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Original Article

Interactive effects of yolk testosterone and carotenoid on prenatal growth and offspring physiology in a precocial bird

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Conditions experienced by individuals during prenatal development can have long-term effects on their phenotype. Maternally transmitted resources are important mediators of such prenatal effects, but the potential interactive effects among them in shaping offspring phenotype have never been studied. Maternally derived testosterone is known to stimulate growth, but these benefits may be counterbalanced by an increase in the production of reactive oxygen species (ROS). Maternally transmitted carotenoids might have the capacity to scavenge ROS and thereby buffer an increase in oxidative stress caused by prenatal exposure to high testosterone levels. Here, we experimentally tested for such interactive effects between maternal yolk testosterone and carotenoid in Japanese quail (*Coturnix japonica*). We found that hatching mass was reduced and reactive oxygen metabolites (ROMs) levels at the end of the period of maximal growth increased in chicks from eggs injected with either testosterone or carotenoid (only a tendency in chicks from testosterone-injected eggs). However, when both egg compounds were manipulated simultaneously, hatching mass and ROM levels were not affected, showing that both carotenoid and testosterone lose their detrimental effects when the ratio between the 2 compounds is balanced. Our study provides the first experimental evidence for interactive effects of 2 maternally derived egg compounds on offspring phenotype and suggests that developmental cues are tightly coadjusted within an egg.

Key words: **growth, maternal effects, metabolic rate, oxidative stress, yolk carotenoids, yolk testosterone.**

INTRODUCTION

Conditions experienced during prenatal development can influence an individual's developmental trajectory and have long-term effects on its physiology, morphology, and behavior, ultimately influencing its fitness (Lindström 1999). Key mediators of such prenatal effects are maternally transmitted developmental cues and resources, such as maternally transmitted hormones (Schwabl 1993), antioxidants (Romano et al. 2008), or immunoglobulins (Gasparini et al. 2001). Among these various maternally transmitted resources that have the potential to influence offspring phenotype, maternal testosterone has been extensively studied (Groothuis et al. 2005; Gil 2008). This work has revealed that offspring originating from an egg with experimentally increased testosterone content grow faster and show an increased begging rate than chicks hatched from control eggs (Schwabl 1993; Groothuis et al. 2005; Gil 2008, but see e.g. Rubolini et al. 2006; Tobler et al. 2007). However, evidence is accumulating that these positive effects of prenatal testosterone exposure on growth and begging might be counterbalanced by costs for the offspring (Groothuis et al. 2005). In particular, recent studies suggest that prenatal testosterone exposure might directly or indirectly (i.e., through an increased growth rate) affect the production of reactive oxygen and nitrogen species, and impair antioxidant defenses (Tobler and Sandell 2009; Treidel et al. 2013, but see Noguera et al. 2011). In accordance with this hypothesis, reduced plasma antioxidant levels (Tobler and Sandell 2009, zebra finch [*Taeniopygia guttata*]) and DNA damage repair efficiency in response to an oxidative challenge (Treidel et al. 2013, domestic chickens [*Gallus gallus*]) have been observed in birds that hatched from testosterone-injected eggs.

Maternally transmitted antioxidant molecules (e.g., carotenoids, vitamin E) might have the capacity to scavenge reactive oxygen species (ROS) produced during development (Surai et al. 2001)

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and/or stimulate antioxidant defenses, and may thus counterbalance a potential increase of oxidative damage caused by prenatal exposure to high testosterone levels. In line with this hypothesis, a positive correlation between levels of yolk testosterone and antioxidants has been found in house finches (*Haemorhous mexicanus*, Navara et al. 2006), suggesting that mothers coadjust these components in the eggs (but see Royle et al. 2001). However, so far, no study has experimentally tested for interactive effects of yolk hormones and antioxidants on offspring phenotype, and only few studies have experimentally investigated the effects of yolk antioxidant levels on offspring phenotype with *in ovo* injections. These studies found that yolk carotenoid injections increased immunocompetence in barn swallows (Saino et al. 2003) and yellow-legged gulls (Romano et al. 2008), enhanced the growth of male yellow-legged gulls from first laid eggs (but depressed the growth of males from last laid eggs; Romano et al. 2008), had no effect on growth in barn swallows (Saino et al. 2003), and had long-term effect on testis size in Japanese quails (Giraudeau et al. 2016b). In the only study where oxidative stress levels were measured, Saino et al. (2011) found that oxidative damage levels increased in response to an increase of egg carotenoid levels in males and in first-laid yellow-legged gull chicks. Thus, high yolk carotenoid levels seem to enhance chick immunocompetence, but the effects on oxidative stress (collected on a single species) and growth appear less clear.

