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Egg yolk antioxidant deposition as a function of parental ornamentation, age, and environment in great tits *Parus major*

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Females can modify phenotype of their offspring through the deposition of biologically active compounds into eggs, including carotenoids, vitamins and other antioxidants. Understanding patterns of deposition is critical for better insight into the significance of maternal effects. Here we investigated how egg yolk antioxidants (lutein, zeaxanthin, β-carotene, vitamin A and E) related to environmental conditions and parental characteristics in great tits *Parus major* using data from three breeding seasons. Male and female traits included condition, age and multiple feather ornaments, both carotenoid- and melanin-based (carotenoid and UV chroma of yellow breast feathers, area of black breast band, white cheek immaculateness). Yolk mass increased with ambient temperature during laying, laying date, and the area of male black breast band. Lutein, zeaxanthin, and vitamin E increased with laying date. Total antioxidants increased with female age, immaculateness of female white cheek patch, and UV chroma of carotenoid-based yellow breast feathers of the social mate. These patterns were thus consistent with 1) environmental effects on yolk mass and composition, 2) higher quality females depositing more antioxidants, and 3) differential allocation of resources in females in relation to male ornamentation. Overall, environmental factors, female traits, and male traits all had an influence on egg yolk characteristics in this socially monogamous songbird.

By modifying prenatal environment of the young, parents can significantly affect phenotype and performance of their offspring (Mousseau and Fox 1998). Birds are particularly interesting model organisms in the context of maternal effects, because embryo development takes place within a sealed system, the egg, whose contents are fixed by the mother at laying. Avian mothers transfer to their eggs many biologically active compounds, including antioxidants (Surai 2002), hormones (Groothuis et al. 2005), antibodies (Hasselquist and Nilsson 2009), and antibacterial enzymes (Shawkey et al. 2008). Thus they modify the composition and quality of their eggs and morphology, physiology, and behavior of their offspring (Surai 2002, Groothuis et al. 2005).

Carotenoids and vitamins count among the most important biologically active egg yolk compounds. Carotenoids can be synthesized only by plants while animals must ingest them with their food. Studies on both domestic and wild birds have demonstrated higher yolk carotenoid concentrations in mothers supplemented with a carotenoid-rich diet (McGraw et al. 2005, Remeš et al. 2007, Tanvez et al. 2009). Carotenoids enhance the intensity of both cell-mediated and humoral immune response (Møller et al. 2000, Chew and Park 2004). They are also effective scavengers of reactive metabolites and thus can curtail the intensity of oxidative stress (Krinsky 2001, but see Constantini and Møller 2008). Carotenoids and vitamin E reduce the susceptibility of yolk lipids to peroxidative damage (Blount et al. 2002) and later protect the developing embryo from oxidative stress (Surai et al. 1996). Upon hatching, yolk-derived carotenoids affect the susceptibility of tissues to oxidative damage (Surai et al. 1996). Egg yolk-derived carotenoids may enhance post-hatching immune and antioxidant capacity (Koutsos et al. 2006), parasite resistance (Ewen et al. 2009), chick growth (Tanvez et al. 2009), and overall post-hatching performance of nestlings (Surai 2002, McGraw et al. 2005, Saino et al. 2008, but see Remeš et al. 2007).

Female birds have been reported to systematically deposit important yolk compounds in relation to various characteristics of themselves, of their social and genetic mates, and of environmental conditions. Deposition of antioxidants into egg yolk was found to be related to female body condition (Groothuis et al. 2006, Navara et al. 2006) and immune status (Saino et al. 2002), female social environment (Verboven et al. 2005, but see Safran et al. 2010), timing of breeding (Szigeti et al. 2007, Safran et al. 2008, Saino et al. 2008, Hargitai et al. 2009), habitat (Hõrak et al. 2002, Isaksson et al. 2008a), food supply (Török et al. 2007), or temperature (Saino et al. 2004). Females might also differentially allocate yolk antioxidants in relation to social mate quality and attractiveness (Saino et al. 2002, Navara et al. 2006, Szigeti et al. 2007, Safran et al. 2008, Ratikainen and Kokko 2010, but see Török et al. 2007).

Identifying robust correlates of egg yolk antioxidant deposition is a prerequisite for better understanding of parental investment strategies, potential role of these substances in adaptive offspring engineering, and the role of environment in modulating patterns of deposition. Here, we investigated correlates of a set of antioxidants (lutein, zeaxanthin, β-carotene, vitamin A and E) in egg yolk in a wild-ranging population of great tits *Parus major* using a large sample of individuals across three breeding seasons. We tested several hypotheses and explored patterns in the data where expectations of the direction of effects were not clear. First, environmental conditions may strongly modulate deposition of egg antioxidants, either facilitating or constraining it. Thus, 1) we studied how timing of breeding, ambient temperature, breeding density, and territory quality predicted yolk mass and deposition of antioxidants. We expected positive correlations of antioxidants with laying day, ambient temperature during laying and territory quality, because these factors should indicate food supply. Insect prey develops progressively with season and thus females laying later have more food available. Higher ambient temperatures allow for more insect activity and thus higher food availability for foraging females. The effect of breeding density could be either negative or positive depending on whether spacing behavior facilitates or constrains food availability. Second, high quality females should allocate more yolk compounds to their eggs (investment hypothesis, Safran et al. 2008). Thus, 2) we studied whether female characteristics presumably related to individual quality (feather coloration, age, condition) predicted yolk mass and deposition of yolk antioxidants. Finally, females should allocate either more (differential allocation, Sheldon 2000) or less resources (differential compensation, Ratikainen and Kokko 2010) into offspring when paired to attractive males. Thus, 3) we studied whether and how social mate characteristics thought to reflect attractiveness (feather coloration, age, condition) predicted yolk mass and deposition of yolk antioxidants.

