THE CHEMICAL IDENTIFICATION OF GENE-CONTROLLED PIGMENTS IN PLATYPOECILUS AND XIPHOPHORUS AND COMPARISONS WITH OTHER TROPICAL FISH[1]

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THIS paper presents a study of the pigments and cells that produce the red, yellow, and orange colors in certain fish with special reference to those cases where genetic analyses have been made of color inheritance. The following is the list of species which we have studied: *Platypoecilus maculatus* and *Xiphophorus helleri* (the swordtail) of the family Poecilidae, *Oryzias* (*Aplocheilus*) *latipes* (the Medaka) of the family Cyprinodontidae, and four species of the Family Osphronemidae: *Macropodus opercularis* L. (the paradise fish), *Colisa lalia* (dwarf gourami), *Colisa fasciata* (striped gourami), and *Betta splendens* (Siamese fighting fish). The classification is summarized in table 3.

All pigments studied are intracellular, being carried by chromatophores which are located in the dermis. The types of chromatophores with which we are especially concerned are the yellow cells, or xanthophores, the red cells, or erythrophores, and a type designated as the xantho-erythrophores (fig. 1) which contain two sharply distinguishable pigments. The xanthophores and erythrophores integrade in species of the family Osphronemidae so that cells of intermediate color may be seen. In these cases, however, the color is uniform throughout the cell, making them quite different from the xantho-erythrophores.

PLATYPOECILUS AND XIPHOPHORUS

These two closely related Mexican fish have been the subject of extensive genetic studies by various authors, especially by BELLAMY, GORDON, KOSSWIG, and BREIDER. GORDON (1931, 1937) may be consulted for the bibliography of the genetics of these fish.

Our studies indicate that the pigments zeaxanthin and lutein produce the yellow color in these fish and that the red is due to erythopterin. BELLAMY (1924, 1928, 1933), FRASER and GORDON (1929), and GORDON (1937a) find that in Platypoecilus the factor for red *R* is sex-linked and the female is the heterogametic sex. Red crossed with gold (FRASER and GORDON 1929) gives typical sex-linked ratios of ZW type with red dominant to gold. The situation for our purposes, however, is more accurately stated

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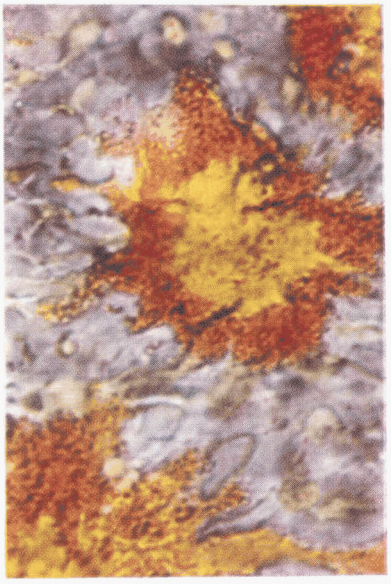
by considering the above as a cross between red and non-red with red dominant over non-red. Similarly, crosses between the autosomal character stippled and gold (stippled is regarded as dominant to gold) is best expressed as a cross between stippled and non-stippled. In these two cases, the stippled and red factors merely conceal the yellow to a certain extent and may therefore be considered to be partially epistatic to the yellow color. GORDON (1927) indicates in his table (page 261) that xanthophores were present in all types, and BELLAMY (1933) on page 523 refers to the typical effects of gene *g* in various combinations. Both these statements we understand to be a recognition of the presence of the yellow pigments in various types other than that known by the fanciers as gold.

Our observations confirm the presence of the yellow pigment in all varieties of Platypoecilus that we have studied. However, while in all non-red types it is the exclusive color of the xanthophores, in the red form the yellow and red pigments are contained in the same type of cell which is called a xantho-erythrophore. Therefore, the presence of the red producing gene brings about the added development of the red pigment in cells which we consider to be homologous to the xanthophore of other types. In the xanthophores, the yellow appears to be present in two forms—as globules about 1 micron in diameter and in solution in the cytoplasm. When cells disintegrate, the globules appear to coalesce. Application of a gentle heat by warming the microscopic mount appears to hasten the process. The xantho-erythrophores are characterized by dense yellow pigment at the center and a red pigment at the periphery of the main body of the cell, but yellow pigment may be diffused in the cytoplasm (Plate 1). This plate is from a natural color photograph taken on a scale immediately after removal from the living fish. It therefore shows an optical section at some depth in the fresh tissue. Observations with reflected light show extraordinarily delicate cell processes of a yellow color which are too minute to contain globules. The red pigment is in the form of granules which may collect on the surface of the droplets of yellow pigment or be free in the cytoplasm. These granules do not coalesce under any conditions observed under the microscope. The chemical nature and methods of identification of the pigments are outlined later in this paper.

