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Ageing and reproduction: antioxidant supplementation alleviates telomere loss in wild birds

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Abstract

Reproduction is inherently costly. Environmental stressors, such as infection and limited food resources, can compromise investment at each breeding attempt. For example, recent data on captive birds showed that increased reproductive effort accelerates ageing. However, the effects of nutritional status and infection on ageing remain unknown. Telomeres function as protective caps at the ends of eukaryotic chromosomes, and changes in telomere length is a commonly used proxy for ageing. To partially address the mechanisms of ageing following reproduction, we supplemented, medicated or administered a combined treatment to wild blue tits (*Cyanistes caeruleus*) breeding in central Spain during 2012. The nutritional supplement consisted of two different antioxidants, whereas the medication was an antimalarial treatment against blood parasites. We evaluated the effect of these manipulations on reproductive success and parasite loads in the first breeding season, and on changes in telomere length between two consecutive breeding seasons. Supplemented birds showed no reduction in blood parasite infections in 2012, although they exhibited higher body mass and fledging success. The antimalarial drugs reduced infections by several parasite species, but this had no effect on fitness parameters. In the following season, telomeres from supplemented birds had shortened less. Altogether, we found that supplementation with antioxidants provided fitness benefits in the short term and reduced telomere loss a year following treatment. Our results provide indirect empirical support for accelerated telomere loss as a cost of reproduction.

Introduction

Telomeres are short tandem repeats of nucleotide sequences at the ends of eukaryotic chromosomes that maintain DNA integrity. They act as 'mitotic clocks', shortening with each round of cell division due to the end replication problem (Watson, 1972). When telomeres reach a critically short length, the cell enters a degenerative process of senescence which is eventually followed by apoptosis (Blackburn, 1991). In fact, ageing was first linked to telomeres in the early 1990s (Harley *et al.*, 1990), and subsequent studies confirmed that, in addition to the process of cell division, other factors accelerate telomere shortening, for example oxidative stress (von Zglinicki, 2002; Epel, 2004). Hence, there is increasing evidence that telomere loss is a good proxy for ageing *in vivo* (Haussmann *et al.*, 2003; Blasco, 2005; Bize *et al.*, 2009; Barrett *et al.*, 2013).

During bird reproduction, higher levels of oxidative stress can be reached through increased parental effort (Alonso-Alvarez *et al.*, 2004; Metcalfe & Alonso-Alvarez, 2010; Christe *et al.*, 2012). When reproductive investment exceeds what is sustainable for parents, the costs on longevity become apparent through accelerated ageing (Santos & Nakagawa, 2012). Thus, studies increasing brood size in a range of organisms have

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shown that costly reproductive events had a negative impact on telomere dynamics on adult (Reichert *et al.*, 2014) and early life (Nettle *et al.*, 2013; Boonekamp *et al.*, 2014; Herborn *et al.*, 2014). The trade-off between investment on current and future reproduction has puzzled evolutionary ecologists for decades (Williams, 1966) and still is a current topic of intense investigation (Roff & Fairbairn, 2007; Creighton *et al.*, 2009; Cox *et al.*, 2010). Traditionally, brood-manipulation experiments have reflected the relationship between investment in reproduction and telomere loss; however, it is currently unknown whether factors alleviating reproductive costs affect telomere shortening in the wild.

Proper nutrition is fundamental to reproductive success: when food is limited in an unfavourable environment, reproduction may be suspended in favour of metabolic processes that ensure survival (Wade *et al.*, 1996). In fact, birds from lower quality territories exhibited higher oxidative stress (van de Crommenacker *et al.*, 2011). Dietary antioxidants, such as vitamin E and methionine, are important during reproduction because they defend against oxidative stress toxicity (Giraudeau *et al.*, 2013). Methionine, an essential amino acid, is also an efficient scavenger of free radicals (Levine *et al.*, 1999; Elias *et al.*, 2005). The positive effects of certain diets on telomere dynamics have been reported in humans (Marin *et al.*, 2012) and mice (Vera *et al.*, 2013), but the effects of supplementation on telomere erosion in the wild are unknown. Moreover, these micronutrients improve immune system functioning, which is essential during breeding (Soler *et al.*, 2003; Brommer, 2004).

