Chapter 7

General discussion

In common with mammals, the mineralized tissue (bone, teeth and scales) in fish contain around 99.5% of total body calcium (Wendelaar Bonga and Pang, 1991). The organization of the skeleton has been the focus of numerous studies as a consequence of its importance in taxonomy and systematics. In recent years partly as a consequence of increased fish aquaculture the skeletal ontogeny of a number of different teleosts has been studied and includes sea bream, (*Sparus auratus*, Faustino and Power, 1998, 1999, 2001) and tilapia (*Oreochromis mossambicus*, Campinho*, et al.*, 2004) the subjects of the present study. However, surprisingly little information is available about skeletal tissue structure and cellular organization in fish and even less is known about the involvement of this tissue in calcium homeostasis. In fact, the mechanisms underlying calcium homeostasis in fish are still not clearly established and the endocrine factors which participate in this process and their mode of action remains to be defined.

One of the objectives of the present thesis was to establish the tissue and cellular organization of cartilage and bone in teleost fish and elucidate if they have a role in calcium balance. The morphology of cartilage and bone was observed during skeletogenesis in sea bream larvae and results showed that in this species bone development occurred by two main mechanisms, endochondral and dermal ossification, although alternative ossification processes were observed in some skeletal elements, such as the gill arches (Chapter 3). The main steps of endochondral and dermal bone development in sea bream are similar to those described in mammals (Kardong, 1998, Marks and Odgren, 2002, Yang and Karsenty, 2002) although the bone formed has a conspicuously different appearance when compared to both cancellous and compact bone in mammals (Dorit*, et al.*, 1991, Kardong, 1998). Mammalian bone is organized in highly ordered units, the osteons, which consist of a series of concentric rings of bone cells and layers of bone matrix (lamellae) which are disposed around a central canal which provides a direct vascular and nerve supply to the tissue (Kardong, 1998, Loveridge, 1999, Sommerfeldt and Rubin, 2001). This organization allows bone to adapt its mass and morphology to subtle changes in mechanical and metabolic demands through modelling/remodelling processes (Loveridge, 1999, Manolagas and Jilka, 1995,

Sommerfeldt and Rubin, 2001). The core region of most mammalian bone contains bone marrow which gives rise to blood cell precursors and also to some bone cell precursors. Three main cell types have been identified in mammalian bone; osteoblasts which are responsible for bone formation and mineralization; osteocytes which are formed when osteoblasts became entrapped in the matrix they secrete and osteoclasts which are responsible for bone resorption. In mammals all these cells are identifiable by their characteristic appearance, localization, affinity for some histological stains and by the presence of specific matrix proteins. For example, the osteoblasts may be present in the osseous structures as rounded active cells, or as inactive flattened cells, associated with bone surfaces, also called lining cells (Manolagas and Jilka, 1995, Marks and Odgren, 2002, Sommerfeldt and Rubin, 2001). In sea bream, the structural organization of bone is distinct from that in mammals. No osteon-like structures were observed and bones seemed to be formed by successive thin layers of mineralized matrix. No blood vessels or nerves were identified in the observed skeletal elements. This different tissue organization may be a consequence of a specific adaptation of fish to an environment where calcium demands and weight-bearing are very different than those experienced by terrestrial vertebrates. In general, the bony tissue observed in sea bream is acellular and no cells are present in the mineralized matrix. However, all osseous structures are covered by a well defined cellular periosteum and it seems likely that this tissue may have an important role in bone turnover. In sea bream, osteoblast-like rounded cells were observed in mineralizing structures and the cellular periosteum covering bones appears to be composed of inactive osteoblasts or osteoblast precursors, in common with what has been described in mammalian bone. The identity of these cells needs to be confirmed and to this end a number of specific osteoblasts markers identified in mammals, such as type I collagen, core binding factor 1 (cbfa 1) and osterix (osx) (Blair*, et al.*, 2002, Ducy and Karsenty, 1998, Nakashima and de Crombrugghe, 2003, Wagner and Karsenty, 2001) have been isolated in the sea bream but still remain to be studied. The osteocytes in terrestrial vertebrates are proposed to be important in sensing the mechanical strains to which bone is subject, and in modulating bone turnover. Fish bone is exposed to mechanical strain associated with locomotion but unlike mammals, fish live in