The genetics of *Xiphophorus helleri* has not as yet been so extensively studied as in Platypoecilus. The two species may be crossed in the laboratory (GORDON 1931b). In some cases when the gene *Sp* (spotted) is present, this cross produces the well known melanotic hybrids. Genetic studies have been made by KERRIGAN (1934), BREIDER (1935), KOSSWIG (1935), GORDON, (1937b, 1940). In Xiphophorus we are again concerned with the red and yellow pigments. There is some reason for believing that the red



DESCRIPTION OF PLATE I

FIGURE 1.—A xantho-erythrophore from *Xiphophorus helleri*. Photograph (Kodachrome) of living cell taken with oil immersion. Magnification ×760 Photograph was taken by MARIAN HEDENBERG.

pigment in certain varieties of Xiphophorus has been introduced by hybridization with Platypoecilus (GORDON 1931, INNES 1935) and that different red types may have had separate origin, one from the original red (Rubra, *R*) in Platypoecilus and another from the red finned yellow platy (gene *Dr*, formerly called *Rf*). It has been suggested that the gene *R* from Platypoecilus spreads the red color over the whole body of the hybrid, while the gene *Dr*, similarly introduced, does not color the ventral throat region (GORDON 1931a). This strain, according to INNES (1935, page 323) arose in 1930. Our material shows this same white throat and quite possibly originates from this same stock. KOSSWIG (1935) uses the symbol *Rb* for the red color. Since there is at present no agreement as to symbolism, we

TABLE 1

Green ("wild")	*rr*	*StSt*	*PP*
Brick red	*RR*	*StSt*	*PP*
Gold	*rr*	*stst*	*PP*
"Red Albino"	*RR*	*StSt*	*pp*
Albino	*rr*	*StSt*	*pp*

Genes *R*=red (may be *Dr* of GORDON), *r*=absence of red; *St*=stippled, *st*=absence of stipple; *P*=pigment factor necessary for development of melanin, *p* inhibits melanin production. Melanophores appear only when genes *St* and *P* both are present.

are using *R* to designate the factor for red in the type we have used. Whatever may have been the origin, the genetic situation appears in many respects similar to that in Platypoecilus.

The wild type green (homologous to stippled in Platypoecilus), when crossed with the gold type, gives an F_2 ratio of 3 green (stippled) to 1 gold (non-stippled) (GORDON 1937b). Here, as in Platypoecilus, xanthophores are present in both types but are not noticeable except in the gold form, so that we may consider the stippled (green) to be epistatic to yellow. Similarly (GORDON 1940), the factor for red need not be regarded as an allele of yellow but as an allele of non-red and as partially epistatic to yellow, because the red pigment of the erythro-xanthophores makes the presence of yellow less obvious. GORDON'S and BREIDER'S work indicates the genetic formulae given in table 1.

All of the five types listed in the table carry yellow pigment. In the brick red and "red albino" it is in the xantho-erythrophore, and in the albino the xanthophores are reduced in size. The "red albino" has a red body color with little or no melanin and has the red eyes of a typical albino. This type was reported arising in a cross described by KOSSWIG (1935), and his results have been duplicated in our laboratory. Of the types listed, brick red and gold (these are both names given by fish fanciers) were the forms used for our chemical analyses of pigments.

ORYZIAS

The genetics of *Oryzias* (*Haplocheilus*) *latipes*, a Japanese fish, commonly known as the Medaka, have been studied by AIDA (1921, 1930, 1936). Colors are produced by melanophores and xanthophores. Types carrying gene *B* owe their dark color to fully developed melanophores, and those having the recessive *b* have melanophores with very little melanin (GOODRICH 1927); this deficiency is interpreted by GOODRICH (1933) to be due to the absence of chromogen, since the 'dopa' reaction indicates the presence of the necessary oxydase. Types carrying gene *R* are yellow due to the well developed xanthophores, and those having the recessive *r* have homologous cells with little or no pigment (GOODRICH 1927). Our chemical analyses show the yellow pigment to be lutein.

