THE PHYSIOLOGICAL EFFECTS OF BRIGHT PLUMAGE COLORATION

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Kristal Alissa Huggins

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THE PHYSIOLOGICAL EFFECTS OF BRIGHT PLUMAGE COLORATION

Kristal Alissa Huggins

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THE PHYSIOLOGICAL EFFECTS OF BRIGHT PLUMAGE COLORATION

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Signature of Author

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Date of Graduation

VITA

Kristal Alissa Huggins, daughter of Kimuel Alonzo Huggins and Leslye Blanchet (Winslow) Huggins was born March 19,1977. She graduated from Eleanor McMain Magnet High School, New Orleans, Louisiana, with Honors in June 1995. She received a Bachelor of Science in Biology with Honors from Xavier University, New Orleans, Louisiana in May 1999. She began graduate school at Auburn University in August 2003. While attending, she worked as a Graduate Teaching Assistant in the Department of Biological Sciences. During her time at Auburn she received the Biological Sciences Departmental Graduate Teaching Award 2006-2007 and the Kenneth Ottis Distinguished Graduate Fellowship Award for outstanding achievement in Physiology/ Molecular Biosciences in 2007-2008.

THESIS ABSTRACT

THE PHYSIOLOGICAL EFFECTS OF BRIGHT PLUMAGE COLORATION

Kristal Alissa Huggins

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Songbirds with extensive, carotenoid-based plumage tend to maintain high levels of circulating carotenoids in plasma, which could represent a potential tradeoff between maintaining brightness at the expense of carotenoid-related toxicities. To test this hypothesis, we maintained American goldfinch (*Carduelis tristas*) males on either a high (n=40) or a low (n=40) dose of lutein/zeaxanthan treatment for 60 days for two years during the time of molt. We took blood samples from animals before, during, and after supplementation, and analyzed the samples for aspartate amino transferase (AST) as a measure of liver and creatine kinase (CK) as a measure of muscle degradation. Additionally, we tested muscle function using a vertical ascent test, a technique that tests the performance capability of the pectoral muscles in birds. We found that AST levels were not affected. In addition, CK levels were significantly higher 60 days after the end of supplementation in animals receiving a high dose of dietary carotenoids ($p = 0.008$), indicating the presence of muscular degradation in these birds. Birds in the same treatment had significantly reduced flight elevations at the same time as the significant

elevation in CK levels ($p=0.008$) at the time creatine kinase levels were elevated indicating that there was a direct effect between increased carotenoid intake, increased creatine kinase levels and outward physical ability.

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I want to thank my mother for her love, understanding and patience while pursuing my passion. Thank you for never questioning why I was still in school. I want to thank my family and friends for their encouragement. And I want to thank my father, grandparents, the Huggins family and Winslow family, my Aunt Violet and Uncle Vernon, because I know that you had everything to do with me becoming a biologist.

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Geoff, I want to thank you for putting up with my avian ignorance and encouraging me throughout the years. Without your dedication to the study of female mate choice and male plumage coloration, I would not have had the experiences and opportunities that I did, thank you.

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ONWARD! UPWARD!

Style manual of journal used: Functional Ecology

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INTRODUCTION

Sexually selected traits have been discussed in terms of their signal value since the early 1900's when Fisher related these traits to increased survivorship and fecundity of the males that possessed them (Fisher, 1915; Fisher, 1930). Since then, these visual traits have been recognized as honest signals in intersexual communication and are used by females to assess the health status and foraging ability of perspective mates (Andersson, 1994; Hill, 2002). Honest signaling and the handicap hypothesis (Andersson, 1994; Zahavi, 1975) predict that a signal will be a reliable indicator of quality when two stipulations are met: the signal must impose a burden on the individual that possesses it and the individual must sufficiently cope with the burden. These qualifiers indicate that the signal must have an associated cost of acquiring or possessing it to have meaning. If this assumption was not true, then every individual would acquire the same advantageous characteristics with relative ease and/or individuals without signals would be chosen just as readily as their signaling competitors.

Visible ornaments in male birds are one such trait: many studies indicate that males with more elaborate expression of ornamental traits gain a mating advantage (Hill,1991). Moreover, as predicted by both honest signaling and the handicap hypotheses, such ornaments have a cost to the individuals who possess them. Some of costs associated with visible ornaments include predation (Promislow et al, 1992; Rosenthal et al, 2001), energetic constraints (Hill, 2000), decreased locomotor ability

(Evans and Hatchwell,1992; Evans and Thomas,1992; Møller, 1989) and redirection of resources (McGraw et al,2004; Saino et al,1999). However, it is often difficult to determine how the actual production and maintenance of ornaments affect individuals, and, thus, the physiological costs incurred by acquiring ornaments are rarely examined.

