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Source: *Evolution*, 54(5):1712-1724.

Published By: The Society for the Study of Evolution

[https://doi.org/10.1554/0014-3820\(2000\)054\[1712:CLAMPE\]2.0.CO;2](https://doi.org/10.1554/0014-3820(2000)054[1712:CLAMPE]2.0.CO;2)

URL: <http://www.bioone.org/doi/full/10.1554/0014-3820%282000%29054%5B1712%3ACLAMPE%5D2.0.CO%3B2>

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CAROTENOID LIMITATION AND MATE PREFERENCE EVOLUTION: A TEST OF THE INDICATOR HYPOTHESIS IN GUPPIES (*POECILIA RETICULATA*)

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Abstract.—Under the indicator models of mate choice, female preferences evolve to exploit the condition-dependence or “indicator value” of male traits, which in turn may cause these traits to evolve to elaborate extremes. If the indicator value of a male trait changes, the payoff function of the female preference for that trait should change and the preference should evolve to a new optimum. I tested this prediction in the guppy, *Poecilia reticulata*, a species in which the indicator value of a sexually selected male trait, carotenoid coloration, varies geographically. Carotenoid coloration is thought to be an indicator of foraging ability and health because animals must obtain carotenoid pigments from their diet. The primary dietary source of carotenoids for guppies is unicellular algae, the abundance of which varies among natural streams because of variation in forest canopy cover. Carotenoid availability limits male coloration to a greater extent in streams with greater forest canopy cover. Thus, the indicator value of male coloration covaries positively with canopy cover. To test the indicator model prediction, I measured genetic divergence in the strength of female preferences for carotenoid coloration between high- and low-carotenoid availability streams in each of three river drainages. Second-generation laboratory-born females were given a choice between full-sib males raised on three different dietary levels of carotenoids. For all six populations, male attractiveness (as determined from the responses of females to male courtship displays) increased with dietary carotenoid levels. However, the strength of female preferences differed between populations in the predicted direction in only one of three river drainages. These results fail to support a crucial prediction of the indicator model. More studies taking an interpopulation approach to studying mate preference evolution are needed before the explanatory value of the indicator models can be rigorously assessed.

Key words.—Carotenoid coloration, condition-dependent indicators, geographic variation, mate choice, sexual selection.

Received October 29, 1999. Accepted May 11, 2000.

Numerous studies provide evidence that females choose mates on the basis of traits that reveal differences between males in condition (reviewed in Andersson 1994), as predicted by the indicator models of mate choice (e.g., Zahavi 1975; Hamilton and Zuk 1982; Kodric-Brown and Brown 1984; Nur and Hasson 1984; Andersson 1986; Pomiankowski 1988; Grafen 1990; Rowe and Houle 1996). These models postulate that females obtain direct or indirect fitness benefits by mating with males in good condition and, consequently, female preferences evolve to exploit the condition-dependence or “indicator value” of developmentally plastic male traits. Other models reverse the direction of causality; male traits evolve to exploit preexisting female preferences (Basado 1990; Ryan 1990; Endler 1992; Endler and Basolo 1998), or female preferences evolve as a genetically correlated response to the selection they impart on male traits (e.g., Fisher 1930; Lande 1981). Under the nonindicator models, condition dependence of male traits may be coincidental or a side effect of past directional selection caused by female preferences (Kirkpatrick 1987; Balmford and Read 1991; Kirkpatrick and Ryan 1991). In theory, both indicator and nonindicator processes could influence the evolution of a given female preference (Fisher 1930; Endler 1980; Pomiankowski 1988; Ryan 1990). Nevertheless, it may be possible to exclude one or more processes in particular cases (e.g., Ryan et al. 1990), with the ultimate goal of evaluating their relative importance (Bradbury and Andersson 1987).

Under the indicator models, stronger female preferences should evolve when male traits are better indicators of the benefits that females could potentially receive by being

choosy (Harvey and Bradbury 1991; Kirkpatrick and Ryan 1991; Andersson 1994). So far, this prediction has only been tested indirectly, by examining the relationship between parasite loads and male secondary sexual trait elaboration across species (e.g., Hamilton and Zuk 1982; Read and Weary 1990; Zuk 1991). Unfortunately, this interspecies approach has run into both theoretical and empirical problems (Read and Harvey 1989; Møller 1990; Balmford and Read 1991; Harvey and Bradbury 1991; Kirkpatrick and Ryan 1991; Zuk 1992). A more direct approach is to examine species in which the indicator value of a secondary sexual trait varies geographically. Given sufficiently low levels of interpopulation gene flow, stronger mate preferences should evolve in populations where the trait is a better indicator. Causal ambiguity can be avoided by choosing systems in which the indicator value of a trait varies in response to extraneous, environmental factors. One such environmental factor is the availability of dietary carotenoid pigments.

Carotenoids are the most common yellow, orange, and red pigments found in the integument of animals (Fox 1979; Goodwin 1984). Carotenoid-based sexual coloration has been shown to correlate with indices of condition (Endler 1980; Hill 1990; Nicoletto 1991; Putnam 1992; Zuk 1992; Frischknecht 1993; Hill and Montgomerie 1994) and to influence mate choice in several species (e.g., Burley and Coopersmith 1987; Kodric-Brown 1989; Hill 1990, 1991; Hillgarth 1990; Milinski and Baker 1990; Zuk et al. 1990; Houde and Torio 1992). Animals cannot synthesize carotenoids (Goodwin 1984; Schiedt 1989) and therefore depend on dietary sources, such as carotenoid-producing plants or bacteria or other ca-

rotenoid-sequestering animals (Fox 1979; Schiedt 1989). There are several possible mechanisms through which carotenoid coloration may serve as an indicator of condition (Thompson et al. 1997; Olson and Owens 1998; Hill 1999; von Schantz et al. 1999); weak animals may ingest carotenoids at low rates (Endler 1980, 1983; Kodric-Brown and Brown 1984; Putnam 1992), parasites may interfere with carotenoid absorption or metabolism (Putnam 1992), or carotenoids in the skin may be mobilized by the immune system to help combat infections (Lozano 1994). However, under most proposed mechanisms, carotenoid scarcity would enhance the indicator value of carotenoid coloration (Grether et al. 1999).

The well-studied color patterns of guppies are expressed only by males and include carotenoid-containing orange spots, the color saturation or chroma of which decreases on low-carotenoid diets (Kodric-Brown 1989; this study), is reduced by parasitic infection (Houde and Torio 1992), and correlates positively with swimming performance (a measure of condition; Nicoletto 1991). In contrast, the area of the orange spots is highly heritable (Winge 1927; Haskins et al. 1961; Houde 1992) and not affected by carotenoids in the diet (Kodric-Brown 1989; this study). Orange spot area and chroma both influence female mate choice (Endler 1983; Kodric-Brown 1985; Houde 1987; Kodric-Brown 1989; Long and Houde 1989; Houdo and Torio 1992) to degrees that vary genetically both within and among populations (Breden and Stoner 1987; Houde 1988, 1994; Houde and Endler 1990; Endler and Houde 1995; Brooks and Couldrige 1999; this study).

The primary source of carotenoids for guppies in nature is attached unicellular algae, the availability of which (among unlogged streams) is largely a function of stream order. High-order streams tend to be wider, make larger gaps in the forest, receive more photosynthetically active light, and contain larger standing crops of algae, but not higher densities of guppies, than low-order streams (Grether et al. 2000). Consequently, guppy populations in the upper reaches of a drainage tend to experience greater carotenoid limitation than those in the lower reaches (Grether et al. 1999). If the female preference for carotenoid coloration has evolved, in part, through an indicator process, the strength of the preference should covary positively with carotenoid limitation. In this paper, I present the results of an experiment designed to test this prediction.

