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

Running headline: Carotenoids, bile pigments, and immunity

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Abstract

Immune function can be modulated by multiple physiological factors, including nutrition and reproductive state. Because these factors can vary throughout an individual’s lifetime due to environmental conditions (e.g., nutrition) or life-history stage (e.g., adult reproduction), 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 at 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 change 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 at 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.

Keywords
Anas platyrhynchos, Biliverdin, Carotenoids, Developmental plasticity, Follicular Development, Immunity, Nitric Oxide
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 (Gallic 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 due to their diverse effects on many components of the immune system. Specifically, increased carotenoid intake and accumulation has 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 (Hôrak 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 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, due to 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 due to the deposition of biliverdin, a pigment found predominately in the 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 if access to dietary carotenoids at different points during development affected 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) from hatch and supplemented their diets with carotenoids during three main developmental periods that correspond to different carotenoid physiology states (see 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 (authors’ unpubl. data). Although females prefer males with more yellow beaks (Omland, 1996a, 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 at adulthood. Female mallard ducks lay relatively large clutches (N typically = 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. Due to 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., alpha-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., 2010), 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

We acquired 46 one-day-old female ducklings of unknown relatedness from Metzer Farms (Gonzales, CA) in December 2009 and housed them as in Butler and McGraw (2009, 2012). Briefly,
ducklings were reared indoors in randomly selected groups of five ducklings per cage (60 x 60 x 60 cm) until they were two weeks old, three per cage until they were four weeks old, and two per cage until they were seven weeks old, at which point all birds were moved outside and individually housed to allow for normal sexual maturation (Butler and McGraw, 2009, 2011). Light:dark regime was 13L:11D while ducklings were housed indoors, and natural photoperiod thereafter (10.5L:13.5D at 7 weeks old to 13.5L:10.5D 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, 2012), 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). This 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 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 (Vanhoutryteghem et al., 2004) required for pigment deposition (McGraw et al., 2002). We prepared diets by mixing a base diet of dry food (Mazuri Waterfowl Starter: Richmond, IN, USA, weeks 0-7; Mazuri Waterfowl Maintenance thereafter) with ORO-GLO dry pigmenter (2% carotenoids by mass, predominately lutein; Kemin AgriFoods North America, Inc., Des Moines, Iowa, USA) suspended in sunflower oil to achieve concentrations of 25 µg/g 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 g 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 hrs 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 five largest follicles in each ovary to the nearest 0.01 mm.

**Adult immune metrics**

When individuals were 19 weeks old (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 0: D0 to D10; Butler and McGraw, 2011, 2012). 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) suspended in 0.1 mL of sterile phosphate buffered saline (PBS; Fisher BP399). Twenty-four hrs later (D1; mean: 23 hrs, 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 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) and 250 µg of keyhole limpet hemocyanin (KLH; Sigma H7017) suspended in 250 µl of sterile ddH2O (Butler and McGraw, 2012). Six days later (D6), we intra-abdominally administered a booster injection consisting of 250 µg of KLH suspended in 250 µl of sterile ddH2O emulsified in 250 µl of Incomplete Freund’s Adjuvant (IFA; Sigma F5506). By collecting blood on D0, D6 and D10 (four days after D6), we were able to quantify primary and secondary humoral immune response to this novel antigen. Specifically, we followed previously published ELISA protocols (Butler and McGraw, 2011, 2012) to quantify KLH-specific antibody production and calculated 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 KLH in sodium carbonate buffer, blocked the wells with 100 µl blocking buffer (PBS with 1%
bovine serum albumin; Sigma B4287) with 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 4ºC, we added 80 µl of HRP-conjugated polyclonal secondary antibody (Bethyl Laboratories, A140-110P) 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 0.5M H2SO4. Within 10 min, we measured absorbance of each well at λ = 450 nm using an iMark Microplate Reader (Bio-Rad Laboratories, UK), with greater absorbances corresponding to a greater IgG titer (see Butler and McGraw (2012) for a more detailed methodology).

