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PIGMENTS OF A COLOR POLYMORPHISM IN A CICHLID FISH

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Abstract—1. *Cichlasoma citrinellum* occurs as gray or gold morphs in lakes in Nicaragua. The gold morph is variable and individuals range from yellow to dark orange.

2. The pigments responsible for the carotenoid coloration are ε-carotene and canthaxanthin. The gray morph contains a carotenoid component and also heavy melanin deposits.

3. The carotenoids are dietary in origin, but their distribution and intensity is presumably under genetic control.

4. The relation of color morphs to the ecological and selective forces which might produce them is discussed.

INTRODUCTION

THE OCCURRENCE of carotenoids in fishes has been widely documented. These pigments provide one of the major sources for the great diversity of colors that exists among this group of vertebrates (Goodwin, 1951; Fox, 1957). One aspect of the biology of pigments is the existence of color polymorphism or polychromatism. These color patterns are especially significant when different social, behavorial or ecological roles can be assigned to the morphs, or when their distribution is associated with some environmental gradient or phenomenon. Further, the biochemistry of the pigments is subject to analysis, and models may be built for the genetic control of their metabolism. Comparative studies may lead to generalizations regarding the evolution of carotenoid metabolism and control mechanisms responsible for their display. One impediment to the closer analysis of such problems is that the occurrence of colorful morphs is at a very low frequency in most species, even though the phenomenon occurs in widely unrelated fishes (e.g. blackbass, Allen & Neil, 1953; minnows, Hubbs & Miller, 1948; grunts, Hulquist, 1967; flatfish, McCormick & Baldwin, 1952; groupers, Moe, 1963; eels, Pavesi, 1894; gars, Phillips, 1958; and sablefish, Phillips, 1952). The common goldfish and koi carp present abundant material; but these animals have been

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subjected to centuries of selection for their bright coloration, making evolutionary interpretations impossible.

Some indication of the nature and control of colors in fish is available. For example, Crozier (1967) studied the distribution and quantitative differences of carotenoids in seven closely related species of *Sebastodes* (= *Sebastes*), a marine rockfish. Both pigment quality and content were correlated with general body color and habitat preference of the species. It is generally known that red species of these predaceous scorpaenid fishes are taken at depths, while species with yellow or orange markings tend to occur in shallow water. Within species, the morphs of a sea horse, *Hippocampus erectus*, were analyzed chemically (Linton & Hamilton, 1964). Color phase differences were due to variation in absolute and relative abundance of carotenoids coupled with the distribution of melanins. The functional role of the color morphs was not determined. A possible relationship between color and factors such as predator avoidance and concealment was investigated in fish by Sumner (1935). He concluded that the chromatophoric mechanisms involved in background matching had a high selective value. The maintenance of a high proportion of brilliantly colored morphs, then, must be done in the face of adverse selection from predators.

In Central America there are several large species of cichlid fishes that have occasional brilliantly colored morphs in various shades of yellow, orange, red or even white, and lack their species-typical melanin markings (*Petenia splendida*, Hubbs, 1935; *Cichlasoma dovii*, *C. managuense* and *C. nicaraguense*, Barlow, personal observation). In two abundant lake species in Nicaragua, *C. labiatum* and *C. citrinellum*, colorful morphs occur in large numbers, comprising up to 8–10 per cent of the adult populations in some lakes, being absent in others. Individual *C. citrinellum* even occur in piebald or mixed color patterns. A possibly parallel situation exists among some African lake cichlids, but the polychromatism there is largely limited to females (Greenwood, 1956; Fryer, 1961). Nothing is known about the pigments of any of these cichlid fishes.

C. citrinellum occurs as two morphs which we call gold and gray. The gold designation is a matter of convenience as this form varies from yellow through orange to red. All else being equal, golds behaviorally dominate the normal gray form. *C. citrinellum* breeds readily in captivity, providing an accessible source of fish. In order to understand the color bases of the morphs we determined the quantitative and qualitative nature of these differences. We report here the isolation and identification of the carotenoid pigments, variation in their concentration and observations on the associated melanins. This work provides an important basis for studies on the regulation of aggressive behavior in this species.

