Developmental Plasticity:

The Influence of Neonatal Diet and Immune Challenges on Carotenoid-Based Ornamental Coloration and Adult Immune Function in Mallard Ducks

by

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ABSTRACT

Conditions during development can shape the expression of traits at adulthood, a phenomenon called developmental plasticity. In this context, factors such as nutrition or health state during development can affect current and subsequent physiology, body size, brain structure, ornamentation, and behavior. However, many of the links between developmental and adult phenotype are poorly understood. I performed a series of experiments using a common molecular currency – carotenoid pigments – to track somatic and reproductive investments through development and into adulthood. Carotenoids are red, orange, or yellow pigments that: (a) animals must acquire from their diets, (b) can be physiologically beneficial, acting as antioxidants or immunostimulants, and (c) color the sexually attractive features (e.g., feathers, scales) of many animals. I studied how carotenoid nutrition and immune challenges during ontogeny impacted ornamental coloration and immune function of adult male mallard ducks (*Anas platyrhynchos*). Male mallards use carotenoids to pigment their yellow beak, and males with more beaks that are more yellow are preferred as mates, have increased immune function, and have higher quality sperm. In my dissertation work, I established a natural context for the role that carotenoids and body condition play in the formation of the adult phenotype and examined how early-life experiences, including immune challenges and dietary access to carotenoids, affect adult immune function and ornamental coloration. Evidence from mallard ducklings in the field showed that variation in circulating carotenoid levels at hatch are likely driven by maternal allocation of carotenoids, but that

carotenoid physiology shifts during the subsequent few weeks to reflect individual foraging habits. In the lab, adult beak color expression and immune function were more tightly correlated with body condition during growth than body condition during subsequent stages of development or adulthood. Immune challenges during development affected adult immune function and interacted with carotenoid physiology during adulthood, but did not affect adult beak coloration. Dietary access to carotenoids during development, but not adulthood, also affected adult immune function. Taken together, these results highlight the importance of the developmental stage in shaping certain survival-related traits (i.e., immune function), and lead to further questions regarding the development of ornamental traits.

For Katherine, Bronx, and Bisquit, for making me eager to bike home every night, away from a job I love.

With a deep respect for the ducks that made this work possible; the biological knowledge and humorous moments that they provided are always to be remembered.

In memory of loved ones lost, Butler and Pullen alike. I am a summation of my experiences, and this milestone in my life is a function of the guidance and love they provided in life and their examples that I aspire toward still.

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PREFACE

INTRODUCTION

In *The Origin of Species*, Darwin (1859) first described multiple differences between the sexes, including several classic examples of sexual selection such as bird song, deer antlers, beetle weapons, and the "gorgeous plumage" of some male birds. He attributed these differences to intra-sexual conflict, with not survival, but successful reproduction as an advantage to the winner. Indeed, a variety of exaggerated traits have been linked to reproductive success. Some of the most common examples include armaments, which allow one sex, typically males, to engage in threat displays or physical altercations with other males to secure access to female mates (e.g., deer antlers, Plard et al. 2011), behavioral displays or vocalizations that increase the likelihood of mating (e.g., anuran calling; Wilczynski and Lynch 2011), and ornaments, or physical structures that vary in some attribute such as size or color and can affect female mate choice (e.g., swordtail tail length; Rosenthal and Evans 1998).

A casual observer will ask how such traits may be under selection, because if they are advantageous, then all individuals in the species should express the trait. In fact, many exaggerated traits are differentially expressed within the population, often with continuous variation (though there are some instances of intrasexual bimorphisms; Hurtado-Gonzales and Uy 2010). There are multiple reasons for these differences. Inter-individual differences in ornament/armament size may simply be a function of allometry; larger animals are able to support larger exaggerated traits (see Plard et al. 2011). Further work

has focused specifically on the expression of ornaments. Zahavi (1975) famously postulated the handicap principle, which states that sexual signals are differentially costly to produce or maintain, and thus only individuals that can survive in spite of this handicap qualify as the highest-quality mates. The specific costs can vary, and may require a high amount of energetic investment (Hasselquist and Bensch 2008), make the individual more susceptible to predation (Endler 1980), or be a target of intra-sexual aggression (van Dongen and Mulder 2007). A specific component of this theory was put forth by Hamilton and Zuk (1982), which stated that parasites in particular can drive the evolution of ornaments, and only individuals that can cope with a high degree of parasitism can express ornaments of high quality. The idea that ornaments can reveal individual quality has expanded to include investigations into other markers of quality, and there is now evidence that ornaments can signal an individual's foraging ability (Karino et al. 2007), parental effort (Senar et al. 2002), and immune function (Peters 2004a), all of which are traits that would allow a female to successfully raise more or higher-quality offspring. Thus, differences in signal expression should be costly if they are to honestly indicate quality, and usually cannot be expressed to the highest degree without a large investment of time, energy, or other limiting resource.

Developmental plasticity

To date, the majority of studies on animal ornamentation has focused on sexually mature adult animals and their concurrent aspects of "quality." For

example, degree of trait exaggeration is tied to an adult's energy reserves (Svensson and Merilä 1998), immune function (Mougeot 2008), or territory quality (Bostrom and Ritchison 2006). However, animals are a product of their genes and their environment, both during adulthood *and development*; in other words, an animal's adult phenotype depends not only upon its current condition, but also the conditions experienced earlier in life (i.e., *in ovo*, as neonates and growing/maturing organisms). This idea of conditions during early life shaping the phenotype of adults is termed developmental plasticity.

Developmental plasticity is a relatively ancestral characteristic in animals. Many insects, including cockroaches (Barrett et al. 2009), damselflies (De Block and Stoks (2008), and butterflies (Saastamoinen et al. 2010), exhibit aspects of developmental plasticity in a variety of morphological or immunological traits. Similar patterns are also found in a variety of vertebrates, including amphibians (Relyea 2002), reptiles (Mugabo et al. 2010), birds (Monaghan 2008), and mammals (Devevey et al. 2010). In many of these studies, developmental plasticity has been demonstrated for naturally selected traits. However, investigators have only recently begun to examine if and how developmental conditions affect sexually selected trait expression in adults.

Nowicki et al. (1998) popularized the framework of developmental plasticity in the context of sexual selection to test if early-life nutrition affected development of neurological structures associated with song production. They found that swamp sparrows (*Melospiza georgiana*) that were food-stressed during development took longer to learn songs and possessed less well-developed song

centers in the brain (Nowicki et al. 2002). Subsequent work with pheasants (*Phasianus colchicus*) showed that nutrition during development can affect ornament color (Ohlsson et al. 2002) at adulthood. However, these studies utilize major changes in either amount of food or protein levels in the food; manipulation of these relatively coarse variables affects nearly every aspect of phenotype due to the ubiquitous utility of protein and energy. In order to examine developmental plasticity of ornamentation, what is needed is a more focused study system that measures the role of discrete units that are directly used in ornamentation.

Carotenoid-based ornamentation

Carotenoids are a group of molecules that are produced in photosynthetic organisms and that vertebrates must acquire from their diet (McGraw 2006). These molecules act as pigments, and are responsible for the red, orange, or yellow coloration of many fish (Grether et al. 2008), lizards (Cote et al. 2010), and birds (McGraw 2006), and their sexually selected bare parts and feathers. Carotenoids also have a number of physiologically beneficial properties, meaning that animals that sequester carotenoids to the integument lose the ability to internally use these molecules. Carotenoids can serve as antioxidants (Woodall et al. 1997), although their biological utility in this regard has been called into question (Costantini and Møller 2008, Cohen and McGraw 2009). There is stronger support for their role in supporting immune function. Correlational studies have linked carotenoid levels to antibody production both *in vitro* (Okai and Higashi-Okai 1996) and *in vivo* (Peters et al. 2004a), to the strength of the

oxidative burst (Macedo et al. 2010), and to the strength of the cutaneous immune response to phytohemagglutinin (Biard et al. 2009). They are also utilized in other systems, including photoprotection (Bouilly-Gauthier et al. 2010) and tuning of photoreceptors (Vorobyev 2003).

Carotenoid access during development has a demonstrable impact on multiple facets of phenotype throughout life. Carotenoid intake during development can affect plumage coloration (Biard et al. 2006) and behavior (Fenoglio et al. 2002) during later stages of development. Effects of carotenoid access early in life can also extend into subsequent life stages. Presence of carotenoids in the yolk affect the ability to incorporate dietary carotenoids into tissue mid-way through neonatal development (4-week-old chicks; Koutsos et al. 2003), and carotenoid access during the nestling period affects carotenoid assimilation ability of adults in zebra finches (*Taeniopygia guttata*; Blount et al. 2003).

Thus, carotenoid-based ornamentation in animals is an appropriate system for studying developmental plasticity of sexually selected traits because carotenoids are tractable, externally acquired, directly used in coloration, and linked to immune function. Additionally, variation in carotenoid intake during development has been linked to differences in phenotype later during development and at adulthood (e.g., Blount et al. 2003). Throughout my dissertation, I sought to explore the developmental plasticity of ornaments and other aspects of phenotype that are associated with carotenoid physiology.

Hypothesis

Because of the aforementioned possible extrinsic limitations of carotenoid accumulation in young and old animals (Blount et al. 2003), I hypothesized that **carotenoid-based ornaments can signal not only current individual quality, but also carotenoid-relevant conditions experienced throughout development**. I tested this hypothesis in mallard ducks (*Anas platyrhynchos*) by rearing them from hatch, examining or manipulating conditions during development, and assessing adult phenotype. Mallards were an appropriate study species for evaluating this hypothesis for multiple reasons. They are easy to rear in captivity without parental involvement, they are commercially available, they have carotenoid-based ornamentation (yellow beaks), and there are a variety of species-specific immune assays that are unavailable for most other species. Additionally, mallards suffer high rates of mortality during development (Talent et al. 1983) and are semi-precocial, and thus largely self-dependent for acquiring enough nutrients to grow quickly and maintain health, and also to avoid predation. Young mallards hatch with black beaks and downy yellow (non-carotenoid-containing) feathers and grow quickly, doubling in mass during each of the first two weeks of life. Males begin to develop alternate (colorful nuptial) plumage at about 10 weeks, and finish by about 16 weeks of age, with a green iridescent head, white neck ring, and brown breast. Beak color fades from black to pale olive by approximately 5 weeks old and then gradually becomes more yellow by the end of acquisition of alternate plumage (Drilling et al. 2002). As adults, female mallards choose as mates those males with beaks that are more yellow

(Omland 1996a,b). Previous work with this species has shown that this beak color is related to adult immune quality, specifically antibody production (Peters et al. 2004b) and sperm velocity (Peters et al. 2004a).

Dissertation outline

I used a series of correlative and experimental studies to examine how early-life experiences were linked to carotenoid-related traits (e.g., coloration, immune function) at adulthood of mallard ducks. These investigations utilized nutritional and immunological manipulations, immunological assays, spectrophotometric quantification of ornamental coloration, and tissue and food carotenoid analyses. This work was organized around three main questions, outlined below.

1) How do carotenoid levels in the diet and tissues of ducklings vary in the wild?

In order to set the stage for eventual manipulative work on carotenoid allocation and accumulation during development in the lab, I first needed to collect data regarding carotenoid levels in the diet and tissues of wild mallard ducklings of a variety of ages. Additionally, because I was interested in the relationships between carotenoid levels and immune status, I also collected data on degree of parasitism and white blood cell counts. I collected ducklings ranging from 1 to 40 days of age from local parks and analyzed the carotenoid content of their diets (gizzard contents), livers (carotenoid storage site), and plasma (circulating levels of carotenoids). I also quantified total leukocyte count, heterophil to lymphocyte ratio (an indicator of stress; Bonier et al. 2007), and rate of internal parasitism

(blood and gut). I predicted that birds who ingested more carotenoids (i.e., in gizzard) would also accumulate more in the body (i.e., in liver and plasma), and, because of the possible health-enhancing role of carotenoids, that higher circulating carotenoid levels would be associated with higher total leukocyte count and reduced levels of parasitism.

2) How do morphological and physiological traits throughout development predict adult coloration and immune function?

For this study, I wanted to assess if metrics collected early in life (e.g., mass, body condition, or circulating carotenoid titer) on unmanipulated mallards would predict aspects of adult phenotype, including immune function, carotenoid accumulation in internal tissues, and carotenoid-based coloration. In captivity, I reared both male and female mallards from 1-day-old to adulthood, and collected data on body mass and tarsus length as well as obtained plasma samples every week for 5 months. After individuals had matured into their nuptial coloration, I then issued a series of immune challenges and quantified beak coloration for all individuals. To address the current-quality-revealing nature of beak color, I predicted that male beak coloration prior to an immune challenge would predict immune response. Lastly, because there is no known signaling function of beak coloration for females, I predicted that no such relationships would exist for these colorful structures.

3) How do early-life nutrition and health affect adult coloration and immune response?

I performed two experiments to test how early-life dietary and health conditions affect adult phenotype, using male mallards reared from hatch in both cases. In the first experiment, I immune challenged mallard ducklings during the early, middle, or late stage of development. I then compared the adult immune function and carotenoid-based ornamentation of these mallards to control animals that did not receive developmental immune challenges. Exposure to immunological stimuli during development can attenuate immune response at adulthood in rodents (Galic et al. 2009). Thus, I predicted that individuals that received immune challenges during the earliest stages of development would have a reduced immune response at adulthood, and that this reduced immune function would be reflected in the honest-signaling beak coloration.

In a second study, I reared male mallards from hatch and utilized a 2X2 factorial experimental design, giving mallards access to either low or high levels of carotenoids throughout the growth period of development and/or during the adult stage. Because developmental and adult conditions can interact to affect adult phenotype (Monghan 2008), I predicted that carotenoid physiology (and thus, immune function) of adults would be greatest for those individuals that experienced similar levels of carotenoids during both development and adulthood. However, I also predicted that those individuals that experienced high levels of carotenoids during any stage would have a more carotenoid-rich beak, as these

individuals might have and increased ability to assimilate carotenoids (Blount et al. 2003) and deposit these molecules in the beak.

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Chapter 1

EARLY- AND LATE-LIFE ACCESS TO CAROTENOIDS AFFECTS ADULT IMMUNE FUNCTION AND MAINTENANCE OF ORNAMENT EXPRESSION

Introduction

The expression of an animal's phenotype at adulthood is not fixed; each trait is a product of the individual's genetic make-up and its developmental history (Scheiner 1993). Thus, ultimate questions regarding an adult animal's behavioral or physiological response to a stimulus are most informed when examining not only the individual's current phenotype, but also its experiences throughout development. Many biologists, including ecologists (Arriero 2009), behaviorists (Fenoglio et al. 2002), and physiologists (Blount et al. 2003), have recognized the importance of the early-life period in the expression of the adult phenotype, demonstrating how factors ranging from hormone levels (Strasser and Schwabl 2004) to diet quality (Blount et al. 2006) to parasite exposure (Bischoff et al. 2009) early in life affect adult ornamentation (Strasser and Schwabl 2004), survival (Monaghan et al. 2011), and investment in reproduction (Blount et al. 2006). However, despite this plethora of research on developmental plasticity, the manner in which the developmental (pre-adult) and adult environments interact to affect fitness is still poorly understood.

Development is a critical period for animals; it is typically characterized by high rates of growth (e.g., Drilling et al. 2002), mortality (Sullivan 1989) and

investment in mechanical structures (Searcy et al. 2004), neurological connections (Bland et al. 2010), and physiological pathways (Sanchez 2006) that will be necessary to survive and reproduce at adulthood. However, investment in such traits is advantageous not only if it increases immediate survival likelihood, but also if it increases chances of survival and reproduction in the future environment. Monaghan (2008) reviewed several hypotheses predicting how low- or high-quality environments during development could interact with low- or high-quality environments during adulthood to maximize fitness. The two most prominent hypotheses are the Environmental Matching and the Silver Spoon hypotheses, while two other unnamed hypotheses incorporate elements of each (see Monaghan 2008 for further details). As the name suggests, the Environmental Matching hypothesis predicts that individuals that experience similar conditions (regardless of the quality of those conditions) during both development and adulthood will have a relatively higher fitness than those that experience mismatched conditions. Alternatively, the Silver Spoon hypothesis predicts that individuals exposed to superior conditions during development and/or adulthood have an increased fitness relative to those that experience poor conditions at any point. More generally, these two hypotheses differ in a fundamental way; the Environmental Matching hypothesis predicts that early- and late-life conditions interact to affect adult fitness, while the Silver Spoon hypothesis does not predict interactive effects.

Support for the Environmental Matching, the Silver Spoon, and the other hypotheses has been mixed. The predictions of the Environmental Matching

hypothesis have been frequently supported within studies on humans (Wells 2007), and many manipulations of diet quality or quantity (Krause et al. 2009; Barrett et al. 2009; Saastamoinen et al. 2010; Dmitriew and Rowe 2011), parasite exposure (Devevey et al. 2010), or stress hormone (e.g., corticosterone) exposure (Spencer et al. 2010) at both the developmental and adult stages in other organisms have yielded support for the Silver Spoon hypothesis. Additionally, manipulation of diet during development can be detrimental in the short term, but full compensation is possible given substantial time during adulthood (Auer 2010). Here, we propose to further test the Environmental Matching and Silver Spoon models of developmental plasticity.

One of the strengths of the studies cited above is the diverse effects of their manipulations on adult phenotype. Differences in food amount (Auer 2010; Saastamoinen et al. 2010; Dmitriew and Rowe 2011) or protein to carbohydrate ratio (Krause et al. 2009; Barrett et al. 2009) can affect an organism's ability to express colorful ornaments (Hill et al. 2009), respond to an immune challenge (Ruiz et al. 2010), survive (Kaftanoglu et al. 2011), and invest in reproduction (Bauerfeind and Fischer 2009). While broad manipulations of diet quality or quantity can be ecologically relevant and may be likely to affect multiple aspect of phenotype, some micronutrients also have myriad effects on phenotype, and these effects can be more tightly linked to specific physiological processes or phenotypic traits. For example, carotenoids are a group of molecules that are generally produced in photosynthetic organisms and that vertebrates must acquire from their diet (McGraw 2006). These molecules act as pigments, and are

responsible for the red, orange, or yellow coloration of many fish (Grether et al. 2008), lizard (Cote et al. 2010), and avian (McGraw 2006) ornaments. These pigments also have antioxidant properties (Woodall et al. 1997; Hõrak et al. 2007), although their importance to antioxidant physiology in animals has been recently questioned (Costantini and Møller 2008; Cohen and McGraw 2009). There is stronger support for their role in supporting immune function (Chew and Park 2004; Aguilera and Amat 2007; Biard et al. 2009). Correlational studies have linked carotenoid levels to antibody production both *in vitro* (Okai and Higashi-Okai 1996) and *in vivo* (Peters et al. 2004a), to the strength of the oxidative burst (Macedo et al. 2010), and to the strength of the cutaneous immune response to phytohemagglutinin (PHA; Biard et al. 2009).

Carotenoid access during development also has a demonstrable impact on multiple facets of subsequent phenotype. Carotenoid access early in life can affect plumage coloration (Biard et al. 2006) and behavior (Fenoglio et al. 2002) during later stages of development. Effects of carotenoids access early in life can also extend into subsequent life stages. Presence of carotenoids in the yolk affect the ability to incorporate dietary carotenoids into tissue mid-way through neonatal development (4-week-old chicks; *Gallus gallus domesticus*; Koutsos et al. 2003a), and carotenoid access during the nestling period affects carotenoid assimilation ability of adults in zebra finches (*Taeniopygia guttata*; Blount et al. 2003).

Here, we tested how dietary access to carotenoids during development and/or adulthood affected immune function and carotenoid-based ornament expression in male mallard ducks (*Anas platyrhynchos*). Males possess a yellow,

carotenoid-pigmented beak (Butler et al. 2011). Females prefer males with more yellow beaks as mates (Omland 1996a,b), and this coloration signals aspects of quality, including sperm quality (Peters et al. 2004a) and ability to mount a humoral immune response (Peters et al. 2004b). Because carotenoid processes differ between ornamental coloration (deposition) and immune function (chemical reactions; Palozza et al. 2010), we hypothesized that adult characters would depend upon developmental and adult conditions differently. More explicitly, we hypothesized that the Environmental Matching hypothesis would apply to adult immune function and carotenoid physiology, as individuals that developed appropriate levels of carotenoid storage or transport (e.g., lipoprotein expression to mobilize carotenoids; Connor et al. 2007) would demonstrate a relatively superior immune response. Alternatively, we hypothesized that beak coloration would reflect the Silver Spoon hypothesis, as individuals with increased access to carotenoids would be more likely to store and subsequently deposit carotenoids in the integument, resulting in more yellow, ornamented beaks (Butler et al. 2011). In accordance with the Environmental Matching hypothesis, we predicted that individuals receiving similar levels of carotenoids during both development and adulthood would maintain constant levels of circulating carotenoids throughout the adult immune challenge, have greater cutaneous immune response to phytohemagglutinin (PHA; Smits et al. 1999), and have a greater humoral response to a novel antigen. While a stronger immune response is not universally indicative of an increased fitness (Graham et al. 2011), these particular metrics have been linked to increased nestling recruitment in songbirds (Cichoń & Dubiec

2005, Moreno et al. 2005, López-Rull et al. 2011; but see Butler et al. 2009) and to more ornamented mallard drakes (Peters et al. 2004a). In accordance with the Silver Spoon hypothesis, we predicted that individuals that receive higher carotenoid diets at any stage would have beaks with more carotenoid-rich coloration. Individuals receiving carotenoid-rich diets early in life may be able to store carotenoids (e.g., carotenoid storage in the liver; Martucci et al. 2004) for subsequent ornamentation, or have an increased assimilation ability during adulthood (Blount et al. 2003), while adults receiving carotenoid-rich diets would be able to produce and maintain beak coloration that reflects a greater amount of carotenoid deposition.

Methods

Experimental protocol and blood collection

We acquired 42 one-day-old male ducklings from Metzer Farms (Gonzales, CA) in December 2010 and housed them as in Butler and McGraw (2009; Butler et al. in press). Briefly, ducklings were reared indoors in randomly selected groups of four ducklings per cage (60 x 60 x 60 cm) until they were 21 days old and two per cage until 52 days 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 (11.5L:12.5D at 52 days old to 14L:10D at 20 weeks old).

Individuals were randomly assigned to one of four treatment groups that varied in dietary carotenoid content. Individuals were placed on either LOW or HIGH carotenoid diets from 2 to 49 days old (DEV period), which encompasses the entire period of growth (Butler and McGraw 2011). To prepare diets, food (Mazuri Waterfowl Starter; Richmond, IN, USA) was mixed with sunflower oil that contained ORO-GLO dry pigmenter (2% carotenoids by mass, predominately lutein; Kemin AgriFoods North America, Inc., Des Moines, Iowa, USA). Treatment levels were based on pilot work that to create diets that contain 3 µg/g (LOW) and 25 µg/g (HIGH) of carotenoids. These amounts represent the first and third quartiles of carotenoid concentration found in wild duckling diets (Butler and McGraw 2010, unpublished data), of which the dominant carotenoid is lutein (Butler and McGraw 2010). At 7 weeks of age, all individuals were transitioned from Waterfowl Starter to Waterfowl Maintenance (Mazuri) with no supplemental carotenoids added. During the DEV period, we measured body mass to the nearest g and tarsus length to the nearest 0.1 mm on a weekly basis. We also collected approximately 200 µl of whole blood in heparinized capillary tubes from ducklings when they were 2 days old prior to initiation of dietary manipulation, and 300 µl when they were 49 days old, prior to cessation of DEV dietary treatment. Blood was stored on ice for several hrs and then centrifuged for 3 min at 10,000 rpm. We then aliquoted plasma into separate microtubes for carotenoid and hemagglutination/hemolysis assays (see below), and stored the plasma at -80 ˚C until analysis.

When individuals were 119 days old (17 weeks old, age at which males have acquired breeding season plumage and are displaying courtship behavior; Drilling et al. 2002), we measured body mass and tarsus length, and collected blood samples as above. Half of each original treatment group was then placed on either LOW or HIGH carotenoid diets that were formulated in the same manner as above, except that we used Waterfowl Maintenance chow. Mallards remained on this diet for the remainder of the experiment (20 weeks old), and hereafter we refer to this period as the ADULT period. This 2X2 design yielded 4 groups: LOW during DEV and ADULT (LL; $N = 11$), LOW during DEV and HIGH during ADULT (LH; $N = 10$), HIGH during DEV and LOW during ADULT (HL; $N = 10$), and HIGH during DEV and ADULT (HH; $N = 11$).

Immune assessment

One week into the ADULT period (to allow enough time for dietary treatment to be reflected in the individual's physiology), we issued a series of immune challenges to assess each individual's immune function (Figure 1). We have described these challenges in detail elsewhere (Butler and McGraw, in press), so here we briefly review our methodologies.

On the first day of the adult immune assessment period (D0; Figure 1), 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, 54 min; s.d. = 16.4 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 = 0.98; Lessells & 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 (Smits et al. 1999; Martin et al. 2006).

Also on D0, we administered an emulsion 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 ddH_2O. Six days later (D6), we administered a booster injection of an emulsion of 250 µl of Incomplete Freund's Adjuvant (IFA; Sigma F5506) and 250 µg of KLH suspended in 250 µl of sterile ddH_2O. By collecting blood on D0, D6 and D10 (four days after D6), we were able to quantify humoral immune response to this novel antigen. Specifically, we used an ELISA (Butler and McGraw 2011, in press) to quantify KLH-specific antibody production, allowing us to measure primary (D6 minus D0) and secondary (D10 minus D0) humoral response.

Both PHA (Sild and Hõrak 2009) and CFA (Zheng et al. 2003) induce systemic increases in nitric oxide (NO) levels, which is a marker of the 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, in press) and followed Sild and 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 $R^2 = 0.994$.

We used a hemolysis-hemagglutination assay (Matson et al. 2005) to assess immune function throughout development and adulthood without affecting immmunodevelopment. Natural antibodies (NAbs) are immunoglobulins (predominately IgM; Matson et al. 2005) that are formed without prior antigen exposure, and their presence can result in the clumping of foreign particles, including red blood cells (hemagglutination). NAbs also interact with complement to lyse foreign cells (e.g., red blood cells; Matson et al. 2005). Therefore, to assess both hemolysis and hemagglutination, we followed the protocol of Matson et al. (2005) with several modifications. We serially diluted 20 µl of plasma in PBS along a row of a 96-well plate, with the final column containing only PBS (negative control). We then added 20 µl of heparinized whole sheep blood (Hemostat Laboratories, Dixon, CA; SBH050) that was diluted 1:100 in PBS to each well. We then incubated parafilm-covered plates at 42 ºC, which approximates the body temperature of mallard ducks (Gray et al. 2008), for 90 min. We then tilted the plates for 20 min at room temperature and scanned them using a flat-bed scanner (Hewlett-Packard Co., ScanJet 3670) at 600 dots per inch to measure hemagglutination. We then left the plates flat at room temperature for 70 min and rescanned for hemolysis (see Matson et al. 2005 for scoring procedures).