Methods

General fieldwork

This work was conducted on three adjacent nest-box plots (188 nest-boxes in total) in a deciduous forest near Grygov (49°31′N, 17°19′E, 205 m a.s.l.) in eastern Czech Republic. The forest is dominated by lime *Tilia* spp. and oak *Quercus* spp. with interspersed ash *Fraxinus excelsior,* hornbeam *Carpinus betulus,* and alder *Alnus glutinosa.* Nest-boxes are placed about 1.6 m above ground and besides great tits are inhabited by collared flycatchers *Ficedula albicollis,* blue tits *Cyanistes caeruleus,* and nuthatches *Sitta europea.*

Fieldwork was carried out between 2005 and 2007 from early April until mid-June. We checked nest-boxes daily to record laying of the first egg and final clutch size. When there were between six and seven eggs laid (i.e. before the incubation started, Remeš unpubl.), we collected the 4th egg (50 eggs in 2005, 45 eggs in 2006, and 66 eggs in 2007). We weighed the egg to the nearest 0.01 g and placed it into the freezer under −20°C. Within one month after the field season ended, we let the eggs thaw under room temperature, separated the yolk, weighed it, and froze it again under −20°C. Yolk antioxidants were analyzed during autumn, but no later than in October. We collected only one egg per clutch because of ethical reasons and also because this population is subject to detailed investigation taking place during chick rearing (including capturing the adults).

During feeding of nestlings (median age of young for females = 7 d, for males = 9 d, range in both cases 6–11 d), we captured parents in the nest-box. We captured females at all the nests (n = 163). However, because of time constraints, we captured males only from a subset of nests (n = 101). We measured their tarsus length with a digital caliper (nearest 0.01 mm) and weighed them on a spring Pesola balance (nearest 0.125 g). We calculated body condition as residuals from the regression of body mass on tarsus length. From each bird we took 10 to 15 yellow feathers from the upper right part of breast for later spectrophotometric analysis. We photographed the bird's white cheek (i.e. right side of the head) and breast with a digital camera (Panasonic DMC-FZ5). While taking a picture of the cheek, the bird was held in a standardized position on its left side; while taking a picture of the breast, the bird was held outstretched by its tarsi and beak and photographed together with a ruler from a standard distance (Matysioková and Remeš 2010a). All measurements and photographs were taken by VR. We determined the age of the birds based on their plumage as one year old or older (Svensson 1992).

We defined breeding density as the number of adjacent nest-boxes (within 2.5 ha centered on the breeding nest-box) occupied by great tit pairs during the formation of the yolk of the sampled egg, i.e. during seven days preceding egg laying (Perrins 1979). Breeding density varied from zero to nine. Every index of breeding density is subjective, but we chose 2.5 ha because this is the largest area reported to be used by great tits during breeding (Wilkin et al. 2006 and references therein) and thus possibly captures well intraspecific behavioral interaction of birds. We defined territory quality as the number of years in which the particular nest-box was occupied by great tits between 2005 and 2009. Territory quality varied between one and five. We defined laying temperature as the mean of average daytime temperatures (from 6:00 to 20:00) during seven days preceding the laying of the sampled egg. Temperatures were obtained from a local weather station (7 km from the study site).

Feather coloration

We chose to analyze the following characteristics of feather coloration: area of the black breast stripe (Norris 1990), carotenoid and UV chroma of yellow breast feathers (Isaksson et al. 2008b), and immaculateness of the white cheek (sensu Ferns and Hinsley 2004). We analyzed photos of breast and cheek in Adobe Photoshop CS3 Extended. We used quick selection tool to roughly delimit the black stripe or the white cheek. Then we manually finished the selection so that it was as precise as possible and measured the surface area of the stripe or cheek. We used a ruler

photographed with every bird to adjust the scale of each photo and to obtain absolute surface area (in cm^2) and in the case of the cheek also perimeter (in cm). We defined stripe surface as the area of the black feathers between the point of inflexion, where the ventral stripe widens to a throat patch, and the posterior end of the stripe (Matysioková and Remeš 2010a). We calculated immaculateness of the white cheek as $4*\pi*(\text{area}/\text{perimeter}^2)$, which served as an index to measure regularity of the cheek's borders. It is equivalent to the index used by Ferns and Hinsley (2004) and the value of 1 indicates perfect circle, whereas lower values (approaching zero) indicate shapes with lower area for a given perimeter. All measurements were taken by BM. To assess repeatability, a different observer measured a subsample of photos. Repeatability, calculated as the intraclass correlation coefficient (Lessells and Boag 1987), was high for both stripe area ($r_i = 0.87$, $p < 0.001$, $n = 75$) and cheek immaculateness ($r_i = 0.89$, $p < 0.001$, $n = 75$).

