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RESEARCH ARTICLE

# Maternally derived yolk antioxidants buffer the developing avian embryo against oxidative stress induced by hyperoxia

**Hannah Watson[1,*], Pablo Salmón[2] and Caroline Isaksson[1]**

## ABSTRACT

In oviparous animals, maternally transferred antioxidants protect the embryo from oxidative damage from high rates of reactive oxygen species (ROS) production incurred by rapid growth. Elevated ROS exposure, beyond that incurred by normal growth, can occur as a result of exposure to exogenous factors (e.g. pollutants, toxins, radiation), increasing the risk of oxidative damage, with potentially adverse consequences for embryonic development and long-term fitness. The capacity of the avian embryo's antioxidant protection system to counter an increased exogenous oxidative threat is poorly understood. We induced an external oxidative challenge via experimental increase in ambient oxygen concentration throughout incubation of wild great tit *Parus major* eggs in the laboratory. At day 11 of incubation, brain tissue revealed no consistent differences in oxidative stress status [as measured by antioxidant levels (superoxide dismutase and total glutathione), lipid peroxidation and telomere length] between control (21% oxygen) and hyperoxic (40% oxygen) embryos. However, the level of vitamin E was significantly lower and lipid peroxidation was significantly higher in yolks of eggs reared under elevated oxygen concentrations. The results suggest that maternally derived yolk antioxidants successfully buffer developing embryonic tissues against an increased exogenous oxidative threat. Furthermore, vitamin E plays a more important role in protecting the embryo than carotenoids. However, the depletion of antioxidants and increased peroxidation of lipids in the yolk could have negative consequences for embryonic development, in particular for the brain and heart that require highly unsaturated fatty acids, and protection against the oxidative burst following hatching.

**KEY WORDS: Antioxidant, Hyperoxia, Lipid peroxidation, Oxidative stress, Prenatal development, Vitamin E**

## INTRODUCTION

In oviparous animals, such as birds, the embryo develops within the confines of the closed egg. The egg, its structure and contents (including the shell, membranes, yolk, albumin, hormones, antioxidants, immunoglobulins) provide, in the presence of external heat and oxygen, the protection and nourishment required for healthy embryonic development (Deeming, 2002; Thompson and Speake, 2002). Maternal transfer of dietary derived antioxidants to the egg yolk provides protection against oxidative stress to the embryo and newly emerged young by detoxifying reactive oxygen species (ROS; Palace and Werner, 2006; Surai, 2002). If not sufficiently countered by antioxidant defences, ROS can lead to damage to macromolecules, such as lipids, proteins and nucleic acids with detrimental consequences for, e.g. membrane function, cellular signalling and cell division (Halliwell and Gutteridge, 2007). Increased oxidative stress has been widely linked to reduced reproductive performance success and survival, accelerated rates of ageing and onset of disease (Bize et al., 2009; Harman, 1956; Monaghan et al., 2009) and thus there should be strong selective pressure for mechanisms to minimise oxidative damage during development.

The avian embryo undergoes rapid growth and consequently experiences very high metabolic rates (Vleck and Bucher, 1998). It is therefore assumed that prenatal growth is associated with high rates of ROS generation, and thereby elevated oxidative stress, due to leakage from the electron transport chain in the mitochondrial membrane (Monaghan et al., 2009). Furthermore, embryonic tissues are rich in unsaturated fatty acids (derived from the lipid-rich yolk and upon which the embryo depends for its energy requirements) that are highly susceptible to attack by ROS (Blount et al., 2000; Noble and Cocchi, 1990). The rapidly growing embryos are therefore vulnerable to high levels of oxidative damage unless armed with highly efficient antioxidant defences (Surai, 2002). In addition to a baseline of high endogenously driven ROS production, embryos may face an additional challenge from exogenous drivers of ROS production, such as ozone, pollutants, toxins and UV radiation (Monaghan et al., 2009; Palace and Werner, 2006).

The avian embryo's protective antioxidant system comprises yolk-derived antioxidants – vitamins E and A, carotenoids and selenium – deposited in the yolk by the female and antioxidants that are synthesised endogenously by the embryo, including the enzymes superoxide dismutase (SOD), catalase and glutathione peroxidase (Surai, 2002; Surai et al., 1996; Yigit et al., 2014). Maternally derived antioxidants are transferred from the yolk to the developing embryo, and high levels of vitamin E and carotenoids accumulate in the liver, particularly during the last few days of development (Surai et al., 1996). The levels of vitamin E in the liver of hatchlings are at least 10 times that of adult hens (Surai, 2000), suggesting maternal allocation could be an adaptive mechanism to counter elevated oxidative stress at hatching triggered by the combination of exposure to atmospheric oxygen, onset of pulmonary respiration and an associated increase in metabolic rate (Vleck and Bucher, 1998). The importance of maternally derived yolk antioxidants has been demonstrated in supplementation studies in both domestic and wild birds. Supplementation of female birds with carotenoids results in higher deposition of carotenoids in the egg yolk, which subsequently reduces susceptibility to lipid peroxidation and positively influences the survival of embryos (McGraw et al., 2005; Surai and Speake, 1998) and enhances immune function in the newly hatched chick (Biard et al., 2007; Haq et al., 1996). Similarly,

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supplementing females with vitamin E reduces susceptibility of tissues, especially the brain, to peroxidation and increases antioxidants in hatchlings (Lin et al., 2005; Surai et al., 1999).

