Chapter 1

General introduction

1.1 General overview

Calcium is a very important factor in many physiological processes such as muscle contraction, nerve excitability, intracellular signal transduction, enzyme activity, cellular secretion, blood coagulation and many membrane and cellular processes (Chester-Jones*, et al.*, 1987, Wendelaar Bonga and Pang, 1991).

In terrestrial and aquatic vertebrates, the extracellular concentration of calcium must be kept constant. Hyper or hypocalcemia have profound effects on physiological processes and may even be lethal for the organism. To maintain extracellular calcium concentrations within narrow limits, an effective homeostatic control mechanism must be present, in which participate several endocrine factors, such as parathyroid hormone, calcitriol and calcitonin, and, in fish, stanniocalcin (Flik*, et al.*, 1995, Wendelaar Bonga and Pang, 1991).

The availability of calcium to animals varies considerably, depending on the environment and their diet. The physiological demand for calcium also varies a lot as, for example, during growth and reproduction. Terrestrial vertebrates depend on their diets as an external source of calcium. Food ingestion is an episodic process which means that calcium availability is variable throughout the day. Calcium is absorbed in the intestine and is transported in the blood to around the body. Its uptake is generally balanced by renal calcium excretion and reabsorption to maintain extracellular calcium levels constant. Fish may obtain calcium not only from their diet but also from the ambient water in which they live, and calcium uptake occurs via the intestine and the gills. The aquatic environment they inhabit provides a continuous supply of calcium although it may occur in concentrations above those of the intercellular fluids, as in seawater (around 10mmol.l^{-1}), or in concentrations similar to or below the internal levels, as in freshwater (0.1-3mmol.l^{-1}) (Bentley, 1998, Chester-Jones*, et al.*, 1987, Flik*, et al.*, 1995, Wendelaar Bonga and Pang, 1991).

The main role of the skeleton is to maintain body shape and provide support. This function is particularly important for animals living on land because they lack the buoyant effect of water. Skeleton has also a very important biomechanical role, through its interaction with muscle, to permit movement. In

terrestrial animals, that have a limited access to calcium the skeleton evolved as an internal reservoir of minerals, mainly calcium, that can be mobilized when extracellular calcium concentration is low and where extracellular calcium can be deposited when extracellular concentration rises (Bentley, 1998, Dorit*, et al.*, 1991, Flik*, et al.*, 1995, Sommerfeldt and Rubin, 2001, Wendelaar Bonga and Pang, 1991). Mammalian bone structure is very well studied and a lot of information is available about calcium regulation in terrestrial vertebrates. In fish, calcified tissues (bone, teeth and scales) contain around 99.5% of total calcium of the organism (Wendelaar Bonga and Pang, 1991) but little is known about their structure or their involvement in calcium homeostasis.

The present introduction will aim to describe skeletal tissue structure and biochemistry. It will start by giving a general overview of the main tissue types, cartilage, bone and fish scales and it will focus on the extracellular matrix of the skeletal tissue, presenting its molecular composition. After, skeletal formation, growth and remodelling and its regulation will be presented and a brief consideration about fish scale development and regeneration will also be presented. Then, the endocrine factors involved in the regulation of calcium homeostasis will be described. Finally, the fish models used in the present work will be presented.

1.2 Vertebrate skeletal tissue

The vertebrate skeleton performs several important roles. It provides support for the body and protects vital organs and it has a mechanical role through its interaction with muscles. The skeleton also plays a key role in calcium and phosphate homeostasis, acting as an internal reservoir of these ions, and in mammals also provides the environment for hematopoiesis, which takes place in the bone marrow (Compston, 2001, Dorit*, et al.*, 1991, Knothe-Tate*, et al.*, 2004, Loveridge, 1999, Marks and Odgren, 2002).

The skeleton in vertebrates is composed of two distinct tissues, cartilage and bone, which are specialized connective tissues in which inorganic salts and proteins have been deposited in the extracellular matrix. The bone and cartilage

have a number of important differences such as cell type and characteristics, the composition of the matrix and its vascularization and the physico-chemical and mechanical properties (Kardong, 1998, Marks and Odgren, 2002).

1.2.1 Cartilage

In mammalian cartilage, the main protein present in the extracellular matrix is type II collagen and the main non collagenous protein is aggrecan, a proteoglycan. The matrix of cartilage is secreted by the chondrocytes which are the cells characteristic of this tissue. Cartilage is an avascular connective tissue which does not receive a direct blood supply, so that nutrients and gases must pass between blood and chondrocytes by long-range diffusion through the intervening matrix (Blair*, et al.*, 2002, Kardong, 1998).

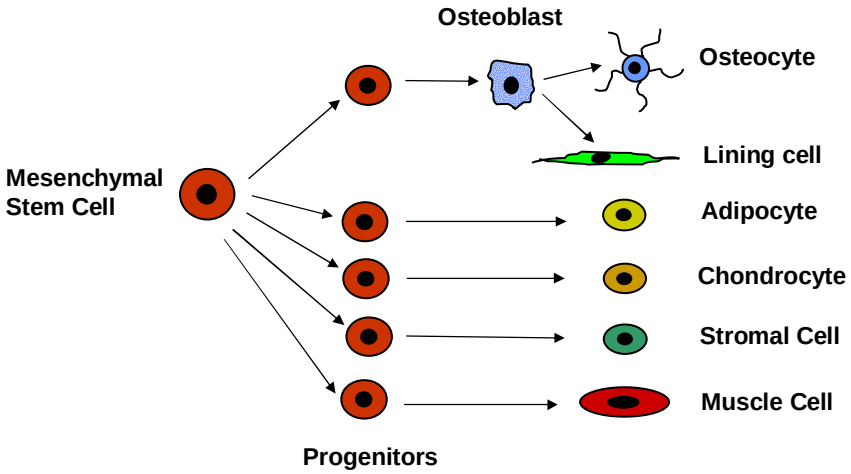

Figure 1.1 – Chondrocyte and osteoblast lineage.

Chondrocytes originate from pluripotent mesenchymal stem cells of the bone marrow which potentially can also give rise to osteoblasts, fibroblasts, adipocytes or muscle cells (Figure 1.1). Common progenitors, called fibroblast colony forming units (CFU), give rise to different colonies of stromal cells which then, depending on the regulatory factors present, differentiate into a specific cell type (Manolagas and Jilka, 1995). During endochondral ossification, chondrocytes undergo a program of proliferation, differentiation, hypertrophy and cell death (apoptosis). In each of these different processes the cartilage cells can be characterized by their specific shape, size and metabolic activities

and by the presence of specific molecular markers (Ducy and Karsenty, 1998, Loveridge, 1999, Wagner and Karsenty, 2001).

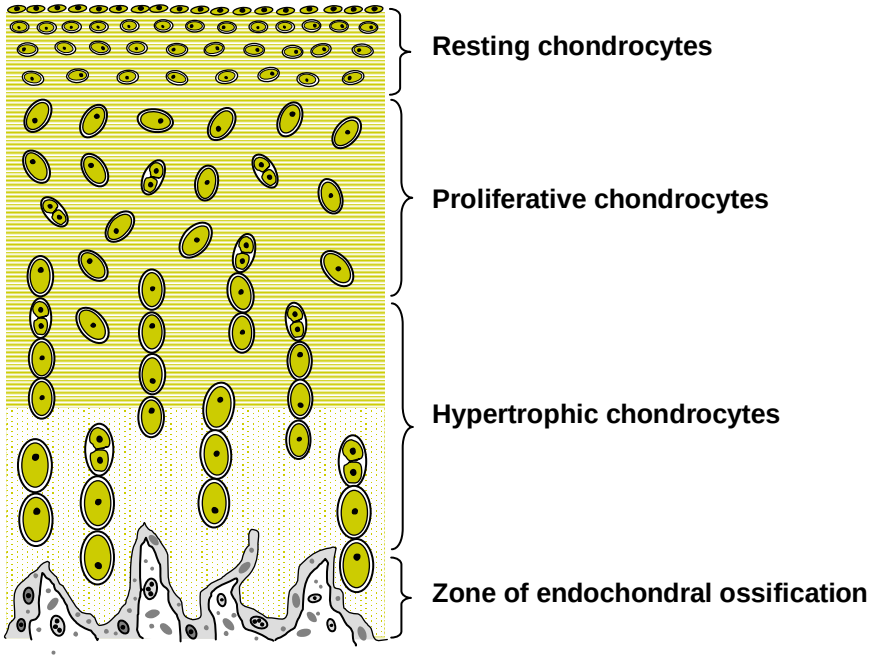

Figure 1.2 – Schematic representation of the growth plate in mammals, presenting the distinct zones of cartilage composed of chondrocytes in different developmental stages.

At the ends of the growing bones of mammals chondrocytes become organized into a structure called the growth plate cartilage, which lies between the forming bone and the epiphyseal cartilage. The growth plate is divided into several different zones composed of chondrocytes in different stages of development (Figure 1.2). These zones are constituted by well defined layers of resting, proliferative and hyperthrophic chondrocytes which participate in longitudinal bone growth (Ducy and Karsenty, 1998, Loveridge, 1999).

1.2.2 Bone

Bone is a highly specialized form of connective tissue in which the extracellular matrix is mineralized. In mammals the inorganic phase of bone is mainly constituted of calcium and phosphate salts (hydroxyapatite). The organic phase of bone extracellular matrix is composed of type I collagen, non collagenous proteins (glycoproteins), proteoglycans, growth factors and cytokines

(Compston, 2001, Kardong, 1998). Three different types of cells can be found in bone, osteoblasts, osteocytes and osteoclasts (Figure 1.3) (Compston, 2001, Kardong, 1998, Sommerfeldt and Rubin, 2001).

Osteoblasts are fully differentiated cuboidal cells found on bone surfaces that produce the extracellular matrix and regulate its mineralization. They express a number of characteristic phenotypic markers such as high alkaline phosphatase (ALP) activity and synthesize collagenous and noncollagenous bone matrix proteins, such as osteocalcin. Osteoblasts also participate in regulating the differentiation of osteoclasts, the bone resorbing cells. In common with chondrocytes, the osteoblasts are derived from primitive mesenchymal cells. Proliferation and differentiation of cells of the osteoblast lineage (Figure 1.1) occur under the influence of several transcription factors such as core binding factor α1 (Cbfa1) and osterix (Osx), growth factors, such as bone morphogenetic proteins (BMPs) and hormones such as, parathyroid hormone (PTH), 1,25 dihydroxyvitamin D_3 and estrogen (Compston, 2001, Mackie, 2003, Marks and Odgren, 2002, Nakashima and de Crombrugghe, 2003, Sommerfeldt and Rubin, 2001, Wagner and Karsenty, 2001, Yamaguchi*, et al.*, 2000).

