

COUNTERGRADIENT VARIATION IN THE SEXUAL COLORATION OF GUPPIES (*POECILIA RETICULATA*): DROSOPTERIN SYNTHESIS BALANCES CAROTENOID AVAILABILITY

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Abstract.—Trinidad guppies (*Poecilia reticulata*) are distributed along an environmental gradient in carotenoid availability that limits the carotenoid content of the orange spots of males. The amount of synthetic red pteridines (drosopterins) in the orange spots covaries with the carotenoid content, such that the ratio of the two types of pigments is roughly conserved across streams. Carotenoids and drosopterins have different spectral properties and thus the ratio of the two types of pigments affects the shape of the orange spot reflectance spectrum. Geographic conservation of the carotenoid:drosopterin ratio suggests that males may be under selection to maintain a particular hue. We tested this hypothesis by comparing the pigmentation and coloration of guppies from six streams in the field to that of second-generation descendants of the same populations raised on three dietary carotenoid levels in the laboratory. The results show clearly that the geographic variation in drosopterin production is largely genetic and that the hue of the orange spots is conserved among populations in the field, relative to the laboratory diet groups. This is a countergradient pattern because genetic differences between populations in drosopterin production mask the effect of carotenoid availability on the hue of the orange spots. The potential for countergradient sexual selection to contribute to reproductive isolation between populations is discussed.

Key words.—Geographic variation, hue, mate choice, plasticity, pteridine, reaction norm, sensory bias, sexual selection.

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Countergradient variation is a geographic pattern in which genetic variation in a trait compensates for environmental variation, such that phenotypic variation among populations is minimized along an environmental gradient (reviewed in Conover and Schultz 1995). Most documented examples of countergradient variation involve the effects of altitude, latitude, or temperature gradients on growth rates (reviewed in Conover and Schultz 1995; see also Arendt and Wilson 1999; Skelly 2004). For example, larvae of the frog *Rana sylvatica* grow more rapidly at lowland sites than at high-elevation sites, but when larvae are raised under identical conditions in the laboratory the mountain larvae grow more rapidly than the lowland larvae (Berven 1982a, b). In this case, the genetic difference in growth rate, likely caused by past selection for rapid growth in the relatively short mountain growing season, only partially masks the influence of the environmental (temperature) gradient.

Secondary sexual characters are excellent candidates for countergradient variation because they tend to be environmentally sensitive and subject to intense selection, but there are surprisingly few documented examples (for one example, see Craig and Foote 2001). Countergradient patterns are inherently difficult to detect because the underlying genetic variation is cryptic. Thus, the paucity of examples may simply reflect a lack of awareness of the phenomenon and its potential importance. One of the most theoretically intriguing aspects of countergradient variation in general, and countergradient variation in secondary sexual characters in particular, is that it could lead to a cryptic form of reproductive isolation between populations in different environments (see Discussion).

In this paper, we document a pattern of countergradient

variation in the sexual coloration of guppies (*Poecilia reticulata*). Guppies are small (< 40 mm) neotropical fish that occur naturally in a wide range of environments, from lowland rivers to headwater streams (Haskins and Haskins 1950; Seghers 1974; Endler 1978). The polymorphic color patterns of males nearly always include orange spots, and females from most wild populations show a preference for males with larger and more chromatic orange spots (Endler 1983; Kodric-Brown 1985, 1989, 1993; Houde 1987, 1988; Houde and Endler 1990; Endler and Houde 1995; Jirotkul 1999; Grether 2000). The xanthophore layer of the orange spots contains two types of pigments: carotenoids, which guppies acquire mainly from consuming unicellular algae, and drosopterins, which the fish synthesize *de novo* from carbohydrates and amino acids (Takeuchi 1975; Grether et al. 1999, 2001a; Hudon et al. 2003). The carotenoid content of the orange spots is constrained by algae availability, which is closely linked to forest canopy cover. Streams that receive more photosynthetically active light contain larger standing crops of algae but not higher densities of guppies, than streams that receive less light (Grether et al. 2001b). Several lines of evidence indicate that guppies in low-light streams are carotenoid-limited (Grether et al. 1999).

Geographic variation in the carotenoid content of the orange spots is mirrored by variation in drosopterin content, such that the ratio of the two types of pigments is roughly conserved across streams (Grether et al. 2001a). This pattern is the opposite of what one would expect if males used drosopterins to compensate for the effects of variation in carotenoid availability on the chroma (color saturation) of the orange spots. The pattern is consistent, however, with the hypothesis that males are under selection to maximize chroma

subject to the constraint of maintaining a particular hue (Grether et al. 2001a). Carotenoids and drosoterins have different spectral properties and thus the ratio of the two types of pigments affects the shape of the reflectance spectrum (Grether et al. 2001a; Hudon et al. 2003). The positive correlation between drosoterin production and carotenoid intake in the field should, in theory, reduce geographic variation in the shape of the orange spot reflectance spectrum and hue, relative to the alternative of drosoterin production not correlating positively with carotenoid intake (Grether et al. 2001a). A female preference for hue has not yet been demonstrated, but guppies of both sexes are innately attracted to orange objects in preference to other colors (including yellow and red), and this sensory bias appears to be linked to the mate preference for orange coloration (Rodd et al. 2002). If drosoterin production has evolved along the carotenoid availability gradient in response to a hue-based female preference, this would be an example of countergradient variation caused by sexual selection (see also Craig and Foote 2001).

Here we test the countergradient sexual selection hypothesis by comparing the pigmentation and coloration of guppies from six streams in the field to that of second-generation descendants of the same populations raised on three dietary carotenoid levels in the laboratory. Data from this experiment enable us: (1) to determine whether the geographic variation in drosoterin production is largely genetic or environmental; (2) to evaluate, empirically, how carotenoids and drosoterins influence the color of the orange spots; and (3) to test the prediction that the hue of the orange spots is geographically conserved in the field relative to the laboratory diet groups.

MATERIALS AND METHODS

Study Populations

The six streams used in this study were selected to cover a range of variation in carotenoid availability while controlling for several potentially confounding factors (Grether et al. 1999, 2001b; Grether 2000). We chose one stream with relatively low carotenoid availability and another with relatively high carotenoid availability in each of three phylogenetically distinct river drainages (Marianne, Paria, Quare) in the Northern Mountains of Trinidad (for grid coordinates, see Grether et al. 2001b). This sampling design helps control for phylogenetic effects to the extent that populations within one drainage are closer to each other genetically than populations in different drainages, as would be expected from the dispersal mode of these fish. To eliminate anthropogenic disturbance and predation as potentially confounding factors, we selected streams in intact old-growth forest above waterfalls that exclude predatory fish except the minor predator *Rivulus hartii*. Previous work on the same streams has shown that in the streams with lower carotenoid availability, guppies ingest carotenoids at lower rates, males have less carotenoids in their orange spots, and there is a steeper trade-off between orange spot area and carotenoid concentration (Grether et al. 1999).

Carotenoid Diet Experiment

The fish used in this experiment were second-generation (G_2) laboratory descendants of fish collected from 10–20

pools in each of the six study streams. In total, 141 wild females contributed offspring to the G_1 generation (15–27 per population). Because females mate multiply in the wild and store viable sperm for up to 8 months (Winge 1937; Carvalho et al. 1996), the number of male founders was probably much larger. In the G_1 generation, the sexes were separated before sexual maturity. After sexual maturity, unrelated G_1 fish were paired to produce full-sib broods of outbred G_2 offspring. For detailed husbandry information, see Grether (2000).

The G_2 broods were raised from birth on a diet containing only trace amounts of carotenoids (see Grether 2000) until the fish could be sexed under a dissecting microscope (5–8 weeks of age, well before male color patterns developed). The males from each brood were divided up as evenly as possible among three 8-L plastic tanks (one to four males per tank) and fed one of three experimental diets (trace, low, or high carotenoid content). To control algae growth in the tanks, the water was treated with 2-chloro-4, 6-bis-(ethylamino)-s-triazine (Algae Destroyer, Aquarium Pharmaceuticals, Chalfont, PA) and changed weekly.