We quantified reflectance spectra of yellow feathers sampled from the breast using standard procedures (Andersson and Prager 2006). We used 10–15 feathers from each bird, which is enough to obtain reliable values from our study species (Quesada and Senar 2006). We used an Avantes AvaSpec-2048 fiber optic spectrometer together with an AvaLight-XE xenon pulsed light source and WS-2 white reference tile. The probe was used both to provide light and to sample reflected light and was held perpendicular to feather surface. We took five readings from different parts of each set of feathers. Feathers were arranged on a black, nonreflective surface so that the underlying surface was completely covered and not visible.

We obtained reflectance (%) from 320 to 700 nm in 1-nm increments. We calculated carotenoid chroma as R700 minus R450, divided by R700, where R700 is reflectance at 700 nm and R450 reflectance at 450 nm. We use carotenoid chroma here because it reflects the amount of yellow carotenoids (lutein and zeaxanthin) in breast feathers in the great tit (Isaksson et al. 2008b). Hue might be a better measure of carotenoid concentration in saturated carotenoid-based colors (Andersson and Prager 2006, p. 82). However, our reflectance spectra had always reasonable reflectance at 450 nm, where lutein and zeaxanthin absorb maximally (females: mean = 14.2%, range: 9.3 to 22.5%, n = 128; males: mean = 14.7%, range: 7.8 to 24.4%, n = 101). This indicates that our carotenoid-based color was not saturated and that is why we used carotenoid chroma. We calculated UV chroma as summed reflectance from 320 to 400 nm divided by total reflectance (i.e. summed reflectance from 320 to 700 nm). We use UV chroma, because UV-based coloration is ubiquitous in birds (Eaton and Lanyon 2003) and has a tight link to courtship signaling (Hausmann et al. 2003). It has been also demonstrated to reflect nutritional condition in great tit nestlings (Jacot et al. 2010). In statistical analyses we always used the average chroma calculated from the five readings from each set of feathers. To assess repeatability of our measurements, in a subsample of feathers we arranged feathers anew and took another five readings and again averaged the carotenoid chroma calculated from them. We calculated repeatability of these two average chroma estimates as an intraclass correlation coefficient (Lessells and Boag 1987), which was sufficiently high (carotenoid chroma: $r_i = 0.85$, $p < 0.001$, $n = 55$; UV chroma: $r_i = 0.90$, $p < 0.001$, $n = 55$).

Analysis of antioxidants

Lutein, zeaxanthin, β-carotene, α-tocopherol and retinol were purchased from Sigma-Aldrich (St. Louis, MI, USA) and Carl Roth (Karlsruhe, Germany). We prepared stock standard solutions of each of the compounds (concentration = 10 μg ml^{-1}) in acetone and methanol (1:1, v/v) and stored them in darkness at 4°C. We controlled the stability of the stock solution of standards daily for one week and observed no change in concentrations. We prepared working solutions daily by mixing and diluting the stock solution with acetone/methanol (1:1, v/v). Samples were homogenized 3 min in 3 ml acetone at ultrasound bath. After centrifugation at 15 000 × g at 4°C for 10 min, the extracts were evaporated to dryness in a rotary vacuum evaporator IKA RV 05-ST with a water bath HB 4 (all from IKA-Werke, Staufen, Germany), dissolved in 500 μl acetone/methanol (1:1, v/v), and injected directly into the HPLC system.

An Agilent 1100 Series HPLC system (Agilent Technologies, Waldbronn, Germany) consisted of an on-line degasser, a binary pump, an autosampler, a thermostated column compartment, a photodiode array UV-VIS detector (working at 280 nm [16 nm SBW], 330 nm [8 nm SBW] and 446 nm [8 nm SBW]), and fluorometric detector (Hewlett-Packard) working at Ex 300 nm and Em 330 nm. LC separation was carried out on a Zorbax SB-CN rapid resolution (75 × 4.6 mm, particle size 3.5 μm), reversed-phase column (Agilent Technologies, USA). The mobile phase consisted of 0.05 mM (v/v) ammonium formate (solvent A) and methanol (solvent B). A linear gradient of the mobile phase was applied as follows: from start 70% B, from start to 10 min 100% B, from 10 to 15 min 100% B, from 15 to 18 min 70% B. The flow rate was 0.7 ml min^{-1} and typically 10 μl aliquots were injected into the column. The column oven temperature was set at 30°C.

