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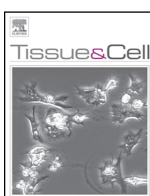

Morphological, immunohistochemical and ultrastructural characterization of the skin of turbot (*Psetta maxima* L.)

L.D. Faílde[a,*], R. Bermúdez[b], F. Vigliano[c], G.A. Coscelli[a,d], M.I. Quiroga[a]

[a] Departamento de Ciencias Clínicas Veterinarias, Universidad de Santiago de Compostela, 27002 Lugo, Spain
[b] Departamento de Anatomía y Producción Animal, Facultad de Veterinaria, Universidad de Santiago de Compostela, 27002 Lugo, Spain
[c] Cátedra de Histología y Embriología, Facultad de Ciencias Veterinarias, Universidad Nacional de Rosario, S2170HGJ, CC 166 Casilda, Argentina
[d] Cátedra de Patología General, Anatomía y Fisiología Patológicas, Facultad de Ciencias Clínicas Veterinarias, Universidad Nacional de Rosario, Bv. Ovidio Lagos y Ruta 33, 2170, Casilda, Argentina

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ABSTRACT

This study was undertaken to identify the normal morphologic, immunohistochemical and ultrastructural features of skin of the turbot (*Psetta maxima* L.). In the turbot skin, three morphologically distinct layers were identified: epidermis, dermis and hypodermis. The epidermis was non-keratinizing, stratified squamous epithelium that varies in thickness from 5 to 14 cells and 60 to 100 μm in size. Goblet cells were seen randomly distributed between malpighian cells in the epidermal layer. These mucous cells were mainly located in the upper third of the epidermis and displayed a spherical to elongated morphology. Dermis was divided in two well-differentiated layers, the superficial stratum laxum and the deeper stratum compactum. Hypodermis was a loose layer mainly composed by adipocytes but we could observe variable amounts of fibroblast, collagen and blood vessels. In turbot two pigmentary layers could be identified: the pigmentary layer of dermis was located between basement membrane and dermis and the pigmentary layer of hypodermis immediately above the muscular layer. Three different types of chromatophores were present: melanophores, iridophores and xanthophores. The main differences observed between groups of fish with different colouration were in the amount of melanophores and xanthophores. The purpose of this article is to provide an overview of normal cutaneous biology prior to consideration of specific cutaneous alterations and diseases in turbot.

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1. Introduction

The skin, the largest organ of the body of the fish, exerts multiple vital protective functions against environmental aggressions and is crucial for communication between individuals (Elliot, 2000; Roberts, 2001; Rakers et al., 2009). It is considered a sensitive organ, and the maintenance of the characteristics of a healthy integument is important from both pathological and economical points of view. Cutaneous lesions are common in fish and ulcerative diseases are one of the most usual pathologies affecting both cultured and wild fish (Noga and Udomkusonsri, 2002). The lesions in the skin of fish cause significant economic losses in aquaculture worldwide because the affected animals cannot be marketed, or its value could decrease (Toranzo et al., 2005; Vilar et al., 2012). Moreover, the epidermal damage provides an access way for the infectious agents and it also produces an osmotic stress that can be life threatening for the fish. Therefore, the knowledge of the structure and morphometry of the healthy skin of fishes is necessary in order to correctly interpret pathological changes (Ferguson, 2006). In addition, fish skin models are being used in dermatology research and as models of a wide variety of human diseases (Rakers et al., 2009).

Colouration in fish takes part in different biological functions like concealment or camouflage, and intraspecific or interspecific communication (Groff, 2001). In addition, pigmentation is an important issue from a commercial point of view since malpigmentation is common in hatchery-reared flatfishes, decreasing the market value of whole fish (Bolker and Hill, 2000). Moreover, achieving a uniform colouration is crucial for fish farms to reach a homogeneous stock of fish suitable for the different market areas, although this goal is difficult because pigmentation is influenced by environment and diet (Fujii, 1993; Bolker and Hill, 2000). For these reasons, aquaculture farms are making a great effort to know the cells and understand factors that influence the pigmentation and thus improve the quality of the final product. Besides, in the last years the pigmentation of the fish skin has gained a growing

* Corresponding author. Tel.: +34 982 820 00022306.
E-mail addresses: luisdaniel.failde@usc.es, danielfailde@hotmail.com (L.D. Faílde).

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attention by the pathologists and producers because cutaneous discoloration has been described associated with some infectious diseases in fish (van Gelderen et al., 2009; Magi et al., 2009; Vilar et al., 2012) and they are considered early lesions (Corrales et al., 2009; Vilar et al., 2012).

Pigmentation in fish is the result of the interaction between different pigmentary cells named chromatophores located in the pigmentary layers of the animals (Bagnara et al., 1968; Bagnara and Hadley, 1973; Prum and Torres, 2003). There are several types of chromatophores involved in the colouration of lower animals, including melanophores (black or brown), xanthophores (orange or yellow), erythrophores (red), leucophores (whitish) and iridophores (metallic or iridescent) (Fujii, 1993, 2000). Recently, a new class of chromatophore named erythro-iridophore has been described (Goda et al., 2011).

Despite the great importance of cutaneous diseases and malpigmentation in turbot, no studies have been addressed on the histology of normal skin in turbot. Therefore, the aim of the current study was to obtain a better characterization and knowledge of the healthy turbot skin, using histological, immunohistochemical and ultrastructural techniques. In addition, description and localization of the chromatophores and cells from the immune system in skin were provided.

2. Materials and methods

2.1. Fish

Fifteen healthy turbot (weight 4492 ± 2.86 g) obtained from a fish farm in Northwest Spain were employed for histological and ultrastrucutral studies. Animals used for histological studies were divided in three groups according with the pigmentary pattern they showed: brown-yellowish, brown-greyish and dark, and one turbot was used to ultrastructural studies.