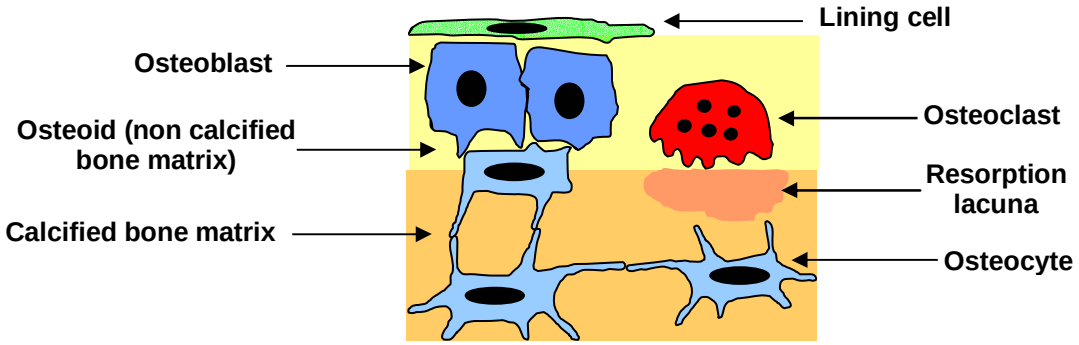

Figure 1.3 – Schematic representation of bone in mammals, presenting the principal cell types and their relative disposition in the tissue.

In mammals, bone surfaces which are not undergoing formation or resorption are covered by flat, elongated, inactive cells, called bone lining cells. They have been considered as inactive precursors of the osteoblasts (Manolagas and Jilka, 1995, Marks and Odgren, 2002, Sommerfeldt and Rubin, 2001). A fraction of osteoblasts become entrapped within the extracellular matrix giving rise to

the osteocytes which are the most abundant cells in bone. These cells have a reduced capacity to produce extracellular matrix. They are in contact with other osteocytes and also with the bone lining cells and osteoblasts through cell processes that are created before and during matrix synthesis (Compston, 2001, Knothe-Tate*, et al.*, 2004, Marks and Odgren, 2002, Sommerfeldt and Rubin, 2001). Osteocytes have a putative role in the response to mechanical stimuli, sensing mechanical strains and modulating activity associated with bone turnover, via several chemical messengers, such as, prostaglandins I_2 and E_2 (PGI_2 and PGE_2) and insulin-like growth factor (IGF) (Compston, 2001, Knothe-Tate*, et al.*, 2004, Loveridge, 1999).

Osteoclasts are large multinucleated cells, derived from the hematopoietic granulocyte-macrophage CFU, which also give rise to monocytes and macrophages (Figure 1.4). Their progenitors move from bone marrow to bone either via the circulation or by direct migration from the marrow. Osteoclasts are formed by the fusion of mononuclear cells and their function is to resorb bone, by dissolving both the mineral and organic bone matrix. They are extremely rare cells in bone but their number increase at sites of active bone turnover. They are usually found attached to the bone surface (Compston, 2001, Manolagas and Jilka, 1995, Marks and Odgren, 2002, Raisz, 1993, Roodman, 1996, Sommerfeldt and Rubin, 2001, Wagner and Karsenty, 2001).

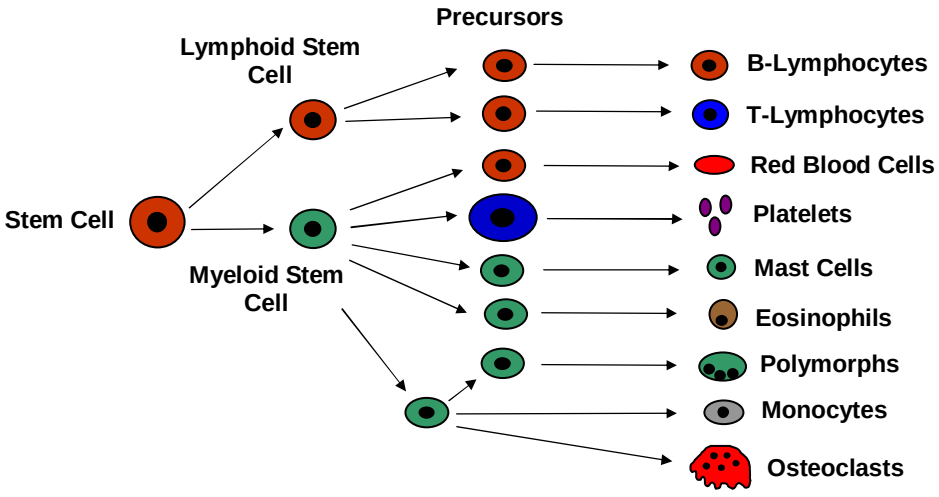

Figure 1.4 – Osteoclast lineage.

In mammals osteoclasts have a unique polarized morphology with four functionally and structurally specialized membrane areas (Figure 1.5) and several membrane and cytoplasmic markers. One of its specialized membrane areas is the “ruffled border” which is a region of the cell membrane with many folds and invaginations, at the interface with the bone surface, where bone resorption takes place. The sealing zone (or clear zone) is a microfilament rich area of the plasma membrane that surrounds the ruffled border and serves as the attachment point of the osteoclast to the bone surface. Attachment is essential for resorption to occur and is mediated by integrins, particularly by the vitronectin receptor ($\alpha_v\beta_3$), which binds matrix proteins containing the RGD (arginine-glycine-aspartic acid) motif (Blair, 1998, Compston, 2001, Loveridge, 1999, Marks and Odgren, 2002, Roodman, 1996, Salo*, et al.*, 1997, Vaananen*, et al.*, 1998, Vaananen*, et al.*, 2000). Other identifiable zones of the osteclastic membrane are the basolateral membrane, which corresponds to a homogenous membrane area, and the functional secretory membrane, which functions as a site for exocytosis of resorbed matrix-degradation products. These degradation products are enclosed in vesicles that are endocytosed from the resorption lacuna, migrate through the cell and, when they reach the functional secretory membrane, are liberated into the extracellular space (Salo*, et al.*, 1997, Vaananen*, et al.*, 2000).

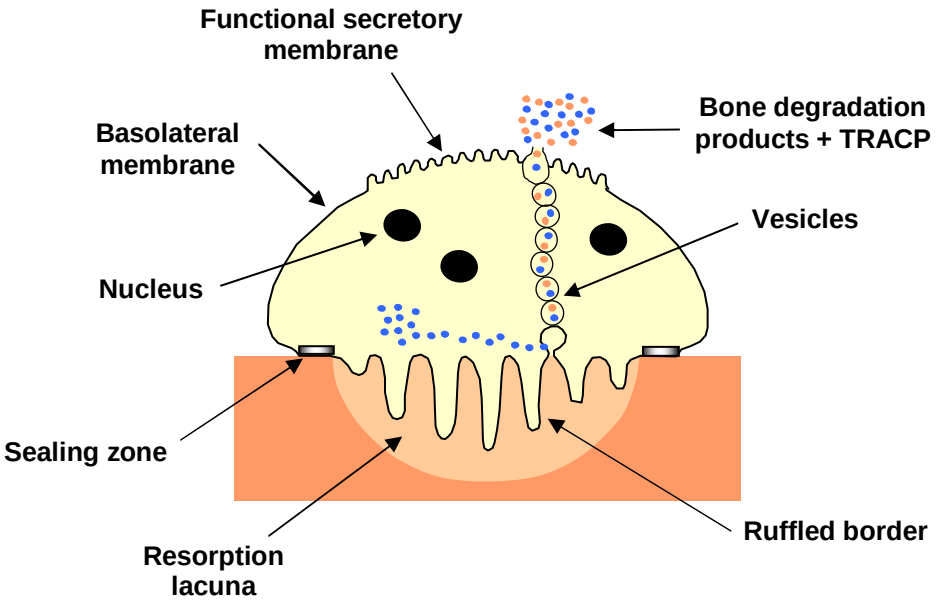

Figure 1.5 – Schematic representation of an osteoclast.

Osteoclasts express several specific enzymes, which are necessary for its function. Tartrate resistant acid phosphatase (TRACP) has been recognized as an osteclastic marker and is proposed to be involved in the resorption process (Oddie*, et al.*, 2000). The major function of TRACP may be dephosphorylation of bone matrix phosphoproteins, such as osteopontin and bone sialoprotein, and in this way osteoclast attachment to the bone surface is modulated (Ek-Rylander*, et al.*, 1994). Osteonectin, a major ECM protein is also a substrate of TRACP (Oddie*, et al.*, 2000). Proteolytic enzymes such as cathepsin K and to a lesser extent cathepsins B, D and L are expressed in osteoclasts and seem to be responsible for the degradation of collagen at low pH (Blair, 1998, Compston, 2001, Gowen*, et al.*, 1999, Loveridge, 1999, Roodman, 1996, Vaananen*, et al.*, 1998). The acidification of the mineralized matrix depends on the production by carbonic anhydrase II of protons which are subsequently delivered across the plasma membrane by a vacuolar type proton pump (V-type ATPase) (Blair, 1998, Compston, 2001, Loveridge, 1999, Roodman, 1996, Vaananen*, et al.*, 1998). Osteoclasts also produce matrix metalloproteinases -1 and -9 which seem to be useful for osteoclast migration (Blair, 1998, Compston, 2001, Loveridge, 1999, Roodman, 1996, Sato*, et al.*, 1998, Vaananen*, et al.*, 1998).

Osteoblasts are present in fish bone but in most teleosts they do not give rise to osteocytes because they do not became entrapped in the bone matrix they produce during bone formation, originating acellular bone (Kardong, 1998). However, there are exceptions and some fish species have cellular bone where osteocytes are present (Meunier and Huysseune, 1992). Osteoclast-like cells have been reported in fish bone (Sire*, et al.*, 1990, Witten, 1997, Witten*, et al.*, 2001) and scales (Persson*, et al.*, 1999) although their function and mode of action are not clear.

1.2.3 Fish scales

Scales are calcified structures that cover part of the body of most bony fishes and the size, number and type of scales is generally related to the way fishes live. Scales may be a heavy coat of armour, a few bony plates on the back, or

form a dense cover of thin flexible scales (Moyle and Cech Jr., 1996). There are various types of scales: the placoid, the ganoid and the elasmoid scales (Figure 1.6). The placoid scales are tiny, tooth-like scales (also called dermal denticles) which occur in sharks. Bony fish have scales which are layered plates, with bony tissue in one of the plates. Ganoid scales, an ancestral form, were present in ancestral chondrosteans. They are thick scales overlain by layers of a dentine-like material (cosmine) and an enamel-like material (ganoine) which are retained nowadays, to some extent, in surviving chondrosteans and in gars. In most neopterygians, scales became thinner and lighter, have lost the cosmine and ganoine layers and the underlying bone was reduced to a thin disc. Such scales are the precursors of the elasmoid scales. As scales became thinner, the trunk and tail of fish could undulate more freely and consequently the swimming efficiency improved. Elasmoid scales are sub-divided into cycloid and ctenoid scales (Figure 1.6). Cycloid scales are round flat scales and ctenoid scales are similar but have tiny, comb-like projections in the posterior (exposed) region of the scales, which probably improve the hydrodynamic efficiency of swimming (Dorit*, et al.*, 1991, Lagler*, et al.*, 1962, Moyle and Cech Jr., 1996, Sire and Akimenko, 2004).