Here, we experimentally manipulated yolk testosterone and yolk carotenoid levels in a 2 × 2 design to examine how these 2 egg compounds interact to shape the morphology and physiology of Japanese quail chicks. In particular, we assessed the potential interactive effects of these 2 egg compounds on hatching success, mass at hatching, growth rate, and oxidative stress (reactive oxygen metabolites [ROMs] and the total plasma antioxidant capacity [TAC]).

Because we were interested to examine the long-term effects of yolk testosterone and yolk carotenoid levels, we also measured whether both of our treatments influenced body mass and resting metabolic rate (RMR) at adulthood (Orledge et al. 2012). Previous experimental studies have shown that prenatal exposure to high testosterone concentrations leads to an increased adult metabolic rate (Tobler et al. 2007; Nilsson et al. 2011; Ruuskanen et al. 2013). The effect of yolk carotenoid levels on metabolic rate, however, has so far never been studied.

We predicted that, compared with controls, offspring from testosterone-injected eggs would grow faster, have a higher metabolic rate, higher ROM levels, and a deficient antioxidant capacity. In contrast, compared with controls, we expected offspring from carotenoid-injected eggs to have a better antioxidant capacity and lower ROM levels. Finally, we predict that experimentally increased yolk carotenoid levels would buffer the negative effects of high yolk testosterone exposure on ROM levels and antioxidant capacity.

METHODS

Egg collection, egg injection, incubation, and hatching

In March 2014, 55 breeding pairs were randomly selected from a Japanese quail population maintained at the University of Zurich, Switzerland. Birds were housed in pairs in cages (122 × 50 × 50 cm, photoperiod of 16:8 light:dark) and received *ad libitum* water and commercial game bird mix low in carotenoid content. Eggs (N = 535) were collected during 2 weeks, and each clutch was randomly assigned to one of the 4 treatments: yolk carotenoid manipulation (C, 14 clutches, 135 eggs), yolk testosterone manipulation (T, 14 clutches, 136 eggs), both yolk carotenoid and yolk testosterone manipulation (CT, 14 clutches, 137 eggs), or a control injection (CO, 13 clutches, 127 eggs). Eggs were injected with either 15 ng of testosterone (17β-hydroxy-4-androsten-3-on, Sigma-Aldrich, Switzerland) dissolved in 15 µL of safflower oil, 15 µg of carotenoids (FloraGLO Lutein 20%, Kemin Foods, Des Moines, IA) dissolved in 15 µL of safflower oil, both testosterone (15 ng) and carotenoids (15 µg) dissolved in 15 µL of safflower oil or with 15 µL of safflower oil as a control (see Tschirren et al. 2005 for a detailed description of egg injection method). The carotenoid lutein was used for the injection because it is the most abundant carotenoid in Japanese quail eggs (Peluc et al. 2012). The doses of testosterone and carotenoids injected represent approximately 1 standard deviation of the published yolk testosterone and yolk carotenoid contents in this species (Hackl et al. 2003; Dvorska and Surai 2004; Niall Daisley et al. 2005; Peluc et al. 2012). Eggs were artificially incubated for 14 days at a temperature of 37.6 °C and 55% humidity and then at 37.6 °C and 80% humidity for the last 3 days.

Forty-one CO-chicks (18 females, 19 males, 3 which could not be sexed), 55 T-chicks (23 females, 30 males, 2 which could not be sexed), 57 C-chicks (26 females, 28 males, 3 which could not be sexed), and 55 CT-chicks (23 females, 26 males, 6 which could not be sexed) hatched. The overall hatching success was 38.6% (CO = 32.3%, T = 40.4%, C = 40.7%, CT = 40.1%), was comparable with previous studies in Japanese quail (Niall Daisley et al. 2005; Okuliarová et al. 2007; Hegyi and Schwabl 2010), and did not differ between treatments ($\chi^2 = 3.29$, $P = 0.36$).

