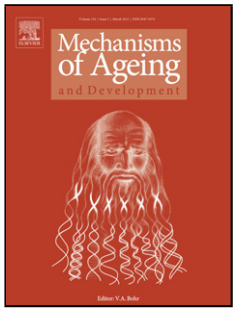

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Environmental conditions can modulate the links among oxidative stress, age, and longevity

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Highlights

- We exposed zebra finch females to challenging environmental conditions
- We examined treatment effects on age-related changes in oxidative stress (OS)
- Levels of damage to DNA and proteins increased with chronological age
- The treatment produced a higher age-related increase in the level of damage to DNA
- We found treatment-specific links among OS and longevity

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Abstract

Understanding the links between environmental conditions and longevity remains a major focus in biological research. We examined within-individual changes between early- and mid-adulthood in the circulating levels of four oxidative stress markers linked to ageing, using zebra finches (*Taeniopygia guttata*): a DNA damage product (8-hydroxy-2'-deoxyguanosine; 8-OHdG), protein carbonyls (PC), non-enzymatic antioxidant capacity (OXY), and superoxide dismutase activity (SOD). We further examined whether such within-individual changes differed among birds living under control (*ad lib* food) or more challenging environmental conditions (unpredictable food availability), having previously found that the latter increased corticosterone levels when food was absent but improved survival over a three year period. Our key findings were: (*i*) 8-OHdG and PC increased with age in both environments, with a higher increase in 8-OHdG in the challenging environment; (*ii*) SOD increased with age in the controls but not in the challenged birds, while the opposite was true for OXY; (*iii*) control birds with high levels of 8-OHdG died at a younger age, but this was not the case in challenged birds. Our data clearly show that while exposure to the potentially damaging effects of oxidative stress increases with age, environmental conditions can modulate the pace of this age–related change.

Keywords: age; longevity; environmental challenging conditions; glucocorticoids; corticosterone; oxidative stress; oxidative damage; antioxidant defences.

1. Introduction

Oxidative stress is a complex, multifaceted state that arises in organisms as a consequence of an imbalance between reactive oxygen species (ROS) produced primarily during aerobic metabolism and the organisms' ROS quenching capacity (Halliwell and Gutteridge, 2015). ROS damage macromolecules, cell components and structures, which, in the absence of repair, can negatively affect performance (Martindale and Holbrook, 2002; Birben *et al.* 2012). The extent to which oxidative stress influences organismal health, ageing and survival will depend both on the level of damage that occurs, and the investment in repair; both of these will vary with species life histories, environmental circumstances and potentially also with age-related changes in investment priorities, and in antioxidant and repair capabilities (Finkel and Holbrook 2000; Monaghan *et al.* 2009; Salmon *et al.*, 2010; Selman *et al.* 2012; Speakman and Garratt, 2014; Speakman *et al.* 2015).

Currently, we know relatively little about age-related changes in oxidative stress within individuals, how this relates to longevity, and how levels vary with environmental conditions. Population-based studies in humans have found only moderate support for a positive correlation between levels of oxidative damage and age, and even less support for age-related changes in antioxidant defences and repair efficiency (see Jacob *et al.* 2013 for a recent review). Robust data on these issues in other vertebrate species remain rare to date (*e.g.* Sohal *et al.* 1994, 1995; Hamilton *et al.* 2001). Most of the studies conducted so far are based on cross-sectional designs (*i.e.* comparing age classes of individuals rather than within-individual changes). Cross-sectional studies, however, can be confounded by differences in the age of death of particular phenotypes in the study population, generally termed "selective disappearance" (e.g. early mortality of poor quality individuals – Nussey *et al.* 2008; Bouwhuis *et al.* 2009). This can mask the true within-individual pattern of age-related variation in oxidative stress (Herborn *et al.* 2015). Though not always possible for a variety of reasons,

repeated sampling of the same individual through time is essential to examine age-related changes in oxidative stress levels. To date only a few studies in the laboratory (Matsuo *et al.* 1993; Alonso-Alvarez *et al.* 2006) and in the wild (Bize *et al.* 2014; Herborn *et al.* 2015) have used such longitudinal sampling designs. These within-individual studies do find that selective disappearance of individuals with high oxidative stress levels does occur (Herborn *et al.* 2015)**,** but generally also find evidence of age-related increases in oxidative stress exposure or age-related decreases in cell resistance to oxidative stress (Matsuo *et al.* 1993; Bize *et al*. 2014).

The quality of the environment might influence both oxidative stress levels at a given age and age-related change in oxidative stress levels. The undemanding conditions that animals generally experience in the laboratory might give rise to very low levels of oxidative stress, whereas these levels might be altered and more variable in more challenging environments (Salmon *et al.* 2010). For example, a recent study in captive black birds showed that individuals exposed to repeated immune and disturbance stressors exhibited higher levels of oxidative damage markers than control birds after one year of treatment (Hau *et al.* 2015). Elevated levels of stress hormones (i.e. glucocorticoids), generally induced by exposure to challenging and unpredictable environmental circumstances (Wingfield and Kitaysky, 2002), have been associated with elevated levels of oxidative damage in vertebrates, as shown in a comprehensive meta-analysis (Costantini *et al.* 2011). However, the effects of environmental conditions on oxidative stress might vary depending on the magnitude and type of environmental challenge.

