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RESEARCH ARTICLE

Immune function is related to adult carotenoid and bile pigment levels, but not to dietary carotenoid access during development, in female mallard ducks

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SUMMARY

Immune function can be modulated by multiple physiological factors, including nutrition and reproductive state. Because these factors can vary throughout an individual's lifetime as a result of environmental conditions (affecting nutrition) or life-history stage (e.g. entering the adult reproduction stage), we must carefully examine the degree to which developmental *versus* adult conditions shape performance of the immune system. We investigated how variation in dietary access to carotenoid pigments – a class of molecules with immunostimulatory properties that females deposit into egg yolks – during three different developmental time points affected adult immunological and reproductive traits in female mallard ducks (*Anas platyrhynchos*). In males and females of other avian species, carotenoid access during development affects carotenoid assimilation ability, adult sexual ornamentation and immune function, while carotenoid access during adulthood can increase immune response and reproductive investment (e.g. egg-laying capacity, biliverdin deposition in eggshells). We failed to detect effects of developmental carotenoid supplementation on adult immune function [phytohemagglutinin-induced cutaneous immune response, antibody production in response to the novel antigen keyhole limpet hemocyanin (KLH), or oxidative burst, assessed by changes in circulating nitric oxide levels], carotenoid-pigmented beak coloration, ovarian development, circulating carotenoid levels or concentration of bile pigments in the gall bladder. However, we did uncover positive relationships between circulating carotenoid levels during adulthood and KLH-specific antibody production, and a negative relationship between biliverdin concentration in bile and KLH-specific antibody production. These results are consistent with the view that adult physiological parameters better predict current immune function than do developmental conditions, and highlight a possible, previously unstudied relationship between biliverdin and immune system performance.

Key words: *Anas platyrhynchos*, biliverdin, carotenoids, developmental plasticity, follicular development, immunity, nitric oxide.

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INTRODUCTION

The mechanisms that control the make-up and responsiveness of the immune system are of widespread interest to those in many scientific disciplines, from medicine (Vesely et al., 2011) to ecology (Martin et al., 2011). Immune function is often modulated by current environmental conditions and physiological state, including reproductive condition (French et al., 2007), hormone levels (Flatt et al., 2008), nutrient availability (Cotter et al., 2011) and their interaction (Ruiz et al., 2010; Cohen et al., 2012). However, immune function can also be shaped by conditions experienced during development (Galic et al., 2009). For example, exposure to an antigen and the resulting increased response to that same antigen later in life is a classic characteristic of the acquired immune response. Additionally, non-antigen-specific immune activation early in life can affect responses to novel antigens later in life (Butler and McGraw, 2012a; Jacot et al., 2005; Star et al., 2009), and factors like hormone exposure (Butler et al., 2010), heat stress (Star et al., 2007) and nutrient availability (Fellous and Lazzaro, 2010) during neonatal development are known to alter subsequent immune function in multiple taxa.

Many nutrients play an active role in triggering and enhancing immune function, including many vitamins (Chew, 1996) and minerals (Cunningham-Rundles et al., 2005). One type of nutrient in particular, carotenoids, has received attention of late because of their diverse effects on many components of the immune system. Specifically, increased carotenoid intake and accumulation have been associated with increased concurrent antibody production (Okai and Higashi-Okai, 1996; Peters et al., 2004), skin swelling response to a phytohemagglutinin (PHA) challenge (Biard et al., 2009) and systemic nitric oxide (NO) levels, which might combat infection as an intracellular signaler and pro-oxidant (Butler and McGraw, 2012a; but see Lin et al., 2012).

Most investigators have examined the role that carotenoids play at only a single life stage – usually adulthood – and links between dietary access to carotenoids and integument coloration (Hill et al., 2009), immune function (Baeta et al., 2008) and oxidative damage (Hõrak et al., 2007) during adulthood have received a great deal of attention. However, carotenoids have physiological consequences at all life stages. The role of carotenoids very early in life is particularly relevant; many young animals, especially reptiles and birds, are of higher quality (e.g. more robust immune function or heavier) when they develop from eggs supplemented with carotenoids (Koutsos et al., 2007) or from eggs laid by carotenoid-supplemented mothers (Berthouly et al., 2008). Additionally, neonatal access to carotenoids can affect subsequent phenotype, including immune function (Butler and McGraw, 2012b) and the ability to assimilate carotenoids at adulthood (Blount et al., 2003). However, little is known about how the timing of differential access

to carotenoids during neonatal development affects adult immune function, despite the importance of testing questions of developmental plasticity using multiple developmental stages (Monaghan, 2008).

Carotenoid pigments also offer profound fitness benefits beyond immunity. Carotenoid-based body colors have been a classic study system for understanding the signaling capacity and evolution of ornamental traits (Svensson and Wong, 2011). In fact, because of their immunological roles, carotenoids can be reliable indicators of the health, condition and genetic quality of potential mates or rivals (Mougeot et al., 2010; Karu et al., 2007; McGraw, 2006). The majority of investigations into the health-signaling role of carotenoids have utilized adult male animals because they more frequently utilize carotenoid-based integument coloration in mate competitions. However, in some systems, females also utilize carotenoid-pigmented ornaments to attract mates (Gladbach et al., 2010) and to signal status (Murphy et al., 2009). Moreover, females deposit carotenoids into the yolks of their eggs (Surai et al., 2001), and dietary carotenoid supplementation of mothers can increase reproductive investment (Blount et al., 2004), leading to the possibility that carotenoids may affect ovarian function. Additionally, recent work has demonstrated that carotenoid supplementation can cause females to lay eggs with more colorful eggshells (i.e. an increase in blue–green appearance as a result of the deposition of biliverdin, a pigment found predominately in bile) (Morales et al., 2011), suggesting a link between dietary access to carotenoids and biliverdin production. Intriguingly, biliverdin has physiological functions that mirror those of carotenoids (McGraw, 2005), including acting as an antioxidant (Kaur et al., 2003) and an anti-inflammatory agent (Nakagami et al., 1993), although the precise physiological links between carotenoid access and biliverdin production are presently unknown.

Here, we performed an experiment with two main goals: (1) to assess whether access to dietary carotenoids at different points during development affects adult carotenoid-associated traits, including immune function, integument coloration and gonad development, and (2) to evaluate how physiological metrics (e.g. circulating carotenoid levels, bile pigment production, gonad development) collected at adulthood correlate with concurrent immune function and carotenoid-pigmented integument coloration in a traditionally understudied female system. To address our first goal, we raised female mallard ducks (*Anas platyrhynchos* L.) from hatching and supplemented their diets with carotenoids during three main developmental periods that correspond to different carotenoid physiology states (see Materials and methods). At adulthood, we then tested whether these transient increases in carotenoid access shaped subsequent carotenoid physiology by measuring circulating carotenoid levels and expression of other carotenoid-associated traits, including immune function and beak coloration. Generally, we predicted that, if adult carotenoid physiology is strongly developmentally plastic (e.g. Blount et al., 2003), dietary access to carotenoids early in neonatal life would have the largest effects on adult circulating carotenoid levels, immune function and coloration. Alternatively, if adult carotenoid physiology is more dependent upon recent carotenoid access, then dietary access to carotenoids at the end of development would have the largest effects on adult circulating carotenoid levels, immune function and coloration. In mallards, both males and females have carotenoid-pigmented beaks (appearing yellow and orange, respectively) that utilize different amounts of multiple carotenoids, predominately lutein and zeaxanthin (M.W.B. and K.J.M., unpublished data). Although females prefer males with more-yellow beaks (Omland, 1996a; Omland, 1996b), there is no known signaling function of female beak coloration.

