# Chapter 6

## Extracellular matrix proteins expression in sea bream (*Sparus auratus*) and tilapia (*Oreochromis mossambicus*) scales

## 6.1 Introduction

Most teleosts are covered by thin lamellar and imbricated scales which are of the elasmoid type (Bereiter-Hahn and Zylberberg, 1993, Fouda, 1979, Onozato and Watabe, 1979, Sire and Arnulf, 1990, Sire*, et al.*, 1997a, Sire and Arnulf, 2000, Sire and Akimenko, 2004). The elasmoid scale is organized in three tissue layers, a basal plate, an external layer and a limiting layer. Elasmoid scales with the same general structure have been described in a number of different teleosts, which include*, Pomatoschistus microps* K. (Fouda, 1979), *Cyprinodon variegates* (Olson and Watabe, 1980), *Hemichromis bimaculatus* (Sire and Meunier, 1981), *Danio rerio,* (zebrafish, Sire*, et al.*, 1997a), *Oncorhynchus mykiss* (rainbow trout, Persson*, et al.*, 1999) and *Cichlasoma nigrofasciatum* (Sire and Arnulf, 2000). The basal layer consists of a thick layer of incompletely mineralized tissue composed of extracellular matrix proteins (ECM) and the elasmodine (numerous layers of collagen fibres organized into a plywood-like structure). The external layer is the first to develop during scale ontogeny and consists of a thin layer of well-mineralized tissue composed of a network of interwoven collagen fibrils, and forms regular elevations along the scale, the *circuli*. This layer does not thicken after its primary deposition and is continuously deposited at the scale margin and contributes to scale growth in diameter. The external limiting layer is deposited late in ontogeny and covers the surface of the external layer in the posterior region of the scale, close to the epidermis. It is highly mineralized, devoid of collagen fibrils and thickens continuously improving scale protection and attachment to the epidermis (Bereiter-Hahn and Zylberberg, 1993, Olson and Watabe, 1980, Sire*, et al.*, 1997b, Sire and Arnulf, 2000, Sire and Akimenko, 2004).

Collagens are the major components of the extracellular matrix together with non-collagenous glycoproteins and proteoglycans (Bosman and Stamenkovic, 2003, Mecham, 1998). The extracellular matrix is an important component of the cellular environment. It offers structural support for cells and can also act as a physical barrier or selective filter to soluble molecules. In addition to forming a complex 3-dimensional architecture around the cells, ECM molecules regulate cell behaviour by modulating cell proliferation and differentiation, interfering with

cell adhesion and movement, response to growth factors, cytokines and signal transduction pathways (Kleinman*, et al.*, 2003, Velleman, 2000). Gene targeting studies of ECM proteins in a range of organisms *in vivo*, demonstrate the importance of cell-matrix interactions during development (Adams and Watt, 1993, Kleinman*, et al.*, 2003, Mecham, 1998). The composition of the ECM and its spatial relationship with cells differs between tissues and at different stages of development (Adams and Watt, 1993, Behonick and Werb, 2003, Kleinman*, et al.*, 2003, Velleman, 2000). In fact, mineralized tissues contain specialized extracellular proteins. These proteins probably enable, enhance and control mineralization. They may also function in the controlled release of minerals to fulfil the demands of the organisms in periods of growth, reproduction and starvation.

Information about ECM proteins in fish scales is scarce and apart from collagen relatively little information exists about the other ECM proteins. A vitamin K-dependent, Gla-containing protein was isolated from *Lepomis macrochirus* (bluegill) and the identity between scale Gla protein and bone Gla protein was established. These studies also suggest a relationship may exist between the concentration of bone Gla protein and the responsiveness of bones (or scales) to calcium demand, as metabolically inactive bones are characterized by high levels of this protein (Nishimoto*, et al.*, 1992).

Bone formation and remodelling are complex processes tightly regulated by mechanical stimuli and by local and systemic factors, such as transcription factors, cytokines, growth factors, prostaglandins and hormones (Bland, 2000, Compston, 2001, Yang and Karsenty, 2002). Estradiol has been shown to induce calcium mobilization from scales and bone in fish species (Mugiya and Watabe, 1977, Persson*, et al.*, 1995, Persson*, et al.*, 1997, Persson*, et al.*, 1998). Furthermore, it was shown that, at least part of the estradiol induced calcium mobilization from scales, in rainbow trout, results from an increase in the osteoclast activity (Persson*, et al.*, 1995, Persson*, et al.*, 1997) indicating that in fish estradiol has an opposite effect when compared to the antiresorptive role in mammals. The detection of estrogen receptor mRNA expression in rainbow trout bone and scales, *in vivo*, suggests that the effects of estradiol on

calcium mobilization may be mediated directly through estrogen receptors expressed in these tissues (Armour*, et al.*, 1997, Persson*, et al.*, 2000).

The objective of the present work was to characterize scale morphology in a marine teleost, the sea bream (*Sparus auratus*) and an euryhaline species, tilapia (*Oreochromis mossambicus*) maintained in fresh water. Subsequently, ECM transcripts were identified in the scales and their molecular organization partially characterized. The target genes selected were those in which the predicted protein product is thought to be important in calcium binding and also of structural importance in cartilage and bone formation. The target genes were isolated from sea bream calcified tissue and their relative abundance determined by RT-PCR. The expression of the ECM proteins in the scales and their putative cellular localization was established by *in situ* hybridization. In addition, to study the possible involvement of estrogen in the endocrine regulation of the mineralization process, the putative localization of estrogen receptors was determined by immunohistochemistry in the scales of sea bream and tilapia and the results obtained were related with the expression of ECM proteins.

## 6.2 Materials and Methods

### 6.2.1 Sampling of scales

Sea bream (*Sparus auratus*) and tilapia (*Oreochromis mossambicus*) scales were plucked with forceps from the caudal region of four individuals of each species and immediately fixed in 4% paraformaldehyde overnight, at 4ºC (section 2.2). Scales were collected from fish in two different stages of development, juvenile sea bream (approx. 50g and 15cm) and tilapia (approx. 10g and 5cm) and adult sea bream (approx. 400g and 30cm) and tilapia (approx. 80g and 15cm). To obtain sagittal sections of skin with scales, samples of the lower jaw of 80 days post hatch (dph) sea bream were collected and fixed in Bouin-Holland solution, for 7 days, at room temperature (section 2.2). Whole scales and the lower jaws were embedded in paraffin following the procedure described in section 2.2 and appendix II and serial sections (8µm) were cut and

mounted on APES (3-aminopropyltriethoxysilane) coated slides (appendix II). Other scales were stored in 70% ethanol at 4ºC until they were used for general histological staining or for whole mount *in situ* hybridization.

### 6.2.2 General histology of scale

To characterize scale morphology, whole scales were stained with haematoxylin and eosin (section 2.3.1), toluidine blue (section 2.3.2) and with alcian blue/alizarin red (cartilage-bone double staining, section 2.3.5). Sagittal sections of the lower jaw of 80dph sea bream were used to characterize scales still embedded in the epidermis/dermis as sections could be processed and remained intact, in contrast to other regions of fish. These sections were stained using Masson's trichrome method (section 2.3.3).

### 6.2.3 RT-PCR of target ECM genes in scales

The relative abundance of five genes encoding five different extracellular proteins characteristic of the ECM of skeletal tissue in terrestrial vertebrates was determined in the sea bream by RT-PCR. The genes analysed included type I collagen, α1 (Col1A1), type V collagen, α2 (Col5A2), fibronectin (FN), osteonectin (OSN) and tartrate-resistant acid phosphatase (TRACP). Total RNA was extracted from adult sea bream scales, opercular bone, cranial bone and kidney using TRI reagent, cDNA was synthesized and RT-PCR reactions were carried out as described in section 2.6. Sea bream specific primers for each of the target genes were designed using the sequence of previously isolated cDNA from different sea bream cDNA libraries and/or based on the available piscine cDNA sequences. The target gene, sequence of the specific primers, and their annealing temperature (Tm) are presented in table 6.1.

A common amplification cycle was utilized for all the primers which differed in annealing temperature and cycle number which were dependent on the gene being studied. The thermocycle was composed of an initial denaturing step for 2 minutes at 95ºC, followed by 25-36 cycles (see Table 6.1) of 30 seconds at 95ºC, 30 seconds at annealing temperature (Tm, see Table 6.1) and 45

seconds at 72ºC, and a final elongation step of 5 minutes at 72ºC. Provisional studies were conducted to ensure template amplification terminated in the logarithmic phase of the reaction. To estimate the amount of cDNA that should be used in each reaction, RT-PCR was carried out using the housekeeping gene 18S ribosomal RNA and appropriate reaction conditions (Section 4.2.5). Amplified reaction products were separated on 1.5% agarose gels containing ethidium bromide (appendix II).

**Table 6.1 –** Specific primers used to amplify each of the target genes by RT-PCR and respective annealing temperature.

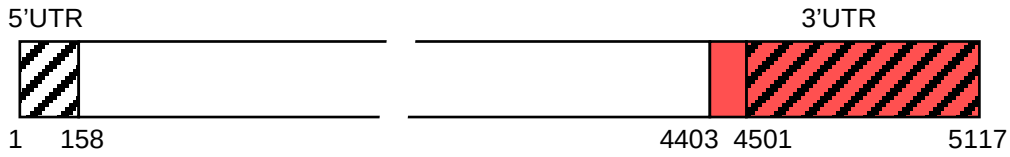
| Gene | Sequences | Cycles | Tm (ºC)* |
|---|---|---|---|
| Col1A1 | 5'-GAGAAGAAGCACGTCTGGTTC-3'<br>5'-CGATGACTGTCTTGCCCCATG-3' | 28 | 65 |
| Col5A2 | 5' ACAAGGACGAGAAGAATGGCAA-3'<br>5' ACAAGAAGCACACTGGCCCG-3' | 25 | 64 |
| FN | 5' CTGGGAAATGGCAAAGGAGAAT-3'<br>5' TTCGGGTCGGAGGCACTCG-3' | 30 | 66 |
| OSN | 5'-GGTCATCGTGGAAGAGCC-3'<br>5'-GCAGGAGGTGTCGTAGGT-3' | 28 | 55 |
| TRACP | 5'-GCCCTTGGTGATAACTTCTACT-3'<br>5'-ACAGCTTCACAGTGTCCAGCAT-3' | 36 | 55 |

*Tm – annealing temperature used in the thermocycling protocol (see above).

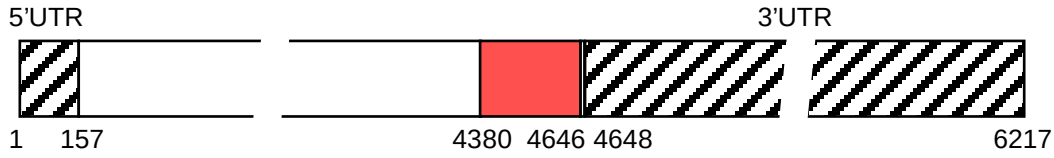
### 6.2.4 *In situ* hybridization

The expression of transcripts encoding ECM proteins was established by *in situ* hybridization in the scales of sea bream and tilapia. The cDNA clone used for riboprobe production, tissue origin of the cDNA, the vector in which cDNA were cloned, the restriction enzyme used to linearize the recombinant vector, the RNA polymerase used to synthesize the riboprobe and the probes approximate size are indicated in table 6.2. The relative localization of the sequence used to generate the riboprobe in the full-length cDNA is presented in figure 6.1.

