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ORIGINAL PAPER

Female oxidative status, egg antioxidant protection and eggshell pigmentation: a supplemental feeding experiment in great tits

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Abstract Oxidative stress has been suggested as a mechanism underlying the costs of reproduction and life history trade-offs. Reproductive activities may lead to high production of pro-oxidants, whose activity can generate oxidative damage when not countered by adequate antioxidant defenses. Because inter-individual differences in the efficiency of the antioxidant system are influenced by an individual's diet, food availability experienced during reproduction may affect the females' antioxidant status and, in birds, their ability to transfer antioxidants into their eggs. Moreover, a female's ability to cope with oxidative stress has been suggested to influence pigment deposition in the eggshell, suggesting a possible signaling function of eggshell maculation. Here we performed a food supplementation experiment in a natural population of great tits (*Parus major*) in order to investigate how nutritional conditions experienced during the egg laying period affect the female's oxidative status and egg investment and how maternal oxidative status and egg antioxidant protection relate to eggshell pigmentation. We show that food-supplemented females had lower oxidative damage levels (ROMs) than non-food-supplemented females. Furthermore, a female's ROMs levels were negatively associated with the levels of yolk antioxidant protection in her eggs, but this negative association was only significant in non-food-supplemented females. This suggests that oxidative stress experienced during reproduction influences the allocation of antioxidants into the eggs. Moreover, we observed a positive relationship between eggshell pigment distribution and maternal and yolk antioxidant protection, suggesting that eggshell pigmentation is a cue of female (and offspring) quality.

Keywords Oxidative stress · Yolk antioxidants · Female condition · Food availability · Eggshell pigmentation · Costs of reproduction

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Introduction

Reproduction is one of the fundamental biological processes in an individual's life, yet it is energetically and metabolically costly (Stearns 1992; Harshman and Zera 2007; Hansen et al. 2013). In oviparous species, for example, the metabolic rate of females increases by up to 27 % during egg laying (Nilsson and Råberg 2001), and both fat and protein reserves become depleted (Williams 2005). Furthermore, during incubation, up to threefold increase of female metabolic rate has been documented (Moreno and Sanz 1994; Bryant 1997; de Heij et al. 2007; Nord et al. 2010).

The metabolic acceleration occurring during reproduction can translate into higher production of reactive oxygen species (ROS) and circulating free radicals (Olsson et al. 2012). The uncontrolled activity of such molecules can cause cell damage and oxidative stress (Halliwell and Gutteridge 2007), which is defined as the imbalance between pro-oxidants and antioxidant defense in favor of the former (Sies 1991). An increase of oxidative damage and a reduction of antioxidant protection

have, for example, been observed in response to an experimental increase of reproductive effort (Wiersma et al. 2004; Travers et al. 2010). Moreover, higher oxidative damage levels were found in breeding individuals as compared to non-breeding individuals (Costantini et al. 2014). The results of these studies suggest that oxidative stress may be a cost of reproduction (Costantini 2008; Metcalfe and Alonso-Alvarez 2010; Metcalfe and Monaghan 2013).

The antioxidant system of animals acts through different lines of defense, which involve endogenous elements (e.g. thiol groups and enzymes) and exogenous compounds acquired through the food (e.g. vitamins and carotenoids) (Catoni et al. 2008). The efficiency of the antioxidant system of an individual may thus be affected by its diet (Catoni et al. 2008; Cohen et al. 2009), in particular by the availability of proteins, which are involved in the synthesis of antioxidant compounds (Li et al. 2002; Alan and McWilliams 2013) and which can influence the activity of various antioxidant enzymes (Fechner et al. 2001). Indeed a low-protein diet has been found to lead to increased oxidative damage and decreased antioxidant activity in both captive and wild-living animals (Feoli et al. 2006; Alan and McWilliams 2013; Giordano et al. 2015).

In birds, a female's physiological and nutritional condition can affect her ability to deposit antioxidants into the eggs (Blount et al. 2002; Saino et al. 2002; Berthouly et al. 2007; Török et al. 2007; Costantini 2010). Egg antioxidants provide protection to the developing embryo against the deleterious effects of free radicals produced during prenatal growth (Surai et al. 1996), and low levels of yolk antioxidants have been shown to impair embryo development (Wilson 1997). Furthermore, low antioxidant availability during embryonic development can negatively affect an individual's reproductive success later in life (Surai 2002), highlighting the crucial role of maternally derived yolk antioxidants in modulating inter-individual variation in fitness.

In birds, eggshells are characterized by a variety of colour patterns created by the deposition of two main pigments: protoporphyrin, which causes red–brown eggshell coloration, and biliverdin, which causes blue–green eggshell coloration (Kennedy and Vevers 1976). Because of the antioxidant properties of biliverdin (Kaur et al. 2003), it has been suggested that the deposition of these pigments into the eggshell may signal a female's antioxidant capacity to her mate ('sexually selected eggshell coloration hypothesis' (SSH); Moreno and Osorno 2003). Indeed some studies found that females with a higher ability of oxidative stress resistance lay eggs with more intense blue–green coloration (Hanley et al. 2008: reviewed in Reynolds et al. (2009)). Protoporphyrin, on the other hand, has pro-oxidant properties (Afonso et al. 1999), and it has been suggested that an intense red–brown shell pigmentation may be related either to higher oxidative tolerance of females capable of efficiently removing protoporphyrin via the shell or to poor physiological condition of females experiencing high levels of circulating protoporphyrin (Moreno and Osorno 2003). However, to date no study has directly tested for a relationship between protoporphyrin-based eggshell maculation and female oxidative condition.

