The effects of captivity on the bill coloration of male Northern Cardinals (*Cardinalis cardinalis)*

by

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Abstract

Male Northern Cardinals (*Cardinalis cardinalis*) possess two distinct carotenoid-based signals, bright red plumage and orangish-red bills. The red ketocarotenoids necessary for both of these color ornaments cannot be synthesized *de novo* but are instead produced by metabolizing yellow carotenoids obtained from the diet. Carotenoid-based ornaments have been shown to be negatively affected by environmental stressors and the health state of birds and thus can serve as signals of individual quality. Carotenoid pigments necessary for bill coloration are continuously produced and replenished. In this study, we tested the hypothesis that holding wild-caught male cardinals in captivity, which previous studies have shown increases the stress level of wild-caught songbirds, would impair metabolism of yellow dietary carotenoids, thus negatively affecting the carotenoid coloration of bills. We captured two groups of male Northern Cardinals. One group was held in cages until the onset of molt, and the others were free-living birds, captured at the onset of molt. For each individual, we measured bill coloration, bill carotenoid content, and circulating carotenoids in the plasma. We found that the bills of both wild and captive cardinals were colored with astaxanthin, α-doradexanthin, canthaxanthin, lutein and zeaxanthin. Free-living birds had more total ketocarotenoids in their bills than those that had been held in captivity. The measured ketocarotenoid content in the bills did not correlate to plasma ketocarotenoid content in either captive or free-living birds. Lastly, bill hue before captivity was predictive of bill hue after captivity, with redder males maintaining more colorful bills in captivity. These data support the hypothesis that red carotenoid-based bill coloration is a condition-dependent trait.

Artificial Intelligence (AI) Use Disclosure Statement

In the preparation of this thesis, the following Artificial Intelligence (AI) tools were used: ChatGPT. These tools were used primarily to correct code in R when running statistical analyses. The author acknowledges full responsibility for the intellectual content of this work and has ensured that all AI-assisted sections have been reviewed and revised for accuracy and appropriate academic style. All AI-generated content was reviewed and validated for relevance, appropriateness, and accuracy before incorporation into the final document to maintain scholarly integrity of this research.

Digital Accessibility Use Disclosure Statement

In the preparation of this thesis, no Digital Accessibility tools were used.

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List of Abbreviations

HPLC	High-performance liquid chromatography
CYP2J19	Cytochrome P450 2J19
BDH1L	3-hydroxybutyrate dehydrogenase 1-like
MTBE	Methyl tert-butyl ether
NaOH	Sodium hydroxide
NaCl	Sodium Chloride
MG	*Mycoplasma gallicepticum*

In many studies of birds, carotenoid-based ornamental coloration has been shown to serve as an honest signal of male quality (Svensson and Wong 2011). Carotenoid-based ornaments can include plumage and bare-part coloration, such as the bill, legs, and eye rings (Iverson and Karubian 2017). Carotenoid-based coloration of bare parts can change quickly to provide information on the social status of the bird, making this flexible ornament potentially an important indicator of the current condition of the bird (Pérez-Rodríguez and Viñuela 2008; Karubian et al. 2011). Most studies linking carotenoid-based ornaments to honest signaling focus on plumage even though bare-parts of birds are more commonly pigmented by carotenoids than plumage (Olson and Owens 2005; Simons et al. 2012), and bare-part coloration is known to serve as a social signal (Karubian et al. 2011).

Carotenoids are fat-soluble pigments that must be absorbed, transported, and deposited into target tissues (Hill et al. 1994). Once ingested, they are taken by scavenger receptors in the epithelial cells of the gut, transported by lipoproteins in the liver and bloodstream, and deposited in the target tissue (Liao et al. 2025; Hill and Johnson 2012). In the case of red coloration, yellow dietary carotenoids are oxidized into red ketocarotenoids before deposition (Hill and Johnson 2012). There are three established pathways from dietary yellow carotenoids to ornamental red ketocarotenoids used in birds (Koch et al. 2025). In this study, we focus on the pathway involving cytochrome P450 2J19 (CYP2J19) and 3-hydroxybutyrate dehydrogenase 1-like (BDH1L). Recent advancements have led to the discovery of these key enzymes necessary for the production of astaxanthin and α-doradexanthin, red ketocarotenoids necessary for coloration in the Northern Cardinal (Barsh 2016).

