Testing the Carotenoid Trade-Off Hypothesis in the Polychromatic Midas Cichlid, *Amphilophus citrinellus*

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ABSTRACT

Many animals use carotenoid pigments derived from their diet for coloration and immunity. The carotenoid trade-off hypothesis predicts that, under conditions of carotenoid scarcity, individuals may be forced to allocate limited carotenoids to either coloration or immunity. In polychromatic species, the pattern of allocation may differ among individuals. We tested the carotenoid trade-off hypothesis in the Midas cichlid, *Amphilophus citrinellus*, a species with two ontogenetic color morphs, barred and gold, the latter of which is the result of carotenoid expression. We performed a diet-supplementation experiment in which cichlids of both color morphs were assigned to one of two diet treatments that differed only in carotenoid content (β-carotene, lutein, and zeaxanthin). We measured integument color using spectrometry, quantified carotenoid concentrations in tissue and plasma, and assessed innate immunity using lysozyme activity and alternative complement pathway assays. In both color morphs, dietary carotenoid supplementation elevated plasma carotenoid circulation but failed to affect skin coloration. Consistent with observable differences in integument coloration, we found that gold fish sequestered more carotenoids in skin tissue than barred fish, but barred fish had higher concentrations of carotenoids in plasma than gold fish. Neither measure of innate immunity differed between gold and barred fish, or as a function of dietary carotenoid supplementation. Lysozyme activity, but not complement activity, was strongly affected by body condition. Our data show that a diet low in carotenoids is sufficient to maintain both coloration and innate immunity in Midas cichlids. Our data also suggest that the developmental transition from the barred to gold morph is not accompanied by a decrease in innate immunity in this species.

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Introduction

A central question in life history evolution is how animals divide limited resources between competing needs such as self-maintenance and reproduction (Partridge and Harvey 1988). In many systems, carotenoid pigments are an example of such a limited resource. Carotenoids are ubiquitous in nature, with more than 600 varieties isolated from natural sources (Britton et al. 2004), yet vertebrates lack the ability to produce carotenoids de novo and must acquire them from the environment, such as through the consumption of photosynthetic or phytophagous organisms (Olson and Owens 1998). The ingestion and subsequent deposition of carotenoids in the integument produces yellow, orange, and red coloration in many animals, which is often amplified by sexual selection (Fox and Vevers 1960; Rothschild 1975; Brush 1990; Hill 1991; Andersson 1994). These ornaments include bills, wattles, and feathers in birds (McGraw 2006; McGraw and Klasing 2006), heads and dewlaps in lizards (Macedonia et al. 2000; Steffen and McGraw 2007), and skin in fish (Evan and Norris 1996; Candolin 1999; Grether et al. 2004). Evidence for the role of carotenoids in coloration comes from numerous studies in which dietary supplementation increased coloration and the concentration of carotenoids in the plasma and integument (McGraw et al. 2002; Blount et al. 2003; McGraw and Ardia 2003; Alonso-Alvarez et al. 2004; Wallat et al. 2005).

In addition to their role in pigmentation, carotenoids also enhance components of the immune system. In mammals, carotenoids stimulate effector T-cell function; enhance macrophage, cytotoxic T-cell, and natural killer cell tumoricidal capacities; and enhance T- and B-lymphocyte proliferation (Bendich 1989; Bendich and Olson 1989; Chew 1993; Jyonouchi et al. 1994). Carotenoids can protect phagocytic cells from autooxidative damage by eliminating detrimental free radicals, as well as increase the rejection of foreign tissues (Seifter et al. 1981; Bendich 1989). The immune systems of fishes and birds are similarly enhanced by carotenoid supplementation (Blount et al. 2003; McGraw and Ardia 2003; Grether et al. 2004; Hõrak et al. 2006; McGraw and Klasing 2006; Clotfelter et al. 2007; but see Navara and Hill 2003).

The carotenoid trade-off hypothesis predicts that, when dietary carotenoids are limited, increased allocation of carotenoids to either coloration or immunity results in decreased

Figure 1. Gold morph of the Midas cichlid, *Amphilophus citrinellus*, showing the six locations where color measurements were taken.

allocation to the other function. Most studies of coloration-immunity trade-offs have been conducted in species in which individuals vary continuously in coloration (e.g., less yellow to more yellow in great tits *Parus major*; Fitze et al. 2007). However, in our previous work, we found that different color morphs of the fighting fish *Betta splendens* utilized carotenoids in strikingly different ways (Clotfelter et al. 2007). Red *B. splendens* on a carotenoid-enriched diet increased in redness, whereas blue fish, which lack the ability to express red coloration, allocated supplemental dietary carotenoids to immunity (mitogenic response to phytohemagglutinin). Studying species with carotenoid-based color morphs may give us unique insights into genetic or developmental controls of carotenoid allocation strategies in animals.

