Red fish, blue fish: trade-offs between pigmentation and immunity in *Betta splendens*

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Carotenoid pigments are responsible for many examples of sexually attractive red, orange, and yellow coloration in animals and play an important role in antioxidant and immune defenses. Because vertebrates cannot synthesize carotenoids, limited dietary availability may impose a trade-off between maintaining ornamental coloration and health. We used an experimental approach to test the carotenoid trade-off hypothesis in the fighting fish *Betta splendens*, by examining whether carotenoid allocation strategies differ among conspecifics that exhibit a gradient of body coloration from blue to red. We found that male redness is underlain by carotenoids and that females preferred to associate with red males over blue males, suggesting a sexually-selected advantage to being red. Moreover, we found strong experimental support for the carotenoid trade-off hypothesis, as individuals that varied in color did not appear to allocate carotenoids equally to both immune response and coloration. Redder fish given supplemental carotenoids increased in both immune response (to a phytohemagglutination challenge) and redness compared with controls. In contrast, bluer fish given supplemental carotenoids did not become more red but instead benefited immunologically more so than either control or redder supplemented fish. These results enhance our understanding of the evolution and plasticity of carotenoid mobilization and utilization pathways in animals. *Key words:* carotenoids, coloration, immune response, pteridines, sexual selection. *[Behav Ecol 18:1139–1145 (2007)]*

Pigment-based colors are common visual signals in the animal kingdom (Needham 1974). Carotenoid pigments are widely used to produce red, orange, and yellow coloration, especially in fishes, lizards, and birds (Evans and Norris 1996; Macedonia et al. 2000; Hill and McGraw 2006a). In many instances, these carotenoid-based colors are sexually attractive to prospective mates (Kodric-Brown 1993; Blount et al. 2003; Maan et al. 2006). Carotenoid pigments also serve a variety of physiological roles (Vershinin 1999), with one major function as an immunostimulant and antioxidant (Bendich 1989; Chew 1993; McGraw and Ardia 2003). Because carotenoid pigments are derived from dietary sources and cannot be synthesized de novo, their availability is under nutritional control in a variety of taxa (Grether et al. 1999; Alonso-Alvarez et al. 2004; Hill and McGraw 2006b). A consequence of this scarcity is that carotenoid allocation is condition dependent (Hill and Montgomerie 1994; von Schantz et al. 1999), which leads to a presumed trade-off between allocation to functions such as coloration and immunity (Lozano 1994; von Schantz et al. 1999; Faivre et al. 2003; Alonso-Alvarez et al. 2004; Peters et al. 2004). Individuals with the brightest colors are presumed to be those that have sufficient carotenoids for meeting both immunological and coloration functions and hence are the healthiest and most desirable mates.

Several types of studies have attempted to elucidate such a carotenoid trade-off. At a very basic level, the fact that dietary supplementation with carotenoids enhances both immunity and coloration (Blount et al. 2003; McGraw and Ardia 2003; Alonso-Alvarez et al. 2004) suggests that carotenoid-limited animals must dedicate carotenoids more to one or

another function or suffer both somatically and sexually. Second, experimental manipulations of health status in animals that deposit carotenoids in bare parts (e.g., beaks, legs, and flesh) have shown that immunocompromised animals fade in color (Faivre et al. 2003; Peters et al. 2004), suggesting that carotenoids are retrieved from colorful tissues to fight pathogenic or parasitic challenges. These studies have not tested the alternative that carotenoid deposition or metabolism was instead impaired by the immune challenge and thus whether trade-offs cause these color changes remains unknown. Third, in perhaps the best test of carotenoid trade-offs to date (Fitze et al. 2007), it was recently found that when 2 types of carotenoids (xanthophylls and carotenes) were provided to nestling great tits (*Parus major*), the carotenoids used in plumage coloration (xanthophylls) were not the carotenoids that influenced immunocompetence (carotenes). Through all this, however, we still await a rigorous, experimental test of the carotenoid trade-off hypothesis in adult animals that display sexually attractive, pigment-based coloration.