MATERIALS AND METHODS

The fish used in this study were bred in artificial ponds in the Life Sciences Building of the University of California, Berkeley. The ponds were supplied with free flowing, fresh water. The staple diet of ground beef heart and trout chow was supplemented with paprika, brine shrimp and commercial β-carotene.

For purposes of analysis the skin and scales were removed from nine individual specimens, chopped into small pieces, weighed and extracted overnight at 4°C in acetone. Two subsequent extractions were made by heating the samples in acetone over a steam-bath for 5 min. The pigments were transferred to *n*-hexane by first evaporating the crude extract, then washing with *n*-hexane three times in a separatory funnel. Usually two washings were adequate for total transfer. After the acetone extraction the skins were immersed overnight in petrol–ether, then heated on steam to extract any non-acetone soluble pigments. Insoluble pigments are discussed below.

The hexane mixtures were fractionated into components by thin-layer chromatography (TLC) on Eastman silica gel plates. Column chromatography of the crude extract on MgO–Celite (1 : 1) was found less effective on the small, individual samples. The samples were applied to the TLC plates with a No. 0 sable hair brush and developed in a standard chromatographic chamber in the solvent systems described by Johnson & Brush (1972). The TLC apparatus was also used as a preparative technique. After standard development the individual bands were removed mechanically from the plate and the individual pigments eluded from the silica gel with a solution of *n*-hexane and 2–5% methanol. To estimate the relative distribution of pigments in the individual fish, mixtures of standardized concentration were spotted on a single plate and the spots recovered and the pigment concentration estimated spectrophotometrically.

To test for the presence of lipid esters, samples were hydrolyzed in 5% alkaline ethanol overnight at room temperature. Samples were transferred to *n*-hexane and the absorption spectra compared to that taken before treatment.

All visible absorption spectra were made on a Cary Model 14 recording spectrophotometer. The concentration of each pigment in g/100 g sample was calculated by the relationship:

$$\text{concentration} = \frac{(A\lambda_{max})\ (\text{volume of fraction})\ (\text{density of hexane})\ (\text{volume of extract})}{E^{1\%}_{1\text{cm}}\ (\text{volume of extract used})\ (\text{wet wt. sample})}.$$

Extinction coefficients of 2200 (canthaxanthin) and 2900 (ε-carotene) were used (Foppen, 1971). Partition coefficients were determined by the method of Petracek & Zechmeister (1956) and the types and numbers of polar side groups (M_{50}) estimated by the method of Krinsky (1963). Infrared absorption spectra for structural studies on individual fractions were made on a Perkin–Elmer Model 257 Grating IR recording spectrophotometer. Spectra were taken in both KBr discs and $CHCl_3$ solutions.

To test for the presence of melanins in the skin after acetone extraction, patches of skin were bathed in a solution of 10% H_2O_2 and observed directly by light microscopy.

All chemicals were reagent grade and used without further processing.

RESULTS

Preliminary tests (Karrer & Jucker, 1950) indicated that the acetone extracted pigments were carotenoids. On TLC two fractions were resolved in hexane–methanol (1 : 1), benzene–methanol (98 : 2 or 25 : 1) and benzene–ethyl acetate (2 : 1). Four fractions were resolved in development in benzene–acetone (98 : 2). The latter solvent system was used regularly for isolation and identification of the pigments. The R_f values, absorption maxima, partition coefficients and M_{50} values for the pigment isolated from the crude extract are summarized in Table 1.

TABLE 1—SUMMARY OF SPECTRAL AND CHEMICAL DATA ON CAROTENOIDS OF *C. citrinellum*

	R_f (benzene–ethylacetate, 2 : 1)	R_f (benzene–acetone, 98 : 2)	Visible absorption		I.r. spectra (peaks)		Partition coefficient hexane (95% MetOH)	M_{50}	Melanin
			In *n*-hexane (λ/μm)	Relative peak intensity	2940, 1470	1740*			
Gray morph	0·80	0·82	468, 440, 416	0·93, 1·0, 7·1	+	–	100 : 0		
		0·57	468, 440, 416	0·93, 1·0, 7·1	+	–	100 : 0		+
	0·74	0·43	467	1·0	+	+	56 : 44	96·4	
		0·35	—	—			—		
Yellow morph	0·80	0·84	467, 439, 415	0·93, 1·0, 0·69	+	–	100 : 0		
		0·56	467, 439, 415	0·93, 1·0, 0·69	+	–	100 : 0		0
	0·74	0·44	467	1·0	+	+	53 : 47	95·8	
		0·37	467						
Orange morph	0·80	0·84	469, 440, 416	0·93, 1·0, 0·60	+	–	100 : 0		
		0·56	469, 440, 416	0·93, 1·0, 0·60			—		0
	0·74	0·44	466	1·0	+	+	53 : 47	95·6	
		0·37	464, 361	1·0, 0·32	+	+	53 : 47	95·6	
Canthaxanthin			468	1·0	+	+	52 : 48 100 : 0		
ε-Carotene			468, 437, 414						

