Chapter 3

Cellular organization of skeletal tissue in sea bream (*Sparus auratus*) larvae

3.1 Introduction

Based upon its pattern of development, two principal types of bone can be distinguished in vertebrates, endochondral bone and intramembranous bone. Endochondral bone (also called cartilage or replacement bone) forms within or from cartilage. Mesenchymal tissue condensations give rise to a cartilage template, which is subsequently replaced by bone. This process occurs in several steps and is accompanied by profound modifications in the cell type and their organization in the tissues and also in the composition of the extracellular matrix. In contrast, intramembranous bone (or dermal bone) develops directly from mesenchymal condensations without a cartilage precursor (Kardong, 1998, Marks and Odgren, 2002, Nakashima and de Crombrugghe, 2003, Sommerfeldt and Rubin, 2001, Triffitt*, et al.*, 1998, Wagner and Karsenty, 2001, Yang and Karsenty, 2002).

Bone histology is quite variable in the vertebrates. Human bone is composed of an extensive osteon system of chondrocytes, osteoblasts, osteocytes and osteoclasts, but this pattern changes, even among mammals. The exact nature of bone in fish is not very well studied but in teleosts it is generally considered to be acellular, although there are some reports which suggest that this may not always be the case (Hughes*, et al.*, 1994, Meunier and Huysseune, 1992). The absence of cells in teleost bone raises questions about how it is remodelled in response to continuous mechanical stress and how skeletal growth accompanies body growth. It has been proposed that changes in fully formed teleost bone are caused by osteoblasts located on the surface of the bone which secrete new matrix (Kardong, 1998).

Bone ontogeny has been studied in numerous fish species generally as a means to increase taxonomic and systematic knowledge and for this reason usually with wild species. The study of skeletal development is also important in aquaculture as cultivation of many teleost fishes is associated with an increase in skeletal abnormalities. Sea bream (*Sparus auratus*) is a widely cultivated species in the Mediterranean and Portugal. The ontogeny of the skeletal system of this species has been extensively described (Faustino and Power, 1998,

1999, 2001). However, relatively little is known about the cellular organization of the diverse elements of its skeleton. As a first step to understanding bone development and identifying the factors involved in its regulation, this study aimed to characterize the transition in cellular organization which occurs during endochondral and dermal bone formation in sea bream.

3.2 Materials and Methods

3.2.1 Sampling

Sea bream (*Sparus auratus*) larvae were collected at 8, 20, 45, 61 and 83 days post hatch (dph) and immediately fixed in 4% paraformaldehyde, overnight, at 4ºC (section 2.2). Gills of juvenile sea bream were also collected and fixed in 4% paraformaldehyde, overnight, at 4ºC (section 2.2).

3.2.2 Whole-mount cartilage - bone double staining

Whole larvae were stained with alcian blue and alizarin red which stains cartilage blue and bone red, respectively, as described in section 2.3.5, although some adjustments were made and are indicated in this section. The younger larvae (8 and 20dph), were macerated in 1% potassium hydroxide solution (appendix I) for 1-2 hours and were immersed in alizarin red (working solution, appendix I) overnight. The older larvae (45, 61 and 83dph) were macerated for 24 hours and were immersed in alizarin red solution for 24 hours. After staining, larvae were washed for 1-2 hours with 1% potassium hydroxide solution, followed by distilled water and finally twice in phosphate buffer (1×PBS, appendix I). Then, larvae were fixed in 4% paraformaldehyde for 20 minutes and washed twice in 1×PBS, for 1 hour. Larvae were transferred to 70% ethanol and stored at 4ºC until they were processed.

3.2.3 Processing

Samples were dehydrated through an ascending series of ethanol (70%, 95%, 100%), cleared in xylene and xylene:paraffin (50:50%, in volume) and embedded in paraffin (for details see appendix II). Serial sections (5 to 8μm) of

paraffin embedded larvae were cut and mounted on APES (3-aminopropyltriethoxysilane) coated slides (section 2.2, appendix II).

To facilitate characterization of soft tissue morphology, after dewaxing and rehydration of sections they were counterstained with haematoxylin and eosin as described in section 2.3.1. To obtain definitive preparations, tissue sections were dehydrated and cleared (appendix II), before mounting in DPX (Fluka) and covering with a clean glass coverslip. Sections were analysed using a microscope (Olympus BH2) coupled to a digital camera (Olympus DP 11).

3.2.4 General histology - gills

Samples of juvenile sea bream gills were dehydrated through an ascending series of ethanol (70%, 95%, 100%), cleared in xylene and xylene:paraffin (50:50%, in volume) and embedded in paraffin (appendix II). Serial sections (5 to 8μm) of paraffin embedded tissue were cut and mounted on APES (3-aminopropyltriethoxysilane) coated slides (section 2.2, appendix II).

To identify cartilaginous tissue sections were stained with alcian blue and haematoxylin. 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. Sections were washed in distilled water and dehydrated by passing 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. Sequential sections to those stained with alcian blue/haematoxylin were stained with Masson´s trichrome stain following the procedure described in section 2.3.3.

3.3 Results

Sea bream larvae (8, 20, 45, 61 and 83dph) were stained using whole mount double staining for cartilage and bone, with alcian blue and alizarin red which stains cartilage (blue) and bone (red), respectively. This approach permitted the identification of cartilaginous and bony structures throughout osteogenic

development. In order to characterize the cellular organization of cartilaginous or bony structures, whole larvae were embedded in paraffin and sagittal sections were cut and stained with haematoxylin and eosin.

The observed structures follow two main developmental patterns of ossification, endochondral ossification and dermal ossification. The process occurring in each of these models is characteristic and is generally conserved in the structures classified as either dermal or endochondral. The ontogeny of cells and tissue in endochondral ossification are described in the pelvic fin, the viscerocranium region and the haemal arch. The principal characteristics of dermal ossification are illustrated using the centra and the caudal fin rays. During skeletogenesis three main types of skeletal tissue were identified, cartilage, bone and a tissue intermediate between cartilage and bone. Cartilage structures were principally identified in the early stages of development. Later in development, the sea bream skeleton became almost totally ossified without any cartilaginous structures present. However, in the fully developed skeleton, a number of structures still retain regions, the location and appearance of which are conserved in all sea bream, which persist as cartilage throughout the fishes' life. These persistent cartilaginous zones are usually associated with the extremities of bony structures, with articulating regions and with areas that will increase in size as the fish grow. These areas can be found, for example, in the neurocranium, the gill bars, the distal radials and the posterior tips of the actinosts of the pectoral fin, and the accessory cartilages of the caudal fin.

In general the overall cellular organization of cartilaginous structures, both those forming the template for bone and those which do not undergo a transition to bone were similar in initial stages of formation. However, it was possible to distinguish several different phases of development in those cartilaginous structures which are eventually substituted by bone. Two principal factors varied, the cellular organization and the chemical characteristic of the extracellular matrix (ECM) as defined by their interaction with the specific histological dyes utilized.

Endochondral bone development

Endochondral bone ossifies from a previously formed cartilaginous template. Once formed, the cartilaginous structure undergoes several changes in its general structure and later the transition from cartilage to bone is identifiable.

The first cartilaginous structures identified appear as solid agglomerations of large and irregular shaped cells, which are closely apposed to each other. All cells are surrounded by a thin layer of ECM which stains intensely with alcian blue suggesting the matrix is rich in mucopolissacharides which have a strong affinity for the dye (Figure 3.1).

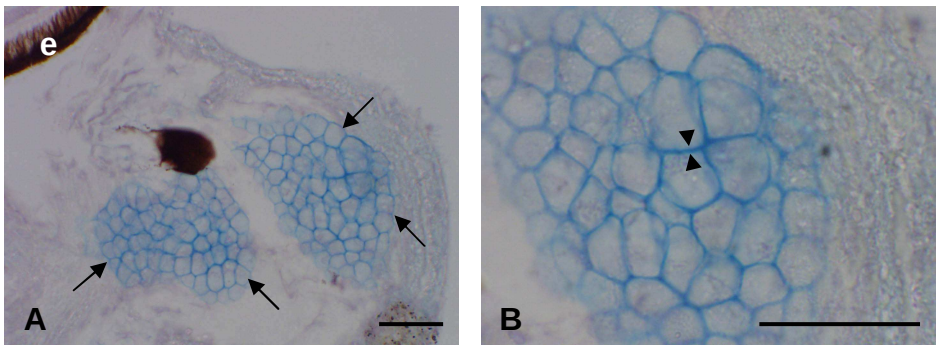

Figure 3.1 – Sagittal sections of alcian blue/alizarin red stained, whole mount larval sea bream 8 days post hatch (dph). Sections were counterstained with haematoxylin and eosin. Image B is a magnification of image A. The incipient cartilaginous structure is the frontal bone which is composed of an agglomeration of large and irregular shaped cells (arrows) surrounded by extracellular matrix (arrowheads) which has affinity for alcian blue. e, eye. Scale bars: 25µm.

