Chapter 5

Ontogeny of osteonectin expression in embryos and larvae of sea bream (*Sparus auratus*)

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5.1 Introduction

Osteonectin (OSN) is an acidic, cysteine-rich, calcium binding glycoprotein first isolated from fetal calf bone matrix (Termine*, et al.*, 1981a) and present in the extracellular matrix of tissues that have a high turnover rate, such as bone (Yan and Sage, 1999). It belongs to a group of matricellular proteins together with thrombospondin 1 and 2, tenascins C and X and osteopontin, which are regulatory macromolecules mediating cell-matrix interactions (Bornstein, 1995) through their capacity to bind to several different ligands (Bradshaw and Sage, 2001, Yan and Sage, 1999). In adult mammals OSN is important in homeostasis of bone, angiogenesis, synthesis and assembly of basement membrane components through the regulation of free Ca^{2+} levels, steroid production, cell migration and cell shape change, tumorigenesis 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). A number of different factors, such as parathyroid hormone (PTH, (Nakajima*, et al.*, 2002) and dexamethasone (Sawhney, 2002) have been shown to regulate OSN and indicate it may be an important intermediate in hormone action. Studies of OSN in mammals have shown that in addition to the functions identified in adults it is also important in a range of key processes during embryonic development. For example, it has an enhanced expression in areas undergoing chondrogenesis, osteogenesis, somitogenesis and angiogenesis (Cowles*, et al.*, 1998, Holland*, et al.*, 1987, Nakase*, et al.*, 1994, Nomura*, et al.*, 1988, Sage*, et al.*, 1989, Sasano*, et al.*, 2000). OSN-null animal models have altered phenotypes presenting osteopenia, cataractogenesis, accelerated dermal wound healing, aberrant dermal collagen fibrils, curly tails and increased fat deposition, underlining the necessity and importance of this protein to normal physiology (Bradshaw and Sage, 2001, Delany*, et al.*, 2000, Yan and Sage, 1999).

In fishes, OSN has recently been isolated from rainbow trout (*Oncorhynchus mykiss*, Tang and McKeown, 1997), goldfish (*Carassius auratus*, Lehane*, et al.*, 1999) and gilthead sea bream (*Sparus auratus*, Redruello*, et al.*, 2005). The tissue distribution of sea bream OSN (sbOSN) has been determined in adult

fish where it is strongly expressed in skeletal structures (Redruello*, et al.*, 2005), suggesting it may have similar functions to those reported in mammalian skeletal tissue. Moreover, using a sea bream fish scale bioassay PTHrP and estrogen treatment were shown to regulate sbOSN expression, suggesting that endocrine factors in teleosts may also directly modify skeletal tissue.

In the present study, as a first step to understanding the role of the endocrine system in fish skeletal development, the ontogenic expression of sbOSN was investigated by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) in sea bream from fertilization to 85 days post hatch (dph). The localization and ontogeny of sbOSN was established by *in situ* hybridization and related to the stage of skeletal development and the status of the main endocrine systems in sea bream embryos and larvae.

5.2 Materials and Methods

5.2.1 Sampling, tissues fixation and preservation

Sea bream eggs were collected every 3 h from fertilization until gastrulation (20 hours post fertilization, hpf), at 6 h intervals until 16 somites (26 until 32hpf) and at 2 h intervals until hatching (45hpf). Larvae were collected at daily intervals from hatching until 10dph, every two days until 20dph and thereafter every five days until 85dph. Samples were frozen in liquid nitrogen and stored at -80 ºC until analysis.

Samples of larvae for histology and *in situ* hybridization were fixed in fresh 4% paraformaldehyde overnight, at 4ºC (section 2.2). Larvae older than 35dph were decalcified in 0.5M EDTA pH 8.0 (appendix I), for 7 to 21 days and washed with DEPC water (appendix I). Samples were then embedded in paraffin and serial sagittal or transverse sections (5µm thick) were cut and mounted on APES (3-aminopropyltriethoxysilane) treated slides (section 2.2).

5.2.2 RNA extraction and semi-quantitative RT-PCR

Total RNA was extracted from pools of sea bream embryos and larvae using

TRI Reagent (section 2.6). The final concentration of total RNA extracted was measured using a GeneQuant Spectrophotometer (Amersham Biosciences) and the quality of extracted RNA was assessed by agarose gel electrophoresis (1.5%, appendix II).

