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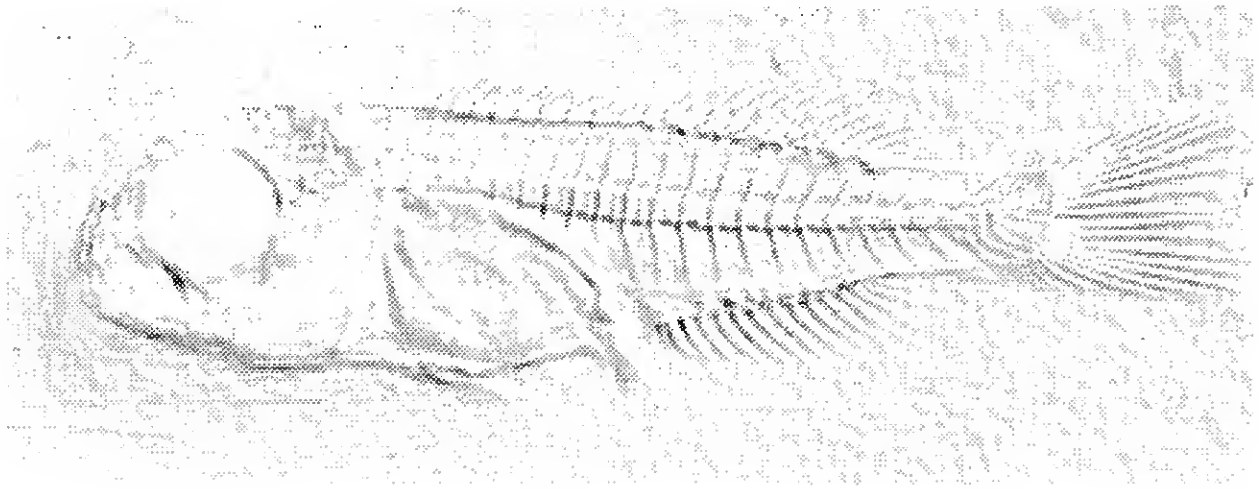
Developmental osteology of the gilthead sea bream (*Sparus aurata*, L.)



Dissertação para a obtenção do grau de doutor em Biologia
especialidade de Fisiologia Animal

Manuel Almeida dos Ramos Faustino

FARO 2001

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Orientadora: *Profª Doutora Deborah Mary Power*

Manuel Almeida dos Ramos Faustino

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2799 T.

O conteúdo desta dissertação é da
exclusiva responsabilidade do autor

(Manuel Almeida dos Ramos Faustino)
mfaust@ualg.pt

À Linda
Aos meus filhos, Joana, André e Alexandre

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SUMMARY

The gilthead sea bream, *Sparus aurata* (family Sparidae) is one of the most frequently used species in the aquaculture of southern Europe. Several studies have been made about the biology and rearing of this species, however little is known concerning the development of the skeletal system.

The normal developmental process of all skeleton (viscerocranial region, vertebral column, pectoral, caudal, pelvic, dorsal and anal fins and associated structures), in sea bream, is described and characterised in detail using the Alcian blue/Alizarin red staining and alkaline phosphatase technique. At hatching sea bream has no cartilaginous or bony structures. The first elements to appear are those related with feeding and manoeuvrability (sclerotic cartilage, jaws, cartilaginous suspensorium, cartilaginous hyoid arch and cartilaginous branchial arches), *i.e.* structures of head skeleton, which permits the opening and closing of the mouth and components of the pectoral fins (cleithrum, and cartilaginous coracoid-scapula) which are essentials for both propulsion and swimming activity. The early formation of these skleletal structures seems to be related with their fundamental importance for larval survival. Dorsal and anal fins and associated structures are the last elements to appear and develop.

Comparing the overall development of the skeletal system of sea bream with other teleosts it is shown that this process is strongly conserved. There is a remarkable similar number of structures and their order of appearance during development has been conserved.

The overall development of the skeleton in sea bream appears to support the theory of saltatory ontogeny which predicts that development does not occur at a constant velocity but is a saltatory process with boundaries arising between progressive changes in form and function.

Ossified structures are classified according to their origin: cartilage replacement bones , which has a cartilaginous precursor and dermal bones which develop within or just beneath the skin without any cartilaginous precursor. The ontogeny of different regional structures revealed that generally, the dermal bones ossify before the cartilage replacement bones.

One of the principal problems that affect the aquaculture of fish is the appearance of specimens with skeletal anomalies which reduce the larval quality and subsequently the final price. Thus, the frequency and nature of anomalies and meristic variations in sea bream are studied and characterised. A detailed analysis is given and show that two regions are most susceptible, the body axis and caudal fin complex. Curiously the anomalies occur predominantly in cartilage replacement bones and it is surprising that this fact has not previously been recorded, which suggests that a new approach is required when these alterations are being considered in fish.

In conclusion, the present thesis provides the basis, about sea bream, for:

i) future comparative morphogenetic and phylogenetic studies;

ii) defining indices of quality;

iii) further improvement of larval production and, probably, a better ecological understanding of this species.

RESUMO

A dourada, *Sparus aurata* (família Sparidae) é uma das espécies mais utilizada na aquacultura do sul da Europa. Diversos estudos têm sido feitos sobre a biologia e cultura desta espécie, no entanto até hoje pouco se conhece sobre o desenvolvimento do seu sistema esquelético.

O normal desenvolvimento de todo o esqueleto (região viscerocranial, coluna vertebral, barbatanas peitorais, caudal, pélvicas, dorsal e anal e estruturas associadas) da dourada, é descrito e caracterizado detalhadamente com a utilização da coloração por azul de Alcian/vermelho de Alizarin e da técnica da fosfatase alcalina. Ao eclodir os exemplares de dourada não apresentam qualquer estrutura cartilagínea ou óssea. Os primeiros elementos a surgir são os que estão relacionados com a alimentação e a mobilidade (esclerótica cartilagínea, mandíbulas, suspensório cartilagíneo, arco hióide cartilagíneo e arcos branquiais cartilagíneos), quer dizer, estruturas da cabeça que permitem abrir e fechar a boca e componentes das barbatanas peitorais (cleitrum e coracóide-escápula cartilagíneo) os quais são essenciais para a propulsão e para a actividade natatória. A precoce formação destas estruturas do esqueleto parecem estar relacionadas com a sua importância fundamental para a sobrevivência das larvas. As barbatanas dorsal e anal, bem como as estruturas associadas são os últimos elementos a surgir e a desenvolver-se.

A comparação entre o desenvolvimento do esqueleto da dourada e outros teleósteos mostra que este é um processo fortemente conservado. Existe uma notável semelhança entre o número de estruturas e a sua ordem de aparecimento, durante o desenvolvimento, tem sido conservada.

O desenvolvimento global do esqueleto, em dourada, parece estar de acordo com a teoria da ontogenia saltatória, a qual postula que o desenvolvimento não é um processo que ocorre a velocidade constante mas sim saltatório, em que surgem fronteiras entre mudanças sucessivas de forma e função.

As estruturas ósseas são classificadas de acordo com a respectiva origem: ossos de substituição, os quais têm um precursor cartilagíneo e ossos dérmicos os quais se desenvolvem no interior do tecido conjuntivo. A ontogenia das diferentes estruturas regionais demonstram que geralmente os ossos dérmicos são os primeiros a ossificar.

Um dos principais problemas que afecta a aquacultura em peixes é o aparecimento de exemplares com anomalias do esqueleto, as quais reduzem a qualidade das larvas e consequentemente o preço final. Desta forma, a frequência e a natureza das anomalias e variações merísticas, em dourada, são estudadas. É fornecida uma análise detalhada, verificando-se que são duas as zonas mais afectadas: o eixo longitudinal do corpo e o complexo caudal. Curiosamente é nos ossos de substituição que as anomalias são mais frequentes, sendo surpreendente que este facto não tenha ainda sido objecto de qualquer referência. Isto leva a que, neste âmbito e em peixes, uma nova e diferente abordagem deva ser feita.

Em conclusão, a presente tese fornece informação essencial, acerca da dourada, para:

i) futuros estudos comparativos, tanto morfogenéticos como filogenéticos;
ii) definir índices de qualidade;
iii) posteriores melhoramentos da produção das larvas e provavelmente para um melhor conhecimento ecológico da espécie.

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Fishes constitute slightly more than one-half of the total number of approximately 48.170 recognized living vertebrate species (Nelson, 1994). Besides their important role in the diet of many people, they represent an important element in the economy of many nations. In 1994 the world aquaculture production of shellfish and finfish reached more than 18.5 million tonnes. Fish production accounted for 13 million tonnes of this total, although surprisingly marine fish only accounted for 3.4 % (FAO, 1996).

It is commom knowledge that teleost larvae produced in hatcheries are frequently of variable quality. The assessment of larval quality is based on a relatively limited number of indices that include alterations in external morphology, such as the appearance and alteration in pigmentation of flatfish (Gartner, 1986), opercular deficiency, osteological and morphological deformities and failure to form a fuctional swimbladder (Paperna, 1978; Kitajima *et al.*, 1981; Barahona-Fernandes, 1982; Chatain & Ounais-Guschmann, 1990; Daoulas *et al.*, 1991; Rodger & Murphy, 1991; Andrades *et al.*, 1996; Hilomen-Garcia, 1997). All these deformities are common in sea bass and sea bream and are directly related to reduced growth and a higher predisposition to disease and stress with consequent high mortality rates. All these aspects directly affect the public image of aquaculture and may decrease the commercial value of reared fish. Within this context characterisation of the development of the skeleton, meristic variation and identification of abnormalities become an important starting point for the development of tools to estimate quality of hatchery

reared larvae and fish. Moreover, it represents the first step to understanding how, why and when deviation from the normal skeletal plan may occur during development.

Skeletal framework

Fishes, amphibians, reptiles, birds and mammals are included in the subphylum Vertebrata (Craniata), phylum Chordata (Nelson, 1994). Organisms are classified as Chordates if they possess a notochord, pharyngeal slits, a dorsal tubular nerve cord and an endostyle (Walker & Liem, 1994). The main characteristic of vertebrates is the presence of a cartilaginous or bony endoskeleton. The cranium protects a well developed brain which together with the spinal cord and nerves forms a sensory system and the vertebral column that replaces the notochord in the adult stage. The vertebrates are distinguished from the other chordates by two principal factors: i) the duplication of the *Hox* gene complex (Marx, 1992; Holland, 1997) and ii) the neural crest, an embryonic tissue from which the first vertebrate skeletal tissue appears to have arisen (e.g., probably dermal bones, teeth, anterior neurocranium and visceral arches) (Noden, 1983; Langille & Hall, 1988; Thorogood, 1988; Nelson, 1994).

The differing physical and chemical properties of water and air have affected the evolution of the vertebrates. In tetrapods (four-footed) such as amphibians, reptiles, birds and mammals, a range of changes occured in all organ systems as an adaptation to emergence onto land and the diverse environments encountered. For example the fish-amphibian transition was much more than a transition from water to land. It was a transition from fins to feet that took

place in the water (Edwards, 1989). Reptiles were the first tetrapods fully adapted to terrestrial habitats and amniotic eggs represented significant adaptations to the dry conditions on land. Birds and mammals, which are among the dominant groups of animals on land, were derived from reptiles and are endothermic tetrapods. Table I summarize some of the major functional differences between fishes and tetrapods (Walker & Liem, 1994; Kardong, 1997; Pough *et al.*, 1999).

Table I. Principal characteristics of fishes and tetrapods.

FISHES	* Presence of fins * Epidermis, without keratine, is alive and metabolically active * Cartilaginous or bony endoskeleton * Numerous bones make up the cranium * Heart with 1 atrium * Single circulation * Nasal sac typically does not open directly into the mouth * Poikilotherms
TETRAPODS	* 2 pairs of pentadactyl (five-digit) limbs * Mostly (including adult terrestrial amphibians) present epidermis with keratin and with the superficial layer of dead and avascular cells * Endoskeleton well ossified * Reduced number of the cranium bones * Adults with lungs * 2 atrium heart (left and right) * Double circulation * Nasal sac open directly into the mouth * Endotherms

Bone, cartilage and other structural materials forms the skeletal framework of vertebrates. The skeleton maintains body shape and together with the muscular system supports and permits movement of the body. In addition to its structural function, calcified skeletal tissues may be of considerable importance as a reservoir of essential ions such as calcium and phosphate both of which are fundamental in numerous biochemical processes (for example muscle contraction, storage and release of energy, Walker & Liem, 1994). The buffering nature of water and its density have allowed the evolution of a variety of body forms with associated muscular and skeletal alterations. The skeletal modifications and adaptations that evolved in different fish species is one of the traditional methods of identifying and classifying fish into distinct groups and is the basis of taxonomic classification. Two main groups of fish can be identified if endoskeleton type is considered, the Chondrichthyes (sharks, chimaeras, rays, skates) and the Osteichthyes. The former group has a persistent cartilaginous skeleton and the latter group has a bony skeleton that results from replacement of embryonic cartilage with bone.

Structural characterization

Cartilage development is one of the earliest morphogenetic steps in the skeletogenesis. The overall process consists of a highly coordinated and orchestrated series of events involving the commitment and differentiation of mesenchymal cells to chondrocytes (Fig. 1). Cartilage includes only two cell types: (i) the chondrocytes, that morphologically are round cells surrounded by an extracellular matrix and within the matrix lacunae and (ii) the perichondral cells in its enveloping mesenchyme. Extracellular matrix, which is synthetised and

secreted by chondrocytes, contains predominantly type II collagen, proteoglycans and glycoproteins (Bertin, 1958; Cormack, 1984; Reddi, 1994; Junqueira *et al.*, 1995).

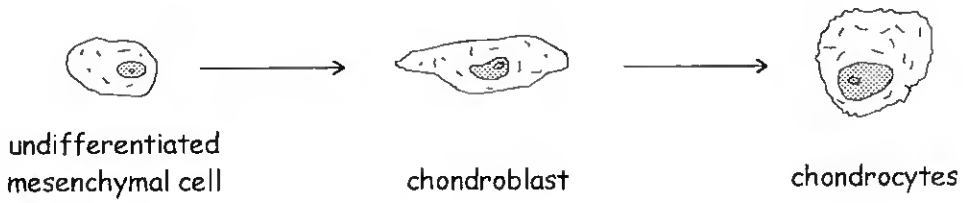

Figure 1. Diagram of chondrocyte genesis.

Proteoglycans (chondroitin sulfate and keratan sulfate) are associated with molecules of hyaluronic acid and the latter complex binds to and interacts with type II collagen (Fig. 2). Furthermore chondronectin (a glycoprotein) binds specifically to type II collagen and proteoglycans to promote the aderence of chondrocytes to the matrix (Cormack, 1984; Junqueira *et al.*, 1995).

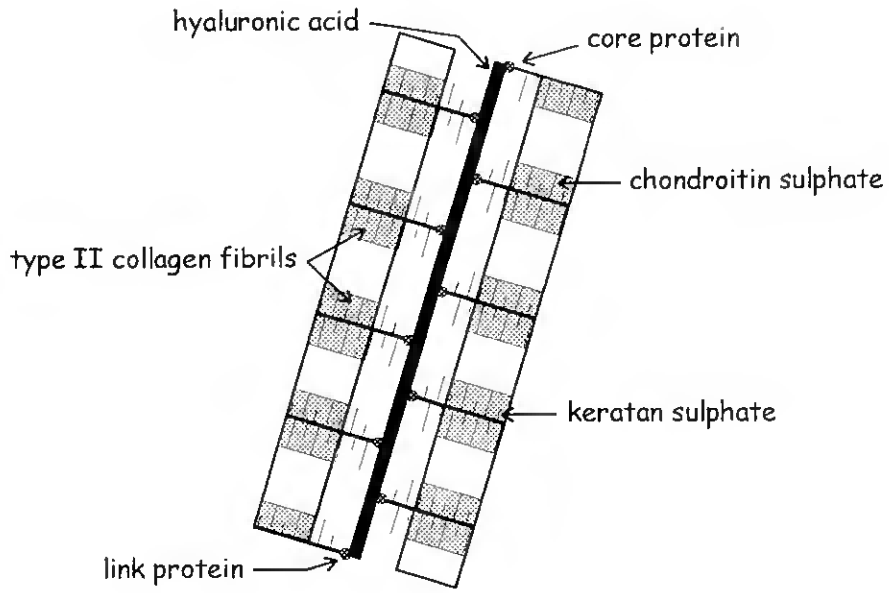

Figure 2. Schematic representation of the molecular organization in cartilage matrix.

Cartilage grows by two independent mechanisms, that may occur separately or simultaneously: appositional growth results from the recruitment of fibroblasts from the perichondrium that covers the cartilage and interstitial growth that results from the mitotic division of chondrocytes. Cartilage lacks blood vessels (is avascular) and transfer of nutrients and oxygen to this tissue occurs by diffusion from the nearby capillary bed of the perichondrium (Cormack, 1984; Junqueira *et al.*, 1995). As mentioned before, perichondral cells participate in the proliferation and differentiation of chondrocytes, however the precise molecular mechanism by which this occurs is largely unknow (Long & Linsenmayer, 1998).

Endochondral bone formation in vertebrates requires precise coordination between proliferation and differentiation of the participating chondrocytes. Endochondral bone formation during vertebrate embryogenesis is a highly regulated process. During this process, young chondrocytes initially undergo rapid proliferation. After proliferation mature chondrocytes produces large amounts of extracellular matrix and subsequently become hypertrophic. The composition and the properties of the cartilage matrix in the hypertrophic zone changes and allows the invasion of blood vessels from the perichondrium and finally the replacement of the cartilage matrix by bone (Reddi, 1981; Vortkamp *et al.*, 1996).

Bone like cartilage, is a specialized connective tissue that arises from mesenchyme (Fig. 3). Bone is surrounded externally by a membranous periosteum and in terrestrial vertebrates is typically made up of three components:

(i) bone cells - osteoblasts, that synthesize the organic matrix, osteocytes, that are important for deposition and resorption of bone and osteoclasts that participate in the bone resorption;

(ii) extracellular organic material or organic matrix, which consist of a

network of type I collagen, proteoglycans and glycoproteins;

(iii) extracellular mineral material, that is predominantly constituted by crystals of hydroxyapatite (calcium and phosphate salts).

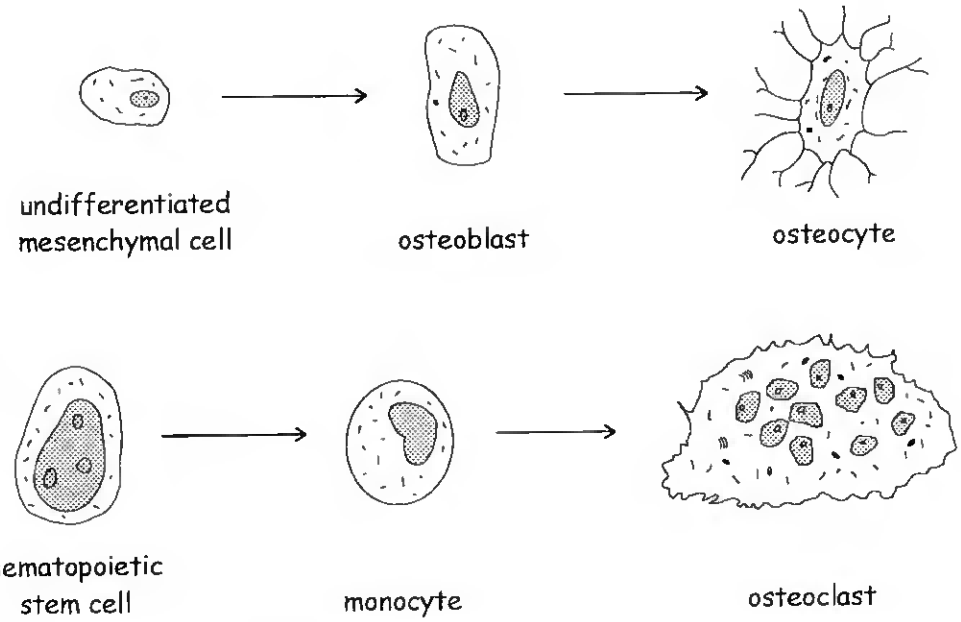

Figure 3. Scheme with the genesis of bony cells.

Bone in terrestrial vertebrates is a highly vascular tissue permeated throughout by blood capillaries and the exchange of gas and metabolites between osteocytes and capillaries depends upon the communication through innumerable tiny canals, the canaliculi, that perforate the matrix (Cormack, 1984; Francillon-Vieillot *et al.*, 1990; Junqueira *et al.*, 1995; de Ricqlès *et al.*, 1991).

Osteoblasts are cuboid-shapped cells that secrete the organic matrix (Junqueira *et al.*, 1995). They are located at the surface of the bone tissue and are in contact with neighboring osteoblasts. The secretion of the organic matrix components occurs at the cell surface, which is in contact with the older bone matrix. Subsequently, calcium salts are deposited in the newly formed matrix

and this process is called bone apposition. Osteoblasts are very rich in alkaline phosphatase. This enzyme participates in the mineralization process and due to this fact is frequently used as an indice of bone formation and matrix mineralization (Doty & Schofield, 1976; Ingleton *et al.*, 1983; Gruber *et al.*, 1988; Rodan, 1992; Komaki *et al.*, 1996).

Osteocytes arise from osteoblasts, they are typical bone cells and occupy lacunae in the matrix which develop as the osteoblasts synthetize new bone. Osteocytes have numerous interconnecting cytoplasmatic projections that provide communication across the bone and are involved in secretion and resorption of bone matrix (Cormack, 1984; Rodan, 1992; Junqueira *et al.*, 1995).

Osteoclasts are multinucleated giant cells directly implicated in bone resorption, they secrete proteases and acids that dissolves the matrix and release bone mineral (Roodman, 1996). Bone resorption is restricted to a specialized surface of the osteoclast membrane that appears as a ruffled border. The interface between the ruffled border of the osteoclast and the bone is known as the "sealing zone" (clear zone) and provides a micro-environment favourable for bone resorption (Cormack, 1984; Rodan, 1992; Junqueira *et al.*, 1995). In this zone the acidic pH solubilizes the bone mineral and the lysosomal enzymes digest the matrix (Baron *et al.*, 1985; Junqueira *et al.*, 1995). Some substances thought to be involved in osteoclast function have been identified and include, for example, integrin receptors that bind to extracellular matrix proteins and vesicular proton ATPase, both of which are abundant in the ruffled border and are necessary for acidification of the resorption space (Rodan, 1992). Resorption by osteoclasts can be determined by measuring tartrate-resistant acid phosphatase activity, which serve as an index of osteoclast activity and bone resorption (Barka & Anderson, 1962; Gruber *et al.*, 1988; Inoue *et al.*, 1995; Roodman, 1996).

The major component of bone mineral is hydroxyapatite a crystalline calcium phosphate matrix. Hydroxyapatite crystals surrounded by proteoglycan aggregates lie along the collagen fibrils. Glycoproteins like sialoprotein and osteocalcin due to their composition have an elevated potential for calcium binding and may be responsible for promoting calcification of bone matrix (Junqueira *et al.*, 1995).

The interaction between the different bone cells types allow bone to repeatedly and continuously undergoe remodelling, i.e. submitted to resorption and deposition processes (Francillon-Vieillot *et al.*, 1990; de Ricqlès *et al.*, 1991) in response to a range of factors.

Aquatic organisms

The structure of bone in aquatic organisms differs from that characterised in terrestrial organisms, for example, the Harversian system characteristic of humans, is practically never encountered in lower vertebrates, especially in bony fishes. Bone in teleost fishes may be cellular as in *Acanthopagrus australis, Pagrus auratus* and *Rhabdosargus sarba* (Hughes *et al.*, 1994) or acellular (lacking osteocytes) as in *Oryzias latipes* (Ekanayake & Hall, 1987), *Aphanius mento* (Stibane, 1992), *Hemichromis bimaculatus* (Sire & Huysseune, 1993), *Oncorhynchus mykiss* (Takagi & Kaneko, 1995), *Oreochromis niloticus* (Witten, 1997). In Osteichthyes, the histological feature of skeletal tissues in bony fish, during normal development, cover a broad spectrum in which cartilage and bone represent the two extremes (Bertin, 1958; Huysseune & Verraes, 1986; Meunier & Huysseune, 1992).

Fishes, unlike terrestrial vertebrates grow throughout their lifetime and

this means that the skeleton must be adapted to undergo almost unlimited growth. Osteogenesis can occur during the life cycle, being interrupted by certain physiological events, such as lack of food (Bertin, 1958), development of the gonads, reproduction or senescence (Balon, 1986; Jobling, 1995; Kamler, 1995). Two main mechanisms for the formation of calcified tissue exist and according to the mechanism used bone may be classified as, i) dermal or membrane bone and ii) cartilage replacement bone. In dermal bone there is direct deposition of bone in connective tissue and in cartilage replacement bone the cartilaginous matrix is progressively replaced by bone (Cormack, 1984; Junqueira *et al.*, 1995).

Endocrine control of calcium metabolism

Calcium (Ca^{2+}) is an element of primordial importance in numerous physiological functions of the vertebrates. Besides its role in bone growth, cellular replication or as a second messenger (Hadley, 1992) it is also involved in the regulation of muscle contraction (Raven & Johnson, 1999).

The exchange of calcium between plasma and bone tissue is a complex process and numerous questions remain about the way in which this occurs.