Here, our objective is to further examine the generality of the carotenoid trade-off hypothesis in a fish species with a naturally occurring color polymorphism, the Midas cichlids *Amphilophus citrinellus*. The natural history of the *A. citrinellus* complex has been extensively studied (Barlow 1973, 1976; Barlow and Wallach 1976; McKaye and Barlow 1976; McKaye 1980; Barlow and McKaye 1982; Meyer 1990). Midas cichlids exhibit two distinct ontogenetic color morphs, commonly referred to as "barred" and "gold." All Midas cichlids begin life as the barred morph, which has prominent melanin-based black bars on a gray background, beneath which are carotenoid pigments. Depending on the population, approximately 8% of individuals undergo a color transformation from barred to gold, during which the melanophores (cells containing melanin) die and expose the underlying xanthophores, which contain carotenoid pigments and produce the orange coloration typical of the gold morph (Barlow 1973; Webber et al. 1973; Dickman et al. 1988). The transformation from barred to gold may be influenced by both genes and environment (water turbidity, depth, and social interactions), though the color transformation is not fully understood (Barlow 1973, 1976). Gold fish are socially dominant when housed with cryptically colored barred individuals (Barlow 1973; Barlow and Wallach 1976; Barlow and McKaye 1982).

The objective of this experiment is to determine whether gold and barred Midas cichlids allocate supplemental dietary carotenoids in different ways. More specifically, we tested the following predictions. (i) Supplemental dietary carotenoids will increase gold coloration in both color morphs, but to a greater extent in gold fish; (ii) supplemental dietary carotenoids will increase innate immune response in both color morphs, but to a greater extent in barred fish, which deposit fewer carotenoids in skin than gold morphs; and (iii) patterns of carotenoid accumulation in blood will reflect these morph differences: barred fish will have relatively more carotenoids in circulation and available for allocation to the immune system or antioxidant activity, while gold fish will have relatively fewer in circulation, again because more pigment is deposited in skin.

Material and Methods

Fish Rearing and Treatment

Wild-caught *Amphilophus citrinellus* (Fig. 1) from the Isletas de Granada region of Lake Nicaragua in southern Nicaragua were obtained and transported to Amherst College (Amherst, MA) in August 2007 via a local commercial exporter (Brian Murillo). Cichlids were individually housed in 38-L tanks on a 12L : 12D photoperiod at 25° ± 2°C. All fish were sexually mature adults (mass: 160.54 ± 7.01 g, total length: 19.93 ± 0.26 cm). The species is sexually monomorphic, and the sex of each fish was determined at the end of the experiment via gonadal inspection (15 females, 34 males). During this period all fish were maintained on a low-carotenoid diet (see below) to control for any initial differences in carotenoid loads (Wang et al. 2006).

In September 2007, fish were randomly assigned to one of two diet treatments with either low or high levels of carotenoids, which were continued for 10 wk (Amar et al. 2000; Grether et al. 2004). Of the 34 males, 19 were on the low-carotenoid diet and 15 were on the high-carotenoid diet. There were 7 and 8 females on the low- and high-carotenoid diets, respectively. Fish diets were made fresh weekly according to recipes described in Clotfelter et al. (2007), and fish were fed to satiation twice daily.

The carotenoids used were β-carotene (0.3% of diet by weight; Sigma Aldrich), lutein (0.2%; General Nutrition Centers), and zeaxanthin (0.01%; General Nutrition Centers). The actual concentrations of carotenoids in the low-carotenoid diet (as measured by high-performance liquid chromatography [HPLC]) were 5.18 $\mu g\ g^{-1}$ β-carotene, 2.34 $\mu g\ g^{-1}$ lutein, and 0.084 $\mu g\ g^{-1}$ zeaxanthin, which are levels comparable to diets used in other fish studies (Grether et al. 2005; Pike et al. 2007*a*). The high-carotenoid diet contained 4,180.2 $\mu g\ g^{-1}$ β-carotene, 1,047.95 $\mu g\ g^{-1}$ lutein, 40.53 $\mu g\ g^{-1}$ zeaxanthin, as well as 521.08 $\mu g\ g^{-1}$ α-carotene.