Amplification of sbOSN by semi-quantitative RT-PCR was carried out with cDNA synthesized from 3µg total RNA obtained from four independent pools of eggs and larvae (50-100mg per pool) from morula to 85dph, using the M-MuLV reverse transcriptase procedure (Gibco BRL, section 2.6). RT-PCR was carried out using 40ng cDNA as template and sbOSN specific primers (sbOSNfw1: 5'-GGTCATCGTGGAAGAGCC-3'; sbOSNrv1: 5'-GCAGGAGGTGTCGTAGGT-3') in a 50µl reaction (section 2.6). The thermocycling protocol was as follows: an initial denaturing cycle for 2 minutes at 95 ºC, followed by 22 cycles of 30 seconds at 95 ºC, 30 seconds at 55 ºC and 45 seconds at 72 ºC, and a final cycle of 5 minutes at 72 ºC. To evaluate the relative levels of expression of sbOSN in different tissues, amplification of 18S ribosomal RNA (rRNA) was carried out to control for variations in the quantity of cDNA used in the PCR. Reaction conditions for 18S rRNA amplification were identical to those for sbOSN with the exception of the primers (18Sfw: 5'-TCAAGAA CGAAAGTCGGAGG-3', 18Srv: 5'-GGACATCTAAGGGCATCACA-3') and the reduced number of cycles to ensure reactions terminated in the exponential amplification phase. The reaction products of sbOSN and 18S rRNA amplified by PCR were separated on agarose (1.25%) gels containing ethidium bromide (appendix II). Signal intensities for PCR products were quantified by densitometric analysis using ImageMaster 1D prime software (v. 2.01, Pharmacia) and the ratio of sbOSN/18S rRNA in each sample was calculated and expressed as arbitrary units.

5.2.3 Histology

Larval sections were stained with haematoxylin and eosin (section 2.3.1) using standard procedures (Stevens, 1990) and tissue morphology and integrity were evaluated. Sections sequential to those used for *in situ* hybridization were also stained with Masson's trichrome staining as described in section 2.3.3. Whole-

mount staining of bone and cartilage was carried out on whole larvae not subject to decalcification using the whole mount cartilage-bone double staining described in section 2.3.5.

5.2.4 Tissue distribution by *in situ* hybridization (ISH)

The full-length sbOSN cDNA (1073 bp) in pBlueScript SK(+) vector was digested with 10U of *Bgl*II endonuclease (Promega) to linearize the DNA and generate a probe (approx. 400 bp) corresponding to the 3' region of sbOSN. The linearized vector was purified and *in vitro* transcription carried out following the protocol described in section 2.4.1. The riboprobe was purified and resuspended in 25µl of RNase and DNase free water (Sigma-Aldrich).

Sagittal and transverse sections of larvae were pre-hybridized at 58ºC for 2 h in hybridization solution and then hybridized overnight at 58ºC in hybridization solution containing approximately 2µl.ml^{-1} of riboprobe. *In situ* hybridization procedure was carried out according to the procedure described in section 2.4.2. Control experiments were performed by treating samples with RNase prior to hybridization with the riboprobe and/or by omitting riboprobe from the reaction. Sections were analysed using a microscope (Olympus BH2) coupled to a digital camera Olympus DP11. Whole-mount *in situ* hybridization was also carried out omitting decalcification with larvae up to 35dph or with the fins of older larvae. Whole-mount samples were treated with proteinase K (10µg.ml^{-1}) for 15 minutes at room temperature, washed with PTW (appendix I) and re-fixed in 4% paraformaldehyde for 15 minutes prior to *in situ* hybridization. The procedure for whole mount *in situ* hybridization was similar to that for sections (section 2.4.2) with the exception that pre hybridization was carried out for 4 hours. Pre hybridization and hybridization were carried out at 58ºC. Stringency washes after hybridization were carried out at 58ºC, twice with 2×SSC, followed by one wash with 1×SSC and 0.2×SSC and washing times were increased to 10 minutes. Hybridized probe was detected using NBT and BCIP as chromagens following the protocol described in section 2.4.2. Stained samples were stored in 100% glycerol.

5.2.5 Statistical analysis

In RT-PCR experiments data are expressed as the mean ± SEM (n = 3 independent samples). Differences in sbOSN expression during development were determined by one-way analysis of variance (ANOVA) followed by the Bonferroni multi-comparison test. The control for between group comparisons was H14 for embryological samples when sbOSN was first detected and D1 (first day in larval series) for larval samples. Statistical significance was considered at $p < 0.05$.

