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RESEARCH ARTICLE

Testing the resource trade-off hypothesis for carotenoid-based signal honesty using genetic variants of the domestic canary

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ABSTRACT

Carotenoid-based coloration in birds is widely considered an honest signal of individual condition, but the mechanisms responsible for condition dependency in such ornaments remain debated. Currently, the most common explanation for how carotenoid coloration serves as a reliable signal of condition is the resource trade-off hypothesis, which proposes that use of carotenoids for ornaments reduces their availability for use by the immune system or for protection from oxidative damage. However, two main assumptions of the hypothesis remain in question: whether carotenoids boost the performance of internal processes such as immune and antioxidant defenses, and whether allocating carotenoids to ornaments imposes a trade-off with such benefits. In this study, we tested these two fundamental assumptions using types of domestic canary (*Serinus canaria domestica*) that enable experiments in which carotenoid availability and allocation can be tightly controlled. Specifically, we assessed metrics of immune and antioxidant performance in three genetic variants of the color-bred canary that differ only in carotenoid phenotype: ornamented, carotenoid-rich yellow canaries; unornamented, carotenoid-rich 'white dominant' canaries; and unornamented, carotenoid-deficient 'white recessive' canaries. The resource trade-off hypothesis predicts that carotenoid-rich individuals should outperform carotenoid-deficient individuals and that birds that allocate carotenoids to feathers should pay a cost in the form of reduced immune function or greater oxidative stress compared with unornamented birds. We found no evidence to support either prediction; all three canary types performed equally across measures. We suggest that testing alternative mechanisms for the honesty of carotenoid-based coloration should be a key focus of future studies of carotenoid-based signaling in birds.

KEY WORDS: Condition-dependent trait, Immunocompetence, Antioxidant, Ornament

INTRODUCTION

An outstanding challenge in behavioral and evolutionary ecology is understanding how ornamental traits can serve as honest signals of individual condition (Higham, 2014). Numerous studies have now documented that a range of ornamental traits are positively associated with aspects of individual health and vitality (Hill, 2014), but the mechanism by which honest signals resist cheating remains contentious (Hill, 2011; Weaver et al., 2017).

One of the most commonly studied classes of ornamentation in animals is carotenoid-based coloration, which includes most of the red, orange and yellow coloration of birds. Many studies have presented evidence that carotenoid-based coloration serves as a reliable signal of condition (reviewed in Svensson and Wong, 2011). The resource trade-off hypothesis, which is currently the most widely accepted hypothesis for how carotenoid-based signals of condition remain honest, hinges on the assumptions that: (1) internal carotenoid pigments provide benefits to immune and/or antioxidant defenses within the body, and (2) animals are limited in the quantity of carotenoids physiologically available (Koch and Hill, 2018). Using this idea, only the highest quality individuals can afford to allocate carotenoids to ornaments (Fig. 1, top).

The resource trade-off hypothesis offers an intuitive explanation for why sick, weak or otherwise low-quality individuals may be constrained from producing high-quality, richly colored carotenoid signals. However, tests of the two central assumptions of the hypothesis – that carotenoids offer physiological benefits, and that they are limited in internal availability – have yielded inconsistent evidence (reviewed in Koch and Hill, 2018). The first assumption has faced particularly strong criticism: while carotenoids are potent antioxidants under a wide range of conditions *in vitro*, it remains uncertain whether they play a significant role in antioxidant defenses *in vivo* in vertebrates (Hartley and Kennedy, 2004; Costantini and Møller, 2008; Pérez-Rodríguez, 2009; Koch et al., 2018). Moreover, while carotenoids are often described as beneficial to immune system function, evidence that carotenoids actually play a positive role in immune defense remains scant and is generally restricted to a handful of studies in mammalian systems (Chew and Park, 2004; Koch and Hill, 2018; Svensson and Wong, 2011). Likewise, there is little empirical evidence to support the assumption that carotenoids are limiting in the diets of wild animals. Much of the evidence for access to carotenoids affecting ornamentation comes from studies of animals supplemented with large doses of carotenoids. These methods have their own complications (Koch et al., 2016a) and while supplementation sometimes appears to alleviate an apparent resource limitation (McGraw et al., 2011), other studies have found no such effects (Navara and Hill, 2003).

A key challenge to studies of the resource trade-off hypothesis is that while researchers can manipulate the size of internal carotenoid resource pools through diet or the potential physiological need for carotenoids through immune or oxidative challenges, it has not been possible to directly manipulate the availability or the allocation of carotenoids within the bodies of animals. Accordingly, there is a clear need for tests in which the costs and benefits of carotenoid allocation

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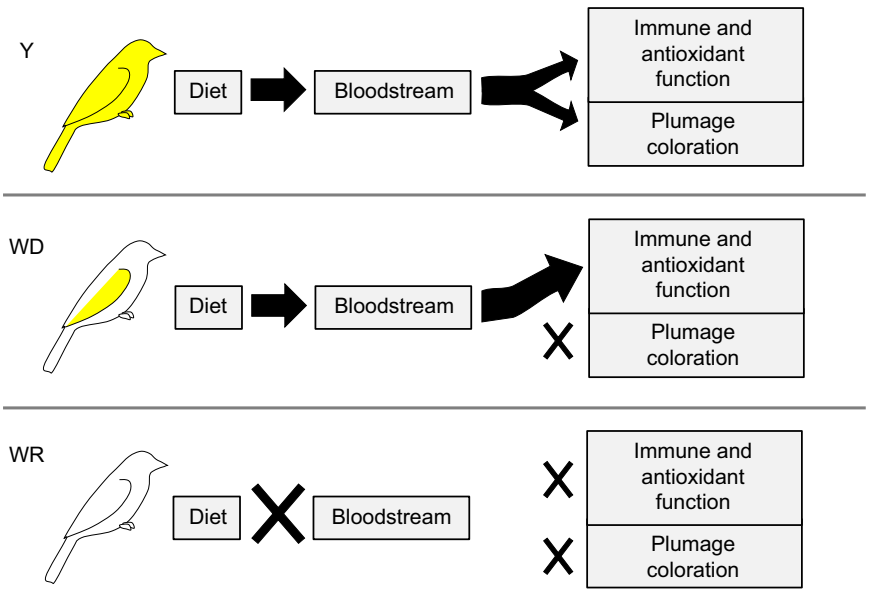

Fig. 1. Hypothetical allocation of carotenoids from diet to tissue to physiological process or coloration. The carotenoid resource trade-off hypothesis proposes that carotenoid pigments absorbed from the diet into the bloodstream must be differentially allocated to either boosting internal processes or coloring ornaments. White dominant (WD) canaries [shown here with yellow wings to distinguish them from white recessive (WR) canaries] do not allocate carotenoids to plumage coloration and therefore can, in theory, allocate all ingested carotenoids to boosting function. WR canaries cannot absorb carotenoids from their diet, so they have no carotenoids to allocate to either process. If we assume that carotenoids are beneficial to internal processes and that depositing them in coloration imposes an allocation trade-off, then immune and antioxidant function will be predicted by the size of the carotenoid allocation arrow pointing to it (or lack thereof): ornament-free WD canaries will outperform ornamented yellow (Y) canaries and carotenoid-rich Y canaries will, in turn, outperform carotenoid-deficient WR canaries.

can be assessed more directly with fewer confounding mechanisms for observed effects. Different types of the domestic canary (*Serinus canaria domestica*) with mutations affecting key carotenoid-related pathways provide an opportunity for such direct tests.