Carotenoid titer and coloration assessment

We analyzed plasma carotenoid content using a hexane:methyl tert-butyl ether extraction method and the high-performance liquid chromatography procedures of McGraw et al. (2008). We quantified total carotenoid titer (predominately lutein and zeaxanthin; Butler and McGraw, in press) for all individuals at the beginning and end of DEV, the beginning of ADULT, and on D0, D1, D6, and D10 of the adult immune assessment period.

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.). On D0, D1, D6, and D10, we measured carotenoid-based beak coloration of adults from λ = 300-700 nm using an Ocean Optics (Dunedin, FL, USA) USB2000 spectrophotometer with a PX-2 pulsed xenon light source. *Sensu* Butler and McGraw (in press), we measured a 1 cm band of the right dorso-lateral surface of the beak between the nares and the beak tip and binned 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, saturation, and hue scores that are most closely correlated with carotenoid content in the mallard beak (B1, negatively related to carotenoid content; S1B, negatively related to carotenoid content; H4b, positively related to carotenoid content; Butler et al. 2011).

Statistics

Several of the variables that we quantified were not normally distributed. These variables (KLH primary response, NO levels on D0, and NO levels on D1) were log-transformed to achieve normality. However, hemolysis and hemagglutination could not be transformed to achieve normality, and the residuals from the described statistical models were also not normally distributed, so we also performed non-parametric tests for comparison (see below). For multivariate analysis of variance (MANOVA) tests, if the Greenhouse-Geisser (G-G) Epsilon was < 0.7, we interpreted the G-G adjusted *P*-value.

To test if circulating carotenoid titer, body mass, tarsus length, hemolysis, and hemagglutination differed as a function of treatment over time, we ran repeated-measures ANOVAs, using all 7 carotenoid data points (pre- and post-DEV, pre-ADULT, and D0, D1, D6, and D10), all 13 body mass points (8 times during DEV plus all 5 ADULT-stage measurements), all 9 tarsus length data points (8 times during DEV plus pre-ADULT), and the four hemolysis and hemagglutination points (pre- and post-DEV, pre-ADULT and D10). Because hemolysis and hemagglutination values could not be transformed to achieve normality, we also used non-parametric Friedman's tests to see if there were differences between treatments within each age class, and within each treatment group as a function of age.

We tested how treatment during DEV, ADULT, and their interaction affected PHA-induced swelling, NO response, primary and secondary KLH response, and beak brightness, hue, and saturation by running separate ANOVAs

for each dependent variable, with DEV, ADULT, and the DEV by ADULT interaction as independent factors. To test if adult beak color (D0) predicted immune response, we performed simple linear regressions with beak saturation, hue, or brightness as the independent variable and PHA-induced swelling, NO response, primary and secondary KLH response, D10 hemolysis, or D10 hemagglutination as dependent variables. Lastly, to test how treatment and immune response may have combined to change adult carotenoid status or beak color, we performed a series of either ANOVAs or analysis of covariance (ANCOVA) models. Specifically, we used ANOVAs to test if the change in circulating carotenoid titer over the first 1, 6, or 10 days of the adult immune assessment differed as a function of treatment. One individual circulated very high levels of carotenoids on the final day of the adult immune challenge (D10), resulting in a non-normal distribution of the data and associated residuals. Analyses with this individual excluded resulted in normally distributed data and residuals, and the statistics were qualitatively similar to those with the individual included. Therefore, because we had no *a priori* reason to exclude this individual, we present results with his data included. We also used ANCOVAs to test if change in beak color (hue, brightness, or saturation) was a function of treatment, using degree of immune response (e.g., PHA-induced swelling) over the same time period as a covariate.

Results

Effects of diet treatment on developmental metrics

Dietary carotenoid treatment affected circulating carotenoid levels (both $F_{1,38} > 14.14$, $P < 0.0006$), with dietary treatment during both DEV ($F_{6,228} = 63.33$, $P < 0.0001$) and ADULT ($F_{6,228} = 26.17$, $P < 0.0001$) interacting with age. There were no effects of dietary treatment, age group, or their interaction on circulating carotenoid levels prior to DEV treatment (all $F_{1,38} < 0.22$, all $P > 0.6$), but DEV HIGH birds circulated higher levels than DEV LOW birds by the end of the DEV period ($F_{1,38} = 334.55$, $P < 0.0001$). Just prior to the ADULT period, there were no differences in circulating carotenoid levels among treatment groups (all $F_{1,38} < 0.59$, all $P > 0.4$), but at all subsequent time points ADULT HIGH birds circulated higher levels of carotenoids than ADULT LOW birds (all $F_{1,38} < 30.18$, all $P < 0.0001$). There were no other significant interactions (all $P > 0.33$; Figure 2).

Dietary treatment during DEV ($F_{1,38} = 0.41$, $P = 0.53$), ADULT ($F_{1,38} = 0.41$, $P = 0.53$), and their interaction ($F_{1,38} = 0.05$, $P = 0.83$)did not affect body mass, nor did any of these main effects interact with age (all $F_{12,456} < 0.58$, all $P > 0.6$). Similarly, there was no effect of dietary treatment during DEV ($F_{1,38} = 0.79$, $P = 0.38$), ADULT ($F_{1,38} = 2.98$, $P = 0.093$), and their interaction ($F_{1,38} = 0.61$, $P = 0.44$) on tarsus length, nor did any of these main effects interact with age (all $F_{8,304} < 1.90$, all $P > 0.13$) to affect tarsus length. Unsurprisingly, there was a strong effect of age on body mass ($F_{12,456} = 1447.51$, $P < 0.0001$) and tarsus length ($F_{8,304} = 4223.69$, $P < 0.0001$), with birds becoming larger as they grew.

According to rmANOVAs that did not have normally distributed residuals (see above), both agglutination ($F_{3,114} = 146.43$, $P < 0.0001$) and lysis ($F_{3,114} = 163.05$, $P < 0.0001$) increased with age, but there was no effect of either dietary treatment (all $F_{1,38} < 2.55$, all $P > 0.12$) or an interaction with treatment and age (all $F_{3,114} < 0.85$, all $P > 0.42$) for either metric. When analyzing these data using non-parametric Friedman's tests, the results were statistically similar, with effects of age within all treatment groups on both agglutination ($\chi^2 = 46.3$, d.f. = 3, $P < 0.0001$) and lysis ($\chi^2 = 43.3$, d.f. = 3, $P < 0.0001$), and no effects of treatment on agglutination or lysis scores at any age (all $\chi^2 < 2.91$, d.f. = 1, all $P > 0.088$).

Effects of dietary treatment on adult-stage metrics

Birds that received the LOW diet during DEV had greater PHA-induced swellings than DEV HIGH birds ($F_{1,38} = 4.84$, $P = 0.034$), but there was no effect of ADULT treatment or the interaction between DEV and ADULT (both $F_{1,38} < 1.99$, both $P > 0.16$; Figure 3) on PHA-induced swelling. Diet treatment during DEV, ADULT, and their interaction did not affect primary KLH response (all $F_{1,38} < 0.32$, all $P > 0.58$), secondary KLH response (all $F_{1,38} < 0.34$, all $P > 0.56$), or NO response (all $F_{1,38} < 2.30$, all $P > 0.14$). Additionally, adult beak color assessed immediately prior to adult immune assessment did not differ by dietary treatment during DEV, ADULT, or their interaction for beak brightness (all $F_{1,38} < 2.88$, all $P > 0.098$), saturation (all $F_{1,38} < 0.46$, all $P > 0.5$), or hue (all $F_{1,38} < 0.67$, all $P > 0.4$).

Adult beak color predicting adult immune function

Beak saturation was related to PHA-induced swelling, with a greater PHA response associated with a more carotenoid-rich beak (lower S1B, $F_{1,40} = 7.76$, $P = 0.0081$; Figure 4). Beak saturation did not predict primary or secondary KLH response, NO response, or hemagglutination or hemolysis on D10 (all $F_{1,40} < 1.54$, all $P > 0.22$) and neither beak brightness nor hue predicted any immune metric (all $F_{1,40} < 2.25$, all $P > 0.14$).

Effects of dietary carotenoid treatment and immune response on change in adult phenotype during the adult immune assessment period

Neither change (all $F_{1,38} < 0.49$, all $P > 0.49$) nor percent change (all $F_{1,38} < 3.01$, all $P > 0.091$) in carotenoid levels during the first 24 hours of the immune challenge (D0 to D1) differed by DEV, ADULT, or their interaction. However, there was a significant interaction of DEV and ADULT on change in carotenoid levels over the first six days of the KLH immune challenge period (D0 to D6; $F_{1,38} = 6.18$, $P = 0.017$), with HH birds decreasing circulating carotenoid levels more than LL, LH, and HL birds (all $P < 0.044$; Figure 5). The result was similar if analyzed as a function of percent change (DEV*ADULT; $F_{1,38} = 5.35$, $P = 0.026$), with HH decreasing circulating carotenoid levels by a greater percentage than both LH and HL (both $P < 0.05$), but not LL ($P = 0.35$). There was no significant effect of DEV, ADULT, or their interaction on change (all $F_{1,38} < 1.14$, all $P > 0.29$) or percent change (all $F_{1,38} < 1.44$, all $P > 0.24$) in circulating carotenoid levels over the course of the 10-day adult immune challenge.

Beak saturation changed in proportion to PHA-induced swelling response ($F_{1,37}$ = 6.32, P = 0.016), with a greater response associated with a loss of carotenoid-based coloration, and no effect of DEV, ADULT, or their interaction (all $F_{1,37}$ < 0.42, all P > 0.5). Beak brightness changed over the course of the first six days of adult immune assessment as a function of adult treatment ($F_{1,37}$ = 4.96, P = 0.032), with ADULT LOW birds decreasing in carotenoid-based coloration more than ADULT HIGH birds (Figure 6a). Change in beak brightness over the course of the 10-day immune assessment differed by the interaction between DEV and ADULT ($F_{1,37}$ = 7.29, P = 0.010), with LH birds decreasing in carotenoid-based coloration more than HH birds (P = 0.0239), and non-significantly decreasing more than LL birds (P = 0.076). There was also a non-significant trend for HL birds to decrease in carotenoid based coloration more than HH (P = 0.054) birds (Figure 6b). All other effects for change in saturation and brightness were not significant (all $F_{1,37}$ < 3.12, all P > 0.086), and there were not significant relationships between change in beak hue and treatment or degree of immune response (all $F_{1,37}$ < 2.58, all P > 0.12).

Discussion

Access to carotenoids during development affected adult immune response and interacted with adult carotenoid access to affect circulating carotenoid levels and maintenance of a carotenoid-pigmented ornament during an immune challenge. We hypothesized that immune responses and carotenoid physiology would follow Environmental Matching predictions, and that ornamentation would follow Silver

Spoon predictions; however, we found no support for such relationships. Instead, our results demonstrate that ornament maintenance most closely follows the predictions of Environmental Matching, and more surprisingly, that individuals exposed to HIGH levels of carotenoids during development either had similar (e.g., KLH-induced antibody production) or reduced (e.g., PHA-induced swelling) levels of adult immune function, depending on the specific immune metric, relative to DEV LOW birds. Similarly, individuals that received HIGH levels of carotenoids during both DEV and ADULT periods had larger decreases in circulating carotenoid levels during those immune challenges without an increase in immune function. Taken together, these findings suggest that HIGH levels of carotenoids during development may serve no inherent benefit at adulthood, and may actually be detrimental in select environments.

Individuals that received LOW diets during development had a greater PHA-induced swelling at adulthood, regardless of adult carotenoid treatment. This finding is not consistent with the Environmental Matching hypothesis, and stands in direct opposition to the Silver Spoon hypothesis, as lower levels of carotenoids are presumed to be indicative of poorer conditions. However, although only occasionally discussed, high levels of carotenoids may actually be detrimental (Olson and Owens 1998). Previous work with American goldfinches (*Carduelis tristis*) has shown that high levels of carotenoids can negatively affect flight performance (Huggins et al. 2010), but in that study, there was a 100-fold increase in carotenoid access between low- and high-carotenoid treatments. Our treatment reflected an approximate 8-fold increase, and circulating carotenoid

levels in HIGH birds were similar to those found in wild ducklings (Butler and McGraw 2010), so it is unlikely that levels of carotenoid supplementation were pharmacological. Therefore, our data demonstrate that individuals that have access to low, but ecologically relevant, levels of carotenoids during development will have an increased cutaneous immune response at adulthood, although the precise mechanism for this result is unknown. PHA-induced swelling reflects multiple components of immune activity (Martin et al. 2006), and thus may be an important immune metric. However, we tested other aspects of immune function, including markers of humoral (primary and secondary anti-KLH antibody production) and innate (NO response, hemagglutination and hemolysis capacity) immune function, and did not find any other treatment differences. Therefore, at least in mallards, access to carotenoids during development seems to affect only one component of the immune system, supporting the expanding view that while carotenoids do increase immune function in several cases (Aguilera and Amat 2007; Biard et al. 2009), they do not necessarily increase response within all axes of the immune system (PHA-induced swelling: Hõrak et al. 2007; antibody production: Smith et al. 2007; markers of innate immunity: Lin et al. 2010).

Interestingly, neither DEV nor ADULT treatment affected adult measures of beak coloration. While access to carotenoids over 10 weeks affects adult drake beak coloration (Giraudeau et al. 2011), it is possible that due to the length of time required for tissue turnover within the beak integument, these differences do not manifest within the first few weeks of supplementation, thus accounting for the lack of effect of carotenoid supplementation during the ADULT stage.

However, the lack of a DEV effect suggests little need for young male ducklings to consume carotenoids during development to ensure maximal expression of beak coloration at adulthood (but see below, Ohlsson et al. 2003).

In accordance with previous studies (Peters et al. 2004b; Butler and McGraw 2010), we found that male beak color prior to an immune challenge was predictive of the degree of immune response, which was related to the loss of carotenoid-based coloration during the time course of the immune response, supporting the putative role of mallard male beak coloration in honestly signaling quality to the female. Such short-term changes to carotenoid-based ornaments have been demonstrated in both mallards (Butler and McGraw 2011) and red grouse (*Lagopus lagopus scoticus*; Mougeot et al. 2010), and future work should test not only for mate preferences for more intensely pigmented carotenoid-based coloration ornaments, but also for a female's ability to detect and make mate-choice decisions using intra-individual differences in coloration over time as a marker of current investment in immunity.

In addition to the correlation between immune response and degree of change in coloration described above, we found dietary treatment effects on change in beak coloration over the first 6 and 10 days of immune challenges. Over the first six days, adult males showed a greater decrease in carotenoid-based coloration if they were currently receiving LOW diets, suggesting that while several weeks of access to carotenoids may not directly affect the absolute level of carotenoid-based coloration (see above), concurrent access may affect the change in coloration during an immune response. Over the full 10 days of adult

immune assessment, however, we detected changes in coloration that show limited support for the Environmental Matching hypothesis; that is, individuals that experienced similar levels of carotenoids during both development and adulthood (LL and HH birds) tended not to decrease in carotenoid-based coloration as much as those that experienced dissimilar diets (LH and HL birds). Some of these relationships were not statistically significant, so these results should be viewed as preliminary findings that require further investigation. Work with red grouse (Martínez-Padilla et al. 2010; Mougeot et al. 2010) and zebra finches (*Taeniopygia guttata*; McGraw et al. 2011) has demonstrated several physiological mechanisms by which carotenoid-based ornamentation may be maintained (e.g., hormone-dependent changes in physiology), highlighting the potential role lability in ornamentation, in addition to average level of ornament expression, may play.

Circulating carotenoid levels decreased throughout the adult immune assessment period in all groups. This finding is consistent with the utilization of carotenoids during an immune response (Koutsos et al. 2003b), although the lack of relationships between adult treatment and most immune metrics, as well as the lack of a control, unchallenged group, does not allow us to experimentally support this position. However, we did detect treatment differences in the change in circulating carotenoid levels decreased, specifically that HH individuals decreased circulating carotenoids levels more than any other group over the first 6 days of the adult immune assessment. While HH individuals had higher initial levels relative to LL and HL (treatment effect of ADULT), and thus may have been able

to decrease levels relatively more without suffering any adverse effects, HH also decreased more than LH. This finding suggests that exposure to low levels of carotenoids during DEV resulted in LH birds' having an increased ability to assimilate or mobilize carotenoids relative to HH birds during an immune challenge. Carotenoids are generally transported by lipoproteins (Allen 1987; Connor et al. 2007), and a potentially fruitful area of research may be to test how developmental access to carotenoids affects lipoprotein profiles in adults exposed to varying levels of carotenoids to discover a possible mechanism for the developmental plasticity of carotenoid physiology.

While our results did not support our predictions, we did uncover multiple examples of how dietary carotenoid content during development affected aspects of adult phenotype, either alone or in concert with carotenoid access during adulthood. Unexpectedly, we uncovered evidence that does not fit any of the traditional models of developmental plasticity (Monaghan 2008). Specifically, DEV LOW birds had a greater PHA-induced swelling, and LH birds more effectively maintained circulating carotenoid levels over the first six days of the immune challenge than HH birds. Taken together, these findings point to an advantage at adulthood of experiencing low-quality conditions during development, even if those adult conditions are high-quality. Thus, we propose that a fifth model of developmental plasticity be considered in future studies: the hard-knock life hypothesis. Specifically, we propose that, if the costs of responding to poor-developmental conditions are low enough, individuals may invest in physiological or neurological processes that provide an adaptive

advantage in all adult conditions, provided that the individual survives the relatively poor developmental conditions (a requirement that was easily met while using captive birds).

There are three important predictions for this hypothesis. First, the costs (in general fitness units) to responding to the poor developmental condition must be smaller than the benefits at adulthood, or else the hypothesis is similar to the Silver Spoon hypothesis. Second, the costs during development must be large enough to decrease the expected lifetime fitness of the individual, most likely via a reduction in short-term survival probability, or selection would act on all individuals to invest in these changes. Third, for the subset of individuals that do survive, the resulting phenotypic changes should yield an increase in fitness in future adult environments. Data supporting this hypothesis have been found not only in this study, but in others as well, with neonatal food deprivation resulting in subsequent increased immune function in lizards (*Zootoca vivipara*; Mugabo et al. 2010), and developmental food restriction in the larval stage yielding adult butterflies (*Bicyclus anynana*) that coped better with forced flight (Saastamoinen et al. 2010). Thus, in addition to testing the hypotheses set forth in Monaghan's (2008) work, future researchers should also test the hard-knock life hypothesis, especially for relatively small, but biologically relevant developmental perturbations, including only moderately pathogenic immune challenges, micronutrient availability, or low levels of physiological stress.

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Figure 1. Timeline of adult immune assessment. Blood samples were collected, and beak color and mass were measured, at all four points (D0, D1, D6, and D10; see text for details). The letter "A" denotes when wing web thickness was measured, "B" denotes when NO level was measured, "C" denotes when KLH-specific antibody titer was measured, and "D" denotes when circulating carotenoid titer was measured. The filled arrow indicates when PHA was injected, and the open arrow indicates when KLH was administered.

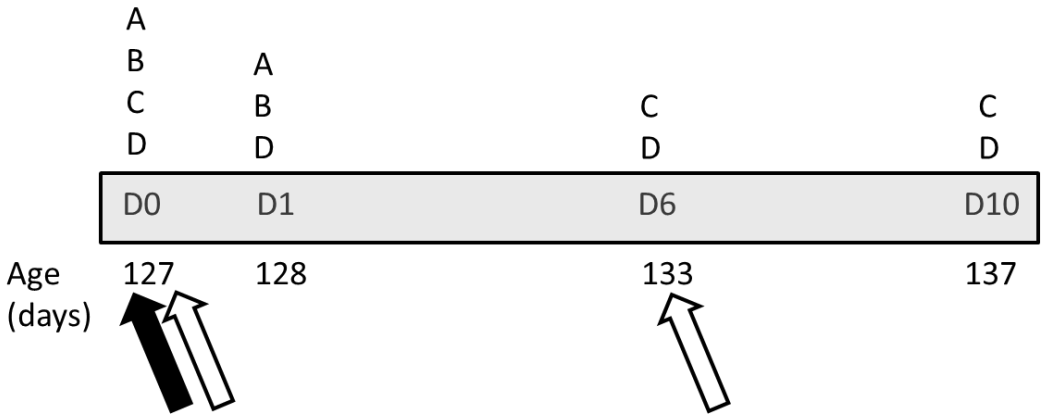


Figure 2. Circulating carotenoid titer as a function of age. Significant differences ($P < 0.05$) within developmental treatment are denoted by #, and within adult treatment by *. Each treatment group is labeled based on access to LOW or HIGH levels of carotenoids during development (2-49 days old; first word) and LOW or HIGH levels at adulthood (119-137 days old; second word). HIGH levels of carotenoids during the first 7 weeks of development increased circulating carotenoid titer by the end of development, but these differences disappeared by the beginning of the adult stage. HIGH levels during adulthood increased circulating carotenoid titer within one week, and levels remained relatively higher throughout the remainder of the study.

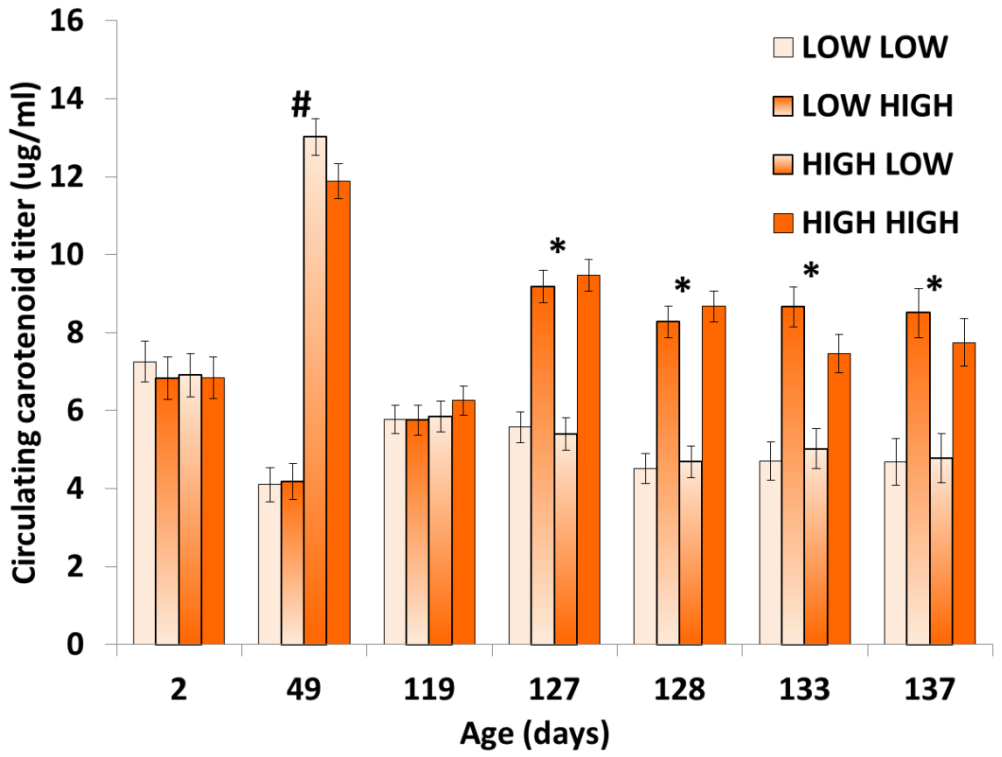


Figure 3. PHA-induced wing web swelling as a function of treatment. The first letter of the treatment denotes whether individuals received LOW (L) or HIGH (H) levels of carotenoids in the diet, while the second letter denotes the diet received at the adult stage, concurrent with the PHA challenge. Those that received LOW diets during development (hatch – 7 weeks old) had larger swellings (*; $P < 0.05$) than those that received HIGH diets.

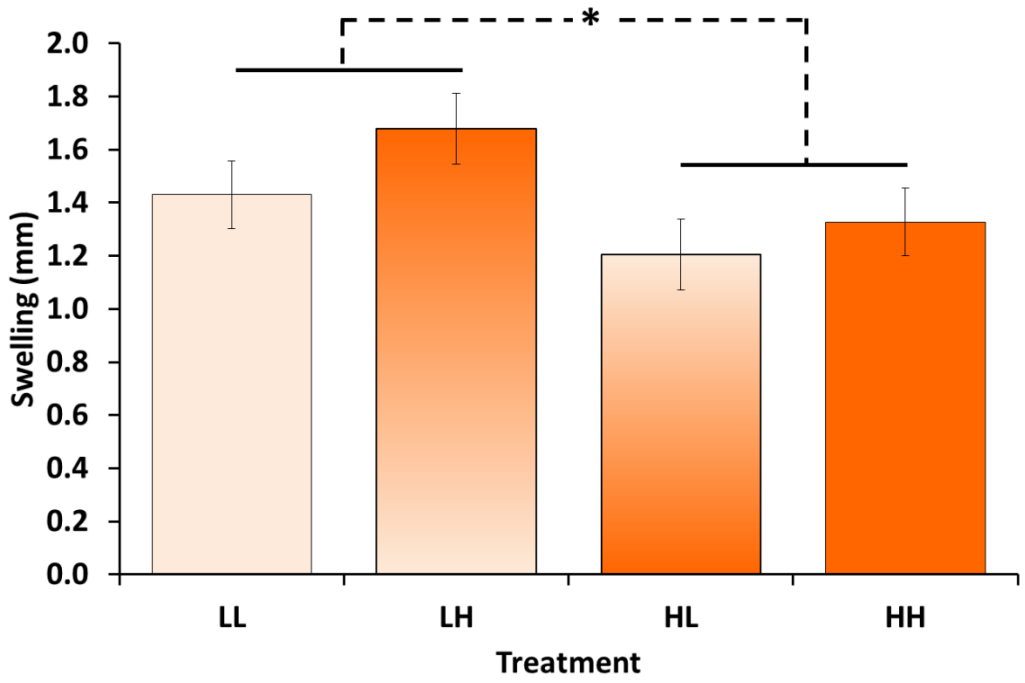


Figure 4. Beak color predicting PHA-induced wing web swelling. Individuals with larger S1B values, or saturation of the blue component of the spectrum (Montgomerie 2008), had smaller PHA-induced swelling responses. Because S1B is inversely correlated with carotenoid content of mallard beaks (Butler et al. 2011), males with more carotenoid-rich beaks had larger PHA-induced swelling responses.