Of the nine carotenoids found in the algae of Trinidad streams (see Grether et al. 1999), only lutein, zeaxanthin, and β -carotene are likely to be usable by guppies for pigmentation the skin (Goodwin 1984; Hudon et al. 2003). The low- and high-carotenoid diets were designed to contain these three pigments, roughly in the same proportions as found in the algae (for a detailed recipe see Grether 2000). Initial high-performance liquid chromatography (HPLC) analyses of these diets (reported in Grether 2000) erroneously suggested that the lutein and zeaxanthin was almost completely degraded during processing of the food (leaving only β -carotene). Subsequent HPLC analyses showed that the diets actually contained all three of the usable pigments in roughly the intended proportions (in the initial HPLC analysis, lutein and zeaxanthin were confused with the spectrally similar pigments diatoxanthin and didinoxanthin; D. Millie, pers. comm.). Based on four replicate samples, the composition of the low-carotenoid diet (mean \pm SE $\mu\text{g g}^{-1}$) was 4.94 ± 0.93 lutein, 3.51 ± 0.10 zeaxanthin, 24.1 ± 9.87 β -carotene (32.5 ± 10.7 total carotenoids), and the composition of the high-carotenoid diet was 185.5 ± 26.6 lutein, 135.7 ± 29.3 zeaxanthin, 39.9 ± 51.0 β -carotene (861.2 ± 7.6 total carotenoids). The total carotenoid content of the trace-carotenoid diet was negligible ($<0.5 \mu\text{g g}^{-1}$).

The fish apparently obtained small amounts of carotenoids from algae, in spite of our efforts to eliminate algae growth in the tanks, because even the fish raised on the trace-carotenoid diet had measurable amounts of carotenoids in their skin. There is no reason to suspect, however, that the amount of carotenoids obtained from algae differed among treatment groups.

After reaching sexual maturity, three males from a given sibship, representing the three diet groups, were placed for 48 h in a tank with females (as part of a concurrent study on mate choice; Grether 2000), during which time they were only fed the trace-carotenoid diet. After the mate choice tests, the color patterns of all males in the sibship were measured and a subset of the males was collected for pigment analyses.

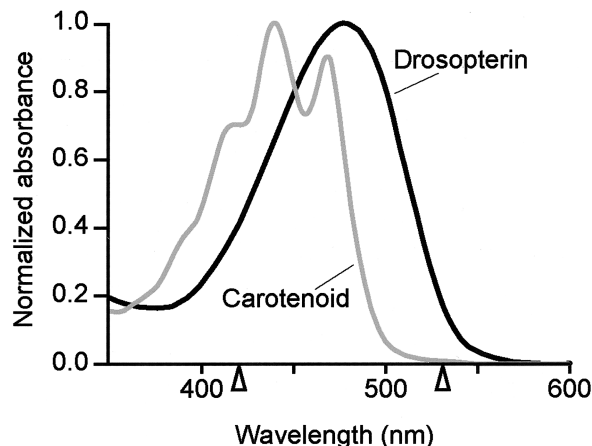


FIG. 1. Normalized absorbance spectra for drosopterins and the main carotenoid in guppy skin (tunaxanthin; modified from fig. 2a in Hudon et al. 2003). Triangles mark the wavelengths used in the simple reflectance ratio (see text).

Color Measurements

Sampling protocol

Color measurements were taken on 231 males in the field (36–41 per stream) and 361 males in the laboratory (17–24 per stream-by-diet combination). In the field, the fish were captured with butterfly nets, transported to our field laboratory in water treated with antibiotics (Fungus Guard, Jungle Products, Jungle Laboratories Corp., Cibolo, TX), stress reducers (Novaqua, Kordon, Novalek Inc., Hayward, CA), and a NH_3 -detoxifier (Amquel, Kordon), and allowed to recover from the stress of capture in 40-L aquaria for ≥ 3 h before the color measurements. In the diet experiment, color measurements were taken shortly after the mate choice tests.

The color measurements involved sedating a male with ethyl 3-aminobenzoate methane sulfonic acid salt (MS-222), photographing both sides his body using a 35-mm camera equipped with a 1:1 macro lens, and then immediately measuring the reflectance spectrum of each orange spot with an Ocean Optics (Dunedin, FL) PS-1000 spectrometer. The area of the orange spots was later measured with a digitizing tablet (for further details see Grether 2000).

Estimation of Color Parameters

The primary effects of carotenoids and drosopterins on reflectance spectra are similar and straightforward. Both types of pigments preferentially absorb short-wave light and thus increase wavelength-biased reflectance (chroma) while reducing total reflectance (brightness). For completeness, we demonstrate both of these effects (see Results). However, our main interest here is to examine color parameters that carotenoids and drosopterins affect in opposite ways, owing to their different absorbance properties (Fig. 1). The most relevant color parameters to analyze would, of course, be those that guppies themselves perceive. Unfortunately, our understanding of the visual system of this species (or any other for that matter) has not advanced to the point where a single color estimation method is clearly superior. For this reason, we used several different techniques. Before describing these

techniques, we provide a brief explanation of basic color terminology (for a more comprehensive explanation see Bradbury and Vehrencamp 1998).

Hue, chroma, and brightness technically refer to how a color stimulus is perceived, not physical properties of the reflectance spectrum, although in practice this distinction is often blurred. To human eyes, reflectance spectra with the same overall shape can vary in brightness and chroma (also known as color saturation) without changing in hue. Hue refers to what we perceive as different categories of color. For instance, a human observer would classify any reflectance spectrum with a unimodal peak at 530 nm as green. Variation in the height of the peak relative to the baseline would be perceived as variation in chroma (pale vs. saturated green), while variation in the height of the whole spectrum would be perceived as variation in brightness (bright vs. dark green).

The techniques that we used to examine the effects of xanthophore pigments (i.e., carotenoids and drosopterins) on the color of the orange spots fall into three categories: (1) physical properties of reflectance spectra; (2) segment classification; and (3) guppy-specific estimates based on cone inputs.

Physical properties of reflectance spectra

Simple reflectance ratio.—The wavelength of peak absorption (λ_{max}) of the carotenoids in the orange spots (ca. 440 nm) lies about 37 nm below that of the drosopterins (ca. 477 nm; Fig. 1). Thus, it should be possible to distinguish the effects of these two classes of pigments on the reflectance spectrum by comparing reflectance at different wavelengths (i.e., below and above the two absorbance peaks). We used the ratio of reflectance at 420 nm to that at 530 nm. The exact choice of wavelengths was arbitrary; other similar ratios (e.g., 410 nm vs. 540 nm) yielded similar results (not shown).

Spectrum correlations.—To determine whether the overall shape of the reflectance spectrum was more similar in the field than when the fish were raised on arbitrary carotenoid levels in the laboratory, we calculated the correlation between pairs of reflectance spectra and compared the mean correlation for different groups (see Fig. 7). Groups in which reflectance spectra are more similar in shape will yield greater mean correlations (Endler 1984). We calculated the mean reflectance spectrum, at 10-nm intervals, separately for each population by diet combination (six mean reflectance spectra for the field, 18 for the lab, 24 in total). We then computed all possible pairwise correlations between the mean reflectance spectra (276 in total) and calculated a mean correlation within each of the four diet groups (i.e., three laboratory diets and field; $N = 15$ correlations per group). To determine whether the observed mean correlations were significantly larger than would be expected under the null hypothesis that diet group membership is unimportant, we used a bootstrap algorithm that sampled 15 correlations at random (with replacement) and calculated the mean correlation. This was repeated 10,000 times and then the observed mean correlation for each diet group was compared to the bootstrapped distribution of mean correlations. *P*-values were computed as the frequency of bootstrap means of the same magnitude or larger than the observed means.

Segment classification

Endler (1990) devised a color space model based on contrasts between four nonoverlapping wavelength intervals (segments). This model provides nonspecies specific estimates of hue and chroma that, to a first approximation, correspond fairly well with human color perception (Endler 1990). For 400–700 nm reflectance spectra, the x -axis of this color space is defined as the proportion of total reflectance over the interval 625–700 nm minus the proportion of reflectance over the interval 475–550 nm. The y -axis is defined as the proportion of reflectance over the interval 550–625 nm minus the proportion of reflectance over 400–475 nm. Hue is a circular statistic (i.e., measured in radians or degrees), defined as the angle from the positive y -axis, clockwise (Endler 1990); chroma is the Euclidian distance of a colored stimulus from the achromatic center of color space.

To compare the interpopulation variation in hue observed in the field to that observed in the laboratory diet groups, we calculated the angular deviation among population mean hues for each group separately and used a bootstrap algorithm to determine whether the observed angular deviations were significantly smaller than would be expected under the null hypothesis that group membership is unimportant. The bootstrap algorithm involved taking random samples of six mean hues, with replacement, from the sample of 24 observed mean hues (one for each population-by-diet group combination) and then calculating the angular deviation for the sample. This was repeated 10,000 times and then the observed angular deviation for each diet group was compared to the bootstrapped distribution of angular deviations. P -values were computed as the frequency of bootstrap angular deviations of the same magnitude or smaller than the observed angular deviations.