Sample Collection

At the beginning of the 10-wk experimental period, fish were removed from their tanks and anesthetized in buffered tricaine methane sulfonate (MS-222; Western Chemical). Integument color was measured as described below, as were body mass (g) and total length (cm). At the end of the experimental period, fish were euthanized in a lethal dose of MS-222. Body mass (g) and total length (cm) were measured again, and we estimated body condition using the equation for Fulton's condition factor (*K*; Bolger and Connolly 1989):

$$K = 10 \times \frac{\text{mass}}{\text{length}^3}.$$

Blood samples were taken from the caudal blood vessels (while fish were still alive) using heparinized syringes, centrifuged for plasma collection, and stored at −80°C. A 1 × 1-cm section of epidermal tissue from the caudal peduncle was removed from each fish and stored at −80°C. Tissue and plasma samples from 46 cichlids (3 samples lost; 24 on the low-carotenoid diet, 22 on the high-carotenoid diet; 17 gold, 29 barred) were later analyzed for carotenoid concentrations using HPLC according to plasma extraction methods in McGraw et al. (2008), skin extraction methods in Clotfelter et al. (2007), and chromatographic methods from experiment 2 in Toomey and McGraw (2007). A variety of carotenoids were detected in skin, including pigments responsible for orange (astaxanthin, canthaxanthin, unidentified ketocarotenoids, and β-carotene) and yellow coloration (tunaxanthin, canary xanthophyll, lutein, and zeaxanthin). Only tunaxanthin, canary xanthophyll, lutein, zeaxanthin, and β-carotene were found in plasma.

Color Measurements

Integument color was measured with a reflectance spectrometer (USB4000; Ocean Optics) while the fish were anesthetized. We assessed reflectance at 5-nm intervals over the wavelength range of 200–800 nm using a 400-μm reflection probe (R400-7; Ocean Optics). The probe was held at a 45° angle 5 mm from the sample (Lahti 2006; Clotfelter et al. 2007). Integration time was set at 100 ms, reflectance was averaged over 100 scans, and boxcar smoothing was set to 50. Reflectance was calculated with reference to a dark and white standard (Labsphere), which was rescanned after every five fish. For each fish, surface color measurements were taken from six body positions (Fig. 1), which were selected because of their variability in carotenoid coloration between morphs and the ease of measurement repeatability. The three anterior positions included points between the eye and jaw, below the jaw, and on the forehead. The three posterior positions include points along the lateral line from the caudal tail to the midbody. The color measurements of all fish were taken on the right side. SPSS (version 15.0) was used to collapse reflectance data using principal components analysis (PCA; Jolliffe 1986). From the spectrophotometer measurements, we analyzed variation among all fish at every 5 nm from 200 to 800 nm. PCA revealed two principal components that accounted for >95% of the variance. Principal component 2 (PC2), which had positive loadings in the 500–700-nm wavelength range, was used as our index of carotenoid coloration in Midas cichlids (Cuthill et al. 1999; Clotfelter et al. 2007). Higher values correspond to more saturated patches of orange coloration.

We found that PC2 values for these six body regions were highly correlated with each other (Pearson's correlation coefficient, $r \geq 0.73$, $P < 0.001$ for each comparison; $n = 58$ fish). We identified two body regions, one anterior (between the eye and jaw) and one posterior (anterior from the caudal peduncle), that were most highly correlated with other body regions, and used reflectance data from these regions to validate our categorical color classification (gold vs. barred). Gold fish had significantly higher PC2 values—greater reflectance in the 500–700-nm range—than barred fish in both the anterior (barred: -0.27 ± 0.14; gold: 0.44 ± 0.24; independent samples *t*-test, $t_{56} = 2.80$, $P = 0.007$) and posterior (barred: -0.25 ± 0.15; gold: 0.41 ± 0.22; $t_{56} = 2.56$, $P = 0.013$) body regions, thus validating the use of a categorical classification system of gold versus barred.

