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Carotenoid limitation of sexual coloration along an environmental gradient in guppies

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Carotenoids produce most of the brilliant orange and yellow colours seen in animals, but animals cannot synthesize these pigments and must rely on dietary sources. The idea that carotenoids make good signals because they are a scarce limiting resource was proposed two decades ago and has become the leading hypothesis for the role of carotenoids in animal communication. To our knowledge, until now, however, there has been no direct evidence that carotenoids are a limiting resource in nature. We showed that carotenoid availability in the wild limits the expression of sexual coloration in guppies (*Poecilia reticulata*), a species in which females prefer males with brighter orange carotenoid-containing spots. Further, the degree of carotenoid limitation varies geographically along a replicated environmental gradient (rainforest canopy cover), which opens new avenues for testing signal evolution theory.

Keywords: coloration; carotenoid availability; carotenoid limitation; secondary sexual characters; geographic variation; *Poecilia reticulata*

1. INTRODUCTION

Carotenoids are the most common red, orange and yellow pigments found in the integument of animals (Fox 1979; Goodwin 1984). Carotenoid-based sexual coloration may reflect the overall condition of fishes and birds (Endler 1980; Hill 1990; Nicoletto 1991; Putnam 1992; Zuk 1992; Frischknecht 1993) and is thought to influence mate choice in several species (e.g. Burley & Coopersmith 1987; Kodric-Brown 1989; Hill 1990, 1991; Hillgarth 1990; Milinski & Baker 1990; Zuk *et al.* 1990; Houde & Torio 1992). Animals acquire carotenoids by consuming carotenoid-producing plants or bacteria (Fox 1979) or by consuming other carotenoid-sequestering animals (Schiedt 1989). No animal can synthesize carotenoids *de novo* (Goodwin 1984; Schiedt 1989). Hence, access to carotenoids may limit the expression of secondary sexual traits in carotenoid-poor environments (Brush & Power 1976; Endler 1980, 1983; Slagsvold & Lifjeld 1985; Hill 1993). This may explain why many animals develop pale coloration when raised in captivity (Brush & Power 1976; Goodwin 1984; Schiedt 1989; Putnam 1992; Hill 1993; but see Hudon 1994). Carotenoid-containing feed supplements often restore normal coloration and are used routinely for this purpose in aviculture and pisciculture (Schiedt 1989; Sommer *et al.* 1992).

Whether carotenoid availability limits secondary sexual coloration in nature is an open question (Hill 1994; Hudon 1994). Carotenoids are ubiquitous in the natural diets of animals (Goodwin 1980, 1984) at concentrations that may be unlikely to limit coloration (Hudon 1994; Thompson *et al.* 1997). On the other hand, species vary in the rate at which they absorb ingested carotenoids (Schiedt 1989), in their ability to convert absorbed carotenoids into usable pigments (Fox 1979; Goodwin 1984; Putnam 1992) and in the rate at which they deposit these pigments in the integument (Brush & Power 1976; Hudon 1991). Thus, even diets rich in total carotenoids may be deficient in the compounds suitable for pigmenting the integument of a particular species. Vertebrates are notoriously inefficient carotenoid assimilators, to the chagrin of fish and poultry farmers (Putnam 1992; Meyers 1994). Farmed salmonids retain just 4–6% of the synthetic carotenoids they ingest and deposit only a fraction of this in their skin (Torrissen *et al.* 1989; Hardy *et al.* 1990). Assimilation of naturally occurring carotenoids must be even lower, since fish convert few natural compounds into usable pigments (Putnam 1992; Sommer *et al.* 1992). These low rates of assimilation do not appear to be a product of internal regulation, because the amount of pigment deposited increases with the concentration of carotenoids in the diet (Bjerkeng *et al.* 1990; Sommer *et al.* 1992). However, assimilation rates are impossible to interpret without field data on carotenoid intake. Some field studies on birds have correlated geographic or temporal variation in coloration with specific components of the diet (Slagsvold & Lifjeld 1985; Partali *et al.* 1987; Ryan *et al.* 1994; Linville & Breitwisch 1997), but direct evidence for carotenoid limitation in nature is lacking (Hudon 1994; Thompson *et al.* 1997).