Here we experimentally manipulated the availability of protein-rich food shortly before and during egg laying in a natural population of great tits (*Parus major*) in order to investigate how food availability during reproduction affects female oxidative status, yolk antioxidant capacity and their relationship with eggshell pigmentation. We predicted that food-supplemented females have enhanced oxidative protection and/or reduced oxidative damage and that females with lower oxidative stress produce eggs with higher antioxidant content. Furthermore, based on Moreno and Osorno (2003) (see above), we predicted to find either a positive or a negative relationship between female oxidative status and eggshell maculation.

Materials and methods

Field procedures

The study was carried out between March and June 2012 in a nestbox breeding population of great tits in a forest dominantly composed of beech and spruce (Zurichbergwald, Switzerland; 47°20′08 ″ N, 8°30′01″ E). The nestboxes (12.5 cm× 12.5 cm×26.5 cm, Type Varia; wooden nestboxes equipped with a waterproof cover) were checked every other day from March onward to monitor the progress of nest building and the beginning of egg laying. At an advanced stage of nest building, but before the first egg was laid (mean±1 standard deviation (SD)=7.1±4.7 days before egg laying), we randomly assigned nestboxes to the food supplementation (F) or to the control group (NF). *F*-nests were supplemented with 15 g of diptera larvae (*Sarcophaga* spp), which represent ca. 80 % of the daily energy need of adult great tits (Gibb 1957) every other day until the clutch was completed. The NF nests were visited and treated as the F nests, but they did not receive extra food. All experimental nests were first breeding events.

In great tits, the time from the beginning of ovum development to its deposition is approximately 4 days (Perrins 1996). Consequently, food-supplemented females experienced increased food availability before the formation of the first egg and during the whole egg laying period. In order to ensure that only the breeding pair had access to the supplemented food, we placed the larvae in a plastic cup, which was attached to the inside wall of the nestbox and not visible from the outside. All larvae were eaten within 2 days (MG, personal observation). We are aware of the possibility that males may have eaten part of the supplementary food. However, the significant effect of the food treatment on female oxidative condition (see

"Results") and egg traits (Giordano et al. 2015) demonstrates that females ate a substantial part of the supplemental food and that this influenced their egg provisioning.

We marked newly laid eggs with a non-toxic marker and collected the fourth egg of each clutch on the day it was laid. On the same day, the egg was weighed, and the yolk was separated from the albumen, weighed and frozen at −80 °C until analysis. Only a subsample of the collected eggs could be used for antioxidant analyses (see below) because some eggs were damaged during transport.

The mean clutch size (± SD) in our study population was 8.2 (±1.3) eggs. The fourth egg is therefore one of the middle eggs of the laying sequence. In great tits, variation in the levels of yolk antioxidants is low within clutches, and yolk antioxidant concentrations do not vary with laying sequence (Isaksson et al. 2008; Remeš et al. 2011; see also Tschirren et al. 2004; Postma et al. 2014 for other egg components). Therefore the antioxidant capacity of the fourth egg can be considered representative for the antioxidant capacity of the clutch.

At clutch completion, a photo of all eggs was taken. Thereto, the eggs were removed from the nest, placed on a plate, and photographed in a standardized way using a Canon EOS 1000D digital camera (Canon Inc., Tokyo, Japan) from a distance of 10 cm. Millimeter paper was photographed together with the eggs to allow for calibration.

Breeding females (F: N=31; NF: N=39) were caught when their nestlings were 10 days old. Females are prone to desert their nest when disturbed during the egg laying or incubation period. For this reason, we caught females only during the nestling stage. Females were weighed with a Pesola spring balance (accuracy of 0.25 g), and a small blood sample (approximately 100 μl) was taken from their brachial vein. The blood was kept cool until centrifugation (10,621×g for 10 min), which happened on the same day. After centrifugation, we separated plasma and red blood cells and stored both at −80 ° C until oxidative stress analyses (see below), which were performed within 4 months after blood sampling.

Female non-enzymatic plasmatic antioxidant capacity

We measured the females' non-enzymatic plasmatic antioxidant capacity (OXY) (F: N=31; NF: N=39) using the OXY-Adsorbent test (Diacron International, Grosseto, Italy) following Costantini et al. (2011). This assay quantifies the ability of a biological matrix (plasma or yolk) to oppose the oxidizing action of hypochlorous acid, one of the strongest oxidant agents in biological systems (Halliwell and Gutteridge 2007). In short, plasma (2 μl) was diluted 1:100 (vol/vol) with distilled water. Then, 2 μl of the diluted plasma was added to 200 μl of a titred HOCl solution and incubated for 10 min at 37 °C. At the end of incubation, 2 μl of chromogen was added, and the absorbance was read at a wavelength of 490 nm (SpectraMax 340PC^{384} Microplate Reader, Molecular Devices, Sunnyvale, CA, USA). Measurements are expressed as mM of HOCl neutralized. Samples were run in duplicate. Repeatability of OXY measures was high (r=0.93, P<0.001, N=70). The interassay coefficient of variation was 5.2 %, and the intrassay coefficient of variation was 3.7 %.

Female glutathione peroxidase activity

Glutathione peroxidase (GPX) activity in the females' red blood cells (F: N=31; NF: N=39) was measured with the Ransel assay (Randox Laboratories, Crumlin, UK) (Paglia and Valentine 1967) according to the manufacturer's instructions. GPX is an antioxidant enzyme which, using thiols as cofactors, catalyzes the reduction of harmful oxidant agents (Arthur 2000). The Ransel assay assesses the ability of GPX to catalyze the neutralization of cumenehydroperoxide via oxidation of glutathione. Briefly, red blood cell samples were diluted 1:40 (vol/vol) with the dilution agent provided with the kit. Then, 200 μl of reagent and 8 μl of cumenehydroperoxide were added to 4 μl of the diluted sample, and after 1 and 3 min, absorbance was read at a wavelength of 340 nm (ThermoFisher, Vantaa, Finland). GPX activity was calculated following the manufacturer's instructions: [(Abs 1 min−Abs 3 min)/2]*15,873. To control for differences in red blood cell concentrations among samples, we measured the protein content using the Bio-Rad Bradford Protein assay kit (Bio-Rad Laboratories, Inc., CA, USA) according to the manufacturer's instructions. The results are expressed as units of GPX per milligram of proteins. Samples were run in duplicate. Repeatability of GPX activity measures was high (r=0.97, P<0.001, N=70). The interassay coefficient of variation was 9.7 %, and the intraassay coefficient of variation was 5.3 %.

