Chapter 2

General materials and methods

The present chapter describes the general methods used in the studies presented in the subsequent chapters. The specific methods and/or conditions relevant to the work reported in subsequent chapters will be specified in a short methods section preceding the results.

2.1 Experimental animals

Sea bream (*Sparus auratus* L.) larvae were obtained from a commercial supplier (Viveiros Vilanova, Lda, Vila Nova de Milfontes, Portugal) where they were cultured in circular tanks with a continuous flow of aerated sea water. Larvae were incubated in 500 L conical tanks at 17-18.5ºC, without illumination until 3 days post hatch (dph). From 3 to 35dph, larvae were cultured in 6000 L circular tanks, at 19-20ºC and with 24 h light. At 35dph larvae were transferred to 15000 L tanks, at 20-22ºC and with a photoperiod of 16 h light/8 h dark. The larvae were fed on algae and rotifers (4 to 22dph), small artemia (10 to 23dph), enriched artemia (18 to 45dph) and inert food (from 20dph).

The adult animals were purchased from commercial suppliers who reared the fish in aquaculture ponds. Fish were transferred and maintained at Estação Marinha do Ramalhete (Universidade do Algarve, Faro, Portugal) under natural annual conditions of water temperature, photoperiod and salinity in tanks with flowing sea water. Fish were fed to satiation on dry pellets formulated for marine fish (Provimi, Portugal).

Adult (2 years) and juvenile (6 months) tilapia (*Oreochromis mossambicus*) were maintained in 500 L closed circuit aquaria in aerated freshwater at 27±1ºC. Approximately 100 L of water were changed every 15 days. Animals were fed to satiation on dry pellets formulated for cichlids (Sorgal, Portugal). Younger animals were fed to satiation on dry pellets formulated for younger cichlids (Nutrafin, Portugal).

Animal care was in accordance with the ethical guidelines of the Animal Behaviour Society (The Association for the Study of Animal Behaviour (ASAB) and Animal Behaviour Society (ABS), 2003) and National legislation.

2.2 Tissue fixation and processing

Adult tissues were collected from fish that were killed with an overdose of 2-phenoxyethanol (1:10000, Sigma-Aldrich). Dissection was carried out immediately in aseptic conditions, maintaining all instruments used in 70% ethanol throughout the process. Tissues for histology and *in situ* hybridization were cut into small pieces (3-5mm) and immediately fixed in fresh 4% paraformaldaheyde (PFA; appendix I), overnight, at 4ºC, washed with sterile 1×Tris Buffered Saline (1×TBS; appendix I) and stored in 70% ethanol, at 4ºC. Alternatively, after fixation tissues were washed with sterile 1×Phosphate Buffered Saline (1×PBS, appendix I) and stored in methanol, at 4ºC. Larvae and juveniles were fixed using the same procedure, immediately after they were collected from the tanks and had been anaesthetized in 2-phenoxyethanol.

Some samples were fixed in Bouin-Holland solution (appendix I) by immersing the tissues in this solution, for 5-7 days, at room temperature. After fixation, tissues were washed twice with tap water and then with sterile water. Finally samples were transferred to 70% ethanol and stored at 4ºC.

Tissues were dehydrated through an ascending series of ethanol (70%, 95%, 100%), cleared in xylene and xylene:paraffin (50:50%, in volume) and embedded in low melting point paraffin wax (for details see appendix II). Serial sections (5 to 8μm) of paraffin embedded tissue and larvae were cut using a microtome (Leica RM 2125 RT) with disposable stainless steel blades (Leica DB 80 L) and mounted on APES (3-aminopropyltriethoxysilane) coated slides (appendix II).

Scales were collected from the caudal region of anesthetized fishes by gently scraping them off with a scalpel or plucking them off using forceps. Scales were fixed in fresh 4% paraformaldehyde and washed as described above.

2.3 General histology

All staining procedures described subsequently were carried out with sections which had been dewaxed and rehydrated. This process was carried out by

immersing sections for ten minutes each, in two xylene baths and then in several ethanol baths of decreasing concentration (100%, 95% and 70%), for five minutes in each solution. The rehydration process was completed by a final immersion in distilled water for five minutes.

2.3.1 Haematoxylin-eosin staining

Haematoxylin-eosin staining is a basic histological procedure that allows the morphological identification of cells and tissues. With this staining procedure, negatively charged nuclei stain purple and the basic cytoplasm stains pink (Stevens, 1990).