OSPHRONEMIDAE

The other fish examined are from genera of a single family of the order Labyrinthici. By various authors they are placed in the family Anabantidae (*cf.* WEBER and DE BEAUFORT 1922), but by JORDAN (1923) they are placed in the more restricted family Osphronemidae. The species studies are found in Southeast Asia. For colored figures see INNES (1935).

Macropodus opercularis has long been known as an aquarium fish. KOSSWIG (1935) and GOODRICH and SMITH (1937) have shown that the dark form is a simple Mendelian dominant to the albino and that the albino completely lacks the melanophores. The red or yellow cells, however, are present in both types. These cells are very similar to each other, and the distinction is apparently due to slight differences in the color of the pigment and not to the presence of two physically different pigments as in Platypoecilus and Xiphophorus. The pigment appears to be in minute droplets, but as in the case of the yellow pigment in the two previously discussed types, these globules may coalesce on disintegration of the cell or when slightly warmed. Our analyses show the presence of the two xanthophylls, violaxanthin and lutein. When these pigments were obtained in solution in our experiments, the solution of violaxanthin was of a slightly more orange color than that of lutein. A preponderance of violaxanthin in certain cells may cause their more reddish color.

Colisa lalia and *Colisa fasciata* were formerly placed in the genus Trichogaster. These two types have markings very similar to those of Macropodus. Our studies show the presence of the same pigments, violaxanthin and lutein.

Betta splendens Regan (the Siamese fighting fish) exhibits a great variety of types, and no satisfactory genetic analysis have yet been made. GOODRICH and MERCER (1934) showed that the dark types are dominant to the light forms (known to fanciers as Betta cambodia and by various trade

names). Red and yellow pigments are present in both varieties, but in "cambodia" the red tends to be largely restricted to the fins. Our analyses shows the red to be erythropterin and the yellow to be lutein.

CHEMISTRY OF THE PIGMENTS

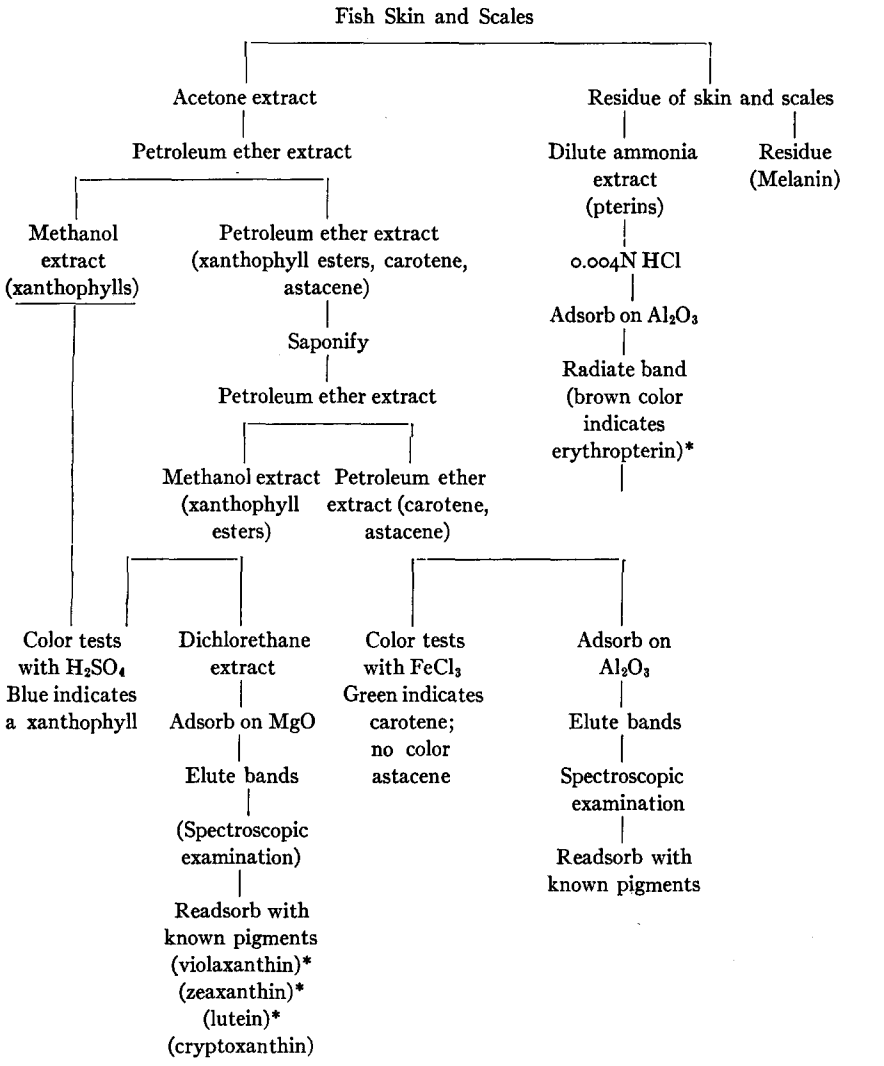
The material for these studies was obtained from laboratory stocks originally purchased from fish fanciers, mostly from M. MATSUNO of Flushing, New York. The chemical studies were made by M. S. ARRICK in consultation with DR. G. A. HILL.