Female red blood cell thiols

We measured thiol-containing compounds in the females' red blood cells (F: N=31; NF: N=39) using the –SHp test (Diacron International, Grosseto, Italy) according to previously published protocols (Costantini et al. 2011). Thiols are antioxidant compounds characterized by a sulfhydryl group (–SH), which reacts with, and scavenges, free radicals. Glutathione is one of the major thiols occurring in animal cells (Bindoli et al. 2008). For the assay, red blood cell samples were diluted 1:200 (vol/vol) with distilled water. Then, 12.5 μl of diluted red blood cells was added to a sulfate buffer (pH 7.6) and allowed to incubate for 3 min at room temperature. After incubation, absorbance was read at 405 nm (ThermoFisher, Vantaa, Finland). Subsequently, 5 μl of chromogen (purchased with the kit) was added, and samples were incubated for 5 min at room temperature. At the end of incubation, the absorbance was read again at 405 nm (ThermoFisher, Vantaa, Finland). Results were expressed as

μmol l^{-1} of –SH groups per milligram of protein. Samples were run in duplicate. Red blood thiols measures were highly repeatable (r=0.97, P<0.001, N=70). The interassay coefficient of variation was 6.7 %, and the intraassay coefficient of variation was 6.4 %.

Because several antioxidant capacity markers were significantly correlated (Pearson product–moment correlation: GPX/thiols: r=0.167, P=0.041, N=70; GPX/OXY: r=0.185, P=0.023, N=70; thiols/OXY: r=−0.084, P=0.303, N=70), we performed a principal component analysis and used the first two principal components (antioxidant capacity biomarkers PC1 and PC2) in the statistical analyses. The first principal component (antioxidant capacity PC1) explained 41.8 % of the variation in female antioxidant status. Antioxidant capacity PC1 describes the component of the female's antioxidant system involving thiols and GPX activity (enzymatic–thiolic component) (loadings: GPX 0.793, thiols 0.792, OXY 0.305). The second principal component (antioxidant capacity PC2) explained further 34.4 % of the variation in female antioxidant status. Antioxidant capacity PC2 describes the non-enzymatic plasmatic antioxidant capacity (loadings: GPX 0.222, thiols −0.234, OXY 0.963). Antioxidant capacity biomarkers were not normally distributed; thus, they were log-transformed before performing the principal component analysis.

Female oxidative damage

We estimated the females' plasma concentration of oxidative damage (F: N=31; NF: N=39) using the colorimetric dROMs test (Diacron International, Grosseto, Italy). This assay measures intermediate oxidative damage molecules (mostly hydroperoxides; Alberti et al. 2000). The assay was performed following previously published protocols (Costantini et al. 2011). In short, plasma (8 μl) was diluted with 200 μl of an acetate buffer (pH 4.8) and an aromatic alkyl-amine (chromogen). The samples were incubated for 75 min at 37 °C. At the end of incubation, the samples were centrifuged (17,950×g for 2 min). The supernatants (190 μl) were pipetted in a microplate, and the absorbance was read at a wavelength of 505 nm (ThermoFisher, Vantaa, Finland). Results are expressed as mM of H_2O_2 equivalents. Samples were run in duplicate. The repeatability of ROMs measures was high (r=0.98, P<0.001, N=70). The interassay coefficient of variation was 10.4 %, and the intraassay coefficient of variation was 5.4 %. Oxidative damage measures (ROMs) were not normally distributed; thus, they were log-transformed prior to statistical analyses.

Yolk antioxidant capacity

We measured the yolk antioxidant capacity (F: N=14, NF: N=11) using the OXY-adsorbent test (Diacron International, Grosseto, Italy) following previously published protocols (Costantini 2010). In short, we extracted the antioxidants in the yolk by diluting an aliquot of yolk (60 mg) with 5 ml of distilled water and vortexing and sonicating this solution for 10 min. The dilution of yolk with water produces an emulsion in which all yolk antioxidants (e.g. carotenoids, vitamin A, vitamin D, vitamin E) are embedded. We then performed the same procedure for a second aliquot of yolk, to which we added 15 μl of a solution of known concentration of OXY standard. The difference in concentration between the second yolk aliquot and the OXY standard was divided by the concentration of the first yolk aliquot to determine the extraction efficiency, which was (mean±SD) 95±1.9 %. The yolk solution was centrifuged (10,621×g for 2 min), and the supernatant was diluted 1:4 (vol/vol) with distilled water. Then, 2 μl of the diluted yolk solution was incubated with 200 μl of titred HOCl for 10 min at 37 °C. At the end of incubation, 2 μl of chromogen (purchased with the kit) was added, and the absorbance was spectrophotometrically read at a wavelength of 490 nm (SpectraMax 340PC^{384} Microplate Reader, Molecular Devices, Sunnyvale, CA, USA). Measurements are expressed as μM of HOCl neutralized per milligram of yolk. Samples were run in duplicate. Yolk OXY measures were highly repeatable (r=0.98, P<0.001, N=25). The interassay coefficient of variation was 7.4 %, and the intraassay coefficient of variation was 5.5 %. Yolk OXY values were not normally distributed; thus, they were log-transformed before performing statistical analyses. Since it was not possible to perform the OXY assay on some eggs, the yolk OXY sample size is lower than the total number of eggs collected.

