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Changes in marine turbot (*Scophthalmus maximus*) epidermis and skin mucus composition during development from bilateral larvae to juvenile flat fish

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Abstract

Alike other flat fish, marine turbot has the particularity that changes from larvae with bilateral symmetry to adult with asymmetry, in terms of the position of the eyes. As expected, the skin configuration of this species is also affected by the development and transformation suffered by fish during metamorphosis. In this context, changes in the epidermis of marine turbot were studied using conventional staining and histochemical techniques using six lectins (UEA-I, PNA, RCA-I, WGA, Con A and SBA). During development from larvae to juvenile (3–300 days post-hatching), the epidermis increased in both thickness and the number of cell layers. In fact, the simple cuboidal epithelium observed in larvae at day 3 already became stratified at days 10–12, which sequentially increase in thickness with fish development. Turbot epidermis is composed basically of four cell types: epithelial and mucous or secretory cells that are present through the development, and pigmented cells and a type that the authors described as club-like cells that appear during and post-metamorphosis. The Alcian blue-periodic acid Schiff (AB-PAS) histochemical method revealed the presence of neutral glycoconjugates in mucous and club-like cells at post-metamorphic stages of fish. Accordingly, lectin analysis showed mucous cells containing glycoproteins rich in fucose (UEA-I labelling) and glycoconjugates rich in the sequence galactose-*N*-acetyl galactosamine (PNA and RCA-I labelling) when this cell type appears. Interestingly, melanophores were observed in the dorsal epidermis of post-metamorphic juveniles. This type of cell contains a black-to-brown pigment that provides the skin the typical colour of this fish species. Changes in mucous coat composition were observed during fish development, which was attributed to different roles of the glycoconjugates.

KEYWORDS
club-like cells, development, histochemistry, metamorphosis, mucous cells, *Scophthalmus maximus*

1 | INTRODUCTION

Skin is considered the larger organ in fishes, and it is able to reflect changes associated with different physiological or environmental conditions occurring during their development. For example, changes at the epidermal level are evident in lamprey when changing from buried-larvae form to free-swimming adult (Rodríguez-Alonso *et al*., 2017); in Atlantic salmon when migrating from fresh water to sea water (O'Byrne-Ring *et al*., 2003); or in halibut when changing its morphology and symmetry to a flatfish adult (Ottesen & Olafsen, 1997). These susceptible stages along fish life cycle determine and shape the conformation of fish skin protective barrier.

Despite the interspecies differences in the types of cells found in the epidermis of teleost fish (Elliot, 2011), most of the authors describe two main types of cells: epithelial or Malpighian cells and mucous or Goblet cells (Elliot, 2011; Faílde *et al*., 2014; Saadatfar *et al*., 2010; Whitear, 1986). There are others cells in the epidermis that could be or could not be secretory, as club or alarm, sacciform and granular cells. These cells are characteristic, although not exclusive, of different fish groups. According to Whitear (1986), granular cells are exclusive for lamprey epidermis. Nonetheless, club and sacciform cells are characteristic of ray-finned fishes (Actinopterygii) and superorder Acanthopterigii, respectively (Elliot, 2011). Moreover, although pigmented cells or chromatophores occur in most of teleost's skin, mainly in the upper layers of the dermis, in elasmobranch and some teleosts, such as flatfish species, chromatophores occur both in dermal and in epidermal layers (Burton, 2010).

The skin of teleost fish is provided by an external mucous coat produced by a layer of live cells, mostly epithelial and mucous cells (Elliot, 2011). Over the past few years, the number of studies on fish skin mucus has been increasing, mainly because of its key role in many fish functions such as ionic regulation, locomotion, communication or defence against parasites and microorganisms (Reverter *et al*., 2017). Many of these studies highlight the protective role of the mucus, as is considered an immunological defence in fish skin (Esteban & Cerezuela, 2015), being also known to produce a variety of antimicrobial compounds (Ebran *et al*., 2000). Nonetheless, the information on the structure and composition of epidermal cells that secrete this mucous coat is still scarce.

Mucus is mainly composed of glycoconjugates with different functions, and its properties are known to be affected by different factors such as diet, starvation or water bacterial load (*e.g*., Landeira-Dabarca *et al*., 2014; van der Marel *et al*., 2010). Mucus composition can also be modified by fish throughout its development or whenever a specific function is required. For example, changes in skin mucus composition during metamorphosis might provide immediate protection to new and potentially harmful conditions not previously experienced by their immune system of early stages of fish (Rodríguez-Alonso *et al*., 2017).

Among mucus glycoconjugates, the predominance of acid or sulphated glycosaminoglycans is related to high viscosity and protection (Bravo *et al*., 2012). Moreover, when bacterial load in the surrounding water is high (thus increasing infection potential), mucous cells usually increase the level of total glycosylation and acidic glycoconjugates (Gómez *et al*., 2013; van der Marel *et al*., 2010). On its part, neutral glycoproteins are more related to the functions of lubrication and adhesion to the substrate (Bravo *et al*., 2012). In this sense, in a histochemical study conducted by Roberts *et al*. (1973), larval plaice epidermis described a change in the nature of mucus glycoconjugates from neutral to acidic after 30 days of hatching. A similar modification in mucus composition was found in the gills of the Atlantic salmon and brown trout when migrating from fresh water to sea water, which was attributed to an increase in mucus viscosity (Roberts & Powell, 2005).

The turbot, *Scophthalmus maximus* L., is a marine flatfish of the family Scophthalmidae, Order: Pleuronectiformes, widespread in the Atlantic Ocean and in the Mediterranean Sea (Froese & Pauly, 2016). Turbot is considered an important commercial species, and its aquaculture is a prominent sector in NW Spain. Although all vertebrates undergo left–right symmetric placement (bilateral symmetry) during embryogenesis, turbot experiences an additional period of post-embryonic asymmetric remodelling. During this post-hatching metamorphosis (*i.e*., 30–40 days after hatching), fish experience a gradual translocation of one eye to the opposite side of the head and the appearance of key neurocranial elements (Al-Maghazachi & Gibson, 1984). Compared to other teleosts, larvae of flat fishes undergo a particularly evident and dramatic metamorphosis, because flat fishes completely reprogram their body to move from the pelagic habitat, in the water column, to the benthic habitat, on the sea floor (do Prado *et al*., 2018).