While a number of studies have investigated drivers of variation in oxidative stress and the oxidative machinery during postnatal development in birds (Alonso-Alvarez et al., 2007; de Ayala et al., 2006; Stier et al., 2015), as well as later-life effects of early-life stress (e.g. Marasco et al., 2013; Noguera et al., 2012), the current knowledge of the regulation of oxidative stress during embryonic development is scarce, especially in altricial species (for studies in precocial species, see Surai, 2000; Surai et al., 1996; Tsunekage and Ricklefs, 2015). Maternal allocation of antioxidants to the egg yolk may represent an adaptive strategy to limit oxidative damage associated with growth to the developing embryo (i.e. maternal effects; Mousseau and Fox, 1998); indeed, across species, antioxidant allocation is positively correlated to embryonic growth rate (Deeming and Pike, 2013). External factors, to which a mother is exposed, such as diet, temperature extremes, pollution and predation pressure, can all affect oxidative balance and act as stimuli facilitating the 'conditioning' of her offspring's oxidative machinery to match the oxidative environment (Costantini, 2014). Whether or not an increased oxidative challenge arising from an exogenous source results in increased oxidative damage to the developing embryo is likely to depend on the capacity of yolk-derived antioxidants to buffer developing tissues.

Increased environmental oxygen concentration has been previously used to successfully induce oxidative stress in a variety of fish and aquatic invertebrates (Lushchak, 2011) and fruit flies (Orr and Sohal, 1992). In birds, the use of hyperoxia to induce oxidative stress is limited to a handful of studies in poultry. Following brief exposure (up to 72 h) to elevated oxygen concentration during prenatal development, Stock et al. (1990) found no differences in lipid peroxidation in embryonic tissues, whereas Wilson and Jaworski (1992) showed reductions in ascorbic acid in the brain, indicating a role for vitamin C in ROS detoxification during embryonic development. Brief hyperoxia also increased SOD activity in several embryonic organs, except the brain (van Golde et al., 1998). While hyperoxic conditions during embryonic development have been shown to increase oxygen consumption and growth rates in poultry (Lourens et al., 2007; Stock et al., 1983), which would further elevate endogenous ROS production, neither oxygen consumption nor growth rate increased in the smaller eggs of bobwhite quail (Williams and Swift, 1988). It is suggested that small eggs are less diffusion-limited than large eggs, due to the higher surface-area-to-volume ratio, and thus embryonic growth is not limited by oxygen uptake (Williams and Swift, 1988; Vleck and Bucher, 1998).

The aim of this study was to determine the capacity of yolk-derived antioxidants to protect the avian embryo from oxidative damage when exposed to an oxidative challenge. Eggs of wild great tits *Parus major* Linnaeus 1758 were incubated under either hyperoxic conditions or normal atmospheric oxygen levels. Hyperoxia was employed to provide an oxidative challenge by inducing a measurable change in oxidative stress without causing significant side effects (as per Koch and Hill, 2017). By using a species that lays small eggs with a high surface-area-to-volume ratio, we do not expect growth to be limited by oxygen and thus did not expect differences in oxygen uptake or growth rate between control and hyperoxic embryos (Williams and Swift, 1988; Vleck and Bucher, 1998). Through the quantification of levels of antioxidants and oxidative damage in brain tissue [the tissue shown to be most susceptible to lipid peroxidation during prenatal development (Surai et al., 1996, 1999)] and yolk, we investigated the capacity for maternally derived antioxidants to buffer the developing embryo from oxidative damage to lipids (concentration of malondialdehyde or MDA) and DNA (relative telomere length).

## MATERIALS AND METHODS

### Study system and experimental procedures

The study was conducted in May 2015 using a nestbox-breeding population of great tits located at Vombs fure, a mixed coniferous/deciduous forest, in southern Sweden (55 deg 39′N, 13 deg 33′E). Nests were visited at least once during nest building and egg laying; assuming that females lay one egg per day, the date of first egg and start of incubation were determined. Two eggs, selected at random, were collected from each of 27 nests with a clutch size of 8±1 eggs on the second day of incubation and transported in a warm insulated box to Lund University, Sweden. All eggs were collected over a period of six days. Eggs were randomly assigned to either a 'control' ($N_{eggs}$=28; $N_{nests}$=14) or 'hyperoxic' ($N_{eggs}$=26; $N_{nests}$=13) group and placed in incubators (Ruvmax, Ödskölt, Sweden) on the day of collection. Eggs from the same clutch were placed in the same group. Neither clutch size (mean±s.d.: control, 8.36±0.7; hyperoxic, 8.38±0.7; $t$=−0.10, $P$=0.919) nor start of incubation (mean±s.d.: control, 3.36±1.7; hyperoxic, 3.15±1.0; $t$=0.38, $P$=0.704) differed between control and treatment nests.