To address our second goal, we used intra- and inter-individual correlations of multiple carotenoid-associated traits to further elucidate the roles that carotenoids may play during adulthood. Female mallard ducks lay relatively large clutches (typically N=6–11 eggs) (Drilling et al., 2002), resulting in a large carotenoid investment during egg laying. This investment can result in a major trade-off in females (Blount et al., 2004) regarding whether to allocate carotenoids to their developing follicles, integument coloration or immune function (*sensu* Blount et al., 2002). We also assessed biliverdin concentration of bile from the gall bladder at adulthood. Because of biliverdin's antioxidant properties, and because carotenoid-pigmented signals may be indicators of other antioxidants (Hartley and Kennedy, 2004), it is possible that biliverdin production may be reflected by the expression of carotenoid-pigmented integumentary coloration. In fact, biliverdin has a greater antioxidant activity than other nutritional antioxidants (e.g. α-tocopherol) (Mancuso et al., 2012), and only slightly lower levels of antioxidant activity than lutein (Di Mascio et al., 1990), the predominant circulating carotenoid in mallards (Butler and McGraw, 2010). These relatively high antioxidant activities of biliverdin would reduce the oxidation rates of carotenoids (Hartley and Kennedy, 2004), thus increasing the pool of carotenoids that can be used for other functions (e.g. integument coloration, immune function). Despite such theoretical links between carotenoid-based signals and biliverdin, and empirical studies demonstrating the effects of dietary carotenoids on biliverdin deposition in eggshells (Morales et al., 2011), we know of no studies that link internal supplies of biliverdin with carotenoid-based coloration or other carotenoid-associated traits.

MATERIALS AND METHODS

Experimental protocol and blood collection

All work with animals was approved under Arizona State University's IACUC, protocol number 10-1094R. We acquired 46 one-day-old female ducklings of unknown relatedness from Metzer Farms (Gonzales, CA, USA) in December 2009 and housed them as described previously (Butler and McGraw, 2009; Butler and McGraw, 2012a). Briefly, ducklings were reared indoors in randomly selected groups of five ducklings per cage (60×60×60 cm) until they were 2 weeks old, three per cage until they were 4 weeks old, and two per cage until they were 7 weeks old, at which point all birds were moved outside and individually housed to allow for normal sexual maturation (Butler and McGraw, 2009; Butler and McGraw, 2011). The light:dark regime was 13 h L:11 h D while ducklings were housed indoors, and natural photoperiod thereafter (10.5 h L:13.5 h D at 7 weeks old to 13.5 h L:10.5 h D at 20 weeks old).

Individuals were randomly assigned to one of four treatment groups that varied in dietary carotenoid content during different periods of development. Individuals were placed on carotenoid-supplemented diets during the period of maximal growth (Early; 3–6 weeks old, N=10) (Butler and McGraw, 2012a), minimal growth and minimal nuptial plumage acquisition (Middle; 8–11 weeks old, N=12), or full acquisition of nuptial plumage (Late; 13–16 weeks old, N=12). Control (N=12) birds did not receive carotenoid-supplemented diets at any point (Fig. 1). The Early period corresponds to an age in wild ducklings when correlations among dietary, circulating and stored carotenoid levels are at their lowest, suggesting a transitional period between carotenoid storage and mobilization physiology (Butler and McGraw, 2010). The Middle period occurs during the stage of the

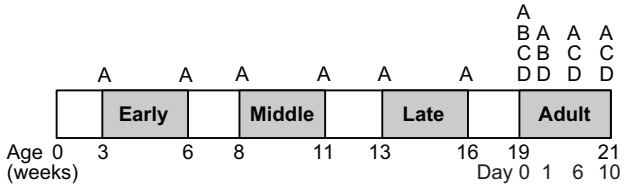

Fig. 1. Timing of sample collection. Circulating carotenoid titer (A) was measured at the beginning of each treatment period (Early, Middle and Late) and at every point of the adult immune assessment period. Both wing web thickness and plasma nitric oxide (NO) concentration (B) were measured prior to phytohemagglutinin (PHA) injection (day 0) as well as 24 h later (day 1). Keyhole limpet hemocyanin (KLH)-specific IgG (C) was measured prior to initial KLH injection and 6 days later (primary response; day 6), at which point a booster KLH injection was administered, and secondary KLH-specific IgG levels were measured 4 days later (day 10). Bill coloration (D) was quantified during every point of the adult period. Bile samples and ovaries were collected from euthanized birds at 22.5 weeks of age.

highest circulating levels of carotenoids in captive-raised ducks (Butler and McGraw, 2011), which may correspond to a period of maximal investment in carotenoid transport mechanisms (e.g. lipoproteins) (Trams, 1969). Lastly, the Late stage corresponds to the period when mallards develop their carotenoid-based integument coloration, which requires upregulation of unidentified physiological mechanisms (Vanhoutteghem et al., 2004) required for pigment deposition (McGraw et al., 2002). We prepared diets by mixing a base diet of dry food (weeks 0–7: Mazuri Waterfowl Starter, Richmond, IN, USA; thereafter: Mazuri Waterfowl Maintenance) with ORO-GLO dry pigmenter (2% carotenoids by mass, predominately lutein; Kemin AgriFoods North America, Des Moines, IA, USA) suspended in sunflower oil to achieve concentrations of 25 $\mu g\,g^{-1}$ of carotenoids (upper quartile of carotenoid concentration in mallard duckling diets in the wild) (Butler and McGraw, 2010). Whenever any treatment group was receiving carotenoid-supplemented diets, all other individuals received food mixed with just sunflower oil as a sham control.

We measured body mass to the nearest gram and tarsus length to the nearest 0.1 mm at the beginning and end of each experimental period, and body mass four times during the adult immune assessment period (see below). Concurrent with times of body mass measurement, we also collected 300 µl (3 weeks old) and 600 µl (all other time points) of whole blood from each individual. Blood was stored on ice for several hours and then centrifuged for 3 min at 16,060 RCF. Next, we aliquoted plasma into separate microtubes for carotenoid, NO and KLH-specific antibody quantification (see below), and stored the plasma at −80°C until analysis. We euthanized all animals when they were 22.5 weeks old, collected ovaries (N=46) *via* dissection and bile samples (N=28) by puncturing the gall bladder, placed these samples on ice, and stored them at −80°C until further analysis. Several days later, we used digital calipers to measure the diameter of the five largest follicles in each ovary to the nearest 0.01 mm.

Adult immune metrics

When individuals were 19 weeks old (the age at which females have finished acquiring breeding season plumage and are displaying courtship behavior) (Drilling et al., 2002), we issued a series of immune challenges over an 11 day period [day (D)0 to D10] (Butler and McGraw, 2011; Butler and McGraw, 2012b). Because individual immune metrics are not always correlated within an individual (Salvante, 2006), we elected to collect multiple metrics in order to generate a more comprehensive assessment of individual immune function. Within this period, we issued a PHA challenge to assess cutaneous immune function (Martin et al., 2006). On D0, we measured the thickness of the patagium (wing web) in duplicate and then injected 0.1 mg of PHA (Sigma L8754, St Louis, MO, USA) suspended in 0.1 ml of sterile phosphate-buffered saline (PBS; Fisher BP399, Waltham, MA, USA); 24 h later (D1; mean: 23 h 56 min, s.d.=19.3 min), we again measured the thickness of the wing web in duplicate. Measurements were significantly repeatable within each day (D0: R=0.95; D1: R=0.99) (Lessells and Boag, 1987), and we calculated swelling response as the difference between average thickness on D1 minus that on D0. A larger swelling response is associated with a more robust cutaneous immune response (Martin et al., 2006; Smits et al., 1999).

