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Original Article

Imperfect past and present progressive: beak color reflects early-life and adult exposure to antigen

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Secondary sexual traits may convey information about individual condition. We assessed the capacity for immune challenge with lipopolysaccharide (LPS) or keyhole limpet hemocyanin (KLH) during the prenatal and early postnatal stages to impact beak color development and expression in captive zebra finches. In addition, we tested whether adult immune challenge impacted beak color, and if early-life experience was influential. Immune challenge with KLH early in life slowed development of red beak coloration, and males challenged with KLH as nestlings had reduced red coloration as adults. Following adult KLH challenge, males exhibited a decline in beak redness. Birds challenged with KLH during development produced more anti-KLH antibodies after adult challenge. There was a significant interaction between young treatment and anti-KLH antibody production; for males not challenged with KLH early in life, individuals that mounted a weaker antibody response lost more red coloration after challenge than males mounting a stronger antibody response. Based on models of avian vision, these differences in beak coloration should be detectable to the finches. In contrast to previous studies, we found no effect of early-life or adult challenge with LPS on any aspects of beak coloration. These results provide evidence that beak color reflects developmental and current conditions, and that the signal is linked to critical physiological processes.

Key words: development, immune challenge, sexual signals, stressor, zebra finch.

INTRODUCTION

Secondary sexual traits are thought to be honest signals of a male's quality (Zahavi 1975; Andersson 1994). These traits have been linked to aspects of condition, including parasite defenses (Hamilton and Zuk 1982; McGraw and Hill 2000; Van Oort and Dawson 2005), resistance to oxidative damage (Perez-Rodriguez et al. 2010), capacity for resource acquisition (Hill and Montgomerie 1994), and production of high-quality sperm (Huuskonen et al. 2009). In addition to reflecting an individual's current condition, there is strong evidence that secondary sexual signals can reflect an individual's developmental history. Work on birdsong in particular has documented the importance of early-life experience for developing song repertoire size or song complexity (e.g., Nowicki et al. 1998; Buchanan et al. 2003; Schmidt et al. 2014), but a growing number of studies document that early-life experience can also affect the expression of visual ornaments, such

as feather and beak color in birds (e.g., Blount et al. 2003; Naguib and Nemitz 2007; Butler and McGraw 2011, 2012a).

Uncovering the mechanisms linking signal expression and individual condition has proven challenging. One system that shows promise for resolving this issue is carotenoid-based displays. Carotenoids are red and yellow plant-derived pigments used by many species in secondary sexual traits (e.g., birds [Badyaev and Hill 2000], fish [Evans and Norris 1996], and reptiles [Steffen and McGraw 2007]), but that also play an important role in physiological processes (e.g., immune function and antioxidant capacity [Olson and Owens 1998; Moller et al. 2000; Blount 2004]). Expression of these carotenoid-dependent traits can, therefore, signal an individual's ability to acquire resources (e.g., Hill 1990), fight off an infection (e.g., Van Oort and Dawson 2005; Mougeot 2008), or cope with high levels of oxidative stress (e.g., Perez-Rodriguez et al. 2010). There is also evidence that carotenoid-based displays can be impacted by the environment and that exposure to stressors such as nutritional deficits and immune challenges can lead to short- and long-term reduction in trait expression (Naguib and Nemitz 2007; Butler and McGraw 2012a). Strength of the signal is not determined strictly by genetic and early environmental factors,

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however, and these displays can change in response to stressors experienced in adulthood (e.g., feather coloration: McGraw and Hill 2000; Hörak et al. 2004; beak coloration: Faivre et al. 2003; McGraw et al. 2011; Rosenthal et al. 2012). Expression of carotenoid-based signals may thus convey information about multiple components of an individual's quality, including genetic condition, developmental condition, and current condition.

In this study, we examined short-term and long-term impacts of early-life and adult exposure to antigens on beak coloration in zebra finches (*Taeniopygia guttata*). The beak of the zebra finch is vascularized (Lucas and Stettenheim 1972) and the color likely depends on carotenoid levels in both living and dead tissue of the beak (*sensu* Butler et al. 2011; Rosenthal et al. 2012). Beak color can change rapidly as carotenoids are deposited into, or pulled from, the living tissue in the beak (Ardia et al. 2010; Rosenthal et al. 2012), suggesting a role for signaling current condition. Both sexes acquire a red-orange beak in the first 2 to 3 months posthatch, but males have significantly redder coloration compared to females which have more orange-colored beaks (McGraw et al. 2011). Beak color has been linked to attractiveness in zebra finches, with females preferring redder beaks (Burley and Coopersmith 1987; De Kogel and Prijs 1996; Blount et al. 2003; Simons and Verhulst 2011). Males, however, appear to prefer females that have intermediate colored beaks (Burley and Coopersmith 1987). Beak tissue is constantly replaced as it is worn down, and to maintain the red or orange coloration, ketocarotenoids (e.g., astaxanthin and canthaxanthin) are deposited into the new tissue (McGraw and Toomey 2010). These ketocarotenoids are produced from substrate xanthophyll carotenoids such as lutein and zeaxanthin, which are acquired from the birds' diet (McGraw et al. 2002). Signals composed of ketocarotenoids may convey more information than signals composed of substrate carotenoids alone and could reflect components of basic metabolic functionality (Hill 2011; Hill and Johnson 2012; Hill 2014). The mitochondrial machinery responsible for the conversion of xanthophyll carotenoids to ketocarotenoids may be shaped during early life and could provide a link between signal expression and early environmental conditions (*sensu* Hill 2011).