Guppy-specific estimates

Basic calculations.—Cone input (quantum catch) estimates are the starting point for making species-specific estimates of color perception. Following Endler (1991), we estimated the photon catch for each cone class from:

$$P_j = \int_{\lambda} Q(\lambda) S_j(\lambda) d\lambda, \quad (1)$$

where $Q(\lambda)$ is the color patch radiance in units proportional to photon flux, $S_j(\lambda)$ is the spectral sensitivity (absorbance) function of photoreceptor class j , P_j is in units proportional to photons, and the integration was done over the wavelengths 400–700 nm. To estimate $Q(\lambda)$, the ambient irradiance spectrum illuminating the colour patch, $I(\lambda)$, must be specified. For the results presented here, we used Endler's (1993) open-cloudy irradiance spectrum, which is a natural forest light spectrum expected under open canopy or cloudy conditions. All of the results reported below were essentially the same for the other four forest light environments described by Endler (1993; results omitted for brevity). The amount of light of a particular wavelength (λ) reaching the receiver's eyes from a color patch is given by:

$$Q(\lambda) = I(\lambda)R(\lambda)T(\lambda), \quad (2)$$

where $Q(\lambda)$ is the patch radiance, in units proportional to photon flux, $R(\lambda)$ is the proportion of incident light of wavelength λ that is reflected by the color patch, and $T(\lambda)$ is the proportion of light of wavelength λ that is transmitted through the water to the receiver's eyes. We used a transmission spectrum for clear water and assumed an absence of veiling light, which approximates the situation under close viewing conditions (see Endler 1991). Absorbance functions ($S_j[\lambda]$) 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 table 11.1 in Nicol and Somiya 1989).

Although guppy cones are responsive to light wavelengths below 400 nm, the halogen light source used in this study did not permit reflectance measurements below 400 nm. Given the absorption properties of carotenoids and drospterins, and in view of the goals of this paper, we do not believe that this is a serious limitation.

Simple cone contrasts.—Guppies should be able to detect variation in the relative amounts of drospterins and carotenoids in the orange spots by comparing the outputs of photoreceptor cones with different peak spectral sensitivities. To calculate guppy-specific cone contrasts, we used two different methods, which we refer to as the cone catch (CC) and cone excitation (CE) methods. The key difference between the methods is that CC contrasts are insensitive to the overall brightness of a stimulus, while CE contrasts first increase and then decrease as the brightness of a stimulus increases relative to the adaptation light (reviewed in Chittka 1992; Grether et al. 2004). CC contrasts were estimated from:

$$D_{jk} = (w_j P_j - w_k P_k) / (w_j P_j + w_k P_k), \quad (3)$$

where w_j and w_k are constants corresponding to how the visual system weighs the output of the different cone classes, and P_j and P_k are the quantum catch estimates for cone classes j and k , respectively (Endler 1991). The w values for guppy cones are unknown and were set to 1.0 for our calculations, following Endler (1991).

Cone excitations were estimated from:

$$E_j = (K_j P_j)^n / [(K_j P_j)^n + 1], \quad (4)$$

where E_j is the excitation level of cone class j , expressed as a proportion of the maximum receptor voltage, and K_j is the reciprocal of the photon catch required to produce half-maximal excitation in cones of class j (Chittka 1992). The value of K_j is assumed to depend on the light spectrum to which the receiver's eyes are adapted, such that all cones reach half-maximal excitation when the receiver's eyes are illuminated by the adaptation light (Laughlin 1981). For the results presented here, we assumed that the illumination light was the same as the adaptation light, which is realistic for the conditions under which guppies normally view each other. The exponents n are photoreceptor and species specific and generally unknown; following Chittka (1992), we set these exponents to 1.0 (but see Vorobyev 1999). CE contrasts were calculated as the difference between the cone excitation values of different cone classes.

With both methods, contrasts between the m cone and the s and uv cones were about equally effective, and better than

the alternatives, for detecting variation in the drosopterin:carotenoid ratio. Because a halogen light source was used for the reflectance measurements, we consider the *s* cone catch estimates to be more reliable than the *uv* cone catch estimates. Therefore, for brevity, only the *m* versus *s* cone contrasts are presented below.

Cone catch brightness and chroma.—In the absence of information on how the visual system compares or weighs the outputs of different cone classes, the sum of all cone catches ($\sum_i P_i$) provides a unbiased estimate of perceived brightness, and the maximum cone contrast (D_{\max}) provides a generalized measure of chroma (Endler 1990). In our dataset of orange spot reflectance spectra, the maximum cone contrast was always between the *l* cone and the *uv* cone, but since our *uv* cone catch estimates were truncated by the use of a halogen light source, we used the contrast between *l* and *s* cones (D_{ls}) as the estimate of D_{\max} . One drawback of using D_{ls} as a measure of chroma is that it leaves out the effects of the xanthophore pigments on the *m* cone (the λ_{\max} of which lies below the drosopterin λ_{\max}). We therefore also calculated a three-cone measure of chroma:

$$D_{lms} = (P_l - P_m - P_s)/(P_l + P_m + P_s). \quad (5)$$

Trichromatic hue.—The concept of hue as an angle in color space has been applied to color space models based on psychophysical studies of humans (Wysecki and Stiles 1982), as well as the cone outputs of nonhuman animals where behavioral data is more difficult to obtain (Goldsmith 1990; Vorobyev et al. 1998). We used a color space model developed by Chittka (1992) based on cone excitations for animals with trichromatic vision (three cone types). Trichromatic color space is a two-dimensional hexagon defined by all possible combinations of three cone excitations. Achromatic spectra (defined as spectra that excite all cone types equally), map to the geometric center of the diagram. To locate a colored stimulus in color space, cone excitation values must be converted into *x*- and *y*-coordinates (using equations in Chittka 1992). Angles between the stimulus and the color space axes can be interpreted as measures of hue. One angle is sufficient to describe the hue vector along which a colored stimulus lies in trichromatic color space.

To compare the interpopulation variation in hue observed in the field to that observed in the laboratory diet groups, we used the same bootstrapping approach as described above for segment classification hue.

Pigment Analysis

We quantified pigments in the orange spots of 90 wild-caught males (15 per stream) and 218 diet experiment males (11–14 per stream by diet combination). Drosopterin and carotenoid content of the orange spots were determined from the absorbance spectra of ethanol and hexane extracts, respectively (for further details, see Grether et al. 1999, 2001a; Hudon et al. 2003).

The color produced by a pigment is directly related to its transmission spectrum, which can be obtained from the absorbance spectrum using the standard equation:

$$T_i(\lambda) = 10^{-A_i(\lambda)}, \quad (6)$$

where $A_i(\lambda)$ is the absorbance at wavelength λ for pigment type *i*. In our analyses of the effects of pigments on color parameters, we used transmittance at the wavelength of maximum absorbance of the pigments ($T_c[\lambda_{\max}]$ and $T_d[\lambda_{\max}]$) as predictor variables. The ratio of the pigment transmittance values, which can be calculated from

$$T_c(\lambda_{\max})/T_d(\lambda_{\max}) = 10^{A_d(\lambda_{\max})-A_c(\lambda_{\max})}, \quad (7)$$

provides an appropriately-transformed measure of variation in the drosopterin:carotenoid ratio. Note that this ratio increases as the drosopterin content of the spots increases relative to the carotenoid content.

To compare the interpopulation variation in the pigment transmittance ratio observed in the field to that observed in the laboratory diet groups, we calculated the variance of population means for each group separately and used a bootstrap algorithm to determine whether the observed interpopulation variances were significantly smaller than would be expected under the null hypothesis that group membership is unimportant. The bootstrap algorithm involved taking random samples of six mean transmittance ratios, with replacement, from the sample of 24 observed mean transmittance ratios (one for each population-by-diet group combination) and then calculating the variance for the sample. This was repeated 10,000 times and then the observed interpopulation variance for each diet group was compared to the bootstrapped distribution of interpopulation variances. *P*-values were computed as the frequency of bootstrap interpopulation variances of the same magnitude or smaller than the observed interpopulation variances.

Quantitative Genetic Analysis of Variation in Drosopterin Production

To examine the sources of variation in drosopterin production, we constructed ANOVAs with population (stream of origin), diet (carotenoid level), and their interaction as fixed effects terms and sibship nested within population as a random effects term. We estimated the broad-sense heritability of drosopterin production from the intraclass correlation divided by the relatedness between full-sibs (0.5). Intraclass correlations were calculated for each population separately, and for all populations pooled, using one-way ANOVAs with sibship as a random effects term. Heritabilities calculated in this way include nonadditive genetic (e.g., dominance) effects as well as common environment (e.g., maternal) effects, and therefore only set an upper limit to the proportion of additive genetic variation in a trait (Falconer 1989). The dependent variables for these analyses, orange spot drosopterin mass (absorbance) and drosopterin concentration (absorbance mm^{-2}), were log-transformed to meet parametric assumptions after being multiplied by 10^3 and 10^4 , respectively, to avoid negative log values.

