Histol Histopathol (1999) 14: 383-390
DOI: 10.14670/HH-14.383
http://www.hh.um.es

Histology and Histopathology
From Cell Biology to Tissue Engineering

Ultrastructural and biochemical analysis of epidermal xanthophores and dermal chromatophores of the teleost *Sparus aurata*

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Summary. We have studied the pigmentary system of the teleost *Sparus aurata* skin by electron microscopy and chromatographic analysis. Under electron microscopy, we found the dermis to contain the three major types of recognized chromatophores: melanophores, xanthophores and iridophores. Melanophores were more abundant in the dorsal region, whereas the iridophores were more abundant in the ventral region. The most important discovery was that of epidermal xanthophores. Epidermal xanthophores were the only chromatophores in the epidermis, something only found in *S. aurata* and in a teleost species living in the Antartic sea. In contrast, the biochemical analysis did not establish any special characteristics: we found pteridine and flavin pigments located mostly in the pigmented dorsal region. Riboflavin and pterin were two of the most abundant coloured pigment types, but other colourless pigments such as xanthopterin and isoxanthopterin were also detected.

Key words: Epidermal-xanthophores, Chromatophores, Teleost-skin

Introduction

Many lower vertebrate animals exhibit complex skin pigmentation patterns due to differential distribution of three basic types of pigment cells: melanophores, xanthophores, and iridophores and an extensive literature exists (Bagnara and Hadley, 1973; Bagnara, 1983; Fujii, 1993).

In recent years, a considerable effort has been made to study different aspects of the cutaneous pigmentary system in fishes (Obika and Meyer-Rochow, 1990; Blanchard et al., 1991; Wright and McBurney, 1992; Obika and Fukuzawa, 1993; Fujii, 1993), amphibians (Bagnara et al., 1988; Pederzoli and Trevisan, 1990; Bukowski et al., 1990; Trevisan et al., 1991; Denéfle and Lechaire, 1992; Fernández and Bagnara, 1993), reptiles (Morrison and Frost-Mason, 1991; Morrison, 1995), and birds (Oliphant et al., 1992; Oliphant and Hudon, 1993). These studies have been mostly carried out using electron microscopy to establish the number, distribution, and characteristics of the chromatophores, as well as their subcellular structures. Concomitantly, biochemical methods have also been applied to determine the chemical nature and occurrence of the main types of pigments responsible for the colour variations in the skin of these animals.

In fishes, as in other poikilotherms, variations in the colour of the skin are the result of the differential distribution of the three above mentioned types of pigment cells. The chromatophores contain intracellular organelles with pigments of different biochemical nature, from the widely occurring melanin to other more simple organic structures such as pteridines, flavins, purines and carotenoids (Fujii, 1993). These pigment cells are present in variable number depending on the skin area and fish species; in addition they show some mobility in the skin under control of the cytoskeletal microtubular network in the cytoplasm (Obika and Fukuzawa, 1993; Kimmler and Taylor, 1995), hormonal stimuli (Fujii, 1993) and the regulation by the sympathetic nervous system (Fujii and Oshima, 1986; Visconti and Castrucci, 1990; Hayashi and Fujii, 1993).

In the light of the extensive literature on poikilotermic pigmentation, it may not seem important to carry out similar studies on additional lower vertebrate species; however, the incentive to do this in this case has been provoked by the recent discovery of a new chromatophore type by Goda and Fujii (1995) the cyanophore. Cyanophores have been found so far only in two species of callionymid fish and the chemical nature of their pigment has not yet been elucidated. Moreover, it seems reasonable to further investigate piscine chromatophores because of the large number of species that exist in both freshwater and marine environments.

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Of these, it is primarily the freshwater chromatophores that have received most attention. We have extensively studied several aspects of melanin-containing cells in the marine teleost, *S. aurata* (Zuasti et al., 1990, 1993); however, we have not examined other chromatophore types in this species. Since *S. aurata* offers considerable advantages for our continuing pigmentary investigations, it seems appropiate to characterize its bright-coloured pigment cells at ultrastructural and biochemical levels.

The pigmentary pattern of *S. aurata* includes three clearly differentiated regions: the dorsal region, which is darkly pigmented and rich in melanin (Zuasti et al., 1990) the ventral region which is white and poor in melanin; and a lateral line with golden iridiscence that gives to this species its common name "gilthead porgy". This peculiar colouration makes this teleost fish a very interesting model for comparison of the number, distribution, and biochemical composition of the pigment contents of the chromatophores. We have found that this wide variation in coloration is misleading in that the structure and disposition of the cells is qualitatively the same in all three regions.

