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Carotenoids buffer the acute phase response on fever, sickness behavior, and rapid bill color change in zebra finches

Deanna R. Broughton[1#], Brent C. Schneider[1#], Kevin J. McGraw[2], and Daniel R. Ardia[1,*],

[1] Department of Biology, Franklin & Marshall College, Lancaster, PA 17604 USA

[2] School of Life Sciences, Arizona State University, Tempe, AZ 85287 USA

[#] Authors contributed equally

* Corresponding author: dardia@fandm.edu

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Summary statement: Carotenoids are colorful pigments traded off between display and health. When we induced a strong immune response, zebra finches supplemented with carotenoids were able to buffer the negative effects of sickness.

ABSTRACT

Carotenoids are finite resources that animals can allocate to self-maintenance, attractiveness, or reproduction. Here we test how carotenoids affect the acute phase response (APR), an intense rapid systemic response characterized by fever, sickness behavior, and production of acute phase proteins, which serves to reduce pathogen persistence. We conducted a 2x2 factorial design experiment in captive adult male and female zebra finches (*Taeniopygia guttata)* to determine the effects of carotenoid supplementation on the intensity of the APR. We measured changes in feeding rate, activity level, and body temperature of the birds. We found that, relative to unsupplemented controls, carotenoid-supplemented birds exhibited less severe reductions in feeding and activity, smaller increases in body temperature, and lower circulating levels of haptoglobin (an acute phase protein) 24 h after inducing an APR. Among supplemented individuals, those with higher blood carotenoid levels exhibited a lower reduction in activity rate after 24 h. Forty-eight hours after APR induction, birds exhibited a significant decrease in plasma carotenoid levels and a decrease in bill hue, with less reduction in hue in carotenoid-supplemented individuals. These results demonstrate that carotenoids can alleviate several important behavioral and physiological effects of an APR and that bill color can change rapidly following induction of the costly APR immune defense. In particular, immune activation may have caused birds to preferentially draw down carotenoids from the bloodstream, ostensibly for use in health. Rapid bill color changes over a 48-hr period support growing evidence that bills may serve as short-term signals of health and condition.

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INTRODUCTION

Organisms often face allocation tradeoffs in response to competing demands for limited resources. Carotenoids are nutrient resources that can be dietarily limiting (Grether et al., 1999; Hill et al., 2002) and may tradeoff between health and attractiveness (Lozano, 1994), as well as other body functions, such as reproduction (Bertrand et al., 2006) or vision (Knott et al., 2010; Toomey et al., 2010). Carotenoids are the pigments directly responsible for generating sexually selected color signals in many animals (Blount et al., 2003; Grether et al., 2005). Carotenoids also can serve antioxidant and immunoregulatory function (Fiedor et al., 2005; Hõrak et al., 2001; McGraw and Ardia, 2003; Saks et al., 2003; Simons et al., 2012). A recent burst of studies on the health role of carotenoids has generally, but not always (Costantini and Møller, 2008; Fitze et al., 2007), produced supporting evidence (Bertrand et al., 2006; Clotfelter et al., 2007; Simons et al., 2012). These data suggest that there are not universal health benefits for carotenoids across animals and immune-system axes, but instead that we must refine our approaches to better isolate particular taxa or mechanistic pathways for which carotenoids and health state are linked. Here we examine a component of the immune system linked to pathogen defense: the acute phase response (APR) (Bonneaud et al., 2003; LeGrand and Alcock, 2012; Martin et al., 2008; Owen-Ashley and Wingfield, 2006). The APR is an intense, rapid, and non-specific systemic response, characterized by fever, sickness behavior, and production of acute phase proteins, which serves to reduce pathogen persistence and increase host survival (Exton, 1997; Hart, 1988; Kluger et al., 1998). However, the APR entails significant energetic costs through raising basal metabolic rates and opportunity costs of reduced physical activity (Adelman and Martin, 2009; Ashley and Wingfield, 2011; Aubert et al., 1997; LeGrand and Alcock, 2012; Roe and Kinney, 1965; Sköld-Chiriac et al., 2014). Cytokines, released by circulating immune cells, trigger the behavioral, immune, and metabolic cascade associated with the APR. Carotenoids have been shown to increase cytokines in birds (Shanmugasundaram and Selvaraj, 2011) and humans (Watzl et al., 1999; Watzl et al., 2005) and thus might modulate the intensity of the APR. The APR is associated with decreased levels of blood carotenoids (Boosalis et al., 1997; Koutsos et al., 2003; Koutsos et al., 2006; Thurnham and Singkamani, 1991).

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A central APR-upregulated hepatic protein, haptoglobin, has strong antioxidant activity (Quaye, 2008) and plays a role in suppressing inflammation (Huntoon et al., 2008; Krueger et al., 2016; Wang et al., 2001). Carotenoids have been found to influence hepatic proteins (Ben-Dor et al., 2005; Zhang et al., 1992) that are upregulated during the APR (Hart, 1988). However, previous investigations into carotenoids and haptoglobin have found differing results even when conducted on the same species (*Gallus gallus*) at different developmental stages. Carotenoid-supplemented 14-d old broiler chickens (*Gallus gallus domesticus*) exhibited smaller increases of haptoglobin, an acute phase protein released during the APR, than unsupplemented chicks (Koutsos et al., 2006). However, carotenoid supplementation had no effect on haptoglobin levels following APR induction in adult red junglefowl (*Gallus gallus*; (McGraw and Klasing, 2006). These results suggest that a better understanding of how carotenoids influence the APR is needed, with explicit measurement of fever, sickness behavior, and haptoglobin.

Here, we expand our understanding of carotenoids and health by investigating how carotenoids affect behavioral, physiological, and bill color responses to the APR in a species with a carotenoid-based signal (Blount et al., 2003; McGraw and Ardia, 2003), the zebra finch (*Taeniopygia guttata* Vieillot). We experimentally supplemented carotenoids through the diet and induced an APR using Complete Freund's Adjuvant (CFA) as an immunostimulator (Freund and McDermott, 1942), using a 2x2 factorial design, to test if and how carotenoids modify the APR. We then measured changes in locomotor activity, feeding rate, body temperature, circulating haptoglobin and carotenoid levels, and bill coloration as a function of carotenoid supplementation and immune activation. If carotenoids play a role in the APR, we predicted first that APR would lead to decreases in circulating blood carotenoid levels (Koutsos et al., 2003). Second, we predicted an interaction between carotenoid supplementation and APR induction, such that all CFA-injected individuals would reduce feeding and increase body temperature, but that carotenoid supplementation would dampen such effects. Third, we predicted that individuals receiving APR induction would have the highest haptoglobin levels, with lower levels in carotenoid-supplemented birds corresponding to lower predicted changes in body temperature and sickness behavior. Finally, we investigated whether APR induction and its putative effects on carotenoids would cause short-term changes in sexually selected carotenoid-based bill color (Blount et al. 2003). Immune system challenges have been found to