Impaired immune function during reproduction may also increase parasitic infections (Møller *et al.*, 2003). Numerous studies have demonstrated that avian malaria-like parasites are widespread (Pérez-Tris *et al.*, 2005; Merino *et al.*, 2008; Szöllösi *et al.*, 2011), and relapses from chronic infections are common during the breeding season (Valkiūnas, 2005). In addition to this, infected individuals result in increased oxidative stress (Isaksson *et al.*, 2013). Malaria is associated with susceptibility to oxidative stress, especially during energetically demanding stages of reproduction such as provisioning (van de Crommenacker *et al.*, 2012). In humans, telomere length shortens with infection and chronic diseases (Ilmonen *et al.*, 2008), but the link between parasitism and ageing in the wild remains understudied. A recent study in a wild warbler population investigated the relationship between chronic malaria and telomere loss and found evidence of the long-term costs of infection on ageing (Asghar *et al.*, 2015). Hence, telomere erosion is likely related to infection status during reproduction in wild birds.

To explore the association between telomere loss, nutritional status and parasitism, we designed a two-fold experiment during two consecutive breeding seasons in blue tits (*Cyanistes caeruleus*). During the first season, one group of birds was administered with a supplement consisting of vitamin E and methionine, another group was treated against blood parasites using antimalarial agents, a third group of birds was treated with both the supplement and medication, and a final group of birds acted as control. With micronutrient supplementation, we expect to generally enhance nutritional status, immune function and protection against antioxidant damage rather than assess the effects of individual compounds (i.e. methionine and vitamin E). In the combined supplement and medication group, parasites are targeted directly with the medication and indirectly through an enhanced immune response and nutritional status with the supplement. Thus, we expect parasitaemia reduction in both treatment groups, with a more acute reduction in the combined treatment group. This experimental design allowed us to investigate whether medication, supplementation and the combination of both (i) reflect improved fitness parameters in the short term and (ii) are efficient in reducing parasite loads. We then evaluated the effect of the treatments on fitness parameters and telomere shortening 1 year after administration of the treatment. If reduced parasitaemia and an increased supply of antioxidants alleviate reproductive costs, we expect to observe less change in telomere length in all experimental groups compared to the control group.

Materials and methods

Sample collection

The study was carried out during the 2012 and 2013 breeding seasons in a Pyrenean oak (*Quercus pyrenaica*) forest in central Spain (Valsaín, Segovia, 40°53′N, 4°01′W, 1200 m a.s.l.). Blue tits had access to a total of 300 wooden nest boxes, with an average occupancy of 25% of nest boxes per year (Fargallo & Merino, 1999). The present population has been under study since 1994 (Fargallo & Merino, 1999). For each season, nest boxes are monitored to determine the impact of infection on host reproduction. Given the high prevalence of blood parasites, treatments could be blindly assigned (del-Cerro *et al.*, 2010).

In 2012, adults were captured at the nest box twice, when nestling age was 3 and 13 days (hatching date = day 0). Birds were ringed, weighed to the nearest gram and aged according to plumage characteristics (Svensson, 1992). Wing (± 0.5 mm; method III following Svensson, 1992) and tarsus (± 0.1 mm) lengths were also recorded. Nestling–provisioning rates were measured on day 10 at a subset of nests, using uniquely identifiable transponders attached to colour rings on the adult's tarsus. An antenna, connected to a data logger (Trovan; EID Iberica, Madrid, Spain), recorded entrances to/exits from the nest between the hours of

6:30 a.m. and 12:00 p.m. On day 15, nestlings were ringed; nestling mass and tarsus length were measured and unhatched eggs counted. After the breeding season, nests were inspected to determine which nestlings had successfully fledged.

At each capture, we obtained a blood sample via the brachial vein. One drop of blood was stored on an FTA card (Whatman International Ltd., Kent, UK), and another was smeared on a slide. Blood smears were immediately air-dried and fixed in ethanol (96%) and then later stained with Giemsa. After sampling the blood, we administered treatments by subcutaneous injections into the belly (each bird received a single injection at each of the two sampling occasions, see Fig. 1). Both individuals from the pair received the same treatment. Sets of four nests that shared similar hatching date (± 1 day) and clutch size (± 1 egg) were assigned to one of the following treatments: antimalarial drug, vitamin–methionine supplement, a combination of both or control. The dosage of antimalarials was considered subcurative based on the dosage for malaria treatment in humans. The treatment consisted of an injection of 0.1 mg of primaquine phosphate (Sigma, St Louis, MO, USA) and 0.125 mg of chloroquine phosphate (Sigma) solubilized in 20% solutol HS 15 (Sigma), which is an innocuous solvent used when conventional vehicles are inadequate (Stokes *et al.*, 2013). The supplement, calculated for a mean body mass of 10 g for adult blue tits, consisted of 0.9 mg α-tocopherol, 0.09 mg of mixed tocopherols and 1 mg of methionine in 20% solutol solution. The third treatment consisted of both antimalarics and supplements (i.e. primaquine, chloroquine, tocopherols and methionine), solubilized in 20% solutol. The control treatment was a 20% solutol solution. In all treatments, a total volume of 0.05 mL was administered per injection.