Control eggs were incubated at 37°C (Haftorn, 1988) and under normal atmospheric conditions (i.e. oxygen concentration of 21%), while hyperoxic eggs were incubated at 37°C and 40% oxygen concentration. Studies from the poultry literature reveal effects on antioxidant status at concentrations of 40–60% (van Golde et al., 1998; Wilson and Jaworski, 1992) but suggest that hatchability starts to decrease above 50–70% (Onagbesan et al., 2008) and growth rate declines above 70% (Stock et al., 1983). One incubator was used for each treatment. The experimental incubator had been modified such that it could be innervated with 60% oxygenated gas, via an inlet valve. Opening and closing of the inlet valve was controlled via an asynchronous serial communication bus (serial input/output eXchange, SIOX solutions Telefrang AB, Västra Frölunda, Sweden) and computer software (adapted from the open source Eclipse SCADA by Gentech Solutions AB, Västra Frölunda, Sweden) in order to maintain an oxygen concentration of 40.0±2.0% in the experimental incubator. In brief, the software continually receives a voltage signal, indicating the oxygen concentration, from an oxygen sensor (KE-50 model, GS Yuasa International Ltd, Kyoto, Japan) situated above the egg plate; if the concentration falls more than 2% below the set level, the inlet valve opens, allowing the input of gas until the set point is reached. Following each opening of the incubator (to add or remove eggs or refill the moisture chamber; see below), the oxygen concentration returned to the set point within 5 min of closing the incubator.

Incubators were set up one week before the start of the experiment to allow for calibration of temperature, humidity and oxygen concentration. Temperature and humidity were recorded every 1–5 min using iButtons (Maxim Integrated Products, San Jose, CA, USA) positioned in the centre of the incubator. Temperature and humidity were checked once per day and adjustments performed as necessary. Throughout the course of the experiment, temperature (mean±s.d.) was maintained at 37.16±0.24°C (control) and 37.15±0.29°C (hyperoxic). Humidity (mean±s.d.) was maintained at 67.51±0.56% (control) and 69.49±1.17% (hyperoxic), with the use of a plastic container filled with ∼450 ml of water (replenished daily and refreshed every 3 days) situated inside the incubator.

On day 11 of incubation (i.e. 9 days after collection and start of artificial incubation), eggs were removed from incubators and

euthanised by freezing. Eggs were weighed before and after artificial incubation. Whole eggs were stored at −80°C for later dissection to isolate yolk and brain tissues for the measurement of antioxidants and oxidative damage that are directly linked to physiological function and individual fitness (Monaghan et al., 2009). While techniques are available for measuring ROS directly, they are limited in scope and application (Salin et al., 2017). Eight embryos (four from each of the control and hyperoxic groups) had not properly developed and were excluded from further laboratory analyses. Procedures were conducted in accordance with Swedish legislation and approved by the Malmö-Lund animal ethics committee; eggs were collected under licence from Naturvårdsverket (the Swedish Environmental Protection Agency).

## Laboratory assays

### Antioxidants in brain tissue

Tissues were first homogenised with 0.01 mol $l^{-1}$ phosphate-buffered saline (PBS) (brain, 1:5; yolk, 1:1). In brain tissue, antioxidant status was characterised via quantification of the endogenous antioxidants – SOD ($N$=43) and total glutathione (tGSH; $N$=45), accounting for both the oxidised and reduced forms of the peptide glutathione. For the measurement of tGSH (see Isaksson, 2013), 10 µl of brain homogenate was mixed with 16 µl of 5% SSA (5-sulfoasalicylic acid) and centrifuged (10 min, 8000 ***g***, 4°C). A 10 µl sample of the supernatant was diluted with 200 µl of GSH buffer (143 mmol $l^{-1}$ $NaH_2PO_4$, 6.3 mmol $l^{-1}$ EDTA, pH 7.4), from which 20 µl was mixed with 170 µl of reaction solution containing 10 mmol $l^{-1}$ 5,5′-dithiobis 2-nitrobenzoic acid and 0.34 U of glutathione reductase in GSH buffer. The reaction was started by the addition of 34 µl of 2 mmol $l^{-1}$ nicotinamide adenine dinucleotide phosphate; following shaking, the absorbance was measured every 30 s for 5 min at 412 nm. Each 96-well plate included a blank and a serially diluted standard curve ranging from 100 µmol $l^{-1}$ to 3.12 µmol $l^{-1}$.

SOD was quantified using a colorimetric assay kit (product no. CS1000, Sigma-Aldrich, Stockholm, Sweden). Homogenised and diluted brain tissue (1:5, as above) was centrifuged (5 min, 1500 ***g***, 4°C), following which the supernatant was removed and diluted (1:10) with Dilution Buffer (provided in kit); 20 µl of the diluted supernatant was used in the assay, according to the manufacturer's protocol. Following the addition of the enzyme working solution, the plate was shaken and the absorbance was measured every minute for 15 min at 450 nm and at 40°C (i.e. approximate body temperature of a bird). SOD activity (U $ml^{-1}$) was calculated relative to a standard curve (included on each plate) ranging from 50 U $ml^{-1}$ to 1.5625 U $ml^{-1}$ and expressed per mg of protein (see below). Protein content was quantified according to the Bradford method (Bradford, 1976) and relative to a standard curve generated from a serial dilution of bovine serum albumin in solution ranging from 1.5 mg $ml^{-1}$ to 0.125 mg $ml^{-1}$. Two hundred microlitres of Bradford reagent was added to 5 µl of supernatant (diluted 1:3 with PBS) on a 96-well plate. Following 30 s shaking and 10 min incubation at room temperature, the absorbance was measured at 595 nm. All samples, standards and blanks were run in triplicate and mean intra-assay variation (means±s.d.) was: tGSH, 2.06±1.24; SOD, 3.95±3.51; protein, 6.73±6.81.