MATERIALS AND METHODS

Study Populations

The fish used in this study were second-generation (F_2) laboratory descendents of fish collected from 10 to 20 pools in each of six streams in the Northern Range of Trinidad in December 1996. The streams were chosen during an extensive survey of several river drainages conducted in April 1996, based on the following criteria: (1) intact primary or old secondary growth forest; (2) relatively homogeneous forest canopy cover; (3) separated from streams differing in canopy cover or predator assemblage by multiple barriers to guppy dispersal, including two or more waterfalls; and (4) no predatory fish, except *Rivulus hartii*. Among streams meet-

ing these criteria, I chose two streams representing the available extremes in forest canopy cover in each of three river drainages (Marianne, Paria, and Quare). The goal was to compare guppy populations exposed to different levels of canopy cover, and thus carotenoid availability (Grether et al. 1999), without the potentially confounding effects of phylogenetic divergence between drainages or differential predation. The possibility of gene flow between study streams was virtually eliminated in the Marianne and Quare drainages by choosing streams in separate upstream branches of the watershed. In the Paria drainage, a small headwater stream was paired with the river it flows into (after a series of waterfalls). Gene flow, if any, from the tributary to the river is probably insignificant because of the large disparity in size between the guppy populations. See Grether et al. (2000) for additional details on the location of the study sites and their physical and ecological characteristics and Grether et al. (1999) for evidence that the guppy populations in these streams differ in the degree of carotenoid limitation.

Care was taken to prevent inbreeding and genetic bottlenecks in the laboratory generations. A total of 141 wild females contributed offspring to the F_1 generation (15–27 per population). The number of male founders was probably much larger because females mate multiply in the wild and store viable sperm for up to 8 months (Winge 1937; Carvalho et al. 1996). In the F_1 generation, the sexes were separated before males developed color patterns or mature gonopodia. After sexual maturity, unrelated F_1 fish were paired to produce full-sib broods of outbred F_2 offspring, which were used in the mate-choice experiment described below.

The laboratory populations were housed in a temperature-regulated (22–26 °C) greenhouse on the UC Santa Barbara campus. Artificial lighting was provided by a combination of incandescent and daylight spectrum fluorescent lights (Duro-Test Vita-lite) timed to turn on at 0645 and off at 1845 hr.; the fish also received diffuse sunlight through the roof. Parental (wild) and F_1 fish were fed a standard diet of live artemia nauplii once each morning and commercial flake food once each afternoon (Tetramin, Tetra Growth, or Tetra Spirulina, depending on the day of the week; Tetra, Blacksburg, VA). Tanks containing fish from different populations were spatially interspersed in the greenhouse.

Carotenoid Diet Experiment

Newborn F_2 fish were housed in single-brood tanks and fed a special flake food diet that was designed to be nutritionally adequate but free of carotenoid pigments (basal diet; see below). The water was treated with 2-chloro-4, 6-bis-(ethylamino)-s-triazine (Algae Destroyer, Aquarium Pharmaceuticals, Chalfont, PA) to retard algal growth (no algae were visible in the tanks). The sexes were separated as early as possible (between 5–8 weeks of age), well before sexual maturity. The females were placed in mixed brood tanks (38 L) of up to 20 individuals and placed on the same standard diet as the F_1 and parental generations. The males from each brood were divided up as evenly as possible among three 8-L tanks (one to four males per tank) and fed one of three experimental diets. The diets were designed to contain different concentrations of the carotenoid pigments found in the

natural diets of guppies but otherwise were identical. Of the carotenoids found in the algae of Trinidadian streams (see Grether 1999), only lutein, zeaxanthin, and β carotene are likely to be usable by fish for pigmenting the skin (Goodwin 1984). The experimental diets were designed to contain these three pigments, roughly in the proportions in which they were found in Trinidadian stream algae (but see below).

The basal (zero-carotenoid) diet contained the following ingredients: spray-dried white fish meal (48.5%), wheat flour (48%), vegetable oil (1%), vitamin premix (1%), and gelatin (1.5%). The estimated protein (40%) and fat (10%) content of this diet is similar to high-quality commercial fish feeds for tropical fish. The vitamin premix included vitamin A palmitate, but no carotenoids. For the low- and high-carotenoid diets, lutein and zeaxanthin were added to the basal diet, in the desired proportions, by combining two aqueous marigold extract products (Cromophyl CL and Cromophyl L RZ-2, Bioquimex Reka, S. A. de C. V., Queretaro, Qro, Mexico). β carotene was added as 10% synthetic β carotene in gelatin beadlets (Rovimix; Hoffman-La Roche, Parsippany, NJ); the amount of pure gelatin was adjusted to keep the composition of the food, exclusive of pigments, constant. The diets were processed into flakes by Ocean Star International, Inc. (Hayward, CA). Based on the literature, I expected degradation of the pigments during processing to be in the range of 5–10% and designed the recipe accordingly. High-performance liquid chromatography (HPLC) of diet samples revealed that degradation was greater than expected, but this was not discovered until after the feeding experiment had begun. Lutein and zeaxanthin were only found in trace amounts, and β carotene was degraded by approximately 80%. The β carotene content of the low- and high-carotenoid diets was 13.84 and 199.02 ppm, respectively. Diatoxanthin and/or didinoxanthin, possible degradation products of lutein and zeaxanthin, were also found in the low- and high-carotenoid diets (4.76 and 153.84 ppm, respectively); these pigments are also found in the natural diets of guppies, but probably cannot be converted into skin pigments (J. Hudon, pers. comm.). The total carotenoid content of the basal diet was < 0.16 ppm. Thus, the concentrations of usable carotenoid pigments in the experimental diets were < 0.16 ppm (zero), 13.84 ppm (low), and 199.02 ppm (high).

Mate-Choice Experiment

To measure female preferences for male carotenoid coloration, I used an open-aquarium design in which groups of males and females were allowed to interact directly (for a discussion of the merits of this design in comparison to others, see Houde 1997). The potential for direct competition between males to influence the results was reduced by using an even sex ratio (3:3), a large test aquarium (120 L), and males that had not been raised together (Endler and Houde 1995; Houde 1997). I also attempted to minimize body size disparities within both male and female groups. Male groups consisted of three full-sib brothers (F_2) each raised on a different dietary carotenoid level. The purpose of using full-sibs was to control, as much as possible, for age, experience, and genetic variation in body size and coloration. Full-sib brothers have very similar color patterns with respect to the

number and sizes of each class of color pattern elements because these traits tend to be controlled by Y-linked genes (Winge 1927; Haskins et al. 1961; Houde 1992). When more than one male was available in a particular sibship-diet combination, for the mate choice tests I chose the subset of males that minimized visible body size disparities.

Female groups consisted of three unrelated, mature virgins (F_2) from a single population. Half the female groups was tested with males from same population; the other half was tested with males from the other population in the same drainage. Females were housed separately from and out of sight of males until they were used in the mate-choice tests because a female's preferences may be influenced by the characteristics of males to whom she previously was exposed (Brooks and Caithness 1995; Rosenqvist and Houde 1997; Hughes et al. 1999). For a detailed explanation of why virgin females are often used in mate choice experiments with guppies, see Houde (1997).