Antioxidant dietary supplements administered orally do not result in increased concentrations of α-tocopherol in plasma compared with controls (Larcombe *et al.*, 2010). However, injectable lipid emulsions are commonly used as dietary supplements in veterinary practice (Driscoll, 2006), and studies with rabbits demonstrate the application of these emulsions as extravascular injectable vehicles for prolonged release of drugs (Wu *et al.*, 2014). The doses of antioxidants administered in the present study were based on the one used in previous studies (Soler *et al.*, 2003; de Ayala *et al.*, 2006). Rats supplemented for 7 weeks with methionine showed oxidative damage of the liver when given excess methionine (Gomez *et al.*, 2009). Therefore, the dose/concentrations used are within the range of what is observed naturally in birds. Besides, based on evidence from Soler *et al.* (2003), these doses effectively reduced *Haemoproteus* parasite infections in magpies. Antimalarial treatments have previously been administered by subcutaneous injection in the same blue tit population, with significant effects on infection (Merino *et al.*, 2000; Martínez-de La Puente *et al.*, 2010), thus validating the use of this application method for all treatments in this study.

During the 2013 breeding season, no treatments were administered. Adults were captured and their blood sampled once (when nestlings were 3 days old, Fig. 1), following the protocol described above.

Parasitological and molecular analyses

For all samples, DNA was extracted from blood using a standard ammonium acetate protocol and stored at −20 °C (Merino *et al.*, 2008). This DNA solution was then purified using silica filters to obtain a higher quality DNA (NZYGel pure, NZYtech Genes and Enzymes, Lisbon, Portugal). DNA samples were quantified by spectrophotometry and adjusted to the same concentration (10 ng μL^{-1}). For the 2012 blood samples (157 individuals from 79 pairs – one male was not captured), we detected and/or quantified the following parasites using two complementary methods (quantitative PCR [qPCR] and microscopic examination of blood smears): *Haemoproteus majoris* haplotype cyan2, *Plasmodium* sp. haplotype cyan1, and *Leucocytozoon* sp. haplotypes leuA, leuA1 and leuB (see Table S1 for GenBank accession numbers). The variable *Leucocytozoon* A includes haplotypes A and A1 (see Supporting Information). In addition, a microscopic examination for the parasite *Lankesterella valsainensis* (Merino *et al.*, 2006) showed it

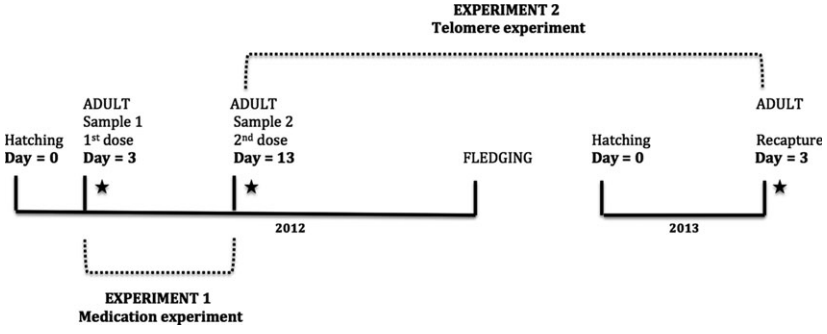

Fig. 1 Schematic figure illustrating the experimental protocol during the two breeding seasons (2012 and 2013). Stages at which the adults were caught and administered with the treatments are shown (1st and 2nd dose). The star indicates that a blood sample was taken. The dashed line indicates which blood sample was used for each analysis (experiments 1 and 2).

was present at very low intensities (16% of samples were infected), thus were quantified only by molecular methods (though qPCR results indicated that 36% of samples were infected). These quantifications were also included in the experiment, although the antimalarial treatment was not specifically targeting this parasite. We used relative qPCR with SYBR green (SYBR Selected Master Mix; Applied Biosystems, Foster City, CA, USA) to amplify a fragment of the cytochrome *b* or 18S rRNA genes using a pair of species-specific primers for each parasite (Table S1). Blood smears were also examined under high magnification (1000×) to quantify the number of *H. majoris* juvenile/mature gametocytes. All blood samples were examined using an Olympus BX41 (Olympus Iberia S.A.U., Barcelona, Spain) light microscope by EPB.