Also on D0, we intra-abdominally administered an emulsion consisting of 250 µl of Complete Freund's Adjuvant (CFA; Difco Laboratories, Detroit, MI, USA) and 250 µg of keyhole limpet hemocyanin (KLH; Sigma H7017) suspended in 250 µl of sterile ddH_2O (Butler and McGraw, 2012b). Six days later (D6), we intra-abdominally administered a booster injection consisting of 250 µg of KLH suspended in 250 µl of sterile ddH_2O emulsified in 250 µl of Incomplete Freund's Adjuvant (IFA; Sigma F5506). By collecting blood on D0, D6 and D10 (4 days after D6), we were able to quantify the primary and secondary humoral immune response to this novel antigen. Specifically, we followed previously published ELISA protocols (Butler and McGraw, 2011; Butler and McGraw, 2012a) to quantify KLH-specific antibody production and calculated the primary (D6 minus D0) and secondary (D10 minus D0) humoral response. To do so, we coated 96-well plates (Fisher 468667) with 100 µl of 0.5 $mg\,ml^{-1}$ KLH in sodium carbonate buffer, blocked the wells with 100 µl blocking buffer (PBS with 1% BSA; Sigma B4287) containing sodium azide (0.1%, Sigma S2002), and subsequently added 100 µl plasma diluted 1:19,000 in blocking buffer with azide in triplicate. After an overnight incubation at 4°C, we added 80 µl of HRP-conjugated polyclonal secondary antibody (Bethyl Laboratories A140-110P, Montgomery, TX, USA) specific for avian IgG that was diluted 1:20,000 in blocking buffer. We then added 100 µl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate, incubated the plate for 20 min at room temperature protected from light, and then added 50 µl of 0.5 $mol\,l^{-1}$ H_2SO_4. Within 10 min, we measured absorbance of each well at λ=450 nm using an iMark Microplate Reader (Bio-Rad Laboratories, Hemel Hempstead, Herts, UK), with greater absorbances corresponding to a greater IgG titer (see Butler and McGraw, 2012a).

Both PHA (Sild and Hõrak, 2009) and CFA (Zheng et al., 2003) induce systemic increases in NO levels, with a greater increase associated with a more robust oxidative burst component of the immune response. Following Sild and Hõrak (Sild and Hõrak, 2009), we quantified NO production in response to these immunostimulants on both D0 and D1. We deproteinized 15 µl of plasma (Butler and McGraw, 2012a) and quantified circulating NO levels (Sild and Hõrak, 2009) on both D0 and D1 using a Greiss reaction, measuring the absorbance at 540 nm. NO response was calculated as the difference between D1 and D0. Absorbance values below the negative blank were assigned a value of 0, and the standard curve had an R^2=0.99.

Carotenoid titer and coloration assessment

We quantified plasma carotenoid content at all 10 time points for which we had collected blood samples (at the beginning and end

of the Early, Middle and Late developmental periods and the adult immune assessment periods D0, D1, D6 and D10; Fig. 1). To do so, we extracted carotenoids from 50 µl of plasma using 1:1 hexane:methyl tert-butyl ether and measured concentrations using high-performance liquid chromatography (HPLC) (McGraw et al., 2008). Detectable amounts of lutein, zeaxanthin and a lutein isomer existed in female plasma at all ages, while smaller amounts of β-cryptoxanthin were sporadically detected. Within each time point, lutein, zeaxanthin, the lutein isomer and β-cryptoxanthin were positively correlated with total carotenoid titer (all R>0.336, P<0.023), except for one instance when β-cryptoxanthin was not (R=0.203, P=0.18). However, β-cryptoxanthin accounted for 1.4% of the total carotenoid titer at this time point, so we elected to use total carotenoid titer at each time point in subsequent analyses.

Ornamental beak coloration in mallards begins to develop by 10 weeks of age (Drilling et al., 2002) and is completed in all birds by 16 weeks (M.W.B., personal observation; J. Metzer, personal communication). At the same time that we collected blood for adult immune assessment (see above), we measured carotenoid-based beak coloration of adults using an Ocean Optics (Dunedin, FL, USA) USB2000 spectrophotometer with a PX-2 pulsed xenon light source to measure reflectance from λ=300 to 700 nm. We measured a 1 cm band of the right dorso-lateral surface of the beak between the nares and the beak tip, and binned all measurements into 1 nm increments using CLRfiles (CLR version 1.05) (Montgomerie, 2008). We then used CLRvars (CLR version 1.05) (Montgomerie, 2008) to calculate the brightness (B1), saturation in the blue portion of the spectrum (S1B), and hue (H4b) scores that are most closely correlated with carotenoid content in the male mallard beak (Butler et al., 2011). Pilot work from females in this study demonstrated that only S1B retained a significant relationship with carotenoid content of the integument in females (r=–0.49, P=0.05), and we report all associated statistics for this color metric. We also performed analyses using both B1 and H4b, but neither variable explained significant variation in any analysis (all P>0.1), except for one analysis examining the relationship between beak coloration and bile pigment concentration (see below).

Bile pigments

Female birds produce biliverdin in both the liver and shell gland, but far greater concentrations are found in the gall bladder than in the shell gland, and gall bladder biliverdin concentration is not related to eggshell coloration in chickens (Zhao et al., 2006). Thus, gall bladder biliverdin concentration may be more related to the individual's general nutritional physiology than levels produced by the shell gland, although this has not been empirically tested. To quantify relative bile pigment concentration from bile, we modified a previously published protocol (Mateo et al., 2004). First, we mixed 35 µl of bile (this amount was within the linear range of absorbance based on pilot work, linear range R^2=0.99) with 565 µl solvent (3 mol l^{-1} HCl:acetonitrile, 5:6), vortexed the solution, and then centrifuged it for 10 min at 16,060 RCF. We then took 0.2 ml of supernatant, added 0.8 ml solvent, and scanned from 330 to 450 nm in a Beckman Coulter DU 52 UV/Vis spectrophotometer (Indianapolis, IN, USA). Samples had a peak absorption at 365 nm (the peak absorbance value for biliverdin) (Mizobe et al., 1997), so we recorded the absorbance at this wavelength and used this value as a metric for bile pigment concentration within bile samples, which is likely driven by biliverdin concentration (over 95% of pigments recovered from mallard bile are biliverdin) (Mateo et al., 2004).

Statistics

We used a repeated-measures (rm) ANOVA to examine the effect of dietary treatment on circulating carotenoid levels, body mass and tarsus length throughout development and adulthood. To test for the effect of dietary treatment during development and of adult carotenoid physiology on adult immune function, we ran a series of ANCOVA models, with dietary treatment as an independent variable, either circulating carotenoid level prior to the immune challenge or change in circulating carotenoid level during the immune response as a covariate, and immune function (primary or secondary KLH-induced antibody production, PHA-induced swelling, NO response) as the dependent variable. We used ANOVA to test for an effect of dietary treatment on adult beak coloration (S1B), ovarian development (function of size of the five largest follicles in the ovary; see below), bile pigment concentration and pre-immune circulating NO levels. To test whether adult beak color prior to an immune challenge was predictive of immune response, we used general linear models (GLMs) with adult immune response metrics as dependent variables and pre-immune beak saturation and the change in beak saturation over the course of the immune response as independent variables. Lastly, we used GLM to test whether measures of immune response (dependent variables), adult circulating carotenoid levels, beak coloration or ovarian development varied as a function of biliverdin or pre-immune NO levels. We performed all statistics with SAS 9.2 (Cary, NC, USA); all variables were either normally distributed or log-transformed (primary and secondary KLH antibody response, pre-immune NO levels, NO response and bile pigment concentration) to achieve normality, and *post hoc* tests were performed by comparing least-squares means. Effect sizes are calculated as Hedges's g, which is commonly referred to as Cohen's d in the literature [see table 1 of Nakagawa and Cuthill (Nakagawa and Cuthill, 2007)].