2.2. Light microscopy

Skin pieces of 1 cm^3 were taken from several locations of the dorsal and ventral surface of turbot skin of 15 specimens. Samples were fixed in 10% neutral buffered formalin, dehydrated in alcohol, cleared in xylene and embedded in paraffin. Thin sections (2–3 μm) were stained with haematoxylin-eosin (H-E), alizarin red, Gallego's trichrome, periodic acid Schiff-alcian blue (PAS-AB) and toluidine blue (Bancroft and Stevens, 1996). For the measurement of epidermis images of the skin were captured using an Olympus DP12 camera connected to a photomicroscope Olympus BX50. The epidermis in the digitized images was measured using Adobe Photoshop 6.0.

Other samples were embedded in cryoprotectant (Tissue Tek®, Netherlands), frozen in liquid nitrogen, cut 30 μm thickness by means of a cryostat at −25 °C, placed in silane coated slides and observed in fresh to prevent the loss of carotenoid pigments during processing of samples.

2.3. Transmission and scanning electron microscopy (TEM and SEM)

For TEM, small pieces from several points of skin of 15 specimens were sampled, fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for 4 h at 4 °C. Afterward, they were post-fixed in 1% osmium tetraoxide in 0.1 M cacodylate buffer, dehydrated and embedded in epoxy resin. Ultrathin sections (80 nm) were stained with uranyl acetate and then observed with a JEOL JEM-1011 transmission electron microscope. Fifteen samples for SEM, after dehydrated, critical point drying was done and finally coating with gold. Samples were observed with a JEOL JSM-6360LV scanning electron microscope.

2.4. Immunohistochemistry

For the immunohistochemical assay paraffin embedded tissues were used, all incubations were performed at room temperature in a humid chamber and all washing procedures consisted of three successive 5 min immersions in phosphate-buffered saline (PBS; 8 mM Na_2HPO_4, 3 mM NaH_2PO_4, 150 mM NaCl, 0.5% (v/v) Tween 20, pH 7.4). Endogenous peroxidase activity was quenched after incubation for 30 min with Peroxidase Blocking Reagent (Dako, Denmark). Antigen retrieval was performed using heat in a pressure cooker, or enzyme treatments (Table 1). Then, sections were washed and incubated with the antibodies indicated in Table 1, washed and incubated for 30 min with an anti-rabbit EnVision+ System Labelled Polymer-HRP (Dako). After rinsing, the sections were finally developed using as chromogen diaminobenzidine (Dako) or Vector VIP (Vector Laboratories, UK), washed in water, counterstained with haematoxylin, dehydrated and mounted. Negative controls were carried out substituting the primary or the secondary antibody for PBS or an irrelevant polyclonal antibody. Adequate sections of mammalian tissues were used as positive controls.

3. Results

On light microscopy, the skin of turbot from both the dorsal and ventral side was organized in three layers: epidermis, dermis and hypodermis (Fig. 1(a)).

The epidermis showed a non-keratinizing stratified squamous epithelium that varied in thickness from 5 to 14 cell layers and 60 to 100 μm in size in both dorsal and ventral side. Malpighian cells were the predominant epidermal cell and displayed different morphologies from basal to superficial location; just upon the basal membrane, epithelial cells were cubical, in the middle of epidermis showed columnar shape and in the upper region they had a flattened morphology (Fig. 1(b)). Ultrastructurally, these cells possessed microridges located in the apical pole (Fig. 1(c)) and were connected to adjacent cells by tight junctions and desmosomes (Fig. 1(c)). In the cytoplasm, epithelial cells contained a large

Table 1
Data of antibodies used in the study.

Primary antibody	Clon/reference	Result	Dilution	Time (h)	Antigen retrieval
CD3	Polyclonal/Dako IR503/IS503	+	Ready to use	2	15′ tris EDTA pH 9
Cytokeratin WSS	Polyclonal/Dako N1512	+	1/500	2	10′ citrate pH 6
Enolase	Polyclonal/Biomeda 215M	–	1/15	2	10′ citrate pH 6
GFAP	Polyclonal/Biomeda V2023	–	1/50	2	15′ citrate pH 6
IgM	Polyclonal/Dr. Karl Pedersen	+	1/10,000	2	15′ citrate pH 6
Lysozyme	Polyclonal/Dra. Ariadna Sitjà	+	1/400	2	5′ proteinase K
N-cadherin	GC 4/Sigma–Aldrich C3865	–	1/50	1	20′ citrate pH 6
S100	Polyclonal/Neomarkers RB-044-A0	+	1/400	2	15′ citrate pH 6
Vimentin	V9/Dako M0725	–	1/200	2	15′ citrate pH 6