The procedure, which is outlined in table 2, is based on methods employed by SUMMER and FOX (1933, 1935), SCHOPF and BECKER (1933 and 1936), STRAIN (1938), and KUHN and BROCKMANN (1932). The brain of the fish was first pithed and the animal then dipped in boiling water for about 20 seconds. STRAIN (1938) found that the oxidation of carotenoid is greatly reduced if the material is put in water of 90–100°C for a short time. The skin and scales were then ground in sand with acetone. The acetone extracted the carotenoid pigments, while melanin and the red pigment later shown to be erythropterin remained in the residue. The acetone soluble pigments were transferred from acetone to petroleum ether by the addition of 10 cc of petroleum ether and water saturated with NaCl. The supernatant petroleum ether was separated and mixed with an equal quantity of absolute methanol. When sufficient water to dilute the alcohol to 80–90 percent was added, the methanol layer separated. The pigments remained in the petroleum ether layer, indicating that they must be carotenes, xanthophyll esters, or astacene, whereas pigments remaining in the methanol would have been xanthophyll. This indicates that the pigments exist as esters in the tissues. The petroleum ether solution was saponified by adding an equal volume of C_2H_5OH-KOH (5 percent) and warming for two hours at 40–50°C. This saponification converts xanthophyll esters into xanthophylls. After saponification, the pigments were extracted with petroleum ether by addition of water saturated with NaCl. Care has to be taken to prevent formation of colloidal solutions at this stage. The petroleum ether extract was again mixed with absolute methanol, and when water was added, two phases formed, and pigment passed into the hypophasic methanol layer. This indicated that the pigments were of the xanthophyll group. If pigment had passed into the petroleum ether layer at this stage, it would be suspected of being a carotene and would be tested by an adsorption method.

The separation of the xanthophyll pigments was accomplished by means of the micro-chromatographic adsorption method. The pigments were transferred to dichloroethane by addition of water saturated with NaCl. An adsorption column three mm in diameter was prepared from a suspen-

TABLE 2

Outline of procedure based on methods of SUMNER *and* FOX *(1933, 1935),* STRAIN *(1938),* KUHN *and* BROCKMANN *(1932)*



* Indicates pigments obtained in this investigation.

sion of a 1:2 mixture of magnesium oxide and Hyflo super-cel[2] in dichloroethane. When the pigment solution was poured on the adsorbent, a wide orange-red band formed at the top of the column. As this was further washed with dichloroethane, bands of pigment separated. Previously

[2] Hyflo super-cel F. A. 501, manufactured by Johns-Manville, is a heat treated siliceous earth for diluting the absorbent.

xanthophyll crystals had been obtained from Eimer and Amend specified as having been extracted from alfalfa. These were tested by the adsorption method, and four bands were obtained. Pigments separated from these bands were examined to obtain the adsorption maxima with a Zeiss "pocket" spectroscope. These gave adsorption maxima corresponding with those given by STRAIN (1938) for violaxanthin, zeaxanthin, lutein, and cryptoxanthin, respectively.