RESULTS

Effects of early-life dietary carotenoid access on developmental variables

Circulating carotenoid levels varied as a function of age ($F_{9,378}$=56.06, P<0.0001), treatment ($F_{3,42}$=12.20, P<0.0001) and their interaction ($F_{27,378}$=75.88, P<0.0001). Ducklings from all treatment groups circulated similar levels of carotenoids prior to experimental supplementation (all P>0.1), and dietary carotenoid supplementation increased circulating carotenoid levels for each treatment group (all P<0.05; Fig. 2). These differences then disappeared 2–3 weeks after the cessation of dietary supplementation for each treatment group, with the exception of individuals from the Early group, which continued to circulate higher levels of carotenoids at the beginning of the Middle treatment period (P<0.05; Fig. 2). Dietary treatment did not affect body mass ($F_{3,42}$=1.91, P=0.14) or tarsus length ($F_{3,42}$=0.73, P=0.54), nor did treatment interact with age for body mass ($F_{18,252}$=1.02, P=0.44) or tarsus length ($F_{15,210}$=0.80, P=0.67).

Effects of early-life dietary carotenoid access and circulating carotenoid titer on adult immune function, beak coloration and ovarian development

Dietary treatment during development did not affect adult beak saturation ($F_{3,42}$=0.35, P=0.79; effect sizes for all treatments <0.42) prior to the adult immune challenge. With circulating carotenoid levels prior to immune assessment as a covariate, dietary treatment also had no effect on primary KLH response, secondary KLH response or PHA-induced swelling (all $F_{3,41}$<1.30, all P>0.29; effect sizes for all treatments <0.8), nor on NO response ($F_{3,41}$=1.68,

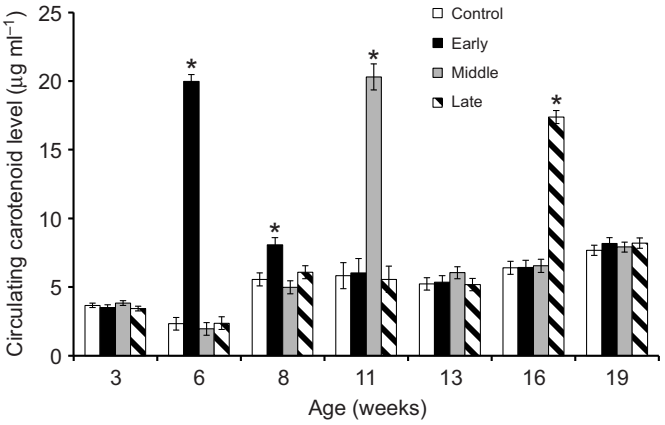

Fig. 2. Circulating carotenoid level as a function of developmental treatment and age. Carotenoid supplementation temporarily increased circulating levels of carotenoids, with levels returning to control levels within 3 weeks. The exception was for birds that received carotenoid supplementation during the Early period; these individuals continued to circulate higher levels of carotenoids for 2 weeks, but not 5 weeks, after treatment cessation.

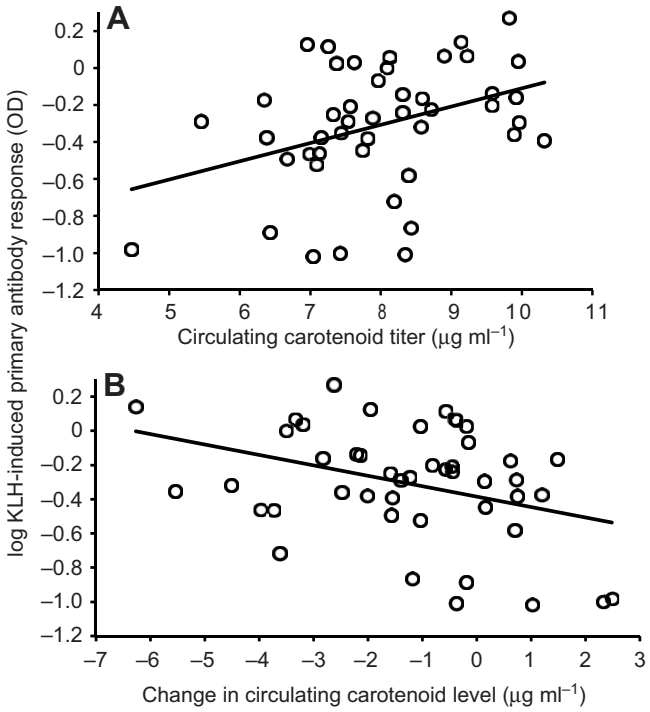

Fig. 3. KLH-specific antibody production as a function of (A) circulating carotenoid titer prior to immune challenge and (B) change in circulating carotenoid levels over the course of the immune response (6 days). In accordance with the immunostimulatory role carotenoids can play, individuals with a greater initial or a greater decrease in circulating carotenoid levels produced a greater primary antibody response to KLH. OD, optical density.

P=0.19; effect sizes for all treatments between 0.65 and 0.90). Circulating carotenoid levels of adults prior to immune assessment positively predicted primary KLH response ($F_{1,41}$=6.54, P=0.014; Fig. 3A), but were not a significant predictor of any other immune metric (all $F_{1,41}$<2.80, all P>0.10).

A principal components (PC) analysis of the size of the five largest follicles in the ovary produced a single PC that had an eigenvalue of 4.70 and an eigenvector that loaded positively and relatively uniformly for all five follicle measurements (all loadings between 0.43 and 0.45), and accounted for 94% of the total variation in follicle size. However, this PC was not normally distributed nor could it be transformed to achieve a normal distribution, and the residuals from an ANOVA showing that dietary treatment did not affect PC value ($F_{3,42}$=0.11, P=0.95) were also not normally distributed. We thus divided our study population into thirds and assigned an ordinal value of 1, 2 or 3 to each individual based on the PC value. A Fisher's exact test showed that dietary carotenoid treatment did not affect rank of follicular development (χ^2_6=0.001, P=1.0).

Beak color as a signal of adult immune function and carotenoid status

Neither beak saturation (S1B) nor change in beak saturation during immune assessment significantly predicted primary or secondary KLH response, PHA-induced swelling or NO response (all $F_{1,44}$<1.90, all P>0.17). There was a non-significant trend for a reduced S1B [associated with greater carotenoid deposition in males (Butler et al., 2011) and in females (see above)] to predict PHA-induced swelling ($F_{1,44}$=3.65, P=0.063). Also, there was a non-significant trend for individuals that showed a greater increase in S1B [which is associated with a loss of carotenoid pigmentation (Butler et al., 2011)] to have a more robust secondary KLH response ($F_{1,44}$=3.56, P=0.066).

Magnitude of adult immune response as a function of change in circulating carotenoid levels over the time course of the immune response

Dietary treatment during development did not affect primary or secondary KLH response, PHA-induced swelling or NO response (all $F_{3,41}$<1.89, all P>0.15) when change in circulating carotenoid levels over the time course of the immune assessment was a covariate. Change in circulating carotenoid levels did not significantly predict KLH secondary response, PHA-induced swelling or NO response (all $F_{1,41}$<1.46, all P>0.23), but a larger concurrent decrease in circulating carotenoid levels was associated with a greater primary KLH response ($F_{1,41}$=7.67, P=0.0084; Fig. 3B).

