere lengths between 2013 and 2014. More positive values meant higher rates of telomere shortening. To account for random variation in the starting telomere length among individuals, $TL_{initial}$ was also included as a covariate in all models. To avoid statistical artifacts due to the regression to the mean, the measure of ΔTL was corrected for this phenomenon following the equation suggested by Verhulst et al. (2013).

In all cases we used mixed models by means of PROC MIXED in SAS 9.3 and restricted maximum likelihood. Degrees of freedom were estimated using the Satterthwaite approximation, thus avoiding possible pseudoreplication. All dependent variables were normally distributed. Given that some females and males were paired, nest was included as a random factor and individuals were used as a sample unit. To obtain minimal adequate models, we elaborated full saturated models including in each case the factors mentioned above, and we sequentially removed the less significant terms following a standard backward stepwise procedure until only significant variables were retained.

Results

There were no significant associations between telomere measures (TL_{2014} and ΔTL) and sex (all $P > 0.10$) or age (all $P > 0.40$) among adults. ΔTL, but not TL_{2014}, was significantly and positively associated with $TL_{initial}$ (for ΔTL: $F_{1,26} = 54.60$, $P < 0.001$; for TL_{2014}: $F_{1,26} = 0.03$, $P = 0.864$). Oxidative stress parameters (MDA, TAS, and total GSH) were not associated with either $TL_{initial}$ (all $P > 0.09$) or age of adults (all $P > 0.19$). In 2014, MDA levels were significantly higher in males than in females ($F_{1,8} = 6.89$, $P = 0.030$; fig. 1*a*), while TAS values were significantly higher in females than in males ($F_{1,4.5} = 21.91$,

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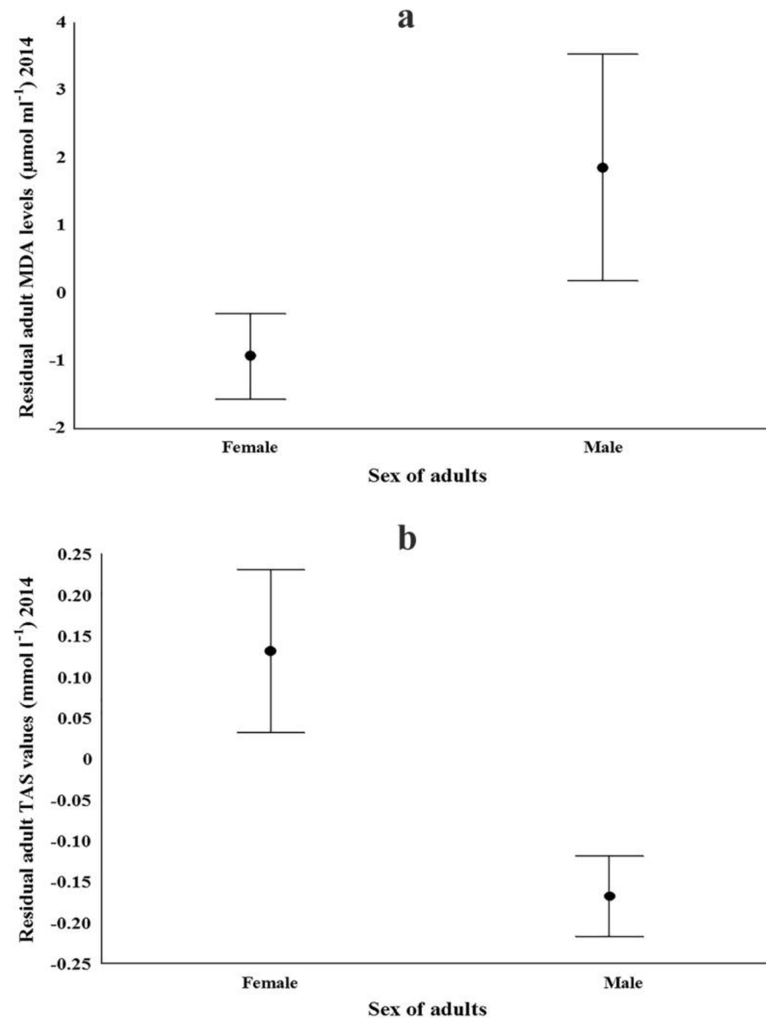

Figure 1. Differences (mean ± SE) between breeding females and males of pied flycatchers (*Ficedula hypoleuca*) in 2014. *a*, Oxidative damage (malondialdehyde [MDA] residuals after controlling for degree of hemolysis). $R^2 = 0.14$. *b*, Total antioxidant status (TAS; residuals after controlling for uric acid levels). $R^2 = 0.25$.

