

# Marginal Differentiation between the Sexual and General Carotenoid Pigmentation of Guppies (*Poecilia reticulata*) and a Possible Visual Explanation

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## ABSTRACT

We present the first detailed analysis of carotenoid pigmentation of the integument of guppies (*Poecilia reticulata* Peters), quantifying variation in carotenoid content and composition of wild guppies from three drainages on Trinidad (1) between the sexual and general pigmentation of males, (2) between the sexes, and (3) geographically in relation to carotenoid availability. We report that the carotenoid pigments in the integument of guppies are predominantly esters of tunaxanthin. The peak wavelength of carotenoids in the orange spots of males lay only ca. 2.8 nm higher than that of pigments outside of the orange spots, and the peak wavelength of carotenoids in the male whole integument does not differ from that in the female whole integument. Carotenoid composition of the general integument of females and the non-orange spot fraction of males, but not of the orange spot fraction of males, varied with diet, correlating with the ratio beta-carotene to lutein in the different streams. Male guppies deposit higher concentrations of carotenoids in their orange spots than in the rest of the integument (five to nine times higher), but not at the expense of the general integument, which was similarly endowed as the general integument of females, even in carotenoid-poor streams. Presumably males absorb/retain more pigments than females. Photoreceptor-based simulations suggest

that tunaxanthin provides both greater brightness and chroma than would 4-keto-carotenoids such as astaxanthin.

## Introduction

### *Carotenoids as Signals*

Carotenoid pigments are responsible for most of the conspicuous yellow, orange, and red colors used by animals in intra-specific (e.g., sexual) and interspecific (e.g., warning) signaling contexts, and they also frequently contribute to crypsis (Needham 1974; Rothchild et al. 1975; Fox 1976; Goodwin 1984; Rothchild et al. 1986). Animals cannot produce carotenoid pigments de novo but are capable of converting ingested carotenoids into new pigments with different absorptive properties before displaying them in the integument (Goodwin 1984; Brush 1990). There have been few attempts to understand why particular species display particular carotenoid pigments. How much of the variation can be explained in terms of the function of the coloration? To what extent does carotenoid use reflect constraints imposed by diet or phylogeny? How much of the variation in carotenoid use reflects variation in visual systems and preexisting sensory biases, or variation in the spectral environment? Although the proximate causes of species differences in carotenoid coloration have been studied at a basic level (e.g., genetic control vs. dietary access; see Fox 1976; Goodwin 1984; Brush 1990), the evolutionary questions remain virtually untouched.

Hill (1996) suggested that red carotenoid displays are more energetically costly to produce and therefore make more reliable signals of phenotypic quality than yellow or orange carotenoid displays. This could explain the wide distribution of red displays in several groups of showy vertebrates. The main premise of Hill's hypothesis is that red keto-carotenoids are less abundant than yellow carotenes and xanthophylls in the diet of most vertebrates or are costly to produce (Hudon 1991; Hill 1996). This hypothesis appears to be supported by Hill's (1996) comparative study of cardueline finches (the degree of sexual dichromatism and the redness of male plumage correlate positively across species in this clade) and by a food-limitation study (controlling for carotenoid intake, better-fed house finches were redder; Hill 2000).

Wedekind et al. (1998) offered an explanation for why male

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three-spined sticklebacks use multiple carotenoid pigments in their nuptial displays. Different pigments may provide different information to females if, for example, they are used by the immune system to combat different types of infections. Presumably, then, the use of a particular combination of carotenoids could be explained by the value of the component pigments as independent signals of condition. So far, to our knowledge, this hypothesis remains untested.

The coloration of poeciliid fishes has been extensively studied in an evolutionary context, but as yet there has been no attempt to explain why particular species display particular pigments. In this article, we present the first detailed analysis of carotenoid pigments in the integument of guppies (*Poecilia reticulata* Peters) and attempt to explain the “choice” of carotenoid pigments by making reference to the extensive base of knowledge on the mating behavior, visual system, ecology, and evolutionary biology of this species.

#### *Study System and Predictions*

Endler (1980) drew attention to the carotenoid-containing orange spots of male guppies by suggesting that they may be indicators of foraging ability and health that females could use to choose high-quality mates. Subsequent studies confirmed that the level of carotenoids in the diet affects the chroma (color saturation) of the orange spots (Kodric-Brown 1989; Grether 2000) and that female guppies prefer males with higher orange chroma (Kodric-Brown 1989; Houde and Torio 1992; Kodric-Brown 1993; Grether 2000). The chroma of the orange spots has also been shown to be reduced by parasitic infection (Houde and Torio 1992) and to correlate positively with swimming performance, a measure of condition (Nicoletto 1991). The main source of carotenoids for guppies in nature is unicellular algae (periphyton), which grows on rocks in the streambed. Streams in the upper reaches of a watershed tend to be more heavily shaded than streams in the lower reaches, resulting in a natural gradient in algae, hence carotenoid, availability (Grether et al. 2001*b*). Waterfalls restrict the movement of guppies between sites, resulting in genetically isolated high-carotenoid-availability and low-carotenoid-availability populations.

In this article, we show that the predominant carotenoids in the skin of guppies are yellow esters of tunaxanthin, a carotenoid not present in the natural diet of guppies (Grether et al. 2001*b*). Thus, the cellular machinery for converting dietary carotenoids into tunaxanthin must have evolved in guppies (or an ancestral species). The orange spots of male guppies are orange, rather than yellow, because they also contain red drosoppterin pigments (Grether et al. 2001*a*), which are synthesized *de novo* from carbohydrates and proteins (Ziegler 1965; Hurst 1980). The relative amounts of these two types of pigments affect the wavelength composition and therefore the perceived “hue” of the orange spots. We have shown previously that the carotenoid content, but not the area, of the orange spots on

male guppies is limited by algae availability to different degrees in different streams (Grether et al. 1999) and that drosoppterin production correlates positively with carotenoid intake (Grether et al. 2001*a*). The positive matching of drosoppterin production to carotenoid intake means that the ratio of these two pigment types, and therefore the hue of the orange spots, is roughly conserved across streams differing in carotenoid availability. This may be because female guppies have a preference for specific hues (Grether et al. 2001*a*; Rodd et al. 2002).

Why do male guppies use yellow tunaxanthin and red drosoppterins to produce orange spots instead of converting ingested carotenoids into orange 4-keto-carotenoids? One possible explanation is that tunaxanthin also serves a utilitarian function, such as crypsis, protection from ultraviolet (UV) light, or immune system enhancement (Needham 1974; Rothchild et al. 1975; Fox 1976; Hairston 1976; Goodwin 1984; Hebert and Emery 1990; Møller et al. 2000). Consistent with this explanation, tunaxanthin is found in the skin of males outside the orange spots and in the drab skin of females (this article). But animals can deposit different types of carotenoids in different parts of their integument (Steven 1948; Hudon et al. 1989; Hudon 1991) and the high concentration of carotenoids in the orange spots of male guppies (Grether et al. 1999) probably exceeds utilitarian needs. Could the absence of orange 4-keto-carotenoids in guppies reflect a phylogenetic constraint? Although 4-keto-carotenoids are commonly found in fishes, they have not been reported in wild poeciliids (but see “Discussion”). If 4-keto-carotenoids are not a biochemical option for guppies, males could still achieve a subtle “red shift” by depositing the carotenoids they ingest into the orange spots without converting them into tunaxanthin. This is because the usable carotenoids in stream algae (beta-carotene, lutein, and zeaxanthin; Grether et al. 2001*b*) absorb at longer wavelengths than tunaxanthin and thus more readily produce orange hues than does tunaxanthin (see Fig. 1*a*, 1*b* for a graphical demonstration).

Another route by which male guppies could achieve a red shift is by increasing the concentration of carotenoids in the orange spots (Fig. 1*c*, 1*d*). In carotenoid-limited populations, however, a male’s ability to adjust hue in this way would be severely limited, especially since guppies appear to meet their general pigmentation needs first, and only deposit surplus carotenoids in the orange spots (this article; see also Grether et al. 1999). This line of reasoning leads to two testable predictions. First, the wavelength of peak absorption of carotenoids in the orange spots should be shifted upward, relative to the skin outside of the orange spots and also relative to female skin, reflecting a greater proportion of unmodified carotenoids in the orange spots. Second, the shift should be greater in low-carotenoid-availability (LCA) streams than in high-carotenoid-availability (HCA) streams, since it is more difficult for fish in the LCA streams to achieve high skin carotenoid concentrations (Grether et al. 1999).