The basipterygium is the supporting structure of the pelvic fin girdle which is located behind the pectoral girdle (Faustino and Power, 1999). It is an endochondral bone and in the developing larvae a cartilaginous structure is readily identified (Figure 3.2) which gradually elongates in an anterior direction towards the cleithrum (Figure 3.3; Faustino and Power, 1999). The principal changes which accompany the development of the cartilaginous template of the basipterygium provide a general overview of the formation of the cartilaginous template of endochondral bone.

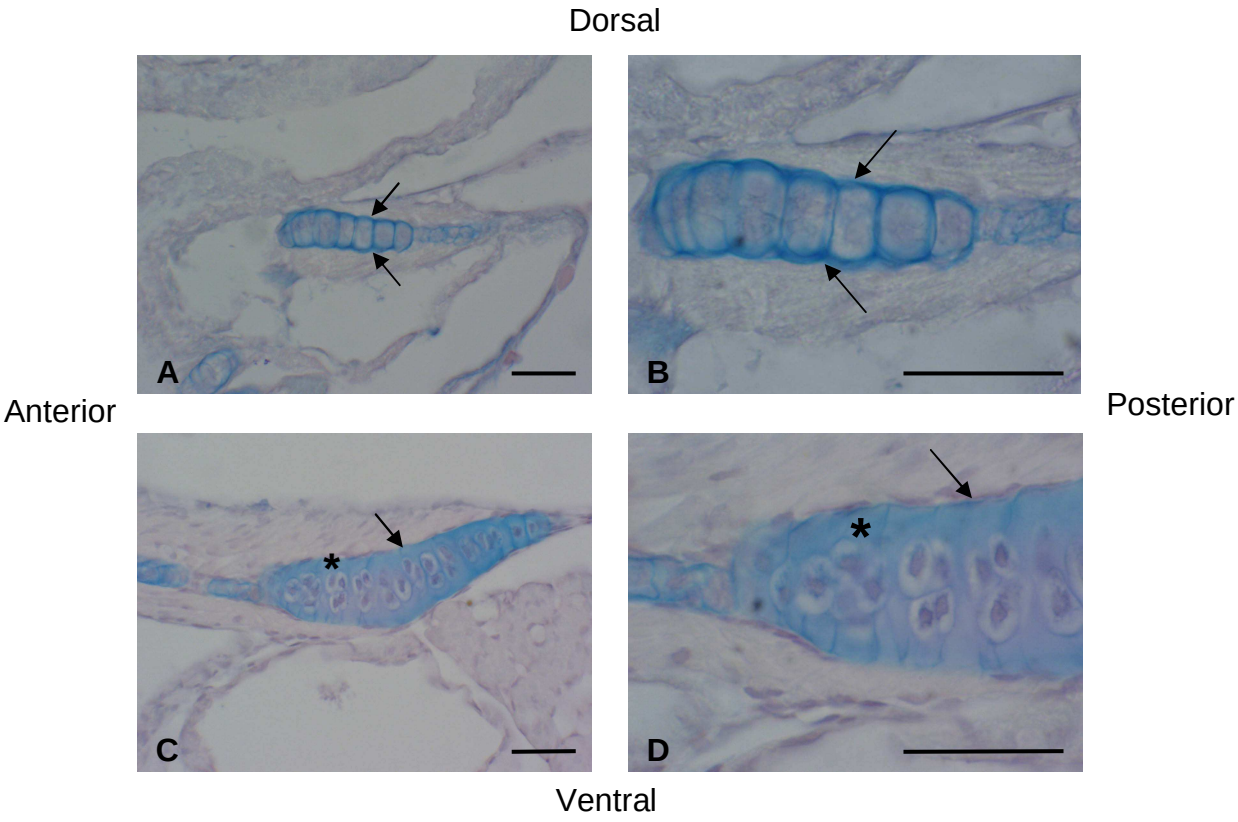

Figure 3.2 – Sagittal sections of whole mount alcian blue/alizarin red stained larval sea bream 8dph, (A and B) and 20dph (C and D), counterstained with haematoxylin and eosin. A) The forming cartilaginous structure of the basypterigium of the pelvic fin in an 8dph larval sea bream. The cartilage stains intensely blue suggesting it is rich in mucopolysaccharides which have a high affinity for alcian blue. B) is a higher power magnification of A) and the segmented organization of the cartilage is clearly visible as each segment is bounded by intense staining blue cartilage (arrows). Cells present in the segments are weakly stained and are in close contact with each other. C) and D) At 20dph, chondrocytes which occupy the lacunae of the segments possess large oval nuclei and are located in the central region of the cartilage template. Cells are bounded by a thick layer of extracellular matrix (blue staining, asterisk). The perichondrium (arrow) is evident as a membrane which surrounds the cartilage template and contains a purple staining monolayer of flattened cells. Scale bars: 25μm.

In the 8dph larvae (Figure 3.2 A and B) the cartilaginous basypterigium has a highly organized appearance and consists of a segmented elongated framework. Each segment is tightly apposed and is delimited by a well defined, thin barrier of intensely blue staining extracellular matrix. The putative chondrocytes occupy the box-like segments and in the first larval stage analysed (8dph) two cells are apparent in each division of the forming basipterygium. The boundary of these cells does not have any affinity for alcian

blue which is confined to the surrounding ECM. At this stage of development the ECM appears to have two principal functions; it separates groups of chondrocytes in different segments and also delineates the outline of the forming structure. A layer of tissue, identified as the perichondrium, surrounds the structure and isolates the chondrocytes from the neighbouring tissues. The characteristic blue colour of the cartilage is a consequence of the association of the dye, alcian blue, with the mucopolissacharides (or proteoglycans) present in the ECM matrix.

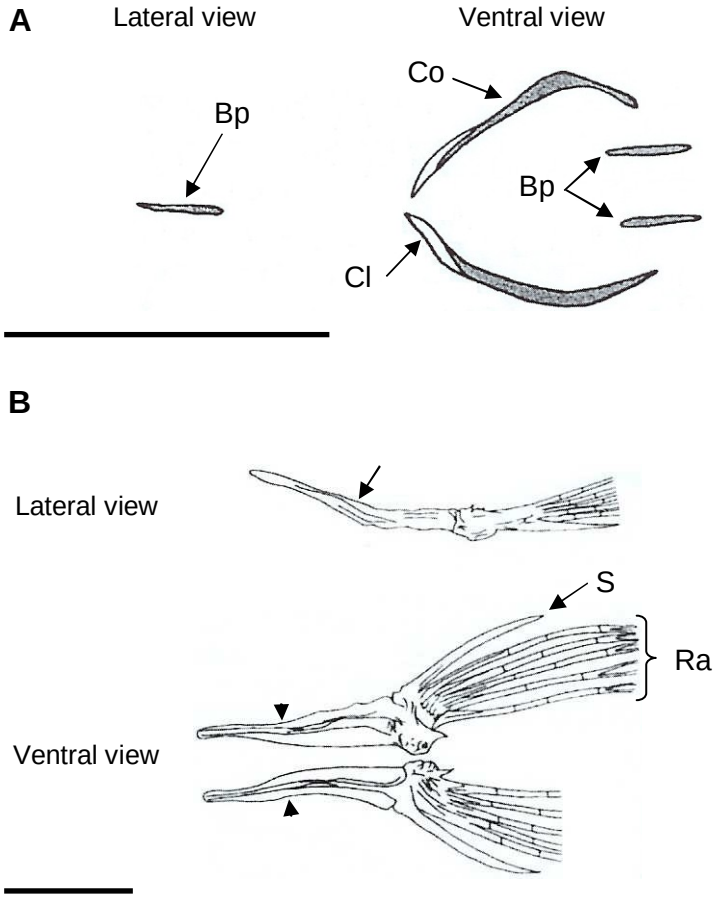

Figure 3.3 – Schematic representation of the initial (A) and final (B) stages of the development of the pelvic fin supports in *Sparus auratus* (adapted from Faustino and Power, 1999). Lateral and ventral dispositions are shown. Grey shaded areas and open areas represent cartilaginous structures and ossified structures, respectively. A) In larvae of 7.9mm standard length (L_S), the cartilaginous basipterygium (Bp) is clearly visible and is an elongated structure which extends in the direction of the cleithrum (Cl). B) In larvae of 16.0mm L_S the pelvic fin and girdle (not shown) are completely developed and the basipterygium, which is indicated by an arrow in the lateral view and by arrowheads in the ventral view, is fully ossified as are all other structures of the fin. Bp, basipterigium; Co, coracoid; Cl, cleithrum; Ra, rays; S, spine. Scale bars: 1.0mm.

As the basipterygium develops further (20dph, Figure 3.2 C and D) and elongates, the segmented organization is retained although the separation between each segment is not so evident because the ECM layer that defines them becomes thinner than it was in the earlier stages of development. The number of chondrocytes increases at each division of the cartilaginous structure and they became bigger relative to those observed in the previous developmental stages. They have a characteristic oval shape with a well delineated nucleus and do not have any affinity with alcian blue. In each segment of the cartilaginous template, the chondrocytes form a disorganized group which is located in the central area of the segments. At this stage, ECM accumulates in the periphery of the developing cartilaginous structure and forms a thick band of blue staining ECM, which is delimited by a well defined perichondrium (Figure 3.2 C and D).