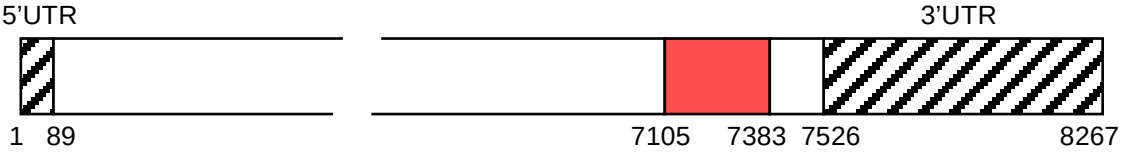
**Type I Collagen, α1 (Col1A1)**



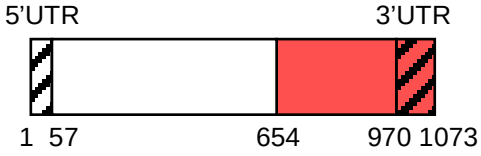

**Type V Collagen, α2 (Col5A2)**



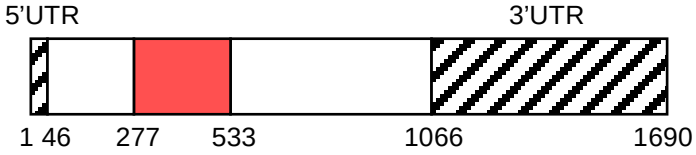

**Fibronectin (FN)**




**Osteonectin (OSN)**




**Tartrate-resistant acid phosphatase (TRACP)**




**Acidic secreted protein in cartilage (ASPIC)**

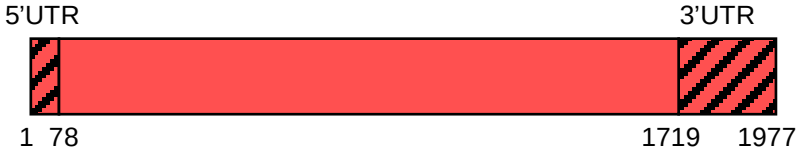



**Figure 6.1 –** Schematic representation of the full-length cDNAs of the sea bream genes. The approximate localization of the cDNA sequence used to generate the riboprobes is indicated in red and the 5' and 3'UTRs are cross hatched.

A characteristic of ECM proteins is that they frequently belong to large families of related proteins which share high sequence conservation in regions encoding functional motifs (eg. conserved calcium binding domain). If probes are generated to regions which are conserved in several different genes it is highly likely they will cross-hybridize to other genes and lead to misinterpretation of target gene distribution. Therefore prior to the production of specific riboprobes for the target genes care was taken (see following paragraph) to select a sequence unique to the target gene.

**Table 6.2 –** Characteristics of cDNA clones used to generate riboprobes for *in situ* hybridization. The cDNA library/tissue origin of the template, the vectors in which genes are cloned, the restriction enzymes used to linearize the recombinant vectors, RNA polymerases used to synthesize the riboprobes by *in vitro* transcription and the approximate size of the probes obtained, estimated by running the probes in agarose gels, are indicated.

| Probes | Origin | Cloning vector | Restriction enzyme | RNA Polymerase | Approximate probe size (bp) |
|---|---|---|---|---|---|
| Col1A1 | Library of intervertebral tissue | pBlueScript SK(+) | *Eco*R I | T7 | 500 |
| Col5A2 | Cloned fragment from intervertebral tissue | pGemT Easy | *Nco* I | SP6 | 300 |
| FN | Cloned fragment from intervertebral tissue | pGemT Easy | *Nco* I | SP6 | 300 |
| OSN | Library of intervertebral tissue | pBlueScript SK(+) | *Bgl* II | T7 | 400 |
| TRACP | Cloned fragment from scales | pGemT Easy | *Sal* I | T7 | 400 |
| ASPIC | Library of pituitary | pBlueScript SK(+) | *Eco*R I | T7 | 1200 |

Database searches using the BLAST algorithm (blastx, Altschul*, et al.*, 1997) confirmed the specificity of the cDNA segment chosen for riboprobe synthesis. The output of the BLAST analysis made it possible to establish if the probe sequence was similar to other related or unrelated genes in the NCBI database, and potential cross-hybridizing sequences were discarded. Examples of the highly significant blast hits obtained for the riboprobe sequence and the

respective E value are presented in table 6.3 (www.ncbi.nlm.nih.gov/), and confirm the high specificity of the gene sequences chosen for riboprobe production.

**Table 6.3 –** Examples of the most significant Blast hits obtained (www.ncbi.nlm.nih.gov/) for each of the gene sequences used for riboprobe production. The accession number and E value, of the sequences which gave the highest probability match with the target gene are indicated. Note - an E value of 0 signifies total identity and the more negative the value the greater the probability of identity with the target gene.

| **Probe** | **Blast hits** | | |
|---|---|---|---|
| | **Definition** | **Accession No.** | **E Value** |
| Col1A1 | type 1 collagen alpha 1 [Paralichthys olivaceus] | BAD77968 | 3e-11 |
| | collagen, type I, alpha 3 [Danio rerio] | NP_958886 | 9e-11 |
| Col5A2 | SI:bY143E18.1 (novel protein similar to human alpha 2 type V collagen (COL5A2)) [Danio rerio] | CAD58730 | 2e-42 |
| | COL5A2 protein [Homo sapiens] | AAH43613 | 2e-32 |
| FN | fibronectin variant 3 [Danio rerio] | AAP80678 | 1e-42 |
| | fibronectin precursor [Bos taurus] | AAD33692 | 1e-32 |
| OSN | SPARC [Oryzias latipes] | AAT01217 | 1e-150 |
| | osteonectin [Rattus norvegicus] | CAA74042 | 1e-140 |
| TRACP | zgc:63825 [Danio rerio] | NP_999938 | 1e-34 |
| | tartrate resistant acid phosphatase 5 precursor [Homo sapiens] | NP_001602 | 4e-26 |
| ASPIC | chondrocyte expressed protein 68 kDa [Homo sapiens] | CAC08451 | 0.0 |
| | cartilage acidic protein 1 [Homo sapiens] | NP_060528 | 0.0 |

For production of riboprobes recombinant cloning vectors were linearized, the DNA purified and *in vitro* transcription carried out following the protocol described in section 2.4.1. All riboprobes synthesized were labelled with digoxygenin.

*In situ* hybridization was carried out with scale sections following the general procedure described in section 2.4.2. Pre-hybridization (2 hours) and overnight hybridization were carried out at 58ºC and high stringency washes were carried out at the same temperature following the general methodology described in section 2.4.2. The probe concentration used in hybridization reactions was approximately 1-3$\mu g.ml^{-1}$. The localization of hybridized riboprobe was established using anti-digoxygenin-AP sera and NBT/BCIP as substrate (section 2.4.2). Colour reactions were allowed to proceed at 37ºC for 2-8 hours, depending on the probe and resulted in a purple/black deposit at the site of riboprobe hybridization. With this method, because of the characteristic of the sections of tissue and scale, it was extremely difficult to obtain results as most of the sections detached from the slides during the hybridization cycle. For this reason whole mount *in situ* hybridization was carried out with isolated scales.

The procedure described in section 2.4.2 was optimized for use with whole scales. *In situ* hybridization with scales was carried out in a 96 multi-well plate (one scale per well) for procedures conducted at room temperature. Before pre-hybridization, samples were treated with proteinase K (0.01$mg.ml^{-1}$, appendix I) for 5 minutes at room temperature, washed twice with PTW, re-fixed with 4% paraformaldehyde (appendix I) and washed again in PTW. High stringency conditions were utilized for hybridization and therefore pre-hybridization and hybridization were carried out at 65ºC. The probe concentration used in hybridization reactions was approximately 3$\mu g.ml^{-1}$. Stringency washes post hybridization were carried out at 65ºC. Samples were washed twice with 2×SSC (appendix I) for ten minutes, washed once with 1×SSC for ten minutes and then with 0.2×SSC for ten minutes. During the pre-hybridization, hybridization and stringency washes (65ºC) scales were maintained between two glass slides to prevent them curling-up. Subsequent washes were carried out following the

general methodology indicated in section 2.4.2. Detection of the probe was carried out using anti-digoxygenin-AP Fab fragments. Scales were incubated in the antibody solution at 4ºC overnight. During this step they were also maintained between two glass slides. NBT/BCIP were used as substrate for colour development (section 2.4.2), which was conducted at 37ºC for between 2-8 hours.

### 6.2.5 Production of antibodies

Polyclonal antibodies were raised against synthetic peptides, corresponding to amino acid sequences of the N- and C-terminal regions of sea bream estrogen receptors α (ERα) and β1 (ERβ1) and the N-terminal region of sea bream estrogen receptor β2 (ERβ2) (Table 6.4 and Figure 6.2). Their origin is also indicated in table 6.4.

**Table 6.4 –** Rabbit anti-sea bream antibodies produced and their origin, notation used and respective peptide sequences.

| Antibody | Notation | Peptide sequence | Origin |
|---|---|---|---|
| Anti-ERα (N-terminal) | 5NTA | RSSVPSSQHSVSREDQC | Sheffield University Krebs Institute |
| Anti-ERα (C-terminal) | 3CTA | SHESPTSPGVLQYGGSRSEC | Sheffield University Krebs Institute |
| Anti ERβ1 (N-terminal) | 5NTB | CSPEKDQSLLQLQKVDSSR | Sheffield University Krebs Institute |
| Anti ERβ1 (C-terminal) | 3CTB | SSRLPRRSPQQETVEQC | Sheffield University Krebs Institute |
| Anti-ERβ2 | KPS | KPSERPSSPRQL | AgriSera |

For the production of rabbit anti-sea bream N- and C-terminal antibodies against ERα and ERβ1, peptides (Table 6.4) were synthesized and conjugated to bovine thyroglobulin using carbo-diimide. The conjugated peptides were emulsified in complete Freund's Adjuvant to generate the rabbit polyclonal anti-sea bream ERs sera. The first injection was followed by a second injection of

conjugate emulsified in incomplete Freund's Adjuvant given 2 months later. The antisera were lyophilized, reconstituted in 2ml of sterile water and stored at -20ºC. Working solutions were kept at 4ºC. N-terminal antibody against ERβ2 was produced by injecting a rabbit with a synthetic peptide (Table 6.4) which was synthesized and conjugated through the N-terminus to keyhole limpet haemocyanin. The conjugate was injected in the rabbit 3 times over 14 weeks (~250µg per immunization).