5.3 Results

sbOSN mRNA was first detected by RT-PCR in embryos at 14hpf (early gastrula) and increased steeply ($p<0.001$) between 20 and 26hpf (equivalent to 4 somite stage; Figure 5.1 A). Although with an upward tendency sbOSN expression was relatively stable until 38hpf and then showed statistically significant upward and downward changes culminating with a maximum just before hatch (45hpf). sbOSN content (Figure 5.1 B) decreased continuously from 1 until 15dph when it reached a minimum followed by a moderate statistically significant increase ($p<0.001$) at day 24, coinciding with the start of ossification, before continuing its downward trend until 85dph.

The sbOSN mRNA expression was determined by *in situ* hybridization in larvae 1dph onwards and its localization and intensity was shown to vary with age (Figure 5.2). sbOSN was first detected in larvae 6dph in the developing dorsal skin and appeared to be low abundance as a weak signal was obtained. At 10dph, sbOSN was detectable, but not intense, in the cartilaginous neurocranium, jaws and branchial arches and in the caudal fin (Figure 5.2). A stronger signal was observed in putative skeletal structures in the dorsal and anal fins although it was still not possible to detect these structures by whole mount bone-cartilage staining (Figure 5.2). At 15dph, sbOSN transcripts continued to be expressed in the same structures as at 10dph, but it was also detectable in the pectoral fin and vertebral centra and arches. It was also expressed in high abundance in the pelvic fin and the perichordal sheath. From

20dph onwards, intense sbOSN expression was detected in osseous structures of both endochondral and dermal origin, such as vertebral arches and the actinosts of the pectoral fins, and vertebral centra, respectively (Figure 5.2, Faustino and Power, 1998, 1999). At this stage, the intense sbOSN signal detected in cartilaginous structures of the viscerocranial skeleton (neurocranium, jaws and branchial arches) preceded the start of ossification which occurred at 25dph.