### Antioxidants in yolk

In yolk ($N$=34), we quantified three non-enzymatic antioxidants, α-tocopherol (vitamin E), zeaxanthin and lutein (both carotenoids), using High-Performance Liquid Chromatography (HPLC; see McGraw et al., 2002). Two hundred microlitres of internal standard containing 1 mmol $l^{-1}$ tocopheryl acetate and 600 µmol $l^{-1}$ retinyl acetate (product nos T3376 and R4632, respectively, Sigma-Aldrich) in acetone was added to 20 mg of yolk, followed by vortexing. After overnight incubation at −80°C, 200 µl of tert-butyl methyl ether was added, followed by vortexing. Samples were centrifuged at 10°C for 5 min (15,000 ***g***), following which the supernatant was transferred to a new tube and dried under nitrogen gas. A second washing step was performed by adding 200 µl of acetone, followed by vortexing and centrifugation; the supernatant was removed and passed through a filter, which was subsequently washed with acetone. Again, samples were dried under nitrogen gas. HPLC was performed using a Waters® 717plus Autosampler (Milford, MA, USA) fitted with a Phenomenex Synergi Hydro-Reverse Phase column (4 µm 80 Å, 250×3 mm+4×2 mm; Torrance, CA, USA), with a mobile phase of methanol/acetonitrile (80:20) and flow rate of 1.2 ml $min^{-1}$ at 40°C. Carotenoids were detected by absorbance at 450 nm and α-tocopherol was detected by fluorescence utilising excitation and emission at 290 nm and 325 nm, respectively. External standard curves enabled quantification of carotenoids (0–90 µmol $l^{-1}$; lutein no. 0133.1 and zeaxanthin no. 0119; both Carote Nature, Münsingen, Switzerland) and α-tocopherol (0–480 µmol $l^{-1}$; product no. T3634, Sigma-Aldrich).

### Lipid peroxidation

Oxidative damage in brain tissue and yolk was quantified via MDA, a marker of lipid peroxidation, by Gas Chromatography–Mass Spectrometry (GC–MS; see Isaksson et al., 2017). MDA was first extracted from 100 µl of the brain (1:5; $N$=44) and yolk (1:1; $N$=32) homogenates (see above): samples were vortexed with 50 µl of *O*-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine hydrochloride (product no. 194484, Sigma-Aldrich; 1 mmol $l^{-1}$ in 1.5 mol $l^{-1}$ sodium acetate, pH 5.0) and incubated at room temperature for 1 h. To this, 300 µl of heptane with internal standard (1-Bromo-3-fluorobenzene) was added. Following vortexing, the lower phase was removed; the upper phase was washed 2–3 times by the addition of 200 µl of distilled water, followed by vortexing and removal of the lower phase. Residual water was removed by adding anhydrous sodium sulphate and extracts were dried under nitrogen gas, leaving a final volume of 40–50 µl. Extracts were analysed using an Agilent 5975 MS (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent 6890 GC and fitted with an Agilent J&W HP-5MS column (length, 30 m; internal diameter, 0.25 mm; film, 0.25 µm). GC conditions consisted of 1 min at 60°C, followed by increases of 15°C $min^{-1}$ to 150°C, and then 10°C $min^{-1}$ to 270°C, and holding there for 5 min.

### Relative telomere length

Relative telomere length (RTL) was measured in DNA isolated from brain tissue ($N$=36) by quantitative real-time PCR (qPCR) using the Mx3005P (Agilent Technologies, Santa Clara, CA, USA). Amplification of telomere sequences was achieved using the primers: Tel1b forward (5′-CGGTTTGTTTGGGTTTGGGTTTG-GGTTTGGGTTTGGGTT-3′) and Tel2b reverse (5′-GGCTTGCC-TTACCCTTACCCTTACCCTTACCCTTACCCT-3′). Sequences of the reference gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), were amplified using primers specific for the great tit (Atema et al., 2013): forward (5′-TGTGATTTCAATGGTGACA-GC-3) and reverse (5′-AGCTTGACAAAATGGTCGTTC-3′). qPCR reactions were performed with a reaction volume of 25 µl containing 10 ng of DNA, 12.4 µl of Supermix (Platinum SYBR-green q-PCR SuperMix-UDG, Invitrogen, Waltham, MA, USA) and 200 nmol $l^{-1}$/200 nmol $l^{-1}$ Tel1b/Tel2b and 100 nmol $l^{-1}$/100 nmol $l^{-1}$ GAPDH-F/GAPDH-R in telomere and

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GAPDH reactions, respectively. Reactions were performed in triplicate and samples were randomly distributed among plates, based on treatment, with siblings included on the same plate. qPCR conditions were: (i) telomere: 10 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; and (ii) GAPDH: 10 min at 95°C, 15 min at 95°C, 30 s at 60°C and 30 s at 72°C. On each plate, a serial dilution (from 20 ng to 1.25 ng) of a reference DNA sample (from embryonic brain tissue) was included as a standard curve. Efficiencies of the reference curve were within the acceptable range for both telomere (mean±s.d., 104.7±2.56) and GAPDH (mean±s.d., 101.53±3.96). Telomere to single copy gene ratios (T:S ratios) were corrected using the Pfaffl method to account for variation in amplification efficiencies between telomere and GAPDH (Pfaffl, 2001). Mean intra- and inter-plate coefficients of variation for T:S ratios were 5.91 and 3.54, respectively.