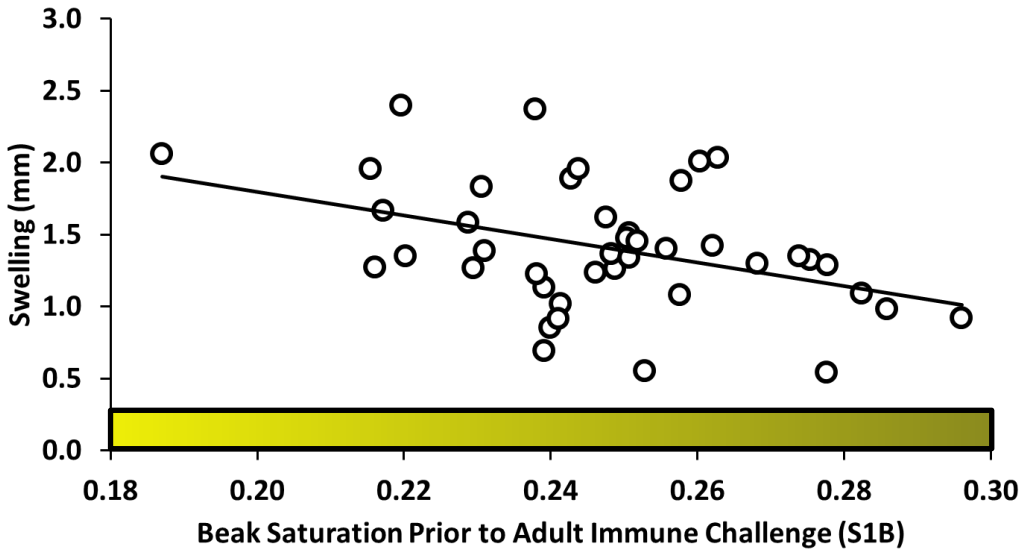


Figure 5. Change in carotenoid titer throughout the adult immune assessment period. Groups are described based on whether they received LOW or HIGH levels of carotenoids during development (2-49 days old; first word) and LOW or HIGH levels at adulthood (119-137 days old; second word). HIGH HIGH birds showed a larger decrease in circulating carotenoid levels over the first six days of the adult immune assessment than all other groups (*; all $P < 0.05$).

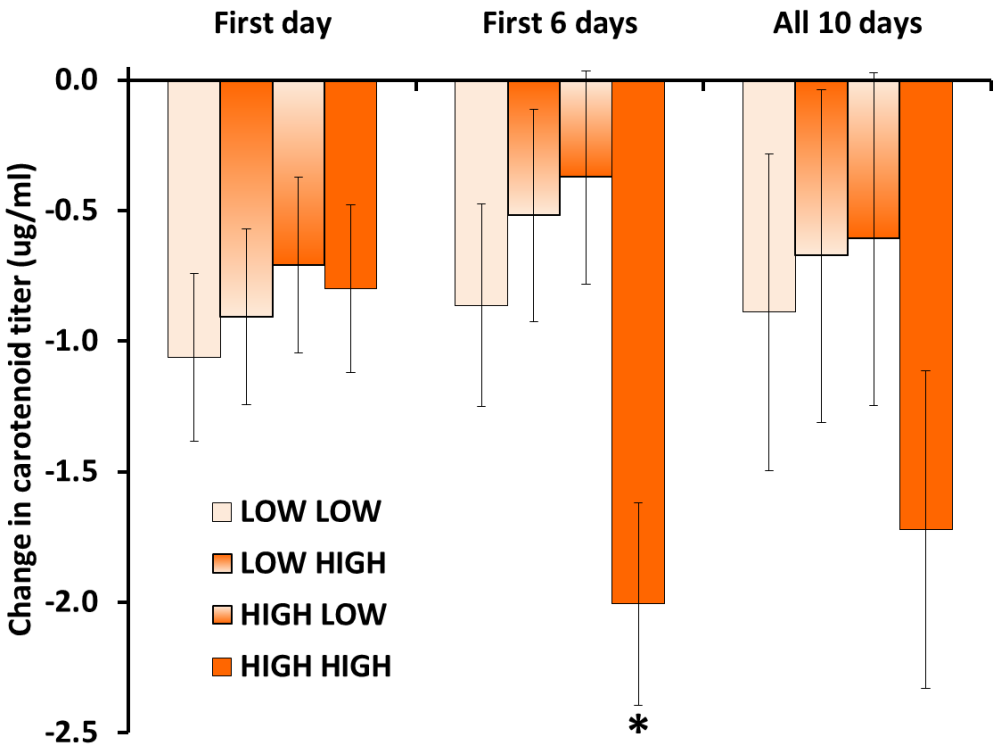
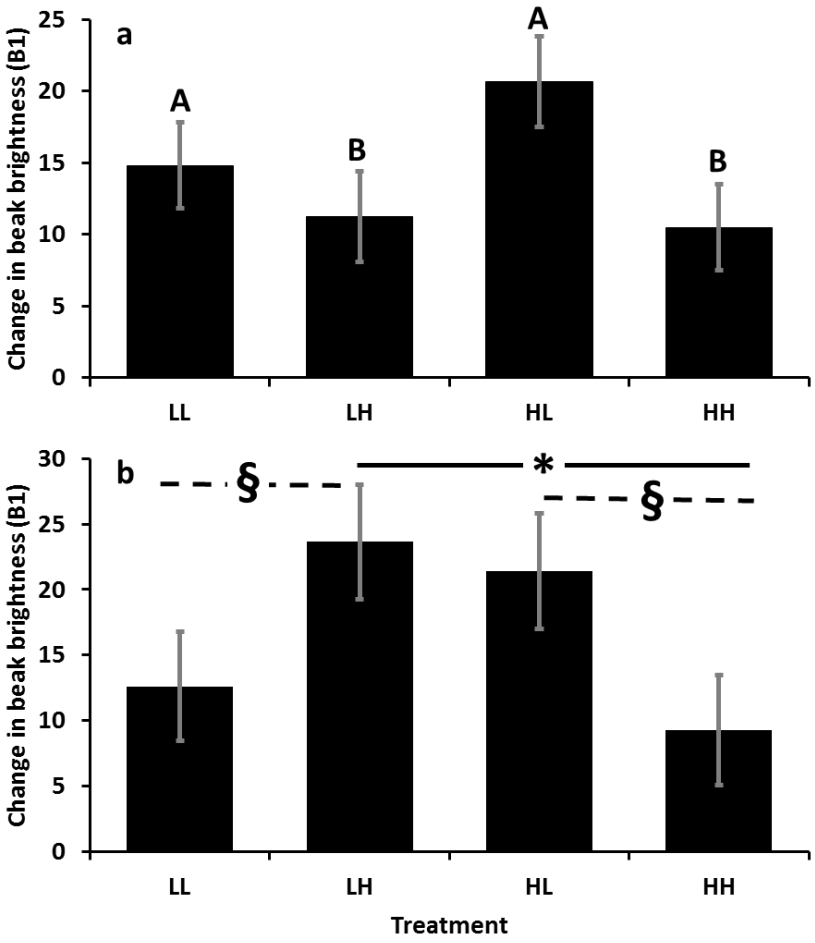


Figure 6. Change in beak brightness over the first (a) 6 and (b) 10 days of the adult immune assessment as a function of treatment. The first letter of the treatment label denotes whether individuals received LOW (L) or HIGH (H) levels of carotenoids in the diet, while the second letter denotes the diet received at the adult stage An increase in brightness is associated with a loss of carotenoid pigment (Butler et al. 2011). Therefore, over the first six days of the adult immune assessment, individuals receiving LOW diets (A) had beaks that became less carotenoid-rich (i.e., increased in brightness) more than those receiving HIGH diets (B; $P < 0.05$). Over the full 10 days of immune assessment, however, LH bird beaks became more carotenoid-depleted than HH bird beaks (*; $P < 0.05$), while similar trends existed between LH and LL birds, and HL and HH birds (§; $0.05 < P < 0.08$).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Chapter 2

CONCLUDING REMARKS

In my dissertation work, I sought to explore if **carotenoid-based ornaments in adult animals not only signal aspects of current quality, but are also developmentally plastic and thus can serve as indicators of early-life nutrition and health state**. To test this hypothesis, I utilized mallard ducks as a study species, due to their amenability of being reared in captivity, previous work that has shown that females prefer males with more carotenoid-rich beaks (Omland 1996a,b), and research showing links between male beak coloration and aspects of quality, including sperm velocity (Peters et al. 2004a) and immune response (Peters et al. 2004b). While developmental plasticity of sexually selected traits has been explored previously in birds (Nowicki et al. 2002; Ohlsson et al. 2002) and invertebrates (Beckers and Schul 2008), this work represents the first attempt to investigate how ornaments reflect developmental conditions within a framework that utilizes the same molecules (i.e., carotenoids) for both ornamentation and beneficial physiological processes, thus providing a more robust framework for generating inferences regarding potential mechanisms and physiological pathways. Specifically, I addressed the following three questions in my dissertation work:

1) How do carotenoid levels in the diet and tissues of ducklings vary in the wild and in relation to current health state?
2) How do morphological and physiological traits throughout development predict adult coloration and immune function?

3) How do early-life nutrition and health state affect adult coloration and immune response?

In answering these questions, I found that carotenoid levels of liver, plasma, and gut contents of wild ducklings co-vary, that body condition (i.e., relative mass when controlling for structural size) measured early in life predicts adult coloration and immune function, and that experiences during development affect adult immune response and maintenance of beak color throughout the adult immune challenge. However, while my data showed that beak color can signal an individual's ability to respond to an immune challenge as an adult, and that adult beak color changes in proportion to the degree of immune response, my results generally did not support my overarching hypothesis regarding developmental plasticity: immunological and dietary-carotenoid perturbations during development did not affect adult beak coloration. However, the adult immune system was affected by both immune challenges and carotenoid access during development, suggesting that this trait is relatively developmentally plastic, generating questions regarding the utility of beak coloration in honestly signaling concurrent and lifetime immune function.

Variation of carotenoid levels in wild ducklings

Animals typically accumulate many carotenoid types from the diet, and apportion them to numerous body tissues, leaving the opportunity for various pigment- and site-specific allocations and tradeoffs (Benito et al. 2011). Yet in most studies,

animals that accumulate more of one carotenoid type accumulate more of others (McGraw 2005), and those that have high concentrations in one tissue type have high concentrations in others (McGraw and Toomey 2010). In all of my studies, circulating carotenoid levels of all carotenoid types were inter-correlated, with the only exceptions being for carotenoid types that made up less than 1% of the total level of carotenoids. Moreover, for captive animals and most of the size classes of wild-caught ducklings, I found high degrees of intra-individual correlations among carotenoid levels within food, plasma, and liver samples. These results are inconsistent with trade-off hypotheses and add further support for the "more-is-better" strategy (Grether et al. 1999). However, for one size class of wild-caught ducklings (those individuals between 100 and 300 grams; approximately 1-3 weeks old), I detected no correlations between carotenoid levels of food, liver, or plasma samples. Thus, it seems that there are shifts in carotenoid physiology during the early stages of development and animals may be faced with an especially sensitive period in shaping adult carotenoid physiology.

Taken together, these findings highlight the importance of investigating carotenoid allocations at multiple life stages. Carotenoid assimilation ability during the juvenile and adulthood stages can be altered by yolk carotenoid levels (Koutsos et al. 2003) and neonatal diet (Blount et al. 2003), respectively, but there has been minimal work examining the mechanics of carotenoid assimilation in animals other than humans (McLean et al. 2005) and on how carotenoid assimilation rates change with age in most avian species, despite calls for investigating developmental plasticity at different stages of development

(Monaghan 2008). Additionally, in avian species the physiological processes by which carotenoids are taken up from food, stored in the liver, and transported are only generally known (high-density lipoproteins: Connor et al. 2007; probably apolipoprotein A-1: Allen 1987). Carotenoid uptake from the gut is generally considered to be a passive diffusive process, although region within the gut (McLean et al. 2005) and the production of specific transporters (Moussa et al. 2008) play a role. However, there has been minimal – if any – solid molecular work demonstrating how carotenoids are deposited into growing feathers or beak integument, and the first steps in identifying candidate genes in determining carotenoid-based integument coloration has only recently begun (Walsh et al. 2011). Thus, investigations that can utilize robust physiological and genetic approaches to explore carotenoid physiology can have a large impact on future research in the areas of animal behavior, sexual selection, and developmental plasticity.

Correlations between developmental conditions and adult color and health

In my correlational study, I found that body condition during growth was a better predictor of adult immune function and coloration than body condition measured later in life, including condition concurrent with immune assessment and ornament expression. I explored multiple explanations for this finding in the chapter itself (mostly physiological in origin), but other, slightly more speculative scenarios exist. For example, while I mention that there could be genetic linkages between color and condition (Gleeson et al. 2005), one research direction that

warrants future study is that selection pressure to express some of these genes may be relatively more intense during the neonatal stage, thus allowing for an increased likelihood of detecting these differences at this particular life history stage. More specifically, young animals are subject to extremely high rates of mortality (Sullivan 1989), and size advantages may be especially important early in life to ensure survival to adulthood. Identification of patterns showing that there is relatively higher selection early in life to express quality (e.g., for the direct benefits of increased survival due to escaping predation), than during the juvenile or even adult stage would promote a suite of new research questions, including those related to maternal allocation decisions, parental care decisions, and parent-offspring conflict. Particular interest may be the evolution of life history strategies that are characterized as "live slow, die old," as one would predict that these species have found a way to relax the relatively increased selection pressure that may be present during neonatal development.

The results from this study also beg the question regarding why there would be selection on a trait to reveal developmental condition relatively more than adult condition. If an ornament is honestly signaling current condition, that should provide females with an advantage when choosing mates (and high-quality males with a high reproductive advantage). So what are the advantages for an individual with an ornament that signals aspects of developmental conditions? First, ornaments that signal quality throughout development and adulthood would, by definition, signal a greater amount of information (i.e., information from a greater number of time points),thus providing females with relatively more honest

signals. Second, these signals would also be less susceptible to cheating. For example, males would not be able to temporarily shift resource allocation toward ornamentation (e.g., during molt to develop more carotenoid-rich feathers that reflect condition just during the molt period). Third, a potential mate would also gather information regarding that individual's rearing conditions. For species with high levels of parental care (a heritable trait; Dor and Lotem 2010), ornament expression may then provide females with information regarding the likelihood of receiving indirect benefits in the form of parental care.

Role of developmental immune challenges in shaping adult health and coloration

The immune system is only partially functional at hatch/birth, but newborn animals must still combat a wide variety of pathogens and parasites. Many vertebrates thus receive some form of immunoprotection from their mother, usually in the form of maternally transferred antibodies (either in milk or yolk). As maternal antibody levels decrease, young animals concurrently develop an endogenous ability to respond to immunological stimuli. During development, immune challenges can shape adult-stage immunocompetence, with the particular manner of response dependent upon the specific point during development that the individual received the immune challenge (Galic et al. 2009). However, also during this developmental period, animals must balance their newfound ability to recognize and destroy pathogens with an ability to recognize and *not* immunologically respond to self. In my dissertation work, I found that immune challenges during development in mallards resulted in differences in immune

function at adulthood, but not in a clear, consistent manner. Specifically, immune challenges during the middle stages of development reduced aspects of innate immunity, while immune challenges during the late stages of development increased acquired, humoral immune function. This seeming contradiction highlights some of the relevant issues in ecoimmunology research today: 1) interpretation of any given immunological metric (Graham et al. 2011) and 2) the frequent lack of correlation or negative relationships among immunological metrics (Salvante 2006; see below).

Despite widespread interest from medical professionals, physiologists, and ecologists in understanding how carotenoids affect the immune system, there are still a large number of important questions regarding the molecular and physiological role(s) that carotenoids can play in ecoimmunology. For example, little is known about how carotenoids molecularly influence immune responses (especially non-provitamin A carotenoids, such as lutein), with most work focusing only on the oxidative burst component of the immune response (Chew and Park 2004; Selvaraj and Klasing 2006), e.g., carotenoids increase the ability of granulocytes, both *in vivo* and *in vitro*, to produce reactive oxygen species (Walrand et al. 2005). However, there is physiological evidence that carotenoids can increase acquired and innate immune responses (Chew and Park 2004), suggesting that future research in this area is warranted.

Several specific immune questions stem from my dissertation work. Because it is recommended that investigators assess multiple types of immune response due to the frequent lack of correlation among immune metrics (Salvante

2006), I assessed both NO response, PHA response, and primary and secondary KLH-specific antibody production concurrently. Unfortunately, I did not have the appropriate control groups (due to the husbandry cost of increasing my sample size) to test whether individuals differentially allocate carotenoids to different types of immune responses. However, such a question is important at both an immunological and life-history level. For example, do individuals allocate these molecules to some immune responses and not others? Which one(s)? Do such allocation patterns differ based on availability of carotenoids (both in the diet or stored in tissues) or the challenges or value of such immune responses across species? Additionally, while all of these questions are most appropriately initially addressed under controlled, laboratory conditions, the impacts of these must subsequently be addressed in the field to test how these differences affect subsequent survival or reproduction.

Interaction of dietary carotenoid access during development and adulthood on adult phenotype

Dietary carotenoid supplementation during development affected adult PHA response, and interacted with adult dietary carotenoid levels to affect carotenoid circulation during an immune response. Counter to my predictions, however, these differences manifested with individuals receiving relatively low levels of carotenoids during development having an increased immune response and elevated circulating carotenoid levels. These data did not fit any of the developmental plasticity models put forth by Monaghan (2008), leading to my

description of a new hypothesis (hard-knock life hypothesis: poor conditions during development may be advantageous in all adult environments, provided that the individual survives the poor developmental conditions; see Chapter 4, Discussion) that could be tested in future experiments.

However, generating more models is just one alternative for future research. Instead, it may be important to more carefully consider what is meant by "poor" and "good" environments during development and adulthood (Monaghan 2008). For example, and apropos to my dissertation, while elevated carotenoid levels have traditionally fallen under the category of a "good" environment, high levels of carotenoids can be toxic (i.e., pro-oxidative) under certain conditions. For example, there is evidence that carotenoids increase the risk of lung cancer (Satia et al. 2009), and decrease flight performance in birds (Huggins et al. 2010). More broadly, there is analogous evidence of such a phenomenon in caloric intake. While the detrimental effects of starvation are self-evident, there are subtleties that are seemingly contradictory. For example, "poor" access to nutrients in the form of caloric restriction increases longevity, which would traditionally be considered a benefit (Mehta and Roth 2009). In other words, more thought is warranted regarding inference of what "high" and "low" quality conditions are for developing animals.

This framework is further complicated by the fact that some developmental perturbations result in advantages for some aspects of phenotype or performance, and disadvantages for others. For example, food restriction during the larval stage of the butterfly *Bicyclus anynana* resulted in smaller adults

(disadvantage), but better coping of forced flight (advantage; Saastamoinen et al. 2010). Results such as these highlight the difficulties in assigning a value of "good" or "poor" to various aspects of developmental nutrition, as without measuring fitness across a range of adult environments, we simply lack the information to make such distinctions.

In future work, I plan to shift my language usage away from qualifiers such as "good" or "poor," and rather utilize treatments such as low, medium, and high, based on the natural range of variation found within those variables in the wild. This framework will have two immediate benefits. First, it will allow me to avoid an *a priori* framework that may be based on assumptions, rather than data, such as how much food is "good." Thus, any results will simply be related to the natural variation present in wild populations, and allow for discussion of alternative strategies, rather than relative quality of values that are "better" for some dependent metrics, and "worse" for others. Second, most biologists assume some form of linearity within their data (e.g., linear regressions, covariates in ANCOVAs, etc.). In reality, though, both too much and too little could be detrimental, and by breaking treatments into groups that represent major blocks along the continuum, I will be able to avoid these assumptions.

GENERAL CONCLUSIONS

Developmental plasticity of adult beak coloration

In my two manipulative studies, wherein I used immune challenges or dietary access to carotenoids to alter developmental conditions, no aspect of beak

color was affected by treatment during development. However, in my correlational study, adult beak color was predicted by body condition during development. Together, these studies suggest that adult ornamentation in mallards is more tightly linked to developmental physiology rather than neonatal environment. This suggests that either genetic effects, maternal effects, conditions experienced during adulthood, or (most likely) some combination of these factors are the primary factors driving ornament expression in mallards.

Interestingly, while I repeatedly found support for the ability of the mallard beak to signal adult immune function (i.e., change in beak color was proportional to degree of immune response), such a relationship was lost in the experiment in which I administered immune challenges during development. There are multiple ways to interpret this finding, but one interpretation suggests that the beak color of mallards reveals current investment in immunity, not how an individual will respond to an immune challenge. In other words, females may use male beak coloration as a marker of real-time information regarding the health state of a potential mate. Such information content fits the life history of mallards. Because males and females establish pair bonds over winter and then breed in the spring (Drilling et al. 2002), females have repeated interactions with males. Thus, there may have been selection for the *lability* of beak coloration to signal *current* immune investment, rather than beak color to signal an individual's ability to respond to a future immune challenge.

It is worth considering if I would have been more likely to find an effect of developmental conditions on adult coloration if I had examined a more fixed

form of coloration, for example carotenoid-based feather coloration. I think it is probable that I would have, for two main reasons. First, carotenoid deposition into feathers is a relatively derived trait, and probably requires a greater number or diversity of specific enzymes and other forms of genetic control to result in that deposition. Such control, with both transcriptional and physiological inputs, provides a larger number of targets upon which developmental plasticity may act. Second, because carotenoids are deposited in feathers only during molt, it may be possible for individuals to "cheat" and deposit disproportionately large quantities of carotenoids into the feathers at the expense of depleting endogenous carotenoid stores (e.g., liver). Selection may have acted against such cheating via a more honest signal that incorporates information across multiple time points, including development.

Developmental plasticity of adult immune response

Adult immune response of mallard drakes is indeed developmentally plastic, with both carotenoid content of the diet and immune challenges during development affecting adult immune function. However, as has been discussed recently elsewhere (Special issue of Functional Ecology, Ecological Immunology, Feb. 2011, 25(1)), immune responses are not always positively correlated, and may frequently be negatively correlated (Salvante 2006). This leads to the next challenge in studying immunology in non-domesticated animals: confronting what each immune metric means in terms of the costs and benefits of surviving and reproducing in the wild. There are many ways to quantify an animal's

immune response, but most are focused on a single component of the incredibly complex and often redundant immune system. Additionally, maximally responding to a particular immune metric is not necessarily associated with the most beneficial response (Graham et al. 2011); both auto-immunity and wasting energy or other resources on unnecessary investment in immune responses can be maladaptive.

Questions regarding the ecological and evolutionary importance of commonly used immune metrics are undoubtedly important. Parasite and pathogen pressure is postulated to have been fundamental to the evolution of many sexually selected traits (Hamilton and Zuk 1982) and may be a natural selection agent at least as potent as predation (Edeline et al. 2008). Thus, research that can more clearly inform the fitness consequences of many of the commonly used immune metrics is critical toward an appropriate understanding of ecological immunology, as is the development of new, informative immunological techniques. However, while such work is necessary for questions within the field of ecological immunology, it is not an end unto itself; rather, such knowledge will allow for hypothesis testing that measures immune function and is grounded in a solid ecological framework.

My dissertation work provides evidence that one such hypothetical framework is both interesting and likely to provide biologically relevant insights. Because both of my developmental perturbation studies demonstrated that adult immune metrics are developmentally plastic, further work should investigate how a wide variety of conditions during development can affect adult immune

function, and how these differences in immune function translate to differences in survival and lifetime reproduction. Such work will greatly increase our understanding of the natural world; many studies have examined ecological phenomena across space, but relatively few have examined within-individual differences across time.

Final conclusions

Much work remains to be done regarding the importance of developmental plasticity to adult survival and reproduction. An animal's phenotype is based on gene expression and the summation of its previous experiences, with the relative importance of those earlier experiences based upon their intensity, frequency, and recency. However, these relationships are not linear; for example, when developing adult fat stores, differences in nutrient access during pre-birth/hatch may be just as important as nutrient access during the month preceding adult assessment of those fat stores. Future work will continue to disentangle the effects of perturbations across life stages on a variety of traits, including ornamentation, immune function, behavior, and physiology. Additionally, future research should incorporate genetic diversity and transcriptome differences across time, both within and among individuals in order to identify the genetic mechanisms responsible for developmental plasticity. The field of examining the ecological and evolutionary significance of developmental plasticity, while studying a multitude of dependent variables, remains full of possibilities.

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APPENDIX A

RELATIONSHIPS BETWEEN DIETARY CAROTENOIDS, BODY TISSUE CAROTENOIDS, PARASITE BURDEN, AND HEALTH STATE IN WILD MALLARD (*ANAS PLATYRHYNCHOS*) DUCKLINGS

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Relationships between dietary carotenoids, body tissue carotenoids, parasite burden, and health state in wild mallard (*Anas platyrhynchos*) ducklings

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ABSTRACT

While rodents and other mammals have traditionally served as models for studying carotenoid physiology, many wild animals from a variety of other taxa utilize carotenoids for self-maintenance and reproduction and accumulate far greater concentrations than those found in mammals. Though we have basic understandings of the control and value of carotenoids in some wild animal systems, many gaps remain. For example, parasites and pathogens impose severe survival constraints on free-ranging organisms, but little is known of how carotenoids work in concert with the immune system to combat natural infectious challenges. Furthermore, due to the high mortality rate from which many young animals suffer, health and carotenoid status during the early stages of development may be critical to survival. The relative importance of dietary versus physiological mechanisms for carotenoid uptake has also been understudied in the wild. To begin to shed light on these issues, we studied relationships between dietary and tissue carotenoids, hematological immune parameters, and endoparasitism of wild mallard (*Anas platyrhynchos*) ducklings at a variety of ages. Lutein, zeaxanthin, β-cryptoxanthin, β-carotene, and canthaxanthin were the most common carotenoids in liver, plasma, and gut contents. We found that, early in development (when food intake is limited), carotenoids were comparatively concentrated in internal tissue (e.g., liver), presumably a carry-over from maternal contributions in yolk, but as ducklings approached independence (and increasingly fed on their own) concentrations were greatest in gut contents. Canthaxanthin concentrations were lower in the plasma and liver of older individuals compared to younger ducklings, even though gut canthaxanthin concentration did not change with age. Additionally, β-carotene was nearly absent from circulation, despite moderate levels within the gut, suggesting a high rate of conversion to retinol. Using principal components analysis, we revealed a correlation between an increased ability to assimilate dietary carotenoids and lower levels of chronic stress (as assessed by lower heterophil-to-lymphocyte ratios) and a correlation between a reduced carotenoid status and increased investment in the immune system (as assessed by higher total leukocyte count). We also found that individuals without parasites had an overall reduced carotenoid status. Thus, we demonstrate age-specific differences in carotenoid allocation in growing animals from a precocial bird species and provide correlational evidence that parasitism and health in wild animals are related to carotenoid status and assimilation ability.