Terrestrial vertebrates take up calcium from the diet, a doubtful and fickle source of calcium. The episodic supply of calcium may have been the driving force for the evolution of bone as a calcium reservoir. This also required the development of a complex system of bone cells and blood vessels to allow both deposition and mobilization of bone minerals. The existence of a calcium reservoir in terrestrial vertebrates allows them to survive in periods of food deprivation or high calcium demands such as during growth or reproduction

(Wendelaar Bonga & Pang, 1992). In terrestrial vertebrates the main hormones that regulate calcium levels in plasma are parathyroid hormone (PTH) synthesized in parathyroid glands and 1,25-dihydroxyvitamin D_3, both are hypercalcemic and a hypocalcemic factor, calcitonin (CL), synthesized by "C" cells in the thyroid (mammals) (Hadley, 1992; Wendelaar Bonga & Pang, 1992). Other hormones such as prolactin (PRL), growth hormones (GH) and estrogens are also involved in this metabolic process but perform minor roles (Wendelaar Bonga & Pang, 1992).

Calcium regulation in fishes differs from terrestrial vertebrates in terms of environmental availability of calcium. In addition to dietary calcium, fishes have continuous access to calcium present in water. Control of calcium homeostasis in fish is a process with some indications but with reduced certainties.

The endocrine control of calcium metabolism in fish is also proposed to be a consequence of the interaction of hyper- and hypocalcemic hormones. However, the hormones involved in calcium regulation in fish differ from those in terrestrial vertebrates. Fish lack a parathyroid gland and little evidence for the existence of this hormone in fish exists. However, the related hormone, parathyroid hormone gene-related peptide (PTHrP), is expressed in the pituitary of both elasmobranchs (Ingleton *et al.*, 1995) and teleosts (Danks *et al.*, 1993) and the gene has recently been cloned in *Fugu rubripes* and *Sparus aurata* (Flanagan *et al.*, 2000; Power *et al.*, 2000) although its function has yet to be determined.

Although 1,25-dihydroxyvitamin D_3 is known to be hypercalcemic the status of 1,25-dihydroxyvitamin D_3 in calcium regulation of fishes is still enigmatic. In fishes CT is produced in the ultimobranchial bodies and although it has been reported to promote bone formation by osteoclasts (Wendelaar Bonga

& Lammers, 1982) its function is still poorly understood. Stanniocalcin (STC) is an important hypocalcemic factor in fish (Hazon & Balment, 1997), it is unique for holosts and teleosts and is produced by the corpuscles of Stannius (Pang, 1974; Wendelaar Bonga & Pang, 1992). It is proposed to reduce active calcium uptake at the gills (Verbost *et al.*, 1993), intestine (Sundell *et al.*, 1992) and probably also in the kidney (Flick *et al.*, 1996).

Developmental ontogeny

In agreement with Balon (1986) the life cycle of fishes, from fertilisation to death, can be divided into five principal periods:

Embryonic period – begin with fertilization of the eggs and is characterised by endogenous feeding;

Larval period – initiated with the transition to the exogenous feeding;

Juvenile period – start as soon as the full differentiation of fins and the regression or replacement of larval organs. This period is characterised by fast growth;

Adult period – begin with the sexual maturation and the production of the first gametes. As the available resources are usually utilised in the development of gonads and reproduction the somatic growth is less than in juvenile period. In the adult period is also clearly visible the changes in external morphology and colour;

Senescence period – the growth is very slow or probably inexistent. This period has a conspicuous decreases in the fertile gametes produced and can last from few days or weeks to several years.

The main factors that control growth are growth hormones secreted by the pituitary gland and steroid hormones from the gonads. However a range of environmental factors such as water temperature, salinity, competition, food availability and photoperiod can interact with each other to influence growth rates.

CHARACTERIZATION OF THE SPECIES USED IN THE STUDY

Morphology

The gilthead sea bream (*Sparus aurata*) is a teleost fish that belongs to the order Perciformes, which is the largest order of Vertebrates and the most diverse of all fish orders. The sea bream is a member of the Sparidae family that includes 29 genera and about 100 species (Nelson, 1994).

Sparidae are characterised by the presence of a single dorsal fin, usually composed of 10-13 spines and 10-15 soft rays, an anal fin with 3 spines and 8-14 soft rays, maxilla covered by a sheath when the mouth is closed, six branchiostegal rays and 24 vertebrae (Nelson, 1994). Jaw teeth are generally well developed and differentiated into conical (canine-like) or flat (incisor-like) teeth in front and rounded, molar-like teeth laterally. They possess a single continuous lateral line, long and pointed pectoral fins, pelvic fins below or just behind pectoral fin bases, with 1 spine and 5 soft rays and the caudal fin is forked (Bauchot & Hureau, 1986).

The taxonomic classification of the sea bream is as folllow (Nelson, 1994):

Division - Teleostei; Subdivision - Euteleostei; Superordem - Acanthopterygii; Series - Percomorpha; Order - Perciformes; Suborder - Percoidei; Superfamily - Percoidea; Family - Sparidae; Genus - *Sparus*; Species - *Sparus aurata* (L.)

Sparidae represent an important group within world fish culture, representing together with Serranidae, 23% of total marine finfish production (New, 1991). Within this group of commercially important species, sea bream production has the highest growth-rate and at present the highest commercial value and occupies a central position in southern European fish farm production. It has a long history of cultivation along the Mediterranean coast, initially under semi-intensive production and more recently in intensive regimes and within Portugal it is one of the main cultivated species (Direcção Geral das Pescas e Aquicultura, 1990-1995, 1997-1999; FAO, 1996). Between 1990 and 1998 Portuguese aquaculture production of sea bream increased from 105 to 1221 tonnes and in 1998 it accounted for 613 tonnes of the farmed fish produced in the Algarve (Direcção Geral das Pescas e Aquicultura, 1990-1995, 1997-1999).

Distribution

Sparidae are marine fish (very rarely brackish and freshwater) that have a wide geographical distribution being found in the Atlantic, Indian and Pacific oceans.

Sea bream, as mentioned above, is an important aquaculture species in southern Europe and the wild fish inhabits the Mediterranean, the east Atlantic from Great Britain to the Canary and Cape Verde Islands; it has also occasionally been found in the Black Sea (Whitehead *et al.*, 1986; Fisher *et al.*, 1987). This

species has a wide ecological range and it may inhabit open coastal marine environments or lagoonar systems with highly variable salinity.

Biology

Sea bream has a characteristic appearance, it has a silvery grey colouration with a darkly pigmented zone at the origin of the lateral line, and it is ovoid in shape (Fig. 4); (Whitehead *et al.*, 1986; Fisher *et al.*, 1987). It's common name, gilthead sea bream, is derived from the presence in adults of a golden curved bar across the forehead that is bordered by two dark zones (Fig. 4). It is essencially a carnivorous species (Whitehead *et al.*, 1986; Fisher *et al.*, 1987), commonly feeding on molluscs, crustaceans, little fishes and sporadically it may also feed on algae (Quéro, 1984), polichaets and insects (Arias, 1980).

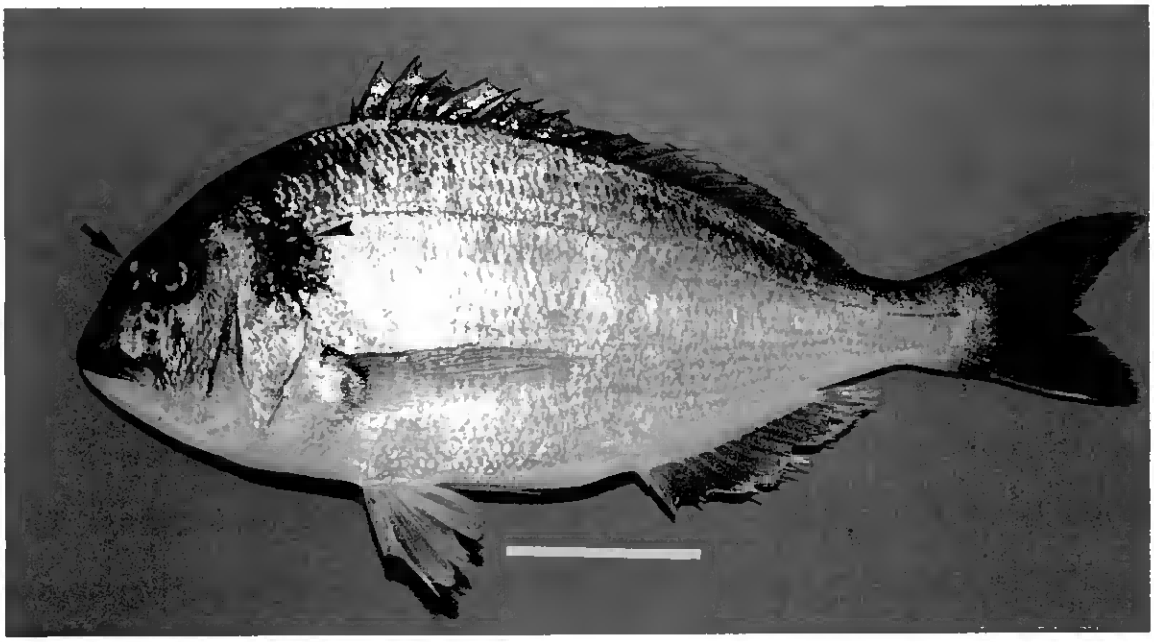

Figure 4. Photograph showing a left lateral view of an adult sea bream. The darker zone at the origin of the lateral line is indicated by an arrowhead and the golden coloured bar across the forehead is indicated by an arrow. Scale bar indicate 5.0 cm.

Adult sea bream reach 30 – 35 cm in length and ocassionally may exceed 70 cm.

Sea bream is a protandrous hermaphroditic species (D'Ancona, 1941; Pasquali, 1941; Zoahar *et al.*, 1978). During the first year of their life all fish are male and under certain environmental conditions the same may occur in the second year. From the first or second year some males undergo a sex change to female (Zoahar *et al.*, 1978: Zoahar *et al.*, 1984). In the Algarve spawning generally occurs from November to February, although manipulation of temperature and photoperiod can change the spawning season.

Embryonic development may also be affected by external factor, such as salinity (Zohar, 1984), photoperiod (Tandler & Helps, 1985) and water temperature (Villani, 1976; Zaki, 1984; Polo *et al.*, 1991; our observations). At 12 °C and 17 °C hatching occurred, respectively, at 72 hours post-fertilization (hpf) and 48-50 hpf (Villani, 1976); at 16 °C it occurs at between 65 and 70 hpf (Zaki, 1984) while at 28 °C it occurs after only 24 hpf (Polo *et al.*, 1991) and at 19 °C, the optimal rearing temperature, hatching starts at 40 hpf (our observations). Table I and Figure 5 summarizes the main developmental steps of sea bream from fertilization to hatching at 19 °C (our observations).

Typically fertilised sea bream eggs are transparent with a single oil globule in the yolk of approximately 1 mm diameter and at hatching sea bream embryos are optically clear, with few melanophores and a primordial finfold around the trunk in the median position (Kiriakos *et al.*, 1994; our observations). Their length ranges from 2.0 mm total length (tip of snout to posterior tip of finfold) (Zaki, 1984) to 2.7 mm notochord length (tip of snout to tip of notochord on small larvae prior to flexure) (our observations) and the yolk sac is oval and measures 1.1 mm in length (our observations). The mouth and jaws are absent and the eyes are unpigmented. The auditory vesicles are clearly visible as is the notochord and the segments (our observations).

Table I. Summary of development of sea bream at 19 °C.

hour post-fertilization	Description of stage
0	Fertilization
0.3	Beginning of swelling
3	Cleavage (16 cells)
9	Blastula (early epiboly)
15	Gastrula (50% epiboly)
19	Segmentation (4 somites)
26	Segmentation (16 somites)
40	Hatching (free embryo)
84	Free embryo (yolk absorption - 50%)
120	Larvae (exogenous feeding)

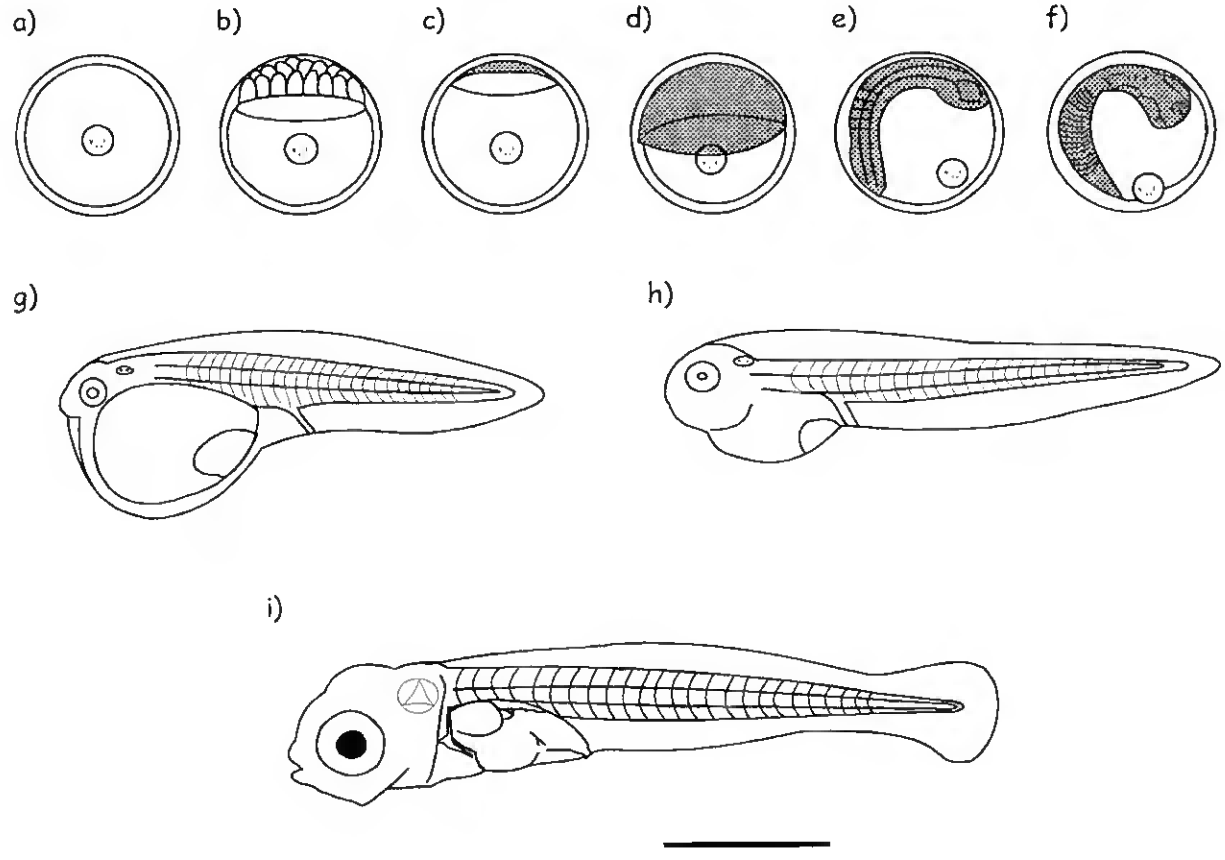

Figure 5. Embryonic and larval sea bream development. (a) 0.3 hours post-fertilization (hpf), beginning of swelling; (b) 3 hpf, 16 blastomeres; (c) 9 hpf, blastula (early epiboly); (d) 15 hpf, gastrula (50% epiboly); (e) 19 hpf, segmentation (4 somites); (f) 26 hpf, segmentation (16 somites); (g) 40 hpf, hatching (free embryo); (h) 84 hpf, free embryo (yolk absorption - 50%); (i) 120 hpf, larvae (exogenous feeding). Scale bar indicate 1.0 mm.

Few hours (3-4 h) after hatching the free embryo (yolk-sac larvae) body is fully straightened and the larvae quickly move to the water surface. During the first 3-4 days after hatching the free embryo is dependent for nutrient on the yolk-sac reserves. The transition to exogenous feeding occurs at about 4 days after hatching (our observation) and is a critical step; failure at this step is frequently responsible for the high mortalities observed in hatcheries (Lumare & Villani, 1970; Person-Le Ruyet & Verillaud, 1980).

At hatching sea bream has no cartilaginous or bony structures (Faustino & Power, 1998, 1999, 2001). This mean that from this time to exogenous feeding a range of cartilaginous or bony elements must develop for survival. The first elements to appear are those which are related with feeding and manoeuvrability, *i.e.* structures of head skeleton which permits opening and closing of the mouth and components of the pectoral fin which are essentials for propulsion and increase the swimming activity. After these important events the osteological development continues with the appearance of the remaining viscerocranial elements, vertebral column and associated elements and the others fin structures (Faustino & Power, 1998, 1999, 2001).

Several reports about sea bream egg and larval development and nutritional requirements have appeared (Villani, 1976; Person-Le Ruyet & Verillaud, 1980; Camus & Koutsikopoulos, 1984; Kadmon *et al.*, 1985; Tandler *et al.*, 1989).

The understanding and characterisation of sea bream development is essential if a rational approach is to be taken to improvement of larval quality. Improvements in quality of larvae will have important implications for the performance of subsequent rearing stages.

THE SCOPE OF THIS THESIS

Numerous studies of cartilage and/or bony skeletal development in wild or reared teleosts exist in the literature (Kendall, 1972; Houde & Potthoff, 1976; Potthoff *et al.*, 1984; Potthoff *et al.*, 1986; Matsuoka, 1987; Collette & Gillis, 1992; Watson & Walker, 1992; Balart, 1995; Adriaens & Verraes, 1997; Hoshino & Amaoka, 1998; Vandewalle *et al.*, 1999), however the objective of such studies has generally been to characterise fish larvae in the wild and thus most of them give an incomplete report of skeletal ontogeny because, i) generally they describe the development of only part of the skeleton, or ii) they refer only to very early stages or iii) are limited only to the structures that persist in the juvenile or adult (Lau & Shafland, 1982; Potthoff *et al.*, 1984; Potthoff *et al.*, 1986; Collette & Gillis, 1992; Balart, 1995; Suda, 1996; Voskoboinikova, 1998).

In recent years as a consequence of the importance of Sparidae to aquaculture, some studies about skeletal development have been reported in the literature. However, generally the studies lack details or fail to cover all of the structures that make up the skeleton (Matsuoka, 1985, 1987; Koumoundouros *et al.*, 2000). In sea bream the only studies that exist are those that describe the abnormalities observed in operculum and caudal fin complex (Kiriakos *et al.*, 1994; Koumoundouros *et al.*, 1997a, b).

The study and ontogenic characterization of skeletal structures provide essential knowledge for further improvement of larval production and, probably, a better ecological understanding of this species. Moreover, characterization of the pattern of skeletal ontogeny is an important step for the identification of factors that can interfere with normal skeletal development (Kwain, 1975; Somasundaram *et al.*, 1984; Bengtsson *et al.*, 1988; Wiegand *et al.*, 1989;

Campbell, 1995; Toften & Jobling, 1996; Lien *et al.*, 1997; von Westernhagen & Dethlefsen, 1997) and for understanding the alterations in the normal developmental process that may give rise to abnormalities.

The scope of the present thesis therefore, was to investigate the ontogeny of the skeleton in the sea bream. In Chapter 2 the normal development of cartilaginous structures in the vertebral column and caudal fin complex and the timing of their transformation into bone is described.

The developmental patterns of cartilage and bone in the remaining fins are characterized in Chapter 3. The results are compared with the development of these structures in related species. The sequential appearance of these fins was also analysed in relation to the need for more complex movements as the larvae grow and need to avoid predators and capture prey.

In Chapter 4 a precise account of the ontogeny of ossification of the viscerocranial skeleton is given. The evolutionary importance of the viscerocranial skeleton is considered and a hypothesis about the timing of ossification is proposed after cross species comparison of this structure.

In addition the origin of ossified tissue, cartilage replacement bone or dermal bone, is indicated for all the structures analysed (Chapters 2, 3 and 4).

In Chapter 5 the frequency of meristic variations and abnormalities were studied and a detailed analysis of abnormalities is presented. Two regions of sea bream are most susceptible to abnormalities, the body axis and the caudal fin complex, both structures which appear early in the ontogeny suggesting that many of the anomalies apparent in the juveniles probably have their onset in early development.

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CHAPTER 2

DEVELOPMENT OF OSTEOLOGICAL STRUCTURES IN THE SEA BREAM (*Sparus aurata*): VERTEBRAL COLUMN AND CAUDAL FIN COMPLEX

Co-author

Deborah M. Power

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ABSTRACT

The development of cartilaginous structures in cultured *Sparus aurata* larvae and the timing of their ossification was studied. In cultivated sea bream larvae the first cartilaginous structure to be identified was hypural 1 at 4.1 mm notochord length (L_N). By 5.3 mm L_N, prior to the onset of ossification, it was possible to distinguish the following cartilaginous structures: all 23 neural arches, all 13 haemal arches and two of the four pairs of parapophyses. The neural arches 1-4 and 15-23 were formed on the notochord and elongated dorsally, while neural arches 5-14 appeared on the dorsal side of the spinal cord and elongated ventrally. Initiation of ossification occurred at 5.7-6.0 mm standard length (L_S) when the cartilaginous ontogeny of the vertebral column was completed. Ossification was coincident with dorsal flexion at the posterior end of the notochord and occurred in a sequential manner: i) dorsoanteriorly, the cartilaginous neural arches and the centra were the first structures to ossify; ii) ventrad at the centre, at 7.0-7.5 mm L_S; iii) posteriorly at 7.1 mm L_S the hypural complex and urostyle (24th centrum) were ossified; and 4) dorsad at the centre (neural arches and spines).

Key words: bone, cartilage, ontogeny, fish larvae.

INTRODUCTION

The sea bream (*Sparus aurata*) is widely cultivated in the Mediterranean and Portugal. As with many other teleosts, cultivation of sea bream is associated with an increased incidence of skeletal malformations, particularly those associated with the vertebral column in the adult (Paperna, 1978; Barahona-Fernandes, 1982; Francescon *et al.*, 1988; Santamaria *et al.*, 1994). The cause and progression of such skeletal malformations in cultivated fish is uncertain and, despite their relatively high frequency in farmed fish, few studies of the cartilaginous-osteological development of cultured fish larvae exist. The majority of published osteological studies are directed at extending basic knowledge of larval taxonomy and systematics (Dunn, 1983) and have been carried out largely on wild species e.g. *Anisotremus virginicus* (L.) (Potthoff *et al.*, 1984), *Scombrolabrax heterolepis* (Roule), *Gempylus serpens* Cuvier, *Nesiarchus nasutus* (Johnson), *Scomber japonicus* Houttuyn (Potthoff *et al.*, 1986), *Engraulis japonicus* (Schlegel) (Balart, 1995). In sea bream, spinal abnormalities are detectable in larval stages (Santamaría *et al.*, 1994; our observations). It is likely that some malformations arise as a consequence of genetic factors and physiological factors, such as inability to inflate the swim bladder (Kitajima *et al.*, 1981; Chatain, 1987), whereas others may be a consequence of environmental factors such as diet, pollution, etc. (Hodson *et al.*, 1980; Weis & Weis, 1989; Hinton *et al.*, 1992).

During vertebrate embryogenesis, cartilage is the first element of the skeleton to form and subsequently, with some exceptions (e.g. interarcual cartilage, basibranchial cartilage, rostral cartilage), becomes bone. Two principal types of bones which differ in their origin can be identified in vertebrates: cartilage replacement bone, which has a cartilaginous precursor, and dermal bone

which develops within or just beneath the skin without any cartilaginous precursor.

The objective of the present study was to chart the normal development of cartilaginous structures in the vertebral column and caudal fin complex in cultured sea bream larvae and the timing of their transformation into bone. Bony structures arising from cartilaginous precursors and dermal bone were also identified. This study will form the basis of future experimental work aimed at elucidating the molecular regulation of osteological development, in order to understand how alterations in this process lead to abnormalities.

MATERIALS AND METHODS

Culture of Larvae

Sparus aurata larvae were hatched and cultured in 0.2m^3 conical tanks gently aerated with a continuous flow of sea water. Hatching occurred 40 h after fertilisation. The larvae were fed on *Brachionus plicatilis* 4 days post-hatching (DPH) when the yolk sac was consumed, and *Artemia* sp. was introduced into the diet from 15 until 40 DPH when dry food was introduced gradually. Light conditions followed a cycle of 12 h light:12 h dark, and the ambient water temperature was 19 $\pm$ 1 ^{o}C.

Experimental Protocol

Larvae were sampled on alternate days from 1 DPH, anaesthetized in MS-222 and fixed overnight at 4 °C in 2% PFA (paraformaldehyde) solution, washed and stored in 70% methanol. Preliminary experiments were carried out to

confirm that length was not altered as a consequence of fixation and subsequent staining methods and to permit the characterisation of normal and abnormal larvae. Abnormal larvae were identified readily and presented anomalies such as loss of structures, altered dimensions of structures and spinal breaks, twists and bends.

Larvae were measured with an ocular micrometer in a stereoscopic microscope (Wild M8). The following measurements were made: notochord length (L_N), tip of snout to tip of notochord on small larvae prior to flexure; standard length (L_S), tip of snout to base of caudal complex on larger larvae in which flexion of notochord has occurred.