### Statistical analyses

All statistical analyses were carried out in R 3.2.4 (http://www.R-project.org/). Data points were excluded if they fell below detection limits or outside of standard curves. Due to the relatively small sample size and some missing measurements, we decided it was best to use the full data set and perform univariate analyses of each response variable, rather than employing a multivariate approach on a reduced data set. Furthermore, with the exception of the three antioxidants measured in yolk (Pearson's correlation coefficient, range: 0.68–0.90), correlations between biomarkers from brain tissue were weak (Pearson's correlation coefficient, range: −0.31–+0.32). General linear mixed models (GLMMs) were fitted using lmerTest (Kuznetsova et al., 2017) to the response variables for brain biomarkers, i.e. SOD, tGSH, MDA and RTL, and yolk biomarkers, i.e. α-tocopherol, zeaxanthin, lutein and MDA. All variables were first assessed for normality and equal variances and transformed where necessary. We first checked for differences in initial egg mass and change in mass during incubation using a GLMM; there were no differences in either initial mass (mean±s.e.m.: control: 1.62±0.017 g, hyperoxic: 1.62±0.034 g; $\beta_{hyperoxic}$=−0.0016±0.043; $F$=0.0013$_{1,18.3}$; $P$=0.972) or mass loss (mean±s.e.m.: control: −0.077±0.0037 g, hyperoxic: −0.066±0.0095 g; $\beta_{hyperoxic}$= 0.010±0.011; $F$=0.92$_{1,40.0}$; $P$=0.343) between the control and experimental groups. Given this finding and that final mass was not available for two eggs, yet initial mass was highly correlated with final mass (Pearson's correlation coefficient: 0.96), we included initial mass in analyses to control for the effect of egg size on biomarkers. Using a hypothesis-led approach, we fitted the same GLMM to each response variable including a fixed factor of treatment (two levels: control or hyperoxic), initial mass (covariate) and a random effect of nest (to control for non-independence between siblings). We considered the need to include a random effect for qPCR plate in the RTL model but this accounted for zero variance and thus it was removed. Due to the potential for outliers to strongly influence outputs in small data sets, we assessed each variable for the presence of outliers, using the modified $z$-score and adopting a conservative threshold score of 3.5 (as recommended by Iglewicz and Hoaglin, 1993). Where outliers were identified, analyses were run both including and excluding these points, and the outputs of both are presented below. The significance of parameter estimates was estimated using conditional $F$-tests based on Satterthwaite approximation for the denominator degrees of freedom.

## RESULTS

We found no significant differences in antioxidant levels between embryonic brain tissue from eggs incubated in normal and elevated oxygen environments: neither the level of SOD (Fig. 1A; $\beta_{hyperoxic}$= −0.094±0.11; $F_{1,21.7}$=0.71; $P$=0.408) nor tGSH (Fig. 1B;