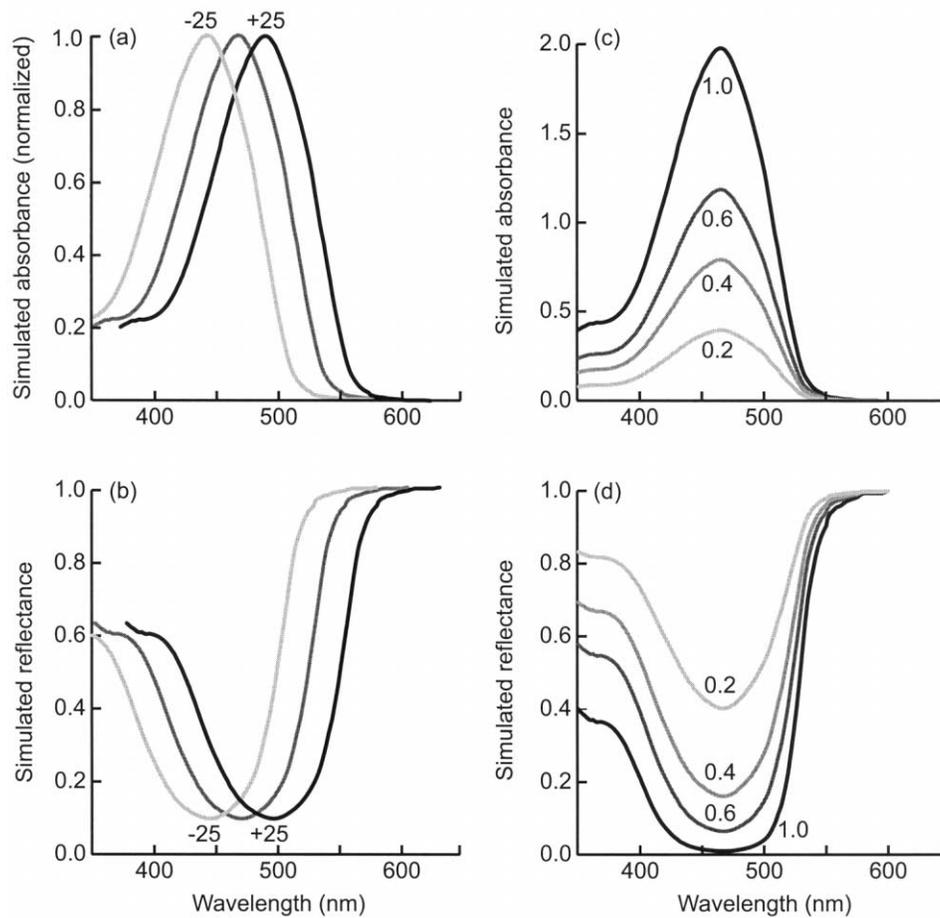


Figure 1. Results of simulations illustrating that an increase in either the wavelength of maximum (peak) absorption or the concentration of a carotenoid pigment has the effect of increasing absorption (*a*, *c*) and reducing reflectance (*b*, *d*) at long (>500 nm) wavelengths, thereby yielding redder hues (at least to human eyes). In *a* and *b*, the peak wavelength is shifted  $\pm 25$  nm relative to prawn astaxanthin. In *c* and *d*, the concentration of the pigment is varied from 20% to 100% of the original astaxanthin extract.

Our focus so far has been on the hue or wavelength composition of orange spot reflectance, but this is only one of the spectral parameters that male guppies may be selected to optimize. Other axes of color variation include chroma (color saturation) and brightness (total reflectance), which can both contribute to the conspicuousness of a color patch (Endler 1990). Chroma refers to the degree to which reflectance from an object stimulates some classes of photoreceptors more than others (see “Material and Methods” for an explicit definition). Red objects, for example, create a greater relative difference between the stimulation of long-wavelength and short-wavelength cone classes than do pink objects. Brightness refers to total photon capture or the sum of photoreceptor inputs (unfortunately, brightness is also sometimes used to mean chroma). Increases in the concentration of pigments in a color patch have the effect of reducing reflectance near the wavelengths of peak absorption, thereby reducing brightness but also increasing chroma. Since carotenoids

vary in their wavelength of maximal absorption, the way in which female guppies weigh variation along the different spectral axes when choosing mates could potentially favor one type of carotenoid pigment over another. Thus, tunaxanthin could potentially be optimal for maximizing attractiveness to female guppies.

To characterize the role of carotenoids in the pigmentation of guppies generally, and to test the predictions stated previously, we quantified variation in the carotenoid pigmentation of wild guppies at three scales: (1) between the sexual and general pigmentation of males, (2) between the sexes, and (3) geographically in relation to carotenoid availability. To understand the significance of these results from the perspective of guppies, we constructed a photoreceptor (cone)-based computer model that takes into account what is known about the visual system of guppies as well as relevant features of the signaling environment (e.g., spectral irradiance). By simulating different pigments and ratios of pigments in the skin, we ex-

amined the effect of variation in pigment composition on guppy-specific measures of brightness and chroma and the trade-off between these two spectral parameters.

## Material and Methods

### Study Populations

Guppies were collected from multiple pools in two streams in each of three river drainages (Marianne, Paria, Quare) in the Northern Mountains of Trinidad (for grid coordinates, see Grether et al. 2001b). We selected streams that were separated from each other by barriers to guppy dispersal, were in intact old-growth forest, and were above waterfalls that excluded predatory fish except the minor predator *Rivulus hartii*. These restrictions eliminated predation and anthropogenic disturbance as potentially confounding variables and ensured that the study streams contained genetically independent populations of guppies (for further details on these populations, see Grether et al. 1999, 2001b; Grether 2000). Previous work on the six streams included in this study showed that guppies ingest carotenoids at lower rates, that males have less carotenoids in their orange spots, and that there is a steeper trade-off between orange spot area and carotenoid concentration in the streams with lower carotenoid availability (Grether et al. 1999).

### Gut Pigment Analyses

Fish collected for gut content analysis (sample 1) were frozen instantly after capture in the field, to stop gut pigment absorption, and stored at  $-80^{\circ}\text{C}$ . Carotenoids were extracted with acetone from the first third of the gut (roughly equivalent to the stomach) from six to eight fish of each sex per stream. The acetone extracts were filtered (Cameo 13N syringe filters, nylon,  $0.45\ \mu$ ; Micron Separations, Westboro, Mass.), evaporated under a flow of nitrogen, and redissolved in the high-performance liquid chromatography (HPLC) mobile phase. High-performance liquid chromatography was carried out as described subsequently. Beta-carotene eluted later than expected and was not measured in Paria fish, so only lutein/zeaxanthin data were used in the analysis. The results for Marianne and Quare were qualitatively the same with beta-carotene included.

### Skin Pigment Analyses

Males collected for skin pigment analyses (sample 2) were gently captured in the field with butterfly nets and transported to our field laboratory in water treated with antibiotics (Fungus Guard, Jungle Products, Jungle Laboratories, Cibolo, Tex.), stress reducers (Novaqua, Kordon, a division of Novalek, Hayward, Calif.), and an  $\text{NH}_3$  detoxifier (Amquel, Kordon). After being allowed to recover for  $\geq 3$  h in 40-L aquariums, the 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 color patch area estimates using a graphics tablet and PASCAL program provided by J. A. Endler. Out of a random sample of 36–41 males per stream, we selected 15 per stream for skin pigment analysis, with the goal of pairing males with the most similar orange area between streams while sampling the full range of orange area within streams.

The skin, exclusive of head and fins, was stripped from the body with surgical instruments, allowed to dry for a few minutes, and divided into orange spot and non-orange spot fractions. Carotenoids in each skin fraction were extracted with acetone, transferred to a new vial, concentrated under a flow of nitrogen to remove the acetone, and redissolved in hexane.

Carotenoid content in each fraction was determined from the absorbance of unsaponified extracts at the peak of absorption (437–446 nm) in hexane on a Beckman DU-65 spectrophotometer using the extinction coefficient  $E_{1\text{cm}}^{1\%}$  for zeaxanthin (2350; Britton 1985). Pigment concentration was calculated by dividing pigment content by patch area. Ultraviolet-visible absorption spectra were also recorded from 350 nm to 550 nm. Carotenoids in extracts and, subsequently, isolated pigments, as well as the products of various chemical tests, were routinely separated on analytical thin-layer chromatography (TLC) plates. Analytical TLC was performed on silica gel (Chromagram sheets, Eastman Kodak, Rochester, N.Y.) using different mixtures of hexane and acetone.