At hatching, chicks were weighed (to the nearest 0.1 g) and marked with a numbered plastic ring for individual identification. They were then reared in mixed treatment groups of 40 chicks for 2 weeks and in groups of 20 chicks for 3 more weeks. At the age of 5 weeks, chicks were released into outdoor aviaries. Chicks received *ad libitum* food and water. Mass measurements were taken at the age of 1, 2, 3, and 5 weeks. In our population, chicks reach their adult skeletal size and body mass at 5 weeks of age (see also van der Ziel and Visser 2001 for a full description of the growth timing in this species). Sex was determined based on plumage characteristics.

Growth rate was estimated for all birds using the mass measured at hatching, and 1, 2, 3, and 5 weeks post-hatching. This period of growth matches the linear part of the growth curve. As an estimate of growth rate, we thus used the coefficient of the linear regression of body mass by age (in days) for each individual. Using this method was strongly supported by the very high adjusted R^2 (mean = 0.958 ± 0.002; N = 193 individuals).

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

Measurements of oxidative stress

At the age of 5 weeks, we drew 200 µL of blood through the alar vein into heparinized capillary tubes. Samples were centrifuged (10 000 g × for 3 min), and plasma was frozen at −20 °C for later analysis. Because the amount of blood collected was insufficient to measure both the levels of d-ROMs and TAC for some of the birds, we measured the ROMs for only 173 individuals and the TAC for 188 individuals. ROMs were measured using the d-ROM test, which quantifies the level of hydroperoxides, compounds that signal lipid and protein oxidative damage (Diacron International, Grosseto, Italy). TAC was assessed using the OXY-adsorbent test, which measures the effectiveness of the blood antioxidant barrier

by quantifying its ability to cope with oxidant action of hypochlorous acid (HClO; Diacron International, Grosseto, Italy). Both assays have been previously described in Haussmann et al. (2012).

Metabolic rate

RMR was measured on 96 adult quails (at the age of 6 months) (CO: 16 females, 18 males; C: 8 females, 12 males; T: 9 females, 12 males; CT: 9 females, 12 males). Because of space limitation in our aviaries, we only kept 96 birds hatched during this experiment for the RMR measurement. Five days prior to the measurement, birds were placed in pairs in cages (122 × 50 × 50 cm) with *ad libitum* food and water. Metabolic rate measurements were performed during the birds' rest phase (6 PM to 8:30 AM), after a 4-h period of fasting to ensure a postabsorptive state. Individuals were placed in a 3.9L plastic metabolic chamber (234 × 165 × 165 mm; Lock & Lock, Hanacobi Co. Ltd., Korea), into a temperature-controlled, dark room within the birds' thermoneutral zone (25–27 °C) (Ben-Hamo et al. 2010). Oxygen consumption rate (VO_2, mL/min) was measured by indirect calorimetry with an 8-channel open-flow respirometry system. Before each trial, the CO_2 analyzer was zeroed using CO_2-free air (N_2; PanGas, Switzerland) and spanned using a 1.002% mol CO_2 mixture (PanGas, Switzerland). The O_2 analyzer was spanned to 20.95% by flushing dry air through the system. During the trials, external air was pumped into the chamber at a flow rate of 1650–1700 mL/min controlled by an 8-channel mass flow meter system (Flow Bar Mass Flow Meter FB-8-1; Sable System). All gas flow connections passed through ultra-low permeability Tygon tubes (internal diameter of 8 mm). Seven of the 8 chambers contained 1 quail, with an empty chamber used as a control. Each recording sequence lasted 45 min with a 5-min measurement of all metabolic chambers, starting and ending with the control chamber. During a sequence, an automatic switch allowed excurrent air from each chamber to be subsampled (250 mL/min; Multiplexer Intelligent RM-8-2; Sable System), dried (magnesium perchlorate; Sigma–Aldrich), and analyzed every second over a 5-min period by a fuel cell O_2 analyzer and a dual wavelength infrared bench CO_2 analyzer (Foxbox, Sable System). Using this set-up, we obtained about 22 sequences per bird. As the equipment took a certain time to adjust between chambers, the first 100 s of each reading was excluded, leaving 200 s per reading. Baseline O_2 and CO_2 were determined by regressing all control chamber readings against time for each 45-min period. Oxygen consumption rates were calculated by comparing oxygen content of the metabolic chamber containing birds (Fe) to the baseline concentrations measured from the control chamber for the same time point (Fi). Given that the mass flow meter was upstream from the metabolic chamber and so CO_2 was not removed from the excurrent air stream, we used the following equation to correct for flow rate (FR) and CO_2 concentration: $VO_2 = FR \times [(FiO_2 - FeO_2) - FeO_2 \times (FeCO_2 - FiCO_2)]/(1 - FeO_2)$ (Lighton 2008). RMR for each bird was determined as the mean of the lowest 60 consecutive seconds of VO_2. Individuals were weighed (±0.1 g) before and after the metabolic rate measurement.