Relationships between non-carotenoid physiological metrics and adult immune function and circulating carotenoid levels

Because of initial difficulties in collecting bile samples, we were able to collect adequate volumes of bile from only a subset of birds that was not evenly divided across dietary treatment groups (Control: N=5; Early: N=5; Middle: N=8; Late: N=10). There was a non-significant trend for biliverdin concentration to differ as a function of dietary treatment ($F_{3,24}$=2.53, P=0.081, effect size Early=1.28; effect size Middle=1.52; effect size Late=0.79), and thus we controlled for this trend by including treatment as an independent variable in subsequent models. Biliverdin concentration was not a significant predictor of either NO response or PHA-induced swelling (both $F_{1,23}$<2.49, both P>0.13), but a lower concentration of biliverdin significantly predicted a greater primary ($F_{1,23}$=13.85, P=0.0011; Fig. 4) and secondary ($F_{1,23}$=7.50, P=0.0117) KLH response. Biliverdin concentration did not predict adult circulating carotenoid levels at any point during the adult immune assessment period (all $F_{1,23}$<1.09, all P>0.31), ovarian development ($F_{1,23}$=1.12, P=0.30) or beak brightness ($F_{1,23}$=2.96, P=0.099). However, biliverdin concentration did predict beak saturation ($F_{1,23}$=7.06,

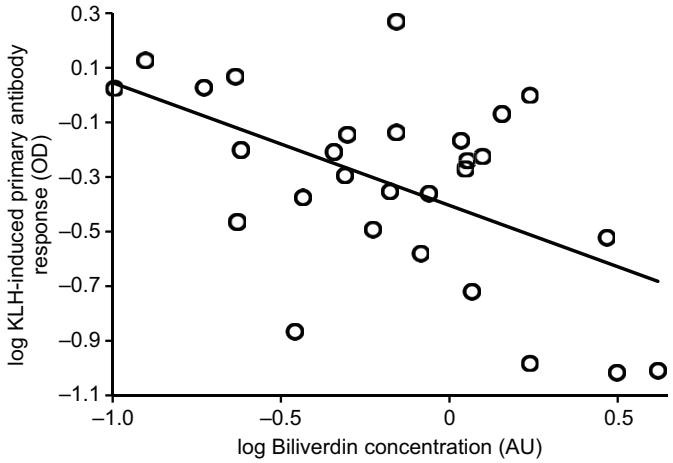

Fig. 4. Primary KLH-specific antibody production as a function of bile pigment concentration. Individuals with greater concentrations of biliverdin (sampled 3.5 weeks post-immune challenge) had lower primary humoral responses. AU, absorbance units.

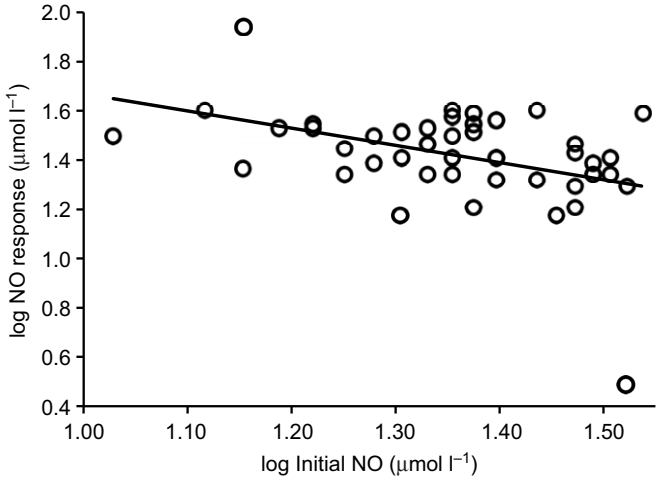

Fig. 5. NO response (change in circulating NO levels) to immunostimulation as a function of NO levels prior to the immune challenge. Individuals that initially circulated higher NO levels produced a lower NO response. If the data point in the lower right-hand corner is excluded, the relationship remains significant ($F_{1,43}$=7.84, P=0.0076).

P=0.014) and hue ($F_{1,23}$=6.13, P=0.021), with birds that had a greater concentration of biliverdin also having beaks that were less saturated (and thus, more carotenoid-rich; M.W.B. and K.J.M., preliminary data) (Butler et al., 2011) beaks.

Circulating NO levels prior to immune assessment did not differ by dietary treatment during development ($F_{3,42}$=0.98, P=0.41; effect sizes for all treatments <0.44), nor did they predict primary or secondary KLH response (both $F_{1,44}$<0.01, both P>0.92). However, higher circulating NO levels prior to immune assessment significantly predicted both a greater PHA-induced swelling ($F_{1,44}$=4.80, P=0.037) and a lower NO response ($F_{1,44}$=9.32, P=0.0038; Fig. 5). Circulating NO levels prior to immune assessment did not predict adult circulating carotenoid levels at any point during the adult immune assessment period (all $F_{1,26}$<1.61, all P>0.21).

DISCUSSION

We found that access to carotenoids during development did not affect adult immune function, ovarian development or carotenoid-pigmented integument coloration in female mallard ducks. However, we did detect significant associations between antibody production and carotenoid status in adults, as found in other work (Butler and McGraw, 2012a), and we also uncovered a novel link between biliverdin production and both antibody response and beak coloration. Thus, both dietarily acquired antioxidants and those produced *de novo* are related to adult immune function. Interestingly, it was biliverdin concentration, but not carotenoid levels, that was related to carotenoid-based beak coloration in this species, highlighting both the possibility that female mallards signal non-carotenoid antioxidant status with carotenoid-pigmented beak coloration (Hartley and Kennedy, 2004) and the potential for biliverdin to have an underappreciated role in vertebrate ecophysiology.

Dietary carotenoid supplementation at different points during development significantly, but temporarily, increased circulating carotenoid levels. Interestingly, carotenoid levels remained elevated in Early birds for an extended period of time, corroborating our suggestion that developmental programming of carotenoid physiology may be most likely during this early stage of development (Butler and McGraw, 2010). However, even these effects persisted only for weeks, not months, and no developmental treatment group differed in adult carotenoid-based beak coloration, multiple metrics of immune function or ovarian development (e.g. by upregulating either carotenoid assimilation or transport ability). Despite previous work with chickens (Koutsos et al., 2003) and zebra finches (McGraw et al., 2005; Blount et al., 2003) showing that carotenoid access during pre-natal and post-natal development, respectively, affects later-life carotenoid physiology and coloration, we found no evidence of such developmentally plastic differences in multiple traits associated with carotenoids (e.g. integument coloration, immune function) in female mallards. These findings also differ from our previous work with male mallards, which demonstrated differences in adult immune function based on carotenoid access during development (Butler and McGraw, 2012b). In contrast to that work, where diets were supplemented for a 7 week period, here we supplemented diets for just 3 weeks in order to test whether there were specific points during development that might induce phenotypic differences. It is possible that such a short-term period did not allow enough time for differences in carotenoid physiology to change sufficiently so as to be detectable at adulthood (Butler and McGraw, 2012b), or that we missed some critical window (Monaghan, 2008) during the first several weeks of neonatal development (*in ovo* or post-hatch).

Though developmental manipulations of dietary carotenoids did not carry over physiologically into adulthood, we uncovered multiple links between adult carotenoid physiology and immune function. Specifically, we found that circulating carotenoid levels prior to an immune challenge positively predicted primary antibody production to KLH, and that a greater decrease in circulating carotenoid levels over the course of the immune response was associated with a greater primary antibody response. This relationship is similar to previous correlational results demonstrated with adult male mallards (Butler and McGraw, 2012a; Peters et al., 2004), and is consistent with an immunostimulatory role of carotenoids, at least regarding antibody production. However, both we (Butler and McGraw, 2012a) and others (Biard et al., 2009) have found the opposite relationship for other immune metrics, particularly PHA-induced swelling (lower levels of carotenoids prior to an immune challenge and greater increase in circulating levels during the immune challenge were

associated with a more robust PHA-induced response). These differences highlight both (a) the need for more in-depth investigations into the physiological role of carotenoids in specific immune responses (e.g. Biard et al., 2009; Takahashi et al., 2011), and (b) the importance of studying multiple immune metrics, as they may not be correlated, or even negatively correlated (Salvante, 2006). Additionally, while a more robust immune response is frequently interpreted as an indicator of a high-quality individual, there are negative consequences of an overly responsive immune system (e.g. auto-immunity, wasted energy), and a higher response is not always adaptive [see Graham et al. (Graham et al., 2011) for a thorough discussion]. To put these results in a naturalistic context, further work is required to investigate how natural variation in antibody production ability relates to survival and reproductive success in the wild.