To isolate individual skin carotenoids, carotenoid extracts of fish originating from the same streams were pooled, although orange spot and non-orange spot fractions were kept separate. Pigments were separated by preparative TLC on precoated plates of silica gel (Anasil G, Analabs, New Haven, Conn.) using a solvent mixture of hexane and acetone (3 : 1). Identifiable colored bands were cut out and pigments eluted from the gel with acetone and transferred to hexane after filtration (Cameo 13N syringe filters, nylon,  $0.45\ \mu$ ; Micron Separations, Westboro, Mass.). Because the pigment differences between streams were quantitative, not qualitative, the pigment bands were pooled across streams.

Isolated pigment bands were characterized spectrally, chromatographically, and chemically, in addition to being compared with known standards. Relative migration on TLC plates ( $R_f$  value, i.e., the ratio of pigment migration to solvent-front migration) was determined in the system hexane : acetone (2 : 1). High-performance liquid chromatography was carried out with a Waters instrument (Waters, Milford, Mass.) equipped with two Waters 501 pumps, a 712 WISP autoinjector, a System Interface Module, and a Lambda Max 481 UV detector. Carotenoid pigments were eluted isocratically on a Zorbax SB-C18 (Rockland Technologies, Chadds Ford, Pa.) reversed-phase column (4.6 mm i.d.  $\times$  250 mm), with a mixture of acetoni-

trile : methanol : dichloromethane (41 : 50 : 9) flowing at 1 mL/min (De Leenheer and Nelis 1992). Peak detection was at 450 nm.

Chemical tests performed for the purpose of identifying specific functional groups of the carotenoid pigments included saponification of carotenyl esters in alkaline (3% KOH) methanol, reduction of carbonyl groups with sodium borohydride in methanol, acetylation of hydroxyl groups with acetic anhydride in dry pyridine, and methylation of allylic hydroxyl groups in acidified (HCl) methanol (Hudon and Brush 1992). A test for carotenoid 5,6-epoxides, as in violaxanthin and tetraxanthin, was also performed following Britton (1985). Standards of canthaxanthin, lutein, and zeaxanthin were provided by Hoffman La Roche, Basel; beta-carotene was obtained from Sigma Chemical, St. Louis. A standard of tunaxanthin was prepared by reduction with sodium borohydride of the main canary-xanthophylls in the western tanager (*Piranga ludoviciana* [Wilson]; Hudon 1991).

The carotenoids in the skin of fish from sample 1 were extracted, quantified, and characterized ( $\lambda_{\max}$ ) in the same manner as sample 2, except that orange spot and non-orange spot fractions were not separated.

#### Stream Carotenoids

We sampled periphyton (attached algae) from stream cobbles in 18–21 pools per stream, both before and after the wet season floods in 1996. Periphyton samples were filtered in the field, stored at  $-80^{\circ}\text{C}$ , and analyzed by HPLC (Millie et al. 1997). Algal standing crops were measured as chlorophyll *a* per square centimeter of cobble (Cattaneo and Amireault 1992). Carotenoid availability was estimated as the amount of lutein, zeaxanthin, and beta-carotene on cobbles divided by guppy biomass. Separate estimates of carotenoid availability were made for each pool and averaged to obtain stream means. Guppy biomass was estimated by measuring the standard length of all guppies in a pool (literally), converting lengths to weights with sex-specific allometric equations, and dividing the sum of the weights by the surface area of the pool (see Grether et al. 2001*b* for details).

#### Computer Simulations

Color is a property of visual systems, not of objects, so it was necessary to make some assumptions about how guppies perceive color. We compared the spectral properties of the carotenoids found in the skin of guppies (tunaxanthin) to those of a common 4-keto-carotenoid (astaxanthin) with a computer model that incorporates the spectral sensitivity functions of guppy cones and lighting conditions in the natural environment. Absorbance spectra of the pigments were obtained from spectrophotometric scans of pigment extracts. Drosopterins were extracted from the eyes of *Drosophila* sp. fruit flies, tun-

axanthin was extracted from the skin of guppies, and astaxanthin was extracted from commercially available black tiger prawns (*Penaeus monodon* Fabricius). Simulated absorbance was calculated for each wavelength using the formula  $A = pC + D(1 - p)$ , where  $C$  and  $D$  are the carotenoid and drosoprotein absorbances (normalized to a minimum of 0 and maximum of 1) and  $p$  is a constant ranging from 0 to 1, which we refer to as the “carotenoid fraction” (Grether et al. 2001*a*). When  $p = 0$ , only drosoproteins contribute to the absorbance spectrum; when  $p = 1$ , only carotenoids contribute to absorbance. Simulated reflectance spectra were obtained by equating reflectance to transmittance with the formula  $T = 10^{-A}$ . This simple model is unrealistic in some respects. The absorbance spectra of the pigments in intact skin cells may differ from those measured from pigment extracts, and reflectance and transmittance would be affected by properties of the skin not included in the model. Nevertheless, we believe this model is suitable for the questions addressed in this article.

To obtain guppy-specific estimates of brightness and chroma, the simulated reflectance spectra were processed to yield estimates of quantum catch by each of the four classes of guppy photoreceptor cones (Endler 1991; see also Chittka 1992 and Vorobyev and Osorio 1998 for similar cone-based approaches). Absorbance functions for guppy cones were calculated from published  $\lambda_{\max}$  values (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). The one cone of guppies is polymorphic ( $\lambda_{\max}$  values of 533, 543, and 572 nm; Archer and Lythgoe 1990), but the simulation results were only weakly sensitive to, and no conclusions were affected by, the choice of one cone. The following  $\lambda_{\max}$  values were used in the simulations presented here: 389 (ultraviolet; UV), 410 (short; S), 465 (middle; M), and 543 nm (long; L).

The photon catch ( $P$ ) for each cone class was estimated from

$$P_i = \sum_k r_k a_k t_k s_{ik},$$

where  $i$  refers to the cone class (UV, S, M, or L),  $r_k$  is the simulated reflectance (transmittance) of the skin pigment or pigment combination at wavelength  $k$ ,  $a_k$  is the ambient irradiance at wavelength  $k$ ,  $t_k$  is the transmission fraction through 0.25 m of clear water at wavelength  $k$  (from Fig. 5 in Endler 1991), and  $s_{ik}$  is the spectral sensitivity or absorbance of cone class  $i$  at wavelength  $k$  (Grether 2000). Ambient irradiance spectra were obtained from J. A. Endler for four different light environments experienced by guppies in nature: early/late, forest shade, cloudy/open, and small gap (see Endler 1991, 1993). The simulations were only weakly sensitive to, and no conclusions were affected by, the choice of ambient irradiance spectrum, so here we present only results for the early/late spectrum (which occurs during the periods of maximum guppy courtship; Endler 1991). In the absence of information on how the

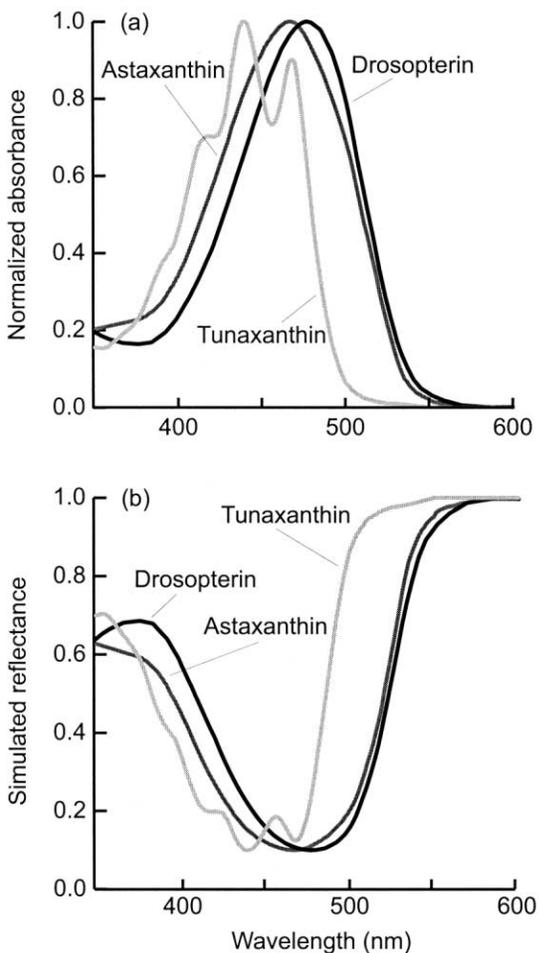


Figure 2. Normalized absorbance spectra (a) and simulated reflectance (b) for drosopterin, tunaxanthin, and astaxanthin. The pigment simulations were based on additive combinations of the absorbance spectra shown in a. See "Material and Methods" for details.