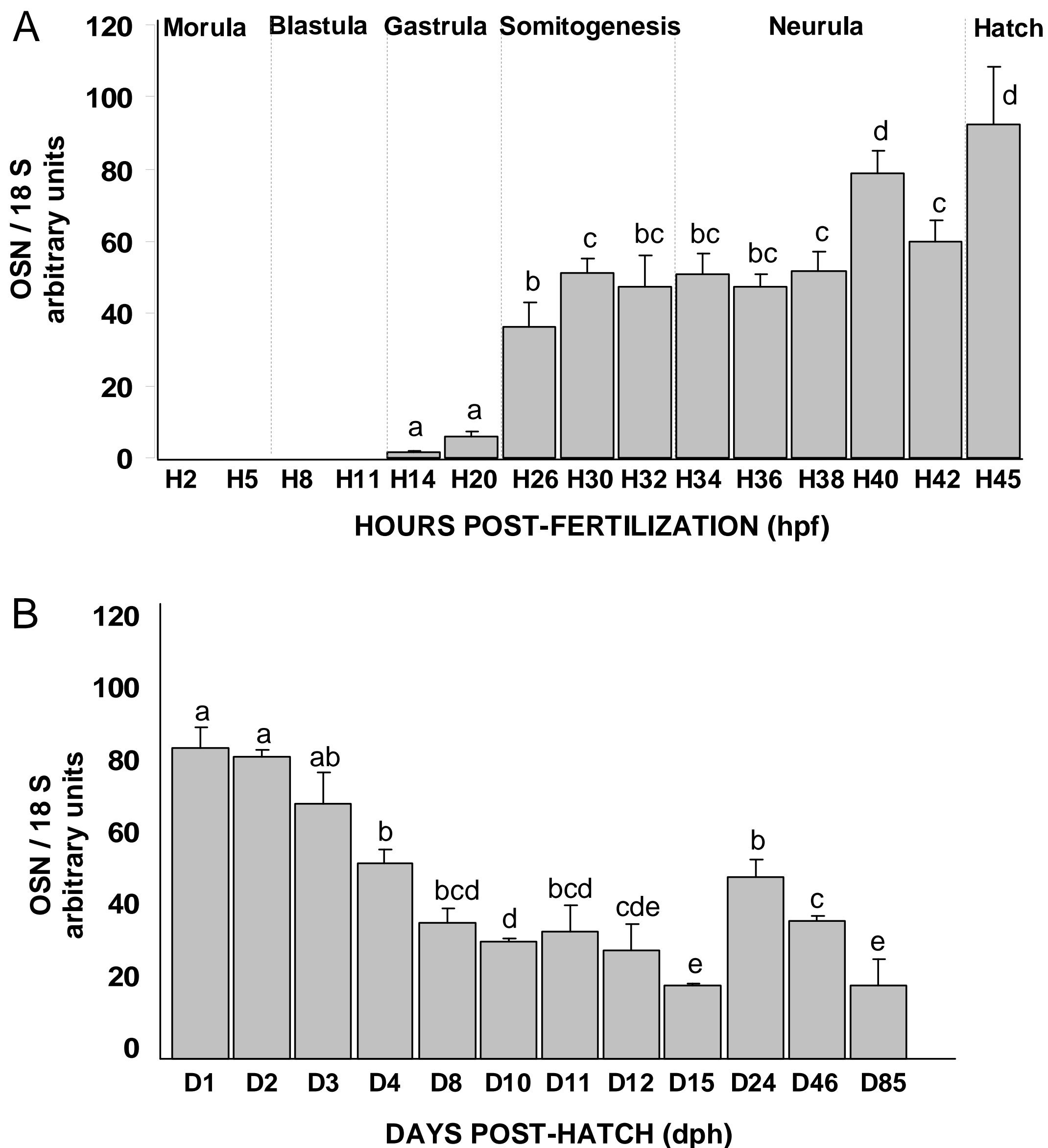

Figure 5.1 - RT-PCR analysis of osteonectin expression during embryo (A) and larval (B) development of sea bream. The bar charts show the ratio between amplified sbOSN and 18S rRNA; The numbers in H2–H45, indicate hours post fertilization and in D1-D85 days post hatch, respectively. Results are the mean ± SEM of three independent samples and are expressed as arbitrary units. Different letters indicate significant differences among groups.

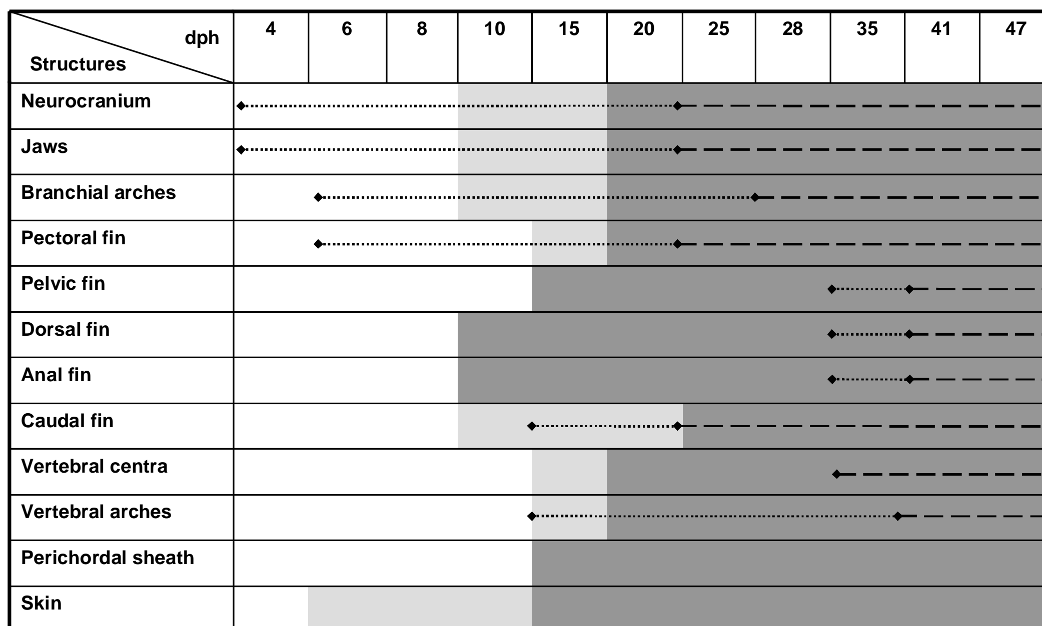

Figure 5.2 - Schematic representation of the onset and relative intensity of sbOSN expression during sea bream larval development determined using ISH. Increased shading intensity of the bars indicates increased sbOSN levels of expression. (…..) Period of cartilage development in the structures studied; (-----) period of ossification, as detected by whole-mount bone-cartilage staining. The perichordal sheath and skin are not part of the skeleton and therefore no representation of cartilaginous or bony structures are given.