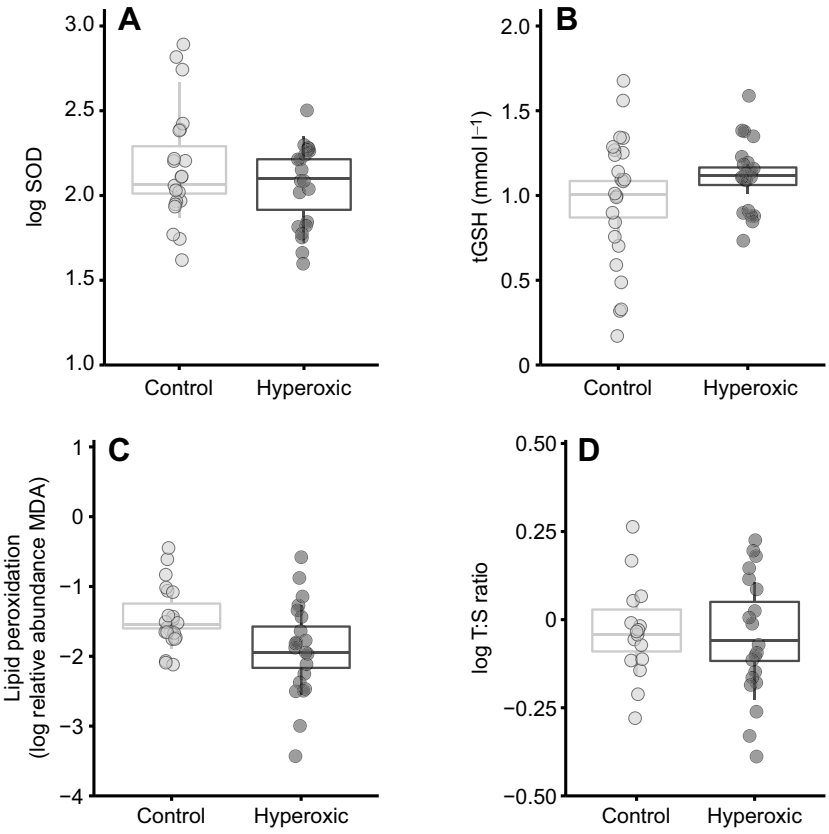


**Fig. 1. Oxidative status of embryonic brain tissue from great tit eggs incubated under control (21% oxygen; light grey) and hyperoxic (40% oxygen; dark grey) conditions.** Antioxidant capacity is quantified by (A) superoxide dismutase (SOD, U mg$^{-1}$ protein; $N$=43) and (B) total glutathione (tGSH; $N$=45), whereas damage is measured as (C) lipid peroxidation (malondialdehyde or MDA; $N$=44) and (D) DNA damage (relative telomere length; $N$=36). Box plots (showing median, 25th and 75th percentiles, and 1.5× inter-quartile range) represent fitted values from general linear mixed models accounting for the fixed effects of treatment and initial egg mass and random effect of nest. Observed values are shown as points.

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$\beta_{hyperoxic}$=0.13±0.11; $F_{1,22.5}$=1.58; $P$=0.222) differed between control and experimental brains. Although there was a tendency for lower lipid peroxidation (MDA) in hyperoxic brain tissue (Fig. 1C; $\beta_{hyperoxic}$=−0.42±0.21; $F_{1,21.0}$=4.05; $P$=0.057), the relationship weakened following exclusion of an outlier (3A; $\beta_{hyperoxic}$=−0.36±0.19; $F_{1,21.4}$=3.60; $P$=0.071). We found no difference in RTL (a marker of DNA damage) between control and hyperoxic embryos (Fig. 1D; $\beta_{hyperoxic}$=−0.013±0.061; $F_{1,21.6}$=0.047; $P$=0.831).

In contrast to embryonic brain tissue, differences were observed in both antioxidant levels and oxidative damage in yolk (Fig. 2). The amount of α-tocopherol (vitamin E) was significantly lower in hyperoxic yolks (Fig. 2A; $\beta_{hyperoxic}$=−1.62±0.71; $F_{1,13.9}$=5.24; $P$=0.038). There were no significant differences in levels of the carotenoids, lutein (Fig. 2B; $\beta_{hyperoxic}$=−0.60±0.34; $F_{1,14.4}$=3.01; $P$=0.104) and zeaxanthin (Fig. 2C; $\beta_{hyperoxic}$=−4.10±2.89; $F_{1,15.2}$=2.02; $P$=0.176), between control and hyperoxic yolks. MDA was significantly higher in the hyperoxic yolks compared with control yolks (Fig. 2D; $\beta_{hyperoxic}$=0.77±0.36; $F_{1,15.1}$=4.60; $P$=0.049) and the relationship was even stronger when three outliers (6A, 12A, 16B) were excluded based on the modified $z$-score ($\beta_{hyperoxic}$=0.63±0.22; $F_{1,13.9}$=8.01; $P$=0.013). Initial mass did not significantly affect any marker (all $P$>0.18), with the exception of yolk MDA, which was higher in yolks from larger eggs (β=1.6±0.77; $F_{1,24.9}$=4.33; $P$=0.048). With the exception of zeaxanthin (mean±s.d., 26.1±5.11), the mean variance associated with the random effect of nest was generally low (range: 0.014–0.31). All results are presented as means±s.e.m. unless stated otherwise.

## DISCUSSION

Although the avian brain is highly susceptible to lipid peroxidation during embryonic development (Surai et al., 1996, 1999), our results show that, when exposed to an exogenous oxidative challenge, yolk-derived antioxidants sufficiently buffer the developing brain from oxidative damage. Following an increased oxidative challenge, induced by incubation under hyperoxic conditions, we found lower levels of the antioxidant, vitamin E, and higher levels of lipid peroxidation in the yolks of great tit eggs, compared with those incubated under normal conditions, whereas we found no differences in antioxidants, lipid peroxidation or telomere length in brain tissue. Although trade-offs between different tissue types may occur, the avian brain has been shown, both *in vitro* and *in vivo*, to be the tissue that has highest susceptibility to peroxidation due to its high lipid content (Surai et al., 1996, 1999). While yolk-derived antioxidants have previously been shown to be important for protecting the embryo from oxidative stress, this knowledge largely comes from the poultry literature and there are very few tests of manipulating oxidative stress to the embryo (Stock et al., 1990; van Golde et al., 1998; Wilson and Jaworski, 1992).