Structures of the viscerocranium, such as the jaw and the ocular cavity (Figures 3.4 and 3.5) illustrate the subsequent steps of endochondral bone development. In the 20dph larva the forming jaw is composed of several regions in different stages of differentiation. Region 1 (Figure 3.4 A, B and C) appears to represent an earlier stage of differentiation, it is formed by an agglomeration of cells which are an irregular shape and are surrounded by a thin layer of mucopolissacharide-rich extracellular matrix with a high affinity for alcian blue, this structure has a similar organization to that observed in the frontal bone of 8dph sea bream larva (Figure 3.1). Region 2 (Figure 3.4 A, B and D) is formed by cells with an elongated shape which remain surrounded by blue staining ECM. A thick layer of matrix from which cells are absent is evident at the limit of the cartilaginous template and is bounded by the perichondrium. The forming jaw is undergoing modifications that produce a tissue with a different organization (defined as region 3 in figure 3.4 A, B and E). These modifications do not occur in the whole structure at the same time. Instead, the changes in tissue characteristics occur in several areas throughout the cartilaginous structure and gradually spread until the whole structure reaches a similar stage of differentiation (region 3). At this stage, chondrocytes within the lacunae appear smaller relative to those observed in earlier stages of cartilage

development and the surrounding matrix is much thicker. Moreover, the ECM in this region does not stain blue which is suggestive of a modification in its composition as the constituent proteins no longer have affinity for the histologic dye, alcian blue. This cartilage-like tissue probably represents a transitional stage between the cartilage identified in the early stages of development and the forming bone. Figure 3.4 (F and G) shows a sagittal section of the jaw of a 45dph larva. In these images, it is possible to identify at the extremity of the structure a region where the ECM stains blue and the cells are large and elongated and in close contact with each other. This cartilaginous region of the 45dph larval jaw has a tissue organization that is very similar to region 2 identified in the 20dph larvae described above and appears to undergoing similar modifications to produce a tissue intermediate between cartilage and bone. In this tissue, cells are smaller, have a well defined nucleus and are enclosed within lacunae present in the enlarging sheets of ECM which surround them. The ECM surrounding the lacunae does not appear to have an affinity for alcian blue. The perichondrium is clearly visible and delimits the skeletal structure and contains elongated cells (Figure 3.4 F and G).

The ocular cavity is another example of a bony structure formed by endochondral ossification (Figure 3.5) and is composed of a thin sheet of bone surrounded by a cellular perichondrium in the adult. In common with the development of the basypterigium the cartilaginous template is a segmented structure in which the matrix delimits each division and has a high affinity for alcian blue. One to two chondrocytes occupy each of the lacunae in the segments and secrete the surrounding matrix which gives the cartilaginous template its characteristic organization. The chondrocytes in each lacunae are separated from each other by a layer of mucopolyssacharide-rich ECM. As the ocular cavity develops the transition of the cartilaginous template into bone appears to initiate at various sites within the structure. At each of these sites a wave of calcification advances in both directions. The onset of calcification is associated with a change in the characteristics of the ECM as it looses its affinity for alcian blue (Figure 3.5 A). A transition zone between the cartilage-like tissue and the bony tissue is identifiable (Figure 3.5 B). Once formed, the bone

does not appear to contain cells (acellular) and stains with alizarin red indicating that the matrix is mineralized (Figure 3.5 B).

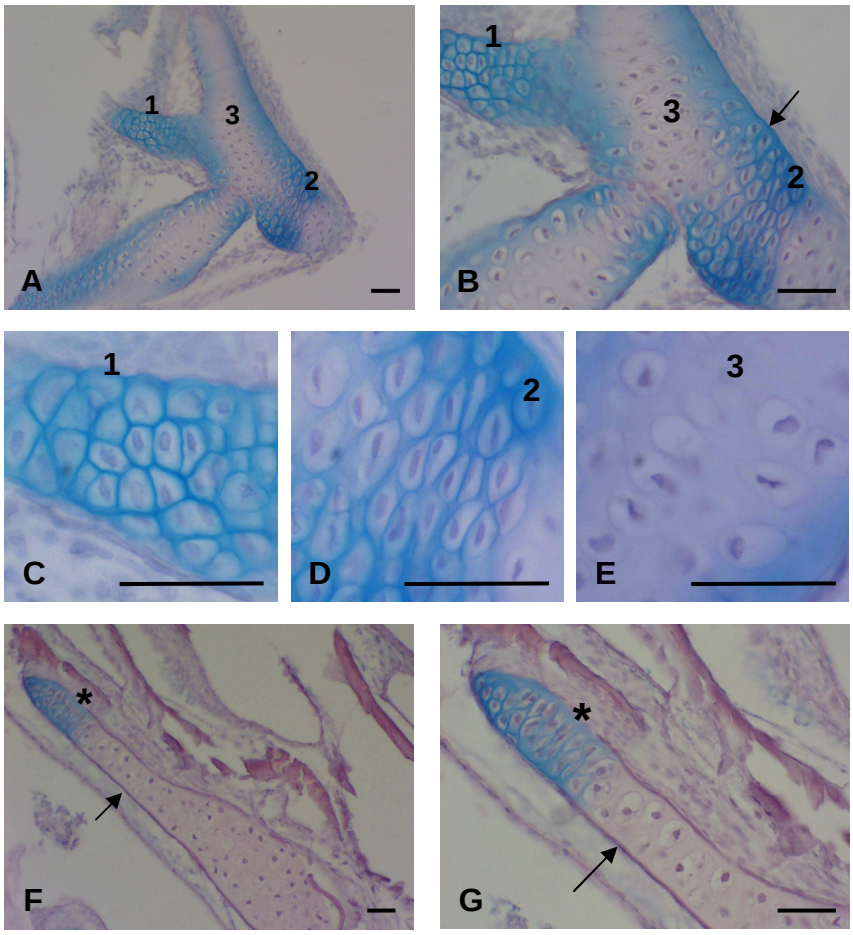

Figure 3.4 – Sagittal section of the jaw region of whole mount alcian blue/alizarin red stained larval sea bream at 20dph (A-E) and 45dph (F and G) and counterstained with haematoxylin and eosin. At 20dph, the cartilaginous template of the jaw is composed of several regions at different stages of development (indicated as regions 1, 2 and 3, in images A and B). B) is a higher power magnification of A) and demonstrates the cellular characteristics of the 3 principal areas identified. Intensely blue staining ECM is accumulating underneath the perichondrium, a membrane that surrounds the whole structure (B, arrow). Region 1 (C) is composed of an agglomeration of closely apposed, irregular shaped cells containing oval nuclei and surrounded by intensely blue staining extracellular matrix, which represents less differentiated cartilage. In region 2 (D) cells retain an irregular form but are elongated and are disposed in a more organized manner. The intensity of staining with alcian blue is diminished suggesting the chemical characteristic of the ECM is changing. In region 3 (E) the ECM has a low affinity for alcian blue and this acellular tissue corresponds to over 50% of the region. The cells have

intensely staining nuclei and are smaller than those detected in region 1 and 2 and are situated in lacunae which are surrounded by a thick layer of ECM. The differentiation of highly cellular cartilage into a less cellular bony precursor appears to start from a central region of the structure and then progresses in a bidireccional manner to the periphery (A and B). Region 3 corresponds to a more advanced stage of endochondral ossification. At 45dph (F and G) the jaw is almost completely formed by a cellular tissue, intermediate between cartilage and bone which possess a tissue organization similar to that observed in region 3 of the 20dph structure. The tissue is formed largely by ECM which has no affinity for alcian blue and contains large lacunae containing cells with intensely staining round nuclei. No sinusoids allowing cellular interactions are evident between lacunae. Moreover, capillaries or nerves are not evident in the tissue. At the anterior extremity of the jaw template, a less developed cartilaginous region is identified by its characteristic blue colour (asterisk). The whole structure is surrounded by a purple staining, cell rich perichondrium (arrow). Scale bars: 25µm.