Eggshell pigmentation

Great tits lay white eggs maculated with protoporphyrin-based red–brown spots. Eggshell maculation was scored on the photographed side of the egg using the criteria described in Gosler et al. (2000) (F: N=209 eggs of 31 clutches, NF: N=271 eggs of 39 clutches). Pigment intensity (I) was scored from 1 (pale pigments) to 5 (dark pigments). Pigment distribution (D) was scored from 1 (pigments concentrated on one part of the egg) to 5 (pigments evenly distributed). Spot size (S) was scored from 1 (small spots) to 3 (big spots). Zero scores were not assigned. All eggs were scored by the same person (MG). The fourth egg, collected to measure yolk antioxidant capacity, was not scored. In order to calculate repeatability of eggshell pigmentation scores, a subset of eggs was scored twice. Repeatability was I: r=0.67, P<0.001, N=130; D: r=0.72, P<0.001, N=130; S: r=0.51, N=130. The mean I, D, S per clutch was calculated and used for subsequent analyses (within-clutch repeatability: I: r=0.87 P<0.001, N=480; D: r=0.79, P<0.001, N=480; S: r=0.51, P<0.001, N=480).

Statistical analyses

Firstly, we used general linear models to test for effects of the food treatment on female antioxidant capacity PC1 and PC2, female oxidative damage (ROMs), female body mass, egg mass, yolk mass, yolk antioxidant capacity, eggshell pigmentation (intensity, distribution and spot size) and clutch size.

Secondly, we fitted a general linear model to assess whether food treatment, female antioxidant capacity PC1 and PC2, female oxidative damage, and their two-way interactions affected yolk antioxidant capacity.

Finally, we used general linear models to test for the effects of the food treatment, female antioxidant capacity PC1 and PC2, female oxidative damage, yolk antioxidant capacity, and their two-way interactions on eggshell pigmentation (intensity, distribution and spot size).

Final models were obtained with a stepwise backward procedure. Non-significant interactions and factors ($P>0.05$) were removed from the models, starting with the least significant interaction term. Residuals from all the models were checked for homoscedasticity and normality. No variance inflation factors equal to or higher than 2 were identified (Marques de Sá 2007). Analyses were performed in JMP 10 (SAS Institute 1989–2007).

Results

Effect of food treatment on female oxidative status

Food-supplemented females had significantly lower levels of oxidative damage than non-food-supplemented females (ROMs: $F_{1,68}=5.976$, $P=0.017$; Fig. 1). No effect of food supplementation on the females' enzymatic–thiolic antioxidant capacity ($F_{1,68}=1.079$, $P=0.303$) or non-enzymatic antioxidant capacity ($F_{1,68}=1.934$, $P=0.169$) was observed. No significant effect of the food supplementation on female body mass was found ($F_{1,68}=1.562$, $P=0.216$).

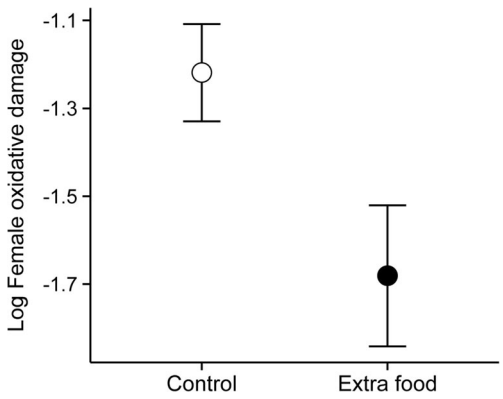

Fig. 1 Effect of food treatment on plasma oxidative damage levels (ROMs; mM H_2O_2 equivalents) in food-supplemented (*filled circle*; $N=31$) and non-supplemented (*open circle*; $N=39$) female great tits. Means± 1 SE are shown

Effect of food treatment on yolk antioxidant status and reproductive investment

Food supplementation did not significantly affect the antioxidant capacity of the eggs ($F_{1,23}=0.298$, $P=0.591$), egg mass ($F_{1,63}=0.114$, $P=0.726$), yolk mass ($F_{1,63}=0.421$, $P=0.519$) or clutch size ($F_{1,68}=2.533$, $P=0.116$). Furthermore, there was no treatment effect on eggshell pigmentation (intensity: $F_{1,68}=0.464$, $P=0.498$; spot size: $F_{1,68}=2.304$, $P=0.134$; distribution: $F_{1,68}=0.488$, $P=0.491$).

Relationship between female oxidative status and yolk antioxidant status

The females' levels of oxidative damage (ROMs) were negatively related to the antioxidant capacity of their eggs ($F_{1,19}=11.207$, $P=0.003$). Moreover, there was a significant interaction effect between the food treatment and the females' levels of oxidative damage on yolk antioxidant capacity ($F_{1,19}=11.203$, $P=0.003$; Fig. 2). The decrease of the egg antioxidant capacity with increasing ROMs levels of the mother was pronounced in non-food-supplemented females ($b=-0.165$; $F_{1,9}=21.437$, $P=0.012$), but weak and non-significant in food-supplemented females ($b=-0.018$; $F_{1,12}=0.096$, $P=0.762$). No relationship between egg antioxidant capacity and female enzymatic–thiolic antioxidant capacity ($F_{1,19}=0.034$, $P=0.855$) or non-enzymatic antioxidant capacity ($F_{1,19}=1.801$, $P=0.195$) was observed.