change bill color over three- to four-week periods in previous studies (Alonso-Alvarez et al., 2004; Faivre et al., 2003), but recent studies in zebra finches, American goldfinches (*Spinus tristis* Linnaeus) and blue-footed boobies (*Sula nebouxii* Milne-Edwards) reported that carotenoid-containing bill or foot tissue can change in color over 48-72 hours (Ardia et al., 2010; Rosen and Tarvin, 2006; Rosenthal et al., 2012; Velando et al., 2006). Here we examined changes in bill color and blood carotenoid levels over a 24-48-hr period, the time frame for the induction of the APR (Adelman and Martin, 2009; Koutsos et al., 2006). We predicted that APR induction would decrease bill redness, due to (1) reduced food (hence carotenoid) intake and or (2) preferential carotenoid allocation away from the bill and instead to immunity (Hill et al., 2009). Zebra finches are sexually-dimorphic and carotenoids serve as a sexually-selected signal that relates to male dominance (Ardia et al., 2010) and female choice (Blount et al., 2003; McGraw et al., 2003; Rutstein et al., 2007). Accordingly, we would predict that males and females might respond differently to our manipulations, with males potentially facing different tradeoffs or levels of physiological activity with regard to the use of carotenoids for both coloration and health (McGraw and Ardia, 2005; McGraw and Toomey, 2010; Mundy et al., 2016). Thus, we also tested for sex differences in responses to the carotenoid and APR treatments; we predicted that males would show greater decreases in bill pigmentation in response relative to females because of higher overall blood carotenoid levels in male zebra finches (McGraw and Ardia, 2005).

MATERIALS AND METHODS

Adult zebra finches, ranging in age from one to three years, were obtained from five commercial breeders and maintained in groups of two or three in small cages (45 cm wide x 61 cm long x 61 cm tall). Ages and sexes were distributed evenly across treatments. The birds received millet seed mix (Animal Care Products, Philadelphia, PA, USA) and drinking water *ad libitum.* Birds were maintained at a constant ambient temperature of 24.0°C $\pm$ 1.0, a relative humidity of 45-60 %, and a 12L:12D photoperiod, to mimic non-breeding conditions.

Individuals were randomly assigned to one of four treatment groups: (1) no carotenoid supplementation, no immune activation (males = 10, females = 10), (2) no carotenoid supplementation, but with immune activation (males = 10, females = 10), (3) carotenoid supplementation, no immune activation (males = 10, females = 10), and (4) carotenoid

supplementation with immune activation (males = 10, females = 8) (See Fig. 1 for experimental design). All birds were housed in male-female pairs except for treatment 4 where two males were housed with a single female; a ninth female started in the study, but was not eventually used in the study because her blood samples were lost. Lutein and zeaxanthin (DSM Nutritional Products, Heerlen, Netherlands), the main carotenoid pigments found in zebra finch diets and internal tissues (McGraw et al., 2003), were supplemented for four weeks, in the form of starch-encapsulated, water-soluble beadlets dissolved in drinking water at a physiologically relevant concentration of 12 μg ml^{-1} (McGraw and Ardia, 2003); control (carotenoid-unsupplemented) animals received plain drinking water. We tested the effectiveness of carotenoid supplementation by examining changes in blood carotenoid levels over the four-week supplementation period before exposure to CFA. A pilot study conducted on individuals not used in this experiment indicated that supplementing carotenoids in the diet raised blood carotenoid levels in supplemented birds relative to controls, with greater increases in males than in females (effect of supplementation: $F_{1,28} = 73.1$, $P < 0.001$, effect of sex: $F_{1,28} = 7.2$, $P = 0.01$; effect of sex x supplementation interaction: $F_{1,28} = 8.6$, $P = 0.001$; Table S1).

After a four-week supplementation period, each bird was moved to its own observation cage (45 x 61 x 61 cm) out of sight of other birds and with food and water, to record feeding activity (from 0900-1100 hrs.) before and during the APR. To record feeding visits, a wooden perch attached to a microswitch (Omron Electronics SS-5GL, Hoffman Estates, IL, USA) and connected to an event logger (Pendant Logger, Onset Computer, Bourne, MA, USA) was placed in such a way as to require birds to perch to feed. To test whether feeding activity from perch counters was an accurate predictor of time spent feeding and total activity level, before starting the experiment we observed seventy 30-minute feeding bouts of individual zebra finches, split evenly between APR-induced and control birds. We found during visual observations that the number of perch counts was strongly positively correlated with overall activity ($\beta = 0.271$, $R^2 = 0.82$, $P < 0.001$; $n = 70$) and with total time spent feeding while on the perch ($\beta = 0.212$, $R^2 = 0.86$, $P < 0.001$; $n = 70$), indicating that activity recorded on perch counters mounted at feeders is predictive of an integrated measure of feeding behavior and overall activity levels.

After an initial 24-hr acclimation period in the observation cage, we stimulated the APR of birds in the immune-activation treatment groups by injecting subcutaneously above the

furcula 100 µl of 1 mg/ml emulsified Complete Freund's Adjuvant (CFA) (Sigma F5881, St. Louis, MO, USA). All other birds were given an injection of 100 µl of Phosphate Buffered Saline. We chose CFA over lipopolysaccharide (LPS), which is often used as an immunostimulant (Martin et al., 2011) because pilot data revealed low APR responsiveness to LPS in our population. Our dosage did not induce localized tissue damage. We measured body mass to the nearest 0.01 g using a digital scale and cloacal temperature within 1 min of handling ($\pm$ 0.01° C) using a 36 g Type-T thermocouple that we inserted 1 mm into the cloaca between 0900 and 1130 hrs. We took three temperature measurements in immediate sequence, which were highly repeatable ($F_{645,646}$ = 14.5, P < 0.001, R = 0.91; Lessells and Boag, 1987), and used the average in statistical analyses. Body temperatures of control individuals were also highly repeatable throughout the study ($F_{123,124}$ = 10.4, P < 0.001, R = 0.81). We compared the acclimatory 24-hr period that occurred prior to injection to the change in feeding behavior 24 and 48 hrs post-injection. Thus overall, cloacal temperature and body mass was measured four times (1) 24-hrs prior to injection; (2) immediately prior to injection; (3) 24-hrs after injection; and (4) 48-hrs after injection. Bill color was measured and blood collected twice: (1) immediately prior to injection and (2) 48-hrs after injection. Capture and handling time at 24-hrs after injection was always less than 2 minutes; at other times handling times were always less than 6 minutes. We collected 50-60 µl of blood with a heparinized micro-hematocrit capillary tube using brachial venipuncture. Blood was immediately stored at 4° C, and within 45 minutes plasma was removed following centrifugation for 10 min. and stored at -80° C. Methods for plasma-carotenoid extractions and HPLC analyses follow those described in McGraw et al. (2003). Briefly, HPLC was used to identify types and amounts of carotenoids in plasma samples, but total carotenoid levels (lutein + zeaxanthin) were used because these two measures are highly correlated (McGraw et al., 2003). We measured plasma haptoglobin concentration 48 hrs after CFA exposure according to manufacturer's instructions using a commercial haptoglobin kit (TP801, Tri-Delta Diagnostics, Morris Plains, NJ, USA). Before assaying haptoglobin, we excluded two plasma samples that were red in color (which suggested red blood cell lysis), as sample hemoglobin contamination leads to artificially high haptoglobin readings. Blood parameters (levels of carotenoids and haptoglobin) were measured on all individuals in all treatments except for four unsupplemented controls, leading to smaller sample size for these analyses.

