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Maternal dietary carotenoids mitigate detrimental effects of maternal GnRH on offspring immune function in Japanese quail *Coturnix japonica*

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Maternal resources deposited in eggs can affect the development of several offspring phenotypic traits and result in trade-offs among them. For example, maternal androgens in eggs may be beneficial to offspring growth and competitive ability, but detrimental to immunocompetence and oxidative stress. In contrast, maternal antioxidants in eggs may be beneficial if they mitigate oxidative stress and immunosuppressive effects of androgens. We investigated possible interactive effects of maternal steroids and carotenoids on aspects of offspring physiology and phenotype, by simultaneously manipulating levels of androgens (via gonadotropin-releasing hormone, GnRH-challenges) and carotenoids (via diet supplementation) in captive female Japanese quail *Coturnix japonica* during egg laying. Carotenoid supplementation of hens, which elevates yolk concentrations of carotenoid and vitamins A and E, enhanced egg hatching success, offspring survival to age 15 d, and size of the bursa of Fabricius in offspring. In contrast, repeated maternal GnRH challenges, which elevated yolk testosterone concentrations, enhanced offspring neonatal size, but negatively affected bursa size. However, interaction among the treatments suggests that the positive effect of maternal carotenoid supplementation on plasma bactericidal capacity was mediated by maternal GnRH challenges. Chicks originating from carotenoid-supplemented hens were less immunosuppressed than those originating from carotenoid-supplemented + GnRH-challenged hens, which were less immunosuppressed than chicks from GnRH-challenged females not supplemented with carotenoids. Females availability of carotenoid enriched diets allows them to enhance the development of offspring immune system via carotenoids and vitamins deposited in egg yolks and offset detrimental effects of androgens deposited by GnRH-challenged females.

Maternal effects comprise the causal influence of the maternal genotype and phenotype on the offspring phenotype (Wolf and Wade 2009). For example, oviparous mothers allocate specific components to eggs, including hormones (Schwabl 1996, Lipar and Ketterson 2000, Eising et al. 2001, Groothuis et al. 2005a) and antioxidants such as carotenoids and vitamins (Surai and Kuklenko 2000, Surai et al. 2001, 2002, Saino et al. 2003, McGraw et al. 2005), which can have long-lasting consequences on offspring health, growth, and survival (Mousseau and Fox 1998, Lindström 1999, Metcalfe and Monaghan 2001). Egg yolk environment, i.e. maternally deposited resources, has a significant effect on several aspects of embryonic development and phenotype determination in avian species (Ho and Burggren 2010, Ho et al. 2011). Moreover, yolk constituents may have interactive effects on development of embryos and neonates (Navara et al. 2006a, Cucco et al. 2008). Because of the broad effects of these compounds on offspring (Ros et al. 1997, Groothuis et al. 2005a, b, McGraw et al. 2005, Gil 2008), knowledge of costs and benefits of their combined effects must be gained to fully assess the adaptive value of maternal resource deposition to eggs (Blount et al. 2002, Groothuis et al. 2005a, Safran et al. 2008).

Much of the research examining control mechanisms and functions of yolk compounds on offspring has approached them separately. From such studies we know for example that avian yolk androgens have direct and positive effects on offspring competitiveness, muscle/skeletal growth, and metabolism (Schwabl 1996, Lipar and Ketterson 2000, Eising et al. 2001, Pilz et al. 2004, Groothuis et al. 2005a, Navara et al. 2005, Navara and Mendonca 2008), and may negatively affect egg hatchability (Navara et al. 2005, Rutkowska and Cichon 2006; but see Rubolini et al. 2006a), embryo development (Boncoraglio et al. 2011), and neonate growth (Groothuis et al. 2005b). Yolk androgens can also indirectly affect offspring through increasing oxidative stress (Folstad and Karter 1992, Duffy et al. 2000, Andersson et al. 2004, Navara et al.

2005) and impairing cell-mediated and humoral immune function (Andersson et al. 2004, Groothuis et al. 2005a, b, Müller et al. 2005, Navara et al. 2005). In birds, androgens may reduce the size of thymus and bursa of Fabricius (Norton and Wira 1977, Olsen and Kovacs 1996). Additionally, elevated T may indirectly affect immune function via enhanced growth rates given the trade-off between growth and immune function, two energetically costly activities (Müller et al. 2005). Other yolk components such as carotenoid pigments can enhance neonate immune function (Krinsky 2001, McGraw and Ardia 2003, Saino et al. 2003, Koutsos et al. 2006) and influence offspring fitness (McGraw et al. 2005, Biard et al. 2007, De Neve et al. 2008). Similarly, vitamins A and E from yolk act as antioxidants that contribute to hatchling health (Haq et al. 1996, Gore and Qureshi 1997). However, little is known of the specific effects of these vitamins on the development of the immune system in embryos (Gore and Qureshi 1997, Surai et al. 1999, 2001).