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, the contrast (c) between long and short cones,

$$P_C = (P_M + P_L - P_S - P_{UV})(P_T)^{-1},$$

provides a suitable, albeit directional, measure of spectral contrast or chroma (Grether 2000). We also calculated the maximum cone contrast ( $D_{\max}$ ), a nondirectional measure of chroma (Endler 1991).

To determine whether our results were specific to guppies or more general, we repeated the previously described procedure for humans (using  $\lambda_{\max}$  values in Merbs and Nathans 1992) and also calculated chroma and hue using the segment clas-

sification method for the human visible spectrum (400–700 nm). Segment classification is a non-species-specific method for classifying color that tends to agree with human perception and allows for comparisons across studies (Endler 1990).

## Results

### Pigment Characterization

Skin extracts produced a single fast-moving band ( $R_f = 0.97$  compared to 0.95 for beta-carotene) on analytical TLC and exhibited absorption spectra typical of common xanthophylls, with distinct maxima at about 415, 440, and 468 nm, the middle peak being the tallest (tunaxanthin in Fig. 2a). On the analytically more powerful HPLC, however, skin extracts yielded several relatively broad, sometimes overlapping, peaks. Separation of these peaks on HPLC in a reasonable time (20 min) necessitated a change of solvent mixture to acetonitrile : dichloromethane : methanol (60 : 37.5 : 2.5). Under these conditions, often more than 20 peaks could be observed in a single run.

Saponification of the skin carotenoids effected a dramatic change in chromatographic behavior (but not of spectral characteristics) of the colored material, which indicates that the carotenoids are esterified: band migration on analytical TLC slowed down to an  $R_f$  of 0.28, matching that of common dihydroxy-carotenoids such as lutein and zeaxanthin, whereas the HPLC pattern was simplified to just a few peaks, with an overbearing peak at 4.7 min that co-eluted with the same dihydroxy-carotenoids.

Because the HPLC method used proved unable to separate zeaxanthin, lutein, and tunaxanthin (contrary to De Leenheer and Nelis 1992; see Craft 1992 for an explanation), the pigments were characterized by chemical and spectrophotometric means following their isolation by preparative TLC. The 12 pooled extracts (orange and non-orange spot fractions of males in each stream) contained three identifiable colored bands, the fastest band containing the vast majority of the pigmentation.

Time-controlled acetylation with acetic anhydride of the saponified carotenoids supported the inference that the main carotenoids in the integument bear two hydroxyl groups, like zeaxanthin and lutein. Unlike zeaxanthin, however, the saponified carotenoids reacted positively to the allylic reaction, which implicates hydroxyl groups in an allylic position to a double bond in position 4 of the carotenoid end-ring (instead of position 5 in common xanthophylls). On the basis of band mobility change on TLC and production of intermediate bands, the bulk of skin carotenoids contained two such allylic hydroxyl groups (lutein has one, zeaxanthin has none). In this respect, the carotenoids match tunaxanthin ( $\epsilon,\epsilon$ -carotene-3,3'-diol), a widely distributed carotenoid in fish (Matsuno and Katsuyama 1976, Goodwin 1984). The carotenoid 5,6-epoxide test was negative.

A significant involvement of tunaxanthin is reinforced by maximal absorption at relatively short wavelengths of most of

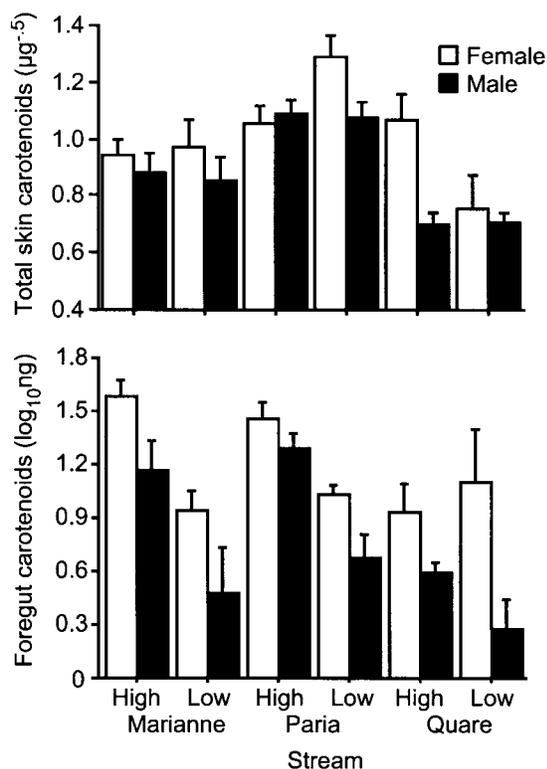


Figure 3. Sex differences in absolute carotenoid content of whole skins and foreguts of guppies from six streams (sample 1). Marianne, Paria, and Quare are the river drainage names; *high* and *low* refer to the within-drainage carotenoid availability categories of the streams. Means + 1 SE are shown.

the guppy skin extracts (ca. 440 nm). Tunaxanthin (various stereoisomers) absorbs maximally at 438 nm in hexane, with deep indentations between absorption peaks (like the guppy pigments; see Fig. 2a), whereas lutein absorbs maximally at 445 nm and zeaxanthin at 449 nm, with shallower, if any, indentations (Britton 1985). Nevertheless, peak absorption at wavelengths longer than 438 nm (whole skin: range 438–443 nm, mean  $\pm$  SE 440.44 nm  $\pm$  0.17,  $n = 66$ ; orange spots: range 438–446 nm, mean  $\pm$  SE 441.5 nm  $\pm$  0.144,  $n = 90$ ; non-orange spots: range 437–440 nm, mean  $\pm$  SE 438.5 nm  $\pm$  0.090,  $n = 90$ ) indicates that lutein, zeaxanthin, or other pigments also occur in the integument of guppies.

One of the minor pigment bands (<99% by absorption) isolated by preparative TLC included an unesterified pigment (pigment C;  $R_f = 0.53$ ) harboring a single hydroxyl group that was allylic with the end-ring double bond. Based on its spectrum (maxima at 414, 441, 470 nm), most molecules in that band probably had two  $\epsilon$  end-rings (with the remainder having one). Another unesterified pigment (pigment B;  $R_f = 0.65$ ) had a single carbonyl group (reduced by sodium borohydride) but no hydroxyl group. Judging from the pigment's spectrum (maxima

at 412, 438, 468 nm) and failure of borohydride reduction to alter the pigment's color, the carbonyl group was not in conjugation with the carotenoid central chain of double bonds. When reduced, the pigment comigrated with pigment C on TLC, suggesting that it is an oxidized derivative of that pigment. The information at hand indicates that pigment B is  $\epsilon, \epsilon$ -carotene-3-one, whereas pigment C is mainly  $\epsilon, \epsilon$ -carotene-3-ol (with some  $\beta, \epsilon$ -carotene-3'-ol). No other keto-carotenoids, such as astaxanthin and canthaxanthin, were detected in the skin of guppies from the streams examined. Thus, guppy skin mainly contains esters of tunaxanthin alongside a few unesterified monosubstituted carotenoids with  $\epsilon$  end-rings.

Although it proved impractical to quantify individual carotenoids in the integument of guppies by HPLC, we were able to monitor interindividual differences in carotenoid composition through variation in the wavelength of peak absorption (peak wavelength) of skin extracts. This approach is valid because the principal pigments involved differ markedly in their wavelength of peak absorption.

#### Sex Differences in Carotenoid Ingestion and Deposition

Females had on average more carotenoids in their skin and foreguts than males (Fig. 3; Table 1). The sex difference in foregut carotenoids diminished but was not eliminated by dividing foregut carotenoid content by body mass (Table 1). Since significant absorption of carotenoids through the gut wall has yet to occur in the foregut (Choubert et al. 1987; Hardy et al. 1990), this indicates that males ingest carotenoids at lower rates than females of the same body mass, which is not surprising given that males spend much of their time searching for and courting females instead of foraging (Houde 1997). However, the sex difference in skin carotenoids was reversed after dividing by body mass (Table 1). This suggests that males deposit a greater fraction of ingested carotenoids in their skin than do females. To check this inference, we examined the ratio of carotenoids in the skin to carotenoids in the foreguts of individual fish. In five of six study streams, males had a greater skin to foregut carotenoid ratio than females (Fig. 4; Table 1). Across streams, the magnitude of this ratio decreased as stream carotenoid availability increased, but only significantly so for males (Fig. 4).