Introduction

Domesticated mammals have traditionally served as model species for investigating the role of carotenoids in human nutrition and health (e.g., mouse [1]; ferret [2]; rat [3]). However, many other animals acquire and use carotenoids, including fishes [4,5], reptiles [6,7], and birds [8], with concentrations often exceeding those in most mammals (e.g., ~1 μg/mL in humans [9], ~30 μg/mL in zebra finches *Taeniopygia guttata* [10]). Thus, these study systems may be ideal for better understanding how animals have come to ingest, uptake, and use carotenoids. In the past five years alone, there have been over 400 refereed journal articles about carotenoids in ecology-related fields (Web of Science, accessed 10 February 2010). Carotenoids can influence health, survival, and reproduction in adult animals [11], and also function as integumentary pigments that generate sexually attractive colors [8], retinal filters that protect the eye from solar damage and tune vision [12,13], and catalysts of development in young animals (as carotenoids are transferred to offspring via the maternal egg yolk [14]).

Even though these various functions have been demonstrated in a range of wild animals, from sea urchins [15] to chickens [16], there are several important gaps in research and theory on carotenoid physiology and functions. First, there has been surprisingly little work on the dietary sources and limitations of carotenoids in wild animals [17,18] and how these relate to various physiological and morphological characteristics of wild animals. This lack of

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information constrains the types of questions that researchers can ask, because the most ecologically relevant studies need to manipulate dietary carotenoid access within a natural range of variation. Second, though the relationship between carotenoids and several aspects of the immune system (leukocyte levels and heterophil:lymphocyte (*H*:*L*)[1] ratio [19], humoral immunity [20], cutaneous immune response [21]) has been well-explored in laboratory animals [22–24], the pathogens and parasites that coevolve with immune systems of wild animals have largely been neglected in the context of carotenoid actions [25], although evidence suggests that parasites can affect carotenoid absorption, circulation, and coloration [26,27]. Third, while attention has often been paid to carotenoids in very young animals (in eggs or very soon after hatching [28]) or in adult animals (as above), comparably less emphasis has been placed on the extended phase of development leading up to sexual maturity. Research in several bird species has shown that even relatively short periods of carotenoid restriction during ontogeny can result in reduced circulating levels of carotenoids at adulthood [29], while carotenoid supplementation during development affects subsequent plumage coloration [30] and immune function [31]. The post-hatch or -birth period of ontogeny is especially extended in many long-lived animals, including humans, leaving the opportunity for many persistent (adult-lasting) impacts of conditions during the growth and maturation phase.

What is now needed to fill these knowledge gaps is a careful description of dietary and tissue carotenoid pools as a function of the parasite and health challenges faced by a wild animal. We have been studying developing mallard ducklings (*Anas platyrhynchos*) as a means of understanding the limits and effects of carotenoid pigments during development [32], mostly via captive experimental manipulations. In this study, we examined the relationship between carotenoid levels in plasma, liver, and gut-content samples of wild mallard ducklings at a variety of ages. Mallards are model organisms for studying ecotoxicology [33,34] and wildlife diseases [35], and the carotenoid-based coloration of adult males signals immunocompetence [36] and is used by females to choose mates [37,38]. However, very little is known about the distribution of carotenoids among tissues in these free-ranging precocial animals [39], the relationship between carotenoid status and health [40], and how these relationships vary throughout the neonatal stage [32].

Methods

In May–June 2007, we used throw-nets to trap thirty ducklings of varying age and unknown sex and relatedness from eight different parks (number of ducklings per park: mean = 3.75, range = 1–12) in Tempe, Arizona, USA. Though wild ducks will sometimes hybridize with domestic releases in parks, we did not detect any aberrant plumage or size discrepancies in the captured ducklings, and we observed relatively few adult domestic or hybrid waterfowl in the parks we visited. At capture, we recorded each individual's body mass and tarsus length and collected 200 μl of blood from the alar vein using heparinized tubes to quantify circulating carotenoid content and variables associated with health, including prevalence of blood parasites and leukocyte density and diversity (see below). After euthanasia, we collected gut-content samples from the gizzard to quantify carotenoid content of an individual's diet, and from within the small and large intestine to quantify endoparasite load. We also collected the liver for carotenoid quantification. Because ducklings were of unknown age, we used mass to assign each duckling to an age group based on life-stage differences in nutrient acquisition. SMALL birds (<50 g, $n = 12$) still possessed a yolk sac. Based on growth data [41], MEDIUM birds (90–300 g, $n = 8$, no individuals weighed 50–90 g) were likely to be less than 3 weeks old, a period in which ducklings get most of their food at the water surface or on land [41], and LARGE birds (300–800 g, $n = 10$) were likely to be over 3 weeks old, at which point ducklings begin subsurface feeding [41].

To quantify the health of an individual, we measured *H*:*L* ratio, total leukocyte count (TLC), and degree of parasitism. We elected to quantify these parameters because carotenoid levels have been linked to *H*:*L* and TLC [19], as well as intestinal parasitism [26], and because these data can be collected at a single time point, thus avoiding animal recapture or laboratory conditions for wild birds. Thus, we created duplicate blood smears that were air-dried, fixed in methanol, and stained with Wright's Giemsa (Vol-U-Sol, Salt Late City, UT). Smears were then analyzed at 1000× using an Olympus BX60 light microscope (Olympus Optical Co., Tokyo). We counted leukocytes in random, non-overlapping fields of view until we reached 100, and then calculated the *H*:*L* ratio (coefficient of variation (CV) = 65.29), which increases during periods of stress [42]. We calculated total leukocyte count (TLC; CV = 65.44) as the number of white blood cells per 10,000 erythrocytes [43]. We also examined intestinal and fecal samples by placing 2–3 drops of sample with 4–5 drops of water on a slide, adding a cover slip, and examining the slide at 40× and 100× under a compound microscope to quantify internal parasite load (roundworms, phylum Nematoda; roundworm eggs; coccidia, phylum Apicomplexa; tapeworms, phylum Platyhelminthes, class Cestoda [44]) based on an ordinal 0–4 classification system (0 = absent, 1 = rare, 2 = uncommon, 3 = common, 4 = abundant; *sensu* [45]). Because variation in parasitism was fairly bimodal (see Discussion), we used this information to categorize individuals by presence or absence of all parasites, and presence/absence of just tapeworms.

To analyze plasma carotenoid content, we used a standard organic-solvent extraction protocol [46]. Briefly, we centrifuged heparinized blood samples for 30 s at 10,000 rpm to isolate the plasma fraction. We then added 500 μl of ethanol to 50 μl of plasma, vortexed the solution, and then added 500 μl of 1:1 hexane:methyl *tert*-butyl ether (MTBE). After a second centrifugation, the supernatant was recovered and dried under a stream of nitrogen, resuspended in mobile phase (42:42:16 methanol:acetonitrile:dichloromethane), and analyzed via high performance liquid chromatography (HPLC). Carotenoid types and amounts were calculated based on elution time and reflectance curves compared to known standards. All isomers for a given carotenoid type were summed together to form a single value (e.g., all lutein *trans* and *cis* isomers are hereafter considered "lutein").

We dried diet samples on weighing paper at 30 °C for 24 h, recorded dry mass to the nearest 0.1 mg with an electronic balance, and then ground the samples using a mortar and pestle in the presence of 1:1 hexane:MTBE. The solution was transferred to a microtube, centrifuged, the supernatant was transferred again, dried down under nitrogen, and saponified to remove fatty-acid esters that can interfere with HPLC analysis (see below). To analyze liver samples for carotenoid content, we excised a portion of the liver (range 0.15–0.34 g) and ground the tissue in the presence of 1:1 hexane:MTBE using a ball grinder (Retsch MM200). We centrifuged the liquid, transferred the supernatant, and dried it down under a stream of nitrogen so that the pigments could be saponified prior to HPLC analysis. To saponify the food and liver samples, we added 1 mL of 0.5 M methanolic NaOH to the dry pigment, vortexed the sample, and capped the tube with nitrogen (to reduce pigment oxidation). We stored the samples at room temperature in the dark for 6 h, vortexing occasionally. We then added 1 mL of saturated sodium chloride, vortexed the sample, added 2 mL of doubly-distilled

[1] *Abbreviations used: H:L*, heterophil:lymphocyte; TLC, total leukocyte count; CV, coefficient of variation; MTBE, methyl *tert*-butyl ether; HPLC, high performance liquid chromatography.

water, vortexed, added 3 mL of 1:1 hexane:MTBE, and then shook the tube vigorously for 1 min. After centrifuging the tube at 3000 rpm for 5 min at room temperature, we transferred the hexane:MTBE supernatant fraction to a fresh tube, evaporated it under a stream of nitrogen, and resuspended the pigment for HPLC analysis similar to the plasma samples.

Carotenoid values were not normally distributed, and transformations of the carotenoid data frequently failed to achieve normality. However, MANOVA models with error degrees of freedom greater than 20, such as the ones presented here, are robust to departures from normality [47]. Therefore, we present MANOVA models using non-transformed carotenoid data to examine differences in carotenoid levels within a sample type, and differences in carotenoid levels among different mass groups (i.e., SMALL, MEDIUM, LARGE) within a sample type. *H*:*L* ratio and TLC were also not normally distributed, but logarithmic and square-root transformations, respectively, resulted in normally distributed variables (Shapiro–Wilks' $W \geqslant 0.92$, $P \geqslant 0.05$). For all non-MANOVA analyses, we used either non-parametric statistics or transformed data to meet normality assumptions. Because there were no overall site differences in dietary carotenoid concentrations (MANOVA, Wilks' $\lambda = 0.12$, $P = 0.66$), we did not include site as a random effect in any model.

To examine which parasite and carotenoid parameters predicted health state, we used multiple regression using stepwise model selection for continuous dependent data (log of *H*:*L* ratio and square root of TLC), and logistic regression using stepwise model selection for nominal dependent data (presence of any intestinal parasites or presence of tapeworms). For multiple regression analyses, predictor variables included principal components of carotenoid levels (see below), while logistic regression analyses included those same principal components with the addition of the log of *H*:*L* and square root of TLC to see if the individual's leukocyte profile was associated with parasitism. We used principal components analysis to generate orthogonal variables from all gut content, liver, and plasma carotenoid levels because we were more interested in general trends stemming from an individual's overall carotenoid status. We utilized the first two (PC1 and PC2) in all future analyses because these were the only two eigenvectors to individually capture more than 10% of the variation (proportion = 42.4% and 26.9%, respectively). Due to eigenvector loadings of PC1 (Table 1), which loaded positively with all dominant carotenoid values, we considered higher PC1 values (hereafter, carotenoid status) indicative of greater carotenoid concentrations across all sample types. We associated higher PC2 values with greater carotenoid assimilation ability because PC2 (hereafter, carotenoid assimilation ability) loaded positively with the dominant plasma and liver carotenoids, but negatively with the dominant gut content carotenoids, which would mean that individuals with a higher PC2 score would have more carotenoids in plasma or liver relative to the diet. All analyses were performed using SAS 9.2 (SAS Institution Inc., Cary, NC, USA), and $n = 30$ for all analyses.

Results

When pooling all animals and samples together into one analysis, total carotenoid concentration was highest in gut contents, followed by liver and plasma (Fig. 1). Lutein, zeaxanthin, β-cryptoxanthin, β-carotene, and canthaxanthin were found in almost every sample, while α-carotene was also found in both the liver and gut contents of many individuals (Table 2). Within all sample types, lutein was the most abundant carotenoid (Fig. 2). However, there were several differences in carotenoid profile among sample types. Within plasma, zeaxanthin was the second most abundant carotenoid, followed by similar levels of β-cryptoxanthin and canthaxanthin and very low levels of β-carotene (Fig. 2). Carotenoid profiles of liver and gut contents (Fig. 2) were qualitatively similar in many respects, with high levels of lutein, moderate levels of zeaxanthin, β-carotene, and canthaxanthin, and low levels of α-carotene. The main difference in carotenoid profile of these two sample types was the relatively high levels of β-cryptoxanthin in liver, despite a near absence of β-cryptoxanthin from the gut contents.

Plasma carotenoid concentration differed by developmental stage (MANOVA, Wilks' $\lambda = 0.23$, $P < 0.0001$), with LARGE ducklings circulating higher levels of β-carotene compared to both MEDIUM and SMALL ducklings (Tukey post-hoc comparisons, all $P < 0.05$; Fig. 3). However, SMALL ducklings circulated higher levels of canthaxanthin than both MEDIUM and LARGE ducklings (Fig. 3). Liver carotenoid concentration also differed by developmental period

Table 1
Eigenvector loadings of principal components for carotenoid status (carotenoid concentration in plasma, liver, and gut contents) of wild mallard ducklings. A greater PC1 was associated with a greater overall carotenoid status, due to the positive loading of all dominant carotenoid types. PC2 loaded positively with the most abundant carotenoids in the plasma and liver, but negatively with those in the gut contents. Therefore, a greater PC2 score was interpreted as a greater carotenoid assimilation ability.

Dependent variable	PC1	PC2
Plasma lutein	0.272	0.073
Plasma zeaxanthin	0.189	0.189
Plasma β-cryptoxanthin	−0.031	0.231
Plasma β-carotene	0.234	−0.151
Plasma canthaxanthin	−0.087	0.266
Plasma total	0.253	0.138
Liver lutein	0.264	0.212
Liver zeaxanthin	0.171	0.340
Liver β-cryptoxanthin	0.034	0.370
Liver β-carotene	0.212	0.156
Liver α-carotene	0.205	−0.132
Liver canthaxanthin	−0.021	0.247
Liver ketocarotenoid	−0.006	0.329
Liver total	0.177	0.333
Gut lutein	0.289	−0.151
Gut zeaxanthin	0.246	−0.164
Gut β-cryptoxanthin	0.232	0.045
Gut β-carotene	0.284	−0.156
Gut α-carotene	0.288	−0.149
Gut canthaxanthin	0.302	−0.080
Gut ketocarotenoid	0.064	0.224
Gut total	0.296	−0.130

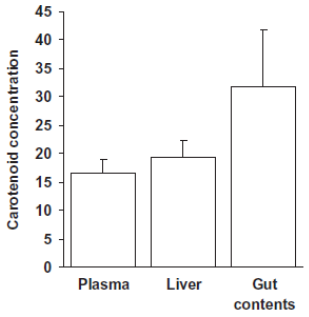

Fig. 1. Mean total carotenoid concentration for types of tissues sampled in mallard ducklings. Concentrations given in μg/L for plasma, and μg/g for liver and gut contents.

Table 2
Identification of carotenoid types. Isomers within a carotenoid type constituted a relatively small proportion of the total level (less than 5% in the plasma, less than 2% in the diet, and less than 17% in the liver). An A superscript denotes comparison to an external standard, while a B superscript denotes reference to published values [68,69].

Carotenoid	Absorption maxima		Peak retention time (min)
Lutein[A]	448	476	6.7
Zeaxanthin[A]	454	479	8.1
β-Cryptoxanthin[B]	454	481	14.4
β-Carotene[A]	456	483	21.3
α-Carotene[B]	452	476	22.1
Canthaxanthin[A]	480		9.2
Unidentified liver ketocarotenoid[B]	464		13.8
Unidentified gut ketocarotenoid[B]	475		7.5

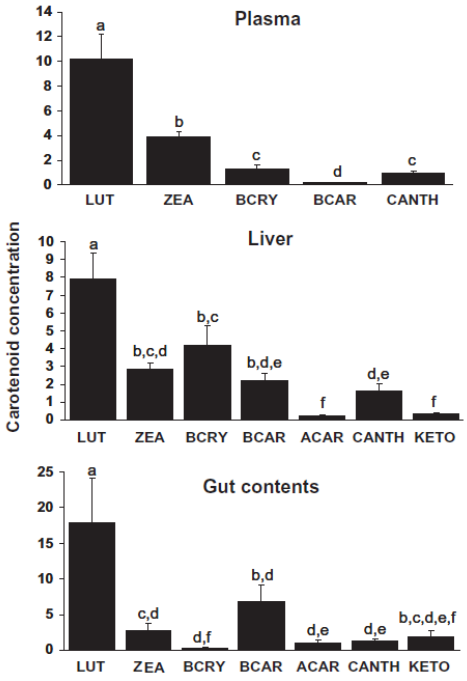

Fig. 2. Mean carotenoid concentration among all ducklings for the predominant carotenoid types found. Concentrations given in μg/L for plasma, and μg/g for liver and gut-content samples. Within a sample type, statistically significantly different ($P < 0.05$ post-hoc test) concentrations by carotenoid type are denoted with a different letter. Abbreviations: LUT, lutein; ZEA, zeaxanthin; BCRY, β-cryptoxanthin; BCAR, β-carotene; ACAR, α-carotene; CANTH, canthaxanthin; KETO, unidentified ketocarotenoid.

(MANOVA, Wilks' $\lambda = 0.13$, $P < 0.0001$). SMALL ducklings had more liver β-cryptoxanthin, canthaxanthin, and an unidentified ketocarotenoid than MEDIUM or LARGE ducklings, less α-carotene than LARGE ducklings, and a greater concentration of total liver carotenoids than MEDIUM ducklings (Tukey post-hoc comparisons, all $P < 0.05$; Fig. 3).

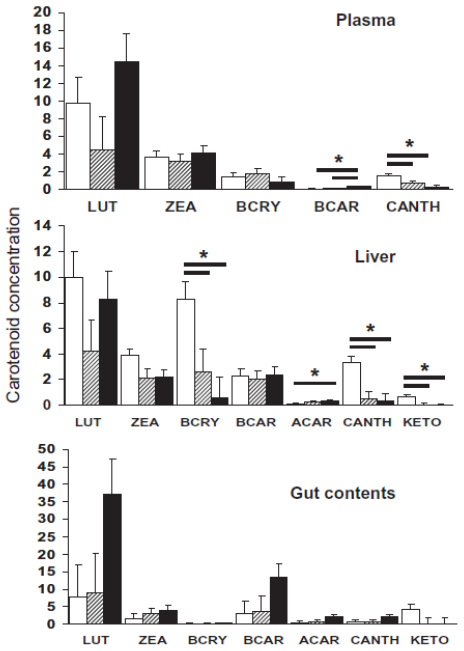

Fig. 3. Mean carotenoid concentration among ducklings by developmental stage for the predominant carotenoid types found, with empty bars denoting SMALL, thatched bars denoting MEDIUM, and filled bars denoting LARGE. Concentrations are given in μg/L for plasma, and μg/g for liver and gut-content samples. Within a sample type, statistically significantly different ($P < 0.05$ post-hoc test) levels within a carotenoid type are denoted with an asterisk and lines highlighting which life-stage groups are different. See Fig. 2 for abbreviations.

Table 3
Within-group correlations between total carotenoid titer among a variety of sample types (liver, plasma, gut contents) in mallard ducklings. Data are presented as Spearman rank correlations (r_S), followed by associated P-values.

Group	Plasma–liver	Plasma–food	Liver–food
All	0.701, <0.0001	0.535, 0.0027	0.662, <0.0001
Small	0.734, 0.0065	0.147, 0.649	0.280, 0.379
Medium	0.190, 0.651	0.357, 0.385	0.619, 0.102
Large	0.903, 0.0003	0.673, 0.033	0.794, 0.0061

Gut carotenoid levels also varied by developmental period (MANOVA, Wilks' $\lambda = 0.35$, $P = 0.03$). However, no univariate ANOVAs were significant, and post-hoc differences within each carotenoid type were non-significant ($P > 0.05$), with the exception of LARGE ducklings ingesting a significantly higher concentration of lutein than SMALL ducklings ($P = 0.042$; Fig. 3).

Correlations between plasma, liver, and gut-content total carotenoid titer changed by developmental stage. Plasma and liver carotenoid content were significantly correlated for SMALL ducklings, while total carotenoid concentration from plasma, liver, and gut contents were significantly correlated for LARGE ducklings (Table 3). No other correlations were significant ($P > 0.05$; Table 3).

H:*L* ratio and TLC were not significantly correlated at any developmental stage (all $|r_S| < 0.11$; all $P > 0.7$). Using stepwise multiple

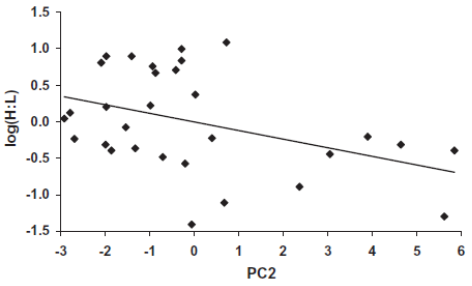

Fig. 4. Linear relationship between PC2 (carotenoid assimilation ability) and the log of *H:L* for all ducklings ($F_{1,28}$ = 5.40, P = 0.0276, R^2 = 0.17).

regression, we found that a greater PC2 score (carotenoid assimilation ability) was negatively related to the log of *H:L* ratio ($F_{1,28}$ = 5.63, P = 0.025; Fig. 4). The square root of TLC was positively related to body mass (P < 0.0001), but negatively related to PC1 (i.e., carotenoid status; P = 0.035; overall model $F_{2,27}$ = 19.24, P < 0.0001). Absence of any intestinal parasites was associated with a lower carotenoid status (Wald Chi-square = 4.34, P = 0.037, point estimate = 0.65), while the absence of tapeworms specifically was associated with a lower TLC (Wald Chi-square = 6.01, P = 0.014, point estimate = 0.46).

Discussion

We found that carotenoid concentration varied by carotenoid type, tissue, and age in wild mallard ducklings, and that carotenoid status was associated with an individual's health. Mallard ducklings suffer high mortality rates; only 25% of ducklings reach 30 days old [48], while once ducklings have fledged (i.e., mass >800 g), the survival rate during the non-hunting period is high (86% chance of survival [49]). Therefore, any advantage during the early-developmental period, including maintaining health or reducing parasite burden, can be especially important to survival. In addition, because developmental conditions can affect adult carotenoid-based coloration in mallards [32] and other birds [50], and thus reproductive success [38], the role of carotenoids during development in wild animals may be particularly important to an individual's fitness.

Lutein was the most abundant carotenoid in the gut contents, liver, and plasma of wild mallard ducklings, which is unsurprising for three reasons: (1) birds tend to accumulate polar carotenoids [51], (2) lutein is the predominant carotenoid in mallard yolks [52], and (3) mallards tend to eat xanthophyll-rich plant material once they are 3 weeks old [41]. β-carotene levels were relatively high in the gut and liver, but relatively scarce in the plasma, which mirrors previous findings in other species [53]. A survey of zoo animals revealed that many birds, including several non-mallard duck species, do not circulate high levels of β-carotene [54], potentially due to hepatic or duodenal conversion of β-carotene into retinol. Interestingly, there was a relatively large amount of β-cryptoxanthin in the liver and a moderate amount in the plasma, even though there were very low levels in the gut contents. This suggests that mallard ducklings are either highly effective assimilators of this relatively scarce carotenoid, that β-cryptoxanthin is not frequently metabolized or utilized beyond being stored, and/or that it is metabolically formed. It is not known what role, if a unique one even exists, β-cryptoxanthin has in mallard physiology, although previous work has demonstrated a link between β-cryptoxanthin and apoptosis and bone resorption in mice [55]. If there is no unique role, β-cryptoxanthin may act similarly to most other carotenoids; that is, it may impact antioxidant status, immune function, or integumentary coloration of the individual, although it is unlikely to drive any of the coloration due to its minimal representation in adult male mallard beaks (unpub. data).

We examined developmental variation in carotenoid status and found that SMALL ducklings circulated more canthaxanthin in plasma and had more canthaxanthin-, β-cryptoxanthin-, and unidentified ketocarotenoid-rich livers than MEDIUM or LARGE ducklings. This suggests that levels of canthaxanthin, β-cryptoxanthin, and the unidentified ketocarotenoid, but not other carotenoids, decrease as a function of age, allowing for several possibilities. For example, assimilation rates may decrease as animals grow (there were no differences in canthaxanthin concentration, which is largely driven by the presence of aquatic prey [56], in the gut contents among different age groups), with duckling receiving most of their canthaxanthin in the form of maternal effects, i.e., the carotenoid-rich yolk. Alternatively, older ducklings may accumulate these particular carotenoids at lower levels, regardless of assimilation ability. Other avian species show differences in carotenoid profile as a function of age [57], although we are among the first to statistically account for any differences in carotenoid content in the diet. Body size itself has been argued to be an important determinant of carotenoid accumulation, with lower levels in larger animals (e.g., due to nutritional scaling principles [58,59]).

The relationship of carotenoid titers among sample types within an individual changed with developmental stage. Lending support for the importance of maternal effects via yolk is the tight relationship between plasma and liver, but not gut-content, carotenoid levels in SMALL ducklings. While all ducklings had food within the gizzard, SMALL ducklings had qualitatively less (MWB – pers. obs.), as is expected when they are still heavily reliant on their yolk sac for nutrition. In chickens, the liver reaches a peak in carotenoid concentration at the time of hatch, with a concomitant decrease in yolk carotenoid concentration [60]. Because gut carotenoid levels did not correlate with either plasma or liver levels, it is probable that the carotenoid status of ducklings during the first few weeks of life is predominantly determined by maternal yolk investment [61]. By the time ducklings had reached the LARGE developmental stage, the carotenoid content of all sample types were intercorrelated, suggesting that by the time they are nearly fully grown, an individual's carotenoid status is dependent upon that individual's ability to acquire carotenoid-rich food. Interestingly, within MEDIUM ducklings, there were no correlations within individuals regarding the carotenoid levels of gut contents, liver, or plasma. One interpretation of this result is that, during this period of development, there is a transition in carotenoid mobilization and allocation strategies. One possible scenario is that circulating plasma levels are driven by carotenoids stored in the liver during the SMALL stage, with a shift toward plasma and liver levels being driven by food levels during the LARGE stage. This framework needs to be experimentally verified, and would highlight the importance of post-natal development in carotenoid physiology [29,62].