In addition to finding links between carotenoid physiology and antibody production, we also found an inverse relationship between biliverdin concentration and ability to produce antibodies. To our knowledge, we are the first to report data linking antibody and biliverdin production in any species, and we detected an inverse relationship; thus, investigating the relationships between biliverdin and antibody production could be a fruitful area for future research, including testing whether this finding is simply correlational or whether there is a mechanistic underpinning. Such mechanistic links are possible, as work with mice has demonstrated that bilirubin (a reduced form of biliverdin) has powerful T-cell immunomodulatory abilities (Liu et al., 2008). However, it is important to note that we sampled biliverdin concentration in the bile, while antibody production occurs in the serum. While it is possible that biliverdin concentration in the bile is correlated with concentration in the serum, we lack the data to test this relationship, and there can be tissue-specific differences in biliverdin production in birds (Zhao et al., 2006), highlighting the need for much more research regarding the avian physiological function of biliverdin beyond eggshell pigmentation.

We also found that levels of NO prior to any immune challenge positively predicted PHA-induced swelling and negatively predicted NO response. Both NO production (Allen, 1997) and elements of the PHA response (Martin et al., 2006) are involved in inflammation, and thus similar physiological pathways may be responsible for this correlation (e.g. higher levels of inducible nitrate synthase) (Takahashi et al., 2011). It is well established that NO production occurs in response to multiple immunological challenges (Bogdan et al., 2000), but the importance of circulating NO levels prior to an immune challenge is more equivocal. Circulating levels are systemically higher in athletes (Banfi et al., 2006) and during periods of infection in chickens (Lillehoj and Li, 2004). The negative relationship that we uncovered between initial NO levels and NO response has previously been identified in common eider ducks (*Somateria mollissima*) (Bourgeon et al., 2007), suggesting that initial NO levels may generally be predictive of an immune challenge-induced NO response. This phenomenon may be due to functional reasons, such as the importance of limiting oxidative damage (Bourgeon et al., 2007), and/or mechanistic reasons, such as a physiological limit to circulating NO levels. However, this systemic metric of pre-immune circulating NO levels also positively predicted the degree of the localized PHA response. Thus, individuals that naturally circulate higher levels of NO may have lower systemic NO responses but greater PHA-induced swelling responses, which is in accordance with the relationship between NO and inflammation.

Based on our data, the carotenoid-pigmented beak of female mallards does not signal an ability to respond to immunological stimuli, in contrast to carotenoid-pigmented patches in fishes (Clotfelter et al., 2007), songbirds (McGraw and Ardia, 2003) and even male mallards (Peters et al., 2004; Butler and McGraw, 2012a). Thus, the physiological control over carotenoid deposition, sequestration and removal in the female mallard beak appears to be uncoupled from multiple immune function-associated physiological parameters. Because female, but not male, mallards show this relationship, future work on the physiological mechanisms underlying bare-part carotenoid-pigmented coloration could investigate the different mechanisms between the sexes within this species. However, the question regarding the function of carotenoid-based beak coloration in female mallards persists. Multiple possible signaling scenarios exist, including signaling species or individual identity (Lorenzo Pérez-Rodríguez, 2008), or dominance status (Murphy et al., 2009). In light of the relationship between biliverdin concentration and beak coloration, honest signaling of other, non-immunological traits (e.g. antioxidant availability) (Bertrand et al., 2006; Hartley and Kennedy, 2004) is possible, if not likely. Alternatively, females may produce a similar (but not identical) trait simply because of their genetic similarity to males of the same species (Kraaijeveld et al., 2007).

In summary, we have demonstrated that supplemental carotenoid access during development does not affect adult coloration or immune function in female mallards, but that carotenoid physiology at adulthood is associated with antibody production even in a species that has a carotenoid-pigmented integument with no known signaling function. Similarly, we found that bile pigment concentration is negatively associated with antibody production, which is a relationship that has not been previously described in animals. Lastly, initial levels of the oxidative molecule NO positively predicted PHA-induced swelling. Together, these results point to a more nuanced relationship between immune function and antioxidants such as carotenoids and bile pigments, with differing relationships evident among different molecule types. Further work that links specific types of antioxidants to precise immune metrics will help to disentangle the myriad ways that antioxidant physiology interacts with immune function.

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AUTHOR CONTRIBUTIONS

M.W.B. and K.J.M. made significant and substantial contributions toward the conception, design and interpretation of the findings of this study, the drafting and revising of the article, and M.W.B. toward the execution of the study.

COMPETING INTERESTS

No competing interests declared.

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REFERENCES

Allen, P. C. (1997). Nitric oxide production during *Eimeria tenella* infections in chickens. *Poult. Sci.* **76**, 810-813.

Baeta, R., Faivre, B., Motreuil, S., Gaillard, M. and Moreau, J. (2008). Carotenoid trade-off between parasitic resistance and sexual display: an experimental study in the blackbird (*Turdus merula*). *Proc. Biol. Sci.* **275**, 427-434.