Although yolk androgens are generally regarded as immunosuppressive, and carotenoids and vitamins as immune enhancers, exposure to a combination of physiologically relevant levels of these compounds may reveal complementary or antagonistic effects. There is evidence of both a complementary and positive association between androgens and carotenoids deposited in eggs, suggesting that females deposit different amounts of those compounds to their eggs to optimize offspring growth, competitiveness, and health (Royle et al. 2001, Navara et al. 2006a, Peluc et al. 2012). However, the interactive effects of those compounds on offspring performance are not yet well understood (Royle et al. 2001). Previous work (Cucco et al. 2008) shows that carotenoids in young birds may act to counter some of the detrimental effects associated with high levels of yolk androgens. However, because such studies have manipulated offspring exposure to androgens via egg injection and carotenoids via diet supplementation to young, it is difficult to understand how maternally allocated compounds may affect offspring performance. Because females have the ability to differentially allocate resources to their eggs (Royle et al. 2001, Navara et al. 2006a, Peluc et al. 2012), it becomes interesting to understand consequences on offspring of maternal deposition of different egg components. Peluc et al. 2012 show evidence that in Japanese quail *Coturnix japonica* carotenoid supplementation and challenges with gonadotropin-releasing hormone (GnRH) to laying female alter deposition of carotenoids and T to eggs in such manner that those compounds were not deposited independently of one another (i.e. yolk T and carotenoids were positively correlated). Peluc et al. (2012) also show that maternal GnRH challenges can increase maternal deposition of yolk carotenoids, because carotenoid supplemented females that were also GnRH-challenged deposited more carotenoids into yolk than females whose diet was supplemented and received a saline injection. Similarly, GnRH-challenged females that were carotenoid supplemented deposited more T into yolk than GnRH-challenged females that received a control diet.

In the present study, we investigated possible additive and interactive effects of maternally transferred steroids, carotenoids, and vitamins on aspects of offspring morphology and immune function. We experimentally induced changes in levels of steroids and/or carotenoids and vitamins (A and E) in eggs by treating captive female Japanese quail with (GnRH) and by providing them with carotenoid (lutein and zeaxanthin) supplements in the diet, respectively. By manipulating maternal diet and reproductive hormones, we focused on the hormone and carotenoid levels that females deposit into eggs. With a 2×2 full factorial design, we evaluated the effects of maternal GnRH challenges and maternal dietary carotenoid supplementation on offspring hatching success, growth, immune function, and survival probability. We predicted that GnRH-challenged females (which deposit higher levels of T in eggs) would produce larger, faster growing, yet immunosuppresed chicks. Given possible health-boosting effects of carotenoids and vitamins, we predicted that chicks from carotenoid-supplemented females would have improved immune function compared to chicks from control females or females receiving GnRH challenges. On the other hand, given the possibility that carotenoids mitigate immunosuppressive effects of T, we expected that chicks from carotenoid-supplemented and GnRH-challenged females would grow faster and would be larger than chicks from non-GnRH-challenged females, yet be less immunosuppresed than chicks from GnRH-challenged, but not carotenoid-supplemented, females.

Methods

Housing and treatment of laying birds

We randomly selected 48 adult female and 24 adult male Japanese quail from a pool of 65 individuals hatched and raised in the laboratory. All selected hens were 12 weeks old, and started laying one egg a day 28 ± 3 d ($n = 48$) prior to initiation of the experiment. Quail included in the study were at their peak of egg production (Ottinger 2001). We housed adults in separate cages at a ratio of two females and one male per cage in an animal indoor room approved by the Institutional Animal Care and Use Committee at North Dakota State Univ. We banded each individual with a unique combination of two colored leg bands. Quail received ad libitum water and a diet of commercial game bird mix (Sprout Meat Maker, Appleton, WI, USA), which contained a small amount of xanthophylls (ca 5 mg kg^{-1}) and carotene (ca 3 mg kg^{-1}). Adult quail were maintained on a light:dark cycle of 14:10 h and ambient temperature of approximately 22 ± 2°C, and were assigned to one of four treatments: GnRH injection, carotenoid supplementation and vehicle injection (saline), both carotenoid supplementation and GnRH injection, and a control injection group (saline) without carotenoid supplementation. Hereafter the four treatments will be referred to as GnRH, carotenoid, GnRH + carotenoid, and control. Females in the same cage were assigned to the same carotenoid treatment but not necessarily the same GnRH treatment.

Carotenoid supplementation of laying quail

A maternal means to elevate yolk carotenoids is dietary supplementation of laying females with these compounds

(McGraw et al. 2005) which increases yolk levels of both carotenoids and vitamin A and E (Peluc et al. 2012). Carotenoid-supplemented quail received two common plant carotenoids from the group of xanthophylls, lutein and zeaxanthin, at a dose of 7.5 μg ml^{-1} dissolved in their drinking water, whereas un-supplemented individuals received no carotenoids in their drinking water. We chose to supplement with xanthophylls because lutein and zeaxanthin are the two major carotenoids in chicken eggs (Surai and Sparks 2001), and also because lutein is a primary carotenoid consumed by birds in the wild, and is routinely fed to commercial poultry to promote product pigmentation of egg yolks and skin (Hernandez et al. 2001). We did not supplement vitamins A and E here, but in a previous study of quail (Peluc et al. 2012), we found that hens that were supplemented with carotenoids laid eggs with elevated levels of both vitamin A and E in the yolk. From a pilot study on the same quail population, we determined that the average daily amount of fluid consumption per individual was 35 ± 5 ml (Peluc et al. unpubl.). Hence treated individuals consumed between 2.25–3.00 mg of carotenoids per day (dose that includes the carotenoids contained in the food). The selected dose is well within the range of doses previously used on carotenoid-supplemented Japanese quail (McGraw 2006: i.e. daily carotenoid consumption of 0.4–4.2 mg). The supplement was given using water-dispersible lutein and zeaxanthin beadlets (at a ratio of 93:7) kindly supplied by DSM Nutritional Products. All drinks were freshly prepared each day using 50 ml of warm water to dissolve the carotenoids and then completing the total solution volume with cool water. Drinks were provided in opaque dispensers to avoid oxidation (Blount et al. 2003). Supplementation in this study began on 18 February 2008 and continued for seven weeks.