We examined the relative influence of prenatal and early postnatal exposure to different antigens on the rate of beak color development (days 50 and 65 posthatch), as well as permanent expression of baseline beak color (3 years posthatch). Additionally, we investigated whether antigen exposure as an adult affected beak color expression and production of antigen-specific antibodies (Ab), and if so, whether early-life exposure to antigen modulated that response. We were also interested in determining whether any differences in beak coloration would be discernible to the finches themselves. Work documenting the spectral sensitivity of different bird species (e.g., Vorobyev and Osorio 1998; Vorobyev et al. 1998; Govardovskii et al. 2000; Hart and Vorobyev 2005) has allowed for the quantitative assessment of signal variation as assessed by birds (e.g., Maia et al. 2013; Jones and Siefferman 2014).

We used 2 functionally distinct antigens for this study; lipopolysaccharide (LPS) and keyhole limpet hemocyanin (KLH). We used LPS and KLH because they differ in how they activate antibody production: LPS is a thymus-independent type 1 antigen, meaning that Ab production is not dependent upon T-cell assistance (Janeway et al. 2001), whereas KLH is a thymus-dependent antigen and production of specific Abs to KLH requires the presence of armed helper T cells (Janeway et al. 2001). Antibody production in response to a secondary injection of LPS typically results in a more rapid, but also more generalized Ab response composed of IgM and antigen-specific IgY, compared to the highly specific IgY

Ab response following secondary KLH injection (Janeway et al. 2001). In previous work, we documented short-term decreases in total Ab levels due to antigen challenge posthatch, as well as suppressive effects of maternal transfer of anti-KLH Abs on the production of anti-KLH Abs following KLH challenge posthatch (Merrill and Grindstaff 2014). We also found evidence of environment matching in hypothalamic–pituitary–adrenal axis function in which KLH-treated offspring of KLH-treated mothers exhibited reduced production of stress-induced corticosterone compared to birds experiencing a mismatch between prenatal and early developmental immune challenge (Merrill and Grindstaff 2015). Together, these results indicate that the treatments resulted in short-term and long-term changes to important physiological processes, both of which have presumed effects on lifetime fitness.

We predicted that 1) early-life antigen challenge (prenatal or postnatal) would result in slowed development of beak color and reduced adult color expression due to the costs of mounting an immune response during development (Bourgeon et al. 2009; Galic et al. 2009), 2) adult challenge with LPS and KLH would induce an antigen-specific Ab response and lead to duller beaks as carotenoids are pulled from the live tissue and circulation to help mount an Ab response (Chew and Park 2004), and 3) the Ab response would be positively related to degree of color loss (Faivre et al. 2003).

METHODS

Study population

Research was approved by the Oklahoma State University Institutional Animal Care and Use Committee (AS107) and complied with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health 1985). Zebra finches were housed in same sex cages of 4 (45W × 45D × 40H cm) to 8 (90W × 45D × 40H cm) birds with ad libitum food, water, and cuttlebone access. Birds were fed a millet seed mix (SunSeed Economy Finch Mix) and once a week received a nutritional supplement consisting of commercial egg food (ABBA 92A, Red Bird Products), hard-boiled chicken egg, and vitamins (Avian Plus, Zoo Med Laboratories). Room lighting was maintained at 16 L:8 D.

Maternal and developmental treatments

Mothers were assigned at random to antigen challenge with KLH (50 µg of KLH [Calbiochem 374817] in 50 µl of autoclaved phosphate-buffered saline [PBS] Sigma P5368), LPS derived from *Salmonella typhimurium* (1.0 mg LPS/kg body weight [Sigma L7261] in 50 µl of PBS), or a 50 µl control injection of PBS. Females were injected twice intra-abdominally (an initial injection and a booster injection at least 35 days after the first injection) prior to production of the clutch resulting in the described young.

Offspring were cross-fostered within 72 h of hatching such that young from natal nests were evenly divided across the 3 treatment groups. This resulted in 9 possible combinations of maternal and offspring treatments. On day 5, nestlings received a primary challenge. All young within a foster nest received the same treatment as the foster mother. KLH-challenged offspring received an intra-abdominal injection of 12.5 µg KLH in 25 µl sterile PBS. LPS-challenged offspring received an intra-abdominal injection of 0.5 mg LPS/kg body weight in 25 µl sterile PBS. Control offspring received an intra-abdominal injection of 25 µl sterile PBS. On day 28, offspring received a secondary challenge with adult female doses. For further information on maternal and developmental

treatments and cross-fostering methods, see Grindstaff et al. (2012) and Merrill and Grindstaff (2014).

Adult offspring antigen challenges and blood sampling

When the birds were approximately 3 years old, they were administered additional antigen challenges with both KLH and LPS to determine if maternal and developmental treatments had long-term effects on antibody responsiveness. All zebra finches were challenged first with LPS, and then at least 3 weeks after the post-LPS blood sample (1 month after challenge), all birds were challenged with KLH, regardless of developmental treatment. For both treatments, zebra finches were dosed intra-abdominally (1 mg/kg body weight). Prior to antigen treatment, blood (50 µl) was collected via the brachial vein from each bird to quantify baseline antibody levels. A posttreatment blood sample was collected 4 days after LPS challenge and 8 days after KLH challenge (Figure 1) to assess antigen-specific antibody levels in response to challenge. These time points targeted the days following the time of greatest cost for each antigen; LPS is a thymus-independent antigen (Janeway et al. 2001) and typically elicits an acute phase response that peaks 24–48 h following injection (Owen-Ashley and Wingfield 2007), but which has carry-over effects (including change in beak coloration) that last for 4–5 days (e.g., Rosenthal et al. 2012; Sköld-Chiriac et al. 2014), whereas KLH is a thymus-dependent antigen (Janeway et al. 2001) that typically does not elicit an acute phase response but does result in a robust antibody response 7 days postinjection (Janeway et al. 2001). Blood samples were collected within 5 min of opening the cage door. Blood samples were spun down in a centrifuge at 5000 rpm for 7 min. Plasma was stored at -80°C until assay.