Banfi, G., Malavazos, A., Iorio, E., Dolci, A., Doneda, L., Verna, R. and Corsi, M. M. (2006). Plasma oxidative stress biomarkers, nitric oxide and heat shock protein 70 in trained elite soccer players. *Eur. J. Appl. Physiol.* **96**, 483-486.
Berthouly, A., Helfenstein, F., Tanner, M. and Richner, H. (2008). Sex-related effects of maternal egg investment on offspring in relation to carotenoid availability in the great tit. *J. Anim. Ecol.* **77**, 74-82.
Bertrand, S., Faivre, B. and Sorci, G. (2006). Do carotenoid-based sexual traits signal the availability of non-pigmentary antioxidants? *J. Exp. Biol.* **209**, 4414-4419.
Biard, C., Hardy, C., Motreuil, S. and Moreau, J. (2009). Dynamics of PHA-induced immune response and plasma carotenoids in birds: should we have a closer look? *J. Exp. Biol.* **212**, 1336-1343.
Blount, J. D. Surai, P. F., Nager, R. G., Houston, D. C., Møller, A. P., Trewby, M. L. and Kennedy, M. W. (2002). Carotenoids and egg quality in the lesser blackbacked gull *Larus fuscus*: a supplemental feeding study of maternal effects. *Proc. Biol. Sci.*, **269**, 29–36.
Blount, J. D. Metcalfe, N. B., Arnold, K. E., Surai, P. F., Devevey, G. L. and Monaghan, P. (2003). Neonatal nutrition, adult antioxidant defences and sexual attractiveness in the zebra finch. *Proc. Biol. Sci.*, **270**, 1691–1696.
Blount, J. D. Houston, D. C., Surai, P. F. and Møller, A. P. (2004). Egg-laying capacity is limited by carotenoid pigment availability in wild gulls *Larus fuscus*. *Proc. Biol. Sci.*, **271 Suppl.**, S79–S81.
Bogdan, C., Röllinghoff, M. and Diefenbach, A. (2000). Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. *Curr. Opin. Immunol.* **12**, 64-76.
Bourgeon, S., Raclot, T., Le Maho, Y., Ricquier, D. and Criscuolo, F. (2007). Innate immunity, assessed by plasma NO measurements, is not suppressed during the incubation fast in eiders. *Dev. Comp. Immunol.* **31**, 720-728.
Butler, M. W. and McGraw, K. J. (2009). Indoor housing during development affects moult, carotenoid circulation and beak colouration of mallard ducks (*Anas platyrhynchos*). *Avian Biol. Res.* **2**, 203-211.
Butler, M. W. and McGraw, K. J. (2010). Relationships between dietary carotenoids, body tissue carotenoids, parasite burden, and health state in wild mallard (*Anas platyrhynchos*) ducklings. *Arch. Biochem. Biophys.* **504**, 154-160.
Butler, M. W. and McGraw, K. J. (2011). Past or present? Relative contributions of developmental and adult conditions to adult immune function and coloration in mallard ducks (*Anas platyrhynchos*). *J. Comp. Physiol. B* **181**, 551-563.
Butler, M. W. and McGraw, K. J. (2012a). Developmental immune history affects adult immune function but not carotenoid-based ornamentation in mallard ducks. *Funct. Ecol.* **26**, 406-415.
Butler, M. W. and McGraw, K. J. (2012b). Differential effects of early- and late-life access to carotenoids on adult immune function and ornamentation in mallard ducks (*Anas platyrhynchos*). *PLoS ONE* **7**, e38043.
Butler, M. W., Leppert, L. L. and Dufty, A. M., Jr (2010). Effects of small increases in corticosterone levels on morphology, immune function, and feather development. *Physiol. Biochem. Zool.* **83**, 78-86.
Butler, M. W., Toomey, M. B. and McGraw, K. J. (2011). How many color metrics do we need? Evaluating how different color-scoring procedures explain carotenoid pigment content in avian bare-part and plumage ornaments. *Behav. Ecol. Sociobiol.* **65**, 401-413.
Chew, B. P. (1996). Importance of antioxidant vitamins in immunity and health in animals. *Anim. Feed Sci. Technol.* **59**, 103-114.
Clotfelter, E. D., Ardia, D. R. and McGraw, K. J. (2007). Red fish, blue fish: trade-offs between pigmentation and immunity in *Betta splendens*. *Behav. Ecol.* **18**, 1139-1145.
Cohen, A. A., Martin, L. B., Wingfield, J. C., McWilliams, S. R. and Dunne, J. A. (2012). Physiological regulatory networks: ecological roles and evolutionary constraints. *Trends Ecol. Evol.* **27**, 428-435.
Cotter, S. C., Simpson, S. J., Raubenheimer, D. and Wilson, K. (2011). Macronutrient balance mediates trade-offs between immune function and life history traits. *Funct. Ecol.* **25**, 186-198.
Cunningham-Rundles, S., McNeeley, D. F. and Moon, A. (2005). Mechanisms of nutrient modulation of the immune response. *J. Allergy Clin. Immunol.* **115**, 1119-1128, quiz 1129.
Di Mascio, P., Devasagayam, T. P. A., Kaiser, S. and Sies, H. (1990). Carotenoids, tocopherols and thiols as biological singlet molecular oxygen quenchers. *Biochem. Soc. Trans.* **18**, 1054-1056.
Drilling, N., Titman, R. and Mckinney, F. (2002). Mallard (*Anas platyrhynchos*). In *The Birds of North America Online*. Ithaca, NY: Cornell Laboratory of Ornithology.
Fellous, S. and Lazzaro, B. P. (2010). Larval food quality affects adult (but not larval) immune gene expression independent of effects on general condition. *Mol. Ecol.* **19**, 1462-1468.
Flatt, T., Heyland, A., Rus, F., Porpiglia, E., Sherlock, C., Yamamoto, R., Garbuzov, A., Palli, S. R., Tatar, M. and Silverman, N. (2008). Hormonal regulation of the humoral innate immune response in *Drosophila melanogaster*. *J. Exp. Biol.* **211**, 2712-2724.
French, S. S., DeNardo, D. F. and Moore, M. C. (2007). Trade-offs between the reproductive and immune systems: facultative responses to resources or obligate responses to reproduction? *Am. Nat.* **170**, 79-89.
Galic, M. A., Spencer, S. J., Mouihate, A. and Pittman, Q. J. (2009). Postnatal programming of the innate immune response. *Integr. Comp. Biol.* **49**, 237-245.
Gladbach, A., Gladbach, D. J., Kempenaers, B. and Quillfeldt, P. (2010). Female-specific colouration, carotenoids and reproductive investment in a dichromatic species, the upland goose *Chloephaga picta leucoptera*. *Behav. Ecol. Sociobiol.* **64**, 1779-1789.
Graham, A. L., Shuker, D. M., Pollitt, L. C., Auld, S. K. J. R., Wilson, A. J. and Little, T. J. (2011). Fitness consequences of immune responses: strengthening the empirical framework for ecoimmunology. *Funct. Ecol.* **25**, 5-17.
Hartley, R. C. and Kennedy, M. W. (2004). Are carotenoids a red herring in sexual display? *Trends Ecol. Evol.* **19**, 353-354.
Hill, G. E., Hood, W. R. and Huggins, K. (2009). A multifactorial test of the effects of carotenoid access, food intake and parasite load on the production of ornamental feathers and bill coloration in American goldfinches. *J. Exp. Biol.* **212**, 1225-1233.
Hõrak, P., Saks, L., Zilmer, M., Karu, U. and Zilmer, K. (2007). Do dietary antioxidants alleviate the cost of immune activation? An experiment with greenfinches. *Am. Nat.* **170**, 625-635.
Jacot, A., Scheuber, H., Kurtz, J. and Brinkhof, M. W. G. (2005). Juvenile immune system activation induces a costly upregulation of adult immunity in field crickets *Gryllus campestris*. *Proc. Biol. Sci.* **272**, 63-69.
Karu, U., Saks, L. and Hõrak, P. (2007). Carotenoid coloration in greenfinches is individually consistent irrespective of foraging ability. *Physiol. Biochem. Zool.* **80**, 663-670.
Kaur, H., Hughes, M. N., Green, C. J., Naughton, P., Foresti, R. and Motterlini, R. (2003). Interaction of bilirubin and biliverdin with reactive nitrogen species. *FEBS Lett.* **543**, 113-119.
Koutsos, E. A., Clifford, A. J., Calvert, C. C. and Klasing, K. C. (2003). Maternal carotenoid status modifies the incorporation of dietary carotenoids into immune tissues of growing chickens (*Gallus gallus domesticus*). *J. Nutr.* **133**, 1132-1138.
Koutsos, E. A., García López, J. C. and Klasing, K. C. (2007). Maternal and dietary carotenoids interactively affect cutaneous basophil responses in growing chickens (*Gallus gallus domesticus*). *Comp. Biochem. Physiol.* **147B**, 87-92.
Kraaijeveld, K., Kraaijeveldsmit, F. and Komdeur, J. (2007). The evolution of mutual ornamentation. *Anim. Behav.* **74**, 657-677.
Lessells, C. and Boag, P. T. (1987). Unrepeatable repeatabilities: a common mistake. *Auk* **104**, 116-121.
Lillehoj, H. S. and Li, G. (2004). Nitric oxide production by macrophages stimulated with *Coccidia sporozoites*, lipopolysaccharide, or interferon-gamma, and its dynamic changes in SC and TK strains of chickens infected with *Eimeria tenella*. *Avian Dis.* **48**, 244-253.
Lin, H.-W., Chang, T.-J., Yang, D.-J., Chen, Y.-C., Wang, M. and Chang, Y.-Y. (2012). Regulation of virus-induced inflammatory response by β-carotene in RAW264.7 cells. *Food Chem.* **134**, 2169-2175.
Liu, Y., Li, P., Lu, J., Xiong, W., Oger, J., Tetzlaff, W. and Cynader, M. (2008). Bilirubin possesses powerful immunomodulatory activity and suppresses experimental autoimmune encephalomyelitis. *J. Immunol.* **181**, 1887-1897.
Mancuso, C., Barone, E., Guido, P., Miceli, F., Di Domenico, F., Perluigi, M., Santangelo, R. and Preziosi, P. (2012). Inhibition of lipid peroxidation and protein oxidation by endogenous and exogenous antioxidants in rat brain microsomes in vitro. *Neurosci. Lett.* **518**, 101-105.
Martin, L. B., Han, P., Lewittes, J., Kuhlman, J. R., Klasing, K. C. and Wikelski, M. (2006). Phytohemagglutinin-induced skin swelling in birds: histological support for a classic immunoecological technique. *Funct. Ecol.* **20**, 290-299.
Martin, L. B., Hawley, D. M. and Ardia, D. R. (2011). An introduction to ecological immunology. *Funct. Ecol.* **25**, 1-4.
Mateo, R., Castells, G., Green, A. J., Godoy, C. and Cristòfol, C. (2004). Determination of porphyrins and biliverdin in bile and excreta of birds by a single liquid chromatography-ultraviolet detection analysis. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **810**, 305-311.
McGraw, K. (2005). The antioxidant function of many animal pigments: are there consistent health benefits of sexually selected colourants? *Anim. Behav.* **69**, 757-764.
McGraw, K. (2006). Mechanics of carotenoid-based coloration. In *Bird Coloration: Volume I, Mechanisms and Measurements* (ed. G. E. Hill and K. J. McGraw), pp. 177-242. Cambridge, MA: Harvard University Press.
McGraw, K. J. and Ardia, D. R. (2003). Carotenoids, immunocompetence, and the information content of sexual colors: an experimental test. *Am. Nat.* **162**, 704-712.
McGraw, K. J., Hill, G. E., Stradi, R. and Parker, R. S. (2002). The effect of dietary carotenoid access on sexual dichromatism and plumage pigment composition in the American goldfinch. *Comp. Biochem. Physiol.* **131B**, 261-269.
McGraw, K. J., Adkins-Regan, E. and Parker, R. S. (2005). Maternally derived carotenoid pigments affect offspring survival, sex ratio, and sexual attractiveness in a colorful songbird. *Naturwissenschaften* **92**, 375-380.
McGraw, K. J., Tourville, E. A. and Butler, M. W. (2008). A quantitative comparison of the commonly used methods for extracting carotenoids from avian plasma. *Behav. Ecol. Sociobiol.* **62**, 1991-2002.
Mizobe, M., Kondo, F., Kumamoto, K., Kanda, Y. and Seguchi, H. (1997). High-performance liquid chromatographic analysis of bilirubin and biliverdin from jaundiced broilers. *J. Vet. Med. Sci.* **59**, 677-680.
Monaghan, P. (2008). Early growth conditions, phenotypic development and environmental change. *Philos. Trans. R. Soc. B* **363**, 1635-1645.
Montgomerie, R. (2008). Clr: Colour Analysis Programs v1.05. Queen's University, Kingston, Canada. http://post.queensu.ca/~mont/color/analyze.html
Morales, J., Velando, A. and Torres, R. (2011). Biliverdin-based egg coloration is enhanced by carotenoid supplementation. *Behav. Ecol. Sociobiol.* **65**, 197-203.
Mougeot, F., Martínez-Padilla, J., Blount, J. D., Pérez-Rodríguez, L., Webster, L. M. I. and Piertney, S. B. (2010). Oxidative stress and the effect of parasites on a carotenoid-based ornament. *J. Exp. Biol.* **213**, 400-407.
Murphy, T. G., Rosenthal, M. F., Montgomerie, R. and Tarvin, K. a. (2009). Female American goldfinches use carotenoid-based bill coloration to signal status. *Behav. Ecol.* **20**, 1348-1355.
Nakagami, T., Toyomura, K., Kinoshita, T. and Morisawa, S. (1993). A beneficial role of bile pigments as an endogenous tissue protector: anti-complement effects of biliverdin and conjugated bilirubin. *Biochim. Biophys. Acta* **1158**, 189-193.
Nakagawa, S. and Cuthill, I. C. (2007). Effect size, confidence interval and statistical significance: a practical guide for biologists. *Biol. Rev. Camb. Philos. Soc.* **82**, 591-605.
Okai, Y. and Higashi-Okai, K. (1996). Possible immunomodulating activities of carotenoids in in vitro cell culture experiments. *Int. J. Immunopharmacol.* **18**, 753-758.
Omland, K. E. (1996a). Female mallard mating preferences for multiple male ornaments I. Natural variation. *Behav. Ecol. Sociobiol.* **39**, 353-360.
Omland, K. E. (1996b). Female mallard mating preferences for multiple male ornaments II. Experimental variation. *Behav. Ecol. Sociobiol.* **39**, 361-366.