Statistical Methods

Multiple regression, with the pigment transmittances ($T_c[\lambda_{\max}]$, $T_d[\lambda_{\max}]$) as the independent variables, was used to examine the separate effects of carotenoids and drosopterins on color parameters. Mean reflectance and total cone catch required a \log_{10} -transformation to meet parametric as-

TABLE 1. Analysis of variation in drosopterin production and orange spot area. Note that the direction of the carotenoid diet effect was negative. See text for further details.

Source of variation	Drosopterin mass (log absorbance)		Drosopterin concentration (log absorbance mm ⁻²)		Orange spot area (mm ²)		df ¹
	F	P	F	P	F	P	
Population	82.79	<0.0001	50.67	<0.0001	20.37	<0.0001	5, 70–71
Carotenoid diet	2.58	0.080	4.08	0.019	0.12	0.883	2, 130
Population × diet	1.70	0.087	1.39	0.190	0.55	0.848	10, 130
Sibship (population)	3.27	<0.0001	3.28	<0.0001	4.59	<0.0001	70, 130
Model	12.49	<0.0001	18.31	<0.0001	9.09	<0.0001	87, 130
R ²		0.92		0.89		0.86	

¹ Approximate degrees of freedom for population were calculated using the Satterthwaite method (Satterthwaite 1946).

sumptions. None of the other variables required transformation. Population (stream of origin) was entered in the models as a fixed effect term to determine whether additional variation in the dependent variables could be attributed to geographically variable factors other than xanthophore pigments. Data from wild-caught and diet experiment fish were pooled for these analyses.

All statistical analyses were carried out using Statview 5.0 (SAS Institute, Inc., Cary, NC), JMP 3.2 (SAS Institute), or Resampling Stats 4.0 (Resampling Stats, Inc., Arlington, VA) on a G4 Macintosh (Apple Computer Inc., Cupertino, CA).

RESULTS

Sources of Variation in Drosopterin Production

In the laboratory, orange spot drosopterin mass and concentration varied significantly among populations and among sibships (Table 1). Because the males from different populations were raised under identical conditions, we can infer from these results that the populations have diverged genetically in drosopterin production. Moreover, as shown in Figure 2B, the mean drosopterin level of a given population in the laboratory was strongly predictive of that in the field. Clearly, most of the variation in drosopterin production among guppy populations in this study is genetic.

The significant sibship term (Table 1) indicates that drosopterin production is genetically variable within populations. The broad-sense heritability (h_b^2) estimates were highly significant for some populations and nonsignificant for others (Table 2). Some of the h_b^2 estimates exceeded 1.0, which may indicate the influence of nonadditive genetic or common environment (e.g., maternal) effects. Alternatively, the high h_b^2 values may be due to sex-linked inheritance of genes affecting drosopterin production (genes that control integumentary coloration are known to cluster on the heterogametic Y chromosome in some guppy populations; Winge 1927; Houde 1997; Brooks and Endler 2001).

Carotenoid diet had a marginally nonsignificant effect on orange spot drosopterin mass (\log_{10} absorbance) and a small but significant effect on drosopterin concentration (\log_{10} absorbance mm⁻²; Table 1). The latter result could not be attributed to an effect of carotenoid diet on the area of the orange spots, because no such effect was observed (Table 1). The relationship between carotenoid levels and drosopterin concentration was negative (i.e., in the opposite direction to the geographic pattern). Males raised on the trace carotenoid

diet had slightly higher concentrations of drosopterins in their orange spots (least square mean \pm SE of log absorbance mm⁻² $\times 10^4$ 1.992 \pm 0.015) than did males raised on the other two diets (low carotenoid: 1.941 \pm 0.015; high carotenoid 1.936 \pm 0.015). In post hoc pairwise comparisons, the difference between the trace and low carotenoid diet groups was marginally nonsignificant (Bonferroni $P = 0.06$) and the difference between the trace and high carotenoid diet groups was marginally significant ($P = 0.033$). These diet-related differences were trivial, however, in comparison to the population and sibship differences. The R^2 for the full model was 0.92 for drosopterin mass and 0.89 for drosopterin concentration (Table 1). Removing both carotenoid diet terms from the model reduced these R^2 values to 0.91 and 0.88. In contrast, leaving carotenoid diet in the model and removing the other terms reduced the R^2 values to 0.004 and 0.01. Thus, the level of carotenoids in the laboratory diets explained at most 1% of the variation in drosopterin content.

As an additional test of whether drosopterin production is linked metabolically to carotenoid deposition, we examined the within-population correlations between the drosopterin and carotenoid contents of the orange spots of the laboratory-raised fish. The correlations ranged from -0.03 to 0.33 (mean \pm SE: 0.11 ± 0.06) for log pigment mass and from -0.11 to 0.26 (mean \pm SE: 0.05 ± 0.05) for log pigment concentration (range of samples sizes: 34–41). Only one of the 12 correlations (the pigment mass correlation for the Quare HCA population) was large enough ($r = 0.33$, $n = 41$) to be significant at an alpha level of 0.1 (with no correction for multiple tests). A comparable analysis of the field data yielded similar results (Grether et al. 2001a).

Genetic Versus Environmental Covariance

If drosopterin production has evolved to compensate for variation in carotenoid deposition in the orange spots, then drosopterin production should be tuned to match the combined effects of carotenoid availability in the field and the population-specific functions relating carotenoid availability to carotenoid deposition. With our sample of six populations, it is not possible to partition the variation among populations in drosopterin production into genetic and environmental carotenoid covariance terms. However, it is possible to measure the effects of these covariances indirectly. Variation among populations in carotenoid deposition within the laboratory diet groups provides a measure of the genetic component of

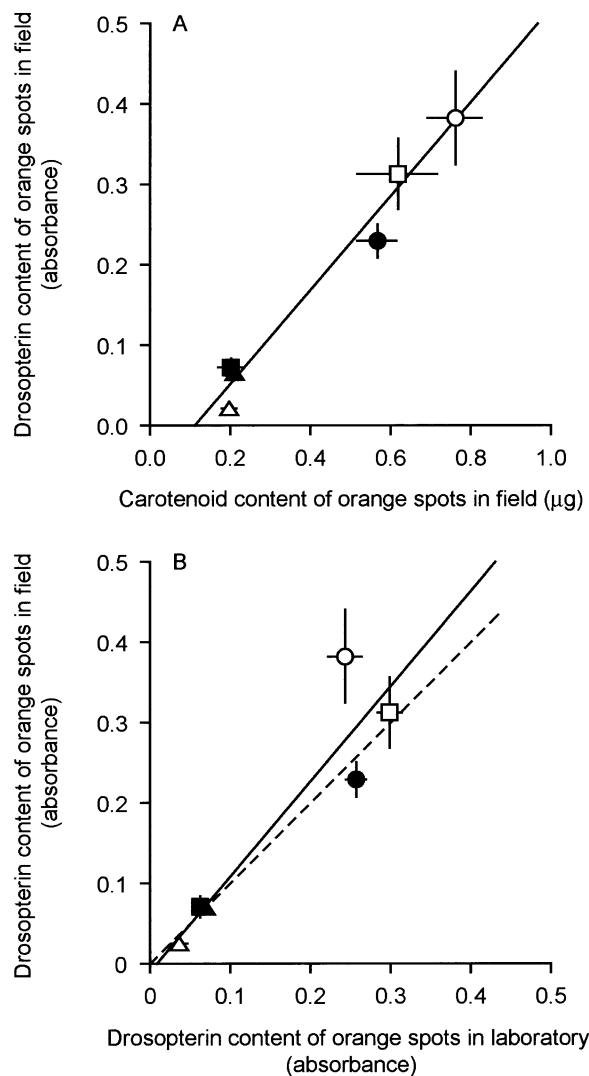


FIG. 2. Orange spot drosoperin content in the field versus (A) carotenoid content in the field and (B) drosoperin content in the laboratory for six guppy populations. Squares, circles, and triangles represent the Marianne, Paria, and Quare drainage basins, respectively, with filled symbols for low-carotenoid-availability streams and open symbols for high-carotenoid-availability streams. Points represent population means. Standard errors are shown if larger than the corresponding symbols ($N = 15$ males per population in the field and 35–41 males per population in the laboratory). The solid lines are from least-squares regression (A: $y = -0.065 + 0.58x$, $R^2 = 0.97$, $N = 6$, $P = 0.0002$; B: $y = -0.009 + 1.18x$, $R^2 = 0.85$, $N = 6$, $P = 0.0084$) and the dashed in (B) line depicts $y = x$. Laboratory diet groups were combined to calculate the overall laboratory mean drosoperin mass for each population; separate analyses by diet group yielded very similar results (not shown). Panel (A) is adapted from Grether et al. (2001a).

variation, while variation among populations in carotenoid deposition in the field includes both components of variation. Therefore, if drosoperin production is only linked to the genetic component of carotenoid deposition, then the drosoperin content of the orange spots should be more strongly correlated with orange spot carotenoid content in the laboratory diet groups than with orange spot carotenoid content in the field. Conversely, if drosoperin production is tuned

to match the combined effects of carotenoid availability and the genetic component of carotenoid deposition, then the reverse should be true, that is, drosoperin production should be most strongly correlated with orange spot carotenoid content in the field. As shown in Table 3, the latter prediction was upheld. The drosoperin content of the orange spots in both the field and in the laboratory correlated better with orange spot carotenoid content in the field than with orange spot carotenoid content in the laboratory diet groups. This pattern of correlations is unlikely to have arisen by chance (one-way ANOVA on the correlations in Table 3, with the diet group for carotenoid content as the categorical variable: $F_{3,12} = 27.42$, $P < 0.0001$; planned comparison between the field and all three laboratory diet groups combined: $t = 5.67$, $P = 0.0001$). These results strongly suggest that drosoperin production is tuned to carotenoid availability in the field, not just to the genetic component of carotenoid deposition.