Confirmatory evidence that these were the pigments concerned was obtained from the order of adsorption on the chromatogram which was to order named above with the first, violaxanthin, being uppermost. This order and the colors corresponded with that reported by STRAIN. Also, the addition of concentrated H_2SO_4 to a dichloroethane solution of the pigment from band 2 gave a blue color for the acid and dichloroethane layer, which is a test for zeaxanthin (STRAIN 1938). Similar treatment of band 3 yielded blue color only in the acid layer, suggesting lutein (STRAIN 1938). When an ethereal solution of band 1 was treated with HCl, a light blue color appeared in the acid layer—a test for violaxanthin (FOX 1936).

The bands obtained from the fish extracts were separated and eluted with ethyl alcohol and transferred to dichloroethane. These separate solutions were then mixed respectively with the separate solution of the known individual alfalfa pigments and again readsorbed on separate columns. When only one band formed, the pigments were judged to be identical.

The red pigment of the erythrophores was extracted by grinding skin and scales with dilute ammonium hydroxide. It had been found very resistant to the solvent action of acetone, methyl alcohol, ethyl alcohol, petroleum ether, acetic acid, formalin, and hexane and had not been removed by boiling. The melanin (soluble in concentrated NH_4OH) remained in the residue. The deep red extract changed to yellow color upon acidification with HCl. These properties suggested a pterin pigment. A 0.004N HCl solution of the pigment was subjected to chromatographic adsorption on aluminum oxide and Hyflo super-cel (1–2) column. A single orange band formed, strongly adsorbed at the top of the column. Radiation of the band with mercury vapor quartz lamp changed it to a light brown color. This indicated that the pigment was probably erythropterin ($C_{19}H_{17-18}(O+H)_{20-21}$).

The above procedure was applied to the various species the following results:

Platypoecilus maculatus: Two varieties of this species (the names are those given by fanciers) were studied. The blood-red "platy" has xantho-erythrophores, and the analysis indicated that the red was erythropterin, and the yellow was due to zeaxanthin and lutein. The Golden Crescent Moon has a gold body color with a black band across the caudal peduncle and a red dorsal fin. The gold pigments were zeaxanthin and lutein, and the

red pigment of the dorsal fin was erythropterin. *Xiphophorus helleri:* "Blood-red" and "brick-red" varieties both have the xantho-erythrophores. The pigments were the same as in Platypoecilus. The red was erythropterin and the yellow zeaxanthin and lutein. The golden swordtail, which also has a red stripe, carried the same pigments. *Oryzias (haplocheilus) latipes:* the yellow type was used and the only pigment other than melanin was lutein. *Colisa lalia, Colisa fasciata,* and *Macropodus opercularis* all showed the presence of violaxanthin and lutein. *Betta splendens:* the yellow was lutein and the red erythropterin. A deep red variety and a yellow bodied type known to fanciers as the red betta and Betta cambodia, respectively, were utilized.

THE NON-MELANIN PIGMENTS OF FISH

In this paper we are chiefly concerned with pigments of the skin other than melanin. KRUKENBERG (1882), by the limited chemical and physical methods then available, described pigments from *Carassius auratus, Cyprinus carpio,* and *Muraena helena.* These were classified as lipochromes, a term since superseded. CUNNINGHAM and MAC MUNN (1893), working on the flat fishes, noticed a similarity of lipochromes in related fish. Since this early period there has been an extensive development of carotenoid chemistry, but this has included very little work on fish. LEDERER (1935) isolated astacene from *Carassius auratus* and *Bery decadactylus.* In Carassius he also discovered small quantities of carotene and xanthophyll. These results, in the course of work preparatory for this investigation, have been confirmed in our laboratory. LEDERER (1935) also found that carotene is present in *Pleuronectus flesus,* xanthophyll in *Scomber scombus,* while both are present in *Cottus bubalis* and *Herophis aequoreus.* FOX (1936) finds a pigment identical or isomeric with taraxanthin in two Pacific fish, the *Fundulus parvipinnus* and *Hypsypops rubicunda.* Pigments have been identified in other parts of fish, as in the muscle of salmon (EULER et al. 1933), in the eyes (LONNBERG 1937), and in various marine animal oils (BURKHARDT et al. 1934).