The carotenoid coloration of the bare parts of birds can change rapidly as the stress-state or health state of a bird changes (Rosenthal et al. 2012). Thus, carotenoid-pigmented bare part coloration may reflect the present health status of a bird (Pérez-Rodríguez and Viñuela 2008). In a study on the red-legged partridge (*Alectoris rufa*), carotenoid-based coloration was found to reflect body condition, with bill redness correlated to body condition (Pérez-Rodríguez & Viñuela 2008). In female American Goldfinches (*Spinus tristis*), bill color serves as a signal of status, mediating interactions among competitors during breeding season (Murphy et al. 2009). In males, bill color is correlated with body condition (Rosen and Tarvin 2006; Hill et al. 2009). These studies indicate that bill color can be a condition-dependent ornament and document the importance of carotenoid-based bill coloration in social signaling, yet the effects of environmental challenges on carotenoid-based coloration have rarely been studied for bare part coloration.

Northern Cardinals are a resident songbird species that are abundant throughout Alabama (Imhof et al. 1976). Males exhibit bright red plumage and orangish bills. Northern Cardinals are an example of birds that primarily utilize metabolically transformed carotenoids for plumage and bill coloration, achieving red coloration through the CYP2J19/BDH1L pathway (Sin et al. 2020). Knowledge of the enzymatic systems that cardinals use to produce red feather pigments makes these birds an excellent model in which to test the hypothesis that red coloration produced via the CYP2J19/BDH1L system can serve as a signal of individual condition.

In this study, we tested the hypothesis that captivity stress would negatively affect the bill coloration of wild-caught male Northern Cardinals. Based on previous observations that carotenoid-based coloration is affected by environmental challenges (Koch et al. 2024), we predicted that captivity would disrupt the conversion of dietary carotenoids to ketocarotenoids and cause a loss of red ketocarotenoids and hence loss of red coloration in the bill. We compared the carotenoid content of the bill tissue between free-living and captive birds. We also compared bill coloration at the time of capture to bill coloration measured after several weeks in captivity. We predicted that 1) total ketocarotenoid content would be lower in birds held in captivity compared to free-living birds, 2) bill carotenoid content would correlate to plasma carotenoid content, and 3) bill hue before captivity would predict bill hue after captivity.

Methods

Bird Capture

We captured 22 adult male Northern Cardinals using Potter traps and mist nets in Auburn, Alabama. All birds used in this experiment were aged as “after-hatch year”, meaning that plumage indicated they were at least in their second calendar year (Pyle 1997). Specifically, between 7-14 July 2023, we trapped 13 male cardinals after they had completed breeding and before they had begun basic molt. We held these 13 males in captivity until they began molting. Blood samples were taken from each bird at capture. An additional 9 free-living birds were captured between 3-28 August 2023, at the onset of molt and sampled immediately.

Experimental Design

Male Northern Cardinals were held in captivity from 15-70 days, depending on how many days after capture they began molt. Birds were held in aviaries outdoors (2.5m x 2.5m x 7.5m), with shade on one third of the cage and branches provided for perching. We housed between 3 and 5 birds per cage. Birds were fed Pennington bird seed consisting of milo, proso millet, sunflower seeds and wheat. Fresh water was provided daily in clean dishes, supplemented with Oasis Vita Drops, FloraGlo lutein (10%) and OptiSharp zeaxanthin (5%). Lutein and zeaxanthin are the primary precursor carotenoids that Northern Cardinals use to produce red ketocarotenoids (McGraw et al. 2001). When birds began molting, a second blood sample was taken, and birds were euthanized. Following euthanasia, bills were collected and stored at -80°C until analysis. For comparison to birds held in cages, we captured 9 free-living males that were molting, took blood samples, and euthanized the birds. After euthanasia, we collected bills from each of these birds and stored the bills at -80°C until analysis.