Statistical analyses

We were mostly interested in testing for potential interacting effects of carotenoid and testosterone injection on the different response variables, rather than for an overall effect of a carotenoid or testosterone injection. We thus considered carotenoid and testosterone treatments as 2 different factors and also considered their second-order interaction effect. To test whether yolk carotenoid and/or testosterone manipulations affect body mass at hatching, growth rate, plasma antioxidant capacity, and ROM levels at the age of 5 weeks, and RMR and body mass at adulthood, we used linear mixed models with the identity of the mother as a random effect and carotenoid treatment (binary variable segregating the 272 eggs injected with carotenoids from the 263 eggs that were not injected with carotenoids), testosterone treatment (binary variable segregating the 273 eggs injected with testosterone from the 262 eggs that were not injected with testosterone), the second-order interaction between the carotenoid and testosterone treatments, sex, egg mass, rank in the laying sequence, and the mother's body mass at laying as fixed effects in all models. For the analysis of antioxidant capacity and ROMs, we also included the mass measured at the age of 5 weeks as a covariate. For the analysis of RMR, we included the body mass measured just before the RMR measurement as a covariate. Furthermore, we also ran a separate analysis on females, including either the number or mass of eggs laid during the 5 days prior the RMR measurement or the number of eggs during the RMR measurement as a covariate. As these variables did not affect the females' RMR, results of these models are not shown.

Plasma antioxidant capacity data were log-transformed to reach homoscedasticity and normality of residuals. For all analyses, we used the Satterthwaite approximation to calculate the denominator's degrees of freedom (Giesbrecht and Burns 1985; McLean and Sanders 1988) and performed backward stepwise elimination of nonsignificant interactions and factors, keeping only significant variables ($P < 0.05$) in the final models, except for carotenoid and testosterone treatments, which were always retained. Estimates were calculated using restricted maximum likelihood, and we performed post hoc Tukey's Honestly Significant Difference (HSD) tests to determine which treatment groups differed from each other. Means ± standard error are given. All analyses were performed in R 3.01 (R Core Team 2013), using the packages "lme4" (Bates et al. 2014) and "lmerTest" (Kuznetsova et al. 2014).

RESULTS

We found significant interaction effects between the carotenoid and testosterone treatments on body mass at hatching and plasma ROM levels at 5 weeks of age (Table 1). Post hoc Tukey's HSD tests showed that an egg injection of either carotenoid or testosterone decreased body mass at hatching, but this effect disappeared when both carotenoid and testosterone were injected simultaneously (Figure 1a). Post hoc Tukey's HSD tests also showed that egg injection of carotenoid significantly increased plasma ROM levels in 5-week-old birds and egg injection of testosterone tended to increase plasma ROM levels at 5 weeks ($P = 0.095$; Figure 1d), but this effect disappeared in individuals originating from an egg where both testosterone and carotenoid were manipulated simultaneously (Figure 1d). In contrast, carotenoid and testosterone treatments had no effects on growth rate (Figure 1c; Table 1), plasma antioxidant capacity in 5-week-old birds (Table 1), body mass at the age of 5 weeks or 6 months (Table 1; Figure 1b), or RMR at the age of 6 months (Table 1).

Body mass of the mother ($F_{3,43} = 0.222$; $P = 0.881$) and egg mass ($F_{3,57.572} = 0.873$; $P = 0.461$) did not differ between treatment groups at the beginning of the experiment. Egg mass was a significant predictor of body mass at hatching, body mass at 6 months, and growth rate, with larger eggs developing faster

Table 1

Effects of testosterone and carotenoid injection in eggs of Japanese quails on body mass at hatching, growth rate, body mass at 6 months, oxidative capacity, ROMs, and RMR