We also uncovered relationships between carotenoid status and health. An increasing PC2 (carotenoid assimilation ability) was associated with lower *H:L* ratio, which is often associated with lower chronic stress, i.e., lower levels of glucocorticoid hormones [42]. This finding is consistent with evidence that male red grouse (*Lagopus lagopus scotticus*) experiencing a stressor (parasitic infection) have increased glucocorticoid levels and decreased carotenoid-based integumentary coloration [63], but extends this phenomenon to ontogeny. We therefore suggest that individuals that are not exposed to chronic stressors (e.g., predation, competition, variable abiotic conditions) may be more effective carotenoid

assimilators. In support of this argument, McGraw et al. [64] found in adult male American goldfinches (*Carduelis tristis*) that nutritional stress (i.e., calorie intake, independent of carotenoid ingestion) compromised the ability of birds to transport carotenoids through the bloodstream. The correlational links presented here should be validated with experimental investigation, however.

We also found that parasite load was related to PC1; individuals with no intestinal parasites had a reduced carotenoid status. A robust immune response in young birds can decrease parasite fecundity [65], and the correlational results presented here could be interpreted as the higher levels of carotenoids in parasitized individuals could be due to an increased preference to forage for carotenoid-rich foods and a mobilization of carotenoids to assist in an immune response. Overall, though, this result runs counter to most other findings in the literature, which show that intestinal parasitism results in a decrease in circulating carotenoid levels ([25,26], but see Ref. [66] for no differences in unsupplemented house sparrows, *Passer domesticus*, and Ref. [67] for a positive relationship between ectoparasitism and carotenoid-based beak color in female zebra finches) and that carotenoid supplementation can reduce parasite replication rate [27]. It should be noted, however, that detectable non-tapeworm intestinal parasitism levels were scored at a level of 1, with the exception of a single 2. In contrast, tapeworm parasitism levels ranged from 3 to 4, meaning that even moderate levels of parasitism were restricted to individuals with tapeworms. When examining the subset of individuals afflicted with tapeworms specifically, we found that those with tapeworms had a higher TLC, suggesting an activation of the immune system. Because TLC was negatively related to carotenoid status (PC1) when controlling for mass, this suggests a different parasite–carotenoid relationship than that found above. Specifically, activation of the immune system in response to intestinal parasitism of tapeworms and/or more intense levels of intestinal parasitism overall may result in a decreased carotenoid status. However, as above, this relationship is correlational, and it should be noted that PC1 did not remain in the final logistic regression modeling the presence of tapeworms.

Here, we present data that carotenoid physiology changes throughout post-natal development in a waterfowl species, as there is a shift from circulating carotenoid levels being correlated to liver carotenoid levels, then to neither liver nor dietary carotenoid levels, and finally to both liver and dietary carotenoid levels, yielding potentional late-life carotenoid assimilation consequences [29]. Further, individual differences in carotenoid status are associated with variation in parasite load and immunological parameters, lending support to the idea that carotenoid access and physiology can shape chances of survival and reproduction in wild animals.

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APPENDIX B

PAST OR PRESENT? RELATIVE CONTRIBUTIONS OF DEVELOPMENTAL AND ADULT CONDITIONS TO ADULT IMMUNE FUNCTION AND COLORATION IN MALLARD DUCKS (*ANAS PLATYRHYNCHOS*)

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ORIGINAL PAPER

Past or present? Relative contributions of developmental and adult conditions to adult immune function and coloration in mallard ducks (*Anas platyrhynchos*)

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Abstract Developmental conditions affect adult physiological processes and phenotypic traits, including those associated with both survival and reproduction. Carotenoids are molecules that generate sexually attractive coloration, and these pigments are acquired throughout life and can affect antioxidant capacity and immunocompetence of young and old animals. However, few studies have tracked carotenoid status and condition during development and into adulthood to understand how ontogeny affects later-life health and coloration of both males and females. We reared male and female mallard ducks (*Anas platyrhynchos*) from hatch to adulthood, measured circulating carotenoid titers and body condition (i.e., size-adjusted body mass) throughout development, and assessed adult immune function and integumentary carotenoid-based beak and foot coloration. We found that adult immune function (wing web swelling response to phytohemagglutinin; PHA) in males was positively correlated with body condition during the growth period of development, rather than adult condition, and similarly that both male and female beak coloration was associated with developmental, rather than adult, body condition. We also found associations between coloration and health during adulthood; males with more carotenoid-rich beaks (a sexually attractive feature) tended to have a more robust adult PHA response and a greater antibody response to a novel antigen, while females with less carotenoid-rich beaks had greater antibody responsiveness at adulthood. In addition, male beak color changed over the course of the 24-h PHA test in proportion to the degree of PHA swelling. However, intensity of foot coloration (a trait of unknown sexual significance) was not associated with any condition, carotenoid, or immune metric for males or females. Taken together, our findings implicate key developmental components to the expression of both survival- and reproduction-related traits at adulthood, but that for a dynamic trait like beak color, there are also important adult conditions that can alter signal expression.

Keywords Body condition · Carotenoid · Coloration · Development · Immune function · Ontogeny · Sexual maturity

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Introduction

Physiological processes and phenotypic expression of adults are often triggered by conditions associated with sexual maturity (i.e., the link between sex steroids, gonadal development, and reproduction; Blas et al. 2010). However, developmental conditions can also play an important role in shaping adult behavior, morphology, and neurophysiology (Monaghan 2008). At a very basic level, growth conditions determine adult body size in species with determinate growth, but there are also examples of conditions during development affecting reproductive (Barrett et al. 2009) and stress-associated (Hayward and Wingfield 2004) physiological processes as well as phenotypic expression of both acoustic (Beckers and Schul 2008; Nowicki et al. 1998) and visual (Naguib and Nemitz 2007; Ohlsson et al. 2002) ornaments. Thus, ontogenetic factors can have broad, long-lasting effects on characteristics related to both survival and reproduction.

Despite the importance of ontogeny to fitness, many studies investigating adult characteristics test only for effects of adult conditions (e.g., Ruusila et al. 2001). This is due to logistical constraints in many cases, including the challenges of tracking individuals throughout life as well as environmental and physiological differences between the life-stages. However, there are some systems and mechanisms that are ideal for tracking from development through adulthood and assaying a broad range of physiological and phenotypic outcomes. Understanding relative contributions of developmental versus adult conditions to fitness is essential to resolving the importance of several key evolutionary phenomena, such as developmental plasticity, heritability, and maternal effects (Monaghan 2008).

Carotenoid pigments are one such system, in that they can be biochemically quantified in the bodies of animals throughout life and serve an array of early- and late-life functions, such as health modulation (Massimino et al. 2003; McGraw and Ardia 2003; Peters et al. 2004a) and bright pigmentation (McGraw 2006), among other roles (e.g., photodefense, Stahl and Sies 2002; visual tuning, Vorobyev et al. 1998; Vorobyev 2003). These molecules are derived from the diet, starting with embryonic transfer (fetomaternal or in ovo, e.g., McGraw et al. 2005) and continuing via food intake as neonates and adults. Adults often express carotenoid-based coloration as an honest, sexually selected signal of an individual's condition (i.e., nutrition and health state; McGraw 2006; Pike et al. 2007; Steffen and McGraw 2009).

Whether such signals reflect developmental and/or adult history of carotenoid status and health is up for debate, although such a phenomenon is plausible given the link between carotenoid status early in life and carotenoid-related traits later in life, as has been most extensively studied in birds. Yolk carotenoid concentration affects carotenoid assimilation in young (chickens, *Gallus gallus*; Koutsos et al. 2003) and adult birds (zebra finches, *Taeniopygia guttata*; Blount et al. 2003). Similarly, yolk carotenoid concentration is related to immune function and coloration in both young (Saino et al. 2000; Fenoglio et al. 2002; Biard et al. 2006) and adult (McGraw et al. 2005) birds. After hatch, neonatal carotenoid supplementation can affect growth (*Perdix perdix*; Cucco et al. 2006), especially while nestlings are being immune challenged (parasitism; O'Brien and Dawson 2008). Thus, because (a) carotenoid exposure early in life is related to carotenoid physiology throughout life and (b) carotenoids can be mechanistically linked to both ornamentation and immune function, tracking carotenoid levels throughout development may explain variation in adult immune function and ornamentation.

Traditionally, most work on ornament development has focused on males (Jacot et al. 2005; Naguib and Nemitz 2007; Ohlsson et al. 2002; Uller et al. 2006, but see Blount et al. 2003), which tend to be the more ornamented sex. However, in a variety of species both males and females have conspicuous traits, including song or colorful patches. While there is some evidence that female coloration can signal reproductive state (Calisi and Hews 2007; Roulin et al. 2001; Setchell and Wickings 2004; Weiss 2002), parasite load (Calisi et al. 2008), or social status (Murphy et al. 2009), conspicuous female coloration may also be due to non-adaptive genetic correlation (Kraaijeveld et al. 2007). By studying the development of immune function and ornamentation of both sexes in the context of carotenoid status, we will be able to evaluate: (1) the correspondence between carotenoid and condition metrics during development to adult immune function and coloration, (2) whether adult carotenoid-based coloration is linked to adult immune function, and (3) whether males and females show similar patterns of developmental plasticity and information content of signals. We predict that, if phenotype at adulthood is dependent upon conditions during development, adult immune function and coloration metrics should be significantly modeled by morphological and carotenoid variables taken during the neonatal stage. Also, if carotenoid-pigmented ornaments honestly signal current health state, then adult coloration should correlate with adult immune metrics. Lastly, we predict that if ornament expression in both sexes reveals similar aspects of quality, then we should see similar correlations between adult phenotype and metrics taken during development in both males and females.

To begin to test these relationships, we reared male and female mallard ducks (*Anas platyrhynchos*) from hatch to adulthood, tracked circulating carotenoid titer and body-size metrics through development, and assessed immune function and color expression at adulthood. The carotenoid-pigmented beak of male mallards is a sexually selected signal of quality, with females preferring males with more yellow beaks as mates (Omland 1996a, b) and male beak color signaling adult immune function and sperm quality (Peters et al. 2004b). Despite the fact that both males and females have carotenoid-pigmented beaks (males: yellow, females: orange) and feet (both sexes: orange), signaling functions in the beak have only been investigated in males. However, female mallards are subjected to high degrees of forced copulations (McKinney et al. 1983), which sometimes result in death (McKinney et al. 1983), although paired females do gain protection through mate guarding (Davis 2002). Thus, honest signaling might allow higher-quality females to attract higher-quality males that would provide superior protection from male antagonism, and it is plausible that the carotenoid-pigmented beak of female mallards serves as an honest signal. To test immunocompetence, we used both the PHA wing web swelling test (Smits et al. 1999) and antibody production in response to the novel antigen

keyhole limpet hemocyanin (KLH). We chose these assays (see more below) because: (a) the PHA test has clear links to early life stages; nestling birds with a greater immune responsiveness to PHA have higher recruitment rates (Cichoń and Dubiek 2005; Moreno et al. 2005; but see Butler et al. 2009), and (b) beak color and circulating carotenoid concentration of male mallard ducks changes significantly with antibody response (Peters et al. 2004a).

Methods

Animal husbandry and sample collection

We acquired 1-day-old male ($n = 27$) and female ($n = 15$) mallard ducklings from Metzer Farms (Gonzales, CA) and reared them in two indoor rooms in randomly selected groups of 3–6 ducklings per cage (60 × 60 × 60 cm) until they were 24 days old, at which point they were singly housed in similar cages. We initially housed them in these groups because they naturally exist in small families during this stage of development and because they show signs of stress when housed singly soon after hatch (MWB, pers. obs). One infrared heat lamp per cage was placed 20 cm from the cage bedding and left on for the first 3 weeks of life to maintain an ambient temperature of approximately 30°C. Photoperiod approximated that which ducks hatched in Arizona in mid-April would experience (13L:11D for the first 5 weeks, followed by 14L:10D for 9 weeks, 13L:11D for 5 weeks, 12L:12D for 4 weeks, and 11L:13D for 4 weeks). Food (Mazuri Waterfowl Starter for the first 7 weeks, and Mazuri Waterfowl Maintenance thereafter) and drinking tap water were provided ad libitum throughout the study.

We collected blood for carotenoid analysis (see below) twice weekly from when ducklings were 6 days old until they were 16 weeks old, and then once a week until they were 22 weeks old. Because all individuals were located within a single room, each individual was exposed to similar amounts of human disturbance (~2.5 h) and direct handling (~5 min) during each sampling period. We also collected blood immediately prior to issuing immune challenges at adulthood (24 weeks old; see below) as well as 10 days later (for assaying antibody response, sensu Hasselquist et al. 2001). To collect blood, we punctured the alar vein and collected approximately 250 µl in heparinized capillary tubes. Within several hours, we centrifuged blood samples for 3 min at 10,000 rpm and stored the plasma at −80°C until analysis.

At the time of blood collection, we measured body mass of each bird to the nearest gram, tarsus length to the nearest 0.1 mm, and calculated body condition (i.e., size-adjusted body mass; Traylor and Alisauskas 2006) as the residuals from the regressions of tarsus length on body mass that we ran separately for each date of data collection (all $F_{1,40} > 20.75$, all $P < 0.0001$). Because we were more interested in general trends across developmental stages rather than week-specific changes that could be more likely to generate spurious results, we calculated average values for three different stages of development: EARLY (weeks 1–6; period of linear growth), MIDDLE (weeks 7–14; period of minimal growth or molt into alternate plumage) and LATE (15–22; period of molt into alternate plumage). These stages represent a protracted timeline of molt compared to individuals that are reared outdoors after 7 weeks of age (Butler and McGraw 2009).

Carotenoid analyses

To analyze plasma carotenoid content, we followed the hexane:methyl tert-butyl ether extraction method and high-performance liquid chromatography procedures of McGraw et al. (2008). Detectable amounts of lutein, zeaxanthin, and a lutein derivative existed in duck plasma at all time points measured, while detectable amounts of canthaxanthin and β-cryptoxanthin existed in small amounts with less regularity. For the MIDDLE and LATE periods, all carotenoid types were positively correlated within individual birds (all $r > 0.45$, all $P < 0.002$), so we used total carotenoid titer for those periods. In the EARLY period, all carotenoid types except for β-cryptoxanthin were positively correlated (all other $r > 0.5$, $P < 0.0002$). However, β-cryptoxanthin accounted for only 0.09% of total carotenoid concentration, so we also used total carotenoid titer to characterize carotenoid status during the EARLY period.

Immune function assessments

To assess adult immune function, we used both a PHA wing web swelling test and a KLH antibody challenge (see above). The PHA test, in which larger swellings are associated with a more robust immune response, was traditionally used as a proxy for cell-mediated immunity (e.g., Lifjeld et al. 2002) but may also assess aspects of the innate immune system (Martin et al. 2006). Both immune challenges were issued concurrently when the ducks were 24 weeks old (i.e., when individuals had completed their nuptial molt), so as not to alter their immunodevelopment. To issue the PHA challenge, we followed the methodology recommended by Smits et al. (1999). We measured patagial thickness with a digital micrometer (Mitutoyo model 293-761-30), injected 0.2 mg of PHA-P (L8754, Sigma) suspended in 0.2 ml of phosphate-buffered saline (PBS), and measured patagial thickness 24 h later (mean 23:54 ± 0:05). Swelling was calculated as the temporal difference in patagial thickness. At the same time that we issued the

PHA injection, we also administered two intramuscular injections, one in each leg (sensu Koenen et al. 2002), of 25 μg KLH (H7017, Sigma) emulsified in a mixture of 50 μl Complete Freund's Adjuvant and 50 μl sterile water per injection (sensu Higgins et al. 2001). We tested antibody production due to previously published links between humoral immunity, carotenoids, and coloration; carotenoid-supplemented greenfinches (*Carduelis chloris*) showed greater antibody production in response to a novel antigen, and they also showed a correlation between antibody response and plumage coloration (Aguilera and Amat 2007).

To calculate antibody response, we used an enzyme-linked immunosorbant assay (sensu Butler and Dufty 2007). We incubated 96-well plates (Fisher 468667) overnight at 4°C with 0.5 mg/ml KLH in sodium carbonate (pH: 9.6) buffer, washed the wells with PBS and Tween 20 (0.05% by volume) and then treated the wells with a blocking buffer (PBS with 1% bovine serum albumin) containing azide (0.1% sodium azide) at room temperature for 2 h. After washing, we added, in triplicate, 100 μl of plasma diluted 1:12,800 in blocking buffer with azide and incubated plates overnight at 4°C. We then washed the plates, added 80 μl of HRP-conjugated polyclonal secondary antibody (Bethyl Laboratories, A140-110P) specific for avian IgG diluted 1:10,000 in blocking buffer, and incubated at room temperature for 2 h. We again washed the plates and then incubated 100 μl of tetramethylbenzidine (TMB) substrate for 15 min in the dark, and then added 50 μl of 1 M H_2SO_4. Within 10 min., we measured absorbance of each well at $\lambda = 450$ nm using SOFTmax PRO (Molecular Devices, CA). Because absorbance values of triplicate samples were repeatable within individuals (Lessells and Boag 1987; pre-inoculation: $R = 0.93$; post-inoculation: $R = 0.99$), we averaged these values and used the difference in optical density between pre- and post-inoculation samples in all subsequent analyses, thus controlling for any intra-individual background in antibody binding.

Beak color quantification

To assess integumentary carotenoid coloration of adult ducks (developing young have melanized and fluorescent, non-carotenoid yellow plumage as well as melanized beaks and legs), we used an Ocean Optics (Dunedin, FL, USA) USB2000 spectrophotometer with a PX-2 pulsed xenon light source to measure reflectance from $\lambda = 300$–700 nm. In duplicate, we measured reflectance of the dorso-lateral surface of the beak halfway between the nares and the beak tip and the medial surface of the medial right toe and binned all spectrophotometric values into 1 nm increments using CLRfiles (CLR version 1.05, Montgomerie 2008). We then used CLRvars (CLR version 1.05, Montgomerie 2008) to calculate brightness (B1), saturation in the blue region of the spectrum (S1B), and hue (H4b), which are the tristimulus scores most closely related to carotenoid content of the male mallard beak (B1 and S1B are negatively related, H4b is positively related; Butler et al. 2010). In addition to data demonstrating that these metrics are most closely associated with beak carotenoid content (Butler et al. 2010), they are also intuitively appealing. B1 measures the relative amount of reflected light, and accounts for overall concentration of light-absorbing pigments (e.g., carotenoids)., S1B, which relates light reflectance between 400 and 510 nm to overall brightness, utilizes the portion of the spectrum that closely corresponds to the maximum absorbance of lutein and zeaxanthin, which are the dominant carotenoids in mallard beak tissue (Butler et al. 2010). We measured beak color at four different time points: (1) before the beginning of the immune assessment (pre-immune), (2) 24 h after PHA injection (post-PHA), (3) 10 days after the KLH injection (post-KLH), and (4) 5 weeks after the beginning of the immune assessment period (end of experiment).

Statistical analyses

To test whether adult coloration and immune function were predicted by morphological variables or carotenoid status during development or adulthood, we used AIC model selection (e.g., Martin et al. 2007) to identify the best model, after which we evaluated the utility of the model based on P and adjusted R^2 values. In addition, when the model with the lowest AIC value contained more than two variables for females or three variables for males, we ruled these models inappropriate, as they had too large a ratio of variables to observations (Kutner et al. 2005). Separately, for males and females, we modeled PHA-induced swelling, beak brightness, hue, and saturation, and foot brightness, hue, and saturation from the independent variables body condition and carotenoid titer from EARLY, MIDDLE, and LATE periods, as well as the carotenoid titer on the day of the immune challenge. For KLH-induced antibody production, we used the same independent variables as well as the difference in carotenoid titer between the pre- and post-inoculation blood samples as well as the PHA-induced swelling. To test whether adult immune function was predicted by beak or foot coloration prior to any immune challenge, we used simple linear regressions to model PHA-induced swelling and KLH-induced antibody production with the independent variables beak brightness, hue, and saturation or foot brightness, hue, and saturation. To test whether the degree of immune response was related to degree of color change, we performed several multivariate analysis of variance (MANOVAs) with either PHA-induced swelling or KLH-induced antibody production as the independent variable, with the differences in hue, saturation, and brightness of

either the foot or beak over the duration of the PHA challenge or KLH challenge, respectively, as the dependent variables. To address the temporal lability of adult beak coloration, we performed several analyses. First, we ran repeated-measures ANOVAs on beak color (hue, saturation, and brightness) during the immune assessment (pre-immune, post-PHA, and post-KLH) to see how immune challenges affect beak coloration. We then ran several linear regressions, with beak color (hue, saturation, and brightness) during the pre-immune period as the independent variables and the same color metrics taken 5 weeks later at the end of the experiment as the dependent variables. Lastly, to assess how carotenoid circulation varied as a function of age and sex, we ran a generalized linear model (GLM) of carotenoid titer during the EARLY, MIDDLE, and LATE period as a function of sex, with a repeated factor of period. We ran all statistics with SAS 9.2 (Cary, NC).

Results

Predictors of carotenoid status during development

We found significant effects of sex ($F_{1,40} = 5.06$, $P = 0.0301$) and developmental stage ($F_{2,80} = 188.15$, $P < 0.0001$) on total plasma carotenoid titer; the sex-by-stage interaction was marginally significant ($F_{2,80} = 3.08$, $P = 0.0515$). Least squares means comparisons revealed that both males and females significantly circulated their highest levels of carotenoids during the MIDDLE stage (all $P < 0.0001$) and the lowest levels at the LATE stage (all $P < 0.0025$). Males and females circulated similar levels at both the EARLY and MIDDLE stages (both $P > 0.1$), but males circulated more than females during the LATE stage ($P < 0.0001$; Fig. 1).

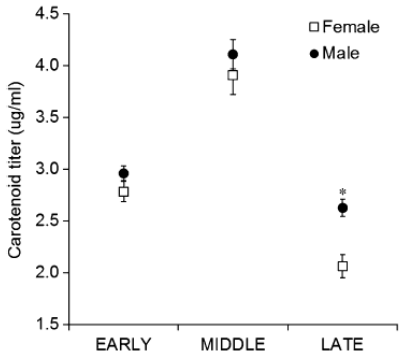

Fig. 1 Mean total circulating carotenoid titer during different developmental stages in maturing male and female mallards. The EARLY stage was characterized by growth, the MIDDLE stage by a lack of growth and by a juvenile molt, and the LATE stage by molt into nuptial plumage. Carotenoid circulation peaked for both sexes in the MIDDLE stage, while males circulated higher levels than females only during the LATE stage ($P < 0.05$, denoted by an asterisk). Male ducklings are denoted by *closed circles* and females by *open squares*. *Error bars* are SEM

Developmental predictors of adult immune function

Male PHA-induced swelling at sexual maturity increased with EARLY body condition ($F_{1,25} = 6.10$, $P = 0.0207$; Fig. 2), while KLH-induced antibody production at adulthood was not significantly modeled by the AIC-selected independent variables (overall model: $F_{2,24} = 2.07$, $P = 0.1488$; both variables $P > 0.05$). Also, female wing web swelling was not significantly modeled by the AIC-selected independent variables ($F_{1,13} = 1.32$, $P = 0.2716$), while female KLH-induced antibody production required too many variables ($n = 7$; Table 1).

Development predictors of adult carotenoid coloration

Male beak saturation (S1B, negatively related to beak carotenoid content) increased with MIDDLE body condition ($P = 0.0377$), but decreased with EARLY body condition ($P = 0.0064$; overall model $F_{2,24} = 4.68$, $P = 0.0192$). AIC-selected variables did not significantly model male beak brightness, beak hue, or foot saturation, brightness, or hue (all $P > 0.05$; Table 2). Female beak brightness increased with MIDDLE body condition ($P = 0.0295$), with a trend to increase with LATE

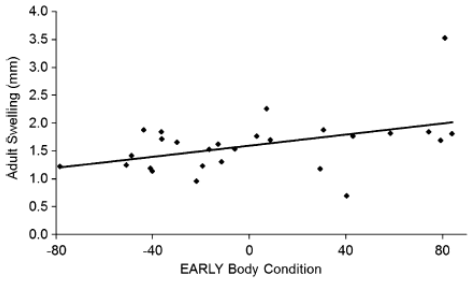

Fig. 2 Adult PHA-induced wing-web swelling as a function of EARLY body condition in male mallards. Body condition, calculated as the residuals of mass on tarsus within each sampling date, was averaged when ducks were 1–6 weeks old. An increasing body condition during the EARLY period was associated with a more robust PHA response at adulthood (24 weeks old). One observation (*upper right*) had a Cook's $D = 1.09$, which suggests it is an outlier. Because it is inappropriate to eliminate an observation just because it appears to be numerically extreme (Zar 1999), we reran the analysis with the observation reassigned a swelling value equal to the next highest value, and the model remained significant ($F_{1,25} = 4.47$, $P = 0.0446$)

Table 1 AIC-selected models predicting adult immune function from morphometrics and carotenoid status throughout development and adulthood in male and female mallard ducks

Dependent variable	Sex	Independent variables	Parameter estimate	*F*	*P*	*df*
PHA-induced swelling	F	EARLY carotenoid	0.33702	1.32	0.2716	1,13
	M	EARLY BC	0.00495	6.10	0.0207	1,25
Ab production	F	EARLY carotenoid	−0.24983	5.20	0.0629	1,6
		MIDDLE carotenoid	−0.66344	60.06	0.0002	1,6
		LATE carotenoid	1.79053	76.74	0.0001	1,6
		LATE BC	0.00611	51.98	0.0004	1,6
		ADULT carotenoid	0.49491	34.81	0.0011	1,6
		Change in carotenoid	0.21485	6.35	0.0453	1,6
		PHA-induced swelling	−0.16635	5.34	0.0600	1,6
	M	ADULT carotenoid	−0.28221	3.69	0.0668	1,24
		Change in carotenoid	−0.22721	3.24	0.0851	1,24

EARLY, MIDDLE, LATE, and ADULT denote age at which metric was taken (see "Methods"). Ab production denotes KLH-specific antibody production, BC denotes body condition, and carotenoid denotes circulating carotenoid titer

carotenoid titer ($P = 0.0788$; overall model $F_{2,12} = 3.16$, $P = 0.0789$), while female beak hue decreased with MIDDLE body condition ($P = 0.0458$), with a trend to increase with LATE body condition ($P = 0.0896$; overall model $F_{2,12} = 2.77$, $P = 0.1027$). Other AIC-selected models for female coloration were non-significant (beak saturation, foot saturation, brightness, and hue; all $P > 0.10$) or required too many variables ($n > 2$; Table 2).

Correspondence between coloration and immunocompetence at adulthood

KLH-induced antibody production was positively predicted by female beak brightness (B1, negatively related to beak carotenoid content; $F_{1,12} = 5.06$, $P = 0.0440$; Fig. 3a), and there was a trend for KLH-induced antibody production to be negatively associated with male beak brightness ($F_{1,25} = 3.00$, $P = 0.0958$; Fig. 3b). In addition, there was a trend for male beak brightness to be negatively associated with PHA-induced swelling ($F_{1,25} = 3.78$, $P = 0.0632$; Fig. 4). All other relationships between beak color and immune response were not significant (all $P > 0.1$), and no component of foot color in either sex related to any metric of adult immune function (all $P > 0.2$; Table 3).