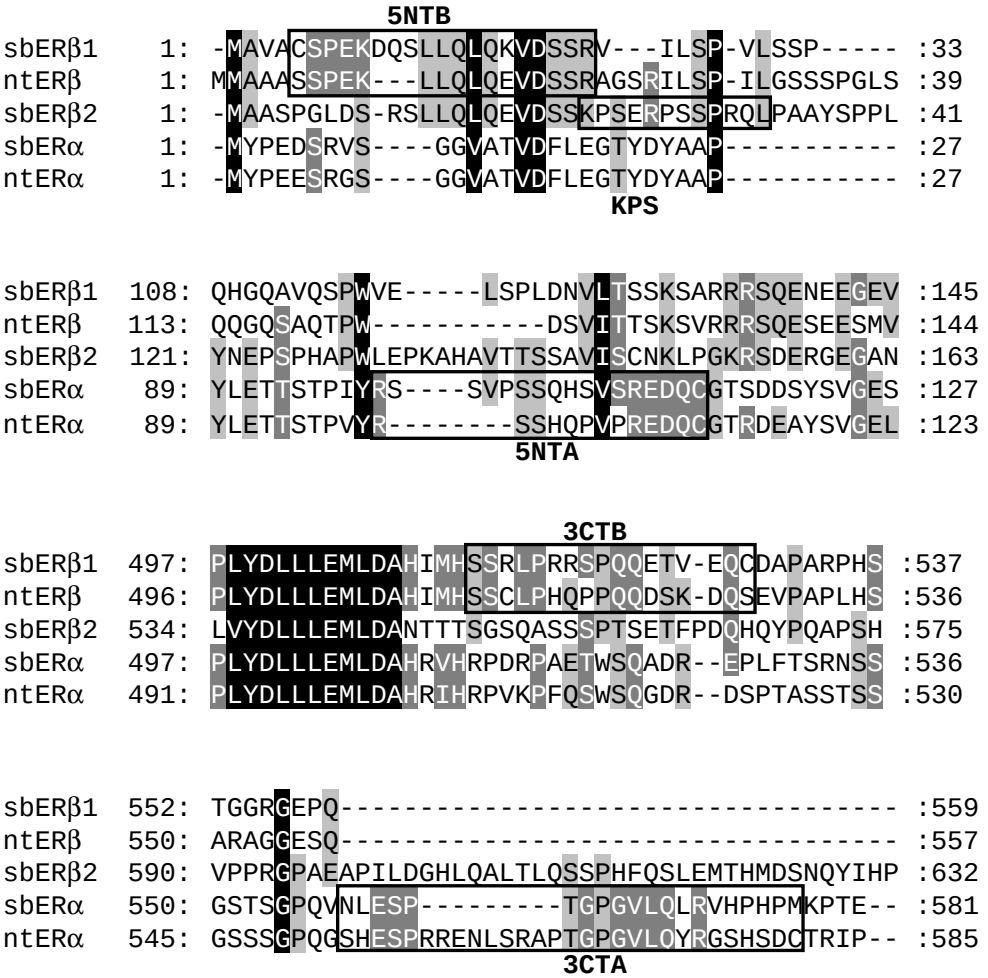



**Figure 6.2 –** Multiple alignment of regions of the sea bream (sb) and tilapia (nt) sequences of estrogen receptors showing the peptides used to produce the antibodies. The peptides used for the production of sea bream antibodies against each estrogen receptor were chosen from a different region of the sequence to avoid cross reaction with the other receptors. Sequences are partially conserved between the two species permitting the use of sea bream specific antibodies with tilapia samples (boxes).

### 6.2.6 Immunohistochemistry

The expression of the estrogen receptors (α, β1 and β2) in the scales of sea bream and tilapia was characterized by whole mount immunohistochemistry following the protocol described in section 2.5.

Several experiments were carried out to optimize the protocol in use. Different antisera concentrations (between 1/200 and 1/1000) were tested until it was verified which was the optimal concentration that should be used for each primary antibody (Table 6.5). In the case of ERα and ERβ1, N- and C- terminal antibodies were mixed in a 1:1 proportion and then used in the final concentration indicated in table 6.5. Time (4 hours or overnight) and temperature (room temperature or 4ºC) of incubation were also tested. Different assays were also carried out with or without agitation throughout the process to check if there was any improvement in the results. Finally, to test the specificity of the antibodies, experiments were carried out incubating the samples without primary antibody (negative control).

**Table 6.5 –** Antibodies used and their respective final concentration.

| Antibody | Concentration |
|---|---|
| Anti-ERα* | 1/500 |
| Anti ERβ1* | 1/1000 |
| Anti-ERβ2 | 1/750 |

*1:1 mixture of N-terminal and C-terminal antibodies

## 6.3 Results

Whole scales of sea bream and tilapia (juvenile and adult) were stained with alcian blue/alizarin red, haematoxylin and eosin, and toluidine blue to characterize scale morphology. Sagittal sections of the lower jaw of 80dph sea bream were stained with Masson's trichrome. The tissue organization evident after histological staining indicates that both sea bream and tilapia scales are of the elasmoid type. Sections of the lower jaw of 80dph sea bream revealed that

the scale is composed of three layers, the basal plate, external layer and limiting layer and this organization is characteristic of elasmoid scales.

There is a clear difference in the ratio of scale size to fish size when isolated scales from sea bream juveniles and adults are compared to those from tilapia juveniles and adults. The scale size difference is clearly evident when the images presented in figure 6.3 are analysed. All the scales in the figure (6.3) have been amplified to the same extent and it is obvious that the scales from a 15cm sea bream juvenile are much smaller than those of a 5cm tilapia juvenile. An additional difference between the sea bream and tilapia scales is that in the latter species the scales are much thicker and also have a greater number of melanocytes and these observations apply to scales isolated from both juvenile and adult fish.

Juvenile sea bream scales are oval in shape, although dimensions and shape change slightly as fish grow and in adult sea bream scales have a more typical elasmoid shape, and are similar to those described in other teleosts. The anterior region of the scale normally anchors it in the dermis and possesses *radii*, which are non-mineralized structures irregularly spaced along the anterior edge of the scale (Figure 6.4). The number of *radii* in different scales is not constant and changes from scale to scale, normally between 7-14 *radii* were detected. The number of *radii* did not appear to be related to the size of the scale or to the age of the animals. In the anterior and lateral regions of the scale the surface is irregular (ornamentation) as a result of concentric *circuli*, which are parallel to the scale margin. The *circuli* arise as a consequence of the accumulation of mineralized material in this region (Figure 6.4). The posterior region of the scale which when attached to the fish protrudes from the dermis, is covered by a single layer of epithelial cells and is lightly pigmented in the adult sea bream. Melanocytes are readily identified in the scale and are characterized by their star-like outline and dense pigmentation. The melanocytes are present in the epidermis which covers the scale and remains attached when scales are plucked from the fish (Figures 6.3 and 6.4). Near the focus and in the posterior region, scale ornamentation appears more irregular and is formed by ridges, in both juveniles and in adults (Figure 6.4).

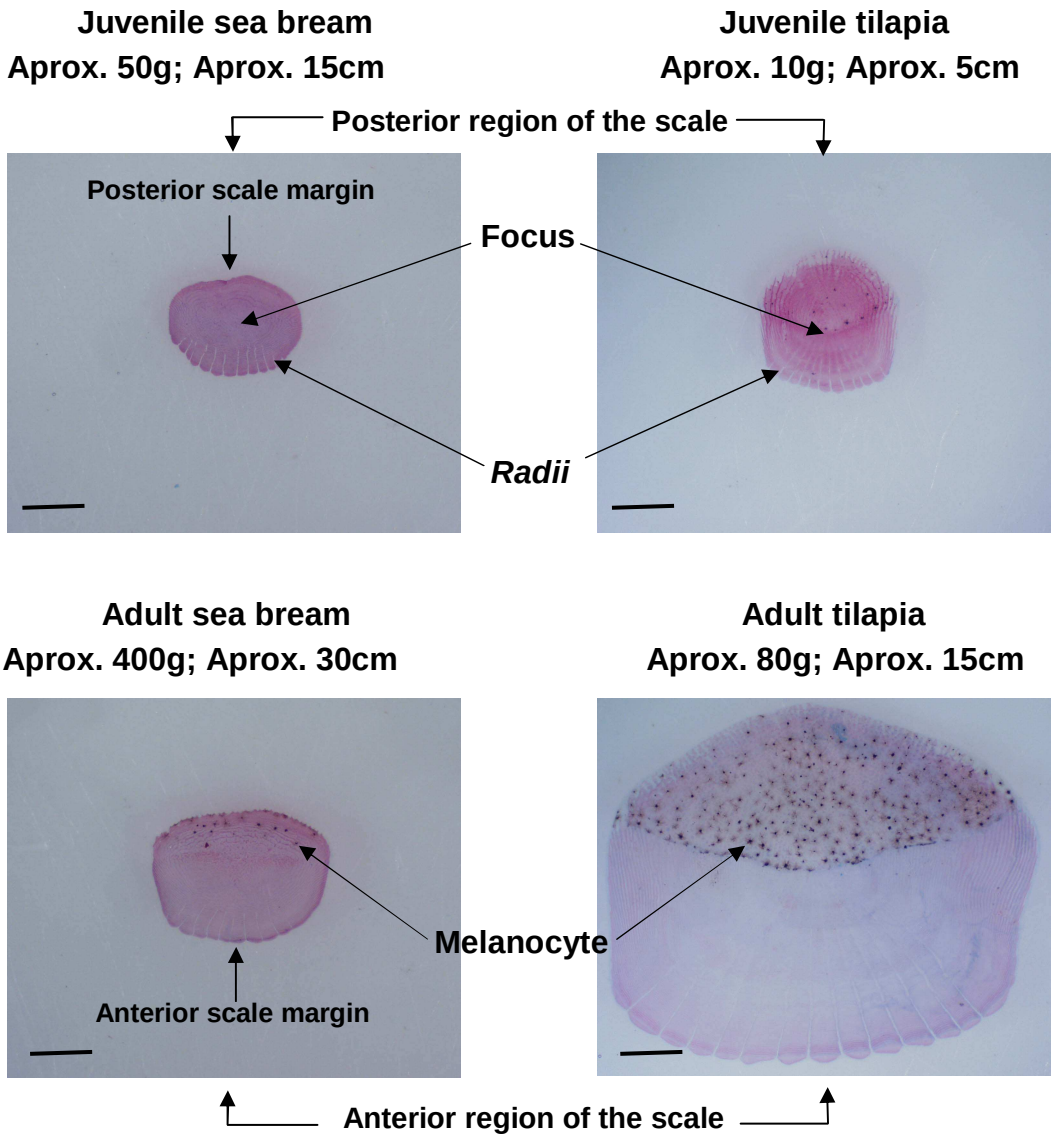



**Figure 6.3 –** Juvenile and adult scales of sea bream and tilapia. Scales are stained with alcian blue and alizarin red (double staining for cartilage and bone). All scales are presented in the same orientation, with the posterior region upwards. Note the difference in the ratio of scale size to fish size, when comparing sea bream and tilapia. Posterior and anterior scale margins, focus, *radii* and melanocytes are indicated with arrows. For more details see figure 6.4. Scale bars: 1mm

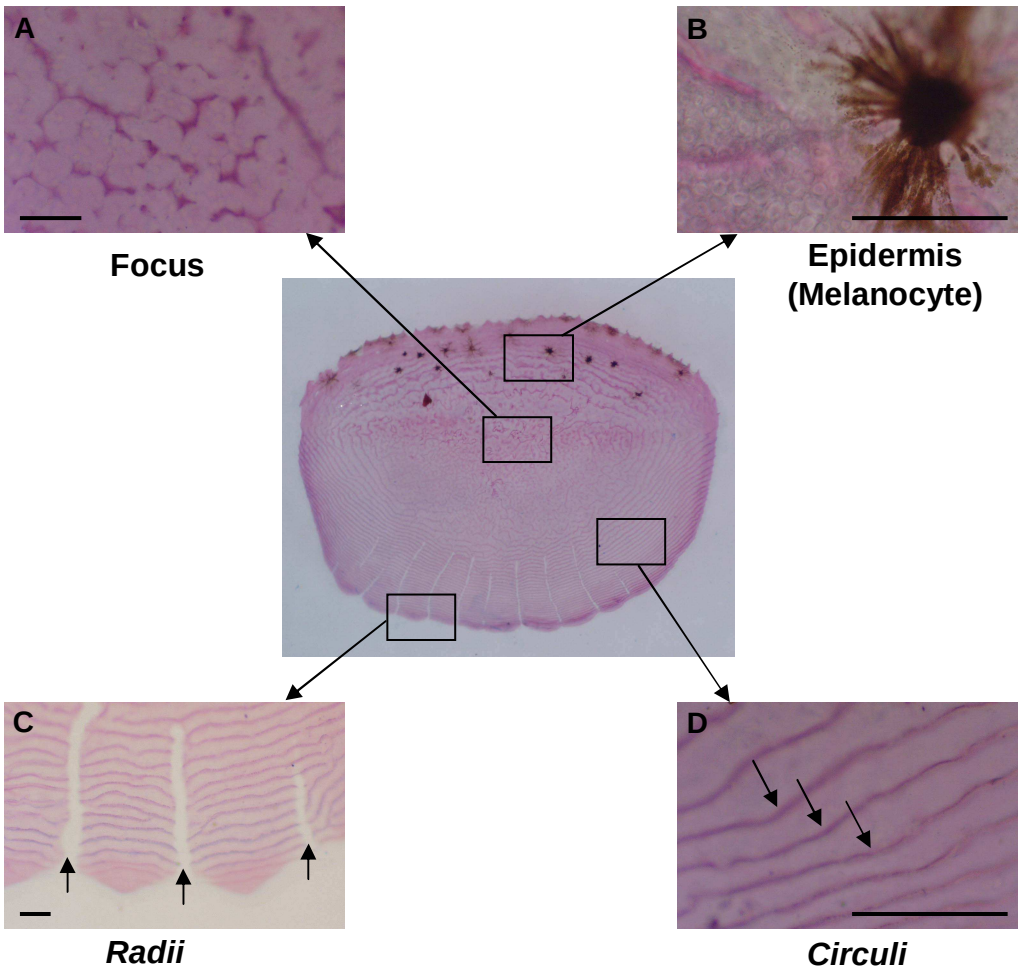



**Figure 6.4 –** Adult sea bream scale stained with alcian blue and alizarin red (double staining for cartilage and bone). The scale is presented with the posterior region upwards. A) Detail of the focus showing the irregular ridges formed by the accumulation of mineralized material. A consequence of this is that the surface structure (ornamentation) in the central region of the scale is irregular. B) Detail of the epidermal layer covering the posterior region of the scale, showing a melanocyte. C) Detail of the anterior margin of the scale. Arrows indicate the *radii* which are radial non mineralized grooves that radiate from the focus and that interrupt the *circili*. D) Detail of the anterior region of the scale. Arrows indicate *circuli* which are mineralized concentric ridges formed during scale growth. These structures are more evident in the anterior and lateral regions of the scales and constitute a more regular surface structure (ornamentation). Scale bars: 25µm.