The outer surface of elasmoid scales has bony-ridges, usually called *circuli*, the distribution and appearance of which may reflect the individual growth pattern. The inner plate of the scale is made of layers of criss-crossing fibrous connective tissue. The first part of the scale to develop is known as the “focus” and as the scale grows it becomes located in a central zone. In many species, grooves (*radii*) radiate from the focus toward one or more margin of the scale (Figure 1.7). Frequently, pigment cells, chromatophores, adhere to the posterior region of the scales (Lagler*, et al.*, 1962).

The diverse scale types have dermal origin (Dorit*, et al.*, 1991, Sire and Huysseune, 2003). Elasmoid scales first appear during development in the dermis as aggregations of cells which then differentiate to form the scale (Lagler*, et al.*, 1962). During scale development five steps can be distinguished: early morphogenesis, late morphogenesis, early differentiation, late differentiation and folding (Sire and Géraudie, 1983, Sire*, et al.*, 1997a, Sire and

Huysseune, 2003, Sire and Akimenko, 2004). The scale developmental sequence, together with morphological evidence for the differentiation of the cells in the dermis and in the basal layer of the epidermis suggests the existence of a genetic cascade that correlates with cell morphology and behaviour in the epidermis and in the mesenchyme (for review, see Sire and Akimenko, 2004). In most cases, scales appear first in the caudal peduncle and spread from there for the rest of the body (Lagler*, et al.*, 1962, Sire and Akimenko, 2004).

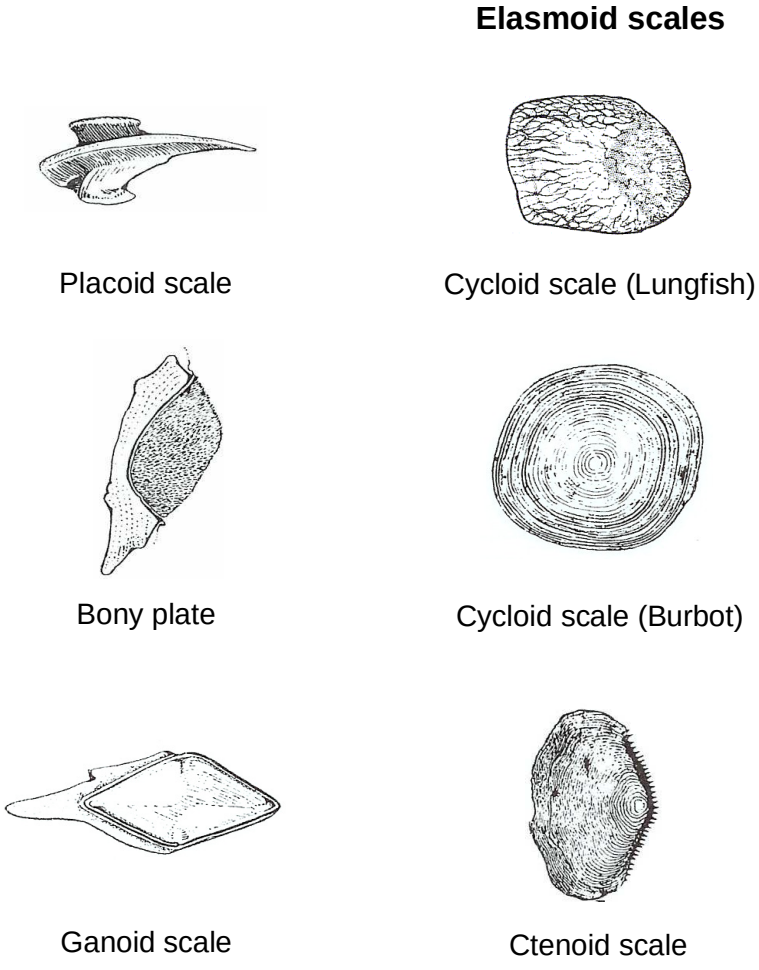

Figure 1.6 – Various types of scales (adapted from Lagler*, et al.*, 1962).

A number of different types of cells have been identified in the scales (Fouda, 1979, Olson and Watabe, 1980). At the scale margin, round or oval shaped marginal cells are present at all ages. Osteoblasts originate from marginal cells on the outer and inner side of the scale and are associated with the *circuli*

present in the external layer. These cells may be responsible for matrix production and for calcification (Olson and Watabe, 1980, Sire and Arnulf, 2000). Associated with the fibrillary (basal) plate are the fibroblasts which also develop from the marginal cells on the inner side of the scale. These cells are associated with the increase in diameter of the collagen fibres of the basal plate and may also participate in calcification, probably in areas where osteoblasts disappear from the outer side of the scale (Olson and Watabe, 1980). Teleost scales also contain osteoclasts that can be mononucleated or multinucleated and these cells have been associated with scale resorption (Persson*, et al.*, 1999, Sire*, et al.*, 1990).

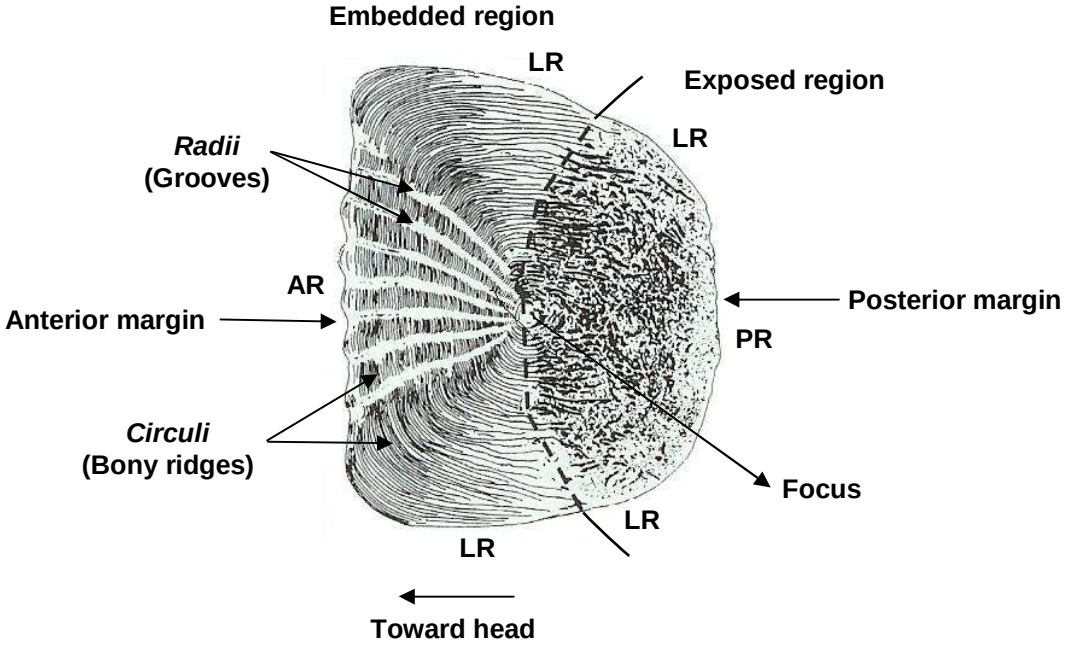

Figure 1.7 – Elasmoid scale (adapted from Sire, 1986).
(AR – anterior region; PR – posterior region; LR – lateral region)

The scales present in fish skin have a protective function and play a role in calcium homeostasis. For this reason when teleost fish lose scales they rapidly replace them by a process that starts a few minutes after scale removal and follows a sequence similar to that occurring in larvae during scale formation (Bereiter-Hahn and Zylberberg, 1993, Quilhac and Sire, 1998)

1.3 Extracellular matrix of vertebrate skeletal tissue

The extracellular matrix (ECM) is a three-dimensional network of heterogeneous macromolecules which provides an instructive and structural environment necessary for cells and tissue (Gustafsson and Fassler, 2000). It offers structural support for cells and can also act as a physical barrier or selective filter for soluble molecules. Moreover, the ECM may act as a storage depot of numerous molecules, such as growth factors and cytokines, which participate in various biological processes that involve the extracellular matrix. These processes include a range of physiological activities related to cell polarity, cell adhesion, morphogenesis, differentiation, migration, proliferation and apoptosis (Adams and Watt, 1993, Gustafsson and Fassler, 2000, Kleinman*, et al.*, 2003, Velleman, 2000).

The composition of the ECM in different tissues is different and is associated with its functional characteristics and developmental stage. A large number of ECM proteins and isoforms have been identified in tissue (Adams and Watt, 1993, Behonick and Werb, 2003, Kleinman*, et al.*, 2003). As the principal focus of the present work is cartilage and bone the following subdivisions will consider ECM composition in these tissues only. Extracellular matrix composition of scales will not be referred due to the scarcity of information available.

1.3.1 Composition of the extracellular matrix - terrestrial vertebrates

The extracellular matrix is composed ultrastructurally of two main layers, the basement membrane and the interstitial matrix. The basic structure of both these layers is defined by a collagen scaffold although the collagens in the two layers are quite different as are their 3-D architectures. In both layers adhesive glycoproteins and proteoglycans adhere to the scaffold and interact with the cells in or adjacent to the matrix. Interaction between matrix proteins and cells is mediated by matrix receptors of which integrins are the major class (Bosman and Stamenkovic, 2003, Gustafsson and Fassler, 2000, Kleinman*, et al.*, 2003). The composition of the extracellular matrix is not static and, through the mediation of numerous proteases and changes in their expression patterns, it is

constantly remodelled in response to cell behaviour and developmental stages. The family of matrix metalloproteases (MMPs) are particularly important in matrix remodelling because through their proteolytic action they contribute to create the cellular environment required during development and morphogenesis. The changes in ECM composition observed, for example, during development make clear that, at a given time and place, the ECM has the potential to provide specific environmental information to cells (Adams and Watt, 1993, Bosman and Stamenkovic, 2003, Kleinman*, et al.*, 2003, Mecham, 1998, Velleman, 2000). The importance of ECM molecules, such as collagens, fibronectin, osteonectin, osteopontin, and many others, in development has been proven by *in vivo* studies, using gene targeting. Mice lacking certain ECM components genes may die before birth or survive exhibiting unique tissue phenotypes (Aszodi*, et al.*, 1998, Gustafsson and Fassler, 2000, Kleinman*, et al.*, 2003).

1.3.1.1 Collagens

Collagens are ubiquitous proteins that provide structure and resiliency to tissue in vertebrates. More than 20 genetically distinct collagens have been identified (Table 1.1) (Adams and Watt, 1993, Aumailley and Gayraud, 1998, Gelse*, et al.*, 2003, van der Rest and Garrone, 1991, Young, 2003).

The collagen fibrils are composed of 3 polypeptides, called α chains that coil into a right-handed triple helix. Collagen exist as homotrimers ($(\alpha_1)_3$) or as heterotrimers constituted of two ($(\alpha_1)_2\alpha_2$) or three ($\alpha_1\alpha_2\alpha_3$) α chains. Each collagen α chain is characterized by a unique repeating structure of Gly-X-Y (where Gly, represents glycine and X and Y two other amino acids, with Y often being a proline).