A visible ornament type that can potentially lend itself to a study of physiological costs is the red and yellow plumage of birds.. The yellow, orange and red hues of some avian plumage are indicative of carotenoid pigmentation (Hill and McGraw, 2006a). Vertebrates cannot produce carotenoids *de novo*, so they obtain them through their diet (Brush, 1981; Hill and McGraw, 2006a). Carotenoids are found abundantly in plant matter, which can make up a large percent of the diet in many birds. Studies investigating the relation ship between fitness and sexually dimorphic carotenoid plumage coloration in birds are numerous. For example, carotenoids are deposited in the plumage and have been shown to correlate to mate choice (Hill, 1990; Hill et al. 2002; MacDougall and Montgomerie, 2003; Sundberg, 1995), increased feeding in parental care (Casagrande et al., 2006; Hill, 1991; Linville et al, 1998), degree of parasitism (Brawner et al., 2000; Hamilton and Zuk, 1982; Møller, 1990), and aspects of immunocompetence and free radical scavenging (Blount et al., 2003; Camplani et al, 1999; Hill, 1999; Navara and Hill, 2003; Saino et al, 1999). More brightly colored males often pair with a mate earlier and have a lower parasite load (Brawner et al., 2000; Hamilton and Zuk, 1982; Hill, 1990; Hill et al., 2002; MacDougall and Montgomerie, 2003; Møller, 1990; Sundberg, 1995). The literature regarding immunocompetence of brightly colored males is inconclusive (Blount et al., 2003; Camplani et al, 1999; Hill, 1999; Navara and Hill, 2003), but studies examining free radical scavenging indicate a positive correlation

between carotenoid uses for immune challenges and depletion of plasma carotenoid concentrations and integument coloration (Alonzo-Alvarez et al., 2004; Saino et al, 1999).

Some studies that have linked social and health differences with carotenoid based ornamentation have suggested that the cost of carotenoid ornamentation is behavioral, reflecting foraging ability, rather than carotenoids imposing a direct physiological cost as an indication of honest signaling (Hill, 1996; Hill, 1999). Studies that have investigated the physiological effects of carotenoids have focused on the beneficial aspects of these compounds (Olsen and Owens, 1998). No studies have focused on the physiological costs of high carotenoid intake alone in reference to carotenoid ornamentation.

Carotenoids have been shown to play a central role in oxidative homeostasis in vertebrate organisms (Chew, 1993; Stahl and Sies, 2003; Surai, 2002; Young and Lowe, 2001). Carotenoids decrease levels of free radical activity in living systems by binding free radicals before they are able to cause tissue damage (El-Agamey et al., 2004; Mortensen et al., 2001; Stahl and Sies, 2003). Physiologically, carotenoids have been shown to directly benefit the immune system (Bendich, 1989; Chew, 1993; Olsen and Owen, 1998) cardiovascular and ocular health (Mayne, 1996). However, these studies also demonstrate that high levels of carotenoids can have detrimental physiological impacts, such as increased tumor cell proliferation (Fiala et al, 2005; Wang et al, 1999). Most studies that show benefits directly tied to carotenoid supplementation are based on relative low doses of carotenoids consumed by mammals that do not display carotenoid-rich ornaments. As addressed by Hill (1999), fish and, especially, bird species with

carotenoid ornamentation require circulating carotenoid levels that are orders of magnitude greater than that of mammalian subjects.

Species that display carotenoid-based ornamental displays naturally accumulate high levels of carotenoids during the period before molt associated with ingestion of carotenoid-rich foods (Hill, 1995; Hill, 1999; McGraw and Gregory, 2004; Negro et al 1998). Because these increases in carotenoid intake and systemic carotenoid content occur only seasonally and greatly surpass the basal amounts for these species, this increase in carotenoid intake can be referred to as hyper-accumulation. The processes of carotenoid hyper-accumulation and carotenoid pigment deposition can be thought of as the potential means, driven by the sexually selective pressures, by which pigmented plumage serves as a signal to potential mates. This accumulation can have a range of positive and/or negative outcomes on systemic tissues, depending on the concentration and types of carotenoids that have accumulated (Zahavi, 1997). If sustaining high levels of carotenoids had negative or adverse effects on physiological homeostatic conditions, the subsequent external plumage saturation would then serve as a beacon for what the individual has overcome.