Response variable	Explanatory variables	Estimate (mean ± SE)	Sum of squares	df	*F*	*P*
Body mass at hatching	Intercept	0.297 ± 0.372				
	Carotenoid treatment	−0.170 ± 0.087	0.023	1,55.09	0.28	0.600
	Testosterone treatment	−0.160 ± 0.087	0.001	1,55.46	0.13	0.723
	Testosterone × carotenoid treatment	0.279 ± 0.124	0.392	1,44.88	5.10	0.029
	Egg mass	0.704 ± 0.028	51.424	1,71.03	611.79	<0.001
	Egg number in the laying sequence	−0.012 ± 0.005	0.556	1,178.10	6.56	0.011
Growth rate (until 5 weeks)	Intercept	0.111 ± 0.043				
	Carotenoid treatment	0.055 ± 0.094	0.041	1,55.74	0.340	0.562
	Testosterone treatment	−0.086 ± 0.094	0.101	1,55.74	0.849	0.361
	Sex	−0.223 ± 0.057	1.840	1,171.64	15.413	<0.001
	Egg mass	0.111 ± 0.043	0.781	1,80.92	6.543	0.012
Body mass at 5 weeks	Intercept	4.541 ± 1.490				
	Carotenoid treatment	1.773 ± 3.209	43.2	1,55.44	0.305	0.583
	Testosterone treatment	−2.976 ± 3.208	121.82	1,55.44	0.861	0.358
	Sex	−8.016 ± 1.953	2385.32	1,171.78	16.850	<0.001
	Egg mass	4.541 ± 1.490	1314.23	1,80.27	9.284	0.003
Body mass at 6 months	Intercept	185.617 ± 29.526				
	Carotenoid treatment	0.377 ± 4.923	1.2	1,55.810	0.006	0.939
	Testosterone treatment	−2.803 ± 4.415	64.2	1,56.110	0.325	0.571
	Sex	−39.204 ± 3.384	26509.3	1,71.309	134.212	<0.001
	Egg mass	6.426 ± 2.416	1398.0	1,55.497	7.078	0.010
Oxidative capacity at 5 weeks	Intercept	5.288 ± 0.045				
	Carotenoid treatment	0.001 ± 0.049	0.000	1,46.811	0.000	0.981
	Testosterone treatment	−0.056 ± 0.049	0.132	1,46.935	0.132	0.256
d-ROMs at 5 weeks	Intercept	−0.042 ± 0.039				
	Carotenoid treatment	0.024 ± 0.013	0.001	1,62.59	0.55	0.462
	Testosterone treatment	0.023 ± 0.012	0.000	1,62.03	0.37	0.545
	Testosterone × carotenoid treatment	−0.036 ± 0.017	0.006	1,48.33	4.17	0.047
	Body mass at 5 weeks	0.001 ± 0.000	0.010	1 156.20	6.80	0.010
RMR at 6 months	Intercept	0.017 ± 0.004				
	Carotenoid treatment	0.365 ± 0.184	1.057	1,42.35	3.943	0.054
	Testosterone treatment	−0.126 ± 0.184	0.126	1,43.01	0.471	0.496
	Body mass at 6 months	0.017 ± 0.004	5.212	1,90.97	19.453	<0.001
	Sex	−1.267 ± 0.193	11.548	1,67.43	43.098	<0.001

Results of linear mixed models including mother identity as a random effect are shown. Final models were obtained by eliminating nonsignificant factors step by step, except for testosterone injection and carotenoid injection, which were always kept in the model. For carotenoid treatment, individuals that were not injected with carotenoids are taken as reference point, so that a positive effect of carotenoid treatment reflects a higher value in individuals injected with carotenoids as compared with individuals that were not injected with carotenoids. Similarly, for testosterone treatment, individuals that were not injected with testosterone are taken as a reference point. Females were taken as a reference point so that a negative effect of sex reflects a lower value in males as compared with females. df, degrees of freedom; SE, standard error.