Despite turbot commercial interest and numerous studies exploring its morphology, physiology and behaviour in adult stage (*e.g*., Al-Maghazachi & Gibson, 1984; Faílde *et al*., 2014), little is known about its skin mucus and epidermis changes throughout larval development, especially, what happens at the epidermal level before and after metamorphosis, a critical stage of turbot life cycle.

The main aim of this work is to characterize the structure and the different cell types of turbot epidermis throughout its development and symmetry swift. A second objective is to analyse the distribution and composition of glycoconjugates in the epithelium of this fish species to contribute to the understanding of its morphological and functional aspects.

2 | MATERIALS AND METHODS

2.1 | Collection of epidermal samples

In this study, individuals of *S. maximus* were used (Rafinesque, 1810) at different developmental stages (Figure 1):

1. Early pre-metamorphic larvae with bilateral symmetry, collected at day 3 after hatching ($n = 5$), 2.5–4 mm total length (L_T);
2. Pre-metamorphic larvae with bilateral symmetry, collected at days 10–12 after hatching ($n = 5$), 10–15 mm L_T;

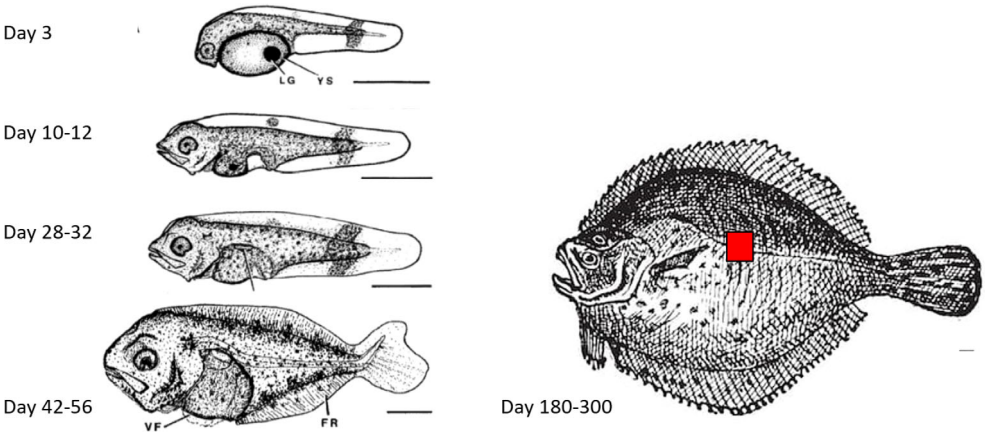

FIGURE 1 Schematic representation of *Scophthalmus maximus* (adapted from Al-Maghazachi & Gibson, 1984) showing the selected body regions and developmental stages sampled in this study. Bar scale at 1 mm

3. Metamorphic larvae with initial external signs of metamorphosis (eye migration), collected at days 28–32 after hatching ($n = 5$), 20–25 mm L_T;
4. Post-metamorphic larvae, with complete dorso-ventral symmetry, collected at days 42–56 after hatching ($n = 5$), 35–55 mm L_T; and
5. Juveniles, flat fish collected at days 180–300 after hatching ($n = 15$), 180–200 mm L_T.

Individuals were obtained at Toralla Marine Science Station (ECIMAT) facilities, Vigo (Spain) in 2016 and 2018.

To obtain epidermal tissue samples, fish were killed in the morning with an overdose of anaesthesia (2-fenoxiethanol), which was administered following the European regulation for the protection of animals used for scientific purposes (86/609/CEE), in addition to guidelines for animal care established by the Spanish Royal Decree 53/2013 and the decree 296/2008 of the Galician Autonomous Government. After killing the fish, larvae and post-metamorphic individuals were directly immersed in Bouin fixative solution. To collect the samples of juvenile fish, samples of 1 cm^2 were also immersed in Bouin fixative solution for 24 h at room temperature. This skin samples were taken from two regions: dorsal and ventral areas (at the midpoint between head and caudal fin, coinciding with the spine) (Figure 1).

2.2 | Histological analysis

After fixation, samples were dehydrated in ethanol series, cleared in xylene and embedded in paraffin wax following standard protocols. Non-consecutive longitudinal sections of 5 μm thick were cut using a rotatory microtome (Leica RM 2255); this stereological transverse-slicing method for quantification of mucous cells is the most common method of sampling skin for histology used in fish. In every sample, 12 series of sections were placed on gelatin-coated slides. Series 1–4 were processed for general staining and the series 5–12 for lectin histochemistry.

To describe the general morphology of the skin, the authors conducted a conventional hematoxylin-eosine staining (H-E). Through which was described cellular types, epidermis thickness and measured mucous cells density using a BX51 Olympus microscope. Epidermis thickness was measured using a microscope scale from the cuticle to the basal lamina (mm). The density of mucous cells and club-like cells – when possible – per fish was estimated from the average number of cells manually counted in three histological sections from each epidermal tissue sample (Nikon 50i microscope, 40×), divided by the area of each section measured with the microscope scale (*i.e.*, number of cells per mm^2 of epidermis).

To distinguish the epidermal cells based on their glucidic content, the authors performed an Alcian blue-PAS technique (AB-PAS) at pH 2.5 to differentiate among cells containing neutral mucins (stained red), acid mucins with sulphated and carboxylated groups (stained blue) and/or mixed cells containing both neutral and acid monosaccharide residues (stained purple). Staining and assemblage were conducted using automatic workstations (Leica ST 5020 and Leica CV 5030, respectively).

Lectin staining techniques were used to investigate the possible changes in the carbohydrate composition of the glycoconjugates of epidermal skin cells throughout marine turbot development. Lectins specifically recognize one or more terminal carbohydrates present in glycoproteins and/or proteoglycans. There are lectins that recognize a single monosaccharide residue and others that recognize several with different affinities (Esteban *et al.*, 2014). The protocol followed was the standard protocol for the detection of glycoconjugates through the use of biotinylated lectins. The histochemistry of lectins was performed according to the technique previously described by Molist *et al.* (2011).