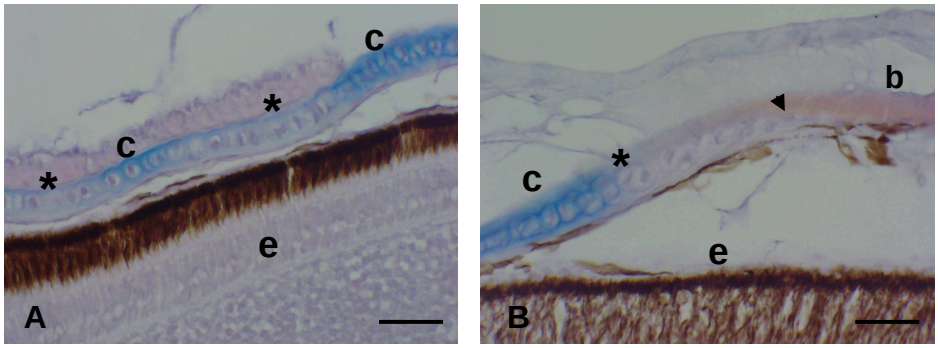

Figure 3.5 – Sagittal sections of whole mount alcian blue/alizarin red stained larval sea bream 45dph (A) and 61dph (B) showing the ocular cavity, and counterstained with haematoxylin and eosin. A) The cartilaginous structure (c) surrounding the eye (e) is formed by a single layer of cells with a segmented organization. At 45dph, two different tissues are identified based upon their affinity for the histological dyes utilized. There is typical cartilage (c) which has a segmented organization and a high affinity for alcian blue and regions with low or no affinity for alcian blue. The latter region retains a cellular segmented structure (45dph, asterisk). At 61dph (B), three distinct regions are identifiable in the ocular cavity, typical cartilage (c) stains blue, a transitional tissue (asterisk) which is cellular but has no affinity for alcian blue and calcified tissue (b) which is acellular and mineralized, as indicates the typical red colour formed when the alizarin red dye binds to the calcium ions present in its matrix. The arrowhead marks the transition region between the cartilage-like tissue and the forming bone. Scale bars: 25µm.

The haemal arch of the vertebra is also formed by endochondral ossification. A cartilage template is formed, although it is less regular or organized compared to that of the pelvic fin and the jaw. In fact chondrocytes secrete a thin barrier of blue staining ECM which appears to surround them (Figure 3.6 A and B) and as the structure develops further it gradually forms a thick layer around lacunae containing cells and at the boundary of the tissue. Gradually the ECM composition changes and although chondrocytes are evident in the tissue the ECM no longer has an affinity for alcian blue indicating its composition is changing (Figure 3.6 C). The cartilage becomes calcified and the cells initially present in the cartilage template are lost and acellular bone is formed. Figure 3.6 (C and D) shows a sagittal section of the haemal arch of a 61dph sea bream larva where different regions of cartilage-like tissue are identifiable. These regions are separated by bony tissue, already mineralized, which suggest that the ossification front is developing in two directions, from the centre to both the dorsal and the ventral extremities of the arch. Haemal arch bone is acellular but is covered by a membrane, the periosteum, which contains flattened cells which are in contact with the underlying bone and may be osteoblasts or osteoblast precursors (Figure 3.6 C).

Based on the observations made with a number of different structures, the main stages of development during endochondral ossification are defined as follows: 1) cell agglomeration, 2) cartilage extracellular matrix production, 3) cell division and differentiation within the cartilaginous template, 4) matrix accumulation at the periphery of the cartilaginous structure, 5) change in ECM characteristics (as indicated by loss of affinity for alcian blue) and 6) bone formation and mineralization.

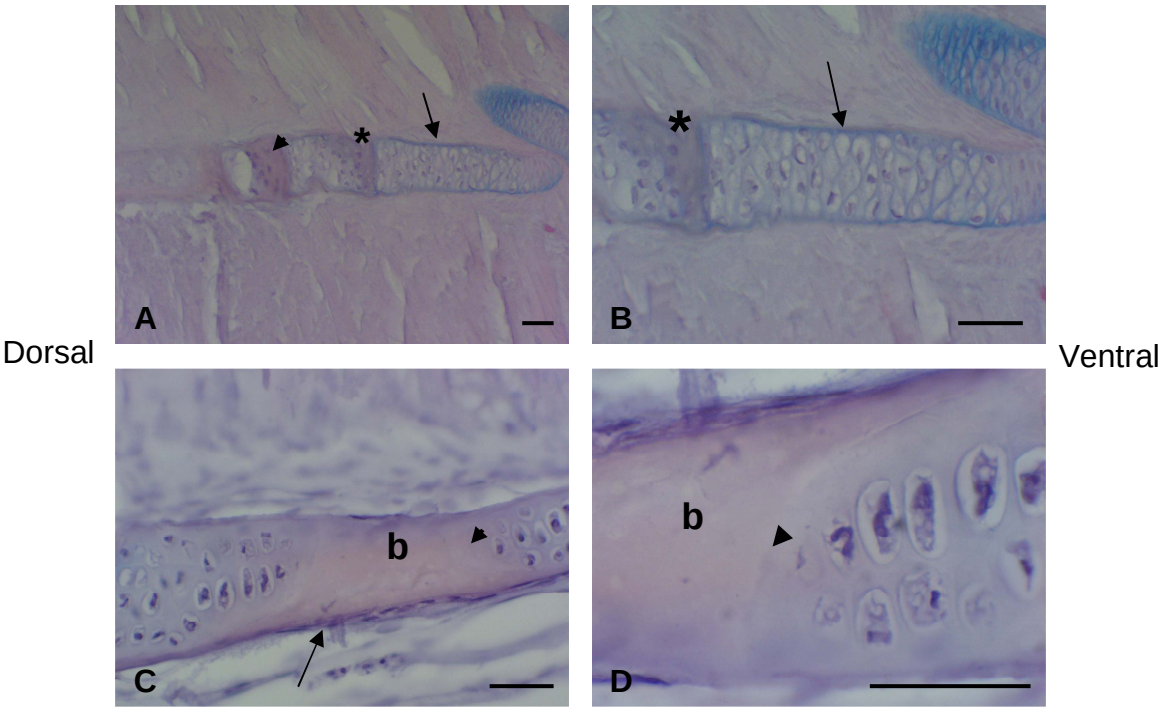

Figure 3.6 – Sagittal sections of the haemal arch in sea bream larvae 45dph (A and B) and 61dph (C and D) stained using the whole mount alcian blue/alizarin red method. Sections were counterstained with haematoxylin and eosin. At 45dph (A and B) relatively little matrix with affinity for alcian blue is evident and irregularly shaped cells, tightly apposed to one and other bounded by a perichondrial membrane (arrow) are visible. The ventral extremity of the arch is less developed and the cells are found within lacunae, surrounded by the ECM. The asterisk identifies a transition zone between cartilage and a cartilage-like tissue that will originate bone. The forming bone is indicated with an arrowhead and is acellular and stains lightly with alizarin red and is mineralizing. In the haemal arches of 61dph (C) sea bream larvae, an advanced stage of the endochondral ossification is noticeable. The haemal arch is composed of tissue intermediate between cartilage and bone, it is characterized by the abundant matrix which lacks affinity with alcian blue and the presence of elongated cells with oval nuclei which are enclosed in lacunae. Cellular regions are separated by acellular bone (b) indicating that ossification of the haemal arch is occurring from the centre of the structure to the extremities. The transition area between the two different tissues is clearly identifiable (D, arrowhead). At this stage, the haemal arch is enclosed by a membrane containing numerous flattened cells, the putative osteoblasts or osteoblast precursors that form the periosteum (arrow). Neither capillaries nor nervous tissue were identified in the developing haemal arches. Scale bars: 25μm.

Dermal bone development

In the fish skeleton the majority of bones are formed by endochondral ossification. However, there is a group of skeletal elements, such as the vertebral centra and the soft rays of the fins which are formed by dermal ossification which do not have an intermediate cartilaginous template. In sagittal sections of the vertebral centrum of 61dph larval sea bream stained with alcian blue and alizarin red the bony tissue that forms the centrum is identifiable by its characteristic red colour which is a consequence of the affinity of calcium in the bone matrix for the histologic dye, alizarin red (Figure 3.7 A and B). The centrum is composed of acellular bone and it is not possible to identify cells within the calcified matrix. The inner surface of the centrum is covered by a layer of connective tissue which separates the bony tissue from the notochord. The outer surface is surrounded by a cellular layer of presumable mesenchymal cells the condensation of which gives rise to the dermal bone. In more developed centra (83dph, Figure 3.7 C and D) the cellular layer is dense at the dorsal and ventral extreme of the vertebral centra, although a periosteum surrounds the structure and contains cells which are in close contact with the centra surface. Presumably the persistence of cells associated with the centra is essential for its increase in size as the fish grows and the nature of these cells still remains to be established.

In all the fins of *S. auratus* the fin rays are dermal structures and an example of their development is provided by the development of the first fin to appear, the caudal fin. The caudal fin in *S. auratus* starts to develop as a cartilage bud (identified as hypural 1, Figure 3.8 A) initially on the ventral side of the notochord and subsequently on the dorsal side and as structure development proceeds, it calcifies. The caudal fin rays are located at the most posterior region of the forming caudal fin skeleton (Figure 3.8). Ossification of the fin rays starts after the formation of their endochondral supporting structures and they do not form from a cartilaginous template (Figure 3.8, Faustino and Power, 1999) which suggests that they are osseous structures of dermal origin.