Effects of Pigments on Color

Reflectance spectra

Given that drosoperin production was only slightly affected by carotenoid intake, the influence of carotenoids on the color of the orange spots can be inferred from the reflectance spectra of fish raised on different dietary carotenoid levels (Fig. 3). In all six populations, carotenoids sharply reduced reflectance below 450 nm but had relatively little effect above 500 nm. This result is consistent with the absorbance properties of the carotenoids in the skin of guppies (Fig. 1). The influence of drosoperins on the color of the orange spots can roughly be inferred by comparing the trace carotenoid reflectance spectra for different populations, although populations may also differ in other color patch components (e.g., iridophores, melanophores).

Spectral shape variables

As expected, carotenoids and drosoperins had opposing effects on the spectral shape variables, as shown by the opposite signs of the multiple regression coefficients (Table 4). Together, the two pigment transmittance variables explained about 50% of the variation in the 420:530 nm reflectance ratio (Table 4). The R^2_{adj} increased to 54% when population was included as a fixed effect term in the model (population term $F_{5,298} = 5.79$, $P < 0.0001$), indicating that a small percentage of the geographic variation in the reflectance ratio might be caused by factors other than xanthophore pigments. Pigment transmittances explained 32–38% of the variation in the m versus s cone contrasts (Table 4). Another 3–6% of the variation was explained by including a population term in the models (both population terms $F_{5,298} > 5.1$, $P \leq 0.0001$), which suggests that the orange spots of males from different populations might differ somewhat in spectral shape even if matched for carotenoid and drosoperin content.

Figure 4, which is essentially a countergradient variation diagram (Conover and Schultz 1995), shows that drosoperins and carotenoids have counterbalancing effects on orange spot spectral shape. The observed uniformity in orange spot spectral shape across populations results from a quantitative match between the two types of pigments. (Note that the

TABLE 2. Drosoperin heritability estimates for six Trinidad guppy populations. Field carotenoid availability categories of the streams are based on Grether et al. (1999).

Drainage	Carotenoid availability in the field	Orange spot drosoperin mass (log absorbance)			Orange spot drosoperin concentration (log absorbance mm ⁻²)			df
		h_b^2	F	P	h_b^2	F	P	
Marianne	high	0.58	2.18	0.056	0.65	2.40	0.037	11, 34
	low	-0.06	0.92	0.539	0.69	2.55	0.028	11, 34
Paria	high	1.55	10.74	<0.0001	1.29	6.18	0.0002	11, 33
	low	0.41	1.75	0.124	0.46	1.86	0.101	11, 34
Quare	high	1.05	4.25	0.0007	0.92	3.50	0.003	13, 40
	low	0.67	2.36	0.033	0.75	2.63	0.019	13, 37
All streams pooled		1.73	19.65	<0.0001	1.62	13.34	<0.0001	75, 217

direction of the effects of the pigments shown in Figure 4 are opposite to those shown in Table 4 because pigment transmittance decreases as pigment content increases.)

Spectral Shape and Hue Convergence

The drosoperin:carotenoid ratio of the orange spots appeared to converge among populations in the field, relative to the laboratory carotenoid diet groups (Fig. 5), and this was confirmed statistically. The observed variance in the pigment transmittance ratio among populations in the field was significantly less than expected by chance (expected $\sigma^2 = 0.118$; observed $\sigma^2 = 0.006$; bootstrap $P = 0.0115$). None of the observed variances among populations in the laboratory were significantly smaller than expected (trace-carotenoid diet $\sigma^2 = 0.160$, $P = 0.69$; low-carotenoid diet $\sigma^2 = 0.025$; $P = 0.15$; high-carotenoid diet $\sigma^2 = 0.028$; $P = 0.18$).

All of the measures of spectral shape and hue that we estimated from reflectance data showed the same pattern of reduced variability in the field. For brevity, we report results only for a subset of those variables. The similarity in overall shape among reflectance spectra in the field, compared to the laboratory diet groups, can be seen in Figure 6. The mean correlation between mean field spectra was significantly greater than expected by chance (expected mean $r = 0.63$; observed mean $r = 0.94$; bootstrap $P < 0.0001$). None of the mean correlations for the laboratory diet groups exceeded chance expectations (trace-carotenoid diet, mean $r = 0.61$, $P = 0.60$; low-carotenoid diet, mean $r = 0.62$, $P = 0.56$; high-carotenoid diet, mean $r = 0.77$, $P = 0.06$).

Angles describing the hue vectors of orange spots in trichromatic cone excitation color space were less variable in the field than in the laboratory diet groups (Fig. 7). The

angular deviation (d) among population mean hue angles was significantly less than expected by chance in the field (expected $d = 38.17$; observed $d = 7.96$; bootstrap $P = 0.0073$) and not in the laboratory diet groups (trace-carotenoid diet $d = 30.16$, $P = 0.18$; low-carotenoid diet $d = 38.44$, $P = 0.45$; high-carotenoid diet $d = 30.98$, $P = 0.20$).

Segment classification hue also varied less among populations in the field than among the same populations in the laboratory diet groups. The angular deviation (d) among mean hues was significantly less than expected by chance in the field (expected $d = 30.72$; observed $d = 4.8$; bootstrap $P = 0.0167$) and not in the laboratory diet groups (trace-carotenoid diet $d = 45.8$, $P = 0.83$; low-carotenoid diet $d = 34.0$, $P = 0.49$; high-carotenoid diet $d = 10.9$, $P = 0.16$).

Brightness and chroma

The multiple regression analysis confirmed that both types of xanthophore pigments decrease brightness and increase chroma of the orange spots (Table 5). Which pigment had the largest influence depended on the color parameter. Drosoperins had the dominant influence on brightness, as measured either as mean reflectance (over 400–700 nm) or total cone catch. Carotenoids had the dominant influence on the l versus s cone contrast (D_{ls}), while drosoperins dominated the other two measures of chroma (D_{lms} and segment classification chroma). When population was added to the models, the R_{adj}^2 for mean reflectance and total cone catch increased by 11.5% and 11.3%, respectively and the R_{adj}^2 for the chroma measures increased by 6.7–7.8% (all population terms $F_{5,298} > 12.0$, $P < 0.0001$). This suggests that some of the geographic variation in brightness and chroma is caused by factors other than xanthophore pigments (e.g., iridophores, melanophores).

As could be deduced from the results presented above, the mean orange spot chroma in the field increased and the mean orange spot brightness decreased, as carotenoid availability in the field increased (Fig. 8). Neither the chroma nor the brightness of the orange spots was significantly more or less variable among populations in the field than expected under the null hypothesis of equally variability in the field and laboratory diet groups (D_{ls} : expected $\sigma^2 = 0.013$, field $\sigma^2 = 0.007$, two-tailed bootstrap $P = 0.48$; $\Sigma_i P_i$: expected $\sigma^2 = 0.032$, field $\sigma^2 = 0.021$, $P = 0.54$; similar results were obtained for D_{lms}). Likewise, none of the interpopulation variances in chroma or brightness of the laboratory diet groups

TABLE 3. Interpopulation correlations between orange spot carotenoid content (μg) and orange spot drosoperin content (absorbance) for $N = 6$ populations. Trace, low, and high refer to the laboratory carotenoid diet groups; field refers to the sample of fish collected in the field. See text for further details.