We tested for carotenoid limitation of sexual coloration in the guppy *Poecilia reticulata*, a small fish native to the tropical forest streams of Trinidad and nearby parts of South America (Endler 1978). In wild populations of

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Table 1. *Comparison of high- and low-light streams within drainages*

(The values shown are mean differences (s.e.) between high- and low-light streams within drainages. Positive values indicate that the high-light stream mean exceeded the low-light stream mean. The *p*-values are for planned within-drainage contrasts from analyses of variance (or covariance) with stream as the main factor, except for the last two rows where the *p*-values are for paired *t*-tests. Note that orange spot area differences between populations were removed by pairing males with similar orange areas. See § 2 for further details.)

	drainage		
dependent variable	Marianne	Paria	Quare
forest canopy openness (%)	1.91 (0.50)****	3.38 (0.50)****	1.67 (0.50)****
ambient light (PAR, $\log_{10}\mu$mol m^{-2} s^{-1})	0.75 (0.18)****	0.94 (0.18)****	0.48 (0.18)**
guppy biomass (g m^{-2})	−0.34 (0.17)*	0.44 (0.17)*	−0.16 (0.17)
algal standing crops (chlorophyll *a*, $\log_{10}$ ng cm^{-1})			
cobbles	1.29 (0.14)****	1.61 (0.14)****	0.40 (0.15)**
tiles	0.32 (0.11)***	1.02 (0.11)****	0.61 (0.10)****
carotenoid availability ($\log_{10}\mu$g mg^{-1})			
cobbles	2.17 (0.36)****	2.07 (0.36)****	0.76 (0.36)*
tiles	0.58 (0.21)**	0.69 (0.20)***	0.74 (0.20)***
foregut carotenoids ($\log_{10}\mu$g)	0.64 (0.15)****	0.52 (0.16)***	0.01 (0.16)
non-spot skin carotenoids ($\log_{10}$ ng mm^{-2})	−0.07 (0.05)	−0.06 (0.05)	−0.09 (0.05)
orange spot carotenoids ($\log_{10}\mu$g)	0.28 (0.08)**	0.19 (0.05)***	−0.03 (0.08)
orange spot area (mm^2)	0.15 (0.16)	0.20 (0.17)	−0.01 (0.04)

$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.0001$.

guppies, colour patterns are expressed only by males and include carotenoid-containing orange spots. Endler (1980) suggested that the orange spots might serve as direct indicators of foraging ability and indirect indicators of health and vigour that females could use to choose high-quality mates. Subsequent studies confirmed that the level of carotenoids in laboratory diets affects the chroma ('brightness') of the orange spots (Kodric-Brown 1989) and that female guppies from some populations prefer males with higher orange chroma (Kodric-Brown 1989, 1993; Houde & Torio 1992). Orange chroma has also been shown to be reduced by parasitic infection (Houde & Torio 1992) and to correlate positively with swimming performance, a measure of phenotypic condition (Nicoletto 1991).

Undisturbed headwater streams in Trinidad are oligotrophic, with rocky beds and clear water (Endler 1980; J. A. Endler, unpublished data). They effectively contain no vascular plants, planktonic algae or filamentous algae. Attached unicellular algae (green algae, blue–green algae and diatoms) appear to be the only significant primary producers (G. F. Grether, personal observation). Plant parts and insects that fall into the streams also contain carotenoids, but at lower concentrations than algae (Goodwin 1980, 1984). The stomachs of guppies from one headwater stream contained approximately equal proportions of algae and benthic invertebrates (Dussault & Kramer 1981). Vascular plant fragments and adult insects constituted less than 1% of the diet. Guppies digest algae, as shown by their growth on a diet comprised solely of unicellular green algae (Dussault & Kramer 1981). When provided excess amounts of both algae and invertebrates, guppies spent 54–88% of their feeding time consuming the algae (Dussault & Kramer 1981). Thus, unicellular algae are the richest carotenoid source available in headwater streams and guppies appear to consume them preferentially.

In this paper, we show that carotenoid availability is lower in streams with greater forest canopy cover and that this results in some populations of guppies being more carotenoid-limited than others.

2. MATERIALS AND METHODS

To examine the effect of canopy cover on carotenoid availability for guppies, we sampled periphyton (attached algae) from natural cobbles and artificial (tile) substrates (Lamberti & Resh 1985) in two streams in each of three drainages (Marianne, Paria and Quare). We selected streams that, besides differing in mean canopy cover, were in intact old-growth forest, separated from each other by barriers to guppy dispersal and above waterfalls that excluded predatory fishes except *Rivulus hartii* (Endler 1978). These restrictions eliminated predation and anthropogenic disturbance as potentially confounding variables and ensured that the study streams contained genetically independent populations of guppies. We measured canopy cover, photosynthetically active light, periphyton standing crops and guppy biomass in 18–21 pools per stream, as described below.