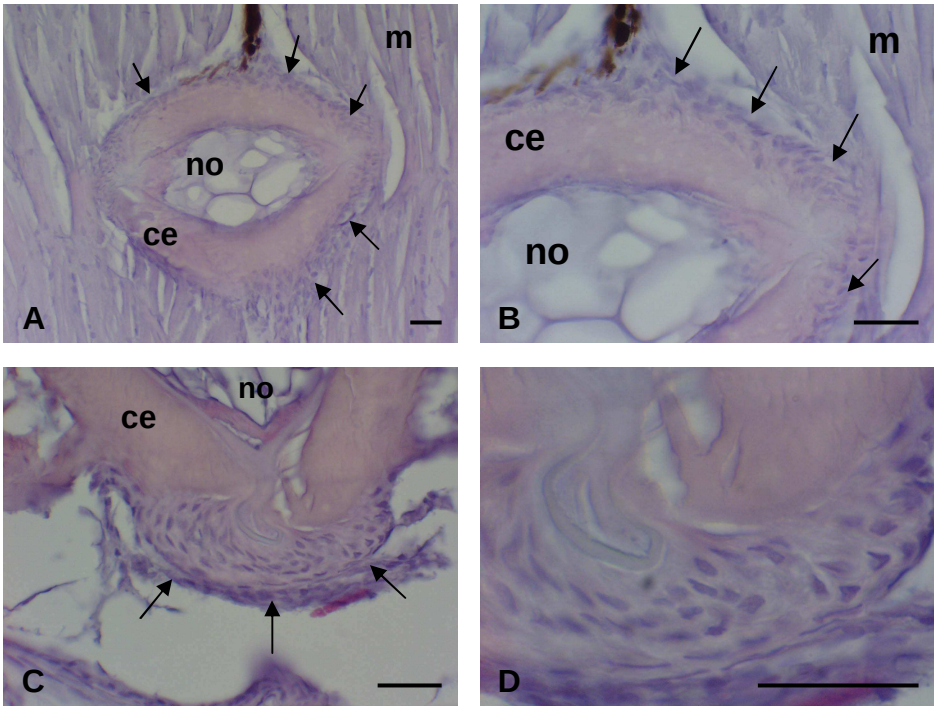

Figure 3.7 – Sagittal sections of the vertebral centrum in sea bream larvae of 61dph (A and B) and 83dph (C and D) stained by the whole mount alcian blue/alizarin red staining method. Sections were counterstained with haematoxylin and eosin. The vertebral centrum (ce) is a dermal bone and in 61dph larvae (A and B) it is well mineralized and is acellular and is surrounded and attached in some regions to skeletal muscle (m). The inner surface of the osseous structure is covered by a layer of connective tissue that separates the bone from the notochord (no). The outer surface of the centrum is surrounded by cell agglomerations (arrows) which are putative mesenchymal cell condensations that are proposed to give rise to dermal bone. In the ventral and dorsal region numerous mesenchymal cells are evident and in the lateral regions far fewer cells are present. Later in development, at 83dph (C and D), the tissue organization of centra is very similar to that observed at 61dph but by this stage the mesenchymal cell condensations are restricted to the dorsal and ventral region of the centrum and other regions are surrounded by a periosteal membrane (arrows in image C). Scale bars: 25μm.

At the anterior end of the forming rays a mass of mesenchyme-like cells are evident (Figure 3.9). These cells are an irregular shape, contain a well defined oval nucleus and are surrounded by extracellular matrix. From this agglomeration of cells, several elongations are evident which project into the forming caudal fin rays (Figure 3.9 A). In the most posterior region of the

forming rays, a layer of mineralized tissue is recognizable by its characteristic red staining with alizarin red (Figure 3.9 B and C). In amongst the irregular shaped cells which form the mass of condensed mesenchyme occasional large round cells are evident (Figure 3.9 B and C). Their morphology suggests that they are putative osteoblasts or osteoblast precursors, the origin of which remains to be resolved. The osteoblasts are responsible for bone matrix production and mineralization which generate the bony tissue that constitutes the rays. At this stage each forming ray is constituted by the condensation of mesenchymal cells in the centre of the structure, surrounded by a layer of newly formed bone which is covered on the outermost surface by a layer of flattened cells, the periosteum (Figure 3.9 C). Later in development (83dph, Figure 3.9 D, E and F) the overall tissue organization is similar to that observed in younger stages. Soft rays are well mineralized and two regions are observable. The inner region of the rays which contact with the remaining mesenchymal cell condensations is lightly stained by alizarin red indicating that is less mineralized than the outer surface which is more intensively stained which is consistent with the notion that the ray forms from mesenchyme condensation which start to calcify first in the outer most layer. The osseous rays are covered by the periosteum which contains flattened cells that separate the bone from the neighbouring tissues (Figure 3.9 F).

Overall the results indicate that dermal bone ossification in sea bream is a multi-step process which includes: 1) cell agglomeration and condensation in the regions in which bony tissue will form, 2) differentiation of mesenchymal cells to generate osteoblast precursors and/or osteoblasts, and 3) production, deposition and mineralization of bone matrix.

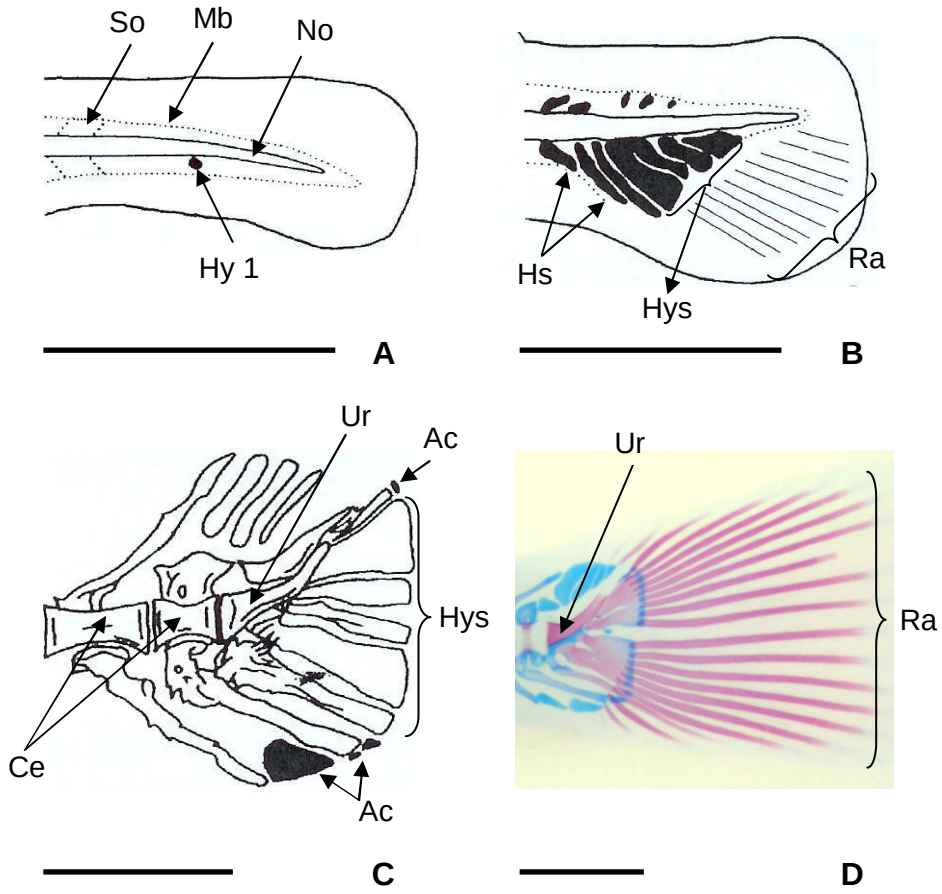

Figure 3.8 – Schematic representation of different phases of the development of the caudal skeleton in *Sparus auratus* (adapted from Faustino and Power, 1998). Shaded areas and open areas represent the cartilaginous structures and the ossified structures, respectively. Image A represents the developing caudal fin bud of a sea bream larva of 4.1mm notochord length, L_N. The first cartilaginous structure, hypural 1 (Hy 1, shaded structure), is formed ventral to the notochord. Image B represents a 5.1mm L_N larva in which the caudal rays (Ra) are represented showing their posterior position in relation to the forming caudal fin complex. Image C (larvae of 16.0mm standard length, L_S) shows all the elements of the caudal complex completely ossified except the accessory cartilages (Ac) which are shaded. Two centra (Ce) are visible and preceed the urostyle (Ur). The fin rays are not represented in the scheme. Image D shows the results of whole mount staining by alcian blue/alizarin red of a sea bream larvae (46dph, corresponding approximately to 12.5mm L_S). Structures of the caudal fin complex which are cartilaginous are stained blue, while bone is stained red. The caudal rays are calcified and present a characteristic red colour which is a consequence of alizarin red binding to calcium ions. At the base of the spines a blue staining cartilaginous region is evident and persists in the juvenile and adult. Ac, accessory cartilage; Ce, centra; Hs, haemal spines; Hys, hypurals; Mb, body margin; No, notochord; Ra, rays; So, somite, Ur, urostyle. Scale bars: 1.0mm.