In this study, we consider three types of canaries with different levels of carotenoid availability and usage. First, the standard yellow lipochrome canary (Y) has feathers that are bright yellow due to pigmentation with carotenoids that are absorbed from the diet, converted into different forms and deposited in growing feathers (Koch et al., 2016b). White recessive (WR) canaries have a mutation that almost completely eliminates their ability to absorb carotenoids from their diet (Toomey et al., 2017). Because vertebrates cannot synthesize carotenoid pigments *de novo*, this mutation results in extreme carotenoid deficiency as well as white plumage (Wolf et al., 2000). Lastly, white dominant (WD) canaries have a mutation that prevents the deposition of carotenoid pigments into feathers during molt. While this mutation has not yet been traced to a specific gene, its phenotypic effects are well known: WD canaries absorb carotenoids and circulate them at the same levels as yellow canaries, but they allocate no carotenoids to ornamentation and have white plumage (Fig. 2). This canary system therefore presents three levels of carotenoid usage: ornamented Y canaries absorb and potentially allocate carotenoids to both feathers and physiological needs; WD canaries absorb and circulate carotenoids but do not allocate them to ornamental feather coloration, potentially leaving more carotenoids for physiological needs; and WR canaries absorb essentially no carotenoids and thus have no carotenoids to allocate to either physiological needs or ornamentation (Fig. 1). By comparing birds with and without internal carotenoid resources (WR versus WD), we can test for the physiological benefit of carotenoids (assumption 1), and by comparing birds with and without carotenoid-based ornamentation (WD versus Y), we can test for a physiological cost to 'spending' carotenoids (assumption 2) as colorants during molt (the period of ornamental carotenoid deposition). Koch et al. (2018) previously tested assumption 1 in WR and Y canaries; here, we take advantage of the WD system to perform a test of assumption 2, that there is a cost of carotenoid allocation.

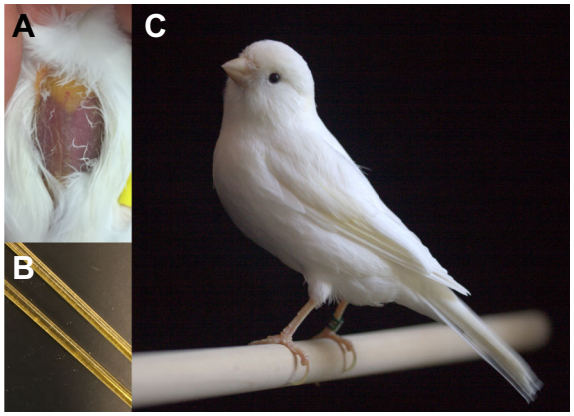

Fig. 2. The white dominant canary possesses internal carotenoids but does not deposit them to color its feathers. White dominant canaries feature yellow subcutaneous fat (A), yellow plasma (pictured in capillary tubes) (B) and white plumage (C).

We compared the performance of WR, WD and Y canaries on several measures of immunocompetence and antioxidant capacity. Based on the framework of the resource trade-off hypothesis, we predicted that during molt, carotenoid-rich, ornament-free WD birds should outperform both carotenoid-deficient WR birds and carotenoid-spending Y birds; however, outside of molt, carotenoid rich WD and Y birds should perform equally well, compared to carotenoid-deficient WR birds.

MATERIALS AND METHODS

Study system and husbandry

We performed our study using a research colony of after-hatch-year canaries [*Serinus canaria domestica* (Linnaeus, 1758)] held at the Auburn University Avian Research Laboratory 1 in Auburn, AL. All procedures were approved by the Auburn University Animal Care and Use Committee (PRNs 2014-2465, 2014-2499, 2015-2724 and 2015-2789). We performed several experimental procedures and analyses from January through August 2016, as described in Fig. 3.

The three canary color types that we studied, WR, WD and Y, are all of the same breed ('color-bred' canaries) and differ only in their carotenoid phenotype. The WD and WR phenotypes are the product of Mendelian dominant or recessive alleles, respectively. While the mutation responsible for the WR phenotype has been isolated to *SCARB1*, which functions in carotenoid absorption (Toomey et al.,

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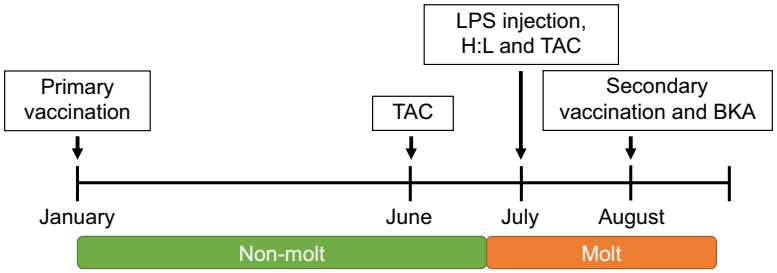

Fig. 3. Timeline of experiments. The molt period of the canaries lasts about two months, from late June to late August. We performed physiological tests of WD, Y and WR canaries during both the molt and non-molt periods in 2016. The exact start date of molt measurements varied according to the molt stage of individual birds, and subsequent measures were staggered to allow adequate time between blood sampling. TAC, total antioxidant capacity; LPS, bacterial lipopolysaccharide; H:L, heterophil to lymphocyte ratio; BKA, bacterial killing assay.

2017), the mutation in WD canaries has not yet been identified. However, decades of observation and careful breeding by aviculturists as well as our own observations and carotenoid analyses demonstrate clearly that WD birds differ from Y birds only in their lack of ornamental carotenoid deposition. Throughout the study, we held all canaries on a carotenoid-controlled diet of mixed canary seed (All Natural Canary Blend, Jones Seed Company; Lawton, OK, USA) coated with a carotenoid-free vitamin powder (AviVita Plus, Avitech Bird Supplies; Frazier Park, CA, USA), which provides adequate dietary carotenoids for yellow birds to fully color their feathers (Koch et al., 2018). The vitamin supplement prevented any symptoms of retinol deficiency in the WR canaries, which cannot absorb retinoid precursor carotenoids from their diet (Wolf et al., 2000).

Carotenoid analyses

Prior to the commencement of the main experiments, we performed carotenoid content analyses on plasma samples taken outside of molt from four WR, four WD, and four Y canaries in our colony; these samples were stored for less than 6 months at −80°C prior to analysis. We also collected skin and feather samples from four birds of each color type that died prior to experimentation; these samples were stored for less than 12 months at −80°C prior to analysis. The carotenoid content of all samples was analyzed using high performance liquid chromatography according to the methods described in Toomey et al. (2017) and Koch et al. (2018).

Vaccination and antibody response

Because there is little conclusive evidence to suggest a direct role of carotenoids in any one immune mechanism in birds (Koch and Hill, 2018; Svensson and Wong, 2011), we selected broad measures of immune system function that inform on multiple aspects of immunocompetence and that have biological relevance to immune defense without causing lasting harm to the birds. First, we tested the canaries' ability to mount an adaptive immune response both after first exposure (primary response) and later exposure (secondary response) to an antigen that stimulates antibody production (through vaccination). Antibody production in a primary response represents the ability of the innate immune system to recognize a novel antigen and induce an adaptive immune response against that antigen, while the secondary response is largely contingent on immunological memory but will also be influenced by the functional state of the innate immune system (Hoebe et al., 2004; Iwasaki and Medzhitov, 2015). Carotenoids have been implicated in boosting lymphocyte proliferation and performance in mammals (reviewed in Chew and Park, 2004), so antibody production is one possible target for finding a physiological benefit to internal carotenoids.

We vaccinated and measured the circulating antibodies of experimental birds twice, both outside (January 2016) and within (late August 2016) the molt period (Fig. 3). Experimental procedures were identical for both periods, although the measurements taken outside of molt represent the first exposure of the birds to the antigen (primary response) and the measurements from molt represent the second exposure (secondary response). Briefly, we first drew a baseline sample of blood (75 µl) from each bird, then injected them intramuscularly with 100 µl of pharmaceutical-grade tetanus vaccine (2 Lf units of tetanus toxoid; also contained 2.7 Lf units of diphtheria toxoid; TENIVAC, Sanofi Pasteur, Lyon, France), dosing 50 µl each into the breast muscles on the left and right sides of the sternum. After 10 days, we drew a second blood sample (75 µl) from each bird. Both blood samples were centrifuged immediately and plasma was stored at −80°C until further analysis. Samples of plasma from before and after vaccination were shipped to Lund University (Lund, Sweden) for anti-tetanus antibody analysis. Anti-tetanus antibody levels were quantified from plasma using previously described enzyme linked immunosorbent assay (ELISA) methods developed for songbirds (Hasselquist et al., 1999; Ilmonen et al., 2000). The net 'antibody response' to tetanus for each individual was calculated as the difference between pre- and post-vaccination measurements, which are reported in milli optical density units per minute (milliOD min^{-1}), as described in Koch et al. (2018).