1.3.1.2 Non collagenous proteins

In addition to collagens, a range of other non-collagenous proteins constitute the ECM. This group is largely constituted of glycoproteins and on the basis of function is divided into two main groups (Table 1.2). Group 1 comprises the adhesive glycoproteins, such as fibronectin, vitronectin and laminin, which form

connections between other ECM proteins and the cells via specific matrix receptors. Group 2 is formed by the matricellular proteins which are extracellular proteins that bind to matrix proteins, cell surface receptors or other molecules, but do not contribute to the structural integrity of the ECM. This group includes thrombospondin, tenascin, osteopontin, osteonectin and several other molecules (Adams and Watt, 1993, Bornstein, 1995, 2001).

Table 1.1 – The collagen family and subfamilies. Molecular composition and representative tissues where each collagen can be found are presented (adapted from Aumailley and Gayraud, 1998, Gelse*, et al.*, 2003, Mecham, 1998, van der Rest and Garrone, 1991).

Type	Molecular Composition	Sub families	Representative tissues
I	$[\alpha_1(I)]_2\alpha_2(I)$	Fibrillar Collagens	Bone, dermis, tendons
II	$[\alpha_1(II)]_3$	Fibrillar Collagens	Cartilage, vitreous body
III	$[\alpha_1(III)]_3$	Fibrillar Collagens	Skin, vessel wall
V	$\alpha_1(V)\alpha_2(V)\alpha_3(V)$	Fibrillar Collagens	Bone, cornea, lung
XI	$\alpha_1(XI)\alpha_2(XI)\alpha_3(XI)$	Fibrillar Collagens	Cartilage, vitreous body
		Non fibrillar Collagens	
IV	$[\alpha_1(IV)]_2\alpha_2(IV)$	Basement memb. forming	Basement membranes
VI	$\alpha_1(VI)\alpha_2(VI)\alpha_3(VI)$	Microfibrillar	Dermis, cartilage
VII	$[\alpha_1(VII)]_3$	Anchoring fibrils	Skin, oral mucosa
VIII	$[\alpha_1(VIII)]_2\alpha_2(VIII)$	Hexagonal network	Endothelial cells
X	$[\alpha_1(X)]_3$	Hexagonal network	Hypertrophic cartilage
IX	$\alpha_1(IX)\alpha_2(IX)\alpha_3(IX)$	FACIT collagens*	Cartilage, cornea
XII	$[\alpha_1(XII)]_3$	FACIT collagens*	Perichondrium, tendon
XIV	$[\alpha_1(XIV)]_3$	FACIT collagens*	Dermis, tendon, lungs
XIX	$[\alpha_1(XIX)]_3$	FACIT collagens*	Human rhabdomyosarcoma
XX	$[\alpha_1(XX)]_3$	FACIT collagens*	Sternal cartilage, tendon
XXI	$[\alpha_1(XXI)]_3$	FACIT collagens*	Blood vessel wall
XIII	$[\alpha_1(XIII)]_3$	Transmembrane collagens	Epidermis, chondrocytes
XVII	$[\alpha_1(XVII)]_3$	Transmembrane collagens	Dermal-epidermal junctions
XV	$[\alpha_1(XV)]_3$	Multiplexins	Fibroblasts, kidney
XVI	$[\alpha_1(XVI)]_3$	Multiplexins	Fibroblasts, keratinocytes
XVIII	$[\alpha_1(XVIII)]_3$	Multiplexins	Lungs, liver

*FACIT – Fibril-associated collagens with interrupted triple helices

Table 1.2 – Examples of non-collagenous proteins present in the extracellular matrix.

	Functions	Structure	Other features	References
Adhesive glycoproteins				
Fibronectin	Cell adhesion, migration, growth and differentiation	Dimeric; with repeat motifs (Type I, II and III)	Contains RGD sequence(1); multiple isoforms	DeSimone, 1994, ffrench-Constant, 1995, Hynes, 1985, Moursi*, et al.*, 1996, Young, 2003
Laminins	Component of basement membranes	Containing α, β, and γ chains	Globular domains; EGF-like(2) repeats; contain RGD sequence(1); 12 isoforms	Aumailley and Gayraud, 1998, Bosman and Stamenkovic, 2003, Mecham, 1998
Vitronectin	Multifunctional protein; more associated with tissue injury and necrosis	Single- or two-chain or fibrillar form	Contains RGD sequence(1)	Adams and Watt, 1993, Mecham, 1998, Seiffert, 1997
Matricellular proteins				
Tenascin	Morphogenesis (embryos); tissue injury (adults)	Complex domain; variable number of repeat motifs	Heptad repeats; EGF-like(2) repeats; fibronectin type III domains; contains RGD sequence(1)	Adams and Watt, 1993, Bosman and Stamenkovic, 2003, Jones and Jones, 2000, Mecham, 1998, Sage and Bornstein, 1991
Thrombospondin (TSP)	Cell-to-cell and cell-to-matrix communication; regulation of cellular phenotype	Homotrimers (TSP-1 and -2), Pentamers (TSP-3, 4 and 5)	Several ligand binding sites for cell surface receptors and protein-protein interactions	Bornstein, 1995
Osteonectin	Role in mineralization of bone and cartilage; participates in cell-matrix interactions	Acidic, cysteine-rich protein	Contains EGF-like(2) and EF hand motifs; Affinity for ionic calcium, hydroxyapatite and type I collagen	Sommer*, et al.*, 1996, Young*, et al.*, 1992, Zhu*, et al.*, 2001
Osteopontin	Participates in the regulation of mineralization; early marker of cells of osteoblast lineage	Phosphorylated sialoprotein rich in polyacidic amino acid sequences	Contains RGD sequence(1)	Sommer*, et al.*, 1996, Young*, et al.*, 1992, Zhu*, et al.*, 2001

(1) RGD sequence - Arginine-Glycine-Aspartic acid sequence; (2) EGF-like – epidermal growth factor-like

1.3.1.3 Other components of the extracellular matrix

Other groups of molecules with particular structural characteristics and specific functions are present in the extracellular matrix, such as proteoglycans, integrins and matrix metalloproteinases (Table 1.3).

Table 1.3 – Other components of the extracellular matrix.

	Functions	Structure	Other features	References
Proteoglycans	Confer specific physical properties to cartilage; involved in molecular interactions at the cell surface and in signal transduction pathways	Multidomain proteins with covalently linked glycosaminoglycans (GAGs)	Ubiquitous and highly abundant	Bosman and Stamenkovic, 2003, Mecham, 1998, Perrimon and Bernfield, 2001
Integrins	Receptors for ECM proteins or for membrane-bound counter-receptors on other cells	Cell surface glycoproteins with the same basic αβ heterodimeric structure	Recognize short peptide sequences such as the RGD (Arg-Gly-Asp) sequence	Adams and Watt, 1993, Aplin*, et al.*, 1998, Bosman and Stamenkovic, 2003
Matrix metalloproteinases (MMPs)	Play a dominant role in ECM degradation	Metalloendopepti-dases; require zinc	MMPs activity is controlled at several levels	Bosman and Stamenkovic, 2003, Kerrigan*, et al.*, 2000, Lee and Murphy, 2004, Visse and Nagase 2003

1.3.2 The extracellular matrix of cartilage and bone

The ECMs of cartilage and bone are unique in their composition, which is a reflection of the distinct functional requirements of each tissue although they share some components such as fibronectin, osteonectin and other regulatory molecules. Fibril-forming collagen is predominant in their composition and type I and type II collagen are characteristic of mammalian bone and cartilage, respectively (Velleman, 2000).

Cartilage contains a mix of proteoglycans in its ECM, such as chondroitin sulphate, heparin sulphate, keratin sulphate and aggrecan. The glycosaminoglycan chains attached to the core protein of the proteoglycans are highly sulphated, negatively charged polymers of disaccharide repeats. The negative charge is important for ionic interactions with water, which is of particular importance in cartilage formation, structure and function (Perrimon and Bernfield, 2001, Velleman, 2000).

In terrestrial tetrapods during the transition from cartilage to bone matrix, the

hypertrophic chondrocytes produce a unique non-cartilaginous ECM containing type X collagen, fibronectin, osteopontin and alkaline phosphatase which is completely replaced by bone (Velleman, 2000). Transformation of cartilage to bone is characterized by several stages which can be distinguished by composition; cartilage → osteoid → bone. Osteoid consists mainly of collagen (approx. 94%). Other proteins, some of them expressed only in bone, such as osteocalcin, are present in the ECM and may have important signalling functions (bone morphogenetic proteins, growth factors, cytokines and adhesive proteins) or have a role during the mineralization process (Sommerfeldt and Rubin, 2001). Calcified bone contains about 25% organic matrix, including cells, 5% water and 70% inorganic mineral (hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$). The highly mineralized nature of bone matrix generates the characteristic rigid functional structure (Velleman, 2000).

In terrestrial vertebrates the mineralization process is initiated a few days after the organic matrix has been laid down and involves hypertrophic chondrocytes or osteoblasts and matrix vesicles containing phosphatases, phospholipids and calcium ions. The mechanism of mineralization is uncertain but probably involves supersaturation of matrix vesicles with calcium which leads to its crystallization. Collagen fibrils, fibronectin and glycoproteins, such as osteonectin and osteopontin, seem to determine the orientation and organization of the bone mineral crystals (Sommerfeldt and Rubin, 2001). Bone matrix also has a unique composition of non-collagenous proteins. Some proteins are specific to the bone matrix, and although some are also present in other connective tissues, they seem to have a specific function in bone (for review, see Young, 2003). Most of the molecules that appear to be involved in initiation and regulation of mineralization are anionic and have structural features that facilitate interaction with mineral, cells and other matrix molecules, and they can have more than one function in bone (Boskey, 1996).

The extracellular matrix of bone and cartilage in aquatic vertebrates is poorly studied. Only a few studies about the genes encoding for ECM components and their distribution and expression patterns exist. Several non-collagenous proteins usually associated with the regulation of cell differentiation and matrix

mineralization in mammalian bone have recently been identified in the extracellular matrix of cartilage and bone of teleost fish, such as osteonectin in *Sparus auratus* (Redruello*, et al.*, 2005) and *Carassius auratus* (Lehane*, et al.*, 1999), and matrix Gla protein (MGP), in *Sparus auratus* (Pinto*, et al.*, 2001, Pinto*, et al.*, 2003, Simes*, et al.*, 2003). This may indicate that similar processes regulate cell differentiation and matrix mineralization in mammalian and teleost fish bone. Considering the importance of ECM molecules for development and maintenance of normal cellular functions in terresetrial vertebrates, a lot of work remains to be done to understand the role of ECM components in aquatic vertebrates.

1.4 Skeletal formation, growth and remodelling in vertebrates

Bony fishes (Osteichthyes) comprise over 95% of all fishes and over half of all vertebrate species. They are grouped into two subclasses: the Sarcopterygii (or lobe-finned fishes) and the Actinopterygii (or ray-finned fishes) which comprise most bony fish species. The subclass Actinopterygii is divided into two infraclasses: the Chondrostei and the Neopterygii. Teleostei are a large division of the Neopterygii that have undergone an extensive adaptative radiation (Dorit*, et al.*, 1991). They are a highly diverse group of fishes that have several characteristics in common, which result from the evolution of actinopterygian fishes towards fishes that are capable of rapid and complex movements, have efficient respiratory systems and are capable of using a wide variety of food (Moyle and Cech Jr., 1996). These advanced features allow teleost fishes to adapt to almost any aquatic environment.