In contrast to this prior work, an ideal system for testing carotenoid trade-offs would be in species that show distinct color morphs that vary in their carotenoid dependency or their ability to mobilize carotenoids (Sinervo and Lively 1996; Craig and Foote 2001; Craig et al. 2005; Pryke and Griffith 2006) and thus may employ different carotenoid allocation strategies. In such a system, a key prediction for the carotenoid trade-off hypothesis would be that individuals that lack or have reduced carotenoid coloration should allocate relatively more dietary carotenoids to their immune system. Moreover, animals with extensive carotenoid coloration should suffer decreased immunocompetence compared with less carotenoid-colored animals given the same level of carotenoid uptake, due to increased allocation to coloration. Therefore, the goal of this study was to test whether genetically based intraspecific differences in body coloration affect relative allocation of carotenoids to coloration versus immune system. We did so by examining the effect of dietary carotenoid

supplementation on a range of color phenotypes in the Siamese fighting fish (*Betta splendens*). Artificial selection on *B. splendens* has produced a range of color morphs, particularly reds and blues, which provides a powerful tool for examining intraspecific carotenoid allocation strategies.

Our initial objectives were to determine the pigment basis for redness in *B. splendens* and the extent to which red body color in males is attractive to females. We used traditional biochemical methods to measure tissue concentrations of carotenoids and pteridines (drosopterins), another class of pigments that can contribute to red and orange coloration in fishes (Dupont 1958; Henze et al. 1977). Pteridines can be synthesized de novo (Hurst 1980); thus, it is important to consider the possibility that fish compensate for carotenoid scarcity by using pteridines as skin colorants (Grether et al. 2001). We predicted that both carotenoids and drosopterins would contribute to red coloration. With respect to female mate choice, we predicted that female *B. splendens* would favor red males over blue males in a dichotomous choice test. Little is known about female preferences for male redness in either wild-type or domestic stocks of this species, but female preferences for carotenoid-dependent coloration in males have been found in many other fishes (Kodric-Brown 1993; Candolin 1999; Maan et al. 2006).

Our main objective, however, was to test the trade-off hypothesis by comparing carotenoid allocation strategies as a function of a fish's initial body coloration. We supplemented dietary carotenoids to individuals over a range of body colors from blue to red (measured with UV–Vis spectrophotometry) in order to determine how the need to devote pigments to skin color affected the ability to allocate carotenoids to coloration at the expense of the immune system. First, we tested whether carotenoid supplementation increased redness and enhanced the inflammatory response to phytohemagglutinin (PHA). Similar work has been done on guppies (*Poecilia reticulata*) (Grether et al. 2004) and salmonids such as rainbow trout (*Oncorhynchus mykiss*) and sockeye salmon (*Oncorhynchus nerka*) (Amar et al. 2000; Craig and Foote 2001; Amar et al. 2004). Based on these studies and those cited above, we predicted that *B. splendens* given supplemental carotenoids would become redder as well as elevate their immune response.

After establishing that carotenoids boost both immune response and coloration (see Results), we tested a central prediction of the trade-off hypothesis: that initial body coloration affects allocation of supplemented carotenoids. Because fish could allocate additional carotenoids to either immune activity or coloration, we predicted that redder individuals would use supplemented carotenoids to augment both coloration and immunity, whereas less- or nonred individuals would use carotenoids to improve immunity and not coloration and thus show greater increases in immune response and smaller changes in coloration compared with redder fish.

MATERIALS AND METHODS

Fish were housed in individual, visually isolated 1-l beakers filled with municipal tap water that had been subjected to reverse osmosis and reconstituted to a conductivity of 100–150 µS. Water was changed at a rate of 25% every other day. Fish were maintained at 27 °C and a 12:12 h light:dark cycle.

Female preferences for male color

To establish whether female *B. splendens* have a mating preference for red males, we conducted a dichotomous mate choice test in the laboratory. We obtained sexually mature, male *B. splendens* from a commercial supplier. We measured standard length (SL) ± 0.01 mm with digital calipers. Five

uniformly red males and 5 uniformly blue males were selected and matched for size (SL red = 37.61 ± 0.56 mm, SL blue = 37.68 ± 0.52 mm; $t_8 = -0.10$, $P = 0.92$). They were also qualitatively matched for temperament, as measured by agonistic responsiveness to their mirror image (Clotfelter et al. 2006). Female mate choice was assessed by placing focal females in a $15 \times 15 \times 30$-cm tank; test males were housed in two $15 \times 15 \times 15$-cm tanks placed perpendicularly to the female choice tank such that they could not see each other. Females acclimated to the choice tank for 5 min, after which time they were allowed to view males for a 5-min prechoice period to ensure that they visited both males before data collection began. We then measured the time spent by females (in seconds) in each third of the tank during a 5-min choice period. The positions of red and blue males were alternated between the left and right tanks to eliminate the effects of potential side biases by females.