The aims of this study were 1) to examine within individual changes in oxidative stress from early- to mid-adulthood; 2) to examine whether challenging environmental conditions influenced oxidative stress levels and/or age-related changes in oxidative stress levels, and 3) to examine whether oxidative stress levels were predictive of survival probability and whether such relationships were altered under more challenging environmental conditions. We used

captive zebra finches (*Taeniopygia guttata*) as our study species. We manipulated the quality of the environment by exposing experimental birds to unpredictable episodes of food withdrawal throughout adulthood (see full details in Marasco *et al.* 2015). In this species, we have previously reported that such challenging environment moderately increased baseline levels of glucocorticoid stress hormone without overall affecting body mass (Marasco *et al.* 2015), leading more surprisingly to improved probability of survival to the age of at least three years (Marasco *et al.* 2015). In this paper, we examine four different markers of oxidative stress in a randomly chosen subset of birds from the experimental population used in Marasco *et al.* (2015): a DNA damage product (8-hydroxy-2'-deoxyguanosine, 8-OHdG), oxidative damage to protein (protein carbonyls), non-enzymatic antioxidant capacity (OXY), and superoxide dismutase (SOD) enzymatic antioxidant activity at two age points (early- and mid-adulthood).

We predicted: 1) that exposure to oxidative stress would increase with age (e.g. Finkel and Holbrook, 2000); 2) that the birds exposed to the challenging environmental protocol would show reduced oxidative damage given our previous finding of their improved survival (Marasco *et al.* 2015), and 3) that individuals showing higher levels of oxidative damage would have shorter lifespans irrespective of the treatment.

2. Material and Methods

2.1 Experimental design

This study was performed on a subset of female zebra finches (*Taeniopygia guttata*) randomly selected from the larger study investigating the long-term effects for mothers (F0), and subsequent generations, of exposure of the F0 generation to challenging environmental conditions mentioned above. Since the main focus of this long-term transgenerational project was on maternal effects, only females were subjected to the treatments. The birds were maintained throughout the experiment at a photoperiod of 14h:10h light:dark cycle and the

temperature was maintained between 20-24°C. The environmental manipulations started when the F0 females were approximately 5 months old (mean ± SEM: 156 ± 1 day old). At this stage, they were fully grown, young adults as sexual maturation is reached by 2.5-3 months of age in the zebra finch (Zann, 1996). From the start of the experiment, females were housed in treatment-specific groups in cages (n = 7-10 per 120 x 50 x 50 cm cage), and randomly assigned to one of two experimental groups: challenging (n = 74) or control (n = 65) environments. Where possible, females that hatched in the same nest were counterbalanced between the two treatment groups and family of origin was taken into account in all analyses.

Females in the challenging environment were denied access to food for a continuous period of almost one third of the daylight hours (4.9h a day), 4 days per week on a random schedule. For the remaining two thirds of the daylight hours, and on the non-treatment days, food was provided *ad libitum*. Access to food in the challenging environment was prevented by placing a textured paper sheet (globular embossed sheets, 180 GSM, 575MM X 485MM - DBM Scotland Ltd) at the bottom of the cages in order to assure full coverage of the food bowls and of any seed food scattered on the floor cage. The floor tray had to be briefly removed from the cage in order to place and to remove the paper sheet. Both control and experimental birds were equally spread in two experimental rooms, meaning that both groups were exposed to the same level of disturbance resulting from experimenters entering the rooms. The removal of the floor tray was a routinely conducted procedure during cage cleaning in both experimental groups.

Birds in the challenging environment were always kept on this food regime other than when breeding (three breeding events at 188 ± 1, 408 ± 1 days, and 653 ± 1 days of age, means ± SEM for all) when they received *ad libitum* food continuously for approximately 2 months. Birds in the control environment were always provided with *ad libitum* food and experienced exactly the same breeding regime as in the challenging environment. We have previously

shown that the treatment had no significant overall effects on body mass, measured up to three years of age, confirming that the random withdrawal of food altered primarily the temporal predictability of resources rather than the daily overall food intake (full details in Marasco *et al.* 2015). Importantly, our environmental manipulation induced changes in the exposure to glucocorticoid stress hormones. We found that at the end of the episodes of food withdrawal the unpredictable food birds showed higher baseline corticosterone (the main avian glucocorticoid) than those in the control conditions (on average 1.4 fold increase), and there was no sign of habituation of this hormonal response over time (full details in Marasco *et al.* 2015). Therefore, our environmental manipulation mimicked a mild/moderate environmental challenge experienced by animals living in unstable environments, such as those with unpredictable foraging conditions. The birds living in these more challenging conditions showed increased probability of survival relative to those in the control conditions based on monitoring to three years of age (treatment: $\exp(\beta) \pm SE(\beta) = 0.53 \pm 0.26$, $z = -2.42$, $p = 0.016$ - Mixed Effects Cox Models; Marasco *et al*. 2015). The positive consequences of the treatment on survival emerged progressively starting from the age of about one year as mortality prior to this point (up to 379 days) was very low in the birds living in both environmental conditions (3.6% controls and 1.8% challenging - Marasco *et al.* 2015). Thus, the data on oxidative stress markers are not biased by mortality of individuals exhibiting a particular phenotype prior to the one year sampling. Since we had full details on the longevity and survival of individual birds living in both environments until three years of life (Marasco *et al.* 2015), we examined whether markers of oxidative stress (full details below) predicted longevity. In this and other studies, differences in age-specific survival patterns are detectable in the zebra finch within this time frame (Monaghan *et al.* 2012; Costantini *et al.* 2014; Marasco *et al.* 2015). The work was carried out under Home Office Project Licence 60/4109.