GnRH challenges and control injections of laying quail

GnRH is a hormone released from the hypothalamus that controls a cascade of hormone secretion events from the pituitary and gonads. Intramuscular injection of GnRH temporarily stimulates the hypothalamo-pituitary-gonadal (HPG) axis, leading to release of luteinizing hormone (LH) from the pituitary, which stimulates steroid hormone secretion from the gonads (Johnson 2000). As a consequence of maternal GnRH challenges, yolk steroid levels also increase (Jawor et al. 2007, Peluc et al. 2012), which provides a maternal means of elevating yolk T levels in the egg. By evaluating effects of maternally deposited compounds to egg yolk as opposed to egg injection we avoid potential changes in offspring development due to injection manipulations. Simply puncturing the egg shell can negatively affect hatchability (Winter et al. 2013), and although most studies do not report precise information on the proportion of eggs damaged by the technique, percentage of eggs lost to injection can vary between 20–40% (Saino et al. 2003, pers. obs.).

We experimentally elevated T levels (within the physiological range; Ottinger and Brinkley 1979, Bertin et al. 2008) in adult quail plasma via GnRH injections. Previous work (Peluc et al. unpubl.) revealed that T concentrations in Japanese quail eggs were at a maximum approximately two weeks after maternal GnRH challenge, after which T concentrations declined. Thus, in an attempt to elevate already high yolk T levels in hens to the upper end of the physiological range, we administered four intramuscular injections of GnRH to each female throughout the experiment (one every 14–16 d) following Jawor et al. (2007). GnRH challenges were performed using an injection of 5 μg cGnRH-I (Sigma L0637) in 50 μl phosphate-buffered saline (PBS) solution (Peluc et al. 2012). Control injections consisted of 50 μl PBS only. We drew blood (for T measurement in plasma) immediately prior to and 30 min after each challenge. Analysis of female plasma revealed that baseline T levels, measured immediately before GnRH injections, did not differ significantly among treatments (mean ± SE: 1.24 ± 0.07 pg μl^{-1}, n = 48; Peluc et al. 2012). However, post-injection T levels were approximately 40% higher in GnRH-challenged females (i.e. GnRH and GnRH ± carotenoid treatments) than in females in the carotenoid and control treatments (Peluc et al. 2012). Treatment did not affect egg laying as all treated females continued laying eggs throughout the experiment at the same rate as control females.

Egg incubation and chicks rearing

Japanese quail, like other domesticated species, can lay eggs nearly continuously throughout the year, but typically will lay in bouts of different lengths that are separated by one or more 'pause days' (Johnson 2000). The time frame of this experiment involved hens in the very early stages of the reproductive cycle, between weeks 12–19, which are characterized by high egg productivity and high receptivity of follicular cells to gonadotropin (Johnson et al. 1986, Johnson 2000, Ottinger 2001). We collected two eggs per week per female for six weeks, starting the week after treatments were initiated, because yolk T and carotenoids were only augmented from the second week onwards (Peluc et al. 2012). We weighed (to the nearest 0.01 g on a digital balance) and identified all collected eggs with permanent marker relative to the originating hen. We could distinguish eggs from the two females in the same cage on the basis of egg shape and colour (Okuliarova et al. 2009); Japanese quail exhibit extremely high between-female and low within-female variation in egg colour, maculation patterns, and shapes (Pike 2011). Before treatments started, we identified the colour pattern of eggs laid by each female, and to ensure identification of which female laid what egg, we housed females with contrasting egg patterns in the same cage. We checked for fresh eggs every day during the morning hours, and we collected the ones laid on Tuesdays and Thursdays to be incubated in weekly intervals (two eggs per female were incubated each week) in a forced air incubator (GQF model 1402) at 37.5°C and 60% relative humidity. On Wednesdays an additional egg per female was collected weekly for composition analysis (details are reported in Peluc et al. 2012). Due to the high repeatability of levels of T and carotenoids in Japanese quail eggs, levels of such compounds in analyzed eggs reflect levels in incubated eggs (Peluc et al. 2012). Before

the expected hatching day, eggs were transferred to another incubator and kept individually, separated by dividers, to allow us to match hatchlings to particular eggs. Hatching was monitored by frequent checks, and newly hatched chicks were marked with an individual combination of coloured leg bands. At this time, we weighed hatchlings to the nearest 0.01 g on a digital balance and measured tarsus length to the nearest 0.1 mm with digital calipers. We evaluated hatching success of 556 eggs layed during the last 6 weeks of experiment. The number of analyzed eggs is slightly smaller than expected (48 hens × 2 eggs per week × 6 weeks = 576 eggs) because in some weeks we collected fewer than two eggs per female due to some eggs found broken in the cages. All chicks hatched within 72 h of each other were housed together in cages that acted as brooding and rearing pens, provided with ad libitum access to water and chick starter mix (Sprout Non-medicated Chick Starter, Appleton, WI, USA), heating from infrared lights, and a light:dark cycle of 12:12 h. To assess postnatal growth, we calculated the difference between tarsus length at hatch and at age 15 d. We only evaluated the effect of treatments on growth for chicks that survived to age 15 d. We determined offspring sex at 15 d of age using sexually dimorphic plumage and inspection of gonad development after chicks were euthanized. Not all young quail survived to age 15 d due to mortality mostly related to internal organ infections. *Escherichia coli* was isolated from internal organs in large numbers/pure culture in over 95% of dead young quail, thus it is possible *E. coli* was the disease agent of those individuals.

