In ovo yolk carotenoid and testosterone levels interactively influence female transfer of yolk antioxidants to her eggs

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ABSTRACT

Mothers can influence prenatal conditions by varying the amount of nutrients, hormones or antioxidants they provide to their developing young. Some of these substances even affect the transfer of these compounds in the next generation, but it is less clear how different maternally transmitted compounds interact with each other to shape reproductive resource allocation in their offspring. Here, we found that female Japanese quail that were exposed to high carotenoid levels during embryonic development transferred lower concentrations of yolk antioxidants to their own eggs later in life. This effect disappeared, when both testosterone and carotenoid concentrations were manipulated simultaneously, showing long-term and interactive effects of these maternally derived egg components on a female's own egg composition. Given that exposure to high levels of testosterone during embryo development stimulates the production of reactive oxygen (ROS) and impairs antioxidant defenses, we propose that carotenoids act as *in-ovo* antioxidants in an oxidatively stressful environment (i.e. when levels of testosterone are high) but might have prooxidant properties in an environment where they are not used to counteract an increased production of ROS. In line with this hypothesis, we previously showed that prenatal exposure to increased concentrations of yolk carotenoids leads to a rise of oxidative damage at adulthood, but only when yolk testosterone concentrations were not experimentally increased as well. As a consequence, antioxidants in the body may be used to limit oxidative damage in females exposed to high levels of carotenoids during development (but not in females exposed to increased levels of both carotenoids and testosterone), resulting in lower amounts of antioxidants being available for deposition into eggs. Since prenatal antioxidant exposure is known to influence fitness-related traits, the effect detected in this study might have transgenerational consequences.

INTRODUCTION

Conditions experienced early in life, and especially those experienced before birth, can affect offspring phenotype in the long term, influencing, among others, their physiology or behavior[1,2]. These developmental conditions are strongly influenced by the amount of nutrients, hormones, antioxidants or immunoglobulins provided by the mothers to their developing young[3]. Some of these maternally-derived resources and developmental cues are known to affect the same offspring traits (e.g. growth rate[2,4]), and it has therefore been hypothesized that maternally-transmitted compounds might interact with each other to shape the offspring's developmental trajectory[5,6,7]. However, to date, such interactive effects have been seldom considered and experimentally investigated in only one prior study, which revealed negative effects of an imbalance between yolk androgens (i.e. testosterone) and antioxidants (i.e. carotenoids) levels on prenatal growth and juvenile oxidative stress levels in Japanese quail[8] (*Coturnix japonica*).

Prenatal exposure to maternally-derived androgens and antioxidants does, however, not only affect juvenile phenotype, but is also known to have long-term consequences on breeding strategies at adulthood. For example, prenatal exposure to experimentally increased yolk androgens levels enhances the development of the nuptial plumage and the frequency of aggressive displays at adulthood[1]. Furthermore, in the only study assessing the long-term effects of yolk antioxidant levels with an experimental approach (i.e. yolk injections), male barn swallows (*Hirundo rustica*) that hatched from eggs with experimentally increased vitamin E levels arrived earlier at their breeding grounds than controls[9]. Different maternally-derived components have thus the potential to interactively shape the offspring's reproductive behavior and reproductive investment at adulthood.

Here we experimentally tested this hypothesis by manipulating yolk lutein and yolk testosterone concentrations in the eggs of Japanese quail using a 2x2 factorial design and assessing their separate and interactive effects on the steroid and antioxidant compositions of eggs laid by the female offspring at adulthood.

METHODS

Adult male and female quails were randomly selected from a captive population maintained at the University of Zurich, Switzerland and housed in pairs in cages. Eggs were collected and each clutch was randomly assigned to one of the four treatments: yolk carotenoid (C) manipulation (injection of 15 μg lutein dissolved in 15μL of safflower oil), yolk testosterone (T) manipulation (15 ng of testosterone), both yolk carotenoid and yolk testosterone (CT) manipulation or a control (CO) injection (injection of 15μL of safflower oil) (see Giraudeau et al. 2016a for a full description of the methods). The doses of testosterone and carotenoids injected represent approximately 1 standard deviation of the published yolk testosterone and yolk carotenoid contents in this species[10,11,12,13]. When five months old, randomly chosen females originating from these manipulated eggs (N= 8 C, 9 T, 8 CT, 15 CO) were weighted (to the nearest g) and housed in pairs in breeding cages with randomly selected males from our breeding population. The fifth egg of each clutch was collected and weighted (to the nearest 0.01g) and the yolk and albumen were separated. The yolk was weighed (to the nearest 0.01g) and then thoroughly mixed. Two yolk aliquots of 1 ml were collected and immediately stored at -80° C until later quantification of yolk antioxidant and testosterone concentrations. See ESM for descriptions of the methods used to extract and analyze yolk testosterone and antioxidant concentrations.