The fish skeleton encompasses a range of structures and includes the notochord, connective tissues, bone, cartilage, non-bony scale and tooth components (such as enamel and dentine), supporting cells of the nervous system and fin rays. The various types of skeletal materials are organized into external and internal skeletal features (Lagler*, et al.*, 1962).

Formation of the skeleton (ossification) in vertebrates occurs, during embryonic development by two mechanisms, intramembranous ossification or

endochondral ossification. In intramembranous ossification, mesenchymal cells condense and differentiate directly into osteoblasts that produce the bone matrix. In endochondral ossification, the condensed mesenchymal cells form cartilaginous templates of the future bones (Figure 1.8). Differentiation, proliferation and hypertrophy follow cell condensation and cells are gradually surrounded by a mineralized extracellular matrix, which favours invasion of blood vessels. After vascular invasion, hypertrophic chondrocytes suffer apoptosis and are replaced by osteoblasts which produce bone extracellular matrix (Compston, 2001, Dorit*, et al.*, 1991, Ducy and Karsenty, 1998, Kardong, 1998, Sommerfeldt and Rubin, 2001, Wagner and Karsenty, 2001).

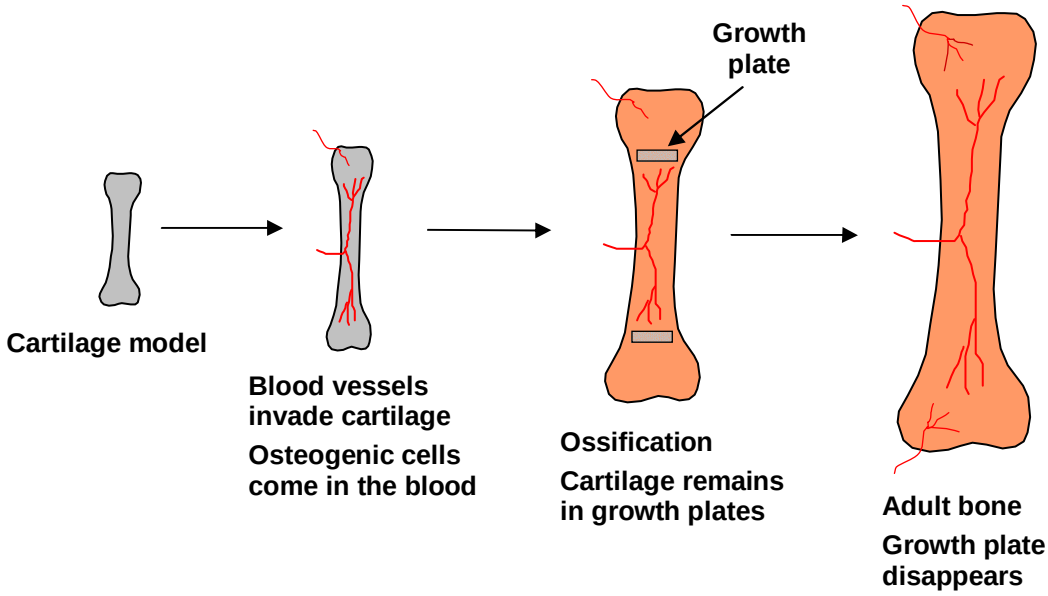

Figure 1.8 – Schematic representation of mammalian endochondral ossification in a long bone.

Hydroxyapatite (calcium phosphate) and other organic salts are deposited in the matrix. In most terrestrial vertebrate these salts are arranged in regular and highly ordered units called osteons (or Haversian system) which consists of a series of concentric rings made up of bone cells and layers of bone matrix around a central canal through which blood and lymphatic vessels, and nerves pass (Kardong, 1998). In general, the degree of mineralization of the bony tissues in Osteichthyes is similar to that of mammalian bone although the degree of mineralization is more variable in fish bone because of its variable

structure (cellular or acellular) and the nature of the aquatic environment (Meunier and Huysseune, 1992).

From the middle of the 19th Century, studies on fish bone have shown that bone tissue displays a great variety of types. A characteristic of bone in modern teleosts is the absence of osteocytes and such bone is called acellular bone (or anosteocytic bone), although ancient teleosts possess cellular bone. However, there are two exceptions, the Protacanthopterygii have acellular bone (except the Salmonidae), although they are considered primitive fishes, and within the Acanthopterygii (modern teleosts), the Thunnidae have cellular bone, which is considered to be a consequence of their high metabolic activity (Meunier and Huysseune, 1992, Moss, 1963). Relatively few studies on bone formation exist in the literature. In the Japanese medaka osteoblasts in acellular bone secrete matrix but they do not become trapped in their own secretion and do not become osteocytes. Instead, they stay on the bone surface and in later stages of bone formation, they only secrete matrix toward the formed bone (Ekanayake and Hall, 1987, 1988). The apparent lack of osteocyte lacunae in fish bone has led to its definition as acellular, although recent studies with Sparidae fishes suggested that the absence of visible or conventional osteocyte lacunae does not mean that the cells themselves are absent (Hughes*, et al.*, 1994). Another type of skeletal tissue in fish is chondroid bone, which is a skeletal tissue with large, random dispersed chondrocyte-like cells, embedded in a mineralized bone-like matrix, usually found in articular areas of the head (Huysseune and Verraes, 1986, Meunier and Huysseune, 1992, Moss, 1963). Chondroid bone is adapted to allow accelerated local growth rate with the demand for a shear-resistant skeletal support and that is more frequent in advanced teleosts (Meunier and Huysseune, 1992). Several chondroid-like tissues that are histologically intermediate between bone and cartilage have been described in mammals although no agreed terminology exists.

Bone remodelling has several roles in bone metabolism. It is used in the regulation of calcium homeostasis, maintains the mechanical competence of the bone matrix and removes areas of microdamage. The process of bone remodelling involves several steps (Figure 1.9). It starts with an activation

phase during which lining cells are removed from bone surface, osteoclast precursors are recruited and attached to bone surface and osteoclasts are activated by the formation of multinuclear bone resorbing cells. This phase is followed by the resorption phase when bone mineral is dissolved and the organic matrix is degraded. Next step is the reversal phase, during which osteoblast precursors invade the resorption site and differentiate into active osteoblasts, and osteoclasts die by apoptosis. Finally, during the formation phase, mature osteoblasts fill the resorption cavity by secreting osteoid which is then mineralized. During this process, the products of bone degradation are removed by endocytosis at the ruffled border and exocytosed into the extracellular area at the functional secretory membrane of the osteoclasts (Blair, 1998, Compston, 2001, Loveridge, 1999, Manolagas and Jilka, 1995, Sommerfeldt and Rubin, 2001, Vaananen*, et al.*, 1998).

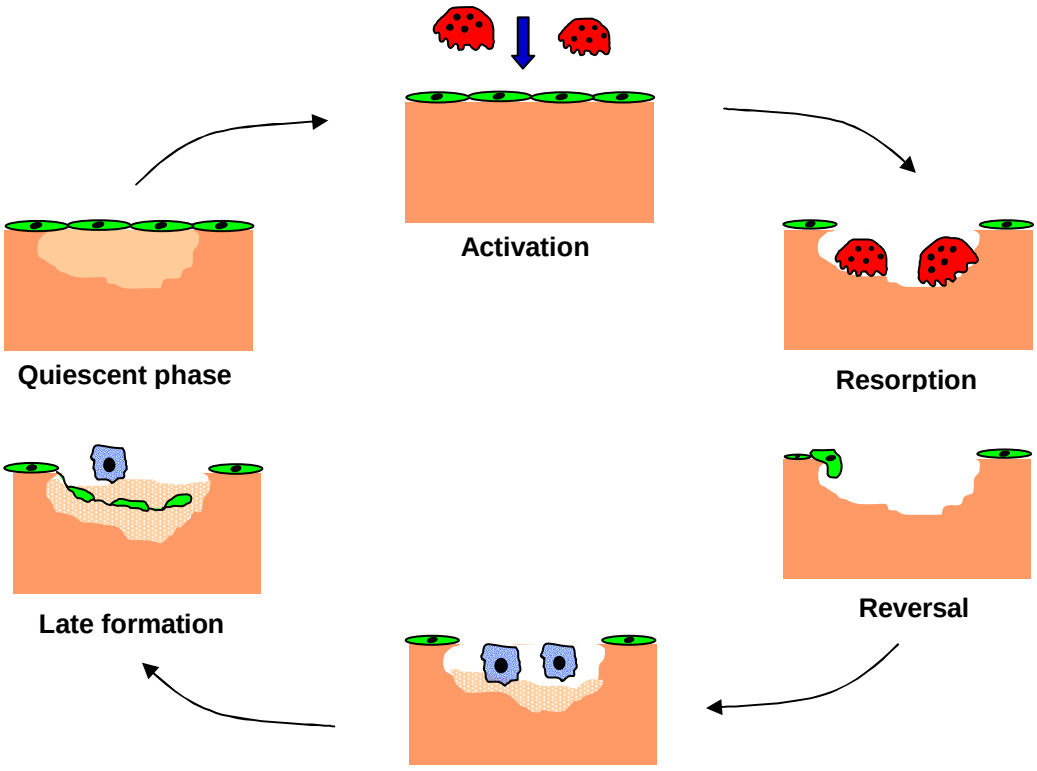

Figure 1.9 – Bone remodelling cycle.

Studies from the beginning of the 20th Century on fish bone have reported resorption under diverse conditions but the role of osteoclasts is controversial (Sire*, et al.*, 1990). Sire *et al.* (1990) demonstrated that osteoclasts are involved in bone resorption both in acellular and cellular bone species. Moreover, they showed that osteoclasts can resorb dermal, perichondral and endochondral bones suggesting that in teleosts osteoclastic activity is independent of the developmental origin of the matrix. The presence of osteoclastic activity in acellular bone suggests that osteocytes are not necessary for the existence of osteoclasts. Ultrastructurally, fish osteoclasts are similar to mammalian osteoclasts although they differ in size and shape. Two different types of osteoclasts have been identified, small flattened osteoclasts, present in areas in which there is limited space for the cell to attack the bone surface, and globular-shaped osteoclasts observed in regions where there is no limitation of space. It has not been possible to establish if flattened osteoclasts are multinucleated, although globular osteoclasts have several nuclei. Such observations suggest that fish and mammalian osteoclasts may have a similar role in bone remodelling (Sire*, et al.*, 1990). In *Oreochromis niloticus*, osteoclasts occur in two forms: mononucleated single isolated cells and cell aggregations (Witten, 1997). Recently, two types of osteoclasts were also identified in *Danio rerio*, mononucleated osteoclasts which appear first in ontogeny and persist in adults, and multinucleated osteoclasts which appear at 40 days post hatch and are the main cell type in adults (Witten*, et al.*, 2001).