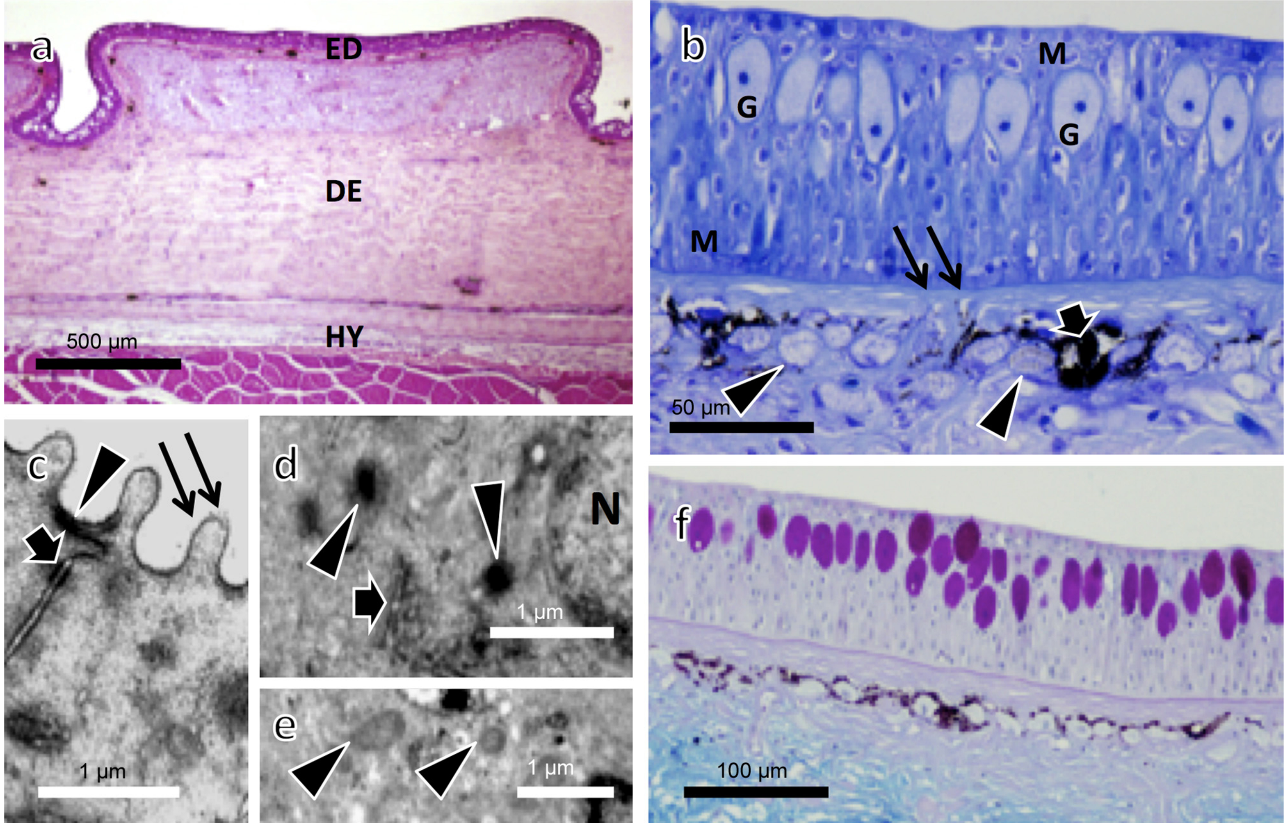

Fig. 1. Skin of turbot. (a) Three layers of skin: epidermis (ED), dermis (DE) and hypodermis (HY). H-E. (b) Malpighian cells (M) and goblet cells (G), basal membrane (double arrow). Melanophore (arrow), iridophores (arrowheads). Toluidine blue. (c) Superficial epithelial cells with tight junctions (arrowhead), desmosomes (arrow) and microridges (double arrow). TEM. (d) Lysosomes (arrowheads), rough endoplasmic reticulum (arrow) and nucleus (N). TEM. (e) Detail of epithelial cell with mitochondria (arrowheads). TEM. (f) PAS positive goblet cells in the epidermis. PAS-AB.

number of mitochondria close to the nucleus, rough endoplasmic reticulum (rER), and lysosomes (Fig. 1(d and e)).

Goblet cells were randomly distributed between Malpighian cells in the epidermis. These mucous cells occupied the entire thickness of the epidermis and displayed a spherical to elongated morphology (Fig. 1(b)). Their pale cytoplasm was dilated due to their granular content and the nucleus was located centrally or in the basal pole of the cell (Fig. 1(b)). These cells showed a strong PAS positive reaction in their cytoplasm (Fig. 1(f)) whereas no positive reaction to the alcian blue was observed. At TEM level, the cytoplasm of goblet cells displayed granular appearance with a large number of secreting vesicles with different electrodensity (Fig. 2(a)) which coalesced in the apical pole prior to discharge their content (Fig. 2(b and c)). Mucous cells were often open to the epidermal surface through an apical pore and were connected by desmosomes to adjacent epithelial cells (Fig. 2(c and d)). In the luminal surface of epithelial cells, numerous microridges formed a typical fingerprint pattern was observed (Fig. 2(d)).

Epidermis was separated from dermis by the basement membrane, which showed light blue colour with Gallego's trichrome staining (Fig. 3(a)). Ultrastructurally, basement membrane displayed two layers, an electron-lucent layer (lamina lucida) and below an electron-dense (lamina densa). Cell membranes of the basal malpighian cells were attached to basement membrane by means of hemidesmosomes (Fig. 3(b)). Immediately under the basement membrane, dense parallel bundles of collagen were firmly attached (Fig. 3(b)).

The dermis was divided in two well-differentiated layers, the superficial stratum laxum and the deeper stratum compactum (Fig. 3(a)). Stratum laxum contained different amounts of collagen fibres, reticulin fibres and pigmentary cells forming a loose layer. Stratum compactum was formed by densely packed bundles of collagen fibres organized mostly parallel to the skin surface (Fig. 3(a)). In some areas, stratum laxum was absent, so that stratum compactum was in direct contact with basement membrane (Fig. 1(a)).

Turbot skin had neither scales nor scale pockets. On the contrary, skin showed bony tubercles with conical shape located in the dermis. These tubercles were composed by a great amount of collagen fibres and small amounts of calcium on the top (Fig. 3(c and d, d inset)).

Hypodermis was mainly composed by loose connective tissue with a high number of adipocytes and a variable amount of fibroblasts, collagen and blood vessels (Fig. 1(a)).

In turbot, two pigmentary layers could be identified: the pigmentary layer of dermis was located between basement membrane and dermis, and the pigmentary layer of hypodermis immediately above the muscular layer. Three different types of chromatophores were present: melanophores, iridophores and xanthophores (Fig. 4(a and b)). Generally, chromatophores were arranged following the same pattern, with the melanophores and xanthophores interrelated in the most superficial level and the iridophores placed immediately under the former.

In unstained fresh sections, xanthophores displayed an orange to yellow colour due to the pigments contained in their cytoplasm. They were randomly distributed between melanophores and in close contact with iridophores throughout the pigmentary layer of dermis (Fig. 4(b and c)). By means of TEM, these cells showed a stellate shape and cytoplasmic granules of several sizes with different electrodensity as well as an irregular nucleus peripherally located (Fig. 4(c)).