Staining for bone and cartilage was carried out on whole mounts using a modification of the method described by Klymkowsky & Hanken (1991). In brief, specimens were rehydrated through a graded series of methanol (70%-25%), followed by two washes (of 20-30 min each) in Tris-buffered saline (pH 7.6). Specimens were stained with Alcian blue 8GX (0.02% in 70% alcohol and 30% glacial acetic acid) and macerated using a 1% aqueous solution of KOH until skeletal elements were clearly visible. Then they were stained with Alizarin red S (stock solution: 1% Alizarin red in 1% KOH). Staining time was variable and depended on the size of the specimen. A total of 164 specimens ranging from 10 DPH (3.1 mm L_N) to 70 DPH (16.0 mm L_S) were observed (Table I). The nomenclature proposed by Matsuoka (1987) is used throughout this chapter.

Staining for bone-specific alkaline phosphatase was carried out using a modification of the Gomori technique (Matsuzawa & Anderson, 1971; Salomon, 1974). β-glycerophosphate was included in the incubation buffer to promote the precipitation of calcium phosphate (Ingleton *et al.* 1983) and silver nitrate was used to visualise insoluble phosphate in bone (Matsuzawa & Anderson, 1971; Salomon, 1974).

Table I. Number of sea bream larvae studied grouped by length.

Length (L_N,L_S - mm)	Number of larvae examined
3.1 - 3.8	10
4.0 - 4.9	20
5.0 - 5.9	20
6.0 - 7.5	20
7.6 - 8.6	20
8.9 - 9.7	16
9.9 - 10.5	15
10.9 - 11.5	15
12.0 - 13.6	15
14.6 - 16.0	13

L_N = 3.1 - 5.6 mm; L_S = 5.7 - 16.0 mm

RESULTS

It was found that the stage of development of cartilage and bone was related more closely to length than to age, although the overall pattern of development did not vary between specimens.

Vertebral column and associated bones

Development typically commenced in four areas of the vertebral column of sea bream: (i) posteroventrad (hypural 1); (ii) ventrad at the centre (anterior haemal arches); (iii) dorsad at the centre (neural arches); and (iv) anterodorsad (neural arches and spines of future centra).

Prior to notochord flexion, which occurred at 5.7-6.0 mm L_S, the first cartilaginous element observed was hypural 1 at 4.1 mm L_N. By 4.4 mm L_N cartilaginous second and third neural arches and eight haemal arches were

visible [Fig. 1 (a)]. Each neural arch consisted of two rod-like structures, one on the right and one on the left laterodorsal sides of either the notochord or spinal cord, and each haemal arch consisted of two small cartilage elements on the right and left lateroventral sides of the notochord. In specimens of 4.9 mm L_N, 10/11 cartilaginous haemal arches and 12/13 neural arches were observed. By 5.3 mm L_N, prior to the onset of ossification, it was possible to distinguish the following cartilaginous structures: all 23 neural arches, all 13 haemal arches and two of the four pairs of parapophyses. Neural arches 1-4 and 15-23 were formed on the notochord and elongated dorsally, while neural arches 5-14 appeared on the dorsal side of the spinal cord and elongated ventrally [Fig. 1 (b)]. The formation of the cartilaginous haemal arch proceeded posteriorly with the exception of haemal arch 13 which was observed prior to haemal arches 10, 11 and 12. The formation of cartilaginous parapophyses proceeded in an anterior direction and by 7.0-7.5 mm L_S four pairs were visible.

Initiation of ossification occurred at 5.7-6.0 mm L_S when the cartilaginous ontogeny of the vertebral column was completed [Fig. 1 (d)], although numerous peripheral structures (such as the pleural ribs) were still forming. Initiation of ossification was coincident with dorsal flexion at the posterior end of the notochord.

Ossification of the vertebral column of sea bream occurred in a sequential manner:

(i) dorsoanteriorly, at 6.0 mm L_S, the cartilaginous neural arches and the centra were the first structures to ossify [Fig. 1 (d)]. Commencing at the second neural arch and proceeding in a posterior direction, the ossification of the centra proceeded at the same rate as that of the first seven to eight neural arches and then ossification of centra proceeded more rapidly;

(ii) ventrad at the centre [Fig. 1 (e)], at 7.0-7.5 mm L_S, four pairs of cartilaginous parapophyses were clearly distinguishable. Their ossification proceeded anteriorly from the fourth to the first;
(iii) posteriorly, at 7.1 mm L_S, the hypural complex and urostyle (centrum 24) ossified at the same time as centra 14 and 15 [Fig. 1 (e)].

The ossification of centrum 1 and neural arch 1, at about 6.6 mm L_S occurred after the formation and ossification of centra 6/7 and neural arch. The ossification of the first haemal arch occurred after the ossification of the respective centra. The onset of ossification was initiated at the centre of each neural and haemal arch and subsequently proceeded in a dorsal and ventral direction. At 9 mm L_S, centrum 21 was present [Fig. 1 (f)] and at about 9.4 mm L_S, the last centrum (23rd) appeared. The ossification of centra 1-21 was initiated at the base of the respective neural arches and subsequently proceeded in a ventral and lateral direction forming a bony ring around the notochord. The exception to this was centra 22 and 23 where ossification initiated at the base of the respective haemal arches and proceeded in a dorsal and lateral manner.

At 9-9.4 mm L_S the cartilaginous pleural ribs appeared, they formed and ossified in an anterior to posterior direction from the third vertebra. Ossified pleural ribs first appeared in specimens of 10.1 mm L_S and by 16.0 mm L_S five pairs were present (Fig. 2). Ossification of the three pairs of epineural bones (previously called dorsal ribs in percoids; Patterson & Johnson, 1995) followed a similar anterior to posterior sequence of development that commenced in specimens of 10.4 mm L_S.

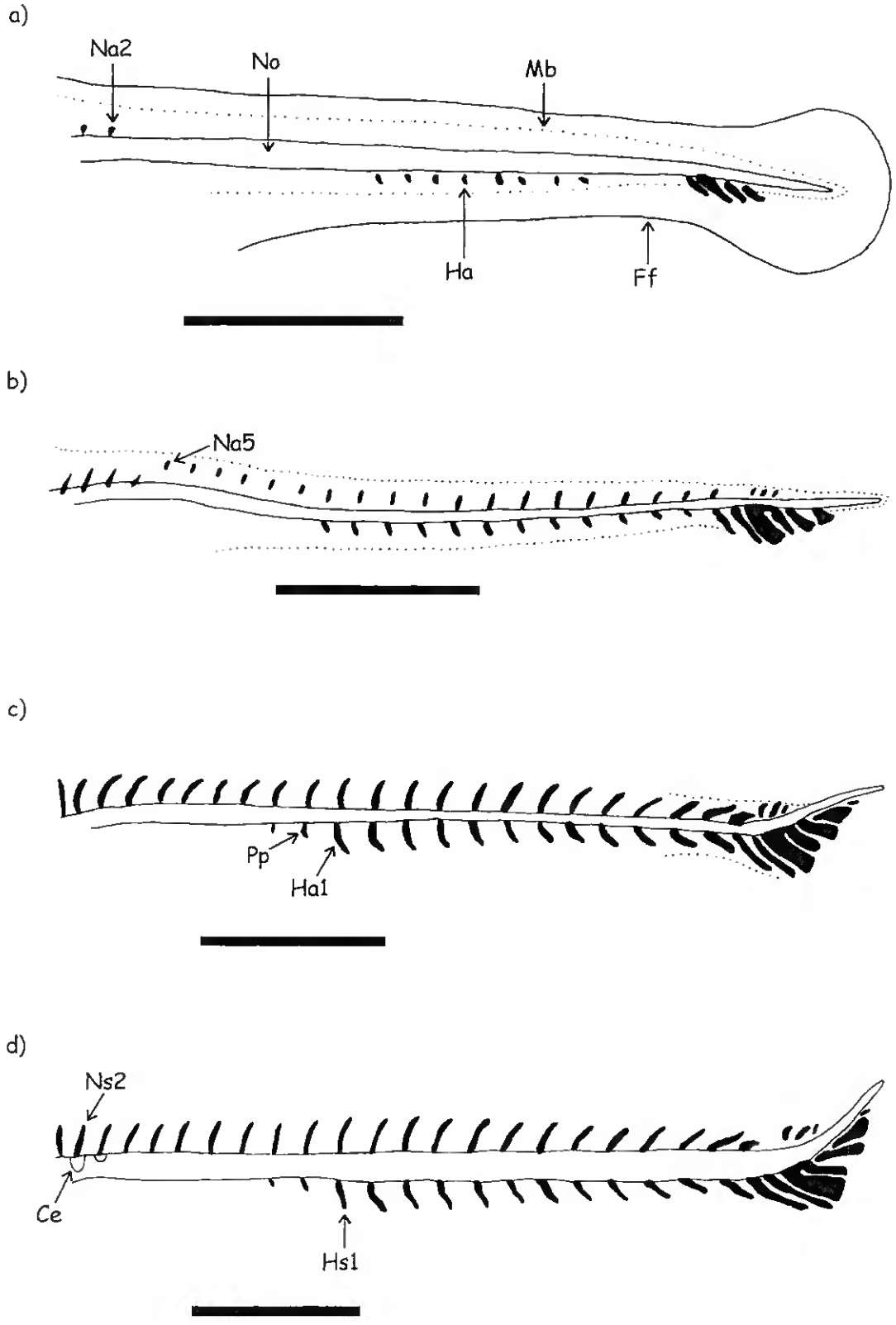

Figure 1. (a-d).

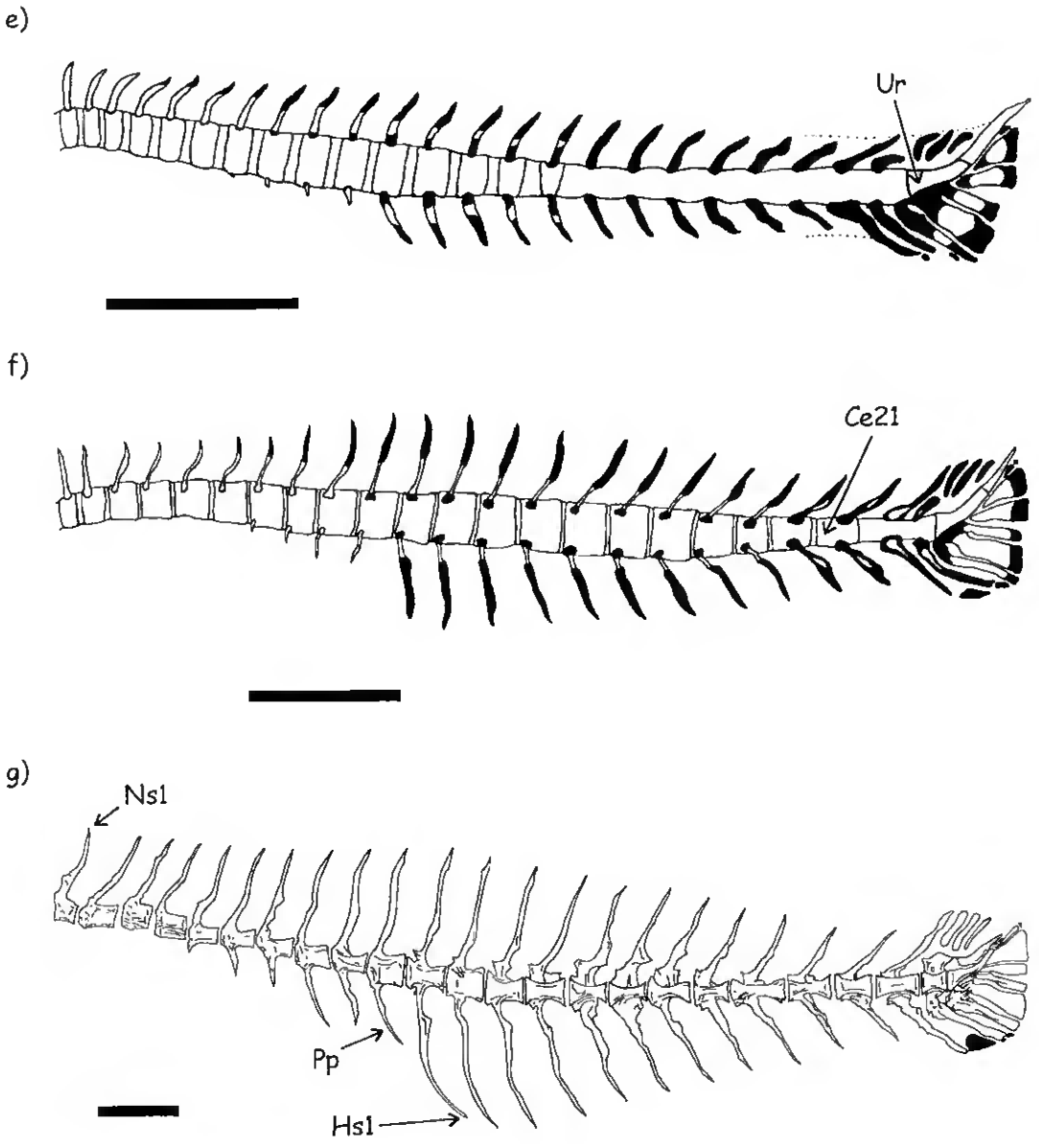

Figure 1. (continued).

Figure 1. Camara lucida drawing charting the development of the principal structures of the vertebral column in cultured sea bream. The sequence of diagrams shows the transition from cartilaginous (dark areas) to ossified (open areas) structures. (a) 4.4 mm L_N, the notochord (No) is straight, neural (Na) and haemal arches (Ha), margin of the body (Mb) and fin fold (Ff) are indicated; (b) 5.1 mm L_N, note that 1-4 and 15-23 neural arches are present on the notochord and elongated dorsally, while 5-14 neural arches appeared on the dorsal side of the spinal cord and elongated ventrally; (c) 5.7 mm L_S, dorsal flexion has occurred, the first parapophyses (Pp) are present and the full complement of neural and haemal arches have developed; (d) 6.0 mm L_S, note initiation of ossification of the centrum (Ce) and neural (Ns) and haemal spines (Hs) are visible; (e) 7.1 mm L_S the urostyle (Ur) is present; (f) 9.0 mm L_S, centrum 21 is indicated; (g) 16.0 mm L_S, ossification of all structures is complete with the exception of accessory cartilages. The dorsal ribs and epineural bones are not indicated in (f) and (g) but are shown in Fig. 2. Scale bars indicate 1.0 mm. See Fig. 3 for developmental sequence of caudal fin.

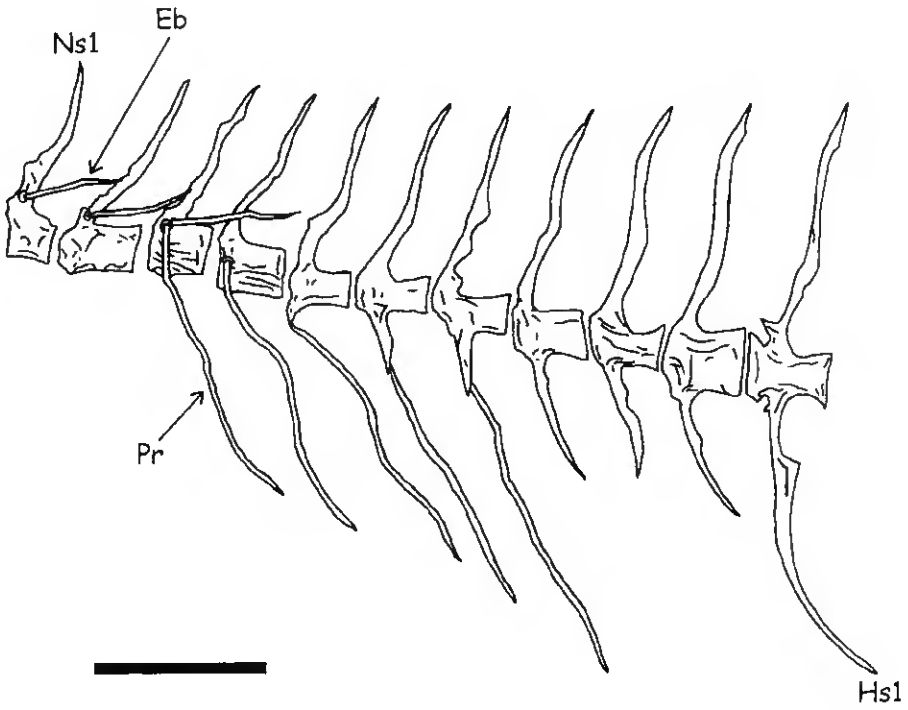

Figure 2. Camera lucida drawing giving details of the ventrolateral abdominal region of the vertebral column illustrating the disposition of the pleural ribs (Pr) and epineural bones (Eb) in sea bream, 16.0 mm L_S. Open areas, ossified structures. For other abbreviations see Fig. 1. Scale bar indicates 1.0 mm.

Caudal fin complex

No caudal elements were detected in larvae ranging from 3.1 to 3.8 mm L_N. At 4.1 mm L_N the caudal complex started to develop as a small cartilage bud (hypural 1) ventral to the unflexed notochord [Fig. 3 (a)]. The parhypural and hypural cartilages 2 and 3 were successively formed at 4.4 mm L_N [Fig. 3 (b)], followed by hypural cartilage 4 at 4.9 mm L_N [Fig. 3 (c)] and hypural cartilage 5 at 5.7 mm L_N [Fig. 3 (e)]. Three epurals formed, the central epural appearing as cartilage at 4.9 mm L_N, just before the anterior and posterior epurals were chondrified at about 5.1mm L_N [Fig. 3 (d)]. By 14.7 mm L_S all three epurals were ossified.

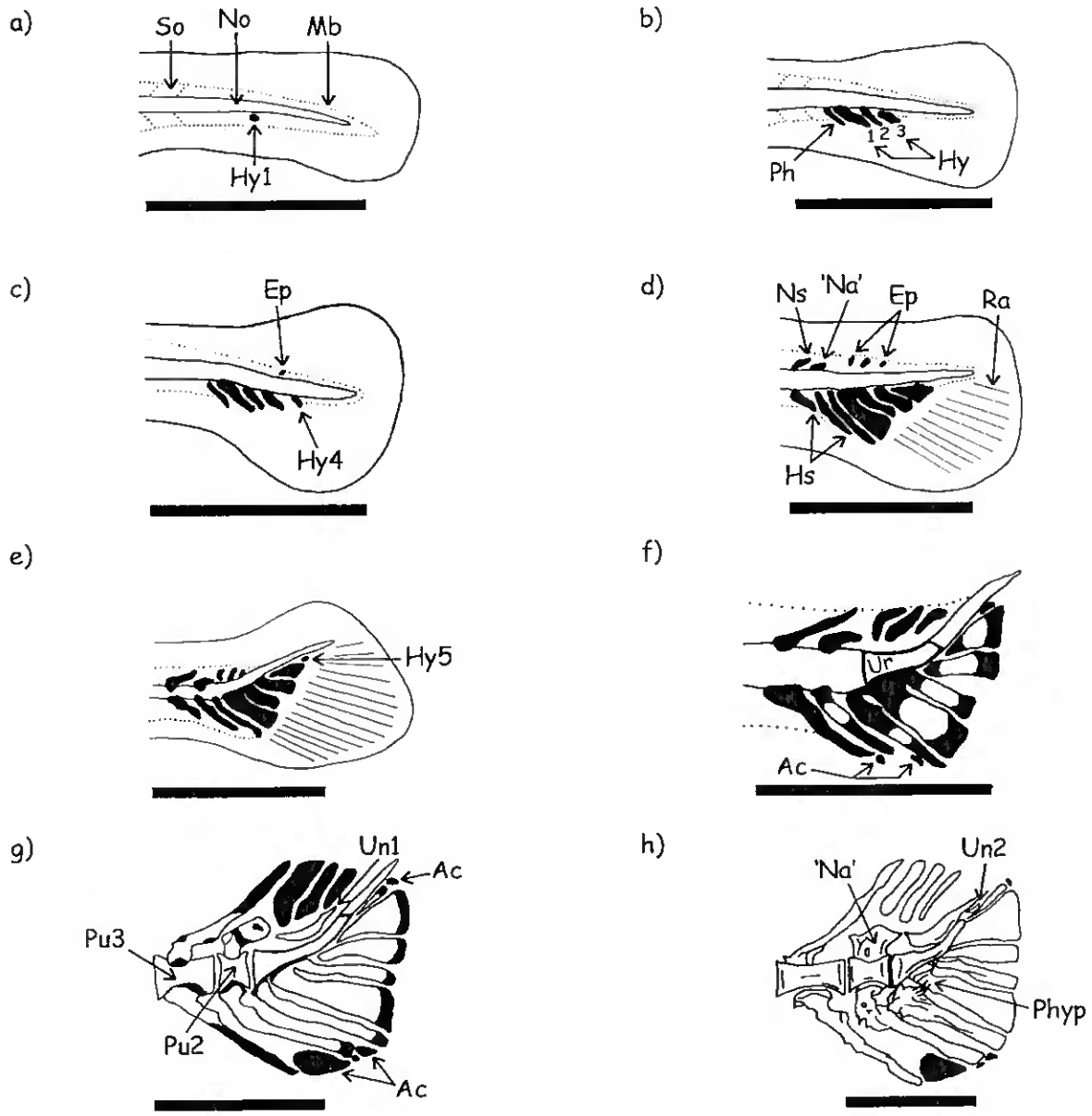

Figure 3. Camera lucida showing the principal phases of development of the caudal skeleton and accessory cartilages in sea bream. Dark areas, cartilaginous structures. Open areas, ossified structures. (a) 4.1 mm L_N, hypural 1 (Hy1) is visible, the somites (So), body margin (Mb) and notochord (No) are also indicated; (b) 4.4 mm L_N, note the presence of parhypural (Ph) and hypurals 1, 2 and 3; (c) 4.9 mm L_N, hypural 4 is present and the first of the three epurals (Ep) is indicated; (d) 5.1 mm L_N, the three epurals are present as is the neural arch (Na), "specialised" neural arch ("Na") and haemal spines (Hs), the rays (Ra) of the caudal fin are also indicated; (e) 5.7 mm L_S, hypural 5 has developed; (f) 7.1 mm L_S, the urostyle (Ur) and the first two accessory cartilages (Ac) are present; (g) 10.4 mm L_S, uroneural 1 (Un1), preural centra 2 and 3 (Pu2 and Pu3) and further acessory cartilages have developed; (h) 16.0 mm L_S, uroneural 2 and parhypurapophyses (Phyp) are present. The rays are not indicated in (f), (g) and (h). For meaning of other abbreviations see Fig. 1. Scale bars indicate 1.0 mm.

Early in the development of the caudal fin complex no cartilaginous junctions were observed between the parhypural, hypural 1 and 2. Later in development, at 5.1 mm L_N, hypurals 1 and 2 fused proximally by a temporary cartilaginous bridge and subsequently, at about 7.1 mm L_S, joined with the parhypural [Fig. 3 (d), (e) and (f)]. All hypurals were once again separated from one another and the parhypural when ossification was completed. Ossification of parhypural and hypurals 1-4 began at about 7.1 mm L_S [urostyle formation occurred at this time; Fig. 3 (f)], while ossification of hypural 5 commenced at 9.7 mm L_S.

The rudiments of the principal rays developed at about 5.1 mm L_N [Fig. 3 (d)]. Ossification of the rays was completed by 7.0 mm L_S and proceeded from their junction with hypurals 2 and 3 in a posterior direction to the periphery of the ray. Additional rays appeared both dorsal and ventral to the first group and underwent the same process of ossification. Seventeen principal rays (9+8) formed and were supported by the hypurals, parhypural, and some accessory cartilages. The rays were segmented posteriad but unsegmented anteriad.

The first uroneural began to form at about 10.4 mm L_S [Fig. 3 (g)] and uroneural 2 appeared at 16.0 mm L_S [Fig. 3 (h)] when all the elements of the caudal complex were ossified except the accessory cartilages. In the present study, four accessory cartilaginous structures were identified. The first appeared at 6.1 mm L_S between the tips of haemal spines 12 and 13; slightly later another structure was observed between the tip of haemal spine 13 and parhypural; at 8.1 mm L_S a third structure appeared posterior to the tip of hypural 5 followed by the fourth and last structure posterior to the tip of haemal spine 13. The caudal fin was the first fin to develop rays in sea bream.

DISCUSSION

This is the first time that the ontogeny of cartilaginous and ossified structures has been reported in cultured larvae of sea bream. Several other Sparidae have been studied, including *Archosargus rhomboidalis* (Houde & Potthoff, 1976) and *Pagrus major* Temminck & Schlegel (Matsuoka, 1987) which show some similarities in cartilage and bone formation to sea bream. Little or no information exists to explain the regulation of cartilage and bone deposition in fishes and several complex patterns of deposition have been described for different taxa. Potthoff *et al.* (1986) defined several general sequences of early development and addition of the cartilaginous neural and haemal arches and spines along the notochord of scombroid fish, e.g. (i) anteriorly dorsad, centrally dorsad, centrally ventrad and posteriorly ventrad with a subsequent merger of the initial areas; (ii) anteriorly dorsad, centrally ventrad, and posteriorly ventrad; then addition is from the anterior in a posterior direction; and (iii) anteriorly dorsad and centrally ventrad with addition in a posterior direction. The sequence of development encountered in the present study of sea bream (Fig. 4) resembles most closely pattern 1, and analysis of the sequence of cartilage development in other taxa suggest they follow to a greater or lesser extent one of these sequences. A greater understanding of the development of cartilage and bone will be important in determining factors and conditions that perturb these processes.