For telomere length analyses, we used samples collected during the second adult capture in 2012 and the only capture in 2013 (Fig. 1). Thus, the sampling universe for this experiment consisted of recaptured individuals only (68 individuals; annual return rate of 43.3%). After molecular screening for multiple parasite species (see above), DNA sufficient for telomere analyses was only available for 51 individuals. These samples represented a balanced proportion of recaptures from 2012 for each treatment group ($N_{control} = 14$, $N_{supplemented} = 12$, $N_{medicated} = 10$, $N_{supplemented + medicated} = 15$); the 2013 recaptures were not significantly skewed to the treatments administered in 2012 (GLM with binomial error distribution, $\chi^2_3 = 213.83$; $P = 0.802$). We used the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as the control single-copy gene. GAPDH primers were specific to the zebra finch but also amplify other bird species (Criscuolo *et al.*, 2009). The use of these primers in our samples was justified by checking nonvariability of the control gene. The melting curves of the control gene cycles confirmed the lack of primer-dimer nonspecific amplification, and the efficiency was close to two for all PCR plates (see the Supporting Information for further details). GAPDH was used as an internal control to normalize the amount of telomere sequence to the amount of DNA in the reaction. Telomere primers Tel1b and Tel2b were used at a concentration of 100 nM; GAPDH-F/GAPDH-R primers were used at 200 nM (Table S1). The final PCR volume was 20 μL containing 10 μL of Light Cycler 480 SYBR Green I Master (Roche, Diagnostics GmbH, Mannheim, Germany) and 20 ng μL^{-1} of DNA. Telomere and GAPDH real-time amplifications were performed on different plates, with each sample run in duplicate. RT-PCR-based methods for estimating telomere length are sensitive to the presence of intersticial telomeric sequences and consequently are not adequate for estimating absolute telomere length (Nussey *et al.*, 2014). However, this is not a problem for our study because we are examining changes in the telomere length from individuals over time, and not absolute telomere length. Telomere PCR conditions were 10 min at 95 °C followed by 30 cycles of 1 min at 56 °C and 1 min at 95 °C. GAPDH PCR conditions were 10 min at 95 °C followed by 40 cycles of 1 min at 60 °C and 1 min at 95 °C. All PCRs were performed in a Light Cycler 480 RT-PCR System (Roche). Each 96-well plate included serial dilutions (40 ng, 10 ng, 2.5 ng, 0.66 ng of DNA per well) of DNA from a reference pool (the internal control) run in triplicate, which were used to generate the standard curves, and a blank control with no DNA. The slopes of the standard curves ranged from −3.649 to −3.460 with a R^2 value between 0.98 and 1.00; efficiencies ranged from 1.885 to 1.976 (see the Supporting Information for further details). The coefficients of variation of the C_q values for the GAPDH and telomere amplifications were < 5% in all samples following Criscuolo *et al.* (2009). Sample-level repeatability within and across plates was > 97.8% for GAPDH and telomere RT-PCR. Quantification cycle values (C_t) were transformed into normalized relative quantities (NRQs) using standard software (see Hellemans *et al.*, 2007 for formulas).

Statistical analyses

All analyses were performed in R v.2.14.0 (R Foundation for Statistical Computing, Vienna, Austria). First, we checked for pre-existing differences between experimental groups in 2012. The full model was evaluated with respect to the significance of each explanatory variable. As treatments were sorted according to timing of breeding and clutch size, medicated and control birds did not differ with respect to laying or hatching date, clutch size, or initial parasitaemia. Only medicated males showed higher infection intensity with *Leucocytozoon* B ($F_{3,65} = 3.1941$, $P = 0.029$) than controls. The conditions prior to the experiment were accounted for by adding initial parasitaemia as a covariate in the subsequent analyses.