The American Goldfinch (*Carduelis trista)* is an excellent species to study the physiological effects of high carotenoid intake. Goldfinches are small passerine birds that exhibit bright, carotenoid-based ornamentation. Males display a range of yellow plumage ornamentation on most of their body that varies from drab pale yellow to bright canary yellow, while females are drabber and more olive in color. The amount of coloration that they display in their plumage is directly related to the amount of carotenoids they are able to ingest during feather production (Hill, 1992; McGraw et. al., 2002). High individual

variation in the levels of carotenoids ingested by these birds in the wild makes them the ideal for studying the physiological effects of high carotenoid doses. In addition, the type and amount of supplementary carotenoids that this species must ingest to attain natural variation has already been reported (McGraw et al., 2001; McGraw et al., 2002; McGraw and Gregory, 2004; McGraw and Hill, 2001). Variation of circulating carotenoids is 80% greater in molting vs. non-molting males (McGraw and Gregory 2004), verifying seasonal hyper-accumulation of carotenoids in this species.

While some carotenoids are ultimately destined for integument, they must first travel through circulation after consumption, exposing a variety of other tissues to carotenoids. Thus, it is necessary to assess how elevated circulating levels of carotenoids affect non-target tissues to investigate if there is a physiological cost associated with these elevated levels. Potential target tissues that should be especially impacted by hyperacculmulation of carotenoids are the liver due to its role in carotenoid metabolism and storage as well as its high level of vascularization, which can potentially lead to increased contact with these compounds (Furr et al, 1997; Parker, 1996) and skeletal muscle tissue because of, again, the high level of vascularization in the endomysium. If liver and skeletal muscle functions are negatively impacted by exposure to sustained, elevated levels of carotenoids, then enzymes indicating tissue degradation should be elevated. If enzymes indicative of both liver and skeletal muscle degradation are elevated, it is suggestive of a general systemic degradative process, but if either organ level enzyme is elevated independently, the negative impact may be isolated to that particular system. Conversely, if carotenoids have a beneficial, anti-oxidant effect, then a decrease in degradative enzymes should occur (Stahl and Sies, 2003; Young and Lowe,

2001). In this study, I tested, whether birds given high levels of carotenoids in their diet displayed changes in enzyme levels that would indicate degradation in either liver and/or skeletal muscle function. Additionally, I tested whether flight muscle performance (as measured by vertical jump performance) would also be affected by high levels of carotenoids in the diet.

METHODS

Collection and Housing

Male American goldfinches were captured in mid January to mid February using mist nets and basket traps around established feeder sites in Alabama. Captured males were kept in flocks (per cage N =20; total N=80) at the Auburn University Aviary in outdoor flight cages (2.31m×2.54m×1.16m). Birds were maintained on *ad libitum* black oil sunflower and thistle seed, which contains negligible amounts of carotenoids. Sulfadimethoxine was supplemented to the water supply as an anticoccidial agent as this species is prone to coccidial infection in captive flocks (Brauner et al., 2000; McGraw and Hill, 2000).

Carotenoid Supplementation

Lutein and zeaxanthin (DSM Nutritional Products), the primary carotenoid pigments found in the plasma and feathers of American goldfinches (McGraw et al., 2002; McGraw and Gregory, 2004) were added to each group's water supply. Carotenoid supplementation consisted of two treatments, low and high levels in a lutein / zeaxanthin ratio of 70:30 diluted in tap water [dosages follow previous carotenoid supplementation studies in American goldfinches [Mc Graw et.al (2002) and Navara and Hill (2003)]. The low treatment contained 0.028 grams of lutein and 0.012 grams of zeaxanthin per gallon of water. The high treatment contained 2.8 grams of lutein and 1.2 grams of zeaxanthin

per gallon of water. Both the high and low treatment groups were supplemented with carotenoid concentrations typical of wild birds (Hill et al., 2000; McGraw and Hill, 2001; McGraw et al., 2002). Carotenoid supplementation was given prior to the molting period and was removed at the end of molt (Middleton, 1977).