The histological methods used in the present study to demonstrate cartilaginous and bony structures are not entirely specific. For example, alizarin red S stains areas of calcium salt deposition (Humason, 1962; Pearse, 1985) but it is not a specific stain for hydroxyapatite, the main mineral phase of bone (Zerekh, 1993), and may indicate merely deposition of calcium salts in

nonossifying embryonic connective tissue. To overcome this problem an assay for bone-specific alkaline phosphatase activity was also used, and its colocalisation with alizarin red staining and the general morphology of the tissue permitted the identification, of bone with more confidence than with alizarin red staining alone.

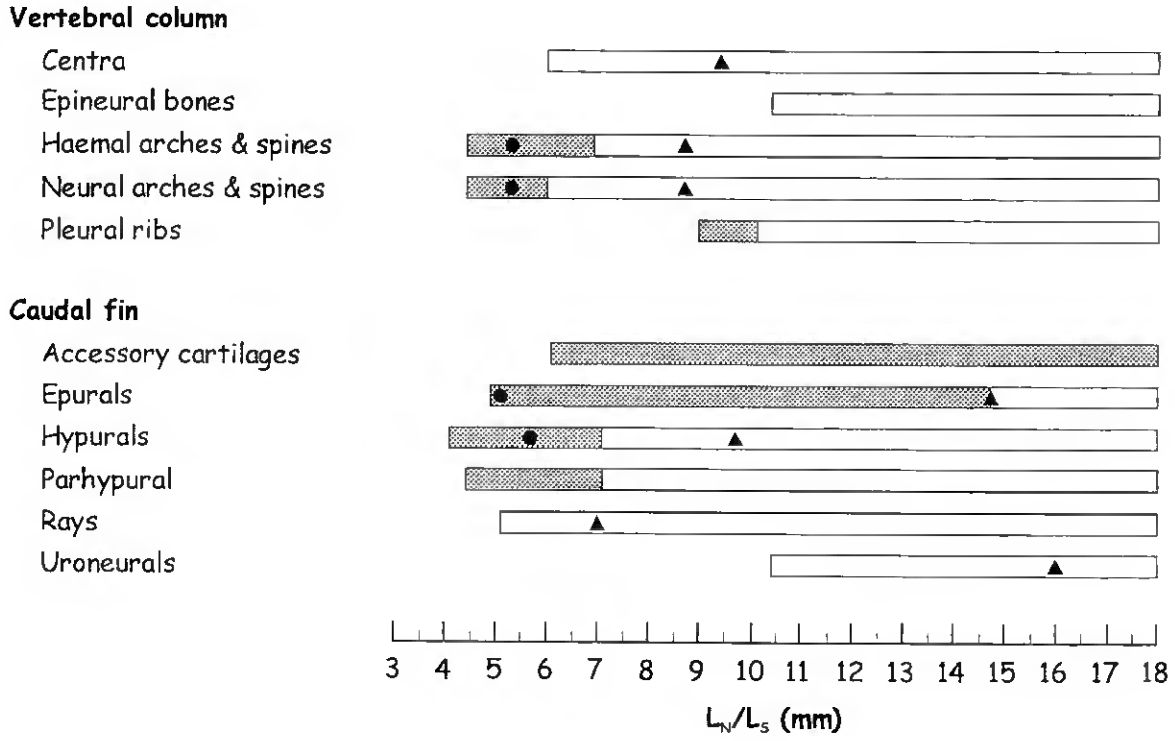

Figure 4. Summary of the ontogeny of the vertebral column and caudal fin complex in cultivated sea bream (0-70 DPH). Stippled areas, cartilage; Open areas, bone; ●, when the full complement of cartilage structures has developed; ▲, when structures are fully ossified.

Prior to the appearance of detectable bony elements, the body axis of sea bream is straight, and flexion of the notochord proceeds shortly prior to ossification when most body structures are composed of flexible cartilage. The first cartilaginous structures to develop anteriorly are the jaws and branchial arches (not discussed in the present article), shortly after cartilaginous hypural 1 and 2 are visible. The early formation of the head skeleton and the caudal

elements may be of fundamental importance for feeding and survival in the period following yolk-sac absorption. Comparison of a range of different teleost larvae shows that flexion occurs within a fairly restricted size range (4.4-6.2 mm). The significance of this observation is uncertain but may be related to the fundamental importance of the caudal elements for manoeuvrability and therefore successful feeding and survival after hatch.

The cartilaginous neural and haemal arches in sea bream developed in a similar way to that reported in *A. virginicus* (Potthoff *et al.*, 1984) and *S. heterolepis* (Potthoff *et al.*, 1986), where the cartilaginous neural arches develop in both anterior and central regions of the notochord and the haemal arches arise initially only in the central region of the notochord. Development in sea bream was, however, more similar to that of the sparid *A. rhomboidalis* (Houde & Potthoff, 1976)), where the first four neural arches arise in the most anterior area of the notochord and the first haemal arch at centrum 7/8, and *P. major* (Matsuoka, 1987) where the first neural arches arise from the area of centra 2 and 3 and the first haemal arch at centra 11. However, the sequence of formation of the cartilaginous neural and haemal arches is generally quite variable from species to species. For example, in *Clupea pallasii* Valenciennes the neural and haemal arches develop initially anteriorly from the penultimate centrum and, from shortly afterwards, development also proceeds posteriorly (Gwyn, 1940). In *Xiphias gladius* L. the neural arches developed antero-dorsad, posteriorly and the haemal arches developed ventrad at the centre, in an anterior and posterior direction (Potthoff & Kelley, 1982; Potthoff *et al.*, 1986). The diversity of patterns described for the development of the cartilaginous neural and haemal arches in teleosts may indicate that their control mechanisms are complex.

The formation of the centra and their subsequent ossification also is characterized by considerable variation among species. In Perciformes such as *S. japonicus* and *Thunnus atlanticus* (Lesson) (Potthoff *et al.*, 1986), dorsal and ventral saddle-shaped ossifications grow toward one another and fuse subsequently on the lateral part of the notochord, whereas in sea bream ossification of the centra proceeds ventrally from an initial ossified area dorso-laterally. The only exception to this pattern in sea bream occurs at centra 22 and 23, where ossification is initiated ventro-laterally at the base of the haemal arches and proceeds in a dorsal direction. Elongation and subsequent constriction of the centra proceeds in a posterior direction, similar to that described in *P. major* (Matsuoka, 1987).

The formation and ossification of the caudal fin in sea bream (Fig. 4) is similar to that described for Clupeiformes, beginning with the appearance of the first hypural and terminating with the ossification of the epural cartilages (Gwyn, 1940; Houde *et al.*, 1974; Johnson & Loesch, 1983). Also, the temporary fusion of hypural elements and their subsequent separation is similar to what has been observed in *Harengula jaguana* Poey (Houde *et al.*, 1974), *Alosa sapidissima* (Wilson) (Johnson & Loesch, 1983) and *E. japonicus* (Balart, 1995), but differs from that described in Pleuronectiformes, where fusion of hypural elements occurs early during caudal development (Balart, 1985).

The delay of completion of the vertebral column is due mainly to the late appearance of the pleural ribs and epineurals. These two skeletal groups increase the swimming activity and manoeuvrability of fishes with growth by increasing the rowing resistance to water. Relatively little information exists about the molecular regulation of the pattern and timing of cartilage differentiation and bone formation and how this interacts with physiological parameters. It is likely that some of the genes responsible for both normal and

abnormal phenotypes of zebrafish *Danio rerio* (Hamilton) (Neuhauss *et al.*, 1996; Schilling *et al.*, 1996) are implicated also in sea bream development. Studies of these genes will be important in understanding how they influence development and how aquaculture practices may affect their expression.

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CHAPTER 3

DEVELOPMENT OF THE PECTORAL, PELVIC, DORSAL AND ANAL FINS IN CULTURED SEA BREAM (*Sparus aurata*)

Co-author

Deborah M. Power

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DEVELOPMENT OF THE PECTORAL, PELVIC, DORSAL AND ANAL FINS IN CULTURED SEA BREAM (*Sparus aurata*)

ABSTRACT

The pectoral fin girdle was the first element of the fins to develop in *Sparus aurata*. By 3.1 mm L_N (notochord length) the cleithrum was ossified and the cartilaginous caracoid-scapula was present. The fin was fully developed at 11.6 mm L_S (standard length) and by 16.0 mm L_S most elements of the fin were ossified. The pelvic fins were the last pair to develop and rudiments of these were first detected at 7.9 mm L_S. The pelvic fin and girdle were completely formed and ossified at 16.0 mm L_S. The development of dorsal and anal fins began at *c.* 6.5-7.0 mm L_S with the formation of 10 cartilaginous dorsal proximal radials and 8 cartilaginous ventral proximal radials. The three cartilaginous predorsals (supraneurals) appeared at 7.7 mm L_S and the ossification of dorsal and anal proximal and distal radials began, respectively, at 10.5 mm L_S and 11.3 mm L_S. Ossified structures in the fins were also classified according to their origin, as being either dermal or endochondral. Finally the chronology of appearance of fin structures in *S. aurata* was compared with that reported for other Sparidae, Engraulidae and Haemulidae.

Key words: *Sparus aurata*, osteology, fins, fish larvae, ontogeny.

INTRODUCTION

Sea bream (*Sparus aurata*) is an important commercial fish in the Mediterranean and Portugal. The larval development and nutritional requirements of this species have been studied extensively (Villani, 1976; Person-Le Ruyet & Verillaud, 1980; Camus & Koutsikopoulos, 1984; Kadmon *et al.*, 1985; Tandler *et al.*, 1989) but information on cartilage and bone development exist largely for the related, wild species, *Archosargus rhomboidalis* (L.) (Houde & Potthoff, 1976) and red sea bream, *Pagrus major* (Temminck & Schlegel) (Matsuoka, 1985, 1987). In Chapter 2 is described the developmental ossification of the spinal column and caudal fin of sea bream and Koumoundouros *et al.* (1997) analysed the normal and abnormal osteological development of this fin in fry. The incidences of body deformation and abnormalities, which appear to be associated with alterations in the skeleton, are reported to be much higher in cultured fish than in wild fish (Paperna, 1978; Barahona-Fernandes, 1982; Francescon *et al.*, 1988; Santamaria *et al.*, 1994). Lack of swim bladder caused by inappropriate zootechnical conditions leads to lordosis in sea bream and sea bass (Chatain, 1994). The origin of other developmental abnormalities such as deformed jaws, lack of jaws and operculum deformities (Barahona-Fernandes, 1982) are largely unknown. The effect on osteological development of selective breeding and the environment in fish culture, is unclear and more studies of skeletal development in cultured fish are required.

The origin of ossified tissue in fish is complex but can be subdivided into two main groups, intramembranous (achondral) which occurs in the absence of a cartilage matrix and gives rise to dermal bone (Cormack, 1984; Junqueira *et al.*, 1995) or cartilage replacement bone (which includes parachondral, perichondral

and endochondral), in which a matrix of cartilage is progressively substituted by bone.

The objective of the Chapter 3 is to characterise normal developmental patterns of cartilage and bone in the fins of a cultured marine teleost, sea bream and compare this with the related wild species. The origin of bony structures in the fins of sea bream was also studied.

MATERIALS AND METHODS

Sea bream (*Sparus aurata*) larvae were hatched and reared in 0.2 m^3 conical tanks with a continuous flow of gently aerated sea water. Hatching occurred 40 h after fertilisation. The larvae were fed on rotifera *Brachionus plicatilis* from day 4 posthatch when the yolk sac was consumed. *Artemia* sp. nauplii, were introduced into the diet from day 15 until day 40 when dry food was introduced gradually. Light conditions followed a cycle of 12h light:12h dark and the ambient water temperature was 19 $\pm$ 1 ^{o}C.

Larvae were sampled on alternate days from day 1 (2.7 mm notochord length, L_N) to day 100 (20.4 mm standard length, L_S) anaesthetized in a 0.01% aqueous solution of MS-222 (ethil *m*-aminobenzoate), fixed overnight at 4 °C in 2% paraformaldehyde (PFA) solution, washed and stored in 70% methanol (Chapter 2).

Larvae (n = 186, Table I) were measured with an ocular micrometer in a stereoscopic microscope (Wild M8). The following measurements were made: notochord length, tip of snout to tip of notochord on small larvae prior to flexure; standard length, tip of snout to base of caudal complex on larger larvae

in which flexion of notochord had occurred. Whole-mount staining of bone and cartilage was carried out using a modification of the Alcian blue/Alizarin red method and alkaline phosphatase as described in Chapter 2.

Drawings of stained larvae were made using a Wild M8 stereoscopic microscope with a camera lucida attachment.

TABLE I. Length frequency distribution of sea bream larvae used in the study.

Length (L_N, L_S - mm)	Number of larvae examined
3.0 - 3.9	10
4.0 - 4.9	20
5.0 - 5.9	20
6.0 - 6.9	12
7.0 - 7.9	17
8.0 - 8.9	13
9.0 - 9.9	17
10.0 - 11.9	27
12.0 - 13.9	15
14.0 - 15.9	12
16.0 - 17.9	11
18.0 - 19.9	7
20.0 - 20.9	5

L_N = 3.1 - 5.6 mm; L_S = 5.7 - 20.9 mm

Terminology used throughout the chapter to describe fin structures, was based upon Houde & Potthoff (1976), Matsuoka (1985, 1987) and Balart (1995).

RESULTS

Development of pectoral fin skeleton

The pectoral girdle was the first of the fin supports to develop in sea bream and at 3.1 mm L_N the needlelike cleithrum, the cartilaginous fin plate and coracoid-scapula complex were present [Fig. 1 (a)]. By 4.7-5.1 mm L_N the cartilaginous fin plate had separated from the coracoid-scapula complex and a crevice had developed in the anterior mid-region of the fin plate [Fig. 1 (b)]. The anterior and posterior process of the coracoid elongated anteriorly and posteriorly respectively, with the latter being more pronounced; simultaneously the scapula elongated in a dorsal direction [Fig. 1 (b)-(d)].

At 5.7 mm L_S a small cartilage, propterygium, was formed above the fin plate cartilage. Cartilaginous distal radials and rays were visible as was the scapular foramen and the lower postcleithrum ossified [Fig. 1 (c)]. Between 6.6 and 7.1 mm L_S the second and third crevices of the fin plate were apparent above and below the first, respectively, and the supracleithrum and posttemporal were detected as ossified structures [Fig. 1 (d)]. Ossification of the upper postcleithrum was apparent between 7.4 and 7.9 mm L_S, whilst four actinosts, nine to 12 cartilaginous distal radials and eight to 10 ossified rays were present in the fin [Fig. 1 (e)-(f)]. At 8.6 mm L_S it was possible to see 11 ossified rays and all the cartilaginous distal radials [Fig. 1 (g)]. The coracoid and scapula ossified simultaneously at 10.0 mm L_S and the actinosts ossified at 10.5 mm L_S in a dorso-ventral direction [Fig. 1 (h)]. The ossified upper and lower supratemporals appeared at 11.4-11.6 mm L_S [Fig. 1 (i)]. The pectoral fin complex was completely formed and almost completely ossified by 16.0 mm L_S. Only the posterior tip of the actinost and a band running across the mid region of the scapula and the distal radials remained as cartilage [Fig. 1 (j)].

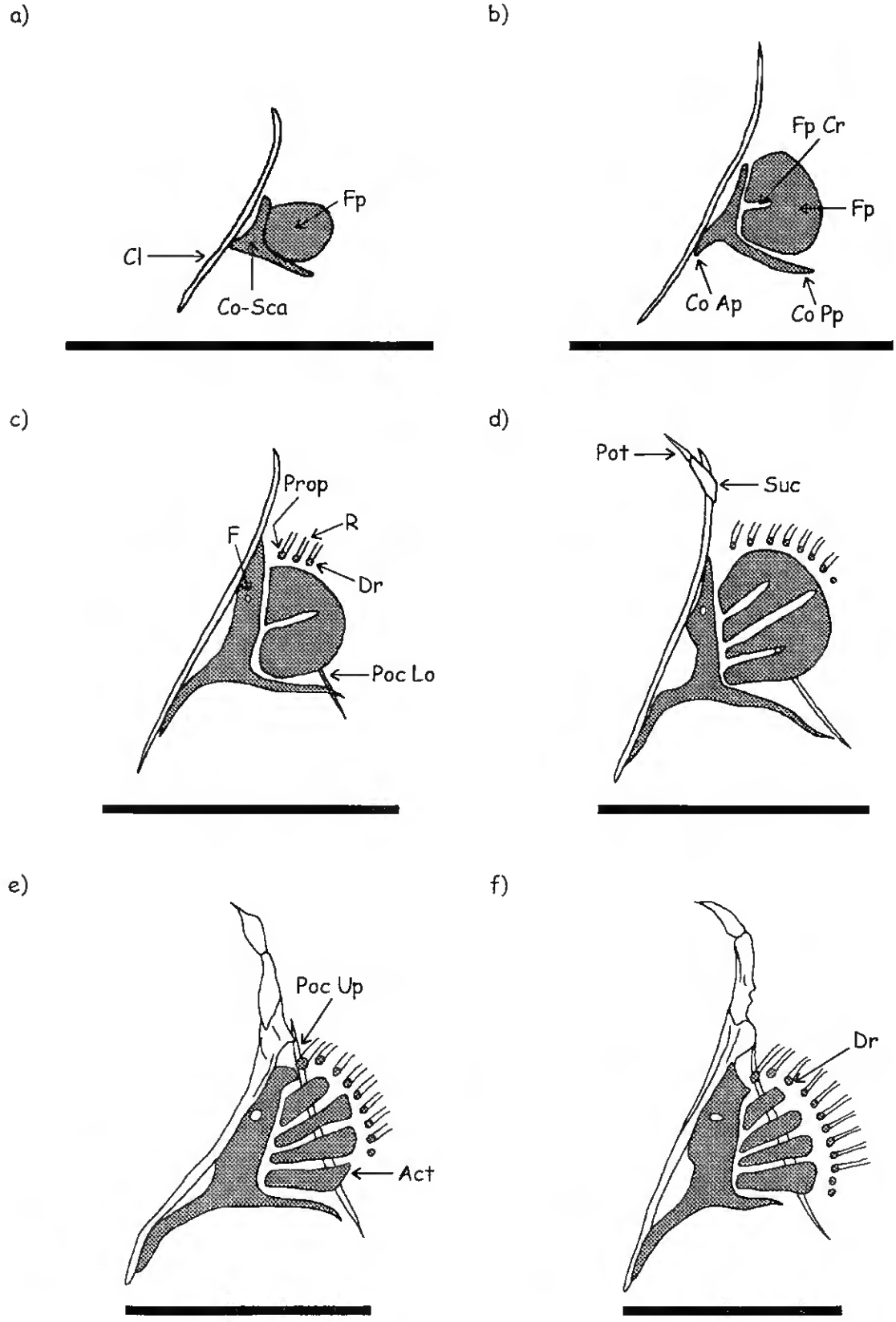
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Figure 1. (a-f).

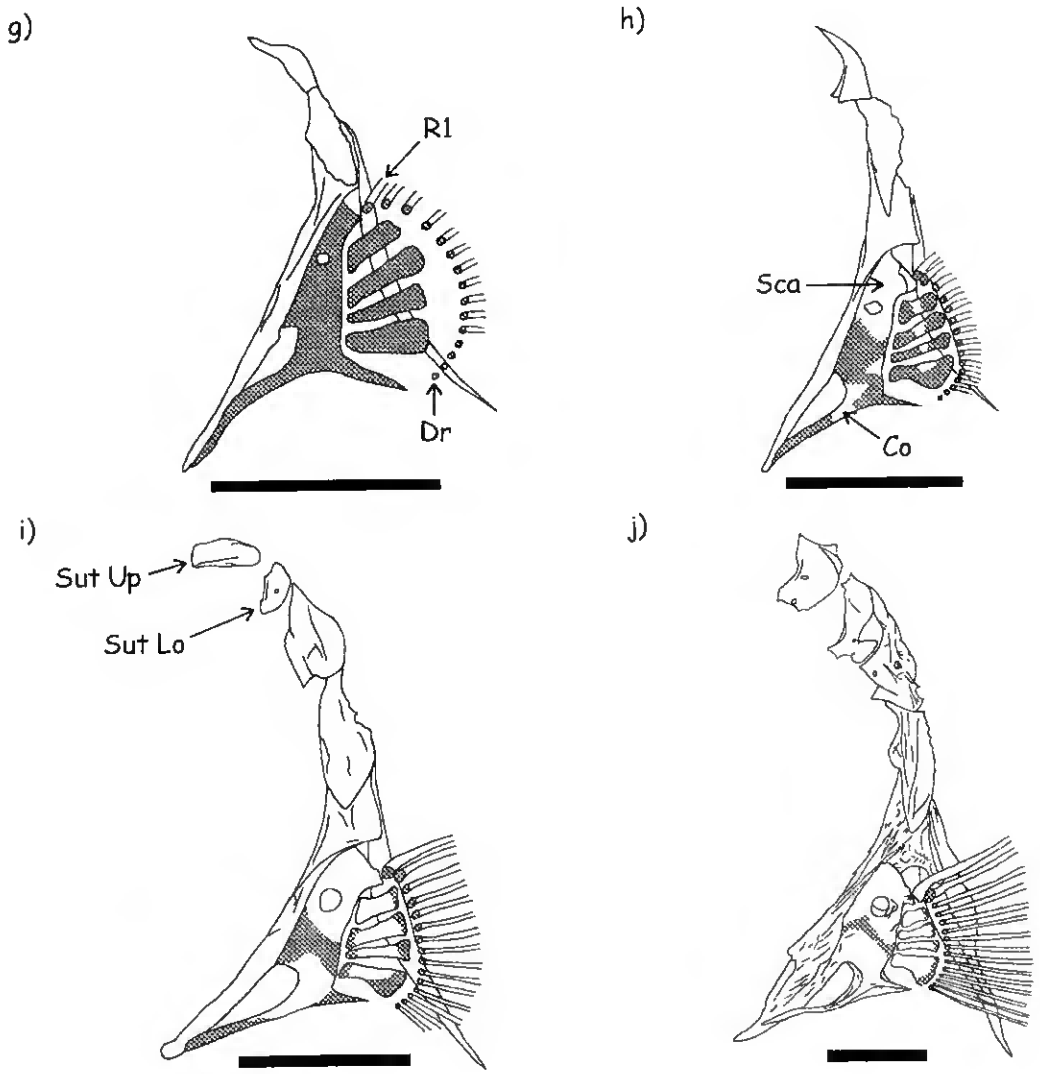

Figure 1. (continued).

Figure 1. Development of pectoral fin supports in sea bream. Lateral view. (a) 3.1 mm L_N, the cartilaginous fin plate and the cartilaginous coracoid-scapula complex are continuous; (b) 4.9 mm L_N, the appearance of the first crevice in the anterior mid-region of the fin plate and the separation of the cartilaginous fin plate from the cartilaginous coracoid-scapula occur; (c) 5.7 mm L_S, the first distal radials and rays are present; (d) 7.1 mm L_S, the second and third crevices become apparent in the fin plate, dorsal and ventral to the first crevice; (e) 7.7 mm L_S, the fin plate is completely divided into four actinosts; (f) 7.9 mm L_S; (g) 8.6 mm L_S; (h) 10.5 mm L_S, note the onset of ossification of the coracoid-scapula and actinosts; (i) 11.6 mm L_S; (j) 16.0 mm L_S, all elements, except the distal radials and a small portion of the scapula are ossified. Grey areas, cartilaginous structures. Open areas, ossified structures. Scale bars indicate 1.0 mm. Act, Actinost; Cl, cleithrum; Co, coracoid; Co Ap, coracoid anterior process; Co Pp coracoid posterior process; Co-Sca, cartilaginous coracoid-scapula; Dr, distal radial; F, scapular foramen; Fp, cartilaginous fin plate; Fp Cr, fin plate crevice; Poc Lo, lower postcleithrum; Poc Up, upper postcleithrum; Pot, posttemporal; Prop, propterygium; R, soft ray; Sca, scapula; Suc, supracleithrum; Sut Lo, lower supratemporal; Sut Up, upper supratemporal.