Bill color was measured as spectral reflectance using a USB2000+ spectrometer and PX-2 pulsed xenon light (Ocean Optics, Dunedin, FL, USA) using a bi-furcated encased fiber-optic probe. The probe was mounted and shielded from external light at a fixed distance of 5 mm perpendicular to the bill. All data were acquired as the proportion of reflectance relative to absolute darkness and a white standard (WS-1, Ocean Optics). We used the programs CLR (1.05) and RCLR (0.09.28) (Montgomerie, 2008; Murphy et al., 2009) to obtain colorimetrics for statistical analyses. Because of the range of possible variables that can be derived from spectral reflectance data (Roberts et al., 2007), we chose *a priori* to measure hue as the midpoint wavelength where $R = (R_{max} + R_{min})/2$ (Montgomerie, 2006) based on previous studies finding links between immunity and carotenoid-based bill hue (Hill et al., 2009; McGraw and Ardia, 2003). Measurements were taken in duplicate on each side of the bill, giving four total measures per individual (repeatability of hue: $F_{219,220} = 36.3$, $P < 0.001$, $R^2 = 0.90$); the average value of hue was used in statistical analyses.

Statistical analyses were conducted in R (R Core Development Team, 2015). Before beginning analyses, variables were tested for assumptions of normality using Darling-Anderson tests. All variables were normally distributed ($A < 0.51$, $P \geq 0.18$), except haptoglobin levels and change in blood carotenoid levels over 48 hrs, which were $\log_{10}$-transformed to achieve normality. We used general linear models (package lmer) to test for factors affecting changes in activity levels, cloacal body temperature, and bill color, and for differences in haptoglobin levels following APR-induction. For all mixed models, we included the main effects of carotenoid supplementation, immune stimulation, and sex, as well as the interaction between the two experimental treatments, as well as the three-way interaction between the two treatments and sex. For each model we included the initial value of each measure at time 0 as a covariate; for change in bill hue, we also included change in activity as a covariate. We included cage as random factor to control for cage effects. P-values for fixed effects from mixed models were obtained via a Wald X-square test via the car package; pairwise Tukey-adjusted comparisons were evaluated using the lsmeans package. To test for differences in plasma haptoglobin concentration between treatments, we used a general linear model with CFA-exposure, carotenoid supplementation, and sex as main effects, as well as their interaction. To test for the effect of carotenoid supplementation of the four-week supplementation period, we used a general linear model that included carotenoid

supplementation and sex as main effects, as well as their interaction. Values reported in the Results section are means ± SE.

RESULTS

Influence Of Carotenoids On Acute Phase Response

Birds exposed to CFA reduced feeding and activity over 24 hours (effect of immune activation: $X^2 = 136.2$, df = 1, $P < 0.001$; Fig. 2), with birds showing higher levels of initial activity also showing higher reduction in activity over 24 hours ($X^2 = 103.1$, df = 1, $P < 0.001$. There was no effect of carotenoid supplementation, sex, or the interaction between carotenoid supplementation and immune activation or the three-way interaction among treatments and sex on the change in activity over 24-hrs (all $X^2 < 3.4$, $P > 0.14$).

Carotenoid-supplemented CFA-injected birds returned activity levels to pre-injection levels at 48 hours post-injection, while the carotenoid-unsupplemented CFA-injected individuals did not (effect of carotenoids $X^2 = 5.1$, df = 1, $P = 0.02$; effect of immune activation $X^2 = 11.5$, df = 1, $P < 0.001$; carotenoids * immune activation interaction $X^2 = 4.3$, df = 1, $P = 0.03$; Fig. 2). There was no effect of sex ($X^2 = 1.3$, df = 1, $P = 0.24$) or activity level at 24 hours ($X^2 = 0.31$, df = 1, P = 057) or a three-way interaction among treatments and sex ($X^2 = 5.74$, df = 3, $P = 0.12$) on change in feeding and activity.

Exposure to CFA increased body temperature (effect of immune activation: $X^2 = 34.7$, df = 1, $P < 0.001$; Fig. 3), but carotenoid-supplemented birds showed a smaller increase in body temperature 24-hrs following CFA exposure compared to immune-stimulated non-supplemented birds (carotenoid supplementation: $X^2 = 3.1$, df = 1, $P = 0.07$; carotenoid * immune activation interaction $X^2 = 4.1$, df = 1, $P = 0.04$; Fig. 3). Individuals with lower body temperatures at the time of immune activation showed the largest increases in body temperature ($X^2 = 6.7$, df = 1, $P = 0.009$). There was no effect of sex ($X^2 = 0.1$, df = 1, $P = 0.74$) or a three-way interaction among treatments and sex ($X^2 = 5.5$, df = 3, $P = 0.13$) on change in body temperature. All four treatments returned to similar levels of body temperature 48 hrs post injection (All $X^2 < 2.1$, Ps > 0.14; Fig. 3).

CFA-exposed birds decreased in body mass after 24 hrs (Fig. 4; effect of immune activation: $X^2 = 32.1$, df = 1, $P < 0.001$), but no effect of carotenoid supplementation ($X^2 = 4.9$, df = 1, $P = 0.06$) or an interaction between treatments ($X^2 = 0.6$, df = 1, $P = 0.45$). There was

no effect of initial body mass (X^2 = 0.7, df = 1, P = 0.40), sex (X^2 = 0.1, df = 1, P = 0.78) or a three-way interaction among treatments and sex (X^2 = 5.1, df = 3, P = 0.15) on change in body mass over 24-hours. Birds that lost mass at 24-hrs regained it by 48 hrs, but carotenoid-supplemented CFA-injected birds recovered to pre-injection mass, while unsupplemented CFA-injected birds did not (Fig. 4).