The excess of carotenoids in the whole skin of males, relative to females, appeared to be entirely due to the high concentration of carotenoids in the orange spots. To validly compare the carotenoid concentration of female whole skin to that of the orange spot skin of males, orange spot carotenoid content was rescaled by multiplying by body area and dividing by orange spot area. Likewise, to compare the carotenoid concentration of female whole skin to that of the non-orange spot skin of males, non-orange spot carotenoid content was multiplied by body area and divided by non-orange spot area. To adjust for sex differences in body size, carotenoid content was divided by

Table 1: Results of ANOVAs to detect sex and stream differences in carotenoid ingestion, deposition, and conversion

Dependent Variable	Sample	Sex	Stream	Stream × Sex	Residual df
Foregut carotenoids ( $\log_{10}$ ng)	1	23.57**** (F > M)	9.41****	1.00	50
Foregut carotenoids/body mass ( $\log_{10}X$ )	1	5.11* (F > M)	7.20****	1.58	50
Total skin carotenoids ( $\mu\text{g}^{-.5}$ )	1	9.14** (F > M)	9.43****	1.93	66
Total skin carotenoids ( $\mu\text{g}^{-.5}$ )	2	4.99* (F > M)	16.76****	6.05****	117
Skin to foregut carotenoid ratio ( $\log_{10}X$ )	1	9.20** (M > F)	7.71****	1.58	48
Total skin carotenoids/body mass ( $X^{-.5}$ )	1	13.29*** (M > F)	16.14****	1.57	66
Total skin carotenoids/body mass ( $X^{-.5}$ )	2	47.52**** (M > F)	13.66****	4.07**	118
Total skin carotenoid absorbance peak (nm)	1	.369 (M = F)	7.72****	.63	54
df		1	5	5	

Note. Values shown are  $F$  statistics. The units and transformation used for each dependent variable are shown in parentheses. The transformations used were those that best minimized departure from parametric assumptions of normality and homoscedasticity. The Sample column indicates which sample of males was used for the analysis (see "Material and Methods"). The direction of the sex difference is shown in parentheses between the Sex and Stream columns. Means and standard errors are presented in Figures 3–5.

\*  $P < 0.05$ .

\*\*  $P < 0.01$ .

\*\*\*  $P < 0.001$ .

\*\*\*\*  $P < 0.0001$ .

body mass, yielding carotenoid concentration estimates in microgram of carotenoids per milligram of body mass. After these corrections, the carotenoid concentration of the orange spots was substantially higher than that of female skin ( $F_{1,118} = 294.48$ ,  $P < 0.0001$ ; Fig. 5), and the carotenoid concentration of male non-orange spot skin was statistically indistinguishable from that of females ( $F_{1,118} = 0.22$ ,  $P > 0.6$ ; Fig. 5). These results suggest that males deposit a greater fraction of ingested carotenoids in the skin than do females and that males route the surplus carotenoids into the orange spots as opposed to shunting carotenoids away from general pigmentation and into the orange spots.

#### Variation in Carotenoid Composition Within and Between the Sexes

Our prediction that the carotenoids in the orange spots would be red-shifted relative to the carotenoids in the non-orange spot skin of males was borne out, although the shift was very small in magnitude. The peak wavelength of carotenoids in the orange spot fraction lay only ca. 2.8 nm higher than that in the non-orange spot fraction (Fig. 6; mean difference  $2.76 \text{ nm} \pm 0.17$ ,  $n = 90$ ; paired  $t$ -test,  $t = 16.68$ ,  $df = 89$ ,  $P < 0.0001$ ,  $n = 90$ ; see "Pigment Characterization" above for ranges and means). By comparison, the addition of an ending double bond in conjugation with the central chain of double bonds adds at least 5 nm to the wavelength of peak absorption of a carotenoid (Scott 1964; Britton 1985). Separately by stream, the peak difference ranged from  $2.07 \pm 0.25 \text{ nm}$  ( $n = 15$ ) in the Quare LCA stream to  $3.40 \text{ nm} \pm 0.39$  ( $n = 15$ ) in the Paria HCA stream (all  $P < 0.0001$ ; Fig. 6). By comparison, the absorbance peak of orange spot drosoperins lay 38.6 nm higher than that of orange spot carotenoids

for males in our sample (paired  $t$ -test,  $t = 137.6$ ,  $df = 89$ ,  $P < 0.0001$ ).

If the coloration of the integument outside of the orange spots of males has a similar function to that of females, such as crypsis, we might expect that the peak wavelength of pigments in the two to be similar. Surprisingly, the whole-skin carotenoid extracts of females were more similar in peak wavelength to the male orange spot extracts than to the male non-orange spot extracts (Fig. 6). On average, the female peak lay

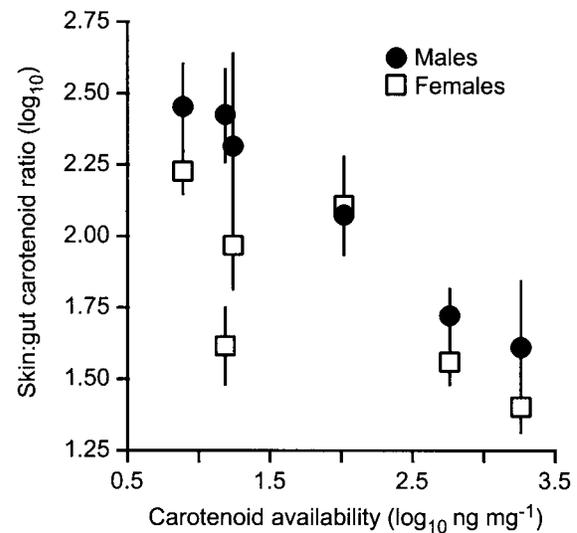


Figure 4. Relationship between carotenoid availability and the skin to foregut carotenoid ratio of male (filled circles) and female (open squares) guppies in six streams (males:  $r = 0.99$ ,  $P < 0.0001$ ; females:  $r = 0.71$ ,  $P = 0.13$ ). Means + 1 SE are shown.

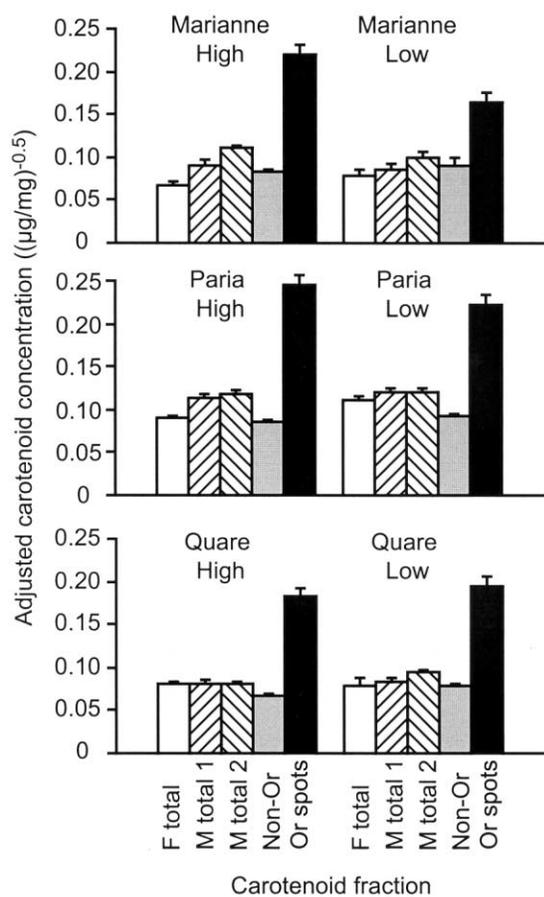


Figure 5. Carotenoid concentration of the integument after adjusting for orange spot area and sex differences in body size (using the method described in the text). Marianne, Paria, and Quare are the river drainage names; *high* and *low* refer to the within-drainage carotenoid availability categories of the six streams. Horizontal axis labels translate as follows: *F total* = female whole skin; *M total 1* = male whole skin (sample 1); *M total 2* = male whole skin (sample 2); *Non-Or* = non-orange spot skin of males (sample 2); and *Or spots* = orange spot skin of males (sample 2). For *F* statistics, see Table 1. Means + 1 SE are shown.