Statistical analysis

It is not clear whether the concentration or the amount of yolk antioxidants is biologically more important (Safran et al. 2008). Thus, as dependent variables, we modeled 1) components from a principal component analysis (PCA) performed on concentrations of yolk antioxidants (see below), total summed concentrations of antioxidants, and 2) total amount of antioxidants per yolk, obtained by multiplying concentrations by yolk mass. Besides yolk antioxidants, we also modeled yolk mass. To use as much data as possible and to avoid overly complex models, we used three sets of variables as predictors in three separate models: 1) environmental factors (laying date, laying temperature, breeding density, and territory quality), 2) female phenotypic traits (UV and carotenoid chroma of yellow breast feathers, area of the black breast band, cheek immaculateness, condition, and age), and 3) male phenotypic traits (the same as in females). Unfortunately, this approach disabled exploring more complex issues, e.g. environment-individual

state interactions, but we felt it was not possible to fit such complex models with the data in hand. Although we report p-values of F-tests, we focus on standardized regression coefficients as measures of effect size.

It is difficult to select important predictor variables when analyzing observational data. When judging importance of individual predictors within our three types of models (i.e. environmental factors, female and male phenotypic traits as predictors), we used F-tests and p-values in full models. We did not use stepwise procedures, because they might lead to biased results (Anderson 2007); moreover, when the predictors are not correlated, parameter estimates from full vs minimum models obtained by stepwise procedures are very close (Grafen and Hails 2002).

Some females were sampled in more than one season. Seven females were sampled in three seasons, 21 females in two seasons, and 100 females in one season only. For the 28 females sampled in more than one year, we calculated repeatability of egg size (calculated on raw values) and composition (calculated on values centered by year, see below) defined as intraclass correlation coefficient (Lessells and Boag 1987). We used Proc Varcomp of SAS and calculated repeatability as: variance component of female/(variance component of female + error variance component). In all models, female and nestbox identity were used as random factors with random intercepts only; we used general linear mixed models (Proc Mixed of SAS). No male was sampled in more than one season, which was certainly caused by much lower number of males captured. Denominator degrees of freedom were calculated by the Satterthwaite method.

Concentrations of all yolk antioxidants except lutein significantly differed among years: lutein ($F_{2,158} = 0.6$, $p = 0.539$), zeaxanthin ($F_{2,158} = 6.7$, $p = 0.002$), β-carotene ($F_{2,150} = 26.8$, $p < 0.001$), vitamin E ($F_{2,158} = 38.0$, $p < 0.001$), vitamin A ($F_{2,158} = 26.2$, $p < 0.001$), PC1 ($F_{2,150} = 4.2$, $p = 0.017$), PC2 ($F_{2,150} = 86.1$, $p < 0.001$), total antioxidants ($F_{2,150} = 29.3$, $p < 0.001$), and antioxidants per yolk ($F_{2,150} = 29.3$, $p < 0.001$). When year was added to our models, its variance inflation factor varied from 4.1 to 5.6, which indicated potential problems with collinearity of predictors (Quinn and Keough 2002). Thus, we centered our dependent variables (i.e. components from PCA, total concentration of antioxidants, and the amount of antioxidants per yolk) within years by subtracting yearly mean from every value. Variance inflation factors in all the models with these centered variables were between 1.0 and 1.7 for all predictors, which indicated that the problems with collinearity were solved. Of course, year was not included as an independent variable in the models with centered dependent variables. We also centered two predictor variables, laying date and laying temperature, which also differed strongly among years (date: $F_{2,160} = 54.4$, $p < 0.001$; temperature: $F_{2,160} = 68.4$, $p < 0.001$).

PCA was run on concentrations of all five antioxidants based on general guidelines by Quinn and Keough (2002). As we did not want antioxidants with higher absolute concentrations to dominate the principal components (PCs) extracted, we ran PCA on the correlation matrix. This explicitly implies that we gave the same weight to all the antioxidants. We did this, because we had no a priory information about the relative importance of individual yolk antioxidants in our species. However, we also used a different approach, namely total antioxidant concentration (see above), which gave more weight to antioxidants with higher concentrations. We used only PCs with Eigenvalues higher than one. To improve interpretability of the PCs (for justification see Quinn and Keough 2002), we performed the Varimax rotation with the extraction of two components. Concentrations of all antioxidants were $\log_{10}$ transformed before submitting them to PCA. Similarly, total antioxidant concentrations and amounts of antioxidants per yolk were $\log_{10}$ transformed in all the models.

Results

Antioxidant concentrations and yolk mass

Table 1 shows descriptive characteristics of great tit eggs. Egg and yolk mass were highly positively correlated, whereas egg mass and relative yolk mass were correlated negatively (Supplementary material Appendix 1, Table S1). Correlations between yolk mass and relative yolk mass on the one side and antioxidant concentrations on the other were weak but consistently negative. This resulted in no overall correlation between yolk mass and the amount of antioxidants per yolk. However, correlations between individual antioxidant concentrations and the amount of antioxidants per yolk were highly positive (Supplementary material Appendix 1, Table S1). These results suggest that the amount of antioxidants in yolks is driven more by antioxidant concentration than yolk mass. Concentrations of individual antioxidants were positively inter-correlated (Supplementary material Appendix 1, Table S1). We performed a PCA

Table 1. Descriptive characteristics of great tit eggs and yolks. Values for individual females from multiple years were averaged before calculation of the mean and median to avoid pseudoreplication (n = 128). For how repeatability was calculated, see Methods.