2.2 Sampling and laboratory analysis

Since we were studying the longevity of the individuals, minimally invasive techniques were used to obtain the required biological samples. In this context, blood sampling offers a good opportunity to gather data on oxidative stress markers in a minimally invasive manner, using either plasma or red blood cells (Stier *et al.* 2015). Levels of oxidative stress in the blood have been shown for instance to correlate to those of organs such as heart, muscle or liver, depending on the biomarker used (Veskoukis *et al.* 2009). Blood oxidative stress markers are also known to exhibit similar responses as other organs to manipulations known to induce oxidative stress such as intense physical exercise, exposure to stress hormones, or acute cold exposure (Pereira *et al.* 2013; Marasco *et al.* 2013; Stier *et al.* 2014a, b).

Small blood samples (up to 140 µl) were taken by venipuncture from the brachial vein prior to the start of the experiment when the birds were 5 months old, and again when the birds were approximately 1 year of age (i.e. mean ± SEM: 380 ± 1 days of age). These birds were therefore in young and mid-adulthood. In the zebra finch, signs of senescence appear from about 2 years of life when the risk of death starts increasing (Monaghan *et al.* 2012; Marasco *et al.* 2015). It was not our intention to examine long-term changes in the oxidative stress markers, but rather to examine how they were influenced by environmental conditions and whether they were predictive of survival over a three year period. All females were in a non-reproductive state at the time of sampling, and over the three year period were allowed to breed at the same age points as reported above. The samples were kept on ice for less than 4hrs before being centrifuged (at 4°C) to separate plasma from red blood cells, and stored at -80 °C until laboratory analyses. We measured two commonly used oxidative stress markers, 8-hydroxy-2'-deoxyguanosine (8-OHdG) in the plasma and protein carbonyls (PC) levels in red blood cells. Additionally, we measured two markers of antioxidant defences in the plasma, the non-enzymatic antioxidant capacity (OXY) and the activity of the superoxide dismutase (SOD) enzyme. 8-OHdG is one of the predominant forms of free radical-induced oxidative lesions on

DNA. At the cellular level, damaged guanine (8-OHdG) can be excised from genomic DNA by specific repair enzymes, and enters the circulation before being eliminated through urine. Consequently, plasma and urinary levels of this marker reflect whole-body oxidative stress status, and will be influenced both by the level of damage and by the rate of repair of such damage (Halliwell and Gutteridge, 2015). Carbonyl groups are introduced into the proteins from free radicals or via reactions with lipid peroxidation products or carbohydrates; damage produced by protein carbonylation cannot be repaired and, therefore, measurements of protein carbonyls (PC) provide a reliable estimate of terminal damage products (Halliwell and Gutteridge, 2015). Measurements of non-enzymatic antioxidants (e.g. vitamins, carotenoids, flavonoids and thiols) in biological tissues provide an indication of the ability of the sample to buffer/counteract oxidants, thus providing an integrated proxy of measurable non-enzymatic antioxidant capacity (OXY). Superoxide dismutase (SOD) is involved in the first step of the antioxidant enzymatic cascade catalysing the dismutation of superoxide radical into oxygen and hydrogen peroxide. The SOD family contain 3 specific isoforms: SOD1 being found in the cytoplasm, SOD2 being targeted to the mitochondria, and SOD3 being actively excreted from the cells to act as an extra-cellular antioxidant (Zelko *et al.* 2002). The four markers were measured using commercial kits following the manufacturers' instructions, using a MultiSkan® Spectrum microplate-reader (Thermo Scientific, USA). The same individual birds were sampled at both 5 months and 1 year of age; due to low blood volume for some birds, we were unable to measure all four oxidative stress markers at 5 months and/or 1 year of age in all collected samples – full details on sample sizes for each oxidative stress marker/age are shown in Table S1 (Supplementary Material).

We measured the circulating concentration of 8-OHdG using a competitive immunoassay (plasma diluted 1:8, OD measurement at 450nm, Assay Designs DNA damage ELISA Kit – Enzo® Life Sciences, USA). DNA damage is expressed as ng of 8-OHdG/mL,

and intra-plate variation based on duplicate samples was low (CV = 7.91 ± 0.51%) as well as inter-plate variation based on a standard sample repeated over plates (CV = 4.83%).

The carbonyl content of red blood cell lysate was quantified using the protein carbonyl ELISA kit (red blood cells diluted 1:6, OD measurement at 450nm, Enzo® Life Sciences, USA). PC content is expressed as nmol/mg of protein, and intra-plate variation based on duplicate samples was low (CV = 5.98 ± 0.47%) as well as inter-plate variation based on a standard sample repeated over plates (CV = 8.92%).

Plasma non-enzymatic antioxidant capacity was measured with the OXY-Adsorbent test (Diacron International, s.r.l, Italy) using 5 µL of 1:100 diluted plasma (OD measurement at 510nm). The OXY adsorbent test quantifies the ability of the plasma antioxidant compounds to buffer a massive oxidation through hypochlorous acid. This assay measures a variety of non-enzymatic antioxidants, including vitamins, carotenoids, flavonoids and thiols (OXY significantly correlate with thiols in the blood: r = 0.65–0.67, Palleschi *et al.* 2007). Antioxidant capacity is expressed as mM of HClO neutralised. Intra-plate variation based on duplicate samples was 4.25 ± 0.30%; inter-plate coefficient of variation based on a standard sample repeated over plates was 3.62%.

The enzymatic activity of SOD in the plasma (predominantly SOD3 variant) was measured using the SOD activity kit (Enzo® Life Sciences, USA) using 25 µL of 1:8 diluted plasma, following manufacturer instructions (OD measurement at 450nm). This test quantifies in vitro the kinetics of inhibition in superoxide formation resulting from SOD antioxidant activity. SOD activity is expressed as units of enzymatic activity (U). Intra-plate variation based on duplicate samples was 8.34 ± 0.55%; inter-plate coefficient of variation based on a standard sample repeated over plates was 8.90%.