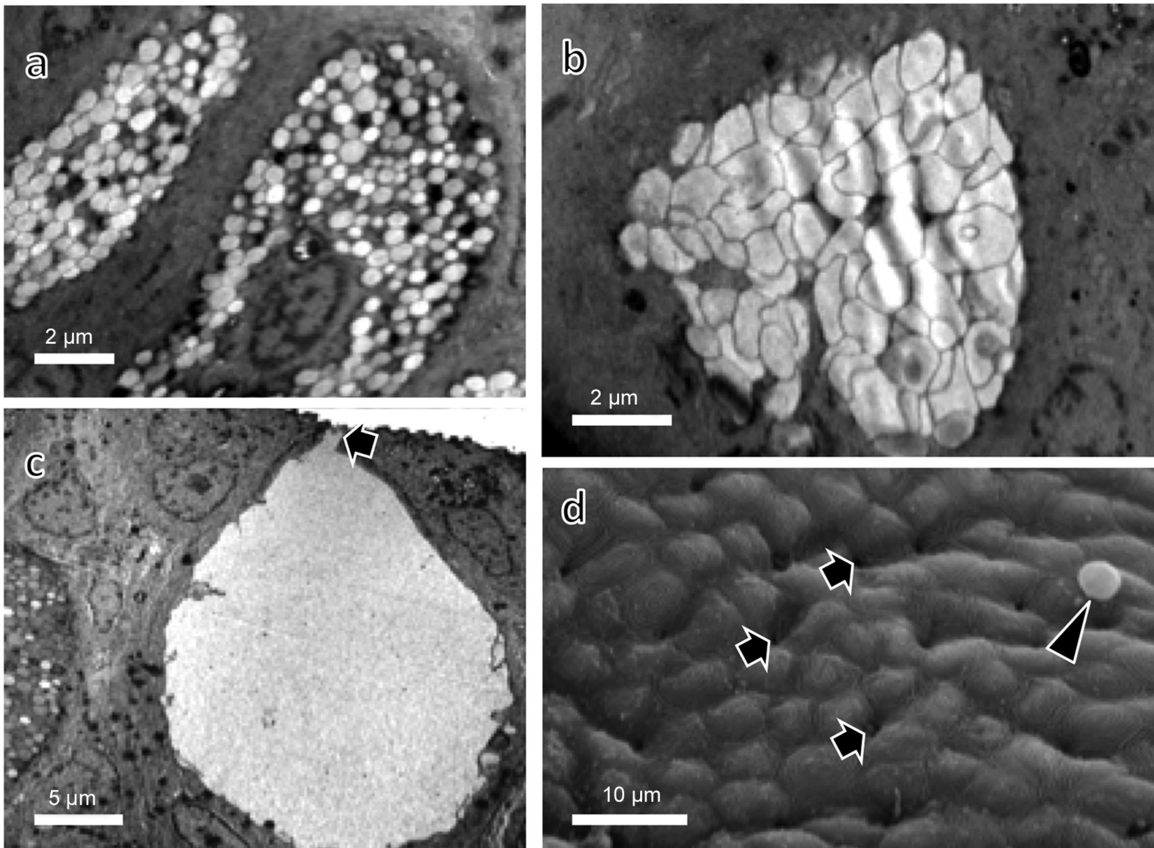

Fig. 2. (a) Goblet cells with a large number of secreting vesicles with different electrodensity and nucleus centrally located. TEM. (b) Goblet cell with secreting vesicles coalescing prior to discharge. TEM. (c) Goblet cell opening to the external surface located in the apical pole of the cell (arrow). TEM. (d) Surface of the skin with microridges and pores of goblet cells (arrows) and mucus (arrowhead). SEM.

Melanophores, pigment cells more abundant in the skin of the turbot, showed a star-shaped form and dark-brown or black colour on light microscopy (Fig. 4(d)) with dendritic processes that established an intimate contact with the iridophores (Fig. 4(a)).

By means of TEM, large numbers of melanosomes containing melanin and showing different electrodensity could be observed within the cytoplasm of melanophores (Fig. 4(e)). The indented nucleus was rounded to oval, with dense chromatin.

Iridophores possessed a rounded to elliptical morphology and the cytoplasm showed a striated appearance in samples stained with toluidine blue (Fig. 4(a)). Ultrastructurally, cytoplasm contained several crystalline platelets slightly electrodense surrounded by a single membrane (Fig. 5(a and b)). Near the nucleus, endoplasmic reticulum with different sizes and flattened to rounded morphology could be seen (Fig. 5(c)). The nucleus was irregular in shape and located central or peripherally (Fig. 5(a)).

The main difference observed between the three turbot groups of study was the amount of melanophores and xanthophores in the skin (Table 2).

The number of iridophores was similar in the three different groups and they were aligned forming a single layer (Fig. 4(a)). Regarding the other chromatophores, fish of dark group had a large amount of melanophores and they could be seen even in the epidermis whereas the amount of xanthophores was scarce (Fig. 6(a)). Fish of brown-yellowish group showed a high number of xanthophores and the number of melanophores was scarce (Fig. 6(b)). Fish of brown-greyish group displayed medium amount of melanophores and xanthophores in the pigmentary layer of the dermis (Table 2).

Finally, antibody anti-S100 showed strong immunostaining in the crystalline platelets of iridophores of the pigmentary layer, and anti-bovine cytokeratin displayed immunopisitivity in the ephitelial cells of the epidermis as well as in the iridophores of the pigmentary layer (Fig. 7(a and b)). Besides, anti-IgM and anti-CD3 showed strong immunostaining in a few cells in the basal epidermis (Fig. 7(c and d)) and anti-IgM also in superficial dermis (Fig. 7(e)).

Antibody anti-CD3 revealed the presence of cells morphologically compatible with T lymphocytes and antibody anti-IgM showed immunopositivity in cells compatible with B lymphocytes

Table 2
Semiquantitative study of chromatophores in the pigmentary layer of the dermis in the dorsal skin (+++ large, ++ medium and + scarce amount of chromatophores). Five specimens of each type were evaluated.