Antigen-reactive antibody ELISAs

Antigen-specific LPS and KLH antibody concentrations were quantified using ELISAs as described previously (Grindstaff et al. 2005; Grindstaff 2008; Merrill and Grindstaff 2014). Briefly, to quantify antigen-specific antibody levels, plates (Nunc Maxisorp) were coated with 0.01 g LPS (Sigma Aldrich cat # L7261) or KLH (Calbiochem cat #: 374805) in 200 ml sodium carbonate–sodium bicarbonate coating buffer (0.15 M, pH 9.6), yielding a concentration of 5 µg/ml. On day 2, plates were washed 3 times with PBS–Tween, blocked with 5% milk powder/PBS–Tween, and then washed twice with PBS–Tween before adding samples and standards. Plasma samples for LPS and KLH were diluted 1:40 in a 1% milk powder/PBS–Tween solution and plated in duplicate. Samples yielding coefficients of variation larger than 10% were

rerun. LPS and KLH calibration pools were created using 10 additional zebra finches from the colony and exposing them to the same antigen treatments as the experimental birds. The calibrator was serially diluted starting at 1:10 and extending to 1:10240. On day 3, plates were washed 3 times and labeled using goat anti-bird IgG horseradish peroxidase (HRP)-conjugated antibody (Bethyl Labs A140-110P) at a concentration of 1:1000. After incubation, plates were washed twice and HRP substrate buffer containing ABTS substrate buffer (Southern Biotech 0202-01), hydrogen peroxide (Fisher Chemical H325-100), and citrate buffer was added. Plates were read at 405 nm in an absorbance microplate reader (Biotek ELx808™) using Gen5 (Biotek), a microplate data collection and analysis software program after 30 min of incubation for LPS and 25 min for KLH. Antibody titers of samples were calculated based on reference to the serial dilution of the calibration pools. The average intra-assay and inter-assay coefficient of variances for the LPS-reactive antibody ELISAs were 8.97% and 12.56%, respectively. The average intra-assay and inter-assay coefficient of variance for the KLH-reactive antibody ELISAs were 7.38% and 7.79%, respectively.

Beak color development

To test the prediction that early-life antigen challenges would slow the development of beak color, we took a total of 12 photographs of the beak of each bird (3 of left side of beak on day 50, 3 of right side of beak on day 50, and again on day 65) under standardized indoor lighting conditions. Photographs were then imported into ImageJ (Abramoff et al. 2004) for analysis. First the beak was digitally outlined by hand on each photo to define the area to be measured. Then a macro was used to calculate the proportion of the beak in the visibly red-orange part of the spectrum. The proportion of the beak visibly in the red-orange part of the spectrum was determined by a set of threshold values for hue, saturation, and brightness (using the ImageJ default method for thresholding with hue ≤ 30 , saturation ≥ 150 , and brightness ≥ 160 on 8-bit color images). Finally, we averaged the proportion values for the left and right sides of the beak on days 50 and 65 for each bird. Calculated values for the proportion of the beak that was red were significantly repeatable on day 50 (mean squares $[MS]_A = 0.278$, $MS_W = 0.009$, $r = 0.83$, standard error $[SE] = 0.02$, $F_{125,623} = 29.93$, $P < 0.001$) and day 65 ($MS_A = 0.064$, $MS_W = 0.003$, $r = 0.77$, $SE = 0.03$, $F_{123,634} = 22.33$, $P < 0.001$).

Beak color assessment in adulthood

To test the prediction that early-life and adult antigen challenges would reduce the expression of adult beak coloration, we used a

Figure 1

Timeline of data collection. Timeline illustrates when different color measures and samples were collected from the zebra finches.

portable spectrophotometer (USB 4000, Ocean Optics, Dunedin, FL) with a deuterium-halogen light source (DH-2000-BAL, Ocean Optics) to measure reflectance between 320 and 700 nm on the left and right side of the upper mandible. Two researchers (L.M. and M.F.N.) collected all color measurements; the probe was always held by L.M., and the bird by M.F.N. Measurements were taken with the probe held at a 90° angle to the beak at a distance of 7.8 mm. Reflectance values were standardized through use of a white standard (WS-1-SL diffuse reflectance standard, Labsphere) and a dark standard (SpyderCube). We used CLRVars (CLR5 version [1.05], [Montgomery 2008](#)) to calculate B2 (brightness, intensity), S1R and S1G (saturation/chroma at high wavelengths), S1U (saturation/chroma at low wavelengths), and H3 and H4b (hue) ([Montgomery 2006](#)). We recorded 2 scores from each side of the upper mandible and averaged the 4 values to create 1 mean beak color value for each of the 6 color variables. We analyzed each aspect of color separately to retain higher resolution in examining the specific impacts of antigen exposure on different aspects of signaling ([Montgomery 2006](#); [Butler et al. 2011](#)). We included multiple metrics because our understanding of how carotenoid concentrations in different tissues (e.g., dead beak tissue, live tissue, blood) contribute to the overall coloration of the beak is still rudimentary, as is our understanding of how immune challenges impact different aspects of the signal. B2 is the mean reflectance from 320 to 700 nm and provides a standardized index of brightness ([Montgomery 2006](#)) in which lower scores correspond with darker red, more carotenoid-rich beaks (McGraw K, personal communication). We used 3 measures of saturation/chroma (S1R, S1G, and S1U) to capture variation at high and low wavelengths ([Montgomery 2006](#)), which is necessary for bimodal reflectance curves like those present in zebra finch beaks ([Bolund et al. 2007](#)), which have peaks at high (red/yellow) and low (UV) wavelengths. For our samples, S1R captures the reflectance from 605 to 700 nm and should provide information about variation in the red, carotenoid-rich end of spectrum. S1G is calculated from 510 to 605 nm and may provide more insight into the more orange/red components of the zebra finch beak. [Butler et al. \(2011\)](#) found S1G to be the only measure to exhibit a relationship with carotenoid levels in the outer, dead portion of the zebra finch beak. S1U (320–400 nm) should capture variation in the UV end of the spectrum, and due to the inverse relationship between reflectance in the UV and red/yellow wavelengths, lower S1U scores indirectly correspond with redder, more carotenoid-rich beaks. We used 2 measures of hue; H3 is the wavelength at which reflectance is halfway between its minimum and its maximum ([Pryke et al. 2001](#)), and [Montgomery \(2006\)](#) recommends H3 as a more reliable measure of hue compared to H1 or H2 because it is less likely to be biased by random fluctuations at high or low wavelengths, whereas work by [Butler et al. \(2011\)](#) suggests that H4b (the arctan of the reflectance for the 4 regions of the light spectrum) may be a better metric for zebra finch beak coloration, although this was assessed from dead beak tissue only. If the finches' beaks change from red to more orange in color in response to antigen challenge then, H4b may capture that variation more accurately than H3 (McGraw K, personal communication).