Innate Immunity

The innate immune system responds nonspecifically to foreign pathogens. Two of the most important of these defenses in teleost fishes are lysozyme and complement proteins, both of which lyse foreign cells (Whyte 2007; Saurabh and Sahoo 2008) and both of which are known to be influenced by carotenoid availability in birds and fish (Amar et al. 2000, 2001, 2004; McGraw et al. 2006; Cucco et al. 2007). We measured lysozyme activity in the plasma of 58 fish collected after 10 wk on the experimental diets according to methods described in Hutchinson and Manning (1996). Dilutions of hen egg-white lysozyme standard (Sigma Aldrich) were prepared in 0.05 M phosphate buffer (pH 6.2). Ten microliters of fish plasma were added in triplicate along with 240 μL of 0.3 mg mL^{-1} lyophilized *Micrococcus lysodeikticus* (Sigma Aldrich) that had been diluted in phosphate buffer. The kinetic assay was read at a wavelength of 490 nm for 10 min. Readings were made on a microplate reader (EL800; BioTek Instruments) at 20.5° ± 1°C, and results are presented in lysozyme units per milliliter.

The alternative complement pathway hemolytic activity

(ACH) was determined from the plasma of 51 fish after 10 wk according to the methods of Yano (1992). Rabbit red blood cells (RaRBCs) were washed three times in isotonic veronal-buffered saline (pH 7.3) containing 0.1% gelatin (GVB) and one time in isotonic veronal-buffered saline (pH 7.3) containing 10 mM Mg^{2+}, 10mM EGTA, and 0.1% gelatin (Mg^{2+}EGTA-GVB). The RaRBCs were resuspended in Mg^{2+}EGTA-GVB to give a concentration of 2.5×10^8 cells mL^{-1}. We added 12.5 mL of RaRBCs to 50 mL of serially diluted plasma in Mg^{2+}EGTA-GVB. The reaction mixtures were incubated at room temperature for 90 min with shaking, and the reaction was stopped by adding 500 μL of isotonic veronal-buffered saline (pH 7.3) containing 20 mM EDTA and 0.1% gelatin (EDTA-GVB). After centrifugation, the extent of hemolysis was estimated by measuring the optical density of the supernatant at 414 nm on a microplate reader. The value $y/(1 - y)$ and the reciprocal of the plasma dilution were plotted on a log-log scale. The reciprocal dilution causing 50% lysis of RaRBCs was designated the ACH50. The results are presented as ACH50 units per milliliter.

Statistical Analysis

Analysis of differences in coloration, immune response, and carotenoid concentrations as a function of diet treatment, color morph category, and fish sex were performed using ANOVA in SPSS (v. 15). We tested for all first- and second-order interactions. Fulton body condition index (see above) was included as a covariate in all analyses, but only significant effects are included in the text. Differences were considered statistically significant at $P < 0.05$. Carotenoid concentration data from both plasma and tissue were log transformed to achieve a normal distribution. Sample sizes differed slightly among analyses because of missing blood or tissue samples, or because fish could not be definitively sexed. Post hoc statistical power was calculated based on small, medium, and large effect sizes (f^2) of 0.02, 0.15, and 0.35 using GPOWER (v. 2; Faul and Erdfelder 1992).

Animal Welfare

All animal protocols used in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of Amherst College.

Results

Coloration and Diet Treatment

After 10 wk on the low- and high-carotenoid diets, gold fish still had significantly higher PC2 scores than barred fish, but there was no effect of diet treatment on integument coloration in either the anterior (morph: $F_{1,40} = 23.2$, $P < 0.001$; diet: $F_{1,40} = 0.18$, $P = 0.67$) or posterior (morph: $F_{1,40} = 17.9$, $P < 0.001$; diet: $F_{1,40} = 0.98$, $P = 0.33$) body regions. There was no main effect of fish sex on coloration, nor were there any significant morph, diet, or sex interactions. Our statistical power to detect small (0.02), medium (0.15), or large (0.35) effect sizes of diet treatment on coloration was 0.16, 0.75, and 0.98, respectively.

Diet and Color Morph Effects on Plasma and Tissue Carotenoids

Across fish in both morph and diet groups, tissue concentrations of carotenoids were positively correlated with anterior ($r = 0.31$, $P = 0.03$) and posterior ($r = 0.34$, $P = 0.02$) PC2