Survey of innate immunity in chicks

We chose two measures of innate immunity that encompass constitutive components of this branch of the immune system: 1) bactericidal capacity of plasma, and 2) size of the bursa of Fabricius. We focused on indices of innate immunity because recent results in chickens indicate that this branch of the immune system is especially influenced by carotenoids, compared with adaptive immunity (Selvaraj et al. 2006). Furthermore, with these two measures we could investigate development (bursa size) and response (bactericidal capacity) of the immune system. The bursa of Fabricius is a primary lymphoid organ unique to birds that is essential for normal development of the humoral immune system, and the major site of B-cell production (Cooper et al. 1965, 1966). The bactericidal activity of plasma is attributable to complement, natural antibodies, and a variety of other pathogen-recognition proteins, which are important humoral components of the immune system in plasma (Matson et al. 2006). One mechanism for carotenoid and vitamin action is that antioxidants protect the humoral components in plasma from oxidative damage, permitting them to destroy bacteria (Alonso-Alvarez et al. 2004). Previous studies suggest that plasma bactericidal capacity and bursal development could be negatively influenced by T and positively afected by carotenoids (Glick 1984, Marsh et al. 1986, McGraw and Klasing 2006, McGraw et al. 2006). For the immunological survey we only used one chick per female per week, yet sample size was reduced to 240 chicks due to mortality prior to age 15 d. Many of the weeks we could not test one chick per female for all females. However, in at least 4 of the 6 weeks we could test one chick per female in 10 females per treatment. When more than one chick was available per female, we randomly chose one of them by assigning each chick a random number and picking the one with the lower number. We chose to sacrifice young at the age of 15 d because it was the youngest age that would allow us to collect minimal data required to evaluate post-natal growth.

We collected blood samples once from each chick at age 15 d, by draining blood immediately after they were euthanized. Because of the high volume of blood needed to perform immunological analyses, samples had to be collected at time of sacrifice (see details on sacrifice age above). Whole blood was centrifuged for 15 min at 15 000 rpm to separate blood cells from plasma, which was aliquoted into 1.5 ml Eppendorf tubes and kept at −80°C until analysis. To evaluate bactericidal activity of plasma we followed the methods used by Matson et al. (2006). Briefly, we added ~600 *Escherichia coli* colony forming units (CFUs, 50 µl) to 20 µl thawed plasma and incubated in 150 µl media (Luria-Bertani broth, EMD Chemicals 1.10285) at 37°C for 45 min. After incubation, we transferred 75 µl aliquots to two agar plates (MacConkey agar), dispersed the solution homogeneously across the plate with a sterile plastic spreader, and incubated the plate for 24 h at 37°C. After incubation we counted the number of bacterial colonies per plate and determined average killing efficiency of the replicate plates for each bird in comparison with control plates prepared with media (170 µl) and *E. coli* (50 µl) only (no plasma). Killing efficiency was highly repeatable for our duplicate samples ($r = 0.84$, $F_{47, 48} = 6.1$, $p < 0.001$) (Lessells and Boag 1987), so we used averages in statistical analyses. Plasma bactericidal activity was expressed as the number of remaining CFUs after incubation of the plasma–bacteria mixture relative to the number of CFUs inoculated on control plates (in the absence of plasma). The differences between the number of viable bacteria after incubation and the number in the initial inoculum are expressed as the proportion killed.

After chicks were euthanized, we measured the length, width, and depth of bursa of Fabricius to the nearest 0.1 mm with calipers and approximated its volume as the product of those three measurements. We chose this method of estimating size instead of weighing the organ for practical reasons, given our knowledge that our measure of bursa volume and weight are highly correlated ($n = 60$ young quail, $r = 0.85$, $p < 0.0001$).

Statistical analyses

Sample size was reduced from expected because of multiple reasons (e.g. eggs failed to hatch, post-hatching mortality). In most cases we could test one chick per female per week, and in praxis we measured traits of 9–12 chicks per treatment per week. We used general linear mixed models to find differences among factors. We tested for effects of carotenoid, GnRH and time (weeks of treatment) and all their interactions. To model the lack of independence of females within cages and the chicks of each female, we added two random effects into the model: cage and

female. According to Littell et al. (1996), this type of modeling pseudo-replication is the design with the highest power. The response variables were: hatching success, offspring survival probability up to 15 d (assuming binomial distribution for both variables and using a logit link function), chick mass at hatch, tarsus length at hatch, bursa volume, chick plasma bactericidal activity at 15 d, and postnatal growth (assuming normal distribution and identity link for all the latter variables). We found no treatment differences in fresh egg mass ($n = 556$; carotenoids: $F_{1,22} = 0.82$, $p = 0.41$; GnRH: $F_{1,22} = 0.52$, $p = 0.73$; time: $F_{5,536} = 0.21$, $p = 0.84$; all interactions $p > 0.15$), so we did not include egg mass in statistical models. We included weight of chicks at age 15 d and sex as covariates when analyzing bursa volume, and included hatching mass and sex as covariates when analyzing plasma bactericidal ability. Those covariates were eliminated from the models when non-significant. In all analyses we simplified the models eliminating in a stepwise manner the non-significant variables and interactions. However, to respect the restrictions imposed by the randomization design, we always kept main effects, and random factors in the model. We only report effects from final models for significant terms. Statistical analyses were performed with Infostat/L package (Di Rienzo et al. 2012) at a significance level of 5%. We performed arcsine transformation on proportion of plasma bactericidal activity prior to analysis to meet normality assumptions. However, to facilitate biological interpretation, we visually present data as untransformed values

Results

Hatching success

Hatching success was enhanced by carotenoid supplementation, whereas it was negatively affected by GnRH treatment (glmm with binomial error distribution; $n = 556$; carotenoids: $\chi^2 = 8.59$, $DF = 1$, $p = 0.0031$; GnRH: $\chi^2 = 6.88$, $DF = 1$, $p = 0.0371$; proportion of total variance explained by random factors: cage = 0.25%, female = 2.71%; Fig. 1).