Carotenoid supplementation

To the human eye, predominant body coloration ranged from blue to purple to red. Fish were paired with respect to these categories of body coloration (see below for spectral analyses of skin color), and then one member of each pair was randomly assigned to the experimental (carotenoid supplemented) and control treatment groups. The carotenoid-supplemented diet (in flake form) contained the following (percent by mass): spray-dried white fish meal (20%), wheat flour (20.2%), vegetable oil (0.9%), vitamins including vitamin A in palmitate form (0.5%), water (58.1%), and β-carotene (0.3%); a similar diet has been used for guppies (Grether et al. 2004). The control diet was identical with the exception that a similar quantity of water was substituted for β-carotene. Both diets were prepared by Ocean Star International, Inc. (Snowville, UT) Sixty-one male *B. splendens* (28 controls and 33 carotenoid supplemented) were used in our diet experiment. Fish were fed a ration equivalent to 5% of their body mass twice daily for 8 weeks. Post hoc comparisons showed that experimental groups did not differ in either initial coloration or body size (see Results).

Color measurements

Before and after the 8-week diet treatment, we measured the reflectance of each fish using an Ocean Optics USB2000 spectrometer connected to a PX-2 pulsed xenon light. Fish were removed from their home beakers and immobilized against a moist sponge. We measured reflectance from a 1-cm diameter region of the left side of the caudal peduncle for 10 s. We assessed reflectance at 5-nm intervals over the wavelength range of 300–700 nm using a 400-µm reflection probe (Ocean Optics R400-7) held at a 45° angle 5 mm from the sample (Lahti 2006). Integration time was set at 100 ms, and reflectance was averaged over 100 scans; boxcar smoothing was set to 5. We standardized measurements with a diffuse tile made of polytetrafluoroethylene that reflects >98% of light over all sampled wavelengths (Ocean Optics WS-1).

We summarized reflectance data using principal components (PCs) analysis (Jolliffe 1986), thought to be the most appropriate means of reducing spectrophotometric data for analysis (Cuthill et al. 1999). We reduced color data to 3 PCs, which explained 96.2% of the variance in the sample, thus creating 3 independent measures of color. PC1 (hereafter "brightness") explained 71.8% of the variance in the sample, loaded negatively across the entire (300–700 nm) range of wavelengths, and corresponded to differences in brightness (Endler 1990). Individuals with high values of PC1 were those with low brightness (i.e., low reflectance). PC2 (hereafter
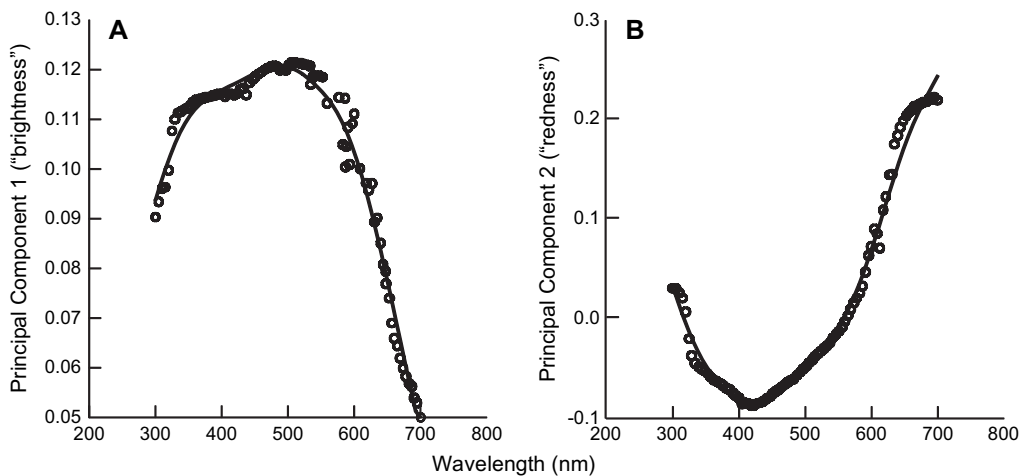
Figure 1
Loadings of 2 PCs from a PCs analysis of light reflected from the caudal peduncle of male *Betta splendens*, plotted against light wavelength (nanometers). (A) PC1 is a measure of skin brightness, in which greater values indicate lower brightness (i.e., reflectance) and (B) PC2 is a measure of redness.