Color discernibility

We used the “coldist” function ([Vorobyev and Osorio 1998](#)) in the R (R Development Core Team 2013) package “pavo” to estimate the color distances between groups with statistically different beak coloration (based on reflectance data) ([Maia et al. 2013](#)). We used “tetra” for the visual system phenotype (applicable for most birds,

including zebra finches, which have tetrachromatic color vision), and a Weber fraction (inverse of the noise to signal ratio) of 0.5 to calculate chromatic contrast (ΔS —shape of the reflectance curve) and achromatic contrast (ΔL —overall percent reflectance) under standard daylight conditions (D65), and an ideal homogeneous background (default) ([Maia et al. 2013](#); [Jones and Siefferman 2014](#)). These distances are calculated in units of Just Noticeable Differences (JND), in which values over 1 are considered to be discernible to the birds ([Vorobyev and Osorio 1998](#); [Vorobyev et al. 1998](#); [Maia et al. 2013](#); [Jones and Siefferman 2014](#)).

Statistical analyses

For all analyses, we used an information-theoretic approach ([Burnham and Anderson 2002](#)) to determine the relative support for models. In our model set, we included the global model containing all variables of interest (see below) as well as candidate models containing variable combinations that were selected based upon a priori hypotheses. We ranked models using Akaike's information criterion corrected for small sample sizes (AICc) and calculated model weights for each model (w_i). We considered models within 2 $\Delta AICc$ of the lowest ranked model as competitive and evaluated their w_i to assess the strength of evidence for each competing model. We also evaluated competitive models (i.e., within 2 $\Delta AICc$ of the lowest ranked model) for “pretending variables,” which are variables that do not contribute to a better fit ([Anderson 2008](#)), by examining model deviance and the inclusion of 0 in the 95% confidence limits for the parameters in those models. Any parameters that included 0 in the 95% confidence intervals (CIs) were rejected as pretending variables ([Anderson 2008](#)), and we report model-averaged parameter estimates for all retained variables. In all model comparisons, we also included an intercept-only model to ensure that we were not selecting models that performed more poorly than the null model. For models in which the intercept-only model was the highest ranked model, we assumed no effect of fixed effects on the dependent variable.

To determine if early-life exposure to antigen impacted development of beak coloration at day 50, we constructed a general linear mixed model (GLMM) with maternal ID as a random effect, maternal treatment, young treatment, sex, and the interaction effects between selected variables as fixed effects ([Supplementary Appendix I](#)). To assess the rate of development, we ran a repeated measures mixed model with maternal ID as a random effect, bird ID as the subject, maternal treatment, young treatment, sex, age (day 50 or 65), and the interaction effects between selected variables ([Supplementary Appendix II](#)) as fixed effects.

For adult beak color expression, we separated analyses into the following categories: pre-LPS exposure beak color, change in beak color after LPS challenge (pre–post), pre-KLH exposure beak color, change in beak color after KLH challenge (pre–post). For the pre-antigen exposure data, we ran GLMMs for each tristimulus color score (B2, S1R, S1G, S1U, H3, H4b), with maternal ID as a random effect, maternal treatment, young treatment, and the interaction effect between them as fixed effects ([Supplementary Appendix III](#)). For the analyses examining change in beak coloration, we ran repeated measures mixed models and broke analyses into 2 distinct groups: one focused on the effect of early-life treatments to determine if pre- or postnatal antigen challenges impacted the magnitude of color change following adult antigen challenge, and one incorporating antigen-specific antibody levels to determine if changes in beak color were linked to the magnitude of the immune response. Models examining change in color

in response to early-life treatment included maternal ID as a random effect, bird ID as the subject, and maternal treatment, young treatment, day (pre or post), and the interaction effects between selected variables as fixed effects (Supplementary Appendix IV). Models examining the change in color in relation to antibody levels included maternal ID as a random effect, bird ID as the subject, and maternal treatment, young treatment, date, change in antigen-specific antibody levels, and the interaction effects between selected variables as fixed effects (Supplementary Appendix V). To determine if adult challenge resulted in an antigen-specific Ab response, we ran repeated measures mixed models as described above with maternal ID as a random effect, bird ID as the subject, maternal treatment, young treatment, day (pre or post), and the interaction effects between selected variables as fixed effects, and anti-LPS Ab levels or anti-KLH Ab levels as dependent variables (Supplementary Appendix VI).

