LAY SUMMARY

Mothers can influence offspring phenotype through the transmission of hormones, immune or antioxidant compounds, but the interactive effects of these resources have never been studied. Here, we show that maternally-transmitted testosterone and carotenoids interact to influence embryo growth and offspring ROMs levels in Japanese quail. These results provide the first experimental evidence for interactive effects of two maternally-derived egg compounds on offspring phenotype and suggests that developmental cues are tightly co-adjusted within an egg.

INTERACTIVE EFFECTS OF YOLK TESTOSTERONE AND CAROTENOID ON

PRE-NATAL GROWTH AND OFFSPRING PHYSIOLOGY IN A PRECOCIAL BIRD

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ABSTRACT

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 ROMs levels were not affected, showing that both carotenoid and testosterone lose their detrimental effects when the ratio between the two compounds is balanced. Our study provides the first experimental evidence for interactive effects of two maternally-derived egg compounds on offspring phenotype and suggests that developmental cues are tightly co-adjusted within an egg.

INTRODUCTION

Conditions experienced during prenatal development can influence an individual's developmental trajectory and have long-term effects on its physiology, morphology and behaviour, 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 *et al.* 2009, Treidel *et al.* 2013, but see Noguera *et al.* 2011). In accordance with this hypothesis, reduced plasma antioxidant levels (Tobler *et al.* 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 produced during development (Surai *et al.* 2001) 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 co-adjust 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.* 2016). 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 x 2 design to quantify how these two egg compounds interact to shape the morphology and physiology of Japanese quail chicks. In particular, we assessed the potential interactive effects of these two egg compounds on hatching success, mass at hatching, growth rate and oxidative stress (reactive oxygen metabolites (ROMs) and the total plasma antioxidant capacity (TAC)). Since 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 at adulthood (Orledge *et al.* 2012). Previous experimental studies have

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102 shown that prenatal exposure to high testosterone concentrations leads to an increased adult

103 metabolic rate (Tobler *et al.* 2007, Nilsson *et al.* 2011, Ruuskanen *et al.* 2013). The effect of

104 yolk carotenoid levels on metabolic rate, however, has so far never been studied.

105 　　We predicted that, compared to controls, offspring from testosterone-injected eggs

106 would grow faster, have a higher metabolic rate, higher ROMs levels and a deficient

107 antioxidant capacity. In contrast, compared with controls, we expected offspring from

108 carotenoid-injected eggs to have a better antioxidant capacity and lower ROMs levels.

109 Finally, we predict that experimentally increased yolk carotenoid levels would buffer the

110 negative effects of high yolk testosterone exposure on ROMs levels and antioxidant capacity.

111

112 **METHODS**

113 *Egg collection, egg injection, incubation and hatching*

114 In March 2014, 55 breeding pairs were randomly selected from a Japanese quail population

115 maintained at the University of Zurich, Switzerland. Birds were housed in pairs in cages (122

116 x 50 x 50 cm, photoperiod of 16h L:8h D), and received *ad libitum* water and commercial

117 game bird mix low in carotenoid content. Eggs (N=535) were collected during two weeks and

118 each clutch was randomly assigned to one of the four treatments: yolk carotenoid

119 manipulation (C, 14 clutches, 135 eggs), yolk testosterone manipulation (T, 14 clutches, 136

120 eggs), both yolk carotenoid and yolk testosterone manipulation (CT, 14 clutches, 137 eggs) or

121 a control injection (CO, 13 clutches, 127 eggs). Eggs were injected with either 15 ng of

122 testosterone (17β-hydroxy-4- androsten-3-on) dissolved in 15μL of safflower oil, 15 μg of

123 carotenoids (FloraGLO Lutein 20%, Kemin Foods, Des Moines, Iowa) dissolved in 15μL of

124 safflower oil, both testosterone (15 ng) and carotenoids (15 μg) dissolved in 15μL of

125 safflower oil or with 15μL of safflower oil as a control (see Tschirren *et al.* 2005 for a

126 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 one standard deviation of the published yolk testosterone and yolk carotenoid contents in this species (Daisley *et al.* 2005, Hackl *et al.* 2003, Dvorska and Surai, 2004, 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 to previous studies in Japanese quail (Daisley *et al.* 2005, Okuliarova *et al.* 2007, Hegyi and Schwabl, 2010), and did not differ between treatments (χ^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 three 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 five 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, 1, 2, 3 and 5 weeks. 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 as a measure of growth rate. Using this method was strongly supported by the very high adjusted R² (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 (10'000g 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 reactive oxygen metabolites for only 173 individuals and the total plasma antioxidant capacity (TAC) for 188 individuals. ROMs were measured using the d-ROMs 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 Haussman *et al.* 2011.