After deparaffined and hydrated, the sections were treated for 1 h with 3% hydrogen peroxide and 40% methanol in phosphate-buffered saline (PBS 0.1 M) with pH 7.4 at room temperature, to inhibit peroxidase and biotin endogenous. Next, sections were washed in PBS and preincubated with bovine serum albumin to minimize nonspecific binding. Consecutive sections were then incubated overnight at 4°C with different biotinylated conjugate lectins which react preferentially with a sugar residue (Vector Laboratories Inc. USA) in fish larvae (days 3 to 28–32), post-metamorphic (days 42–56) and in juvenile (days 180–300) at a concentration of 10 μg ml^{-1}, using PBS as the buffer solvent. The authors of this study selected the following lectins: fucose-binding lectin (UEA-I),

mannose-binding lectin (Con A), terminal sequence galactose (β 1-3), *N*-acetylgalactosamine-binding lectin (PNA) galactose and *N*-acetylgalactosamine-binding lectin (RCA-I), *N*-acetylglucosamine-binding lectin (WGA), and *N*-acetylgalactosamine-binding lectin (SBA).

Subsequently, sections were washed in PBS and incubated with avidin–biotin complex (ABC kit) at room temperature for 1 h (diluted 1/100, Vectastain Elite PK 6200; Vector Laboratories Inc.). The reaction products were visualized with diamino-benzidine-hydrogen peroxidase system DAB (0.5 mg mol^{-1} 3, 3′-diaminobenzidine; SIGMA FAST Tablets) and 0.01% H_2O_2 in PBS for 5–10 min. Sections were counterstained with Mayer's hematoxylin. To verify the specificity of the lectins, other sections were preincubated with each conjugated lectin and the respective 0.2 M inhibitory sugar solution (Brooks, 2017). Moreover, no DAB reaction was observed when the lectins were removed from this protocol.

Differences in the location of the reaction products were observed using a microscope (Olympus BX51 microscope) and described accordingly. Photographs were taken using an Olympus DP71 digital camera, and contrast and brightness parameters were adjusted using GNU image manipulation programme (GIMP).

2.3 | Statistical analysis

Before conducting any statistical analysis, the authors tested for the assumption of normality (Kolmogorov–Smirnov Z-test, $P > 0.05$) and homogeneity of variances (Levene's test, $P > 0.05$). To test the differences in cell (mucous and club-like cells) densities (dependent variable) among developmental stages (factor), the authors ran an ANOVA test using Statistica 24.0 (IBM Software) for each cell type. Finally, *post hoc* pair-wise comparisons between factors were made with a Tukey's test.

3 | RESULTS

3.1 | General morphology of the epidermis

Throughout metamorphosis-symmetry switch from larvae to juvenile, the epidermis of *S. maximus* increases in both thickness and the number of cell layers (Figure 2). The thickness underwent a gradual increase, which was linked with the number of cell layers. Fish larvae

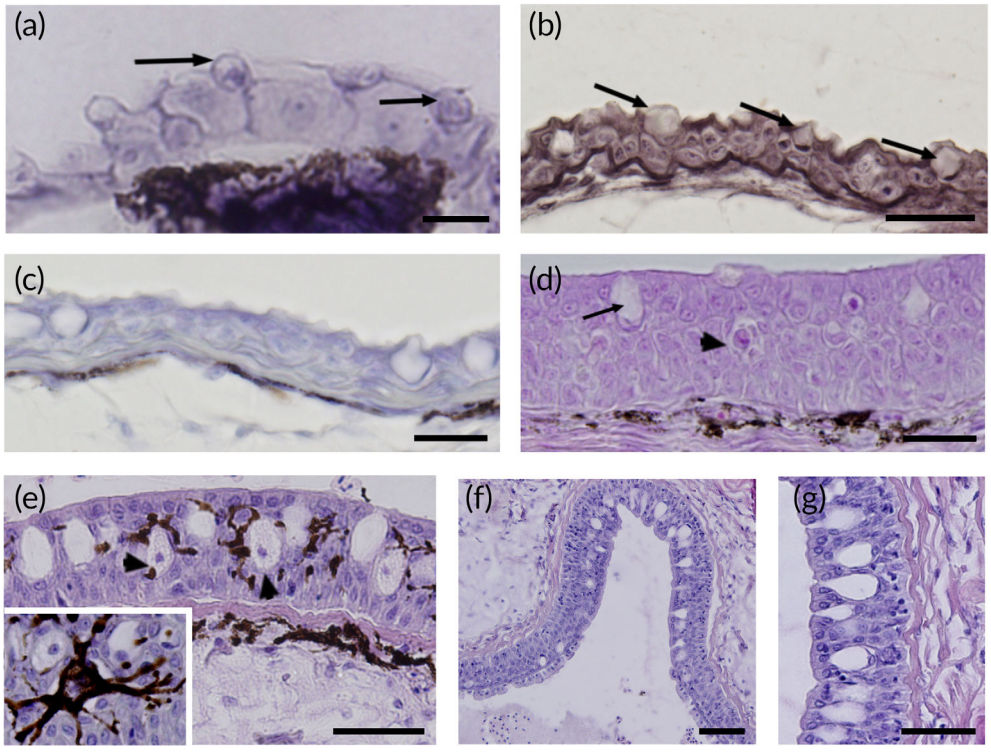

FIGURE 2 Histological sections of the epidermis during larvae-juvenile development (*i.e.*, metamorphosis) of *Scophthalmus maximus* stained with haematoxylin and eosin. (a) Day 3 post-hatching, epithelial cells form a simple cubic epithelium and rounded mucous cells are protruding the epithelium (arrows). (b) Days 10–12, the epithelium becomes bi-stratified and mucous cells are still located protruding the epithelium (arrows). (c) Days 28–32, the numbers of layers are two to three and mucous cells are keeping the same location. (d) Days 42–56, post-metamorphic fish larvae, the thickness of the epidermis considerably increases because of the increase in the number of cells. At this stage, club-like cells appear (arrowhead), and mucous cells are integrated with an elongated shape in the epithelium. (e) In the dorsal area of juvenile epidermis (days 180–300), club-like cells differentiate from mucous cells because of rounded nucleus and slightly eccentric (arrowheads). Pigmented cells appear in the epidermis. Inset: Magnification in pigmented cell or chromatophore with ramifications in the epidermis. (f and g) In the ventral area of juvenile epidermis, there are no chromatophores (epidermis and dermis). Ventral epithelium contains mostly mucous cells. Scale in (a) 10 μm; in (b–d) 20 μm; in (e, g) 50 μm and in (f) 100 μm