Tilapia scales of both juveniles and adults have a typical elasmoid scale shape (almost quadrangular) and as the fish grow and the dimension of the scales increase there is no alteration to the general shape. In scales collected from adult tilapia, the posterior region is heavily pigmented and the covering epidermis contains numerous melanocytes (Figure 6.3). In juvenile tilapia the shape of the scale is similar to that observed in the adult but the epidermal layer contains relatively few melanocytes (Figure 6.3). The non-mineralized *radii* vary between 10 and 15 (adult tilapia) and 8 and 11 (juvenile tilapia) in the scales observed. In common with sea bream scales, *radii* are irregularly spaced along the anterior region, most of them originating close to the focus, and the variable number does not seem to be related to scale size. The concentric *circuli* in tilapia scales are more evident in the anterior region and are intensely stained with alizarin red which is specific for divalent ions, such as calcium. The staining for *circuli* at the margin of the scale (3-4 *circuli*) is much more noticeable and may suggest that this region is more mineralized. In the scales of the younger fishes the margin of the posterior region did not stain with alizarin red, indicating it is not mineralized.

In scales from adult sea bream and adult tilapia stained with alcian blue and alizarin red a very light blue stain is evident. Alcian blue is a cationic histological dye that stains mucopolysaccharides or glycoaminoglycans and the blue stain in scales is probably due to the presence of mucopolysaccharides, in mucus that remains on the scales after they are removed from the fish.

*In vivo* the anterior region of the scale is inserted into the scale pocket in the dermal layer of the skin and this organization is clearly evident in figure 6.5 which shows a sagittal section of the skin from sea bream juveniles which have been stained with Masson's trichrome. The posterior region of the scale protrudes out of the dermis and is covered by an epidermal layer which may be unicellular or composed of several different layers of cells (Figure 6.5). The posterior region of the scale which projects from the dermis overlaps with the anterior region of the neighbouring scale and this way produces a continuous coat of overlapping scales which totally cover the exterior surface of the fish with the exception of restricted regions which do not bear scales (eg. frontal

head region). Transverse sections through the scale (Figure 6.5) allows its organization to be observed and a well mineralized osseous layer (red stain in figure 6.5) is sandwiched between an upper limiting layer of connective tissue (stained green, Figure 6.5) and a lower basal layer (stained green, Figure 6.5) which contains the putative scale-forming cells.

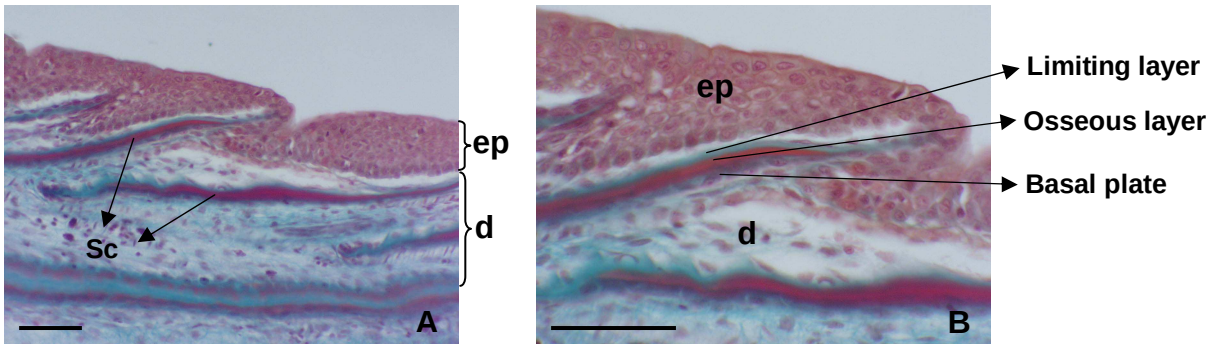



**Figure 6.5 –** Saggital section from the lower jaw of a juvenile sea bream (80 days post hatch (dph)) stained with Masson´s trichrome showing scales *in situ*. Connective tissue is stained green and mineralized tissue is stained bright red. The posterior region of the scales (Sc) is to the right. A) The anterior scale region is inserted in the dermis (d). The posterior scale region protrudes from the dermis and is covered by the epidermis (ep). Each posterior scale region overlaps the anterior region of the neighbouring scale. B) This image is a higher amplification of the image presented in A. The three main layers that constitute the scales are identifiable. Below the epidermis there is a limiting layer of connective tissue (green). Underneath, is a mineralized osseous layer (stained red) which overlays the basal layer, which stains green and where the putative scale-forming cells are identifiable. Scale bars: 50μm.

The mRNA expression of six ECM proteins, type I collagen, α1 (Col1A1), type V collagen, α2 (Col5A2), fibronectin (FN), osteonectin (OSN), tartrate-resistant acid phosphatase (TRACP) and acidic secreted protein in cartilage (ASPIC), was determined by whole mount *in situ* hybridization. These genes were chosen because they have important structural (Col1A1 and Col5A2) and regulatory (OSN and FN) functions in skeletal tissue and are used as markers for the presence and/or activity of cell types important in cartilage and bone development and remodelling, such as the chondrocytes (ASPIC) and the

osteoclasts (TRACP). Their relative expression intensity is presented in table 6.6 and their general distribution in the scales of juvenile sea bream and tilapia is schematically represented in figures 6.6 and 6.7, respectively. Localization and intensity of the detectable signals vary with the probe and with the species studied but the overall distribution and signal intensity did not differ greatly between juvenile and adult fishes, of the same species.

Haematoxylin-eosin and toluidine blue staining of sea bream and tilapia scales allowed the identification of different cell types which expressed the target transcripts of the present study with differing intensity (Figures 6.8-6.11, see below). Cells with a large nuclei and containing relatively little cytoplasm (osteoblast-like cells) are distributed in all regions of the scale. These osteoblast-like cells are also located in the space between the *circuli* in the anterior region of the scale (Figures 6.8 C and E, 6.9 C, 6.10 B and D). Near the scale margin the osteoblast-like cells seem to have a more flattened shape (Figures 6.8 E and 6.10 B). In addition, a second type of cells is also identifiable in anterior and posterior regions of sea bream and tilapia scales. The latter cell is characterized by its round shape and much smaller nuclei compared to the osteoblast-like cells (Figure 6.11 B).

**Table 6.6 –** Relative expression intensity of the target genes analysed by whole mount *in situ* hybridization. Results are presented for the anterior (Ant.) and posterior (Post.) regions of the scales of juvenile and adult sea bream and tilapia.

| Genes | Juvenile sea bream | | Adult sea bream | | Juvenile tilapia | | Adult tilapia | |
|---|---|---|---|---|---|---|---|---|
| | **Ant.** | **Post.** | **Ant.** | **Post.** | **Ant.** | **Post.** | **Ant.** | **Post.** |
| Col1A1 | +++ | + | +++ | + | +++ | +/- | +++ | +/- |
| Col5A2 | + | + | - | +/- | - | - | - | - |
| FN | +/- | ++ | +/- | + | - | - | - | - |
| OSN | +++ | + | +++ | + | ++ | +/- | ++ | +/- |
| TRACP | + | + | +/- | +/- | - | +/- | - | + |
| ASPIC | - | - | - | - | - | - | - | - |

(-) not detected, (+/-) very low abundance, (+) low abundance, (++) high abundance and (+++), very high abundance

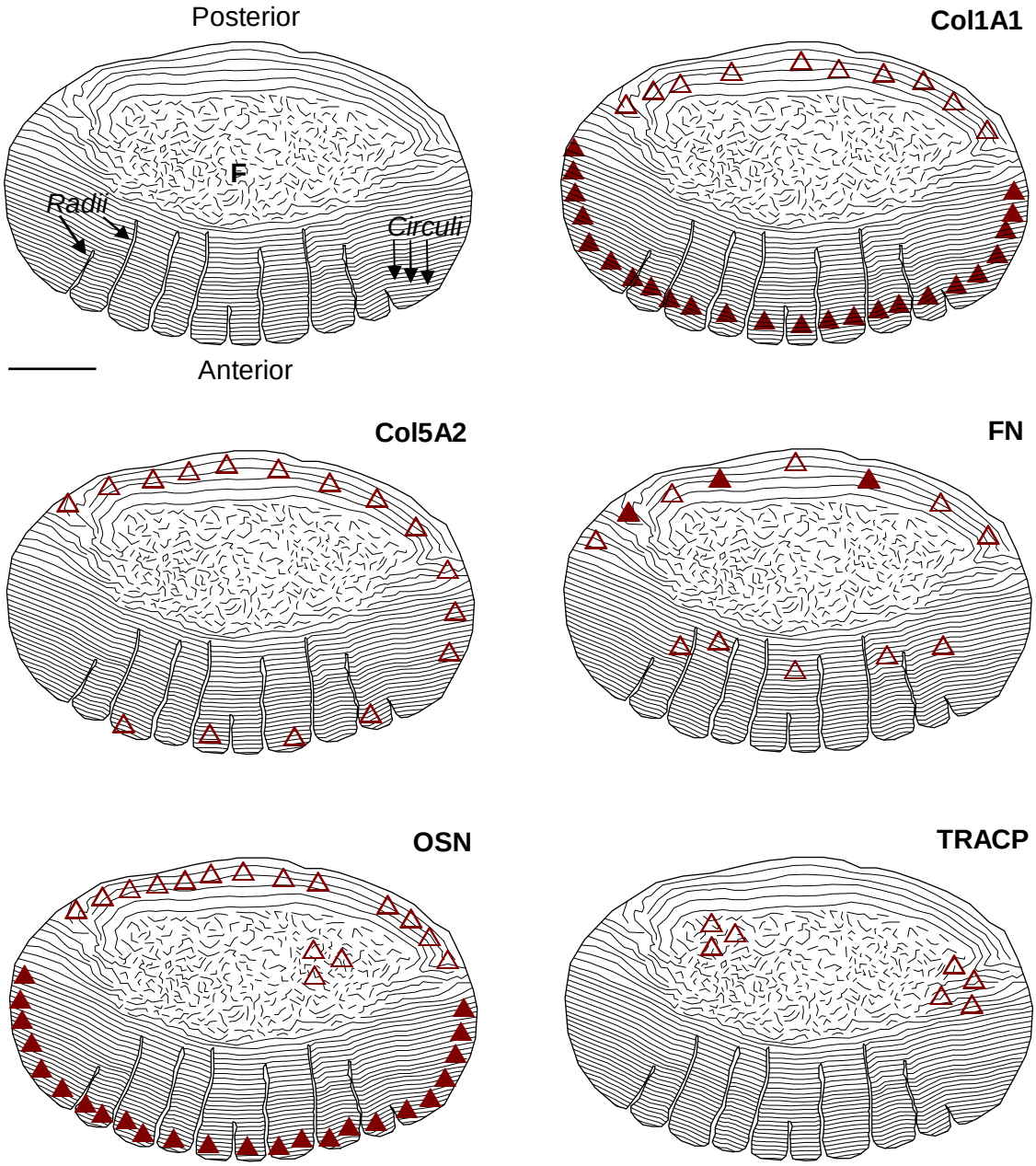



**Figure 6.6 –** Schematic representation of a juvenile sea bream scale. The relative distribution and intensity of the signal detected by *in situ* hybridization with the probes type I collagen, α1 (Col1A1); type V collagen, α2 (Col5A2); fibronectin (FN); osteonectin (OSN) and tartrate-resistant acid phosphatase (TRACP) is represented. The localization of the symbols shows the general distribution pattern observed in the scales analysed. Acidic secreted protein in cartilage (ASPIC) was not detected in sea bream scales. (▲) represents an intense signal and (△) represents a weak signal. F- focus. Scale bar: 500μm.