We did not have sufficient numbers of females in all treatment groups for the adult beak coloration data ($n = 2$ for females with Maternal Treatment = KLH; $n = 2$ for females with Young Treatment = LPS), therefore, analyses were run on males only. In addition, for adult color analyses, we consolidated treatments by antigen exposure. For pre- and post-antigen analyses, we simplified analyses by grouping maternal treatment based on exposure to the focal antigen: “LPS: Yes or No” and “KLH: Yes or No” and did the same for young treatment. Two extreme outliers (>3 interquartile range; Tukey 1977) were removed from analyses; one individual following adult LPS challenge (this was most likely a result of measurement error of beak coloration, *sensu* Montgomerie 2006), and the other prior to adult KLH challenge. This individual exhibited a significantly duller beak than all other birds and died shortly after the adult challenges.

For all models, we used maximum likelihood, and for all GLMMs, the denominator degrees of freedom were approximated using the containment method (Littell et al. 2006). For all repeated measures models, the denominator degrees of freedom were approximated using the Kenward-Rogers method (Littell et al. 2006). All variables were checked for normality of residuals and homogeneity of variance prior to analyses. All antibody data were log-transformed prior to analysis, and all analyses were run in SAS 9.4 (Cary, NC).

RESULTS

Development of beak coloration

The strongest model for beak coloration at day 50 was “young treatment” alone and had more than twice the support of the next best model ($w_i = 0.583$ vs. 0.237 for young treatment + sex; Supplementary Appendix I). Young exposed to KLH had the lowest levels of red coloration, and control offspring had the highest, with LPS-treated offspring possessing intermediate levels (Figure 2). Of the 3 treatments (including control), only KLH had CIs that did not overlap 0 (estimate: -0.118 ; lower CI: -0.196 , upper CI: -0.04), suggesting that KLH treatment had the largest effect on beak color development. The CIs for sex overlapped 0, indicating that the sexes did not differ in proportion of the beak that was red on day 50.

For the change in beak coloration from day 50 to day 65, the best model was that which incorporated the interaction between young treatment and day (Supplementary Appendix II). This model had more than twice the support of 3 competing models ($w_i = 0.327$ vs. 0.14 and 0.133) all of which contained “day” as an effect, as well as additional effects determined to be pretending variables. The proportion of red coloration in the beak increased for all birds, but the magnitude of this change differed by treatment (Figure 2), because baseline levels varied by treatment. At day 65, all birds were at approximately the same level of beak redness ($\sim 85\%$), whereas the beaks of KLH-treated offspring were an average of 28% red on day 50, compared to 36% and 39% for LPS and control offspring, respectively (Figure 2).

Adult antibody response

LPS challenge did not result in a change in anti-LPS Ab levels from pre- to postchallenge because sampling date did not contribute significantly to model fit (Supplementary Appendix VIA). KLH challenge did result in an increase in anti-KLH Ab levels from pre- to post-KLH challenge (Supplementary Appendix VIB; Figure 3). Young and maternal treatments were pretending variables and models 2–4 were removed. “Young treatment*day” was the only candidate model remaining (Supplementary Appendix VIB). Baseline levels of anti-KLH Ab did not differ between birds exposed to KLH as nestlings and those that were not (KLH:

Figure 2

Average proportion ($\pm$ SE) of an individual bird's beak that was red on days 50 and 65 posthatch grouped by young treatment. Birds were challenged with KLH ($n = 36$), LPS ($n = 43$), or PBS (control; $n = 47$) on days 5 and 28 posthatch. KLH-treated birds had beaks with less red coloration on day 50 than the other 2 groups, but all 3 treatments were similar on day 65, at which point an average of approximately 85% of a bird's beak was red. Data here include both male and female zebra finches.

Figure 3

Change in KLH-specific Ab from pre- to post-KLH challenge during adulthood by young treatment. Birds were grouped by whether they were challenged with KLH or not on days 5 and 28 posthatch. Adult KLH challenge occurred at approximately 3 years of age, and postchallenge measures were assessed 8 days after KLH exposure. There was no difference in anti-KLH Ab levels prechallenge, but males that were exposed to KLH as nestlings ($n = 7$) mounted a more robust Ab response than males that were not exposed to KLH as nestlings ($n = 31$).

estimate: -0.891 ; lower CI: -1.251 ; upper CI: -0.531 ; No KLH: estimate: -0.887 ; lower CI: -1.331 ; upper CI: -0.442), but birds exposed to KLH as nestlings mounted a stronger Ab response than birds not exposed to KLH as nestlings (change from pre- to post-KLH: KLH: estimate: -0.891 , SE ± 0.179 ; No KLH: estimate: -0.467 , SE ± 0.086) (Figure 3).

Adult beak coloration

Baseline adult beak coloration was not influenced by early-life exposure to LPS; the intercept-only model ranked highest for B2, S1G, S1U, and H4b and was second ranked for S1R and H3, in which the intercept-only models were within $0.2 \Delta\text{AICc}$ and had similar weights (Supplementary Appendix IIIA). However, adult baseline beak color was influenced by exposure to KLH as nestlings. For both B2 and S1R, young treatment alone was the best explanatory variable (Supplementary Appendix IIIB), such that birds exposed to KLH as nestlings had less red beaks as adults than birds not exposed to KLH as nestlings (Figure 4). Young treatment was the highest ranked model for S1U but did not significantly outperform the next 2 models, including the intercept-only model ($\Delta\text{AICc} = 0.8$). In addition, estimates for young treatment, maternal treatment, and their interaction had CIs that overlapped 0 (young treatment: estimate: 0.008 ; lower CI: -0.018 ; upper CI: 0.034 ; maternal treatment: estimate: -0.013 ; lower CI: -0.044 ; upper CI: 0.019 ; mat trt*young trt: estimate: 0.018 ; lower CI: -0.016 ; upper CI: 0.051). Young treatment was also the top-ranked model for H4b, but the estimate for H4b had CIs that overlapped 0 (estimate: 0.009 ; lower CI: -0.015 ; upper CI: 0.033). The intercept-only model was the highest ranked model for H3 (Supplementary Appendix IIIB).