	Mean	SD	Median	Repeatability
Egg mass (g)	1.66	0.13	1.66	0.77
Yolk mass (g)	0.34	0.03	0.34	0.42
Relative yolk mass (%)	20.47	1.58	20.42	0.37
Lutein (μg g^{-1})	57.56	39.23	47.40	0.25
Zeaxanthin (μg g^{-1})	1.76	1.47	1.36	0.05
β-carotene (μg g^{-1})	1.40	1.99	0.88	0.14
Vitamin E (μg g^{-1})	216.38	133.44	179.05	<0.01
Vitamin A (μg g^{-1})	13.62	7.75	12.00	<0.01
Total antioxidants (μg g^{-1})	294.76	169.75	245.70	<0.01
Antioxidants per yolk (μg)	99.38	56.92	81.30	<0.01

on antioxidant concentrations. PC1Antioxidant correlated strongly with lutein (component loading 0.82) and zeaxanthin (0.87), less so with vitamin E (0.47), and only weakly with vitamin A (0.31) and β-carotene (−0.16); it explained 35.5% of variance. PC2Antioxidant correlated strongly with vitamin E (component loading 0.70), vitamin A (0.75) and β-carotene (0.78), weakly with lutein (0.29), and not at all with zeaxanthin (−0.04); it explained further 34.8% of variation. Repeatability of egg size was quite high, whereas that of yolk composition was low to zero (Table 1).

Environmental factors

Yolk mass was larger late in the season and increased with laying temperature. Overall concentration of antioxidants was not related to any of the environmental factors. However, concentrations of lutein and zeaxanthin, and to a lesser extent of vitamin E, increased with later laying dates (Fig. 1a). Although yolk mass increased with laying temperature, the amount of antioxidants per yolk did not increase significantly (Fig. 2; Supplementary material Appendix 1, Table S2). This was certainly caused by consistently negative correlations between yolk mass and antioxidant concentrations (Supplementary material Appendix 1, Table S1). Although only two of the correlations were statistically significant, their consistently negative direction might have been enough to weaken the relationship between laying temperature and the amount of antioxidants per yolk.

Female and male traits

Concentrations of vitamin E, vitamin A, and β-carotene increased with cheek immaculateness of females. This means that females with more regular white patch on cheeks deposited more antioxidants. Older females deposited higher concentration and amount of total antioxidants per yolk. This was driven by lutein, zeaxanthin and vitamin E, as witnessed by a higher PC1Antioxidant score in older females (Fig. 3a; Supplementary material Appendix 1, Table S2). Yolk mass increased with the area of male black breast band. Concentration and amount per yolk of total antioxidants increased with UV chroma of yellow breast feathers of males (Fig. 3b). This was driven by lutein, zeaxanthin and vitamin E, as witnessed by a positive relationship of PC1Antioxidant to male UV chroma (Fig. 3b; Supplementary material Appendix 1, Table S2). Although yolk mass increased with the area of male black breast band, the amount of antioxidants per yolk did not increase significantly. This might be again ascribed to generally negative correlations between yolk mass and antioxidant concentrations (see above and Supplementary material Appendix 1, Table S1).

It is important to note that as environmental factors, female traits, and male traits were generally not correlated (Supplementary material Appendix 1, Table S3), the results of independent modeling using these three sets of predictors were genuine and not confounded.

Discussion

Antioxidant concentrations

Concentrations of carotenoids and vitamin E in our population of great tits counted among the highest values reported for 112 species of bird, whereas the concentration of vitamin A was higher than any other value among the same 112 species (Biard et al. 2009). Concentrations of antioxidants were similar to values reported by other studies of the great tit (Estonia: Hõrak et al. 2002, Sweden:

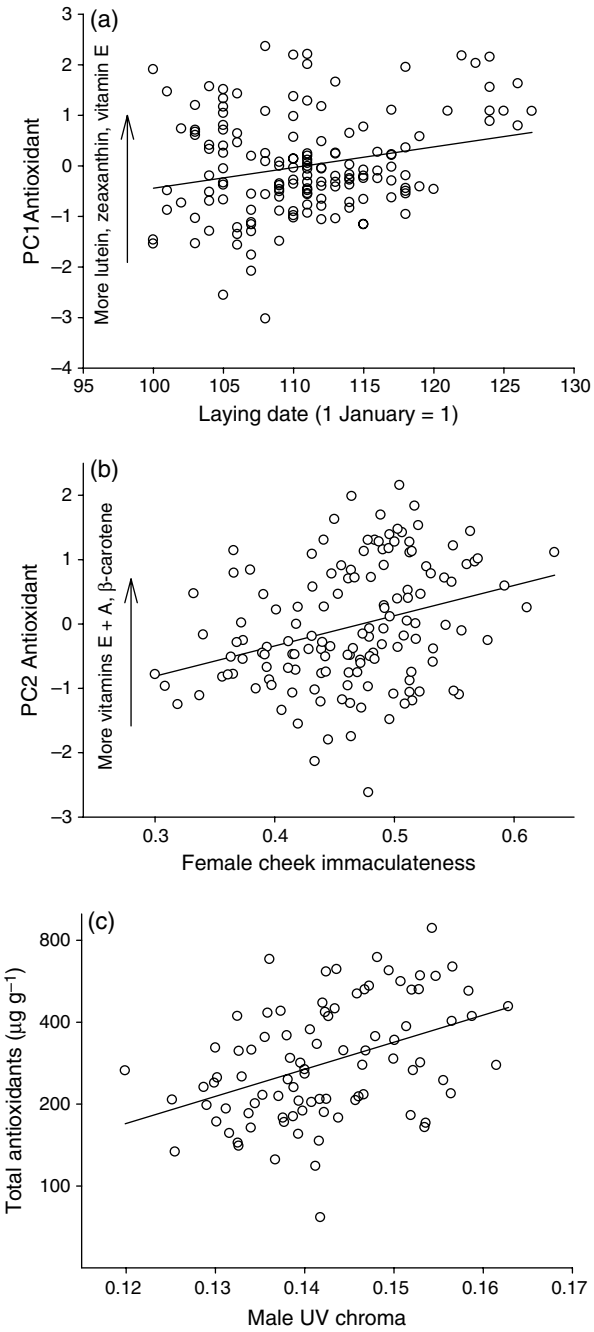

Figure 1. Relationships between (a) PC1Antioxidant and laying date, (b) PC2Antioxidant and female white cheek immaculateness (defined in Methods), and (c) total antioxidants (log scale) and UV chroma of male yellow breast feathers (defined in Methods).

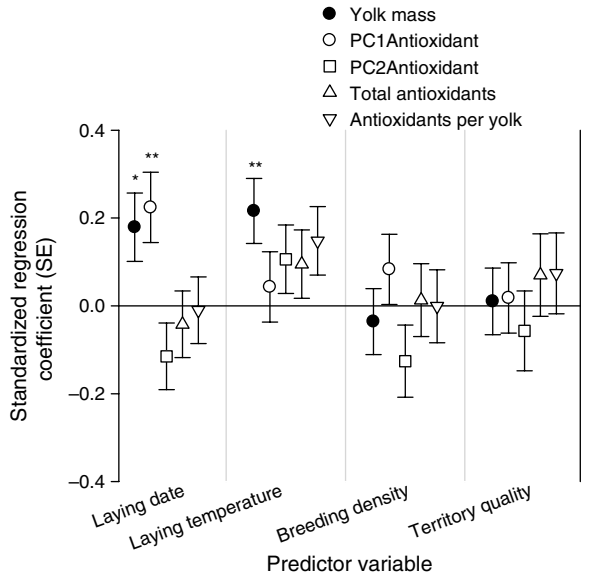

Figure 2. Summary of the linear mixed models relating yolk mass and composition to the factors of the environment. Full results of modeling are available in Supplementary material Appendix 1, Table S2. Asterisks denote significance at $^{**}p < 0.01$, $^{*}p < 0.05$.

Isaksson et al. 2008a, France: Møller et al. 2008). Interestingly, our mean value of 62.2 μg g^{-1} (SD = 43.5, median = 47.0; sum of lutein, zeaxanthin and β-carotene) is ca 1.5× higher than carotenoid concentration of 40.5 μg g^{-1} in a nearby population of great tits lying ca 18 km apart (Remeš et al. 2007). Our concentration of vitamin E was only slightly higher than the values reported by Hõrak et al. (2002) and Møller et al. (2008), whereas our concentration of vitamin A was almost 5× higher than in Møller et al. (2008).

Concentrations of individual antioxidants were generally positively correlated in our population of great tits (Supplementary material Appendix 1, Table S1). Similarly, concentrations of carotenoids and vitamin A and E were strongly positively correlated in plasma of great tits in Estonia (Hõrak et al. 2004). These antioxidants were positively inter-correlated also in yolks of the collared flycatcher (Hargitai et al. 2006). On the interspecific level, concentrations of egg yolk carotenoids and vitamin A and E were positively correlated across 112 species of birds (Biard et al. 2009). All these results suggest that nutrition-derived antioxidants vary together within and across species, which possibly results from the common dietary origin of all these compounds.