$P = 0.007$; fig. 1*b*). The remaining oxidative stress variables did not differ between sexes (all $P > 0.06$).

TL_{2014} was correlated with adult MDA levels in 2013, independently of sex (table 1; fig. 2), but it was affected by a significant interaction between TAS and sex of adults in 2014 (table 1; fig. 3). Moreover, TL_{2014} was positively correlated with laying date in 2014 ($F_{1,11} = 13.30$, $P = 0.004$). ΔTL was significantly affected by an interaction between MDA levels and sex of adults in 2014 (table 1). This association was positive and significant for males ($F_{1,1} = 819.45$, $P = 0.022$), while ΔTL was not significantly correlated with MDA levels in females ($F_{1,7} = 0.818$, $P = 0.396$). Moreover, laying date and clutch size in 2014 were positively correlated with ΔTL (for laying date: $F_{1,11} = 44.50$, $P < 0.001$; for clutch size: $F_{1,11} = 30.74$, $P < 0.001$).

Maternal and paternal TL_{2014} were not correlated with nestling oxidative stress variables (table 2). Independently of sex, ΔTL in adults was significantly and positively associated with MDA levels of their nestlings in 2014 (table 2; fig. 4). We also found positive associations between ΔTL and laying date and between ΔTL and clutch size (for laying date: $F_{1,12} = 40.85$, $P < 0.001$; for clutch size: $F_{1,12} = 25.75$, $P < 0.001$) as well as a difference in ΔTL between sexes of adults, which was weakly higher in females than in males ($F_{1,12} = 5.62$, $P = 0.035$). Moreover, ΔTL was affected by a significant interaction between MDA levels of nestlings in 2014 and sex of adults (table 2). This association was positive for both sexes, being nonsignificant for males and females separately (for males: $F_{1,3} = 6.973$, $P = 0.078$; for females: $F_{1,7} = 0.908$, $P = 0.372$).

Discussion

We have conducted an observational study to establish associations between telomere measures in breeding individuals and sex, age, laying date and clutch size, and oxidative status of parents and nestlings in a pied flycatcher population. Our results

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Table 1: Summary of mixed models analyzing associations of telomere length measured in 2014 (TL_{2014}) and telomere shortening (ΔTL) with oxidative variables of adult individuals in 2013 and 2014 in pied flycatchers (*Ficedula hypoleuca*)

	TL_{2014}		ΔTL	
	F (df)	*P*	*F* (df)	*P*
2013:				
MDA	4.39 (1, 22)	.048*	3.65 (1, 18.5)	.072
MDA × sex	.36 (1, 16)	.558	.036 (1, 16)	.558
TAS	.54 (1, 14)	.476	.54 (1, 14)	.476
TAS × sex	.28 (1, 13)	.607	.28 (1, 13)	.607
Total GSH	3.03 (1, 25)	.094	2.62 (1, 22)	.119
Total GSH × sex	.93 (1, 23)	.346	.83 (1, 20)	.372
2014:				
MDA	.97 (1, 10)	.349	1.08 (1, 11)	.322
MDA × sex	1.12 (2, 13)	.356	3.98 (2, 11)	.049*
TAS	1.91 (1, 11)	.195	2.62 (1, 10)	.137
TAS × sex	4.28 (2, 11)	.042*	.14 (1, 7)	.716
Total GSH	.58 (1, 24)	.454	.28 (1, 21)	.605
Total GSH × sex	.00 (1, 18)	.956	.00 (1, 18)	.956

Note. All statistical tests were controlled for laying date, clutch size, sex and age of adults, and initial telomere length. Nest was included as random factor. GSH = glutathion; MDA = malondihaldehyde; TAS = total antioxidant status.