A similar profile was observed in the development of the pectoral fin where a low intensity sbOSN expression was detected at 15dph when most elements were still cartilaginous. At 20dph an increase in sbOSN expression was observed just before the start of ossification which occured at 25dph (Figure 5.2). In sea bream larvae at 46dph, sbOSN expression was still high but expression was mainly confined to cartilaginous distal radials associated with the soft rays of the fins (Figure 5.4 D).

Osteonectin expression was detectable in the caudal fin throughout its development in sea bream larvae. In general lower levels of sbOSN expression were associated with the formation of cartilaginous structures such as the hypurals and parahypural (Figure 5.4 B). Higher levels of sbOSN expression were associated with ossification of the caudal fin complex which began at 25dph (Figure 5.2, Faustino and Power, 1998). At 46dph sbOSN expression was detected at the junction of ray segments and an intense signal was found

at the cartilaginous junction between the rays and the hypurals (not shown).

Starting at 10dph an intense sbOSN signal was detected in the dorsal and ventral regions of the finfold where subsequently the pelvic, dorsal and anal fins developed. According to a previous study (Faustino and Power, 1999), the first cartilaginous structures were expected to appear in these fins of sea bream larvae around 20dph (approximately 6.5-7.0mm standard length, Ls) but in the current study these structures were only identified at 35dph (approximately 8.1mm Ls, Figure 5.2). The sbOSN mRNA expression remained intense when ossification of these structures began at approximately 41dph (corresponding to approximately 10.5mm Ls, Figure 5.2, Faustino and Power, 1999).

At 47dph, sbOSN expression was detected in cartilaginous structures associated with the dorsal fin (Figure 5.3 B) and with the jaw (Figure 5.3 D). In both cases, sbOSN was expressed in large cells with an irregular outline, the presumptive chondrocytes. In addition to its presence in skeletal structures, sbOSN mRNA was also detected but in lower abundance in the nerve cord, skin, perichordal sheath and in kidney tubules (Figures 5.3 F and 5.5).

In the vertebra, sbOSN transcripts were detected for the first time at 15dph (Figure 5.3). The level of sbOSN expression increased at 20dph, well in advance of the period when ossification first started at 35dph and 41dph for vertebral centra and arches, respectively (Figure 5.2).

5.4 Discussion

The endocrine regulation of bone metabolism in fish is virtually unstudied and advances are hampered by the lack of information about the basic cellular and molecular organization of bone in teleosts. The present study focuses on the relative abundance and the tissue distribution of sbOSN transcripts in relation to skeletal formation in larvae of sea bream. The levels of sbOSN mRNA underwent large changes during embryogenesis and larval development, with low levels, of sbOSN first being detected by RT-PCR in eggs at early gastrulation (see Figure 5.1 A).