Pérez-Rodríguez, L. (2008). Carotenoid-based ornamentation as a dynamic but consistent individual trait. *Behav. Ecol. Sociobiol.* **62**, 995-1005.
Peters, A., Delhey, K., Denk, A. G. and Kempenaers, B. (2004). Trade-offs between immune investment and sexual signaling in male mallards. *Am. Nat.* **164**, 51-59.
Ruiz, M., French, S. S., Demas, G. E. and Martins, E. P. (2010). Food supplementation and testosterone interact to influence reproductive behavior and immune function in Sceloporus graciosus. *Horm. Behav.* **57**, 134-139.
Salvante, K. G. (2006). Techniques for studying integrated immune function in birds. *Auk* **123**, 575-586.
Sild, E. and Hõrak, P. (2009). Nitric oxide production: an easily measurable condition index for vertebrates. *Behav. Ecol. Sociobiol.* **63**, 959-966.
Smits, J., Bortolotti, G. R. and Tella, J. L. (1999). Simplifying the phytohaemagglutinin skin-testing technique in studies of avian immunocompetence. *Funct. Ecol.* **13**, 567-572.
Star, L., Nieuwland, M. G. B., Kemp, B. and Parmentier, H. K. (2007). Effect of single or combined climatic and hygienic stress on natural and specific humoral immune competence in four layer lines. *Poult. Sci.* **86**, 1894-1903.
Star, L., Juul-Madsen, H. R., Decuypere, E., Nieuwland, M. G. B., de Vries Reilingh, G., van den Brand, H., Kemp, B. and Parmentier, H. K. (2009). Effect of early life thermal conditioning and immune challenge on thermotolerance and humoral immune competence in adult laying hens. *Poult. Sci.* **88**, 2253-2261.
Surai, P., Speake, B. and Sparks, N. (2001). Carotenoids in avian nutrition and embryonic development. 1. Absorption, availability and levels in plasma and egg yolk. *Jpn. Poult. Sci.* **38**, 1-27.
Svensson, P. A. and Wong, B. B. M. (2011). Carotenoid-based signals in behavioural ecology: a review. *Behaviour* **148**, 131-189.
Takahashi, K., Takimoto, T., Sato, K. and Akiba, Y. (2011). Effect of dietary supplementation of astaxanthin from *Phaffia rhodozyma* on lipopolysaccharide-induced early inflammatory responses in male broiler chickens (*Gallus gallus*) fed a corn-enriched diet. *Anim. Sci. J.* **82**, 753-758.
Trams, E. G. (1969). Carotenoid transport in the plasma of the scarlet ibis (*Eudocimus ruber*). *Comp. Biochem. Physiol.* **28**, 1177-1184.
Vanhoutteghem, A., Londero, T., Ghinea, N. and Djian, P. (2004). Serial cultivation of chicken keratinocytes, a composite cell type that accumulates lipids and synthesizes a novel beta-keratin. *Differentiation* **72**, 123-137.
Vesely, M. D., Kershaw, M. H., Schreiber, R. D. and Smyth, M. J. (2011). Natural innate and adaptive immunity to cancer. *Annu. Rev. Immunol.* **29**, 235-271.
Zhao, R., Xu, G. Y., Liu, Z. Z., Li, J. Y. and Yang, N. (2006). A study on eggshell pigmentation: biliverdin in blue-shelled chickens. *Poult. Sci.* **85**, 546-549.
Zheng, C. L., Ohki, K., Hossain, A., Kukita, A., Satoh, T. and Kohashi, O. (2003). Complete Freund's adjuvant promotes the increases of IFN-gamma and nitric oxide in suppressing chronic arthritis induced by pristane. *Inflammation* **27**, 247-255.