After hydration, slides were immersed in Harris haematoxylin solution (appendix I) for 30 seconds, washed in tap water, immersed in an aqueous 1% eosin Y solution (appendix I) for 30 seconds and washed in distilled water. To obtain definitive preparations, tissue sections were dehydrated through an ascending series of ethanol and cleared in xylene, as described in appendix II, before mounting in DPX (Fluka) and covered with a clean glass coverslip.

2.3.2. Toluidine blue staining

Toluidine blue is a metachromatic dye that stains tissue different colours, according to the concentration of polyanions present.

Once hydrated, tissue sections were immersed in a 1% aqueous toluidine blue solution, at pH 4.5 (appendix I) for 45 minutes (Witten and Hall, 2003), washed in distilled water and immediately mounted in glycerol gelatine (Sigma-Aldrich).

Almost all tissues, such as the muscle and the epithelial tissue, stain dark blue but cartilage is stained purple and calcified bone is stained light pink.

2.3.3 Masson's Trichrome staining

This method relies upon two similar acid dyes (acid fuchsin and light green) to provide a differential visualization of tissue elements.

Masson´s trichrome staining was carried out according to the protocol described by Witten and Hall, 2003. Dewaxed and rehydrated tissue sections were immersed in Mayer's acid haematoxylin (MSH-16, Sigma-Aldrich) for ten minutes, washed with tap water for another ten minutes and rinsed in distilled water. Sections were then immersed in freshly prepared xylidine ponceau solution (appendix I) for two minutes, rinsed in distilled water, treated with 1% phosphomolybdic acid (appendix I) for four minutes and rinsed again in distilled water. Finally, sections were stained with a solution of light green (appendix I), for 90 seconds. The excess dye was cleaned from the slide, which was then rapidly passed (approximately 10 seconds in each solution) through an ascending series of ethanol (70, 95 and 100%), cleared with xylene (twice for 10 minutes) and mounted in DPX (Fluka) and covered with a clean coverslip.

Mineralized tissue such as bone matrix is stained red while non-mineralized connective tissues is stained green.

2.3.4 Alcian-haematoxylin-van Gieson staining

Alcian blue 8 GX is a dye that binds to mucopolysaccharides allowing the identification of cartilaginous tissue rich in such molecules through a characteristic blue staining. The van Gieson technique allows the differentiation between collagenous tissues which stain red and the other tissues which stain yellow. By combining alcian blue and the van Gieson staining procedures it is possible to identify cartilage and collagenous tissues simultaneously. The use of haematoxylin allows the visualization of the nuclei of cells which stain purple.

Hydrated tissue sections were immersed in an alcian blue 8 GX solution (appendix I), washed in tap water, immersed in Harris haematoxylin (appendix I) for 1 minute and washed again in tap water. Then sections were immersed in van Gieson solution (appendix I) for 3 minutes. Slides were blotted dry, rapidly passed (approximately 10 seconds in each solution) through an ascending series of ethanol solutions (70, 95 and 100%), cleared with xylene (twice for 10 minutes) and mounted in DPX (Fluka) and covered with a clean glass coverslip.

2.3.5 Whole-mount cartilage - bone double staining

Whole-mount staining of bone and cartilage was carried out on whole larvae and scales which had not been subject to decalcification using a modification of the alcian blue/alizarin red method (Faustino and Power, 1998).

Larvae and scales were rehydrated using an ethanol series of decreasing concentration (75, 50 and 25%) and immersing for 15 minutes in each solution, and finally washing in distilled water for 15 minutes. Samples were immersed for 2 hours in alcian blue 8 GX solution (appendix I) and then washed twice with absolute ethanol for 15 minutes. These washes were followed by a rehydration procedure during which the samples were immersed for 15 minutes in a series of ethanol solutions with decreasing concentrations (95, 70, 40 and 15%). Once hydrated, samples were transferred to a 1% potassium hydroxide solution (appendix I) for maceration, where they were left until they became soft and transparent and sunk to the bottom of the sample bottle. Prior to the proceeding process, liquid reagents were always discarded and, between different solutions, the samples cleaned with absorbent paper. Subsequently, samples were immersed overnight in alizarin red S working solution (appendix I) in order to stain bone.

Finally, samples were washed for approximately 1 hour (or until they sunk to the bottom of the reagent flask) in a series of solutions of 0.5% potassium hydroxide/glycerol (3:1, 1:1 and 1:3) and were then stored in glycerol at room temperature in the dark.