Diet group for carotenoid content	Diet group for drosoperin content			
	Trace	Low	High	Field
Trace	0.74	0.76	0.71	0.69
Low	0.90	0.92	0.87	0.89
High	0.88	0.90	0.86	0.79
Field	0.94	0.95	0.90	0.99

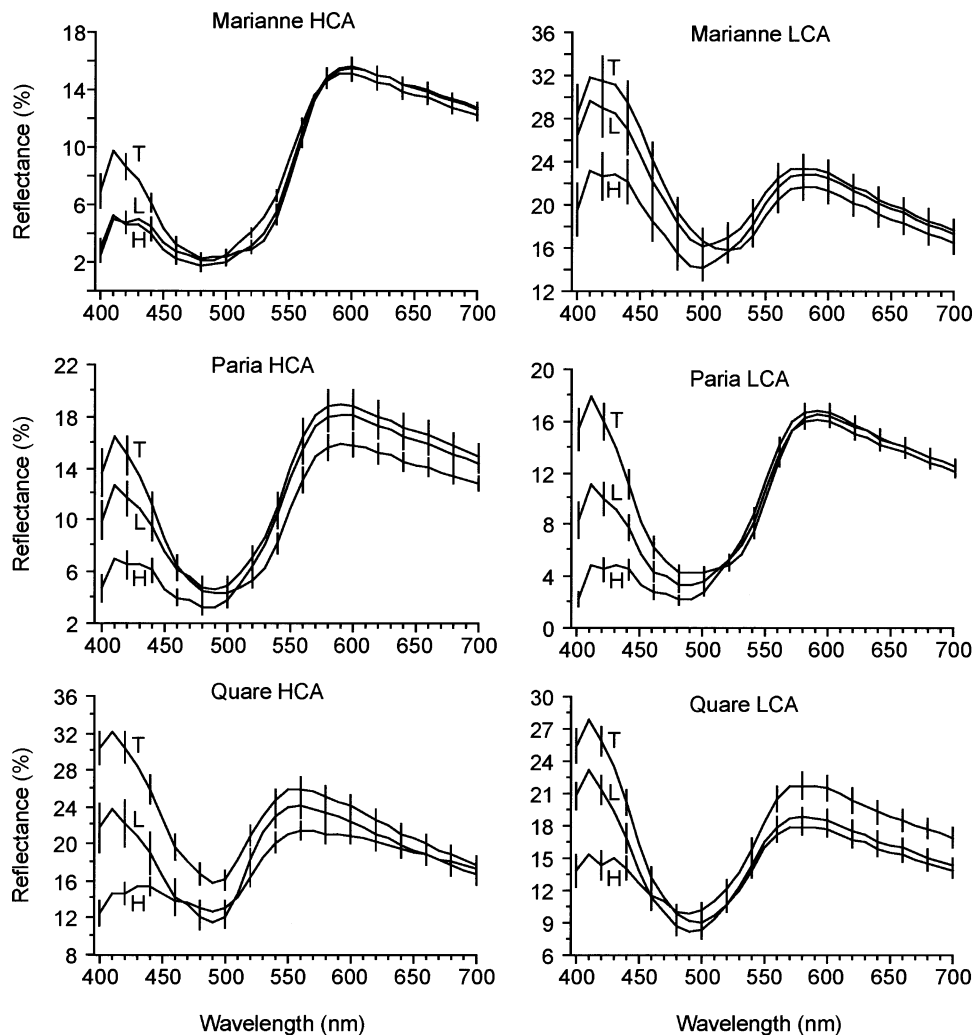


FIG. 3. Orange spot reflectance spectra of guppies in the laboratory, grouped by population. The plotted values indicate the mean reflectance at 10-nm intervals; standard error bars are shown on every other mean. Letters indicate the carotenoid diet level corresponding to the nearest reflectance curve (T, trace; L, low; H, high). Marianne, Paria, and Quare are the names of the river basins. LCA (low) and HCA (high) indicate the field carotenoid availability category of the population.

deviated significantly from the null expectation (D_{ls} : trace $\sigma^2 = 0.005$, $P = 0.30$; low $\sigma^2 = 0.010$, $P = 0.78$; high $\sigma^2 = 0.010$, $P = 0.78$; $\sum_i P_i$: trace $\sigma^2 = 0.051$, $P = 0.18$; low $\sigma^2 = 0.042$, $P = 0.48$; high $\sigma^2 = 0.033$, $P = 0.90$; similar results were obtained for D_{lms}).

DISCUSSION

The results of our common-garden experiment confirmed that the spectral shape and hue of the sexually selected orange spots of males guppies was conserved among populations in the field, relative to the same populations raised on fixed levels of carotenoids in the laboratory (Figs. 6, 7), and that this pattern is a direct consequence of the geographic covariation between drosopterin production and carotenoid deposition (Fig. 4). At the proximate level, geographic covariation between drosopterin production and carotenoid deposition could be caused by genetic divergence between populations in drosopterin synthesis rates, a reaction norm linking drosopterin synthesis to carotenoid availability, or a

combination of these two mechanisms. Our results show clearly that most of the geographic variation in drosopterin production is genetic. Orange spot drosopterin production varied significantly among populations and among sibships (Table 1), and the mean drosopterin level of a given population in the laboratory was strongly predictive of that in the field (Fig. 2). We have no evidence for a positive effect of carotenoid intake on drosopterin production within individual fish. If anything, carotenoid intake had a small negative effect on drosopterin production (see Results). Our results suggest that drosopterin production is highly heritable, at least in some guppy populations, and therefore should respond rapidly to selection.

Chance historical factors, such as genetic drift, are unlikely to explain the observed geographic pattern because our sampling design controlled for phylogeny (see first paragraph of Materials and Methods). We are aware of three plausible selection-based hypotheses. First, metabolic costs of drosopterin synthesis may favor reduced investment in drosopterin

TABLE 4. Multiple regression analysis of the effects of carotenoids and drospterins on three measures of orange spot spectral shape. The $b_i \pm SE$ are regression coefficients and standard errors. $N = 306$ fish. CC, cone catch; CE, cone excitation. Note that pigment transmittance decreases as the pigment content of the orange spots increases. For further details, see Materials and Methods.

Variable	Drospterin Transmittance (λ_{max})			Carotenoid Transmittance (λ_{max})			R^2_{adj}
	$b_1 \pm SE$	$F_{1,303}$	P	$b_2 \pm SE$	$F_{1,303}$	P	
Reflectance ratio (420/530)	-2.30 ± 0.20	130.77	<0.0001	3.04 ± 0.17	306.41	<0.0001	0.50
CC contrast (D_{ms})	0.28 ± 0.04	61.79	<0.0001	-0.42 ± 0.03	185.28	<0.0001	0.38
CE contrast ($E_m - E_s$)	0.02 ± 0.01	6.83	0.0094	-0.06 ± 0.01	125.74	<0.0001	0.32

production in the low-carotenoid-availability streams (Grether et al. 2001a). Consistent with this hypothesis, guppies grow more slowly and mature at smaller sizes in streams with lower algae (hence carotenoid) availability (Grether et al. 2001b). However, if the cost of drospterin production was high enough to override attractiveness benefits, we would expect food limitation in the laboratory to have a measurable effect on drospterin production, independent of body size, and this

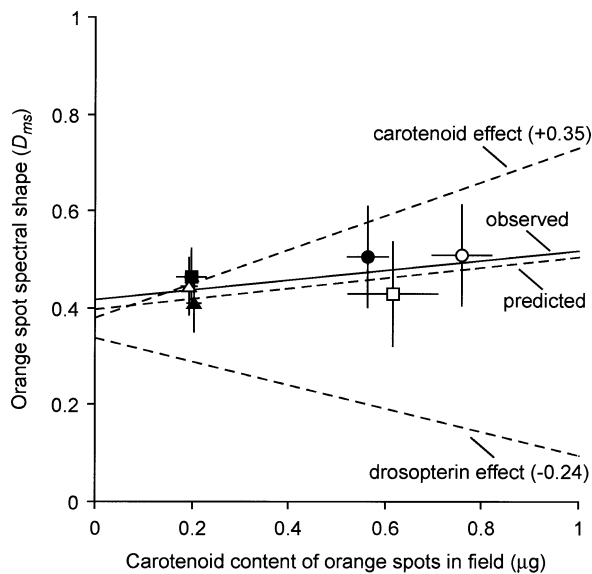


FIG. 4. Countergradient variation diagram showing the counterbalancing effects of carotenoids and drospterins on orange spot spectral shape along the carotenoid availability gradient. Plotted points represent the mean D_{ms} ($\pm SE$) of the orange spots of six guppy populations in the field ($N = 36-41$ males per population). Population symbols are explained in the legend of Figure 2. The upper dashed line represents the effect of carotenoids on D_{ms} and the lower dashed line represents the effect of drospterins on D_{ms} , as estimated from the multiple regression model in Table 4 ($D_{ms} = 0.46 + 0.28 T_d[\lambda_{max}] - 0.42 T_c[\lambda_{max}]$). The middle dashed line represents the model prediction when $T_d(\lambda_{max})$ and $T_c(\lambda_{max})$ were set to the observed population mean field values. Similar results were obtained for the other spectral shape variables in Table 4. The horizontal axis (carotenoid content of the orange spots in the field; mean $\pm SE$) summarizes both environmental and genetic influences on carotenoid deposition ($N = 15$ males per population). To estimate the drospterin effect, $T_c(\lambda_{max})$ was set to 1.0, and to estimate the carotenoid effect, $T_d(\lambda_{max})$ was set to 1.0 (note that a transmittance value of 1.0 corresponds to a pigment content of zero). All lines are from least squares regression through the observed or predicted population means. The drospterin and carotenoid effect values shown in the figures are the slopes of the corresponding lines. The slopes of the observed and predicted lines are 0.10 and 0.11, respectively. The slope of the observed line was not significantly different from zero ($P = 0.21$).

appears not to be the case. After adjusting for the effects of food levels on size at maturity, males raised from birth on a low-food diet did not have less drospterins in their orange spots than males raised on a high-food diet (G. F. Grether, G. K. Kolluru, and J. Hudon, unpubl. data).