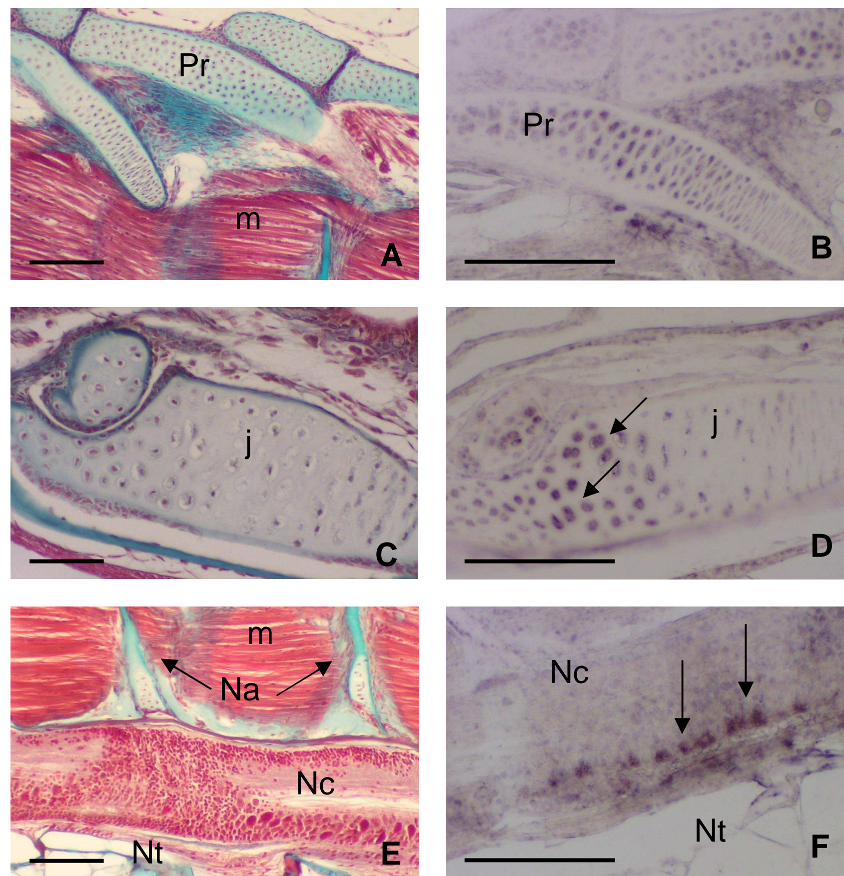

Figure 5.3 - Masson´s trichrome staining of sections of a 47dph sea bream larvae (A, C, E) and ISH with a sbOSN riboprobe of adjacent sections (B, D, F). A) Dorsal fin in which cartilaginous proximal radials (Pr) are identified by the green colour characteristic of non-mineralized connective tissues; B) sbOSN mRNA expression in the presumptive chondrocytes present in the cartilaginous proximal radials of the dorsal fin; C) jaw (j) with cartilaginous structures stained green; D) sbOSN transcripts were detected in some chondrocytes in the proximal region of the cartilaginous structure of the jaw; E) vertebral column with cartilaginous neural arches (Na); F) sbOSN was highly expressed in some cells of the perichordal sheath at the border between the nerve cord and the notochord. m - muscle, Nc - nerve cord, Nt - notochord. Scale bars: 100µm.

This resembles the situation in *Xenopus* embryos where OSN transcripts are first detected during late gastrulation/early neurulation (Damjanovski*, et al.*, 1994). At this stage, OSN is proposed to be involved in the regulation of the cell-cell and cell-matrix interactions, facilitating cell migration and cell shape changes, characteristics of this period of embryological development. sbOSN was detected in all subsequent larval stages of sea bream although maximal levels occurred at hatching, raising questions about its potential role in this process. A series of maximum and minimum points complete the sbOSN

expression profile during sea bream larvae development. In mammalian and amphibian embryos a similar expression profile has also been observed, although the exact function of OSN in different developmental stages still remains to be established (Damjanovski*, et al.*, 1994).

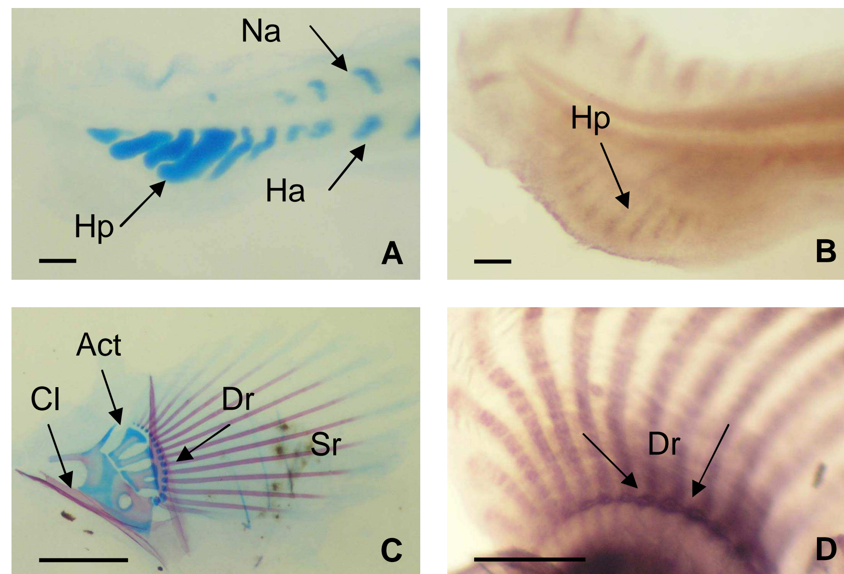

Figure 5.4 - Alcian blue/alizarin red whole mount staining of 20dph and 46dph sea bream larvae (A and C) and ISH with a sbOSN riboprobe (B and D). A) alcian blue staining revealed the hypurals (Hp) of the caudal fin, the neural (Na) and haemal arches (Ha) of the vertebra were all cartilaginous and these structures are indicated with an arrow; B) sbOSN mRNA was detectable in low abundance in the hypurals; C) the ossified cleithrum (Cl) of the pectoral fin is evident by its red staining and is indicated by an arrow; the actinosts (Act) were undergoing ossification as evidenced by regions of blue and red staining; the distal radials (Dr) are cartilaginous structures (blue) present in the base of the soft rays (Sr) that are undergoing ossification (red); D) the most intense expression of sbOSN is in the distal radials of the pectoral fin. Scale bars: 100μm.