Blood Sampling for Creatine Kinase and Aspartate Amino Transferase

Blood was taken from subset of birds from each of the high and low carotenoid supplemented groups taken at capture, the day before supplementation began, 7 days on carotenoid supplementation, 30 days on carotenoid supplementation, 60 days on carotenoid supplementation, 30 days after carotenoid supplementation was removed, and 60 days after carotenoid supplementation was removed (Fig.1). Birds were caught with butterfly nets from outdoor aviary enclosure and placed individually in brown paper bags until sample was collected. All birds were bled within 1 hour of capture. Venapuncture was performed by nicking the brachial (wing) vein and collecting the blood using heparanized microhematocrit tubes. The blood samples were refrigerated until centrifuged (within the hour). Samples were centrifuged at 14,000 rpm for 6 min. Plasma was stored at –20 ºC until assayed for muscle and liver enzymes.

Measurement of Liver and Muscle Enzymes

Plasma enzyme analysis was performed using Roche automated clinical chemistry analyzers at Auburn University School of Veterinary Medicine Clinical Pathology Laboratory. To test liver function, we measured plasma concentration of aspartate amino transferase (AST). This enzyme a reliable serum indicator of hepatocellular damage in

birds (Harr, 2002). In addition, creatine kinase (CK) was measured as an indicator of systemic muscle degradation (Harr, 2002).

Muscle Performance Testing

To test for functional signs of skeletal muscle degradation, we tested pectoral muscle performance. The pectoralis is the largest muscle group in all flighted birds at an average of 40% of body mass (Hartman, 1961). Muscle performance was tested using a novel measure, the vertical flight performance. The vertical flight test was performed by placing a single bird in a rectangular Plexiglas® chamber measuring 122cm (h) x 20cm (w) x 20cm (d). This width and depth was 2.5 cm smaller than the average goldfinch wingspan (22.86 cm Sibley, 2000), preventing birds from flying at an angle and thus limiting them to vertical flight while inside the chamber. The external surface of the chamber was marked every 15cm to record the height each bird achieved (Fig 2). The birds were given 30 seconds to become familiar with their surroundings and were then stimulated by a research technician approaching the chamber. Subsequently, the first 2 attempted flight heights were recorded. Vertical flight challenges were recorded prior to carotenoid supplementation, 30 days on carotenoid supplementation, 60 days on carotenoid supplementation and 60 days post carotenoid removal for the 2006 year experiment and only for 60 days post carotenoid removal sample period for the 2005 year experiment (Fig.1).

Statistical Analysis

All plasma enzyme values as well as the vertical flight performance measure were tested for normality. None of these variables were normally distributed by year. Thus we log transformed the 2004 AST values to achieve normality and the effects of carotenoid treatment groups by sample date were analyzed using a two way ANOVA. Log transformation did not normalize CK values nor the performance measures, thus non-parametric statistics were used for these analyses. Because CK values did not differ between years data from the 2004 and 2005 experiments were combined (Mann Whitney U test, $p>0.05$). We used a Kruskal-Wallis test to determine the effect of sample period for each carotenoid treatment and a post hoc non-parametric multiple comparison test was used to determine which sample periods differed from one another (Sokal and Rohlf, 1995). We tested the effect of treatment on vertical flight performance using a Mann-Whitney U test within sample period and between sample periods.

RESULTS

Liver and Muscle Enzymes

AST levels rose significantly between the time of capture and the captive pre-supplementation period (when no carotenoids were present in the diet; $F_{1,20}$= 5.20, p= 0.033). After carotenoid supplementation began, AST plasma levels significantly decreased in both the low and high treatment groups ($F_{3,77}$ = 4.82, p = 0.004;) from the levels observed when birds were initially held during the non-supplemented period (Fig. 3). AST plasma levels remained low and did not differ significantly between treatment groups (high carotenoid supplementation vs. low carotenoid supplementation) at any time during the supplement and post supplement period ($F_{2,1,73}$ = 1.08, p = 0.36).

Initially, CK levels rose significantly between capture and captive period prior to supplementation (pre-supplement) when no carotenoids were present in the diet (Z value= -3.16, p= 0.0003). Both high and low treatment groups showed a significant decrease between captive pre-supplement levels of CK and those observed 7 days after supplementation began (H corrected for ties =36.28, p< 0.001). There was no significant difference in CK levels between groups in the 7 and 30 day supplement sample periods. However, 60 days after supplement began, both treatment groups exhibited a significant increase in CK (H corrected for ties= 34.22, p<0.0001). Sixty days after supplement ended, birds that had received high carotenoid treatment still exhibited significantly higher levels of CK than those who had been kept on lower levels of carotenoid

supplement (Z = - 2.64, p= 0.008)(Fig. 4). This was the only time period that differed significantly between high and low carotenoid treatment groups.