Each group of males was tested in separate sessions, on consecutive days, with two groups of females: one from the same population as the males and the other from the other population in the same drainage (the order alternated between tests). Usually two observation aquaria were in operation at once; when possible, I tested groups of males from different populations in the same drainage simultaneously. The tests were conducted in a windowless room maintained on the same light/dark cycle as the greenhouse lights. The observation aquaria were covered with natural, multicolored gravel on the bottom and brown paper on three sides; observations were made from the fourth side. Each aquarium was illuminated from the top with one daylight spectrum fluorescent tube (Duro-Test Vita-Lite) and four 15-W incandescent bulbs. Otherwise the room was dark, to maximize visibility of the fish to the observer and to minimize the visibility of the observer to the fish.

On the morning of the day before a mate-choice trial, the males chosen for testing were released into an observation aquarium after their color patterns were studied and sketched. Even full-sib males usually differ in the location or presence of one or more color spots; I had no trouble telling males apart based on their color patterns. Females were released into the observation aquarium shortly after the males. The first observation session began the next morning between 0700 and 0800. I made three replicate focal samples of 5 min per male per session (for a total of 18 focal samples, or 1.5 h per male group), alternating between males in a predetermined order. A minimum of 0.5 h elapsed between consecutive focal samples on a given male. Behavioral observations were recorded on a laptop computer using an event recorder program written in TrueBASIC 5.1 (code available from the author on request). Courting males perform discrete "sigmoid" displays, and the responses of females to these displays can easily be scored (for a detailed description, see Houde 1997). Following Houde and Endler (1990; Endler and Houde 1995), male attractiveness was measured in two ways: (1) the proportion of a male's sigmoid displays that elicited a sexual ("gliding") response from a female; and (2) the mean intensity of female responses on a rank scale from 0 (none) to 5 (copulation). Female preferences for male

carotenoid coloration were inferred from the relationship between male attractiveness and dietary carotenoid levels.

A total of 228 males were used in the mate-choice tests, 36 from each Paria and Marianne population and 42 from each Quare population. The number of females used was twice the number of males. Sample sizes differ slightly between analyses because some data were missing for three Quare males; one male jumped out of the observation tank between tests and died, one male performed no sigmoid displays when tested with females from his own population, and one male accidentally was not photographed for spot area measurements.

Male Characters

The coloration, weight, and length of males from given sibship were measured immediately after the second mate-choice trial. Males used in the mate-choice trials were measured first, followed by their untested brothers. To avoid order effects, males were measured in each of the three possible diet sequences (e.g., zero-low-high) one-third of the time. Males were sedated with ethyl 3-aminobenzoate methane sulfonic acid salt (MS-222) and photographed on both sides of the body under tungsten light using tungsten-corrected slide film (Kodak Ektachrome 160T). Later, the slides were projected onto white paper, the outline of the body and tail and all color-pattern elements were traced, and the tracings were digitized to obtain area estimates using a graphics tablet and PASCAL program provided by J. A. Endler. The total area for each color class on the body and tail was calculated and converted to proportions of body or tail area for analysis. The conversion to proportions was done to make the color class variables independent of body and tail size. Eight classes of color elements were recognized: orange, yellow, green, blue, purple, white, black, and fuzzy black. Orange and yellow tail spots are easily distinguished, but on the body these colors grade into one another and so were grouped together as orange. Fuzzy black spots can change dramatically in size during courtship (Baerends et al. 1955); black spots have sharper edges and change less or not at all. This distinction is more difficult to make for tails than for bodies, and because the results for black and fuzzy black tail spots were qualitatively the same, these color classes were combined for the analysis presented here. Blue, green, and purple tail spots were not observed frequently enough for meaningful statistical analysis.

Immediately after photographs were taken, the reflectance spectrum of each orange spot on both sides of the body was measured with a diode-array spectroradiometer (Ocean Optics PS-1000; Dunedin, FL). Individual orange spots were isolated by holding the fish up to a 1.3-mm aperture in a horizontally mounted, razor-thin steel plate. The aperture was illuminated at an angle of 45° relative to the dorsal surface of the fish by a quartz fiber optic connected to a voltage-regulated halogen lamp. Reflected light was transferred to the spectroradiometer by a fiber optic detector placed directly above the aperture at the distance at which the aperture edges matched the acceptance field of the detector (1.7 mm). Reflectance at each wavelength interval was calculated by reference to a white standard (Oriol Spectralon, Stratford, CT),

which was scanned immediately before each fish. Each orange spot was scanned three times and the replicate spectra were averaged. Mean reflectance spectra were processed to yield estimates of quantum catch by each of the four classes of guppy photoreceptor cones (see Endler 1991). Absorbance functions for guppy cones were calculated from the published λ_{\max} -values of 389 (UV), 410 (s), 465 (m), and 543 nm (l; Archer and Lythgoe 1990) using equations and parameters provided in Stavenga et al. (1993) and an optical density of 0.3 (based on Nicol and Somiya 1989, Table 11.1). Following Endler (1991), the photon catch for each cone class was estimated from:

$$P_{ij} = \sum_k r_{jk} a_k t_k s_{jk}, \quad (1)$$

where i refers to the cone class (UV, s, m, or l), r_{jk} is the mean reflectance of orange spot j at wavelength k , a_k is the ambient irradiance at wavelength k above the observation aquarium, t_k is the transmission fraction through 0.25 m of clear water at wavelength k (from Endler 1991, fig. 5), and s_{jk} is the spectral sensitivity or absorbance of cone class j at wavelength k . Spot-size weighted mean values were calculated for each male from:

$$P_i = \left(\sum_j W_j P_{ij} \right) \left(\sum_j W_j \right)^{-1}, \quad (2)$$

where W_j is the area of orange spot j . In the absence of information on how the visual system of guppies weighs input from different cone classes, the total cone photon catch $P_T = \sum P_i$ provides the best estimate of perceived brightness (Endler 1991). Because carotenoids primarily absorb short-wavelength light, I used $P_C = (P_m + P_l - P_s - P_{UV})(P_T)^{-1}$ as a measure of spectral contrast or chroma. Other measures of chroma, such as Endler's (1991) maximum cone contrast, gave similar results (not shown) but have the disadvantage of being nondirectional. For the present application, it was desirable to use a measure of chroma that increased monotonically with dietary carotenoid levels.

After the reflectance scans, males were measured from their lower jaw to caudal peduncle (standard length) with calipers (± 0.01 mm digital readout) under a microscope, patted with a Kimwipe to remove excess water, and weighed to 0.1 mg. Mass divided by the cube of standard length was used as an index of condition.

Analysis Details

Carotenoids in the diet were expected to affect the chroma and brightness of the orange spots, but not the other male characters. To test this assumption, I constructed separate analyses of variance (ANOVAs) for each character, with fixed diet and population terms and a random-effects sibship term nested within population, using JMP 3.2.2 (SAS Institute, Inc., Cary, NC). If significant diet or diet by population effects were detected, I examined pairwise comparisons of diet levels within populations. The Bonferroni multiple comparisons procedure was used for adjusting P -values within each ANOVA. Using an experimentwise error rate across all characters would have been overly conservative, given the number of characters and the expectation that certain characters

TABLE 1. Results of ANOVAs to detect effects of dietary carotenoid levels on male characters while controlling for genetic variation within and among populations. The values shown are *F*-statistics. Where carotenoid diet effects were significant, the direction of the change between carotenoid levels is indicated in parentheses, e.g., (+) indicates that the dependent variable mean was greatest on the low-carotenoid diet. Sibship was entered as a random-effects term, nested within population. See text for variable definitions.

