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ORIGINAL PAPER

Carotenoid supplementation and GnRH challenges influence female endocrine physiology, immune function, and egg-yolk characteristics in Japanese quail (*Coturnix japonica*)

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Abstract Androgens and carotenoids circulating in plasma affect the physiology and behavior of vertebrates. Much is known about control mechanisms and functions of each of these substances, yet their interactive effects are not well understood. Here we examine possible additive, multiplicative, and interactive effects of testosterone and carotenoids on female endocrine physiology, immunocompetence, and investment in eggs by simultaneously manipulating levels of testosterone [via gonadotropin releasing hormone (GnRH) challenges] and carotenoids (via diet supplementation) in captive female Japanese quail (*Coturnix japonica*). Females were randomly assigned to one of four treatments: carotenoid supplementation, GnRH challenge, GnRH challenge + carotenoid supplementation, or control. Carotenoid supplementation significantly increased circulating plasma carotenoid levels and acquired immune system performance, but not innate immunity. GnRH challenges elevated circulating testosterone and carotenoid levels, and induced immunosuppression in females. However, females in the GnRH challenge + carotenoid supplementation treatment had higher cell-mediated immune responses than control females and similar responses to those of carotenoid-supplemented females. Hence, availability of carotenoids in female quail seemed to counteract immunosuppressive effects of GnRH challenges. Our results provide further evidence for synergistic effects of carotenoids and testosterone on endocrine physiology and immune function in female birds. Elevated plasma testosterone or carotenoids levels resulted in increased deposition of those compounds to eggs, respectively. Furthermore, because we found that concentrations of testosterone and carotenoids in yolks were correlated within each treatment group, differential deposition of hormones and carotenoids in eggs may not only respond to surrounding social and environmental conditions, but also to other components of the egg.

Keywords Acquired immunity · Innate immunity · Maternal effects · PHA · Testosterone · Vitamin E · Yolk carotenoids · Yolk steroids

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Introduction

The functions and control mechanisms of several biomolecules that affect physiological and behavioral traits of animals have traditionally been viewed and studied separately. Such is the case of androgens and carotenoids (Ketterson et al. 2009; McGraw and Ardia 2003; Staub and DeBeer 1997), which are known to have widespread effects on both adult and offspring phenotypes (Gil 2008; Groothuis et al. 2005; McGraw et al. 2005; Ros et al. 1997; Surai et al.

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2001b). To date, effects of each of these molecules have been well-studied in birds, both in adulthood and during development, when birds are exposed to maternal sources of these compounds (Eising et al. 2006; Groothuis et al. 2005; McGraw et al. 2005; Schwabl 1993; Surai et al. 2001a, b). Recent studies suggest that androgens and carotenoids can have interactive effects in adult birds (Blas et al. 2006; McGraw et al. 2006a; McGraw and Parker 2006; Peters 2007), and in eggs and chicks (Royle et al. 2001; Safran et al. 2008). Yet, knowledge of costs and benefits associated with interactions of these molecules in mothers and offspring are still uncertain (Cucco et al. 2008; McGraw and Ardia 2007; McGraw et al. 2006a; Safran et al. 2008).

The effects of androgens on morphology, physiology and behavior have been well documented in adult males (Casto et al. 2001; Ketterson and Nolan 1999; Saino et al. 1995; Strasser and Schwabl 2004; Wingfield et al. 1990), but few studies have examined the role of androgens in adult female vertebrates (Staub and DeBeer 1997; Ketterson et al. 2005). Similar to males, circulating testosterone levels in female birds are elevated during breeding (Ketterson and Nolan 1999), which is often coupled to the timing of aggressive conflicts (Hegner and Wingfield 1987; Langmore et al. 2002; Smith et al. 2005; Zysling et al. 2006). Experimental studies reveal associated costs of elevated testosterone to females, including decreased survival, increased stress, impaired molting (Ketterson et al. 2009; Klukowski et al. 1997), and changes in reproductive attributes like nest-defense, egg-laying, ovulation, and fecundity (Cawthorn et al. 1998; O'Neal et al. 2008; Rutkowska et al. 2005). Furthermore, some studies show that experimentally elevated testosterone levels in females can suppress humoral and cell-mediated immunity (*Sturnus vulgaris*, Duffy et al. 2000; *Junco hyemalis*, Zysling et al. 2006). However, others have shown elevated testosterone to be immunosuppressive in the laboratory but not in the field (*Malurus cyaneus*, Peters 2000), or found no association between testosterone and immunity (Hasselquist et al. 1999; Roberts et al. 2004).

In contrast to androgens, carotenoids and a range of lipid-soluble vitamins (i.e., vitamins A and E) can affect adult health and reproduction by acting as immunostimulatory molecules (Alonso-Alvarez et al. 2007; McCay 1985; McGraw and Ardia 2003; Peters 2000; Saino et al. 2003; Surai et al. 1999a, b). Carotenoids are not produced by animals and must be acquired from the diet (Goodwin 1986). It is likely that health benefits occur through changes in gene regulatory and cellular communication functions, especially in tissue regeneration and the immune response through membrane stabilization (Young and Lowe 2001). Fitness benefits of carotenoids occur both in mothers (e.g. health, reproduction; Blount et al. 2004), and in young who receive more carotenoids during development (e.g. health, growth, survival; Ewen et al. 2009; Newbrey and Reed 2009; Tanvez et al. 2009).

In addition to separate androgen- and carotenoid-related processes, interactive effects between these biomolecules have been demonstrated in adults, eggs, and hatchlings of several fish and bird species. One such example is the androgenic control of carotenoid pigmentation in fishes and birds (Jayasooriya et al. 2002; McGraw et al. 2006a). In male birds, testosterone up-regulates circulating lipoproteins, which are plasma carriers for carotenoids, thereby increasing carotenoid bioavailability (Blas et al. 2006; McGraw et al. 2006a). Because carotenoids act as potent immune-stimulants (Blount et al. 2003b), an important interaction between testosterone and carotenoids is the ability of carotenoids to physiologically offset any immune costs of elevated testosterone. For example, in zebra finches (*Taeniopygia guttata*), circulating levels of testosterone are implicated in the active mobilization of circulating carotenoids, which in turn boost immune function (McGraw and Ardia 2007). Although androgens may suppress female immune responsiveness (Casto et al. 2001; Duffy et al. 2000) in similar ways as they do in males, in the one experimental test of this in female birds elevated testosterone did not enhance bioavailability of carotenoids in zebra finches (McGraw 2006b). Immune-boosting effects of testosterone may be sex-specific (McGraw and Ardia 2005), but the interaction between androgens and carotenoids in relation to health are rarely studied in females (Ketterson et al. 2005; McGraw and Ardia 2007) and deserve further investigation.