1.97 nm higher than the male non-orange spot peak (two-way ANOVA; sex  $F_{1,112} = 154.60$ ,  $P < 0.0001$ ; sex  $\times$  stream  $F_{5,112} = 2.85$ ,  $P = 0.018$ ; Fig. 6). The significant sex by stream interaction indicates that the magnitude of the peak difference varied among streams, but in all cases the female peak exceeded the male non-orange spot peak (range of peak difference: 1.6–2.9 nm). On average, the male orange spot peak lay 0.78 nm higher than the female whole skin peak (sex  $F_{1,112} = 5.30$ ,  $P = 0.0002$ ; sex  $\times$  stream  $F_{5,112} = 3.06$ ,  $P = 0.013$ ), but in two streams the female peak was actually slightly higher than the male orange spot peak (Fig. 6). The peak wavelength of whole skin carotenoid extracts did not differ significantly between the sexes (Table 1; Fig. 6).

#### Geographic Variation in Carotenoid Composition in Relation to Carotenoid Availability

Our prediction that the carotenoids in the orange spots would be red-shifted to a greater degree in the LCA streams than in the HCA streams was not upheld. The trend was in the opposite direction in all three river drainages (Fig. 6), but significantly so only in the Quare drainage (planned comparison  $P = 0.013$ ). Overall, geographic variation in the peak wavelength of orange spot carotenoids was significant ( $F_{5,84} = 3.07$ ,  $P = 0.014$ ), however. Stream means ranged from 440.3 nm in the Quare LCA stream to 442.1 nm in the Paria HCA stream. The peak wavelength of orange spot carotenoids did not generally correlate, across streams, with the availability of usable carotenoids ( $r = 0.366$ ,  $P = 0.48$ ,  $n = 6$ ).

Significant geographic variation was also found in the peak

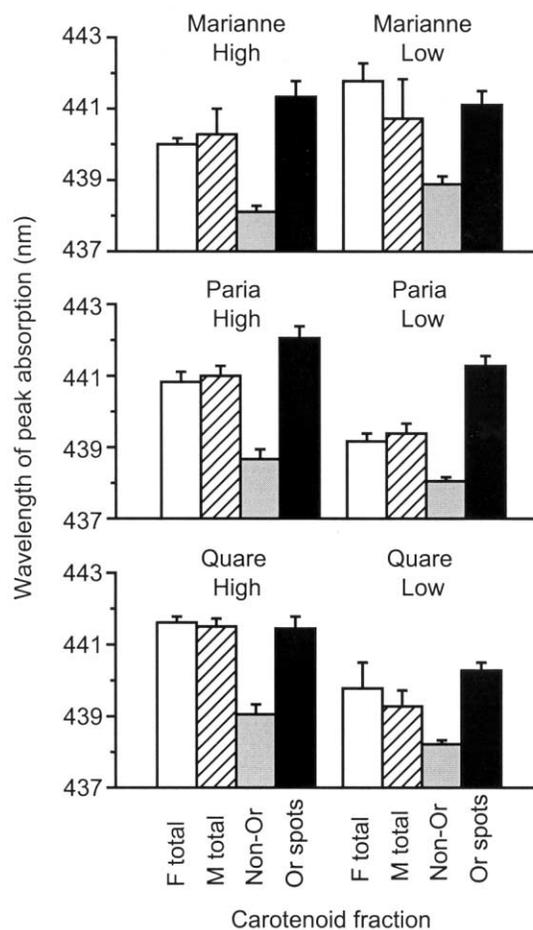


Figure 6. Peak wavelength of carotenoids in the integument of guppies from six streams. Horizontal axis labels translate as follows: *F total* = female whole skin; *M total* = male whole skin (sample 2); *Non-Or* = non-orange spot skin of males (sample 2); and *Or spots* = orange spot skin of males (sample 2). For *F* statistics, see the text and Table 1. Means + 1 SE are shown.

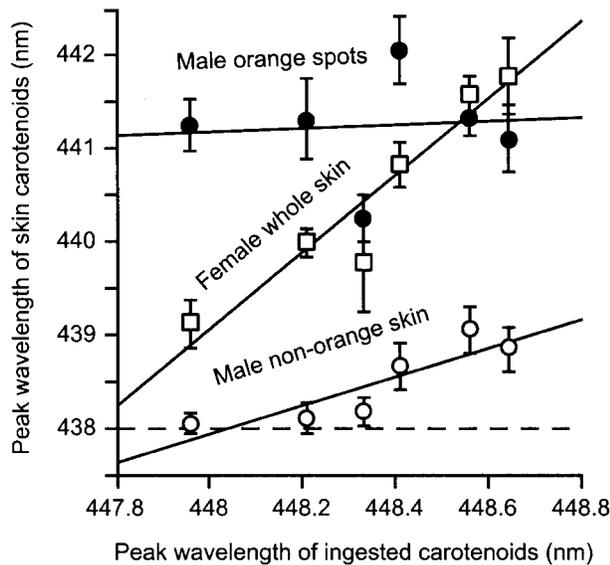


Figure 7. Relationship between the peak wavelength of carotenoids in guppy integument and the peak wavelength of carotenoids available in the periphyton of different streams. The peak wavelength of ingested carotenoids was calculated as the arithmetic means of the peak wavelength of usable carotenoids weighted by their abundance in the diet (see "Material and Methods"). The peak wavelength of skin carotenoids was strongly correlated with the peak wavelength of ingested carotenoids for females (*open squares*;  $y = -1404.011 + 4.114x$ ;  $r = 0.95$ ,  $P = 0.003$ ) and the non-orange spot fraction of males (*open circles*;  $y = -247.79 + 1.531x$ ;  $r = 0.88$ ,  $P = 0.02$ ), but not the orange spot fraction of males (*filled circles*;  $r = 0.09$ ,  $P = 0.9$ ). Linear regression equations were fitted by the least squares method. The order of the streams, from left to right: Paria low, Marianne high, Quare low, Paria high, Quare high, and Marianne low, where *high* and *low* refer to the within-drainage carotenoid availability categories of the streams. The stippled line at 438 nm represents the asymptote wavelength afforded by tunaxanthin, the end product along the biochemical pathway.

wavelength of male non-orange spot carotenoids (males  $F_{5,84} = 4.42$ ,  $P = 0.0013$ ) and in female whole skin carotenoids ( $F_{5,28} = 8.318$ ,  $P < 0.0001$ ). For the non-orange spot fraction, the peak wavelength was slightly longer in the HCA stream than in the LCA stream in the Paria (438.7 vs. 438.1 nm, planned comparison  $P = 0.038$ ) and Quare (439.1 vs. 438.2 nm,  $P = 0.003$ ) drainages, but the difference went in the opposite direction in the Marianne drainage (438.1 nm compared with 438.9,  $P = 0.012$ ). The same pattern of intradrainage peak wavelength differences was observed in female whole skin carotenoid extracts (Paria: 440.9 vs. 439.1 nm,  $P = 0.001$ ; Quare: 441.6 nm vs. 439.8 nm,  $P = 0.003$ ; Marianne: 440.0 vs. 441.8 nm,  $P = 0.003$ ).

The peak wavelength of whole-skin extracts was more similar between the sexes within streams than among streams within a sex, as reflected by a significant correlation between the sexes across streams ( $r = 0.927$ ,  $P = 0.029$ ,  $n = 6$ ). This suggests an

effect of the local environment. Variation in the types of carotenoids obtained in the diet could potentially affect the types of carotenoids deposited in the integument. To examine this possibility, we calculated the average peak wavelength of usable carotenoids in periphyton as

$$(449C + 449Z + 445L)(C + Z + L)^{-1},$$

where  $C$ ,  $Z$ , and  $L$  represent the concentrations of beta-carotene, zeaxanthin, and lutein, respectively ( $\text{ng}/\text{cm}^2$  of cobble substrate), and the values in the numerator are the absorbance peaks of the respective pigments (nm). This equation provides an estimate of what the peak wavelength of carotenoids in the integument of guppies would be if the periphyton carotenoids were deposited unmodified in the integument. Since beta-carotene and zeaxanthin have identical absorbance peaks, the ratio  $(C + Z) : L$  provides an equivalent way to look at these data.