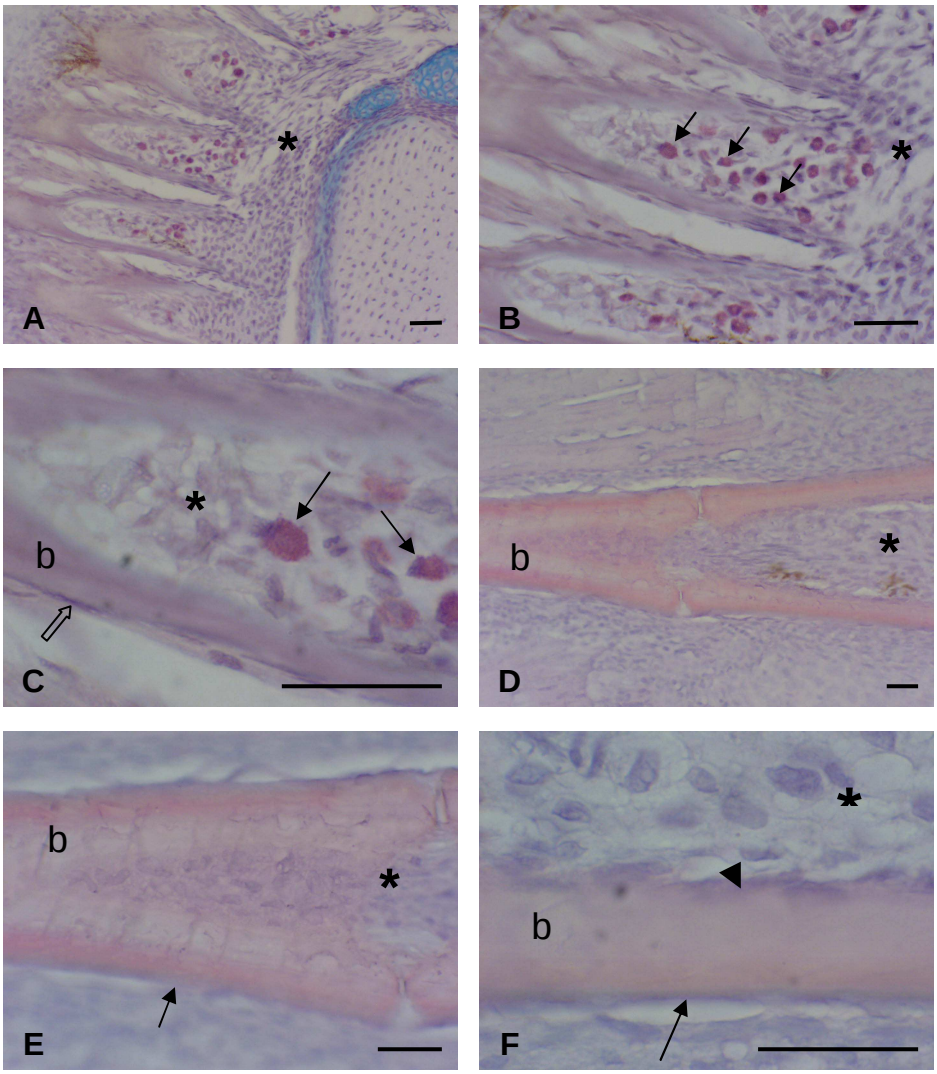

Figure 3.9 – Sagittal sections of the soft rays of the caudal fin sea bream larvae of 61dph (A-C) and 83dph (D-F) stained using the alcian blue/alizarin red whole mount staining technique. Sections were counterstained with haematoxylin and eosin. Soft rays are osseous structures of dermal origin, proposed to form from cellular condensations of the mesenchyme. At 61dph (A-C), mesenchymal cell agglomerations are identifiable in the posterior end of the caudal fin (asterisk). Big rounded cells with a small peripheral nucleus, the putative osteoblasts or osteoblast precursors are located in these regions (arrows). Bony tissue (b) is identifiable by its characteristic red colour which is a consequence of alizarin red binding to calcium ions present in the bone matrix. The outer surface of the bone is covered by a membrane composed of connective tissue and flattened cells (open arrow in image C) which constitutes the periosteum. Neither capillaries nor nervous tissue was detected in this tissue. At 83dph (D-F) the soft rays of the caudal fin have a similar tissue organization to those of 61 days with the exception that putative osteoblasts are far less abundant. At the base of the ray an agglomeration of cells project into the centre of the ray (asterisk) and is overlaid by an outer bony layer. Mineralized bone (b) is readily identifiable by its red colour although two distinct regions are visible. The outer region of the bone is darker red suggesting that is more mineralized which is consistent

with the notion that the ray forms from mesenchyme condensation which start to calcify first in the outer most layer. Image F shows that both the inner (arrowhead) and the outer (arrow) surfaces of the bone are covered by a cellular membrane which is evident by its purple colouration. Scale bars: 25μm.

Alternative processes of bone development

In the present work, in addition to endochondral and dermal mechanisms of bone formation, there are skeletal tissues that appear to develop through alternative mechanisms in the sea bream. The gill arch is a structure in which skeletal tissue develops by an alternative process and the characteristics of the resulting skeletal structure differs from that of dermal or endochondral structures.

The gills develop early during larval ontogeny as a consequence of their fundamental importance for respiration. The gill arch provides the support upon which the gill filaments are arrayed and part of it persists as cartilage throughout the fishes' life.

The overall ontogeny of the cartilage seems to be similar regardless of its final developmental destination. While it is not possible to establish the exact composition of the ECM, its strong reaction with alcian blue indicates that it contains abundant mucopolissacharides (or proteoglycans). The first cartilaginous structures to appear have a similar appearance and ontogeny to those present in the basipterygium. A structure with a well defined segmental organization is formed with each segment containing groups of cells, the putative chondrocytes (Figure 3.10 A and B). Each segment is delimited by an ECM layer which separates them and also surrounds the whole structure. In a later stage of development (20dph, Figure 3.10 C and D) the ECM accumulates mainly in the periphery of the cartilaginous structure. The ECM between the cell lacunae forms a thin layer and segments are in close apposition. The cartilaginous structure of the gill arch is surrounded by a layer of connective tissue that is identified as the putative perichondrium.