* Presence of i.r. peak at 1740 indicates keto-group.

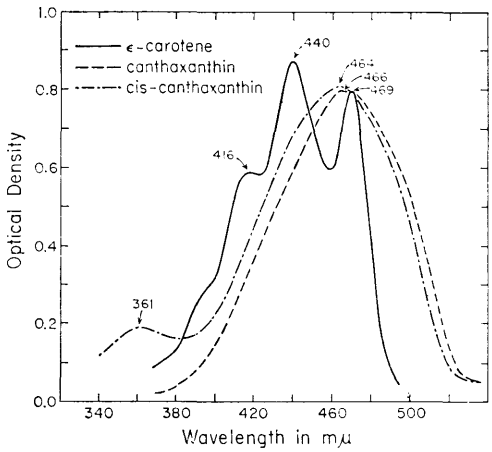

FIG. 1. Absorption spectra of carotenoid pigments isolated from *C. citrinellum*. The intense absorption in the 350–500 μM range explains the yellow to red hue of the fish skin. The relationship between reflectance and transmission spectra has been studied in avian plumage (Johnson & Brush, 1972) but not fish skin.

Identification

Absorption spectra of the major chromatographic fractions indicated the presence of a carotene and a xanthophyll (Fig. 1). Infrared spectral data agreed, indicating the basic polyene structure with a keto group present in one fraction but not the other (Brügel, 1966). The partition coefficients, chromatographic behavior and relative polarity data were consistent with these observations. From these data, and co-chromatography, we have identified the major pigments in *C. citrinellum* as ε-carotene and canthaxanthin.

The minor bands produced in the benzene–acetone solvent system were indistinguishable from the associated major ones in i.r. spectra, partition coefficient and M_{50} values. In the visible spectra major absorption peaks were shifted slightly toward the shorter wavelengths; there was an additional minor peak at 361 μm (Fig. 1). These spectral characteristics are generally associated with cis-isomers (Karrer & Jucker, 1956; Isler, 1971). Isomerization has been reported previously for canthaxanthin (Cooney *et al.*, 1966). On the basis of the spectral evidence, and apparent structural similarity, we conclude that the additional (e.g. minor) bands produced by the benzene–acetone (98 : 2) solvent system were cis-isomers of the major bands.

Distribution

Carotenoid pigments were present in the integument of all fish used in this study. The gray (normal) morphs also contained considerable amounts of melanin, as determined by direct observation of granules and bleaching with peroxide. Presumably the melanins served, to a great extent, to mask the carotenoids. Melanophores were not observed in the skin of the gold morph.

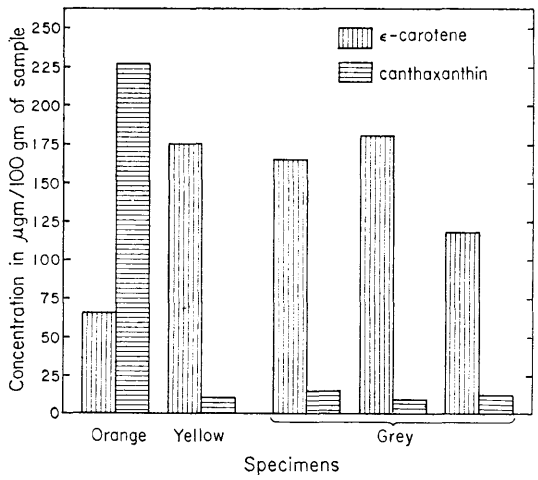

FIG. 2. Concentration and distribution of carotenoid pigments in gold and gray morphs of *C. citrinellum*.