2.3 Data Analysis

We first tested the impact of age and treatment on oxidative stress markers using repeated linear mixed models (SPSS v20.0). We included age (i.e. 5 month *vs*. 1 year), treatment (control *vs*. challenging environment) and their interaction as fixed factors. We included bird identity as the repeated subject and bird family as a random effect. We also included body mass as a covariate, and checked for potential interactions between body mass and treatment. We sequentially removed non-significant terms ($p > 0.05$) from the final models, starting with the interactions. In preliminary exploratory analyses we considered approaches to reduce data dimensionality and therefore multiple testing. However, co-variation among the oxidative stress markers was very low for both the 5 months and 1 year measurements (-0.03 < Pearson r < 0.28) and Principal Component Analysis failed to effectively reduce data dimensionality (% of variance explained by the first 2 Principal Components was relatively low, respectively 32% and 28%).

Next, we performed analyses to test whether variation in the oxidative stress markers predicted survival (measured from one to three years of age; only one control bird in the subset of birds used in this study died of natural causes during the first year of life and this was excluded from the survival analyses) and/or explain variation in the age at which death occurred within the pool of females that died. In these analyses, we only included birds that died naturally, or were culled on welfare grounds after veterinary assessment verified that their death was imminent due to intrinsic causes rather than accidental death (2 control birds and 1 bird in the challenging environment were excluded from survival analyses for this reason). We tested the relationships between survival (binary data (0/1), oxidative stress, and treatment using Generalized Linear/Mixed Models (GLMs/GLMMs) with a binomial error structure and logit function in R (R 3.1.3, Rcore team, 2014 - package "lme4", Bates *et al.* 2015). We performed separate binomial models for each marker (*i.e.* 8-OHdG; PC; OXY, or SOD) to test whether the 1 year measurements (~224 days after the start of the challenging treatment),

predicted survival over the next two years of life, and to test if oxidative stress influenced probability of survival differently in the two treatment groups. In addition, we performed General Linear Models (LMs) to investigate whether oxidative stress marker measurements (at 1 year) explained a significant amount of the variation in the age at death, using the 34 birds that had died between 1 and 3 years of age (18 out of 62 individuals in the control environment, 29.03%; 16 out of 73 individuals in the challenging environment, 21.92%). Fixed factors entered in both GLMs/GLMMs and LMs models were: treatment (control *vs.* challenging), oxidative stress marker at 1 year (i.e. 8-OHdG; PC; OXY, or SOD) and their interaction; when possible, family id was entered as random factor. In preliminary analyses we included body mass (at 1 year) as a covariate in each model performed and we considered the two way- and three-way interactions of body mass with treatment and the oxidative stress marker. Body mass and the interactions tested were either not significant or could not be ascertained due to poor model performance. Consequently, we removed body mass from the final survival models since it never improved explanatory power. Model fit of the survival binomial models was evaluated via graphical check of the binned plot residuals versus the fitted values (Zuur *et al.* 2009). We also tested the "delta" oxidative stress markers (i.e. change between 5 months and 1 year corrected for the regression to the mean effect following Kelly and Price, 2005 – see details in Supplementary Material) as explanatory factors instead of the actual oxidative stress levels at 1 year of age. These analyses are reported in Supplementary Material for clarity.

We chose to perform separate models for each marker (using the 1 year measurements, or the delta marker index values) to maximise statistical power as we were unable to measure all four oxidative markers in all the collected samples as reported above (full details in Table S1). Nevertheless, the same significant patterns were obtained with inclusion of all four oxidative stress markers, together with the interactions of each marker with the treatment, when performing two separate binomial models (one using the 1 year measurements, and the other

one using the delta marker index values; variance inflation factors among the covariates were ≤ 1.1 for both models) (data not shown). This model strategy was not achievable for the model testing age at death due to the relatively limited sample size.

Unless otherwise specified values are reported as mean ± SEM with *p*-values ≤ 0.05 being considered as significant, and the analyses were performed using the software SPSS v20.0. We reported effect size for significant factors, in order to provide an evaluation of the magnitude of the biological effect of interest according to recommendations of Nakagawa and Cuthill (2007). We used two kinds of effect size measures depending on the statistical model used. We used g_{Hedges} (Hedges and Olkin, 1985) for the models testing age and treatment effects, and for the binomial survival models; we refer to small (0.2), moderate (0.5) and large (0.8) effects according the widely used nomenclature (Nakagawa and Cuthill, 2007; Cohen 1988). We used partial eta-squared for the models testing age at death, and refer to small (0.01), moderate (0.06) and large (0.14) effects (Cohen 1988).

3. Results

3.1 Effect of age and environmental conditions on oxidative stress markers

We found a significant increase with age both for 8-OHdG ($p < 0.001$, $g_{Hedges} = 0.73$ (moderate), Table S2a, Figure 1a) and PC ($p < 0.001$, $g_{Hedges} = 0.47$ (moderate), Table S2b, Figure 1b). The age-related change in plasma 8-OHdG levels was influenced by environmental circumstances (treatment x age interaction: $p = 0.004$, $g_{Hedges} = 0.59$ (moderate), Table S2a). 8-OHdG levels increased with age in both groups, but the age-related increase was greater in the birds living under challenging environmental conditions (Figure 1a). In contrast, we found no significant effect of the environmental circumstances on protein damage levels or on its age-related increase (Table S2b, Figure 1b).