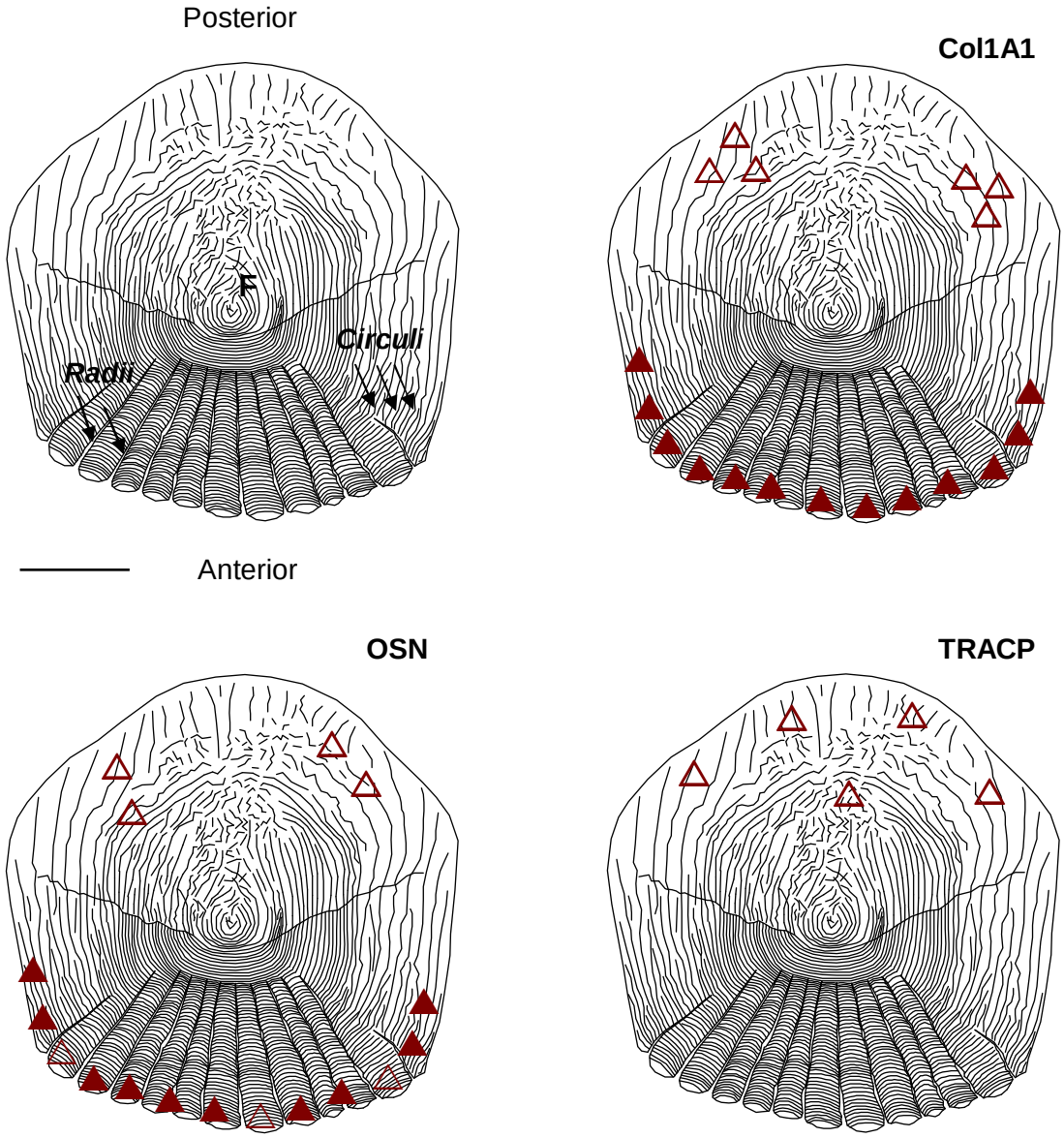



**Figure 6.7 –** Schematic representation of a juvenile tilapia scale. The relative distribution and intensity of the signal detected by *in situ* hybridization with the probes type I collagen, α1 (Col1A1); osteonectin (OSN) and tartrate-resistant acid phosphatase (TRACP) is represented. The localization of the symbols shows the general distribution pattern observed in the scales analysed. Acidic secreted protein in cartilage (ASPIC); type V collagen, α2 (Col5A2) and fibronectin (FN) were not detected in tilapia scales. (▲) represents an intense signal and (△) represents a weak signal. F- focus. Scale bar: 500μm.

Type I collagen alpha 1 chain (Col1A1) mRNA is abundantly expressed in both sea bream and tilapia scales (Table 6.6). The signal is most intense in the anterior region of the scale, accumulating near the margin and near the most anterior region of the *radii* (Figures 6.6 and 6.7). In both adult and juvenile sea bream scales a more generalized but weaker signal is detectable in the posterior region (Figure 6.6). In both tilapia and sea bream, Col1A1 mRNA expression is observed in two principle cell types distinguished by their characteristic morphology; flattened cells near the scale margin (Figure 6.8 E and F) and more rounded cells, with large nuclei and scarce cytoplasm (osteoblast-like cells), that seem to be associated with the basal plate (Figure 6.8 C and D). In order to establish if the Col1A1 is in the covering epidermal layer or in cells in the scales, toluidine blue staining was carried out. This staining reveals that the polyhedric cells which form the covering epidermal layer of the scale (Figure 6.8 A) do not hybridize with the Col1A riboprobe. No signal is detected when this cell layer is focussed (Figure 6.8 B and D) and the cytoplasm appears transparent, although an “out of focus” generalized purple background is evident. Focussing through the scale (Figure 6.8 D) makes it possible to establish the origin of the Col1A1 hybridization signal which is an intense signal associated with oval shaped cells possessing a large nucleus in the putative basal layer as identified by the haematoxylin-eosin staining (Figure 6.8 C). Transverse sections (not shown) of scales which hybridized with Col1A1 confirmed that the stained cells are in fact located in the basal plate layer, which contains the cells important in scale formation.

Type V collagen (alpha 2 chain, Col5A2) is undetectable in tilapia scales and is expressed in very low abundance in sea bream scales (Table 6.6, Figures 6.6 and 6.7). In both juvenile and adult sea bream scales Col5A2 mRNA is detected sporadically in flattened cells near the scale margin both in the anterior and posterior regions, and appears to be colocalized in the same type of cells that express Col1A1. In sea bream scales from young fish the signal is slightly more abundant (Table 6.6).

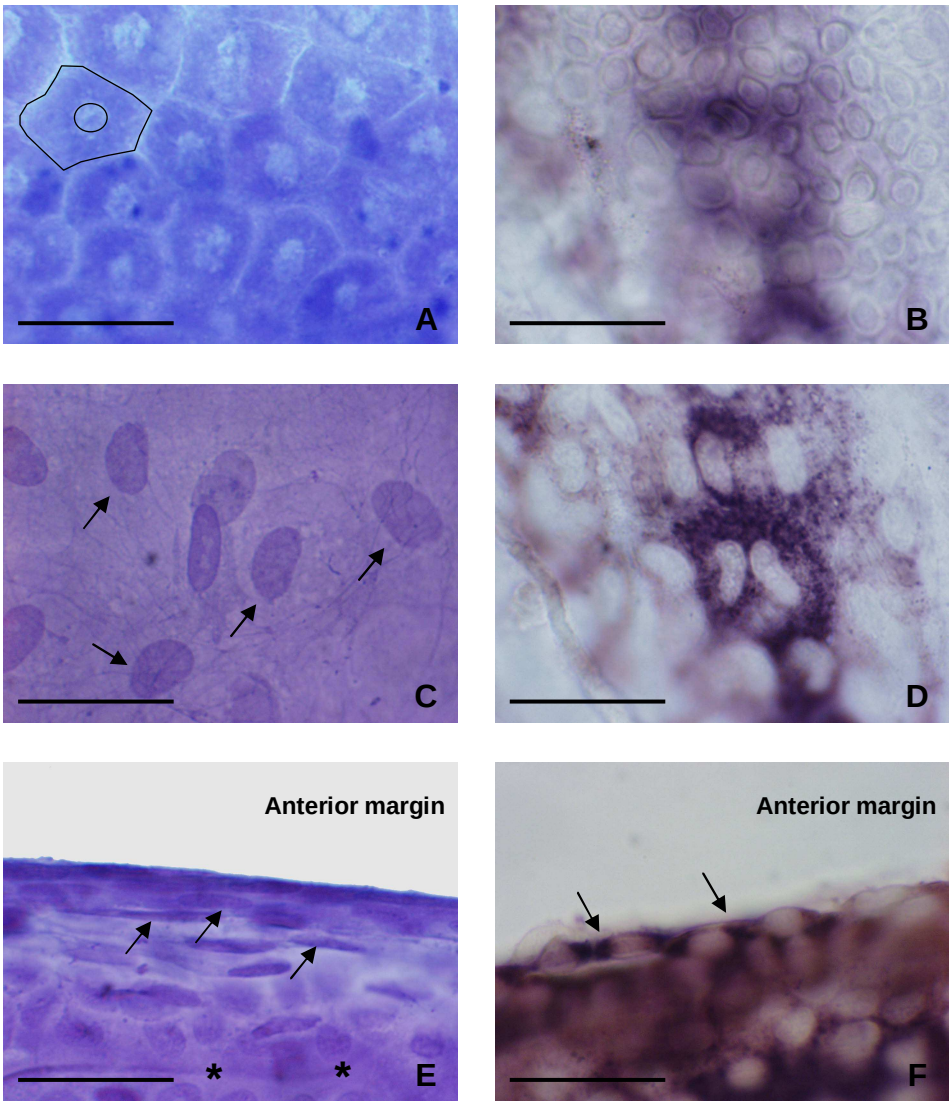



**Figure 6.8 –** General histology (A, C, E) and *in situ* hybridization of Col1A1 (B, D F) in juvenile sea bream. A) Juvenile sea bream scale stained with toluidine blue. Detail of the epidermis covering the anterior region of the scale showing the large polyhedric cells, one of which is outlined in black, that form the epidermal layer. C) and E) Juvenile sea bream scale stained with haematoxylin and eosin. In C the anterior scale region is shown in which large rounded cells with big nuclei (arrows) associated with the basal scale layer are visible. Near the anterior scale margin (E) another cell type is visible with a more flattened appearance (arrows). *Circuli* are identified by asterisks. Juvenile sea bream scales strongly stained for Col1A1 in the anterior scale region (B, D, F). Image B presents the epidermal cells in A and demonstrates they do not hybridize with Col1A1 and that the reaction appears to be in other cells. mRNA expression was detected in the rounded cells of the basal layer (D) and in the flattened cells of the scale anterior margin (F, arrows). It is clear that the cells of the epidermis do not stain for Col1A1 although a generalized purple stained is evident which correspond to the signal detected in the basal layer cells (D). Scale bars: 25µm.