	Carotenoid diet	Population	Carot. diet × pop.	Sibship	Carot. diet × sibship	<i>R</i> ²
Orange body chroma (<i>P_c</i>)	122.65**** (+)	14.38****	4.06****	3.77****	0.90 ns	0.87
Orange body brightness (<i>P_γ</i>)	26.63**** (−)	57.68****	3.39***	5.06****	0.88 ns	0.93
Yellow tail area ¹	80.55**** (+)	4.09**	1.92*	2.57****	1.88****	0.89
White tail area ²	16.70**** (−)	4.51**	1.50 ns	1.13 ns	2.10****	0.81
Blue body area ¹	0.75 ns	10.11****	1.58 ns	3.42****	0.86 ns	0.79
White body area ¹	1.50 ns	2.30 ns	2.18*	7.44****	0.88 ns	0.84
Green body area ¹	0.15 ns	1.11 ns	1.06 ns	4.18****	0.95 ns	0.77
Sharp black body area ²	2.46 ns	2.94*	0.33 ns	2.90****	0.72 ns	0.70
Fuzzy black body area ²	5.26** (+)	13.14****	0.87 ns	1.93***	0.98 ns	0.77
Purple body area ²	0.47 ns	7.52****	0.32 ns	3.98****	1.06 ns	0.83
Black tail area	2.48 ns	4.76***	0.74 ns	5.65****	1.38*	0.88
Orange body area ¹	0.69 ns	30.81****	2.08*	5.27****	0.99 ns	0.92
Orange tail area ²	0.58 ns	4.65***	1.73 ns	8.47****	0.85 ns	0.86
Body area	1.67 ns	4.88***	0.53 ns	5.78****	0.81 ns	0.82
Tail area	2.67 ns	2.28 ns	0.68 ns	6.91****	0.98 ns	0.84
Standard length	2.06 ns	5.09***	0.55 ns	5.32****	1.05 ns	0.84
Condition index	0.62 ns	8.14****	1.28 ns	2.74****	1.09 ns	0.80
mean df ³	2, 160.9	5, 71	10, 158.4	69, 143.3	138, 131.2	224, 131

ns, *P* > 0.05; * *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001; **** *P* < 0.0001.

¹ Square-root transformed.

² Log₁₀ (*x* + 0.01)-transformed.

³ Satterthwaite method.

would be affected by carotenoids in the diet and others would not.

I tested for population differences in the strength of female preferences for male carotenoid coloration in ANOVAs with dietary carotenoid level entered as a categorical variable (zero, low, or high), and also in analyses of covariance (ANCOVAs) with log₁₀ β carotene concentration (log βC) as a covariate (using 0.16 ppm for the zero-carotenoid diet). Separate models were constructed for each drainage with male attractiveness as the dependent variable and female population, male population, male sibship nested within male population, and carotenoid diet (or log βC) as independent variables. The carotenoid diet (or log βC) by female population interaction quantifies population differences in the strength of female preferences. Nonsignificant covariate interaction terms were removed sequentially from the ANCOVA models before testing the log βC by female population term for significance. ANCOVA is potentially more powerful than ANOVA but assumes a linear relationship between male attractiveness and log βC. Because the ANOVA and ANCOVA results were quite similar, I present complete results only from the ANOVAs.

Multiple regression was used to determine which of the carotenoid-dependent characters most strongly influence female choice. Male characters were regressed simultaneously on residuals of male attractiveness. Attractiveness residuals were obtained from the ANOVA models of Table 2 after removing all carotenoid diet terms. This procedure, which removed variation in male attractiveness associated with sibship and population, made the multiple regression equations more comparable across populations.

To meet parametric assumptions, male characters were transformed as indicated in Table 1, attractiveness measure

1 was arc-sine-square-root transformed, and attractiveness measure 2 was square-root transformed.

RESULTS

Effects of Dietary Carotenoids on Male Characters

Table 1 summarizes the effects (or lack thereof) of the experimental diets on all measured male characters, after controlling for variation among populations and among sibships within populations. Orange body chroma (*P_c*) increased with the level of carotenoids in the diet, whereas orange body brightness (*P_γ*) decreased (Table 1). These results are consistent with the spectral absorbance properties of carotenoids (Goodwin 1984). The magnitude of the effect of diet on chroma varied among populations, as reflected by a significant population-by-diet interaction, but all populations responded significantly in the same direction (Fig. 1A). Orange body brightness decreased significantly at higher dietary carotenoid levels in four of the six populations (Fig. 1B). Yellow tail area increased sharply with the level of carotenoids in the diet, whereas white tail area decreased (Table 1). This probably reflects conversion of white spots to yellow spots when carotenoids are present in the diet. From the zero- to the low-carotenoid levels, the proportion of the tail covered with yellow spots increased significantly for all six populations (Fig. 2). No further significant changes in yellow tail area occurred from the low- to high-carotenoid levels. Each of these carotenoid-dependent variables also showed significant variation within and among populations.

The remaining variables did not show compelling carotenoid-dependent variation (Table 1). Body area, tail area, standard length, and the condition index varied significantly among populations and among sibships within populations,

TABLE 2. Analysis of variation in male attractiveness. The carotenoid diet × female population term tests for population differences in the strength of female preferences. Marianne, Paria, and Quare are the drainage basin names. The attractiveness measures are defined in the Methods.

	Marianne		Paria		Quare	
	F	df ¹	F	df ¹	F	df ¹
(A) Attractiveness measure 1						
Carotenoid diet	32.26****	2, 44	38.30****	2, 44	25.94****	2, 53.8
Male population	0.82 ns	1, 22	1.03 ns	1, 22	0.48 ns	1, 26.3
Female population	19.59**	1, 22	12.30**	1, 22	0.36 ns	1, 26.3
Male pop. × female pop.	0.23 ns	1, 22	0.36 ns	1, 22	2.69 ns	1, 26.3
Carot. diet × male pop.	0.32 ns	2, 44	0.15 ns	2, 44	1.57 ns	2, 53.8
Carot. diet × female pop.	1.23 ns	2, 44	3.91*	2, 44	1.07 ns	2, 50
Carot. diet × male pop. × female pop.	0.53 ns	2, 44	0.01 ns	2, 44	1.00 ns	2, 50
Sibship (male pop.)	1.43 ns	22, 9.4	0.78 ns	22, 17.1	1.13 ns	26, 21.7
Carot. diet × sibship (male pop.)	0.75 ns	44, 44	0.94 ns	44, 44	1.11 ns	52, 50
Female pop. × sibship (male pop.)	1.25 ns	22, 44	2.12*	22, 44	1.80*	26, 50
<i>R</i> ²	0.75		0.84		0.82	
(B) Attractiveness measure 2						
Carotenoid diet	42.96****	2, 44	63.34****	2, 44	53.9****	2, 54.3
Male population	0.11 ns	1, 22	0.08 ns	1, 22	0.371 ns	1, 26.2
Female population	30.10****	1, 22	23.99****	1, 22	0.587 ns	1, 26.2
Male pop. × female pop.	0.32 ns	1, 22	0.41 ns	1, 22	2.357 ns	1, 26.2
Carot. diet × male pop.	1.34 ns	2, 44	1.11 ns	2, 44	0.112 ns	2, 54.3
Carot. diet × female pop.	0.86 ns	2, 44	1.40 ns	2, 44	0.02 ns	2, 50
Carot. diet × male pop. × female pop.	0.18 ns	2, 44	0.29 ns	2, 44	0.44 ns	2, 50
Sibship (male pop.)	0.83 ns	22, 22.6	0.65 ns	22, 22.4	1.559 ns	26, 19.2
Carot. diet × sibship (male pop.)	1.22 ns	44, 44	1.21 ns	44, 44	0.84 ns	52, 50
Female pop. × sibship (male pop.)	3.51***	22, 44	3.33***	22, 44	2.252**	26, 50
<i>R</i> ²	0.90		0.90		0.84	

ns, *P* < 0.05; * *P* < 0.05; ** *P* < 0.01; ****P* < 0.001; *****P* < 0.0001.
¹ Satterthwaite method.