Material and methods

Young, healthy adult specimens of the teleost *S. aurata* were used in the present study. These fish were caught at the Oceanographic Institute (Murcia, Southeastern Spain). They were sacrificed and the dorsal, lateral and ventral skin was immediately removed and excised for ultrastructural and biochemical studies.

The samples prepared for electron microscopy observations were fixed for 2h at 4 °C in 2% glutaraldehyde buffered at pH 7.4 in a 0.1M sodium cacodylate solution containing 0.05M $CaCl_2$. The fragments were postfixed for 1h in a 1% osmiun tetroxide-buffered solution, washed with 0.1M cacodylate buffer, dehydrated and embedded in an Epon resin. Ultrathin sections were obtained using an LKB ultratome III, and they were stained with uranyl acetate and lead citrate. Finally, the samples were examined in a Zeiss EM 10C electron microscope.

The presence of flavins and pteridines in the *S. aurata* skin was explored by thin-layer chromatography (TLC), according to Pederzoli and Trevisan (1990). Fresh skin samples of around 1 cm^2 were weighed and immediately homogenized in a 70% ethanol solution, using a ratio 1:10 (w/v, g/ml). The homogenization was carried out in a Politron (Kinematika, Switzerland) by 4 strokes of 15 sec, with the power setting at position 7.

The homogenates were then centrifuged at 12000 g for 10 min in an Eppendorf table centrifuge to separate the non-solubilized material and connective tissue from the hydroalcoholic solution. The supernatants were maintained at 4 °C in the dark and immediately applied to TLC plates. The chromatographies were run for 1h in the dark on HPTLC 5631 silica gel plates (Merck), previously activated at 100 °C, using a mixture of n-propanol/n-butanol/7% ammonium hydroxide (1/2/1) as solvent. 10μl of the biological samples and 2 μl of the standard pigment solutions (0.1 mg/ml in 70% ethanol) were applied to the TLC plates.

The standard solutions were freshly prepared and protected from light due to their photosensitivity. Immediately after the chromatography, the plates were observed under UV light and photographed to determine the Rf values of the fluorescent spots. Pigments in the fish skin samples were identified by comparison to the commercially available standards: flavin adenine mononucleotide, flavin adenine dinucleotide, riboflavin, neopterin, pterin, bathopterin, xanthopterin, isoxanthopterin and pterin-6-carboxylic acid. All of them were purchased from Sigma Chem. Co. (St. Louis, MO, USA). The analysis was qualitative rather than quantitative, and the abundance of the different pigments was presented as a scale ranging from (+, fairly visible fluorescent spot, lower concentration than the standard) to (+++, very prominent spot, higher concentration than the standard).

Results

As is generally the case for the integument of fishes, the dermis of *S. aurata* was found to contain the three types of pigment cells: melanophores, iridophores and xanthophores. In contrast, the epidermis of the *S. aurata* was quite peculiar in that only xanthophores could be found. Concerning the dorsal and ventral skin pattern, melanophores were the most numerous pigment cells in dorsal skin, whereas iridophores were most abundant in the ventral skin. The mid lateral golden line showed numerous xanthophores. Hence, all three dermal regions had qualitatively the same structure and cell distribution.

In Fig. 1 we can see the distribution of chromatophores in the dermis. Close to the basal lamina there were iridophores surrounded by melanophores (in general, we could also find melanophores surrounded by iridophores, see Fig. 2). Near these cells, but deeper the xanthophores were found (Fig. 1, insert). Between all these cells collagen fibers of the connective tissue of the dermis were always found.

Melanophores rich in melanin granules were located in the dermis, beneath the basal lamina of the epidermis, but none were observed in the epidermis. The morphology of these cells was irregular, with many cytoplasmic processes which contained melanosomes (Fig. 2). The nuclei were centrally located and showed peripheral chromatin and nuclear envelope with slight indentations. There were a large number of dark melanosomes full of melanin, which were the most common organelle in the cells throughout the cytoplasm. The shape of these melanin granules was rounded or oval, with uniformly electron-dense pigment (Figs. 2, 3). Other subcellular structures, such as small mitochondria, scarce lipid droplets, endoplasmic reticulum, Golgi apparatus and small vesicles could also be observed (Fig. 3).