The two markers of antioxidant defences did not show a consistent age-related pattern. Plasma non-enzymatic antioxidant defences (OXY) was significantly influenced by the interaction between environmental circumstances and age ($p = 0.042$, $g_{Hedges} = 0.47$ (moderate), Table S2c). Birds facing the challenging environmental conditions did not significantly differ from those in the control environment before the start of the experiment, but ended up with significantly higher OXY levels (Figure 1c). SOD levels were influenced by age and environmental conditions as revealed by the significant interaction between experimental treatment and age ($p = 0.020$, $g_{Hedges} = 0.48$ (moderate), Table S2d), but the pattern was the opposite of that observed for OXY (Figure 1d). SOD activity of control birds significantly increased with age, while it was not the case for birds facing the challenging environmental conditions.

3.2 Oxidative stress, environmental conditions and probability of survival

At 1 year of age, neither plasma 8-OHdG nor red blood cell PC levels significantly predicted the proportion of birds surviving to at least three years old in either environment (Table S3a, b). While there was no significant interaction between environmental conditions and OXY at 1 year in predicting probability of survival up to three years of age (Table S3c), we found that SOD levels at 1 year predicted survival in an environment-specific fashion ($p = 0.03$; Table S3d). The magnitude of this interaction effect was overall weak: birds in the control conditions showed a higher probability of death as SOD activity increased, while the opposite trend was observed in the challenging conditions (Figure 2; control alive: 4.203 ± 0.157, control dead: 4.687 ± 0.300, $g_{Hedges} = 0.44$ (small); challenging environment alive: 4.220 ± 0.177, challenging environment dead: 3.603 ± 0.265, $g_{Hedges} = 0.49$ (moderate). Analysing our data using the age-related change in oxidative stress instead of the 1 year values yielded similar results (see supplementary Table S5), except for a marginally significant trend for delta PC index to predict mortality across both treatment groups ($p = 0.06$; $g_{Hedges} = 0.51$ (moderate)). Birds showing

higher age-related increases in PC were more likely to die (delta PC index: alive birds: -0.005 ± 0.004; dead birds, 0.013 ± 0.010).

3.3 Oxidative stress, environmental conditions and age of death

We found evidence of a different relationship between 8-OHdG levels at 1 year and age of death in the different environments (interaction: $p = 0.004$, partial eta-squared = 0.25 (large); full model output in Table S4a). Birds living in the control conditions that died young had relatively high 8-OHdG levels ($r = -0.63$, $p = 0.005$; Figure 3), whilst there was no such relationship in the birds living under more challenging environmental conditions ($r = 0.30$, $p = 0.27$; Figure 3). Neither PC, OXY, nor SOD levels at 1 year explained variation in the age of death in either environment (Table S4b-d). Analysing our data using the age-related change in oxidative stress instead of the 1 year value yielded similar results (see supplementary Table S6).

4. Discussion

The results from our longitudinal study clearly show that exposure to oxidative stress increases with age, and that environmental conditions can modulate the links among oxidative stress, age, and longevity. Specifically, we found that: (1) oxidative stress increases from early- to mid-adulthood in zebra finch females since we found an age-related increase in both plasma 8-OHdG and red blood cells protein carbonyls; (2) the age-related increase in plasma 8-OHdG was greater in the birds maintained under the more environmentally challenging conditions (unpredictable food availability compared with *ad lib* food); (3) age-related changes in antioxidant defences were influenced by environmental conditions since enzymatic defences (SOD) increased with age in the control but not in the challenging environment, while the opposite was true for non-enzymatic antioxidant levels; (4) levels of 8-OHdG at 1 year were not predictive of probability of survival up to three years of age in either environment, but were

negatively related to the age of death in the control environment only; (5) a higher age-related increase in protein oxidative damage was moderately linked to lower survival probability, irrespective of environmental conditions; (6) SOD levels at one year were predictive of survival, but in opposite directions between control (negative) and challenging (positive) environments.

Focusing first on the effect of age *per se* in the birds living in the control environmental conditions throughout the experiment, we found an intra-individual increase in plasma 8-OHdG levels and red blood cells protein damage, but also an increase in plasma SOD activity. This increase in oxidative stress indicators with age is overall in line with our first prediction (i.e. that exposure to oxidative stress would increase with age). Plasma non-enzymatic antioxidant capacity did not change with age, but this is probably not surprising under *ad libitum* food in laboratory conditions, since this marker is influenced at least to some extent by the dietary intake of antioxidants (Cohen *et al.* 2007). Our results suggest unambiguously that exposure to oxidative stress increases with age from quite early in adult life, since our oxidative stress measurements covered the early- to mid-adulthood of the birds (i.e. from 5 months to 1 year). The results of our longitudinal study are in accordance with a meta-analysis based on cross-sectional studies in rodents which showed an overall age-related increase in oxidative damage to DNA in these species (Møller *et al.* 2010). Our marker of DNA damage in the plasma (8-OHdG) might reflect both damage and repair capacity. However, given that protein damage levels in the plasma also increased with age in our birds, this suggests that the age-related increase in 8-OHdG reflects an age-related increase in oxidative damage to DNA. Recent cross-sectional studies in humans on this topic are equivocal since one study showed an age-related increase in both DNA damage and repair (Soares *et al.* 2015), while another showed an age-related decrease in DNA repair capacity (Løhr *et al.* 2015). Such mixed results emphasise the need for comprehensive longitudinal studies investigating age-related changes

in both DNA damage and repair capacities. Interestingly, our results are somewhat in contrast with recent findings in wild European Shags (*Phalacrocorax aristotelis*), a relatively long-lived bird species. In this species, an increase in oxidative stress exposure, was only evident at old age (Herborn *et al.* 2015). Such inter-specific differences might arise as a consequence of life-history related differences in the pattern of investment in somatic tissue defences. Our zebra finch results suggest that the age-related increase in 8-OHdG and protein carbonyls levels is probably more linked to an increase in ROS production than a defect in endogenous protective mechanisms, since we found an up-regulation of plasma SOD antioxidant activity with age. The only study available to date on age-related changes in ROS production in birds found no evidence for an increase in ROS production using a cross-sectional approach (Stier *et al.* 2015). However, as mentioned already, such approach is likely to be biased by the reduced survival of individuals exhibiting high ROS production during early life, giving the erroneous impression that that ROS production does not change with age.