Metabolic rate

Resting 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 (122x50x50cm) with *ad libitum* food and water. Metabolic rate

176   measurements were performed during the birds' rest phase (6pm-8:30am), after a four hours

177   period of fasting to ensure a post-absorptive state. Individuals were placed in a 3.9l plastic

178   metabolic chamber (234 x 165 x 165 mm, Lock & Lock, Hanacobi Co. Ltd., Korea), into a

179   temperature-controlled, dark room within the birds' thermoneutral zone (25-27°C) (Ben-

180   Hamo *et al.* 2010). Oxygen consumption rate (VO_2, $ml.min^{-1}$) was measured by indirect

181   calorimetry with an eight-channel open-flow respirometry system. Before each trial, the CO2

182   analyzer was zeroed using CO2-free air (N2, PanGas,Switzerland) and spanned using a 1.002

183   % mol CO2 mixture (PanGas,Switzerland). The O2 analyzer was spanned to 20.95% by

184   flushing dry air through the system. During the trials, external air was pumped into the

185   chamber at a flow rate of 1650-1700 $ml.min^{-1}$ controlled by an eight-channel mass flow meter

186   system (Flow Bar Mass Flow Meter FB-8-1, Sable System, USA). All gas flow connections

187   passed through ultra-low permeability Tygon tubes (internal diameter of 8 mm). Seven of the

188   eight chambers contained one quail, with an empty chamber used as a control. Each recording

189   sequence lasted 45 minutes with a five minutes measurement of all metabolic chambers,

190   starting and ending with the control chamber. During a sequence, an automatic switch allowed

191   excurrent air from each chamber to be subsampled (250 $ml.min^{-1}$; Multiplexer Intelligent RM-

192   8-2, Sable System, USA), dried (magnesium perchlorate, Sigma-Aldrich, USA) and analyzed

193   every second over a five minutes period by a fuel cell O2 analyzer and a dual wavelength

194   infrared bench CO2 analyzer (Foxbox, Sable System, USA). Using this set-up, we obtained

195   about 22 sequences per bird. As the equipment took a certain time to adjust between

196   chambers, the first 100 seconds of each reading was excluded, leaving 200 seconds per

197   reading. Baseline O2 and CO2 were determined by regressing all control chamber readings

198   against time for each 45 minute period. Oxygen consumption rates were calculated by

199   comparing oxygen content of the metabolic chamber containing birds (Fe) to the baseline

200   concentrations measured from the control chamber for the same time point (Fi). Given that the

201 mass flow meter was upstream from the metabolic chamber and so CO_2 was not removed

202 from the excurrent air stream, we used the following equation to correct for flow rate (FR)

203 and CO_2 concentration: $VO_2 = FR * ((FiO_2 - FeO_2) - FeO_2 * (FeCO_2 - FiCO_2))/ (1 - FeO_2)$

204 (Lighton 2008). Resting metabolic rate (RMR) for each bird was determined as the mean of

205 the lowest 60 consecutive seconds of VO_2. Individuals were weighed ($\pm$ 0.1g) before and

206 after the metabolic rate measurement.

207

208 *Statistical analyses*

209 We were mostly interested in testing for potential interacting effects of carotenoids and

210 testosterone injection on the different response variables, rather than for an overall effect of a

211 carotenoids or testosterone injection. We thus considered carotenoid and testosterone

212 treatments as two different factors, and also considered their second order interaction effect.

213 To test whether yolk carotenoid and / or testosterone manipulations affect body mass at

214 hatching, growth rate, plasma antioxidant capacity and ROMs levels at the age of five weeks,

215 and RMR and body mass at adulthood, we used linear mixed models (LMM) with the identity

216 of the mother as a random effect and carotenoid treatment (binary variable segregating the

217 272 eggs injected with carotenoids from the 263 that were not injected with carotenoids),

218 testosterone treatment (binary variable segregating the 273 eggs injected with testosterone

219 from the 262 that were not injected with testosterone), the second order interaction between

220 the carotenoid and testosterone treatments, sex, egg mass, rank in the laying sequence and the

221 mother's body mass at laying as fixed effects in all models. For the analysis of antioxidant

222 capacity and ROMs, we also included the mass measured at the age of five weeks as a

223 covariate. For the analysis of RMR, we included the body mass measured just before the

224 RMR measurement as a covariate. Furthermore, we also ran a separate analysis on females,

225 including either the number or mass of eggs laid during the five days prior the RMR

10

226 measurement or the number of eggs during the RMR measurement as a covariate. As these

227 variables did not affect the females' RMR, results of these models are not shown.