Total antioxidant capacity

To test for any detectable effect of carotenoid presence, absence or ornamental deposition on antioxidant defenses, we assessed a measure of hydrophilic antioxidant capacity. While carotenoids are lipophilic and therefore may contribute only indirectly to this measure of antioxidant capacity (Tomášek et al., 2016), similar tests of hydrophilic antioxidant capacity have been common in studies of the resource trade-off hypothesis, yielding inconsistent results (Alonso-Alvarez et al., 2008; García-de Blas et al., 2016; Hõrak et al., 2010; Morales et al., 2009). Resolving whether or not carotenoids contribute significantly to antioxidant capacity is a priority for testing the resource trade-off hypothesis (Koch and Hill, 2018). During both molt (July–August 2016) and non-molt (June 2016) periods, we measured total antioxidant capacity in plasma samples using the TAC kit (OxiSelect Total Antioxidant Capacity Assay Kit, Cell BioLabs; San Diego, CA, USA; Fig. 3). We diluted 5 µl of plasma in 15 µl of phosphate-buffered saline (PBS) in duplicate for each individual, and report results in units of µmol l^{-1} copper reduction equivalents (CREs; Koch et al., 2018). For the measurements during molt, we used plasma collected after the immune challenge described below so that we captured TAC during a state of potential immune-induced oxidative stress (Costantini and Møller, 2009).

We also attempted several methods of quantifying oxidative damage in our canary plasma (d-ROMs and ELISAs for protein carbonyls or 4-hydroxynonenal). However, none of these assays was sensitive enough for accurate measurement in the small quantities of plasma that we were able to collect from our birds.

LPS challenge

To expand on our measure of adaptive immunity, we tested two different aspects of innate immunity; specifically, we tested the

response of live birds to bacterial lipopolysaccharide (LPS), a potent innate immune stimulant and non-specific innate immune defenses against bacteria associated with soluble immune proteins in plasma (see next section). First, during molt (July–August 2016), we challenged birds with bacterial LPS. LPS is commonly used to invoke an acute innate immune response in animals without causing lasting disease. In songbirds, LPS injection has generally been found to affect body temperature, food consumption and body mass (Owen-Ashley and Wingfield, 2006), as well as causing an increase in oxidative stress (Costantini and Møller, 2009). We quantified response to LPS using these broad measures of sickness response to capture any effect of carotenoid presence or absence (or allocation of carotenoids to ornaments) on overall acute innate immune response symptoms. Carotenoid-based coloration (Rosenthal et al., 2012) and circulating carotenoid levels (Alonso-Alvarez et al., 2004; Sild et al., 2011) have been found to decrease in response to LPS challenge in songbirds, although the mechanism causing that decrease remains uncertain.

We monitored the molt stage of each bird from July through the end of August and challenged birds when they had emerging pinfeathers, were beginning to show loose feather plumes across the majority of their ventral and/or dorsal sides, and displayed evidence of molted wing and tail feathers. Full details of the LPS challenge procedure are described in Koch et al. (2018). Briefly, we isolated a subset of birds in experimental cages the day before LPS injection, then dosed them the following morning with an intra-abdominal injection of 1 mg ml^{-1} LPS from *E. coli* (O55:B5; List Biological Laboratories, Campbell, CA, USA) dissolved in PBS. We recorded the mass and body temperature (using a Leaton Digital Thermocouple Thermometer inserted ∼1 cm into the vent; Shenzhen DeXi Electronics, Shenzhen, China) of each bird immediately before and 8 h after injection. Also 8 h after injection, we collected 150 µl of blood in two heparinized capillary tubes, and immediately spread one drop on a microscope slide for cell counts; the remaining blood was centrifuged to extract plasma and red blood cell samples, then stored at −80°C until further analysis (TAC measurement, described above). Finally, we provided birds with a known quantity of seed for 24 h before and then 24 h following injection, which we weighed to calculate food consumption before and after the challenge.

From the blood smear collected after LPS injection, we also measured the ratio of heterophils (the avian analog to mammalian neutrophils) to lymphocytes. This measure, called H:L ratio, has previously been found to be a general indicator of immune activation in birds such that a higher value indicates greater activation (Al-Murrani et al., 2006; Davis et al., 2004; Gross and Siegel, 1983), and has been related to carotenoid-based color expression in songbirds (Maney et al., 2008). To collect this measure, we first fixed and stained (Hema 3 Fixative and Solutions, Fisher Scientific, Pittsburgh, PA, USA) the slides. We then used standard techniques for avian blood cell counting and type identification to determine the number of heterophils and lymphocytes present in 10,000 total cells per individual (estimated based on total slides viewed and average cell counts per three representative slide views with a single layer of cells). We divided total number of heterophils by total number of lymphocytes to calculate the H:L ratio.

Bacterial killing assay

To follow up our results from the LPS challenge, we performed an additional innate immune response measure, isolating the complement-dependent antibacterial defenses of the plasma (Demas et al., 2011; Merle et al., 2015). Again, while the mechanisms by which carotenoids may be directly involved in plasma bacterial killing ability are not known, this measure has previously been found to increase in birds supplemented with increased dietary carotenoids (Leclaire et al., 2011; McGraw et al., 2011) and is therefore a promising target for detecting possible benefits of carotenoids. On a subset of plasma extracted from the pre-vaccination blood sample during molt in August 2016 (Fig. 3), we performed a modified microplate-based plasma bacterial killing assay (French and Neuman-Lee, 2012) as described in Koch et al. (2018). Briefly, we tested bacterial killing of the canary plasma against a bacterial solution (*E. coli* ATCC 8739, reconstituted from pellet in sterile PBS; Microbiologics Epower, St Cloud, MN, USA) diluted to 1×10^5 CFUs. We mixed 80 µl of diluted canary plasma (diluted 1:4 in sterile PBS) with 8 µl of the bacterial solution, then incubated the mixture at 37.4°C for 30 min. After incubation, we plated 20 µl of each sample in triplicate on a 96-well microplate, added 125 µl of sterile tryptic soy broth to each well, then measured absorbance at a wavelength of 600 nm. After 12 additional hours of incubation at 37.4°C, we took a second reading at 600 nm. On each plate, we also tested positive controls (bacteria but no plasma), negative controls (plasma but no bacteria, to test for contamination) and 'FBS controls' (containing sterile fetal bovine serum instead of canary plasma, to control for non-immune interactions between bacteria and plasma components, which appeared to boost bacterial growth).

To assess the results, we first calculated an average net absorbance for each individual and control by subtracting baseline values from 12 h values. We then eliminated any absorbance readings that differed more than 10% from the other two values for an individual and averaged the remaining net absorbance values for each individual. We divided this final net absorbance for each individual by the final net absorbance of the FBS controls on the same microplate to obtain a value for percent difference in bacterial growth between samples and positive control. This 'percentage bacteria killed' value for each individual was used in further analyses. However, we found that individuals tended to consistently either completely kill (<10% bacterial growth compared to FBS positive controls) or completely fail to kill (>90% bacterial growth compared to FBS positive controls) their bacterial challenge, so we also performed a binomial regression analysis to assess statistical patterns in the data. For this analysis, we excluded data points with values between 10 and 90% of bacteria killed so that all remaining individuals could be categorized as having fully killed or fully failed to kill their bacterial challenge.

Statistics

For all measurements examined, we used ANOVA to test for significant differences in response measurements based on color type, sex, or the interaction of sex and color type. We tested for differences between the sexes because measurements used in our study, such as those of immune system function, have previously been found to differ between the sexes in songbirds with carotenoid-based coloration (Love et al., 2008; McGraw et al., 2011). However, given that sex and the interaction of sex and color type played little role in our results (Tables S1–S4), we focus on differences in performance among the three color types. We used the Tukey *post hoc* method to test pairwise comparisons between WR versus WD and WD versus Y results for each measure. For measures collected outside of molt only (TAC and primary antibody response to tetanus), we also ran identical analyses in which we pooled the measurements of WD and Y color types and compared them against WR ('carotenoid-rich' versus 'carotenoid-poor'); because allocation of carotenoids to feathers only occurs during molt, WD and Y birds should not differ outside of molt. For LPS injection results only, we also performed two-tailed paired *t*-tests to assess whether injection

Journal of Experimental Biology (2019) 222, jeb188102. doi:10.1242/jeb.188102

significantly affected mass, food consumption or temperature across all canaries. Preliminary results indicated that baseline values for food consumption, body temperature and mass may differ between sexes or colors, so our ANOVAs of these LPS effects also included a term for initial pre-injection values. Finally, for bacterial killing assay analyses, we tested for significant differences using binomial regression (on categorical data coded as 'fully killed' or 'fully failed to kill'), as well as ANOVA (on continuous data of percentage bacteria killed). All statistical analyses were performed in R v3.5.0 (www.r-project.org/). All reported error values are s.e.m.