Effects on blood carotenoids and haptoglobin levels

Blood carotenoid levels significantly decreased over a 48-hr period in CFA-exposed birds relative to controls (X^2 = 116.6, df = 1, P < 0.001; Fig 5). Carotenoid-supplemented birds undergoing immune activation exhibited a much larger decrease in blood carotenoid levels than did unsupplemented immune-activated birds (effect of supplementation X^2 = 3.4, df = 1, P = 0.06; carotenoid * immune activation interaction X^2 = 26.15, df = 1, P < 0.001), as birds with higher initial levels showed much stronger declines over time (X^2 = 34.9, df = 1, P < 0.001). There was no effect of sex (X^2 = 0.1, df = 1, P = 0.99) or a three-way interaction among treatments and sex (X^2 = 2.8, df = 3, P = 0.41) on change in blood carotenoid levels over 48-hours.

Birds exposed to CFA had higher plasma haptoglobin levels than did non-CFA injected birds. Within CFA-injected treatments, carotenoid-supplemented birds had lower haptoglobin levels at 48-hrs post injection compared to non-supplemented CFA-injected birds (Fig. 6; effect of immune activation: X^2 = 50.4, df = 1, P < 0.0001; carotenoids: X^2 = 13.9, df = 1, P < 0.001; immune activation x carotenoid supplementation: X^2 =7.1, df = 1, P = 0.007), but with no effect of sex (X^2 = 1.1, df = 1, P = 0.28).

Effects Of Immune Activation And Carotenoid Supplementation On Bill Color

Four weeks of carotenoid supplementation increased bill redness in both sexes relative to control birds (Table S2). After exposure to CFA, bill hue declined strongly in both CFA-injected treatments (X^2 = 32.7, df = 1, P < 0.001; Fig. 7), with a much stronger decline in unsupplemented CFA-injected birds compared to carotenoid-supplemented CFA-injected birds (effect of carotenoid supplementation: X^2 = 23.4, df = 1, P < 0.001; carotenoid * immune activation interaction: X^2 = 5.8, df = 1, P = 0.01). There was no effect of sex (X^2 = 3.0, df = 1, P = 0.08) or a three-way interaction among treatments and sex (X^2 = 5.1, df = 3, P = 0.16) on

change in bill hue over 48-hours. There was no effect of change in activity and feeding over 48-hours on change in bill hue ($X^2 = 0.8$, df = 1, P = 0.36), but birds with the brightest bills at the start showed the greatest decline ($X^2 = 48.9$, df = 1, $P < 0.001$)

DISCUSSION

We found support for our predictions that carotenoids dampen effects of an acute phase response (APR) on fever, sickness behavior, circulating haptoglobin levels, and bill coloration. Overall, the effects suggest a link between carotenoids and health state. Typically, the APR leads to anorexia and reduced water intake (Sköld-Chiriac et al., 2014), in part to reduce micronutrient access by bacterial pathogens (Klasing, 1984), with resultant large reductions in body mass. Our carotenoid-supplemented birds exposed to CFA exhibited a lower reduction in activity and more rapid return to pre-APR activity levels, suggesting carotenoids ameliorated the behavioral responses of birds to APR. Similarly, carotenoid supplementation led to a smaller increase in body temperature, thus reducing the energy cost associated with temperature elevation. Lastly, carotenoid supplementation led to lower levels of an acute phase protein (haptoglobin) than seen in unsupplemented birds exposed to CFA. In addition, CFA exposure lead to rapid reductions in bill color, which were in turn correlated with changes in blood carotenoid levels. Our results are the first study, to our knowledge, to study these questions in a colorful songbird and are novel in the integration of physiological measures of body temperature and haptoglobin with a sexually selected signal of quality.

We believe our design was effective in detecting an APR. Key symptoms of the APR are fever and reduced activity/feeding (Adelman and Martin, 2009; Hart, 1988; Sköld-Chiriac et al., 2014). Our use of automated perch counters revealed a reduction in feeding and activity within 24-48 hrs after CFA injection. In addition, we found cloacal temperature to be both a repeatable intraindividual trait and to increase in response to CFA exposure. Thus, here we report that CFA exposure lead to both a decrease in feeding and an increase in body temperature (Adelman and Martin, 2009; Hart, 1988; Sköld-Chiriac et al., 2014), as well as higher levels of haptoglobin (Gabay and Kushner, 1999) compared to controls. In addition, CFA-injected birds exhibited classic phenotypic symptoms of sickness, including fluffed feathers, mass loss, and a hunched posture over a 24-48 hr period (pers. obs.), without mortality. Thus, we are confident that physiologically relevant CFA exposure in our study

elicited an APR and led to changes in physiology and behavior distinctly different from control birds that were given PBS injections.

The potential benefits and costs of a carotenoid-buffered APR are difficult to interpret. Generally, carotenoid supplementation leads to enhanced immune responses when individuals are challenged with a mitogen or novel antigen (Blount et al., 2003; McGraw and Ardia, 2003), both of which elicit immune responses that have low activation costs (Lee, 2006; Martin et al., 2008). The APR, in contrast, is considered a highly costly response in terms of energy, immunopathology, and opportunity costs (Adelman and Martin, 2009; Owen-Ashley et al., 2006). In many life-history contexts, there is variation in the strength of the APR, presumably due to varying costs and benefits of activating such a costly defense (Lee et al., 2005; Martin et al., 2008). Here we report that carotenoids led to a milder APR response, associated with lower temperature increases, a smaller reduction in feeding, lower levels of haptoglobin, and greater ability to maintain bill redness. These reduced energetic and nutrient costs, as outlined earlier, thus reduce the cost of an APR. One benefit could be the ability to maintain a signal of quality, bill redness, which does not show as large a decrease in redness in carotenoid-supplemented birds that mount an APR.. More study is clearly needed to understand the long-term costs and benefits of carotenoid-buffering of the APR. It is also important to note that other components of the supplementation could also have provided health benefits. Carotenoids are delivered in beadlets that also contained small amounts of tocopherol, an antioxidant (Di Mascio et al., 1991). While we cannot rule out that there were effects of both carotenoids and tocopherol, we are confident that carotenoids are an important driver of the response we observed as previous work found strong carotenoid effects when compared to beadlets alone (McGraw et al., 2005).