but there were no significant diet effects or diet-by-population interactions. The same was true for most remaining color spot classes, including orange tail area. Orange body area showed a significant diet-by-population interaction, but within-population diet level comparisons revealed only one marginally significant difference; in the low-carotenoid-availability Quare population, males raised on the low-carotenoid diet had greater orange body area than those raised on the zero-carotenoid diet (*P* = 0.04). White body area also showed

a significant diet-by-population interaction; within-population comparisons revealed that white body area was greater on the low-carotenoid diet than on the zero-carotenoid diet for the high-carotenoid-availability Paria population (*P* = 0.04) and greater on the high-carotenoid diet than on the low-carotenoid diet for the low-carotenoid-availability Paria population (*P* = 0.02). Fuzzy black body area also showed a weak and inconsistent association with diet. Overall, fuzzy black body area was significantly greater on the low-carotenoid diet than on the high-carotenoid diet (*P* = 0.005), but there was no significant difference between zero- and low-carotenoid diets (*P* = 0.68) or zero- and high-carotenoid diets (*P* = 0.14). Within-population comparisons revealed only one significant difference: in the low-carotenoid-availability Marianne population, fuzzy black body area was greater on the low-carotenoid diet than on the zero-carotenoid diet (*P* = 0.04).

All results reported above were qualitatively the same when absolute areas were used for the color class characters instead of proportions, and similar results were obtained when the number of spots of a given color class was used in place of the sum of spot areas (not shown).

Female Preferences for Carotenoid Coloration

The level of carotenoids in the diet of males had highly significant positive effects on male attractiveness to females from all six populations (Fig. 3, Table 2). In multiple-comparisons tests, males raised on the high-carotenoid diet were significantly more attractive to females than males raised on the zero-carotenoid diet (Table 3). The carotenoid diet-by-

TABLE 3. Multiple comparisons test results from the ANOVAs of Table 2. *P*-values for comparisons of male attractiveness between carotenoid levels, corrected for multiple comparisons (within rows), are shown. The carotenoid availability column indicates the field source population of the females with which the males from a given drainage were tested.

Drainage	Carotenoid availability	Carotenoid level comparison		
		Low vs. zero	High vs. low	High vs. zero
(A) Attractiveness measure 1				
Marianne	low	0.0735	0.3183	0.0009
	high	0.0001	0.7878	< 10 ⁻⁵
Paria	low	0.0015	< 0.0001	< 10 ⁻⁸
	high	0.1104	0.2052	0.0006
Quare	low	0.0042	> 0.9	0.0006
	high	0.0003	0.1149	< 10 ⁻⁶
(B) Attractiveness measure 2				
Marianne	low	0.0018	0.0111	< 10 ⁻⁷
	high	< 10 ⁻⁵	0.1746	< 10 ⁻⁸
Paria	low	< 10 ⁻⁶	0.0042	< 10 ⁻¹¹
	high	< 10 ⁻⁵	0.0978	< 10 ⁻⁸
Quare	low	< 10 ⁻⁵	0.6738	< 10 ⁻⁶
	high	< 10 ⁻⁵	0.9384	< 10 ⁻⁶

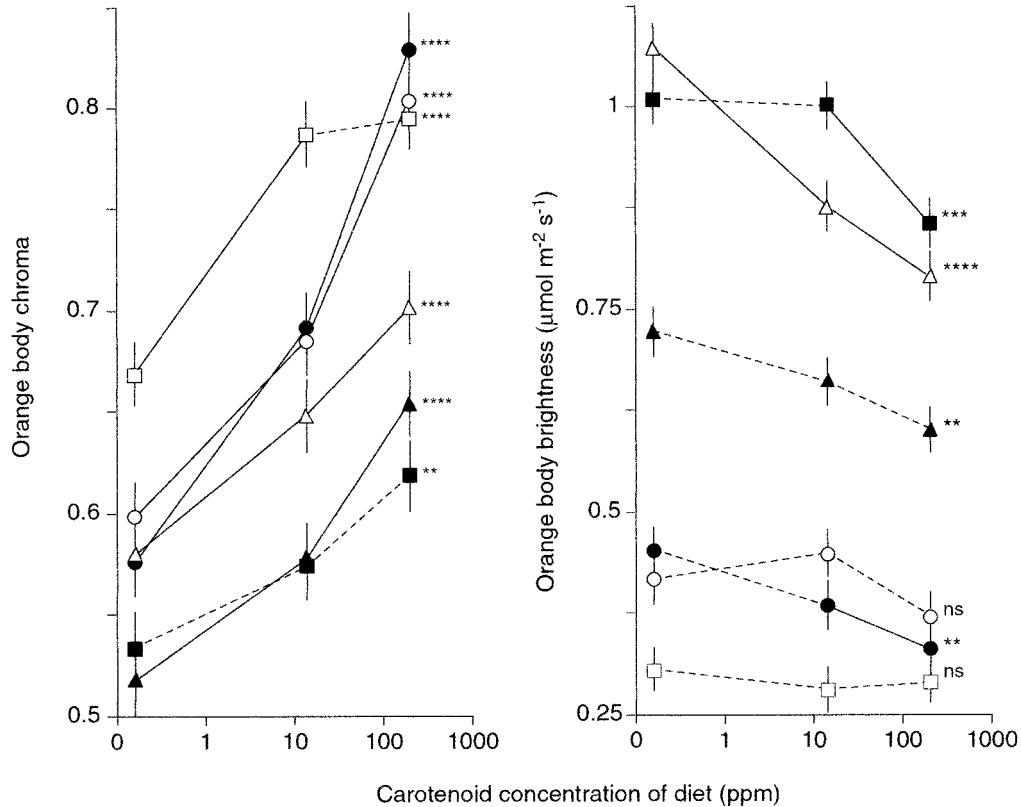


FIG. 1. Effect of dietary carotenoid levels on (A) orange body chroma (P_C) and (B) orange body brightness (P_T). Squares, circles, and triangles represent the Marianne, Paria, and Quare drainages, respectively, with filled symbols for low-carotenoid-availability streams and unfilled symbols for high-carotenoid-availability streams. Points represent least-squares means \pm 1 SE from the ANOVAs in Table 1. Means connected by solid lines are significantly different ($P < 0.05$); means connected by dashed lines are not significantly different ($P > 0.05$). Asterisks indicate the significance level of the zero- versus high-carotenoid level comparison after Bonferroni corrections (** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; ns, $P > 0.05$).