*Significant difference ($\alpha = 0.05$).

showed sex differences in levels of oxidative damage and antioxidant capacity in breeding adults. We found a negative association between telomere length and oxidative damage shown by adult individuals in the previous season. Moreover, we found that telomere length was positively associated with antioxidant defenses in breeding females, while telomere shortening was positively correlated with oxidative damage in males in the present year. Both sexes showed a positive correlation between telomere shortening and nestling MDA levels, although the correlation was significant only for breeding females.

Sex-specific telomere length has been documented in many taxa (Barrett and Richardson 2011; Olsson et al. 2011; Young et al. 2013), particularly in species with a large dimorphism in body size and often related to differences in growth trajectories. In the case of pied flycatchers, sexual dimorphism is mainly based on nuptial plumage, being very subtle in body size (Lundberg and Alatalo 1992). This could explain the lack of differences in telomere measures between the two sexes in this species.

For common terns (*Sterna hirundo*), it has been previously reported that individuals with shorter telomeres arrive and start breeding earlier in the season and raise larger broods. This association is explained by individual consistency in reproductive performance, implying that consistently successful individuals pay a cost in terms of telomere shortening (Bauch et al. 2013). Here we found that telomere shortening was higher for those individuals that had larger clutches and that laid their eggs later in the present year. Larger broods and later broods may be more costly to raise (e.g., Moreno et al. 1998; Alonso-Alvarez et al. 2004; Arnold et al. 2004; Costantini et al. 2014; Reichert et al. 2014; but see López-Arrabé et al. 2016). Moreover, these associations were significant only in 2014, and the lack of a relationship between telomere shortening and past reproductive performance may indicate that in this case current conditions are more important in telomere dynamics.

The negative association between telomere length and MDA levels in breeding individuals in the previous season could imply that oxidative conditions during past reproductive events may be particularly important in determining telomere length in adults. Thus, individuals with higher levels of oxidative damage would

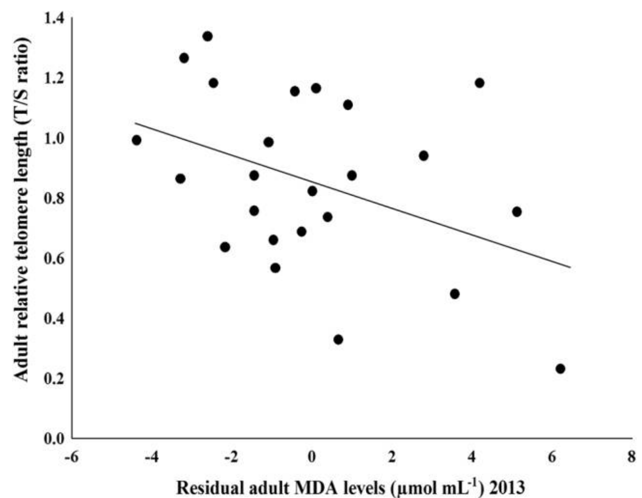

Figure 2. Association between relative telomere length and oxidative damage levels (malondialdehyde [MDA] residuals after controlling for degree of hemolysis) in adult pied flycatchers (*Ficedula hypoleuca*) in 2013 (least squares linear regression: $F_{1,22} = 4.39$, $P = 0.048$, $R^2 = 0.17$).

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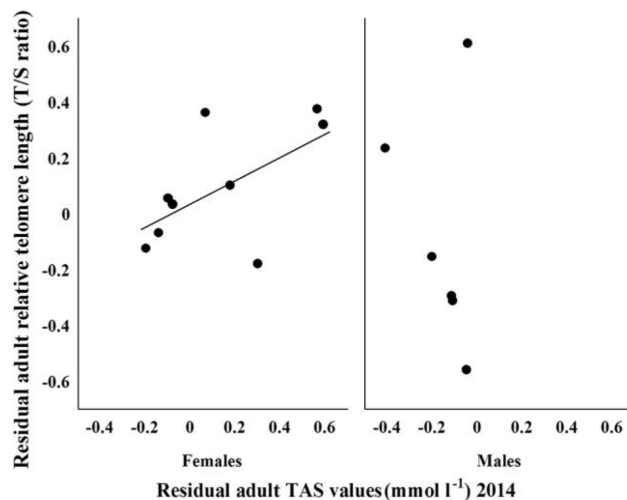