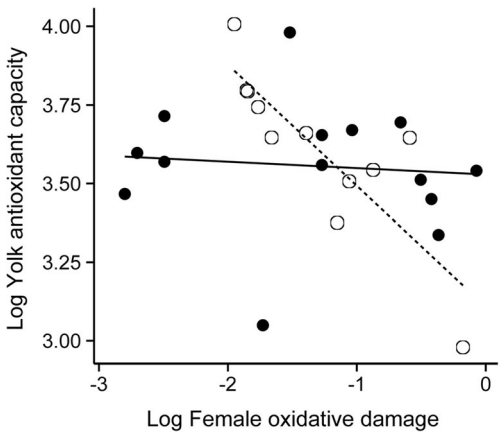

Fig. 2 Relationship between female plasma oxidative damage (ROMs; mM H_2O_2 equivalents) and yolk antioxidant capacity (yolk OXY; μM HOCl neutralized mg^{-1} yolk) in food-supplemented (*filled circle*, *solid line*; $N=14$) and non-supplemented (*open circle*, *dotted line*; $N=11$) females

Relationships among eggshell pigmentation, female oxidative status and yolk antioxidant status

Eggshell pigment distribution was significantly positively related to female enzymatic–thiolic antioxidant capacity (b= 0.505; $F_{1,22}$=8.495, P=0.008; Fig. 3a). Females with a higher blood antioxidant capacity laid eggs with a more homogenous spot distribution. Similarly, pigment distribution was significantly related to the antioxidant capacity of the yolk (b= 0.391; $F_{1,22}$=5.09, P=0.034; Fig. 3b). Eggs with a higher yolk antioxidant capacity had a more homogenous shell spot distribution. No relationships between eggshell pigment distribution and female non-enzymatic antioxidant capacity ($F_{1,21}$= 1.555, P=0.226) or female oxidative damage ($F_{1,20}$=0.192, P=0.666) were observed.

No relationships between pigmentation intensity and female antioxidant capacity (enzymatic–thiolic antioxidant capacity: $F_{1,21}$=1.350, P=0.258; non-enzymatic antioxidant capacity: $F_{1,19}$=0.001, P=0.977), female oxidative damage ($F_{1,22}$=1.359, P=0.256) or yolk antioxidant capacity ($F_{1,23}$= 1.222, P=0.281) were observed.

Similarly, no relationships between spot size and female antioxidant capacity (enzymatic–thiolic antioxidant capacity: $F_{1,66}$=0.924, P=0.339; non-enzymatic antioxidant capacity: $F_{1,64}$=0.052, P=0.821), female oxidative damage ($F_{1,65}$= 0.121, P=0.729) or yolk antioxidant capacity ($F_{1,19}$=0.034, P=0.855) were detected. None of the interactions between food treatment and female or egg traits (P>0.091 in all cases) significantly affected eggshell pigmentation (D, I, S), and they were therefore not retained in the final models.

Discussion

Here we experimentally tested in a wild bird population how nutritional conditions experienced during the egg laying period affect female oxidative status and the allocation of antioxidants into the eggs. Moreover, we tested the hypothesis that protoporphyrin-based eggshell pigmentation relates to a female's oxidative condition and her antioxidant investment into the eggs.

The level of oxidative damage (ROMs) in the females' circulation during the nestling rearing period was strongly influenced by the food treatment. Females that received extra food during the egg laying period had less oxidative damage than non-food-supplemented females.

Whereas circulating levels of oxidative damage were reduced when females received extra food, we observed no difference in antioxidant protection between treatment groups. One explanation for these findings is that the food supplementation led to a reduction in the production of oxidizing agents. Given that the extra food was provided within the nest, food-supplemented females may, for example, have reduced their flying and food searching time. Indeed physical activity can cause an imbalance in the levels of fatty acids, with an increase of unsaturated fatty acids, molecules susceptible to oxidative damage (Porter et al. 1995; Nikolaidis and Mougios 2004). Furthermore, the contraction of skeletal muscles occurring during flying can result in an accelerated production of oxidizing agents, such as superoxide (Powers and Jackson 2008).

An alternative explanation is that the food supplementation might not have reduced the production of oxidizing agents, but it may have boosted a (unmeasured) component of the antioxidant machinery, for example, uric acid (Alan and McWilliams 2013) or catalase (Rindler et al. 2013). In fact, protein levels in the diet have been shown to affect a variety of antioxidant pathways (e.g. enzymatic activity (Fechner et al. 2001), production of uric acid (Alan and McWilliams 2013) and synthesis of thiol-containing compounds (Li et al. 2002)). Moreover, we cannot exclude the possibility that the food supplementation induced short-lasting beneficial effects on the measured antioxidant components, not persisting long enough to be detected. Finally, the food supplementation might have affected the birds' cell membrane composition, making them less susceptible to oxidative damage (Hulbert et al. 2007).

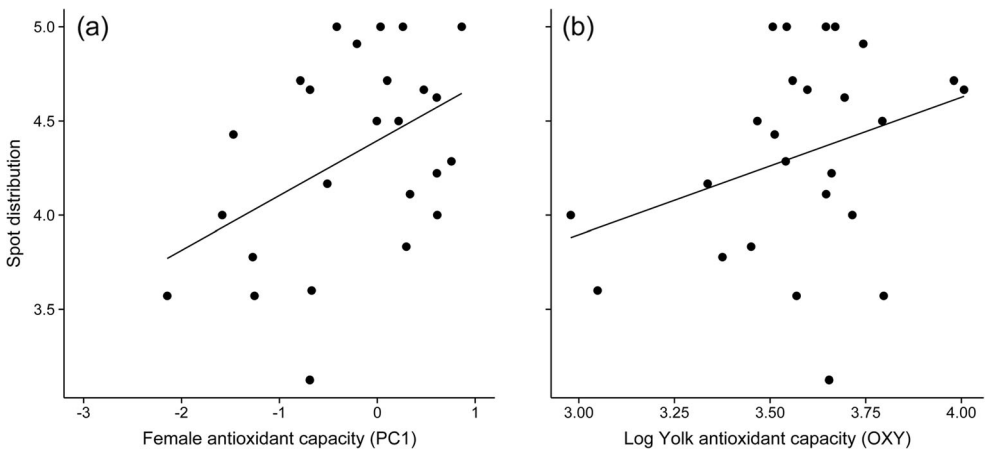

Fig. 3 Relationships between eggshell pigmentation distribution and **a** female enzymatic–thiolic antioxidant capacity (*PC1*) and **b** yolk antioxidant capacity (*OXY*; μM HOCl neutralized mg^{-1} yolk)

Although the mechanism behind the lower levels of oxidative damage observed in food-supplemented females remains currently unknown, our data clearly show that the benefits of having extra food available during egg laying can carry over and can still be detected during the nestling rearing phase. These results imply that an individual's oxidative balance is sensitive to relatively short-term changes in territory quality, meaning that an increase in breeding territory quality can translate in a reduction in the oxidative cost of reproduction (van de Crommenacker et al. 2010).