Two modes of bone resorption have been described in teleosts, a deep lacunar resorption, caused by the multinuclear cells and a flat resorption caused by mononuclear cells (Sire*, et al.*, 1990, Witten*, et al.*, 2001). Tartrate-resistant acid phosphatase (TRACP) normally used as a marker for osteclastic activity in mammals can be used as a marker for the activity of bone resorbing cells in fish (Persson*, et al.*, 1995, Persson*, et al.*, 1999, Witten, 1997, Witten*, et al.*, 2001).

1.5 Regulation of skeletal development and remodelling

Development of the skeleton is a stepwise set of processes, each step depending on the step before, but each involving different cellular processes,

such as migration, adhesion, proliferation and growth (Hall and Miyake, 2000). Each step is tightly controlled and interactions between the cell and its environment play a major role in maintaining the expression of differentiation-specific genes (Adams and Watt, 1993).

The regulation of skeletal development involves regulation of cell differentiation, bone formation and bone remodelling. The latter process is mediated by systemic hormones and local factors (Compston, 2001, Guise and Mundy, 1998, Khosla, 2001).

Mesenchymal precursor cell condensations involve several steps, initiation, establishment of boundary conditions, cell adhesion, proliferation, growth and and cessation of growth (Behonick and Werb, 2003, Hall and Miyake, 2000). These condensations take the shape of the skeletal element for which they will serve as template and the constituent cells begin to differentiate into chondrocytes or osteoblasts (Behonick and Werb, 2003). Extracellular matrix proteins such as fibronectin, N-cadherin, N-CAM, noggin, syndecan and tenascin-C are involved in this process (Behonick and Werb, 2003, Hall and Miyake, 2000). The bone morphogenetic protein (BMP) family is a subgroup of molecules within the transforming growth factor β (TGFβ) superfamily that induce differentiation of pluripotent mesenchymal cell lines into chondrocytes (Yamaguchi*, et al.*, 2000, Yoon and Lyons, 2004). The effect of BMPs in chondrogenesis appears to be mediated by sox 9, a gene crucial to chondrogenesis and to chondrocyte function (Blair*, et al.*, 2002, Ducy and Karsenty, 1998, Hall and Miyake, 2000, Kobayashi and Kronenberg, 2005, Wagner and Karsenty, 2001, Yang and Karsenty, 2002), and to the expression of type II and X collagens (Canalis*, et al.*, 2003, Yoon and Lyons, 2004). Transcription factor sox 9 is expressed predominantly in mesenchymal condensations and prehypertrophic chondrocytes and has been identified as being required for formation of normal mesenchymal condensations, for conversion of mesenchymal cells to chondrocytes, for chondrocyte proliferation and for suppression of premature conversion of these chondrocytes to hypertrophic chondrocytes.

The status of chondrocytes influences ECM production and is regulated by parathyroid hormone-related protein (PTHrP) and Indian hedgehog (Ihh). Resting chondrocytes produce type II collagen. Proliferating chondrocytes also produce type II collagen and parathyroid hormone-related protein (PTHrP). Prehypertrophic cells have reduced production of type II collagen and start synthesizing type X collagen and Indian hedgehog (Ihh). Finally, hypertrophic chondrocytes secrete type X collagen and matrix metalloproteinase MMP-13 (Behonick and Werb, 2003). PTHrP secreted by the cells of the perichondrium bind to the receptor PTH1R expressed on prehypertrophic chondrocytes to suppress their differentiation. Ihh upregulates PTHrP synthesis thereby inhibiting chondrocyte hypertrophy (Behonick and Werb, 2003, Ducy and Karsenty, 1998, Strewler, 2000, Wagner and Karsenty, 2001, Yamaguchi*, et al.*, 2000). Matrix metalloproteinases secreted by hypertrophic chondrocytes are important regulators of the ossification process as they contribute to the alteration of the ECM environment by partially degrading the matrix surrounding the hypertrophic cells (Behonick and Werb, 2003, Blair*, et al.*, 2002, Ducy and Karsenty, 1998). The extracellular matrix produced by hypertrophic chondrocytes has a specific composition, which includes vascular endothelial growth factor (VEGF) that favours its invasion by blood vessels (Behonick and Werb, 2003, Blair*, et al.*, 2002, Wagner and Karsenty, 2001). Vascularization of bone is important and marks the beginning of bone tissue formation.

Osteoblast-competent stem cells occur throughout the body but differentiation is limited to the skeleton which provides an appropriate environment (Blair*, et al.*, 2002). Indian hedgehog (Ihh) and core binding factor α1 (Cbfa1) have been shown to control osteoblast differentiation. Cbfa1 is the earliest and most specific marker of osteoblast differentiation. It binds to and activates the promoter of most genes expressed in osteoblasts, such as osteocalcin, bone sialoprotein, osteopontin and α1 type I collagen. Cbfa1 has a dual role in cells of the osteoblastic lineage, regulating not only osteoblastogenesis but also the function of mature osteoblasts (Behonick and Werb, 2003, Blair*, et al.*, 2002, Canalis*, et al.*, 2003, Ducy and Karsenty, 1998, Kobayashi and Kronenberg, 2005, Nakashima and de Crombrugghe, 2003, Wagner and Karsenty, 2001,

Yamaguchi, *et al.*, 2000, Yang and Karsenty, 2002). Cbfa1 is required with osterix (Osx) for osteoblast differentiation during both endochondral ossification and intramembranous bone formation (Kobayashi and Kronenberg, 2005). BMPs are one of the most potent local factors that regulate osteoblast differentiation. BMP-1 through -6 are expressed by osteoblastic cell lines. An important function of BMPs is to induce the differentiation of mesenchymal cells towards cells of the osteoblastic lineage to promote osteoblastic maturation and function (Canalis, *et al.*, 2003, Yamaguchi, *et al.*, 2000).

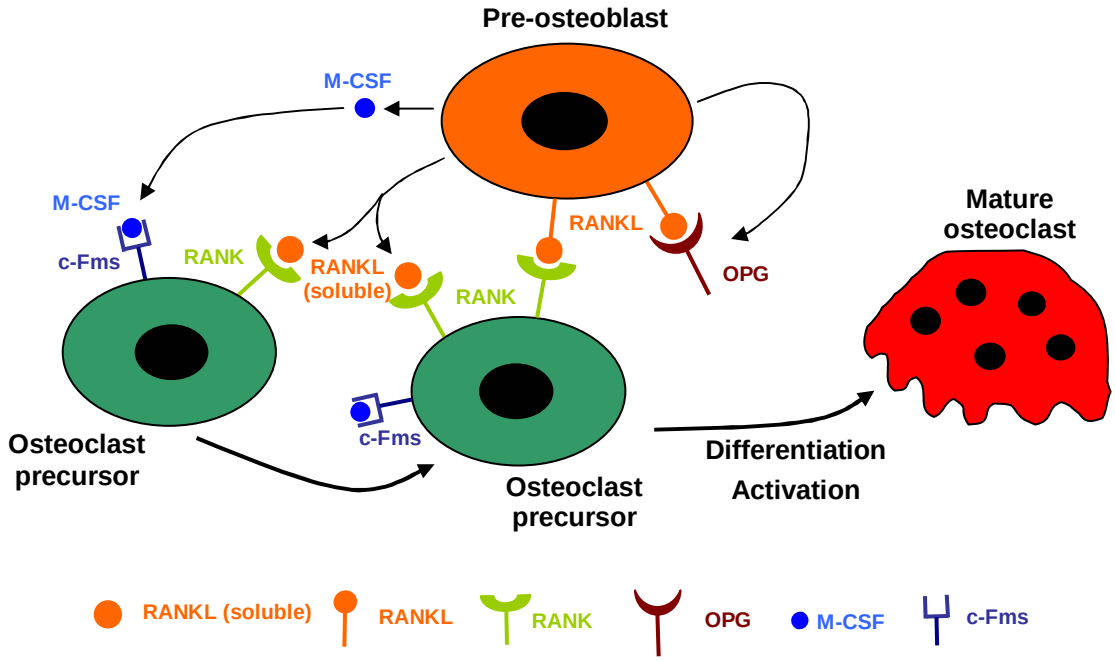

Figure 1.10 – RANKL/RANK/OPG system.

Growth factors such as macrophage colony-stimulating factor (M-CSF) and RANKL (receptor activator of nuclear factor (NF-κB) ligand), and transcription factors such as Pu.1 and RANK (NF-κB), important in hematopoietic cell differentiation, influence myeloid derived osteoclast differentiation (Blair, *et al.*, 2002, Ducy and Karsenty, 1998, Kobayashi and Kronenberg, 2005, Wagner and Karsenty, 2001, Yang and Karsenty, 2002). The system OPG/RANKL/RANK (in which OPG means osteoprotegerin) is the dominant, final mediator of osteoclastogenesis (Figure 1.10). RANKL molecule is

produced by osteoblastic stromal cells, and is able to bind to both the RANK receptor, which is expressed by osteoclast precursors, and to osteoprotegerin (OPG) produced by osteoblasts, which is a soluble receptor. Binding of RANKL to RANK mediates a cell-cell contact between osteblasts and osteoclast precursors that stimulates osteoclast differentiation and bone resorption (Ducy and Karsenty, 1998, Hofbauer, 1999, Khosla, 2001, Wagner and Karsenty, 2001). Bone morphogenetic proteins (BMPs) also influence osteoclastogenesis through their influence on RANKL and OPG (Canalis*, et al.*, 2003).

1.6 Regulation of scale development and regeneration

Relatively little information exists about the mechanisms regulating scale formation, scale regeneration and scale resorption. The few studies on scale development and regeneration that exist point to an epidermal-dermal interaction that mediates the genetic control of morphogenesis and differentiation during scale formation (Quilhac and Sire, 1998, Sire*, et al.*, 1997a, Sire and Arnulf, 2000).

Sonic hedgehog (shh) is a gene which has been implicated in the development of many organisms. Its expression has been studied during scale development in zebrafish and the results suggest that basal epidermal cells use this molecule to regulate scale forming cells (Sire and Akimenko, 2004). The comparison of alizarin red-stained and whole mount *in situ* hybridized specimens of the same size/age reveals that shh expression correlates with the pattern of squamation and that its transcripts are never detected in skin regions devoid of scales. Moreover, longitudinal sections show that the basal epidermal cells, which do not directly face developing scales never express shh. This gene is expressed in a similar pattern in the scales and fin rays, in a subset of cells of the basal epithelial layer adjacent to scale- or bone-forming cells. This particular location suggests that the targets of the short-range signalling molecule shh are the scale-forming cells, responsible for the extension of the posterior margin (Sire and Akimenko, 2004). Sonic hedgehog belongs to the Hedgehog gene family which also includes Indian hedgehog (Ihh), which is involved in the regulation of chondrocytes and osteoblast differentiation, through its interaction with PTHrP

and BMPs respectively. The expression of shh in the scales may indicate that scale-forming cells produce other ECM proteins, besides BMPs and PTHrP, which may participate in the regulation of matrix production and scale mineralization.