During the last few days of embryonic development, large quantities of antioxidants – notably carotenoids and vitamin E – are transferred from the yolk to the embryo, where they are pivotal for maintaining redox homeostasis and limiting oxidative damage in the period immediately following hatching (Surai et al., 1996). While maternally transferred carotenoids are known to play a key role as a free-radical scavenger, reducing lipid peroxidation during embryonic development and in the first few days after hatching (McGraw et al., 2005; Surai and Speake, 1998), vitamin E has been shown to be a more superior antioxidant (Hartley and Kennedy, 2004; Surai et al., 1996, 1999). Our results support that theory because hyperoxic-exposed yolks showed greater depletion of vitamin E compared with carotenoids and relative to controls. Increased vitamin E in yolk, and subsequently in embryonic tissues, was shown to be effective in reducing the susceptibility of tissues to peroxidation *in vitro* (Surai et al., 1999), whereas an *in vivo* study demonstrated that low provision of vitamin E to the developing

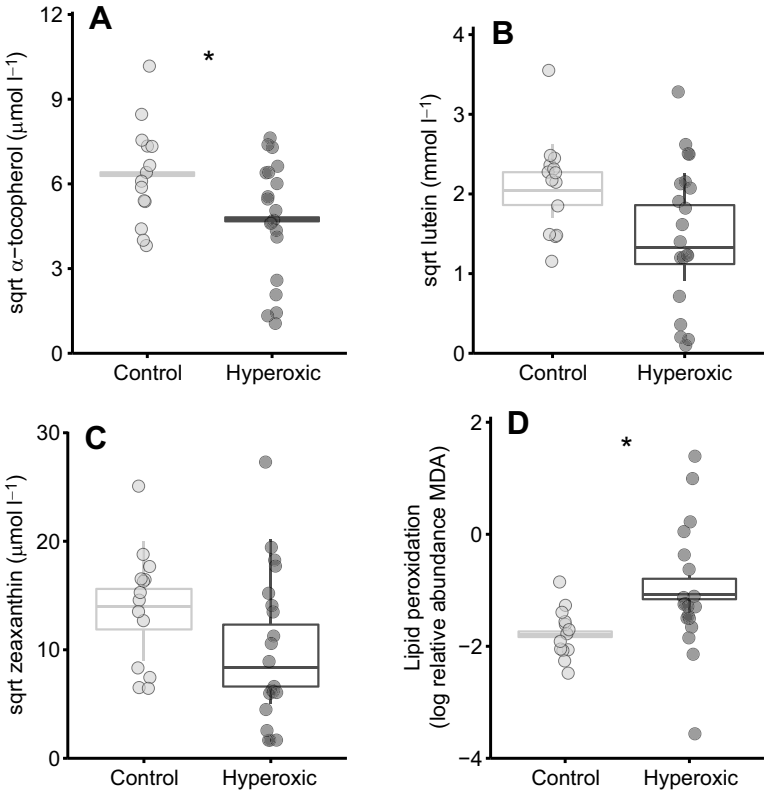


**Fig. 2. Oxidative status of yolks from great tit eggs incubated under control (21% oxygen; light grey) and hyperoxic (40% oxygen; dark grey) conditions.** Antioxidant capacity ($N$=34) is quantified by (A) α-tocopherol (vitamin E), and the carotenoids (B) lutein and (C) zeaxanthin (all square-root transformed; sqrt), whereas damage ($N$=32) is measured as (D) lipid peroxidation (malondialdehyde or MDA). Box plots (showing median, 25th and 75th percentiles, and 1.5× inter-quartile range) represent fitted values from general linear mixed models accounting for the fixed effect of treatment and initial egg mass and random effect of nest. Observed values are shown as points and significance at the 0.05 level is indicated by an asterisk.

chick embryo leads to development of encephalomalacia, as a result of peroxidative damage to the cerebellum (Mezes et al., 1997). This highlights the importance of maternally derived antioxidants for normal chick development, especially under high pro-oxidant environmental conditions.

Despite the clear benefit to the embryo imparted by the yolk's antioxidants when exposed to an oxidative challenge, the elevated oxidation and subsequent depletion of lipids, and/or depletion of yolk-derived antioxidants could have costs for the newly hatched chick. Since the developing avian embryo and newly hatched chick rely on lipids from the yolk for nourishment (Noble and Cocchi, 1990), elevated peroxidation of yolk lipids may limit the availability of the highly unsaturated lipids to the embryo or chick and create a nutritional deficit. Furthermore, it is the long-chain polyunsaturated fatty acids (PUFAs) – essential as components of cell membranes and signalling molecules, and for development of the brain and heart (Speake et al., 1998; Surai et al., 1996) – that are most susceptible to oxidation (Larsson et al., 2004).

Upon hatching, oxidative stress increases markedly as a result of exposure to atmospheric oxygen, onset of pulmonary respiration and an associated increase in metabolic rate (Vleck and Bucher, 1998). For wild birds living in environments with high levels of air pollution (e.g. nitrogen oxides and tropospheric ozone), the effect could be exacerbated as a consequence of an even greater elevation in endogenous ROS production, upon hatching. If antioxidants are limiting, the increased rate of depletion of antioxidants and PUFAs may pose a constraint on allocation to storage for protection during the crucial periods of hatching and early postnatal development. Negative links have previously been shown between antioxidant level and oxidative damage in postnatal development; e.g. shag chicks (*Phalacrocorax aristotelis*) with lower antioxidant capacity suffered higher levels of lipid peroxidation (Noguera et al., 2012), highlighting the importance of the availability of antioxidants for preventing oxidative damage during postnatal development. Such physiological constraints in early life may influence senescence and reproductive lifespan, and thus fitness (Cohen et al., 2017). Indeed, Noguera et al. (2012) showed that postnatal oxidative stress was associated with reduced probability of recruitment in shags. However, the observed depletion of yolk-derived antioxidants to protect the embryo during development may still be adaptive if it maximises fitness under the given environmental conditions. In other words, even if the depletion of maternally transferred antioxidants comes at a cost to physiological function later in life, if it enables normal embryonic development and successful hatching, depletion will be selected for over preservation of yolk antioxidants.