In fetal and early neonatal rat and mouse, the developmental profile suggests OSN is important in the development of the skeleton and in other structures undergoing rapid cell growth and proliferation (Cowles*, et al.*, 1998, Holland*, et al.*, 1987, Mothe and Brown, 2001, Nakase*, et al.*, 1994, Sage*, et al.*, 1989). OSN-null mice develop a low turnover osteopenia, have a decreased matrix apposition rate and decreased osteoblast and osteoclast numbers, suggesting that this protein is important for normal remodelling and maintenance of bone mass in mammals (Delany*, et al.*, 2000). Studies with osteoblastic cell lines

derived from OSN-null osteoblasts showed that these cells do not achieve a fully differentiated phenotype. These cells do not survive stress as well as wild type cells, contain fewer mineralized nodules and are less responsive to PTH, suggesting that OSN is essential for normal bone remodelling, maintenance of bone mass and bone quality (Delany*, et al.*, 2003).

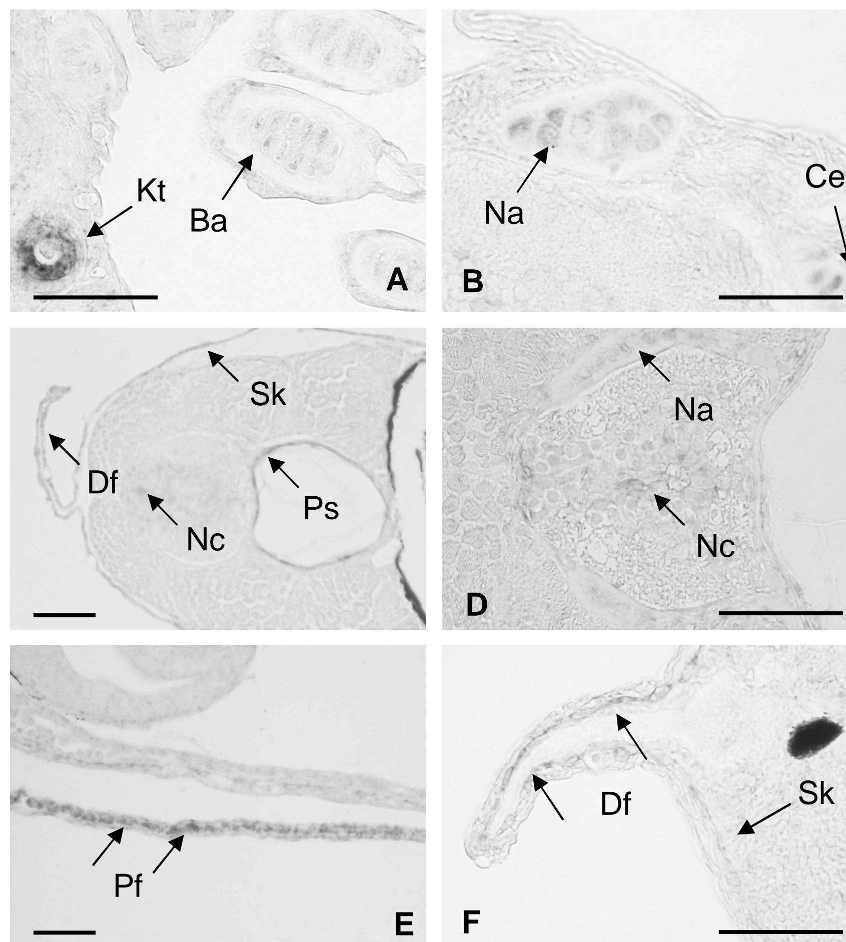

Figure 5.5 - sbOSN mRNA expression in transverse sections of sea bream larvae of 15 (A-C) and 25 (D-F) dph. A) sbOSN transcripts were detected in the kidney tubule (Kt) and branchial arches (Ba); B) the developing centrum (Ce) and neural arch (Na) of the vertebral column contained OSN transcripts; C) sbOSN was present in low abundance in the skin (Sk), perichordal sheath (Ps) and developing dorsal fin (Df) and in occasional cells scattered in the nerve cord (Nc); D) In 25dph sea bream larvae, the neural arch and some cells present in the nerve cord had a low but detectable expression of sbOSN mRNA; E) epithelial cells in the membrane of the pectoral fin (Pf) and F) dorsal fin and the developing skin contained abundant sbOSN expression. Scale bars: 50μm.