Previous studies on captive and wild birds have linked an increased reproductive effort with a reduced ability to neutralize free radicals. Captive zebra finches (*Taeniopygiaguttata*) females, for example, showed a reduced red blood cell antioxidant capacity and lower antioxidant enzyme activity when rearing enlarged broods (Alonso-Alvarez et al. 2004; Wiersma et al. 2004). Similarly, great tits rearing an experimentally enlarged brood had a lowered resistance to oxidative stress (Christe et al. 2012). We found that food-supplemented and non-food-supplemented females had a similar level of reproductive investment (in terms of egg mass, yolk mass, yolk antioxidant capacity and clutch size; see also Giordano et al. 2014), but food-supplemented females experienced significantly lower levels of oxidative stress. This suggests that the supplemented food allowed females to counter the oxidative costs of reproduction and that extra nutrients derived from the supplementation were used for self-maintenance rather than for increasing reproductive investment.

Whereas the food supplementation did not directly influence yolk antioxidant deposition, we found that it influenced the relationship between a female's levels of oxidative damage and the yolk antioxidant capacity of her eggs. Females with higher plasma oxidative damage levels deposited lower amounts of antioxidants in their eggs, suggesting that oxidative stress may constrain the female's reproductive investment. This negative association between female oxidative damage and egg antioxidant investment was evident only in non-food-supplemented females, whereas food-supplemented females were able to sustain a high deposition of yolk antioxidants, regardless of their circulating levels of oxidative damage. Being able to sustain high levels of antioxidant deposition may be advantageous because low levels of yolk antioxidants can impair embryo development (Wilson 1997), and a reduced yolk antioxidant deposition may negatively affect the nestlings' survival prospects (McGraw et al. 2005; Marri and Richner 2014).

It has been proposed that females may signal their oxidative condition to the male by modulating the deposition of eggshell pigments, thereby eliciting a higher paternal effort (Moreno and Osorno 2003). Most studies that tested this hypothesis focused on biliverdin-based eggshell colours (e.g. Morales et al. 2008), whereas the signaling function of protoporphyrin-based eggshell pigmentation is less well understood.

Support for a potential signaling function of protoporphyrin-based maculation comes from a recent study in great tits, which showed that the intraclutch increase in pigmentation darkness was positively related to female quality (i.e. body size and presence of red cell lysis) (De Coster et al. 2013). In contrast with this result, Martínez-de la Puente et al. (2007) showed a negative relationship between eggshell spottiness and female condition (i.e. immunoglobulin levels) in blue tits (*Cyanistes caeruleus*), a species closely related to great tits. However, these studies did not test for a relationship between eggshell pigmentation and female oxidative condition.

In our study, no relationship between pigmentation intensity or spot size and female oxidative status was detected. But interestingly, we found a significant relationship between the distribution of protoporphyrin-based pigments on the eggshell and both yolk and female antioxidant capacity. Eggs with high yolk antioxidant capacity and eggs produced by females with high enzymatic–thiolic antioxidant capacity had more homogenously distributed spots. To the best of our knowledge, this is the first observation of a relationship between female oxidative status and eggshell pigmentation in a species with protoporphyrin-based eggshell coloration.

In blue tits, another species with protoporphyrin-colored eggs, males were found to adjust their parental effort according to eggshell pigment distribution (Sanz and García-Navas 2009), with an increase of the daily paternal provisioning rate for nestlings hatched from eggs with more evenly distributed shell spots. It suggests that males are able to discriminate eggshell patterns, also in cavities and nestboxes, and that eggshell pigment distribution influences paternal investment.

However, besides a potential signaling function, there are alternative explanations for the relationship between eggshell pigment distribution and female and egg oxidative status observed in our study. For example, eggshell pigmentation can influence eggshell strength (Gosler et al. 2005), and females in good physiological condition might simply be able to produce more robust eggs. Although the possible physiological mechanisms and costs behind this process are not known, our results suggest that females with high antioxidant capacity may be able to deploy protoporphyrin more widely through the shell, with possible benefits for shell stability. In order to disentangle the function behind eggshell maculation, future work should simultaneously and experimentally test the alternative hypotheses (i.e. signaling vs. structural function).

In conclusion, our results show that (1) food availability experienced during the laying period can influence the oxidative cost of reproduction in great tit females and (2) the female's oxidative status and her nutritional condition influence the deposition of antioxidants into the eggs in an interactive way. Moreover, we found that (3) eggshell spot distribution relates to female and yolk antioxidant status, thereby supporting the hypothesis that eggshell maculation is a cue of female quality.

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Ethical standards All procedures were conducted under license from the Veterinary Office of the Canton of Zurich, Switzerland (195/2010) and the Federal Office for the Environment, Switzerland (324).

Conflict of interest The authors declare that they have no conflict of interest.