The xanthophores were also dendritic cells

distributed in the dermis as well as in the epidermis. Dermal xanthophores were mostly elongated and surrounded by collagen fibers. They showed a prominent heterochromatic nucleus, a cytoplasm with very few mitochondria, scarce endoplasmic reticulum, and a Golgi apparatus. The most characteristic property of these cells was that in the cell body, as well as in the dendritic processes, we found carotenoid vesicles and pterinosomes. The pterinosomes are classified as immature and mature according to the degree of lamellar differentiation (Obika, 1993). The majority of xanthophores in the dermis of the *S. aurata* contained carotenoid vesicles (Fig. 4a) and immature pterinosomes with fibrous material of different electrondensity (Fig. 4b) in their cytoplasm. Mature pterinosomes, characterized by the presence of vesicles containing well developed concentric lamellar structures, were scarce.

Epidermal xanthophores were located between the keratinocytes over the basal layer of the epidermis. They were morphologically similar to the dermal xanthophores. These xanthophores showed dendritic processes reaching the keratinocytic layers. The main difference was that we found mature pterinosomes with partially formed lamellar structures in the cytoplasm and in the dendritic processes (Fig. 5).

The iridophores were more abundant in ventral than dorsal skin, but they were again located exclusively in the dermis and usually close to melanophores. Their shape was ellipsoidal, rounded or elongated, but with a number of dendritic processes interdigitated with the processes of the melanophores (Figs. 2, 6). These cells had an irregular, indented, ellipsoid lobed nucleus which was centrally located. The main characteristic of the iridophores was the presence of reflecting platelets in their cytoplasm including their dendritic processes. These organelles were arranged as stacks of 5-10 rounded platelets with variable orientation. Each platelet was surrounded by a single membrane that probably contains a high concentration of purines. However, the contents of these subcellular structures were removed by the dissolution during fixation, so that they often appeared as empty spaces or holes on the micrographs, but in well preserved preparations they could be observed filling at least part of the space (Fig. 2). The cytoplasm of iridophores also contained mitochondria, endoplasmic reticulum and small vesicles distributed through the cytoplasm. Golgi apparatus was scarcely found (Fig. 6).

The biochemical analysis of the pigment content of the integument revealed that the pigmented dorsal region