RESULTS

Carotenoid analyses

As expected, we found WR canaries lacked significant carotenoids in plasma (0.75±0.18 µg ml^{-1}), skin (0.03±0.03 ng mg^{-1}) and feathers (no carotenoids detected). WD canaries similarly lacked carotenoids in their skin (0.01±0.07 ng mg^{-1}) and feathers (0.01±0.01 ng mg^{-1}), but have carotenoid-rich plasma (23.6±7.1 µg ml^{-1}). Y canaries possessed relatively carotenoid-rich feathers (0.12±0.04 µg mg^{-1}), skin (0.15±0.07 µg mg^{-1}) and plasma (20.3±10.6 µg ml^{-1}).

Vaccination and antibody response

Outside of molt, in the primary response to vaccination with tetanus, we found no significant differences in performance among birds based on color type (Fig. 4, Table 1), sex or their interaction (all P>0.11; Table S1, Fig. S1). Comparing 'carotenoid-rich' (WD and Y) canaries to 'carotenoid-poor' (WR) for these results collected outside of molt revealed that carotenoid-rich canaries had lower antibody responses than carotenoid-poor canaries ($F_{1,28}$=4.75; P=0.04). During molt, in the secondary response, there was a significant effect of sex (P=0.046): males, on average, had a stronger antibody response (1.84±0.11 milliOD min^{-1}) than did females (1.48±0.22 milliOD min^{-1}). Color type or the interaction of color type and sex had no significant effects on secondary antibody response (both P>0.23; Table S1, Fig. S1).

Total antioxidant capacity

Both within and outside of molt, WD, WR and Y canaries did not differ significantly in total antioxidant capacity (both P>0.41; Table 1; Fig. 4). There was a significant effect of sex on TAC, but only within molt (P=0.04; P=0.47 outside of molt); molting males had greater average TAC (2001±266 CRE) than females (1208±143 CRE;

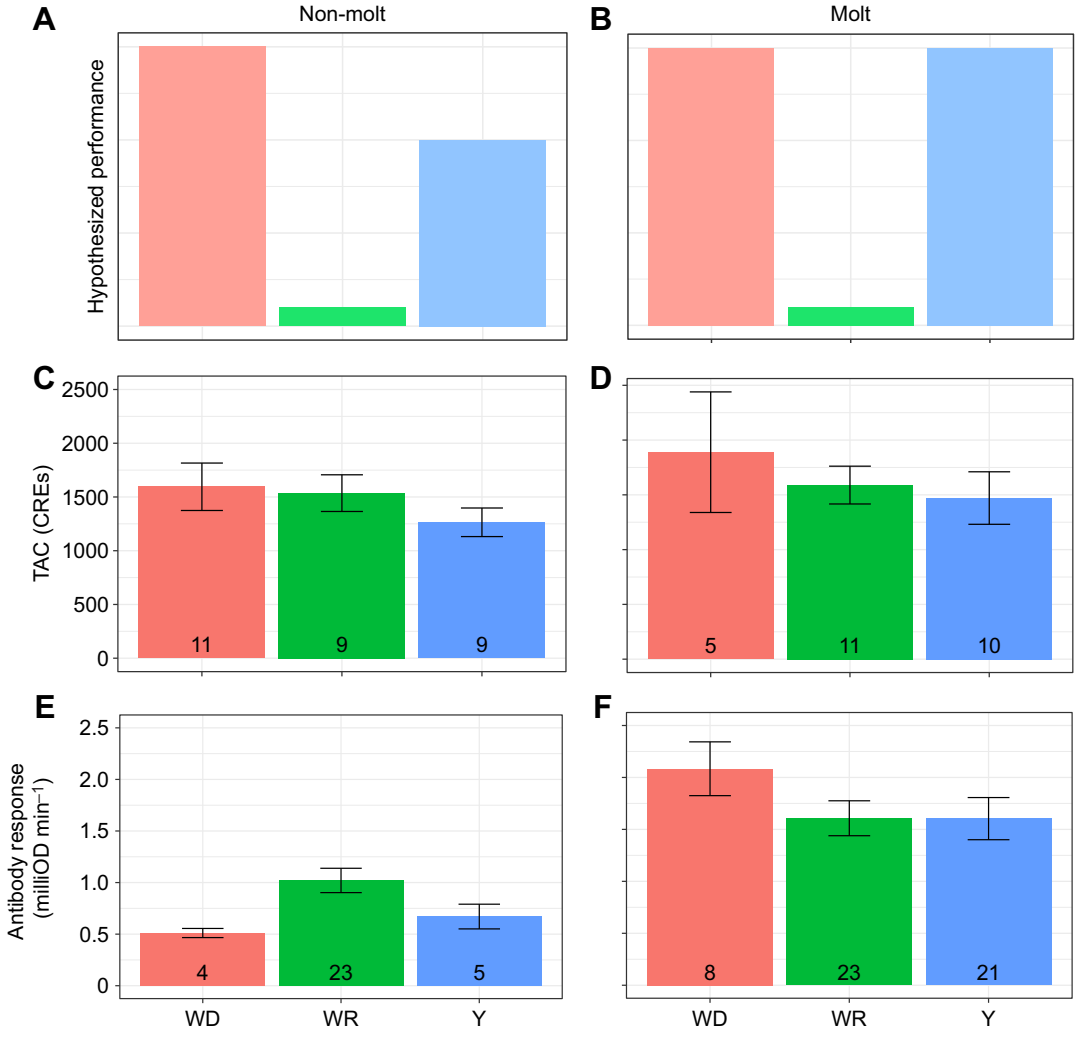

Fig. 4. Hypothesized and actual results of two physiological measures taken during and outside of molt. Based on the carotenoid resource trade-off hypothesis, the predicted patterns in the data are clear: WD and Y birds should feature strong physiological performance outside of molt (A), but WD birds should outperform Y birds within molt (B); WR birds should always perform poorly. We found none of these expected patterns in measures of total antioxidant capacity (TAC) (C,D; ANOVA P>0.4 for color type) and antibody response to tetanus (primary response outside of molt, E; secondary response during molt, F; ANOVA P>0.1 for color type). Sample sizes are printed in the bars of C–F; values are means±s.e.m. CRE, copper reduction equivalent.

Journal of Experimental Biology (2019) 222, jeb188102. doi:10.1242/jeb.188102

Table 1. Results of statistical analyses of the effects of canary color type on immune or antioxidant performance

		Overall model (color type)		*Post hoc* WR versus WD		*Post hoc* Y versus WD	
Stage	Measurement	*F* (d.f.)	*P*	Difference	*P*	Difference	*P*
Non-molt	Primary antibody response	1.255 (2,26)	0.273	0.51	0.17	0.16	0.88
	TAC (CRE)	0.93 (2,23)	0.41	−59.2	0.97	−330.5	0.41
Molt	Secondary antibody response	1.58 (2,46)	0.24	−0.48	0.34	−0.48	0.34
	LPS-mediated mass change (g)	4.42 (2,50)	0.017	0.52	0.034	0.29	0.35
	LPS-mediated temperature change (°C)	0.73 (2,28)	0.49	−0.26	0.73	−0.34	0.60
	LPS-mediated food consumption change (g h^{-1} g^{-1})	0.51 (2,54)	0.60	−0.53	0.66	−0.51	0.69
	TAC (CRE)	0.52 (2,20)	0.60	−300.1	0.74	−418.6	0.58
	H:L	2.354 (2,45)	0.11	0.17	0.11	0.16	0.15
	BKA (% killed)	1.85 (2,47)	0.17	24.8	0.34	33.7	0.14

The overall model refers to the main ANOVA results, while *post hoc* results refer to pairwise comparisons between focal color types using the Tukey method. In the *post hoc* results, a negative difference indicates that WD birds had lower values than WR or Y birds. Degrees of freedom (d.f.) values indicate the degrees of freedom of the model and the error, respectively. BKA, bacterial killing assay; CRE, copper reduction equivalent; H:L, heterophil to lymphocyte ratio; LPS, lipopolysaccharide; TAC, total antioxidant capacity; WD, white dominant; WR, white recessive; Y, yellow.