We showed that living in a challenging environment was associated with marked alterations in an individual's oxidative status (i.e. significant interaction between treatment and age for 3 markers out of 4). While our challenging environmental conditions did not affect protein carbonyl levels in the red blood cells, 8-OHdG levels in the plasma increased more with age in the birds living in the challenging environment. Moreover, birds exposed to the challenging conditions showed reversed within-individual changes in the antioxidant markers (OXY and SOD) relatively to the control birds. Interpretation of the latter results is not straightforward, but the pattern we found might be related to environmentally-induced differences in the balance between endogenously produced (e.g. SOD) and dietary antioxidants. The elevation in plasma 8-OHdG levels is not in accordance to our second prediction (i.e. that the birds exposed to the challenging environmental protocol would show reduced oxidative damage given our previous finding of their improved survival), but we have

to keep in mind that such elevated 8-OHdG levels might reflect both an increase in DNA damage and/or repair. Interestingly, the challenging environment was able to break the negative relationship observed between 8-OHdG and the age of death in the control environment. This is of particular interest given that the birds exposed to the challenging environment did on average show increased life expectancy relative to those in the control environment (Marasco *et al.* 2015). Together, our results suggest that birds living in the challenging environment were likely to be more resistant to oxidative stress. In support of this, a variety of mild stressors, generally producing moderate increases in exposure to glucocorticoids as in our study, can extend lifespan via activating various cellular stress response pathways (Fontana and Partridge, 2015), including those implicated in increasing organismal ability to repair oxidatively damaged DNA (Guo *et al.* 1998; Cabelof *et al.* 2003). This idea would also be in agreement with experimental evidence showing that the effects of environmental stressors generally follow an inverted-U dose-response curve as a function of stress severity, with mild-moderate stressors being salutary and more severe stressors procuring negative fitness effects (recently reviewed by Sapolsky, 2015). The beneficial effects of our challenging environment on the survival up to three years (Marasco *et al.* 2015) and the lack of difference in protein oxidative damage between the treatment groups further support the idea that living under challenging environmental conditions might have activated adaptive transcriptional and post-transcriptional responses that altered ROS-dependent signalling state rather than oxidative damage per se (Holmstrom and Finkel, 2014).

There was suggestion from our analyses that the chances of survival were marginally reduced in the birds showing high within-individual increases in red blood cell protein carbonyls with age, irrespective of their environment. Although being only marginally significant, this result gives limited support to our third prediction (i.e. that individuals showing higher levels of oxidative damage would have shorter lifespans irrespective of the treatment)

given that protein carbonylation is unequivocally a marker of damage. The weakness of the effect suggests that the age-related increase in oxidative damage to proteins that had occurred by one year of age, and irrespective of environmental quality, was not sufficiently detrimental to account for much of the variation in subsequent survival. Our data are not surprising considering the mixed findings in the literature about the ability of known markers of oxidative damage to predict survival prospects. For example, studies in mice genetically modified to increase exposure to ROS via altering specific antioxidants, generally showed no effect on the probability of survival, but rather contributed to the progression of diseases (Salmon *et al.* 2010). The recent growing body of work in un-manipulated individuals primarily carried out in free-living bird populations has also shown mixed results (reviewed in Costantini, 2014), with some studies supporting the idea of negative association among markers of oxidative damage and survival (Freeman-Gallant *et al.* 2011; Noguera *et al.* 2012, Costantini *et al.* 2015a, Herborn *et al.* 2015), and others reporting no association (Beaulieu *et al.* 2011; Stier *et al.* 2014c; Costantini *et al.* 2015b). The variable outcomes may be due to several factors, including species-specific patterns, differences in life-stages between individuals (e.g. breeding *vs*. non-breeding animals), age differences, or other confounding environmental factors, and differences relating to the measures of oxidative damage. Our longitudinal study was conducted under controlled environmental conditions, hence removing some of these potentially confounding factors. More direct manipulations of ROS exposure are needed in order to fully understand the links between oxidative damage and longevity.

In conclusion, oxidative stress exposure, indicated by circulating levels of 8-OHdG and protein carbonyls, does increase with chronological age and this process is likely to start at least from early/mid adulthood. Such an increase in oxidative stress levels was probably associated with an increase in ROS production rather than an age-related deterioration in antioxidant defences. Our study also showed that the protracted exposure to an environmental

challenge led to higher increases in levels of 8-OHdG in the plasma, but this specific point deserves further investigation to tease apart the relative contribution of oxidative damage *per se* and of DNA repair capacity. We showed that elevated plasma levels of 8-OHdG were not linked to survival or longevity under challenging environmental conditions, contrary to what was observed in the control environmental conditions in which high 8-OHdG levels were negatively correlated to the age of death. Given the beneficial effects of our challenging environmental conditions on survival in our study population (Marasco *et al.* 2015), it appears that such challenging environment promoted to some extent resistance to oxidative stress, perhaps through activating repair capabilities – this idea remains to be experimentally validated. As a final comment, we propose that future studies exposing individuals to different degrees of environmental challenge will be extremely useful in elucidating potential non-linear relationships between environmentally-mediated oxidative stress and its associated costs and benefits at the organismal level.