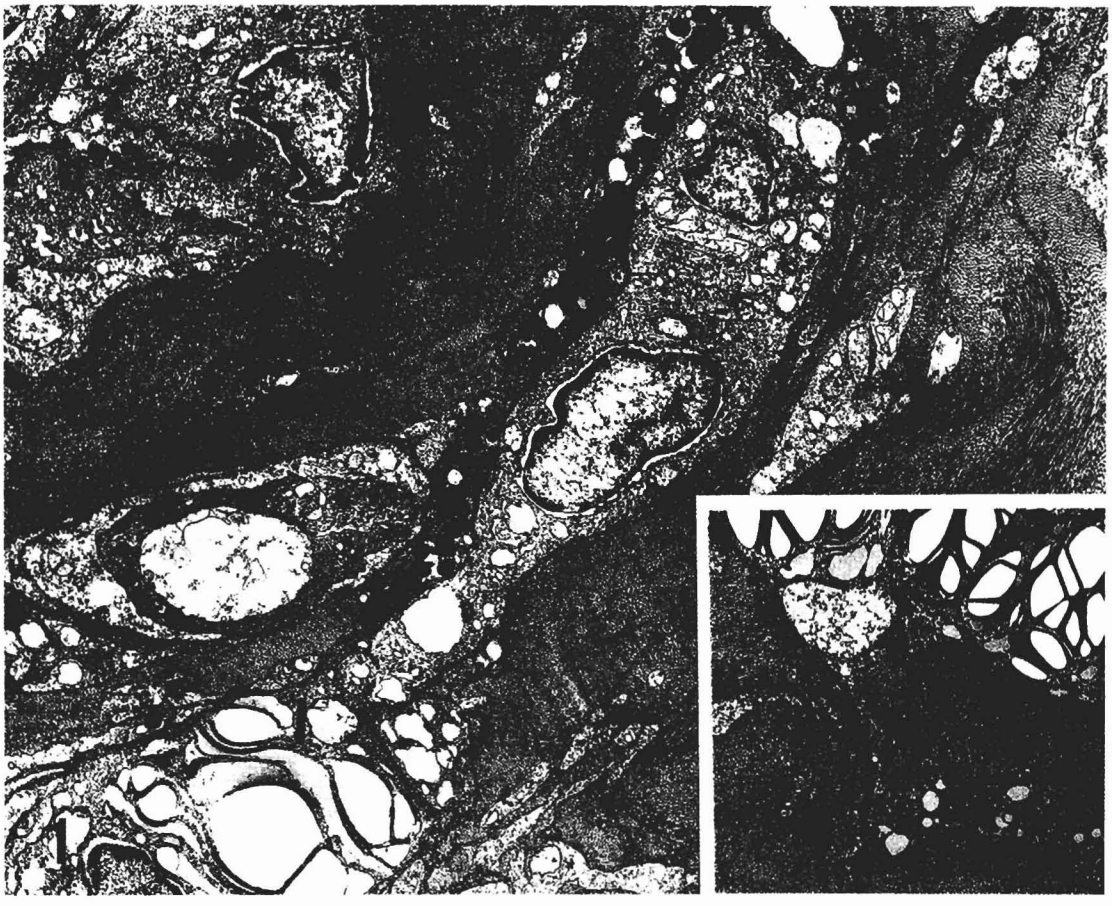

Fig. 1. Electron micrograph of the chromatophores located in the dorsal dermis, beneath the basal lamina (bl) of the epidermis. The processes of the melanophores (M) surround an iridophore (I). c: ollagen fibers. x 6,000. Insert: Deeper in the dermis we find xanthophores (X). x 4,000

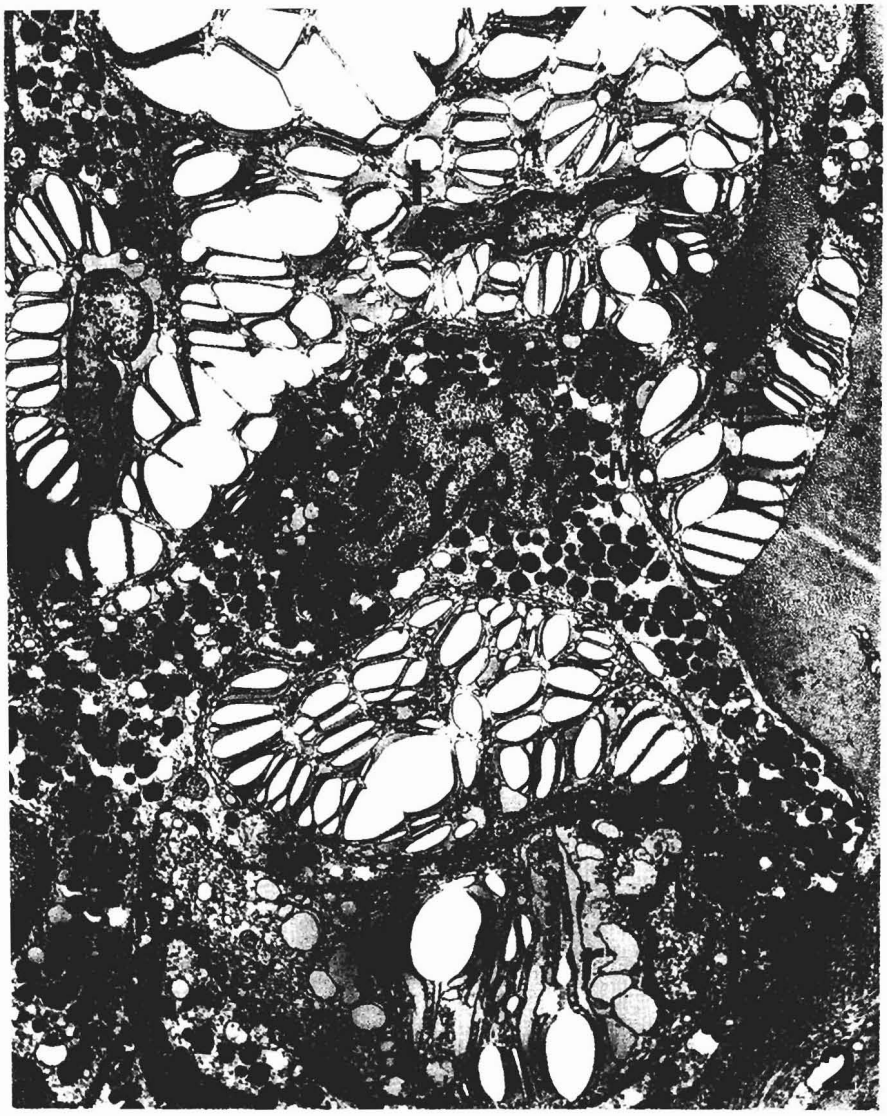

Fig. 2. Dermal melanophore (M) with dendritic processes interdigitated with numerous iridophores (I). In the lower part of the picture the subcellular structures of reflecting platelets (r) can be seen. x 6,500