(a) *Light and periphyton pigments*

Canopy openness was measured from hemispherical photographs (Ter Steege 1996) taken from the centre of pools. Photosynthetically active radiation (PAR) was measured at midday (11.00–13.00) when clouds did not block the sun. One set of cobbles and tiles was harvested 46 days after the tiles were placed in the streams and a second set was harvested at 92 days. Periphyton samples were filtered in the field, stored at −80 °C and analysed by high-performance liquid chromatography (HPLC) (Millie *et al*. 1997). Algal standing crops were measured as chlorophyll *a* per square centimetre of substrate (Cattaneo & Amireault 1992). Carotenoid availability was estimated as the amount of lutein, zeaxanthin and β-carotene in periphyton divided by guppy biomass. The other carotenoids we observed

in the periphyton (fucoxanthin, didinoxanthin, diatoxanthin, violaxanthin, neoxanthin and siphonein) can probably not be converted into skin pigments by fish (Goodwin 1984). Guppy biomass was estimated by measuring the standard length of all guppies in a pool, converting lengths to weights with sex-specific allometric equations and dividing the sum of the weights by the surface area of the pool. We tested for stream differences in the variables described in this section in ANOVAs, with stream as the main factor and pools as the unit of replication within streams. Variables were $\log_{10}$-transformed as required, to meet parametric assumptions (see table 1).

(b) *Gut and skin pigments*

Fishes collected for gut content analysis were immediately frozen in liquid nitrogen after capture in the field to stop digestion and stored at −80 °C. Carotenoids were extracted from the first third of the gut (approximately equivalent to the stomach) from six to eight fishes of each sex per stream and analysed by HPLC. The dominant peaks were identified as β-carotene, lutein and/or zeaxanthin, fucoxanthin and chlorophyll, based on known standards, relative position and fluorescence. β-carotene eluted later than expected and was not measured in Paria fishes, so only lutein/zeaxanthin data were used in the analysis; the results for Marianne and Quare fishes were qualitatively the same with β-carotene included. We tested for stream differences in foregut carotenoid content using ANCOVA, with stream, sex and $\log_{10}$(body weight) as factors, to remove the potentially confounding effects of stream differences in mean body size; removing sex and weight from the model did not affect the significance of the stream contrasts.

Males collected for the skin pigment analyses were photographed under anaesthetic (MS-222) within hours of field capture, frozen instantly and stored at −80 °C. Orange body spots were measured from slides using image analysis software (Houde & Endler 1990) and summed to yield orange area. 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 removed surgically and divided into orange spot and non-orange spot fractions. Carotenoids were extracted with acetone, transferred to hexane and quantified with a spectrophotometer (Hudon & Brush 1992). We tested for stream differences in the carotenoid content of the orange spots using paired *t*-tests in which males with similar orange area were paired ($n = 8$, 12 and 14 pairs in the Marianne, Paria and Quare drainages, respectively), to eliminate the potentially confounding effects of stream differences in mean orange area. Other statistical analyses used the full sample of 15 males per stream.

3. RESULTS AND DISCUSSION

In all drainages, algae but not guppies were consistently more abundant and, thus, carotenoid availability for guppies was greater in the stream receiving greater amounts of light (table 1). Across drainages, canopy openness explained 87–92% of the variation among streams in algal standing crops which, in turn, explained 83–98% of the variation in carotenoid availability (figure 1). Carotenoid ingestion by guppies, as determined from foregut extractions, was greater in the high-light stream than in the low-light stream in the Marianne and Paria drainages (table 1). In the Quare drainage, carotenoid availability was relatively low in both streams (figure 1) and foregut carotenoid content did not differ between streams (table 1). Taken together, these results provide compelling evidence for variation among streams in carotenoid availability.