Colouration	Melanophores	Iridophores	Xanthophores
brown-yellowish	+	+++	+++
brown-grayish	++	+++	++
dark	+++	+++	+

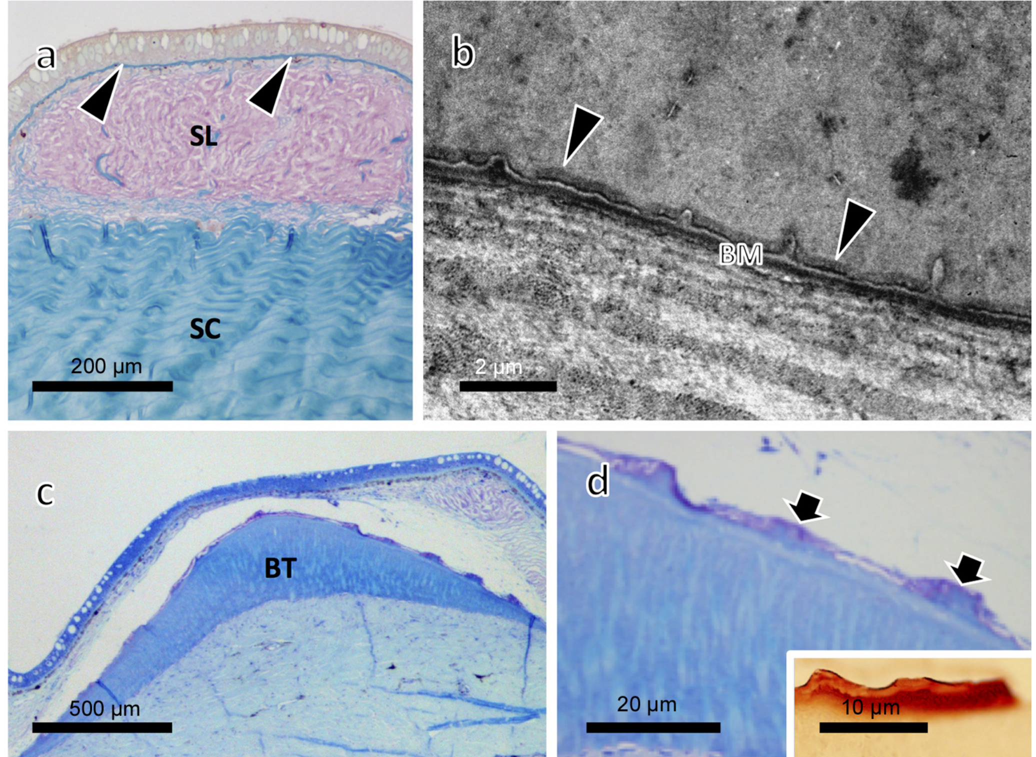

Fig. 3. (a) Basement membrane (arrowheads), dermis with stratum laxum (SL) and stratum compactum (SC). Gallego's trichrome. (b) Basement membrane (BM) with several bundles of collagen and hemidesmosomes (arrowheads). TEM. (c) Bony tubercle (BT) displayed typical conical shape. Gallego's trichrome. (d) Detail of external layer of the tubercle with calcium deposits (arrows). Toluidine blue. (inset) Detail of calcium deposits stained with Alizarin red.

and plasma cells. On the other hand, anti-lysozyme antibody stained cells into the vessels in the superficial dermis (Fig. 7(f)). These cells showed round morphology and the cytoplasm strongly stained. The rest of antibodies did not show positive results (Table 1).

The main differences between ventral and dorsal skin were in the goblet cells of the epidermis because in the dorsal skin the amount of these cells was higher and occasionally we can observed clusters of these cells, while in the ventral appear randomly distributed. The other difference was in the pigmentary layer of the dermis because the iridophores were the only type of chromatophores detected in the ventral skin.

4. Discussion

The current work describes the main morphological features of turbot skin at histological and ultrastructural level, as well as the immunohistochemical properties of different cutaneous components.

Although there are inter-species differences in teleost skin (Ferguson, 2006), the histology and ultrastructural characteristics of integument in turbot are similar to that described in the majority of teleost species (Arellano and Sarasquete, 2006; Guerra et al., 2006; Saadatfar et al., 2010).

The thickness of the epidermis from experimental turbot did not exceed 100 μm in any case. This value is lower than that reported by Groff (2001) for benthonic fish.

Different profiles of epidermal cells have been described along the epidermis in different species of fishes (Hawkes, 1974a; Harris and Hunt, 1975). These findings are in agreement with those observed in the current study, where different morphologies in the epithelial cells were detected depending on the location in the epidermis. In addition, these cells showed positive immunostaining with anti-cytokeratin antibody according to described in other fish species (Bunton, 1993; Schaffeld et al., 2005; Mauger et al., 2009). The intercellular connections like tight junctions and desmosomes observed in turbot have also been reported in plaice and lamprey (Downing and Novales, 1971; Roberts et al., 1972; Matoltsy and Bereiter-Hahn, 1984).

Different types of epidermal-secreting cells have been described in various species of fish (Ostrander, 2000; Groff, 2001) however, only goblet cells were identified in the present work. These mucous cells occupied the entire thickness of the epidermis and the mucus sheet that they produce may help to avoid abrasive lesions when the fish are partially buried in sand, gravel or sediment or in touch with other surfaces or specimens in fish holding-tanks.

Although chromatophores have been described in fishes (Hawkes, 1974b; Fujii, 1993; Ferrer et al., 1999; Arellano and Sarasquete, 2006; Ferguson, 2006; Zarnescu, 2007), the histological studies are limited. Among pigment cells, melanophores are the best-known type and their morphological and structural characteristics in turbot were similar to those described in other fish species (Groff, 2001).

The main feature of iridophores in the turbot skin was the reflecting platelets in the cytoplasm, which are responsible for their silvery appearance (Fujii, 1993). Ferrer et al. (1999) suggested that these platelets are derived from endoplasmic reticulum in *Sparus aurata*. In the current study similar process may occur, since endoplasmic reticulum with different sizes and morphologies could be seen located close to the nucleus. Furthermore, turbot iridophores showed round profile but in *S. aurata* and paddlefish (*Polyodon spathula*) have stellate morphology because they possess cytoplasmic processes (Ferrer et al., 1999; Zarnescu, 2007).