One additional consequence of these biomolecules in females is their effects on offspring, which are not necessarily the same as those for mothers. There is some suggestion that females can balance the costs and benefits associated with androgen and carotenoid deposition in eggs, thus making adaptive investments in offspring that can enhance offspring survival (Gil et al. 1999; Pilz et al. 2003; Schwabl 1993). Depending on the ecological context, elevated yolk testosterone has advantageous, detrimental, or no effects on offspring behavior, development, and fitness (Gil 2003; Groothuis and Von Engelhardt 2005). For example, yolk androgens may elevate competitiveness and growth after hatching, which provides an advantage when competing with siblings, but elevated androgens may also be immunosuppressive and cause oxidative damage during rapid growth (Daisley et al. 2005; Groothuis et al. 2005; Ketterson and Nolan 1999; Okuliarova et al. 2007). In comparison, carotenoids deposited into the yolk are stored in embryonic tissues (e.g. adipose, liver) and protect against potentially harmful oxidative processes that occur during development (Surai and Speake 1998; Surai et al. 1999b). Similarly, yolk carotenoids enhance immune function in newly hatched chicks (Haq et al. 1996; Krinsky 2001;

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McGraw and Ardia 2003; Saino et al. 2003; Peluc et al., in review). The presence of both androgens and carotenoids in yolk may create complex, interactive effects on maternal investments to offspring and offspring development. Furthermore, because of effects on females and offspring, allocation of these resources to eggs versus a female's own physiological and behavioral function should be such that mothers balance their own benefits and costs as well as costs and benefits to offspring in a way that maximizes fitness (Navara et al. 2006a).

In this study, we investigated possible additive, multiplicative, and interactive effects of maternal steroids and carotenoids on female endocrine physiology, immune function, and the allocation of these compounds to egg yolks. We manipulated levels of both testosterone [via gonadotropin releasing hormone (GnRH) challenges] and carotenoids (via diet supplementation) in captive female Japanese quail (*Coturnix japonica*). GnRH is a hormone released from the hypothalamus that controls a cascade of hormone secretion events from the pituitary and gonads. Intramuscular injections of GnRH temporarily stimulate 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). GnRH challenges are often used to assess the reproductive condition of individuals (Goymann and Wingfield 2004; Hirschenhauser et al. 2000; Lacombe et al. 1991; Moore et al. 2002; Soma and Wingfield 2001), but recently GnRH challenges have been used to quantify seasonal and individual variation in capacity for steroid hormone synthesis (Jawor et al. 2007). One consequence of a GnRH challenge is elevation of yolk steroid levels (e.g., testosterone), which has the potential to influence attributes of offspring via the yolk. This provides an opportunity to examine steroid and carotenoid effects on mothers and offspring, which could be beneficial to both, detrimental to both, or create conflict between mothers and offspring (Gil 2003; Groothuis and von Engelhardt 2005; Ketterson et al. 2005). Here we predicted that elevated testosterone or carotenoid levels in female plasma are related to elevated deposition of those compounds in egg yolks. We expected to see an immune-enhancing effect of elevated carotenoid levels in plasma, and immune-suppressing effect of elevated plasma testosterone in adult females. However, because carotenoids and androgens can exert mechanism-specific actions on different lines of immune defense in birds (e.g., Biard et al. 2007; McGraw and Ardia 2007; McGraw and Klasing 2006; Tobler et al. 2010; Tschirren et al. 2005; Zysling et al. 2006), we measured responses of both innate and acquired immunity. Furthermore, we predicted that females exposed to higher levels of both testosterone and carotenoids would have higher immunocompetence than those exposed to high levels of testosterone alone because of synergistic effects between these two compounds (Navara et al. 2006a).

Materials and methods

Experimental design

Housing and treatment of laying birds

We evaluated the effects of GnRH and carotenoid supplementation in a captive population of Japanese quail. Previous studies of Japanese quail indicate they provide an appropriate model system to evaluate these interactive effects because of the ability to manipulate plasma levels of carotenoids through diet (McGraw 2006a) and testosterone through GnRH challenges (Peluc et al., unpublished). Furthermore, their life history is such that it allows us to evaluate effects on reproductive investment because they mature in 7–8 weeks of age and start reproducing and produce fertile eggs nearly daily (Huss et al. 2008) when provided with appropriate photoperiods (Robinson and Follett 1982). We randomly selected 48 adult female and 24 adult male Japanese quail for study from a pool of individuals hatched and raised in the laboratory. All individuals selected were 12 weeks of age, and all females started laying 28 ± 3 days prior to the start of the experiment. We housed adults in separate cages at a ratio of two females and one male per cage in an animal-approved indoor room at North Dakota State University. We banded each individual with a unique combination of two colored leg bands. Quail received an ad libitum diet of water and commercial game bird mix (Sprout Meat Maker, Appleton, WI, USA), which contained a minimal amount of xanthophylls (ca. 5 mg/kg). Female quails were maintained on a light:dark cycle of 14:10 h and ambient temperature of approximately 22 ± 2°C, and were randomly 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 housed in the same cage received the same water treatment (carotenoids or no carotenoids), but were not necessarily in the same injection (GnRH or saline) treatment.

Carotenoid supplementation

Carotenoid-supplemented birds received two common plant carotenoids, lutein and zeaxanthin, at a dose of 7.5 μg ml^{-1} drinking water, whereas unsupplemented individuals received no carotenoids in their drinking water. From a pilot study on the same quail species, we determined that the average daily amount of fluid consumption per individual was 35 ± 5 ml. Hence treated individuals consumed between 2.25 and 3 mg of carotenoids per day.

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The selected dose is well within the range of doses previously used on carotenoid-supplemented Japanese quail (McGraw 2006a: 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 Ltd. (Parsippany, NJ). All drinks were freshly prepared each day using cool water and were provided in opaque dispensers to avoid oxidation (Blount et al. 2003a). Supplementation in this study began on 18 February 2008 and continued for 7 weeks.

GnRH challenges and control injections

The ability to increase plasma testosterone levels in response to a GnRH challenge seems to be related to the responsiveness of the ovary to GnRH (e.g., presence of hierarchical follicles) at the time of the experimental manipulations. For example, Jawor et al. (2007) found that ovarian response to GnRH-induced increases in LH was significant during egg development in female dark-eyed juncos, whereas females failed to increase plasma testosterone in response to GnRH during other stages of reproduction. In the present study, we chose to challenge female quail during the egg-laying stage to ensure ovary receptivity to the treatment. All females treated were actively laying one egg daily prior to the start of the experiment and continued laying eggs throughout the 7-week experiment.

Results from a previous experiment (Peluc et al., unpublished) indicated that GnRH injections to female quail significantly elevated plasma testosterone levels within the physiological range previously observed in this species (Bertin et al. 2008; Hackl et al. 2003; Ottinger and Brinkley 1979). The same experiment indicated that testosterone concentrations in Japanese quail eggs were at a maximum (36 ± 2 pg mg^{-1}) approximately 2 weeks after maternal GnRH challenge, after which testosterone concentrations declined. Thus, in an attempt to elevate already high yolk testosterone levels in quail 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 days) following Jawor et al. (2007). Briefly, we collected an initial blood sample from each individual from the alar vein (300–600 µl) into Microvette (Sarstedt) tubes treated with lithium heparin and stored them cold (4°C) until processing. Then we immediately administered an intramuscular GnRH or saline injection in the right pectoral muscle of the birds. GnRH challenges were performed using an injection of 5 µg cGnRH-I (Sigma L0637, St. Louis, MO, USA) in 50 µl phosphate-buffered saline (PBS) solution. Control injections consisted of 50 µl PBS (saline) only. We collected a second blood sample 30 min post-injection. Birds were returned to their cages following injections and between sample collections. Whole blood was centrifuged to separate plasma from blood cells, and plasma was aliquoted into three separate 1.5-ml Eppendorf tubes and kept at −80°C until analysis for testosterone, carotenoids, and immune function (see below).