Effect of immune response on short-term change in beak and foot color

Male beak coloration changed during the PHA incubation in relation to the amount of PHA-induced swelling (Wilk's Lambda $F_{3,23} = 4.58$, $P = 0.0118$), with larger swellings associated with increases in brightness ($F_{1,25} = 6.50$, $P = 0.0173$; Fig. 5) and hue ($F_{1,25} = 4.54$, $P = 0.0431$). Degree of swelling was not related to change in beak coloration for females (Wilk's Lambda $F_{3,11} = 0.13$, $P = 0.9397$), nor to change in any measure of foot color for either sex (all $P > 0.1$). Degree of KLH-induced antibody production was not related to change in beak or foot coloration for either sex (all $P > 0.15$; Table 4), except for a trend for foot saturation to increase with antibody production in females ($F_{1,12} = 4.50$, $P = 0.0554$).

Beak brightness changed over the course of immune assessment, with brightness decreasing over the 24-hr PHA incubation, but then increasing to higher levels at the end of the 10-day KLH incubation ($F_{2,78} = 27.86$, $P < 0.0001$). Males had brighter beaks at all time points than females ($F_{1,39} = 9.39$, $P = 0.0039$). Similarly, beak saturation decreased during the PHA incubation, but increased to higher levels at the end of the KLH incubation ($F_{2,78} = 49.80$, $P < 0.0001$). There was a trend for females to have beaks that were more saturated in the blue region of the spectrum than males at all time points ($F_{1,39} = 3.30$, $P = 0.0771$). Also, while females had greater (more right-shifted, i.e., oranger) hues than males at all time points ($F_{1,39} = 68.63$, $P < 0.0001$), hue did not change over the time course of the immune challenges ($F_{2,78} = 2.19$, $P = 0.1193$). There were no significant sex × time interactions for any beak color metric (all $P > 0.5$).

Female beak brightness, saturation, and hue values at the end of the experiment were not significantly associated with those same metrics taken 5 weeks prior, immediately before adult immune assessment (all $P > 0.4$). However, male beak brightness ($F_{1,25} = 4.56$, $P = 0.0428$) and saturation ($F_{1,25} = 10.58$, $P = 0.0033$; Fig. 6), but not hue ($P = 0.4202$), values at the end of the experiment were significantly positively associated with those same variables measured prior to the adult immune assessment.

Table 2 AIC-selected models predicting adult coloration from morphometrics and carotenoid status throughout development and adulthood in male and female mallard ducks

Structure	Dependent variable	Sex	Independent variables	Parameter estimate	*F*	*P*	*df*
Beak	S1B	F	EARLY carotenoid	0.01019	2.10	0.1714	1,13
		M	EARLY BC	−0.000175	8.94	0.0064	1,24
			MIDDLE BC	0.0001099	4.84	0.0377	1,24
	B1	F	MIDDLE BC	0.31485	6.10	0.0295	1,12
			LATE carotenoid	37.81404	3.69	0.0788	1,12
		M	EARLY BC	−0.18792	2.79	0.1077	1,23
			MIDDLE BC	0.18832	3.80	0.0636	1,23
			ADULT carotenoid	16.50595	2.96	0.0993	1,23
	H4b	F	MIDDLE BC	−0.00929	4.97	0.0458	1,12
			LATE BC	0.00973	3.42	0.0896	1,12
		M	EARLY BC	0.0007627	0.86	0.3636	1,25
Foot	S1B	F	EARLY BC	−0.000496	8.64	0.0135	1,11
			LATE BC	0.0003022	6.66	0.0257	1,11
			ADULT carotenoid	0.02732	8.94	0.0123	1,11
		M	ADULT carotenoid	−0.01399	2.13	0.1580	1,25
	B1	F	LATE carotenoid	20.81611	2.19	0.1691	1,10
			EARLY BC	−0.39151	2.92	0.1176	1,10
			MIDDLE BC	0.62308	5.52	0.0403	1,10
			LATE BC	−0.47722	3.28	0.1012	1,10
		M	ADULT carotenoid	−13.23117	1.54	0.2270	1,25
	H4b	F	MIDDLE carotenoid	−0.15298	3.19	0.1085	1,9
			LATE carotenoid	0.25391	1.42	0.2639	1,9
			MIDDLE BC	−0.00976	9.42	0.0133	1,9
			LATE BC	0.01241	8.24	0.0186	1,9
			ADULT carotenoid	0.35274	5.15	0.0497	1,9
		M	LATE carotenoid	−0.09862	1.30	0.2664	1,25

S1B, B1, H4b denote saturation, brightness, and hue, respectively. EARLY, MIDDLE, LATE, and ADULT denote age at which metric was taken (see "Methods"). Ab production denotes KLH-specific antibody production, BC denotes body condition, and carotenoid denotes circulating carotenoid titer

Discussion

We found that body condition during development was a better predictor of adult coloration and immune function than was adult body condition. Specifically, male adult PHA-response was significantly modeled by EARLY body condition, while beak color of adult males (saturation metric: S1B) and females (brightness metric: B1, hue metric: H4b) was modeled by body condition metrics taken during the EARLY and/or MIDDLE stage. More specifically, male body condition in the EARLY developmental stage was more predictive of adult beak saturation than was MIDDLE body condition (lower *P* value), implying that the EARLY stage was relatively more important. Consistent with our findings, prior studies of other duck species show that duckling body condition is a better predictor than adult body condition of adult survival (Traylor and Alisauskas 2006; white-winged scoters, *Melanitta fusca deglandi*) and recruitment (Christensen 1999; common eiders, *Somateria mollissima*), though this is not always the case (e.g., wood ducks, *Aix sponsa*, Hartke et al. 2006; northern pintails, *Anas acuta*, Richkus et al. 2005; tufted duck, *Aythya fuligula*; common pochard, *Aythya ferina*, Blums et al. 2005). The relative importance of neonatal body condition to adult proxies for fitness has been demonstrated in non-avian taxa as well. In rabbits (*Oryctolagus cuniculus L.*), for example, male adult competitive performance was more accurately predicted by neonatal body condition than by that of adults (Rödel and von Holst 2009). Thus, our data are consistent with the observation that body condition during development is more tightly correlated to measures of adult phenotype than condition at adulthood.

While we did not explicitly test in our correlational study how neonatal or adult body condition mechanistically relates to adult immune function or coloration, there are several possible broad explanations. Individuals with a genetic makeup that promotes a greater body condition

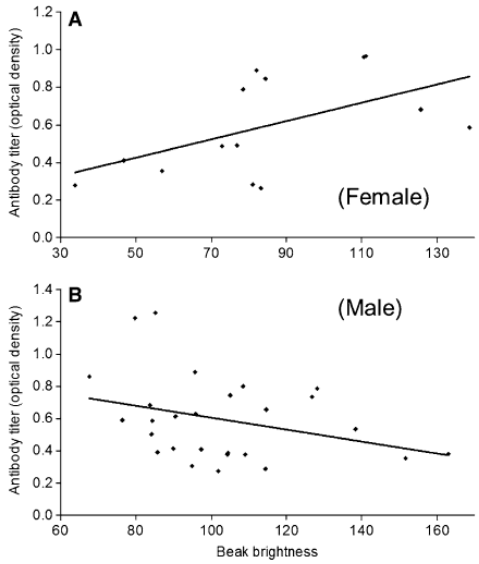

Fig. 3 KLH-specific antibody response at adulthood as a function of female (**a**) and male (**b**) beak brightness (B1: total reflectance) in adult mallards. Because brighter beaks have less pigment, and therefore fewer carotenoids (i.e., presence of more light-absorbing carotenoids renders a tissue less bright; Butler et al. 2010), males that had less carotenoid-rich beaks tended to have lower antibody responses (*negative slope*), while females with less carotenoid-rich beaks had greater antibody responses (*positive slope*)

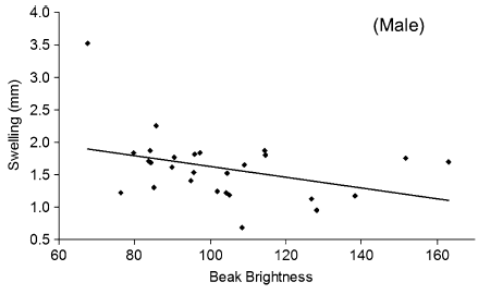

Fig. 4 PHA-induced wing-web swelling as a function of beak brightness (B1: total reflectance) in adult male mallards. Males that had brighter, and therefore less carotenoid-rich, beaks tended to have smaller PHA-induced swellings. The extreme value at *upper left* was not a statistical outlier (Cook's $D < 1$)

may also have genes that facilitate a more robust immune response or increased ability to deposit carotenoids in integument. At present, the specific genes or linkages are undefined, although a significant genetic covariance between body condition and immune function has been identified in another avian species (*T. guttata*; Gleeson et al. 2005). Alternatively, maternal effects such as hormone levels during development can affect growth (Hull et al. 2007), pigment accumulation (Blas et al. 2006), and sexually selected traits of adults (Strasser and Schawbl 2004), although more work is needed to elucidate the relationship between hormones and carotenoids during development. Similarly, carotenoid content of yolks can affect neonatal health (Saino et al. 2003) and adult sexually selected traits (McGraw et al. 2005), while carotenoid supplementation of chicks affects both growth and immune function (Cucco et al. 2006). These putative nutritional and physiological mechanisms warrant further experimental testing in mallards.

Body condition metrics from the EARLY and MIDDLE period were more predictive of adult coloration and immune function than those same metrics taken during either the LATE or adult stage. This finding is not due to a spurious statistical finding based on a larger variance in body condition at the earlier stages; in fact, the size of the standard deviation increased from EARLY to ADULT stage (42.6, 58.3, 62.4, and 68.4, respectively). Rather, we posit a physiological basis for this finding. Those individuals that more effectively assimilate, accumulate, and/or utilize nutrients early in development, when growth (and by association, body condition) is important, may also have these same physiological abilities later in life when the mobilization of nutrients in response to an immune challenge is required. Similarly, those with greater body condition (and potentially, nutrient assimilation and utilization) during growth may also have an improved ability to deposit carotenoids in the integument, although we did not test for specific physiological mechanisms.

In addition to our developmental results, we provided further evidence for the ability of the mallard drake beak to honestly signal current immunocompetence (Peters et al. 2004a, b), in that there was a trend for an individual's PHA response and KLH-specific antibody response to positively correlate with a less bright, and thus more carotenoid-pigmented (Butler et al. 2010), beak. Thus, males can signal aspects of both ontogenetic and post-maturity quality with the expression of their colorful sexual ornament. However, unlike in males, females possessing darker beaks had a weaker antibody response at adulthood. More ornamented individuals sometimes, although rarely, do have higher parasite levels (*T. guttata*; Burley et al. 1991) and reduced immune function (Garvin et al. 2008), but our correlational finding for females is difficult to interpret both mechanistically and functionally, given that we know nothing of any signal function for female beak color in this species and do not yet know causal links between early- and late-life carotenoid and health states. Our result may mechanistically signify current, direct carotenoid allocation

Table 3 Statistical relationships between adult immune function and adult coloration in male and female mallards

Structure	Dependent variable	Independent variable	Sex	Parameter estimate	*F*	*P*	*df*
Beak	Ab production	B1	F	0.00488	5.06	0.0440	1,12
			M	−0.00373	3.00	0.0958	1,25
		S1B	F	2.57586	0.13	0.7240	1,12
			M	−6.25577	2.73	0.1111	1,25
		H4b	F	−0.28289	2.49	0.1409	1,12
			M	0.41314	2.61	0.1187	1,25
	PHA-induced swelling	B1	F	0.0001502	0.00	0.9716	1,13
			M	−0.0082147	3.78	0.0632	1,25
		S1B	F	−15.907437	2.36	0.1488	1,13
			M	0.3588992	0.00	0.9642	1,25
		H4b	F	0.2133064	0.52	0.4826	1,13
			M	0.3917981	0.55	0.4648	1,25
Foot	Ab production	B1	F	0.00475	1.50	0.2438	1,12
			M	0.0023	1.29	0.2664	1,25
		S1B	F	−0.57182	0.02	0.8822	1,12
			M	2.54689	1.31	0.2624	1,25
		H4b	F	−0.44939	1.27	0.2812	1,12
			M	−0.1844	0.48	0.4954	1,25
	PHA-induced swelling	B1	F	−0.0047779	0.55	0.4707	1,13
			M	0.0050675	1.61	0.2158	1,25
		S1B	F	0.5341283	0.01	0.9293	1,13
			M	2.7483459	0.37	0.5462	1,25
		H4b	F	0.3731083	0.35	0.5634	1,13
			M	0.1298688	0.06	0.8097	1,25

S1B, B1, H4b denote saturation, brightness, and hue, respectively. Ab production denotes KLH-specific antibody production

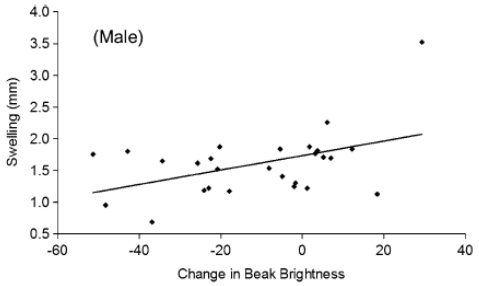

Fig. 5 PHA-induced wing-web swelling in adult male mallards as a function of the change in beak brightness over the concurrent 24-hour period during which PHA was administered. Males that had the greatest decrease in brightness, and therefore a possible increase in carotenoid pigmentation, had the smallest PHA-induced swellings. One observation (*upper right*) had a Cook's $D = 1.36$. As in Fig. 2, we reran the analysis with the observation reassigned a swelling value equal to the next highest value, and the model, while no longer significant, did tend toward significance ($F_{1,25} = 4.02$, $P = 0.0558$)

away from beak to boost immune system demands, or that more colorful females prioritize other immune-system components (only a subset was studied here). On the functional level, beak color variation in females may positively reveal an individual's condition during development or non-health attributes (e.g., social status; Murphy et al. 2009) at adulthood, since we found links between ontogenetic condition and adult coloration, but not immunity, in females. Because our study was conducted under standard captive conditions, in the future it will be important to investigate these relationships in wild birds as well.

In both adult males and females, foot coloration did not correlate with any aspect of individual quality, in contrast to studies of blue-footed boobies (*Sula nebouxii*, Torres and Velando 2007; Velando et al. 2006), nor did change in foot color significantly correlate with any immunological measure, except for a trend for foot saturation in females to increase with antibody production. To our knowledge, there are no studies of the control or function of foot coloration in mallards, despite it being such a conspicuous feature, though one study found that leg band color in female mallards influenced brood social dynamics, in that ducklings with bands similar to the mother were dominant within the brood, regardless of band color (Poisbleau et al. 2010). Our negative results are not consistent with the hypothesis that mallard foot coloration signals quality,

Table 4 Statistical relationships between adult immune function and change in adult coloration over the duration of the immune challenge in male and female mallards

Structure	Independent variable	Dependent variable	Sex	Parameter estimate	*F*	*P*	*df*
Beak	PHA-induced swelling	Change in B1	F	2.80878728	0.05	0.8256	1,13
			M	18.0726067	6.50	0.0173	1,25
		Change in S1B	F	0.00374353	0.26	0.6190	1,13
			M	0.00192759	0.22	0.6442	1,25
		Change in H4b	F	−0.09580379	0.37	0.5560	1,13
			M	0.19174533	4.54	0.0431	1,25
	Ab production	Change in B1	F	−48.8810445	1.78	0.2069	1,12
			M	4.19372223	0.02	0.8797	1,25
		Change in S1B	F	−0.01323436	1.15	0.3038	1,12
			M	0.00281613	0.21	0.6525	1,25
		Change in H4b	F	0.88984804	2.05	0.1779	1,12
			M	−0.31396839	1.46	0.2380	1,25
Foot	PHA-induced swelling	Change in B1	F	8.235033	0.15	0.7065	1,13
			M	−25.6639528	2.66	0.1155	1,25
		Change in S1B	F	−0.00725384	0.24	0.6357	1,13
			M	−0.01025099	0.55	0.4671	1,25
		Change in H4b	F	−0.02030423	0.05	0.8219	1,13
			M	0.01893075	0.09	0.7665	1,25
	Ab production	Change in B1	F	−3.7786093	0.02	0.8884	1,12
			M	−3.87987452	0.03	0.8643	1,25
		Change in S1B	F	0.03781574	4.50	0.0554	1,12
			M	−0.00964251	0.32	0.5749	1,25
		Change in H4b	F	0.10833902	0.72	0.4139	1,12
			M	0.0692587	0.26	0.6164	1,25

S1B, B1, H4b denote saturation, brightness, and hue, respectively. Ab production denotes KLH-specific antibody production

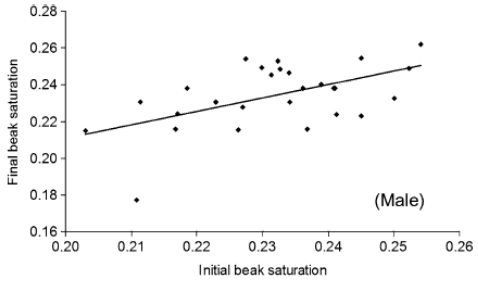

Fig. 6 Relationship between saturation (S1B: reflectance ratio) values of male beaks taken prior to any immune challenges (24 weeks old) and 5 weeks later (29 weeks old). Males with higher saturation values at 24 weeks old also had higher saturation values at 29 weeks old. The extreme value at *lower left* was not a statistical outlier (Cook's $D < 1$)

though many possible signaling roles (e.g., species recognition) have yet to be investigated.

Drake beak color changed in proportion to the degree of PHA response, becoming less carotenoid-rich with a greater immune response over the course of 24 h. Thus, in male mallards, beak color may signal not only developmental history and general adult quality, but also current quality within the adult stage. This finding speaks to the rapid, dynamic nature of avian bare-part color ornaments like beaks (Gautier et al. 2008) and their ability to capture information about an individual's health state in real time. Avian bare parts can change color over short periods in response to food stress (blue-footed boobies; Velando et al. 2006) and in proportion to humoral immune response (mallards; Peters et al. 2004a). While bare parts have not previously been shown to change with PHA challenge (blackbirds, *Turdus merula*; Biard et al. 2009), in that study color was scored ordinally by researchers using color cards, thus possibly reducing the precision of color measurement. Interestingly, we show that, even though bill color changed in proportion to an immune response, beak coloration of individuals before any immune assessment and 5 weeks after initiation of immune assessment was significantly related. Thus, developmental conditions may comparatively fix animals within a narrow range of color expression into adulthood, within which dietary, health, or other

perturbations after sexual maturity may work to more subtly change an individual's color.

While we were able to establish relationships between body condition, coloration, and immune function, no measurement of circulating carotenoid content from any time point entered the statistical models as a predictor of adult phenotype. This is not necessarily evidence that these molecules are not developmentally important, as all individuals were receiving the same diet under standard housing conditions. Experimental assessment of carotenoid access is warranted; even though individuals received the same diet, circulating levels of carotenoids varied over time, suggesting that carotenoid physiology changed as a function of age for both sexes. Furthermore, sex-dependent differences manifested only in the LATE stage, suggesting that carotenoid access during this period may be an essential component of the development or maintenance of sexually selected ornaments. In wild ducklings, carotenoid content of the food can vary widely (Butler and McGraw 2010). Thus, in light of our findings highlighting the relative importance of ontogeny, future research should examine how variation in carotenoid access and physiology early in life affects development of adult coloration and immune function.

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APPENDIX C

DEVELOPMENTAL IMMUNE HISTORY AFFECTS ADULT IMMUNE FUNCTION BUT NOT CAROTENOID-BASED ORNAMENTATION IN MALLARD DUCKS

Summary

1. Sexually selected traits often honestly advertise aspects of individual quality, such as immune function. Such traits have traditionally been thought to reveal real-time information (e.g., current health state), but they may also reflect immunological conditions experienced during ontogeny, which can fundamentally shape survival prospects, adult immune function, and reproductive performance.

2. We tested the effects of immune challenges (injections of sheep red blood cells) during neonatal development on adult immune function and ornamental coloration in male mallard ducks (*Anas platyrhynchos*). Mallards have a carotenoid-pigmented beak that plays a role in mate choice, and because carotenoids are also associated with immune responsiveness, carotenoid accumulation in body tissues may change as a function of immunological state during development and into adulthood.

3. We found that mallards that received immune challenges at the completion of growth (8-10 weeks old) had a reduced proinflammatory response (as measured by the local swelling response to phytohemagglutinin; PHA) as adults, compared to control birds and those receiving immune challenges at other developmental time points. Mallards that received immune challenges during the acquisition of nuptial plumage (13-15 weeks old) had an increased humoral immune response as adults, as assessed by antibody production to keyhole limpet hemocyanin (KLH). Body mass, adult beak color, and carotenoid circulation were not affected by immune challenges issued at any point during development.

4. Circulating carotenoid levels prior to adult immune challenge and change in carotenoid titer over the course of the adult immune assessment period were correlated with degree of response to PHA and KLH at adulthood, as well as with nitric oxide production, which is associated with innate immunity. However, the direction of these correlations varied by type of immune assessment. Surprisingly, unlike in several previous studies, we found no link between beak coloration and adult immune function.

6. These findings demonstrate the importance of developmental immune challenges in shaping adult immune response, although the precise mechanisms remain unidentified. Moreover, we found support for the hypothesis that carotenoids are linked to immune function, due to the relationship between multiple components of the immune system and both initial and change in circulating carotenoid levels.

7. However, our study does not support the hypothesis that sexual signal expression reveals developmental immune history. Taken together, our findings suggest that the relationships between carotenoid physiology, carotenoid-based ornamentation, and immune function are complex.

Keywords: Carotenoid pigments, Coloration, Developmental plasticity, Immune function, *Anas platyrhynchos*

Introduction

Sexual selection has favored the elaboration of ornaments that help animals secure higher-quality or a greater number of mating opportunities (Andersson 1994). The honest advertisement model of sexual selection posits that ornaments, such as antlers, horns, and bright colors (Lailvaux et al. 2005; Ezenwa & Jolles 2008; Cogliati, Corkum & Doucet 2010), reflect an individual's quality by being costly enough that only the highest-quality individuals can produce and/or maintain the ornament (Allen & Levinton 2007). Fitness-related information communicated by ornament expression includes territory quality (Bostrom & Ritchison 2006), parental ability (Senar, Figuerola & Pascual 2002), genetic quality (Hale et al. 2009), and immune function (Peters et al. 2004a). Due to the putative role of parasites in mediating the evolution of ornamentation (Hamilton & Zuk 1982), the ability of ornaments to reveal immune function has been studied extensively.

Natural variation in an adult's ornamentation is often positively correlated with its degree of immune response (Peters et al. 2004a; Mougeot 2008), and immune challenges can alter concurrent ornament expression (Peters et al. 2004b; Gautier et al. 2008). However, both immune function (Fair, Hansen & Ricklefs 1999; Galic et al. 2009) and ornamentation (Ohlsson et al. 2002; Naguib & Nemitz 2007; Beckers & Schul 2008) can be sensitive to developmental conditions. Thus, ornamentation may signal not only current ability to respond to an immune challenge (Peters et al. 2004a), but also immunological status during development (Spencer & MacDougall-Shackleton 2011). Multiple hypothetical

scenarios exist; first, ontogenetic immune challenges may impose costs on sensitive, growing organisms (Bourgeon, Maho & Raclot 2009; van de Crommenacker et al. 2010), which compromise their ability to meet energetically demanding processes at adulthood, such as mounting an immune response (Marais, Maloney & Gray 2011) or bearing ornaments (Allen & Levinton 2007). Conversely, early-life immune challenges may be necessary for immunodevelopment and promote optimal, life-long performance of the adult immune system (Strachan 2000). Thus, although the full suite of physiological mechanisms mediating the relationships between early-life immune challenges and adult ornamentation remains unclear, a potentially fruitful first step is to investigate a system that utilizes the same molecules for both ornamentation and an effective immune response. To this end, many researchers have turned to carotenoid-pigmented ornaments when examining links between ornamentation and immune function (McGraw 2006; Peters et al. 2004a,b).

Carotenoids are diet-derived molecules that serve a variety of functions in animals, including tuning visual systems (Vorobyev 2003; Lind & Kelber 2009) and conferring photoprotection (Stahl & Sies 2002). These molecules also are responsible for many vibrant sexually selected color ornaments (McGraw 2006) and can affect the responsiveness of the immune system, including antibody production (Chew & Park 2004) and cutaneous immune function (response to phytohemagglutinin: PHA; Biard et al. 2009). Due to their antioxidant properties (Kelman, Ben-Amotz & Berman-Frank 2009), carotenoids may also protect an individual's tissues during the oxidative burst (release of oxidative molecules,

e.g., nitric oxide; NO) of the innate immune response (Sild & Hõrak 2009). Because carotenoids must be acquired from the diet and play diverse physiological and fitness roles, only the highest-quality individuals acquire sufficient carotenoid levels and allocate them to the integument without suffering the costs of removing these molecules from other physiologically beneficial processes (Lindström et al. 2009). Thus, investigating the impact of developmental immune challenges within a carotenoid framework provides a unique opportunity to mechanistically link early-life immune challenges with adult ornamentation and immune function, using a common pigmentary currency.

The specific stage at which developmental perturbations occur can differentially affect the expression of traits in neonates and adults (e.g., Butler et al. in press). For example, access to carotenoids both *in ovo* and during development increased growth of nestling canaries (*Serinus canaria*), but response to PHA (see below) increased only when carotenoids were provided *post ovo*, regardless of *in ovo* carotenoid access (Tanvez et al. 2009). Additionally, because of the unique life-history periods characterizing growth (van der Ziel & Visser 2001), immune investment (Møller & Haussy 2007), molt (Vézina et al. 2009), and ornamental trait expression (Moen, Pastor & Cohen 1999), it is possible that health perturbations during distinct ontogenetic stages may differentially shape the development and expression of adult ornamentation or immune function.