References

Afonso S, Vanore G, Batlle A (1999) Protoporphyrin IX and oxidative stress. Free Radic Res 31:161–170

Alan RR, McWilliams SR (2013) Oxidative stress, circulating antioxidants, and dietary preferences in songbirds. Comp Biochem Physiol B 164:185–193

Alberti A, Bolognini L, Macciantelli D, Caratelli M (2000) The radical cation of N, N-diethyl-para-paraphenylendiamine: a possible indicator of oxidative stress in biological samples. Res Chem Intermed 26: 253–267

Alonso-Alvarez C, Bertrand S, Devevey G, Prost J, Faivre B, Sorci G (2004) Increased susceptibility to oxidative stress as a proximate cost of reproduction. Ecol Lett 7:363–368

Arthur JR (2000) The glutathione peroxidases. Cell Mol Life Sci 57: 1825–1835

Berthouly A, Helfenstein F, Richner H (2007) Cellular immune response, stress resistance and competitiveness in nestling great tits in relation to maternally transmitted carotenoids. Funct Ecol 21:335–343

Bindoli A, Fukuto JM, Forman HJ (2008) Thiol chemistry in peroxidase catalysis and redox signaling. Antioxid Redox Signal 10:1549–1564

Blount JD, Surai PF, Nager RG, Houston DC, Møller AP, Trewby ML, Kennedy MW (2002) Carotenoids and egg quality in the lesser black-backed gull *Larus fuscus*: a supplemental feeding study of maternal effects. Proc R Soc Lond B 269:29–36

Bryant DM (1997) Energy expenditure in wild birds. Proc Nutr Soc 56: 1025–1039

Catoni C, Peters A, Schaefer HM (2008) Life history trade-offs are influenced by the diversity, availability and interactions of dietary antioxidants. Anim Behav 76:1107–1119

Christe P, Glaizot O, Strepparava N, Devevey G, Fumagalli L (2012) Twofold cost of reproduction: an increase in parental effort leads to higher malarial parasitaemia and to a decrease in resistance to oxidative stress. Proc R Soc Lond B 279:1142–1149

Cohen AA, McGraw KJ, Robinson WD (2009) Serum antioxidant levels in wild birds vary in relation to diet, season, life history strategy, and species. Oecologia 161:673–683

Costantini D (2008) Oxidative stress in ecology and evolution: lessons from avian studies. Ecol Lett 11:1238–1251

Costantini D (2010) Complex trade-offs in the pigeon (*Columba livia*): egg antioxidant capacity and female serum oxidative status in relation to diet quality. J Comp Physiol B 180:731–739

Costantini D, Monaghan P, Metcalfe NB (2011) Biochemical integration of blood redox state in captive zebra finches (*Taeniopygia guttata*). J Exp Biol 214:1148–1152

Costantini D, Meillere A, Carravieri A et al (2014) Oxidative stress in relation to reproduction, contaminants, gender and age in a long-lived seabird. Oecologia 175:1107–1116

De Coster G, De Neve L, Lens L (2013) Intra-clutch variation in avian eggshell pigmentation covaries with female quality. J Ornithol 154: 1057–1065

de Heij ME, van der Graaf AJ, Hafner D, Tinbergen JM (2007) Metabolic rate of nocturnal incubation in female great tits, *Parus major*, in relation to clutch size measured in a natural environment. J Exp Biol 210:2006–2012

Fechner A, Bohme CC, Gromer S, Funk M, Schirmer RH, Becker K (2001) Antioxidant status and nitric oxide in the malnutrition syndrome kwashiorkor. Pediatr Res 49:237–243

Feoli AM, Siqueira IR, Almeida L, Tramontina AC, Vanzella C, Sbaraini S, Schweigert ID, Netto CA, Perry MLS, Goncalves CA (2006) Effects of protein malnutrition on oxidative status in rat brain. Nutrition 22:160–165

Gibb J (1957) Food requirements and other observations on captive tits. Bird Study 4:207–215

Giordano M, Groothuis TGG, Tschirren B (2014) Interactions between prenatal maternal effects and posthatching conditions in a wild bird population. Behav Ecol 25:1459–1466

Giordano M, Costantini D, Tschirren B (2015) Sex-specific effects of prenatal and postnatal nutritional conditions on the oxidative status of great tit nestlings. Oecologia 177:123–131

Gosler AG, Barnett PR, Reynolds SJ (2000) Inheritance and variation in eggshell patterning in the great tit *Parus major*. Proc R Soc Lond B 267:2469–2473

Gosler AG, Higham JP, Reynolds SJ (2005) Why are birds' eggs speckled? Ecol Lett 8:1105–1113

Halliwell B, Gutteridge JMC (2007) Free radicals in biology and medicine, 4th edn. Oxford University Press, Oxford

Hanley D, Heiber G, Dearborn DC (2008) Testing an assumption of the sexual-signaling hypothesis: does blue-green egg reflect maternal antioxidant capacity? Condor 110:767–771

Hansen M, Flatt T, Aguilaniu H (2013) Reproduction, fat metabolism, and life span: what is the connection? Cell Metab 17:10–19

Harshman LG, Zera AJ (2007) The cost of reproduction: the devil in the details. Trends Ecol Evol 22:80–86

Hulbert AJ, Pamplona R, Buffenstein R, Buttemer WA (2007) Life and death: metabolic rate, membrane composition, and life span of animals. Physiol Rev 87:1175–1213

Isaksson C, Johansson A, Andersson S (2008) Egg yolk carotenoids in relation to habitat and reproductive investment in the great tit *Parus major*. Physiol Biochem Zool 81:112–118

Kaur H, Hughes MN, Green CJ, Naughton P, Foresti R, Motterlini R (2003) Interaction of bilirubin and biliverdin with reactive nitrogen species. FEBS Lett 543:113–119

Kennedy GY, Vevers HG (1976) Survey of avian eggshell pigments. Comp Biochem Physiol B 55:117–123

Li J, Wang H, Stoner GD, Bray TM (2002) Dietary supplementation with cysteine prodrugs selectively restores tissue glutathione levels and redox status in protein-malnourished mice. J Nutr Biochem 13:625–633