Muscle Performance Analysis

Vertical flight performance rose significantly 7 days after supplementation began in the high carotenoid concentration treatment from when they were initially tested during the pre-supplementation captivity period (Z= -4.175, p<0.0001). Subsequently, at the 30 day sample during supplementation, vertical flight performance significantly decreased in the high carotenoid treatment group (Z =-4.826, p <0.0001). By the end of the experiment (during the 60 day post carotenoid supplementation period) the high carotenoid treatment group performed at a significantly lower height than the low carotenoid treatment group (Z= -2.629, p= 0.0086) (Fig.5).

DISCUSSION

Our study found that carotenoid ingestion appeared to have the predicted beneficial effects on several physiological and performance measures, but these were only apparent in the short-term. In contrast, longer-term exposure to carotenoids generated unexpected and potentially detrimental responses in these same parameters, which extended, especially in birds exposed to high levels of carotenoids, beyond the treatment itself, suggesting a significant, long-term cost to acquiring and maintaining extremely colorful plumage.

Initially, when carotenoids, regardless of dose, were re-introduced into the goldfinches' diet, they seemed to promote salutary physiological changes. For example, enzymes indicative of liver and skeletal muscle function (AST and CK, respectively), declined significantly (Figs.3&4). This decline may reflect the antioxidant properties of dietary carotenoids. Most studies have shown that carotenoids are essential to thwart free radical attack on tissues of the body, especially in their role as scavengers of singlet molecular oxygen and peroxyl radicals (Stahl and Sies, 2003). Since the birds were initially held in captivity on a diet with very little carotenoid content, the supplementation, (which elevated carotenoid intake several fold over the seed diet) potentially restored oxidative homeostasis. Further support for the short-term beneficial effects of carotenoid ingestion was also found in the results from our flight performance trials. Flight performance was enhanced with the addition of carotenoid supplementation

in both high and low treatments, with the high carotenoid treatment groups performing significantly better a week after the introduction of carotenoids (Fig. 5). In a similar performance study with zebra finches (*Taeniopygia guttata*), birds that received carotenoid supplementation (in the lower concentration range for the species) exhibited shortened response time in take-off performance to a stimulus and shortened flight time in general when compared with birds not receiving any carotenoid supplementation (Blount and Matheson, 2006). Both our and the Blount and Matheson (2006) studies show that dietary supplementation with carotenoids have positive effects in flight performance following a short period of supplementation over no carotenoid supplementation. However, in our study, the beneficial effects of carotenoid ingestion were transitory and, as the treatment continued, diametrically opposite results were observed.

Creatine kinase, the enzyme indicative of skeletal muscle degradation, exhibited a steady increase after supplementation began and continued throughout the remainder of the study for both carotenoid supplemented groups (Fig. 4). CK remained elevated even after birds were no longer being supplemented, being significantly elevated in animals that had been maintained under high carotenoid supplementation. The significant elevation of CK levels in the high carotenoid treatment group versus the low treatment group, sixty days after removal of supplemented carotenoids, suggests that there is a long-term cost associated with this type of persistent carotenoid ingestion leading to carotenoid hyper-accumulation. Case studies in humans have shown that prolonged (i.e. months) diet supplementation of carotenoid related lipid soluble substances, such as Vitamin A, can induce chronic disease such as severe liver damage or acute toxicity with

symptoms ranging from vomiting and blurred vision to increased cerebrospinal fluid pressure and lack of muscular coordination (Allen and Haskell, 2002).

The surprising finding that there still was a residual, significant elevation in plasma creatine kinase (indicative of skeletal muscle deterioration) in birds who had been exposed to high levels of carotenoids even 60 days after the supplementation ended suggests a potential substantive physiological cost to the extended exposure to high levels of carotenoids necessary to produce colorful plumage. When we tested whether the observed significant elevation in CK translated to a performance deficit, we found that, indeed, at the time point and the treatment group in which CK levels were elevated, flight performance was adversely affected (Figures 4&5).