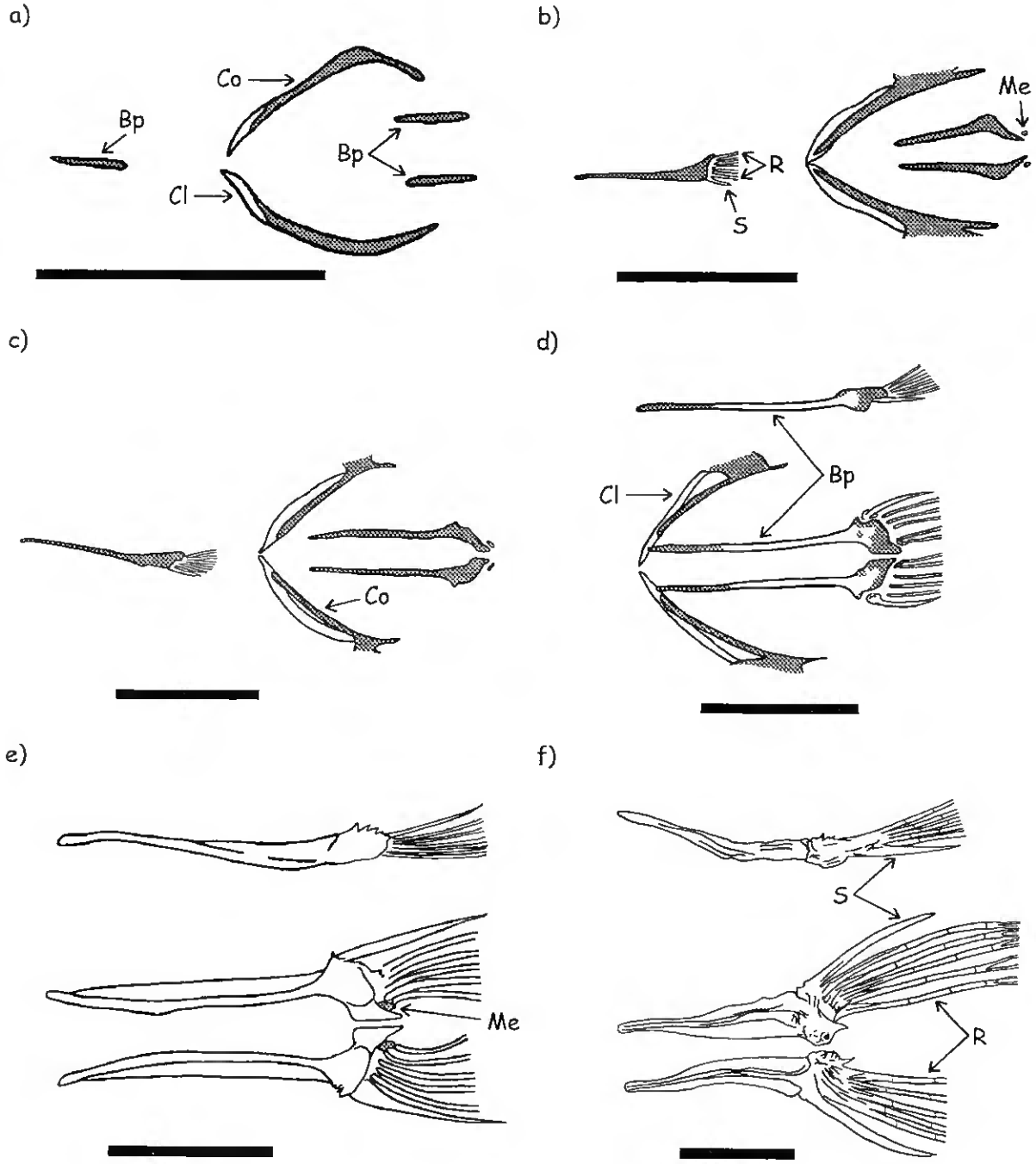

Figure 2. Development of the pelvic fin supports in sea bream. Ventral and lateral view. The spine and rays are not indicated in ventral view of (b) and (c). (a) 7.9 mm L_S, the cartilaginous basipterygium elongates towards the cleithrum; (b) 9.4 mm L_S, the metapterygium appears at the posterior tip of the basipterygium; (c) 11.4 mm L_S, spines and rays ossified; (d) 13.6 mm L_S, the basipterygium reaches the cleithrum; (e) 14.7 mm L_S, basipterygium ossifed; (f) 16.0 mm L_S, development of pelvic fin and girdle completed; note that the rays are segmented and bifurcated. Grey areas, cartilaginous structures. Open areas, ossified structures. Scale bars indicate 1.0 mm. Bp, Basipterygium; Me, metapterygium; S, spine. For meaning of other abbreviations see Fig. 1.

Development of pelvic fin skeleton

The pelvic girdle in sea bream consists of paired basipterygia, each of which supports one spine and five rays.

In sea bream of 7.9 mm L_S the cartilaginous basipterygium, which is located behind the pectoral girdle, gradually elongated in an anterior direction towards the cleithrum [Fig. 2 (a)]. The five rays and spine were visible first at 9.4 mm L_S and a small cartilaginous structure, the metapterygium, appeared next to the posterior tip of the basipterygium [Fig. 2 (b)]. At 11.4 mm L_S the tip of the basipterygium approached the cleithrum [Fig. 2 (c)]. At 13.6 mm L_S the basipterygium reached the cleithrum and was fully ossified in all but the anterior third and base [Fig. 2 (d)]. By 14.7 mm L_S the basipterygium was completely ossified but the metapterygium persisted as cartilage [Fig. 2 (e)] until 16.0 mm L_S when it fused with the ventral basal half of the innermost fin ray and then ossified. The pelvic fin and girdle were completely formed and ossified at 16.0 mm L_S, and the tips of the rays were bifurcated [Fig. 2 (f)].

Development of the dorsal and anal fin skeleton

The dorsal fin of adult sea bream comprises 22 pterygiophores, 11 spines and 13 rays. The anal fin comprises 12 pterygiophores, three spines and 11 rays.

At 6.5-7.0 mm L_S 10 cartilaginous proximal radials were present dorsally [Fig. 3 (a)], between the 11th and 17th neural spines. Ventral to these, were eight cartilaginous proximal radials [Fig. 3 (a)], between the second and seventh haemal spines. The formation of the remaining 12 dorsal proximal radials proceeded both anteriorly and posteriorly relative to the existing elements [Fig. 3 (b)]. Distal radials of the dorsal and anal fins, first appeared at 7.0 mm L_S [Fig. 3 (b)] and by 7.7 mm L_S the full complement of the cartilaginous proximal and

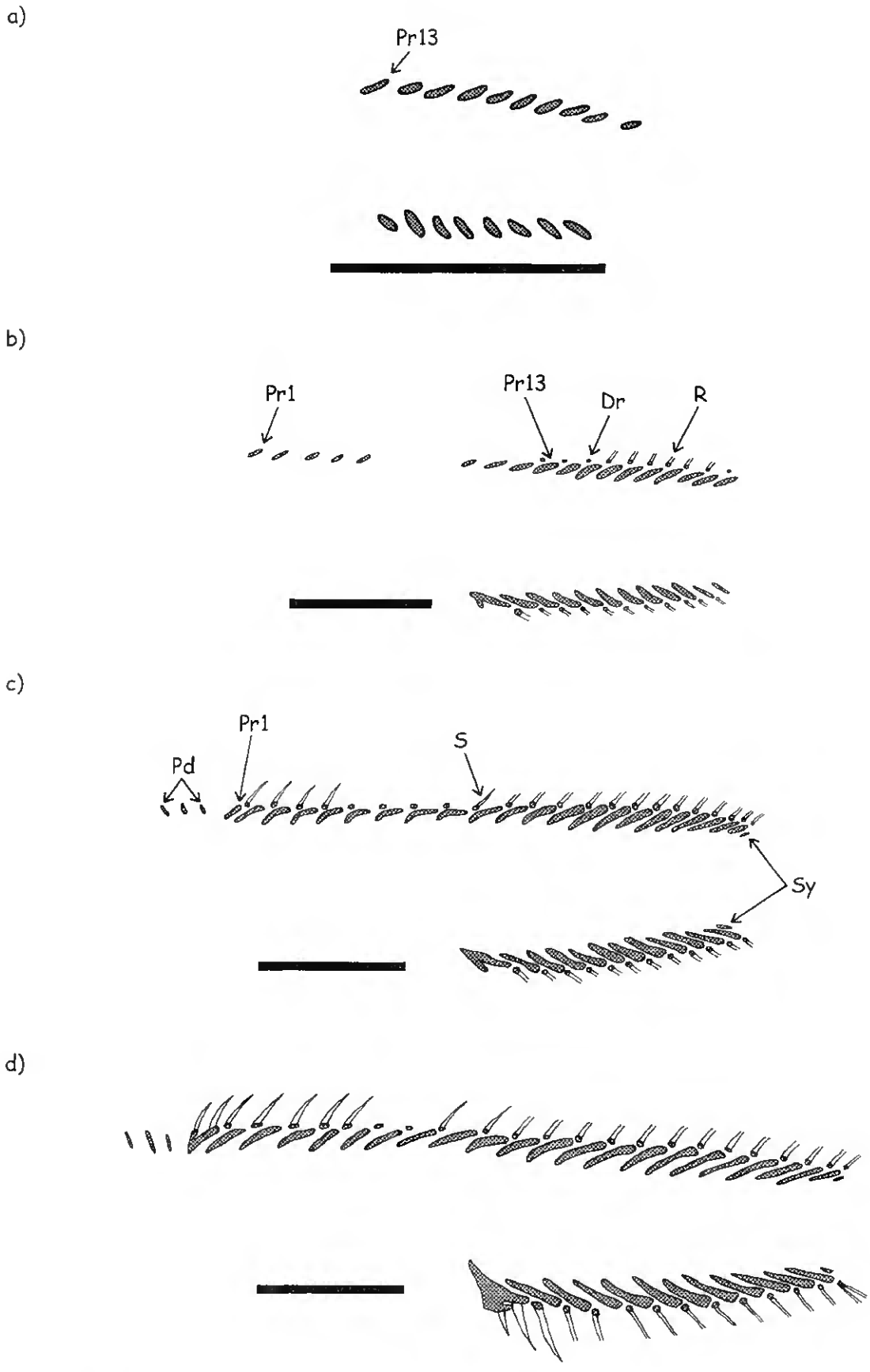

Figure 3. (a-d).

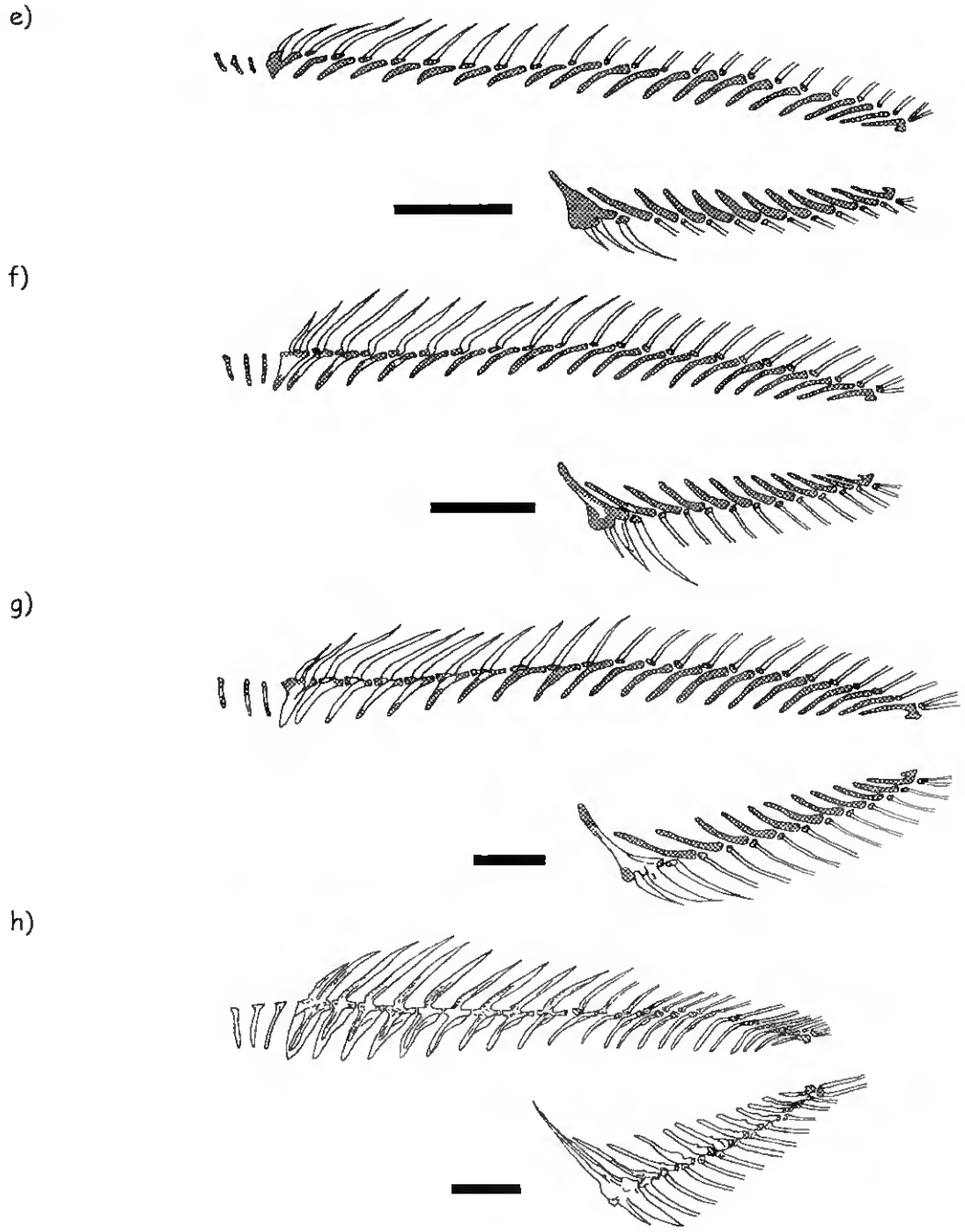

Figure 3. (continued).

Figure 3. Development of dorsal and anal fin supports in sea bream. Lateral view, left side. (a) 6.6 mm L_S, note the first group of dorsal proximal radials and anal proximal radials; (b) 7.0 mm L_S, the second group of dorsal proximal radials appears anteriorly; (c) 7.7 mm L_S, three predorsals and the cartilaginous stay are present; (d) 8.6 mm L_S, all elements of the anal fin supports are present; (e) 9.4 mm L_S, all elements of the dorsal fin supports are present; (f) 10.5 mm L_S, note the onset of ossification; (g) 11.6 mm L_S, ossification of predorsals initiates; (h) 16.0 mm L_S. Grey areas, cartilaginous structures. Open areas, ossified structures. Scale bars indicate 1.0 mm. Pd, Predorsal; Pr, proximal radial; Sy, stay. For meaning of other abbreviations see Figs. 1 and 2.

distal radials of these fins was visible [Fig. 3 (c)] and three predorsals cartilages were also present [Fig. 3 (c)]. Growth of the spines and rays of both the dorsal and anal fins began at 7.0 mm L_S and by 9.4 mm L_S all elements were present [Fig. 3 (e)]. The rays were segmented, and the distal tips were frayed but subsequently (>17.0 mm L_S) bifurcated. The dorsal and anal fin stays were visible at 7.7 mm L_S as cartilaginous structures close to the most posterior proximal radials of the dorsal and anal fins, to which they fused ultimately at 9.4 mm L_S [Fig. 3 (c)-(e)].

The ossification of the dorsal and ventral cartilaginous proximal radials, in sea bream, began at 10.5 mm L_S and proceeded anteriorly and posteriorly [Fig. 3 (f)]. Ossification of the dorsal and anal cartilaginous distal radials began at 11.3 mm L_S and continued in an anterior to posterior direction. Simultaneously, the three predorsals also ossified [Fig. 3 (g)]. The dorsal and anal fins were fully developed and ossification was nearing completion at 16.0 mm L_S [Fig. 3 (h)].

In addition to describing the ontogeny of the fins in sea bream, the origin of the bony structures (dermal or cartilage replacement bone) was also determined (Table II). It is of interest to note that the composition (cartilage or bone) of common structures, for example, spines and rays or radials, of the fins studied was always the same. In the pectoral fin, the cleithrum and associated structures (supracleithrum, postcleithrum and temporals) are dermal bone and no cartilage intermediate is formed.

TABLE II. Origin of bone in the pectoral, dorsal, anal and pelvic fin skeleton (D, dermal bone; C, cartilage replacement bone).

Structure	Bone type	Structure	Bone type
Pectoral fin skeleton		Distal radials	C
Cleithrum	D	Spines	D
Coracoid	C	Rays	D
Scapula	C	Anal fin skeleton	
Actinosts	C	Proximal radials	C
Supracleithrum	D	Distal radials	C
Posttemporal	D	Spines	D
Lower postcleithrum	D	Rays	D
Upper postcleithrum	D	Pelvic fin skeleton	
Lower supratemporal	D	Basipterygium	C
Upper supratemporal	D	Metapterygium	C
Rays	D	Spine	D
Dorsal fin skeleton		Rays	D
Proximal radials	C		

DISCUSSION

In the sea bream the pectoral fin and fin support were the first structures to develop and are used for propulsion in early larval stages. The development of the caudal, pelvic, dorsal and anal fins, which are essential for more complex movements, occurs later and is associated with a reduction in the locomotor role of the pectoral fin.

The pectoral fin bud, which consisted of a rudimentary coracoid-scapula cartilage to which the fin plate joins, was visible soon after hatching. The subsequent fin development observed in cultured sea bream was similar to that described in wild sparids, for example *Pagrus major* (Matsuoka, 1985, 1987) and

Archosargus rhomboidalis (Houde & Potthoff, 1976), the fin rays forming dorsally to ventrally. This pattern of development appears to have been conserved in all teleostean fishes (Richards *et al.*, 1974; Sreekumari, 1976; Potthoff *et al.*, 1984; Balart, 1995). The early development of the pectoral fin and fin support is probably related to the importance of this skeletal group for swimming manoeuvrability of the larvae which begin exogenous feeding 4-5 days after hatching.

During ontogeny of sea bream the coracoid develops both anterior and posterior processes. The development of the posterior process has been reported widely in all teleosts. However, the enlargement of the anterior process observed in sea bream seems to be restricted to higher teleosts (Houde & Potthoff, 1976; Potthoff, 1980; Potthoff & Kelly, 1982; Potthoff *et al.*, 1984; Balart, 1995; Matsuoka, 1985, 1987). Goodrich (1922) proposed that this was a specialisation to strengthen the support of the pectoral fins in larvae before complete development of the dermal girdle.

Several proposals exist for the origin of the propterygium (or uppermost distal cartilage, scapular radial, upper cartilaginous pectoral radial). In sea bream pectoral fins the propterygium which supports the superior ray was completely separated from the fin plate, in contrast to reported development in *Xiphias gladius* (L.) (Potthoff & Kelley, 1982), *Anisotremus virginicus* (L.) (Potthoff *et al.*, 1984) and Clupeiformes (Goodrich, 1922; Balart, 1995) where the propterygium is suggested to arise as an outgrowth of the scapula. The origin of the propterygium in sea bream is similar to that reported in the sparid *P. major*, it has an autogenous origin above the cartilage blade (Matsuoka, 1985, 1987).

In common with other teleosts, (generally wild) the pelvic fin and basipterygia were the last structures to develop in sea bream. Pelvic fin ray

cartilaginous development was completed before that of the pectoral fin ray; however, ossification occurred at a similar rate in these two structures. All the rays observed in these structures were segmented. Bifurcation of the rays in the pelvic fin occurred at 16.0 mm L_S and at 20.4 mm L_S in the pectoral fin (with the exception of propterygium which is not a ray and is not bifurcated or segmented).

Throughout development of sea bream, predorsal bones show no evidence of serial homologies, neither with the pterygiophores nor with the neural spines.

The overall pattern of development of the dorsal and anal fins and fin support in teleosts is similar. The proximal radials are the first structures to develop, followed by distal radials and then rays and spines (Kohno & Taki, 1983). However, within each group of structures the development pattern is complex and at this level differences are observed between species. In sea bream the first cartilaginous proximal radials to develop are posterior and subsequently develop in an anterior direction. A similar development pattern was also observed for the rays. This is in contrast to the pattern reported in other perciform families where dorsal fin rays or spines develop first anteriorly (Matsumoto *et al.*, 1972; Potthoff, 1975; Collins *et al.*, 1980; Richards & Saksena, 1980). In Clupeiformes the pattern of formation of these fins is uncertain but does not appear to correspond to that observed in sea bream or other teleosts (Balart, 1995). Interestingly, no middle radials were detected in any of the specimens of sea bream examined (up to 20.4 mm L_S). The failure to detect this structure may be related to the size of specimens studied, particularly as middle radials were detected only in the sparid, *P. major* at 45.4 mm total length (L_T) (Matsuoka, 1985, 1987).

In common with what has been observed widely among teleost fish, the first dorsal and anal proximal radial in sea bream were enlarged. The

organization of the anterior radials in sea bream is similar to that in *Morone americana* (Fritzsche & Johnson, 1980), *A. virginicus* (Potthoff *et al.*, 1984) and the sparids *A. rhomboidalis* (Houde & Potthoff, 1976) and *P. major* (Matsuoka, 1985, 1987); the anteriormost dorsal and anal pterygiophore (each pterygiophore consists of a proximal and a distal radial) supports two spines in secondary association and one spine in a serial association (Houde & Potthoff, 1976; Potthoff *et al.*, 1984, Matsuoka, 1985, 1987). Some controversy surrounds the development of the first dorsal proximal radial in teleosts. Several authors have proposed that the proximal radial arises from the fusion of two cartilaginous elements (Fritzsche & Johnson, 1980; Potthoff *et al.*, 1984), while others have proposed it arises as a single element (Balart, 1985, 1995; Matsuoka, 1985, 1987). In the present study, the first dorsal proximal radial arose usually as a single element, as has also been reported in *P. major* (Matsuoka, 1985, 1987) but which conflicts with observations in *A. virginicus* (Potthoff *et al.*, 1984). However, in some specimens of sea bream the first proximal radial appeared to arise from two cartilaginous elements, suggesting that both processes may occur normally. Further studies of this structure in sea bream and other sparids may help to resolve the origin of the first proximal radial in this family.

The ontogeny of the fins and their ossification in cultured sea bream was similar to that observed in the wild sparids *A. rhomboidalis* and *P. major* (Table III). Comparison of fin ontogeny in sparids with that reported in other teleosts demonstrates that a common developmental pattern exists. The sequence of appearance of each fin and the development of structures within a given fin are constant (Table III). Moreover, the origin of the bone (endochondral or dermal) in each of the fin structures also appears to be constant (Table II) (Houde & Potthoff, 1976; Potthoff *et al.*, 1984; Matsuoka, 1987; Watson & Walker, 1992;

TABLE III. Sequence of appearance of the principal fin structures for each of the following fins, pectoral, dorsal, anal and pelvic, in several fishes; for each cell in the table, a semicolon separates structures that appeared at different times.

	Pectoral fin skeleton	Dorsal fin skeleton	Anal fin skeleton	Pelvic fin skeleton
Sparus aurata (Sparidae) present work	Cleithrum, coracoid-scapula and fin plate; lower postcleithrum; distal radials; rays; supracleithrum; posttemporal; upper postcleithrum; actinosts; lower and upper supratemporals	Proximal radials; distal radials, rays and spines	Proximal radials; distal radials, rays and spines	Basipterygium; rays and spine; metapterygium
Pagrus major (Sparidae) (Matsuoka, 1987)	Cleithrum, coracoid- scapula and fin plate; supracleithrum; posttemporal, lower postcleithrum, rays, upper postcleithrum; actinosts; lower and upper supratemporals	Proximal radials; distal radials, rays and spines	Proximal radials; distal radials, rays and spines	Basipterygium; rays and spine; metapterygium
Archosargus rhomboidalis (Sparidae) (Houde & Potthoff, 1976)	Cleithrum, coracoid-scapula and fin plate; lower and upper postcleithrum; actinosts	First dorsal: proximal radials; distal radials and spines Second dorsal: proximal radials; distal radials and rays	Proximal radials; distal radials, rays and spines	Pelvic fin supports; rays
Engraulis japonicus (Engraulididae) (Balart, 1995)	Cleithrum, coracoid-scapula and fin plate; supracleithrum; upper posttemporal; lower posttemporal; rays; distal radials; actinosts	Proximal radials; distal radials and rays	Proximal radials; distal radials and rays	Basipterygium; metapterygia; rays
Anisotremus virginicus (Haemulidae) (Potthoff *et al.*, 1984)	Cleithrum, coracoid-scapula and fin plate; posttemporal; supracleithrum; lower and upper postcleithrum; distal radials; rays; actinosts; supratemporal; intertemporal	First dorsal: proximal radials; distal radials and spines Second dorsal: proximal radials; distal radials and rays	Proximal radials; distal radials; rays and spines	Basipterygium; rays and spines

Balart, 1995). When a graph is constructed of cumulative percentage of fin structures present in sea bream larvae, against average length (Fig. 4), it is very similar to that observed for *P. major* (Matsuoka, 1987). The result appears to support the theory of saltatory ontogeny (Balon, 1979, 1981, 1984, 1985, 1986), which suggests that development is not a gradual but a saltatory process, through a combination of qualitative changes in form and function, creating boundaries between a succession of quantitative intervals. Three principal phases of cartilaginous/osteological development can be identified in sea bream (Fig. 4), ≤ 3.1 mm L_N; 3.1 mm L_N - 11.6 mm L_S; and > 11.6 mm L_S. The first phase corresponds to the yolk-sac stage (or free embryo) and the

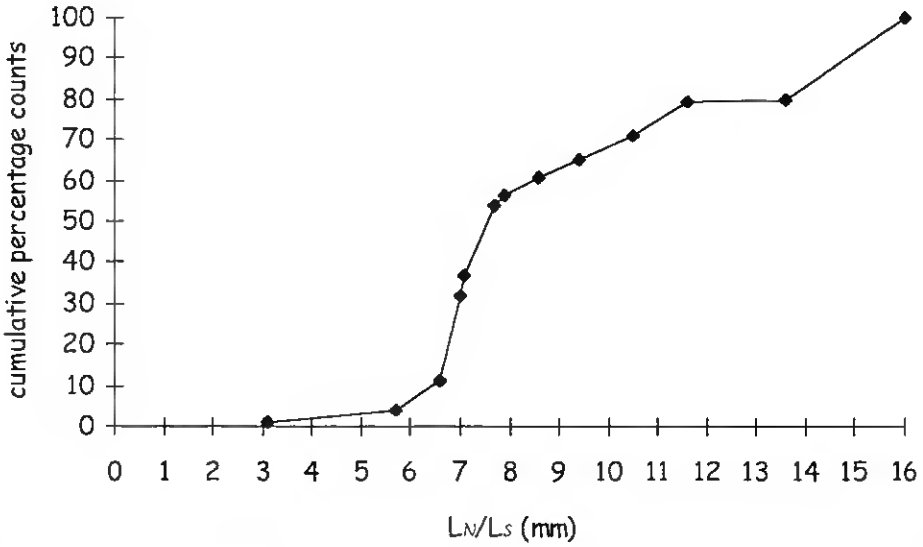

Figure 4. Relationship between cumulative percentage of the counts of structural changes against length. Cumulative percentage count was determined using the following principal events for all the fins and associated structures: (i) initial appearance of cartilage structures, (ii) the presence of the full complement of cartilage structures, (iii) onset of ossification, (iv) completion of ossification. The number of larvae analysed for each length are indicated in Table I.

cartilaginous/osteological structures which develop are those which are necessary for exogenous feeding, the bones of the head (discussed in the

Chapter 4) which permit opening and closing of the mouth and the structures which will ultimately support the pectoral fins. The second phase is associated with the larval phase, when most of the fin structures form and ossify, leading naturally to the transition to the juvenile stage. In sea bream the third distinct phase, the change of slope at 11.6 mm L_S (Fig. 4), occurs before rays segment, suggesting that it may not always be appropriate to use the appearance of segmented rays as an indicator of transition from larvae to juvenile (Snyder, 1976).