"redness") explained 17.2% of the variation and loaded negatively between 320 and 520 nm and highly positively between 600 and 700 nm, thus making PC2 an assessment of red versus blue coloration. Higher values of PC2 were redder individuals. Plots of PC loadings versus wavelength are shown in Figure 1. We validated PC2 as an index of redness by regressing the final PC2 value for each fish on the wavelength at peak reflectance from our reflectance curves, which yielded a significantly positive relationship ($R^2 = 0.10$, $F_{1,58} = 6.71$, $P = 0.012$). The reflectance curves we obtained from red fish were qualitatively similar to those for the red ventral coloration of threespine sticklebacks (*Gasterosteus aculeatus*) (Rush et al. 2003; Rick et al. 2004).

Immune response

We assessed generalized cell-mediated immunity using exposure to PHA, measured as an inflammatory response (Martin et al. 2006). We previously validated this technique for use in *B. splendens* (Ardia and Clotfelter 2006) by comparing the inflammatory response of fish injected with PHA with those injected with saline. Each individual was anesthetized in tricaine methanesulfonate and placed on a wet sponge under a 6.3× dissecting microscope. On the right side of the caudal peduncle, 3–5 scales were removed to mark the injection site for consistent measurements. Prior to injection, the thickness of the caudal peduncle at the location of scale removal was measured with a digital micrometer (±0.001 mm accuracy) 3 times ($F_{98,196} = 34.3$, $P < 0.0001$, repeatability = 0.94). After measurements, each individual was injected at the location of scale removal with 4 µg of PHA (L-8751, Sigma–Aldrich, St. Louis, MO) in 2 µl of phosphate-buffered saline. After 24 h, each fish was anesthetized again and the thickness of the tissue at the location of injection and scale removal was remeasured (repeatability $F_{60,120} = 22.1$, $P < 0.0001$, repeatability = 0.84). The response of each individual was recorded as the difference (in millimeters) between postinjection thickness and preinjection thickness (Smits et al. 1999).

Pigment analysis

At the end of the carotenoid supplementation period, we euthanized fish and immediately removed a 0.5 × 0.5-cm section of dermis and epidermis from the caudal peduncle; tissue samples were stored at −80 °C until analysis. Thawed tissue was then ground in 2 ml methyl *tertiary*-butyl ether (MTBE) for 2 min in a mixer mill (McGraw et al. 2003). The jar was then rinsed with 1 ml MTBE to remove any residual pigment

and combined with the 2 ml extract in a 9 ml screw-cap glass tube. We then added 2 ml of 1% NH_4OH to the tube, vortexed it for 1 min, and then centrifuged it for 5 min at 3000 rpm. This method partitioned the carotenoids into the top (MTBE) layer and the pteridines into the bottom (NH_4OH) layer. We used absorbance spectrophotometry separately on the 2 fractions to determine carotenoid and pteridine concentrations based on standard calculations (McGraw et al. 2002). Carotenoids in the MTBE fraction absorbed light maximally at 447 nm and presumably were yellow xanthophylls; pteridines in the ammonium hydroxide fraction absorbed maximally at 490 nm and were presumably drosopterins (Grether et al. 2001). Thus, in our calculations, we used 2550 as the extinction coefficient for xanthophyll carotenoids (Bauernfeind 1981) and 10 000 as the extinction coefficient for drosopterins (Wilson and Jacobsen 1977).

Statistical analysis

All variables met the assumptions of parametric statistics. We used SAS 9.1.3 to conduct statistical analyses. We tested the effect of color on mate choice using a mixed model analysis of variance (ANOVA) and compared initial allocation of fish to treatments by color using an unpaired *t*-test. The effect of supplementation on immune response, coloration, and tissue pigment concentrations was tested using analysis of covariance, with initial fish coloration and SL included as covariates. Thus, we report least square means that account for the effects of these covariates. We included interaction terms in the models that corresponded to our a priori predictions. Tests were 2 tailed, and differences were considered significant at $P < 0.05$. Means are shown with ±standard error (SE).