female population interaction, which measures population differences in preference strength, was significant only for the Paria drainage and only using attractiveness measure 1 (Table 2). Based on attractiveness measure 1, females from the low-carotenoid-availability Paria population had significantly stronger preferences for carotenoid coloration than females from the high-carotenoid-availability Paria population, as predicted by the indicator hypothesis. However, there were nonsignificant trends in the opposite direction in the other two drainages (Fig. 3). The mean attractiveness of males (a measure of female receptivity) was influenced by the source population of the females, but not by the source population of the males. In the Marianne drainage, females from the high-carotenoid-availability population showed higher levels of receptivity than females from the low-carotenoid-availability population, but the reverse was found in the Paria drainage, and females from the two Quare populations did not differ in receptivity. The lack of significant male population-by-female population interactions indicates that females were not more attracted to males from their own population than to males from the other population in the same drainage. The lack of significant carotenoid diet-by-male population interactions indicates that the strength of female preferences for carotenoid coloration was not affected by whether females were tested with males from their own population

or with males from the other population in the same drainage. The results were qualitatively the same with \log_{10} β carotene concentration as a covariate; the covariate-by-female population interaction was significant only for the Paria drainage and only for attractiveness measure 1 (attractiveness measure 1: Marianne $F_{1,94} = 1.94$, $P = 0.167$; Paria $F_{1,94} = 7.88$, $P = 0.006$; Quare $F_{1,108} = 2.07$, $P = 0.153$; attractiveness measure 2: Marianne $F_{1,94} = 0.42$, $P = 0.516$; Paria $F_{1,94} = 2.55$, $P = 0.114$; Quare $F_{1,108} = 0.03$, $P = 0.874$). No other covariate interaction terms were significant ($P > 0.05$).

Multiple regression of the carotenoid-dependent male characters on male attractiveness residuals suggested that female preference functions were similar between streams within drainages (Table 4). The amount of variation in male attractiveness explained by the carotenoid-dependent male characters was greatest in the Paria drainage (36–51%) and least in the Marianne drainage (13–18%). Between streams within drainages, the R^2 -values were similar in magnitude, thus providing further evidence for a lack of a relationship between carotenoid availability and the strength of female preferences. Orange body chroma was the best predictor of male attractiveness for the low-carotenoid-availability Marianne population and for both Paria populations. Yellow tail area was the best predictor for the high-carotenoid-availability Marianne population and the low-carotenoid-availability Quare

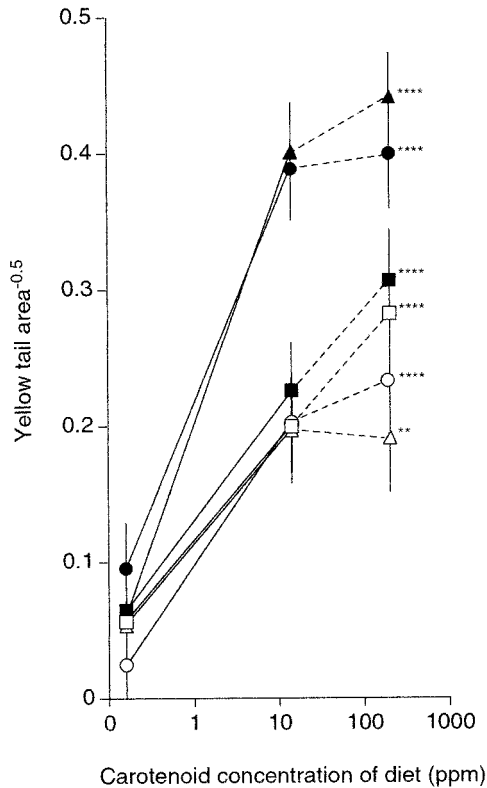


FIG. 2. Effect of dietary carotenoid levels on relative yellow tail area. Symbols and other conventions follow Figure 1.

population. Although carotenoids in the diet reduced the brightness (P_T) of the orange spots (Fig. 1B), all significant partial regression preference slopes for this character were positive.

Preference slopes (partial regression coefficients) for orange body chroma and brightness were positively correlated across populations (Table 5, off the diagonal), even though these characters responded in opposite directions to the level of carotenoids in the diet (Fig. 1) and were negatively correlated both within populations ($-0.75 \leq r \leq -0.45$, $54 \leq N \leq 69$, all $P < 0.0001$) and across population means ($r = -0.84$, $P = 0.036$, $N = 6$). Preference slopes based on attractiveness measure 2 for orange body chroma and yellow tail area were also positively correlated. Preference slopes based on attractiveness measure 1 were strongly correlated with those based on attractiveness measure 2 for all characters except white tail area (Table 5, on the diagonal). There was no evidence that female preferences and male characters were correlated across populations. None of the preference slopes in Table 4 correlated significantly with the population means for the corresponding male characters (all $P \geq 0.2$, $N = 6$). I also examined the correlation between the ratio of the orange chroma and brightness preference slopes and ratio of the population means of these characters (see Discussion). Based on attractiveness measure 1, the ratios were significantly correlated ($r = 0.82$, $P = 0.048$), but based on attractiveness measure 2, they were not ($r = 0.70$, $P = 0.133$).

DISCUSSION

As with several other species, it is clear that male guppies acquire carotenoid pigments from their diet and use them to attract females, but it is not clear why females are attracted. The leading hypothesis has been that female preferences for carotenoid coloration have evolved because carotenoids are a limiting resource, the accumulation of which serves as a direct indicator of male foraging ability and an indirect indicator of health and vigor (Endler 1980; Olson and Owens 1998). The assumptions underlying this hypothesis are supported more strongly for guppies than for any other species (see introduction). To test the hypothesis, I made phylogenetically controlled comparisons of female preferences for carotenoid coloration between guppy populations experiencing different degrees of carotenoid limitation. Females from all six populations showed highly significant preferences for males raised on higher dietary carotenoid levels, and in only one of three river drainages did female preferences differ genetically between populations in the predicted direction (Fig. 3).

To my knowledge, this was the first attempt to directly test the prediction that female preferences evolve in response to the indicator value of male traits. The prediction is based on the assumptions that female preferences are genetically variable and that the ratio of benefits to costs of female preferences is greater when the targeted male traits are better indicators of condition. In support of the first assumption, artificial selection experiments suggest that female preferences are heritable (Houde 1994; Brooks and Coullidge 1999) and common-garden experiments have shown that they vary genetically among populations (Breden and Stoner 1987; Houde 1988; Houde and Endler 1990, Endler and Houde 1995; this study). The assumption that the benefits of female preferences for carotenoid coloration increase with carotenoid scarcity is an integral part of the foraging ability indicator mechanism, that is, if this assumption is wrong, the foraging ability indicator hypothesis is also wrong. This is a critical point, which I now develop more fully.

As shown in Figure 4, the chroma of the orange spots of male guppies is a decelerating function of the rate of carotenoid intake. This is consistent with studies showing that the fraction of ingested carotenoids retained by fish decreases as the rate of carotenoid ingestion increases (Choubert and Storbakken 1989; Bjerkeng et al. 1990; Putnam 1992; Meyers 1994). Carotenoid deposition in the spots of male guppies may be further constrained by total orange area, which is developmentally fixed. In any case, a given natural population should fall somewhere along the decelerating carotenoid dose-response curve. Variation among males in foraging ability and therefore carotenoid intake should have a larger effect on carotenoid deposition and color for populations in the steep part of the curve than for populations in the region where carotenoid deposition is closer to saturating. As the mean rate of carotenoid intake increases, the signal:noise ratio should decrease, and differences between males in foraging ability should become more difficult for females to detect.