Yolk samples

To analyze yolk testosterone and carotenoid contents, we collected one egg per female on the day before carotenoid and GnRH treatments were initiated ($n = 48$), and one egg per female every week for 7 weeks after treatments were initiated ($n = 336$). Eggs were collected fresh during the morning hours. To ensure that we could identify the originating hen for each egg, before treatments started we documented the color pattern of eggs laid by each female. Previous observations of eggs laid by the quail under study indicate that there is great variability among females in the egg coloration pattern, although there is consistency of color pattern within females. Accordingly, we housed females with contrasting egg patterns in a single cage. Thus, we were able to effectively determine the origin of each egg laid. We separated fresh yolks from eggshell and albumen and aliquoted yolk into two 1.5 ml Eppendorf tubes, maintained at −80°C for later analysis. Samples for carotenoids were placed in darkened tubes to prevent light degradation of the samples.

Immune measures

We used two different measures of immune function to evaluate both the innate and acquired immune systems: (1) bactericidal assay, and (2) PHA-induced wing-web swelling.

Bactericidal assay We tested the bactericidal activity of adult plasma with an assay that measures constitutive, innate immunity (Matson et al. 2006). Specifically, this assay measures the ability of complement, natural antibodies, and a variety of other pathogen-recognition proteins (but not cellular components) to destroy bacteria. One reason for selecting this assay was that carotenoid supplementation previously was found to enhance bactericidal activity of blood in society finches (*Lonchura domestica*, McGraw et al. 2006b) and red jungle fowl (*Gallus gallus*, McGraw and Klasing 2006). We followed the methods of Matson et al. (2006) using *Escherichia coli* bacteria (V1 isolate, Wooley et al. 1993). Briefly, we added ~600 *E. coli* colony forming units (CFUs, 50 µl) to 20 µl of plasma incubated in 150 µl media (Luria–Bertani broth, EMD Chemicals 1.10285, Gibbstown, NY, USA) at 37°C for 45 min. After incubation, we transferred 75-µl aliquots of each sample to two agar plates (MacConkey agar, EMD Chemicals

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1.00205, Gibbstown, NY, USA), 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 the 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). Repeatability for killing efficiency was moderately high for our duplicate samples ($r = 0.77$, $F_{47,48} = 7.2$, $P < 0.01$; Lessells and Boag 1987), so we used averages in statistical analyses.

PHA-induced wing-web swelling At the end of the 7-week experiment, we evaluated one aspect of the acquired immune system by assessing the inflammatory response of birds to a novel and harmless foreign body [phytohemagglutinin (PHA)]. This is an immune challenge that has been widely used as an indicator of the immune-competence of an individual (Blount et al. 2003a; El-Lethey et al. 2003; Saino et al. 2003; Wayland et al. 2002) and is enhanced by carotenoid supplementation in chickens (*Gallus domesticus*, Koutsos et al. 2006) and zebra finches (Blount et al. 2003a; McGraw and Ardia 2003). We subjected all female quail to one immune challenge at the end of the seven-week experiment. A 1.0 cm patch on the left midpatagium was cleared of feathers. Three measures of thickness were taken using a pressure-sensitive digital micrometer (Mitutoyo 293-369) to obtain an average pre-injection measurement. The bare skin was swabbed with alcohol, and 50 mg of (PHA)-P (Sigma L8754, St. Louis, MO, USA) in 50 ml PBS was injected subcutaneously using a 27-gauge needle. Injection dosages were extrapolated according to weight from the amounts used in a variety of bird species in a study by Smits and Williams (1999), which also showed that a control injection is not required to accurately assess the swelling response to PHA in birds; thus no control injection was performed. Twenty-four hours after the initial injection, we measured the injection site three times to obtain the wing-web swelling in response to the mitogen, because further swelling after this time point is unlikely to occur (Lees and Peres 2008). We subtracted the average thickness of the wing-web post-injection from the average thickness of the wing-web pre-injection to determine immunoresponsiveness (sensu Smits et al. 1999). Repeatability of wing-web swelling measurements was moderately high (pre-injection: $r = 0.68$, $F_{47,48} = 5.72$, $P < 0.05$; post-injection: $r = 0.59$, $F_{47,48} = 4.67$, $P < 0.05$).

Yolk and plasma testosterone analyses

We measured testosterone concentrations in yolk samples using radioimmunoassay (RIA) following the protocol established by Schwabl (1993) and modified by Boonstra et al. (2009). Briefly, hormones were extracted from 0.03 g of yolk homogenate dissolved in 1 ml of water. We added 4 ml of petroleum:diethyl ether (30:70, v/v) to the sample, vortexed for 5 min, and let the phases separate at room temperature for 20 min. The ether phase was decanted after snap-freezing the aqueous phase in an ethanol bath at −30°C. This procedure was repeated twice more and all ether phases were combined in a single tube, and evaporated to dryness under a stream of nitrogen. Dried extracts were re-dissolved in 1 ml 90% ethanol and kept at −20°C overnight. Samples were then centrifuged for 10 min (4°C, 2,000 RPM) to further remove proteins. We saved the ethanol phase, added 2 ml of hexane, and recovered the ethanol phase again. Samples were dried in a water bath at 40°C under a stream of nitrogen before re-suspending the extracts in PBS buffer containing gelatin (PBSg), which were used directly for RIA analysis (i.e., no column chromatography). Pooled yolks from multiple females were used to create PBSg buffer containing yolk stripped of hormones and lipids as a background for the standard curves. We used the protocol established by Wingfield et al. (1984) for stripping plasma of steroids to remove steroids from egg yolks. This stripped yolk buffer allowed us to create a standard curve that contained proteins or any other compounds not removed during the extraction process, which provides an appropriate comparison for our extracted samples.

We measured testosterone concentrations in plasma samples using RIA following the protocol established by Wingfield and Farner (1979). We extracted hormones from approximately 150 µl plasma dissolved in 250 µl double distilled water. We added 5 ml of distilled dichloromethane to each sample, vortexed them and let the samples sit for 2 h, after which time we removed the dichloromethane phase, evaporated and re-suspended the extracts in PBSg. We used this suspension directly for RIA analysis. Recovery rates were evaluated by spiked samples (20 µl labeled steroid; approximately 2,000 cpm) prior to the extraction process. We ran duplicate samples in nine RIAs for yolk and four for plasma. Inter- and intra-assay variation (based on internal standards) of yolk testosterone were 6.96 and 10.50%, respectively. Inter- and intra-assay variation (based on internal standards) of plasma testosterone were 3.01 and 6.86%, respectively, as measured from duplicates.

Plasma and yolk carotenoid analyses

We measured lipid-soluble carotenoids and vitamins (A and E) in plasma and yolk using high-performance liquid chromatography (HPLC). Carotenoids were extracted from ca. 0.05 g thawed yolk or 50 µl plasma using 500 µl ethanol and 500 µl tert-butyl methyl ether (TBME) and vortexing

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the solution for 20 s after the addition of each solvent. The tube was centrifuged for 15 s at 10,000 RPM, at which point the supernatant was removed and evaporated to dryness. We re-suspended the residue in 200 µl mobile phase (methanol:acetonitrile:dichloromethane, 42:42:16, v/v/v) and injected 50 µl into a Waters Alliance 2695 HPLC system (Waters Corporation, Milford, MA) fitted with a Waters YMC Carotenoid 5.0 µm column (4.6 mm × 250 mm) and a built-in column heater set at 30°C. We used a three-step gradient solvent system to analyze both xanthophylls and carotenes in a single run, at a constant flow rate of 1.2 ml/min: first, isocratic elution with 42:42:16 (v/v/v) methanol:acetonitrile:dichloromethane for 11 min, followed by a linear gradient up to 42:23:35 (v/v/v) ethanol:acetonitrile: dichloromethane through 21 min, held isocratically at this condition until 25 min, and finishing with a return to the initial isocratic condition from 25 to 29.5 min. We used a Waters 2996 photodiode array detector and collected data from 250 to 600 nm. We identified pigments by comparing their respective retention times and absorbance maxima (λ_{max}) to those of reference carotenoids run as external standards. Three carotenoids (lutein, zeaxanthin and β-cryptoxanthin) and two vitamins (A and E) were identified in egg yolks, whereas only lutein and zeaxanthin were identified in plasma.