Moreover, carotenoid levels declined in circulation, and presumably in the bill (i.e. color fading), as a function of the APR, which is consistent with the only other avian study examining the APR and changes in plasma carotenoid levels (McGraw and Klasing, 2006). The fact that bill color was reduced in carotenoid-supplemented, CFA-induced birds suggests that even high levels of blood carotenoids are not sufficient for health support in the face of an APR. Such an APR-driven decrease in body carotenoids suggests that (1) the decreased feeding rate of CFA-treated birds provided them with fewer dietary carotenoids to accumulate in the body, (2) immune activation diverted carotenoid supplies away from bill and to immune tissues, perhaps as antioxidants or immunomodulators (see more below), (3) the APR impaired

metabolism of red carotenoids at the beak (McGraw and Toomey, 2010), and/or (4) a change in blood flow of the vascularized tissue beneath the bill (Butler et al., 2011), because the pigmented, keratinized beak tissue is dead and birds cannot retrieve red pigments from bill tissue for other uses in the body. The effect of immune activation was the main influence on reduction in bill hue, as we found no link between individual reduction in feeding and reduction in bill color.

One consequence of fever is the production of reactive oxygen species (ROS) (Finkel and Holbrook, 2000; Kluger et al., 1998; Roe and Kinney, 1965). Carotenoids can serve as antioxidants and free radical scavengers and may thus reduce the damage caused by ROS production (Edge et al., 1997; McGraw, 2005). The response we observed may also be due to changes in physiological mechanisms triggered by activation of the APR. Multiple physiological systems, particularly the nervous and endocrine system, work together to adjust the severity of the APR (Adelman and Martin, 2009). Carotenoid levels may play a role in influencing responsiveness to inducing the APR. Thus, carotenoids themselves may play no direct role through the immune system in influencing the intensity of the APR, but may affect organismal-level tradeoffs of costs and benefits, which in turn lead to differing investments in APR. Carotenoid levels may interact with hormones levels, such as glucocorticoids or testosterone, which may in turn affect APR response. For example, increased testosterone leads to increased blood carotenoid levels in red-legged partridges (*Alectoris rufa*) (Blas et al., 2006), while elevated corticosterone leads to decreased carotenoid levels in red grouse (*Lagopus lagopus*) (Mougeot et al., 2010). A further possibility is the interaction of carotenoids with gap-junctional communication (Stahl and Sies, 1998), which in turn could affect the severity of the APR through reduced signaling leading to an attenuated inflammation response. These possible interactions are speculations and are intended to frame future research on how carotenoids may influence the costly but effective APR immune response.

Because we elicited the APR using an adjuvant containing inactivated bacterial pathogens rather than a real pathogen, our manipulation suggest that carotenoids reduce the energetic cost of the APR, independent of any downstream pathways related to pathogen combat *per se*. Some studies do, however, also link carotenoid pigment accumulation to pathogen/parasite resistance (Dawson and Bortolotti, 2006). Given that the APR involves a set of complex and integrated responses and that our study examined only a subset of these, it is

unclear if other aspects of the APR are also affected. The fact that circulating haptoglobin levels were lower in carotenoid-supplemented birds from our study suggests high levels of haptoglobin are not needed. Haptoglobin works, in part, by weakening pathogen ability to bind iron, a key growth factor for proliferation of pathogens (Dobryszycka, 1997; Drain et al., 2001; Gutteridge, 1987; Imrie et al., 2004). Further research is needed to test whether higher haptoglobin levels are not needed if carotenoids are diverted to support the immune system performance as both carotenoids and haptoglobin scour reactive oxygen species, a physiological function of both (Dobryszycka, 1997; Edge et al., 1997; Quaye, 2008). The influence of carotenoids on haptoglobin levels during an APR is equivocal in other studies, however. In young broiler chickens, carotenoids reduced increases of haptoglobin (Koutsos et al., 2006) similar to our study, but in a smaller study of adult junglefowl (McGraw and Klasing, 2006) no relationship between carotenoids and haptoglobin was reported. The next step is to examine a link between APR intensity, actual pathogen/parasite resistance, and the molecular actions of carotenoids on specific immunological components.

The fact that bill color changed markedly over a 48-hr period supports growing evidence that bills may serve as very short-term signals of quality (Ardia et al., 2010; Hill, 2006; Hill et al., 2009; Rosenthal et al., 2012). For example, male American goldfinches show fading in bill color when housed in captivity for 24 hrs (Rosen and Tarvin, 2006). Immune activation leads to rapid (7 days or less) bill or foot color changes in mallards (*Anas platyrhynchos* Linnaeus) (Peters et al., 2004) and blue-footed boobies (Velando et al., 2006). In addition, in a separate study we found that injection of zebra finches with testosterone led to significant increases in bill hue and chroma over a 4-day period (Ardia et al., 2010). The dynamic nature of carotenoid-based integuments, such as bills and legs, suggests that they represent a condition-based signal potentially different from more frequently considered feather color ornaments. However, the rapid changing of bill color suggests that the long-term signaling value of sensitive, real-time indicators may be low and/or different, as integrated phenotypic traits, such as feathers, may better reflect overall condition or quality (Merrill et al., 2016).

Interestingly, we found no sex differences in our results, in contrast to previous work finding sex differences in the effects of carotenoids on immunity (Grether et al., 2004; McGraw and Ardia, 2005). Both males and females exhibited similar APR responses and had similar

reductions in bill color following APR. Even though males generally maintain higher levels of blood carotenoids than females (McGraw and Ardia, 2005), it appears that both sexes may draw down carotenoids from integumentary investment at similar levels, at least in response to an APR. More research is needed to determine sex differences in allocations to different immunological or pathogenic threats. Overall, our results demonstrate that carotenoids buffer the acute phase response and that bill color, a sexually selected signal in zebra finches, can change rapidly following induction of the costly APR immune defense.

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Author contributions D.A., B.S., and D.G. equally conceptualized the study and performed the experiments; K.M. conducted carotenoid analyses; D.A. carried out data analysis and wrote the first draft of the manuscript; All authors contributed to revising the manuscript.

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Figures

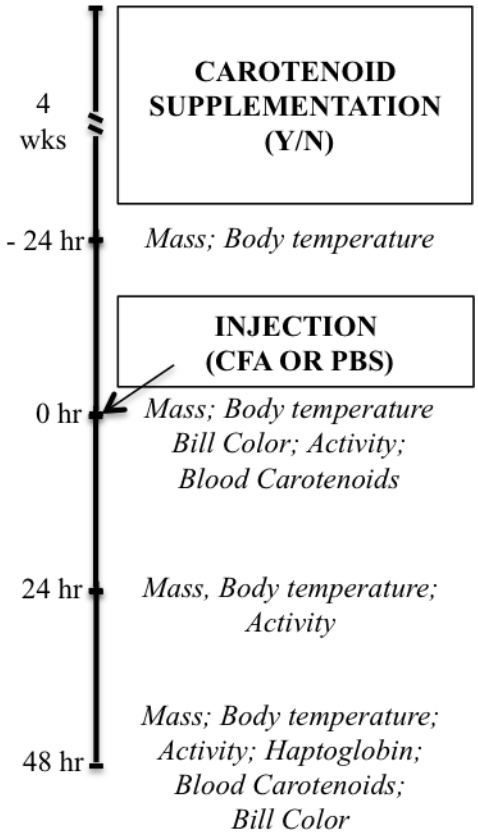

Figure 1. Experimental timeline for study. Main treatments are in bold; main measurements are italicized.