RESULTS
Pigment basis of coloration

To understand the basic relationship between pigments and skin color in this species, we analyzed data for control fish only. Tissue pigment concentrations were highly significant predictors of fish brightness (PC1) ($F_{2,22} = 18.5$, $P < 0.0001$). Drosopterins were positively correlated with PC1 ($F_{1,22} = 15.8$, $\beta = 0.67$, $P = 0.001$), indicating that high levels of drosopterins were found in fish with low brightness. Carotenoids had no relationship to final brightness ($F_{1,22} = 1.2$, $\beta = 0.18$, $P = 0.29$). Skin pigment concentrations were also significant predictors of fish redness ($F_{2,22} = 4.1$, $P = 0.03$), which varied with a continuous distribution defined by PC2.
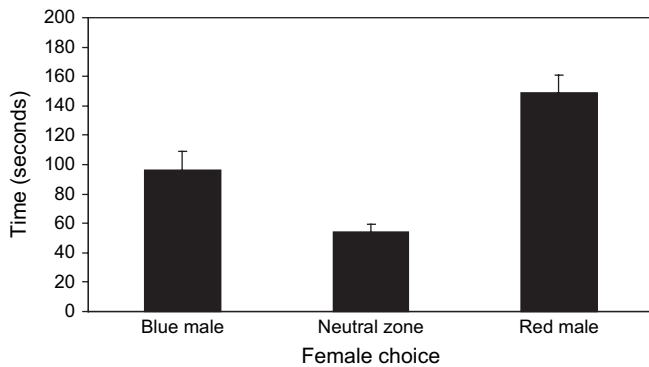
Figure 2
Female *Betta splendens* ($N = 23$) spent more time ($F_{1,44} = 9.36$, $P = 0.004$) associating with red males than they did with blue males.

Redder fish had more carotenoids ($F_{1,22} = 5.4$, $\beta = 0.54$, $P = 0.03$) but fewer drosopterins ($F_{1,22} = 7.5$, $\beta = -0.64$, $P = 0.01$).

Supplementation of *B. splendens* with dietary carotenoids had weak effects on the concentrations of skin carotenoids (controls = 5.16 ± 0.45 µg/g, $N = 27$; supplemented = 5.96 ± 0.33 µg/g, $N = 32$; $t_{57} = -1.46$, $P = 0.15$) and drosopterins (controls = 3.95 ± 0.49 mg/g, $N = 26$; supplemented = 5.15 ± 0.52 mg/g, $N = 33$; $t_{57} = -1.65$, $P = 0.10$), tending to increase both carotenoid and pteridine levels. However, dietary carotenoid supplementation increased skin carotenoid concentrations in fish that were red at the start of the experiment (initial PC2 score) (effect of supplementation: $F_{1,55} = 7.6$, $P = 0.008$; initial PC2 score: $F_{1,55} = 4.4$, $P = 0.04$; initial PC2 × supplementation: $F_{1,55} = 6.8$, $P = 0.01$). These fish also had more drosopterins in their skin, but their drosopterin concentrations increased only marginally due to carotenoid supplementation (effect of supplementation: $F_{1,53} = 2.7$, $P = 0.10$; initial PC2 score: $F_{1,53} = 4.3$, $P = 0.04$); there was also no interaction between initial PC2 score and the effect of supplementation ($F_{1,53} = 0.08$, $P = 0.78$). When we tested only fish with initial positive redness (PC2) scores, we found a significant effect of carotenoid supplementation on skin carotenoid concentrations ($t_{28} = 2.7$, $P = 0.04$; controls = $5.17 + 0.41$, $N = 13$; supplemented = $6.79 + 0.37$, $N = 18$) but no difference in skin drosopterin concentrations ($t_{28} = -0.6$, $P = 0.54$; controls = $4.75 + 0.7$, $N = 13$; supplemented = $5.34 + 0.6$, $N = 18$).

Female preference for red coloration in males

Female *B. splendens* ($N = 23$) spent significantly more time in the side of the tank nearest the red male (149.09 ± 12.12 s) as with the blue male (96.48 ± 12.21 s) (Figure 2; mixed model ANOVA $F_{1,44} = 9.36$, $P = 0.004$). Female preference was unaffected by which pair of male stimulus fish we used (removal of term led to no change in −2 log likelihood ratio). Note that this was the only experiment in which fish were categorized dichotomously (red vs. blue) rather than continuously (PC2).