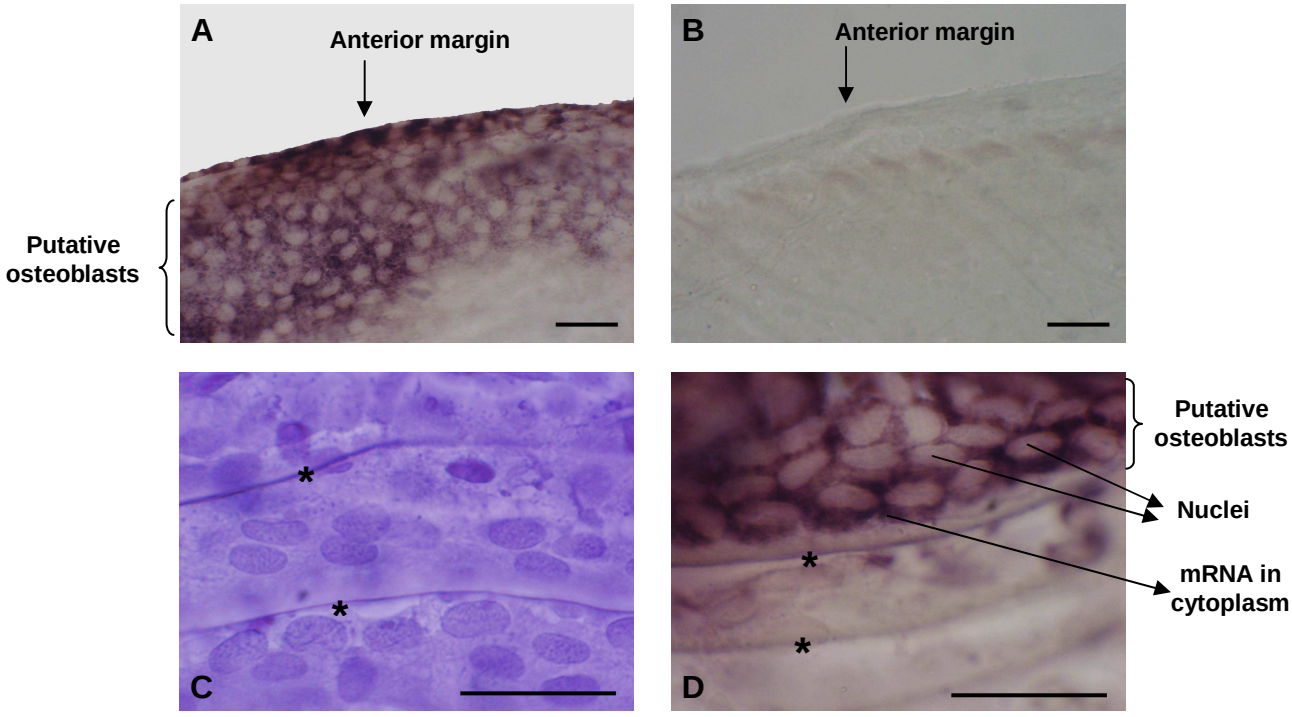



**Figure 6.9 –** *In situ* hybridization of OSN in juvenile sea bream (A) and tilapia (D). High expression of OSN mRNA was detected in the anterior margin of the juvenile sea bream scale (A). Staining occurred in the cytoplasm of flattened cells at the scale margin and also the more rounded cells, associated with the basal layer. The rounded cells which hybridize with OSN riboprobe have big nuclei and little cytoplasm as is evident when juvenile sea bream scales are stained with haematoxylin and eosin (C). The morphology of cells hybridizing with OSN suggests that they are putative osteoblasts (A and D). No signal was detected in the negative control in which hybridization was carried out without OSN probe in a juvenile sea bream scale (B). *Circuli* are identified by asterisks in images C and D. Scale bars: 25μm.

A very intense expression of osteonectin mRNA is observed in both sea bream and tilapia scales although in tilapia signal intensity is slightly lower than in sea bream (Table 6.6). The difference in signal intensity may just be a consequence of using heterologous sea bream riboprobes in tilapia. The signal distribution and thus gene expression follows a pattern very similar to that of the Col1A1 mRNA expression. Osteonectin expression is more frequent and intense near the anterior and lateral margins of the scale and is sporadic, with less intensity, in the posterior region (Figures 6.6 and 6.7). Osteonectin expression is detected in the putative osteoblast-like cells that express Col1A1 mRNA (Figure 6.9 C

and D) and also in the flattened cells near the anterior margin (Figure 6.9 A) which express Col1A1 and Col5A2.

Tilapia scales did not express fibronectin transcripts presumably because of low sequence similarity of this gene in these species, and resultant lack of riboprobe hybridization (Table 6.6). In sea bream, FN mRNA is detected in high abundance (Table 6.6) in the flattened cells located in the scale posterior margin (Figures 6.6 and 6.10 A and B). Signal is also detected in osteoblast-like cells identified between the calcified *circuli* in the anterior region (Figures 6.6 and 6.10 C and D). In the posterior region of the scale (Figure 6.10 E) dispersed cells which differed in appearance from putative osteoblasts and appear fibroblast-like cells also expressed fibronectin. They have a big nucleus and possess lateral elongations in where fibronectin mRNA expression is concentrated (Figure 6.10 E). Fibronectin expression seems to be present in the same type of cell that express Col1A1, Col5A2 and OSN, although expression is located in a different region of the cell as the most intense staining of FN transcripts is associated with the posterior region while the other transcripts are mainly expressed in the anterior region of the scale.

TRACP mRNA is detected in the scales of both species studied (Table 6.6), although it is slightly more abundant in sea bream. A hybridization signal is evident in sporadic, small groups of round cells in the posterior region of sea bream scales, far from the scale margins (Figure 6.11 A). These cells are morphologically different (Figure 6.11 B) from all the other cell types detected in the scales with the other riboprobes. This appears to suggest that TRACP is expressed by another type of cell and in agreement with its use as an osteoclast marker in other vertebrates it seems most likely that the cells may be putative osteoclasts. In tilapia, TRACP mRNA expression is weaker than in the sea bream scale but the cells that hybridize with the TRACP riboprobe are similar in appearance to those in sea bream.

It is not possible to detect ASPIC transcripts in either sea bream or tilapia scales using *in situ* hybridization.

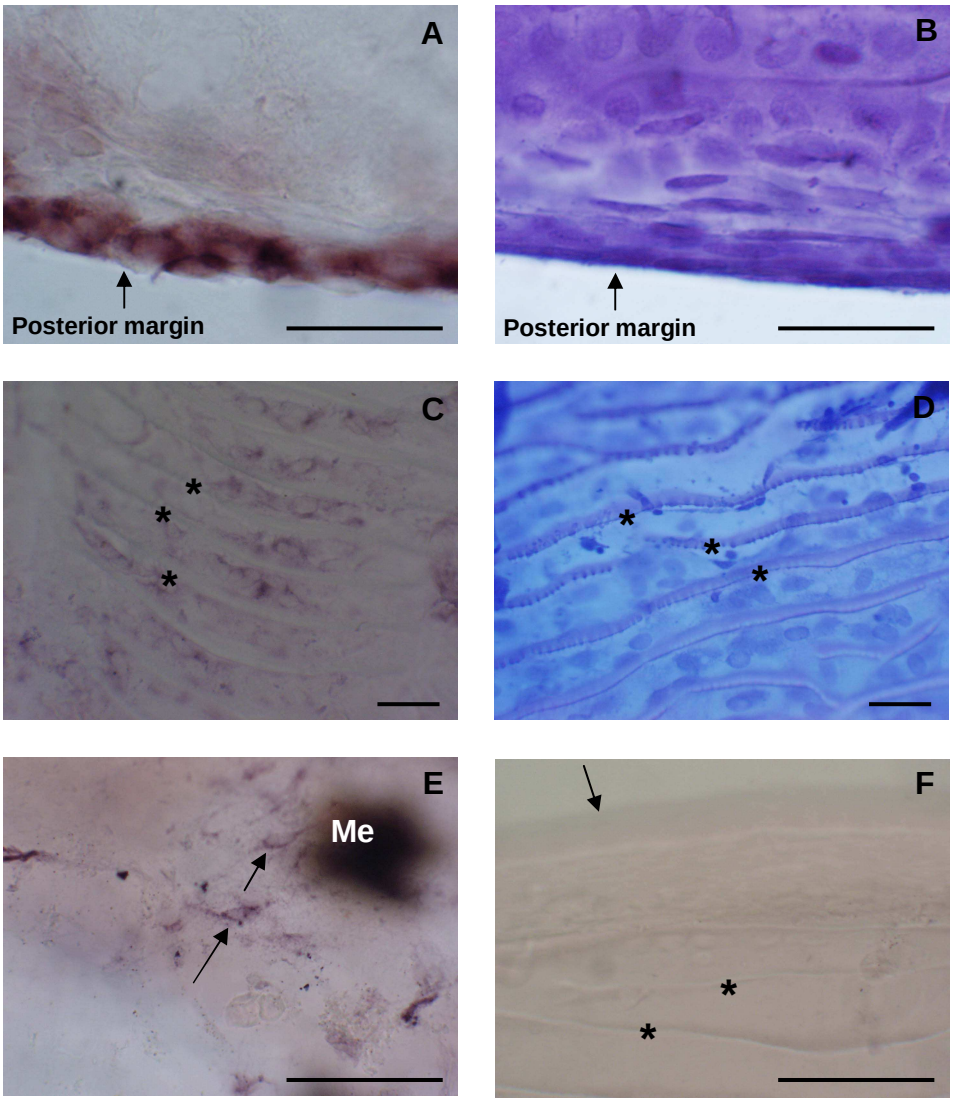



**Figure 6.10 –** *In situ* hybridization of fibronectin in juvenile (A) and adult (C and E) sea bream scales. An intense signal was detected in the marginal flattened cells in the posterior margin of the juvenile sea bream scale (A). Image B shows the detail of a juvenile sea bream scale stained with haematoxylin and eosin where the flattened marginal cells are evident. Image D shows details of the anterior region of an adult sea bream scale stained with toluidine blue where it is possible to identify the *circuli* which stain light pink (mineralized tissue). Between the *circuli* there are osteoblast-like cells, with large nuclei, which also express at low levels fibronectin mRNA (C). In the posterior region of adult sea bream scales sporadic cells with big nuclei and lateral elongations (fibroblast-like cells) were detected. They express fibronectin and the signal seems to be associated with the lateral elongations of the cells (E, arrows). A melanocyte (Me) is identifiable by its dark colour but it seems to be in a different layer as in this image it is out of focus. A negative control was carried out without riboprobe (F) and no hybridization signal was detected either in the margin of the scale (arrow) or between the *circuli*. Asterisks identify the *circuli* in images C, D and F. Scale bars: 25µm.