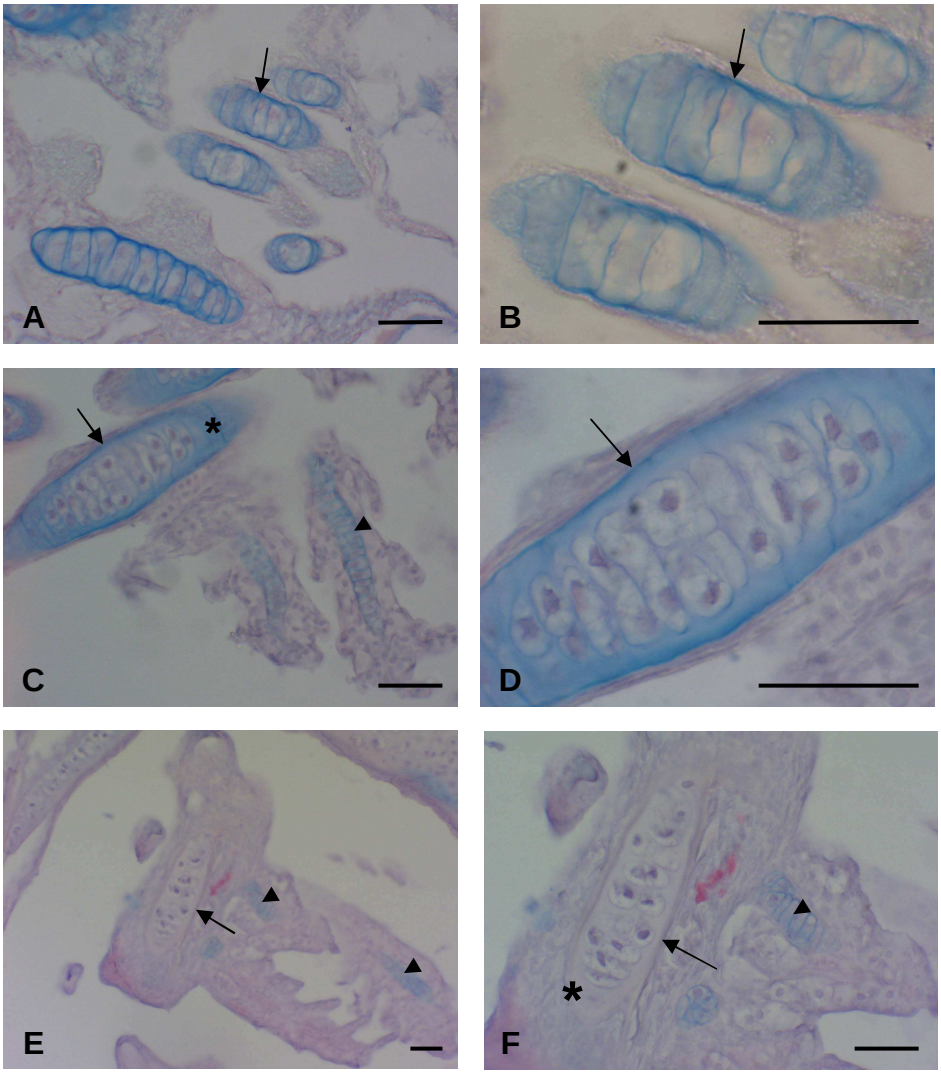

Figure 3.10 – Sagittal sections of the gill region of sea bream larvae 8dph (A and B), 20dph (C and D) and 45dph (E and F) stained by the whole mount alcian blue/alizarin red technique. Sections were counterstained with haematoxylin and eosin. The forming cartilaginous structure is the gill arch. At 8dph (A and B) has an organized segmented appearance, the divisions of which are formed by blue staining extracellular matrix (ECM). Within each segment there are groups of cells. The gill arch is surrounded by the perichondrium (arrow). Later in development, at 20dph (C and D) the cells forming the gill arch are in close contact with each other and disposed in an organized manner. The cells are large and regular in appearance and contain a small round nucleus. The ECM accumulates underneath the perichondrium and is thickest at the tips of the arches (asterisk). Along the primary filament of the gills there is a row of cartilage cells (E, arrowhead). In 45dph sea bream larvae (E and F), the cell organization and the chemical composition of the ECM change. Big cells with well defined nuclei are enclosed in lacunae which are surrounded by a thick layer of ECM (asterisk). The ECM no longer stains blue and instead has a slight affinity for alizarin red, indicating that mineralization of the structure is occurring. The gill arches are bounded by a layer of tissue (arrow) which is not

cartilaginous as it does not stain blue but has a high affinity for haematoxylin. The primary filaments remain cartilaginous in all stages of larvae examined (arrowhead). Scale bars: 25µm.

In common to the developmental process observed during endochondral ossification, the cartilaginous tissue that forms the gill arches appears as the structure differentiates to change its chemical composition. Matrix components loose their affinity for the alcian blue dye and at this stage the tissue forming the gill arch has no affinity for alcian blue and is not stained (Figure 3.10 E and F). However, the primary filaments of the gills apparently remain as cartilage throughout the fishes' life and retain affinity for alcian blue. Along the filaments it is possible to observe a well defined segmented cartilage structure with a characteristic blue ECM, with chondrocytes occupying the lacunae. The cartilage that forms the gill arch does not appear to follow the characteristic transition to bone observed during endochondral ossification.

To better establish the final organization of the tissue that forms the gill arches, sagittal sections of juvenile sea bream gills were prepared and stained with alcian blue/haematoxylin, to identify cartilaginous tissue and with Masson's trichrome staining, to identify mineralized connective tissue. The gill arches of juvenile sea bream seem to be formed by a tissue with a cellular organization which differs from that observed in cartilage and in bone. Cells isolated in lacunae present in abundant ECM which accounts for approximately 80% of the tissue. The matrix that accumulates around the cells in juveniles probably has an intermediate composition between cartilage and bone matrix. In contrast to the staining characteristics of the gill arches in larvae they do not stain blue with alcian blue, or red with Masson's trichrome indicating that it is not a typical cartilaginous or bony mineralized matrix (Figure 3.11 A and B). The composition of the ECM making up the gill arches in juvenile and adult sea bream remains to be established.

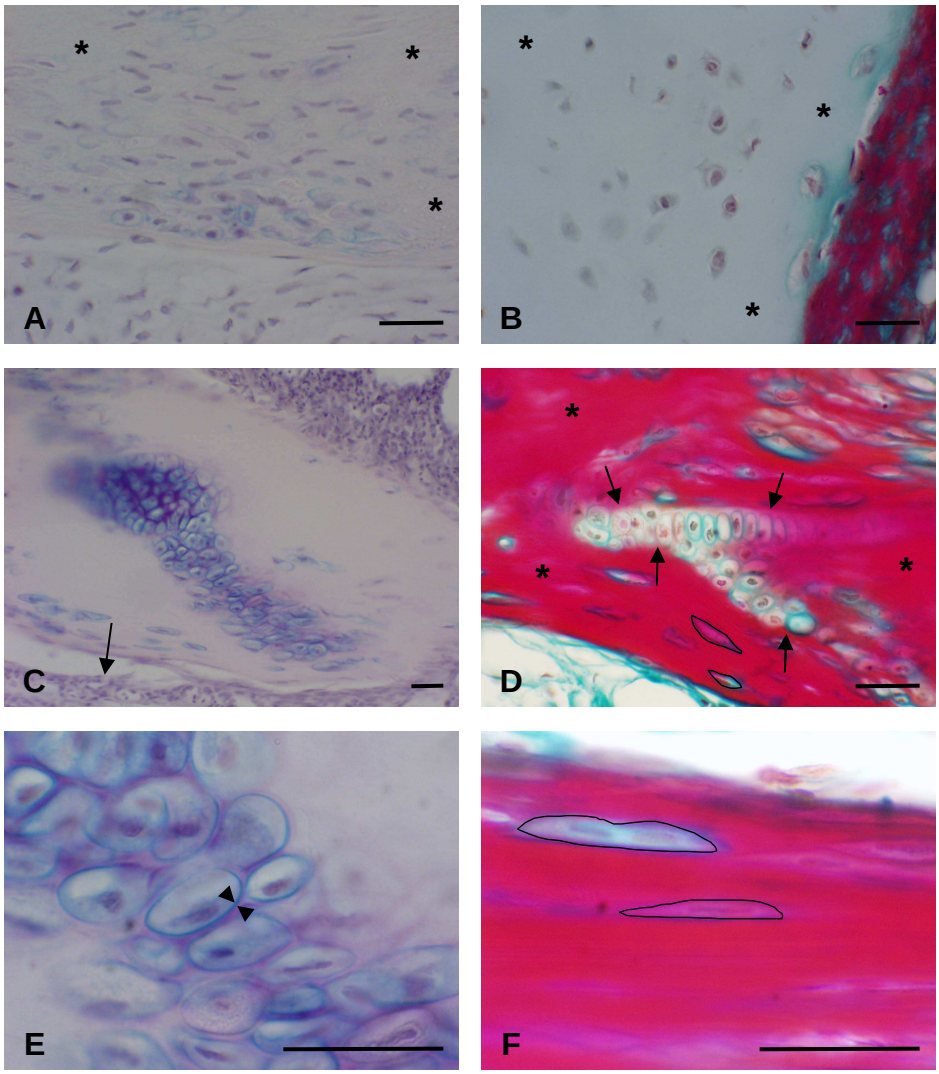

Figure 3.11 – Sagittal sections of gill arches of juvenile sea bream stained with alcian blue and haematoxylin (A, C and E) and with Masson's trichrome staining (B, D and F). In juvenile sea bream (80 days post hatch) the organization and structure of the tissue that forms the gill arch is intermediate between cartilage and bone (A and B). The extracellular matrix is acellular (asterisks) and has no affinity for alcian blue indicating that it does not have mucopolissacharides characteristic of cartilage (A). The ECM of the gill arch (asterisks) does not seem to be mineralized as it does not stain red with Masson´s trichrome (B). Moreover, in contrast to endochondral and dermal bone it contains cells with varying morphology in the tissue matrix. The gill arch is surrounded by a thick layer of fibroblast-like cells (C, arrow). D) In the transition area between the gill arch and the gill primary filaments several mineralized regions (asterisks) are identified by their intense red stain. Within the mineralized matrix of this tissue there are many groups of cells, with different morphology. Big elongated chondrocytes-like cells are enclosed in lacunae (D, arrows) and are encircled with a layer of intensely blue staining matrix (C and E, arrowheads). Closer to the periphery of the mineralized regions of the gill arch there are small groups of flattened cells, two of which are outlined in black (D and F) which appear to reside in lacunae present in the matrix. Scale bars: 25μm.

In the gill sections stained with Masson's trichrome several regions of intensely mineralized tissue were identified by their characteristic red colour. In this bone-like tissue many cells are widely dispersed in the mineralized matrix (Figure 3.11 D and F). The morphology of the majority of these cells are chondrocyte-like, as they are elongated, with big nuclei, and are enclosed in well defined lacunae which are surrounded by mucopolyssacharide-rich tissue and organized in groups that are spread within the mineralized matrix (Figure 3.11 C and E).

3.4 Discussion

Mammalian bone develops as a consequence principally of two well described processes: endochondral ossification and dermal ossification. The way in which these two processes occur during development and the associated modifications in tissue organization are well studied in mammals (Kardong, 1998, Marks and Odgren, 2002, Nakashima and de Crombrugghe, 2003, Sommerfeldt and Rubin, 2001, Triffitt*, et al.*, 1998, Wagner and Karsenty, 2001, Yang and Karsenty, 2002). Studies on skeletal ontogeny in sea bream have shown that in this species bone is of both endochondral and dermal origin (Faustino and Power, 1998, 1999, 2001). However, the modifications occurring at the tissue and cellular level during ossification have not been described in this species or in other teleosts. In the present study the tissue and cellular modifications which occur in osseous structures of endochondral origin (the frontal bone, the basypterigium of the pelvic fin, the jaw, the haemal arch and the ocular cavity) and dermal origin (vertebral centra and the soft rays of the caudal fin) have been characterized in the sea bream.