Two lines of evidence presented in Grether et al. (1999) indicate that the populations of guppies in this study occupy

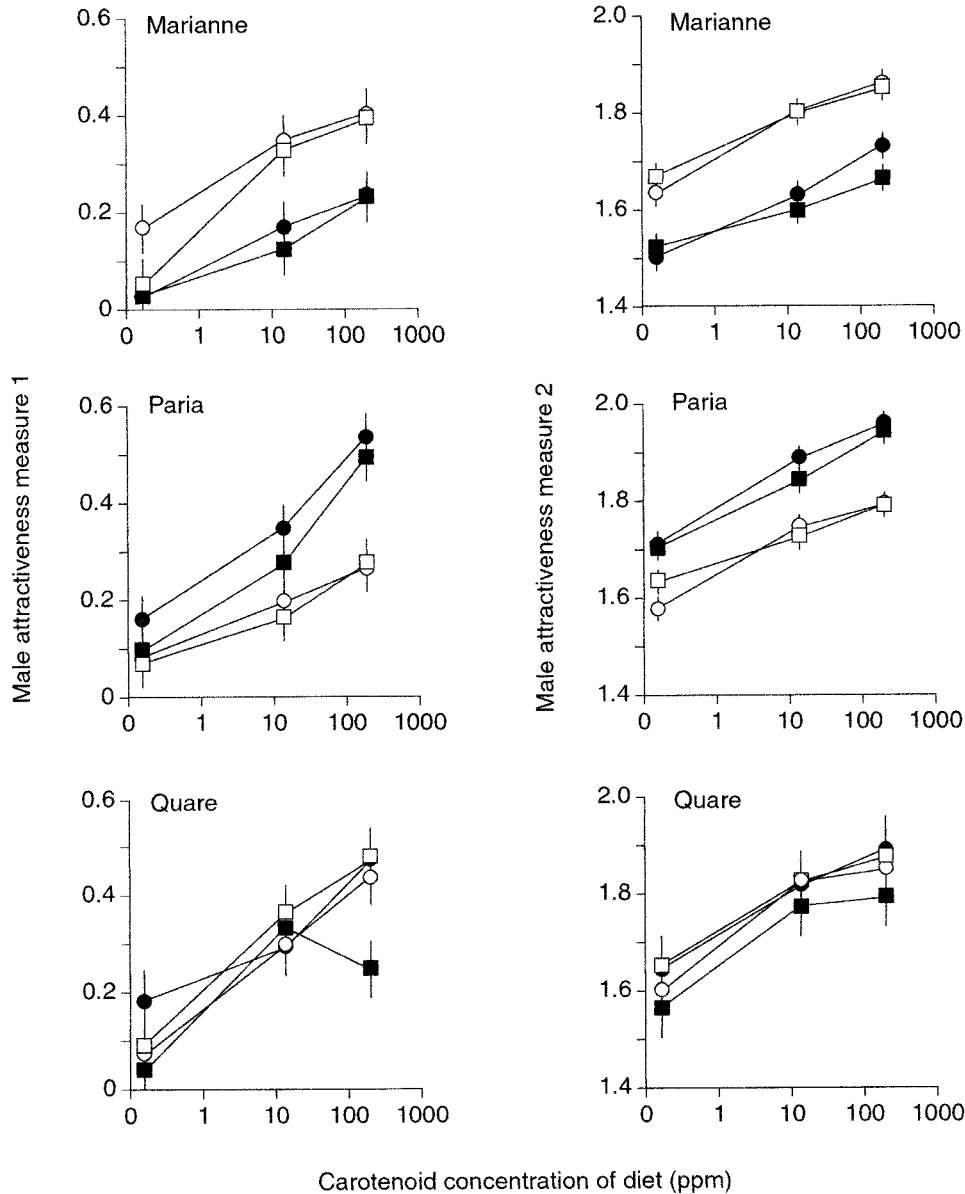


FIG. 3. Female preference functions for male carotenoid coloration based on two different attractiveness measures. Filled circles represent the attractiveness of males from the low-carotenoid-availability streams to females from the low-carotenoid-availability streams, and unfilled circles represent the attractiveness of males from the low-carotenoid-availability streams to females from the high-carotenoid-availability streams. Conversely, filled squares represent the attractiveness of males from the high-carotenoid-availability streams to females from the low-carotenoid-availability streams, and unfilled squares represent the attractiveness of males from the high-carotenoid-availability streams to females from the high-carotenoid-availability streams. Points represent least-squares means \pm 1 SE from the ANOVAs in Table 2. Multiple comparison test results are shown in Table 3.

different positions on the carotenoid dose-response curve. First, fish taken from the high-carotenoid-availability streams had larger amounts of carotenoids in their foreguts (stomachs), higher concentrations of carotenoids in their orange spots, and higher skin:foregut carotenoid ratios than guppies from the high-carotenoid-availability streams. Second, the within-stream correlation between total orange spot area and the total amount of carotenoids in the spots was significantly positive for the high-carotenoid-availability streams, but not for the low-carotenoid-availability streams. This implies that carotenoid deposition is closer to saturating in the high-ca-

rotenoid-availability streams. As argued above, it follows that carotenoid coloration is a better indicator of foraging ability in the low-carotenoid-availability streams.

The validity of this study as a test of the foraging ability indicator hypothesis therefore hinges on how the costs of female preferences vary with carotenoid limitation. Low-order streams with low carotenoid availability also have relatively low food availability, as shown most clearly by stream differences in female growth rates in the field (Grether et al. 2000). Magurran and Seghers (1994; Magurran 1998) have argued that females may need to devote a greater proportion

TABLE 4. Multiple regressions of male attractiveness residuals on carotenoid-dependent male characters. Standardized partial regression coefficients and R^2 are shown. Drainage and carotenoid availability identify the field source population of the females. N is the number of males used in the female choice tests.

Drainage	Carotenoid availability	Orange body chroma (P_c)	Orange body brightness (P_b)	Yellow tail area	White tail area	R^2	N
(A) Attractiveness measure 1							
Marianne	low	0.27§	0.12	0.13	-0.22§	0.13*	72
	high	0.25	0.03	0.27*	-0.04	0.13*	72
Paria	low	0.49****	0.24*	0.30**	-0.10	0.37****	72
	high	0.47****	0.17	0.24*	-0.17	0.36****	72
Quare	low	0.14	-0.11	0.17	-0.13	0.14**	81
	high	0.23*	-0.08	0.17	-0.16	0.20**	82
(B) Attractiveness measure 2							
Marianne	low	0.29§	0.09	0.21§	-0.15	0.14*	72
	high	0.28§	0.05	0.28*	-0.12	0.17*	72
Paria	low	0.51****	0.26*	0.39***	-0.11	0.48****	72
	high	0.55****	0.23*	0.33**	-0.18*	0.51****	72
Quare	low	0.23*	-0.11	0.23*	-0.17	0.27****	81
	high	0.21§	-0.07	0.24*	-0.17	0.23***	82

§ $P < 0.1$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

of their time to foraging in low-order streams than in high-order streams. If so, the costs of being choosy may increase with carotenoid limitation and offset any increase in the benefits.