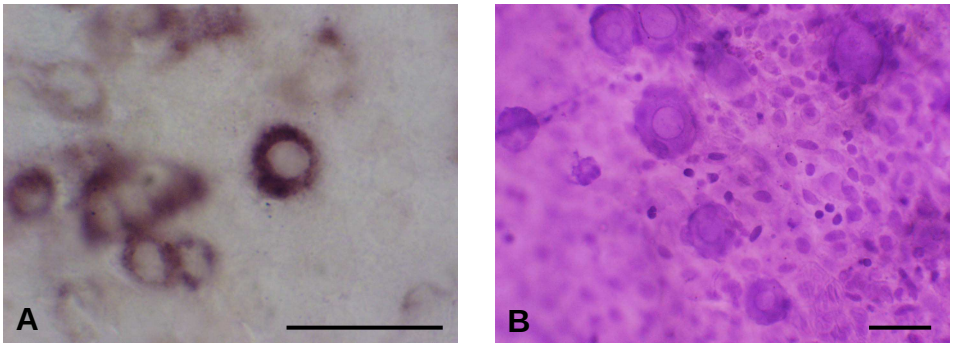



**Figure 6.11 –** *In situ* hybridization of TRACP in juvenile sea bream. A) Expression was detected in the cytoplasm of big round cells which appear isolated or in small groups in the posterior region of the scale, near the focus. Image B shows a detail of the posterior region of an adult tilapia scale where it is possible to observe the morphology of this type of cells, which are different from the cells presented in the previous figures. Same region in juvenile sea bream (A) and adult tilapia (B) scales which accounts for the difference in the cell size, although position, morphology and probe hybridization characteristics suggest they are the same cell type. The fact that these cells express TRACP mRNA and have a different morphology from putative marginal cells and osteoblasts identified in the scales (see previous figures) suggests that they are putative osteoclasts. Scale bars: 25µm.

In addition to cellular localization, the relative abundance of the genes of interest has been determined by RT-PCR. The results obtained indicate that all the target genes are expressed in adult sea bream scales (Figure 6.12) although at different levels. In agreement with the intensity of the *in situ* hybridization reaction, Col1A1 and osteonectin are the most abundant transcripts in scales. However, to detect OSN expression in the scales it was necessary to increase the number of cycles used in the PCR amplification program relative to that used with other sea bream tissues which suggests that there are fewer cells expressing osteonectin in the scales than in the other mineralized tissues. In order to establish the skeletal specific nature of the target genes chosen for this study RT-PCR was also conducted with opercular bone and cranial bone which served as a positive control for skeletal tissue. Kidney was used as a representative non-skeletal tissue. All target genes studied were amplified in calcified tissue. However, Col5A2 ans OSN were also present in low abundance in the kidney. Two reaction products were obtained

with Col5A2 primers, one of the expected size (approx. 300 bp) which corresponds to Col5A2 and the identity of the second band is currently being determined. Negative RT-PCR controls in which cDNA is omitted, or in which mRNA substituted cDNA in the reaction failed to give a product.

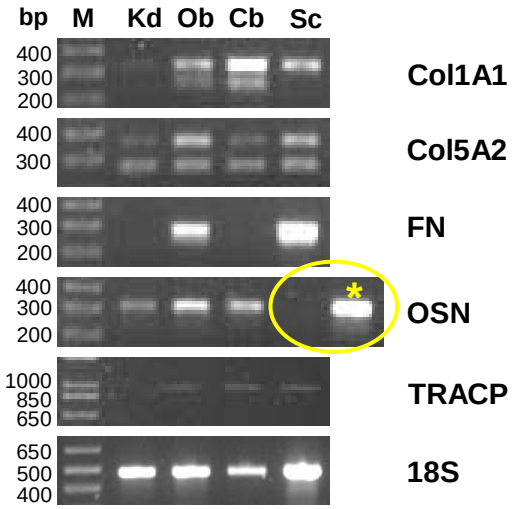



**Figure 6.12 –** RT-PCR amplification of type I collagen, α1 (Col1A1), type V collagen, α2 (Col5A2), fibronectin (FN), osteonectin (OSN) and tartrate-resistant acid phosphatase (TRACP) in adult sea bream kidney, opercular bone, cranial bone and scales. The images show the reaction products of specific PCR for the studied genes and 18S ribosomal RNA separated by agarose gel (1.5%) electrophoresis. Opercular bone (Ob) and cranial bone (Cb) were used as positive controls for mineralized tissues and kidney (Kd) was used as a non-skeletal tissue. All the studied genes are expressed in the scales as demonstrated by the presence of reaction products. OSN expression was detected in all tissues studied using 28 amplification cycles with the exception of the scales (yellow circle), in which 36 cycles were utilized (asterisk). M – marker, Kd – kidney, Ob – opercular bone, Cb – cranial bone, Sc – scale.

The expression of the estrogen receptors (α, β1 and β2) was determined by whole mount immunohistochemistry. Their relative expression intensity is presented in table 6.7.

The three estrogen receptors are expressed in both species but the location and signal intensity vary with the species and the age of the animals. Estrogen receptor β1 is weakly expressed in sea bream and almost undetectable in tilapia (Table 6.7). In juvenile sea bream a few rounded cells in the scale

posterior region express this receptor (Figure 6.13 D and E). In juvenile tilapia a very weak signal is detected in sporadic cells present in the anterior region, between the *circuli* (Figure 6.13 F). These cells have a similar morphology to those that express TRACP mRNA (Figure 6.11) which were identified as putative osteoclasts. Both species express the estrogen receptor α (ERα) although the signal is stronger in sea bream, in both juvenile and adult fishes (Table 6.7). In juvenile sea bream the signal is detected in rounded cells located in the scale posterior region (Figure 6.13 B) while in adult sea bream the ERα is mainly expressed by isolated cells present in the scale anterior region (Figure 6.13 A). ERα is expressed in the putative osteoclasts which also express ERβ1. Estrogen receptor β2 is abundant in both species and in both the anterior and posterior regions of the scales (Table 6.7). In juvenile tilapia, sporadic unidentified elongated cells, located between the *circuli* strongly express ERβ2 (Figure 6.13 G). In the posterior region a different type of cells, with lateral projections, express with low intensity ERβ2 (Figure 6.13 H). In juvenile sea bream, rounded cells of the scale posterior region, similar to the putative osteoclasts, also express ERβ2 (Figure 6.13 I). Negative controls in which the primary antibody was omitted had no detectable signal (Figure 6.13 C).

**Table 6.7 –** Relative expression intensity of the estrogen receptors (α, β1 and β2) analysed by whole mount immunohistochemistry. Results are presented for the anterior (Ant.) and posterior (Post.) regions of the scales of juvenile and adult sea bream and tilapia.

| **Proteins** | **Juvenile sea bream** | | **Adult sea bream** | | **Juvenile tilapia** | | **Adult tilapia** | |
|---|---|---|---|---|---|---|---|---|
| | **Ant.** | **Post.** | **Ant.** | **Post.** | **Ant.** | **Post.** | **Ant.** | **Post.** |
| ERα | +/- | ++ | ++ | +/- | +/- | +/- | - | +/- |
| ERβ1 | +/- | + | +/- | +/- | +/- | - | - | - |
| ERβ2 | - | ++ | +/- | + | ++ | + | ++ | + |

(-) not detected, (+/-) very low abundance, (+) low abundance, (++) high abundance and (+++), very high abundance

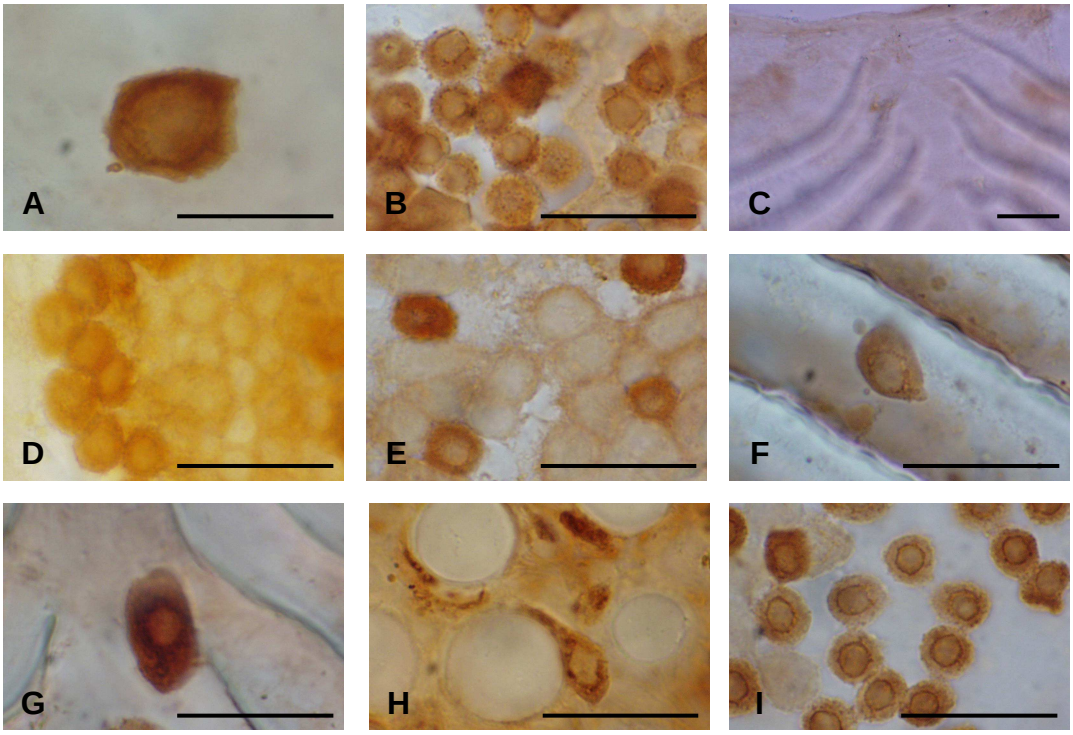



**Figure 6.13 –** Whole mount immunohistochemistry of estrogen receptors α, β1 and β2 in sea bream and tilapia scales. ERα expression was identified both in isolated cells in the scale anterior region of adult sea bream (A) and in groups of cells of the posterior region of the scale of juvenile sea bream (B) similar to those that express both ER β1 and ER β2. ER β1 expression was detected in juvenile sea bream both in cells near the scale posterior margin (D) and dispersed in the posterior region (E). Isolated cells located between the *circuli* in the scale anterior margin of juvenile tilapia express ER β1 with low intensity (F). High expression of ER β2 was detected in isolated cells of the scale anterior region of juvenile tilapia (G). In the posterior region, cells with different morphology also express ER β2 (H). In juvenile sea bream, expression of ER β2 was detected in groups of rounded cells located in the scale posterior region (I). A negative control was carried out without the primary antibody (C) and no signal was detected. Scale bars: 25μm.

## 6.4 Discussion

Sea bream (*Sparus auratus*) and tilapia (*Oreochromis mossambicus*) scale morphology in juvenile and adult fishes, is similar and typical of that described for the elasmoid scale type in other teleosts (Bereiter-Hahn and Zylberberg, 1993, Fouda, 1979, Onozato and Watabe, 1979, Sire and Arnulf, 1990, Sire*, et*

*al.*, 1997a, Sire and Arnulf, 2000, Sire and Akimenko, 2004). In both species, scale size increases as the fish grows and, in the sea bream, scale shape is variable and changes slightly from the juvenile to the adult form. In contrast, the shape of the scale in tilapia is maintained during growth, in common with what occurs in *Hemichromis bimaculatus*, where the general shape does not change markedly during ontogeny despite the increase in dimension and their surface structure change (Sire, 1986).