a buoyant aquatic environment where they are essentially weight free and this may explain the rare occurrence of osteocytes in fish bone (Hughes*, et al.*, 1994, Meunier and Huysseune, 1992, Moss, 1963). More studies are necessary to establish in what conditions fish bone turnover occur, the cell types involved and how the process is mediated. In mammals bone resorbing osteoclasts are derived from haematopoietic precursors which move from the bone marrow to bone where they differentiate into active cells and contribute to bone turnover and calcium mobilization. Osteoclast resorbing activity is clearly associated with tartrate resistant acid phosphatase (TRACP). TRACP activity and expression have been used as a marker for osteoclastic activity both in mammals and is fish (Oddie*, et al.*, 2000, Persson*, et al.*, 1995, Persson*, et al.*, 1999, Suzuki*, et al.*, 2000, Witten, 1997, Witten*, et al.*, 2001). In mammals, bone remodelling is important in maintaining bone matrix composition, in calcium homeostasis and in removing microdamaged areas of bone (Blair, 1998, Sommerfeldt and Rubin, 2001, Vaananen*, et al.*, 1998). In fish bone and scales, although osteoclasts appear to be present, their function and mode of action are not clear yet. The origin of osteoclasts in fish, which lack bone marrow, also remains to be established and it will be of interest to determine if the head kidney in fish which is the principal focus of haemopoietic tissue gives rise to osteoclast precursors and how these cells migrate to bone.

In the gill arches of juvenile sea bream skeletal tissue with a different organization from typical cartilage and bone in other regions was observed. In the gill arch a cartilage-like tissue with an extracellular matrix composition that seems to be intermediate between cartilage and bone is observed. The initial stages of development of this tissue are similar to what is observed in endochondral bone, however, it does not undergo the final transition to bone. Other highly mineralized tissue, with chondrocyte-like cells spread within its matrix, resembling chondroid bone (Meunier and Huysseune, 1992), is also present in the gills (chapter 3). More studies will be necessary to clearly establish the origin and role of these distinct skeletal tissues and their osteological development.

Bone extracellular matrix (ECM) is produced by osteoblasts and is composed of

numerous proteins important for skeletal development. Changes in ECM composition occur during the transition of cartilage to bone, during bone endochondral development. It has been demonstrated in mammals that certain proteins present in the bone matrix have specific functions which are related to the regulation of bone matrix mineralization. Moreover, some ECM proteins have been used in mammals as molecular markers of specific cell types. The composition of the ECM in fish skeletal tissues has not been described. The characterization of the expression pattern of ECM proteins in fish during skeletogenesis and in adult tissues will be an important step in the identification of the origin of the diverse cell types present in the fish skeleton and also for the elucidation of their role in bone development and turnover.