Another possibility that could rescue the foraging ability indicator hypothesis is that female preferences may themselves be carotenoid dependent. Perhaps carotenoid availability varies sufficiently within streams (among pools or seasonally) to select for a reaction norm of female preferences, instead of fixed population differences. This could explain the consistently strong preferences measured in this study because all females were initially fed a zero-carotenoid diet before being switched to a standard laboratory diet. There is some evidence that animals can detect and respond to ingested carotenoids. For example, caterpillars of the parsnip webworm (*Depressaria pastinacella*) are less likely to avoid exposure to ultraviolet light when carotenoids are included in their diet (Carroll et al. 1997). In any case, the hypothesis that a female's mate preferences may depend on the level of carotenoids in her diet is both interesting and testable.

One unexpected result of this study was that preference slopes for orange spot chroma and brightness were positively correlated across populations (Table 5), even though these characters responded in opposite directions to the level of carotenoids in the diet (Fig. 1) and were negatively correlated both within populations and across population means. This suggests that female responses to these two traits are not correlated expressions of a single preference for carotenoid

coloration, but instead reflect separate preferences that, with respect to carotenoid deposition, conflict with each other. Preferences for orange and black spots in guppies have been shown to have separate genetic bases (Brooks and Coullidge 1999), but this study provides the first evidence for multiple "orange" preferences. Variation in the relative strength of orange chroma and brightness preferences might explain some of the variation among populations in the color of the orange spots. To examine this possibility, I examined the relationship between the ratio of the preference slopes for orange spot chroma and brightness and the ratio of the corresponding character means. The ratios were positively correlated across populations, providing preliminary evidence that male coloration may be shaped, in part, by the relative strength of conflicting female preferences.

On a trait-by-trait basis, however, preference slopes and character means were not correlated in this study. The drainage with the greatest divergence in the strength of female preferences (Paria) showed the least divergence in male characters (Figs. 1-3). With a larger sample of populations ($N = 11$), Endler and Houde (1995) found significant positive correlations between the preference slopes and population means for three of the numerous characters they examined: orange spot area, black spot number, and color contrast. As those authors noted, however, neither a correlation nor a lack of correlation between female preferences and mean male trait values sheds much light on how female preferences evolve,

TABLE 5. Correlations between preference slopes for the carotenoid-dependent male characters. Below the diagonal are correlations between partial regression coefficients for attractiveness measure 1. Above the diagonal are correlations between partial regression coefficients for attractiveness measure 2. On the diagonal are correlations between coefficients for the two different attractiveness measures ($N = 6$).

Character	Orange body chroma	Orange body brightness	Yellow tail area	White tail area
Orange body chroma	0.96****	0.94**	0.86*	0.15
Orange body brightness	0.92**	0.98****	0.80§	0.38
Yellow tail area	0.66	0.56	0.91**	0.46
White tail area	-0.02	-0.05	0.73	0.64

§ $P < 0.1$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

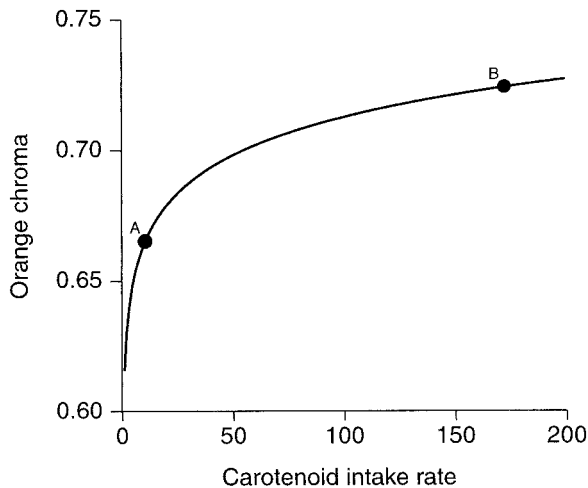


FIG. 4. Carotenoid dose-response curve for the orange spots of guppies. The line represents the linear regression of orange body chroma on the \log_{10} of dietary carotenoid concentration (ppm), plotted on a linear scale, for all populations combined (Fig. 1 shows each population separately on a \log_{10} scale). Points A and B represent the mean rates of carotenoid intake in two hypothetical streams. Variation in carotenoid intake would have a larger effect on chroma in stream A than in stream B (see Discussion).

because both patterns are consistent with nearly all models of sexual selection.

The results of this study bear indirectly on the question of whether predation risk selects for weaker mate preferences in guppies. Houde and Endler (1990; Endler and Houde 1995) found a loose, negative association between predation risk and the strength of female preferences, which, as they pointed out, was somewhat confounded with geography. This contrasts sharply with the consistent differences between high- and low-predation sites in numerous other traits (reviewed in Endler 1995). To explain the lack of a clear effect of predation on mate preference evolution, Magurran (1998) suggested that high food availability at high-predation sites may reduce the costs to females of spending time choosing mates, thus counteracting the increased risk of predation. My study compared female preferences between sites differing in food availability, but not predation. Based on the foraging cost hypothesis alone, female preferences should have been stronger where food availability was higher, but no such pattern emerged (Fig. 3). Adding carotenoid limitation as a factor still does not explain why high-predation sites do not have consistently weaker mate preferences than low-predation sites, because high-predation sites probably also have high carotenoid availability.

Another line of evidence suggests that female preferences may not evolve as readily as other traits in guppies. Endler and Houde (1995) found that females tended to be more receptive to males from their own population than to males from populations in other river drainages. Using a similar experimental design, I found that females were not more receptive to males from their own population than to males from other populations in the same drainage (Table 2). Taken together, these studies suggest that mate preferences are phylogenetically constrained in comparison to life-history traits, coloration, and antipredator behaviors, which have been

shown to evolve very rapidly in guppies (Endler 1995; Reznick et al. 1997).

Some of the most genetically differentiated guppy populations show no signs of mate preference divergence (Magurran et al. 1996). Explanations have focused on male dispersal and sneaky mating behavior, which facilitate gene flow (Magurran 1998), and ecological and geological factors that curb differentiation (Endler 1995). But the answer may be more specifically related to constraints on mate preference evolution itself. One possibility is that the benefits and costs of female preferences in a mating context are trivial in comparison to their benefits and costs in some other context, such as searching for food. If so, preferences would not be expected to closely track the indicator value of male traits or predation risk, or, for that matter, to coevolve with male traits through the Fisherian or chase-away processes (Fisher 1930; Lande 1981; Holland and Rice 1998).

For guppies and several other species, all of the assumptions appear to have been met for mate preferences to evolve through the indicator process (Møller and Alatalo 1999), but we still lack direct evidence.

ACKNOWLEDGMENTS

For advice and help of various kinds I thank M. Alkins-Koo, M. J. Bryant, H. A. Bryga, G. Clevén, M. Cummings, D. N. Reznick, J. A. Endler, J.-G. Godin, R. M. Grey, J. Groff, J. Hudon, M. Jirotkul, M. Lamon, W. J. McClintock, F. H. Rodd, J. Zerr, and 35 undergraduate research interns at UCSB. For helpful comments on an earlier version of the manuscript, I thank G. E. Hill and an anonymous reviewer. J. A. Endler and M. Cummings provided essential equipment; Bioquimex Reka and Hoffman-La Roche donated carotenoid pigments; Ocean Star International, Inc. produced and donated the experimental diets; and D. F. Millie and L. Boihem analyzed the experimental diets by HPLC. I thank P. Bacon and the Department of Zoology at the University of West Indies for sponsorship; the Trinidad and Tobago Ministry of Food Production, Marine Exploitation, Forestry, and the Environment for permits to collect guppies; and the Water and Sewage Authority for permission to work in the Quare drainage. The project was funded by a postdoctoral fellowship from the National Science Foundation and NSF grant DEB 9508198 to J. A. Endler.

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