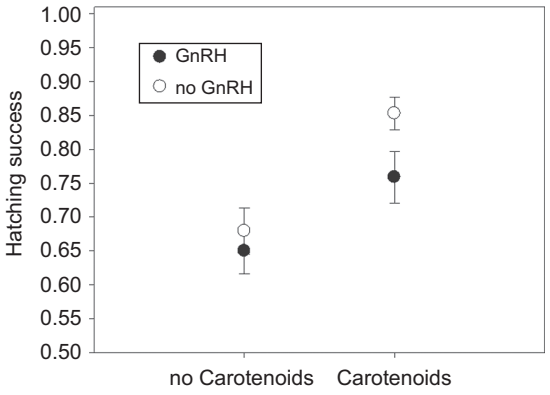

Figure 1. Mean (± SE) hatching success of eggs produced by GnRH-challenged (n = 138), carotenoid-supplemented (n = 141), GnRH-challenged + carotenoid-supplemented (n = 137), and control females (n = 140).

Hatching success for eggs laid by GnRH + carotenoid treated females was intermediate between hatching success of eggs from carotenoid-only treated hens and that of eggs from GnRH challenged hens (Fig. 1).

Size at hatch and postnatal growth

Chicks hatched from eggs produced by GnRH-challenged females were heavier than chicks from control females throughout the study, whereas carotenoid supplementation did not affect hatching mass ($n = 409$; carotenoids: $F_{1,22} = 1.79$, $p = 0.244$; GnRH: $F_{1,22} = 4.85$, $p = 0.0317$; time: $F_{5,379} = 3.72$, $p = 0.025$; proportion of total variance explained by random factors: cage = 0.05%, female = 1.17%; Fig. 2A). Tarsus length at hatch was also affected by GnRH treatment to females. Chicks that hatched from eggs produced by GnRH-challenged females also had significantly longer tarsi than chicks from vehicle-injected females ($n = 409$; carotenoids: $F_{1,22} = 0.38$, $p = 0.55$; GnRH: $F_{1,22} = 6.11$, $p = 0.015$; proportion of total variance explained by random factors: cage = 0.07%, female = 0.98%; Fig. 2B). Postnatal growth between hatching and day 15 was not affected by experimental treatments to hens, and time was the only variable accounting for significant differences in tarsus length

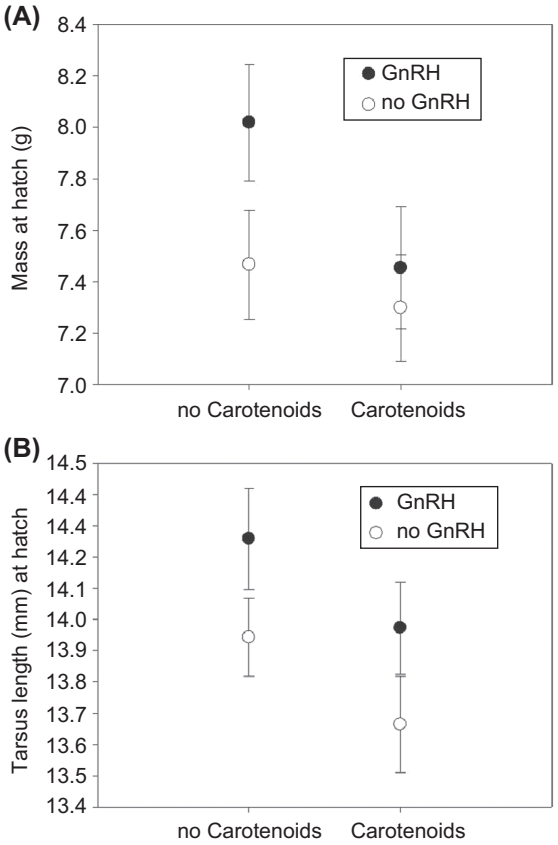

Figure 2. (A) mean (± SE) mass at hatch of chicks from eggs produced by GnRH-challenged (n = 90), carotenoid-supplemented (n = 120), GnRH-challenged + carotenoid-supplemented (n = 104), and control females (n = 95); (B) mean (± SE) tarsus length at hatching of chicks from GnRH-challenged (n = 90), carotenoid-supplemented (n = 120), GnRH-challenged + carotenoid-supplemented (n = 104), and control females (n = 95).

(n = 409; carotenoids: $F_{1,22} = 0.08$, $p = 0.774$; GnRH: $F_{1,22} = 0.36$, $p = 0.554$; time: $F_{5,379} = 15.42$, $p = 0.0001$; proportion of total variance explained by random factors: cage = 0.38%, female = 0.78%).