Since both CK levels and flight performance were detrimentally affected 60 days after the supplementation ceased, it is important to consider where these observed responses would potentially coincide in the life history of this species. Our supplementation period coincided with the entire natural molt cycle of the species, a time in which goldfinches are normally ingesting high amounts of carotenoids. Supplementation ended at the end of molt, which, again, mimics the time period where goldfinches naturally discontinue their hyperphagic consumption of carotenoids. After molt is completed, goldfinches normally begin their breeding cycle. Thus, the elevated levels of CK and the reduced flight performance we observed in animals that had been in the high carotenoid group would occur at the beginning of the breeding season. During this time period, these males are displaying courtship behaviors, engaging in nest building, and increased foraging, all activities that require flight (Middleton, 1993). In addition to these behaviors possibly being affected by depressed ability to fly efficiently,

predator escape could also be potentially hindered during the season when they are most active and obvious due to their breeding plumage. While this study documented direct physiological and performance changes, we are unsure as to the mechanism by which they occur. Accumulated carotenoids have negative free radical oxidative effects on tissues in a prooxidative state but have positive free radical depleting effects when functioning as an antioxidant (Mortensen et al., 2001; Palozza et al., 1995; Palozza, 1998; Russell, 2000; Russell, 2004; Stahl et al., 2002; Stahl and Sies, 2003). The effect of carotenoid intake on free radical creation and depletion in animals displaying carotenoid-based ornaments warrants further investigation.

In contrast to the elevation in CK and the decline in vertical flight performance, AST levels which are indicative of liver degradation, did not differ significantly either by treatment group or time (i.e. all sampling events during and following supplementation). Previous studies have shown the liver to have the highest concentration of carotenoids in the body (Schmitz et al, 1991). The liver has also been shown to be a storage (Koustos et al., 2003) and processing (Yeum and Russell, 2002) site for ingested carotenoids. In fact, the liver has a large number of receptors used in sequestering carotenoids and has the ability to breakdown certain carotenoids by oxidative cleaving for utilization for other biological processes (Yeum and Russel, 2002). Since this organ routinely deals with greater amounts of carotenoids than other tissues as part of its normal function, it may be adapted to withstanding carotenoid hyper-accumulation and may actually have protective mechanisms that skeletal muscle tissue does not.

Our study is the first to demonstrate negative impacts of carotenoid supplementation associated with longer periods of carotenoid intake, with birds exposed

to higher concentrations of dietary carotenoids showing reduced flight performance when compared to those exposed to lower levels of carotenoids. This finding coincides with the premise of honest signaling and the handicap hypothesis; the more pronounced a trait becomes, the detriment imposed by that trait that the individual has to overcome should be equal in magnitude (Lotem, 1993). The Blount and Matheson study (2006), which showed a beneficial outcome to carotenoid supplementation, used levels consistent with the lower range of values encountered in wild populations. It has been shown that American goldfinches exhibit higher ratio of plasma to diet carotenoids than Zebra finches (McGraw2005) and Zebra finches have a more diverse carotenoid plasma profile than do American goldfinches (McGraw et. al,2003; McGraw,2004). In addition, Zebra finch carotenoid signaling is concentrated in the red bill of the males (Burley et al. 1992). Bill coloration is also a more transitory trait, that can change with current environmental condition (Burley et al, 1992), than permanently or seasonally deposited plumage coloration (as in goldfinches). Unlike the zebra finches, American goldfinches exhibit an increased plasma to diet ratio of carotenoids, homogeneity of circulating carotenoids (McGraw, 2004) and create a more long lasting and extensive plumage signal. Therefore, it is possible that they are exposed to higher levels of carotenoids for longer time periods than zebra finches and, thus, could face a larger and more long-lasting reaction to these that is use to enhance fitness and would now serve to signal honest status.

Our observations suggest that we must investigate both the positive and negative impacts of ornaments on the individual displaying them. Acquisition of carotenoid pigmented integument follows the Handicap hypothesis as an honest signal in this species exhibiting both positive and negative effects. Most studies concerned with this type of

sexually selected signal focus on the positive aspects associated with this bright coloration, but the negative aspects must be investigated as thoroughly to understand the signal meanings in their entirety.