Figure 3. Association between relative telomere length (residuals after controlling for laying date) and total antioxidant status (TAS; residuals after controlling for uric acid levels) in adult female and male pied flycatchers (*Ficedula hypoleuca*) in 2014 (least squares linear regression: for females, $F_{1,6} = 9.075$, $P = 0.024$, $R^2 = 0.35$; for males, $F_{1,3} = 0.133$, $P = 0.739$, $R^2 = 0.06$).

suffer greater rates of telomere loss, modulating their length. Moreover, the positive relationship found between telomere length and TAS values in breeding females in the same year suggests an important protective role for the antioxidant barrier on telomere integrity. Likewise, those males showing higher oxidative damage—that is, higher MDA levels—in the current season suffered greater telomere shortening rates, which is consistent with oxidative stress being involved in accelerated telomere shortening and aging (Finkel and Holbrook 2000). Telomeric DNA is rich in guanine and particularly susceptible to oxidative damage. There is previous evidence that oxidative stress accelerates telomere shortening (Von Zglinicki 2002; Houben et al. 2008; but see Nettle et al. 2015). Sex differences in these associations could result from the differences in oxidative stress levels shown by females and males. Such a difference between sexes in redox status during the breeding season is likely to be mediated by sex steroids, with testosterone and estrogens increasing and reducing, respectively, the production of reactive oxygen and nitrogen species (Gupta and Thapliyal 1985; Viña et al. 2005), which could in turn affect levels of antioxidant defenses and oxidative damage. In addition, males and females experience different life-history trade-offs, likely resulting in variable strategies to deal with redox homeostasis (Costantini 2008; Metcalfe and Alonso-Alvarez 2010). Higher antioxidant levels in females may reflect a higher investment in cellular maintenance or, alternatively, an upregulation in response to higher cellular reactive oxygen species during chick rearing (Alonso-Alvarez et al. 2004; Wiersma et al. 2004; Isaksson 2013). However, the lower MDA levels found in females seems to support the first scenario, suggesting that they are better protected against oxidative stress than males, the latter showing greater telomere shortening rates at high levels of oxidative damage. Thus, these associations could be reflecting differences between sexes in reproductive physiology, affecting redox balance (Isaksson 2013) and aging.

Table 2: Summary of mixed models analyzing associations of telomere length measured in 2014 (TL_{2014}) and telomere shortening (ΔTL) of adult individuals with nestling oxidative variables in 2013 and 2014 in pied flycatchers (*Ficedula hypoleuca*)

	TL_{2014}		ΔTL	
	F (df)	*P*	*F* (df)	*P*
Nestlings 2013:				
MDA	.75 (1, 23)	.394	.75 (1, 23)	.394
MDA × sex	.06 (1, 20)	.812	.06 (1, 20)	.812
TAS	.17 (1, 23)	.687	.17 (1, 23)	.687
TAS × sex	.39 (1, 22)	.693	.16 (1, 22)	.693
Total GSH	.02 (1, 18)	.894	.02 (1, 18)	.894
Total GSH × sex	1.34 (2, 25)	.280	1.82 (2, 22)	.186
Nestlings 2014:				
MDA	1.38 (1, 16)	.257	11.56 (1, 12)	.005*
MDA × sex	2.39 (1, 11)	.150	5.92 (1, 12)	.031*
TAS	.10 (1, 15)	.757	1.05 (1, 13)	.324
TAS × sex	.07 (1, 9)	.804	.07 (1, 9)	.804
Total GSH	.73 (1, 14)	.408	.93 (1, 12)	.353
Total GSH × sex	1.14 (2, 14)	.347	1.62 (2, 12)	.238

Note. All statistical tests were controlled for laying date, clutch size, sex and age of adults, and initial telomere length. Nest was included as random factor. GSH = glutathione; MDA = malondihaldehyde; TAS = total antioxidant status.