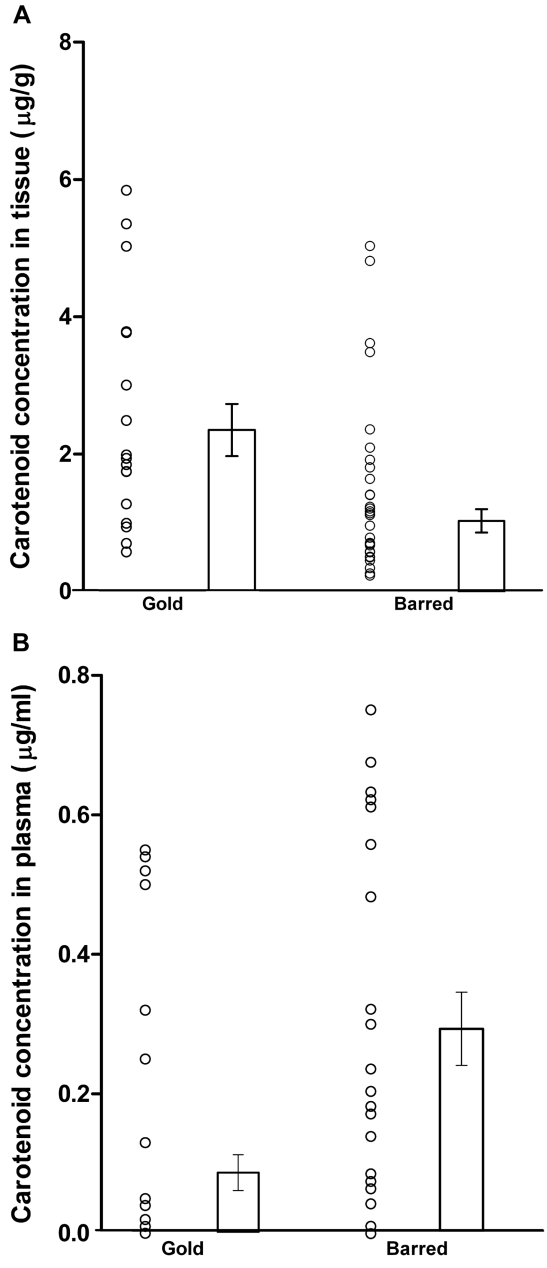

Figure 2. Gold fish (*A*) had more total carotenoids in their tissues than did barred fish ($F_{1,38} = 13.41$, $P = 0.001$) but (*B*) had fewer total carotenoids in plasma ($F_{1,37} = 16.84$, $P < 0.001$). Open circles represent data points from individual fish.

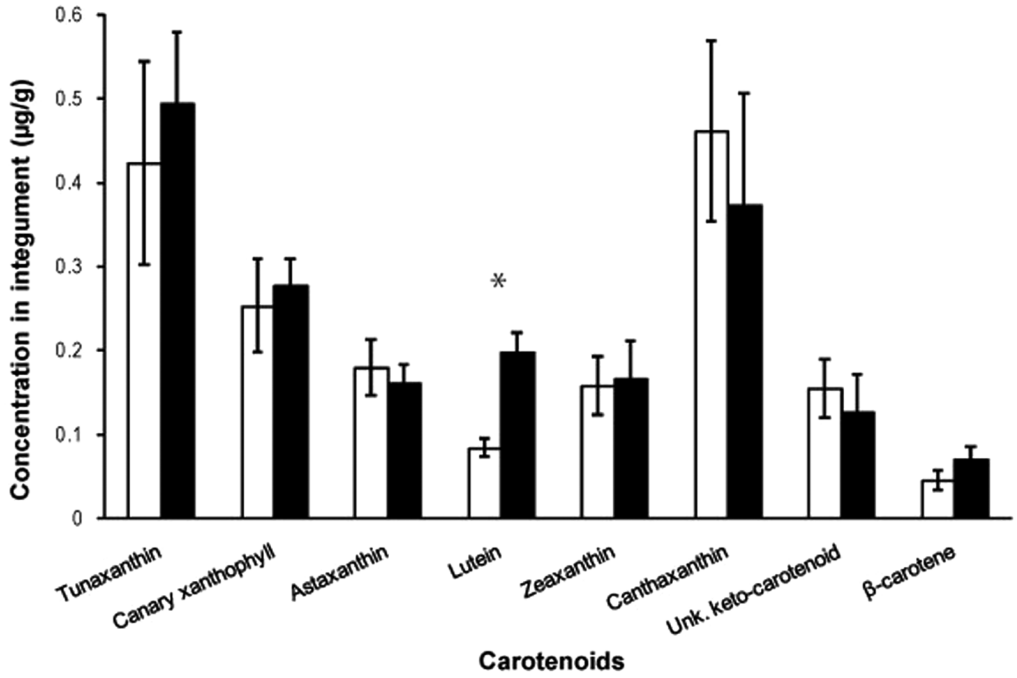

Figure 3. Carotenoids identified in Midas cichlid integument and their relative concentrations. White bars represent fish on the low-carotenoid diet, and black bars represent fish on the high-carotenoid diet supplemented with β-carotene, lutein, and zeaxanthin. The total carotenoid concentration in the skin did not differ between fish in the two diet treatment groups ($F_{1,42} = 0.25$, $P = 0.62$); only lutein was significantly different between the two groups.