The main stages of endochondral ossification were established based on cell organization in the tissue and on the modifications in chemical composition of the extracellular matrix, defined according to its capacity to bind alcian blue, a dye that has a strong affinity for the mucopolyssacharides normally present in cartilaginous tissues. In general, regardless of the structure observed, the progression of endochondral ossification appeared to follow a similar pattern. Condensation of matrix secreting cells, was followed by cell division and

differentiation to produce large cells with the characteristic appearance of chondrocytes. This transition was associated with an increase in the matrix:cell ratio within the cartilaginous template and a final modification of the ECM chemical composition with an associated loss in affinity for alcian blue. Finally, ECM mineralization commenced, cell number in the tissue fell rapidly, although the mechanism by which this occurred has not been established, and bony mineralized tissue formed.

In mammals, endochondral ossification is mainly associated with the long bones. In these bones, three distinct regions are recognized during bone development: the diaphysis which is the middle region of the bone, the epiphysis which corresponds to the bone extremities and the metaphysis (also called epiphyseal plate or growth plate) which is located between the diaphysis and the epiphysis. Mammalian endochondral bone development starts when mesenchymal cells condense and differentiate to form a cartilage template surrounded by a perichondrium. Then, in the region of the diaphysis, cells on the inner surface of the perichondrium become osteoblasts and start depositing a bone collar in which calcium salts accumulate in the matrix to calcify the cartilage. The alteration in the extracellular matrix during calcification favours chondrocyte apoptosis and the invasion of the matrix by blood vessels. Finally, osteoblasts appear, establishing the primary centre of ossification, and produce bone matrix which replaces cartilage. New bone formation starts in the diaphysis region and continues in the metaphysis. The metaphysis is the active area of cartilage growth, calcification, cartilage removal and bone deposition where distinct regions of resting, proliferating and hyperthrophying chondrocytes are recognizable and allow the bone to grow in length. In mammals, secondary centres of ossification arise in the epiphyses and ossification follows similar steps to that observed in the primary centre (Kardong, 1998, Marks and Odgren, 2002, Yang and Karsenty, 2002).

Dermal bone is formed from mesenchymal cell aggregations. In sea bream it was observed that these aggregations are located in the places where bony tissue is going to be formed and appear to serve as a template for the osseous structure. Bone forming cells, osteoblasts, differentiate directly from the

mesenchymal cells or are mobilized from other regions of the developing animal and the bone matrix is produced and mineralized. The bony tissue which is most distant from the mesenchymal cell condensations is more mineralized than the bony layers which are closer to the mesenchyme (Figure 3.9) which suggests that matrix deposition and mineralization occurs in successive layers. As new bone is formed, the area of mesenchymal cells is reduced although it seems that a restricted region of condensed mesenchymal cells remains associated with the bony structures to permit their continuous growth during the animal's lifetime.

In mammals dermal ossification appears to follow a similar process to that observed in sea bream in the present study, although it is better characterized. Dermal or intramembranous ossification in mammals begins when mesenchyme which is compacted into sheets of membranes starts to condense and is quickly invaded by blood vessels. Between the compacted cells ground substance is accumulated and dense bars of bone matrix are deposited within this substance. At this stage, osteoblasts become evident. Matrix bars become more numerous and gradually replace the initial ground substance. Dermal bone growth proceeds in mammals by the accumulation of successive layers of new bone on the surface of the existing bone matrix bars (Kardong, 1998, Marks and Odgren, 2002, Yang and Karsenty, 2002).

In mammals, bones can be classified into four main groups: long, short and irregular, which are formed by endochondral ossification, and flat bones which are formed by intramembranous ossification. Mammalian bone is a highly organized tissue and, according to its visual appearance, it may be classified as cancellous or spongy bony, and compact or cortical bone. Cancellous bone has a porous appearance and is formed by bars and extensions that project into the bone marrow. Its functional unit is the trabeculae which contain osteocytes, canaliculi and lamellae but no central canal. Cancellous bone can be found, for example, in the inner part of long bones, closer to the bone marrow cavity, and in the middle region of the flat bones. Compact bone has a dense appearance is composed of small repeating units called osteons (Haversian system) in which bone cells, osteocytes, and their matrix are disposed in concentric rings

around a central canal through which pass the nerves and the blood vessels. Compact bone constitutes the outer regions of the long and flat bones, surrounding the cancellous bone (Dorit*, et al.*, 1991, Kardong, 1998).

Fish bone in common with mammalian bone is composed of both a living cellular component and nonliving matrix. Vertebrate bone histology varies a lot and even among mammals may differ significantly (Kardong, 1998). Relatively little information is available about the structural organization of fish bone and no consensual nomenclature exists to describe its organization (Hughes*, et al.*, 1994, Meunier and Huysseune, 1992, Moss, 1963). The observations in the present study suggest that the final structure of dermal bone between mammals and fish may be similar, however there is relatively little structural similarity between fully developed endochondral bones. In fact tissue analogous to cancellous or compact bone in mammals was not observed in the sea bream. A range of skeletal tissues which vary with the species and the type of bone considered and sometimes with a particular region of the bone have been described. Fish bone has been designated as acellular or anosteocytic bone when its mineralized matrix is deprived of osteocytes (Ekanayake and Hall, 1987, 1988, Kardong, 1998) or cellular, when osteocytes are detected although it does not form organized units such as those found in mammalian bone (Hughes*, et al.*, 1994, Meunier and Huysseune, 1992, Moss, 1963). Designations such as chondroid bone (Meunier and Huysseune, 1992), tubular bone and Sharpey fibre bone (Hughes*, et al.*, 1994), woven-fibred bone and parallel-fibred bone (Sire and Huysseune, 1993) have been used to describe the skeletal tissues observed in fish. The diversity of bony tissues present in fish is probably a consequence of the developmental and functional constraints that affect fish bone formation (Meunier and Huysseune, 1992). More studies are necessary to establish the metabolic characteristics and cellular organization of fish bone. In mammals the bone structure revealed by histology allows a clear distinction between dermal and endochondral bone but this does not appear to be the case in fish.

The sea bream bony structures observed in this study, both of endochondral and dermal origin, begin to develop from cellular agglomerations which, in the

end, give origin to bone. In these structures, formed bone has an acellular appearance as no cells were identified within the mineralized bone matrix. The osseous structures in sea bream are covered by inner and outer cell layers which may contribute to bone turnover although this was not established in the present study. In the case of the dermal bones studied, the vertebrate centra and fin rays, cell agglomerations remain associated with the bony tissue possibly to allow growth in girth and length. In the gills, skeletal tissues which could not be classified as dermal or endochondral were identified (Figures 3.10. and 3.11), suggesting that the gill arches are composed of a tissue intermediate between cartilage and bone. Cells are dispersed in the ECM although they seem to be distant from each other and it was not possible to establish if they contact other cells via sinusoids. The extracellular matrix of the gill arch in juveniles and adults, in contrast to larvae, does not stain blue with alcian blue which indicates an alteration in its chemical composition (Figures 3.10. and 3.11). Several areas of a highly mineralized tissue were observed in a transition region between the gill arch and the gill primary filaments. Groups of chondrocytes-like cells are spread in the mineralized tissue (Figure 3.11).

Mature chondroid bone is described as a skeletal tissue with large, randomly dispersed chondrocytes-like cells embedded in a mineralized bone-like matrix (Meunier and Huysseune, 1992) which suggest that the tissue identified in the sea bream gills may be chondroid bone. Chondroid bone in teleosts has been principally associated with sites subject to mechanical stresses. It has been considered that this skeletal tissue meets the support, resistance and movement demands to which certain bones, such as articular bones, are subjected (Meunier and Huysseune, 1992). Gills are exposed to a constant water flow and consequently they probably have a high turnover rate which may explain the presence of chondroid-like bone in the gills and the different histological characteristics of the tissue identified in the gill arches.

In conclusion, the present work showed that in sea bream osteogenesis occurs by two main processes, endochondral and dermal ossification which are multi-step mechanisms that follow similar steps to those described for mammals although other alternative osteological pathways seems to occur, namely in the

gill arches. In most cases the formed bone has an acellular appearance except in the gill arches where an alternative skeletal tissue was identified. It was observed that the composition of the extracellular matrix changes during osteogenesis, and studies to characterize ECM proteins and their expression in fish skeletal tissues may contribute to understanding the origin of the diverse skeletal tissue. In particular, ECM proteins that have been used as molecular markers for mammalian bone and cartilage and their respective specific cells may contribute to understanding the character and evolution of fish skeletal tissues.