Carotenoid supplementation boosts red coloration

Post hoc comparisons showed that we allocated fish to treatment groups irrespective of their initial brightness (control = -0.90 ± 1.34, $N = 28$; supplemented = -0.27 ± 1.53, $N = 33$; $t_{59} = -0.31$, $P = 0.76$), redness (control = -0.59 ± 0.78, $N = 28$; supplemented = 0.19 ± 0.67, $N = 33$; $t_{59} = -0.77$,
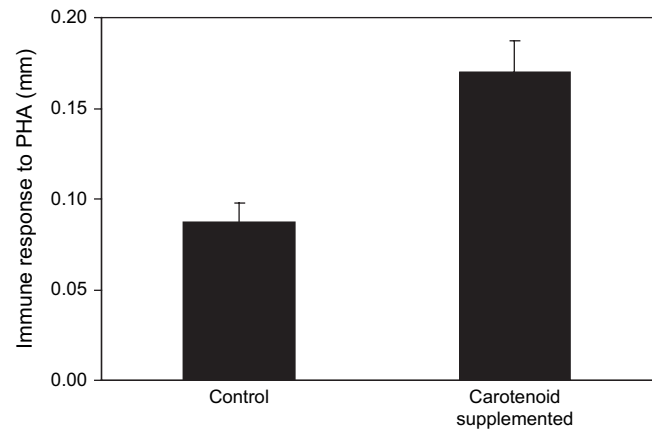
Figure 3
Fish supplemented with dietary carotenoids ($N = 33$) were able to mount a significantly greater immune response to the PHA injection than were the fish on the control diet ($N = 28$; $F_{1,58} = 12.68$, $P = 0.001$).

$P = 0.45$), or SL (control = 37.56 ± 1.45 mm; supplemented = 39.06 ± 0.34 mm; $t_{59} = -1.09$, $P = 0.28$).

We were able to measure initial (before carotenoid supplementation) and final brightness and redness in 60 (27 controls and 33 supplemented) of the 61 fish (erroneous final color measurement on one fish resulted in a significant outlier; Cook's distance = 0.76). The carotenoid trade-off hypothesis assumes that the delivery of supplemental carotenoids will enhance coloration, and consistent with this, we found that fish supplemented with carotenoids became significantly more red (PC2) (least square mean ± SE change in redness; controls = -2.13 ± 0.51, $N = 27$; supplemented = 0.33 ± 0.46, $N = 33$; $F_{1,54} = 43.76$, $P < 0.0001$). Carotenoid supplementation did not, however, induce a significant change in skin brightness (PC1) (control = 0.43 ± 15.04, $N = 27$; supplemented = 1.58 ± 15.24, $N = 33$; $t_{59} = -0.30$, $P = 0.77$).

Carotenoid supplementation boosts immune response

A second key assumption of the trade-off hypothesis is that carotenoid supplementation boosts immune activity. Also consistent with this, we found that carotenoid supplementation significantly increased the immune response of male *B. splendens*, as measured by the swelling of the caudal peduncle in response to PHA injection (Figure 3; mean postinjection swelling in mm ± SE: control = 0.087 ± 0.01 mm, $N = 28$; supplemented = 0.17 ± 0.02 mm, $N = 33$; carotenoid supplementation group: $F_{1,58} = 12.68$, $P = 0.001$). We obtained similar results when we used the ratio of preinjection swelling to postinjection swelling as our dependent variable (data not shown).

Initial coloration affects trade-offs between immune response and coloration

In support of a key prediction for the carotenoid trade-off hypothesis—namely, that animals with less carotenoid-dependent coloration should devote comparatively more carotenoids to an immune response than should animals with more such coloration—we found that initial body coloration affected allocation strategies of carotenoids between coloration and immunity. Fish with initially low PC2 values (more blue) showed a larger increase in immune activity (PHA response) than did redder fish when supplemented with carotenoids (Figure 4;
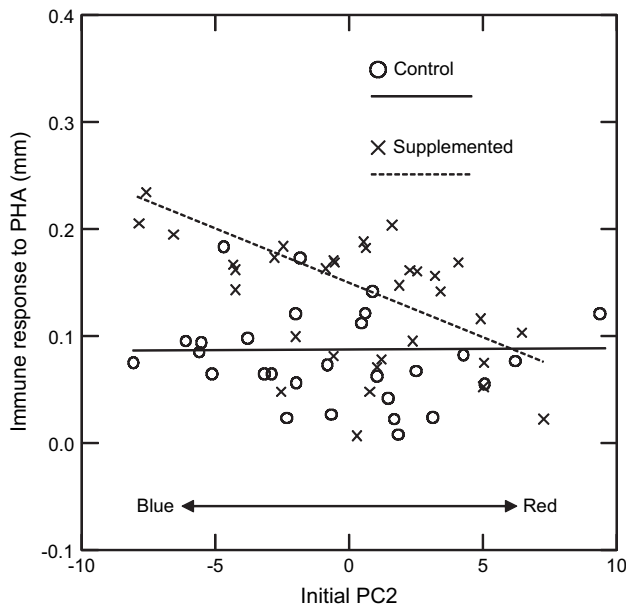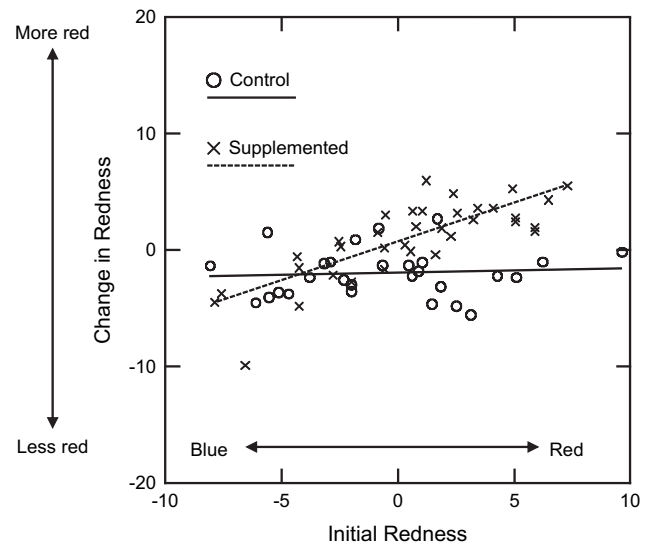
Figure 4
Fish that were initially bluer (negative PC2 values; see text for explanation) and received the carotenoid-supplemented diet showed a larger boost in immune activity than did redder fish (positive PC2 values) or than did fish on the control diet (overall model: $F_{4,56} = 7.83$, $P < 0.0001$; initial redness: $F_{1,56} = 6.78$, $P = 0.01$; initial redness × supplementation: $F_{1,56} = 7.59$, $P < 0.01$; supplementation: $F_{1,56} = 15.5$, $P = 0.0002$).