Next, we examined the effect of treatment on the intensity of the infection with linear or generalized linear models (GLMs), depending on the most appropriate error distribution. When there was evidence of overdispersion (Zuur *et al.*, 2009), likelihood ratio tests were used to compare the negative binomial and the analogous Poisson model, which confirmed that the negative binomial was more appropriate than the Poisson model. These analyses aimed to test the variation in parasitaemia with respect to the treatment; thus, individuals that remained uninfected during the course of the experiment (samples 1 and 2 from season 2012) were removed from the analyses. In any case, when the analyses included these individuals, the same conclusions were reached. All models included initial parasitaemia, sex, experimental group and the interaction between sex and experiment. By introducing initial intensity as a covariate in the analyses, we controlled

for the pretreatment differences (Merino *et al.*, 2000). The dependent variable (final parasite intensity) was log-transformed when necessary, and residuals examined to check for compliance with each test's assumptions. The differences between treatment groups were evaluated using a *t*-test with Bonferroni correction or a sign test for related samples. When the assumptions of normality were violated and the variance between groups was highly different, we used the sign test to evaluate the difference in the median of parasitaemia between sampling occasions (Gibbons & Chakraborti, 1992). We also accounted for pretreatment differences by matching repeated observations of the same subject. Full models were evaluated with respect to the significance of each explanatory variable. The effect size of the difference in parasitaemia for each treatment group was computed as the Cliff's delta for nonparametric effect size estimates with the 'orddom' package in R (Rogmann, 2013).

In 2012, we also investigated the effect of treatment on host fitness. For the adults, changes in body mass were examined through an ANCOVA with initial body mass, tarsus length and sex as explanatory variables. Differences in female provisioning rates between treatments were tested using a Kruskal–Wallis test. Males were discarded from the analyses due to loss of transponders. For the analyses on reproductive success, we used generalized linear models (GLMs) with binomial error for the proportion of hatched young that reached 15 days of age (fledging success). The effect of the adults' treatment on nestling body mass was checked using a linear mixed effect model in order to account for nonindependence of brood mates (nest as random effect). Tarsus length and hatching date were included as covariates. In all cases, full models were evaluated with respect to the significance of each explanatory variable. After multiple testing on the same data, we used the false discovery rate (Benjamini & Yekutieli, 2001) to correct all *P*-values from the resulting models (see Table S2 for uncorrected *P*-values).

Finally, we explored the effect of treatment on telomere shortening. The variance is usually used as a measure of spread in ordinary least squares regression, but it is particularly sensitive to outliers, especially with low sample size. Two points in the combined supplemented and antimalarics group and one point in the vitamin-only group appeared to have high leverage in our data set, excluding these data points generated further heteroskedastic problems. Therefore, we fitted an ordinal logistic regression to the complete data set to control for skew and high-leverage data points (see the Supporting Information for further details on the statistical analyses). The rate of telomeric change was calculated as the difference in telomere lengths between 2012 and 2013, corrected for regression to the mean following the equation suggested by Verhulst *et al.* (2013). We used the change in telomere length as a dependent variable to control for pretreatment differences in telomere length (median test differences for the control vs. supplemented group, one-sample sign test: $s = 2$ P-value = 0.01) and treatment as explanatory variable. Sex or the interaction between treatment and sex was not included due to reduced sample size. Fledgling success or nestling body mass in 2013 was not tested because nestlings were included in a different experimental design that year.

Results

Efficacy of treatments in 2012

Supplementation had a positive effect on adult body mass. After correcting for initial body mass, sex and tarsus length, the ANCOVA revealed a significant effect of the treatment on final body mass ($F_{3,136} = 3.98$, $P < 0.000$, $N_{\text{control}} = 33$, $N_{\text{supplemented}} = 41$, $N_{\text{medicated}} = 35$, $N_{\text{supplemented + medicated}} = 37$). The natural reduction in body mass during the breeding season was mitigated only after supplementation with antioxidants (pairwise *t*-test with Bonferroni correction, control vs. supplemented group: $P < 0.000$ and control vs. combined group: $P = 0.02$, Fig. 2). This decrease was significantly different between the sexes ($F_{1,136} = 1.06$, $P = 0.003$, $N_{\text{female}} = 77$, $N_{\text{male}} = 69$), but not between treatment and sex ($F_{3,136} = 0.16$, $P = 0.74$, $N = 146$). After correcting for tarsus length, nestling body mass in 2012 was not affected by the treatment of the adults ($F_{3,81} = 0.61$, $P = 0.74$, $N = 86$ nests), nor were female provisioning rates (Kruskal–Wallis test: $\chi^2_3 = 1.74$, $P = 0.74$, $N = 36$)). However, fledgling success was affected by the treatment ($\chi^2_3 = 23.67$, $P = 0.0001$,