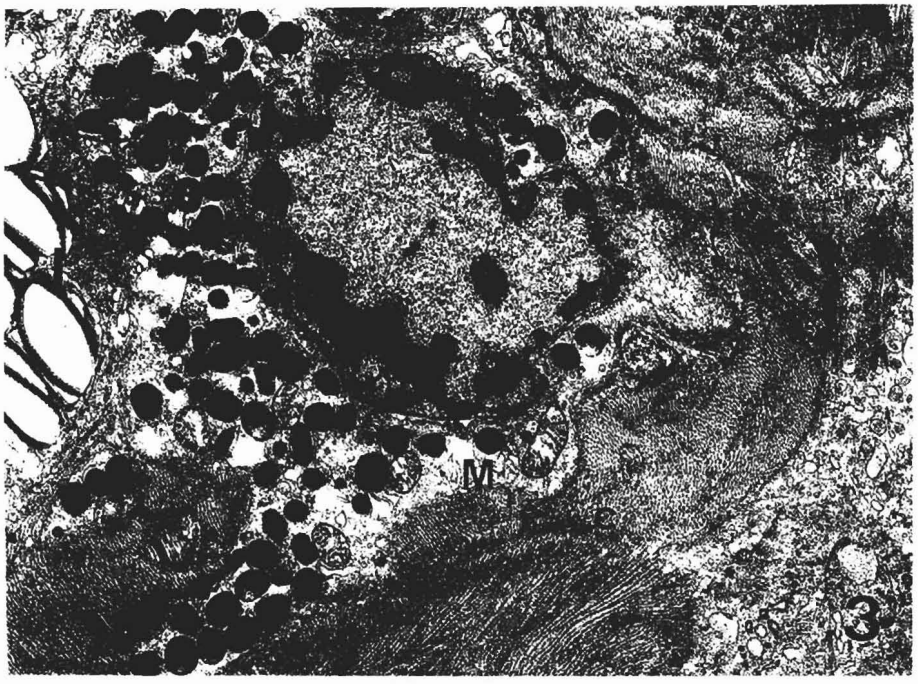

Fig. 3. There are numerous melanosomes in the cytoplasm of the melanophore (M). Other organelles are scarce. I: ridophores; c: collagen fibers. x 12,000

of *S. aurata* is richer than the ventral region not only in melanin, but also in other unrelated pigments. They included pteridines and flavins, which were especially evident in young animals, where the extraction by the hydroalcoholic solution was more efficient. The analyses revealed the presence of significant amounts of flavin adenine dinucleotide, pterin, and riboflavin. We also detected noticeable amounts of xanthopterin and isoxanthopterin (Table 1), presumably associated to xanthophores, as reported elsewhere, and another two unidentified compounds. The definitive location of these pigments in iridophores should await for the separation of pure xanthophores and iridophores.