LPS exposure in adulthood did not result in changes to any aspect of beak coloration (Supplementary Appendices IVA and VA). In all instances, the null, intercept-only model either provided the best fit or other models did not provide a significantly better fit.

Adult exposure to KLH resulted in changes to B2, S1R, S1U, and H4b from pre- to postchallenge (Supplementary Appendix IVB), such that beaks became less red over the 8 days following challenge. In the models examining the effects of early-life exposure to KLH, the highest ranked models for B2, S1R, S1U, and

H4b were young treatment and date. B2, S1R, S1U, and H4b changed at a similar rate for birds exposed to KLH as nestlings and those not exposed to KLH as nestlings (Figure 4). Among models examining the relationship between change in anti-KLH Ab levels and change in color, we found that the interaction between young treatment and change in Ab levels was the best ranked model for B2, S1R, and S1U, while the intercept-only model was highest ranked for S1G, H3, and H4b (Supplementary Appendix VB). For B2 and S1R, the second ranked model was change in Ab plus the interaction between maternal treatment and change in Ab, which differed from the top model by 1.2 and 1.7 AICc , respectively. CIs for change in anti-KLH Ab and the interaction between maternal treatment and change in anti-KLH Ab overlapped 0 in both model sets, suggesting that these variables were not responsible for the variation observed. Among birds not exposed to KLH as nestlings, KLH-specific antibody production was related to change in the color metrics B2, S1R, and S1U (B2: estimate: -0.008 ; lower CI: -0.016 ; upper CI: -0.001 ; S1R: estimate: 0.021 ; lower CI: 0.006 ; upper CI: 0.035 ; S1U: estimate: -0.005 ; lower CI: -0.011 ; upper CI: 0.001), in which birds that mounted weaker Ab responses lost more red color from their beaks than birds that mounted a more robust Ab response (Figure 5). CIs overlap 0 for S1U, but the trend is the same as for B2 and S1R, and the overlap is minimal. There were no relationships between the change in Ab levels and change in beak color among birds exposed to KLH as nestlings (B2: estimate: 0.003 ; lower CI: -0.005 ; upper CI: 0.011 ; S1R: estimate: -0.010 ; lower CI: -0.027 ; upper CI: 0.006 ; S1U: estimate: 0.006 ; lower CI: -0.001 ; upper CI: 0.013), although sample sizes were limited ($n = 7$).

Color discernibility

Using the avian vision models to compare the baseline beak coloration between males treated with KLH as nestlings and those that were not, we found a nonsignificant difference (i.e., JND values < 1.0) for chromatic contrast (ΔS : JND = 0.876), and a significant difference for achromatic contrast (ΔL : JND = 2.827). We also compared all males from pre-KLH to post-KLH and calculated JND values of 2.021 for ΔS , and 4.447 for ΔL . All comparisons with JND values > 1 should be detectable by the birds. Changing background light to ideal made no quantitative or qualitative difference. We did not examine color distance for birds treated with LPS as nestlings or adults treated with LPS due to the lack of statistical changes in beak coloration for these birds.

DISCUSSION

Early-life immune challenge can impact a wide range of physiological (e.g., Lemke and Lange 1999; Grindstaff et al. 2006; Karrow 2006; Pihlaja et al. 2006; Merrill and Grindstaff 2014), cognitive (e.g., Bilbo and Schwarz 2009; Meyer et al. 2009; Grindstaff et al. 2012), and morphometric parameters (Klasing and Leschinsky 1999; Grindstaff 2008; Butler and McGraw 2012a); however, relatively few studies have examined both short- and long-term effects of early-life challenges due to logistical constraints, and fewer still have investigated the relative role of maternal challenge compared to early postnatal challenge. In this study, we examined the impact of prenatal and early postnatal immune challenges on beak coloration in zebra finches and found that exposure to KLH as a nestling can result in both short-term and long-term reductions in beak color expression. We also found that exposure to KLH as an

Figure 4

Change in beak coloration from pre- to post-KLH adult challenge for B2, S1R, S1U, and H4b grouped by young treatment (KLH or no KLH). Beaks of males exposed to KLH as nestlings ($n = 9$) were less red than the beaks of males not exposed to KLH as nestlings ($n = 34$) both prior to, and following, adult challenge with KLH. Males from both treatments became duller on average from pre- to postchallenge, and the magnitude of change was similar between the treatments. Dark arrows indicate the direction in which values correspond to redder beaks; for example, smaller values for brightness are associated with redder, more carotenoid-rich beaks, whereas higher values for saturation at high wavelengths are associated with redder beaks. Bars represent mean $\pm$ SE for the young treatments.

Figure 5

Relationship between change in beak coloration and change in anti-KLH antibody (Ab) levels following adult KLH challenge among males not exposed to KLH as nestlings ($n = 30$). Males that mounted more robust Ab responses lost less red coloration from their beaks than males that mounted weaker Ab responses (dark arrows denote direction in which values are associated with redder color).

adult elicits the production of anti-KLH Abs, and that individuals exposed to KLH as nestlings mounted a stronger Ab response than birds not exposed to KLH as nestlings. This result indicates that birds exposed to KLH as nestlings retained immunological memory of the antigen (i.e., memory T cells), enabling them to mount a stronger antigen-specific response 3 years later. To our knowledge, this is the longest demonstrated example of immunological memory established during development for a passerine bird.