Levels of yolk antioxidants were positively correlated within eggs, so we performed a principal component (PC) analysis and used yolk antioxidant PC1 in statistical analyses (see ESM for correlations among antioxidants and posthoc analyses of the separate antioxidants). PC1 explained 58% of the variation in yolk antioxidant concentrations (ESM).

In total 30 families (6 C, 7 T, 8 CT, 9 CO) were included in this study. Because some families produced more than one daughter (mean ± SD: 1.3 ± 0.7 daughters per family; range 1-4), family means were used in the statistical analyses to account for the non-independence of siblings. We analyzed the effect of exposure to manipulated concentrations of yolk carotenoid and testosterone during embryo development on a female's adult body mass and the composition of her eggs using linear models that contained yolk testosterone manipulation, yolk carotenoid manipulation and their interaction as fixed effects. The interaction was removed from the final model if it was non-significant. Yolk mass was included as a covariate in the analyses of yolk components to account for treatment effects on yolk size, and therefore the total content of egg components (see Results). All statistical analyses were performed in R 3.01 (R Core Team, 2013).

RESULTS

Females originating from testosterone-injected eggs laid heavier eggs (mean ± 1SD: T/CT: 12.27 ± 0.83 g; C/CO: 11.47 ± 0.80 g; Fig. 1) that contained heavier yolks (T/CT: 3.71 ± 0.44 g; C/CO: 3.29 ± 0.48 g); however, these variables were not affected by the yolk carotenoid manipulation (Fig. 1, table 1). We found no effect of the egg manipulations on adult body mass (table 1).

Yolk testosterone concentrations in the eggs laid by offspring were not significantly influenced by the testosterone or carotenoid manipulations (table 1). In contrast, there was a significant interaction effect between the yolk carotenoid and testosterone manipulations on yolk antioxidant

concentrations (PC1) in a female's eggs (table 1; Fig. 1). Females hatched from carotenoid-injected eggs laid eggs with lower yolk antioxidant concentrations, but only if the yolk testosterone concentration experienced during embryo development was unmanipulated (Tukey contrast: $p = 0.049$; all other contrasts $p > 0.156$; figure 1). Yolk mass was significantly negatively associated with yolk antioxidant concentrations (PC1) (b = -1.775, Table 1). When the effects of yolk manipulations were tested for each antioxidant separately, we found the same significant interactive effect of *in ovo* testosterone and carotenoid treatments on neoxanthin, violaxanthin, and zeaxanthin concentrations in eggs laid by the offspring (ESM).

DISCUSSION

This study provides the first experimental evidence that two maternally derived egg components have interactive long-term effects on a female's reproductive investment at adulthood. Female Japanese quail that were exposed to high carotenoid levels during embryonic development transferred significantly lower concentrations of yolk antioxidants to their own eggs, but this effect disappeared when both testosterone and carotenoid concentrations were manipulated simultaneously *in ovo*. We previously showed a similar interactive effect of yolk testosterone and carotenoid manipulation on reactive oxygen metabolite levels at the end of the growth period (5 weeks old birds). Prenatal exposure to high concentrations of yolk carotenoids increased oxidative damage levels at adulthood, but only when yolk testosterone concentrations were not experimentally increased as well[8], indicating that prenatal conditions (i.e. levels of yolk antioxidants) have long-term effects on an individual's oxidant/antioxidant balance. As a consequence, we propose that circulating antioxidants in the body may be used to limit oxidative damage in females exposed to high levels of carotenoids during development, resulting in lower

amounts of antioxidants being available for deposition into eggs later in life. Alternatively, or in addition, prenatal exposure to high carotenoid levels might shift the trade-off between self-maintenance and reproduction towards a reduced reproductive investment during the first breeding event, as we have previously shown in males (i.e. reduced testis size[14]).