The fact that length rather than age seems to be a better index of the development of skeletal bone structures in teleosts is higly relevant to aquaculture or fisheries where environmental and ecological conditions can lead to drastic alterations in growth rates.

The genetic mechanisms underlying the synchrony between size and bone structure development merit further study.

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CHAPTER 4

OSTEOLOGIC DEVELOPMENT OF THE VISCEROCRANIAL SKELETON IN SEA BREAM (*Sparus aurata*): ALTERNATIVE OSSIFICATION STRATEGIES IN TELEOST FISH

Co-author

Deborah M. Power

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OSTEOLOGIC DEVELOPMENT OF THE VISCEROCRANIAL SKELETON IN SEA BREAM (*Sparus aurata*): ALTERNATIVE OSSIFICATION STRATEGIES IN TELEOST FISH

ABSTRACT

The ontogeny of the viscerocranial skeleton of *Sparus aurata* larvae was studied from 1 to 90 days post-hatching. In the smallest specimens analysed at 2.7 mm L_N no cephalic elements were present and at 3.1 mm L_N the following cartilaginous structures were visible: trabecula cranii, auditory capsule, Meckel's cartilage, quadrate, hyosymplectic cartilage, sclerotic, hypohyal, ceratohyal epihyal cartilage, interhyal, hypobranchial 1 and ceratobranchial 1. The only structure ossified at this size is the maxillary and the next ossified structures to appear are the preopercle and opercle at about 3.7 mm L_N. The last bones to appear are infraorbital 2 and 6 at 15.1 mm L_S. The first cartilaginous elements and stuctures to ossify in *S. aurata* appear to be related with functional requirements, so that structures involved directly in feeding and breathing generally appear and ossify before those that are not. The ontogeny of different regional structures revealed that generally the dermal bones ossify before the cartilage replacement bones. Comparison of *S. aurata* viscerocranial skeleton ontogeny with that of phylogenetically distant fish demonstrates that different ossification strategies exist in higher and lower teleost fish.

Key words: neurocranium, jaws, hyoid arch, branchial arches, ontogeny, comparative study, ossification, *Sparus aurata*.

INTRODUCTION

The sea bream (*Sparus aurata*) is an important commercial marine teleost fish in the Mediterranean and southern Europe. Numerous reports about *S. aurata* egg and larval development and nutrition exist (Villani, 1976; Person-Le Ruyet & Verillaud, 1980; Camus & Koutsikopoulos, 1984; Kadmon *et al.*, 1985; Tandler *et al.*, 1989). Recently several reports have appeared cataloguing the osteological abnormalities encountered frequently in the operculum and caudal fin complex in cultured sea bream larvae (Kiriakos *et al.*, 1994; Koumoundouros *et al.*, 1997*a*, *b*). However, such reports have focused largely on the end-point, the abnormal structure, and do not provide the means by which to understand the basis of their appearance. A clear account with sufficient detail of the normal pattern of chondrification and ossification is an important step in the identification of factors, genetic or environmental, which may interfere with the normal process (Kwain, 1975; Somasundaram *et al.*, 1984; Bengtsson *et al.*, 1988; Wiegand *et al.*, 1989; Campbell, 1995; Toften & Jobling, 1996; Lien *et al.*, 1997; von Westernhagen & Dethlefsen, 1997) and for determining the underlying mechanisms which give rise to abnormalities. Detailed accounts were made about the development of the vertebral column and caudal complex (Chapter 2) and fins (Chapter 3) in sea bream. The present chapter describes the development of the neurocranium, jaws, suspensorium, opercular series, hyoid arch and branchial arches (regions of the viscerocranial skeleton) and completes the description of the normal development of the skeleton in sea bream. A range of reports focusing on development of the viscerocranial skeleton of teleost fish exist in the literature (Langille & Hall, 1987; Watson & Walker, 1992; Matsuoka, 1997; Adriens & Verraes, 1998) but most of them refer either to the early

stages (Adriaens & Verraes, 1997; Kimmel *et al.*, 1995; Vandewalle *et al.*, 1992, 1999), are incomplete or are limited to the development of bones present in juvenile and adult fish (Lau & Shafland, 1982; Potthoff *et al.*, 1984; Balart, 1985; Collette & Gillis, 1992; Johnson *et al.*, 1996; Suda, 1996; Doyle, 1998; Parenti & Thomas, 1998; Voskoboinikova, 1998). Moreover, the diversity of teleost fish makes it difficult to generalize about bone development, particularly when the developing structure is as complex as the viscerocranial skeleton. A few studies exist documenting the development of the viscerocranial skeleton in Sparidae, but they are not very detailed and generally do not cover the development from hatching (day 0) to the juvenile stages (Houde & Potthoff, 1976; Mook, 1977; Matsuoka, 1985; Koumoundouros *et al.*, 2000).

In the present chapter a precise account of viscerocranial skeleton ontogeny is given and the origin of bones and timing of ossification is characterized for the sea bream. This study forms the foundation for future studies into morphogenesis and deviations from normal developmental patterns in the sea bream. It provides additional detailed information for studies of percoid phylogeny and classification. Finally several different models for development of the viscerocranial skeleton in teleosts are discussed after carrying out cross species analysis.

MATERIALS AND METHODS

The general procedure for larval culture and sampling has been referred (Chapter 2). In brief, larvae were hatched (40 h after fertilization) and reared in a continuous flow of sea water at 19 ± 1 °C. They were fed rotifera

(*Brachionus plicatilis*) from 4 days post-hatching (DPH) to 15 DPH, *Artemia* sp. nauplii from 16 DPH to 40 DPH and thereafter larvae were fed dry food.

Sampling was carried out on alternate days from 1 DPH and 10-15 specimens were sampled into 2% PFA (paraformaldehyde), after anaesthesia in 0.01% MS-222. Larvae fixed overnight at 4 °C were washed and transferred to 70% methanol for storage. A total of 180 larvae (Table I) ranging from 1 DPH (2.7 mm notochord length, L_N) to 90 DPH (17.9 mm standard length, L_S) was observed and analysed for the description of viscerocranial skeleton ontogeny.

TABLE I. Length-frequency distribution of sea bream larvae observed and analysed.

Length (L_N, L_S - mm)	Number of larvae
2.7 - 2.9	6
3.1 - 3.9	10
4.0 - 4.9	20
5.0 - 5.9	20
6.0 - 6.9	12
7.0 - 7.9	17
8.0 - 8.9	13
9.0 - 9.9	17
10.0 - 11.9	27
12.0 - 13.9	15
14.0 - 15.9	12
16.0 - 17.9	11

L_N = 2.7 - 5.6 mm; L_S = 5.7 - 17.9 mm

Larvae were measured using a stereoscopic microscope equipped with an ocular micrometer (Wild M8) and the following measurements were recorded for each larva: notochord length, tip of the snout to tip of notochord on small larvae prior to flexure; standard length, tip of snout to base of the caudal complex on larger

larvae in which flexion of notochord had occurred. Drawings of stained specimens were made with the aid of a Wild M8 stereo microscope equipped with a camera lucida.

Whole-mount staining of bone and cartilage was carried out using a modification of the alcian blue/alizarin red method and alkaline phosphatase (Chapter 2).

The nomenclature used to describe skeletal structures was based upon de Beer (1937), Harrington (1955), Daget (1964) and Matsuoka (1985). Table II lists the terms used and the source. The viscerocranial skeleton is complex and composed of numerous elements which can be grouped into several regions. Frequently the development of the viscerocranial skeleton has been described by considering each of these regions (Matsuoka, 1985; Collette & Gillis 1992; Suda, 1996). In the present study, a similar approach is taken and the regions considered are: neurocranium (ethmoid, orbital, otic and basicranial regions), jaws (maxillary, premaxillary, rostral cartilage, angular, dentary and retroarticular), suspensorium (ectopterygoid, endopterygoid, hyomandibular, metapterygoid, palatine, quadrate and symplectic), opercular series (preopercle, opercle, subopercle and interopercle), hyoid arch (basihyal, branchiostegal rays, ceratohyal, epihyal, hypohyals, interhyal and urohyal) and branchial arches (basibranchials, ceratobranchials, hypobranchials, epibranchials, interarcual cartilage and pharyngobranchials).

TABLE II. List of terms of skeletal structures used (a superscript indicates the source).

Auditory capsule [1]	Frontal [2]	Parachordal [1]
Angular [2]	Gill raker [2]	Parasphenoid [2]
Basibranchials [2]	Hyomandibular [3]	Parietal [2]
Basibranchial, isolated cartilage	Hyosymplectic cartilage [1]	Pharyngobranchials [1]
of [4]	Hypobranchials [1]	Premaxillary [2]
Basihyal [1]	Hypohyal cartilage [1]	Preopercle [4]
Basioccipital [2]	Hypohyal, lower [2]	Prootic [2]
Basisphenoid [2]	Hypohyal, upper [2]	Pterosphenoid [2]
Branchiostegal rays [2]	Infraorbitals [3]	pterotic [3]
Ceratobranchials [1]	Interarcual cartilage [4]	Quadrate [1]
Ceratohyal [1]	Intercalar [3]	Retroarticular [2]
Ceratohyal cartilage [2]	Interhyal [2]	Rostral cartilage [2]
Dentary [2]	Interopercle [4]	Sclerotic, anterior [2]
Ectopterygoid [2]	Lacrimal [2]	Sclerotic cartilage [1]
Endopterygoid [2]	Lamina orbitonasalis [1]	Sphenotic [3]
Epibranchials [1]	Maxillary [2]	Subopercle [4]
Epihyal [2]	Meckel's cartilage [1]	Supraoccipital [2]
Epiotic [2]	Metapterygoid [3]	Symplectic [1]
Epiphysial tectum [4]	Nasal [3]	Taenia marginalis [1]
Ethmoid [2]	Occipital arch [1]	Trabecula [1]
Ethmoid plate [2]	Opercle [4]	Urohyal [2]
Ethmoid, lateral [2]	Palatoquadrate cartilage [2]	Vomer [3]
Exoccipital [2]	Palatine [3]	

[1] de Beer (1937), [2] Harrington (1955), [3] Daget (1964), [4] Matsuoka (1985)

RESULTS

DEVELOPMENT OF THE CARTILAGINOUS TEMPLATE

In the smallest specimens analysed at 2.7 mm L_N no cartilaginous or bony viscerocranial elements were noticeable, although by 3.1 mm L_N [Figs 1 (a); 2 (a); 3 (a); 4 (a) and 5 (a)] the following cartilaginous structures were visible: trabeculae, ethmoid plate, parachordal cartilage, auditory capsule, basal plate, Meckel's cartilage, quadrate, hyosymplectic cartilage, sclerotic, hypohyal, ceratohyal epihyal cartilage, interhyal, hypobranchial 1 and ceratobranchial 1.

The trabeculae [Figs 1 (a)-(c) and 2 (a)-(c)], two thin structures aligned medially that extend posterolaterally, are positioned between the eyes. Anteriorly the two parts fuse medially to one another to form the trabecula communis [Fig. 1 (a)-(c)] and anterolaterally it forms the ethmoid plate [Figs 1 (a)-(c) and 2 (a)-(c)]. At the posterior end the trabeculae are joined by the parachordal cartilages [Fig. 2 (a), (b)] and enclose the hypophyseal fenestra [Fig. 2 (a), (b)]. The parachordal cartilages skirt the anterior tip of the notochord [Fig. 2 (a)-(c)] and form the basal plate [Fig. 2 (a), (b)] which connects with the auditory capsules [Figs 1 (a) and 2 (a)]. Meckel's cartilage is positioned anterolaterally in a plane ventral to the trabeculae, with the posterior end bent downwards [Fig. 3 (a)]. As the larvae grow the posterior end of Meckel's cartilage extends and articulates in a shallow socket with the anteroventral tip of the quadrate cartilage [Fig. 3 (b)].

The lamina orbitonasalis [Figs 1 (b), (c) and 2 (b), (c)] begins to extend upward in larvae of *c.* 4.1 mm L_N. The structure appears as a dorsal projection of the anterolateral border of the ethmoid plate and is fused dorsally to the anterior taenia marginalis [Figs 1 (c) and 2 (c)].

The epiphysial tectum [Figs 1 (b), (c) and 2 (b), (c)] is visible first at 3.7 mm L_N dorsally at mid-orbit and later forms the junction of the anterior [Figs 1 (c) and 2 (c)] and posterior [Figs 1 (b), (c) and 2 (b), (c)] taenia marginalis. When the epiphysial tectum appears first it is incomplete and fuses subsequently to form one element and develops a median posterior projection, the taenia tecti medialis [Fig. 1 (d)]. The taenia marginalis anterior begins to grow anteroventrally from the epiphysial tectum to the anterolateral part of the ethmoid plate and fuses with the lamina orbitonasalis. The taenia marginalis posterior which is near the anterior extension of the auditory capsule grows anteriorly to the middle of the orbit and subsequently the epiphysial tectum connects the rigth and left sides.

The occipital arches [Figs 1 (b) and 2 (b)] are anterodorsal projections of the posterior end of the parachordal cartilages which appear in specimens of about 3.7 mm L_N.

The rostral cartilage is an ovoid structure [Fig. 3 (c)-(f)] that appears at 5.7 mm L_S and is located at the superior end of the premaxillary adjacent to the ethmoid.

The development of the viscerocranial skeleton is more complex than any other skeletal structure in sea bream and a further degree of complexity arises as a consequence of the origin of each of the structures. The origin of the ossified tissue was determined and sub-divided into two main groups. Bones that ossify around or within a cartilage matrix, cartilage replacement bone, and bones that develop directly within connective tissue, dermal bones. The viscerocranial skeleton of sea bream is composed of 83 cartilage replacement bones and 51 dermal bones (Table III).

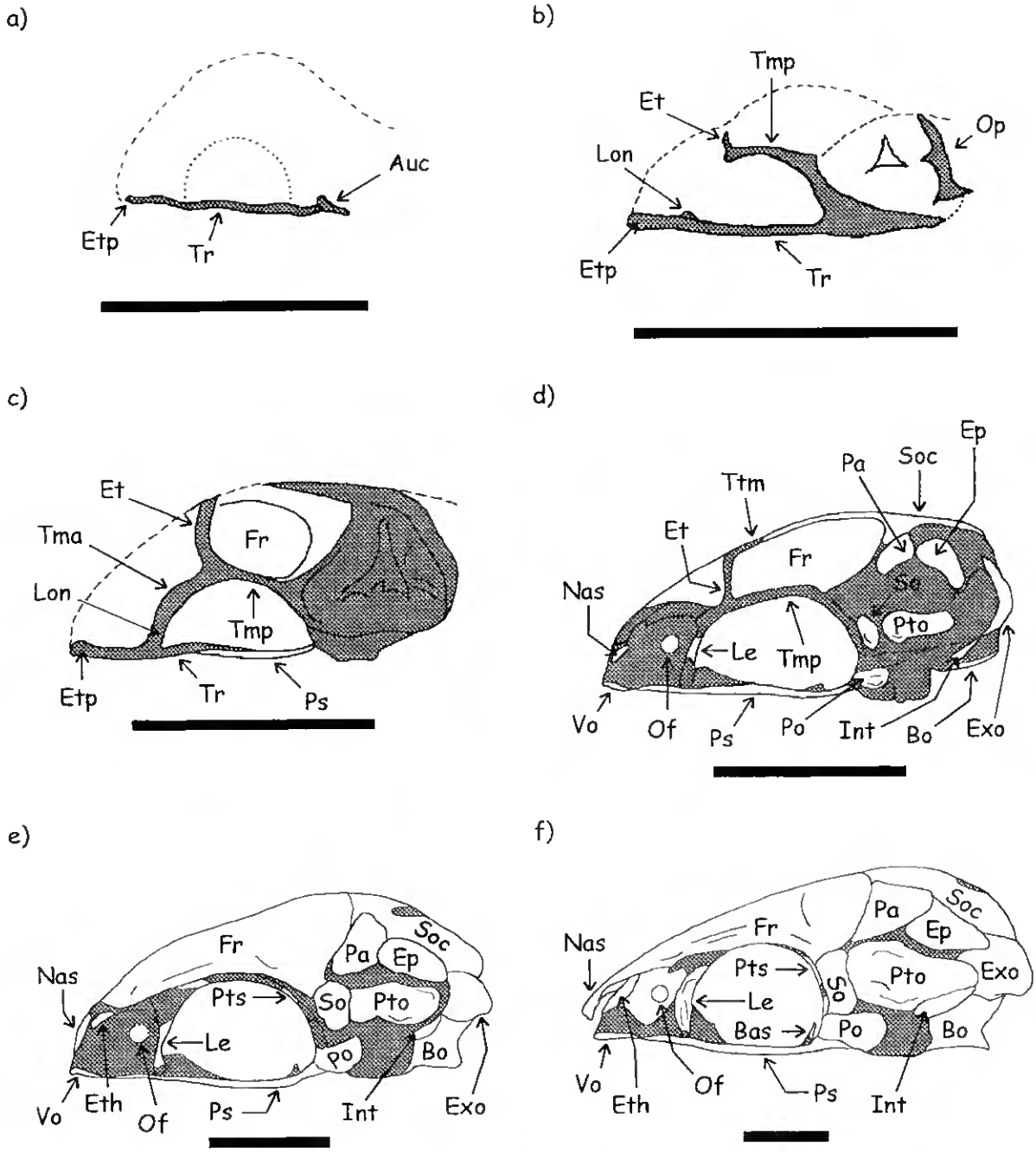


Figure 1. Development of the neurocranium in sea bream. Left lateral view. Infraorbitals and sclerotic are shown in Fig. 3. (a) 3.1 mm L_N; (b) 4.1 mm L_N; (c) 5.7 mm L_S; (d) 8.7 mm L_S; (e) 11.6 mm L_S; (f) 17.9 mm L_S. Cartilage, grey shading; ossifying structures, open areas; scale bars indicate 1.0 mm. Auc, Auditory capsule; Bas, basisphenoid; Bo, basioccipital; Ep, epiotic; Et, epiphysial tectum; Eth, ethmoid; Etp, Ethmoid plate; Exo, exoccipital; Fr, frontal; Int, intercalar; Le, lateral ethmoid; Lon, lamina orbitonasalis; Nas, nasal; Of, olfactory foramen; Op, occipital arch; Pa, parietal; Po, prootic; Ps, parasphenoid; Pto, pterotic; Pts, pterosphenoid; So, sphenotic; Soc, supraoccipital; Tma, taenia marginalis anterior; Tmp, taenia marginalis posterior; Tr, trabecula; Ttm, taenia tecti medialis; Vo, vomer.

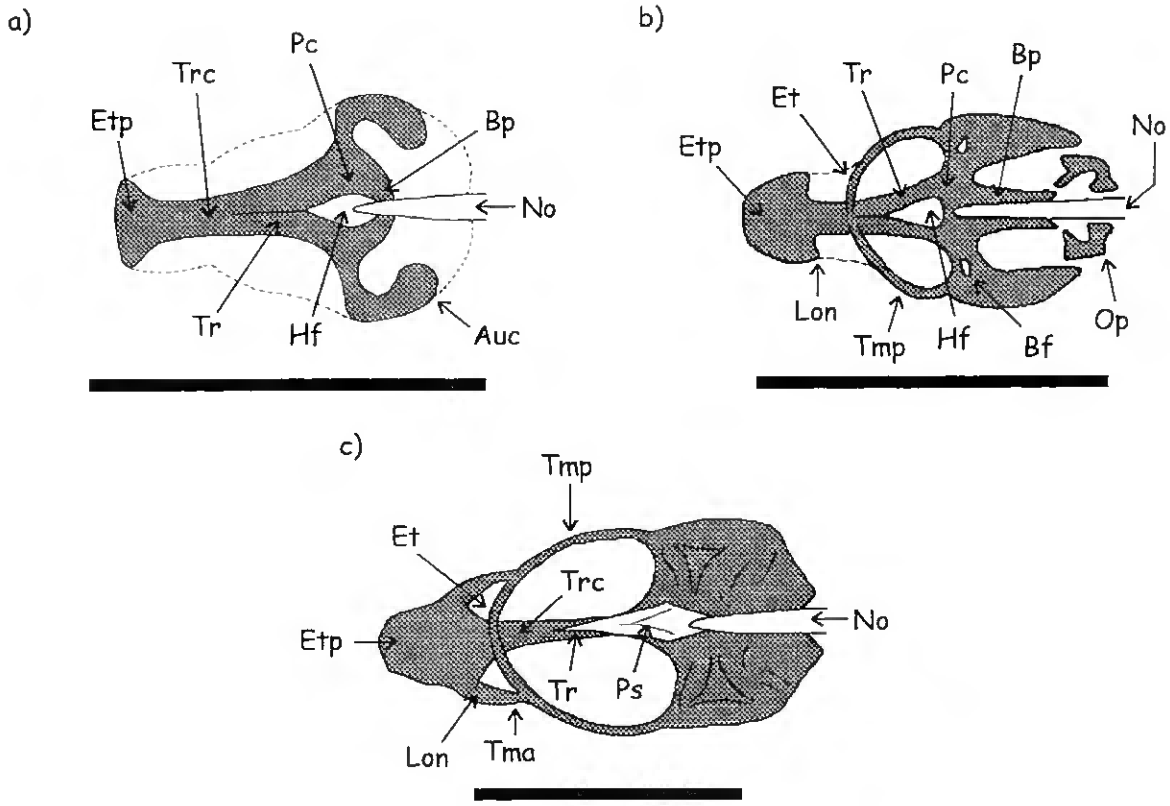


Figure 2. Development of the neurocranium in sea bream. Dorsal view. Frontals are not shown. (a) 3.1 mm L_N; (b) 4.1 mm L_N; (c) 5.7 mm L_S. Cartilage - grey shading. Ossifying structures - open areas. Scale bars indicate 1.0 mm. Auc, auditory capsule; Bf, basicapsular fenestra; Bp, basal plate; Et, epiphysial tectum; Etp, Ethmoid plate; Hf, hypophyseal fenestra; Lon, lamina orbitonasalis; No, notochord; Op, occipital arch; Pc, parachordal cartilage; Ps, parasphenoid; Tma, taenia marginalis anterior; Tmp, taenia marginalis posterior; Tr, trabecula; Trc, trabecula communis.

Description of viscerocranial skeleton ontogeny follows a regional approach to explore possible patterns of ossification and facilitate developmental comparison between different species. All the bones in the viscerocranial skeleton of sea bream are bilaterally paired with the exception of basibranchials, basihyal, basioccipital, basisphenoid, parasphenoid, supraoccipital, urohyal and vomer. The description of chondrogenesis and ossification of structures within regions is in alphabetical order and unless indicated otherwise all developing paired ossifications are described unilaterally (Table III, lists paired and unpaired structures).