Statistical procedures

We performed separate repeated-measures analyses of variance (ANOVA) to test the effects of treatment (carotenoid supplementation, GnRH challenges, and their combination) on wing-web swelling, plasma and yolk testosterone concentrations, plasma and yolk carotenoid concentrations, and bacterial-killing ability of plasma. Treatment and date (date of challenge, or date of sample collection) were included as fixed factors, cage ($n = 24$) was included as a random effect, and female individual identity was included as a random effect nested within cages. The effect of time between challenge and egg collection (either 7 or 14 days) was examined with a model that included cage as random effect and female nested within cages. We used Tukey's post hoc tests when significant differences from ANOVAs were detected. Additionally, to test whether each GnRH challenge was effective in elevating testosterone levels, we performed paired t tests that compared initial (baseline) plasma testosterone to post-challenge plasma testosterone. A compound symmetrical covariance structure was used for the repeated measures of plasma testosterone levels in response to GnRH challenges, in order to calculate the within-individual correlation coefficient, which is a measure of repeatability (Lessells and Boag 1987). To further understand relationships among response variables, we ran correlations within treatment groups, between immune measures and changes in plasma and yolk carotenoid concentrations for individuals over the course of the 7-week treatment period. Similarly, we ran correlations to compare testosterone levels in yolk to baseline levels in plasma, post-challenge plasma, and integrated response to GnRH challenge (post-challenge plasma testosterone − baseline plasma testosterone). We log transformed data when necessary to meet normality assumptions. However, to facilitate biological interpretation, we present untransformed values in figures and tables. All statistical analysis was conducted with program JMP (SAS Institute 2006).

Results

Effects of treatments on female endocrine, carotenoid, and immune status

Plasma testosterone response to GnRH challenges

Female baseline testosterone levels, measured immediately before GnRH injections were administered, did not differ significantly among treatments ($R^2 = 0.71$, treatment: $F_{3,24} = 1.17$, $P = 0.33$; cage: $F_{20,24} = 2.59$, $P = 0.02$, Fig. 1a). Testosterone levels post-injection were significantly higher in GnRH-challenged females (i.e., GnRH and GnRH + carotenoid treatments) than in females injected with saline in the carotenoid and control treatments ($R^2 = 0.57$, treatment: $F_{3,132} = 5.16$, $P = 0.001$; date: $F_{3,132} = 1.97$, $P = 0.01$, treatment by date: $F_{9,132} = 3.97$, $P = 0.05$, cage, $F_{20,24.61} = 0.94$, $P = 0.54$, individual female, $F_{24,132} = 1.84$, $P = 0.04$; Fig. 1b). Indeed, paired t tests on the same individual between baseline and post-injection plasma testosterone levels revealed that plasma testosterone levels increased on each GnRH-injected female at each of the four challenge time-points (GnRH: $r = 0.63$, $t_{46} = 5.48$, $P < 0.0001$; GnRH + carotenoid: $r = 0.63$ $t_{46} = 5.57$, $P < 0.0001$). However, testosterone levels after saline injections did not differ significantly from baseline levels in carotenoid (paired samples t test; $r = 0.01$, $t_{46} = -0.10$, $P = 0.85$) or control females (paired samples t test; $r = 0.04$, $t_{48} = 0.26$, $P = 0.74$). Similarly, the response to GnRH challenges (i.e., difference between baseline and post-injection plasma testosterone levels) was significantly higher in females injected with GnRH than carotenoid-supplemented or control females; ($R^2 = 0.38$, treatment: $F_{3,132} = 5.29$, $P = 0.002$, date: $F_{3,132} = 0.49$, $P = 0.68$, treatment by date: $F_{9,132} = 1.18$, $P = 0.31$, cage: $F_{20,25.50} = 1.12$, $P = 0.38$, female: $F_{24,132} = 0.75$, $P = 0.78$; Fig. 1c). Repeatability of response to GnRH challenges was relatively low ($r = 0.33$, $F_{22,24} = 1.80$, $P = 0.03$). Baseline testosterone levels and

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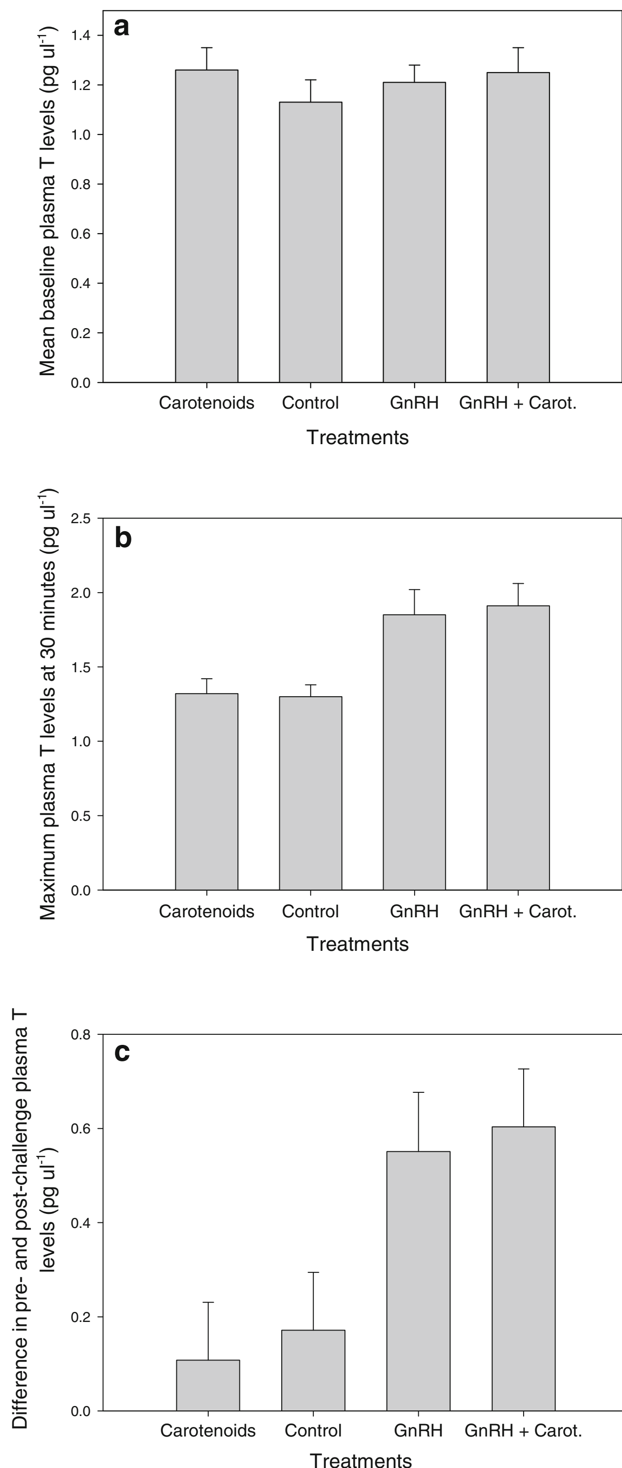

Fig. 1 Plasma testosterone levels in adult female Japanese quail: **a** before and **b** 30 min after GnRH challenge, and **c** challenge mean response (post- pre-challenge testosterone levels), relative to treatment. Values for each treatment are mean (±SE) obtained from the four challenges performed on the females

post-GnRH challenge levels were correlated within individuals in both GnRH and GnRH + carotenoid treatments (Pearson correlation; GnRH: $r = 0.66$; GnRH + carotenoid: $r = 0.77$, $P < 0.001$), but not for the other treatments (carotenoids: $r = 0.16$, $P = 0.09$; control: $r = 0.12$, $P = 0.12$).