Color differences among individuals within the morphs were studied semi-quantitatively on TLC. The distribution of pigments in five individuals is shown in Fig. 2. The coloration of individuals was determined by a mixture of the red and yellow pigments. Both gray individuals and yellow individuals of the gold morphs had approximately similar amounts of ε-carotene. In the more intensely colored orange individuals there was a slight increase in ε-carotene, but a dramatic increase in canthaxanthin. The gray morph varied widely in coloration when breeding. Their throats, eyes and the interspaces between bar markings on the flanks vary from pale yellow through canary yellow to orange and even red. The individuals used in this study were uniformly gray. In the gold morph there was excellent correspondence between the total carotenoid present and the overall color intensity.

DISCUSSION

The colors of *C. citrinellum* result from deposition of both melanin and carotenoid pigments. The difference between the gold and the normal gray morphs is due to the presence of masking melanin in gray fish. Within the gold morph variation in color from yellow through orange to red is due to an increasing concentration of a keto-carotene. We have identified the pigments as ε-carotene and canthaxanthin.

Canthaxanthin is distributed widely in animals. ε-Carotene, which is common in algae, was first reported from animals by Fox & Hopkins (1965) and specifically from fish by Crozier & Wilkie (1966). It may be distributed widely in fishes, occurring commonly as the 3,3′-dihydroxy derivative. It could be the source of reports of "lutein" or "taraxanthin-like" pigments in fishes (Crozier & Wilkie, 1966). The absolute and relative concentration of these pigments may provide

only a superficial explanation of the mechanisms involved in the production of color polymorphisms. Much of the display, in some species, obviously depends on short-term regulation of chromatophores (Fujii, 1967). The picture is further complicated in *C. citrinellum* in that the apparent color brightens and seems to shift from yellow toward orange with the onset of breeding. In the sibling species, *C. labiatum*, the bright morphs occur in shades of red, ranging from pink to dark red. At the present we have no data on their pigments.

Another consideration is the presence of carotenoids in the diet. In nature *C. citrinellum* is omnivorous and eats algae, snails, crustaceans and fishes. In captivity and on standard fare, the skin color fades slowly. The laboratory fish were given food that contained several potential carotenoids (see Materials and Methods) and which restored much of the color. One of the items, paprika, contained capsanthin. Another item, brine shrimp, is known to contain canthaxanthin plus traces of other carotenoids including pirardixanthin (Krinsky, 1965). Pirardixanthin was identified tentatively as 3,3′-dihydroxy-ε-carotene (Crozier & Wilkie, 1966). In fishes generally, pigmentation of the flesh and skin can be affected by diet (for example, see Saito & Regier, 1971, for a specific case involving diets containing crustacean material). The quality, amounts and stability of stored pigments, plus the general effect of diet and background color were summarized by Fox (1957) for several species of euryhaline or marine fish.

It is not clear why different individuals in a population selectively deposit certain of the carotenoids in their diet. While the intensity of the color may change with diet, some *C. citrinellum* are clearly more yellow and others orange. Further color mixtures, such as orange around the eyes of white fish, appear to be heritable. Existing models for the control of plumage carotenoids in birds imply a genetic basis for the difference (Brush & Seifried, 1968; Brush, 1970). Since all the fish in this study presumably had equal access to the food, a genetic role is not inconsistent with our observations.

It is difficult to explain the distribution of color polymorphisms in various animal groups, and fishes are no exception. Colorful exteriors tend to be more common in visual, diurnal animals such as birds than in nocturnal, secretive forms. Despite the fact that many fish are colorful, especially those species whose distribution is tropical shallow waters, the occurrence of color polymorphisms is relatively low. The only case where the selective role of the different morphs is even partly understood is the three-spined Stickleback (*Gasterosteus aculeatus*) (McPhail, 1969; Semler, 1971). Here the pigments and their potential control mechanisms have been described (Brush & Reisman, 1965). Yet there is a considerable paucity of information on the chemical basis of polychromatisms, their genetic control and their ecological and evolutionary roles in fishes.

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Key Word Index—Cichlasoma citrinellum; carotenoids; ε-carotene; canthaxanthin; ecological coloration.