after hatching and into larger birds (see Table 1). In contrast, egg mass did not predict plasma antioxidant capacity at 5 weeks of age ($P = 0.519$), plasma ROMs at 5 weeks of age ($P = 0.840$), or RMR at the age of 6 months ($P = 0.172$). In addition, larger birds had significantly higher levels of plasma ROMs at 5 weeks of age (see Table 1). Females grew significantly faster and were significantly heavier at 5 weeks and 6 months of age than males (mean body mass 5 weeks after hatching in males = 177.300 ± 1.394 g; in females = 186.871 ± 1.889 g, mean body mass 6 months after hatching in males = 221.589 ± 2.650 g; in females = 266.260 ± 3.329 g; see also Table 1). However, hatching mass ($P = 0.397$), antioxidant capacity ($P = 0.839$), or plasma ROMs ($P = 0.256$) did not differ between sexes. Chicks hatched from eggs laid earlier in a female's laying sequence were bigger (Table 1). In contrast, the rank in the laying sequence did not affect growth rate ($P = 0.701$), mass at the age of 5 weeks ($P = 0.380$) or 6 months ($P = 0.156$), plasma antioxidant capacity ($P = 0.052$), plasma ROMs ($P = 0306$), or RMR ($P = 0.882$). Mother's body mass at laying did not affect any of the tested variables (hatching mass: $P = 0.560$; growth rate: $P = 0.133$; body mass at the age of 5 weeks: $P = 0.128$; body mass at the age of 6 months: $P = 0.226$; plasma antioxidant capacity: $P = 0.340$; plasma ROMs: $P = 0.101$; RMR: $P = 0.165$). Finally, none of the tested variables affected the plasma antioxidant capacity at 5 weeks of age, and RMR at the age of 6 months was only affected by sex and body mass, with females and heavier birds having higher RMR scores (mean RMR in males = 4.276 ± 0.121; in females = 6.297 ± 0.095; Table 1).

DISCUSSION

Despite the large number of studies published on the importance of maternally transmitted compounds in transgenerational