Blood Collection

Blood was collected via venous puncture in the brachial vein using a 28-gauge sterile needle. After collection, blood was spun at 2,000 g for 15 minutes to separate plasma. Plasma was stored at -80°C until extraction and analysis.

Carotenoid Extraction

Carotenoids were extracted from the colorful sheath (carotenoid-containing portion) of the bills which were shaved with a scalpel. Mass of the shavings was recorded. To a microtube with the shavings, a 1:1 ratio of hexane:MTBE was added, and the sample was ground using a microtube pestle until the solvent turned a red/orange color, confirming presence of carotenoids. Samples were then centrifuged at 5,000 g for 7 minutes. The supernatant was carefully removed and placed into a new tube without disturbing the pellet. If color was still present in the bill shavings, the previous steps were repeated until the supernatant no longer changed color during the pulverizing process. Samples were placed into a CentriVap to evaporate the supernatant to dryness. Samples were then resuspended in 100 uL of ethanol and saponified. Saponification was performed by adding 1 mL of 0.02 M methanolic NaOH to the resuspended extract, capping the samples under nitrogen and incubating in the dark for 4 hours at room temperature. Once saponification was complete, the samples were moved into 15 mL vials. To the 15 mL vials, 1 mL of saturated NaCL, 2 mL of distilled deionized water, and 2 mL of hexane:MTBE were added. Samples were mixed by inverting the tubes for 30 seconds. 15 mL vials were centrifuged at 2,000 g for 5 minutes. The upper solvent fraction was then transferred to a 2 mL microtube and evaporated to dryness in a CentriVap. Samples were capped with nitrogen and stored at -80°C until High-Performance Liquid Chromatography (HPLC) analysis. For plasma carotenoid extraction, 250 uL of ethanol was added to anywhere from 3-10 uL of plasma, vortexed for 5 seconds, then 250 uL of hexane:MTBE was added, followed by vortexing for another 5 seconds. Samples were centrifuged at 10,000 rpm for 3 minutes. The supernatant was then transferred to a snapcap tube. All steps were repeated 2-3 times to ensure maximum carotenoid recovery. Samples were then dried, capped with nitrogen and stored in -80°C until analysis.

HPLC Analysis

Carotenoid analysis was performed using HPLC following methods described previously (Toomey et al. 2015). Samples were resuspended in 200 mL of methanol:acetonitrile 1:1 (vol:vol). Using an autosampler, we injected 50 mL of each extract into a Shimadzu HPLC using a YMC carotenoid 5.0 - mm column (4.6 mm x 250 mm, YMC). Pigments were separated using a gradient mobile phase of acetonitrile:methanol:dichloromethane (44:44:12) (vol:vol:vol) for 0-11 minutes then changed to acetonitrile:methanol:dichloromethane (35:35:30) (vol:vol:vol) from 11-21 minutes followed by a return to isocratic conditions through 36 minutes. These mobile phases were pumped at a constant rate of 1.2 mL/min and conditions stayed at room temperature for the entire run. Samples were monitored at 445 and 480 nm, the wavelengths at which non-ketocarotenoids and ketocarotenoids are detected, and identified and quantified as described previously (Toomey et al. 2015).

Photo Data Analysis

All birds in the cage treatment group were photographed with a Canon 7D camera as RAW images under standard lighting with a color chip included in each photo within one hour of capture and again before being euthanized via decapitation. Free-living birds were photographed within one hour of capture. We extracted hue data from photos using Adobe Photoshop, using the lasso tool to draw a polygon on the lower mandible. The same area of the bill was measured for each individual, intentionally avoiding areas of glare.