The blues, greens, purples, and silver white of many fish is due in part or wholly to reflection and diffraction and refraction of light from guanine crystals. (See discussion by RAUTHER 1927.) Blue pigment, however, has been described in Australian parrot fish of the genus Odax by FRANCIS (1875) who made some chemical studies. GOODRICH and HEDENBURG (1941) have noted a similar pigment in Bermuda parrot fish, and ZEYNECK (1901, 1902, 1912) considers the blue pigment in a Mediterranean wrasse *Crenilabrus parvo* to be a carotenoid albumen.

Pigments which upon further investigation we think may prove to be pterins have been described in numbers of fish. BALLOWITZ (1913) found

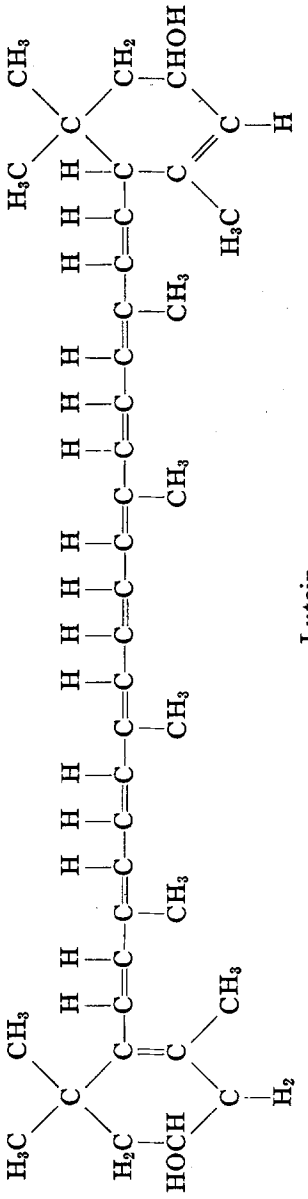
in certain fresh water fish (*Fundulus chaperi, Haplocheilus chaperi, Pantodon bucholsi*) a red pigment not soluble in alcohol or ether; he reported also the brown-red alcohol resisting cells in Xiphophorous and *Betta rubra*. BECHER (1924) found similar cells in Essox. KOSSWIG (1935) noted the presence of these relatively insoluble pigments in Platypoecilus and Xiphophorus and suggested that they might belong in the melanin series, but he reports no chemical investigation. A similar suggestion is made by BREIDER (1936). We do not know of any previous descriptions of a pterin from the integuments of fish. They have been found most abundantly in insects and among the metabolic products of vertebrates. (See review by LEDERER 1940.)

As noted above, it has been suggested that the red pigment of Platypoecilus and Xiphophorus may belong in the melanin series. It is true that certain of the transformation products between tyrosine and melanin have a brown or red color (hallochrome), but the possibility that the red pigment may be a melanin seems to us to be improbable on the basis of the results of our investigation.

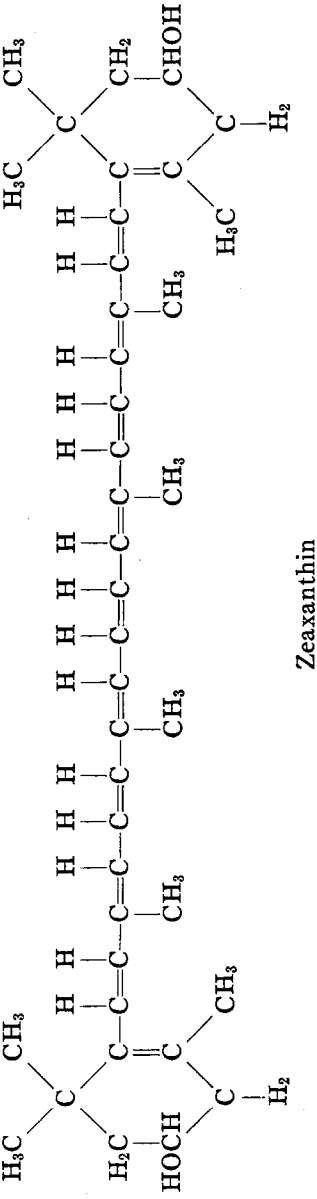
DISCUSSION

The three carotenoid pigments found in these fish are clearly similar xanthophylls. They were lutein (xanthophyll) $C_{40}H_{56}O_2$, zeaxanthin $C_{40}H_{56}O_2$, and violaxanthin $C_{40}H_{56}O_4$. The first two differ only by the position of a double bond. The structural formulae of these two is given below; that of violaxanthin is not known.