228   Plasma antioxidant capacity data were log-transformed to reach homoscedasticity and

229 normality of residuals. For all analyses, we used the Satterthwaite approximation to calculate

230 the denominator's degrees of freedom (Giesbrecht and Burns 1985, McLean and Sanders

231 1998), and performed backward stepwise elimination of non-significant interactions and

232 factors, keeping only significant variables ($p < 0.05$) in the final models, except for carotenoid

233 and testosterone treatments, which were always retained. Estimates were calculated using

234 restricted maximum likelihood, and we performed post-hoc Tukey HSD tests to determine

235 which treatment groups differed from each other. Means ± SE are given. All analyses were

236 performed in R 3.01 (R Core Team 2013), using the packages "lme4" (Bates et al. 2008) and

237 "lmerTest" (Kuznetsova et al. 2014).

238

239 **RESULTS**

240   We found significant interaction effects between the carotenoid and testosterone

241 treatments on body mass at hatching, and plasma ROMs levels at five weeks of age (Table 1).

242 Post-hoc Tukey HSD tests showed that an egg-injection of either carotenoids or testosterone

243 decreased body mass at hatching, but this effect disappeared when both carotenoid and

244 testosterone were injected simultaneously (Figure 1a). Post-hoc Tukey HSD tests also showed

245 that egg injection of carotenoid significantly increased plasma ROMs levels in five weeks old

246 birds and egg injection of testosterone tended to increase plasma ROMs levels at five weeks

247 (p = 0.095, Figure 1d), but this effect disappeared in individuals originating from an egg

248 where both testosterone and carotenoid were manipulated simultaneously (Figure 1d). In

249 contrast, carotenoid and testosterone treatments had no effects on growth rate (Figure 1C,

Table 1), plasma antioxidant capacity in five weeks old birds (Table 1), body mass at the age of five weeks or six months (Table 1; Figure 1b) or RMR at the age of six 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 six months and growth rate, with larger eggs developing faster after hatching and into larger birds (see Table 1). In contrast, egg mass did not predict plasma antioxidant capacity at five weeks of age (p = 0.519), plasma ROMs at five weeks of age (p = 0.840) or RMR at the age of six months (p = 0.172). In addition, larger birds had significantly higher levels of plasma ROMs at five weeks of age (see Table 1). Females grew significantly faster and were significantly heavier at five weeks and six months of age than males (mean body mass five weeks after hatching in males = 177.300 ± 1.394g; in females = 186.871 ± 1.889, mean body mass six months after hatching in males = 221.589 ± 2.650 g; in females = 266.260 ± 3.329g; 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 five weeks (p = 0.380) or six 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 five weeks: p = 0.128; body mass at the age of six months: p = 0.226; plasma antioxidant capacity: p = 0.340; plasma ROMs: p = 0.101; BMR: p = 0.165). Finally, none of the tested variables affected the plasma antioxidant capacity at five weeks of age, and RMR at the age of six 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 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 ROMs 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 (Okuliarova *et al.* 2007). In addition, a reduced mass has also been found in 12 days-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 prooxidant-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 post-hatch, Treidel *et al.* 2013) and a transient impairment of the antioxidant defenses in male zebra finches ten days after hatching (Tobler *et al.* 2009, but see Noguera *et al.* 2011). Similarly, birds originating from a testosterone injected egg tended to have increased ROMs levels in our study. A fruitful next step would be to examine how embryo exposure to testosterone influences growth factor expression, ROS production and antioxidants defenses before hatching.

The consequences of yolk carotenoid manipulations have been less explored (Saino *et al.* 2003, 2011, Romano *et al.* 2008) 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 ROMs 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

325　negatively affect ROMs levels after hatching and also have deleterious effects before birth.

326　This is remarkable because the injected carotenoid dose was well within the natural range

327　(Peluc *et al.* 2012) and yolk carotenoid levels after injection were not unnaturally high since

328　females were fed with a low-carotenoid diet during the whole experiment.