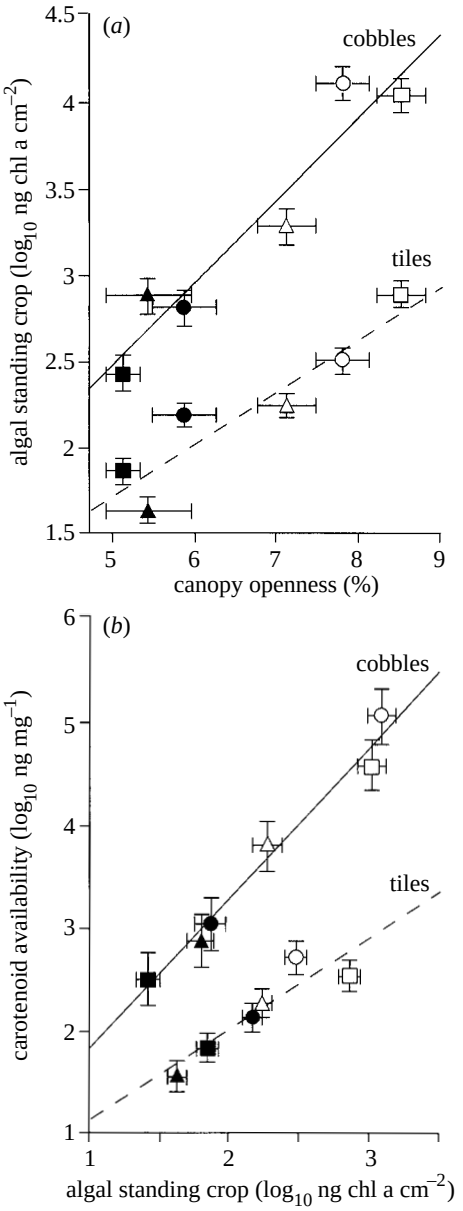

Figure 1. Variation among streams in algal biomass and carotenoid availability. Squares, circles and triangles represent the Marianne, Paria and Quare drainages, respectively, with filled symbols for low-light streams and open symbols for high-light streams. Points represent least-squares means ± 1 s.e. (see § 2 and table 1) and lines are from least-squares regressions. Solid lines are for the natural cobble substrates (points are displaced vertically by +1 in (*a*) and +2 in (*b*)) and dashed lines are for tiles (no displacement). (*a*) Algal standing crop versus canopy openness (cobbles $r^2 = 0.92$, $t = 6.6$, d.f. = 4 and $p = 0.001$; tiles $r^2 = 0.87$, $t = 5.2$, d.f. = 4 and $p = 0.003$). (*b*) Carotenoid availability versus algal standing crop (cobbles $r^2 = 0.98$, $t = 13.9$, d.f. = 4 and $p = 0.0001$; tiles $r^2 = 0.83$, $t = 4.4$, d.f. = 4 and $p = 0.006$).

Three lines of evidence suggest that carotenoid availability limits carotenoid deposition in the orange spots of

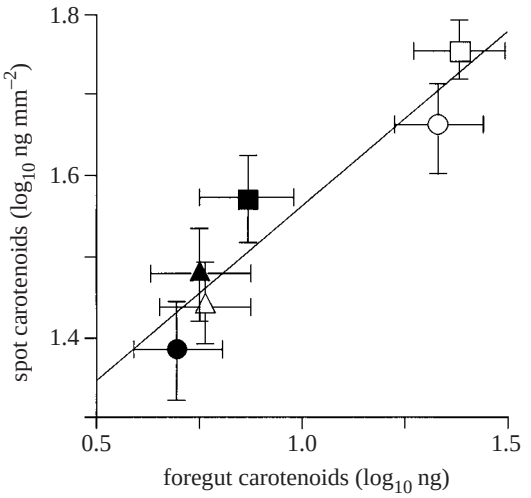

Figure 2. The relationship between orange spot carotenoid concentration and foregut carotenoid content across stream means ± 1s.e. ($r^2 = 0.89$, $t = 5.7$, d.f. = 4 and $p = 0.002$). Symbols follow figure 1.

male guppies. First, the carotenoid content of the orange spots showed the same pattern of variation among streams as the foregut carotenoid content, i.e. greater in the high-light stream in the same two drainages (figure 2 and table 1). This was not a generalized response to carotenoid loads or ambient light levels, because the mean carotenoid concentration in the skin outside of the orange spots did not differ between high- and low-light streams (table 1). We infer that guppies meet their general pigmentation requirements first and selectively deposit excess absorbed carotenoids in the orange spots. The carotenoid demand of sexual coloration was much higher than that of general pigmentation; the carotenoid concentration was 5.4–8.9 times higher in the orange spots than elsewhere in the skin (paired *t*-tests, $n = 15$ and $p \leqslant 0.001$ for all streams).

Second, if dietary carotenoids are scarce, larger orange spots should be more difficult to fill with pigment (Hill 1993), resulting in a negative within-stream correlation between total orange spot area and the concentration of carotenoids in spots. (The orange area is highly heritable (Houde 1992) and is not affected by the carotenoid content of the diet (Kodric-Brown 1989).) As shown in figure 3*a*, the magnitude of this correlation was greater and, hence, carotenoid limitation was greater in streams with lower carotenoid availability. Similarly, if carotenoid availability truly limits deposition, the total amount of carotenoids in orange spots should be independent of total orange spot area. This was the case for the three low-light streams, where the carotenoid content and orange area were uncorrelated (Marianne $r = 0.41$, $p = 0.13$; Paria $r = -0.06$, $p = 0.84$; Quare $r = 0.05$ and $p = 0.86$; all $n = 15$), but not for the high-light streams, where the correlation was positive (Marianne $r = 0.77$, $p = 0.0004$; Paria $r = 0.65$, $p = 0.008$; Quare $r = 0.55$, $p = 0.04$; all $n = 15$).