2.4 mRNA tissue distribution by *in situ* hybridization

In situ hybridization (ISH) is an excellent and very sensitive tool for the examination of gene expression in histological tissue sections allowing the detection of mRNA contained in a single cell. With this technique cell relationships are maintained and it is possible to identify which cell types are expressing the gene of interest (Wilcox, 1993). The basis of a hybridization assay is the specificity of the interaction of a probe with target nucleic acid. A hybrid can be formed when complementary strands of nucleic acids from

different sources form a stable duplex structure. In a hybridization assay, the two sources are the target molecule (sample) and the probe (a known fragment of nucleic acid with a label that can be detected). Duplex structures may be composed of DNA:DNA, DNA:RNA or RNA:RNA, in order of increasing stability (Kenny-Moynihan and Unger, 2002). Riboprobes (RNA probes, complementary with the mRNA of the sample) have proven to be more sensitive than cDNA probes or synthetic oligonucleotides (Wilcox, 1993, Wilkinson, 1993). There are also multiple ways to label the probes, using radioactive or non-radioactive nucleotides (Kenny-Moynihan and Unger, 2002, Wilcox, 1993, Wilkinson, 1993). Although non-radioactive probes are less sensitive than radioactive probes (Wilcox, 1993), they are safer to handle and generally the methodology for their use is less time consuming. Riboprobes can be rapidly produced from cloned DNA and used in ISH assays which involve the following general steps:

- synthesis of a labelled RNA probe
- fixation and pre-treatment of tissues
- hybridization of probe to the tissues
- removal of non-hybridized probe
- visualization of the probe (Wilkinson, 1993).

2.4.1 Riboprobe synthesis

Single-stranded RNA probes (riboprobes) can be synthesized by *in vitro* transcription using one of the several commercially available cloning vectors (phagemids), which contain promoters for the highly specific bacteriophage DNA-dependent RNA polymerases SP6, T7 and T3. The most commonly used transcription vectors are pBlueScript (T3 and T7 promoters, Stratagene) and pGEM (SP6 and T7 promoters, Promega). Once the DNA is cloned in one of these vectors, the vector is linearized with a restriction enzyme such that the RNA probe obtained by transcription is complementary (antisense) to the target mRNA and lacks plasmid sequences (Wilkinson, 1993). These single-strand RNA probes have several advantages. No self-hybridization is possible, favouring maximal interaction with target sequence and non-specifically bound RNA probe can be removed (Kenny-Moynihan and Unger, 2002). The restriction enzyme chosen to linearize the vector should generate a 5'-overhang

or a blunt end so that the transcripts initiate on the correct strand. If a 3' overhang is generated (Table 2.1), the polymerases will transcribe both strands of the insert by either initiating on the 3' overhang, initiating at the promoter and "turning the corner" at the end of the template or by aberrant (non-promoter) initiation (Schenborn and Mierendorf Jr, 1985).

Table 2.1 - Restriction enzymes which generate 3'-overhangs which should be avoided to linearize vectors to synthesize riboprobes for *in situ* hybridization.

Restriction enzymes	Restriction Site
Aat II	GACGT$^{\downarrow}$C
Apa I	GGGCC$^{\downarrow}$C
Bbu I	GCATG$^{\downarrow}$C
Bst98 I	CATG$^{\downarrow}$
Cfo I	GCG$^{\downarrow}$C
Hae II	($^{A}_{G}$)GCGC$^{\downarrow}$($^{T}_{C}$)
Hha I	GCG$^{\downarrow}$C
Hsp92 I	CATG$^{\downarrow}$
Kpn I	GGTAC$^{\downarrow}$C
Nsi I	ATGCA$^{\downarrow}$T
Pst I	CTGCA$^{\downarrow}$G
Pvu I	CGAT$^{\downarrow}$CG
Sac I	GAGCT$^{\downarrow}$C
Sac II	CCGC$^{\downarrow}$GG
Sgf I	GCGAT$^{\downarrow}$CGC
Sph I	GCATG$^{\downarrow}$C

To synthesize the riboprobes used in the present work the vector containing the clone of interest was digested with the appropriate restriction enzyme depending on the orientation of the cloned DNA in the vector (Figure 2.1). The generation of specific probes for *in situ* hybridization will be described in the methods section of the appropriate chapters of this thesis.