Statistical Analysis

All statistical analysis was performed with R version 4.5.2.“[Not] Part in a Rumble”. Individual carotenoid concentrations were determined utilizing a standard curve to calculate concentrations from HPLC reports. Analysis performed included T-tests, Paired t-tests, Mann Whitney U Tests, Levene’s Test for Variance, Spearman’s Rank Correlation, Wilcoxon-signed rank tests, and Shapiro-Wilk test of normality. Some birds were photographed using different paint chip standards, so code controlled for difference in paint chip in bill hue calculations. The effect of captivity on bill color was analyzed using measured carotenoid concentrations and bill hue scores. Significance was established at $p<0.05$ for all analyses. All figures were generated in R.

Results

We detected α-doradexanthin, astaxanthin, canthaxanthin, lutein, and zeaxanthin in the bill tissue of male Northern Cardinals. At the onset of molt in both free-living and captive males, the dominant carotenoid was astaxanthin, followed by α-doradexanthin, canthaxanthin, zeaxanthin, and lutein (**Figure 1**).

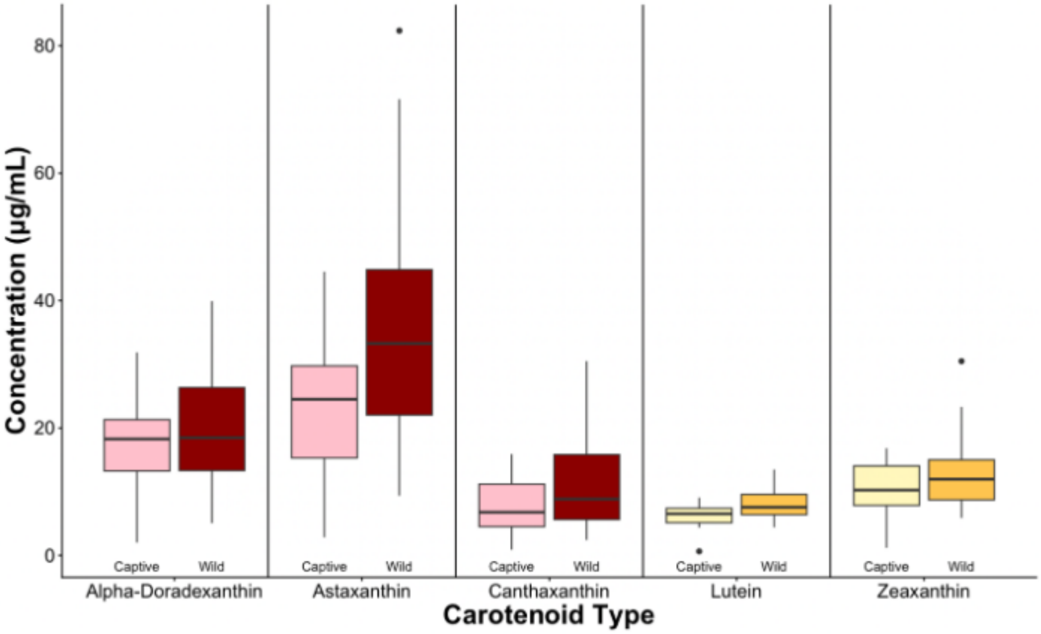

Figure 1. Boxplot of carotenoid concentrations (ug/mL) detected in free-living and captive male Northern Cardinals. The bottom of each box represents the 25th percentile. The top of the box represents the 75th percentile. Lines inside each box indicate the median. Whiskers represent variability in the dataset, and any isolated dots outside of the boxes are outliers. Carotenoids detected include α-doradexanthin, astaxanthin, canthaxanthin, lutein and zeaxanthin. The boxes are colored to represent treatment type (captive or free-living) and carotenoid type (dietary or metabolized) (Red = ketocarotenoids in wild birds; pink = ketocarotenoids in captive birds; dark yellow = dietary carotenoids in wild birds; light yellow = dietary carotenoids in captive birds).