329　　　　Alternatively, the negative effects of the *in ovo* carotenoid injection on hatching mass

330　may be due to a reallocation of resources from growth to immune system development (Saino

331　*et al.* 2003, Soler *et al.* 2003). Unfortunately, we did not measure immunocompetence in our

332　study, but previous work on barn swallows has shown that nestlings hatched from lutein-

333　injected eggs had a larger T-cell mediated immune response compared with control nestlings

334　development (Saino *et al.* 2003). Thus, by depositing higher yolk carotenoid concentrations in

335　eggs mothers may be able to boost offspring health (at the detriment of growth) in pathogen-

336　rich environments. In line with this hypothesis, Jacob *et al.* (2015) have recently shown that

337　an experimental decrease of the nest bacterial density led to a reduction in the levels of

338　carotenoids transferred to the yolk and an increased growth rate in great tits (*Parus major*).

339　　　　Interestingly, hatching mass and ROMs levels were not affected when both egg

340　compounds were manipulated simultaneously, showing that both carotenoid and testosterone

341　lose their detrimental effects during prenatal development when the ratio between these two

342　compounds is balanced. This result suggests that the egg is an integrated system where several

343　components (including hormones and antioxidants) interact (Surai 2002, Saino *et al.* 2011)

344　and an imbalance between these components leads to a disequilibrium of this system. It also

345　suggests that mothers may co-adjust different egg components in the eggs (Postma *et al.*

346　2014) to achieve an optimal outcome for the offspring. Testosterone and carotenoids appear to

347　be two crucial elements of this integrated system since no detrimental effects have been

348　observed when both of these compounds were injected simultaneously, even though other

349　components (e.g. corticosterone, vitamin E, immunoglobulins etc) remained unmanipulated.

15

Evidence for an effect of maternally-transmitted testosterone on post-natal growth are mixed. While 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 resting metabolic rate has been observed in nestling and adult zebra finches (*Taeniopygia 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, while no effect of a similar manipulation was detected in black-headed gulls (*Larus ridibundus,* Eising *et al.* 2003). Since we did not find any long-term effect of the testosterone injections and only a non-significant trend for an effect of carotenoid injections on resting metabolic rate (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 since our study is, so far, the only one examining the effect of yolk carotenoid levels on metabolism.

374   In conclusion, our study provides the first experimental evidence for interactive effects

375   between yolk testosterone and carotenoids on hatching mass and oxidative damage levels at

376   the end of the period of maximal growth, suggesting that different maternally-derived

377   components are tightly co-adjusted within an egg. Manipulating only one egg component in

378   isolation, as is usually done, might thus disturb the fragile equilibrium between different egg

379   compounds, potentially leading to spurious results.

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389   **Data Accessibility**

390   Analyses reported in this article can be reproduced using the data provided by Giraudeau et al.

391   (2016).

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599 Table 1.

600 Effects of testosterone and carotenoid injection in eggs of Japanese quails on body mass at hatching,

601 growth rate, body mass at six months, oxidative capacity, ROMs and RMR. Results of linear mixed

602 models including mother identity as a random effect are shown. Final models were obtained by

603 eliminating non-significant factors step by step, except for testosterone injection and carotenoid

604 injection, which were always kept in the model. For carotenoid treatment, individuals that were not

605 injected with carotenoids are taken as reference point, so that a positive effect of carotenoid treatment

606 reflects a higher value in individuals injected with carotenoids as compared to individuals that were

607 not injected with carotenoids. Similarly, for testosterone treatment, individuals that were not injected

608 with testosterone are taken as a reference point. Females were taken as a reference point so that a

609 negative effect of sex reflects a lower value in males as compared to females.

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 five 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 six 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
dROMS at 5 weeks	Intercept	-0.042 ± 0.039				
	Carotenoid treatment	0.024 ± 0.013	0.001	1,62.59	0.55	0.462

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	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 five 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 six 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

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612　FIGURE 1. Effects of carotenoid and testosterone injections in eggs of Japanese quails on hatching

613　mass (a), body mass at adulthood (b), growth (c) and ROMs (d). Different letters indicate statistically

614　significant differences (Tukey's HSD, $p < 0.05$). Note that non-significant differences always had $p >$

615　0.200, except for the difference in ROMs between the control and the testosterone groups where the

616　difference was marginally significant (Tukey HSD: $p = 0.095$). Means ± SE are presented.