values, meaning that gold fish had more tissue carotenoids than barred fish ($F_{1,38} = 13.41$, $P = 0.001$; Fig. 2*A*). Our high-carotenoid diet, however, did not cause a significant increase in overall levels of tissue carotenoids ($F_{1,41} = 0.26$, $P = 0.61$; Fig. 3). The power of this analysis to detect small, medium, and large effects was 0.16, 0.73, and 0.97. Lutein, the more concentrated of the two xanthophylls supplied to the fish, was significantly higher ($P < 0.001$) in the tissue of animals on the high-carotenoid diet. Females had higher tissue levels of carotenoids than males ($F_{1,38} = 7.41$, $P = 0.01$), and there was a marginally significant treatment × sex interaction ($F_{1,38} = 4.22$, $P = 0.047$).

Plasma carotenoid concentrations were negatively correlated with PC2 values in the anterior region ($r = -0.35$, $P = 0.018$) and the posterior region ($r = -0.29$, $P = 0.050$), meaning that gold fish had fewer plasma carotenoids than barred fish ($F_{1,37} = 16.84$, $P < 0.001$; Fig. 2*B*). Fish on the high-carotenoid diet had higher overall levels of plasma carotenoids than the control fish (ANOVA, $F_{1,37} = 21.98$, $P < 0.001$; Fig. 4). Plasma levels of the three carotenoids in our high-carotenoid diet were higher in fish on this diet than in fish on the low-carotenoid diet (lutein, $P = 0.007$; zeaxanthin, $P = 0.035$; β-carotene, $P = 0.035$), and these fish had nonsignificantly higher levels of tunaxanthin ($P = 0.06$). Female fish had marginally fewer plasma carotenoids than did males ($F_{1,37} = 2.95$, $P = 0.09$). There were no significant morph, diet, or sex interactions.

Trade-Offs between Coloration and Immunity

Fish on the low-carotenoid diet did not differ in their lysozyme activity from those on the high-carotenoid diet ($F_{1,40} = 0.51$, $P = 0.48$), and gold fish did not have significantly reduced lysozyme responses compared to barred fish ($F_{1,40} = 1.08$, $P = 0.30$; Table 1). The power of this analysis to detect small, medium, or large effects of diet treatment or color morph on lysozyme activity was 0.16, 0.75, and 0.98, respectively. Body condition was a positive predictor of lysozyme activity ($F_{1,40} = 7.51$, $P = 0.009$). There was no main effect of fish sex on lysozyme activity, nor were there any significant morph, diet, or sex interactions.

Alternative complement pathway hemolytic activity was not affected by fish color morph ($F_{1,33} = 0.22$, $P = 0.64$), diet treatment ($F_{1,33} < 0.01$, $P = 0.99$), or body condition ($F_{1,33} = 0.16$, $P = 0.69$; Table 1). The power of this analysis to detect small, medium, and large effects of diet treatment or color morph on complement activity was slightly lower: 0.15, 0.69, and 0.96, respectively. As with lysozyme activity, there was no main effect of fish sex, nor were there any significant morph, diet, or sex interactions.

Discussion

Dietary carotenoid supplementation failed to influence carotenoid-based skin coloration in Midas cichlids of either color morph, despite the fact that plasma carotenoid levels were sig-