Importantly, the transfer of lower concentrations of yolk antioxidants to eggs was only observed in females that experienced increased carotenoid but unmanipulated testosterone levels during embryo development. Recent evidence suggests that embryonic exposure to high levels of testosterone stimulates the production of reactive oxygen and nitrogen species (ROS/NS), and impairs antioxidant defenses[15,16]. We propose that carotenoids might act as antioxidants in an oxidatively stressful environment (i.e. when levels of testosterone are high) but might have prooxidant properties in an environment where they are not used to counteract an increased production of ROS/NS (previous studies have demonstrated such pro-oxidant properties of carotenoids[17]). Thus, contrary to individuals only exposed to increased concentrations of carotenoids at the embryonic stage, females exposed to increased levels of both testosterone and carotenoids would not suffer from increased levels of oxidative stress (as observed in [8]) and would be able to allocate similar levels of antioxidant to their eggs then control females.

Under this hypothesis, mothers should also co-adjust the deposition of carotenoids (and potentially also of the other maternally-derived antioxidants) to the levels of androgens deposited in the eggs to achieve an optimal outcome for the offspring. A first examination of these relationships at the inter-specific level revealed that high concentrations of testosterone are associated with high concentrations of the antioxidant vitamin E in eggs[18]. Further studies should explore the potential relationships between levels of various maternally-derived hormones that might stimulate ROS/NS production in offspring (i.e androgens, glucocorticoids) and the egg antioxidant system.

In addition to a significant interaction effect between experimentally manipulated yolk carotenoid and testosterone concentrations on a female's antioxidant deposition into eggs later in life, we found that females originating from testosterone-manipulated eggs increased their breeding investment by laying heavier eggs with heavier yolk than females hatching from eggs in which testosterone has not been manipulated. This result is in line with the finding of Müller *et al.* (2009) who found that female canaries (*Serinus canaria*) hatching from testosterone-manipulated eggs laid more eggs than control females (but see [19]). Two main hypotheses have been proposed to explain long-lasting effects of yolk androgens on female breeding performance. First, embryonic exposure to maternal androgens might promote hormone production or responsiveness (via increased androgen receptor densities) at later life stages[19,20]. Second, maternally derived androgens can positively influence muscle development[21], begging behavior[22], and growth of chicks[2]. Since female breeding performance has been shown to benefit from favorable early-life conditions in several species[23], the long-lasting effect of yolk androgens levels on maternal reproductive investment might be the indirect consequence of early growth conditions[24]. The latter is an unlikely explanation for the patterns observed in our study, however, as we found no effect of the manipulations on adult body mass and that prenatal growth was negatively, rather than positively, influenced by an experimental increase of yolk testosterone concentrations[8]. Instead, it suggests that the long-term effect of prenatal exposure to high levels of testosterone on egg size is due to direct long-term effects on a female's physiology.

To conclude, our study demonstrates long-term interactive effects of two maternally derived egg compounds on a female's egg composition at adulthood. Since prenatal antioxidant exposure is known to influence several fitness-related traits in birds[4], the effect detected in this study might have transgenerational consequences.

Ethics

All procedures conform to the relevant regulatory standards and were conducted under licences provided by the Veterinary Office of the Canton of Zurich, Switzerland (195/2010; 14/2014; 156).

Data accessibility

Data have been submitted as a supplementary file.

Authors' contributions

M.G. and A-K.Z. collected the data; M.G. and B.T. designed the study; B.T. analyzed the data. M.G. wrote the manuscript and all authors edited the manuscript. KJM analyzed yolk carotenoid concentrations and A-K.Z., M.O and M.Z analyzed yolk testosterone concentrations. All authors agree to be held accountable for the content therein and gave final approval for publication.

Competing interests

No competing interests.

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25. LEGENDS

FIGURE 1: Long-term effects of yolk testosterone and yolk carotenoid manipulations on egg mass and the deposition of yolk antioxidants (PC1).