at day 3 showed a simple cuboidal epithelium with a unicellular layer that ranges between 8 and 10 μm (Figure 2a). On days 10–12, the epidermis slightly increased to 10–12 μm and already showed two layers (Figure 2b). On days 28–32, the epidermis measured 15–20 μm of thickness and became a bi- or tri-layered (Figure 2c). After metamorphosis, a stratified cuboidal multilayer epidermis of six to eight cell layers and 30–40 μm thick was observed (days 42–56) (Figure 2d) and increased until 60 μm at juvenile stage, both in dorsal (Figure 2e) and ventral areas (Figure 2f,g). Epidermis showed four cellular types: epithelial cells, mucous or secretory cells, pigmented cells or chromatophores and club-like cells (Figure 2). Nonetheless, epithelial and mucous cells are present through the development, but club-like and pigmented cells appear later (during and post-metamorphosis).

The morphology of the epithelium, the types of cells and the composition of its glycoconjugates also change during fish development. All these changes are more pronounced during the metamorphosis period. The basic element of the epidermis is the epithelial cells. A thin acellular layer, the cuticle, covers the epidermis along the development. Mucous cells are clearly visible at day 3 (Figure 2a) and remain as an important component of epidermis through the whole turbot's development. These mucous cells are interspersed among the epithelial cells and show several variations in shape at different fish stages. In pre-metamorphosis larval stages, mucous cells have a round shape with a clear cytoplasm and a slightly eccentric nucleus and are located protruding to epithelial cells (Figure 2a–c). Nonetheless, after metamorphosis the mucous cells have an elongated shape and a flattened nucleus that is located in the most basal portion of the cell. They are also integrated into the epithelium without protruding in it but in an upper position (Figure 2d). In juvenile turbot, these secretory cells in dorsal area are scattered along epidermis surface (Figure 2e). Nonetheless, in ventral area these are more concentrated in epidermal folds (Figure 2f,g).

Mucous cells' density changed with developmental stages ($F_{4,69} = 10.628$, $P < 0.001$), decreasing through the first stages of fish development until metamorphosis (Figure 3). Interestingly, although ventral mucous cells' densities in juveniles (days 180–300) were similar to values obtained in larvae ($P = 0.742$), densities in dorsal areas sharply increased fourfold ($P < 0.001$).

Although much less abundant than mucous cells, a cellular type, characterized by their round shape, dense and small nucleus located slightly eccentric, and the lack of opening to the epidermal surface, was also differentiated by the authors (Figure 2d,e). These morphological characteristics are common in club cells from actinopterygids; nonetheless, the authors described as club-like cells because of the presence of granules in the cytoplasm, instead of filaments and/or a vacuole (Herikson & Maltoltsy, 1968) as in true club cells. Moreover, these cells can be differentiated from sacciform cells typical of acanthopterygids by its peripheral nucleus, vacuoles at the margin of the cytoplasm and usually open at the surface of the skin by an apical pore (Mittal *et al*., 1981). The club-like cells appear only after metamorphosis when the fish has a dorso-ventral symmetry. At this stage, club-like cells are distinguished from the mucous cells because of the morphology in granules, nucleus position and the lack of connection to the epidermal surface. They are mostly present in the middle and upper layers of the epidermis, and only in dorsal area of juveniles (Figure 2d,e), where their density is close to mucous cells in the ventral area at the same stage ($P = 0.959$; Figure 3).

Integumentary colours are primarily dependent on the presence of pigment cells or chromatophores in the skin. These cells usually occur in the dermis in the upper loose connective tissue layer (Figure 2a–d). Nonetheless, after metamorphosis and symmetry switch, chromatophores are also visible in the dorsal area of the epidermis (Figure 2e, inset). They show a star-shaped and multiple

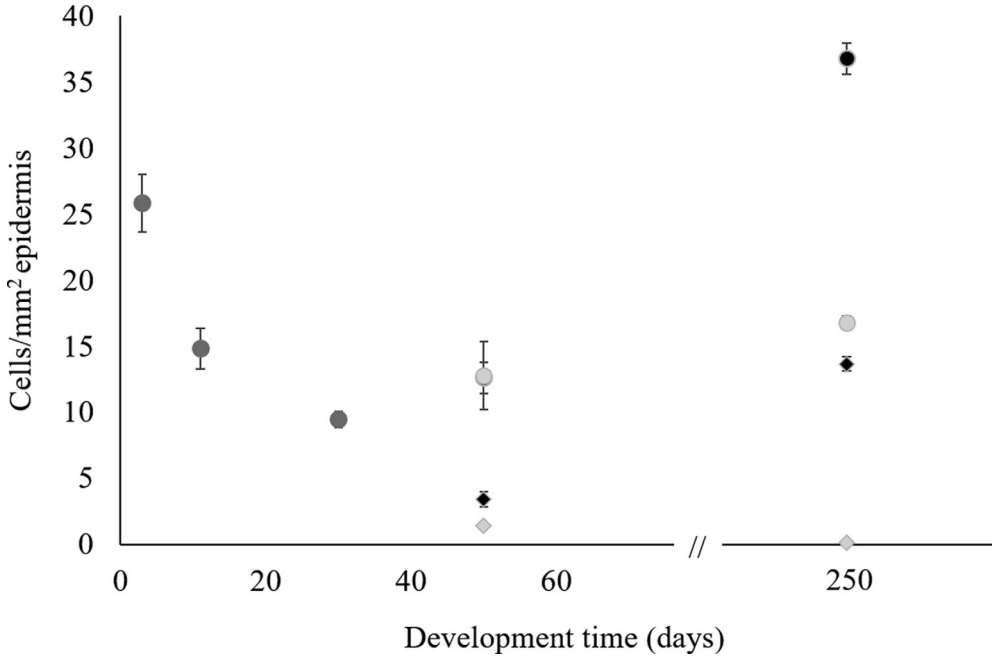

FIGURE 3 Density (cells/mm^2) ± S.E. of mucous cells -○-(circle), and club-like cells -◇-(rhombus shape) in the epidermis along development. Colours differentiate developmental stages and epidermal areas: pre-metamorphic (dark grey) and dorsal (black) and ventral (light grey) post-metamorphic. Density of mucous cells decreases until metamorphosis and then the number of cells is similar in the ventral part, but increases considerably in the dorsal part

branched processes extending from the cell body. The pigment they contain has a black-to-brown colour indicating that these cells are melanophores.