The peak wavelength of carotenoids in female integument correlated strongly across streams with the average peak wavelength of usable carotenoids in periphyton ( $r = 0.954$ ,  $P = 0.0031$ ,  $n = 6$ ; Fig. 7), with the  $(C + Z) : L$  ratio ( $r = 0.949$ ,  $P = 0.0038$ ), and with the log of the  $(C + Z) : L$  ratio ( $r = 0.966$ ,  $P = 0.0017$ ). Removing beta-carotene from the  $(C + Z) : L$  ratio reduced noticeably the magnitude of the correlation ( $r = 0.813$ ,  $P = 0.049$ ,  $n = 6$ ), whereas removing zeaxanthin from the ratio increased the correlation slightly ( $r = 0.963$ ,  $P = 0.0020$ ). This would seem to indicate that beta-carotene has a stronger influence on peak wavelength of skin carotenoids than does zeaxanthin. Similar results were obtained for the non-orange skin of males (Fig. 7). In contrast, the peak wavelength

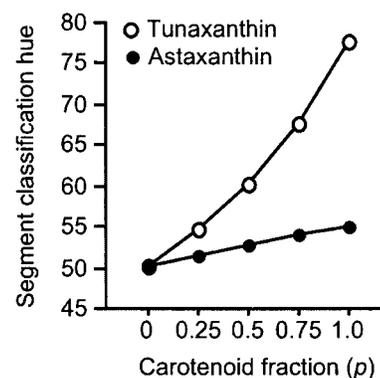


Figure 8. Simulation results showing the effect of carotenoid type (tunaxanthin, *open circles*; astaxanthin, *filled circles*) and the carotenoid fraction ( $p$ ) on segment classification hue. Segment classification hue is a circular statistic measured in degrees, where 0 = pure red, 90 = yellow, 180 = green, and 270 = blue, as perceived by humans (Endler 1990). Carotenoid fraction refers to the relative amounts of carotenoids and drospterins;  $p = 0$  for pure drospterin spectra, and  $p = 1$  for pure carotenoid spectra.

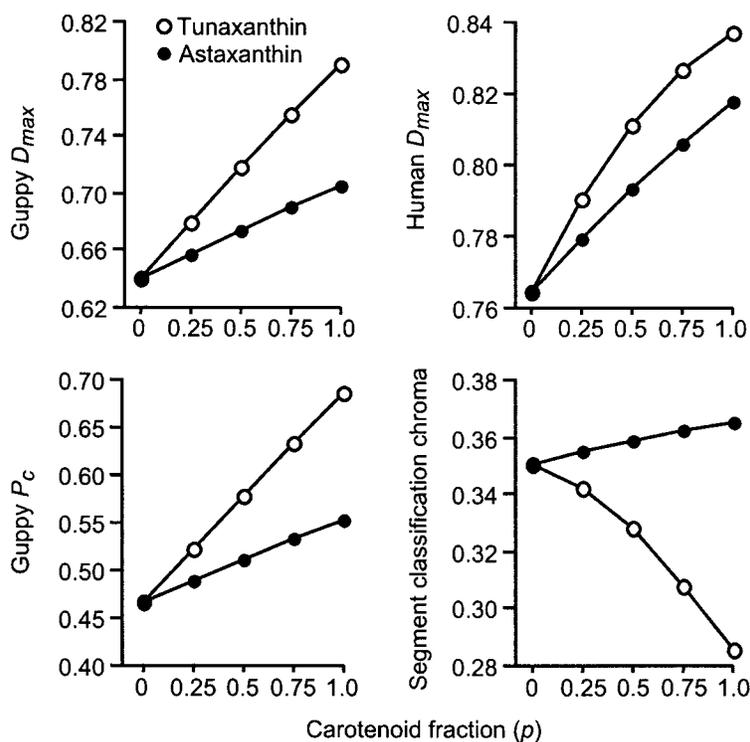


Figure 9. Simulation results showing the effect of carotenoid type (tunaxanthin, open circles; astaxanthin, filled circles) and the carotenoid fraction ( $p$ ) on four different measures of chroma. As defined in “Material and Methods,” guppy  $P_c$  and guppy  $D_{max}$  are cone-based measures specific to guppy vision, human  $D_{max}$  is specific to humans, and segment classification chroma is non-species-specific. Carotenoid fraction refers to the relative amounts of carotenoids and drosopterin;  $p = 0$  for pure drosopeterin spectra, and  $p = 1$  for pure carotenoid spectra.

of the orange spots of males did not appear to be related to variation in periphyton carotenoid composition (Fig. 7). These results were not confounded with variation among streams in the absolute amounts of usable carotenoids present in the periphyton (data not shown).

#### Computer Simulations

Tunaxanthin absorbs maximally at shorter wavelengths (438 nm) than either astaxanthin (467 nm) or drosopeterins (477 nm) and therefore reflects more long wavelength light (>450 nm) and less short wavelength light (<450 nm) than the other two pigments (Fig. 2). The absorbance and reflectance curves of astaxanthin are shifted only about  $-10$  nm relative to those of drosopeterins, and the spectra produced by these two pigments are quite similar in shape (Fig. 2). As one might expect, substituting tunaxanthin for drosopeterins in the simulations resulted in a rapid shift toward a yellower reflectance spectrum, whereas substituting astaxanthin for drosopeterins had a more subtle effect on hue (Fig. 8).

Less intuitively, the photoreceptor-based simulations showed that tunaxanthin has a more potent effect on chroma than would astaxanthin as perceived by guppies and, to a lesser

degree, by humans (Fig. 9). Both carotenoids were more potent in their effects on chroma than were drosopeterins (as indicated by the positive slopes). This was true for both the directional ( $P_c$ ) and nondirectional ( $D_{max}$ ) measures of chroma. Interestingly, reliance on the segment classification version of chroma would have led to nearly opposite conclusions (a caveat for the use of this popular non-species-specific measure of chromaticity; Fig. 9).

Total cone catch or brightness increased sharply when tunaxanthin was substituted for drosopeterins in the simulations. In contrast, substituting astaxanthin for drosopeterins had a weak negative effect on brightness (Fig. 10).

#### Discussion

##### Variation in Carotenoid Composition and Abundance

The carotenoid pigments in the orange spots of male guppies were very similar to those in the non-orange skin of both sexes (mostly esters of tunaxanthin), which is surprising given that the orange spots contrast conspicuously with the remainder of the integument and are absent altogether in females. Nevertheless, the carotenoids in the orange spots differed from those in non-orange skin in terms of pigment concentration, peak

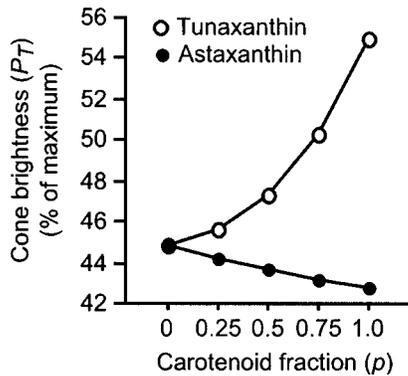


Figure 10. Simulation results showing the effect of carotenoid type (tunaxanthin, *open circles*; astaxanthin, *filled circles*) and the carotenoid fraction ( $p$ ) on cone brightness ( $P_T$ ), expressed as a percentage of the maximum possible cone catch for guppies. Carotenoid fraction refers to the relative amounts of carotenoids and drosoperterins;  $p = 0$  for pure drosoperterin spectra, and  $p = 1$  for pure carotenoid spectra.

wavelength, and the dependence of these parameters on the environment. It is probable that the orange spots and outlying skin differ in other ways, for example, properties of the iridophore layer, that contribute to the color difference (G. F. Grether, personal observation).

The concentration and peak wavelength of carotenoids in the skin of guppies were found to depend, respectively, on the amount and composition of carotenoids in the algal food base (periphyton), although these effects differed somewhat between the sexes and between the orange and non-orange skin of males (Fig. 7). The males in our sample had 5.4–8.9 times more carotenoids in their orange spots per square millimeter of skin surface than in the rest of their integument. This was not accomplished at the expense of the general integument, which was endowed similarly to the general integument of females. Instead, males appear to use more of the carotenoids they ingest than do females and channel the “surplus” carotenoids into the orange spots. In LCA streams, males have relatively low concentrations of carotenoids in their orange spots, but elsewhere in the integument the concentration of carotenoids does not differ between LCA and HCA streams (Grether et al. 1999; this article). This suggests that general pigmentation needs are met before sexual ones even though the amounts of carotenoids found in the orange spots are greater than those in the general pigmentation. Apparently, carotenoids are not used in a strict order related to amounts used but instead according to an adaptive hierarchy. Carotenoid pigments are used for a variety of other purposes and occur in a wide range of tissues, as well (Needham 1974). Other proposed functions of carotenoids, such as immune system enhancement (Lozano 1994; Møller et al. 2000), may rank higher in priority than general pigmentation but require even smaller quantities of carotenoids.