277 FIGURE 1

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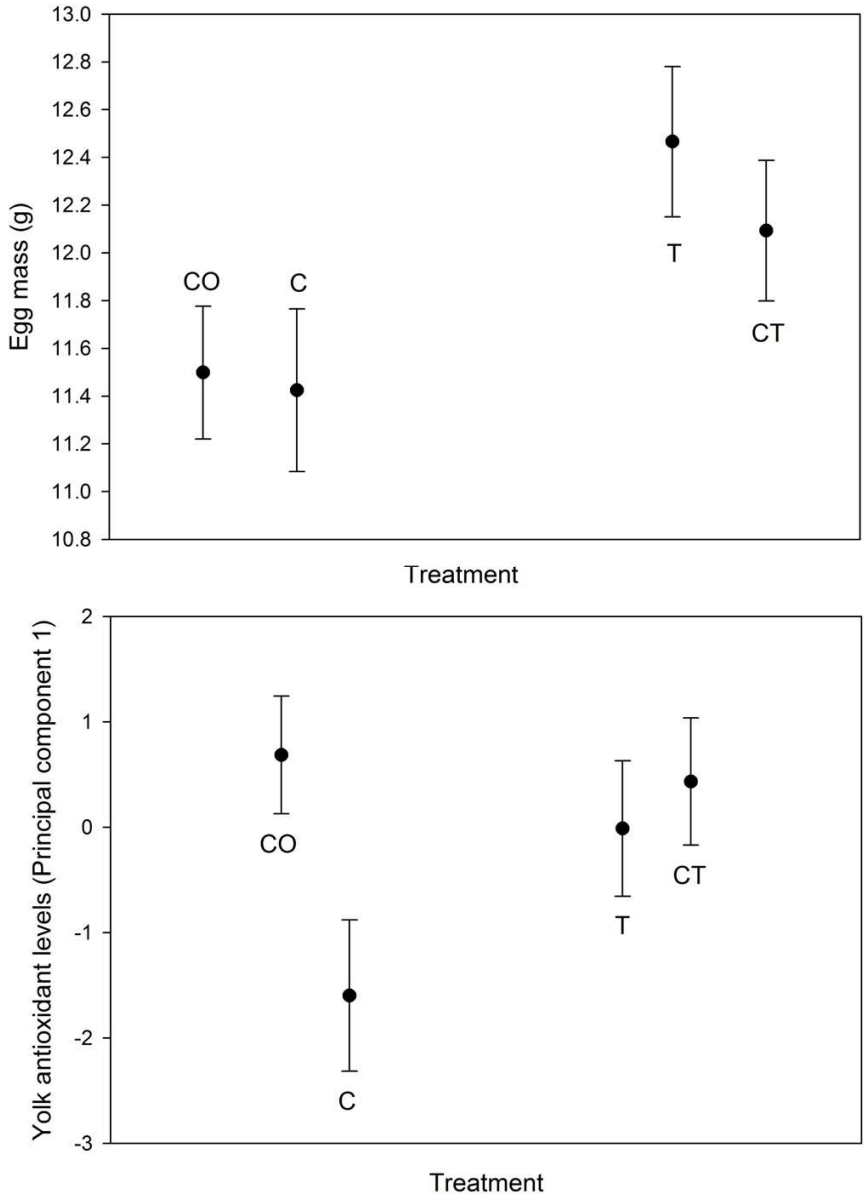

280 **Table 1.** Long-term effects of exposure to manipulated levels of yolk testosterone and yolk
281 carotenoid during embryo development on body mass and egg composition at adulthood.

		F	DF	P
Body mass (g)				
	Carotenoid manipulation	0.164	1, 27	0.688
	Testosterone manipulation	0.016	1, 27	0.901
	Interaction	0.300	1, 26	0.588
Egg mass (g)				
	Carotenoid manipulation	0.555	1, 27	0.463
	Testosterone manipulation	7.064	1, 27	0.013
	Interaction	0.235	1, 26	0.632
Yolk mass (g)				
	Carotenoid manipulation	0.416	1, 27	0.524
	Testosterone manipulation	5.958	1, 27	0.025
	Interaction	0.441	1, 26	0.513
Yolk testosterone (pg / mg yolk)				
	Carotenoid manipulation	0.060	1, 26	0.808

	Testosterone manipulation	0.765	1, 26	0.390
	Interaction	0.137	1, 25	0.714
	Yolk mass (g)	0.141	1, 26	0.710
Yolk antioxidant PC1				
	Carotenoid manipulation	1.296	1, 25	0.266
	Testosterone manipulation	0.219	1, 25	0.644
	Interaction	4.889	1, 25	0.030
	Yolk mass (g)	5.297	1, 25	0.036

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