3.2 | Histochemistry

Using AB and PAS histochemistry, it was possible to distinguish between glycoconjugates containing acidic (AB positive, blue colour) and neutral (PAS positive, red colour) carbohydrates. When both types of carbohydrates are co-expressed, a purple colour was observed.

Across pre-metamorphic larval stage, no cells are stained with AB-PAS histochemistry. In contrast, the thick basal lamina shows a strong reaction to PAS. Moreover, the staining in the thin cuticle is weakly PAS positive (Figure 4a). The labelled pattern is different after metamorphosis, and mucous and club-like cells are stained with PAS from the days 42–56 of the development (Figure 4b). In addition, in juvenile it is clearly visible that secretion of mucous cells is PAS positive (Figure 4c,d), pointing the neutral nature of glycoconjugates that constitute the mucous coat. The same composition of glycoconjugates is present in club-like cells visible in the dorsal area of the skin (Figure 4c).

The presence of L-fucose, α-mannose, α-galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine monosaccharides was observed in marine turbot epidermis using the following lectins: UEA-I, Con A, PNA and RCA-I, WGA, SBA (Table 1). The pattern of staining for the lectins Con A and WGA is similar in the different stages of the life cycle, whereas the staining for UEA-I, PNA and RCA-I undergoes some variations in the transitional period between the pre- and post-metamorphic stages, in which symmetry swift represents a key process of change.

The UEA-I labelling was observed in mucous and epithelial cells (Table 1) but in different stages of the development. The presence in the mucous cells of L-fucose is visible for the first time at the end of the pre-metamorphic period (days 28–32; Figure 5a) and also in post-metamorphic larvae (Figure 5b) and juveniles (Figure 5c,d). Nonetheless, epithelial cells, particularly those located more superficially in the epidermis, appear positive after metamorphosis (Figure 5b). The intensity of this labelling decreases dramatically in juvenile stage (Figure 5c,d).

The pattern of staining for Con A – mannose – and WGA – N-acetylglucosamine – along studied stages is similar (Table 1). Mucous and club-like cells are negative along the life cycle; in contrast, epithelial cells show positivity to both lectins from the basal to the apical layers (Figure 5e,f). In post-metamorphic individuals (Figure 5e), this labelling is stronger than in juvenile (Figure 5f). Nonetheless, in both stages the apical layer is especially intense than the rest of the epithelial layers.

The same pattern in mucous and club-like cells is also observed for PNA and RCA-I lectins – galactose-*N*-acetylgalactosamine. In pre-metamorphic larvae, these lectins label mucous cells from day 3 (Figure 5g), but the staining disappears after metamorphosis (Figure 5h). In this stage instead of mucous cells, the club-like cells show an intense staining that remains in the juvenile stage (Figure 5i). In the case of epithelial cell, the pattern varies. PNA that detects the terminal sequence shows the positivity from the post-metamorphic stage to juveniles (Figure 5i). Nonetheless, RCA-I labels the apical

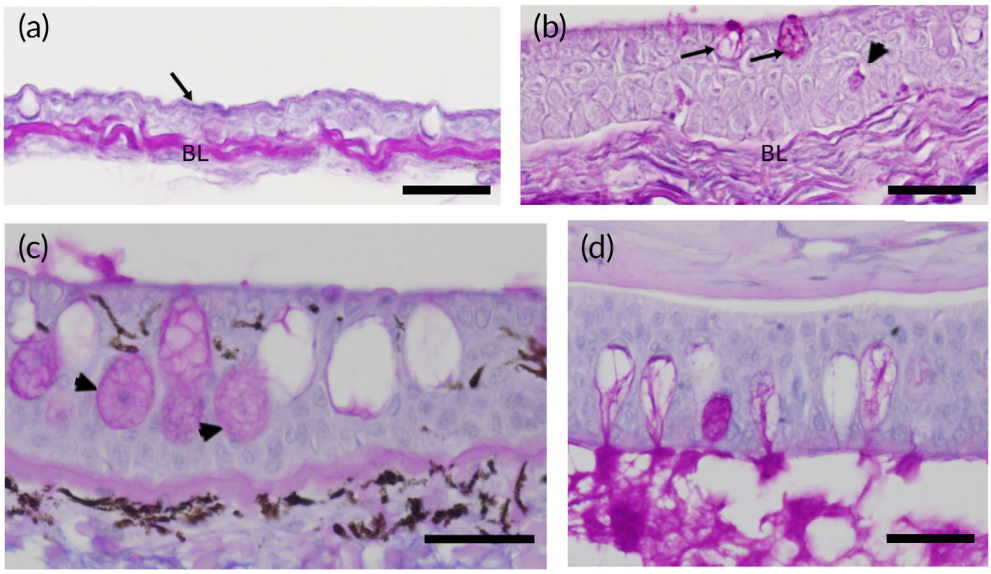

FIGURE 4 Histological sections of different developmental stages of the epidermis of *Scophthalmus maximus* stained with Alcian blue-periodic acid Schiff (AB-PAS). (a) Day 28 post-hatching, mucous cells are negative to AB-PAS in contrast to the PAS positivity of the basal lamina (BL) and cuticle (arrow). (b) Post-metamorphic days 42–56, mucous (arrow) and club-like cells (arrowhead) showing reactivity to PAS staining. (c) In the dorsal area of juvenile epidermis (days 180–300) granular cells (arrowheads) differentiate from mucous cells (arrow) because of the presence of a rounded nucleus slightly eccentric. They are located in the middle layers of the epidermis separated from the epithelial surface by superficial layers. (d) The majority of the cells in the ventral area of juvenile epidermis are mucous cells. Scales 40 μm