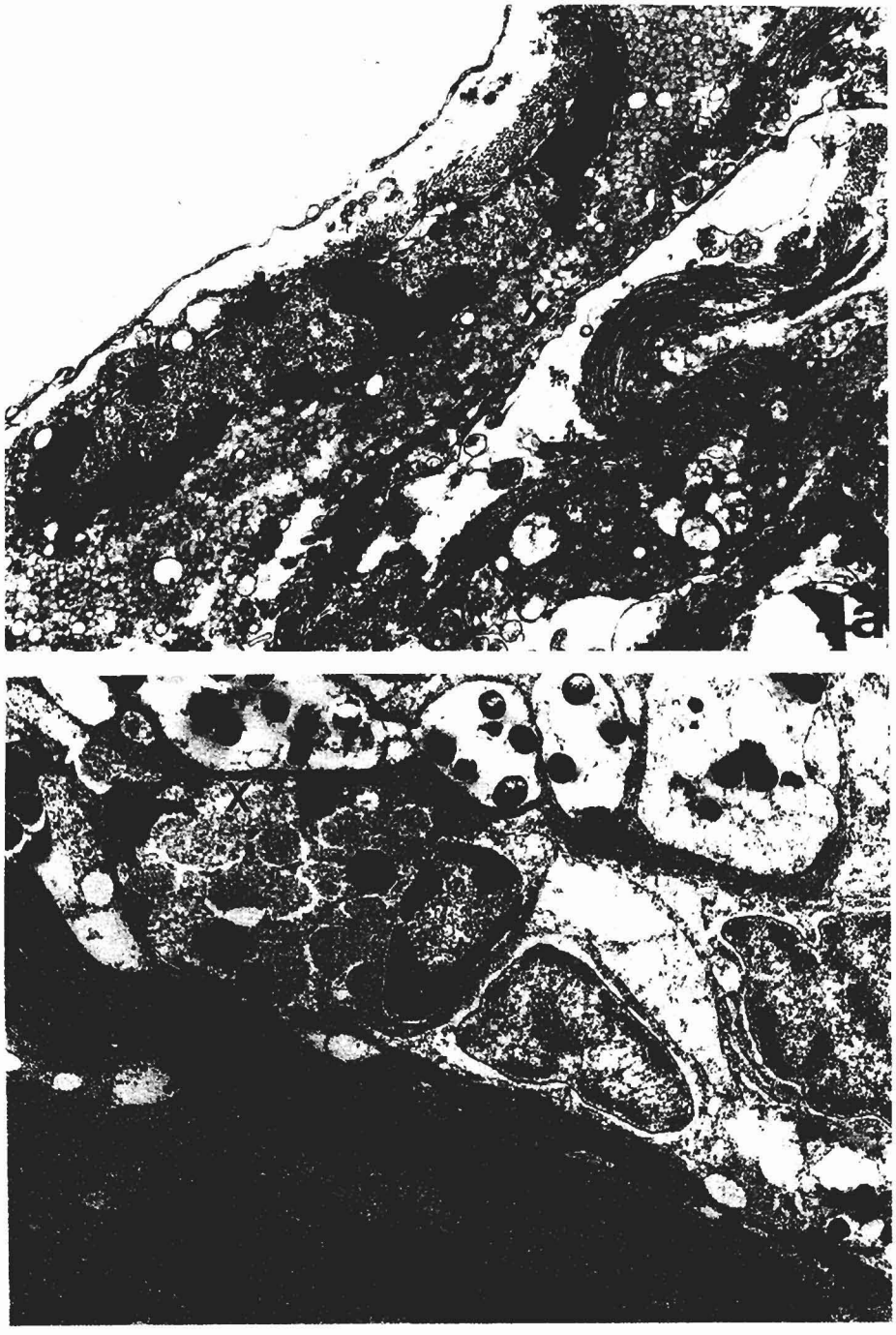

Fig. 4. Dermal xanthophores (X). **a.** In the cytoplasm there are numerous carotenoid vesicles. x 12,500. **b.** The pterinosomes contain fibrous material of different electrondensity. x 1,000

Discussion

S. aurata is a teleost fish which contains the three major types of chromatophores in its skin: melanophores, xanthophores and iridophores. The ultrastructure of these pigment cells has been studied and compared with those reported in other lower vertebrates. The melanophores of *S. aurata* were located in the dermis, and as is often the case with fish (Fujii, 1993), epidermal melanophores were absent, just as with most poikiloterme. Melanophores were much more abundant in the pigmented dorsum than in the white ventral skin. This distribution is similar to that found in other fish species (Kasukawa and Oshima, 1987; Obika and Meyer-