In the cytoplasm of xanthophores two different organelles were identified according to the electrodensity they showed. Similarly, Obika and Meyer-Rochow (1990) described carotenoid vesicles

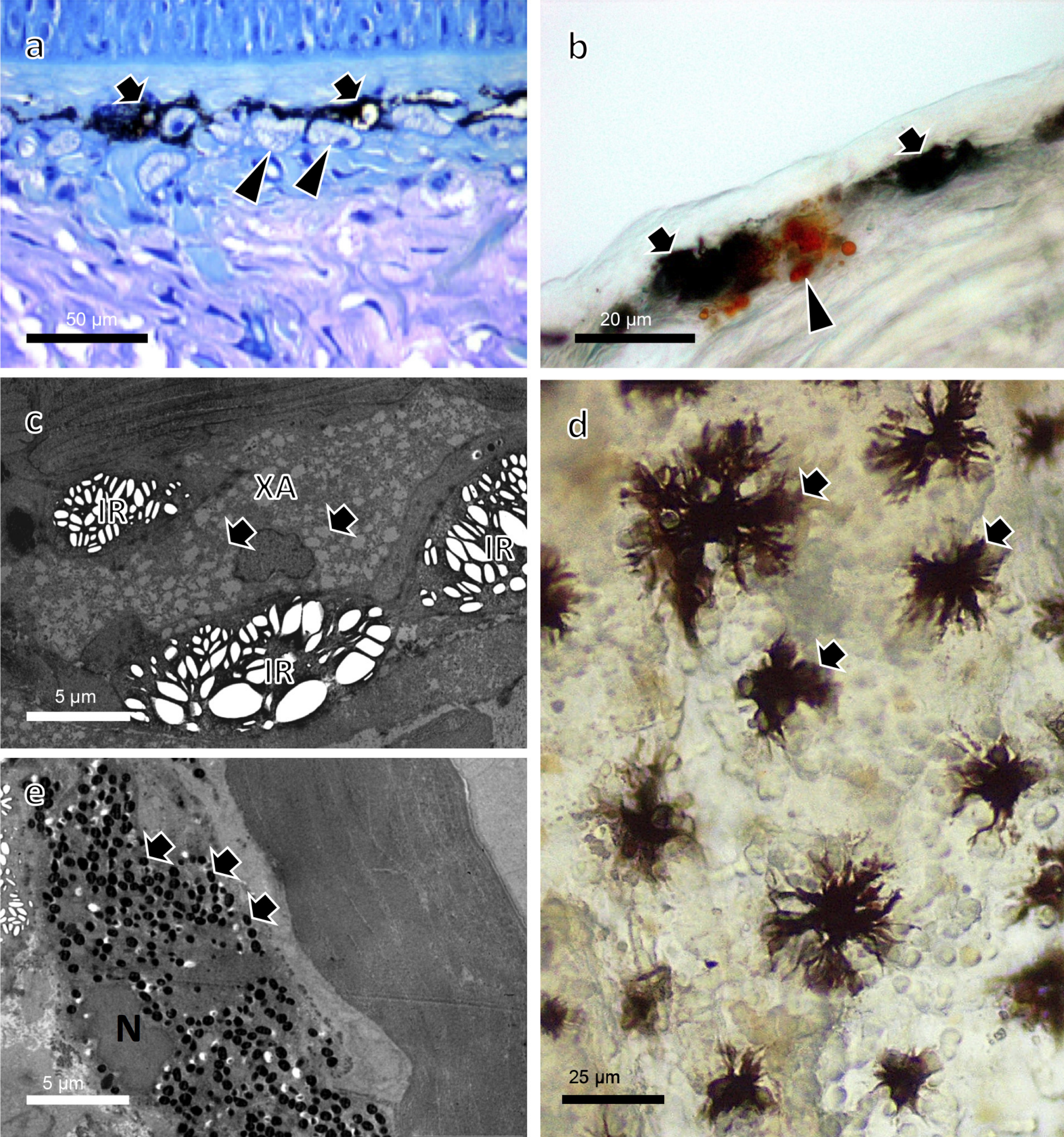

Fig. 4. (a) Pigmentary layer of dermis with melanophores (arrows) and iridophores (arrowheads). Toluidine blue. (b) Pigmentary layer of dermis with melanophores (arrows) and xathophores (arrowhead). Fresh section. (c) Xanthophore (XA) containing cytoplasmic granules of different electrodensities (arrows) and in close contact with iridophores (IR). TEM. (d) Melanophores showing typical stellate morphology (arrows). Fresh section. Stereoscopic microscope. (e) Melanophore with several melanosomes (arrows) full of melanin in the cytoplasm and nucleus (N) peripherally located. TEM.

and immature pterinosomes in the cytoplasm of dermal xanthophores.

Bagnara et al. (1968) defined the dermal chromatophore unit as the combination of xanthophores, iridophores and melanophores following a vertical pattern. In fact, in zebrafish a vertical combination of these pigment cells have been described (Hirata et al., 2005) with xanthophores in the uppermost layer, iridophores in the intermediate layer and melanophores in the basal layer. In the fish analyzed in this experiment a vertical order of dermal chromatophores was observed with melanophores and xanthophores located in the uppermost layer and iridophores in the basal layer.

The distribution and relationships of the turbot chromatophores along the skin may be responsible for skin colour changes observed in the adaptation of the fish to the seabed. Finally, the semiquantitative study performed, showed noticeable differences in number and location of chromatophores in the three groups of turbot. Fujii (1993) and Bolker and Hill (2000), attributed these variations to environment and dietary factors, but further studies would be necessary to know the aspects that affect the cutaneous colouration in turbot.