TABLE 1 Lectins used to stain epidermis in different areas and developmental stages of marine turbot (*Scophthalmus maximus*)

			Pre-metamorphic larva (days 3–32)	Post-metamorphic larva (days 42–56)	Juvenile(days 180–300)	
					Dorsal	Ventral
UEA I	Fucose	Apical epidermis	–	++	+	+
		Basal epidermis	–	–	–	–
		Mucous cells	+(d32)	+	+	+
		Granular cells	NA	–	–	NA
Con A	Mannose	Apical epidermis	+++	+++	++	++
		Basal epidermis	+++	++	+	+
		Mucous cells	–	–	–	–
		Granular cells	NA	–	–	NA
PNA	Gal – NAc.Gal terminal	Apical epidermis	–	++	+	+
		Basal epidermis	–	+	++	++–
		Mucous cells	++	–	–	–
		Granular cells	NA	++	++	NA
RCA	Gal – NAc.Gal	Apical epidermis	+	++	–	–
		Basal epidermis	–	+	+	++
		Mucous cells	++	–	–	–
		Granular cells	NA	++	++	NA
WGA	NAc.Glu	Apical epidermis	+++	+++	++	++
		Basal epidermis	+++	++	+	+
		Mucous cells	–	–	–	–
		Granular cells	NA	–	–	NA
SBA	NAc.Gal	Apical epidermis	+++	+++	++	+++
		Basal epidermis	–	–	+	–
		Mucous cells	–	–	–	–
		Granular cells	NA	+	+	NA

Note: "+" indicates the presence and "–" indicates the absence of staining. The number of symbols (+) indicates the intensity of staining.

epithelial layers of pre-metamorphic stage and all the epithelium – apical and basal layers – in post-metamorphic larva, but then the reaction changes to the most basal layers in juveniles.

Finally, SBA – *N*-acetylgalactosamine – is negative for mucous cells but positive for club-like cells when it appears after metamorphosis, and is always positive for the apical epithelial layer of the epidermis (Table 1 and Figure 5k–m).

4 | DISCUSSION

Despite the great diversity of teleost fish, the modifications in morphology and thickness of the epidermis throughout development are similar in most of the teleost. Accordingly, this study revealed that from pre-metamorphic larvae to juvenile, the epidermis of marine turbot had undergone remarkable changes, especially when symmetry metamorphosis occurred.

The increase in epidermis thickness reported in the present study is comparable to estimates described in other species (Fletcher *et al*., 1976; Rodríguez-Alonso *et al*., 2017)). Along the development, turbot epidermis varies from 8 μm and one layer at day 3 to 60 μm and six to eight layers at juvenile stage. Moreover, Faílde *et al*. (2014) pointed out that in adults the thickness of the epidermis did not exceed 100 μm with a maximum number of 14 layers. Therefore, the increase in the thickness of the epidermis continues from juvenile to adult. As previously observed for other fish species, the increase in thickness observed in the turbot during its development could be attributed to the increase in the number of cell layers (Elliot, 2011; Rodríguez-Alonso *et al*., 2017). Moreover, this increase is known to vary depending on species, age, region of the body and environmental conditions (Elliot, 2011).