Second, the geographic pattern could be the result of a nonlinear trade-off between the effects of chroma and brightness on male attractiveness. If the optimal balance of chroma and brightness depends on ambient lighting, for example, if brighter displays were favored in darker environments (e.g., Marchetti 1993), this could select for reduced pigment deposition in darker streams. However, this hypothesis predicts that drospterin production should be more closely related to stream differences in ambient light than to stream differences in carotenoid intake. Instead, orange spot drospterin content correlated more strongly with carotenoid intake as measured from gut content analyses ($r = 0.93$, $P = 0.0048$, $N = 6$ stream means; Grether et al. 2001a) than with ambient

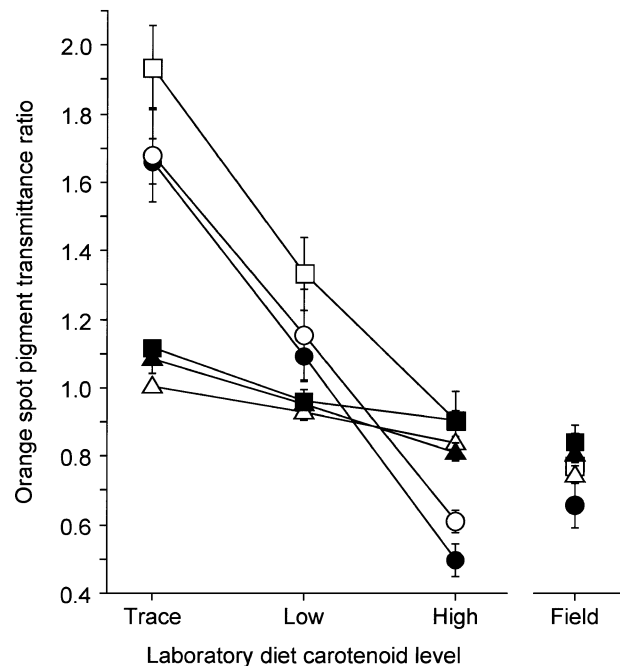


FIG. 5. Convergence in the orange spot drospterin:carotenoid ratio among populations in the field relative to the laboratory carotenoid diet groups. Plotted values represent mean $\pm SE$; in some cases the standard errors are smaller than the plotted symbols ($N = 35-41$ males per population in the laboratory and 15 males per population in the field). See Figure 2 legend for a key to the population symbols. The pigment transmittance ratio is defined in equation (7).

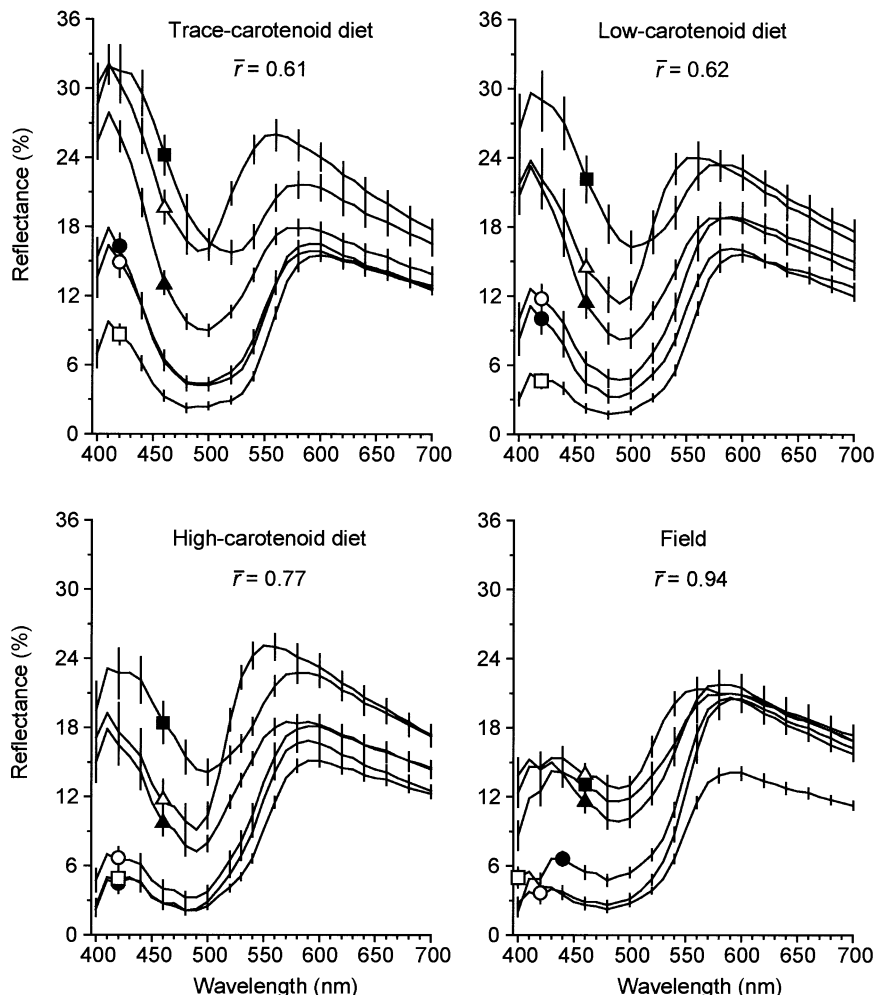


FIG. 6. Orange spot reflectance spectra of guppies in the field and laboratory, grouped by diet. The plotted values indicate the mean reflectance at 10-nm intervals; standard error bars are shown on every other mean ($N = 17\text{--}24$ males per population in the laboratory and 36–41 males per population in the field). Symbols identify the drainage and carotenoid availability level in the field, following the scheme described in Figure 2. The r -values are the mean correlations between mean reflectance spectra (see Results for statistical test results).

light (total irradiance over 400–700 nm at midday; $r = 0.02$, $P = 0.98$) or forest canopy openness ($r = 0.59$, $P = 0.25$) as determined in Grether et al. (2001b). Moreover, the geographic variation in orange spot brightness (as estimated by total cone catch) was only weakly correlated with ambient light ($r = -0.15$, $P = 0.79$, $N = 6$ stream means) and canopy openness ($r = -0.47$, $P = 0.37$).

Third, a female preference for hue could explain the observed geographic pattern and the results presented in this paper strongly support this hypothesis. By all measures, the spectral shape and hue of the orange spots were conserved among populations in the field, relative to the laboratory diet groups. This is a countergradient pattern because genetic differences between populations in drosopterin production mask the effect of carotenoid availability on the spectral shape and hue of the orange spots (see Fig. 4). Experiments are currently underway to determine whether female guppies actually do prefer males with a particular carotenoid:drosopterin ratio in their orange spots, independent of total pigment content.

A male who matched drosopterin synthesis to his own carotenoid intake would clearly have an advantage if females preferred a particular hue, so it is reasonable to ask why drosopterin production is only linked to carotenoid availability at the population level and not within individual fish. One possible explanation is that the biochemical pathways involved in drosopterin synthesis and carotenoid drosopterin cannot be linked physiologically, or at least that no genetic variation in such a link has yet appeared in guppies. We are not aware of any data bearing directly on this question.