Both PHA (Sild and Hõrak, 2009) and CFA (Zheng et al., 2003) induce systemic increases in nitric oxide (NO) levels, with a greater increase associated with a more robust oxidative burst in the immune response. Following 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, 2012) and followed Sild & Hõrak (2009) to quantify circulating NO levels 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 R² = 0.99.

Carotenoid titer and coloration assessment
We quantified plasma carotenoid content at all ten time points for which we had collected blood samples (at the beginning and end of the EARLY, MIDDLE, and LATE developmental periods, adult immune assessment periods D0, D1, D6, and D10). 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 (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 (MWB, pers. obs.; J. Metzer, pers. comm.). 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 $\lambda = 300-700$
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 ($r = -0.49478$, $P = 0.05$), 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 the shell gland, and gall bladder biliverdin concentration is not related
to eggshell coloration (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 protocol from 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
(3N 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 330-450 nm in a
Beckman Coulter DU 52 UV/Vis spectrophotometer (Indianapolis, IN). 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 analysis of variance (rmANOVA) 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 analysis of covariance (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 ANOVAs 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 if adult beak color prior to an immune challenge was predictive of immune response,
we used general linear models (GLM) 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 if 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), all variables were either normally distributed or log-transformed to achieve normality
(primary and secondary KLH antibody response, pre-immune NO levels, NO response, and bile
pigment concentration), and post-hoc tests were performed by comparing Least Squares Means
(LSMeans). Effect sizes are calculated as Hedges’s $g$, which is commonly referred to as Cohen’s $d$ in
the literature (Nakagawa and Cuthill, 2007; Table 1).

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 after supplementation (all $P < 0.05$; Fig. 2). These differences then disappeared 2-3 weeks
after cessation of dietary treatment 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 ($F_{3,41} = 1.68, P = 0.19$; effect sizes for all treatments between 0.65-0.90). 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 was 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 = 4.70, had 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 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 PC value. A Fisher’s Exact test showed that dietary carotenoid treatment did not affect rank of follicular development ($\chi^2_6 = 0.001, P = 1.0$).

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

Neither beak saturation (S1B) nor change in beak saturation during immune assessment predicted primary or secondary KLH response, PHA-induced swelling, or NO response (all $F_{1,44} < 1.90$, all $P > 0.17$), with the following exceptions. 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 ($F_{1,44} = 3.56, all P = 0.066$).

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 level over the time course of the immune assessment was a covariate. Change in circulating carotenoid level 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

Due to 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 ($F_{1,23} = 7.06$, $P = 0.014$) 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; pilot work, 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, 2012), 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 were 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 three weeks in order to test if 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 during the first several weeks of neonatal development (*in ovo* or post-hatch; Monaghan, 2008).

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 Butler and McGraw (2012) and Biard et al. (2009) 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., autoimmunity, wasted energy), and a higher response is not always adaptive (see 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 if this finding is simply correlational or if 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 in circulating NO levels. However, this systemic metric of pre-immune circulating NO levels also positively predicted 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 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, 2007), 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) are possible, if not likely. Alternatively, females may produce a similar (but not identical) trait simply due to their genetic similarity to males of the same species (Kraaijeveld et al., 2007).
In summary, we demonstrated that supplemental carotenoid access during development did 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 carotenoid-pigmented integument with no known signaling function. Similarly, we found that bile pigment concentration was 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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Figure 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 (ADULT). Both wing-web thickness and plasma nitric oxide concentration (B) were measured prior to PHA injection (Day 0) as well as 24 hours later (Day 1). 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 four days later (Day 10). Bill coloration was quantified during every point of the ADULT period. Bile samples and ovaries were collected from euthanized birds at 22.5 weeks of age.
Figure 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 three weeks. The exception was for birds that received carotenoid supplementation during the EARLY period; these individuals continued to circulate higher levels of carotenoids for two, but not five, weeks after treatment cessation.
Figure 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. 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.
Figure 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.
Figure 5. Nitric oxide (NO) response (change in circulating NO levels) to an 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 ($F_{1,43} = 7.84, P = 0.0076$).