Here, we manipulated immune activation and tracked carotenoid status during development in male mallard ducks (*Anas platyrhynchos*; Fig. 1) to

investigate the extent to which adult carotenoid-based ornamentation and immune function was dependent upon ontogenetic immune challenges. Mallard drakes are ideal for such a study because they have a yellow, carotenoid-pigmented beak that is used in mate choice (Omland 1996a,b) and that reflects adult immune function (Peters et al. 2004a). Also, we examined multiple *in vivo* components of the immune system that have been associated with carotenoid physiology (Biard et al. 2009; Macedo et al. 2010), both during development and at adulthood, to quantify intra-individual differences in immunoresponsiveness (Salvante 2006). Specifically, we quantified circulating immunoglobulin (predominately IgG) levels (to confirm humoral response to immune challenges), cutaneous immune response to PHA, antibody response to the novel antigen keyhole limpet hemocyanin (KLH), and the NO response (component of innate immunity) to the simultaneous administration of these two immunostimulants. The PHA test was traditionally considered to reflect cell-mediated immune function (e.g., Smits, Bortolotti & Tella 1999), but histological evidence suggests that it may also incorporate aspects of innate immunity, specifically proinflammatory potential (Martin et al. 2006; Vinkler, Bainová & Albrecht 2010). While the mechanisms that would underlie the developmental plasticity of these immune metrics are poorly defined at present, evidence in rats has shown that neonatal immune challenges attenuate adult febrile and neuroimmune response (Galic et al. 2009), and that there is a critical window (middle of postnatal development) during which neonatal immune challenges affect the adult immune response (Spencer et al. 2006).

We predicted that immune challenges during development would 1) decrease circulating carotenoid levels during development, 2) decrease the immune response of individuals later in life (*sensu* Spencer et al. 2006; reviewed in Galic et al. 2009), due to costs of mounting an immune response (Bourgeon, Maho & Raclot 2009; van de Crommenacker et al. 2010), and 3) decrease the intensity of carotenoid-based ornamentation in proportion to the predicted decreased immune function of adults. Additionally, we predicted that adult immune response would be positively related to current circulating carotenoid levels, and that the degree of immune response would correlate with a decrease in circulating carotenoid levels (Biard et al. 2009). To test these predictions, we reared males from hatch to adulthood, immune-challenged individuals at different points during development, measured circulating carotenoid levels, body mass, and circulating IgG titer throughout development and adulthood, and quantified adult immune function and ornamentation. Specifically, we manipulated immune activation of immature ducks using non-infectious immune-system challenges (Complete Freund's Adjuvant: CFA; Sheep red blood cells: SRBC; see below), which do not mimic any particular pathogenic infection *per se*, but provide the advantage of controlling the degree of the challenge compared to the broad range of pathogenicity that would result from inoculation with live infectious organisms. However, it is noteworthy that ducks are susceptible to a variety of pathogens, including parasites (Ballweber 2004; Butler and McGraw 2010), bacteria (Lillehaug et al. 2005), and viruses (Latorre-Margalef et al. 2009; Lillehaug et al. 2005), some of which can induce mortality (Ballweber 2004).

Because CFA contains *Mycobacterium* components, and SRBCs are relatively large structures with a large number of epitopes, our developmental immune challenges generally model bacterially and/or humorally recognizable immune challenges.

Materials and Methods

Experimental protocol and blood collection

We acquired 48 one-day-old male ducklings from Metzer Farms (Gonzales, CA) in December 2008 and reared them as in Butler and McGraw (2009, 2011; see Supporting Information for additional information). Individuals were randomly assigned to one of four treatment groups based on the developmental stage at which we administered injections. Individuals in experimental groups received one injection of SRBC each week for three consecutive weeks. SRBC are a non-pathogenic insult that elicits a humoral immune response (Baelmans et al. 2005) and decreases circulating carotenoid levels in adult mallards (Peters et al. 2004b). Individuals in the EARLY group (n = 12) received injections at 3, 4, and 5 weeks old, which is the period of exponential growth (Fig. S1). MIDDLE (n = 12) individuals received injections at 8, 9, and 10 weeks of age, which is when growth is complete but the nuptial molt has not begun. LATE individuals (n = 12) received injections at 13, 14, and 15 weeks old, which is when individuals are molting into nuptial plumage (birds typically begin exhibiting courtship behavior and are thus sexually mature at 16 weeks of age; Drilling et al. 2002). A series of three injections was given to each

bird at its respective time period to simulate a low-intensity, but extended, immune challenge. The first injection consisted of 0.2 ml packed SRBC (Innovative Research, suspended at 10% in saline) that was emulsified in 0.5 ml CFA (Difco Laboratories, Detroit, MI) and administered intra-abdominally posterior to the keel (*sensu* Hanssen et al. 2004). The second and third injections were similarly administered, but consisted of 0.2 ml packed SRBC emulsified in 0.5 ml Incomplete Freund's Adjuvant (IFA, which lacks the bacterial components present in CFA and thus minimizes chances of injection-site inflammation; Sigma F5506). We also had a CONTROL group (n = 12) consisting of individuals that did not receive injections at any point during development (to avoid inadvertently eliciting an immune response; Adler et al. 2001).

We collected approximately 300 µl of whole blood using heparinized capillary tubes from all individuals at the beginning and end of each treatment period (e.g., the beginning of week 3 and the end of week 5; Fig. 2) by puncturing the alar vein. Whole blood was stored on ice for several hrs and then centrifuged for 3 min at 10,000 rpm. We then aliquoted plasma into separate microtubes for carotenoid, circulating immunoglobulin levels, and NO assessment (see below), and stored the plasma at -80 ˚C until analysis. We also collected blood from experimental individuals 24 hrs after the first injection of the treatment regime to assess carotenoid and NO concentrations, and from all individuals immediately prior to adult immune assessment (18 weeks old; see below), as well as 1, 6, and 10 days after the initial adult immune challenge (see below) to quantify changes in carotenoid, NO, and KLH-specific antibody levels. At each time of blood

collection, we also measured body mass of each bird to the nearest gram to test whether immune challenges affected growth.

Developmental immune metrics

Because humoral immune responses result in antibody production, we confirmed that SRBC treatments stimulated the immune system by quantifying circulating immunoglobulin levels. We assessed circulating IgG titer (*sensu* Karell et al. 2008) at four time points (the beginning of the EARLY, MIDDLE, LATE, and adult immune assessment periods) using an enzyme-linked immunosorbant assay (ELISA) and an avian IgG-specifc secondary antibody (see Supporting Information for details). Duplicate samples analyzed for each individual were significantly repeatable within each time period (all $R > 0.97$), so average values were used. If absorbance values of plasma-filled wells were lower than the average of the control blanks on that plate, the plasma sample was assigned an absorbance value of 0.

To quantify NO response to immune stimulation during development, we quantified circulating levels of NO before SRBC/CFA administration and 24 hrs later. We followed the protocol described in Sild and Hõrak (2009), but amended it for larger (15 µl) plasma volumes (see Supporting Information for further information). Nitric oxide response was calculated as the NO concentration post-immune challenge minus the NO concentration prior to immune assessment to control for baseline differences among individuals; a greater NO response is indicative of a more robust innate immune response.

Adult immune metrics

When ducks were 18 weeks old (designated the "pre-adult challenge" period), we administered a 24-hr PHA wing-web swelling test (Smits et al. 1999; see Supporting Information for further information), with wing-web thickness measured pre-challenge and 24 hrs later. A larger swelling is associated with a more robust immune response. We quantified NO response (see above) from plasma collected at the time of both wing-web thickness measurements.

To further assess adult immune function, we quantified humoral immunity, or antibody production to a novel antigen. Specifically, we quantified the humoral response to KLH (*sensu* Butler & McGraw 2011; see Supporting Information for further information). We administered injections of 0.2 ml SRBC and 0.25 ml of 1 mg/ml aqueous KLH (Sigma H7017) emulsified in 0.25 ml IFA intra-abdominally at two time points: concurrently with the PHA injection and as a booster 6 days later. We then calculated antibody response using an ELISA similar to the circulating IgG ELISA described above (see Supplementary Material for further information). Absorbance values across triplicate samples were significantly repeatable within individuals (Lessells & Boag 1987; pre-inoculation: $R = 0.76$; primary response: $R = 0.997$; secondary response: $R = 0.994$), so we calculated an average value for each sample and subtracted the pre-inoculation value from both post-inoculation samples, thereby controlling for any intra-individual variation in background antibody binding.

Carotenoid titer and coloration assessment

To analyze plasma carotenoid content, we followed the hexane:methyl tert-butyl ether extraction method and high-performance liquid chromatography procedures of McGraw, Tourville & Butler (2008). We quantified total carotenoid titer (see Supporting Information for further information) for all individuals at the beginning and end of each developmental period (e.g., EARLY: beginning of week 3 and end of week 5), as well as four times during adult immune assessment (pre-adult challenge and 1, 6, and 10 days after the pre-adult challenge).

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 assessments (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 = 300-700 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, saturation, and hue scores that are most closely correlated with carotenoid content in the mallard beak (B1, negatively related to carotenoid content; S1B, negatively related to carotenoid content; H4b, positively related to carotenoid content; Butler, Toomey & McGraw 2011).

Statistics

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). The assumptions of equal variance were met, and post-hoc tests were performed by comparing Least Squares Means (LSMeans). To test the effects of immune treatment on duckling measures (e.g., circulating immunoglobulin concentration, body mass, circulating carotenoid titer), we used a repeated-measures analysis of variance (rmANOVA), with age as the repeated measure. For those groups that received immune challenges during development, we used an ANOVA to test for differences among groups (EARLY, MIDDLE, and LATE) in the 24 hr NO response to the SRBC/CFA injection and in the change in carotenoid titer during the same period.

To test for treatment differences in adult immune function, we ran a series of analyses of covariance tests (ANCOVAs), with treatment as the main effect, circulating carotenoid titer as a covariate, and PHA-induced swelling response and primary and secondary KLH-induced antibody concentration as dependent variables. To test for effects of changes in carotenoid titer during adult immune challenges on adult immune function, we ran another series of ANCOVAs, with developmental treatment as the main effect, change in carotenoid titer over the 1, 6, or 10 day period, respectively, as a covariate, and NO response, PHA-induced swelling response, or primary or secondary KLH-induced antibody concentration as dependent variables. We tested the effect of developmental immune treatment on adult coloration by using a multivariate analysis of variance (MANOVA), with

treatment as the independent variable and brightness, hue, and saturation as the dependent variables.

To test whether adult beak color predicted adult immune function, we ran multiple regressions with color variables (brightness and hue only, due to the correlation between brightness and saturation; R = -0.34, $P = 0.0168$) as the independent variables and adult circulating immunoglobulin concentration, NO response, PHA-induced swelling response, and primary and secondary KLH-induced antibody concentration as dependent variables. Lastly, we also tested for effects of immune response and treatment on change in beak color using several MANCOVAs. Specifically, developmental immune treatment was the main effect, immune response (NO response, PHA-induced swelling response, or primary or secondary KLH-induced antibody concentration) was a covariate, and change in beak hue, saturation, or brightness over 1 (PHA and NO), 6 (primary KLH response), or 10 (secondary KLH response) days were the dependent variables.

Results

Effects of early-life immune challenges on developmental variables

Circulating immunoglobulin concentration differed by age (Wilks' $\lambda = 0.218$, $F_{3,42} = 50.28$, $P < 0.0001$), developmental immune treatment ($F_{3,44} = 3.46$, $P = 0.024$), and their interaction (Wilks' $\lambda = 0.331$, $F_{9,102.37} = 6.54$, $P < 0.0001$). LSMeans comparisons revealed that all treatment groups circulated similar IgG levels prior to the EARLY period (all $P > 0.6$), but that birds showed increases in

IgG levels following their respective developmental immune treatments (Fig. 3). In other words, as predicted, EARLY birds circulated higher levels than CONTROL birds prior to the MIDDLE period ($P = 0.032$), EARLY ($P = 0.0008$) and MIDDLE ($P = 0.0004$) birds circulated higher levels than CONTROL birds prior to the LATE period, and MIDDLE ($P = 0.0069$) and LATE ($P = 0.0015$) birds circulated higher levels prior to adult immune assessment than CONTROL birds, while EARLY birds tended to circulate higher levels ($P = 0.080$). These results indicate that our non-pathogenic challenges had their intended, stimulatory effects on immune function.

Body mass changed as a function of age (Wilks' $\lambda = 0.0096$, $F_{6,39} = 666.24$, $P < 0.0001$) and treatment ($F_{3,44} = 3.57$, $P = 0.021$), with no significant interaction (Wilks' $\lambda = 0.546$, $F_{18,110.79} = 1.47$, $P = 0.12$; Fig. S1). However, LSMeans comparisons revealed that, while MIDDLE birds were generally less massive than CONTROL birds, this difference existed prior to the MIDDLE immune treatment and remained for the duration of the study (all $P < 0.012$). EARLY and LATE groups did not differ from CONTROL birds at any time (all $P > 0.05$).

Circulating carotenoid titer changed as a function of age (Wilks' $\lambda = 0.0401$, $F_{5,40} = 191.34$, $P < 0.0001$), but contrary to our predictions, not by treatment ($F_{3,44} = 0.30$, $P = 0.82$) nor their interaction (Wilks' $\lambda = 0.796$, $F_{15,110.82} = 0.64$, $P = 0.84$). Carotenoid levels were lowest before and after the EARLY period, moderately higher at the end of the LATE period, and highest at the

beginning and end of the MIDDLE period and the beginning of the LATE period (Fig. 4).

Immune-challenged birds during development showed a non-significant trend for different NO responses as a function of age ($F_{2,33} = 3.03$, $P = 0.062$). Post-hoc tests showed that EARLY birds had a significantly lower NO response during the first 24 hrs of the immune challenge than LATE birds ($P = 0.020$). Immune challenges during development also reduced circulating carotenoid titers during the subsequent 24 hrs ($t_{35} = -5.16$, $P < 0.0001$), but there were differences by age ($F_{2,33} = 4.54$, $P = 0.018$), with significant decreases occurring during the MIDDLE (mean ± s.e.; -1.49 ± 0.39 µg/ml; $P = 0.0005$) and LATE (-1.95 ± 0.39 µg/ml; $P < 0.0001$), but not EARLY (-0.35 ± 0.39 µg/ml; $P = 0.37$) periods.

Effects of early-life immune challenges and circulating carotenoid titer on adult immune function and coloration

Contrary to our predictions, adult PHA-induced swelling was negatively related to circulating carotenoid titer prior to immune challenge ($F_{1,42} = 5.93$, $P = 0.019$), but in accordance with our predictions, did differ by developmental immune treatment ($F_{3,42} = 4.39$, $P = 0.009$), with MIDDLE birds having smaller swellings than CONTROL birds ($P = 0.0049$). Secondary KLH-specific antibody response was positively predicted by circulating carotenoid titer prior to immune challenge ($F_{1,42} = 9.26$, $P = 0.004$) and differed by treatment ($F_{3,42} = 3.61$, $P = 0.021$), with LATE birds having larger antibody responses as adults than CONTROL birds ($P = 0.013$). Circulating carotenoid titer prior to immune

challenge was positively related to primary KLH-specific antibody response ($F_{1,42}$ = 6.10, P = 0.018) and negatively to NO response ($F_{1,42}$ = 7.91, P = 0.0074; Fig. 5), although treatment was not significant for either immune metric (both P > 0.17). Contrary to our predictions, developmental immune treatment did not affect adult beak color (Wilks' λ = 0.9424, $F_{9,102.37}$ = 0.28, P = 0.98).

Beak color as a signal of adult immune function

Contrary to our predictions, neither beak brightness nor hue were related to any metric of adult immune function (PHA-induced swelling, circulating immunoglobulin titer, primary and secondary KLH-specific antibody response; all P > 0.3), although there was a non-significant trend for birds with more carotenoid-rich beaks to have stronger NO responses (brightness: $F_{1,45}$ = 2.88, P = 0.097; hue: $F_{1,45}$ = 3.18, P = 0.081).

Magnitude of adult immune response as a function of the change in circulating carotenoid levels and beak coloration over the time course of the immune response We found that change in carotenoid titer over the first 24 hrs of the adult immune challenge positively predicted swelling response ($F_{1,42}$ = 4.33, P = 0.043) and NO response ($F_{1,42}$ = 8.51, P = 0.0056; Fig. 6), while developmental immune treatment significantly predicted adult wing web swelling ($F_{3,42}$ = 3.50, P = 0.023; LATE and MIDDLE had smaller swellings than CONTROL birds: both P < 0.05; Fig. 7a) and tended to predict NO response ($F_{3,42}$ = 2.36, P = 0.084). Neither developmental immune treatment ($F_{3,42}$ = 1.03, P = 0.39) nor change in

adult carotenoid titer ($F_{1,42} = 2.53$, $P = 0.1191$) was associated with primary KLH-specific antibody response in adults. However, change in carotenoid titer ($F_{1,42} = 15.00$, $P = 0.0004$) and developmental immune treatment ($F_{3,42} = 3.42$, $P = 0.026$; Fig. 7b) were significantly associated with secondary KLH-specific antibody response, with LATE birds having higher responses than CONTROL birds ($P = 0.0029$) and a trend for EARLY birds to have higher responses than CONTROL birds ($P = 0.079$).

Neither developmental immune treatment nor magnitude of PHA or KLH immune response predicted change in beak coloration (hue, saturation, or brightness) over the course of the adult immune assessments (all $P > 0.28$). However, a larger NO response was significantly associated with the beak becoming brighter (less carotenoid-rich; Butler, Toomey & McGraw 2011) over the corresponding 24 hrs ($F_{1,42} = 10.44$, $P = 0.0024$), regardless of treatment ($F_{3,42} = 0.19$, $P = 0.90$).

Discussion

The determinants of immune function in human and non-human animals alike continue to receive scientific attention from both basic and applied researchers. However, developmental conditions, ranging from stress to nutrition to parasite burden, have been comparatively neglected as predictors of immune function until recently (e.g., Guzmán et al. 2009). Immune challenges early in life have been found to decrease health state in adult rats (Spencer et al. 2006; Galic et al. 2009), but such a relationship is unstudied in other non-human animals. Here, we

demonstrate that immune challenges during development did not alter growth patterns but did affect adult immune function, and that both age during development and the type of adult immune metric are vital to understanding this relationship.

We found that immune challenges during development affected two different immunological responses in adult ducks. Following the evidence in rats (Spencer et al. 2006; Galic et al. 2009), we predicted that immune challenges during development would reduce adult immune function, and we found some support for this prediction; the PHA response of adults from the MIDDLE and LATE groups was reduced compared to controls. Lower PHA responses in the nestling stage have been tied to decreased recruitment rates in several species of songbirds (Cichoń & Dubiec 2005, Moreno et al. 2005, but see Butler et al. 2009), suggesting developmentally immune-challenged mallards may realize negative consequences to mounting an immune response during development (*sensu* Hanssen et al. 2004).

In contrast to our PHA results, however, we found that LATE birds had an *increased* humoral response at adulthood relative to controls. An increased ability to produce antibodies in response to an antigen may be associated with higher levels of survival (Star et al. 2007). Thus, the higher humoral immune response of LATE birds may be predictive of an increased survival likelihood, generating a conflicting prediction to the PHA result. This finding, in combination with conflicting evidence regarding within-life repeatability of individual immune function (repeatable: Addison, Ricklefs & Klasing 2010; non-repeatable: Love et

al. 2008), highlights the necessity of both testing this hypothesis in the field and underscoring that a greater immune response does not always result in a more (or less) fit phenotype (Graham et al. 2011).

Unlike the other immune treatment groups, birds from the EARLY group did not differ from CONTROLS at the adult stage for any carotenoid, color, or immune metric. This occurred despite the fact that early-life immune challenges affected neonatal physiology (circulating IgG was elevated in EARLY birds for at least 7 weeks). Thus EARLY birds, while possessing functioning immune systems, may not possess fully developed immune systems (Glünder, van der Ven & Foulman 2004), or may be investing in growth at the expense of immune response (Brommer 2004), yielding a lower relative investment in immune function. Alternatively, differences apparent in MIDDLE and LATE birds could have been due to cumulative, carry-over effects of the immune challenges, and it is possible that if we had performed adult immune assessments several weeks earlier (e.g., when individuals were 16 weeks old), we also would have been able to detect differences in PHA, NO, or KLH response in EARLY birds (*sensu* McBride & Shuman 1988). However, it is unlikely that the treatment effects on MIDDLE and LATE birds are due exclusively to carry-over effects, as these birds had not been immune challenged for 8 and 3 weeks prior to adult immune assessment, respectively, which is enough time for inflammatory immune responses to have subsided to near baseline levels (Adler et al. 2001; Biard et al. 2009).

Perturbations to the immune system alter carotenoid status in several bird species (neonates: Koutsos, Calvert & Klasing 2003; adults: McGraw & Ardia, 2003; Peters et al. 2004b; Biard et al. 2009), but in our developmental study immune challenges did not affect circulating carotenoid levels during development outside of the immediate 24 hrs post-initial immune challenge. We see four possible explanations for this result: (1) we did not collect blood samples frequently enough to detect any longer-term effects of our challenges, (2) immune challenges during development do not utilize carotenoids in the same way that immune challenges do in adult mallards (Peters et al. 2004b), (3) lower levels of circulating carotenoids relative to wild duckling (Butler and McGraw 2010) did not allow us to detect an effect, or (4) ducklings were able to circulate constant levels of carotenoids either by releasing carotenoid stores from other body tissues (e.g., liver, adipose) or increasing carotenoid uptake from diet. However, despite finding no links between immune treatment and circulating carotenoid levels throughout development, immune responses at adulthood showed several strong relationships with circulating carotenoid levels and changes in carotenoid levels. In particular, we found that responses to the two 24 hr. immune assessments (PHA and NO) were negatively associated with circulating carotenoid titer prior to assessment, and that birds that had greater primary and secondary humoral responses had higher circulating carotenoid titers prior to assessment. Thus, an individual with a relatively higher amount of circulating carotenoids would be likely to have a less robust PHA or NO response, but a more robust KLH response. This finding is congruent with work in chickens showing that markers

of innate immune response are higher in carotenoid-depleted birds (Meriwether et al. 2010), although this relationship can switch if there are low circulating levels of fatty acids (Selvaraj & Klasing 2006).

These relationships may also reflect state-dependent differences in immune response. One interpretation of these data is that an individual with greater amounts of circulating carotenoids may prioritize an acquired immune response (specific antibody response; Aguilera & Amat 2007). However, an individual with lower circulating levels, and presumably fewer carotenoids in other tissues (McGraw & Toomey 2010), may not have the ability to invest in a large, carotenoid-expensive, long-term response. Such an individual may then rely upon investing carotenoids in more inflammatory, innate immune responses, reflected in higher PHA and NO responses; this relationship between circulating carotenoid levels and the PHA response has been identified previously (Biard et al. 2009). However, because we issued both challenges concurrently, we are unable to directly test this hypothesis, and further work regarding the mechanistic role that carotenoids play in the different components of the immune response (e.g., antibody production, inflammatory response) are required before this idea can be tested thoroughly.

Conversely, the *change* in carotenoid titer over the course of the adult immune response was positively correlated with PHA and NO response, but negatively with secondary KLH response. While this may initially seem contradictory, these data are consistent with the scenario postulated above. If the humoral immune response is more carotenoid-expensive, then carotenoid levels

should be somewhat depleted with a larger humoral response, while the putatively less carotenoid-expensive PHA and NO responses could promote an individual to mobilize carotenoids from other tissues, thus transiently buffering the decrease in circulating carotenoid titer. While this experiment was not designed to explicitly test for differential carotenoid investment among immune response types or for the prioritization of acquired or innate immune responses based on carotenoid status, the above data suggest that these areas might be fruitful areas of further research. Systems of particular interest include 1) species with carotenoid-based ornamentation, especially those populations that are exposed to a variety of pathogens and have limited access to carotenoids, and 2) taxa that utilize carotenoids differently across life-history stages (e.g., based upon breeding status, wherein females deposit large quantities of carotenoids in yolks at the expense of their own carotenoid stores; Karadas et al. 2005; McGraw, Adkins-Regan & Parker 2005).

Contrary to our predictions, we did not find any relationships between adult male mallard beak color and adult immune function. It is unlikely that this finding is due to an artifact of housing conditions, as we have identified such relationships in our previous work on mallards (Butler & McGraw 2011). Therefore, because immune challenges during development significantly altered the immune responses of adults but these differences in immune function were not reflected in adult beak coloration, it is possible that immune challenges during development decoupled associations between beak carotenoid allocation (i.e., coloration) and immune function. While no mechanism has yet been identified,

this finding is similar to previous work that has shown that early-life diet manipulations can result in a decoupling of plasma carotenoid levels and beak color in zebra finches (*Taeniopygia guttata*; Blount et al. 2003). We can speculate as to adaptive reasons for this finding (e.g., immune challenged animals may be more likely to shift carotenoid resources to invest in reproduction (coloration) rather than self (immune function; bet-hedging, reviewed in Childs, Metcalf & Rees 2010)), but such explanations remain to be tested. We look forward to future research that builds upon the foundation of this work, using both laboratory and field studies to test the mechanisms and functional consequences of neonatal immune challenges upon adult survival- and reproduction-related traits.

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Figure 1. A male mallard in nuptial plumage, with the carotenoid-pigmented yellow beak clearly visible in the online color version (photo: M.W. Butler).

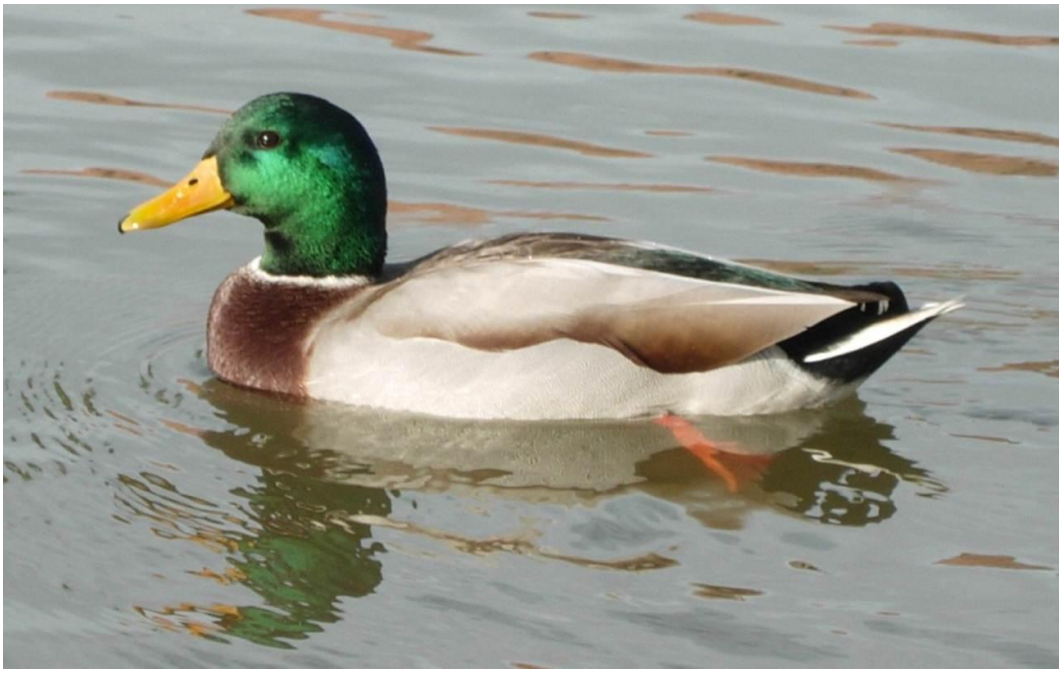


Figure 2. Timing of sample collection. Circulating IgG (A) was measured at the beginning of each treatment period, while plasma nitric oxide concentration (B) was measured at the beginning of each period and 24 hrs later. Circulating carotenoid titer (C) was measured at the beginning and end of each treatment period, 24 hrs after initial treatment injection, as well as at all points in the adult phase. Wing web thickness (D) was measured prior to PHA injection (*Day 0*) as well as 24 hours later (*Day 1*). KLH-specific IgG (E) 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*).