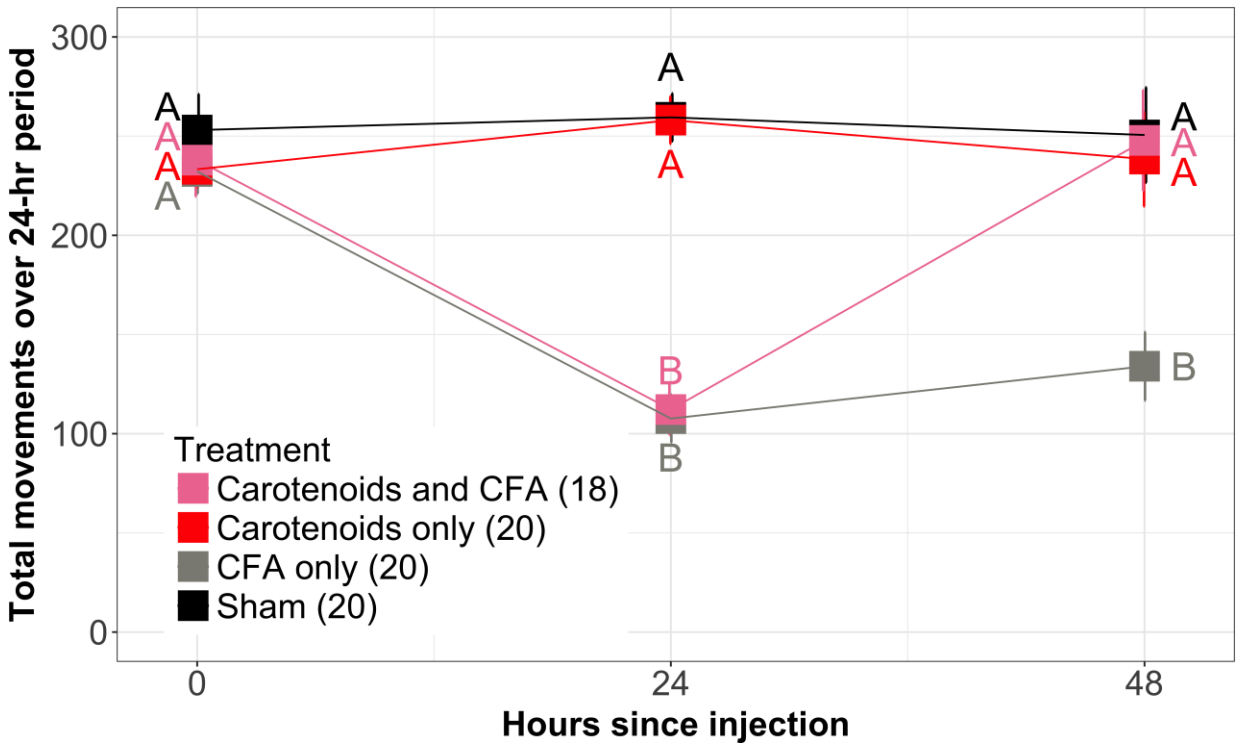

Figure 2. Behavioral responses of zebra finches over the 48-hr period after the induction of a systemic inflammatory response through injection with Complete Freund's Adjuvant (CFA). Carotenoids = birds who were supplemented with carotenoids for four weeks prior to injection. Activity levels were recorded using automated perch counters attached to feeding dishes. Values shown are least square means +/- SE; letters refer to Tukey-corrected differences at $P < 0.05$ from GLM mixed models, with samples sizes in parentheses.

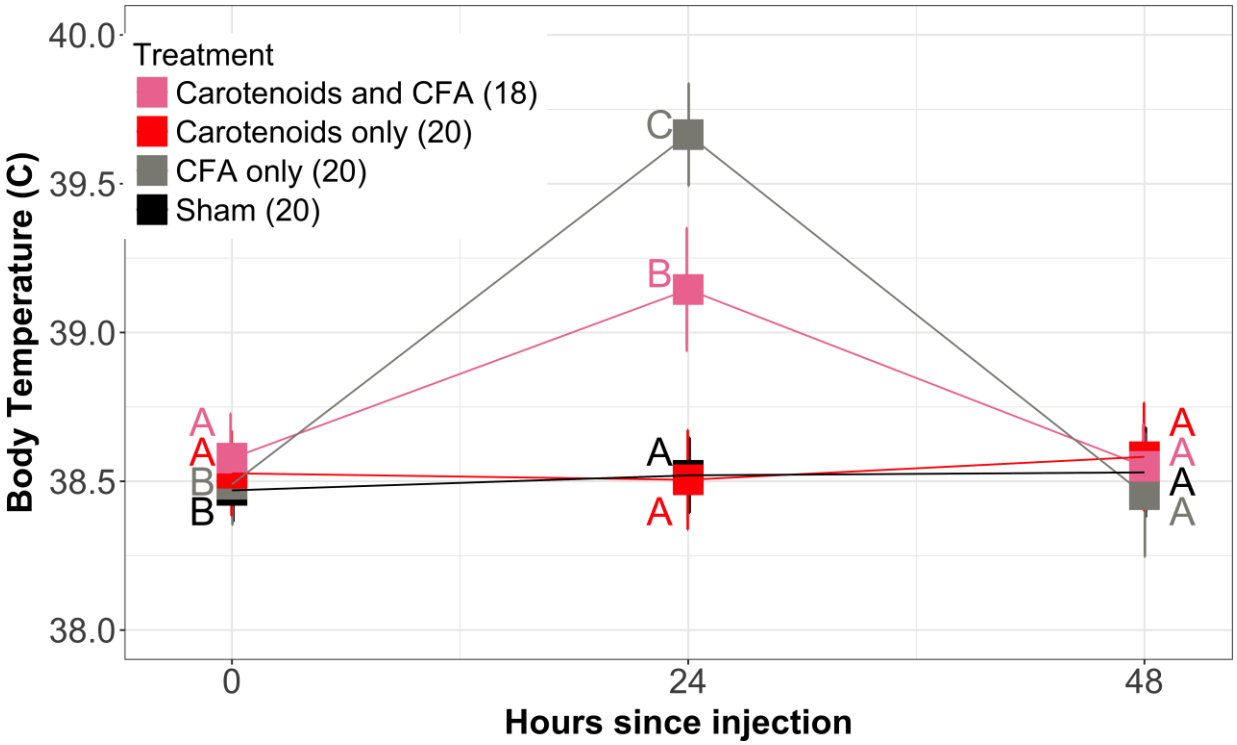

Figure 3. Cloacal body temperature over the 48-hr period following the induction of a systemic inflammatory response through injection with Complete Freund's Adjuvant in zebra finches. See legend of Fig. 2 for additional information about the figure.