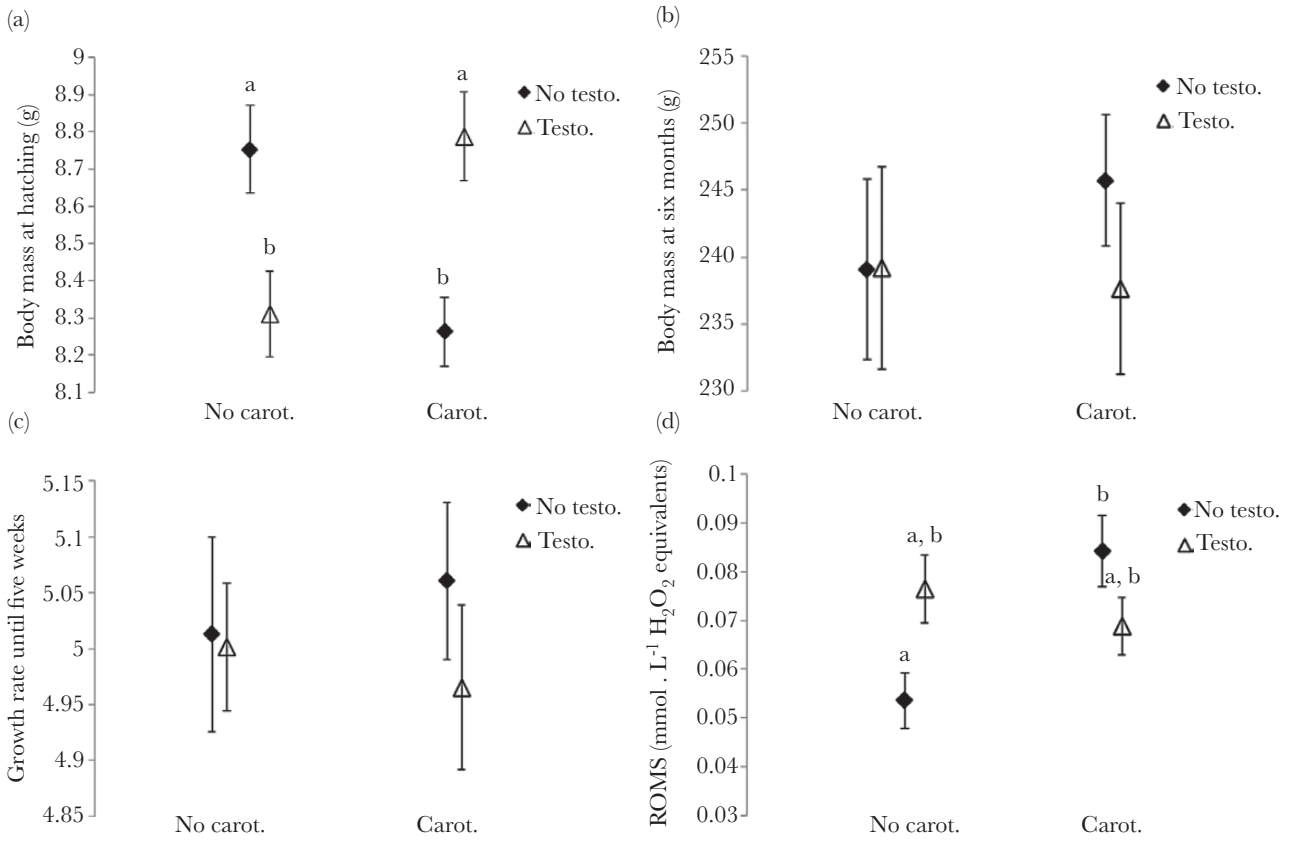

Figure 1

Effects of carotenoid and testosterone injections in eggs of Japanese quails on hatching mass (a), body mass at adulthood (b), growth (c), and ROMs (d). Different letters indicate statistically significant differences (Tukey's HSD, $P < 0.05$). Note that nonsignificant differences always had $P > 0.200$, except for the difference in ROMs between the control and the testosterone groups where the difference was marginally significant (Tukey's HSD, $P = 0.095$). Means ± standard error are presented.

developmental plasticity in various taxa (Uller 2008), so far potential interactive effects of these compounds on offspring development and phenotype are poorly understood. In birds, several yolk compounds are known to influence the same offspring phenotypic traits, making interaction effects between egg components a likely scenario. Here, we explored for the first time interactive effects between yolk testosterone and carotenoids by a simultaneous *in ovo* manipulation and examination of the effects on growth, oxidative stress, and metabolism.

We found that independent manipulations of yolk testosterone and yolk carotenoid levels significantly reduced hatching mass and increased ROM levels at the end of the period of maximal growth (only a trend in chicks from testosterone-injected eggs). These results differ from most previous studies (in numerous species and using various testosterone dosages) where hatching mass has not been affected by testosterone injections (Schwabl 1996; Sockman and Schwabl 2000; Andersson et al. 2004; Tschirren et al. 2005; Rubolini et al. 2006; Tobler et al. 2010; Noguera et al. 2011). However, it is in accordance with another study in Japanese quail where a similar detrimental effect of testosterone injections on hatching mass has been found (Okuliarová et al. 2007). In addition, a reduced mass has also been found in 12-day-old chicken embryos from eggs injected with testosterone (Henry and Burke 1999). In mammals, fetal exposure to testosterone has also been shown to reduce birth weight in rats (Wolf et al. 2002), sheep (Manikkam et al. 2004), and humans (Carlsen et al. 2006), but not in mice (de Catanzaro et al. 1991). The reason why embryo development is affected by exposure to testosterone in some species but not others remains unknown, and we can only speculate about the mechanisms underlying the embryo growth reduction observed in our study. One possible explanation is that increased levels of yolk testosterone might have influenced the pro-oxidant–antioxidant balance and/or the embryo's susceptibility to oxidative stress, with negative consequences for embryo growth. In line with this hypothesis, testosterone *in ovo* injections led to a reduced DNA damage repair efficiency in chicken (at days 17 and 18 posthatch, Treidel et al. 2013) and a transient impairment of the antioxidant defenses in male zebra finches 10 days after hatching (Tobler and Sandell 2009, but see Noguera et al. 2011). Similarly, birds originating from a testosterone-injected egg tended to have increased ROM levels in our study. A fruitful next step would be to examine how embryo exposure to testosterone influences growth factor expression, ROS production, and antioxidant defenses before hatching.

The consequences of yolk carotenoid manipulations have been less explored (Saino et al. 2003; Romano et al. 2008; Saino et al. 2011), and, to the best of our knowledge, our study is the first to show that these maternally transmitted compounds can negatively affect embryo growth (i.e., mass at hatching). However, contrary to our prediction and the general idea that carotenoids are beneficial due to their presumed ability to scavenge ROS and/or stimulate immunocompetence during development (Blount et al. 2002; Saino et al. 2003), carotenoid injection negatively influenced

hatching mass and increased ROM levels at the end of the period of maximal growth in our study. Previous studies in adult birds have shown that at high concentrations, carotenoids can lose their antioxidant activity and can have harmful pro-oxidant properties through single-electron oxidations or reductions (Palozza et al. 1995; Palozza 1998; Martin et al. 1999; Russel 1999; Hartley and Kennedy 2004; Huggins et al. 2010; Simons et al. 2014). For example, Huggins et al. (2010) showed that high intake of carotenoid pigments in American goldfinches (*Spinus tristis*) led to an increase in creatine kinase, an indicator of skeletal muscle breakdown, and a reduction in vertical flight performance. Our result adds to this growing literature, showing that carotenoids can negatively affect ROM levels after hatching and also have deleterious effects before birth. This is remarkable because the injected carotenoid dose was well within the natural range (Peluc et al. 2012) and yolk carotenoid levels after injection were not unnaturally high because females were fed with a low-carotenoid diet during the whole experiment.