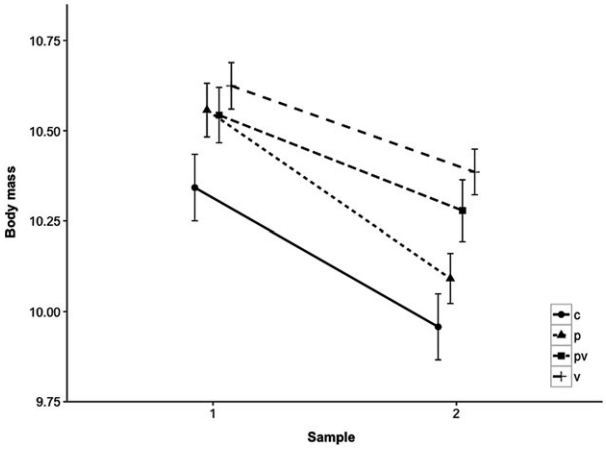

Fig. 2 Change in adult body condition index with respect to treatment and sampling occasion. Bars denote standard error. Codes: control = c, antimalarial drugs = p, supplement = v, antimalarial and supplement = pv. Sample refers to initial sample (1 = pretreatment) and final sample (2 = post-treatment).

$N = 72$ nests), with the supplemented group having significantly more fledgling success compared to the control group (pairwise *t*-test with Bonferroni correction, control vs. supplemented, $P = 0.043$, Fig. 3).

Medication treatment was effective in reducing *Haemoproteus* parasitaemia when initial intensity was included as a covariate (negative binomial GLM, $\chi^2_3 = 21.77$, $P = 0.0002$, $N_{control} = 26$, $N_{supplemented} = 33$, $N_{medicated} = 29$, $N_{supplemented + medicated} = 29$). Control birds naturally experienced a reduction in the number of mature parasites; however, a significant decrease was observed in birds administered with antimalarial drugs alone (sign test, $s = 8$, $P = 0.012$, effect size ES = −0.66) and in combination with antioxidants (sign test, $s = 9$, $P = 0.031$, effect size ES = −0.41). This treatment was also effective against *Leucocytozoon* B (negative binomial GLM, $\chi^2_3 = 19.35$, $P = 0.0006$, $N_{control} = 27$, $N_{supplemented} = 28$, $N_{medicated} = 25$, $N_{supplemented + medicated} = 29$). In this case, parasitaemia by *Leucocytozoon* B was significantly reduced in birds receiving antimalarial drugs alone (sign test, $s = 6$, $P = 0.0073$, effect size ES = −0.52), whereas control birds experienced an increase in infection intensity. Finally, *Lankesterella* infections were also significantly affected by medication (ANCOVA, $F_{3,57} = 8.42$, $P = 0.0003$, $N_{control} = 17$, $N_{supplemented} = 17$, $N_{medicated} = 19$, $N_{supplemented + medicated} = 13$) when antimalarics (pairwise *t*-test with Bonferroni correction, $P = 0.0238$) and antimalarics with supplement (pairwise *t*-test with Bonferroni correction, $P = 0.0099$) were administered. Infections by *Plasmodium* (ANCOVA, $F_{3,70} = 1.81$, $P = 0.74$, $N_{control} = 20$, $N_{supplemented} = 24$, $N_{medicated} = 14$, $N_{supplemented + medicated} = 21$) and *Leucocytozoon* A (Poisson GLM, $\chi^2_3 = 2.61$, $P = 0.71$, $N_{control} = 34$, $N_{supplemented} = 36$, $N_{medicated} = 33$, $N_{supplemented + medicated} = 37$) were unaffected by any treatment.

Change in telomere length between 2012 and 2013

All recaptured individuals from 2012 ($n = 54$) had reduced telomere lengths in 2013, except for three individuals. Overall, telomere lengths were reduced by 16.44% in the control, 16.33% in the supplemented plus antimalarics, 11.56% in the antimalarics and 6.43% in the supplemented group. Administration of these treatments in 2012 had an effect on telomere shortening, based on the ordinal logistic regression (likelihood ratio score vs. null model, $\chi^2_3 = 12.62$, $P = 0.01$, $N = 51$). Supplemented birds showed significantly less change in telomere length (Wald's $Z = -3.14$, $P = 0.002$) (Fig. 4).

The effect of treatment in 2012 was not reflected in breeding parameters in 2013, such as clutch size ($\chi^2_3 = 7.19$; $P = 0.84$), hatching date ($\chi^2_3 = 13.69$; $P = 0.75$) or laying date ($\chi^2_3 = 27.19$; $P = 0.5$).