The panel of antibodies used in the study was chosen to characterize the different components of skin. Firstly, cytokeratin

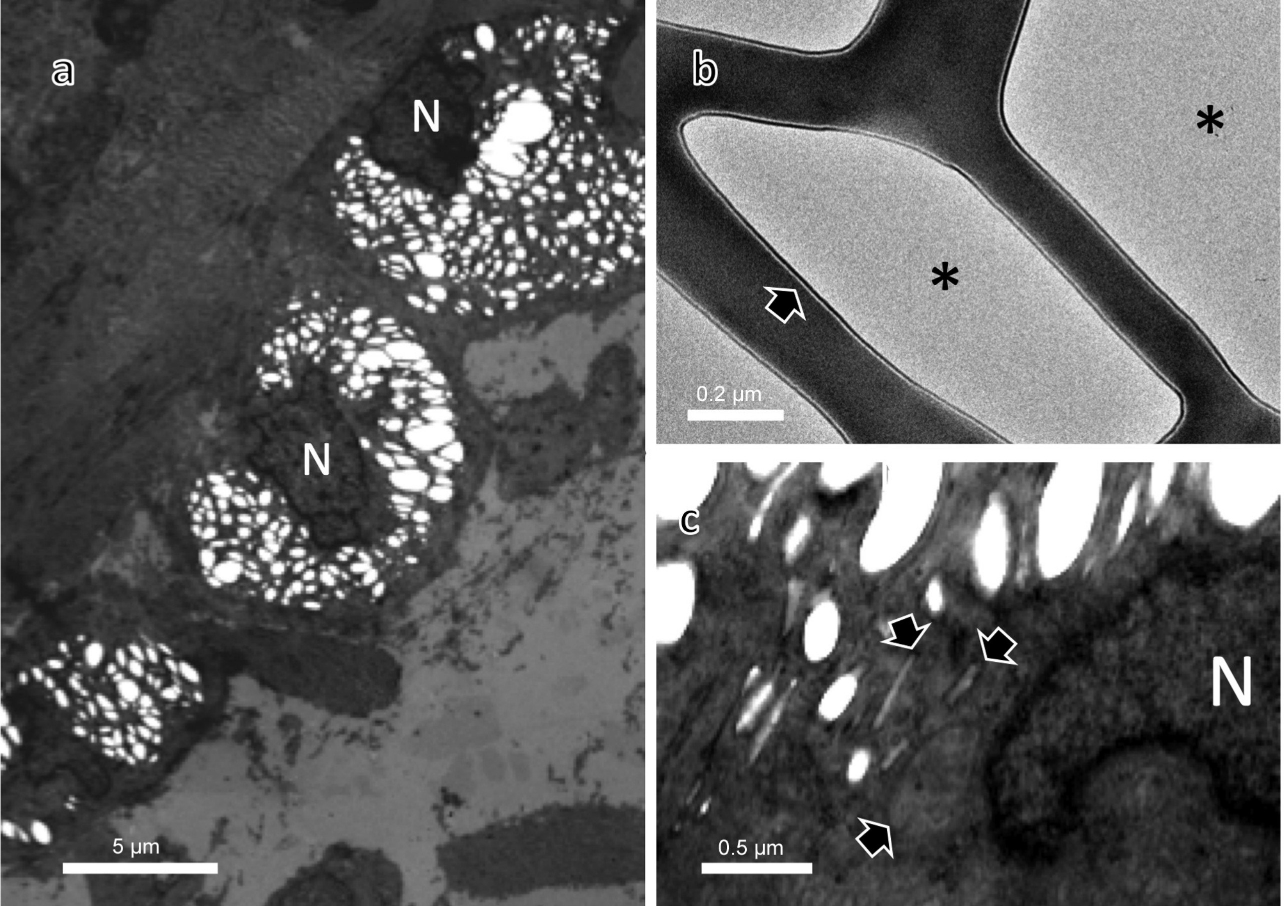

Fig. 5. (a) Iridophores in the pigmentary layer with cytoplasm showing empty spaces and nuclei (N) located centrally or peripherally. TEM. (b) Crystalline platelets (asterisks) in the cytoplasm of iridophores surrounded by a single membrane (arrow). TEM. (c) Endoplasmic reticulum cisterns with different morphologies (arrows) near to the nucleus (N). TEM.

(epithelial cells) and vimentin (mesenchymal cells) are commonly used in mammals to characterize the different cell types populating the skin (Kanitakis, 2002; Mauger et al., 2009) as well as S-100 to mark, among other, different cells in the epidermis (Valcayo, 1993; Böni et al., 1997; Kanitakis, 2002). In the current study, positive results were obtained with cytokeratin antibody that showed immunopositivity in epithelial cells and iridophores; but vimentin antibody not shown immunostaining. These results are in accordance with Rakers et al. (2011) and Grunow et al. (2013) who described that the characterization of epidermal and mesenchymal cells based on cytokeratin and vimentin antibodies is not as clear as in mammals. Secondly, the chromatophores are derived from neural crest as well as glia and neurons (Quigley et al., 2004, 2005), therefore, a battery of antibodies which immunostained neural

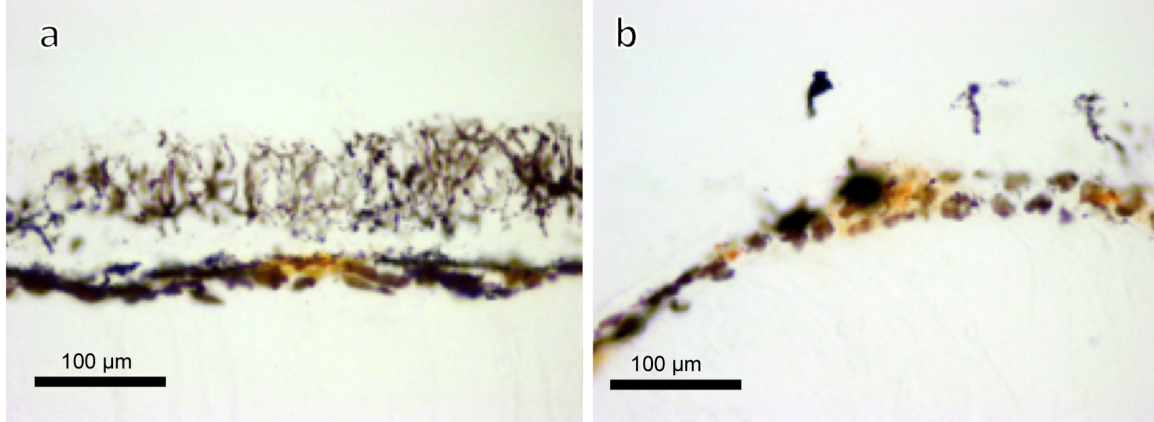

Fig. 6. (a) Dark turbot with melanophores in the pigmentary layer and in the epidermis. Fresh section. (b) Skin of brown-yellowish turbot with high amount of xanthophores in the pigmentary layer. Fresh section. (For interpretation of the references to color in figure legend, the reader is referred to the web version of the article.)