The linearization reaction was carried out for 1.5 hours at 37ºC. The reaction medium was composed of an appropriate volume of plasmid solution, 1µl of restriction enzyme, 2µl of the respective optimal buffer and the final volume of the reaction (20µl) was completed with sterile water. To purify the linear DNA the reaction volume was increased to 100µl with sterile water, 100µl of basic

phenol (pH 8.0) added and incubated at room temperature for 35 minutes, and mixed occasionally. The mixture was centrifuged at 12000rpm for 10 minutes, at room temperature and the aqueous phase was collected and the volume measured. The phenol extracted DNA was precipitated by adding one tenth of its volume of sodium acetate (3M, pH 5.2, appendix I) and 2.5 times its volume of 100% ethanol, mixing the tube and leaving at -20ºC, overnight (or at -80ºC for at least 1 hour). The DNA was collected by centrifuging the tube at 12000rpm for 20 minutes, at room temperature and discarding the supernatant. The pellet of precipitated DNA was washed twice with cold 70% ethanol, centrifuged at 12000rpm for 10 minutes, at room temperature, dried and suspended in 20µl of RNase and DNase free water (Sigma-Aldrich). The yield and quality of the linearized DNA was assessed by agarose gel electrophoresis and visualized by staining with ethidium bromide (appendix II), prior to utilization for riboprobe synthesis.

Once purified, the linear DNA served as the template for the *in vitro* transcription reactions to synthesize the digoxygenin labelled riboprobe. Depending on the cloning vector utilized and insert orientation, 20U of T3, SP6 or T7 RNA polymerase (Promega) was added to the reaction mixture. The reaction mix was composed of 1µl of digoxygenin or biotin RNA labelling mix (Roche), 4µl of 5×transcription buffer (Promega), 1µl of 100mM DTT (dithiothreitol, Promega), 0.2µl of 10mg.ml^{-1} BSA (bovine serum albumin, appendix I) and 0.5µl RNA Guard (Promega). An appropriate volume of linear DNA, corresponding to approximately 1µg.ml^{-1} was added to the reaction mixture and the volume was completed to 20µl with sterile water. Riboprobe synthesis was carried out at 37ºC for 1.5 hours and then stopped with 2µl of 0.2M EDTA (appendix I).

The synthesized riboprobe was selectively precipitated with 2.5µl of 4M lithium chloride (appendix I) and 75µl of 100% ethanol, at -20ºC overnight (or -80ºC for at least one hour). The precipitated riboprobe was then centrifuged at 12000rpm for 20 minutes, at 4ºC and the supernatant was discarded. The pelleted riboprobe was washed twice with cold 70% ethanol, centrifuged at 12000rpm for

10 minutes, at 4ºC, and dried and suspended in 25µl of RNase and DNase free sterile water (Sigma-Aldrich).