For most passerine birds, the critical period of development appears to be truncated into the span of a few weeks, during which they experience rapid morphological, neurological, and physiological changes as they transition from egg to subadult. Perturbations to normal development may impact a variety of parameters (see Monaghan 2008, 2014 for discussion of maladaptive and potentially adaptive consequences of early-life stressors), including sexual signals (Spencer et al. 2003; Alonso-Alvarez et al. 2004; Butler and McGraw 2011, 2012a; but see also Butler and McGraw 2012b). On day 50 posthatch, birds exposed to KLH as nestlings were developmentally delayed compared to birds that had not received KLH, with respect to percent of the beak that had changed from black to red (Table 1). This is a more generalized assessment of

beak color expression than examining the tristimulus color parameters with a spectrophotometer, but it represents a functional measure of signal development. In the period between day 50 and day 65, however, birds challenged with KLH as nestlings caught up with the birds not challenged with KLH and exhibited comparable levels of beak redness on day 65 (Figure 2). It is unclear whether the KLH-treated birds exhibited greater rates of carotenoid deposition during this period in order to “catch-up” to the non-KLH birds (*sensu* Lindström et al. 2005), or whether the non-KLH birds naturally slowed or stopped carotenoid deposition at some point during this 15-day period as part of the natural cycle of beak coloration. The fact that on average, the beaks of non-KLH treated birds were still below 100% red (approximately 85%) on day 65 suggests that birds were still in the process of depositing carotenoids into the beak, and that KLH-treated birds had exhibited some compensatory development during the 15-day period. We did not collect data on the tristimulus color values, so we cannot say whether the beaks were in fact equivalent at day 65 in aspects of brightness, saturation, and hue. There is evidence that carotenoid deposition can be affected by circulating hormones such as testosterone (Ardia et al. 2010) and corticosterone (McGraw et al.

Table 1
Summary of key findings

Age category	Dependent variable	Independent variables	Effect
Developmental	Beak color 50 DPH Δ Beak color (50–65 DPH)	Yng Trt Day $\times$ Yng Trt	Yng Trt = KLH birds had reduced % beak redness All beaks got redder, rate of increase higher for Yng Trt = KLH
Adulthood	Baseline adult Ab (LPS)	None	None
	Δ Adult Ab (LPS)	None	None
	Baseline adult Ab (KLH)	None	None
	Δ Adult Ab (KLH)	Day $\times$ Yng Trt	KLH Ab levels increased from pre- to post-adult KLH challenge, rate of increase was greater for Yng Trt = KLH birds
	Baseline adult beak color	Yng Trt	B2, S1R, ΔL affected by Yng Trt = KLH; B2 and S1R values indicated less red color in the beaks for Yng Trt = KLH, and there was significant difference in beak color space (ΔL) for those birds compared to Yng Trt $\neq$ KLH
	Δ Adult beak color pre- to post-LPS	None	None
	Δ Adult beak color pre- to post-KLH	Day, Yng Trt $\times$ Δ Adult Ab (KLH)	B2, S1R, S1U, H4b, ΔL , ΔS all changed from pre- to post-KLH challenge (becoming less red); $\Delta B2$ and $\Delta S1R$ were inversely related to ΔKLH Ab

Major findings for each dependent variable of interest. During development, the percent of an individual zebra finch's beak that was red was assessed at 50 days posthatch (DPH) and again at 65 DPH. Young treatment (Yng Trt) was KLH, LPS, or control. The change in percentage red (Δ Beak color) was assessed from 50–65 DPH. When birds were at least 3 years old, they were challenged with LPS and 3 weeks or more later, with KLH, and both beak coloration and antibody (Ab) levels were assessed prior to (baseline) and following (4 days for LPS; 8 for KLH) each immune challenge. For measures of adult beak coloration, we used tristimulus color parameters and avian color vision model metrics: B2 is a measure of brightness, S1R is a measure of saturation or chroma at high wavelengths, S1U is saturation at low wavelengths, H4b is hue, ΔL is achromatic contrast, and ΔS is chromatic contrast.

2011), and in prior work with these birds, we found an effect of early-life antigen treatment on the production of corticosterone (CORT) during adulthood (Merrill and Grindstaff 2015). The differences in rate of beak color development may have been linked to short-term changes in hormone production such as CORT, although this idea should be explicitly tested.

Beyond short-term effects, immune challenges during development may permanently shape an organism's phenotype (Monaghan 2008), including the expression of secondary sexual traits (Nowicki et al. 1998; Naguib and Nemitz 2007; Butler and McGraw 2011; Schmidt et al. 2014). Here, we found that challenge with KLH during early life led to duller beaks in adult male zebra finches (Table 1). This result suggests that beak color is an honest signal and expression can be used by females and other males to gauge a male's developmental condition (*sensu* Spencer and MacDougall-Shackleton 2011). The long-term impact of KLH exposure during early life suggests that the underlying physiology responsible for beak color expression is permanently altered. Beak coloration in zebra finches is ketocarotenoid based (Hill 2002; McGraw and Toomey 2010), which means production of the color depends not just upon carotenoid acquisition and assimilation, but also upon the metabolic transformation of dietary carotenoids. These birds were housed in indoor cages with ad libitum access to food, which suggests that access to dietary carotenoids was not likely to have been limited. Beak coloration, therefore, may represent an honest signal of metabolic efficiency (*sensu* von Schantz et al. 1999; Blount 2004). If so, this signal might provide information about many aspects of a male's condition, including resistance to parasites, capacity for dealing with stressors, and ability to acquire and process resources (*sensu* Hill 2011, 2014).