Finally, the ratio of carotenoids in the skin to that in the foregut decreased as carotenoid availability increased (figure 3*b*). We are aware of two possible reasons for this result, both of which imply that carotenoid availability limits carotenoid deposition in the skin. The declining

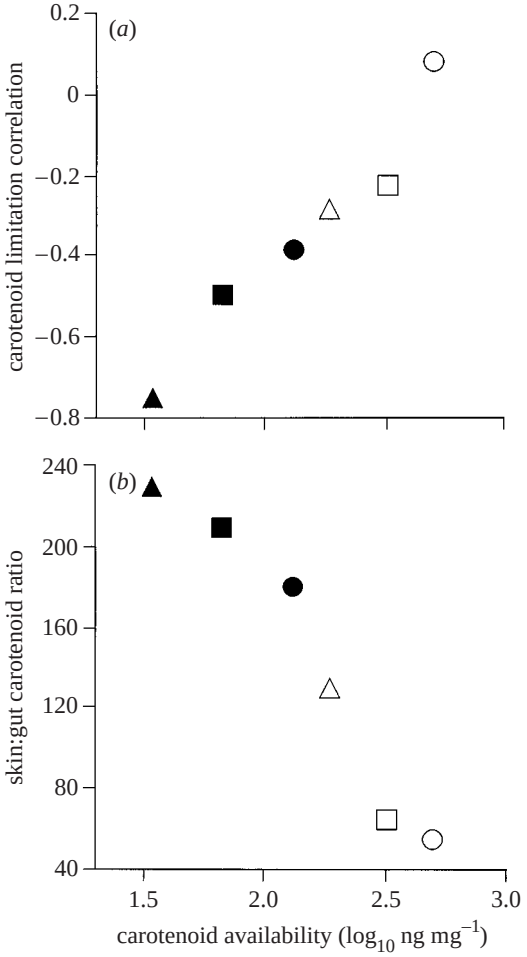

Figure 3. The relationship between carotenoid availability and (*a*) the within-stream correlation between orange spot carotenoid concentration and total orange area ($r^2 = 0.94$, $t = 8.2$, d.f. = 4 and $p = 0.0006$), and (*b*) the mean ratio of skin carotenoid content to foregut carotenoid content ($r^2 = 0.94$, $t = 7.56$, d.f. = 4 and $p = 0.0008$). Symbols follow figure 1.

ratio may reflect a diminishing returns dose–response curve, as has been observed in carotenoid feeding experiments with salmonid fish (Choubert & Storebakken 1989). As the carotenoid content of feed increases, the fraction of carotenoids retained by the fish decreases (Choubert & Storebakken 1989). Alternatively, this result may reflect genetic differences between populations in carotenoid assimilation rates, as would be expected if selection for more efficient assimilation was stronger in the streams with lower carotenoid availability.

To our knowledge, this study provides the first direct support for the popular view that carotenoids make honest signals because they are a scarce limiting resource (Endler 1980; Hill 1991; Gray 1996; Olson & Owens 1998). Recent hypotheses based on the health benefits of carotenoids (Lozano 1994; Olson & Owens 1998; Wedekind *et al.* 1998) also become more plausible if dietary carotenoids are scarce. High levels of carotenoids in the skin may signal not only past good health but also the potential to respond to future health challenges (Lozano 1994). A related hypothesis views carotenoids in the integument as costly displays that only healthy individuals can afford (Olson & Owens 1998). Either way, carotenoid scarcity should enhance the signal value of

carotenoid coloration. Our results do not rule out other mechanisms through which carotenoids may signal health (Hudon 1994; Thompson *et al.* 1997; Von Schantz *et al.* 1999; Zahn & Rothstein 1999).

Our finding that carotenoid limitation varies geographically suggests a possible direct test of the indicator models of mate choice (Andersson 1994); stronger female preferences for carotenoid-based male traits should evolve in populations where carotenoids are more limiting. The Fisherian and sensory drive models make no such prediction (Andersson 1994). If carotenoid limitation does affect mate preference evolution, as the indicator models predict, mate preferences may diverge among populations of species distributed along productivity gradients.

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