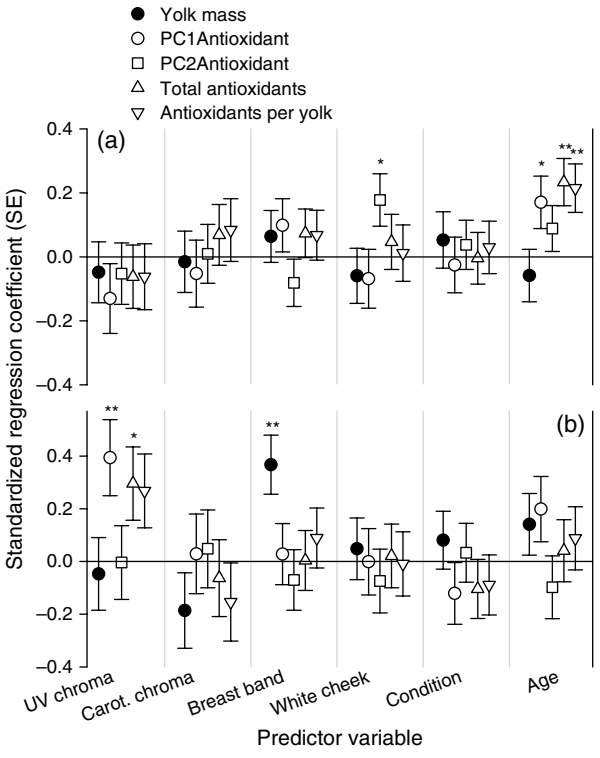

Figure 3. Summary of the linear mixed models relating yolk mass and composition to (a) female traits and (b) male traits. Full results of modeling are available in Supplementary material Appendix 1, Table S2. Asterisks denote significance at $^{**}p < 0.01$, $^{*}p < 0.05$.

Environmental factors

Increasing yolk mass with laying date and temperature has been observed in various other populations of European tits (Perrins 1979), as well as other bird species, for example the barn swallow *Hirundo rustica* (Saino et al. 2004). When temperatures are low, females are supposed to be constrained by low activity of insect food during egg formation and/or by a need to devote more energy to maintenance metabolism and less to egg formation. Alternatively, increasing yolk mass with laying date might be a strategy to provide extra prenatal resources for offspring. Food supply, foraging success (Naef-Daenzer and Keller 1999) and survival prospects of fledging great tits decline with season (Perrins 1979, Naef-Daenzer et al. 2001) and thus it might be adaptive to boost offspring performance with extra resources (Krist 2011). However, in our study increases of yolk mass with laying date and temperature did not translate into more antioxidants per yolk, which was caused by consistently negative correlations between yolk mass and antioxidant concentrations (Supplementary material Appendix 1, Table S1).

Concentrations of lutein, zeaxanthin, and to a lesser extent vitamin E increased with laying date. This seasonal effect might have been caused by seasonal changes in food supply and/or quality (Perrins 1979). For example, yolk carotenoid concentration was positively associated with caterpillar supply at the time of egg formation in the collared flycatcher (Török et al. 2007). The role of diet in affecting antioxidant concentrations is also supported by the finding that plasma carotenoid concentration in great tits increased during the day, probably reflecting the dietary input of carotenoid-rich food items and the respective accumulation of carotenoids in the plasma during the day (Hõrak et al. 2004). Similar proximate effects operating over longer time scales are capable of mediating relationships between plasma antioxidant concentrations in females and season, provided that antioxidant concentrations in major food items, or relative proportion of food items with differing antioxidant concentrations, change over time. For instance, concentrations of lutein and β-carotene differed between moth and sawfly larvae, the main food of the great

tit, in Finland (Sillanpää et al. 2008). Similarly, concentrations of antioxidants differed between *Operophtera* caterpillars, *Erannis* caterpillars and spiders, the main food of blue tits, in Scotland (Arnold et al. 2010). Then any changes in the abundance of these food items over time could generate seasonal changes in carotenoid supply. Moreover, concentrations of antioxidants in *Operophtera* caterpillars also changed with season. Consequently, given that plasma and yolk antioxidant concentrations correlate positively in great tits (Isaksson et al. 2008a), the above-mentioned proximate effects could have led to a seasonal change in the concentration of egg yolk antioxidants observed in our population (Fig. 1a).

Female traits

Female ornaments were shown to indicate condition, individual quality and parental investment in a broad range of species (Amundsen and Pärn 2006). We expected that yolk characteristics would be related to female coloration, based on the evidence from other populations of great tit that feather ornaments are condition-dependent and signal individual condition and quality (Hõrak et al. 2001, Ferns and Hinsley 2004). We confirmed this expectation only for cheek immaculateness, where females with more regular shape and borders of white cheek patch deposited more vitamin E, vitamin A, and β-carotene. More immaculate great tits were shown to be competitively superior and breed earlier (Ferns and Hinsley 2004). Thus, it seems that immaculate white patches signal not only superior social competitiveness, but also ability or willingness to invest more into a breeding attempt. On the contrary, there were no relationships between yolk antioxidants and other female feather ornaments. This agrees with previous findings from our population, where neither yellow breast feather color nor the size of the black breast stripe correlated with an index of condition or female incubation intensity (Matysioková and Remeš 2010a, b). Moreover, carotenoid chroma of breast feathers did not predict an ability of females to cope with energetic stress (assessed by a handicapping experiment, Matysioková and Remeš 2011).

Older females deposited higher concentrations and amounts of antioxidants, which seemingly agrees with the investment hypothesis (Safran et al. 2008). However, we aged females based on their plumage, which enables to determine only broad age categories of one year old vs older birds (Svensson 1992). Thus, our observation of older females depositing more antioxidants agrees with a general observation of improved breeding success of older birds compared to first year breeders (Sæther 1990). However, we were not able to study changes in antioxidant deposition in older birds of known age, which could reveal signs of plateau or even senescent decline in maternal investment (Bowhuis et al. 2010).