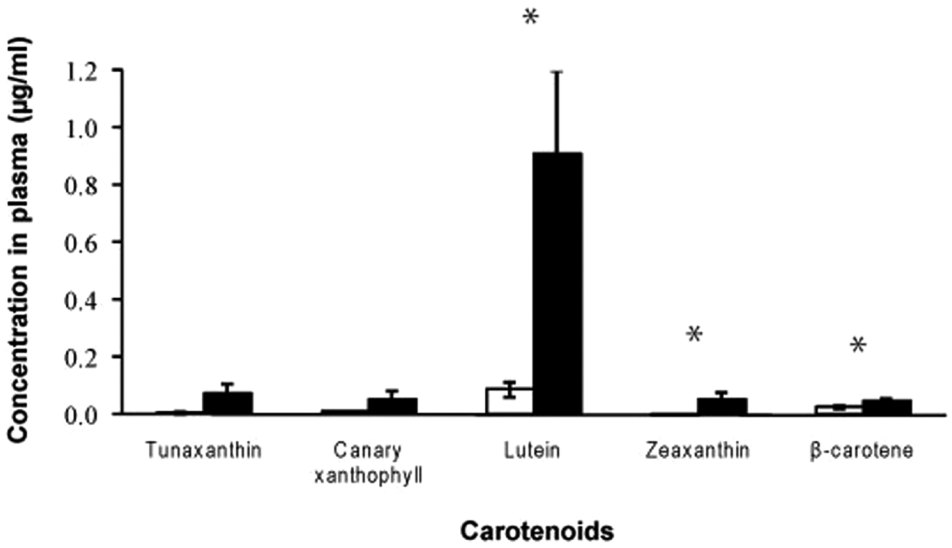

Figure 4. Carotenoids identified in Midas cichlid plasma and their relative concentrations. White bars represent fish on the low-carotenoid diet, and black bars represent fish on the high-carotenoid diet supplemented with β-carotene, lutein, and zeaxanthin. The total carotenoid concentration in plasma was significantly higher in fish on the carotenoid diet than in fish on the control diet ($F_{1,42} = 24.1, P < 0.001$). Significant differences in individual carotenoids are indicated by an asterisk.

nificantly elevated as a result of our high-carotenoid diet. Gold-colored cichlids had higher PC2 values than barred fish at the start and conclusion of the 10-wk experiment, regardless of diet treatment. Furthermore, we found that lysozyme activity and alternative complement pathway hemolytic activity, two important measures of innate immunity in teleosts, were not significantly different between gold and barred fish, or between fish on the two diets. Thus, we found no evidence of morph-dependent differences in carotenoid allocation under conditions of high carotenoid availability. Our results are consistent with the hypothesis (Barlow 1976) that the color transformation is under relatively strict genetic control and not limited by access to carotenoids. Unlike Webber et al. (1973), however, we found that gold morphs had higher levels of carotenoids in their integument than barred morphs, whereas the opposite was true of plasma carotenoids. Differences between our results and those of Webber et al. (1973) may be attributable to numerous factors, including the duration of the study, the experimental diet used, and our use of wild-caught versus captive-bred individuals.

Our results contrast with two previous studies that have demonstrated intraspecific differences in coloration-immune trade-offs between fish on low- and high-carotenoid diets. Grether et al. (2004) found that the immune response of male

Table 1: Innate immunity in Midas cichlids as a function of ontogenetic morph (gold vs. barred) or diet treatment (low vs. high carotenoids)

	Lysozyme Activity (lysozyme units mL^{-1})	Complement Factor (ACH50 units mL^{-1})
Morph:		
Gold	11,931.81 ± 1,033.73	87.95 ± 11.39
Barred	13,186.60 ± 621.61	81.64 ± 7.02
F	1.08	.22
df	1, 40	1, 33
P	.30	.64
Diet treatment:		
Low carotenoid	12,989.42 ± 973.38	84.88 ± 10.83
High carotenoid	12,128 ± 716.83	84.70 ± 7.87
F	.51	<.01
df	1, 40	1, 33
P	.48	.99

Note. Values represent estimated marginal means that account for the effects of body condition and fish sex.

guppies (*Poecilia reticulata*), which possess striking carotenoid-dependent coloration, was more dependent on dietary carotenoids than was true for females, which lack such coloration. Clotfelter et al. (2007) demonstrated that artificially selected color morphs of *Betta splendens* differed in their use of supplemental dietary carotenoids: fish with greater carotenoid coloration increased in redness, while fish with reduced carotenoid coloration mounted a more robust immune response. These studies may have found positive carotenoid-immunity relationships because the techniques the authors used (allograft and mitogenic challenge, respectively) stimulate, at least in part, the adaptive immune system. Our negative result may be attributed to the fact that we used in vitro assessments of innate immunity, which may be more influenced by body condition than by carotenoid intake per se because of the nutritional and metabolic costs of maintaining the immune system (Lochmiller and Deerenberg 2000; Kurtz et al. 2007). Indeed, we found that lysozyme activity was strongly influenced by fish body condition. However, Amar et al. (2000, 2001, 2004) found that supplementing the diets of rainbow trout (*Oncorhynchus mykiss*) with β-carotene or astaxanthin increased both alternative complement and lysozyme activity, whereas supplementation with astaxanthin only increased lysozyme activity. They found no significant increase in growth in trout on the carotenoid-supplemented diets, which was consistent with our findings that body condition did not differ between cichlids on the low- and high-carotenoid diets.