Countergradient Variation in Other Secondary Sexual Characters

We know of only one other well-documented example of countergradient variation in a secondary sexual character, and it also involves geographic variation in carotenoid availability. Pacific salmon (*Oncorhynchus nerka*) exist as two reproductively isolated morphs: anadromous sockeye, which

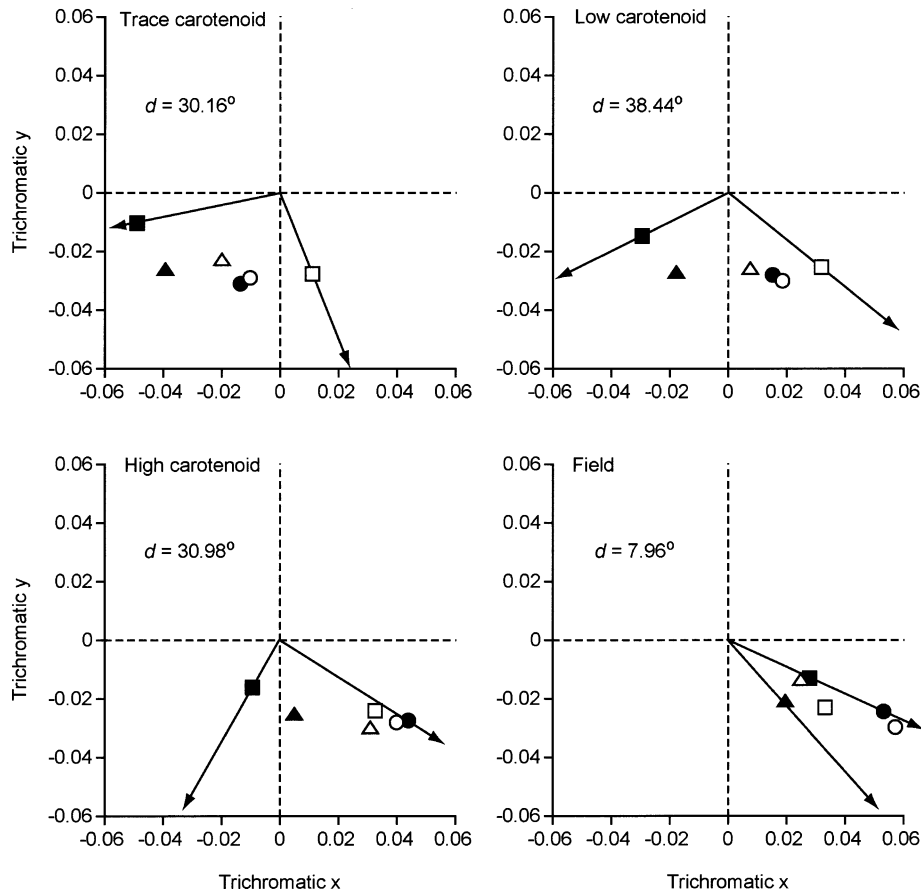


FIG. 7. Cone excitation color space analysis of variation in orange spot reflectance spectra. Each panel corresponds to a different carotenoid diet. Each point represents a population mean value of trichromatic x and y (as defined in Materials and Methods). Symbols identify the drainage and carotenoid availability level in the field, following the scheme described in Figure 2. The dashed lines cross at the achromatic center of segment classification color space. The solid lines with arrows represent the two most extreme hue vectors in each panel. The d -values are the angular deviations among population mean hue angles. Bootstrap P -values corresponding to the d -values are given in Results.

mature in the Pacific Ocean and return to lakes and rivers to spawn, and nonanadromous kokanee, which remain in freshwater lakes throughout their lives (Craig and Foote 2001 and references therein). Kokanee are thought to have evolved repeatedly from residuals, sockeye that fail to migrate to the ocean. At sexual maturity, sockeye and kokanee both display intensely red carotenoid-based breeding coloration, but this similarity in coloration masks an important difference between the environments in which the two morphs develop. Carotenoid availability for salmon is probably lower in the

oligotrophic lakes inhabited by kokanee than in the ocean where sockeye normally develop (Craig and Foote 2001). Residuals, the ancestral form of kokanee, are largely green at sexual maturity, as a result of developing in the low-carotenoid lacustrine environment. Red breeding coloration has repeatedly evolved in kokanee, through genetic changes in carotenoid assimilation efficiency (kokanee are three times more efficient at assimilating ingested carotenoids than are sockeye; Craig and Foote 2001). In short, it appears that the environmentally induced change in the phenotype (red to

TABLE 5. Multiple regression analysis of the effects of carotenoids and drosopterin on orange spot brightness and chroma. Note that pigment transmittance decreases as the pigment content of the orange spots increases. The $b \pm SE$ are regression coefficients and standard errors. $N = 306$ fish.

Variable	Drosopterin Transmittance (λ_{max})			Carotenoid Transmittance (λ_{max})			R^2_{adj}
	$b \pm SE$	$F_{1,303}$	P	$b \pm SE$	$F_{1,303}$	P	
Mean reflectance	0.56 ± 0.05	134.77	<0.0001	0.06 ± 0.04	2.62	0.107	0.42
Total cone catch	0.81 ± 0.04	176.48	<0.0001	0.16 ± 0.05	8.94	0.003	0.51
Seg. class. chroma	-0.42 ± 0.03	148.75	<0.0001	-0.20 ± 0.03	41.82	<0.0001	0.55
D_{ls} cone contrast	-0.07 ± 0.04	3.34	0.068	-0.39 ± 0.03	137.72	<0.0001	0.43
D_{lms} cone contrast	-0.32 ± 0.03	86.08	<0.0001	-0.20 ± 0.03	45.17	<0.0001	0.47

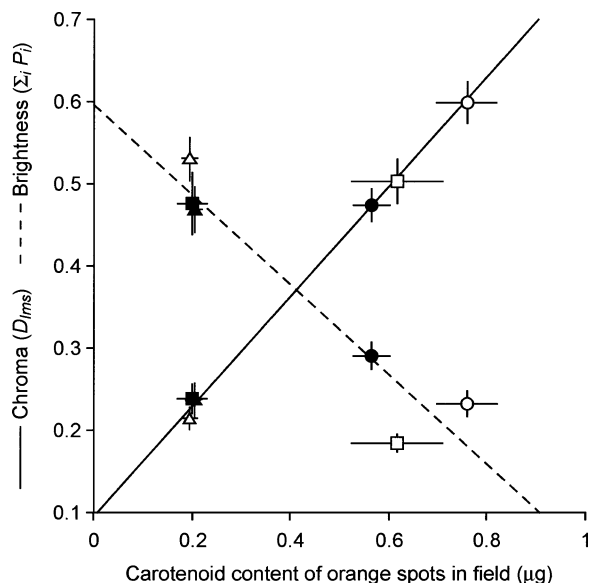


FIG. 8. Geographic variation in orange spot chroma and brightness along the carotenoid availability gradient. Plotted points represent the means (\pm SE) of six guppy populations in the field ($N = 36\text{--}41$ males per population). Population symbols are explained in the legend of Figure 2. The solid line represents the least squares regression of chroma on orange spot carotenoid content ($y = 0.10 + 0.67x$, $R^2 = 0.99$, $N = 6$, $P < 0.0001$), where chroma is measured as the contrast between the l cone and the m and s cones (D_{lms}). Similar results were obtained with other measures of chroma (e.g., D_{ls} and D_{max}). The dashed line represents the least squares regression of brightness on orange spot carotenoid content ($y = 0.60 + 0.55x$, $R^2 = 0.90$, $N = 6$, $P = 0.004$), where brightness is measured as total cone catch ($\Sigma_i P_i$). Some standard errors are smaller than the symbols; the symbols for two streams overlap.

green) that occurs when Pacific salmon invade freshwater lakes has been reversed repeatedly by countergradient selection, perhaps caused by a hue-based mate preference (Foote et al. 2004).

For another possible example of countergradient variation resulting from geographic variation in carotenoid availability and a coloration-based mate preference, see Hill (1994) and related papers (Hill 1993, 1995; Hill et al. 2002, Hill and McGraw 2004; but see Grether 1995).

Countergradient Variation and Reproductive Isolation

Countergradient selection has the potential to lead to a cryptic form of reproductive isolation between populations evolving in different environments (see also Carroll et al. 2001; Craig and Foote 2001). This differs from the usual scenario of local adaptation reducing gene flow between populations in that, with countergradient selection, the optimum phenotype may be the same in the two environments. Consider, for example, that a low-drosopterin male guppy who dispersed downstream over a barrier waterfall as a juvenile, from a low-carotenoid-availability site to a high-carotenoid-availability site, would develop orange spots with an abnormally high carotenoid:drosopterin ratio. Upstream male migrants, on the other hand, would develop abnormally low carotenoid:drosopterin ratios. If female guppies indeed prefer males with orange spots of the normal hue, male interpop-

ulation migrants would suffer a mating disadvantage, and so would their hybrid male offspring. This form of incipient reproductive isolation could not be detected, however, with standard laboratory mate choice tests. To be valid, mate choice tests would have to be carried out using true inter-population migrants or fish raised on diets designed to precisely match the carotenoid availability that a migrant would encounter.

This species, however, may not be a particularly good candidate for reproductive isolation through countergradient selection. Guppy populations at opposite extremes of the carotenoid availability gradient are usually separated from each other by populations with intermediate levels of carotenoid availability (G. F. Grether, unpubl. data). Thus, sexual selection against migrant phenotypes should be relatively weak. The effects of countergradient selection on reproductive isolation are likely to be strongest in situations in which the environmental gradient is steep.

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