We found that free-living males had a significantly higher mean concentration of total ketocarotenoids compared to captive males (free-living males = 78.67 ug/mL;

captive males = 48.93 ug/mL; t = -2.57; df = 15.68; **p = 0.0302**). Assumptions of normality and equal variance were tested using the Shapiro-Wilk normality test and Levene's Test for Homogeneity of Variance, ensuring $p > 0.05$ (Shapiro-Wilk normality test p = 0.32; p = 0.98; Levene's Test for Homogeneity of Variance p = 0.093) (**Figure 2**).

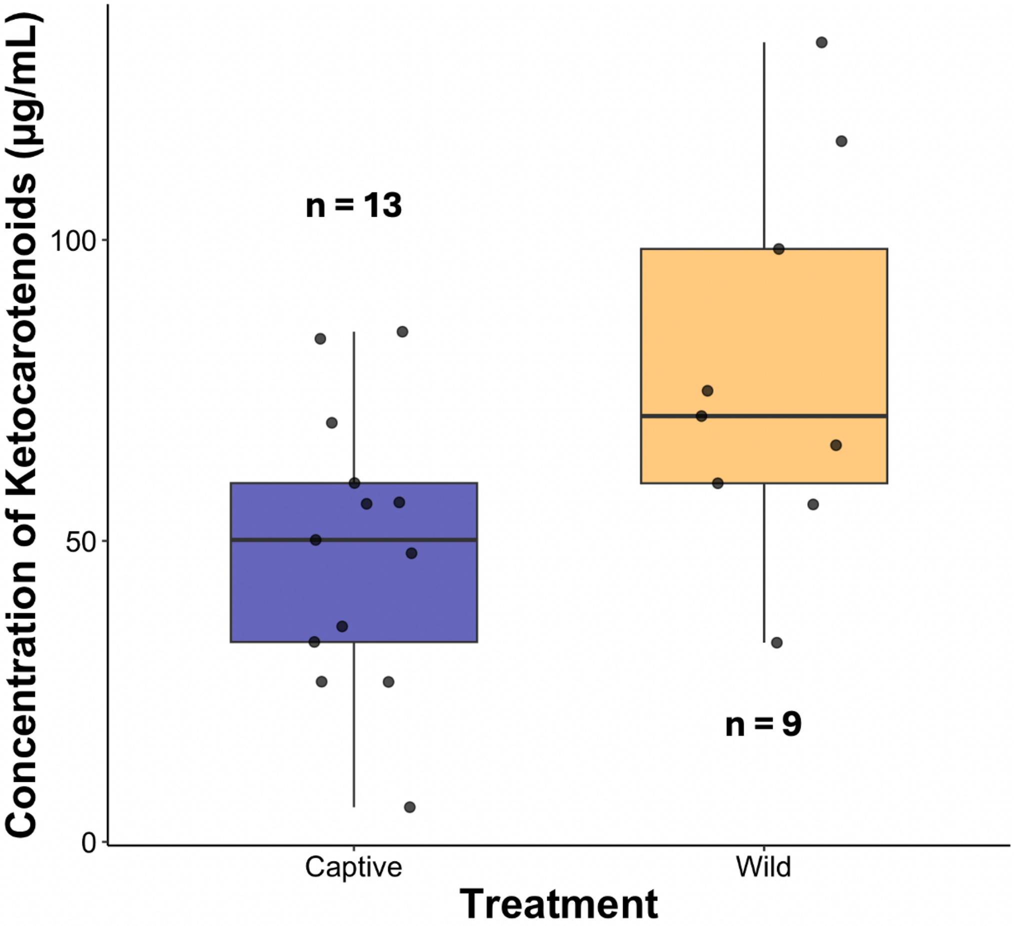

Figure 2. Boxplot showing concentrations of ketocarotenoids in free-living and captive male Northern Cardinals. The bottom of each box represents the 25th percentile. The top of the box represents the 75th percentile. Lines inside each box indicate the median. Whiskers represent variability in the dataset, and any isolated dots outside of the boxes are outliers. Free-living Northern Cardinals had a higher mean concentration of total ketocarotenoids when compared to captive males (**p=0.0302**).