Innate immunity in chicks

Carotenoid supplementation as well as GnRH challenges to females influenced bactericidal ability of chick plasma (Fig. 3A; n = 240; carotenoids: $F_{1,20} = 468.73$, $p = 0.0001$; GnRH: $F_{1,20} = 188.35$, $p = 0.0001$; time: $F_{5,210} = 8.96$, $p = 0.0001$; GnRH × carotenoid: $F_{1,20} = 182.03$, $p = 0.0001$; carotenoid × time: $F_{1,210} = 4.67$, $p = 0.0006$; proportion of total variance explained by random factors: cage = 0.84%, female = 3.26%). As evidenced by the significant interaction between the main factors, the positive effect of carotenoids on plasma bactericidal ability is weaker in GnRH-challenged females than in absence of GnRH treatment. The enhancement in plasma bactericidal ability produced by carotenoids relative to the control group decreased with time (Fig. 3B). Bursa size at age 15 d was also affected by maternal treatments (n = 240; chick mass at d15: $F_{1,213} = 54.34$, $p = 0.0001$; chick sex: $F_{1,213} = 8.33$, $p = 0.004$; carotenoids: $F_{1,22} = 9.62$, $p = 0.0032$; GnRH: $F_{1,22} = 9.59$, $p = 0.0034$; proportion of total variance explained by random factors: cage = 0.59%, female = 2.31%). Chicks hatched from eggs produced by carotenoid-supplemented females had significantly larger bursa of Fabricius than chicks from eggs produced by unsupplemented females (Fig. 3C). Chicks hatched from eggs produced by GnRH-challenged females had significantly smaller bursa of Fabricius than chicks from eggs produced by unchallenged females. Heavier individuals had significantly larger bursas, and female chicks had consistantly larger bursas than males (mean female bursa volume = 53.5 ± 1.92 mm^3, n = 101; mean male bursa volume = 46.9 ± 2.03 mm^3, n = 139).

Survival to age 15 d

Carotenoid supplementation and GnRH challenge strongly interacted to influence the proportion of chicks that survived to age 15 d (Fig. 4; n = 409; carotenoids: $\chi^2 = 5.08$, DF = 1, $p = 0.024$; GnRH: $\chi^2 = 2.70$, DF = 1, $p = 0.098$; GnRH × carotenoids: $\chi^2 = 6.07$, DF = 1, $p = 0.004$; proportion of total variance explained by random factors: cage = 0.66%, female = 2.84%).

Discussion

We evaluated the impact of two maternal physiological manipulations on offspring morphology and fitness. Dietary carotenoid supplementation of egg-laying female quail had positive effects on egg hatching success and one of the immune function parameters in chicks (bursa size).

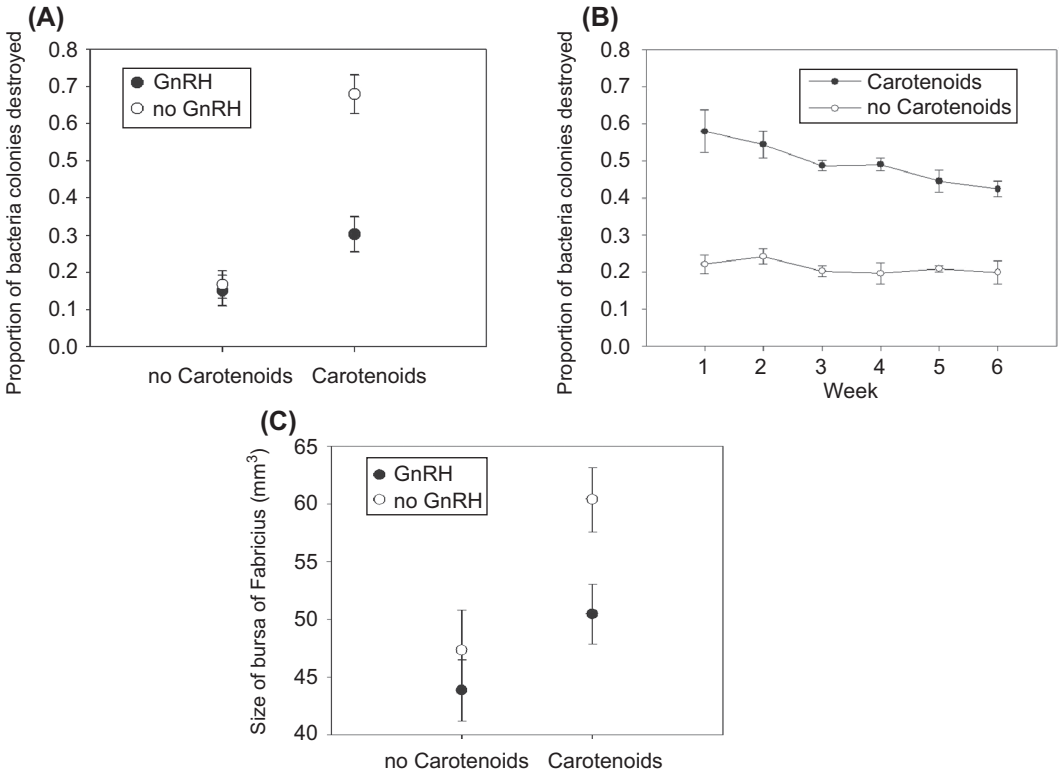

Figure 3. (A) mean (± SE) proportion of bacterial colonies destroyed when exposing plasma of 15 d old chicks from GnRH-challenged (n = 53), carotenoid-supplemented (n = 64), GnRH-challenged + carotenoid-supplemented (n = 63), and control females (n = 60), relative to bacteria colony forming units inoculated in control plates; (B) mean (± SE) proportion of bacterial colonies destroyed when exposing plasma of 15 d old chicks from carotenoid-supplemented and control females during 6 weeks of treatment; (C) mean (± SE) size of the bursa of Fabricius for 15 d old chicks from GnRH-challenged, carotenoid-supplemented, GnRH-challenged + carotenoid-supplemented, and control females.