Another potential cost of depletion of maternally derived antioxidants may be paid for, in the short-term, by the immune system. Chicks cannot synthesise their own antibodies until several days after hatching; during this vulnerable period, they rely on passive immunity provided by immunoglobulin G (IgG), which is deposited in the yolk by the female (Kowalczyk et al., 1985). Chicks hatched from hens fed supplemental vitamin E showed higher levels of IgG, which is thought to be due to antioxidants protecting IgG from catabolism (Haq et al., 1996). While our study shows that yolk-derived vitamin E and carotenoids may be able to protect the developing embryonic tissues from oxidative damage, the depletion of antioxidant stores could put greater demand on the chick's own immune system post-hatching. Without passive immunity, the chick may still be able to respond to an immune challenge by activating a humoral response, but this in turn produces ROS, subsequently giving rise to further tissue damage such as lipid peroxidation (Costantini and Møller, 2009). Furthermore, micronutrient deficiencies during embryonic development could negatively impact development of the immune system (Klasing, 2018). Increased investment of resources into the immune system is also expected to be traded off against other functions, such as growth, which could have negative effects on fitness (Lochmiller and Deerenberg, 2000).

While hyperoxia has been used to induce oxidative stress in aquatic organisms and flies, the technique of increasing ROS exposure via elevated oxygen concentration in birds has been limited to a handful of studies in poultry and thus only precocial species (Stock et al., 1990; van Golde et al., 1998; Wilson and Jaworski, 1992). Attempts to experimentally increase oxidative stress in wild altricial birds, during adulthood, have been made via the administration of the herbicide, paraquat. Meitern et al. (2013) reported fatalities among 50% of paraquat-treated birds, whereas a dose–response study undertaken by Isaksson and Andersson (2008) found even relatively low doses caused reduced alertness and fatigue. The use of hyperoxia is expected to present similar challenges to paraquat and other toxins, i.e. identifying the appropriate dose and accounting for adverse side effects (Koch and Hill, 2017). Indeed, there is extensive literature in poultry science revealing diverse effects of exposure to elevated levels of oxygen during embryonic development including differential growth rates of organs and changes in hatchability; however, it is important to note that effects generally only become negative once oxygen exceeds 70% (McCutcheon et al., 1982; Onagbesan et al., 2008; Stock et al., 1983), far higher than the concentration employed in the present study. Here, we have demonstrated the successful application of hyperoxia to induce oxidative stress, as demonstrated by concurrent reduction in antioxidants and elevation of lipid peroxidation in the yolk, in a wild altricial bird species. Although only one incubator was used for each of the treatment and control, given the low variance in temperature and humidity, we are confident that the observed differences were indeed driven by the large differences in oxygen exposure. Importantly, we increased ROS exposure independently of growth rate. While embryonic growth in poultry appears to be oxygen-limited towards the end of incubation, and both hypoxia and hyperoxia during this stage decreases and increases growth, respectively (e.g. Metcalfe et al., 1984), small eggs may not be diffusion-limited in the same way as the larger eggs of poultry, due to a larger surface-area-to-volume ratio (Vleck and Bucher, 1998). Furthermore, the risk of development failure was no higher in embryos incubated in hyperoxic conditions, confirming no lethal effects of the treatment. However, we cannot exclude the possibility of effects that may not be evident until later in life and further studies are required to better understand the potential for adverse side effects.

Our study presents novel evidence for yolk-derived antioxidants protecting the embryo from oxidative damage in response to an environmentally driven oxidative challenge in a wild, altricial bird. The results demonstrate that the avian yolk is capable of bearing the burden of an elevated oxidative challenge, without the embryo having to compromise its endogenous antioxidant supply or suffer oxidative damage to tissue lipids and DNA. This furthers our understanding of the importance of maternally derived yolk antioxidants for providing protection to the developing embryo. However, the fitness consequences of the observed depletion of yolk antioxidants are unclear. Furthermore, the extent to which embryos are mere passive recipients of maternally derived egg components or whether they can modulate their transfer and utilisation remains poorly understood (Williams and Groothuis, 2015). Variation in embryo energetics, metabolism and development rates suggest that embryos might be

able to modulate the relationship between egg composition and postnatal phenotype (Starck and Ricklefs, 1998; Vleck and Vleck, 1996) and this is a field that demands closer investigation.

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**Competing interests**
The authors declare no competing or financial interests.

**Author contributions**
Conceptualization: H.W., C.I.; Methodology: H.W., P.S., C.I.; Validation: H.W.; Formal analysis: H.W.; Investigation: H.W., P.S.; Resources: H.W., P.S., C.I.; Data curation: H.W., P.S.; Writing - original draft: H.W.; Writing - review & editing: H.W., P.S., C.I.; Visualization: H.W., P.S.; Supervision: H.W., C.I.; Project administration: H.W., C.I.; Funding acquisition: H.W., C.I.

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**Data availability**
Data are available from figshare (Watson et al., 2018): https://doi.org/10.6084/m9.figshare.6216293.

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