There was no significant relationship between bill carotenoids and plasma carotenoids. This pattern held for total ketocarotenoids and yellow (dietary) carotenoids in both free-living (Spearman's rank correlation test p = 0.974, p = 0.430) and captive (Spearman's rank correlation test p = 0.744, p = 0.2) birds. (**Figure 3**).

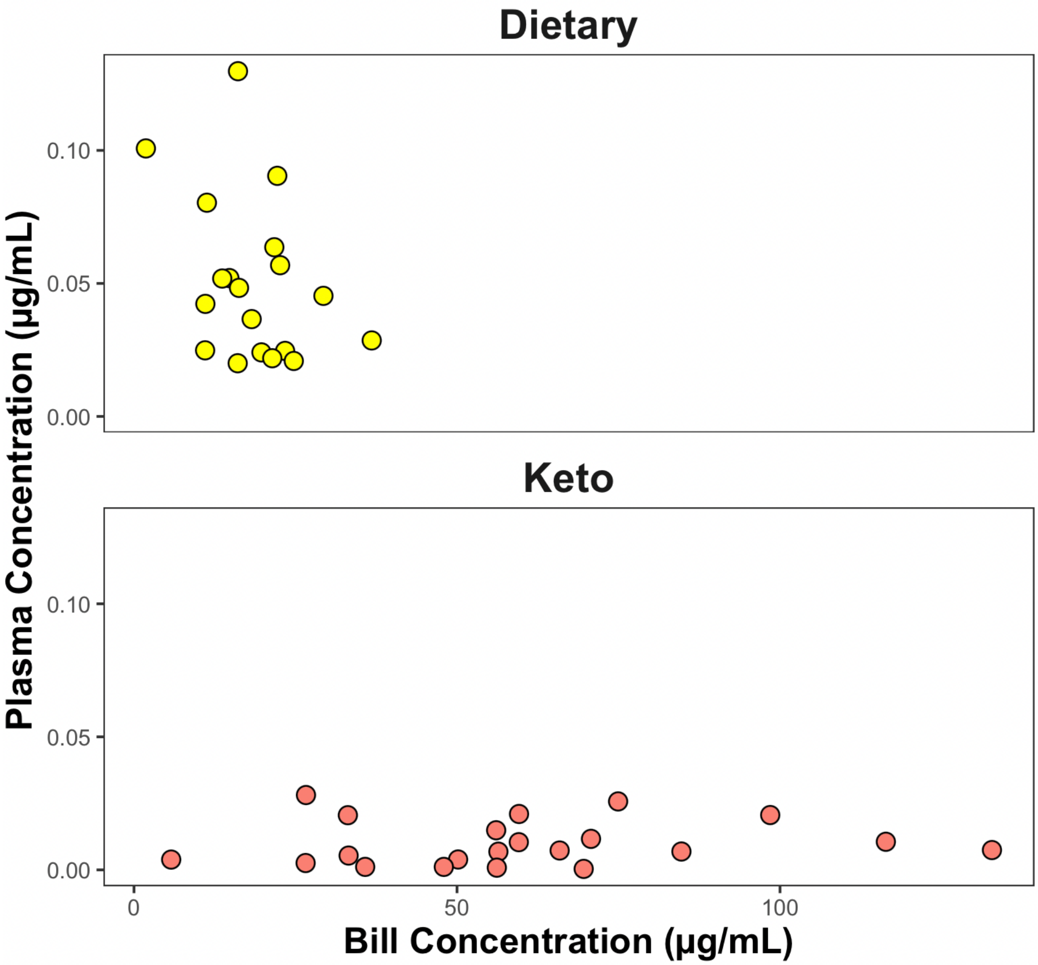

Figure 3. Scatterplots showing the relationship between dietary (yellow) and metabolized (red) carotenoid concentrations in bill and plasma samples.