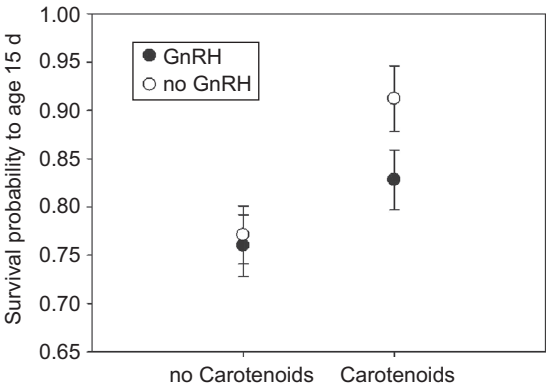

Figure 4. Mean (± SE) proportion of chicks that survived to age 15 d from eggs produced by GnRH-challenged (n = 90), carotenoid-supplemented (n = 120), GnRH-challenged + carotenoid-supplemented (n = 104), and control females (n = 95).

On the other hand, the positive effect of maternal carotenoid supplementation on chick survival probability to age 15 d was reduced by maternal GnRH challenges. Similarly the positive effect of maternal carotenoid supplementation on plasma bactericidal ability declined with time during the study, and it was reduced by GnRH challenges to females. Yet, chicks originating from GnRH + carotenoid treated hens had a better bactericidal response than chicks from GnRH-challenged not carotenoid supplemented hens. In contrast, maternal GnRH challenges enhanced neonatal size, and negatively affected the growth of the bursa of Fabricius in chicks.

We have evidence that carotenoid supplementation and GnRH challenges to laying females increased carotenoids and T yolk deposition, and furthermore that yolk deposition of such compounds is not independent of one another (Peluc et al. 2012). Results presented by Peluc et al. (2012) show that GnRH + carotenoid female quail deposited more carotenoids into yolk than carotenoid supplemented (not challenged) females, and deposited more T into yolk than GnRH (not diet supplemented) females. This allocation pattern may just imply the passive deposition of both compounds (i.e. females deposit greater amounts of those compounds when their level in circulation is higher, Schwabl 1993, Bortolotti et al. 2003). However, an adaptive explanation for the correlation would be that females actively allocate androgens and carotenoids to egg yolks, which benefit their offspring. The trade-off of diminished availability of these compounds to females would be the benefit of higher quality of offspring through enhanced development and competitive ability, or protection against oxidative stress during development.

Because we did not manipulate egg constituents directly (i.e. did not inject carotenoids or T on eggs), we cannot exclude the possibility that treatments altered other aspects of hen physiology and deposition of egg compounds (i.e. other steroid hormones, antioxidants, other nutrients, or antibodies) that may have also affected offspring performance. Yet, our data corroborates the findings of other studies (Surai et al. 2003, Groothuis et al. 2005, Karadas et al. 2006, Koutsos et al. 2007, Meriwether et al. 2010, Saino et al. 2011), in that developmental and immunological changes observed in Japanese quail chicks are likely related to changes in yolk composition (i.e. amounts of T, carotenoids, and vitamins A and E). Similarly, we cannot attribute changes in chick performance to variation in egg quality through time, as we did not find egg mass differences attributable to time (i.e. egg laying position) or treatments. Although egg quality may be affected by laying order in supernumerary clutches (Heaney et al. 1998, Nager et al. 2000, Williams and Miller 2003, Mand et al. 2007, Verboven et al. 2010, but see Cucco et al. 2011), species-specific relationships between egg quality and laying sequence result in a variety of patterns (Aparicio 1999). For example, previous studies in Japanese quail suggest that precocial birds with long laying sequences display relatively low intra-female differences in yolk T concentrations (Gil and Faure 2007, Bertin et al. 2008, Okuliarova et al. 2009). Nevertheless, although we did not find strong temporal variation in yolk carotenoids or T levels (Peluc et al. 2012) or other constituents (yolk mass, albumen mass, shell mass; Peluc et al. unpubl.), we cannot exclude the possibility that other parameters of egg quality not measured here could have been affected by egg laying position and ultimately affect chick performance.

We observed that maternal carotenoid supplementation enhanced egg hatching success, whereas maternal GnRH-challenges resulted in higher egg hatching failure. This is not unexpected as negative effects of in ovo T on egg hatching have been reported (Sockman and Schwabl 2000, Navara et al. 2005, Rutkowska and Cichon 2006, Boncoraglio et al. 2011). Conversely, maternally deposited yolk carotenoids may enhance hatchability by lowering the activity of potentially harmful reactive-oxygen species during embryonic development (McGraw et al. 2005), or by enhancing eggs antibacterial activity (Cucco et al. 2007). Similarly, enzymatic antioxidants such as vitamin E scavenge ROS and protect cells from oxidative damage (Finkel and Holbrook 2000). Yet, one interesting result here is that hatching success of eggs produced by GnRH + carotenoid-treated hens was higher than that of eggs from GnRH females receiving control diet. This suggests that the combination of resources deposited in eggs from GnRH + carotenoid-treated hens interact in such way that one may mitigate detrimental effects of the other.