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Figure captions

Figure 1. Effects of age and treatment on four oxidative stress markers in adult zebra finch females: (a) plasma levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG), (b) protein carbonyl (PC) levels in red blood cells, (c) plasma non-enzymatic antioxidant capacity (OXY) and (d) plasma superoxide dismutase (SOD). Control environment is shown in black and challenging environment in grey. Means are reported ± SEM and different letters indicate significant differences, i.e. $p \leq 0.05$ (see text and Table S2 for details about statistics).

Figure 2. Estimated probabilities of mortality in relation to plasma levels of superoxide dismutase (SOD) at 1 year of age in the control (black line) and in the challenging (grey line) environments; dashed lines in black or grey, for control treatment or challenging environments respectively, represents 95% confidence intervals obtained by adding or subtracting variance of the random factor family id to the predictor function). Observed individual values (0 alive /1 dead) are represented by black circles for control and grey triangles for challenging environment. Elevated SOD activity was associated with higher mortality rates in the control females whereas the opposite trend was observed in the birds exposed to the challenging environmental conditions (treatment x SOD, $p = 0.03$).

Figure 3. Relationship between age of death and plasma levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG) measured at 1 year of age. Graphical representation of the significant interaction ($p = 0.004$) between treatment (black: control environment; grey: challenging environment) and 8-OHdG in predicting age of death. The interaction is driven by a significant negative correlation between age of death and 8-OHdG in the control environment ($r = -0.628$, $p = 0.005$), whilst no significant correlation was found in the challenging environment ($r = 0.303$, $p = 0.272$).

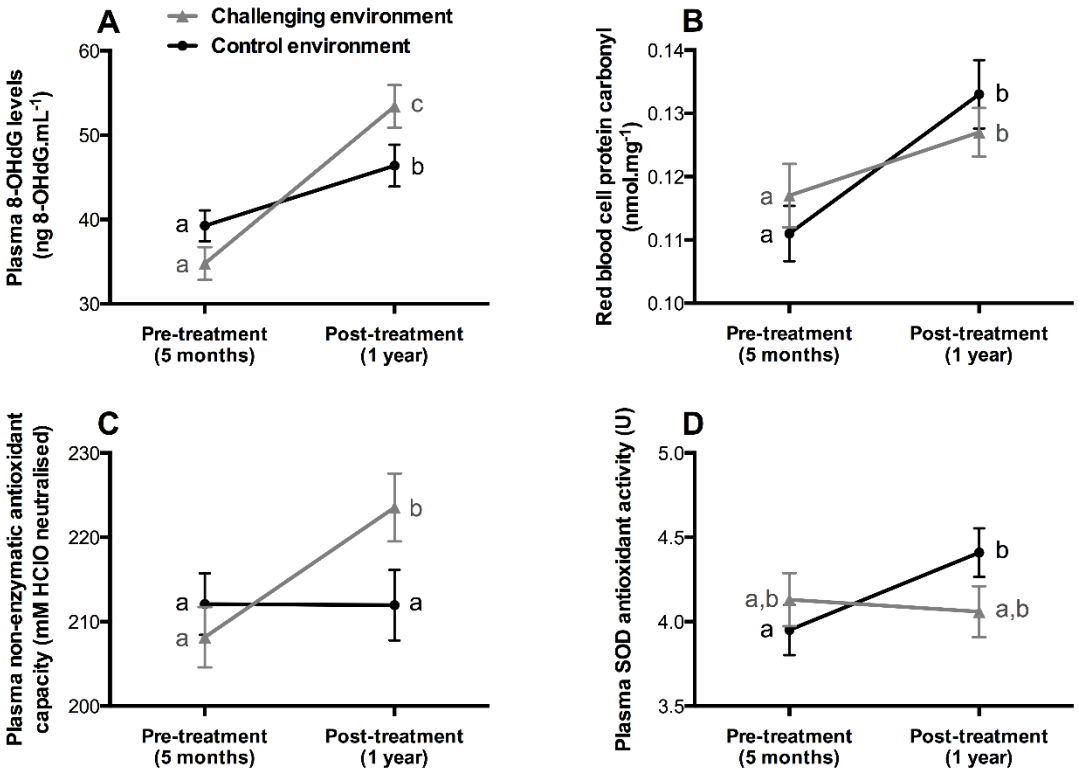

Figure 1.