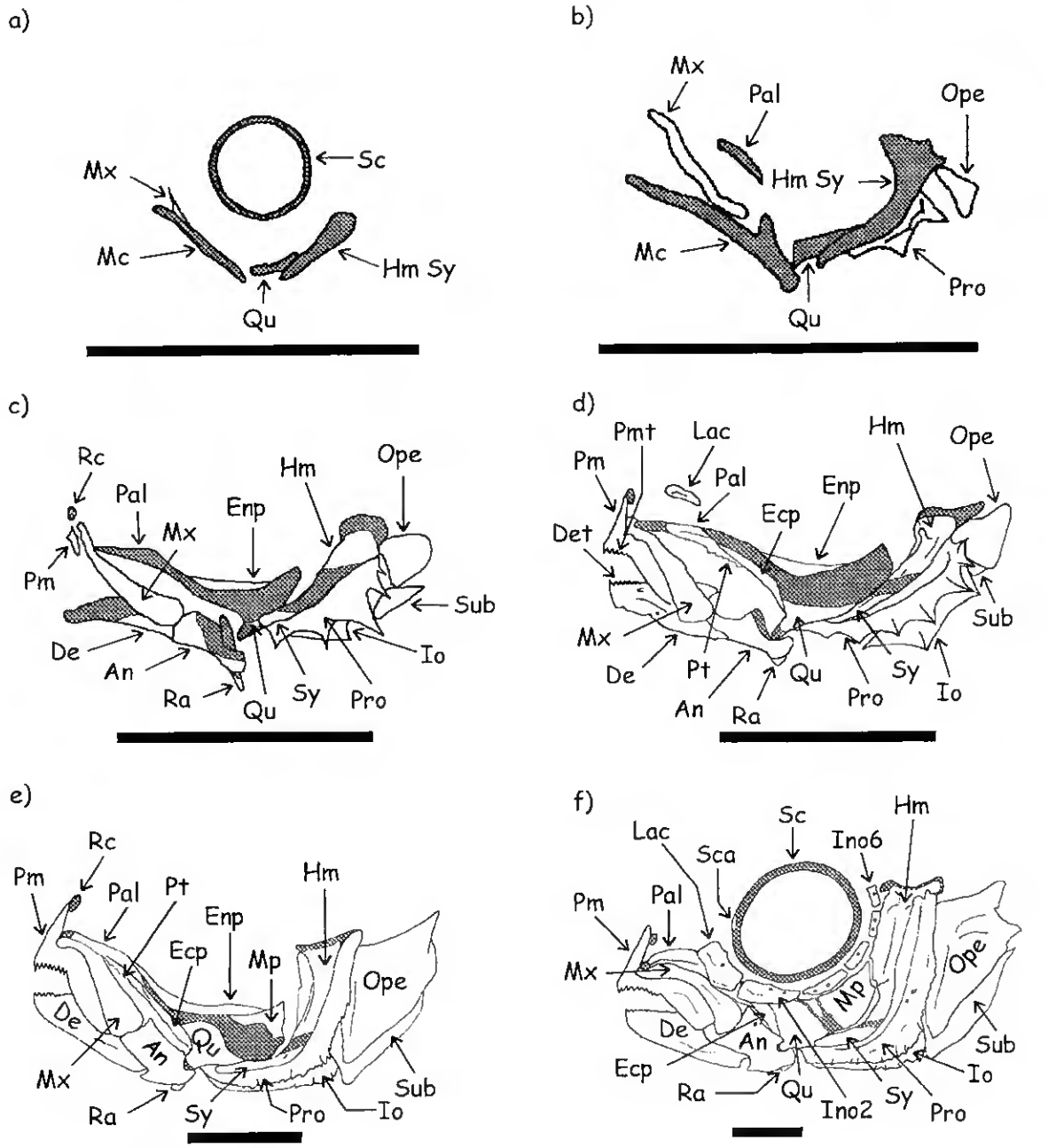


Figure 3. Development of the jaws, suspensorium, opercular series, sclerotic and infraorbital bones in sea bream. Left lateral view. Sclerotic is not shown in (b-e), lacrimal is not shown in (e) and endopterygoid is not shown in (f). (a) 3.1 mm L_N; (b) 4.1 mm L_N; (c) 5.7 mm L_S; (d) 8.7 mm L_S; (e) 11.6 mm L_S; (f) 17.9 mm L_S. Cartilage - grey shading. Ossifying structures - open areas. Scale bars indicate 1.0 mm. An, angular; De, dentary; Det, dentary teeth; Ecp, ectopterygoid; Enp, endopterygoid; Hm, hyomandibular; Hm Sy, hyosymplectic cartilage; Ino, infraorbital; Io, interopercle; Lac, lacrimal; Mc, Meckel's cartilage; Mp, metapterygoid; Mx, maxillary; Ope, opercle; Pal, palatine; Pm, premaxillary; Pmt, premaxillary teeth; Pro, preopercle; Pt, palatine teeth; Qu, quadrate; Ra, retroarticular; Rc, rostral cartilage; Sca, anterior sclerotic; Sc, sclerotic; Sub, subopercle; Sy, symplectic.

TABLE III. Type and number of bones present in the different regions of the viscerocranial skeleton of sea bream. Dermal bones indicated in bold, cartilage replacement bones are in normal text.

Region and bone		Region and bone	
Neurocranium		Symplectic	paired - 1
Basioccipital	single - 1	Jaws	
Basisphenoid	single - 1	Angular	paired - 1
Epiotic	paired - 1	**Dentary**	paired - 1
Ethmoid	paired - 1	**Maxillary**	paired - 1
Exoccipital	paired - 1	**Premaxillary**	paired - 1
Frontal	paired - 1	Retroarticular	paired - 1
Infraorbitals (2-6)	paired - 5	Opercular series	
Intercalar	paired - 1	**Interopercle**	paired - 1
Lacrimal	paired - 1	**Opercle**	paired - 1
Lateral ethmoid	paired - 1	**Preopercle**	paired - 1
Nasal	paired - 1	**Subopercle**	paired - 1
Parasphenoid	single - 1	Hyoid arch	
Parietal	paired - 1	Basihyal	single - 1
Prootic	paired - 1	**Branchiostegal rays (1-6)**	paired - 6
Pterotic	paired - 1	Ceratohyal	paired - 1
Pterosphenoid	paired - 1	Epihyal	paired - 1
Sclerotics	paired - 1	Hypohyal, lower	paired - 1
Sphenotic	paired - 1	Hypohyal, upper	paired - 1
Supraoccipital	single - 1	Interhyal	paired - 1
Vomer	single - 1	**Urohyal**	single - 1
Suspensorium		Branchial arches	
Ectopterygoid	paired - 1	Basibranchial (1-3)	single - 3
Endopterygoid	paired - 1	Ceratobranchial (1-5)	paired - 5
Hyomandibular	paired - 1	Epibranchial (1-4)	paired - 4
Metapterygoid	paired - 1	Hypobranchial (1-3)	paired - 3
Palatine	paired - 1	Pharyngobranchial (1-4)	paired - 4
Quadrate	paired - 1		

NEUROCRANIUM

Ossification is discussed in relation to the four principal regions, ethmoid, orbital, otic and basicranial.

ETHMOID REGION

This region is composed of the ethmoid, lateral ethmoid, nasal and vomer. In sea bream these bones ossify in the following sequence: vomer, lateral ethmoid, nasal, ethmoid.

Ethmoid [Fig. 1 (e), (f)]

The ethmoid appears first at 9.3 mm L_S as a pair of slender ossified structures adjacent to the nasal in the ethmoid plate. The paired ethmoid grow and fuse eventually to form a median ossified sheet which meets the anterior border of the frontal, posterodorsally.

Lateral ethmoid [Fig. 1 (d)-(f)]

The lateral ethmoid is a paired structure on either side of the head which is visible first as a thin ossified border along the posterior edge of the cartilaginous lamina orbitonasalis at *c.* 6.9 mm L_S. These structures grow laterodorsally to reach the adjacent frontal and articulate with the adjacent lacrimal.

Nasal [Fig. 1 (d)-(f)]

The nasal appears first at 8.1 mm L_S as a small flat bone, anterior to the lateral ethmoid. The nasal articulates with the anterolateral edge of the frontal.

Vomer [Fig. 1 (d)-(f)]

The vomer begins to ossify by 6.5 mm L_S and appears as two small and slender ossifications on the anterolateral margins of the ethmoid plate and is connected with the parasphenoid posteriorly.

ORBITAL REGION

The orbital region includes the basisphenoid, frontal, infraorbitals, lacrimal, pterosphenoid and sclerotics. The orbit is surrounded anteriorly by the posterior wall of the lateral ethmoid, dorsally by the frontal and posteriorly by the pterosphenoid, sphenotic, prootic, lacrimal and infraorbital bones. Both left and right orbits are partially separated by the basisphenoid and the sclerotic bones enclose the eyeballs. In sea bream the sequence of ossification of the completely formed cartilaginous structures in this region are as follows: frontal, lacrimal, pterosphenoid, infraorbitals 3-5, basisphenoid, sclerotic anterior, infraorbitals 2 and 6.

Basisphenoid [Fig. 1 (f)]

The basisphenoid appears first at 8.7 mm L_S as a small cartilaginous structure located above the dorsal surface of the parasphenoid and begins to ossify at 12.7 mm L_S.

Frontal [Fig. 1 (c)-(f)]

The frontal forms the largest portion of the dorsal surface of neurocranium and appears first at 5.1 mm L_N as a very thin and elongated bony lamella above the posterior taenia marginalis. Ossification of this structure

occurs in three directions simultaneously, medially toward the midline, posteriorly along the taenia marginalis and towards the sphenotic and parietal and anteriorly towards the nasal.

Infraorbitals [Fig. 3 (d), (f)]

Infraorbitals 3, 4 and 5 begin to ossify simultaneously at 10.8 mm L_S. Infraorbital 2 and infraorbital 6 (dermosphenotic; Nelson, 1969) are the last to form at 15.1 mm L_S. Infraorbitals 2 and 3 are elongated slightly curved structures, infraorbital 4 has a similar form but is smaller and these structures form respectively, the ventral, posteroventral and posterior border of the orbit. Infraorbitals 5 and 6 are the smallest structures in the series and form respectively, the posterior and posterodorsal margin of the orbit. All the infraorbitals have a bony canal that encloses the lateral sensory system and extends from one end of the bone to the other. The bony canal of each infraorbital contains one or more small pores.

Lacrimal (*lachrymal, lacrymal, infraorbital 1*) [Fig. 3 (d), (f)]

The lacrimal is located at the anterior border of the orbit and is the largest and the first infraorbital to ossify at 8.3 mm L_S. The lacrimal appears initially as a flat, triangular shaped bone which as ossification proceeds enlarges and grows upward. The lacrimal articulates with the lower end of the lateral ethmoid.

Pterosphenoid [Fig. 1 (e), (f)]

The pterosphenoid forms the posterodorsal margin of the orbit and begins to ossify at 9.7 mm L_S. Initially it appears as a thin and slender ossification along the posteroventral margin of the posterior taenia marginalis.

Subsequently the ossification spreads dorsally to the frontal and posteriorly to the sphenotic and prootic.

Sclerotic [Fig. 3 (a), (f)]

At 3.1 mm L_N the lateral edge of the eye capsule is surrounded by a very thin sclerotic cartilage. The anterior sclerotic bone appears in larvae of 14.7 mm L_S as small disk-shaped bones. The posterior sclerotic bone was not present in any of the specimens analysed.

OTIC REGION

This region consists of the epiotic, intercalar, parietal, prootic, pterotic, sphenotic and supraoccipital. Ossification of the structures in this region occurs in the following order: prootic, pterotic, parietal and epiotic, supraoccipital and sphenotic, intercalar.

Epiotic [Fig. 1 (d)-(f)]

The epiotic is visible first at 7.7 mm L_S as a small, thin ossified structure. It is bounded dorsomedially by the supraoccipital and is overlapped anteriorly by the parietal and overlaps the exoccipital posteriorly.

Intercalar [Fig. 1 (e), (f)]

The intercalar is a small concave bone that ossifies in the connective tissue at 8.5 mm L_S. It forms part of the posterior border of the neurocranium interposed between the pterotic and exoccipital.

Parietal [Fig. 1 (d)-(f)]

The parietal forms part of the posterior roof of the neurocranium and ossifies at the same time as the epiotic at 7.7 mm L_S. It develops anteriorly toward the frontal, medially toward the supraoccipital, posteriorly toward the epiotic and laterally toward the dorsal edge of the pterotic.

Prootic [Fig. 1 (d)-(f)]

The prootic ossifies slightly before the pterotic and epiotic at 6.9 mm L_S. Each prootic is bordered ventrally by the parasphenoid, overlaps anterodorsally with the sphenotic and is separated posterodorsally from the pterotic by a thin strip of cartilage.

Pterotic [Fig. 1 (d)-(f)]

The pterotic is localized posterior to the sphenotic and ossifies slightly after the prootics at 7.4 mm L_S. Ossification extends anteriorly toward the sphenotic, posteroventrally toward the intercalar, posteriorly toward the exoccipital. The pterotic is separated dorsally from parietal and epiotic by a thin layer of cartilage.

Sphenotic [Fig. 1 (d)-(f)]

The sphenotic is visible first at 7.9 mm L_S as a small and circular ossification posterior to the posterior taenia marginalis. It grows dorsally to the frontal, posteriorly to the pterotic and ventrally overlaps with the prootic.

Supraoccipital [Fig. 1 (d)-(f)]

The supraoccipital forms the dorsomedian portion of the posterior end of the neurocranium and ossifies at the same time as the sphenotic at 7.9 mm L_S.

The supraoccipital is bordered laterally by the frontals and parietals and posteriorly by the epiotics and exoccipitals.

BASICRANIAL REGION

In sea bream larvae the basicranial region is formed by three structures which ossify in the following order: parasphenoid, basioccipital and exoccipital.

Basioccipital [Fig. 1 (e), (f)]

The basioccipital begins as two small filaments of bone, along the notochord, in the posterior basicranial region at 6.5 mm L_S. Ossification proceeds dorsally towards the exoccipital and laterally in the direction of the otoliths. Posteriorly the basioccipital forms a condyle that attaches to the first vertebral centrum.

Exoccipital [Fig. 1 (d)-(f)]

The exoccipital is visible first along the posterior edge of the cartilaginous occipital arch at the same time as the basioccipital at 6.5 mm L_S. The paired exoccipitals meet posterodorsally and are joined anterodorsally with the epiotic and supraoccipital and articulate laterally with the intercalar and pterotic. Viewed ventrally the exoccipital articulate anteriorly with the prootic, medially with the basioccipital and laterally with the intercalar. The exoccipitals viewed posteriorly form the lateral walls of the foramen magnum.

Parasphenoid [Figs 1 (c)-(f) and 2 (c)]

The parasphenoid, a long and thin fusiform bone with tapered anterior and posterior tips, is located in the connective tissue between the trabeculae and begins to ossify at 4.9 mm L_N. During its ontogeny this bone elongates and articulates subsequently anteriorly with the vomer, forming the ventral axis of the skull. At 6.0 mm L_S, the parasphenoid begins to develop ascending wings in the dorsoposterior end that connect with the ventral prootics. The parasphenoid forms the ventral border of the orbits and is connected dorsally with the lateral ethmoids, basisphenoid and basioccipital.

JAWS

The jaws are composed of the upper jaw (maxillary, premaxillary and rostral cartilage) and lower jaw (angular, dentary and retroarticular). Teeth erupt from the premaxillary and dentary. In sea bream the sequence of ossification is as follows: maxillary, dentary, angular and retroarticular, premaxillary.

Angular [Fig. 3 (c)-(f)]

The angular is visible first at 5.1 mm L_S along the lateral surface of the posterior half of Meckel's cartilage. Ossification spreads anteriorly as a thin plate along the lateral face of the dentary. Posterodorsally the angular possesses a shallow groove for articulation with the condyle of the quadrate and is bordered at the posteroventral margin by the retroarticular.

Dentary [Fig. 3 (c)-(f)]

The dentary appears as bone on the anterior half of Meckel's cartilage at 4.6 mm L_N. Subsequently the dentary extends posteriorly and meets the angular. Dentary teeth are visible first at 6.7 mm L_S.

Maxillary [Fig. 3 (a)-(f)]

The maxillary is visible first at 3.1 mm L_N. This structure ossifies as a thin sliver of bone on the lateral face of the upper jaw. It develops a short anterior ascending process that extends laterally and articulates with the posterior margin of the premaxillary. The posteroventral end contacts with the posterodorsal edge of the dentary.

Premaxillary [Fig. 3 (c)-(f)]

The premaxillary starts to ossify at 5.7 mm L_S as a curved flat structure close to, and in front of the upper part of the maxillary. The premaxillary develops a small ascending process that covers the rostral cartilage at its anterior end. A long posterior process of the premaxillary projects ventrally and downwards ending in a round tip and contacts with the dorsolateral edge of the dentary. The posterior extremities of the ascending process of both premaxillaries are close together medially and fit into the ethmoid. Small premaxillary teeth are present at 6.7 mm L_S.

Retroarticular [Fig. 3 (c)-(f)]

The retroarticular is a rhomboid structure that starts to ossify at 5.1 mm L_S on the posteroventral tip of the Meckel's cartilage. The retroarticular is attached to the posteroventral margin of the angular.

Rostral cartilage [Fig. 3 (c)-(f)]

The rostral cartilage is present at 5.7 mm L_S as an oval structure near the upper ends of the maxillary and premaxillary and does not undergo ossification.

SUSPENSORIUM

The suspensorium includes the ectopterygoid, endopterygoid, hyomandibular, metapterygoid, palatine, quadrate and symplectic. Ossification of the structures in this region occurs in the following order: hyomandibular and symplectic, quadrate and endopterygoid, palatine and ectopterygoid, metapterygoid.

Ectopterygoid [Fig. 3 (d)-(f)]

The ectopterygoid is formed as a very thin ossification along the lower border of the anterior palatine cartilage at 7.4 mm L_S. The anterior borders of the quadrate reaches the posterior border of the ectopterygoid at 8.7 mm L_S and subsequently overlaps it.

Endopterygoid [Fig. 3 (c)-(e)]

The endopterygoid starts to ossify at 5.4 mm L_N as a sliver of bone along the posterodorsal margin of the palatine and quadrate cartilages. It is attached anteriorly to the palatine and ventrally to the quadrate and metapterygoid.

Hyomandibular [Fig. 3 (c)-(f)]

At 3.1 mm L_N an undivided hyosymplectic cartilage is present and by 4.9 mm L_N the dorsal region of this structure begins to ossify generating the

hyomandibular, near the facial nerve foramen. The ossification extends dorsally and laterally and the ossified hyomandibular articulates dorsally with the ventral edges of sphenotic and pterotic. Posteriorly the hyomandibular fuses with the preopercle and laterally it articulates with the opercle. The ventral border of hyomandibular is connected to the upper surface of the symplectic by a thin band of cartilage which is a vestigial of the hyosymplectic.

Metapterygoid [Fig. 3 (e), (f)]

Ossification of the metapterygoid begins in the upper posterior end of the quadrate cartilage at 9.3 mm L_S. The metapterygoid has a quadrangular shape and forms a cartilaginous suture with the endopterygoid.

Palatine [Fig. 3 (b)-(f)]

At 4.1 mm L_N the mid-region of the palatine is chondrified and by 7.4 mm L_S the palatine begins to ossify along the anterior ventral margin. The ossification spreads ventrally through the palatine which articulates with the ectopterygoid and endopterygoid. The anterior tip of the palatine remains cartilaginous and attaches to the maxillary. At 8.7 mm L_S a few small temporary teeth are visible on the posterior ventral edge of the palatine.

Quadrate [Fig. 3 (a)-(f)]

The quadrate is present as cartilage at 3.1 mm L_N and its ossification, which extends in a dorsal and ventral direction, begins at 5.4 mm L_S. The ossified quadrate attaches: (i) posterolaterally with the symplectic and the lower part of the preopercle; (ii) dorsally with the posteroventral margin of the endopterygoid and (iii) anteriorly to the lower lateral border of the

ectopterygoid. The condyle of the quadrate articulates anteroventrally with the angular.

Symplectic [Fig. 3(c)-(f)]

The symplectic ossifies and is visible first at 4.9 mm L_N in the ventral region of the hyosymplectic cartilage. The process of ossification extends dorsally toward the hyomandibular and ventrally toward the quadrate. The symplectic attaches posteroventrally with the anterodorsal edge of the preopercle and anterodorsally with the quadrate. The region of the symplectic which articulates with the hyomandibular remains cartilaginous even in the largest specimens analysed (largest = 17.9 mm L_S).

OPERCULAR SERIES

Ossification of the structures which make up the opercular series occurs in the following order: preopercle and opercle, subopercle and interopercle.

Interopercle [Fig. 3 (c)-(f)]

Ossification of the interopercle, a slender roughly triangular shaped structure with a single small spine, becomes evident first at 5.4 mm L_N. Ossification proceeds ventrally and more spines appear, but in later stages (11.6 - 17.9 mm L_S) these spines are resorbed and become vestigial. The interopercle is overlapped anterolaterally by the preopercle and overlaps the anterolateral margin of the subopercle .

Opercle [Fig. 3 (b)-(f)]

The opercle is visible first above the preopercle at 3.7 mm L_N, as a flat triangular shaped bone, located near the posterior margin of the hyosymplectic cartilage. The opercle articulates anteriorly with the hyomandibular, is overlapped on its anterior border by the posterior half of the preopercle and overlaps the dorsal part of the subopercle.

Preopercle [Fig. 3 (b)-(f)]

The preopercle is a crescent-shaped bone that begins to ossify at 3.7 mm L_N in the connective tissue posterior and ventral to the hyosymplectic cartilage. The preopercle articulates with the quadrate, symplectic and hyomandibular and overlaps the anterolateral margin of the interopercle and opercle. Initially the preopercle has two spines on the posterior margin and in the subsequent stages five (5.7 mm L_S) and seven (8.7 mm L_S) spines are apparent. The preopercle spines are visible in early stages (4.1 mm L_N - 8.7 mm L_S), but later (11.6 - 17.9 mm L_S) become vestigials like the interopercle spines.

Subopercle [Fig. 3 (c)-(f)]

The subopercle is a flat roughly triangular structure that begins to ossify at 5.4 mm L_N near the ventral border of the opercle. The ossification extends ventrally and the subopercle is overlapped dorsally by the opercle and anterolaterally by the interopercle. The corners of the subopercle become rounded with the exception of the posterodorsal corner which has a tapered tip.

HYOID ARCH

The hyoid arch is composed of the basihyal, branchiostegal rays, ceratohyal, epihyal, hypohyals (upper and lower), interhyal and urohyal. In sea bream the sequence of ossification is: branchiostegal rays 1-4, ceratohyal, branchiostegal rays 5 and 6, basihyal, lower hypohyal and urohyal, epihyal, upper hypohyal and interhyal.

Basihyal [Fig. 5 (c)-(f)]

At 5.7 mm L_S the basihyal is present as cartilaginous triangular shaped structure and at 6.9 mm L_S the posterior tip of the basihyal is ossified. Ossification spreads throughout the cartilage with the exception of the anterior tip which remains cartilage even in the largest specimens analysed (17.9 mm L_S). The basihyal articulates with the upper and lower hypohyals laterally and with basibranchial 1 posteriorly.

Branchiostegal rays [Fig. 4 (b)-(e)]

The branchiostegal rays become apparent first in specimens of 4.6 mm L_N and develop ventrally in a posterior (epihyal) to anterior (ceratohyal) direction. By 5.7 mm L_S four branchiostegal rays were visible and in specimens of 6.0 mm L_S the full complement of rays (6) are present.

Ceratohyal [Fig. 4 (b)-(e)] *and Epihyal* [Fig. 4 (c)-(e)]

The ceratohyal and epihyal are present in specimens of 3.1 mm L_N as a single cartilaginous structure, the ceratohyal epihyal cartilage. At 5.7 mm L_S the ceratohyal begins to ossify in the mid-region of the ceratohyal epihyal cartilage.

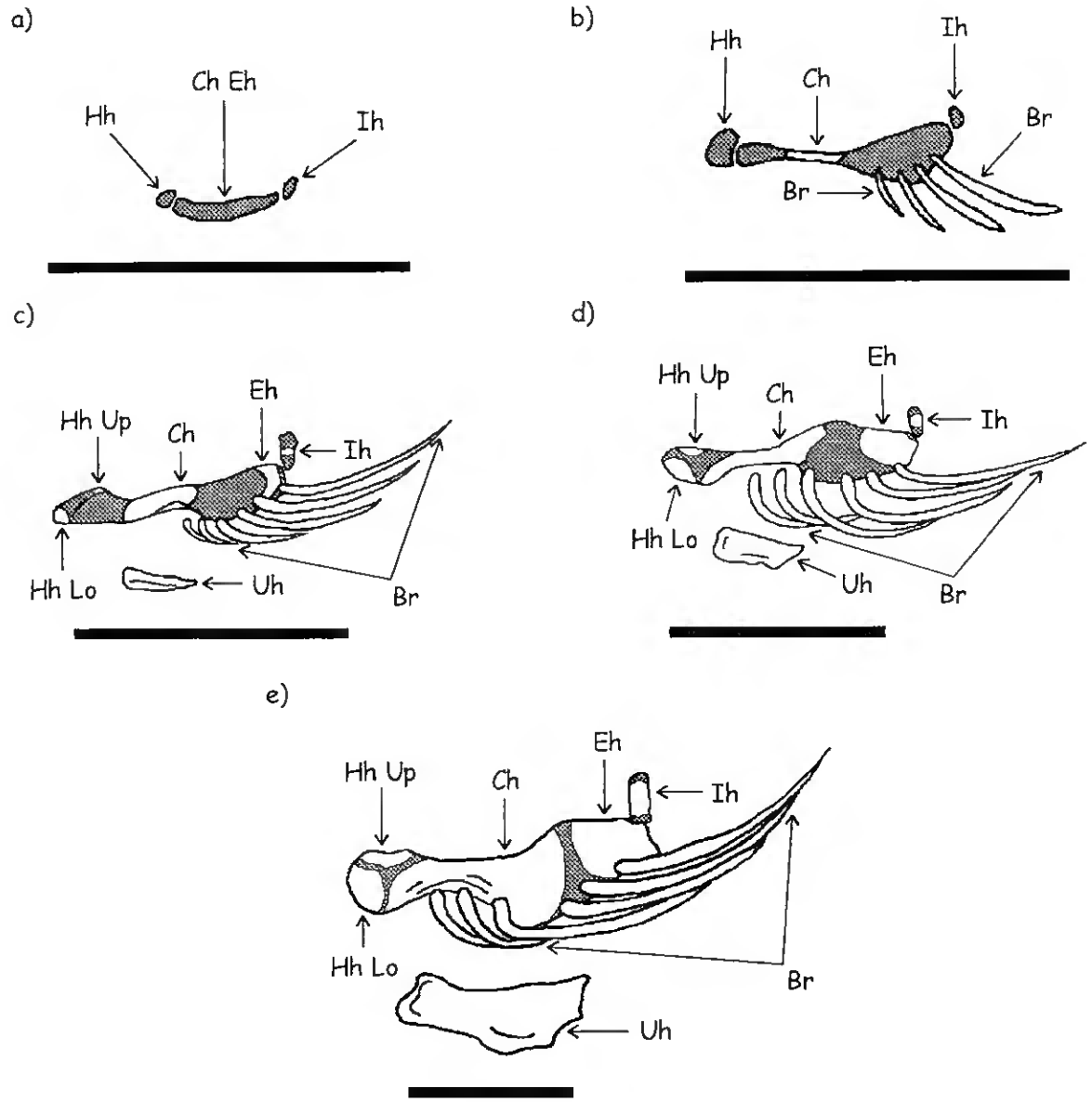


Figure 4. Development of the hyoid arch in sea bream. Left lateral view. Basihyal is shown in Fig. 5. (a) 3.1 mm L_N; (b) 5.7 mm L_S; (c) 8.7 mm L_S; (d) 11.6 mm L_S; (e) 17.9 mm L_S. Cartilage - grey shading. Ossifying structures - open areas. Scale bars indicate 1.0 mm. Br, branchiostegal rays; Ch, ceratohyal; Ch Eh, ceratohyal epihyal cartilage; Eh, epihyal; Hh, hypohyal; Hh Lo, lower hypohyal; Hh Up, upper hypohyal; Ih, interhyal; Uh, urohyal.