It was pointed out above that there were both red and orange cells in *Macropodus opercularis*, and this is also the case in *Colisa lalia*. These chromatophores seem to intergrade, although the intermediate cells are certainly less numerous than those of the two extremes. It may be that the two colors are due to the differing proportions of the two compounds lutein and violaxanthin and that one is a transformation product from the other. The intensity of color of these fish, and of other fish, seems to vary with age, metabolic conditions, and the breeding season or exposure to colored backgrounds (SUMNER 1940). These changes are not due merely to expansion or contraction of the cells but to the amount, intensity, and color of the pigment. A basis of such color changes may well be the existence of a series of transformable compounds such as those observed here. Many "improved" color strains of fish have been developed by fanciers, and it would be of interest to know of the existence of genetic color modifiers which may control these. So far, no genetic analyses have been made. It is perhaps not impossible, however, that fish may furnish material among the vertebrates which may show correlation between genetic constitution and the chemistry of pigments such as is known to exist in plants. (See reviews by LAWRENCE and PRICE 1940; SCOTT-MONCRIEFF 1937.)



Lutein



Zeaxanthin

TABLE 3

	YELLOW	RED OR ORANGE
Order Cyprinodontes		
Family Poeciliidae		
Platypoecilus maculatus (Platyfish)	Lutein Zeaxanthin	Erythropterin
Xiphophorus helleri (Swordtail)	Lutein Zeaxanthin	Erythropterin
Family Cyprinodontidae		
Subfamily Fundulinae		
Oryzias (Aplocheilus) latipes (Medaka)	Lutein	(No red pigment)
Order Labyrinthia		
Family Osphronemidae		
Macropodus opercularis (Paradise fish)	Lutein and violaxanthin	
Colisia lalia (Dwarf gourami)	Lutein and violaxanthin	
Colisia fasciata (Striped gourami)	Lutein and violaxanthin	
Betta splendens	Lutein	Erythropterin

As a matter of interest in relation to the problem of gene action it should be pointed out that in fish, as in many other forms, there is no "all or nothing" reaction. Most light colored fish have a few melanophores. There may possibly be a complete absence in a few true albino types as in Macropodus and in Xiphophorus (KOSSWIG 1935). Even the albinos, however, have xanthophores reduced in size and probably in number as compared with the yellow types. The gold types of Platypoecilus and Xiphophorus have a few xantho-erythrophores. We have no evidence of the quantitative action of a dominant gene causing incomplete dominance.

The pigment identifications reveal an expected similarity in the closely related types, Platypoecilus and Xiphophorus, which both possess the same three pigments—lutein, zeaxanthin, and erythropterin. Three of the four species of the family Osphronemidae—*Macropodus opercularis*, *Colisa lalia*, and *Colisa fasciata*—carry the pigment lutein and violaxanthin. The fourth species, *Betta splendens*, which is morphologically somewhat removed from the other three species, has a different assortment of pigments, lutein and erythropterin. Oryzias has only lutein. Thus there appears, ex-

cept possibly in the case of *Betta splendens*, a fair correlation with expectation of similarities based on taxonomic position. (See table 3.)

SUMMARY

Chemical identification was made of pigments present in the following fish: *Platypoecilus maculatus*, *Xiphophorus helleri*, *Oryzias latipes*, *Macropodus opercularis*, *Colisa lalia*, *Colisa fasciata*, and *Betta splendens*.

The pigments (other than melanin) appear to be the carotenoids, lutein (xanthophyll), zeaxanthin, violaxanthin, and the pterin, erythropterin. The carotenoids probably exist in the tissues in the form of esters.

The distribution of these pigments in relation to various mendelian color varieties of Platypoecilus, Xiphophorus, Oryzias, Macropodus, and Betta is outlined.

Platypoecilus and Xiphophorus varieties carrying the gene controlling the red color possess a peculiar cell, the xantho-erythrophore. This appears to be a xanthophore in which erythropterin is laid down in addition to the lutein and the zeaxanthin always present and is therefore the result of the specific action of this gene.

The relation of the distribution of pigments to the taxonomic position of the species is discussed. In the main, closely related types have the same pigments.

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