Adult KLH challenge also resulted in a reduction in beak color expression from pre- to post-antigen challenge (Table 1). Moreover, we documented a relationship between anti-KLH Ab production and the change in beak coloration among birds not exposed to KLH as nestlings, in which birds that mounted

a stronger Ab response exhibited a less pronounced decrease in carotenoid-dependent expression (B2, S1R) than birds that mounted a weaker Ab response. This result suggests that when immunological memory is removed from the equation, the ability to mount an Ab response is positively correlated with the ability to maintain red beak coloration in zebra finches. The relationship between these processes may depend upon the rates of carotenoid ketolation (Ge et al. 2015), rates of Ab production, and an interaction with circulating hormones. If CORT can lead to a reduction in beak coloration (*sensu* McGraw et al. 2011), high-quality males may have the ability to buffer against increases in circulating CORT.

Butler and McGraw (2012b) found that baseline bill saturation in mallards (*Anas platyrhynchos*) was positively correlated with their cutaneous immune response, although they failed to find a relationship between Ab levels in response to KLH challenge and aspects of bill color or circulating carotenoid levels. Faivre et al. (2003) demonstrated that blackbirds (*Turdus merula*) challenged with sheep red blood cells (SRBCs) exhibited a rapid decline in yellow coloration, but that, in contrast to our findings, the likelihood of becoming duller was positively linked to the strength of anti-SRBC response. The yellow coloration of the blackbird and mallard beaks is dependent upon carotenes and zeaxanthins (blackbird: Faivre et al. 2003), and xanthophylls and zeaxanthins (mallards (Butler and McGraw 2012b), and is not dependent upon ketocarotenoids as is beak coloration in zebra finches. The yellow beak may represent more basic components of resource acquisition (Hill 2002), rather than processes related to metabolic efficiency (Hill 2014; Ge et al. 2015).

We found that the differences we detected should be discernible to the birds themselves (Vorobyev and Osorio 1998; Vorobyev et al. 1998). Determining whether the focal species is capable of distinguishing the differences that are detectable statistically is an important, but often neglected, component of examining variation in signal expression, and here we provide one of the first examples

of an experimental manipulation resulting in statistically and discernibly different signals.

We failed to detect an effect of maternal treatment on color expression or Ab production in this study. This finding suggests that postnatal experience may have a more profound impact on the phenotype over the long term. Our prior work indicated that KLH challenge had a greater impact on short- and long-term physiology than LPS challenge, and our current work corroborates those findings. We found no change in beak color from pre- to post-LPS challenge, which differs from the results of Rosenthal et al. (2012), in which challenge with LPS resulted in a decrease in beak luminance (brightness) and hue in American goldfinches beginning within 72 h of LPS injection and lasting for the duration of the 6-day study. Wild-caught birds were used in that study and all birds exhibited a reduction in beak luminance and hue within the first 2 days of capture, although the magnitude of decrease was greater for LPS-treated birds compared to controls. We also did not document a detectable change in anti-LPS Ab from pre- to postchallenge, suggesting that the adult LPS treatment did not elicit a robust response, at least over the 4-day period between challenge and sample collection. All birds were exposed to LPS in adulthood as part of a different study a minimum of 1 month prior to the adult antigen challenges for this work. In addition, LPS is found in the cell walls of gram-negative bacteria, including ubiquitous strains of *Escherichia coli*, and *Salmonella*, both of which may exist in captive zebra finch colonies. The combination of adult exposure and potential long-term environmental exposure could have negated any differences among treatment groups, or from pre- to post-adult challenge, although the relatively high dosage in the adult challenge should have elicited a response.

The question of what metrics to use when examining zebra finch beak coloration is still under debate, but our data suggest that B2, S1R, S1U, and H4b reflect changes in zebra finch beak color in response to immune challenge, and that the finches can discern these differences. B2 is a broadly used metric to assess average brightness across the range of reflectance values, and our results support the use of that parameter for zebra finch beaks. Zebra finch beaks exhibit reflectance peaks at both high wavelengths (red/yellow end of the spectrum) and low wavelengths (UV end) (Bolund et al. 2007), and we used different measures of saturation/chroma to capture variation at both peaks. S1R and S1G capture different aspects of the high end of the spectrum (S1R should capture truer red color, whereas S1G should capture more orange coloration). The documented differences in adult baseline S1R between young treatments and from pre- to post-adult KLH challenge indicate that immune challenges may predominantly impact the red coloration of the beak, rather than more orange/yellow coloration. H3 did not capture any variation in beak coloration related to early-life or adult experience, whereas H4b changed from pre- to post-adult KLH challenge. These results differ from Butler et al. (2011), who failed to find a relationship between the carotenoid content of the zebra finch beak and any tristimulus color metric (although S1G was close to significant). This discrepancy may be due to the fact that carotenoids were measured in the dead outer tissue of the beak for that study, but that beak coloration is likely also due to carotenoid levels in the living tissue and in the blood (*sensu* Butler et al. 2011).

Our study highlights important questions that remain regarding sexual signals. For example, how does the receiver discriminate between an individual with a poor developmental history, but no current infections, and an individual with a good developmental

history but that is currently fighting an infection? Is one more important than the other? Perhaps it does not matter to the receiver, and either one may indicate a lower quality individual. The greater magnitude of variation from pre- to post-KLH challenge in adulthood compared to the differences between males that were exposed to KLH as nestlings and those that were not suggests that current condition may play a larger role in beak signal expression, although mate choice experiments could help shed light on these questions. Dynamic signals such as beak coloration may be broad signals that provide females (and potentially competing males) with immediate information about both developmental history and current condition. Other components of a male's display may provide additional information about aspects of developmental condition (e.g., song repertoire size [Nowicki et al. 1998]) and current condition (e.g., song rate [Buchanan et al. 1999]). Our data provide compelling evidence documenting the dual nature of beak color in zebra finches, in which both early-life experience and current condition play important roles in signal expression.

SUPPLEMENTARY MATERIAL

Supplementary material can be found at <http://www.behco.oxfordjournals.org/>

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