The carotenoid composition of the non-orange skin of both

sexes was correlated with the relative abundance of the known usable carotenoids lutein, beta-carotene, and, to a lesser extent, zeaxanthin in the periphyton (Fig. 7). An association could arise if these carotenoids were deposited unmodified in the integument, or in this case served as precursors for the carotenoids present in the skin. Tunaxanthin is the main carotenoid present in the skin of guppies (and the end product along a carotenoid biochemical pathway in fish), but other carotenoids were present as well.

Lutein can be expected to contribute to the deposition of tunaxanthin in the skin of guppies because it is a direct precursor of that carotenoid (Miki et al. 1985). The contribution of beta-carotene is harder to explain, as it is many steps removed from tunaxanthin and not a known precursor of the yellow carotenoid. However, the identification of two probable metabolites of beta-carotene on the path to tunaxanthin in the skin of the fish examined (compounds B and C) suggests that such conversion may be taking place. Compounds B and C may appear in the skin because of their relatively high hydrophobicity.

Beta-carotene, and probably other dietary carotenoids, also yields pigments absorbing at longer wavelengths than tunaxanthin, judging from the peak wavelength of skin carotenoids above 438 nm (tunaxanthin’s peak wavelength) in many fish. These were apparent particularly in streams with higher relative abundance of beta-carotene. Dietary carotenoids may be expected to contribute to different degrees to skin pigment composition as a function of the ability of guppies to absorb, transport, and process these carotenoids.

The slope of the relationship between skin and dietary carotenoids suggests that a slight increase in the peak wavelength of ingested carotenoids would result in a disproportionately greater increase in the peak wavelength of skin carotenoids (note the different scale of the horizontal and vertical axes in Fig. 7). This observation would seem to indicate that the pigment composition of the general integument, especially in females, is very sensitive to variation in the relative abundance of lutein and beta-carotene in the diet. This sensitivity was further borne out by the highly significant correlation between peak wavelength of skin carotenoids and the beta-carotene/lutein ratio of the periphyton. The latter result could indicate that beta-carotene or a metabolite counterbalances lutein in some of the processes involved, although other explanations are possible. Whether this norm of reaction as a function of pigment availability in the diet is adaptive or not will require further investigation.

Another surprising result of the present study was that the pigmentation of the non-orange spot fraction of male guppies is not equivalent to that of the general integument of females, considering that the two may play similar roles, such as crypsis or protection from UV light, for example. Instead the pigment composition of the integument of females was consistently closer to that of the orange spot fraction of males than to that of the

skin outside of the orange spots. At a physiological level, the difference in pigment composition of the general integument of males and females could arise from the processes responsible for the differentiation in pigment composition between the two color fractions of the males if, for example, the two skin areas of males differed in their selectivity of carotenoid uptake, the orange spots preferentially absorbing longer wavelength-absorbing carotenoids, leaving shorter wavelength absorbing pigments for the rest of the integument or vice versa. Functionally, the difference in pigment composition of males may also provide for added visual contrast between the orange spots and the surrounding skin.

Although carotenoid composition of the non-orange spot fraction of males and the general integument of females was correlated with diet, that of the orange spot fraction of males was not. This suggests that carotenoid composition in the orange spot of males is controlled by different factors than those operating in the general integument.

#### *Tunaxanthin versus Keto-Carotenoids*

We now return to the question raised in the "Introduction" of why guppies and apparently other poeciliids (Goodrich et al. 1941) use yellow tunaxanthin and red drospterins to produce orange spots instead of using orange 4-keto-carotenoids (which are commonly found in vertebrates; Goodwin 1984). First we consider in more detail the nonadaptive hypothesis that tunaxanthin use reflects a phylogenetic constraint.

Although it is not generally possible to assess the full metabolic repertoire of a given species, the capabilities of related species may be illuminating. No species of poeciliid fish is known to produce keto-carotenoids naturally, but interspecific hybrids between *Xiphophorus helleri* and *Xiphophorus maculatus* with the "red dorsal fin" (*Dr*) mutation produce and deposit 4-keto-carotenoids in their general integument (although the red coloration of the *Dr* segregants is caused by drospterins, not keto-carotenoids; Rempeters et al. 1981). This suggests that the capacity to convert xanthophylls into 4-keto-carotenoids could evolve in poeciliids, if it were selectively advantageous. Nonetheless, it remains plausible that the absence of 4-keto-carotenoids in guppies reflects a phylogenetic constraint.

However, if male guppies were under strong selection to use carotenoids absorbing at longer wavelengths than tunaxanthin, it would seem to be a small evolutionary step to deposit one or more of the periphyton carotenoids (beta-carotene, zeaxanthin, or lutein) directly into the skin. Each of these pigments can produce orange hues at lower concentrations than tunaxanthin, without the need for biochemical transformation. We found evidence of these pigments (or their metabolites) in the orange spots, but the "red shift" was very small in magnitude (about 2.8 nm; Fig. 6) and did not differ significantly between LCA and HCA streams. In short, the evidence that male guppies are under strong selection to put orange carotenoids in their spots is lacking. We must therefore consider other possible

reasons, besides phylogenetic constraints, for the use of tunaxanthin.

When combined with melanins, colorless pteridines, and structural pigments, tunaxanthin can produce the types of yellowish and greenish tones associated with concealment in shallow water. In *Sebastodes* rockfishes, for example, tunaxanthin predominates in the olive drab forms that live close to the substratum in shallow waters, whereas astaxanthin predominates in the forms that inhabit open waters (Crozier 1967). The difference in carotenoid usage in this group appears to be related to the contrasting spectral qualities of the two environments (Crozier 1967). Tunaxanthin may well contribute to background matching (crypsis) in female guppies, but this does not explain its presence in the orange spots of males, which in low predation streams (such as the ones used in this study) are thought to be under strong sexual selection for increased visibility (reviewed in Houde 1997).

It might be argued that tunaxanthin offers effective protection from UV light in the shallow streams inhabited by guppies because it absorbs shorter wavelengths than most other carotenoids. But although the concentration of carotenoids in the orange spots of males was highest in the better-lit streams, the concentration of carotenoids in the general integument did not differ between light environments—hardly an adaptation for increasing protection against UV irradiation (Grether et al. 1999). Furthermore, carotenoids do not appear to be particularly well suited to protect the integument from light irradiation compared with other classes of pigments present in fish, such as pteridines, purines, and melanins (Armstrong et al. 2000).

The key to the puzzle of why male guppies use tunaxanthin in their sexual display may lie in the eyes of female guppies. Our photoreceptor-based simulations suggest that if the total amount of pigment were held constant, males could achieve the highest levels of chroma and brightness by using tunaxanthin alone (Figs. 9, 10). In nature, the situation may be that carotenoids are available in limited supply, whereas drospterins can be produced at a cost. Our simulations suggest that fewer micrograms of drospterins would be needed to reach a given level of chroma if the ingested carotenoids were converted into tunaxanthin instead of into keto-carotenoids. Moreover, the inherent trade-off between chroma and brightness is less severe with tunaxanthin than it would be with astaxanthin. For a given level of chroma, a drospterin-tunaxanthin spot would be brighter, as perceived by female guppies, than a drospterin-astaxanthin spot. This discussion would not be complete, however, without a consideration of hue.

The wavelength composition or hue of a single pigment's reflectance spectrum depends on pigment concentration (Fig. 1c, 1d). The dual pigment system in guppies (i.e., yellow tunaxanthin and red drospterins) may have evolved as a mechanism for maintaining a roughly constant hue across (or within) environments varying in carotenoid availability. Indeed, dro-

sophterin production is greater in streams where carotenoid availability is greater (the opposite of what would be predicted if guppies were selected to achieve a given level of chroma; Grether et al 1999). This hypothesis presupposes some benefit of maintaining a particular hue. Guppies of both sexes are innately attracted to orange objects in preference to objects of other colors (including yellow and red) and the degree of attraction to orange is predictive of the strength of the female preference for orange coloration in males (Rodd et al. 2002). It remains to be determined which hues of orange are preferred by female guppies. We predict these are the hues achieved, on average, by male guppies in nature.

We previously suggested that drosophterins may have initially evolved in poeciliid fishes as 4-keto-carotenoid mimics (Grether et al. 2001a). Once drosophterins appeared, selection on chroma, brightness, or hue could subsequently have favored a switch from 4-keto-carotenoids to tunaxanthin. This admittedly speculative evolutionary scenario predicts that the disappearance of keto-carotenoids from the integument of poeciliids (or their ancestors) should coincide, on a phylogeny of the group, with the appearance of drosophterins.

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