Sea bream and tilapia both have scales in which non-mineralized *radii* interrupt the accumulated mineralized material which forms the *circuli*, in the anterior and lateral regions of the scales. These *circuli* constitute the scale surface structure (ornamentation) and have been proposed to reflect the growth rhythm of the fish (Sire and Géraudie, 1983). Surface ornamentation in the posterior region of the scales of both sea bream and tilapia, appears irregular as a result of the randomly deposited ridges of mineralized material. In *Hemichromis bimaculatus* the ridges continuously increase throughout the fish's life, sometimes fusing to each other, and in this way contributing to the thickening of the oldest ontogenic areas, close to the focus (Sire, 1986). Most scale ornamentation has an anchoring function, and either helps maintain the scale in its dermal pocket (role of *circuli* in the anterior region) or maintain the epidermis attached to the surface of the posterior region. Other structures found on the scale play a role in surface hydrodynamics (Sire and Arnulf, 2000). In sea bream scale *circuli* are more regularly spaced than those observed in tilapia and this may reflect the pattern of fish growth and/or the different anchoring and hydrodynamic needs of each species. Comparison of the ornamentation pattern among wild and captive specimens of *Hemichromis bimaculatus* suggests that the water flowing around the fish may influence the genesis of ornamentation patterns (Sire, 1986).

Teleost fish have the capacity to adapt to different environments and conditions. Scales which have a protective function probably evolved in teleosts as an adaptation to their habitat. The differences observed in the ratio of scale size to fish size in tilapia and sea bream may be a consequence of this adaptive process. Thin scales allow fish to move more freely and improve swimming efficiency (Lagler*, et al.*, 1962, Moyle and Cech Jr., 1996) but possibly

accumulate less calcified material, and are a less efficient calcium reservoir. Sea bream, a marine teleost, has much thinner scales than tilapia, a freshwater fish and it has not been determined in the present study if the differing thickness is related to mineral accumulation in the scales. The thicker tilapia scales may represent an important calcium reservoir which can be mobilized in periods of higher calcium demand. This may reflect the fact that sea bream inhabit a marine environment in which calcium availability is much higher than the freshwater that tilapia inhabit. More studies are necessary in both marine and freshwater species to determine if there is a relation between scale thickness and calcium content. Moreover, the capacity to accumulate and mobilize calcium from the scales in marine and freshwater species and the mechanisms, which regulate this process, still remain to be established.

Elasmoid scales are composed of three main layers, basal, external and limiting and different types of cells have been associated with each layer (Fouda, 1979, Olson and Watabe, 1980). Osteoblasts (also called scleroblasts), responsible for matrix production and its mineralization, are associated with both the scales basal layer (inner side) and the *circuli* of the scale (osseous external layer) (Olson and Watabe, 1980, Sire and Arnulf, 2000). Fibroblasts are associated with the basal plate and have principally been linked to the increase in diameter of the collagen fibres but may also participate in the calcification process (Olson and Watabe, 1980). Marginal cells are present in the scale margin. These cells are round or oval in shape and usually maintain their structure at all ages. Resorbing cells, osteoclasts (or scleroclasts), have also been identified in teleost scales. They can be mono or multinucleated cells (Persson*, et al.*, 1999, Sire*, et al.*, 1990). In sea bream and tilapia scales, osteoblasts and marginal cells are present (Figures 6.8, 6.9 and 6.10) as are sporadic, infrequent fibroblast-like cells (Figure 6.10) and putative osteoclasts (Figure 6.11). The identification of the different cell types based on morphology and expression of mRNA encoding ECM proteins is discussed in more detail in the following paragraphs.

The present study establishes for the first time in fish scales the expression and cellular localization of the mRNA encoding six extracellular matrix proteins; type

I collagen, α1 (Col1A1); type V collagen, α2 (Col5A2); fibronectin (FN), osteonectin (OSN), tartrate-resistant acid phosphatase (TRACP) and acidic secreted protein in cartilage (ASPIC). The proteins studied are important components of the extracellular matrix of skeletal tissue in which they play different roles. Characterization of their expression in scales facilitates the identification of different cell types present in the scales because some of them have been used as cell markers in vertebrates. Moreover, their presence in the scales may signify that they participate in scale formation possibly with functions similar to those in mammalian skeletal tissue.

Type I and type V collagens are fibrillar collagens which act as structural components of bone matrix (Aumailley and Gayraud, 1998, Gelse*, et al.*, 2003, Mecham, 1998, van der Rest and Garrone, 1991). Studies with cultured cells indicated that type I collagen, together with alkaline phosphatase, is a molecular marker of early-stage osteoblast differentiation (Nakashima and de Crombrugghe, 2003). The basal plate of goldfish (*Carassius auratus*) scales are composed of collagen fibrils organized in superimposed layers parallel to the scale surface and arranged in a plywood-like structure (Nicolas*, et al.*, 1997). Type I collagen is the major component of goldfish scales but type V collagen is also present (Zylberberg*, et al.*, 1992). Type I collagen in fish is encoded by three different genes and both $\alpha1(I)[\alpha2(I)]_2$ and $\alpha1(I)\alpha2(I)\alpha3(I)$ heterotrimers are present. These heterotrimers are synthesized by scale osteoblasts (also called hyposquamal scleroblasts) that form a continuous layer lining the basal surface of the scales (Nicolas*, et al.*, 1997, Zylberberg*, et al.*, 1992). In the present study sea bream and tilapia scales express type I collagen and the intense signal detected suggests that in common with observations in goldfish, this molecule is an important component in these species. In contrast, type V collagen is only detected in sea bream and has a much lower expression than type I collagen. It remains to be established if the failure to detect type V collagen in tilapia scales is due to the probe specificity or to very low levels of type V collagen in this species. In the sea bream both collagens are expressed in the basal plate and the location and morphology of the cells with an intense type I collagen expression suggests they are probably the scale-forming osteoblasts.

Osteonectin is a matricellular protein, abundant in bone extracellular matrix and has been associated with the regulation of bone mineralization in mammals (Sommer*, et al.*, 1996, Young*, et al.*, 1992, Zhu*, et al.*, 2001). OSN mRNA has previously been shown to be expressed in skeletal tissues of adult sea bream (Redruello*, et al.*, 2005) and in both cartilaginous and osseous tissue during sea bream ontogeny (Estêvão*, et al.*, 2005) suggesting that it may also play a regulatory role in fish skeletogenesis. Osteonectin mRNA is highly abundant in sea bream and tilapia scales, both in juvenile and adult specimens. Its expression is colocalized with expression of type I collagen in the putative osteoblasts (Figure 6.9). The presence of osteonectin in putative osteoblasts of fish scales suggests that in common with observations in mammalian bone (Mackie, 2003), osteonectin may play a role in regulation of scale formation and mineralization.

Fibronectin is a major constituent of the extracellular matrix, with important functions related to cell adhesion, migration, growth and differentiation (DeSimone, 1994, ffrench-Constant, 1995, Hynes, 1985, Liu*, et al.*, 2003, Moursi*, et al.*, 1996, Young, 2003). Fibronectin is present in developing bone in mammals and is synthesized by mesenchymal cells, chondrocytes and osteoblasts and is associated with developing skeletal matrix (Weiss and Reddi, 1981). Moreover, fibronectin has been shown to play an important role in the progressive differentiation of osteoblasts, possibly promoting the recruitment or migration of osteoblast precursors (Moursi*, et al.*, 1996). In the sea bream scale the results of both *in situ* hybridization and RT-PCR revealed it has a high abundance and is expressed by a number of different cell types (Figure 6.10) located in different regions of the scale. Fibronectin is most abundant in scale osteoblasts located in the posterior region of the scale and not in those of the anterior region with a high expression of collagen type I and V and osteonectin. The pattern of distribution in the scale of fibronectin is consistent with its role in osteoblast differentiation (Moursi*, et al.*, 1996, Weiss and Reddi, 1981), as it is localized in regions of the scale important in growth, development and remodelling. The failure to detect fibronectin in tilapia scale is probably a consequence of its failure to hybridize with the sea bream riboprobe particularly

as species-specificity has been reported for fibronectin isoforms (Liu*, et al.*, 2003).

Acidic secreted protein in cartilage (ASPIC, also called chondrocyte expressed protein-68 (CEP-68) or CRTAC) is expressed in human cultured chondrocytes and has been identified as a stable marker for cultured chondrocytes and also as a marker of chondrocyte differentiation, and may complement type II collagen as a specific chondrocyte marker (Steck*, et al.*, 2001). Sea bream and tilapia scales do not express ASPIC mRNA which is unsurprising as scales are of dermal origin which means that they originate from mesenchymal condensations and a cartilage intermediate does not form. So that the presence of chondrocytes in fish scales is improbable, making the absence of ASPIC unsurprising. The relatively recent identification of ASPIC in fish means its function has yet to be established and studies will be required to determine if ASPIC expression is detected in skeletal tissues and if it is involved in skeletogenesis.

Tartarte-resistant acidic phosphatase (TRACP) has been used as a marker for osteoclasts and is proposed to be involved in bone resorption in mammals (Oddie*, et al.*, 2000). In fish, TRACP has also been used as an osteoclast marker in bone (Witten, 1997, Witten*, et al.*, 2001) and scales (Persson*, et al.*, 1995, Suzuki*, et al.*, 2000). The involvement of TRACP in the resorbing process of bone and its use as a marker for osteoclastic activity suggests that the cells that express TRACP mRNA in sea bream and tilapia may be osteoclasts or osteoclast precursors. The existence of osteoclasts in the scales is expected as their activity had been previously described using enzyme assays in other teleost scales, such as the rainbow trout (Persson*, et al.*, 1995, Persson*, et al.*, 1997, Persson*, et al.*, 1999), the goldfish and the nibbler fish (Suzuki*, et al.*, 2000, Suzuki and Hattori, 2002).

Estrogen receptors ($\alpha$, $\beta$1 and $\beta$2) expression was determined by immunohystochemistry in sea bream and tilapia scales of juvenile and adult animals. Signal location and intensity vary with both the species and the age, and with the receptor type studied. These preliminary results suggest that estrogen receptor isoforms may have different roles in different species and

during the fishes' lifetime but further studies must be carried out to better establish the function of each receptor type in the regulation of scale turnover. The present study showed that in most cases the cells that express the estrogen receptors also express TRACP mRNA suggesting they are osteoclasts. This may indicate one of the mechanisms by which estrogen brings about a rise in plasma calcium. Modulation of osteoclasts through binding to the estrogen receptors could ultimately influence scale turnover and calcium mobilization. Previous studies in rainbow trout (Armour*, et al.*, 1997, Persson*, et al.*, 1995, Persson*, et al.*, 1997), goldfish (Mugiya and Watabe, 1977, Suzuki*, et al.*, 2000, Suzuki and Hattori, 2002), killifish (Mugiya and Watabe, 1977) and nibbler fish (Suzuki*, et al.*, 2000) have shown that estradiol induces osteoclastic activity and the resorption of scale calcium although they did not establish a direct effect of estrogen on scale cells. Estrogen receptor mRNA expression was detected in rainbow (Armour*, et al.*, 1997) and goldfish (Suzuki and Hattori, 2002) scales. High affinity-low capacity estrogen binding was also identified in rainbow trout scales (Persson*, et al.*, 2000) suggesting the existence of estrogen receptors in teleost scales which indicates that estrogen could act directly on scale cells to influence scale formation and calcium mobilization.

In conclusion, the present study permitted the identification of several cell types in sea bream and tilapia scales of both juvenile and adult animals, which express different ECM mRNA, with different intensities. The capacity to produce the ECM proteins studied indicates they are involved in scale formation and/or remodelling and that they may have a role in scale matrix production and mineralization. To better understand the importance of the ECM in the regulation of fish scale formation and calcium mobilization, further studies need to be carried out to establish if other factors important in regulating mammalian bone matrix, such as osteopontin and osteocalcin are present. This work also demonstrated that estrogen receptors are expressed in scale cells of sea bream and tilapia, putatively identified as osteoclasts or osteoclast precursors, suggesting that the estrogen is one of the endocrine factors involved in the regulation of scale turnover and calcium mobilization. The way in which other endocrine factors influence scale metabolism and ECM protein production will provide insight into how they contribute to calcium balance.

**Acknowledgments of practical work**

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