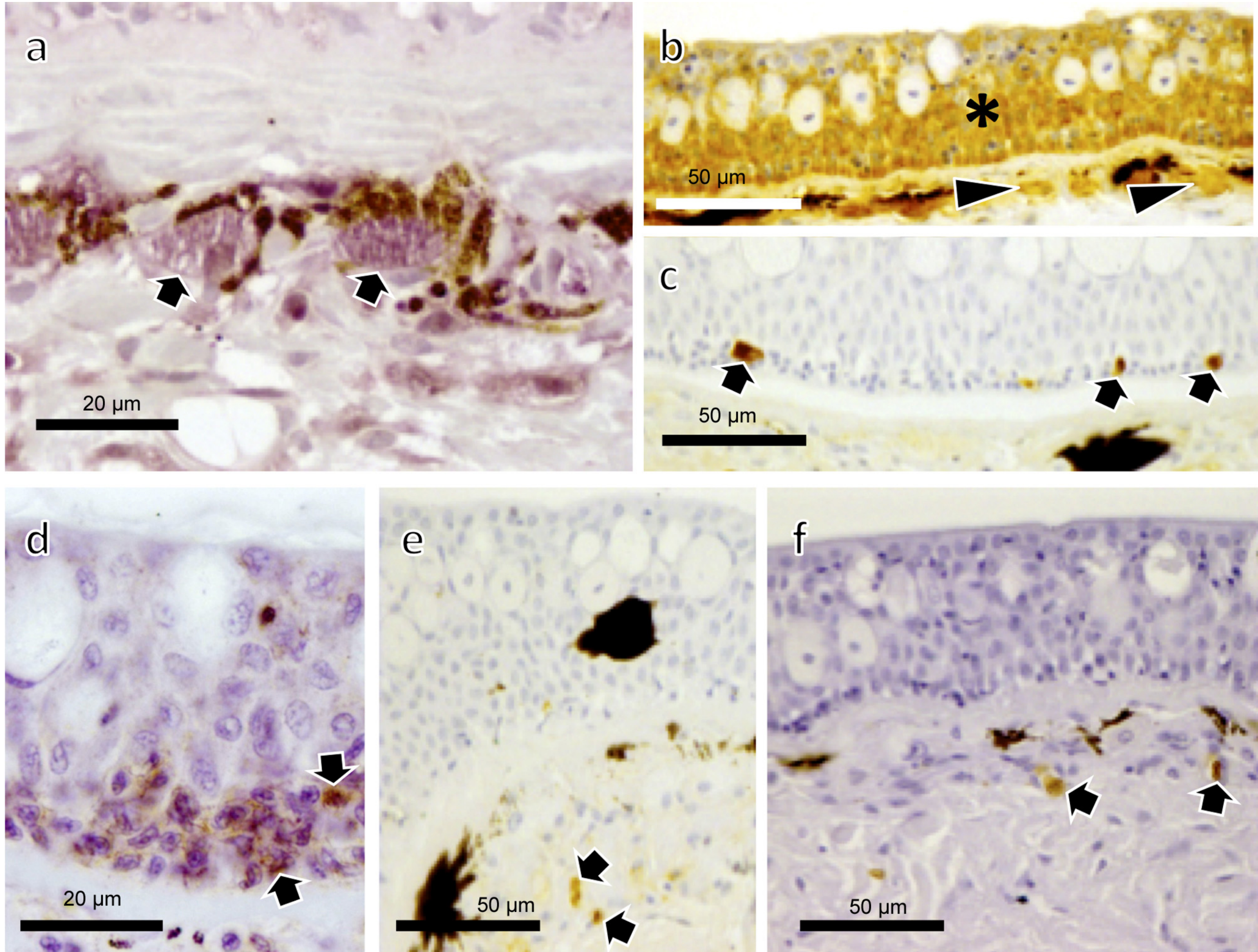

Fig. 7. (a) Crystalline platelets in the cytoplasm of iridophores positive against antibody anti-S100 (arrows). IHC, Vector VIP. (b) Epithelial cells (asterisk) and iridophores (arrowheads) stained with the antibody anti-bovine cytokeratin. IHC. (c) Ig+ cells in the basal epidermis in turbot (arrows). IHC. (d) Lymphocytes in the basal epidermis in turbot (arrows) immunostained with antibody anti-CD3. IHC. (e) Ig+ cells in superficial dermis in turbot (arrows). IHC. (f) Immunostaining cells against antibody anti-lysozyme in the vessels of superficial dermis in turbot (arrows). IHC.

cells in mammals were tested (Enolase, GFAP and N-Cadherin). Unfortunately, in turbot cross reactivity was not observed with these antibodies.

Finally, lysozyme, IgM and CD3 were included to assess the immune cells present in healthy skin and all these antibodies give positive immunostaining. The presence of these antibodies in different organs of turbot could be related with the non-specific and specific immune defense mechanisms of turbot as proposed several authors in other fish (Uribe et al., 2011). In particular, lysozyme is a bacteriolytic enzyme that has been detected in serum, secretions, mucous membranes and tissues rich in leucocytes in salmonids (Grinde et al., 1988; Lie et al., 1989). IgM is the predominant immunoglobulin in teleost and have an important role in inhibiting the entry of pathogens (Alexander and Ingram, 1992; Rombout et al., 1993; Aranishi and Nakane, 1997; Boshra et al., 2006; Saurabh and Sahoo, 2008).

In summary, the characterization of histological, ultrastructural and immunohistochemical features of the skin in healthy turbot have been assessed in the current study. This work has provided news insights that are basics to understand the behaviour of the skin and apply this knowledge in the early diagnoses and prevention of cutaneous diseases.

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