Osteonectin (OSN) is one of the major non-collagenous proteins present in mammalian bone matrix. It is a regulatory glycoprotein that has been implicated in bone formation and mineralization in mammals (Sommer*, et al.*, 1996, Young*, et al.*, 1992, Zhu*, et al.*, 2001). Osteonectin expression was characterized during skeletal ontogeny in sea bream larvae (Chapter 5) and in adult sea bream tissues (Chapter 4) by RT-PCR and *in situ* hybridization (ISH). During sea bream development, the expression pattern of OSN mRNA varied substantially throughout embryogenesis and larval development suggesting, that in common with observations in mammals, it may be involved in a multitude of processes such as angiogenesis, cell migration and cell shape change, synthesis of basement membrane components and wound healing (Brekken and Sage, 2000, Chen*, et al.*, 1991, Cowles*, et al.*, 1998, Holland*, et al.*, 1987, Nomura*, et al.*, 1988, Porter*, et al.*, 1995, Sage*, et al.*, 1989) in addition to skeletal development in teleost fish. In sea bream larvae, OSN mRNA expression was observed by ISH in both dermal and endochondral skeletal structures and both during chondrogenesis and osteogenesis which suggests that OSN may have a role in bone formation and mineralization similar to what is described in mammalian embryos (Chen*, et al.*, 1991, Holland*, et al.*, 1987, Mothe and Brown, 2001, Nakase*, et al.*, 1994). In sea bream adult tissues, osteonectin is most abundant in calcified tissues, although it is also occasionally detected in low abundance in soft tissues. The potential role of OSN in sea bream soft tissues has not been considered in the present study. The

expression of OSN in fully developed calcified tissues suggest that this protein has a role in tissue mineralization and turnover, similar to that described in mammals (Delany*, et al.*, 2000, Delany*, et al.*, 2003). Clearly characterization and elucidation of the role of other ECM proteins during both sea bream development and in adult tissue will be essential in order to better understand the involvement of ECM proteins in skeletal formation, turnover and calcium balance in fish.

Fish scales are skeletal elements, of dermal origin, that provide protection and due to their high calcium content may be involved in calcium homeostasis. In the present work, sea bream and tilapia scale morphology of juvenile and adult animals was described (Chapter 3). Scales of both species are of the elasmoid type, and are similar to those described in other teleosts (Bereiter-Hahn and Zylberberg, 1993, Fouda, 1979, Onozato and Watabe, 1979, Sire and Arnulf, 1990, Sire*, et al.*, 1997, Sire and Arnulf, 2000, Sire and Akimenko, 2004). The study of ECM proteins including type I collagen, α1 (Col1A1), type V collagen, α2 (Col5A2), fibronectin (FN), osteonectin (OSN), tartrate-resistant acid phosphatase (TRACP) and acidic secreted protein in cartilage (ASPIC), permitted the establishment of their expression pattern in scales of juvenile and adult animals and helped in the identification of a number of different cell types (Chapter 6). Type I collagen and osteonectin expression have previously been associated with osteoblasts in mammals and fish (Mackie, 2003, Marks and Odgren, 2002, Sommerfeldt and Rubin, 2001), TRACP is used as a marker of osteoclast activity (Oddie*, et al.*, 2000, Persson*, et al.*, 1995, Persson*, et al.*, 1999, Witten, 1997, Witten*, et al.*, 2001) and ASPIC is used as a markers of chondrocytes in humans (Steck*, et al.*, 2001). The results obtained in the present study allowed the identification of putative osteoblasts and osteoclasts in sea bream and tilapia scales and showed that signal intensity and location varies with species and with the probe studied. No great differences in gene expression exist between juvenile and adult scales of the same species.

Type I and type V collagens are structural components of bony tissues in mammals (Aumailley and Gayraud, 1998, Gelse*, et al.*, 2003, Mecham, 1998, van der Rest and Garrone, 1991) which were detected in teleost juvenile and

adult scales. This observation supports the idea that scale matrix has a similar composition to that of mammalian bone. The identification of osteoblasts and osteoclasts in the scales, the scale tissue organization and calcium content, and the fact that scales can be easily removed from fish and cultured make the scales a good model to characterize skeletal ECM composition, the endocrine factors that regulate bone formation and turnover, and the involvement of skeletal tissue in calcium homeostasis in fish. The regulation of skeletal development involves the regulation of cell differentiation, bone formation and bone remodelling and is a complex process in which both endocrine and local factors participate (Compston, 2001, Guise and Mundy, 1998, Khosla, 2001). Similarly, it is expected that the regulation of scale development involves the regulation of scale cell differentiation and scale turnover. Regulatory proteins, such as fibronectin and osteonectin are also expressed in sea bream scales. The effect of endocrine factors that may be involved in the regulation of bone formation and calcium homeostasis can be evaluated for example, by studying how these factors affect the expression pattern of these regulatory proteins involved in skeletogenesis.