Fig. S2). The interaction of sex and color type had no significant effect on TAC either during or outside of molt (P>0.17; Table S2, Fig. S2). An analysis pooling WD and Y performance outside of molt (see above) also revealed no significant differences between carotenoid-rich and carotenoid-poor canaries ($F_{1,51}$=1.36, P=0.72; Table S2).

Response to LPS challenge

The initial values of each individuals' mass, temperature or food consumption always influenced the magnitude by which its values changed after LPS injection (all P<0.001), and WR and WD (but not Y and WD) canaries also differed significantly in their initial body temperature (P=0.02; Table S3, Fig. S3). Color type, sex or their interaction had no other significant effects on baseline body temperature, food consumption or mass, although there was a trend toward a difference between the sexes in initial food consumption (P=0.087) and between the color types in initial mass (P=0.078; all other P>0.1; Table S3).

Among all canaries, LPS injection induced a loss of mass (average decrease: 1.20 g; t=17.18, d.f.=56, P<0.001) within 24 h, and a body temperature increase (average increase: 0.46°C; t=6.67, d.f.=34, P<0.001) within 8 h, but no change in food consumption over 24 h (average increase: 0.11 mg consumed g^{-1} body mass h^{-1}; t=0.56, d.f.=60, P=0.58). We found that color type of the canary significantly affected the magnitude of LPS-induced loss of mass (P=0.02; Table 1). This is driven by differences between WR and WD birds: WR canaries tended to lose more mass than WD canaries, although there was no such pattern between Y and WD birds (Table 1). Sex had no significant effect on change in mass, but there was a trend toward a significant difference in change in body temperature between males and females (average temperature increase: females, 0.58±0.09°C; males, 0.33±0.10°C; P=0.052) and there was a significant difference in change in food consumption between males and females (average change in females: −0.51±0.36 mg consumed h^{-1} g^{-1} body mass; in males: 0.16±0.24 mg consumed h^{-1} g^{-1} body mass; P=0.027). Neither temperature increase nor change in food consumption differed among color types or with the interaction of sex and color type (Table 1, all P>0.17; Table S3, Fig. S3). The ratio of heterophils to lymphocytes present in blood smears prepared 8 h after LPS injection also did not differ between color types, sexes or their interaction (all P>0.11; Table 1; Table S3, Fig. S4).

Bacterial killing ability

We found no significant differences in bacterial killing ability between the sexes, among the colors or with their interaction, either in a binomial regression analysis where individuals were coded as having either killed (>90% bacterial clearance) or failed to kill (<10% clearance) their challenge (all P>0.53) or in an ANOVA of percentage clearance (P>0.10; Table 1; Table S4, Fig. S5).

DISCUSSION

In this study, we used canaries with mutations in key carotenoid pathways to test two of the central assumptions of the carotenoid resource trade-off hypothesis: (1) carotenoids provide physiological benefits, and (2) animals trade off subtraction of physiological benefits with production of ornamentation as carotenoids are allocated to ornaments (Koch and Hill, 2018). After comparing performance across several measures of immunocompetence and one measure of antioxidant capacity in WD versus Y (ornament-free versus ornamented) and WD versus WR (carotenoid-rich versus carotenoid-free) canaries, we found no empirical support for either assumption.

These comparisons offer what are perhaps the most direct tests to date of whether internal carotenoid resources provide significant physiological benefits to birds and whether allocating these carotenoid resources to ornamental plumage coloration decreases these benefits. While most previous tests of the resource trade-off hypothesis have relied on experimental manipulations, such as modified dietary carotenoid intake and/or physiological challenge, our experimental groups were fed identical diets and held under identical conditions. Indeed, Y, WR and WD canaries appear functionally identical in all aspects of phenotype except carotenoid usage because they are variants of the same breed of canary, differing only in mutations that affect carotenoid coloration.

The patterns in our data do not conform to the predictions of the resource trade-off hypothesis: there were no consistent trends toward WD canaries performing better than Y canaries during molt or better than WR canaries outside of molt. Despite uneven sample sizes due to the difficulty in locating WD canaries, our data did not exhibit consistent trends in the direction predicted by the resource trade-off hypothesis, making it unlikely that a greater sample size would significantly alter our conclusions. The only measurements in which we detected a significant effect of color type or carotenoid phenotype on response were: (1) carotenoid-rich types (WD and Y) exhibited lower primary antibody response to tetanus outside of molt than did carotenoid-poor WR canaries; (2) WR canaries had higher baseline body temperature prior to LPS injection; and, (3) WR canaries lost more mass during LPS challenge than did WD canaries, although WR birds also tended to have higher baseline mass and the magnitude of their mass loss is similar to that of Y

canaries. None of these relationships suggest a major role of internal carotenoids in boosting physiological response or of a cost to depositing carotenoids as ornamental colorants during molt. We nevertheless encourage future studies to explore other potential avenues of physiological benefit of carotenoids, such as lipophilic antioxidant capacity, which has recently been proposed to be the best method for detecting the potential antioxidant effects of carotenoids (Tomášek et al., 2016). Moreover, isolating the genetic mutation(s) responsible for the WD phenotype would be extremely valuable both for describing the mechanistic origin of the 'ornament-free' canary and for advancing our understanding the genetic basis of variation in plumage ornaments.

Our observations corroborate patterns that we found in our previous analysis comparing only WR and Y birds (Koch et al., 2018). In that study, we were also able to assay the levels of one endogenous antioxidant (glutathione) in WR and Y red blood cells, and we found no difference between the birds; however, without a more comprehensive panel of measurements, we cannot rule out the possibility that WR birds may have compensatory mechanisms to circumvent their lack of carotenoids. Indeed, the trend toward higher average body mass among WR birds (compared with WD and Y birds) that we recorded at the start of our immune challenge experiment in this study suggests some underlying differences may exist between the color types. Previous genetic exploration of the WR canaries compared them to yellow canaries of vastly different genetic lines (e.g. lines bred for varying body conformation) in order to isolate the genetic regions responsible for the WR phenotype (Toomey et al., 2017). Further genetic analyses of closely related WR and WD/Y canaries, such as those tested in our experiments, will be important to rule out whether WR birds have been selected for compensatory adaptations to their physiological lack of carotenoids. However, given that we found no consistent differences in performance among the three color types of canaries we examined here or between WR and Y canaries in our previous study (Koch et al., 2018), we consider it unlikely that we have overlooked any substantial sources of variation among these birds.

Our results provide an important new piece of evidence in the discussion surrounding the honesty of carotenoid-based signals, but a definitive explanation for condition-dependent coloration remains elusive. One possibility is that retinol (vitamin A) may play a greater role than previously assumed in the physiological benefits that have been attributed to carotenoids (Hill and Johnson, 2012; Koch et al., 2018). By providing all our birds with a supplement that includes retinol, we prevented retinoid deficiency in the WR birds, which cannot absorb the carotenoid precursors to retinol (Wolf et al., 2000). However, in wild birds, individuals low in carotenoids are unlikely to have access to other sources of retinol, so low-carotenoid birds may also be low in retinol (and vice versa; Simons et al., 2015). Given that retinol has a wide range of well accepted health benefits in vertebrates (Hill and Johnson, 2012), it is plausible that some of the health benefits that have been credited to carotenoids themselves arise because carotenoids are a valuable source of retinoids.