Plasma response to carotenoid supplementation

Dietary carotenoid supplementation resulted in a significant increase in plasma lutein and zeaxanthin concentration. Overall, females in the carotenoid and GnRH + carotenoid treatments had the highest levels of carotenoids in their plasma, whereas plasma carotenoid levels in females from the GnRH treatment did not differ from those of control females ($R^2 = 0.97$, treatment: $F_{3,66} = 55.16$, $P < 0.001$, date: $F_{2,66} = 70.08$, $P < 0.001$, treatment by date: $F_{6,66} = 7.31$, $P = 0.02$, cage: $F_{20,22.37} = 9.87$, $P = 0.01$, female: $F_{23,66} = 0.47$, $P = 0.97$; Table 1). The significant difference in plasma carotenoid levels among dates of blood collection implied strong variability through time, with an increase shortly after supplementation started, a continued increase throughout the experiment, and a moderate decline after supplementation stopped (Fig. 2).

Effect of treatments on immune function

Females in the carotenoid and GnRH + carotenoid treatments had enhanced acquired immune function. Those females mounted a significantly higher response to PHA than controls, whereas the lowest response was found in females from the GnRH treatment ($R^2 = 0.79$, treatment: $F_{3,24} = 11.15$, $P = 0.001$, cage: $F_{20,24} = 1.15$, $P = 0.048$; Fig. 3). On the other hand, treatments did not affect plasma bacterial-killing ability of females ($R^2 = 0.41$, treatment: $F_{3,70} = 0.56$, $P = 0.63$, date: $F_{3,70} = 3.80$, $P = 0.03$, treatment by date: $F_{6,70} = 0.78$, $P = 0.58$, cage: $F_{20,24.49} = 0.68$, $P = 0.80$, female: $F_{23,70} = 1.02$, $P = 0.45$).

Relationships between immune function and plasma carotenoid and T levels

PHA responses were positively correlated with circulating carotenoid levels in the carotenoid (Pearson correlation; lutein: $r = 0.45$, $P = 0.02$; zeaxanthin: $r = 0.44$, $P = 0.03$) and GnRH + carotenoid treatments (Pearson correlation; lutein: $r = 0.48$, $P = 0.01$; zeaxanthin: $r = 0.46$, $P = 0.03$). PHA responses were not correlated with circulating carotenoid levels in the GnRH (lutein: $r = 0.24$; zeaxanthin: $r = -0.12$, both $P > 0.45$) or control treatments (lutein: $r = 0.23$; zeaxanthin: $r = 0.15$, both $P > 0.25$). Testosterone, measured as baseline levels, post-challenge levels, or response to GnRH challenges, was not correlated with wing-web swelling in any of the treatment groups (all $P > 0.55$). Likewise, plasma levels of carotenoids or testosterone did not correlate significantly with the plasma bacterial-killing ability in any of the four treatment groups (all $P > 0.30$).

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Table 1 Effects of experimental treatments on concentrations of different plasma carotenoids (μg ml^{-1}) in breeding female Japanese quail

Carotenoids	R^2	$F_{(3,66)}$	*P* value	Treatment	Mean ± SE
Lutein				GnRH + carotenoids a	11.94 ± 0.62
	0.95	55.81	0.001	Carotenoids b	6.24 ± 0.61
				GnRH c	1.30 ± 0.64
				Control c	1.18 ± 0.63
Zeaxanthin				GnRH + carotenoids a	1.90 ± 0.10
	0.93	20.35	0.001	Carotenoids b	1.16 ± 0.10
				GnRH bc	0.81 ± 0.10
				Control c	0.76 ± 0.10
Total carotenoids				GnRH + carotenoids a	13.84 ± 0.67
	0.97	55.16	0.001	Carotenoids b	7.40 ± 0.66
				GnRH c	2.11 ± 0.68
				Control c	2.01 ± 0.71

Statistical results are from repeated-measures linear models with treatment and date of blood collection included as fixed factors, cage included as a random effect and female individual identity included as a random effect nested within cages. Treatments followed by the same letter were not significantly different at a *P* level of 0.05

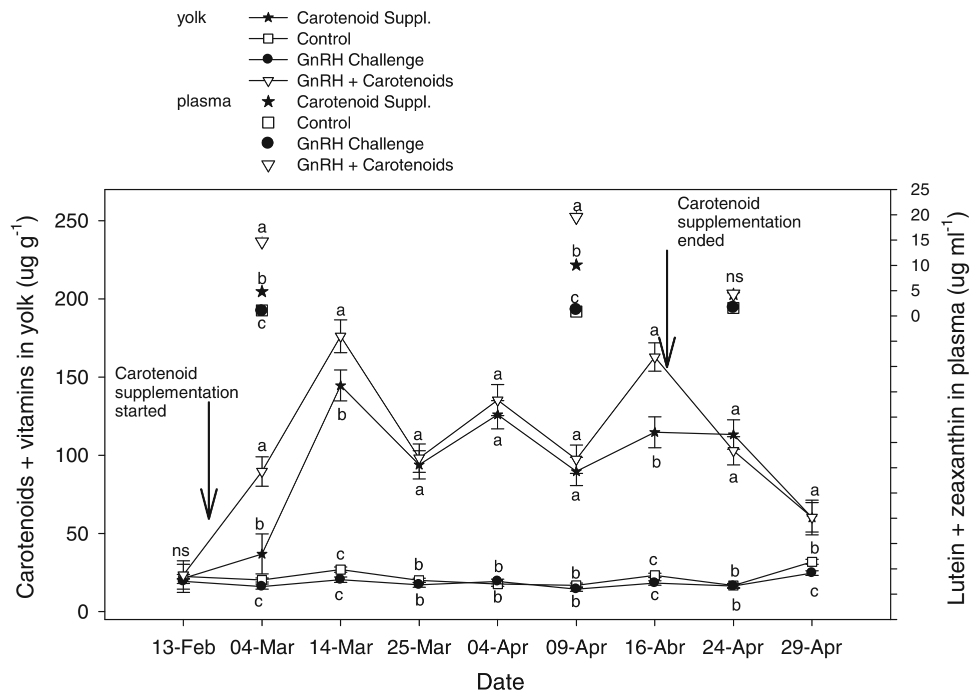

Fig. 2 Deposition of carotenoids and vitamins in egg yolks (primary *y*-axes) and plasma levels of lutein and zeaxanthin (secondary *y*-axes) through time from GnRH-challenged, carotenoid-supplemented, GnRH + carotenoid, and control females. Values for each treatment are means (±SE). *Letters* depict significant differences at a 0.05 alpha level among treatments within dates. *Arrows* show dates when carotenoid supplementation started and ended