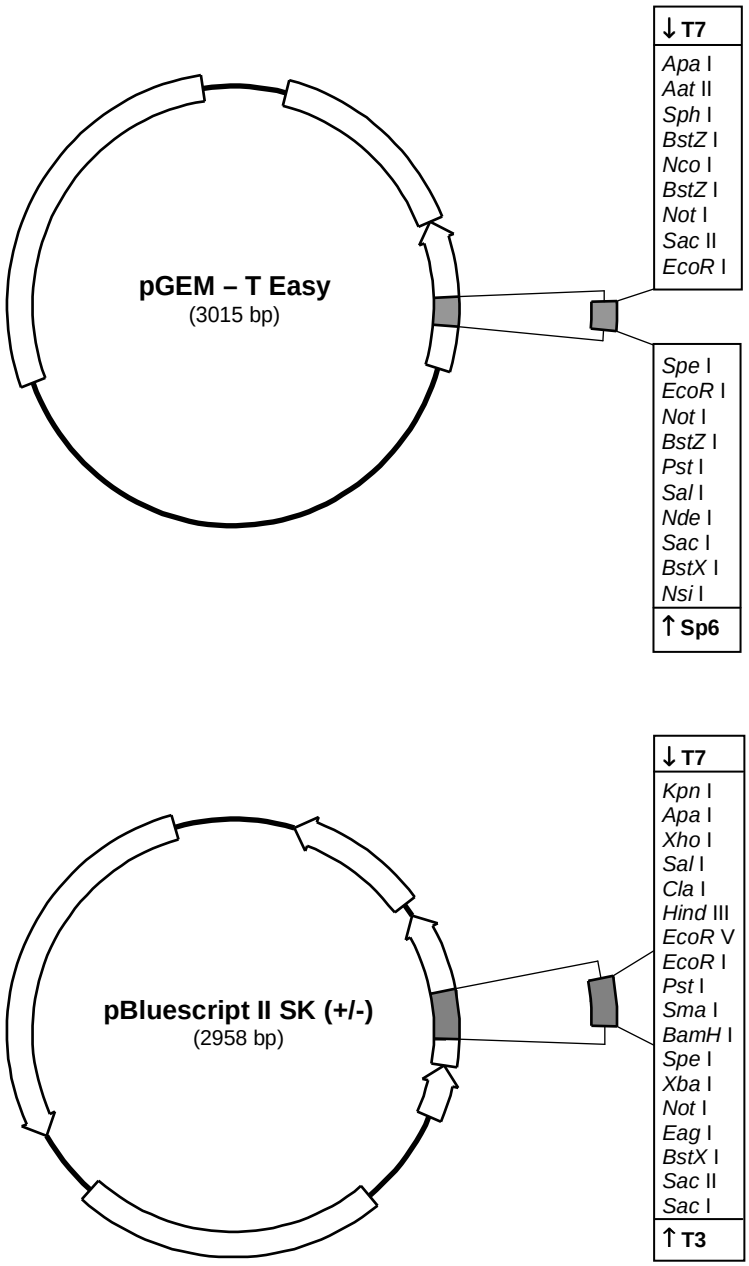

Figure 2.1 – Schematic representation of the cloning vectors used in the present work, pGem-T Easy (Promega) and pBluescript SK(+/-) (Stratagene), presenting the restrictions enzymes flanking the multiple cloning site (grey box) (adapted from pGEM – T and pGEM – T Easy vector systems, Technical Manual, Promega and pBluesript II phagemid vectors, instruction manual, Stratagene).

The appropriate volume of linear DNA necessary for synthesis of labelled riboprobe was determined by initially carrying out *in vitro* transcription of riboprobe with different concentrations of linear DNA and non-labelled nucleotides (ATP, CTP, GTP and UTP) at a final concentration of 0.4mM in the reaction. The yield of the transcription reaction was assessed by agarose gel electrophoresis and visualization of the riboprobe with ethidium bromide (appendix II)

All solutions used for *in vitro* transcription reactions were prepared with DEPC treated water to avoid RNase contamination which would cause the degradation of RNA.

2.4.2 *In situ* hybridization

Tissue sections prepared as described above (section 2.2) were dewaxed by immersion in xylene (2×10 minutes) and hydrated by immersion in ethanol in decreasing concentrations (100%, 95% and 70%) for 5 minutes. Finally sections were washed in PTW (Phosphate-Tween Buffer, appendix I) for approximately five minutes to ensure tissue was completely hydrated.

In some cases (referred to in the methods section specific for each of the subsequent chapters of this thesis) when whole mount *in situ* hybridization was carried out, tissue was treated with proteinase K (0.01mg.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.

Sections were covered with hybridization solution (50% formamide, 4×SSC, 0.1% torula RNA, 0.01% heparin, 1×Denhart's, 0.1% tween-20, 0.04% CHAPS, appendix I) and pre-hybridized at the hybridization temperature (varying between 56ºC and 58ºC, according to the probe in use), for 2 to 4 hours. Tissues were then hybridized overnight, at the same temperature, in hybridization solution containing approximately 3μg.ml^{-1} of riboprobe, covered with *parafilm* (to prevent evaporation of the probe), in a humidified box.