Maternal GnRH-challenges enhanced neonatal size but did not affect offspring growth. We did not find evidence of strong egg size differences among treatments. Hence the differences in neonatal size may result from increased prenatal growth rate due to higher T levels in eggs produced by GnRH-challenged females (Peluc et al. 2012). Yolk androgens have positively affected embryonic growth rates and development in other avian species (reviewed by Groothuis et al. 2005a). Yet, yolk androgens may affect growth differently at different stages of development (Sockman and Schwabl 2000, Andersson et al. 2004, Rubolini et al. 2006b, Cucco et al. 2008, Hegyi and Schwabl 2010). Possibly in our study system an age-specific effect of yolk androgens on growth may be related to environmental circumstances such as food availability and degree of sibling competition (Müller et al. 2005). Ad libitum access to food in our experiment may have diminished the impact of com-

petition for resources and account for the lack of differences in post-hatching growth rates across treatments (Navara et al. 2006b, Müller et al. 2008). When food is more limited, larger offspring may have outcompeted smaller ones (Navara et al. 2006b).

We have showed that maternal GnRH challenges are likely to negatively impact aspects of immunity in quail chicks, whereas maternal carotenoid supplementation is mostly related to enhanced immune performance in offspring quail. For example, we observed independent and positive effects of maternal carotenoid supplementation on the size of bursa of Fabricius, whereas offspring from females receiving GnRH challenges had smaller bursa than offspring from unchallenged females. The bursa is particularly sensitive to increased T levels (Glick 1983), which may disrupt embryonic development and growth (Hirota et al. 1976, Click 1980, Glick 1983, 1984, Olsen and Kovacs 1996). Elevated levels of vitamin E, on the other hand, promote the development of this immune organ (Gore and Qureshi 1997), and its deficiency is known to depress its growth (Marsh et al. 1986) and reduce circulating lymphocytes in chicks (Dietert et al. 1983). However, the combined effects of maternal GnRH and caroternoid treatments resulted in chicks with intermediate bursa size. Once again this may be evidence that maternal yolk T and carotenoids can offset each others effects. Similarly, plasma bactericidal ability of offspring seems to be affected by maternal carotenoid supplementation and GnRH challenges, providing evidence of synergistic effects of those compounds on this aspect of chick immune performance (Royle et al. 2001, Safran et al. 2008). The negative impact of maternal GnRH challenges on plasma bactericidal capacity was highly mediated by carotenoid supplementation to females. When females are not fed carotenoids GnRH-challenge has no negative effect on bactericidal ability of chick plasma. However, when females are carotenoid-supplemented, GnRH has a negative effect on chick plasma bactericidal ability. This last observation may well be related to the fact that females receiving a carotenoid supplemented diet deposited more T in their eggs than not supplemented females (Peluc et al. 2012).

Besides independent effects of maternally transferred resources to offspring, our results emphasize that maternal effects on embryo development and offspring immune performance differ depending on the combination of quantity and quality of the resourses deposited in the yolk. Previous studies had proposed possible interactive effects of maternal yolk androgens and carotenoids on chick performance (Royle et al. 2001, Navara et al. 2006a, Rubolini et al. 2006a, Török et al. 2007, Safran et al. 2008). Yet, to our knowledge experimental tests of such hypothesis are lacking. Cucco et al. (2008) examined the combined effect of prenatal (ovo injection) exposure to T and carotene dietary supplements to young grey partridge *Perdix perdix*. They observed that negative effects on immunity of high dose in ovo T are reduced in chicks fed a rich beta-carotene diet. Our study corroborates the complementary and opposing effects of carotenoids and T on chick immune function. Yet, by physiologically manipulating maternal resource allocation to eggs we took into account the fact that a female may alter several egg components simultaneously, and that effects of such compounds on offspring performance depend on their combination in quantity and quality. It is evident from results by Peluc et al. (2012) that resource allocation to eggs is a complex phenomenon, and different resourses are not independently deposited to the yolk (e.g. more carotenoids are deposited to eggs of GnRH challenged than not challenged females). Female birds are thought to fine tune development of individual offspring by adjusting the relative amounts of yolk androgens and yolk antioxidants (Royle et al. 2001, Groothuis et al. 2006, Williamson et al. 2006, Peluc et al. 2012). Yet, linking offspring performance with patterns of maternal deposition, can be challenging because trade-offs between mother and offspring and pleiotropic interactions among different egg components are hypothesized to result in opposite effects (Royle et al. 2001). If females can differentially allocate androgens and antioxidants to egg yolk to enhance offspring performance, the adaptive value of such deposition patterns should be validated by fitness proxies in chicks, such as size at hatching, growth and immunity. Such evidence has been provided in this study. Here, we show a direct connection between maternal deposition of yolk carotenoids and hormones and their positive, negative and interactive effects on offspring performance.

We have provided evidence that early maternal effects mediated by differential deposition of antioxidants (carotenoids and vitamins A and E) and T to eggs (Peluc et al. 2012) are able to affect offspring immune response and viability (Karadas et al. 2005). Future studies should examine effects of the yolk environment as an integrated multi-component system (i.e. considering an array of biomolecules deposited by the female). Such an approach will help evaluate costs and benefits of the combined influences of egg yolk constituents, and will further enhance our understanding of the the mechanism and adaptive significance of maternal resources allocated to eggs.

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