1.7 Endocrine regulation of calcium homeostasis

Calcium homeostasis in vertebrates is regulated by systemic hormones that include calciotropic hormones such as parathyroid hormone (PTH), calcitriol or 1,25-dihydroxy vitamin D_3 ($1,25(OH)_2D_3$) and calcitonin (CT) (Figure 1.11) and, in fish, also stanniocalcin, and other hormones such as estrogen and thyroid hormones (Wendelaar Bonga and Pang, 1991). These hormones exert their effects on various target organs involved in calcium metabolism which include bone, kidney, gastrointestinal tract, and also scales, in fish (Compston, 2001, Guise and Mundy, 1998, Khosla, 2001, Withers, 1992). Although it has only been studied in a few teleosts, it is clear that calcium metabolism in aquatic vertebrates is different from that of terrestrial vertebrates, because the availability of calcium varies with the environment. The wide range of calcium regulating mechanisms in fish is probably a consequence of their biological diversity. For example, fish have the ability to live in fresh or salt water containing variable levels of calcium and they may have bony or cartilaginous skeleton. In contrast to tetrapods most fish have ultimobranchial gland (that produce calcitonin) and corpuscles of Stannius (that produce stanniocalcin, a hypocalcemic hormone) both tissues important in calcium regulation (Bentley, 1998, Wendelaar Bonga and Pang, 1991). The relative importance of endocrine factors in calcium homeostasis in fish is not well established.

Parathyroid hormone (PTH) is an 84 amino acid peptide secreted from the parathyroid glands, in tetrapods, in response to a decrease in extracellular calcium concentrations which is sensed by calcium sensing receptors in the glands (Bentley, 1998, Chester-Jones*, et al.*, 1987, Swarthout*, et al.*, 2002).

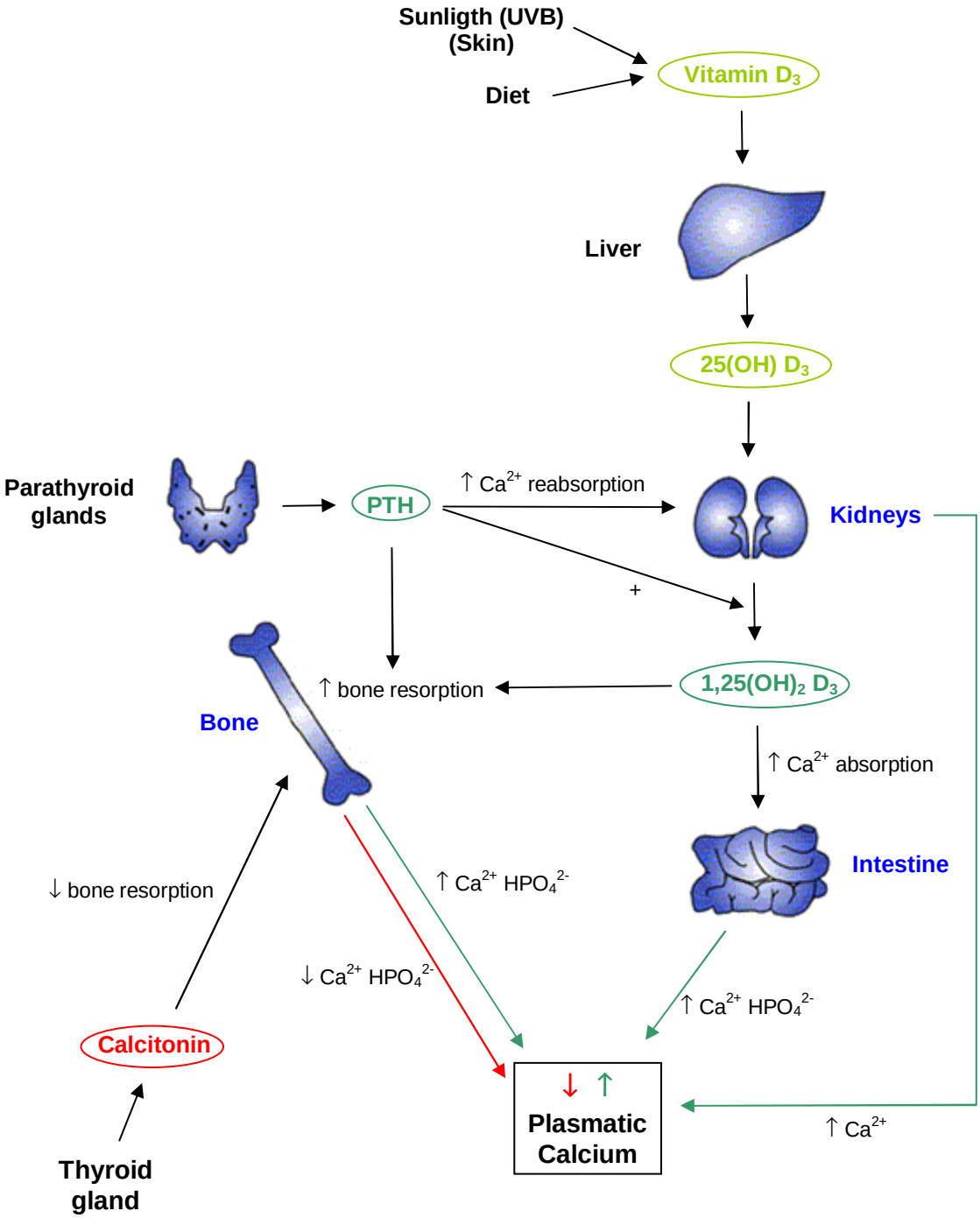

Figure 1.11 – A scheme presenting the interaction between the principle organs and hormones involved in calcium homeostasis in mammals.

The biological action of PTH is well characterized in tetrapods and occurs when it binds to specific receptors (PTHR1 and PTHR2) in bone cells and the kidney (Figure 1.11) (Karaplis and Goltzman, 2000, Philbrick*, et al.*, 1996, Swarthout*, et al.*, 2002). PTH causes a rise in plasma calcium through bone resorption, reabsorption of calcium in the kidney, and stimulation of $1,25(OH)_2D_3$ production. When plasmatic calcium concentrations increase, negative feedback causes a drop in PTH production (Karaplis and Goltzman, 2000). Binding of the 1-34 amino terminal region of PTH to its receptors up- or down-regulates the production of osteoblastic factors and genes (Table 1.4) (Karaplis and Goltzman, 2000, Philbrick*, et al.*, 1996, Swarthout*, et al.*, 2002). PTH can have both anabolic and catabolic effects *in vivo* in bone, the balance of which depends on the physiological status of the animal and on the PTH treatment (continuous or intermittent) (Swarthout*, et al.*, 2002).

Fish do not appear to have parathyroid glands nor to secrete PTH (Bentley, 1998, Chester-Jones*, et al.*, 1987, Ingleton, 2002, Withers, 1992). However, parathyroid hormone-related protein (PTHrP) a paracrine factor expressed in many tissues has been identified (Ingleton, 2002). In mammals, it is expressed in cells of the osteogenic lineage in bone and cartilage. PTHrP, in common with PTH, binds to, and activates PTHR1 receptor (Philbrick*, et al.*, 1996, Swarthout*, et al.*, 2002). PTHrP is particularly important in chondrocyte differentiation and, in PTHrP knock-out mice, chondrocytes do not proliferate normally and bones are shortened (Strewler, 2000) while overexpresion of PTHrP in mice, causes a delay in chondrocyte maturation, apoptosis is suspended and islands of chondrocytes persist within mature bone (Philbrick*, et al.*, 1996, Strewler, 2000). PTHrP has been cloned in *Fugu rubripes* (Power*, et al.*, 2000) and *Sparus auratus* (Flanagan*, et al.*, 2000) and has been shown to be a hypercalcemic factor in larval stages (Guerreiro*, et al.*, 2001). It is not established yet if PTHrP has a role in calcium metabolism in fish bone and scales. Treatment of sea bream scales with (1-34)-PTHrP results in a strong decrease of osteonectin mRNA expression, suggesting that even if PTHrP does not have a direct effect on bone and scale metabolism, it may regulate some key genes involved in matrix production and mineralization (Redruello*, et al.*, 2005).

Table 1.4 – Examples of genes stimulated or inhibited by PTH in mammals.
(adapted from Swarthout*, et al.*, 2002)

Genes
Stimulated
MMP-13 (Collagenase-3)
RANKL
M-CSF
IL-6
IGF-I
Osteocalcin
Inhibited
Type I collagen
Osteopontin
Osteonectin
Alkaline phosphatase

Calcitriol or 1,25-dihydroxy vitamin D_3 ($1,25(OH)_2D_3$) is an active metabolite of vitamin D_3. Its precursor, vitamin D_3 (cholecalciferol), in mammals is obtained from the diet or formed in the skin from 7-dehydroxycholecalciferol, by ultraviolet irradiation. Hydroxylation to 25-hydroxyl vitamin D_3 occurs in the liver and then, in the proximal renal tubules, $1,25(OH)_2D_3$ is formed by 1α-hydroxylation, as a response to decreased concentrations of plasmatic calcium, and by stimulation of PTH (Guise and Mundy, 1998, Jones*, et al.*, 1998). Target organs (Table 1.5) for $1,25(OH)_2D_3$ are the intestine, the kidney and the bone. In bone the effects of $1,25(OH)_2D_3$ are mediated by different types of receptors, the rapid effect is mediated by a membrane bound receptor and the slow effect by the nuclear vitamin D receptor (VDR) (Farach-Carson, 2001, Hofbauer*, et al.*, 1998, Yasuda*, et al.*, 1999). Relatively little is known about the physiology of vitamin D_3 metabolites in fish. Teleost fish store vitamin D_3 in the liver, usually in greater quantity in marine than in freshwater species (Bentley, 1998, Chester-Jones*, et al.*, 1987). It is not clear yet if these fishes contain the same hydroxylation system as mammals (Chester-Jones*, et al.*, 1987). No consensus exists about the effects of $1,25(OH)_2D_3$ in fish although vitamin D_3 appears to act in the gastrointestinal tract, the kidney and the bone (Bentley, 1998).

Table 1.5 – Actions of $1,25(OH)_2D_3$ on its target organs and respective effect on calcium plasmatic concentration (Farach-Carson, 2001, Guise and Mundy, 1998, Hofbauer*, et al.*, 1998, Jones*, et al.*, 1998, Schlaeppi*, et al.*, 1997, Yasuda*, et al.*, 1999).

Organ	Action	Effetc
Intestine	↑ Calcium and phosphate absorption	↑ Calcium plasmatic concentration
Kidney	↑ PTH action (promoting renal calcium reabsorption)	↑ Calcium plasmatic concentration
Bone	***Rapid effect*** - ↑ bone resorption ↑ influx of calcium into osteoblasts ↑ synthesis of RANKL ↓ synthesis of OPG ***Slow effect*** -↑ bone formation ↑ OPG mRNA synthesis ↓ type I collagen synthesis ↑ expression of osteocalcin, osteopontin and VEGF	↑ Calcium plasmatic concentration ↓ Calcium plasmatic concentration

Increased concentration of extracellular calcium in mammals results in an increase in calcitonin production by the parafollicular cells (or C-cells) of the thyroid glands (Figure 1.11), probably mediated by a calcium-sensing receptor. Calcitonin is a 32 amino acid peptide that rapidly inhibits osteoclastic bone resorption through direct binding to calcitonin receptors present in the osteoclasts (Guise and Mundy, 1998, Jones*, et al.*, 1998).