High stringency washes to remove non-specifically bound probe were carried

out at the hybridization temperature, twice with 2×SSC (appendix I) for five minutes and then with 1×SSC for five minutes. Tissues were washed twice at room temperature with 2×SSC containing 0.12% CHAPS, followed by 2×SSC and PTW (50:50, in volume) and finally PTW. Tissue section were covered with 2% blocking solution (appendix I) containing 10% sheep serum (Sigma) and incubated for 3 to 5 hours at room temperature. Detection of the probe was carried out using anti-digoxigenin-AP Fab fragments (Roche) (1/100 to 1/600 in 1% blocking solution, appendix I). Sections were covered with the antibody solution, sealed with *parafilm* and incubated overnight at 4ºC. Sections were then washed by immersion in two baths of a solution of Tris-NaCl (appendix I) for five minutes in each bath. Colour development was carried out by first equilibrating the sections with the developing buffer (appendix I) by immersion for 10 minutes and subsequently sections were immersed in the developing solution which contained the chromagens, 4.5µl.ml^{-1} of NBT (4-nitroblue tetrazolium chloride, Sigma-Aldrich) and 3.5µl.ml^{-1} of BCIP (5-bromo-4-chloro 3-indolylphosphate, Roche). The time taken for colour development varied with the probe and tissue utilized and reactions were carried out at 37ºC for between 1 to 24 hours. Colour development was stopped by immersing the sections in 1×PBS. Tissues were then fixed with 4% paraformaldehyde, for 15 minutes at room temperature, washed twice with 1×PBS for 5 minutes and twice with distilled water and mounted in glycerol gelatine (Sigma) and covered with a clean glass coverslip. Sections were analysed using a microscope (Olympus BH2) coupled to a digital camera (Olympus DP 11).

Because many variables exist in the *in situ* hybridization procedure, controls are very important to monitor each assay for validity. The interpretation of a precipitated product as evidence of an identified segment of nucleic acid requires that all other explanations be eliminated (Kenny-Moynihan and Unger, 2002). Positive and negative controls should be run to make sure that the positive results obtained in the samples have been correctly evaluated (Kenny-Moynihan and Unger, 2002, Wilcox, 1993). Sense probes (with the same label and used in similar concentrations) are often used as negative controls but sometimes they can give a positive hybridization reaction or a high background

(Wilcox, 1993). To overcome such problems a range of other negative controls can be used, such as the pre-digestion of the tissues with RNase as hybridization of riboprobes depends on the presence of mRNA in the tissues (Wilcox, 1993). In the present study two main types of control experiments were performed to verify the specificity of the signals obtained. After hydration, samples were treated with RNase (0.02mg.ml^{-1} in 1×PBS) for 30 minutes at 37ºC and washed twice with PTW. Then, *in situ* hybridization was carried out following the protocol described. Other *in situ* hybridization experiments were carried out omitting riboprobe from the reaction to demonstrate that there was no interference due to the reagents.

2.5 Immunohistochemistry

The basis of immunohistochemical assays is the specific binding of an antibody to its corresponding antigen. The binding is dependent on noncovalent interactions between the antibody and target antigen. The majority of antibodies used for immunohistochemistry (IHC) are of the immunoglobulin G class. Several factors such as time, temperature and pH during the incubation will influence the binding of antibody to antigen. Antibody dilution is also a critical factor in the antigen-antibody reaction. Antibody should be used at as high a dilution as possible not only for economic reasons but also to avoid false results due to high antibody content and to decrease background staining (Kenny-Moynihan and Unger, 2002). The optimum concentration should allow the primary antibody to bind to the antigen using both Fab components of the molecule to avoid antibody dissociation in subsequent steps of the process. The antibody dilution should also permit interaction of the primary antibody with only a proportion of the antigen binding sites to ensure that the secondary antibody Fab components binds to the Fc component of the primary antibody. There are a range of methods which can be used but all are based on direct or indirect immunohistochemical detection.

Indirect immunohistochemistry, the methodology used in the present work, exploits the natural capacity of immunoglobulin to act as an antigen. In this method, the primary antibody is raised in one species and is not conjugated.

Immunoglobulins from the species in which the primary antibody was produced are then used as an immunogen in a second species resulting in antibodies recognizing immunoglobulin in the primary serum. Immunoglobulin from the secondary serum is conjugated to a detection system (Kenny-Moynihan and Unger, 2002). In the present studies, the primary antibodies utilized were raised in rabbits and the secondary antibody was raised in sheep and was peroxidase conjugated.