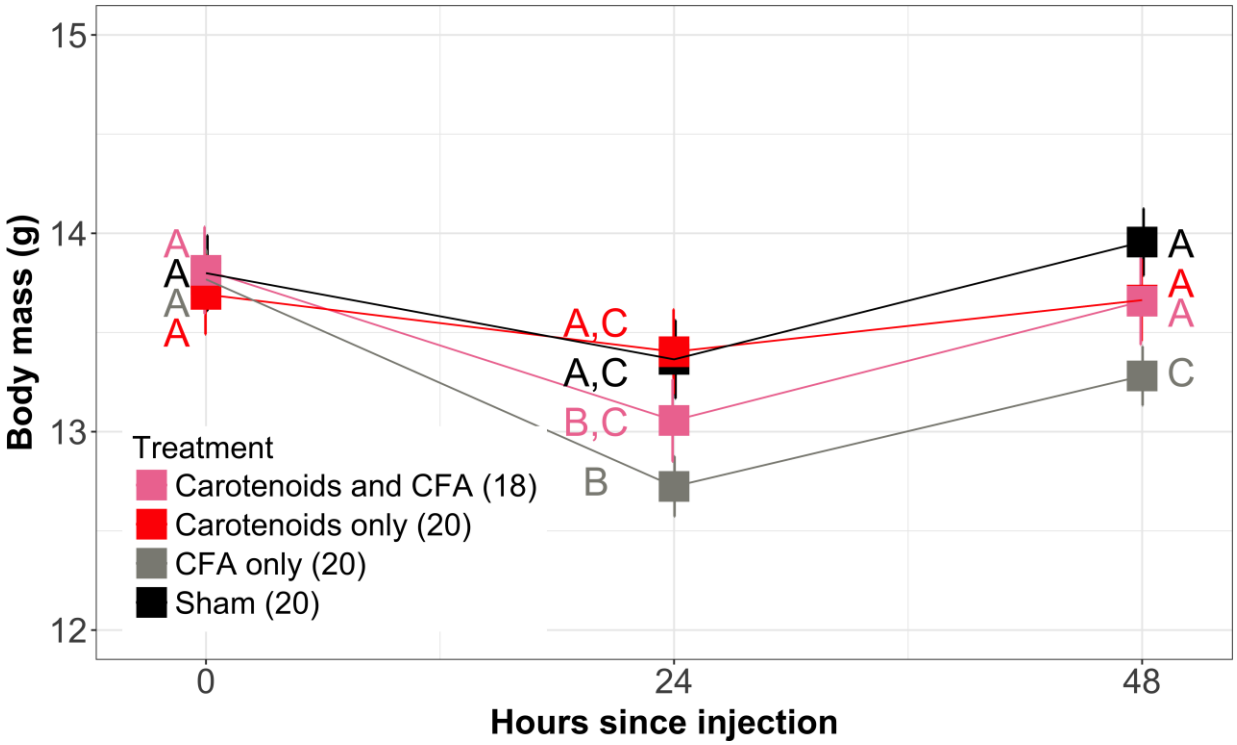

Figure 4. Body mass over the 48-hr period following the induction of a systemic inflammatory response through injection with Complete Freund's Adjuvant in zebra finches. See legend of Fig. 2 for additional information about the figure.

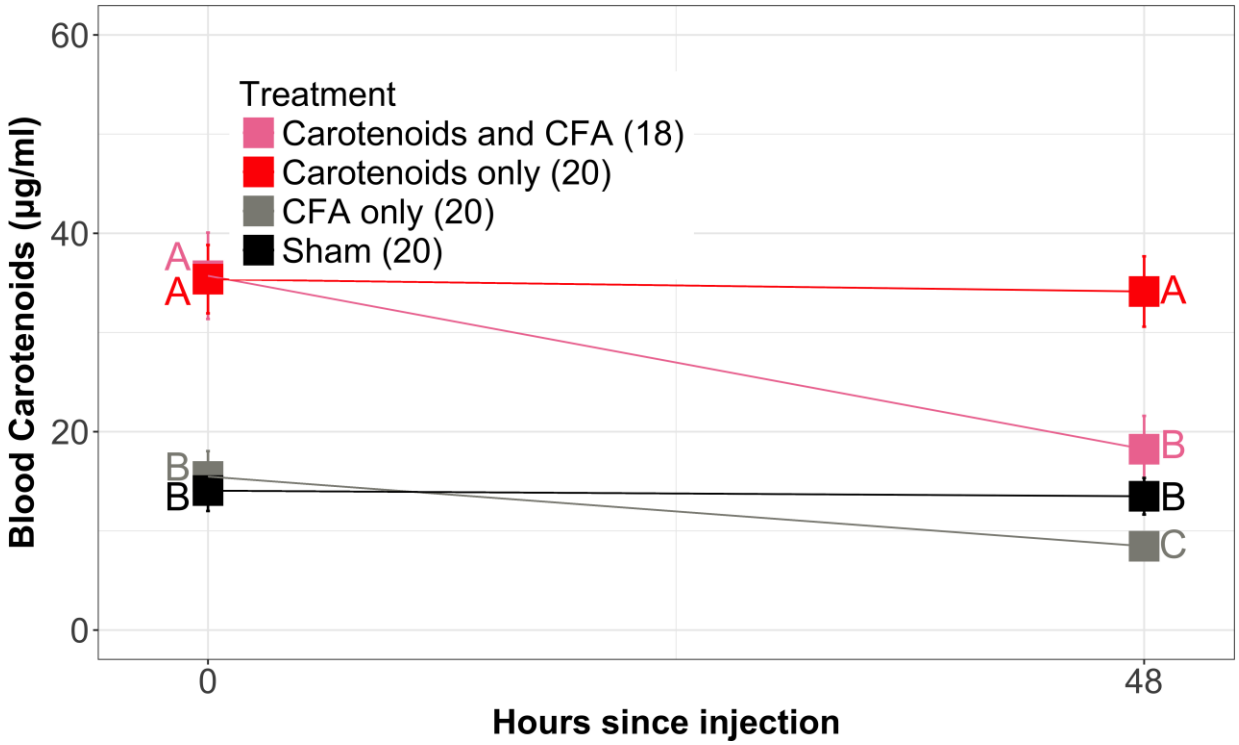

Figure 5. Blood carotenoid levels over the 48-hr period following induction of a systemic inflammatory response through injection with Complete Freund's Adjuvant in zebra finches. See legend of Fig. 2 for additional information about the figure.