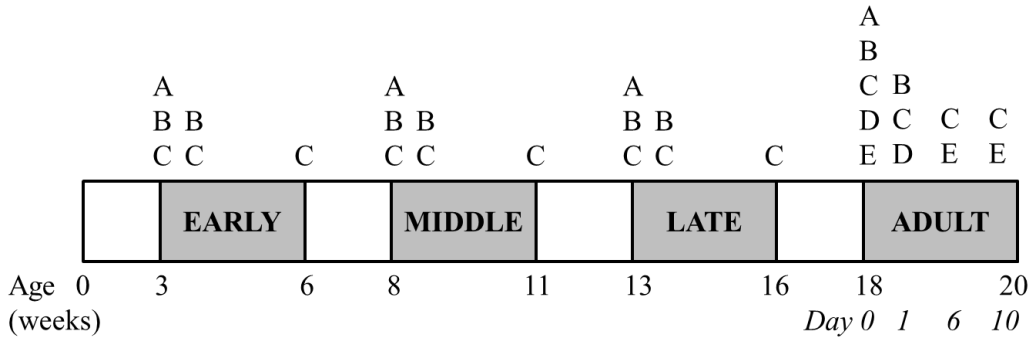


Figure 3. Circulating immunoglobulin levels as a function of age and treatment in male mallards. All individuals circulated similar levels of IgG prior to SRBC immune challenge, but immune challenges resulted in a lasting increase in circulating antibody titer relative to CONTROL birds. For example, birds that were immune challenged during the EARLY period were circulating higher levels of IgG before the MIDDLE period. Differences within age groupings are significant at $P = 0.05$ (denoted by *).

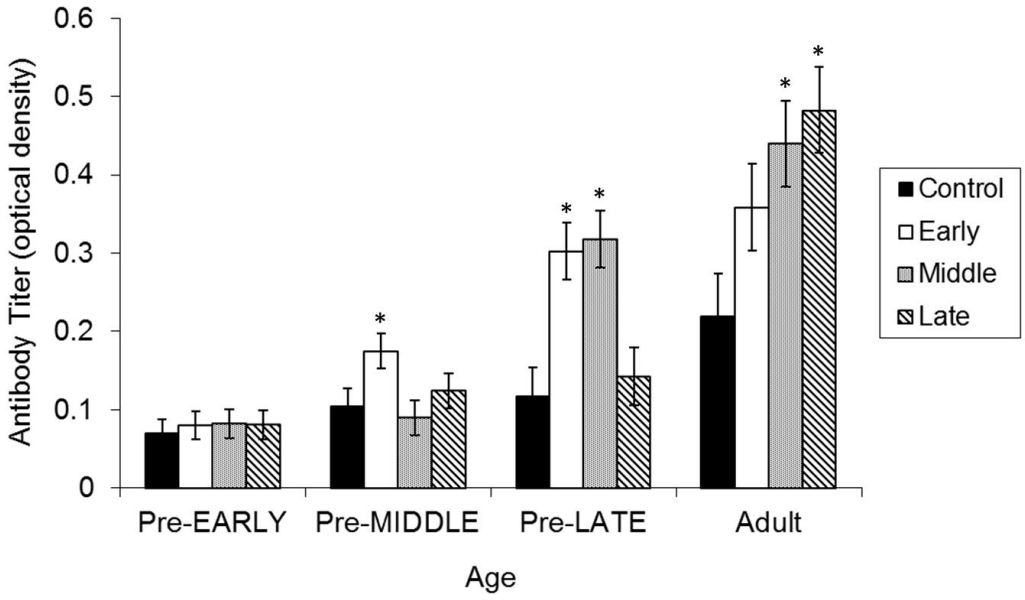


Figure 4. Total circulating carotenoid titer as a function of age in male mallards. Carotenoid titer peaked at the end of the MIDDLE period, and was lowest during the EARLY period. Means sharing a letter are not significantly different ($P > 0.05$).

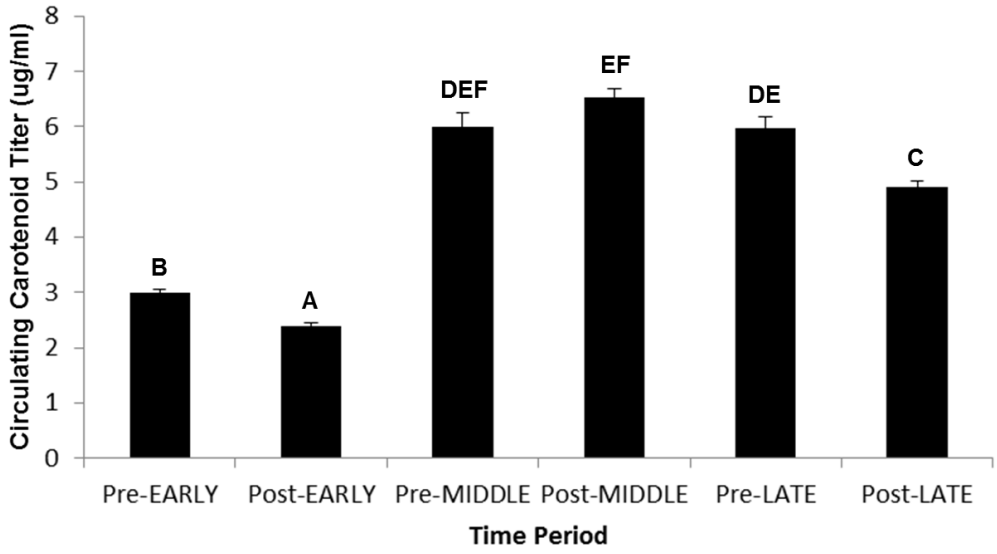


Figure 5. Nitric oxide response of adult male mallards as a function of circulating carotenoid titer prior to immune assessment. Higher levels of circulating carotenoids prior to immune assessment were associated with a lower NO response.

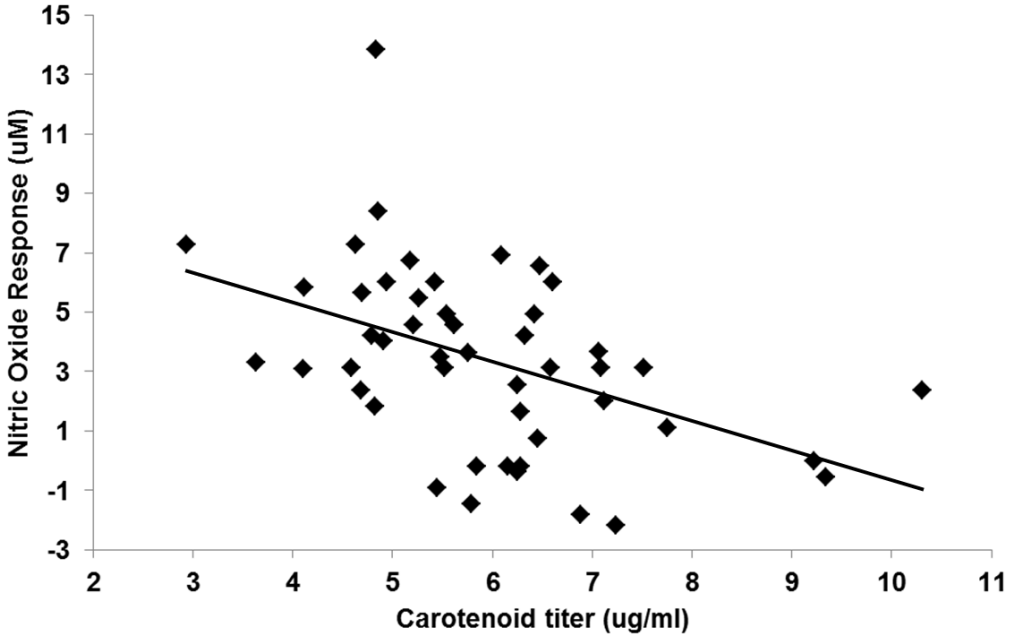


Figure 6. Nitric oxide response of adult male mallards as a function of change in circulating carotenoid titer over the course of the immune assessment. During the 24 h period of NO production in response to an immune challenge, a greater change in carotenoid circulation was associated with a relative higher NO response. Thus, individuals that increased circulating carotenoid levels had greater NO responses than those that showed a decrease in circulating carotenoid levels.

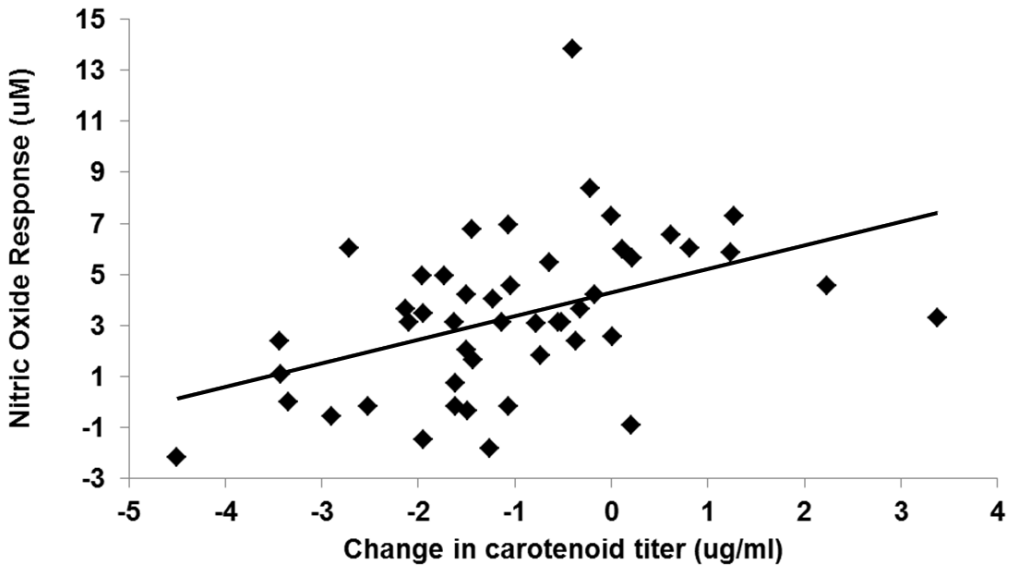
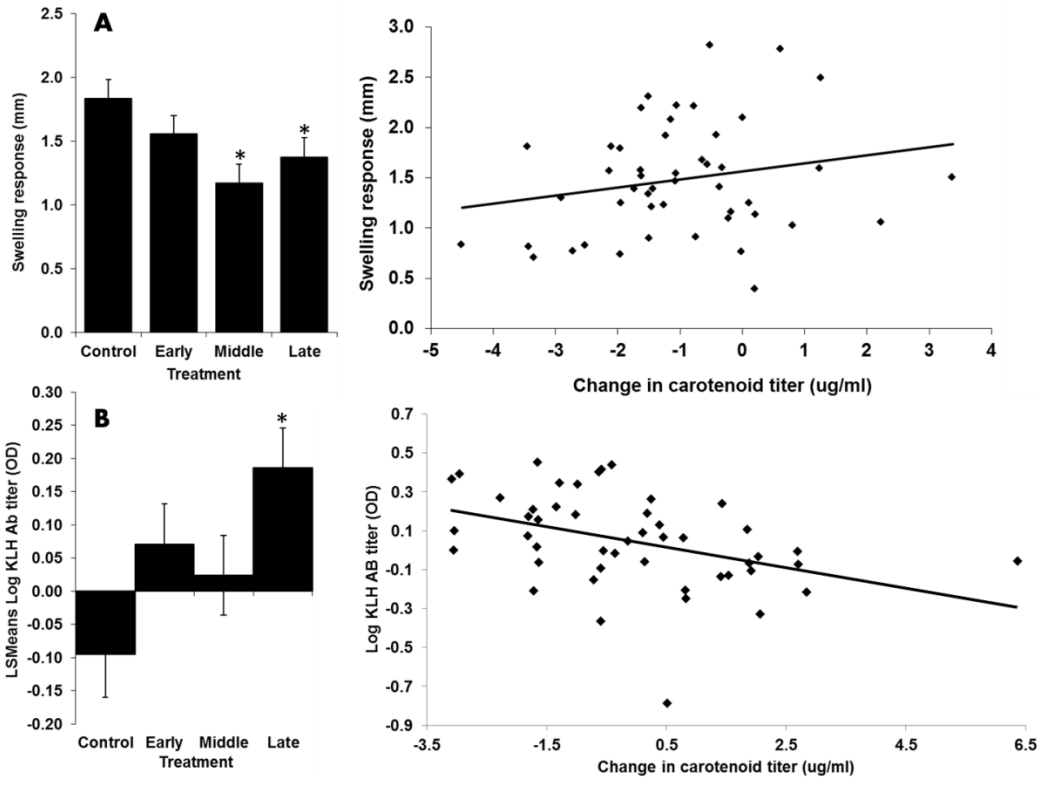


Figure 7. (A) PHA swelling response and (B) KLH-induced antibody production of adult male mallards as a function of developmental immune treatment and change in circulating carotenoid titer during the duration of the adult immune assessment period. Relative to control birds, MIDDLE and LATE birds had reduced swellings and LATE birds had higher humoral responses ($P < 0.05$; *). Change in carotenoid titer was positively related to swelling and negatively related to humoral response for all birds.



Appendix S1: Further details regarding methodology

Husbandry

Ducklings were reared in two indoor rooms in randomly selected groups of four ducklings per cage (60 x 60 x 60 cm) until they were 20 days old, three per cage until 23 days old, and two per cage until 45 days old, at which point all birds were moved outside and individually housed to allow for normal sexual maturation (Butler and McGraw 2009, 2011). Infrared heat lamps were positioned approximately 20 cm from the front of the cage from hatch until 52 days old to maintain a 30 ºC area within the cage. Light:dark regime was 13L:11D while ducklings were housed indoors, and natural photoperiod thereafter (10.5L:13.5D at 45 days old to 13.5L:10.5D at 20 weeks old). When the birds were three weeks old, they were individually tattooed with a unique number on their left tarsi. Food (Mazuri Waterfowl Starter for the first 7 weeks, and Mazuri Waterfowl Maintenance thereafter) and drinking tap water were provided *ad libitum* throughout the study.

Circulating IgG ELISA

To perform this assay, we incubated 96-well plates (Fisher 468667) at 4 ºC overnight with 100 µl of anti-avian IgG antibody (Bethyl, A140-110A) diluted 1:50 in sodium carbonate buffer (pH 9.6). We then emptied the plates and added 100 µl blocking buffer (PBS with 1% bovine serum albumin; Sigma B4287) with sodium azide (0.1%, Sigma S2002) and incubated at room temperature for 2 hrs.

We then washed the plate three times with wash buffer (PBS and 0.05% Tween 20; Arcos 23336) and added, in triplicate, 100 µl plasma diluted 1:280,800 in blocking buffer with azide and incubated the plate overnight at 4 ºC. Next, we washed the plates five times with wash buffer and added 80 µl HRP-conjugated polyclonal secondary antibody (Bethyl Laboratories, A140-110P) specific for avian IgG that was diluted 1:20,000 in blocking buffer. The plate was then incubated for 2 hrs at room temperature and washed five times with wash buffer. We then added 100 µl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate, incubated the plate for 30 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 $\lambda = 450$ nm using an iMark Microplate Reader (Bio-Rad Laboratories, UK), with greater absorbances corresponding to a great IgG titer.

Nitric oxide assay

We added 15 µl plasma to 40 µl 75 mmol ZnSO4 and 50 µl 55 mmol NaOH, centrifuged samples for 10 min at 16,000 RCF, and combined 80 µl supernatant with 80 µl glycine buffer (pH = 9.7). We then added two activated cadmium granules and shook the samples at maximum force (G24 Environmental Incubator Shaker, New Brunswick Scientific Co., Inc., Edison, NJ) for 15 min. Next, we combined 120 µl supernatant with 120 µl Greiss reagent (Sigma G4410), shook samples at maximum force for 15 min, and transferred 200 µl of each sample to a 96 well plate. We then read the absorbance of each well at $\lambda = 542$ nm using a µQuant plate reader (BioTek Instruments, Inc., Winooski, VT), and converted

absorbance values to NO concentration based on standard curves run with each assay.

PHA wing-web swelling test

We measured the thickness of the patagium (wing-web) with a digital micrometer (Mitutoyo model 293-761-30) in duplicate and subsequently injected 0.2 ml of 1 mg/ml PHA (Sigma L8754) suspended in sterile phosphate-buffered saline (PBS; Fisher BP399) at the site of measurement. Twenty four hrs later, we measured patagial thickness in duplicate. Because thickness measurements were significantly correlated (pre-injection: $R = 0.97$, $P < 0.0001$; 24 hrs later: $R = 0.99$, $P < 0.0001$), we calculated the average for each time point and defined swelling as the difference between the two thicknesses.

KLH-specific IgG ELISA

This ELISA was similar to the circulating IgG ELISA described above, but with the following modifications. Instead of coating the plates with anti-avian IgG antibody, we began the ELISA by incubating each well overnight with 100 µl of 0.5 mg/ml KLH in sodium carbonate buffer. We diluted the plasma samples 1:12,100 and the HRP-conjugated polyclonal secondary antibody 1:10,000 based on pilot work. Finally, after the addition of the TMB, plates were incubated for 20 min at room temperature, protected from light.

Carotenoid quantification

As found previously, detectable amounts of lutein, zeaxanthin, and a lutein derivative existed in duck plasma at all time points, while smaller amounts of ß-cryptoxanthin were sporadically detected (Butler and McGraw 2010). Within each time point, lutein, zeaxanthin, the lutein derivative, and ß-cryptoxanthin were positively correlated with total carotenoid titer (all $R > 0.513$, $P < 0.0002$), except for one instance when ß-cryptoxanthin was not ($R = 0.283$, $P = 0.0511$). However, ß-cryptoxanthin accounted for 0.03% of the total carotenoid titer at this time point, so we elected to use total carotenoid titer at each time point in subsequent analyses.

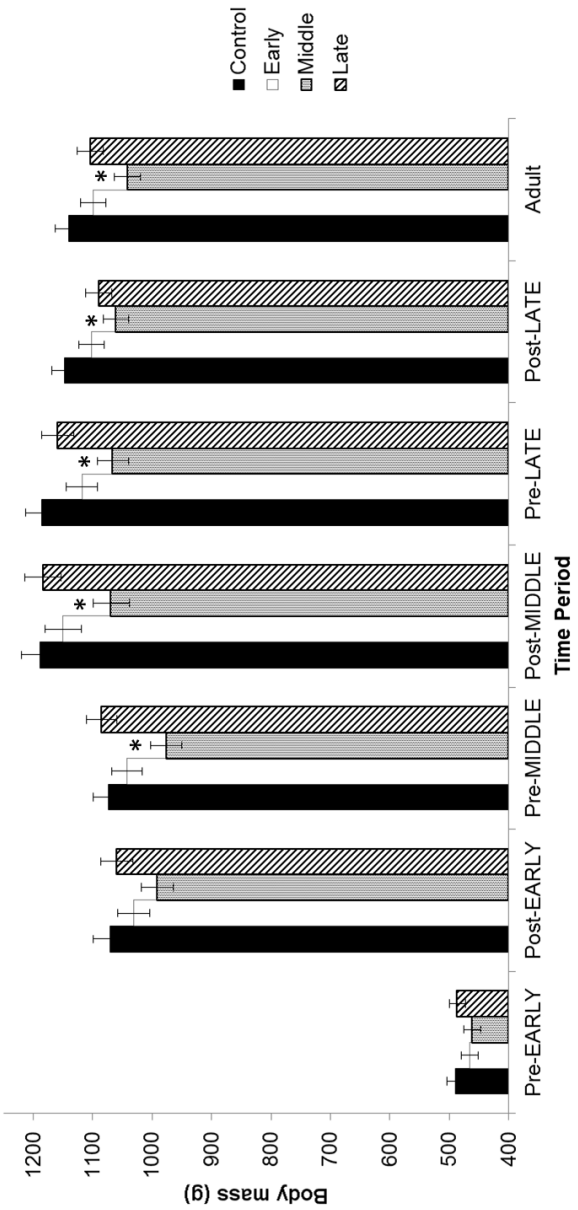
References

Butler, M.W. & 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). Journal of Comparative Physiology B, 181, 551-563.

Butler, M.W. & McGraw, K.J. (2010) Relationships between dietary carotenoids, body tissue carotenoids, parasite burden, and health state in wild mallard (Anas platyrhynchos) ducklings. Archives of Biochemistry and Biophysics, 504, 154-160.

Butler, M.W. & McGraw, K.J. (2009) Indoor housing during development affects moult, carotenoid circulation and beak colouration of mallard ducks (Anas platyrhynchos). Avian Biology Research, 2, 203-211.

Butler, M.W. & McGraw, K.J. (2010) Relationships between dietary carotenoids, body tissue carotenoids, parasite burden, and health sta Figure S1. Body mass as a function of age for all treatment groups. Although there was no significant effect of the age-by-treatment interaction term on body mass (see Results), MIDDLE birds were significantly smaller at multiple time points (*; $P < 0.05$), including prior to treatment.



APPENDIX D

APPROVAL LETTERS FROM INSTITUTIONAL ANIMALCARE AND USE COMMITTEE

Institutional Animal Care and Use Committee (IACUC)
Arizona State University

Tempe, Arizona 85287-3503
(480) 965-2179 FAX: (480) 965-8013

Animal Protocol Review

Protocol Number:	07-910R
Protocol Title:	Development of immune function and coloration in mallards
Principal Investigator:	Kevin McGraw
Date of Action:	12/07/2006

The animal protocol review was considered by the Committee and the following decisions were made:

- ☐ The original protocol was APPROVED as presented.
- ☒ The revised protocol was APPROVED as presented.
- ☐ The protocol was APPROVED with RESTRICTIONS or CHANGES as listed below. The project can only be pursued, subject to your acceptance of these restriction or changes. If you are not agreeable, contact the IACUC Chairperson immediately.
- ☐ The Committee requests CLARIFICATIONS or CHANGES in the protocol as described below. Approval is contingent upon review and approval of the required revisions by the IACUC Chair.
- ☐ The protocol was approved, subject to the approval of a WAIVER of provisions of NIH policy as noted below. Waivers require written approval from the granting agencies.
- ☐ The protocol was DISAPPROVED for reasons outlined in the attached memorandum.
- ☐ The Committee requests you to contact _____ to discuss this proposal.
- ☐ A copy of this correspondence has been sent to the Vice President for Research.

RESTRICTIONS, CHANGES OR WAIVER REQUIREMENT:

Approved number of Animals: 255 Mallard ducks

Approval Period: 12/07/2006 – 12/06/2009

Signature: _____ Date: **12/07/2006**
IACUC Chair or Designee

Investigator
cc: IACUC Office, IACUC Chair, ORSPA

Institutional Animal Care and Use Committee (IACUC)
Office of Research Integrity and Assurance
Arizona State University

Tempe, Arizona 85287-1103
(480) 965-2179 FAX: (480) 965-7772

Animal Protocol Review

ASU Protocol Number:	**10-1094R**
Protocol Title:	**Development of immune function, coloration, and behavior of mallard ducks.**
Principal Investigator:	**Kevin McGraw**
Date of Action:	**11/19/2009**

The animal protocol review was considered by the Committee and the following decisions were made:

- ☒ The original protocol was APPROVED as presented.
- ☐ The revised protocol was APPROVED as presented.
- ☐ The protocol was APPROVED with RESTRICTIONS or CHANGES as noted below. The project can only be pursued, subject to your acceptance of these restriction or changes. If you are not agreeable, contact the IACUC Chairperson immediately.
- ☐ The Committee requests CLARIFICATIONS or CHANGES in the protocol as described in the attached memorandum. The protocol will be reconsidered when these issues are clarified and the revised protocol is submitted.
- ☐ The protocol was approved, subject to the approval of a WAIVER of provisions of NIH policy as noted below. Waivers require written approval from the granting agencies.
- ☐ The protocol was DISAPPROVED for reasons outlined in the attached memorandum.
- ☐ The Committee requests you to contact _____ to discuss this proposal.
- ☐ A copy of this correspondence has been sent to the Vice President for Research.
- ☐ Amendment was approved as presented.

RESTRICTIONS, CHANGES OR WAIVER REQUIREMENT:
Approved # of Animals: Mallard Ducks Pain **Level:** C **Species:** 288
Approval Period: 11/19/2009 - 11/18/2012

Signature: _____ Date: 11/19/09
IACUC Chair or Designee

Original: Principal Investigator
cc: IACUC Office
IACUC Chair

APPENDIX E

COAUTHOR PERMISSIONS FOR INCLUSION OF PUBLISHED WORKS

November 10th, 2011

To whom it may concern,

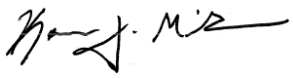
I, Kevin McGraw, grant Michael Butler permission to include, as part of his dissertation, our manuscripts:

Butler MW and KJ McGraw. 2010. Relationships between dietary carotenoids, body tissue carotenoids, parasite burden, and health state in wild mallard (*Anas platyrhynchos*) ducklings. *Archives of Biochemistry and Biophysics* 504:154-160.

Butler MW and KJ McGraw. 2011. Past or present? Relative contributions of developmental and adult conditions to adult immune function and coloration in mallard ducks (*Anas platyrhynchos*). *Journal of Comparative Physiology B* 181:551-563.

Butler MW and KJ McGraw. In press. Developmental immune history affects adult immune function but not carotenoid-based ornamentation in mallard ducks. *Functional Ecology*.

Sincerely,



Kevin J. McGraw