The absence of a clear relationship between carotenoids and innate immunity in Midas cichlids can also be interpreted in light of ambiguous results from bird studies. Carotenoid supplementation in birds has been demonstrated to have inconsistent effects on innate immunity (McGraw and Klasing 2006; McGraw et al. 2006). Furthermore, the importance of carotenoids as antioxidants in birds has been challenged by several recent studies (Costantini et al. 2007; Isaksson et al. 2007; Costantini and Møller 2008; Isaksson and Andersson 2008). Available data suggest that carotenoids are important antioxidants in fish (Wang et al. 2006) and thus would be predicted to correlate positively with overall health, but clearly the robustness of the carotenoid-immunity relationship merits further investigation across a range of taxa.

Our results might have been affected by complex interactions among carotenoids. We found more carotenoid pigments in the integument of Midas cichlids than did Webber et al. (1973), including tunaxanthin, canary xanthophyll, lutein, zeaxanthin, β-carotene, astaxanthin, and canthaxanthin. It is generally assumed that circulating plasma carotenoid levels are associated with tissue carotenoid concentrations, but this may not necessarily be the case. Aside from the competitive interactions between individual carotenoids, the expression of carotenoids is also controlled by the differential uptake of carotenoid molecules, both in the gut and at the tissue site (Parker 1989). Amar et al. (2004) noted in their study of trout that different carotenoids exhibit differences in absorption, tissue distribution, and retention. The authors commented that salmonids preferentially absorb and deposit more polar carotenoids, particularly astaxanthin, in favor of less polar carotenoids such as canthaxanthin, zeaxanthin, or carotenes (Amar et al. 2004). Their finding suggests that the ability to absorb and deposit carotenoids may be taxon specific.

It is possible that the negative results reported here are due to the duration of the experiment or because our low-carotenoid diet was not sufficiently limiting, but the available literature does not suggest these to be confounding factors. The duration of our experiment (10 wk) was similar to that used in several other studies that found positive coloring effects of carotenoids in fishes (Evans and Norris 1996; Amar et al. 2000; Grether et al. 2004; Wang et al. 2006; Clotfelter et al. 2007; Baron et al. 2008). Doolan et al. (2009) monitored changes in red and yellow coloration in Australian snapper (*Pagrus auratus*) fed supplemental astaxanthin and found significant color changes after as few as 3 wk on the high-carotenoid diets, and they also found that color expression reached a plateau after only 6 wk. Furthermore, Wang et al. (2006) found that characins (*Hyphessobrycon callistus*) fed diets supplemented with astaxanthin and/or β-carotene had increased antioxidant capacity after 8 wk, suggesting that our 10-wk experiment duration was sufficient to demonstrate carotenoid enhancement of innate immunity if it were to occur. With respect to our experimental diets, the concentration of total carotenoids in our low-carotenoid diet was similar to "low" diets used by Grether et al. (2005) and Kolluru et al. (2006), and less than "low" diets used by Pike et al. (2007*a*, 2007*b*). Thus, we believe our low-carotenoid diet was sufficiently limiting as to enforce a carotenoid trade-off in the Midas cichlids.

In conclusion, we found that gold Midas cichlids had higher levels of tissue carotenoids than their barred counterparts, which had higher carotenoid reserves in blood plasma. Neither morph changed color in response to supplemental β-carotene, lutein, and zeaxanthin. Innate immunity was not different between the color morphs, nor was it increased by the high-carotenoid diet. We can draw two general conclusions from this research. First, Midas cichlids are able maintain both coloration and innate immunity on a diet consisting of low levels (<10 μg/g) of carotenoids, suggesting that dietary carotenoids are not typically limiting in nature for this species. Second, although gold and barred morphs allocated more carotenoids to their integument and plasma, respectively, gold fish were not constrained in their ability to mount an innate immune response. Additional research is necessary to further test the carotenoid trade-off hypothesis, particularly in species with genetically determined color morphs.

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