Another possibility is that the basic premise of the resource trade-off hypothesis is incorrect and carotenoids play no important functions in vertebrates other than social signaling. A physiological cost to absorbing or converting carotenoid pigments from the diet could maintain condition dependence in carotenoid-based signals without requiring carotenoids to play a direct role in boosting physiological processes (Weaver et al., 2018); however, we found no consistent evidence of such physiological costs to carotenoid uptake or conversion in our WD or Y canaries. An alternative hypothesis for the basis of carotenoids as honest signals that invokes no physiological functions for carotenoids is the shared pathway hypothesis (Hill, 2011). This hypothesis proposes that the quality of external coloration can be linked to the quality of vital cellular processes if both coloration and physiological well-being are dependent on the functionality of shared mechanistic pathways. By this idea, individual 'condition' is defined as the performance of cellular processes that drive variation both in honest signals and in the qualities they signal, and carotenoids themselves are not required to have any physiological function besides being colorants (Weaver et al., 2017). However, testing the shared pathway hypothesis requires isolating and testing the performance of that shared pathway; although a potential shared cellular process has been proposed – mitochondrial function (Hill, 2014; Koch et al., 2017) – it awaits empirical testing. We encourage future research to critically test the resource trade-off hypothesis as an explanation for condition dependence in carotenoid-based signals in birds and to devise new means of more directly testing the functions of carotenoids within the animal body. Until such time as techniques for manipulating specific genetic pathways in vertebrates are widely accessible, domestic birds with existing genetic mutations in key carotenoid pathways offer a rich and accessible system for such studies.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: R.E.K., G.E.H.; Methodology: R.E.K., M.S., A.N.K., D.H., M.B.T., G.E.H.; Formal analysis: R.E.K.; Investigation: R.E.K.; Resources: G.E.H.; Data curation: R.E.K.; Writing - original draft: R.E.K.; Writing - review & editing: R.E.K., M.S., A.N.K., D.H., M.B.T., G.E.H.; Supervision: A.N.K., G.E.H.; Funding acquisition: R.E.K., A.N.K., D.H., G.E.H.

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Table S1. Average anti-tetanus antibody responses for the primary response (outside of the molt season) and secondary response (during molt) for WD, WR, and Y canaries.

	Descriptive results			ANOVA results		
	Color type	Sample size	Average response ± SE (milliOD/min)	Variable	F (df)	P
Primary response (non-molt)	WD	3 F, 1 M	0.51 ± 0.04	Color type	2.358 (2,26)	0.114
	WR	14 F, 9 M	1.02 ± 0.12	Sex	1.255 (1,26)	0.273
	Y	3 F, 2 M	0.67 ± 0.12	Interaction	0.783 (2,26)	0.467
Secondary response (molt)	WD	6 F, 2 M	2.08 ± 0.26	Color type	1.164 (2,46)	0.321
	WR	10 F, 13 M	1.61 ± 0.17	Sex	4.190 (1,46)	0.046
	Y	7 F, 14 M	1.60 ± 0.20	Interaction	1.476 (2,46)	0.239

F = female, M = male; df = degrees of freedom (numerator, denominator).

Table S2. Average total antioxidant capacity of plasma samples from WD, WR, and Y canaries, inside and outside of molt.

	Descriptive results			ANOVA results		
	Color type	Sample size	Average response ± SE (CRE)	Variable	F (df)	P
Non-molt	WD	3 F, 8 M	1595 ± 221	Color type	0.925 (2,23)	0.411
	WR	2 F, 7 M	1536 ± 171	Sex	0.541 (1,23)	0.469
	Y	2 F, 7 M	1265 ± 133	Interaction	1.908 (2,23)	0.171
Molt	WD	2 F, 3 M	1889 ± 550	Color type	0.516 (2,20)	0.605
	WR	7 F, 4 M	1589 ± 173	Sex	4.821 (1,20)	0.040
	Y	6 F, 4 M	1471± 240	Interaction	0.346 (2,20)	0.711

CRE = copper reduction equivalents; F = female, M = male; df = degrees of freedom (numerator, denominator).

Table S3. Baseline physiological metrics and their LPS-mediated changes in WD, WR, and Y canaries during molt.

	Descriptive results			ANOVA results		
	Color type	Sample size	Average response ± SE	Variable	F (df)	P
Initial mass (g)	WD	6 F, 2 M	22.73 ±1.26	Color type	2.691 (2,5)	0.078
	WR	10 F, 16 M	25.40 ± 0.72	Sex	1.008 (1,51)	0.320
	Y	7 F, 16 M	23.60 ± 0.59	Interaction	0.526 (2,51)	0.594
Change in mass (g)	WD	6 F, 2 M	-0.85 ± 0.19	Color type	4.421 (2,50)	0.017
	WR	10 F, 16 M	-1.37 ± 0.01	Sex	0.242 (1,50)	0.625
	Y	7 F, 16 M	-1.14 ± 0.10	Interaction	2.258 (2,50)	0.115
				Initial value	12.536 (1,50)	<0.001
Initial body temperature (°C)	WD	4 F, 3 M	40.67 ± 0.20	Color type	4.552 (2,29)	0.019
	WR	8 F, 7 M	41.22 ± 0.12	Sex	0.007 (1,29)	0.932
	Y	6 F, 7 M	40.97 ± 0.09	Interaction	0.099 (2,29)	0.906
Change in body temperature (°C)	WD	4 F, 3 M	0.60 ± 0.11	Color type	0.733 (2,28)	0.490
	WR	8 F, 7 M	0.45 ± 0.11	Sex	4.109 (1,28)	0.052
	Y	6 F, 7 M	0.41 ± 0.12	Interaction	0.371 (2,28)	0.693
				Initial value	16.262 (1,28)	<0.001
Initial food consumption (mg consumed / hour / g body mass)	WD	6 F, 2 M	2.79 ± 0.43	Color type	0.376 (2,55)	0.688
	WR	11 F, 17 M	3.08 ± 0.26	Sex	3.032 (1,55)	0.087
	Y	8 F, 17 M	2.76 ± 0.34	Interaction	2.347 (2,55)	0.105
Change in food consumption (g consumed / hour / g body mass)	WD	6 F, 2 M	0.34 ± 0.51	Color type	0.510 (2,54)	0.603
	WR	11 F, 17 M	-0.19 ± 0.23	Sex	5.185 (1,54)	0.027
	Y	8 F, 17 M	-0.17 ± 0.40	Interaction	1.811 (2,54)	0.173
				Initial value	19.724 (1,54)	<0.001
Heterophil to lymphocyte ratio (post-LPS only)	WD	6 F, 3 M	0.17 ± 0.039	Color type	2.354 (2,45)	0.107
	WR	9 F, 15 M	0.34 ± 0.039	Sex	0.556 (1,45)	0.460
	Y	6 F, 12 M	0.33 ± 0.058	Interaction	0.244 (2,45)	0.785

Negative values for change in mass, temperature, or food consumption indicate that measurements decreased in value after LPS injection. F = female, M = male; df = degrees of freedom (numerator, denominator).

Table S4. Bacterial killing ability of WD, WR, and Y canaries during molt.

Descriptive results			ANOVA results			Binomial GLM results		
Color type	Sample size	Average response ± SE (Percent bacterial killing), fraction of individuals who fully-killed their challenge	Variable	F (df)	P	Variable	Z	P
WD	5 F, 3 M	39.05 ±17.35, 3/8	Color type	1.845 (2,47)	0.169	Intercept	-0.444	0.657
						Color type (WR vs. WD)	0.365	0.715
						Color type (Y vs. WD)	0.011	0.991
WR	10 F, 10 M	58.22 ±10.56, 12/20	Sex	0.395 (1,47)	0.533	Sex	-0.188	0.851
Y	6 F, 15 M	65.49 ± 9.77, 14/21	Interaction	2.472 (2,47)	0.095	Interaction (WR vs. WD by Sex)	0.634	0.526
						Interaction (Y vs. WD by Sex)	-0.011	0.992

Individuals were considered to have "fully-killed their challenge" if they had a percentage of bacteria killed greater than 90%. Results are presented both for an ANOVA performed on continuous data of percent bacterial killing, and a binomial generalized linear model (GLM) on categorical data indicating whether or not an individual fully-killed their challenge. F = female, M = male; df = degrees of freedom (numerator, denominator).