Parathyroid hormone-related protein (PTHrP) is expressed in mammalian cells of bone and cartilage and is important in the regulation of chondrocyte differentiation (Philbrick*, et al.*, 1996, Strewler, 2000, Swarthout*, et al.*, 2002). Although it has been cloned in teleost species (Flanagan*, et al.*, 2000, Power*, et al.*, 2000) it has not yet been established if PTHrP participates in calcium turnover in fish bone and scales. Using an *in vitro* scale bioassay it was shown that PTHrP, both at physiological and pharmacological concentrations, significantly decreased OSN mRNA expression in sea bream scales, suggesting that one way in which PTHrP may regulate scale mineralization is through the regulation of OSN expression (Chapter 4). The effect of other endocrine factors on OSN expression should also be studied using this bioassay. This methodology could also be used to determine the effect of endocrine factors on the expression of other regulatory and structural ECM proteins identified in the present thesis, not only in adult scales but also in scales at different ontogenetic stages. To establish the mode of action of PTHrP the expression and cellular localization of receptors for this protein should also

be characterized by *in situ* hybridization and/or immunohistochemistry.

Estrogen has been suggested in rainbow trout, goldfish, killifish and nibbler fish to increase osteoclastic activity as indicated by an increase in measurable TRACP activity (Armour*, et al.*, 1997, Mugiya and Watabe, 1977, Persson*, et al.*, 1995, Persson*, et al.*, 1997, Suzuki*, et al.*, 2000, Suzuki and Hattori, 2002) and to decrease OSN mRNA expression in rainbow trout (Lehane*, et al.*, 1999) bone and scales. The results may indicate that estrogen may participate in the regulation of matrix production and mineralization during reproduction when the demand for calcium is high. Moreover, the actions of estrogen appear to be directly via estrogen receptors expressed in bone and scale cells (Armour*, et al.*, 1997, Persson*, et al.*, 2000, Suzuki and Hattori, 2002). Another objective of the present work was to characterize the expression of the estrogen receptors (α, β1 and β2) in sea bream and tilapia scales by immunohistochemistry. It was shown that the estrogen receptors are expressed in scales of juvenile and adult sea bream and tilapia although expression patterns vary with species and age. Results also suggest that estrogen receptors are expressed in the putative osteoclasts identified in the scales (Chapter 6). These results support the idea that estrogen may be involved in fish scale formation and/or mineralization and in calcium homeostasis. To determine the possible function of each receptor type in scale turnover further studies must be carried out.

In conclusion, the results obtained in the present thesis indicate that skeletal tissue organization in fish differs significantly from what is observed in mammals and it seems probable that the difference may be an evolutionary adaptation to the specific aquatic environment they inhabit. A number of questions have been raised by the results reported in the present thesis which remain to be answered. The results obtained suggest that calcium present in bone and/or scales may be mobilized when calcium demand increases despite the fact that fish are surrounded by a calcium rich environment. The physiological basis of this process remains to be explored but it seems likely from the results of the present study that calcium mobilization may be mediated by extracellular matrix proteins and occurs under endocrine regulation. However, more studies must be carried out to determine which ECM proteins are involved in each step of

bone development and how local and endocrine factors regulate their expression, promoting or inhibiting calcium deposition in the bone/scales matrix. In this context it will also be important to establish the origin of bone cell precursors and the reason why osteocytes are apparently absent from fish bone matrix. The use of specific markers to identify the different bone cell types will be very useful to clearly identify the cells present during skeletogenesis. Another avenue of research is to establish which factors participate in bone cell differentiation and/or apoptosis regulation and the consequences of these processes on bone turnover.