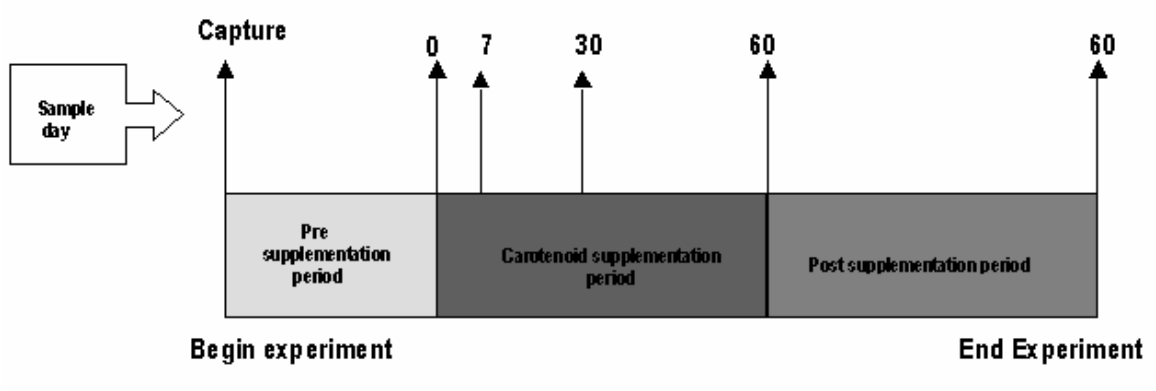

Figure 1**.** Experimental timeline for plasma aspartate amino transferase and creatine kinase enzyme sampling. In 2005, vertical flight challenges were also done for the day 60 post supplementation sample period. In 2006, vertical flight challenges were done for all sample periods on all indicated sample dates (0 day through 60 day post supplementation period).

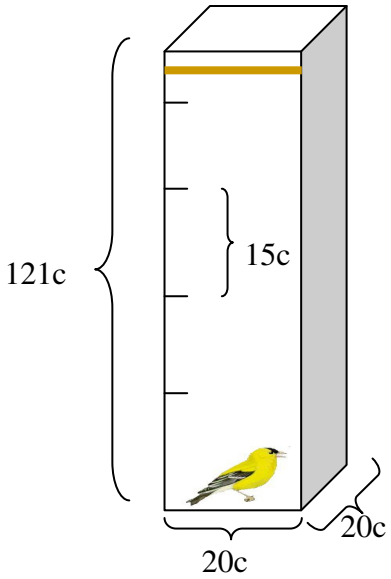

Figure 2. Chamber used for vertical flight performance testing.

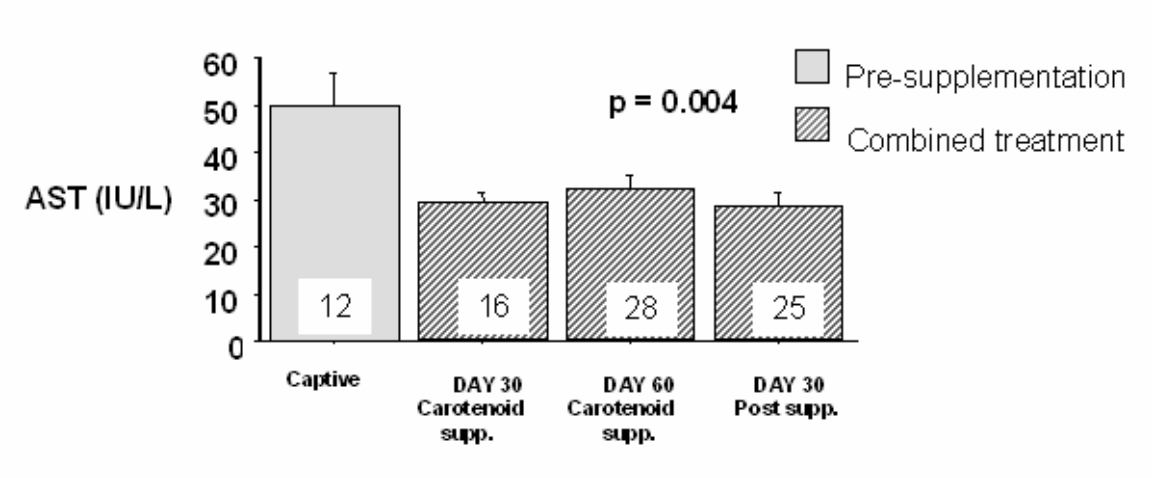

Figure 3. Mean values of plasma amino aspartate plasma content of birds from combined treatments (i.e. high and low carotenoid diets) across sampling periods over the course of the experiment.