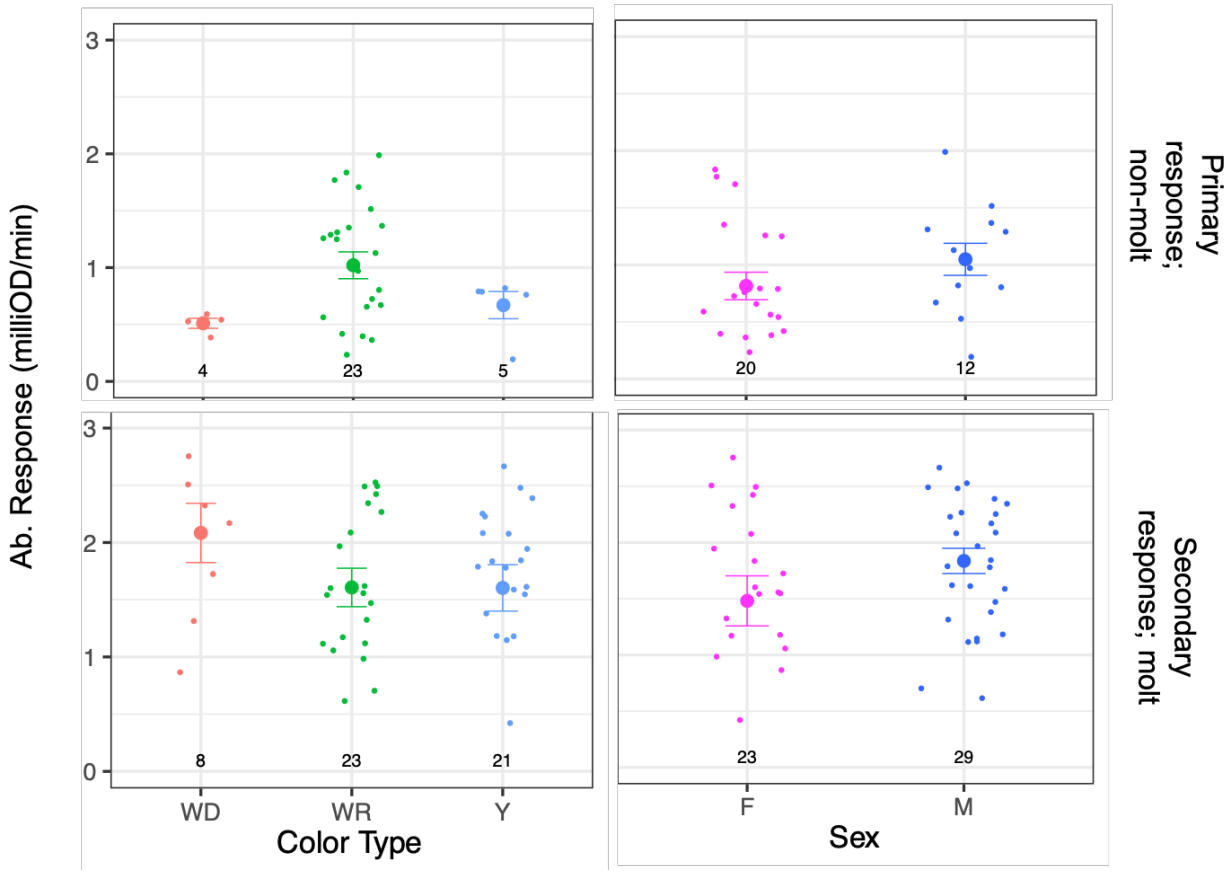

Figure S1. Mean ± SE anti-tetanus antibody responses of the three color types (left panel) and the two sexes (right panel). Small points represent individual raw data; numbers at the base of each panel represent sample sizes.

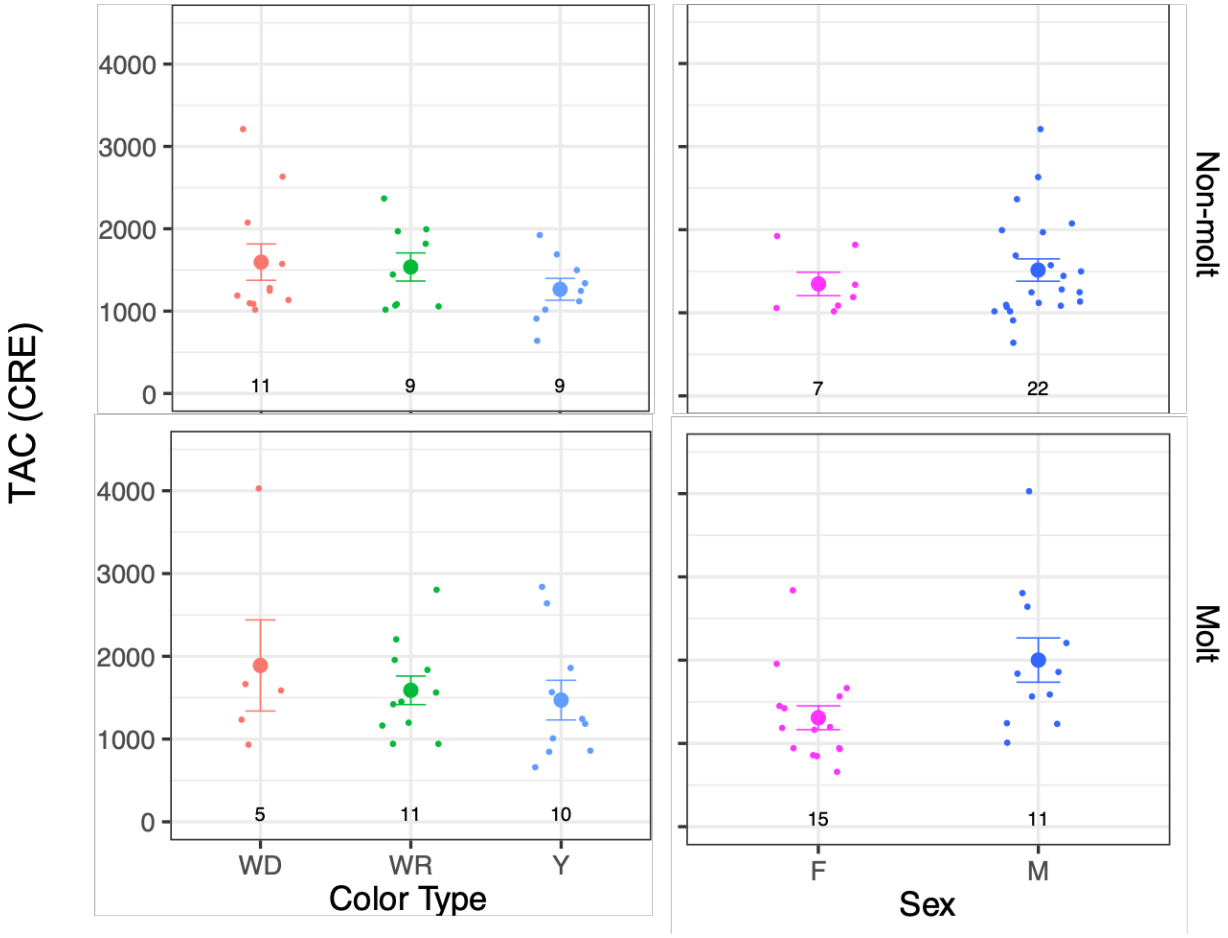

Figure S2. Mean ± SE total antibody capacity (TAC) of the three color types (left panels) and the two sexes (right panels). Small points represent individual raw data; numbers at the base of each panel represent sample sizes.