Male traits

Two resource allocation strategies of females were identified. First, females should allocate more resources into offspring when paired with higher-quality males, indicated for instance by their ornaments, a strategy known as differential allocation (Sheldon 2000). Second, females might instead boost performance of the offspring of lower-quality males, a strategy called differential compensation (Ratikainen and Kokko 2010). Several studies of yolk antioxidants in wild birds reported patterns consistent with both differential allocation and compensation. First, blue tit females produced eggs with more carotenoids when mated to ultraviolet attractive males (Szigeti et al. 2007). Second, female house finches *Carpodacus mexicanus* deposited significantly more total antioxidants into eggs sired by less attractive males (Navara et al. 2006). Similarly, in an Italian population of the barn swallow, mates of tail-shortened males had a larger lutein concentration in their eggs compared with those of control and tail-elongated males (Saino et al. 2002). Finally, there was no relationship of yolk volume or carotenoid concentration to the area of white forehead patch of males in the collared flycatcher (Török et al. 2007). There was also no relationship between carotenoid concentration and male tail length or throat color in a US population of the barn swallow (Safran et al. 2008).

Females in our population deposited more antioxidants when mated to males with more intense UV chroma of yellow breast feathers. They also laid larger yolks for males with larger black band area, although this did not translate into more yolk antioxidants due to negative correlations between yolk mass and antioxidant concentrations (Supplementary material Appendix 1, Table S1). These patterns are consistent with differential allocation strategy, provided carotenoid- and melanin-based male ornaments in great tits indicate male quality and consequently higher reproductive value of the offspring. Size of the black breast band was positively related to the social status of the bird (Lemel and Wallin 1993) and to the frequency (Norris 1990) and intensity of nest defense (Quesada and Senar 2007). Thus, black stripe signals the ability of the male to win agonistic intraspecific encounters and defend offspring. It may also indicate the ability of male to provide superior parental care (Norris 1990). UV chroma of yellow breast feathers in great tits reflects the content of carotenoids in feathers (Jacot et al. 2010). Thus, saturation of carotenoid-based coloration, including UV chroma, might signal the superior foraging ability of individuals in terms of food quality or quantity (Møller et al. 2000) as has been demonstrated in the guppy *Poecilia reticulata* (Karino et al. 2007). There are no tests of this hypothesis in birds, but it has been demonstrated that great tits prefer carotenoid-rich diet over carotenoid-poor food items (Senar et al. 2010). Moreover, in a Swedish population of great tit nestling plumage carotenoid chroma was predicted by the chroma of the rearing father (after cross-fostering of the young) indicating that foraging ability of the social male might have significant effects on offspring phenotype (Isaksson et al. 2006).

Besides benefits of superior offspring feeding and defense, females might gain from more ornamented males also indirect, genetic benefits and allocate yolk antioxidants accordingly. However, patterns of allocation based on indirect benefits might be obscured by multiple paternities within the population. If a large part of offspring were sired outside of the social bond, or if social and genetic mates systematically differed in their ornaments, patterns revealed

without accounting for extra-bond paternity could be misleading. Fortunately, both of these potential sources of complication seem to be negligible in the great tit. First, rates of extra-bond paternity are reported to be comparatively low in this species, accounting for $<10\%$ of offspring (Krokene et al. 1998, Strohbach et al. 1998, Lubjuhn et al. 1999, Otter et al. 2001). Second, social and genetic mates do not usually differ in feather ornaments (Krokene et al. 1998, Strohbach et al. 1998). The only exception was a Japanese population (a different subspecies, *P. m. minor*), where 16.6% of offspring were sired outside of the social bond and extra-pair sires had larger black breast band than social mates (Kawano et al. 2009).

Conclusions

Whether the concentration or the total amount of a compound per yolk is more important for developing offspring remains an open question (Safran et al. 2008). Heavier eggs contained higher total carotenoid concentrations in the great tit (Isaksson et al. 2008a) and the yellow-legged gull *Larus michahellis* (Saino et al. 2008). On the contrary, there was no correlation between egg mass and antioxidant concentrations in the black-headed gull *Larus ridibundus* (Groothuis et al. 2006). In our study, correlations between yolk mass and antioxidant concentrations were not significant but overall were slightly negative (Supplementary material Appendix 1, Table S1). This caused the observation that although yolk mass increased with certain factors (laying temperature, male breast band area), total yolk antioxidants did not (Fig. 2, 3). On the contrary, antioxidant concentration was higher in older females and this translated into more total antioxidants per yolk (Fig. 3a). These findings suggest that offspring may originate from eggs with different combinations of yolk mass, antioxidant concentrations and amounts per yolk, with possibly different consequences for their performance.

The main findings of our study were as follows. 1) All antioxidants were positively inter-correlated within yolks. Correlations of antioxidants with yolk mass were weak. 2) Environmental factors, female traits, and male traits all influenced egg yolk characteristics. 3) Low repeatability of yolk composition suggests that it changes from year to year based on prevailing environmental and social conditions. 4) Large variation among females in egg yolk antioxidants identified in this population of a socially monogamous songbird suggests that there is a substantial potential for adaptive offspring engineering in relation to environmental and social factors.

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Supplementary material (Appendix J5402 at <www.oikosoffice.lu.se/appendix>). Appendix 1.

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