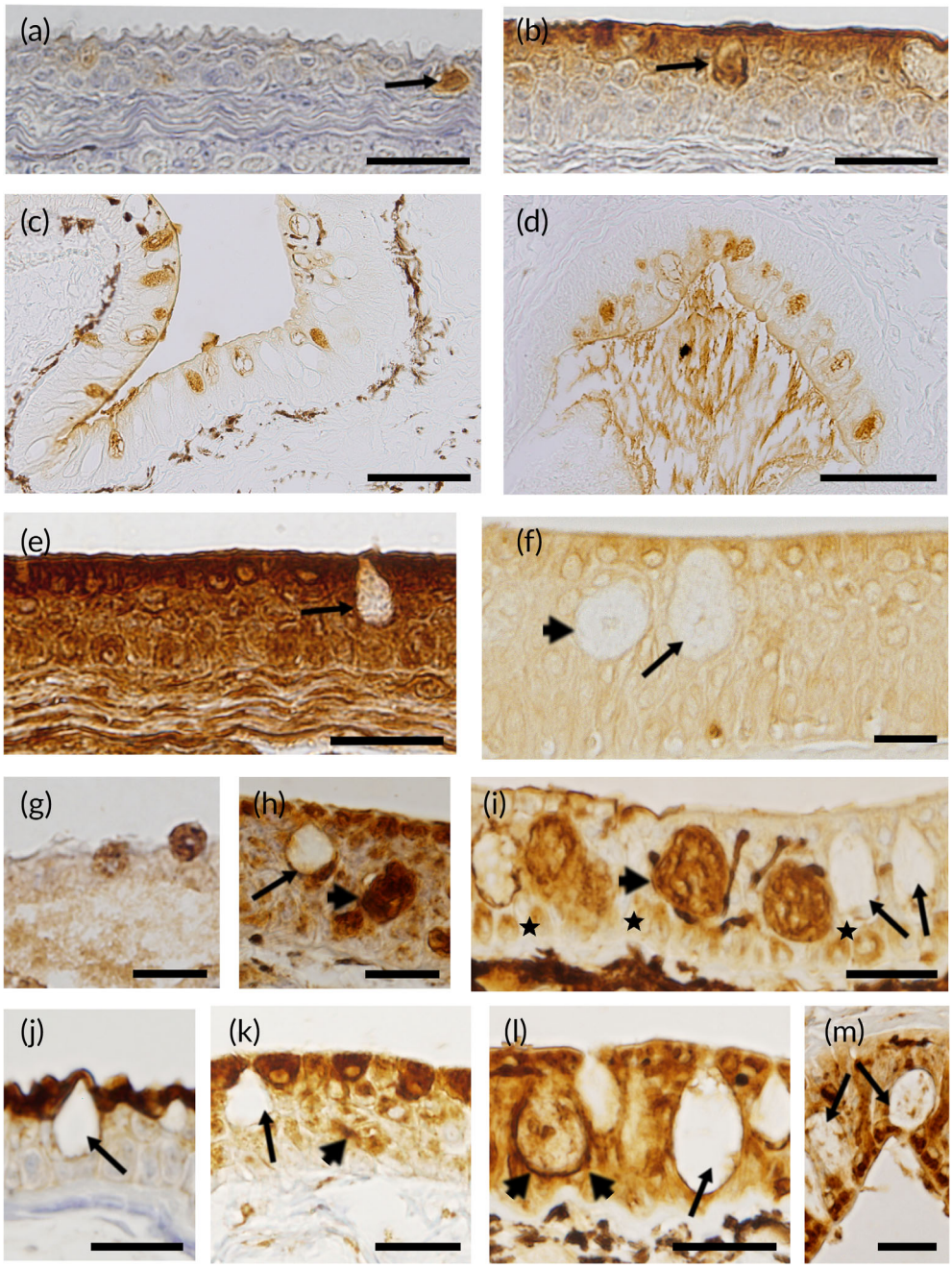

FIGURE 5 Lectin-binding sites in histological sections of the epidermis of *Scophthalmus maximus* during development. (a) Photomicrograph through the epidermis of a 32 day pre-metamorphic larvae, showing UEA-I staining in mucous cells (arrow). (b) UEA-I stains mucous cells (arrow) and the apical layers of the epidermis in post-metamorphic stages. (c and d) Mucous cells of the dorsal (c) and ventral (d) epidermis are positive to UEA-I; nonetheless, the intensity of the staining of epithelial cells decreases in juvenile stage. (e and f) The Con A staining of mucous (arrows) and club-like cells (arrowheads) is negative along the life cycle including the post-metamorphic larvae of 42–56 days (e) and juvenile stages (f). Nonetheless, the epithelial cells show a strong reactivity from the basal to the apical epithelial layers. The intensity decreases in juvenile stage (f). (g–i) The pattern of staining for PNA (g and h) and RCA (i) is similar. Mucous cells are positive in day 3 (g) and along the larval pre-metamorphic stage, but they become negative in post-metamorphic stage (arrow, h) in which the club-like cells show reactivity to both lectins (arrowhead). In this stage epithelial cells from basal to apical layers are positive being the staining stronger in the latter layer than the former. In the dorsal part of the epidermis at the juvenile stage (i) the pattern is the same than in post-metamorphic stage; nonetheless, only the epithelial cells from the basal layer show reactivity (asterisk). (j–m) The SBA staining is negative for mucous cells (arrow) from pre-metamorphic (j) to post-metamorphic (k), including the dorsal (l) and ventral (m) epidermis from juvenile stage, but positive for the club-like cells (arrowhead) and the apical layer of epithelial cells. Scale in (a, b, e, i, k, l, m) 40 μm; in (c, d) 100 μm; in (f, g, h, j) 20 μm

Four types of cells have been observed in turbot's epidermis, which, in a gradient of abundance, were epithelial, mucous, club-like and pigmented cells. The first two types are always present in the epidermis, but pigmented cells appear along larval development, and club-like cells are observed only after metamorphosis. Nonetheless, previous studies on the epidermis of postspawning adult turbot only described two types of cells – epithelial and mucous cells (Faílde *et al*., 2014).

4.1 | Epithelial cells

Conventional AB-PAS histochemical staining revealed that these cells were negative to both dyes, either throughout development (*i.e*., larva to juvenile) or in adult postspawning Although this result may indicate that these cells have no secretory function (Rodríguez-Alonso *et al*., 2017), they are labelled with some lectins, indicating the presence of glycoproteins and proteoglycans. These inconsistent results could be explained by the higher sensitivity of lectins histochemical method to reveal a number of glycoconjugates compared to the identification provided by conventional methods (Danguy *et al*., 1988; Zaccone *et al*., 2001). In fact, the contribution of epithelial cells, mainly the superficial layers, to mucus secretion has been described in a number of teleost fish (Elliot, 2011; Zaccone *et al*., 2001).

Lectin analysis also showed epithelial cells containing glycoproteins with mannose (Con A labelling) and fucose (UEA-I labelling) in different stages of the turbot development. Con A was positive in all the epidermis layers throughout fish development, even if juveniles revealed a gradient in intensity from basal to superficial layers. Accordingly, the presence of mannose residues in epithelial cells has been found in several teleost fish species such as in the superficial layer of blenny (*Blennius sanguinolentus*; Zaccone *et al*., 1985), spread in all epidermis of plaice (*Solea senegalensis*; Sarasquete *et al*., 1998) or intermediate epidermal layers of trout (*Oncorhynchus mykiss*; Burkhardt-Holm, 1997). In contrast to mannose, fucose (positive UEA labelling) was only present after fish metamorphosis, decreasing the intensity of the staining in juveniles. This presence of fucose only in the post-metamorphic stage of turbot may reflect a change in environment and microhabitat, related to flatfish benthonic habits and could contribute to increase the lubricity of the skin (*e.g*., Ottesen & Olafsen, 1997). In fact, fucose residues have been mainly described in the epithelium of sessile species to facilitate its adhesion to the substratum (Bravo *et al*., 2012).

Lectin analysis also revealed the presence of other glycoconjugates containing *N*-acetylglucosamine or sialic acid (WGA labelling), *N*-acetyl galactosamine (SBA labelling) and the sequence galactose – *N*-acetylgalactosamine (PNA and RCA-I labelling). The presence of sulphated glycosaminoglycans, which are constituents of proteoglycans, is known to be responsible for increasing skin mucus viscosity (Bravo *et al*., 2012).

4.2 | Mucous cells

The second most common cell type in turbot epidermis is involved in secretory function (Esteban & Cerezuela, 2015; Whitear, 1986). The same range of mucous cells' density has been found in other flatfish species like Atlantic halibut (Ottesen & Olafsen, 1997), plaice (Fletcher *et al*., 1976; López-Vidriero *et al*., 1980) and flounder (Fletcher *et al*., 1976). Nonetheless, a dramatic increase in the number of mucous cells was observed during fish metamorphosis (from pre- to post-metamorphic stages). This fact may reflect a need for enhanced mucus production when adult fish colonize the sand-bottom environment (Rodríguez-Alonso *et al*., 2017). Moreover, the low number of mucous cells in pre-metamorphic larval epidermis compared to the adult stage suggests that fish is less protected in terms of mucus production than in metamorphosed fish (Ottesen & Olafsen, 1997). Besides changes during development, the number of mucus cells in turbot juveniles also varied depending on the region of the fish body, being higher in dorsal than ventral areas. Similar result has been found in a histomorphometry study conducted by Mohamed *et al*. (2020) in two teleosts (*Otolithes ruber* and *Huso huso*) and one elasmobranch (the catfish *Pangasius hypophthalmus*) fish. Nonetheless, in these species mucous cells were scattered along the superficial layers, whereas in marine turbot, mucous cells appeared randomly distributed in dorsal area and clustered or concentrated in ridges in ventral areas.