Female allocation of resources to egg yolks

Yolk testosterone

Yolk testosterone concentrations prior to GnRH challenges ranged between 4.01 and 27.61 pg mg^{-1} (mean ± SEM 10.56 ± 1.46 pg mg^{-1}), and did not differ significantly among treatments ($F_{3,46} = 0.92$, $P = 0.43$). Experimental treatments and time did affect yolk testosterone concentration ($R^2 = 0.67$, treatment: $F_{3,273} = 15.13$, $P < 0.0001$, date: $F_{7,273} = 41.67$, $P < 0.001$, treatment by date: $F_{21,273} = 1.16$, $P = 0.01$, cage: $F_{20,26.23} = 1.44$, $P = 0.01$, female: $F_{25,273} = 0.28$, $P = 0.16$). The increase in yolk T above initial levels was moderately repeatable ($r = 0.49$, $F_{45,46} = 2.98$, $P = 0.025$). A Tukey's post hoc test showed that females in the GnRH + carotenoid treatment deposited more testosterone in their eggs than females in the GnRH treatment, and both groups deposited more testosterone than carotenoid-supplemented or control females, whereas yolk levels from females in the carotenoid treatment did not differ from control eggs (Fig. 4). When considering only those eggs laid by GnRH-treated females, significantly

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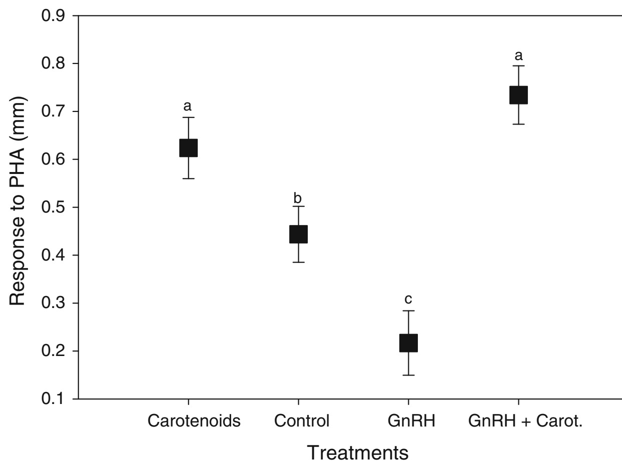

Fig. 3 The acquired immune system of female Japanese quail had differential responses to a foreign compound (PHA) relative to the treatment received (GnRH-challenged, carotenoid-supplemented, GnRH + carotenoid, and control females). Values for each treatment are means (±SE). *Letters* depict significant difference at a 0.05 alpha level

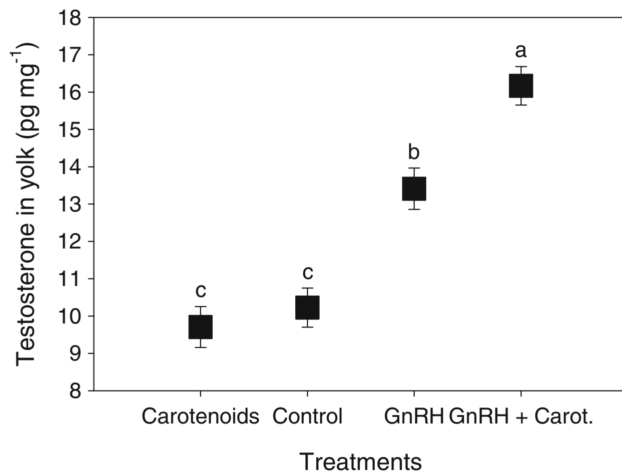

Fig. 4 Female Japanese quail differentially deposited testosterone in their eggs relative to the treatment to which they were assigned (GnRH-challenged, carotenoid-supplemented, GnRH + carotenoid, and control females). Values for each treatment are means (±SE). *Letters* depict significant difference at a 0.05 alpha level

more testosterone was deposited in eggs laid 14 days after GnRH injections than on eggs laid 7 days after challenges ($R^2 = 0.35$, time between challenges: $F_{2,153} = 4.13$, $P = 0.01$, cage: $F_{11,13.07} = 2.58$, $P = 0.053$, female: $F_{2,153} = 4.13$, $P = 0.01$.

We found a significant and positive correlation between response to GnRH injection (i.e., change in plasma testosterone levels) and yolk testosterone concentration (Pearson correlation; $r = 0.50$ $P = 0.003$, Fig. 5). There was no significant correlation between yolk testosterone concentration and either baseline ($P = 0.37$) or post-challenge plasma testosterone concentration ($P = 0.19$).

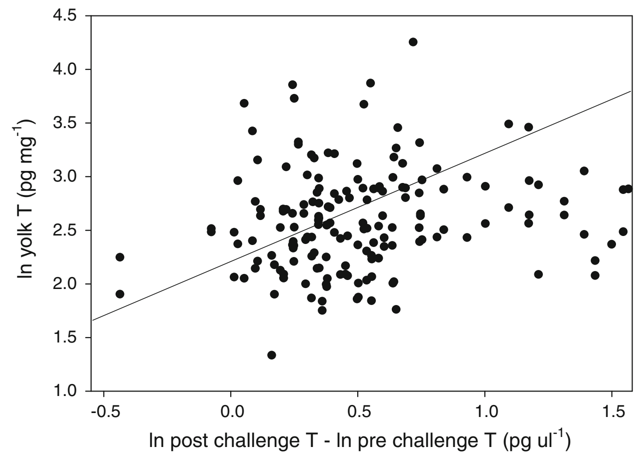

Fig. 5 Relationship between plasma testosterone levels (in response to GnRH challenges) and yolk testosterone levels in female Japanese quail. The *line* represents the best fit of data points for the relationship between the cited variables

Yolk carotenoids and vitamins

There was no significant difference in yolk carotenoid or vitamin levels among treatments before diet supplementation ($R^2 = 0.43$, treatment: $F_{3,24} = 1.77$, $P = 0.17$; cage: $F_{20,24} = 0.68$, $P = 0.80$; mean ± SEM, lutein: 21.66 ± 0.68 μg g^{-1}; zeaxanthin: 10.60 ± 0.40 μg g^{-1}; β-cryptoxanthin: 0.19 ± 0.01 μg g^{-1}; vitamin A: 10.49 ± 0.29 μg g^{-1}; vitamin E: 4.70 ± 0.14 μg g^{-1}). However, we found significant treatment effects on yolk concentrations of carotenoids and vitamins once supplementation started ($R^2 = 0.77$, treatment: $F_{3,117} = 82.81$, $P = 0.0001$, date: $F_{3,117} = 2.10$, $P = 0.10$, treatment by date: $F_{9,117} = 1.72$, $P = 0.09$, cage: $F_{20,24.8} = 0.81$, $P = 0.67$, female: $F_{24,117} = 0.96$, $P = 0.52$, Table 2). Increase in yolk carotenoids above initial levels was moderately repeatable ($r = 0.49$, $F_{47,48} = 3.03$, $P = 0.03$). Vitamin A levels were significantly lower in eggs from females in the GnRH treatment relative to the other three treatments. Vitamin E, zeaxanthin, and β-cryptoxanthin concentrations were significantly higher in eggs from females in the carotenoid and GnRH + carotenoid treatments relative to eggs from control and GnRH treatments. In contrast, lutein was significantly higher in eggs from females in the GnRH + carotenoid treatment, followed by eggs from females in the carotenoid treatment; eggs from females in the GnRH and control treatments contained similar lutein levels.