Discussion

Telomere shortening has recently been used as a biomarker for cellular ageing processes in birds (Bize *et al.*, 2009; Barrett *et al.*, 2013). As hypothesized, antioxidant supplementation had positive effects on telomere dynamics, supporting the idea that nutritional status plays an important role in cellular functioning during reproduction. Recently, Reichert *et al.* (2014) showed

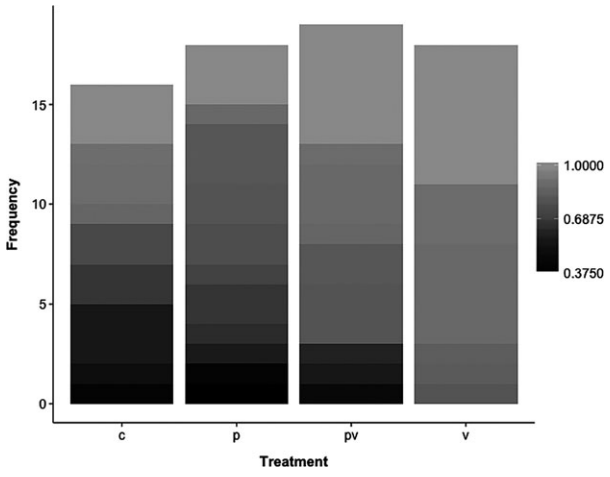

Fig. 3 Fledging success. For representation purposes, we show the frequency of nests with its particular fledging success proportion. For each treatment group, the frequency of nests represents a continuous proportions ranging from 1 (all nestlings fledged) to 0 (no nestlings fledged). Codes: control = c, antimalarial drugs = p, supplement = v, antimalarial and supplement = pv.

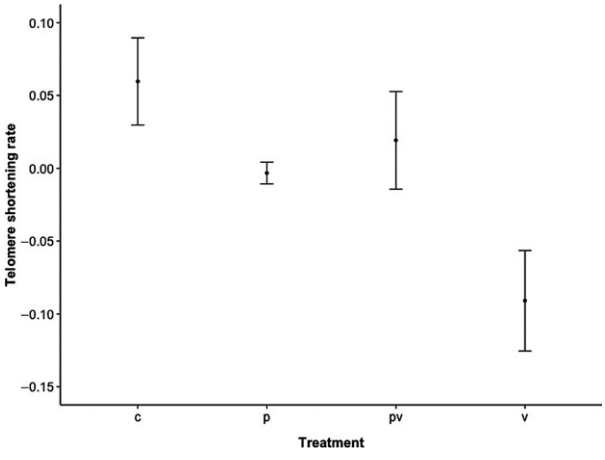

Fig. 4 Telomere shortening rate with respect to treatment. The change in telomere length is corrected for regression to the mean using the equation suggested by Verhulst *et al.* (2013). The equation uses the corrected mean centring of the values from 2012 and 2013 measurements; thus, the rate is set to a central zero. Codes: control = c, antimalarial drugs = p, supplement = v, antimalarial and supplement = pv.

that a single costly reproductive event shortened adult zebra finches' telomeres, which were not restored 1 year later. Our results in blue tits provide the first experimental evidence that antioxidants, such as vitamin E and methionine, decelerate telomere shortening *in vivo*. Supplementation alleviated the costs of reproduction, in such a way that telomere loss was significantly reduced 1 year after administration. For example, by providing extra reserves for methylation, methionine could help maintain DNA integrity and thus counteract telomere shortening (Ligi, 2011). The role of methionine in maintaining genome integrity has been suggested previously. In humans, low reserves of mediators in the DNA methylation pathway correlate with shorter telomeres (Benetti *et al.*, 2007; Fenech, 2012). Restriction of amino acids other than methionine has also been shown to prevent telomere shortening in rat livers (Tanrikulu-Kucuk & Ademoglu, 2012). In another study, a single intraperitoneal injection of a synthetic antioxidant modulated DNA methylation in rats (Vanyushin *et al.*, 1998). Supplementation with vitamin E could also explain the positive effects seen on telomere dynamics in this study. Dietary intakes of vitamin E were associated with longer telomeres in humans (Xu *et al.*, 2009), and *in vitro* experiments in human skin fibroblasts demonstrate that vitamin E restores telomerase activity and protects against telomere erosion (Makpol *et al.*, 2010). The reduction in telomere loss may be the result of an increased antioxidant capacity. Indeed, subcutaneous injection of the antioxidant melatonin increased antioxidant activity in rat liver (Manikonda & Jagota, 2012). To confirm this hypothesis in blue tits, future experimental work that also included measuring circulating levels of antioxidants is needed. Surprisingly, the experimental group that received the combined treatment (primaquine, chloroquine, methionine and vitamin E) did not have a significant change in telomere shortening compared with the control birds. Primaquine is used to remove human malaria at both the tissue and blood stages of the parasite; chloroquine works mainly against the blood stage of the parasite (Baird & Rieckmann, 2003). The use of such drug associations for the treatment of human (Desjardins *et al.*, 1988) and avian malaria (Graczyk *et al.*, 1994) has been described before; but to date, there is no record of these compounds being used together with vitamin and methionine supplementation. The lack of an impact on telomere loss may be attributed to a negative interaction between the medication and antioxidants. Therefore, future experimental work in this blue tit population will focus on supplementation to better understand how telomere maintenance mechanisms may benefit from an enhanced nutritional status.