The functional significance of OSN in fish bone development is intriguing although currently it is difficult to establish parallels between teleost and mammalian bone formation, as there is relatively little information about this process in fish. Superficially, bone formation in fish appears to occur by the same general process observed in mammals and two types of bone, intramembranous (or dermal) bone and endochondral bone have been described (Kardong, 1998, Sommerfeldt and Rubin, 2001).

Intramembranous ossification occurs during embryonic development by the direct transformation of mesenchymal cells into osteoblasts and in endochondral ossification embryonic mesenchyme transforms into cartilage and subsequently undergoes mineralization (Marks and Odgren, 2002, Sommerfeldt and Rubin, 2001). The source of calcium for bone mineralization in fish has not been established, although in sea bream larvae (30dph) regions undergoing mineralization such as the jaws and fin epithelia are particularly rich in chloride cells which may allow direct calcium uptake from the surrounding water in these areas (Guerreiro*, et al.*, 2004). As OSN binds up to 8 Ca^{2+} with low affinity its expression in sea bream skin which is rich in chloride cells may be related to its function as an extracellular reservoir of Ca^{2+} as has been previously proposed in developing amphibian and mouse skin (Holland*, et al.*, 1987, Huynh*, et al.*, 2000, Sage*, et al.*, 1989).

OSN expression in sea bream larvae is present in both intramembranous (e.g. vertebral centra) and endochondral (e.g. jaw and vertebral arches) skeletal elements of sea bream larvae. Two principal OSN expression patterns were observed in sea bream larvae irrespective of the bone origin. In the first group, which included the pelvic, dorsal and anal fins, sbOSN expression preceded chondrogenesis. In the second group which included the neurocranium, jaws, branchial arches, pectoral and caudal fins and vertebral centra and arches the onset of sbOSN expression preceded or was coincident with calcification (Figure 5.2). The ontogeny and localization of sbOSN in the developing skeleton of sea bream larvae suggests it plays a role in this process, during both chondrogenic and osteogenic differentiation and mineralization. This is similar to the reported role of OSN in rat and mouse embryos (Mothe and

Brown, 2001, Nakase*, et al.*, 1994), porcine fetuses (Chen*, et al.*, 1991) and newborn mice (Holland*, et al.*, 1987). The regulation of OSN by hormones, such as parathyroid hormone related protein (PTHrP), a key factor in this developmental process remains to be established in vertebrates, although in sea bream scales OSN expression is downregulated by PTHrP (Redruello*, et al.*, 2005).

Significant advances in the identification of endocrine factors which regulate skeletal development in tetrapods have come from studies with knock out mice. Analysis of the consequences of knocking out prolactin receptor (PRLR), estrogen receptor or PTH/PTHrP receptor has demonstrated that the respective ligands are involved in skeletal development (Clément-Lacroix*, et al.*, 1999, Coss*, et al.*, 2000, Karaplis and Vautour, 1997). Cell culture studies have shown that continuous treatment of osteoblasts with PTH results in decreased expression of many genes involved in bone formation, including OSN, but genes involved in bone resorption are increased (Swarthout*, et al.*, 2002).

The function of the endocrine system in skeletal formation in fish or even in early larval development still remains to be established. It is well established that fish eggs contain substantial quantities of maternal hormones although it is still uncertain if they have a role in embryo and larval development (Power*, et al.*, 2001).

Immediately post hatch (48hpf) sea bream larvae are small and poorly formed with immature organs and tissues. Despite this a few days after hatching a number of endocrine glands can be identified; for examples, the pituitary gland is evident in the embryo although endocrine cells detectable by immunohistochemistry are only present several days after hatch (Power and Canario, 1992), this is also the case with thyroid follicle (Power, 2001). Receptors for both prolactin and thyroid hormone have also been detected in the sea bream prior to hatch indicating the important elements for transduction of hormone activity are also present (Llewellyn*, et al.*, 1998, Nowell*, et al.*, 2001, Santos*, et al.*, 2003). Such observations indicate that in sea bream a number of endocrine axis may be functional prior to the development of the skeleton at

around 4dph. The presence in sea bream embryos and larvae of hormones shown in mammals to have a key role in skeletal development raises interesting questions about the role of such endocrine factors in skeletal development in fish.

Acknowledgments of practical work

M. D. Estêvão carried out histology and *in situ* hybridization and acknowledges B. Redruello for the RNA extraction and semi-quantitative RT-PCR.