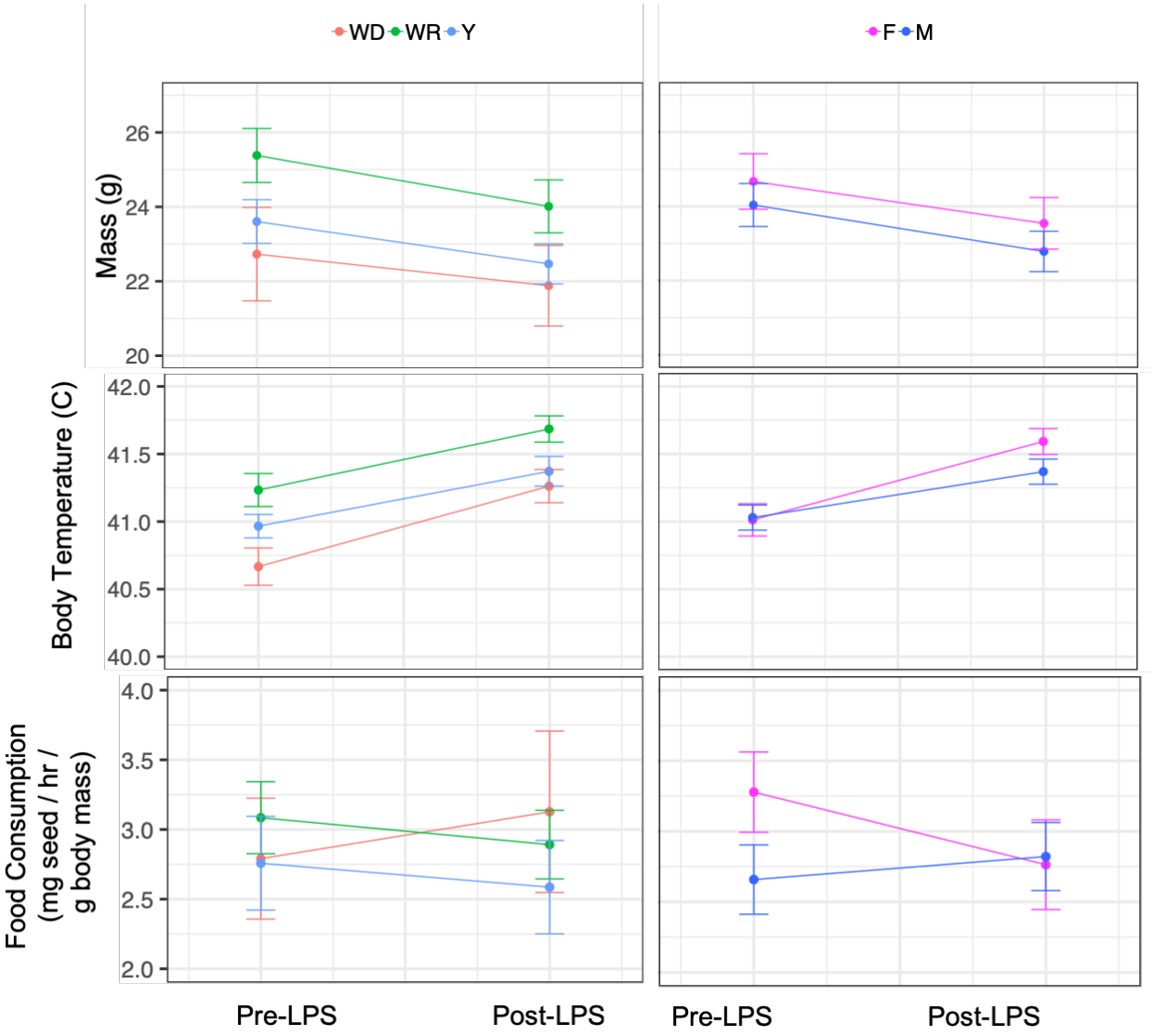

Figure S3. Mean ± SE measurements taken prior to or after bacterial lipopolysaccharide (LPS) injection.

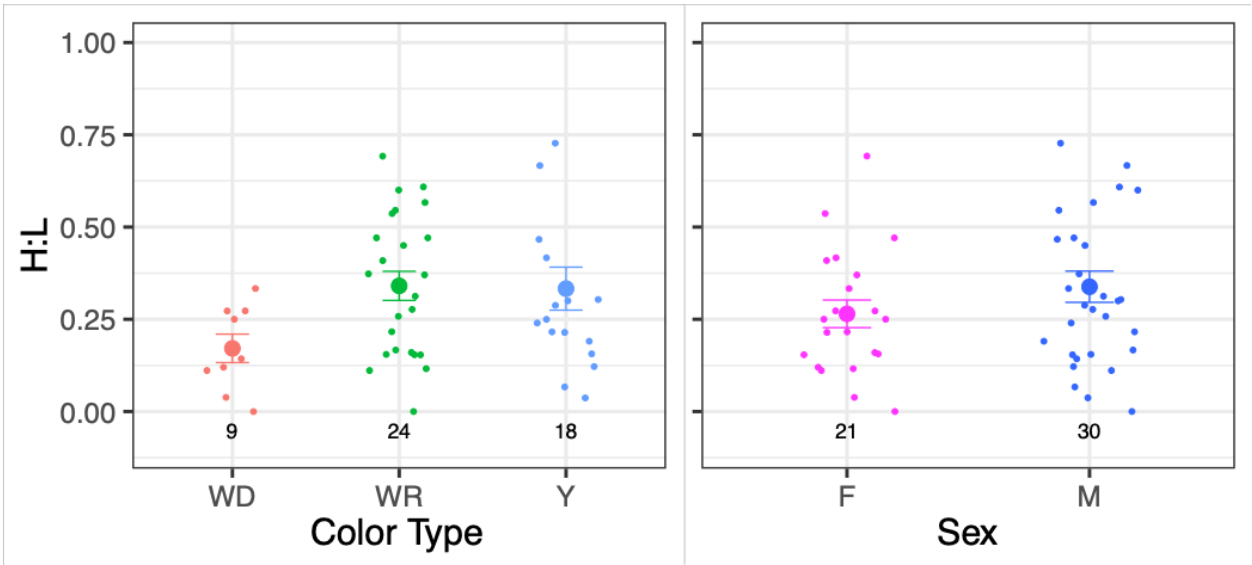

Figure S4. Mean ± SE heterophil to lymphocyte ratio of the three color types (left panels) and the two sexes (right panels). Small points represent individual raw data; numbers at the base of each panel represent sample sizes.

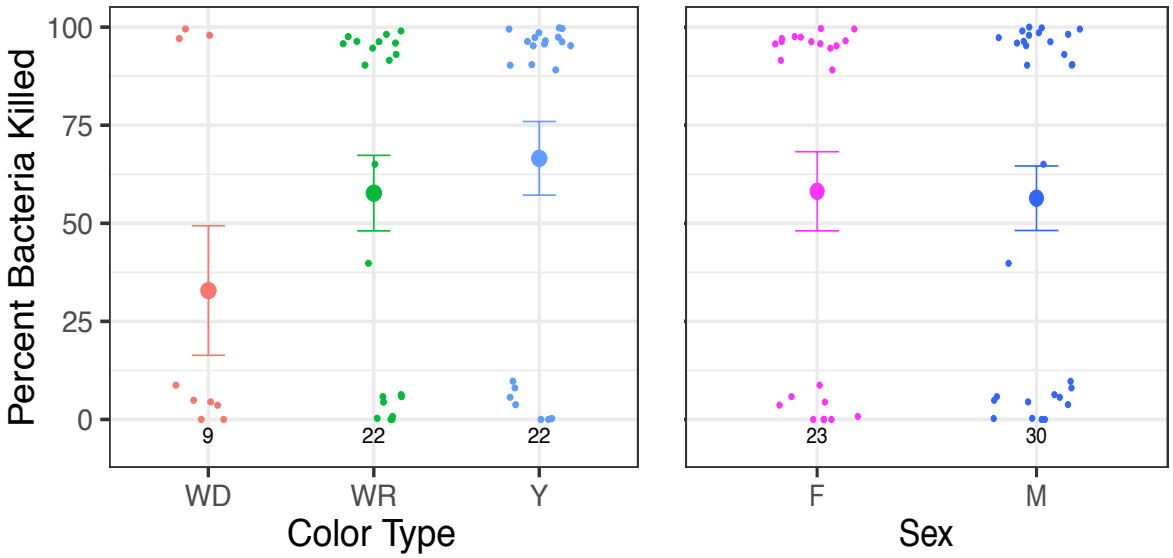

Figure S5. Mean ± SE bacterial killing capacity (percent bacteria killed relative to positive controls). Small points represent individual raw data; numbers at the base of each panel represent sample sizes.