overall model: $F_{4,56} = 7.83$, $P < 0.0001$; initial redness: $F_{1,56} = 6.78$, $P = 0.01$; initial redness × supplementation: $F_{1,56} = 7.59$, $P < 0.01$; supplementation: $F_{1,56} = 15.5$, $P = 0.0002$). Carotenoid supplementation increased redness (least square mean ± SE change in redness; controls = −2.13 ± 0.51, $N = 27$; supplemented = 0.33 ± 0.46, $N = 33$; $F_{1,54} = 43.76$, $P < 0.0001$) but only in fish that were initially more red (Figure 5; initial redness: $F_{1,54} = 116.6$, $P < 0.0001$; initial redness × supplementation: $F_{1,54} = 85.3$, $P < 0.0001$). Supplementation decreased fish brightness, as indicated by higher PC1 values (least square mean ± SE change in brightness; controls = −1.12 ± 0.82, $N = 27$; supplemented = 2.58 ± 0.73, $N = 33$), such that reflectance in less-bright fish decreased even further in response to supplementation than in fish with higher initial brightness (initial brightness: $F_{1,54} = 166.6$, $P < 0.0001$; initial brightness × supplementation: $F_{1,54} = 10.8$, $P = 0.001$). Neither immune responses nor SLs were significantly related to changes in redness or brightness when they were included as covariates ($F \leq 1.2$, $P \geq 0.17$).

DISCUSSION

Organisms are predicted to make trade-offs when resources are scarce and serve multiple functions. Carotenoids are thought to be an example of a scarce resource; they must be acquired through the diet, and they provide benefits to both health and sexually selected coloration. We report experimental evidence demonstrating carotenoid trade-offs in *B. splendens*; male *B. splendens* displayed different carotenoid allocation strategies based on their initial coloration. Unlike other species in which this trade-off has been examined, where the ability to maintain carotenoid-based coloration is condition dependent and results in a range of red and less-red phenotypes, male *B. splendens* have genetically determined color morphs. Redder individuals (positive PC2 values) provided

Figure 5
Carotenoid supplementation increased redness (PC2; see text for explanation), and the change in redness varied depending on initial redness (initial redness: $F_{1,54} = 116.6$, $P < 0.0001$; initial redness × supplementation: $F_{1,54} = 85.3$, $P < 0.0001$).

with supplemental carotenoids showed an increased inflammatory response to PHA and greater redness, whereas bluer individuals (negative PC2 values) showed no change in coloration and instead mounted an even greater immune response. In other words, because bluer fish were faced with an inherently more relaxed carotenoid trade-off for health versus coloration (fewer carotenoids devoted to color), they apparently diverted their accumulated pool of carotenoids more to one function (immune response) than to the other (color).