In tracking the bill coloration of individual birds at the time of capture versus after being held in cages, we found that bill hue of individuals decreased significantly following captivity (p=0.0097). Hue is represented by an angle on the color wheel, with red at 0° and yellow at 60° (Hill and McGraw, 2006, Chapter 3). A higher value for hue indicates a less red bill in birds (Hill et al., 2019). All males but one decreased in bill hue post captivity. Six out of 10 males maintained their bill redness and hue ranking throughout captivity, while 4 out of 10 failed to maintain their bill redness.

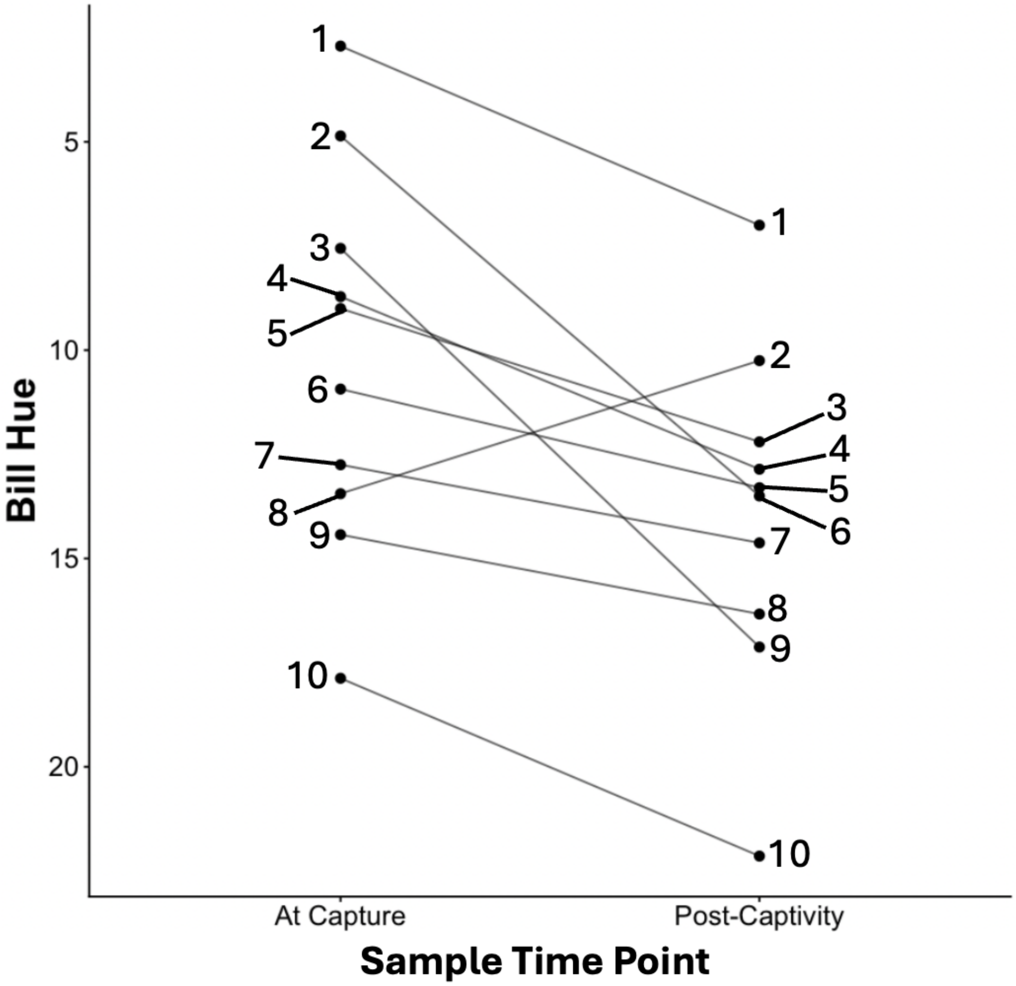

Figure 4. A figure representing the hue (a unitless position on a color wheel) of the bills of individual male Northern Cardinals at the time of capture and after week in captivity. Lines connect the data points for the same birds in the two treatment groups.