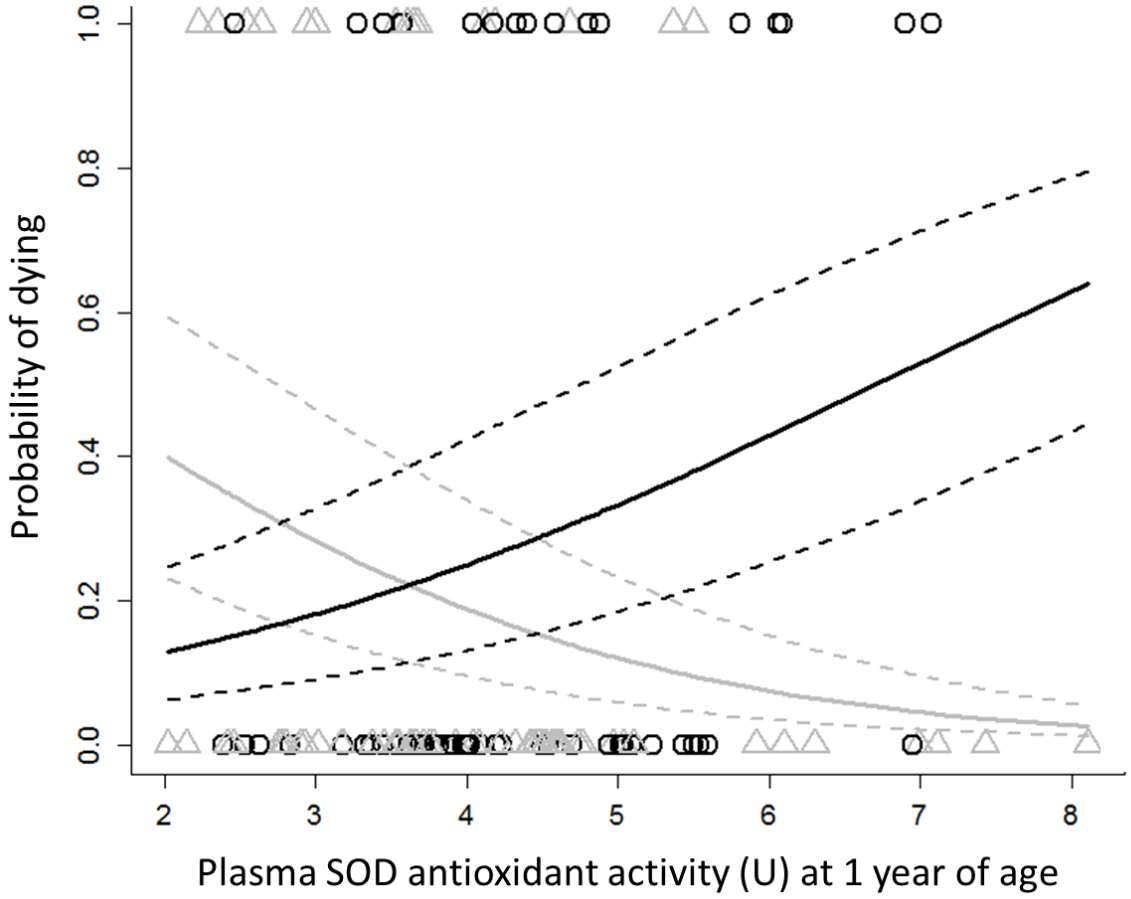

Figure 2.

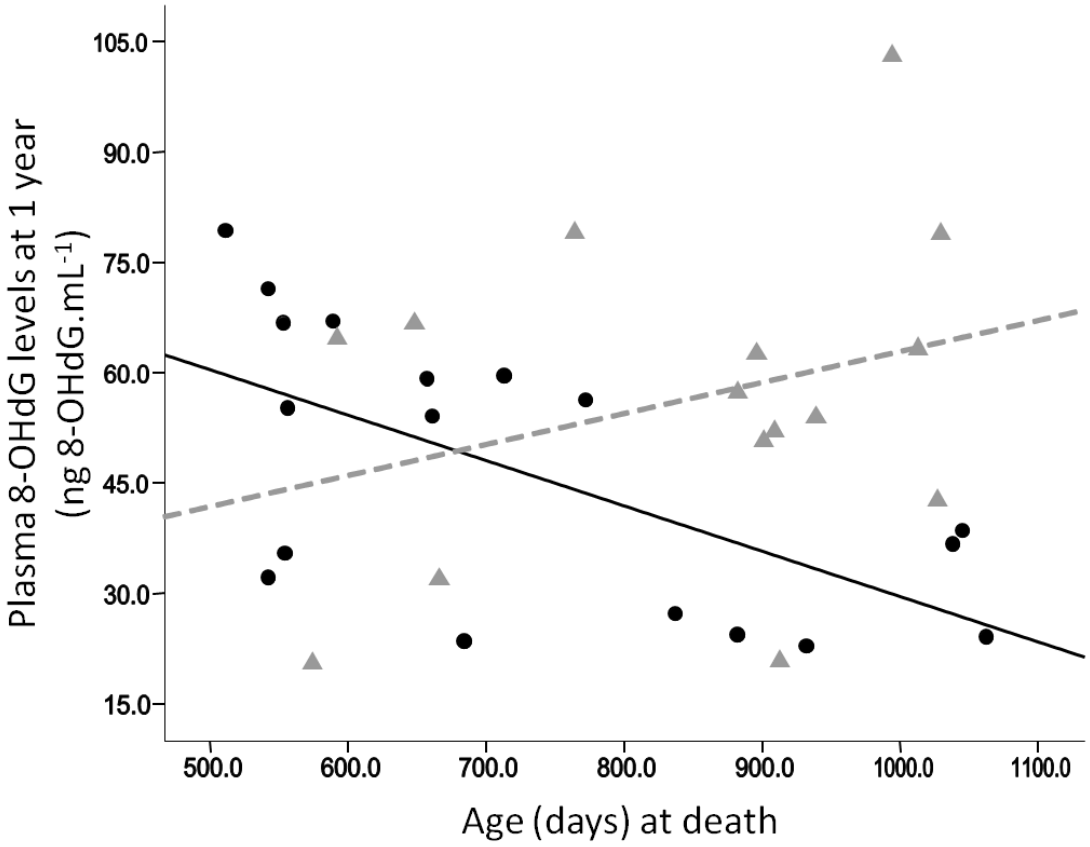

Figure 3.