Role of carotenoids in coloration, mate choice, and immunity

We first found support for 3 key assumptions of the carotenoid trade-off hypothesis in *B. splendens*: that carotenoid pigments 1) are used for coloration, 2) are a predictor of female preference, and 3) boost both immunity and coloration when in abundance. We found that coloration in *B. splendens* is underlain by both carotenoids and pteridines, as is true for many other red or orange color patches in fish (Henze et al. 1977) and lizards (Macedonia et al. 2000). Carotenoid-supplemented fish became redder in color, and naturally redder fish had higher carotenoid concentrations in their skin as a result of the supplementation. Furthermore, carotenoid supplementation decreased fish brightness, providing further evidence that dietary carotenoids were allocated to skin coloration. There was also evidence that redder fish had greater drosopterin concentrations in their skin. Grether et al. (2001) reported that skin pteridine concentrations in Trinidadian guppies covaried positively with natural carotenoid availability due to population genetic differences in drosopterin content. Grether et al. (2005) also found that experimental manipulations of dietary carotenoids can marginally and inversely impact skin drosopterin concentrations. Clearly, more work is needed to better understand the complementarities and competitions between these 2 classes of integumentary colorants.

Second, we report a role of red coloration in sexual selection in this species. Female *B. splendens* display a preference for associating with red males. Such female preferences for carotenoid-dependent signals in males have been reported in other fishes (Kodric-Brown 1993; Candolin 1999; Maan et al.

2006) but have not been previously reported for *B. splendens* or any member of its perciform family (Osphronemidae).

Finally, we demonstrated that dietary supplementation with carotenoids significantly increased the ability of male *B. splendens* to mount an inflammatory response to PHA injection. Our results provide additional evidence for the immunoenhancing role of carotenoids in vertebrates and that environmental scarcity of carotenoids may lead to the evolution of allocation strategies. Moreover, our use of the generalized swelling response to PHA as a metric of immune response (Ardia and Clotfelter 2006) complements the humoral measures (Amar et al. 2004) or allografting outcomes (Grether et al. 2004) used in other fish studies.

Experimental support for the trade-off hypothesis: the effect of initial coloration

In our key test of the carotenoid trade-off hypothesis, we provided evidence that individuals vary in their carotenoid allocation strategy depending on their degree of carotenoid-based skin coloration. We found that redder fish appeared to allocate their supplemental carotenoids to both immune response and color, as they increased in redness over the supplementation period and increased their inflammatory response to PHA relative to control fish. In contrast, bluer fish (individuals with negative PC2 scores) given supplemental carotenoids did not change color but instead mounted a greater inflammatory response to the PHA challenge than observed in either control or redder supplemented fish.

This study is the first to demonstrate that, within members of the same sex, individuals whose coloration is less carotenoid based have a qualitatively different carotenoid allocation strategy than do redder conspecifics. Grether et al. (2004) have previously shown that carotenoid enhancement of immunity in guppies is sex specific because males—and not females—have carotenoid-based coloration. Most studies that have attempted to shed light on carotenoid trade-offs in animals have examined species whose yellow-to-red coloration is purely carotenoid based and is environmentally (condition) dependent (Blount et al. 2003; McGraw and Ardia 2003; Alonso-Alvarez et al. 2004; Peters et al. 2004). In such systems, carotenoid trade-offs have been more difficult to evaluate because there is no obvious group of animals that is constrained in carotenoid allocation (i.e., even drab animals can divert supplemental pigments to both immunity and coloration). While we realize that this genetic color polymorphism has been derived through artificial selection, we believe that these results provide a model for examining intra- and interspecific differences in carotenoid allocation strategies, as artificially selected fish represent extremes of a naturally occurring color gradient. Wild *B. splendens* possess both blues and reds to varying degrees, and many of the domestic fish we used in this study fell along a similar continuum. Furthermore, we showed that even the bluest fish had carotenoids in their dermis, demonstrating their ability to develop carotenoid-based pigmentation. Thus, selection for uniformly blue and red coloration in domestic strains led to associated changes in carotenoid allocation strategies, leading to changes in both coloration and carotenoid usage. Applying this approach to examining differences among populations or closely related species that differ in the extent of carotenoid-based coloration may help elucidate the rate and extent of change in the costs and benefits of allocating carotenoids to coloration versus immune response.

Overall, our results indicate that body coloration in polychromatic species can have a strong effect on carotenoid allocation strategies. Research investigating the role of evolutionary trade-offs between sexual ornaments and immunity

should examine underlying differences in carotenoid allocation strategies that may be caused by differences in coloration.

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