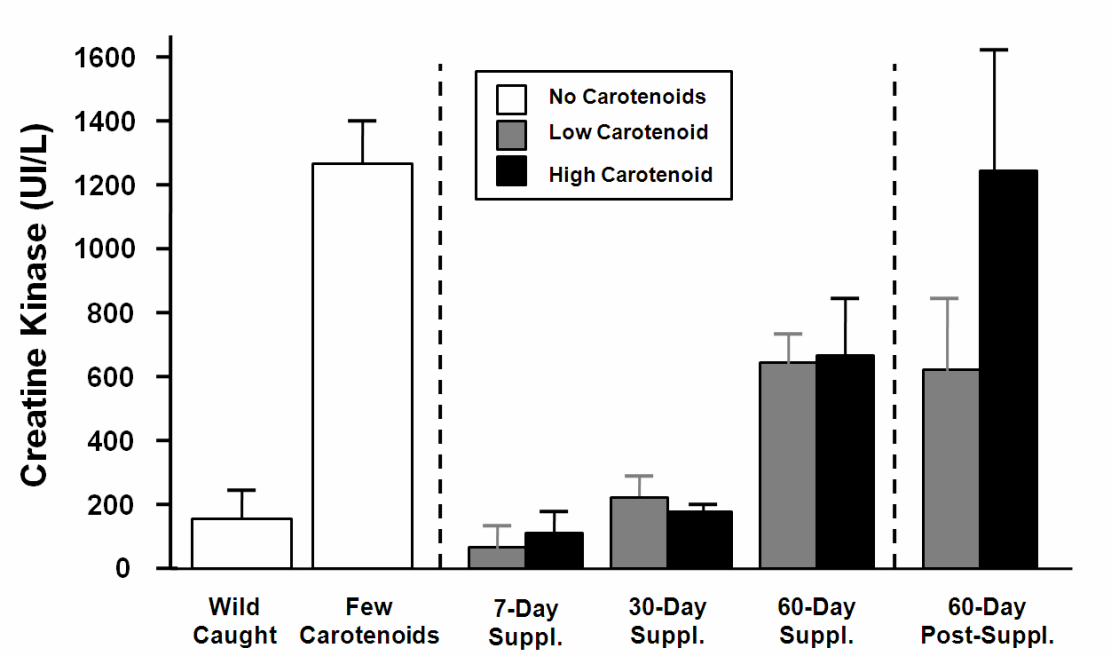

Figure 4. Mean values of plasma creatine kinase concentrations split by diet carotenoid treatment across sampling periods over the course of the experiment. Dashed lines indicate the beginning and end of carotenoid supplementation. The "few carotenoid period" refers to the being fed the natural seed diet which contains trace amounts of carotenoid pigments.

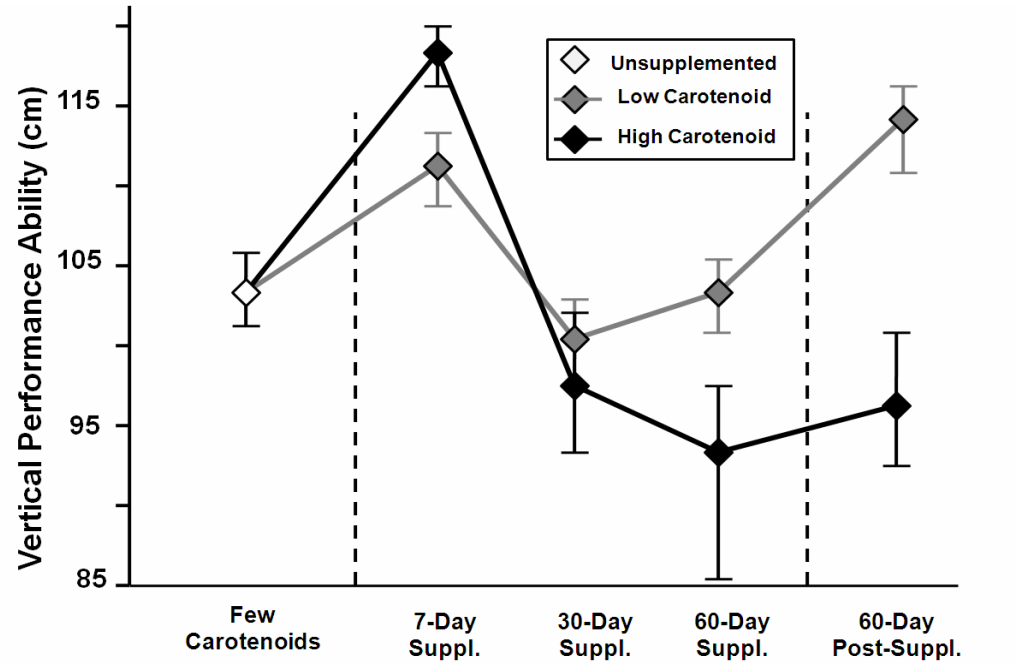

Figure 5. Mean vertical flight performance values split by treatment across sampling periods over the course of the experiment.

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