Yolk carotenoid and vitamin concentrations varied through time (Fig. 2). Females in the carotenoid and GnRH + carotenoid treatments had increased yolk vitamin and carotenoid concentrations immediately following supplementation and continued to allocate higher levels of those compounds until the treatment ceased. Females in the

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Table 2 Effects of experimental treatments on concentrations of all measured carotenoids and vitamins in yolk (μg g^{-1}), in breeding female Japanese quail

Carotenoids and vitamins	R^2	$F_{(3,117)}$	P value	Treatment	Mean ± SE
Vitamin A				GnRH + carotenoids a	10.55 ± 0.29
				Carotenoids a	10.52 ± 0.29
	0.61	22.41	0.0001	GnRH b	8.58 ± 0.28
				Control a	10.25 ± 0.31
Vitamin E				GnRH + carotenoids a	5.54 ± 0.13
				Carotenoids a	5.55 ± 0.13
	0.63	30.29	0.0001	GnRH b	4.04 ± 0.13
				Control b	4.35 ± 0.14
Zeaxanthin				GnRH + carotenoids a	15.01 ± 0.39
				Carotenoids a	13.44 ± 0.40
	0.74	42.68	0.0001	GnRH b	9.35 ± 0.40
				Control b	10.74 ± 0.42
β-cryptoxanthin				GnRH + carotenoids a	0.49 ± 0.02
				Carotenoids a	0.43 ± 0.02
	0.70	34.41	0.0001	GnRH b	0.27 ± 0.02
				Control b	0.29 ± 0.02
Lutein				GnRH + carotenoids a	107.87 ± 2.61
				Carotenoids b	95.93 ± 2.69
	0.77	82.81	0.0001	GnRH c	18.64 ± 2.70
				Control c	21.23 ± 2.75

Statistical results are from repeated-measures linear models with treatment and date of egg collection included as fixed factors, cage included as a random effect and female individual identity included as a random effect nested within cages. Treatments followed by the same letter were not significantly different at a P level of 0.05

GnRH + carotenoid treatment had consistently higher carotenoid and vitamin levels in their yolks than females in the carotenoid treatment (Fig. 2).

Correlation between yolk T and carotenoid levels

Correlations between concentrations of yolk testosterone and carotenoids or vitamins were only significant in the GnRH + carotenoid treatment (lutein, $r = 0.32$, $P = 0.003$; zeaxanthin, $r = 0.35$, $P = 0.007$, β-cryptoxanthin, $r = 0.30$, $P = 0.001$, and vitamin E, $r = 0.35$, $P = 0.030$), with the exception of vitamin A ($r = 0.12$, $P = 0.65$). For the rest of the treatment groups, the correlations between testosterone and carotenoid or vitamin concentrations deposited in egg yolks were not significant (all $P > 0.15$).

Discussion

The simultaneous administration of a GnRH challenge and supplemental carotenoids in the diet in captive female Japanese quail during egg-laying effectively increased plasma levels of testosterone and carotenoids, and further induced significant changes in testosterone, carotenoid, and vitamin levels in egg yolks. These manipulations also initiated significant changes in maternal immune function.

The direct role of testosterone in avian immunity remains a matter of debate and evidence supporting immunosuppressive influence of testosterone is equivocal (Duffy et al. 2000; Hasselquist et al. 1999; Lindström et al. 2001; Peters 2000; Roberts et al. 2004; Saino et al. 1995; Zuk and Johnsen 1998; Zysling et al. 2006). Although the role of testosterone on female birds has received considerably more attention in the last few years (Clotfelter et al. 2004; Elekonich and Wingfield 2000; Groothuis and von Engelhardt 2005; Jawor et al. 2006, 2007; Langmore et al. 2002), testosterone-mediated immunomodulation is rarely studied in females (Ketterson et al. 2005; McGraw and Ardia 2007). In Japanese quail, high plasma testosterone levels in males have been found to be both immunosuppressive and energetically costly (Boughton et al. 2007). In this experiment we show that GnRH challenges administered to female Japanese quail have immunosuppressive effects, possibly due to elevated plasma testosterone. Because GnRH is a releasing hormone that signals a cascade of hormones involved in reproduction, the immunosuppressive effects detected may have been due to other hormones. For example, elevated testosterone can also result in increased levels of corticosterone (Casto et al. 2001; Ketterson et al. 1991; Klukowski et al. 1997; Schoech et al. 1999), which may directly suppress immune function (Roberts et al. 2009). Similarly, the aromatization of testosterone to estradiol could be the cause of the immunosuppression observed here (Leitner et al. 1996; Owen-Ashley et al. 2004). The agent directly involved in immunosuppression deserves further study; however, we can say that stimulation of the

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HPG axis with a hypothalamic releasing hormone effectively changes immune function. This is consistent with life history theory, which predicts that allocation to reproductive function can result in trade-offs with functions of self-maintenance (Stearns 1989). The acquired immune system of female Japanese quail (measured as cell-mediated immune response to PHA) was compromised by GnRH challenges, but not the innate immune function (measured as the plasma ability to destroy bacteria). This suggest that the relationships between an active HPG axis and immune function is more complex than typically portrayed, as it varies depending on the species studied and the immune parameter investigated (e.g., Deviche and Cortez 2005; Hasselquist et al. 1999). For example, the impact of hormones on the immune system might vary across species because species differ in hormonal profiles during the breeding season (Wingfield et al. 1990). Furthermore, studies examining the relationship between hormones and the immune system tend to cover only one branch of the immune system. Most studies focus on specific responses from the acquired arm of the immune system (Casto et al. 2001; Hasselquist et al. 1999), and less often they measure non-specific responses associated with innate immunity (Zuk et al. 1995). Previous work suggests that an understanding of the effects of hormones on immunocompetence requires characterizing multiple indices of immunity (Lochmiller 1995; Sheldon and Verhulst 1996; Zuk and Johnsen 1998), because higher response of one component of the immune system does not imply greater overall resistance (Adamo 2004). Our results also highlight the challenge of obtaining a 'general' measure of immunocompetence and stress the importance of measuring different aspects of the immune system (Adamo 2004; Mendes et al. 2006).