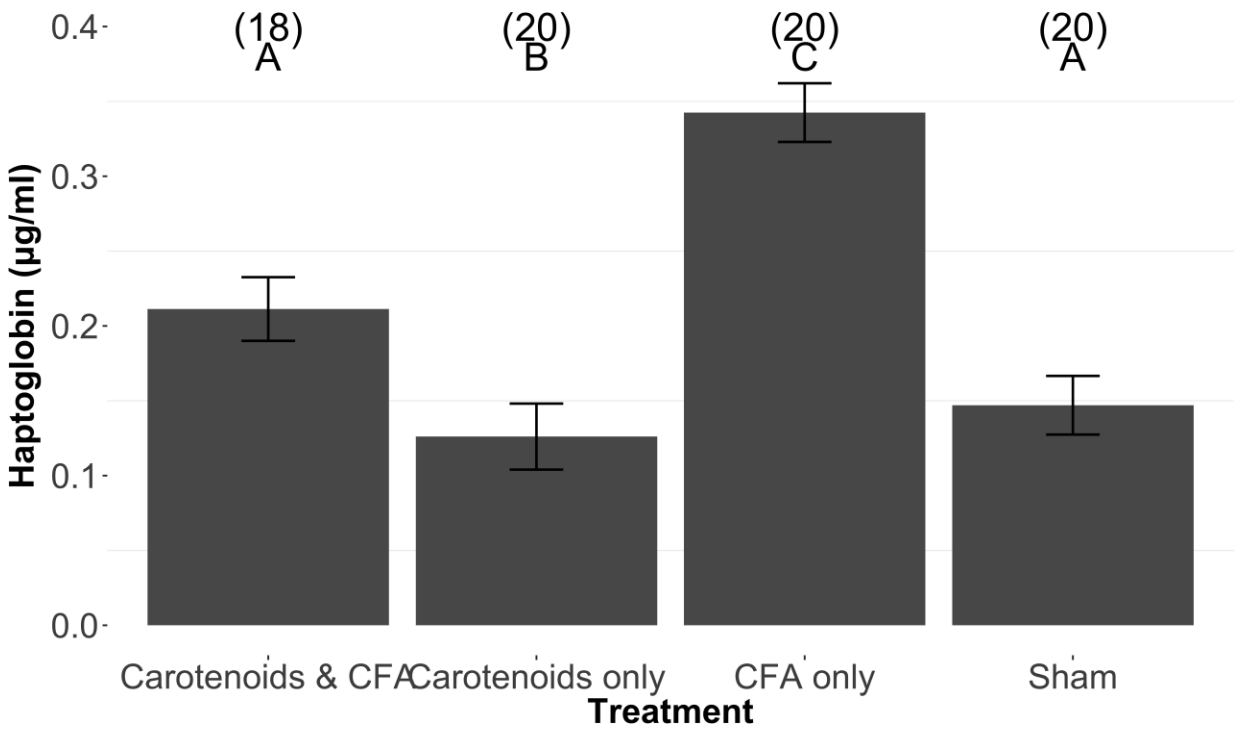

Fig. 6. Haptoglobin levels 48-hrs following induction of a systemic inflammatory response through injection with Complete Freund's Adjuvant in zebra finches. See legend of Fig. 2 for additional information about the figure.

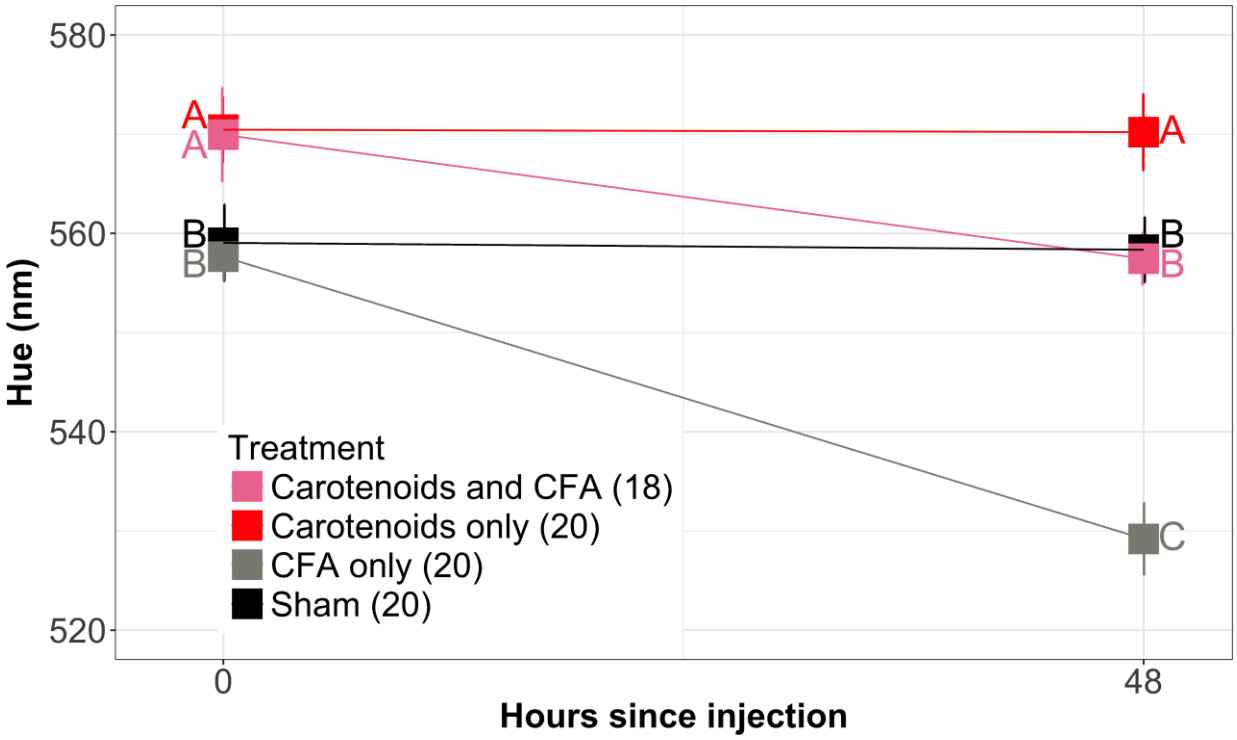

Figure 7. Bill hue (wavelength of maximum reflectance) over the 48-hr period following induction of a systemic inflammatory response through injection with Complete Freund's Adjuvant in zebra finches. See legend of Fig. 2 for additional information about the figure.