The benefits associated with supplementation also appeared during the 2012 breeding season. Birds suffered from decreased body mass during reproduction, but the supplemented birds benefited from a lower decline in body condition. Supplementation may have boosted the birds' performance (i) by reducing oxidative stress with the antioxidant properties of vitamin E and methionine (Alonso-Alvarez *et al.*, 2004) or (ii) by providing extra nutritional requirements. Indeed, we found that supplementation increased the probability that all nestlings in the brood fledged. This is consistent with previous findings using methionine supplementation in passerines (Soler *et al.*, 2003; Brommer, 2004). However, when we tested overall performance during reproduction (i.e. food provisioning rates), we did not find a significant effect of the treatment.

Methionine-fed magpie nestlings harboured less blood parasites (Soler *et al.*, 2003). Therefore, we hypothesized that supplementation would increase the immune response against parasites resulting in a decline in parasite load. However, in blue tits, no significant effect on infection status was found after supplementation. To our knowledge, this is the first experiment that has combined vitamin E and methionine with an antimalarial treatment; therefore, other factors may explain the lack of an effect. For example, vitamin E may have provided nutritional and antioxidant properties to developing parasites (Müller, 2004), promoting growth. A similar pattern has been observed in studies where well-fed parasitized hosts experienced higher mortality than underfed hosts (Pulkkinen & Ebert, 2004). In great tits (*Parus major*), female fleas that fed on food-supplemented hosts laid significantly more eggs (Tschirren *et al.*, 2007). In contrast, experimentally infected captive canaries did not experience higher parasitaemia after supplementation (Cornet *et al.*, 2014). Conflicting results regarding the effects of supplementation on parasitism highlight the need for further studies to better understand the ecology of host–parasite interactions. In any case, it is clear that, in blue tits, antioxidant supplementation improved fitness despite not having a significant effect on parasite load.

Faster telomere shortening rates have been related to stress (von Zglinicki, 2002) or diseases in humans and other mammals (Cawthon *et al.*, 2003). Contrary to expectations, the antimalaric treatment administered to adult blue tits had no effect on telomere shortening rates. The lack of an apparent relationship between parasitism and telomere loss may be explained by several factors. Firstly, blood parasites may have long-term detrimental effects on ageing processes that only become evident with time (Martínez-de La Puente *et al.*, 2010). Secondly, antimalaric drugs cause a range of side effects in humans. For example, combinations of primaquine and chloroquine over long periods of time can cause pruritus and anaemia (Kondrashin *et al.*, 2014). In birds, harmful side effects of such combination of drugs are unknown. In previous studies of this bird population, primaquine treatment successfully

reduced malarial parasite infections and improved fitness (Merino *et al.*, 2000; Martínez-de La Puente *et al.*, 2010). However, during the 2012 breeding season, both primaquine and chloroquine were used, and a different effect on blood parasite reduction was observed. Thus, the use of these drugs possibly masked a positive effect on telomere erosion in medicated blue tits.

Overall, we showed positive effects of antioxidant supplementation during the reproductive attempt and 1 year after. The short-term effects were seen in body mass and fledgling success, whereas the long-term effect was evident by a slower rate of telomere shortening. These findings constitute experimental evidence for linking nutritional status during a costly reproductive event and ageing. One of the costs of increased reproductive effort is an accelerated ageing rate as revealed by telomere erosion (Reichert *et al.*, 2014). In this study, without manipulating reproductive effort, we showed that improving nutritional status reduced telomere erosion 1 year after the breeding attempt. In the future, particular attention should be given to supplementation experiments and telomere maintenance mechanisms to understand how costs of reproduction affect ageing.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Primer sequences used in the present study.

Table S2 Corrected and uncorrected *P*-values from multiple testing.

Data S1 Supporting information.

Data deposited at Dryad: doi:10.5061/dryad.6m68b

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