*Significant difference ($\alpha = 0.05$).

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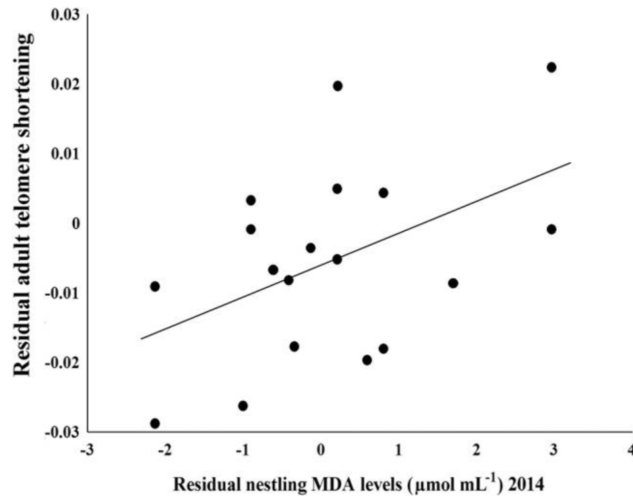

Figure 4. Association between telomere shortening in adult individuals (residuals after controlling for laying date, clutch size, and sex of adults) and oxidative damage levels (malondialdehyde [MDA] residuals after controlling for degree of hemolysis) in nestlings of pied flycatchers (*Ficedula hypoleuca*) in 2014 (least squares linear regression: $F_{1,12} = 11.56$, $P = 0.005$, $R^2 = 0.23$).

Parents can influence offspring phenotype through genetic and nongenetic effects. In iteroparous animals, reproductive performance generally changes with age (Clutton-Brock 1984), suggesting age-dependent parental effects (Torres et al. 2011). Moreover, the quality and survival of chicks might also be influenced by the deteriorating quality of both male and female germline DNA at older ages (Velando et al. 2008). The strong positive association found between parental telomere shortening and nestling MDA levels may be interpreted as an example of how parental physiological aging could affect offspring quality in terms of oxidative stress. Short telomeres in birds and humans are generally associated with poor health and/or survival, suggesting that individuals that are best at avoiding telomere-shortening processes such as oxidative stress attain the highest fitness (Bauch et al. 2013). Moreover, there is previous evidence that older parents produce offspring of low fitness (Schroeder et al. 2015). Accordingly, parents showing lower signs of cellular senescence—using telomere shortening as a proxy—could be able to invest more resources in their offspring. This could have a positive effect on offspring oxidative status, reducing their oxidative damage levels during a critical phase like early development. On the other hand, parents and nestlings share rearing and environmental conditions that could affect the association of physiological traits among relatives simultaneously, increasing telomere shortening in adults and oxidative damage in their nestlings at the same time (López-Arrabé et al. 2016).

It is particularly interesting that the association between nestling oxidative stress and parental telomere shortening arose despite the lack of association between telomere measures and the age of adults. This reinforces the idea that it is the degree of somatic senescence, rather than chronological age itself, that affects nestling development. This lack of association between age and telomere measures could indicate that telomere shortening does not occur at a constant rate with age. In fact, it has been suggested previously that the highest rate of telomere shortening takes place in early life, when somatic growth takes place (Hall et al. 2004; Salomons et al. 2009). Unfortunately, our estimates of telomere shortening were restricted to the adult age, when such a period of high growth rate has come to an end. However, we must take into account that mortality could be higher for those individuals with very rapid telomere loss, and samples of older birds would therefore be biased toward individuals with slow telomere shortening (Hall et al. 2004).

In conclusion, we have found associations between telomere dynamics and different components of oxidative status—oxidative damage and antioxidant capacity—in breeding pied flycatchers. Moreover, we have shown sex-specific covariation between these traits and some reproductive performance indicators. This study also presents novel results on the relationship between parental telomere shortening and offspring oxidative damage. Understanding the mechanisms underlying associations between aging and oxidative status in breeding birds and across generations is essential to fully understand the importance of oxidative stress and telomere dynamics in shaping individual phenotypes.

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