Marques de Sá JP (2007) Applied statistics. Springer, Berlin

Marri V, Richner H (2014) Yolk carotenoids increase fledging success in great tit nestlings. Oecologia 176:371–377

Martínez-de la Puente J, Merino S, Moreno J, Tomás G, Morales J, Lobato E, García-Fraile S, Martínez J (2007) Are eggshell spottiness and colour indicators of health and condition in blue tits *Cyanistes caeruleus*? J Avian Biol 38:377–384

McGraw KJ, Adkins-Regan E, Parker RS (2005) Maternally derived carotenoid pigments affect offspring survival, sex ratio, and sexual attractiveness in a colorful songbird. Naturwissenschaften 92:375–380

Metcalfe NB, Alonso-Alvarez C (2010) Oxidative stress as a life-history constraint: the role of reactive oxygen species in shaping phenotypes from conception to death. Funct Ecol 24:984–996

Metcalfe NB, Monaghan P (2013) Does reproduction cause oxidative stress? An open question. Trends Ecol Evol 28:347–350

Morales J, Velando A, Moreno J (2008) Pigment allocation to eggs decreases plasma antioxidants in a songbird. Behav Ecol Sociobiol 63:227–233

Moreno J, Osorno JL (2003) Avian egg colour and sexual selection: does eggshell pigmentation reflect female condition and genetic quality? Ecol Lett 6:803–806

Moreno J, Sanz JJ (1994) The relationship between energy-expenditure during incubation and clutch size in the pied flycatcher *Ficedula hypoleuca*. J Avian Biol 25:125–130

Nikolaidis MG, Mougios V (2004) Effects of exercise on the fatty-acid composition of blood and tissue lipids. Sports Med 34:1051–1076

Nilsson JA, Råberg L (2001) The resting metabolic cost of egg laying and nestling feeding in great tits. Oecologia 128:187–192

Nord A, Sandell MI, Nilsson JA (2010) Female zebra finches compromise clutch temperature in energetically demanding incubation conditions. Funct Ecol 24:1031–1036

Olsson M, Healey M, Perrin C, Wilson M, Tobler M (2012) Sex-specific SOD levels and DNA damage in painted dragon lizards (*Ctenophorus pictus*). Oecologia 170:917–924

Paglia DE, Valentine WN (1967) Studies on quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 70:158–169

Perrins CM (1996) Eggs, egg formation and the timing of breeding. Ibis 138:2–15

Porter NA, Caldwell SE, Mills KA (1995) Mechanisms of free-radical oxidation of unsaturated lipids. Lipids 30:277–290

Postma E, Siitari H, Schwabl H, Richner H, Tschirren B (2014) The multivariate egg: quantifying within- and among-clutch correlations between maternally derived yolk immunoglobulins and yolk androgens using multivariate mixed models. Oecologia 174:631–638

Powers SK, Jackson MJ (2008) Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. Physiol Rev 88:1243–1276

Remeš V, Matysiokova B, Klejdus B (2011) Egg yolk antioxidant deposition as a function of parental ornamentation, age, and environment in great tits *Parus major*. J Avian Biol 42:387–396

Reynolds SJ, Martin GR, Cassey P (2009) Is sexual selection blurring the functional significance of eggshell coloration hypotheses? Anim Behav 78:209–215

Rindler PM, Plafker SM, Szweda LI, Kinter M (2013) High dietary fat selectively increases catalase expression within cardiac mitochondria. J Biol Chem 288:1979–1990

Saino N, Bertacche V, Ferrari RP, Martinelli R, Møller AP, Stradi R (2002) Carotenoid concentration in barn swallow eggs is influenced by laying order, maternal infection and paternal ornamentation. Proc R Soc Lond B 269:1729–1733

Sanz JJ, García-Navas V (2009) Eggshell pigmentation pattern in relation to breeding performance of blue tits *Cyanistes caeruleus*. J Anim Ecol 78:31–41

SAS Institute (1989–2007) JMP, Version 10. Cary, NC

Sies H (1991) The role of reactive oxygen species in biological processes. Klin Wochenschr 69:965–968

Stearns SC (1992) The evolution of life histories. Oxford University Press, New York

Surai PF (2002) Natural antioxidants in avian nutrition and reproduction. Nottingham University Press, Nottingham

Surai PF, Noble RC, Speake BK (1996) Tissue-specific differences in antioxidant distribution and susceptibility to lipid peroxidation during development of the chick embryo. Biochim Biophys Acta 1304: 1–10

Török J, Hargitai R, Hegyi G, Matus Z, Michl G, Peczely P, Rosivall B, Toth G (2007) Carotenoids in the egg yolks of collared flycatchers (*Ficedula albicollis*) in relation to parental quality, environmental factors and laying order. Behav Ecol Sociobiol 61:541–550

Travers M, Clinchy M, Zanette L, Boonstra R, Williams TD (2010) Indirect predator effects on clutch size and the cost of egg production. Ecol Lett 13:980–988

Tschirren B, Richner H, Schwabl H (2004) Ectoparasite-modulated deposition of maternal androgens in great tit eggs. Proc R Soc Lond B 271:1371–1375

van de Crommenacker J, Horrocks NPC, Versteegh MA, Komdeur J, Tieleman BI, Matson KD (2010) Effects of immune supplementation and immune challenge on oxidative status and physiology in a model bird: implications for ecologists. J Exp Biol 213: 3527–3535

Wiersma P, Selman C, Speakman JR, Verhulst S (2004) Birds sacrifice oxidative protection for reproduction. Proc R Soc Lond B 271: S360–S363

Williams TD (2005) Mechanisms underlying the costs of egg production. Bioscience 55:39–48

Wilson HR (1997) Effects of maternal nutrition on hatchability. Poult Sci 76:134–143