The physiological function of calcitonin, produced by the ultimobranchial gland in fish, is not completely defined. Calcitonin is proposed to be secreted as a consequence of a rise of blood calcium levels (Sasayama, 1999) and appears to have a hypocalcemic effect, in fish with cellular bone and by influencing calcium uptake across the gills. The responses of teleost fish to administrated calcitonin are variable and depend on the dose used, the environmental and physiological conditions of the fish, and the species. It seems, that unlike terrestrial vertebrates, this hormone is not essential for the rapid control of extracellular calcium concentrations, but it may activate osteoblasts and promote bone mineral deposition and also have a role in skeleton protection during periods of high calcium demand (Bentley, 1998, Wendelaar Bonga and Pang, 1991). Evidence for a skeletal protective effect of calcitonin is the

decrease in osteoclastic activity in scales of goldfish and nibbler fish (Suzuki*, et al.*, 2000).

Estrogen in addition to its central role in reproduction is also involved in growth, differentiation and function in many target tissues (Compston, 2001). The specific nuclear actions of estrogens are determined by the structure of the hormone, the isoform of the estrogen receptor involved, the characteristics of the target gene promoter, and the balance of coactivators and corepressors that modulate the final transcriptional response to the complexes of estrogen and estrogen receptors (Gruber*, et al.*, 2002). Osteoblasts, osteoclasts and osteocytes express estrogen receptors, although their concentration is lower than in reproductive tissues. In bone, estrogen inhibits bone resorption predominantly by its effects on osteoclast number and activity. It seems that these effects are mediated by locally produced factors such as TGF-β, IL-1, IL-6, TNFα, M-CSF, GM-CSF and PGE_2 (Bland, 2000, Compston, 2001, Nilsson*, et al.*, 2001, Riggs, 2000, Riggs*, et al.*, 2002).

Estrogen is also involved in calcium metabolism in fish although it seems to have the opposite effect to that in mammals. Estrogen increases osteoclastic activity, and consequently the resorption of scales in rainbow trout (Persson*, et al.*, 1995), 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, Suzuki and Hattori, 2002) although in sea bream it seems to have no effect (Guerreiro*, et al.*, 2002). In the Atlantic salmon during sexual maturation and spawning there is an increase in the osteoclastic activity possibly as a consequence of increased steroid production (Persson*, et al.*, 1998). In goldfish and killifish bone estrogen suppresses calcium deposition without stimulating bone resorption (Mugiya and Watabe, 1977). The action of estrogen in bone and scale resorption seems to be mediated by estrogen receptors (ERs) expressed in these tissues. ER mRNA expression and high-affinity, low capacity binding capacity was detected in rainbow trout bone and scales (Armour*, et al.*, 1997, Persson*, et al.*, 2000). In sea bream, ERs were not detected in bone by RT-PCR (Socorro*, et al.*, 2000). Furthermore, estrogen caused a dose dependent reduction in osteonectin mRNA in goldfish scales, suggesting that

this hormone may act on cells responsible for matrix production and mineralization through the regulation of the expression of extracellular proteins involved in those processes (Lehane*, et al.*, 1999). Melatonin, a hormone produced by the pineal gland, also decreases both osteoclastic and osteoblastic activity in goldfish scales and may suppress the action of estrogen, by reducing ER mRNA expression (Suzuki and Hattori, 2002).

The corpuscles of Stannius are present in most bony fish (Teleostei and Holostei). They are small organs associated with the renal tissue. Two different types of cells (type 1 and type 2) may be present in the corpuscles which have the appearance of peptide-secreting cells and contain secretory granules (Chester-Jones*, et al.*, 1987, Withers, 1992). Type 1 cells are present in marine fish while in fresh water and euryhaline fish both types are present. The type 1 cells are probably the site of synthesis of stanniocalcin, a glycoproteic hormone (formerly called hypocalcin or teleocalcin) that is a relatively fast-acting hormone that lowers plasma calcium concentrations. The removal of the corpuscles of Stannius has a hypercalcemic effect accompanied by reduced calcium excretion and increased osteoclastic activity and calcium mobilization from bone (Wendelaar Bonga and Pang, 1991). Stannioclacin appears to act principally by decreasing the influx of calcium ions across the gills of teleost fish. It has also been shown to promote the absorption of phosphate from the renal proximal tubule (*in vitro*). Such actions could facilitate the deposition of calcium ions into bone contributing to the hormone's hypocalcemic effect (Bentley, 1998, Sasayama, 1999, Wendelaar Bonga and Pang, 1991). Teleostean and holostean fishes have a dual hypocalcemic system as both ultimobranchial glands and corpuscles of Stannius are present. It has been suggested that stanniocalcin is responsible for control of blood calcium levels in marine fishes, but that calcitonin is responsible for fine-tuning (Sasayama, 1999).

Thyroid hormones (THs) have a key role in normal skeletal, development, growth and maintenance of adult bone mass (Bassett and Williams, 2003, Yen, 2001). Thyroid receptors TR-α1, -α2 and -β1 are expressed in resting and proliferative chondrocytes. It has been shown that thyroxine (T_3) regulates the

rate of chondrocytes differentiation by interfering with the Indian hedgehog-PTHrP feedback loop (Bassett and Williams, 2003). TR-α1 and -α2 are also present in osteoblasts and T_3 stimulates osteoblasts activity both directly and indirectly via numerous growth factors and cytokines (Bassett and Williams, 2003). The osteoclastic bone resorptive actions of T_3 seem to be mediated indirectly, by locally produced factors (for review, see Bassett and Williams, 2003).

Besides the local regulation of bone remodelling, achieved by autocrine and paracrine mechanisms, recent genetic studies have shown that there is a central control of bone formation, mediated by a neuroendocrine mechanism, involving leptin. Leptin seems to inhibit bone formation without directly contacting the osteoblast but instead, via hypothalamus where leptin receptor is particularly abundant, but the molecules that directly convey the information to the osteoblasts remain to be identified (Amling*, et al.*, 2000).

1.8 Fish models used in the present work

In the present work two fish models were used to study the ontogeny and expression of extracellular proteins. Considering the wide variability of teleost species and their capacity to adapt to different environments, a marine teleost (gilthead sea bream, *Sparus auratus*) and a fresh water teleost (tilapia, *Oreochromis mossambicus*) were chosen.

1.8.1 Gilthead sea bream

The gilthead sea bream (*Sparus auratus*) is a perciform fish which belongs to the Sparidae family and can grow up to 70cm in length and 2-3kg in weight (Figure 1.12). It is a carnivorous demersal fish that is common in the Mediterranean Sea, is present along the Eastern Atlantic coast from Great Britain to Senegal, and is rare in the Black Sea. It is euryhaline and eurythermal and tolerates both marine and brackishwater environments such as coastal lagoons and estuarine areas, particularly during the initial stages of its life cycle. It is a very important commercial species for South-Atlantic and Mediterranean

fisheries and aquaculture (Morales, 1983; www.fishbase.org; www.fao.org) and due to its economic value numerous biological studies have been conducted related with its growth and metabolism, energy homeostasis and immune function (Pérez-Sánchez, 2000, Pérez-Sánchez*, et al.*, 2002).

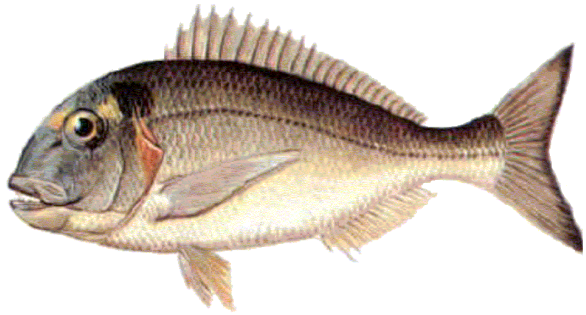

Figure 1.12 – Scheme of gilthead sea bream (*Sparus auratus*).

Sea bream is a protandrous hermaphrodite with a breeding season ranging from October to February in Southern Portugal (Condeça, 2001). Its gonad morphology and steroidogenesis are well described (Condeça, 2001, Condeça and Canário, 1999, 2001). All individuals mature sexually as males at year one, when they are approximately 350g, but in subsequent years animals undergo sex reversal to female. Born in the sea during wintertime, the juveniles typically migrate in early spring towards protected coastal waters in search for abundant food and milder temperatures (trophic migration). In late autumn they return to the open sea, where the adult fish breed (Morales, 1983; www.fishbase.org; www.fao.org). Calcium homeostasis has been relatively poorly studied, although skeletal ontogeny is well documented (Faustino and Power, 1998, 1999, 2001). There is no information available about skeletal tissue organization or composition or about the factors regulating skeletogenesis.

1.8.2 Tilapia

Mozambique or Java Tilapia (*Oreochromis mossambicus*, also known as *Tilapia mossambica*) is a cichlid fish (Order Perciformes; Figure 1.13) that is native to Africa and the Middle East and has been reared in Africa, Europe and throughout the Pacific. It is an omnivorous species, grows in fresh water but has a very wide salinity tolerance and is sexually dimorphic. Tilapia are a good

model for the study of physiological responses to changes in salinity because the modification of ion and water regulatory mechanisms can be monitored as the fish are acclimated to either increasingly dilute or concentrated conditions. In Mozambique tilapia, the transition between hypo- and hyperregulation permits the study of rate, retention, and elimination adjustments in response to changes in salinity (Bijvelds*, et al.*, 1997, Lin*, et al.*, 2001, Vonck*, et al.*, 1998).

Tilapia can grow at temperatures between 17 and 35ºC and growth stops at temperatures below 12ºC. Temperatures below 9ºC are lethal for this species. At temperatures above 22ºC (27-28ºC is the optimal temperature) spawning continues throughout the year. In the reproductive season, the males dig a nest at the bottom of the pond where the female lays the eggs. Then, female mouth breed eggs which hatch within 3 to 5 days after fertilization. Larvae stay inside the progenitors' mouth until the yolk sac is absorbed, which can take 9-10 days, when they reach the juvenile stage. Juvenile tilapia mature sexually 2-3 months after fertilization when they are 6-10cm in length.

Figure 1.13 – Image of a Mozambique tilapia (*Oreochromis mossambicus*).

Environmental factors such as water temperature, gender, food supply and population density are very important in determining fish size (Bardach*, et al.*, 1972, Morales, 1983). For example, in normal growth conditions in aquaculture, a male can reach about 700g in 8-9 months (Popma and Masser, 1999). Skeletal development and the influence of temperature on growth are well characterized in *Oreochromis mossambicus* (Campinho*, et al.*, 2004) but the cellular organization of skeletal tissue and the mechanisms which regulate skeletogenesis are still unknown for this species.