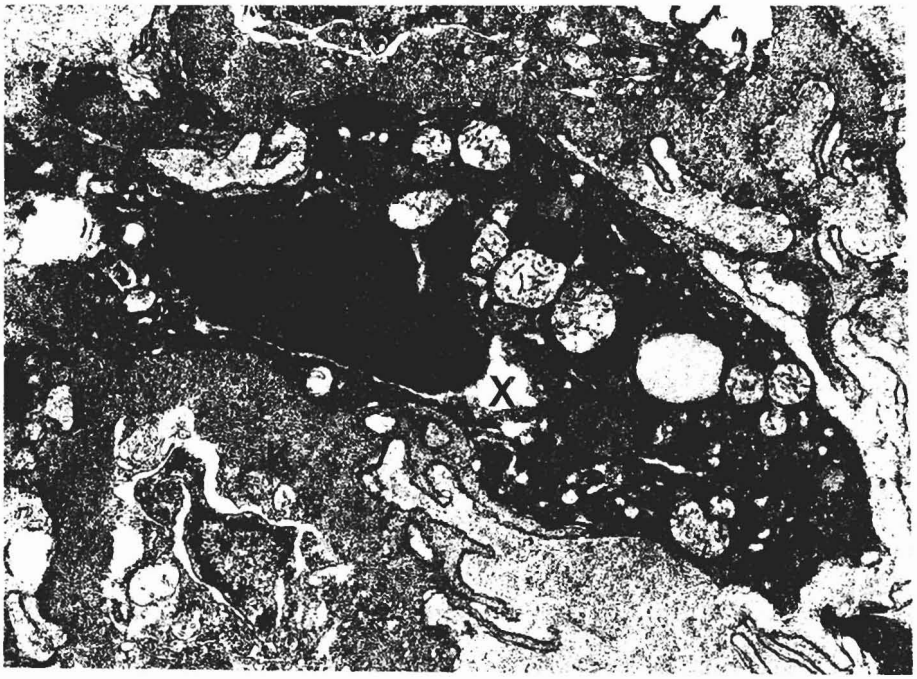

Fig. 5. Epidermal xanthophore (X) located between keratinocytes (K). In the cytoplasm there are immature and mature pterinosomes with lamellar structures. x 18,000

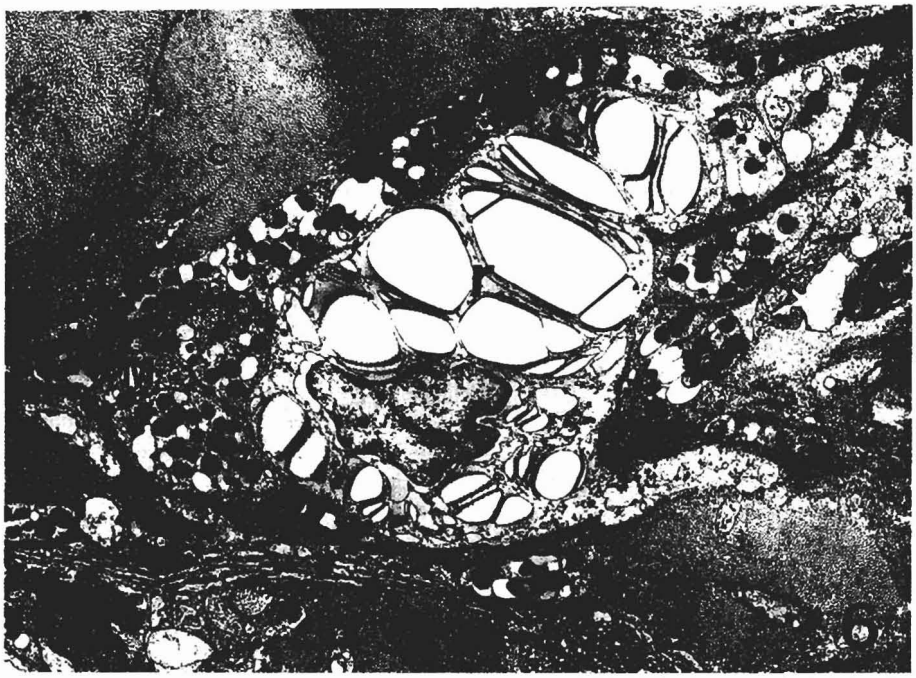

Fig. 6. Dermal iridophore of the ventral skin characterized by the numerous stacks of reflecting platelets (r) in the cytoplasm. c: collagen fibers; M: dendritic processes of melanophores. x 6,000

Table 1. Non-melanin pigment found in dorsal skin of *Sparus aurata*

STANDARD PIGMENTS	R_f	OCCURRENCE IN *SPARUS AURATA*
Bathopterin	0.33	-
Flavin adenin mononucleotide	0.46	-
Flavin adenin dinucleotide	0,08	+++
Isoxanthopterin	0.21	+
Neopterin	0.20	-
Pterin	0.36	+++
Pterin-6-carboxylic acid	0.12	-
Riboflavin	0.43	+++
Xanthopterin	0.14	+

-: not detected; +: traces; +++: abundant.

Rochow, 1990; Zuasti et al., 1990), amphibians (Denéfle and Lechaire, 1992; Fernández and Bagnara, 1993), and reptiles (Gopalakrishnakone, 1986; Morrison, 1995).