Dietary carotenoid supplementation of female Japanese quail significantly increased plasma carotenoid levels and wing-web swelling. Moreover, we detected a positive relationship between plasma levels of lutein and zeaxanthin in supplemented individuals and response to PHA challenge. At this time we cannot distinguish between the relative effects of these two pigments on immunocompetence, but our results do suggest that xanthophylls in general serve as immunoenhancers in female Japanese quail. These results contribute to growing evidence that indicates xanthophylls increase immune defense in birds (e.g., McGraw and Ardia 2003; McGraw et al. 2011; Sepp et al. 2011). Carotenoid supplements did not, however, alter the bactericidal ability of female plasma, suggesting that the innate immune system is less sensitive to carotenoid supplementation. In contrast, increased carotenoid accumulation translated into a stronger bactericidal activity of their blood towards *E. coli*, but a lack of responsiveness to PHA in male society finches (McGraw et al. 2006b). Differences in carotenoid immunomodulation among species may be a function of sex, taxon, domestication, or the immune parameter tested. McGraw and Klasing (2006) observed that carotenoid-supplemented male, but not female, red jungle fowl showed enhanced plasma bactericidal competence relative to unsupplemented birds. They suggested that males may prioritize constitutive innate immunity more than females (McGraw et al. 2006a, b) and thus devote more carotenoids to such a defense, whereas increased availability of carotenoids in adult females may preferentially boost the acquired arm of the immune system. Similarly, in a study of northern bobwhite (*Colinus virginianus*, Peluc et al., unpublished), dietary carotenoid supplementation significantly increased PHA-induced wing-web swelling in females relative to control and failed to enhance bactericidal ability of their plasma. The results here presented emphasize the need for more studies that examine in detail the immunomodulatory effects of carotenoids in females versus males, which will enhance our understanding of the diverse effects that carotenoids can have on different components of avian immune systems.

In addition to the independent effects of testosterone and carotenoids on immune function, our results support the hypothesis that the interaction between testosterone and carotenoids in relation to immune performance is relevant for female birds. In male birds testosterone circulating in plasma can increase carotenoid bioavailability by upregulating circulating lipoproteins (Blas et al. 2006; McGraw et al. 2006a). Yet, to our knowledge, this has not been observed in female birds (McGraw 2006b). Here we found higher concentrations of lutein and zeaxanthin in plasma of GnRH-challenged and carotenoid-supplemented females than in females that only received a carotenoid supplement (i.e., received a saline injection). This result suggests that plasma steroid concentrations influence the level of carotenoids circulating in plasma in female Japanese quail. Carotenoid availability to females in our study seemed to counteract the immunosuppressive effects of GnRH challenges. Females that were GnRH-challenged but had access to a carotenoid-supplemented diet showed a stronger cell-mediated immune response than control females, and similar to that of carotenoid-supplemented females. The ability of carotenoids to counteract immunosuppression mediated by testosterone has also been observed in other species (Blas et al. 2006; McGraw and Ardia 2007; McGraw et al. 2006a; McGraw and Parker 2006; Peters 2007), although such studies have addressed those effects only on males. Results of our study suggest that the elevation of plasma testosterone via GnRH challenges in females promotes carotenoid accumulation, which in turn is able to combat any immunosuppressive effects of steroids. This mechanism could provide breeding females with a means for mitigating

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costs of elevated steroids to themselves as well as to their developing offspring.

GnRH-challenges elevated testosterone levels in yolk in addition to plasma. Females not only showed consistent increases in plasma testosterone after injections, but also showed a strong and positive covariation between testosterone levels in plasma and yolk across successive challenges. Yolk testosterone concentration covaried positively with the magnitude of the increase in testosterone in plasma following GnRH injections. Hence, yolk testosterone concentration seemed to relate to the overall response of the hypothalamus–pituitary–gonad (HPG) axis to GnRH challenge rather than absolute levels of plasma testosterone either before or after challenge. Previous work in birds has shown that yolk testosterone levels can covary with circulating testosterone levels in the plasma (Schwabl 1996). Moreover, yolk testosterone increases when plasma testosterone is elevated experimentally (Clotfelter et al. 2004; Hackl et al. 2003; Lopez-Rull and Gil 2009; Rutkowska et al. 2005). The cause of the variation in responsiveness of the HPG axis to GnRH among females is unclear at this time, but the fact that the strength of a female's response covaries with yolk testosterone levels suggest that females are able to deposit "extra" testosterone in eggs in response to environmental factors that stimulate the HPG axis, perhaps providing them with a mechanism to avoid costs of this extra testosterone in their own circulation.

Yolk allocation of testosterone may be an adaptive form of parental favoritism or an adverse by-product of endocrine processes during egg formation. In a variety of species studied in the wild, higher levels of yolk testosterone are associated with female aggressive interactions or higher breeding densities (Caprio et al. 2009; Mazuc et al. 2003; Pilz and Smith 2004; Schwabl 1997; Whittingham and Schwabl 2002). However, it is not known if aggressive interactions may stimulate the release of GnRH in females, which could in turn initiate increases in the levels found in yolk (Elekonich and Wingfield 2000; Hau et al. 2000; Jawor et al. 2006; Langmore et al. 2002; Navara et al. 2006a; Smith et al. 2005). In such case, the deposition of androgens in the eggs could serve as an adaptive method of regulating circulating androgen levels in the female, preventing potentially disruptive elevations in circulating androgen concentrations during a particularly sensitive period in the reproductive cycle (Navara et al. 2006b). On the other hand, in certain situations, the ability to deposit additional testosterone in yolks might promote benefits in their offspring, such as enhancement of their growth or competitive ability (Eising et al. 2001; Gil 2003). Although specific mechanisms that drive correlations between female plasma and yolk testosterone are unclear, this study is in agreement with others (Jawor et al. 2007) in that the elevation of testosterone production in the gonad consequently results in measurable levels of testosterone in egg yolks.

Carotenoids are also biologically active compounds that may mediate maternal effects when deposited into the egg yolk (Biard et al. 2007; Blount et al. 2002; Groothuis et al. 2006; Rutkowska et al. 2007; Tanvez et al. 2009). Here we found three lines of evidence that allocation of different resources to egg yolks may not be independent of one another, as deposition of testosterone to yolk was closely related to deposition of carotenoids and vitamins. First, GnRH + carotenoid-treated females deposited more yolk testosterone than females from the GnRH-challenged treatment. Second, we found significantly more carotenoids and vitamins in eggs from females that received both a GnRH challenge and a supplemented diet than in eggs from females that were supplemented with carotenoids only. Third, concentration of carotenoids and vitamins in egg yolks was significantly and positively correlated with yolk testosterone concentration of eggs. Results of previous studies are controversial, in that positive, negative, and even no relation has been observed between androgens and carotenoids allocated to yolk (Cucco et al. 2008; Groothuis et al. 2006; Navara et al. 2006a; Royle et al. 2001; Safran et al. 2010; Safran et al. 2008; Verboven et al. 2005). The allocation pattern observed here for Japanese quail suggests two hypotheses; one is that the correlation between yolk testosterone and carotenoids is an epiphenomenon of a single mechanism responsible for allocation of both compounds to yolks (passive deposition). In this case, females are expected to deposit greater amounts of yolk compounds when there are more of these compounds in circulation (Bortolotti et al. 2003; Schwabl 1993). Not mutually exclusive is an adaptive explanation for the correlation, in which case we would expect females to actively deposit androgens and carotenoids into avian egg yolks. The trade-off of diminished availability of these compounds to themselves is the benefit of higher quality of offspring through enhanced development and competitive ability, or protection against oxidative stress during development. Moreover, the differential allocation of these physiologically relevant molecules to egg yolks indicates that their deposition is affected not just by surrounding social and environmental conditions (Gil et al. 2007; Groothuis et al. 2005; Kingma et al. 2009; McGraw et al. 2005; Safran et al. 2008, 2010; Saino et al. 2003; Surai et al. 2001c), but also by adaptive allocation of egg components. Future studies looking at resource allocation to eggs should consider analyzing a variety of egg contents simultaneously, which may provide more insights into possible interactions among resources and mechanisms of deposition. This will further help us elucidate the strategic basis behind resource allocation to eggs and the level of control that female birds may have over offspring quality.

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