Alternatively, the negative effects of the *in ovo* carotenoid injection on hatching mass may be due to a reallocation of resources from growth to immune system development (Saino et al. 2003; Soler et al. 2003). Unfortunately, we did not measure immunocompetence in our study, but previous work on barn swallows has shown that nestlings hatched from lutein-injected eggs had a larger T-cell-mediated immune response compared with control nestlings development (Saino et al. 2003). Thus, by depositing higher yolk carotenoid concentrations in eggs, mothers may be able to boost offspring health (at the detriment of growth) in pathogen-rich environments. In line with this hypothesis, Jacob et al. (2015) have recently shown that an experimental decrease of the nest bacterial density led to a reduction in the levels of carotenoids transferred to the yolk and an increased growth rate in great tits (*Parus major*).

Interestingly, hatching mass and ROM levels were not affected when both egg compounds were manipulated simultaneously, showing that both carotenoid and testosterone lose their detrimental effects during prenatal development when the ratio between these 2 compounds is balanced. This result suggests that the egg is an integrated system where several components (including hormones and antioxidants) interact (Surai 2002; Saino et al. 2011) and an imbalance between these components leads to a disequilibrium of this system. It also suggests that mothers may coadjust different egg components in the eggs (Postma et al. 2014) to achieve an optimal outcome for the offspring. Testosterone and carotenoid appear to be 2 crucial elements of this integrated system because no detrimental effects have been observed when both of these compounds were injected simultaneously, even though other components (e.g., corticosterone, vitamin E, immunoglobulins) remained unmanipulated.

Evidence for an effect of maternally transmitted testosterone on postnatal growth is mixed. Although some studies found a clear increase in growth rate in chicks from testosterone-injected eggs (Eising et al. 2001; Pilz et al. 2004; Muriel et al. 2015), others found no (Rubolini et al. 2006; Tobler et al. 2007) or even a negative effect of experimental *in ovo* injections of testosterone on growth (Sockman and Schwabl 2000). Similarly, yolk carotenoid injections had some complex effect on growth in yellow-legged gulls (i.e., it enhanced the growth of males from the first laid eggs but depressed the growth of males from the last laid eggs; Romano et al. 2008) and had no effect in barn swallows (Saino et al. 2003). We found no indication that growth rate was influenced by testosterone or carotenoid injection. Together, it suggests that the effects of maternally transmitted compounds on growth are complex and may be context dependent (Muriel et al. 2015).

Long-term effects of yolk testosterone and carotenoid manipulations on adult metabolism are still poorly understood, and contrasting results have been found when the effects of yolk testosterone manipulation on metabolism were examined. An increased RMR has been observed in nestling and adult zebra finches (*T. guttata*, Tobler et al. 2007; Nilsson et al. 2011) and in adult pied flycatchers (*Ficedula hypoleuca*, Ruuskanen et al. 2013) hatched from testosterone-injected eggs, whereas no effect of a similar manipulation was detected in black-headed gulls (*Larus ridibundus*, Eising and Groothuis 2003). Because we did not find any long-term effect of the testosterone injections and only a nonsignificant trend for an effect of carotenoid injections on RMR (measured at the age of 6 months), our results are in line with the latter, indicating that in precocial species adult metabolism may not be influenced by maternally transmitted compounds. However, future studies should confirm these results because our study is, so far, the only one examining the effect of yolk carotenoid levels on metabolism.

In conclusion, our study provides the first experimental evidence for interactive effects between yolk testosterone and carotenoids on hatching mass and oxidative damage levels at the end of the period of maximal growth, suggesting that different maternally derived components are tightly coadjusted within an egg. Manipulating only one egg component in isolation, as is usually done, might thus disturb the fragile equilibrium between different egg compounds, potentially leading to spurious results.

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