Mucous cells showed PAS positivity in post-metamorphic and juvenile turbot, indicating the presence of neutral glycoproteins. Moreover, the lectin histochemical analysis showed the presence of glycosaminoglycans rich in the sequence galactose-*N*-acetylgalactosamine (labelling with PNA and RCA-I) at early larval stages (up to day 32), but mucous cells were mainly composed of L-fucose (UEA-I labelling) during metamorphosis (beyond day 32). Usually, fish mucus layer consists of a fluid of complex composition in which glycoproteins and glycosaminoglycans are mixed (Landeira-Dabarca *et al*., 2014; Rodríguez-Alonso *et al*., 2017; Whitear, 1986; Zaccone, 1983); nonetheless, these changes may reflect a shift in function. This result indicates that during pre-metamorphic fish stage, mucous cells (rich in glycosaminoglycans) increase viscosity to enhance protection and defence against pathogens (Díaz *et al*., 2010), could be a very important protective material for the vulnerable larval stages. Then, the shift of content from glycosaminoglycans to glycoproteins rich in L-fucose at the end of pre-metamorphic stage until juveniles could reflect the adaptation of the animal to the new asymmetry, as well as the modification of their behaviour, from an active and pelagic larva to a more inactive burrowing, but predatory adult (Ottesen & Olafsen, 1997; Roberts *et al*., 1973). This new composition of the mucus would provide an increase in lubrication to adapt to its new habitat. A change in lectin-binding sites has been described during the epidermal maturation processes in humans (Reano *et al*., 1982). However, the chemical nature of the mucus produced by mucous cells may occur during larval metamorphosis (Ottesen & Olafsen, 1997 in halibut; Rodríguez-Alonso *et al*., 2017 in lamprey), or in the case of the gill's mucous cells when the fish change the habitat from fresh water to sea water (Roberts & Powell, 2005), or even in response to pathogenic infection (Gómez *et al*., 2013).

4.3 | Club-like cells

Unlike club-like cells, the true club cells of some fishes do not stain with conventional PAS/AB technique (Al-Banaw *et al*., 2010; Halbgewachs *et al*., 2009; Wisenden & Smith, 1997), or with a wide battery of the carbohydrate-specific lectins (Al-Banaw *et al*., 2010). This fact could be explained by the fact that the cytoplasm of true club cells contains few vesicles and is filled with a fibrillar material (Herikson & Maltoltsy, 1968). Nonetheless, turbot club-like cells have an acidophilic cytoplasm which is granular in appearance, sharing this characteristic with sacciform cells (Elliot, 2011). Club-like cells were apparent only after metamorphosis in the dorsal area of juvenile fish, but were not observed in postspawning adults (Faílde *et al*., 2014). The variation in number of club cells among different areas of the epidermis and along the life cycle of turbot has also been described in some cyprinoids that lose their club cells during the spawning season (Smith, 1976). Lectin analysis indicated that club-like cells in marine turbot contained glycoconjugates rich in galactose and *N*-acetylgalactosamine. The content of true club cells of some fish species is mainly composed of chondroitin and keratan sulphate (Ralphs & Benjamin, 1992) that are polymers of galactose, *N*-acetylgalactosamine and *N*-acetylglucosamine chains. Usually, club cells are associated with alarm function, as its content is released through skin damage during a predation event and evoke an anti-predator strategy in conspecifics (Hintz *et al*., 2017). This utility could explain the presence of these cells only in postmetamorphic turbot dorsal area, which is the region exposed to potential predators in benthonic flatfish. Nonetheless, more recent genetic studies (*e.g*., Pandey *et al*., 2021) have assigned to club cells a primary role in immune function because of the identification of certain molecules, rather than an alarm signalling that might have evolved secondarily.

4.4 | Pigmented cells

According to other studies, the chromatophores observed in marine turbot were mainly located between the basal lamina and the dermis (Burton, 2010; Faílde *et al*., 2014). Nonetheless, in the present study chromatophores integrated in the middle of epithelial cells (epidermis) of dorsal area were revealed. This one-side distribution of chromatophores may be a consequence of turbot benthonic life adaptation to sandy bottom, as a camouflage system. In fact, the epidermal chromatophores have been considered to be not involved in rapid colour change (Bagnara & Hadley, 1974), although they do show physiological responsiveness to background change (in flounder, Burton, 1981). Nevertheless, the lack of epidermal chromatophores in postspawning turbot adults described by Faílde *et al*. (2014) might suggest that these pigmented cells are exclusive of dorsal epidermis in juveniles and disappear throughout the development. In fact, Burton and Fletcher (1983) found in flounder that epidermal chromatophores can be lost for a short time during postspawning epidermal thinning.

The significance of glycoconjugate composition changes and the proportion of the different cell types during fish life cycle underline the importance of the innate immune cellular function of the epidermis in turbot. This study sheds new light on the morphology and components contained in epidermal cellular types in marine turbot, and contribute to better understand the changes that imply a sensitive process such as metamorphosis. Although these features are believed to underlie different functions, more research is still required to determine more precisely the role of different mucus compositions at different stages of the marine turbot life cycle. Furthermore, the study of fish proteome to reveal specific molecules, its changes as a response to infections and possible local signalling networks in fish skin entails a considerable task for future years.

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AUTHOR CONTRIBUTIONS

The conceptual idea was detailed by M.A. and P.M, whereas A.L-D., C.S.R.A. and P.M. participated in data collection, processing of samples and preparation of figures and table. Finally, M.A., A.L-D. and P.M. contributed to the writing of the manuscript.

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