Iridophores were also located exclusively in the dermis, usually close to melanophores, but they were more numerous in ventral than in dorsal skin, as reported for other species of fish (Gundersen and Rivera, 1982; Kasukawa and Oshima, 1987; Obika and Meyer-Rochow, 1990; Wright and McBurney, 1992). These chromatophores are highly variable in appearance, and a relationship between shape and mobility has been proposed (Kasukawa and Oshima, 1987; Matsuno and Iga, 1989). *S. aurata* showed two types of dermal iridophores, either dendritic or rounded ellipsoids. Most of them were dendritic, and their dendritic processes were often interdigitated with those of melanophores. Their cytoplasm was filled with reflecting platelets, which is responsible for their silvery appearance. Furthermore, the ventral region of the fish, which contained a preponderance of iridophores appears silvery. *S. aurata* iridophores displayed well developed smooth and rough endoplasmic reticulum, but the Golgi apparatus was scarce. These observations suggest that the single-membrane reflecting platelets are derived from the endoplasmic reticulum (Bagnara et al., 1979; Kamishima, 1981), rather than from double-membrane saccules associated with Golgi apparatus (Morrison and Frost-Mason, 1991; Wright and McBurney, 1992).

In contrast to melanophores and iridophores, xanthophores in *S. aurata* were located in both the dermis and the epidermis. This is remarkable because up to now, epidermal xanthophores have only been described in the Antarctic teleost *Trematomus bernacchi* (Obika and Meyer-Rochow, 1990). In *S. aurata*, the morphology of dermal and epidermal xanthophores was similar, although they differed in that we only found mature pterinosomes in the epidermal xanthophores. The dermal xanthophores contained carotenoid vesicles and immature pterinosomes, as described in other species of fish (Wakamatsu et al., 1987; Obika and Meyer-Rochow, 1990), but mature pterinosomes were scarce. On the other hand, in epidermal xanthophores mature pterinosomes predominated. It has been proposed that differences in the type of pterinosomes present could reflect different physiological states of the fish with respect to age or reproductive condition (Blanchard et al., 1991).

Concerning the localization of the skin pigments, it is clear that melanin is mostly located in melanophores (Zuasti et al., 1990). According to the well established literature (Bagnara, 1983; Bagnara and Hadley, 1973), xanthophores should contain basically pteridine pigments, and iridophores purines. However, chemical and functional distinction between pterinosomes and reflecting platelets is not as clear-cut as was believed a few years ago (Oliphant and Hudon, 1993). In fact, Bagnara et al. (1988) have reported pteridine pigments in isolated amphibian reflecting platelets. Moreover, the relative abundance of these pigments among species varies. The nature of these non-melanin pigments has been described in a variety of species of lower vertebrates, including both iris and integumental pigmentation (Bagnara and Hadley, 1973; Oliphant, 1987). Isoxanthopterin, neopterin, pterin, sepiapterin and xanthopterin are the most abundant pigments found in fishes (Wakamatsu et al., 1987; Blanchard et al., 1991). However, these are colourless or pale yellow pigments, so the golden colour might be due to carotenoids rather than to pteridines.

In our system, the high content in riboflavin found in *S. aurata* skin suggests that this compound could contribute to the colour of the dorsal region of this fish. Some of the pteridine pigments that we found in *S. aurata* could also possibly contribute to the final colour of the fish by means of structural colour. These pigments could be in crystalline form, possibly associated with purines in the reflecting platelets of the iridophores, as Bagnara et al. (1988) showed in tadpole chromatophores.

Isoxanthopterin and xanthopterin were identified in *S. aurata* samples, but no pterin-6-carboxylic acid, a common breakdown product of other pteridines was found. Thus, the composition of the hydroalcoholic extraction mixtures seems to be an authentic reflection of the actual "in vivo" content, free of artifacts due to decomposition or photodestruction of the samples during the extraction procedure and TLC analysis.

Essentially, we found that pigment cells were located in the dermis of the fish, although some xanthophores were also found in the epidermis. We have also established the nature and relative concentrations of several pteridin and flavin pigments present in the dorsal and ventral regions.

Acknowledgements. We are grateful to Professor J.T. Bagnara for his interest in this work and his critical comments. This work has been partially supported by the grant no. PB94/1158 from the DGICYT, Spain.

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Accepted September 1, 1998