Whole mount immunohistochemistry was carried out in sea bream (*Sparus auratus*) and tilapia (*Oreochromis mossambicus*) whole scales, fixed in 4% paraformaldehyde as described above (section 2.2) and stored in methanol at 4ºC. Scales were transferred to a 0.3% hydrogen peroxide solution (in methanol) for at least 1 hour at room temperature to inactivate endogenous peroxidase. Then they were rehydrated through a series of 1×PBS (appendix I) solutions containing increasing concentrations of methanol (25, 50 and 75%, in methanol), for 10 minutes in each solution, and finally immersed in Phosphate-Triton Buffer (PBST, appendix I) for 10 minutes. Scales were immersed in Phosphate-Carregenin-Triton Buffer (PCT, appendix I) with 10 % sheep serum (Sigma-Aldrich) for 1 hour and then transferred to the primary antibody solution and incubated overnight at room temperature. The antibodies used and respective dilutions will be indicated in the specific methods section of the subsequent chapters. After incubation in the primary antibody solution, scales were washed for 15 minutes each in three baths of PBST at room temperature and transferred to the secondary antibody solution (anti-rabbit IgG peroxidase conjugate, Sigma), 1/50 in PCT buffer, for 45 minutes at room temperature and washed again three times with PBST for 15 minutes in each solution. Up to this point scales were always slowly agitated. They were then immersed in the developing solution (1 tablet of DAB (Sigma *fast dab* kit) and 1 tablet of urea H_2O_2 (Sigma *fast dab* kit*)* in 1000µl of distilled water) in the dark, until appropriate colour development was achieved. The colour reaction was stopped by washing twice in PBST for 10 minutes in each solution and subsequently immersing in distilled water for 10 minutes prior to mounting in glycerol gelatine and covering with a clean glass coverslip.

2.6 Semi-quantitative RT- PCR

The polymerase chain reaction permits amplification *in vitro* of specific gene sequences (RNA and DNA) so that an elevated number of copies of the target gene are obtained in a short period of time. DNA and two primers are combined in a salt solution with dNTPs and a heat stable DNA polymerase enzyme. The primers (13-21 bases) match a sequence selected in the target DNA. The reaction mix is rapidly heated to DNA denaturing temperatures (95℃) and cooled to a temperature at which the polymerase functions optimally. Each thermal cycle generates copies of the sequence lying between the primers in an exponential fashion. In the case of RT-PCR, the RNA extracted from the cells is used as the template for the production of a complementary sequence of DNA (cDNA) and then the PCR is used to amplify this DNA molecule. The amount of DNA can be quantified by running PCR's with similar amplification protocols using an housekeeping gene such as 18S ribosomal RNA or β-actin. The amplified products are separated on agarose gels, their signal quantified by densitometry and the ratio between the molecule of interest and the housekeeping gene (present in an almost constant concentration in different cells) is calculated.

In the present work, total RNA was extracted from individual samples of adult sea bream tissues using TRI reagent (Sigma-Aldrich) and homogenizing with a sterile glass homogenizer. For skeletal tissues, mechanical disruption in liquid nitrogen was carried out prior to RNA extraction. Tissues used for the studies reported in subsequent chapters will be indicated in the methods section of each chapter.

First strand cDNA synthesis was performed using 3μg of total RNA and the M-MuLV reverse transcriptase procedure (Gibco BRL). The reaction was allowed to proceed for 1.5 hours, at 37ºC. A negative control in which reverse transcriptase was omitted was also prepared. RT-PCR reactions were carried out using approximately 50ng of template cDNA and 10pmol of specific primers in a *Taq* polymerase-based 50μl reaction (Promega). The primers and the amplification protocol (iCycler, BioRad) used for the various genes studied will

be presented in the methods section of the appropriate chapters. Amplified products were separated on 1.25% agarose gels containing ethidium bromide (appendix II) and their signals were quantified by densitometry using ImageMaster 1D prime software (v. 2.01, Pharmacia Biotech).

2.7 Northern blot

In this method, the RNA extracted from cells is separated by agarose gel electrophoresis and transferred to a membrane and probed with radioactive RNA or DNA. This method permits estimation of the abundance and the size of the RNA transcripts present in tissue samples.

In the present study 10μg of total RNA per tissue were hybridized using a specific radiolabelled cDNA probe. RNA samples were run on a 1.25% agarose gel (appendix II) and then transferred to a nylon membrane (Hybond XL, Amersham Biosciences) by capillary transfer using 20×SSC buffer (appendix I). Probe was labelled with ^{32}P by random priming using a rediprime II kit (random prime labelling system, Amersham Biosciences). Membranes were pre-hybridized for 2 hours with hybridization buffer (appendix I) and then hybridized with the radiolabelled probe at 60ºC, overnight. Free radiolabelled probe and non-specifically bound probe was removed using high stringency washes. Probe hybridization was determined by autoradiography by exposing membranes to an appropriate film (Kodak BioMax MS Film) at -80ºC.