Discussion

In many birds, carotenoid coloration is a signal of individual condition (Hill and McGraw 2006, Chapter 5). Such condition-dependent signaling has been observed in many studies on plumage coloration (Hill 2002), but there are relatively few studies of red bills signaling condition. Here, we tested the condition dependency of red bills in Northern Cardinals. We identified the dominant carotenoids present in the bills of male cardinals to be α-doradexanthin, astaxanthin, and canthaxanthin, all metabolized carotenoids that are known products of the CYP2J19/BHD1L pathway (Toomey et al. 2022). Dietary carotenoids were also present, but in much smaller amounts than metabolized carotenoids. The low presence of dietary carotenoids is expected if the conversion of dietary carotenoids to ketocarotenoids is efficient (Hill 2014). Birds cannot synthesize carotenoids *de novo*, so yellow dietary carotenoids must be metabolized to red ketocarotenoids to produce red ornamentation (Hill et al. 1994). As the ratio of red to yellow pigments increases, the tissue becomes redder (Inouye et al. 2001).

Consistent with our hypothesis that stress during feather growth will cause loss of red coloration, free-living cardinals had a significantly higher mean concentration of total ketocarotenoids present in the bill—and redder bills— than captive birds. The loss of red coloration in the bills of captive cardinals likely reflects a disruption in the CYP2J19/BDH1L pathway that male cardinals use to produce red carotenoid pigments.

We predicted that there would be a positive correlation between circulating carotenoids in the plasma and carotenoids present in the bill because of the direct blood supply to the fleshy bill. The presence of a direct blood supply suggests that carotenoid pigments can be delivered to the living bill tissue, thus altering bill coloration (Rosenthal et al. 2012). This circulating supply of carotenoids necessary for coloration may be responsible for the ability of the bill to rapidly change carotenoid content and coloration (Ardia et al. 2010). Contrary to what we predicted, however, we did not observe a positive relationship between carotenoids circulating in the plasma and carotenoids present in the bill. Considering the site of dietary carotenoid metabolism is not well-established, and with previous research showing CYP2J19 expression in the epidermal tissue of passerines (Alonso-Alvarez et al. 2022), this finding suggests that metabolism of ketocarotenoids necessary for bill coloration may take place directly in the bill tissue, rather than elsewhere in the body and carried to the bill as previously hypothesized (Del Val et al. 2009). This is consistent with recent work suggesting that there may be a relationship between the type of ornament and the tissue used to metabolize dietary carotenoids to ketocarotenoids (Alonso-Alvarez et al. 2022).

Lastly, and consistent with our prediction, the hue of the bills of male cardinals became less red (more orange) following weeks in captivity. Moreover, birds that had brighter bills at the time of capture retained relatively brighter bills in captivity. These results suggest an innate difference in ability to endure stressful conditions, consistent with the shared pathway hypothesis (Hill 2011). This is also consistent with a previous study on House Finches in which the coloration of birds at the time of capture predicted ability to recover from infection with *Mycoplasma gallicepticum* (MG) (Hill and Farmer 2005).

Among birds with carotenoid-based ornaments, those with bill coloration are exhibiting a signal that can change rapidly in response to both social and environmental stressors. By documenting a reduction in carotenoid content and bill hue in response to captivity in male Northern Cardinals, this study further supports the hypothesized links between ornamentation, vital cellular processes, and the innate ability of individuals to handle stress. This study has broad implications for the response of the CYP2J19/BDH1L pathway to stress and understanding the role of the bill as an honest signal. Continuing important research to further our understanding of carotenoid-based coloration has the potential to uncover the evolutionary truth behind coloration and the mechanisms responsible for it.

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