Ossification extends anteriorly and posteriorly across the cartilage although a cartilaginous band between the ceratohyal and epihyal still persists in specimens of 17.9 mm L_S. The anterior end of the ceratohyal articulates with the upper and lower hypohyals.

At 7.4 mm L_S the epihyal begins to ossify at the posterior end of the ceratohyal epihyal cartilage and the ossification progresses in an anterior direction across the cartilage. The epihyal articulates posterodorsaly with the interhyal.

Hypohyals [Fig. 4 (a)-(e)]

At 3.1 mm L_N the hypohyal is present as a single cartilaginous structure. The lower hypohyal begins to ossify at 7.1 mm L_S in the lower part of the hypohyal cartilage and at 8.7 mm L_S the upper hypohyal begins to ossify in the upper portion. Both hypohyals remain connected to each other by a thin cartilaginous strip and articulate with ceratohyal.

Interhyal [Fig. 4 (a)-(e)]

The cartilaginous interhyal is present at 3.1 mm L_N near the posterior tip of the ceratohyal epihyal cartilage. Ossification initiates in the mid-region at 8.7 mm L_S and spreads dorsally and ventrally. In specimens of 17.9 mm L_S the ventral and dorsal tips of the interhyal remain cartilaginous and the ventral region articulates with the posterodorsal end of the epihyal.

Urohyal [Fig. 4 (c)-(e)]

The urohyal is an unpaired ossification of the tendon of the sternohyoideus muscle (Ridewood, 1904; de Beer, 1937; Arratia & Schultze, 1990) that appears initially as a thin sliver of bone at 7.1 mm L_S. The urohyal is localized ventral to the basibranchial area and connects anteriorly with the lower hypohyal, as ossification proceeds this structure gradually extended vertically.

BRANCHIAL ARCHES

This complex is formed by the lower (basibranchials, ceratobranchials, hypobranchials and isolated cartilage of basibranchial) and upper (epibranchials, interarcual cartilage and pharyngobranchials) branchial arches. The ceratobranchials and epibranchials support a series of gill rakers. These structures ossify in the following order: ceratobranchial 5, ceratobranchials 3 and 4, ceratobranchials 1 and 2, epibranchials 1-4 and pharyngobranchial 3, basibranchials 1-3, hypobranchial 3 and pharyngobranchials 2 and 4, pharyngobranchial 1, hypobranchials 1 and 2.

Basibranchials [Fig. 5 (b)-(f)]

The cartilaginous basibranchial (copula 1; de Beer, 1937) is present at 3.5 mm L_N. In specimens of 8.9 mm L_S the first, second and third pairs of basibranchials begin to ossify. The first pair of basibranchials ossify posterior to the basihyal and anterior to the first pair of the hypobranchials, the second pair of basibranchials ossify posterior to the first pair of basibranchials and anterior to the second pair of the hypobranchials and the third pair of basibranchials ossify posterior to the second pair of basibranchials and anterior to the third pair of hypobranchials. A thin cartilaginous band remains between the basibranchials in the region were they articulate with the hypobranchials.

Ceratobranchials [Fig. 5 (a)-(f)]

The ceratobranchials appear as cartilage in the following order, the first pair at 3.1 mm L_N and the remaining four pairs at 3.5 mm L_N. Ossification is evident first at 6.0 mm L_S in the fifth ceratobranchial pair and is followed by the third and fourth ceratobranchial pairs at 6.5 mm L_S. At 6.9 mm L_S there is

onset of ossification of the remaining structures, the first and second ceratobranchial pairs. A few teeth are present at 6.0 mm L_S on the fifth ceratobranchial pair and at 5.9 mm L_S gill rakers become evident on the first two ceratobranchial pairs. In specimens between 11.6 and 17.9 mm L_S, gill rakers develop on the anterior and posterior border of the first three ceratobranchial pairs and on the anterior border of the fourth ceratobranchial pair. A dermal tooth plate develops and fuses to the dorsal face of the fifth ceratobranchial pair in specimens between 8.7 and 17.9 mm L_S.

The ceratobranchial pairs 1, 2 and 3 articulate at the anterior tip through cartilage with the posterior end of their respective hypobranchials. The fourth ceratobranchial pair attaches *via* its anterior cartilaginous tip to the isolated cartilage of basibranchial. The posterior end of the first four pairs of ceratobranchials articulates through cartilage with the four pairs of epibranchials.

Epibranchials [Fig. 5 (b)-(f)]

Between 3.7 and 4.1 mm L_N the four pairs of cartilaginous epibranchials develop and at 7.1 mm L_S ossification initiates in the middle of each structure. Epibranchial 1 develops an anterior process, epibranchial 2 a medial process and epibranchials 3 and 4 a posterior process. On the anterior ventral side of epibranchial 2 a toothplate appears. A few gill rakers develop on the outer row of the epibranchial 1 and 2 at 9.1 mm L_S. The anterior ends of epibranchials 1-4, which persists as cartilage, connect with there respective pharyngobranchials 1-4. The posterior tip of each epibranchial also remain cartilaginous and articulate with the posterior ends of ceratobranchials 1-4.

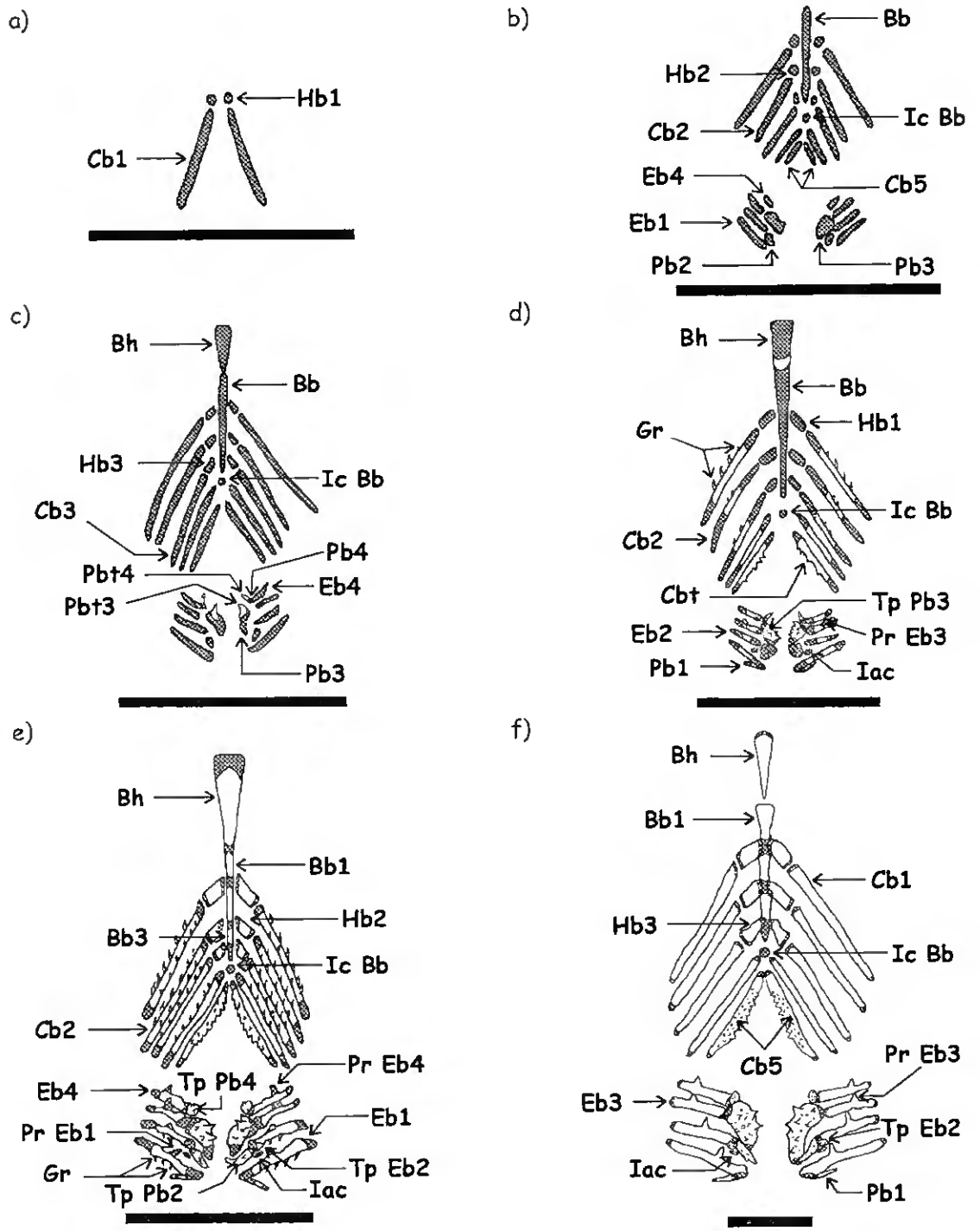


Figure 5. Development of the upper and lower branchial arches in sea bream. Upper and lower branchial arches are shown, respectively, in ventral and dorsal views. The gill raker of ceratobranchials 1-4 and epibranchials 1-2 are not shown in (f). (a) 3.1 mm L_N; (b) 4.1 mm L_N; (c) 5.7 mm L_S; (d) 8.7 mm L_S; (e) 11.6 mm L_S; (f) 17.9 mm L_S. Cartilage - grey shading. Ossifying structures - open areas. Scale bars indicate 1.0 mm. Bb, basibranchial; Bh, basihyal; Cb, ceratobranchial; Cbt, ceratobranchial teeth; Eb, epibranchial; Gr, gill raker; Hb, hypobranchial; Iac, interarcual cartilage; Ic Bb, isolated cartilage of basibranchial; Pb, pharyngobranchial; Pbt, pharyngobranchial teeth; Pr Eb, process on epibranchial; Tp Eb, tooth plate of epibranchial; Tp Pb, tooth plate of pharyngobranchial.

Hypobranchials [Fig. 5 (a)-(f)]

At 3.1 mm L_N the first pair of hypobranchials is present as cartilage and by 3.5 mm L_N the second and third pair appear. Ossification of the hypobranchials begin in the middle of each cartilage structure and spreads outward. Hypobranchial 3 is the first structure to ossify at 9.1 mm L_S followed by hypobranchials 1 and 2 at 9.7 mm L_S. The hypobranchials 1-3 connect anteriorly *via* a persistent cartilaginous region with the respective adjacent basibranchial and posteriorly with the first three ceratobranchials.

Interarcual cartilage [Fig. 5 (d)-(f)]

The interarcual cartilage (Allis, 1915) is present in specimens of about 6.5 mm L_S and is localized between epibranchial 1, epibranchial 2 and pharyngobranchial 2.

Isolated cartilage of basibranchial [Fig. 5 (b)-(f)]

The isolated cartilage of basibranchial (copula 2; de Beer, 1937) becomes evident first in specimens of 3.5 mm L_N and persists as cartilage. This structure attaches to the anterior end of the ceratobranchial 4.

Pharyngobranchials [Fig. 5 (b)-(f)]

The cartilaginous pharyngobranchials are not all evident at the same time. The earliest pharyngobranchials to appear are 2 and 3 in specimens of 3.7 mm L_N, followed by pharyngobranchial 4 at 5.7 mm L_S and by pharyngobranchial 1 (suspensory pharyngeal; Iwai & Nakamura, 1964) at 6.1 mm L_S. Pharyngobranchial 3, the largest element in the series, is the first to ossify at 7.1 mm L_S, followed by pharyngobranchials 2 and 4 at 9.1 mm L_S and finally pharyngobranchial 1 at 9.3 mm L_S. A few pharyngobranchial teeth are present on the pharyngobranchial

3 at 5.1 mm L_N and by 5.7 mm L_S a single tooth appears on the pharyngobranchial 4. The tooth plate develops on the ventral side of pharyngobranchial 3 by 8.7 mm L_S and by 9.3 mm L_S the tooth plate of pharyngobranchials 2 and 4 is visible also. Pharyngobranchials 1-4 connect posteriorly through a persistent cartilaginous region with the anterior tip of the respective epibranchials 1-4.

DISCUSSION

Ontogenesis of the viscerocranial skeleton in sea bream is a complex process that involves the development and ossification of more than 134 bones, which constitute the fully developed viscerocranial skeleton in sea bream. The ossification process is separated readily into 26 steps (Fig. 6) if structures are grouped according to the initiation of ossification. In common with what has been observed in other fishes (Jollie, 1975; Langille & Hall, 1987; Vandewalle *et al.*, 1992; Watson & Walker, 1992; Adriens & Verraes, 1998; Wagemans *et al.*, 1998), the majority of dermal bones in sea bream is present prior to ossification of the cartilaginous viscerocranial skeleton template. The dermal bones associated with the lateral sensory system are an exception and in sea bream, they are generally the last bones to appear. The first eight ossification steps in sea bream include 31 of the 51 dermal bones, but only 14 of 83 cartilage replacement bones. The development of the viscerocranial skeleton in sea bream appears to be more plastic than the development of the vertebral column or fins (Chapters 2 and 3) and far greater variation was observed between specimens in the order and rate of ossification within the groups of structures identified. However, the overall ontogenetic plan and the final structure of the

viscerocranial skeleton was strongly conserved. The ontogenesis of the viscerocranial skeleton in sea bream appears to comply with the model of saltatory ontogeny proposed by Balon (1979, 1981, 1984, 1985, 1986), in that it is a continuous process but the rate is variable (i. e. development does not follow a linear pattern).

The sea bream hatch while still relatively undeveloped and have few or no cartilage or bony structures. This means that from hatching to mouth opening (4-5 DPH), a range of cartilaginous or bony structures must develop if the larva is to survive. At initial mouth opening, sea bream larvae are equipped with: jaws (maxillary and Meckel's cartilage), cartilaginous suspensorium (hyosymplectic and quadrate), cartilaginous hyoid arch (hypohyal, ceratohyal epihyal and interhyal) and cartilaginous branchial arches (hypobranchial 1, ceratobranchial 1 and slightly after basibranchials 1-3, ceratobranchials 2-5 and hypobranchials 2 and 3) all structures related with opening or closing the mouth and expanding or narrowing the oral and branchial areas. The organization of the structures involved in mouth opening and subsequent feeding in sea bream are consistent with a feeding mechanism involving sucking (Alexander, 1970), the anterior tip of the hyoid arch is located ahead of the posterior ends of Meckel's cartilage, a condition that would generate a stronger negative pressure in the oral cavity leading to sucking feeding. Effective feeding is essential for survival and it seems likely that large changes in the normal development of the viscerocranial skeleton could influence larval survival profoundly.

Relatively few studies documenting abnormalities of the viscerocranial skeleton in aquaculture species exist. The abnormalities which have been reported in sea bream are associated principally with the operculum (Koumoundouros *et al.*, 1997*b*), while in sea bass *Dicentrarchus labrax* (L.) abnormalities in the lower and upper jaw bone, frontal and operculum have been

	Length (L_N, L_S - mm)
Mx	3.1
↓	
Ope ↔ **Pro**	3.7
↓	
Br 3 ↔ **Br 4** ↔ **Br 5** ↔ **Br 6** ↔ **De**	4.6
↓	
Hm ↔ **Ps** ↔ Sy	4.9
↓	
An ↔ **Fr** ↔ Ra	5.1
↓	
Enp ↔ **Io** ↔ Qu ↔ **Sub**	5.4
↓	
Ch ↔ **Pm**	5.7
↓	
Br 1 ↔ **Br 2** ↔ Cb 5	6.0
↓	
Bo ↔ Cb 3↔ Cb 4 ↔ Exo ↔ **Vo**	6.5
↓	
Bh ↔ Cb 1 ↔ Cb 2 ↔ Le ↔ Po	6.9
↓	
Eb 1 ↔ Eb 2 ↔ Eb 3 ↔ Eb 4 ↔ Hh Lo ↔ Pb 3 ↔ **Uh**	7.1
↓	
Ecp ↔ Eh ↔ Pal ↔ Pto	7.4
↓	
Ep ↔ **Pa**	7.7
↓	
So ↔ Soc	7.9
↓	
Nas	8.1
↓	

Lac	8.3
↓	
Int	8.5
↓	
Hh Up ↔ Ih	8.7
↓	
Bb 1 ↔ Bb 2 ↔ Bb 3	8.9
↓	
Hb 3 ↔ Pb 2 ↔ Pb 4	9.1
↓	
Eth ↔ Mp ↔ Pb 1	9.3
↓	
Hb 1 ↔ Hb 2 ↔ Pts	9.7
↓	
Ino 3 ↔ Ino 4 ↔ Ino 5	10.8
↓	
Bas	12.7
↓	
Sca	14.7
↓	
Ino 2 ↔ Ino 6	15.1

Figure 6. Sequence of ossification of the viscerocranial skeleton in sea bream related to length (L_N and L_S, mm). Structures are listed in order of appearance. Arrows represent the progression of ossification and in each step, when several bones are present, they are listed in alphabetic order. The bones connected by ↔ may develop simultaneously or individually but in no particular order. Dermal bones are indicated in bold; cartilage replacement bones are indicated in normal text. L_N = 3.1 - 5.6 mm; L_S = 5.7 - 17.9 mm. An, angular; Bas, basisphenoid; Bb, basibranchial; Bh, basihyal; Bo, basioccipital; Br, branchiostegal rays; Cb, ceratobranchial; Ch, ceratohyal; De, dentary; Eb, Epibranchial; Ecp, ectopterygoid; Eh, epihyal; Enp, endopterygoid; Ep, epiotic; Eth, ethmoid; Exo, exoccipital; Fr, frontal; Hb, hypobranchials; Hh Lo, lower hypohyal; Hh Up, upper hypohyal; Hm, hyomandibular; Ih, interhyal; Ino, infraorbitals; Int, intercalar; Io, interopercle; Lac, lacrimal; Le, lateral ethmoid; Mp, metapterygoid; Mx, maxillary; Nas, nasal; Ope, opercle; Pa, parietal; Pal, palatine; Pb, pharyngobranchials; Pm, premaxillary; Po, prootic; Pro, preopercle; Ps, parasphenoid; Pto, pterotic; Pts, pterosphenoid; Qu, quadrate; Ra, retroarticular; Sca, anterior sclerotic; So, sphenotic; Soc, supraoccipital; Sub, subopercle; Sy, symplectic. Uh, urohyal; Vo, vomer.

reported (Barahona-Fernandes, 1982). Interestingly the frequency of abnormalities reported for the neurocranium are low or non-existent, compared with other regions of the body. It is unclear if this low frequency arises: (i) as a consequence of their lethal character so that affected individuals do not survive past early larval stages or (ii) because the plasticity of this region permits deviations from normal development without large alterations in phenotype. Clearly more detailed studies of these possibilities will be required to resolve this question.

The development of the viscerocranial skeleton shows a remarkable degree of conservation between species, probably as a consequence of functional constraints. In common with the ontogeny described in sea bream, the first bony structures to appear are generally associated with feeding and breathing (Fig. 6). In the barbel *Barbus barbus* (L.) (Vandewalle *et al.*, 1992) the first elements are the opercle, the dentary, the maxillary and branchiostegal rays. In catfish *Chrysichthys auratus* (Geoffroy Saint-Hilaire) (Vandewalle *et al.*, 1995) the dentary, opercle and two pairs of branchiostegal rays appear first, followed by the maxillary. In turbot *Scophthalmus maximus* (L.) (Wagemans *et al.*, 1998), a pleuronectiform, the preopercle appears first, followed by maxillary, dentary and opercle. In sargo *Anisotremus davidsonii* (Steindachner) (Watson & Walker, 1992) the first structures to appear are the premaxillary, maxillary, dentary, preopercle and opercle. In salema *Xenistius californiensis* (Steindachner) (Watson & Walker, 1992) the maxillary appears first, followed by the premaxillary, dentary and branchiostegal rays. In contrast, ceratobranchial 5, opercle, parasphenoid and branchiostegal rays are the first structures to appear in zebrafish *Danio rerio* (Hamilton) (Cubbage & Mabee, 1996).

In spite of the strong conservation of the ontogenesis of the viscerocranial skeleton the timing is very variable among Teleostei and in some

species the development of the viscerocranial skeleton initiates prior to hatching with the appearance of cartilaginous [sea trout *Salmo trutta* (L.) (de Beer, 1937), *B. barbus* (Vandewalle *et al.*, 1992)] or ossified elements in the head region [fork-tailed catfish *Arius graeffei* (Kner & Steindachner) (Rimmer, 1985), sea catfish *Galeichthys feliceps* (Valenciennes) (Tilney & Hecht, 1993)]. In other species, including sea bream, *A. davidsonii* and *X. californiensis* (Watson & Walker, 1992), African catfish *Heterobranchus longifilis* (Valenciennes) (Vandewalle *et al.*, 1997), sea bass *Lates calcarifer* (Bloch) (Kohno *et al.*, 1996*a*), milkfish *Chanos chanos* (Forsskål) (Kohno *et al.*, 1996*b*), African catfish *Clarias gariepinus* (Burchell) (Adriaens & Verraes, 1997), *C. auratus* (Vandewalle *et al.*, 1999), Japanese medaka *Oryzias latipes* (Jordan & Snyder) (Langille & Hall, 1987), skull development begins after hatching.

Comparison of the development of the viscerocranial skeleton in sea bream with other Sparidae, red sea bream *Pagrus major* (Temminck & Schlegel) (Matsuoka, 1985), common dentex *Dentex dentex* (L.) (Koumoundouros *et al.*, 2000) and sheepshead sea bream *Archosargus probatocephalus* (Walbaum) (Mook, 1977), demonstrated a remarkable level of conservation between the ontogeny of these species. For example, in sea bream, the development of the osteoneurocranium, important for exogenous feeding in teleosts, initiates with the parasphenoid, followed by the frontal and then the basioccipital, exoccipital and vomer. *P. major* and *D. dentex* share a similar pattern with the exception that in the former the parasphenoid and frontal ossify at the same time (Matsuoka, 1985) and in the latter the parietal and supraoccipital start to ossify before the vomer (Koumoundouros *et al.*, 2000). In *A. probatocephalus* (Mook, 1977) the ossification of parasphenoid and basioccipital is simultaneous as is the frontal and occipital area. In contrast, the frontal appears after the parasphenoid, basioccipital, exoccipital and prootic in *A. davidsonii* (Watson &

Walker, 1992) and in *X. californiensis* the parasphenoid, basioccipital and exoccipital ossify at the same time (Watson & Walker, 1992). The main difference observed between the Sparidae described is the timing of onset and completion of ossification, for example in *A. probatocephalus* the onset of ossification occurs between 2.0 and 3.0 mm L_S, compared with 3.1 mm L_N in sea bream, 2.9 mm L_T (total length) in *P. major* and 3.6 mm L_T in *D. dentex*. The ontogenetic conservation is probably related to the similar requirements for survival of the early life stages of these species.

In order to determine if ontogenesis of the viscerocranial skeleton is conserved between fish of different phylogenetic origin (Fig. 7), a comparison was made of this process using the data available from previously published studies (Langille & Hall, 1987; Watson & Walker, 1992; Matsuoka, 1997; Adriens & Verraes, 1998). The species selected were sea bream and *A. davidsonii* (Perciformes), *O. latipes* (Beloniformes), *C. gariepinus* (Siluriformes) and Japanese sardine *Sardinops melanostictus* (Temminck & Schlegel) (Clupeiformes) [Fig. 8 (a)-(i)]. The number of species considered is not exhaustive, but includes representatives of several different orders in which sufficient detail of viscerocranial skeleton ontogeny is reported. Comparison of viscerocranial skeleton ontogeny in the selected species cannot be matched totally for each region as in some species, *O. latipes, C. gariepinus* and *A. davidsonii*, structures are lacking or have not been described. Several general differences in viscerocranial skeleton development were identified when species were compared: (i) the number of elements present in each region of the viscerocranial skeleton is relatively well conserved, (ii) conservation of ontogeny is high for some regions and low for others, (iii) the sequence of ossification of cartilaginous structures varies between species and (iv) the timing and duration of ossification is very different between the species compared [(Fig. 9 (a)-(j)].

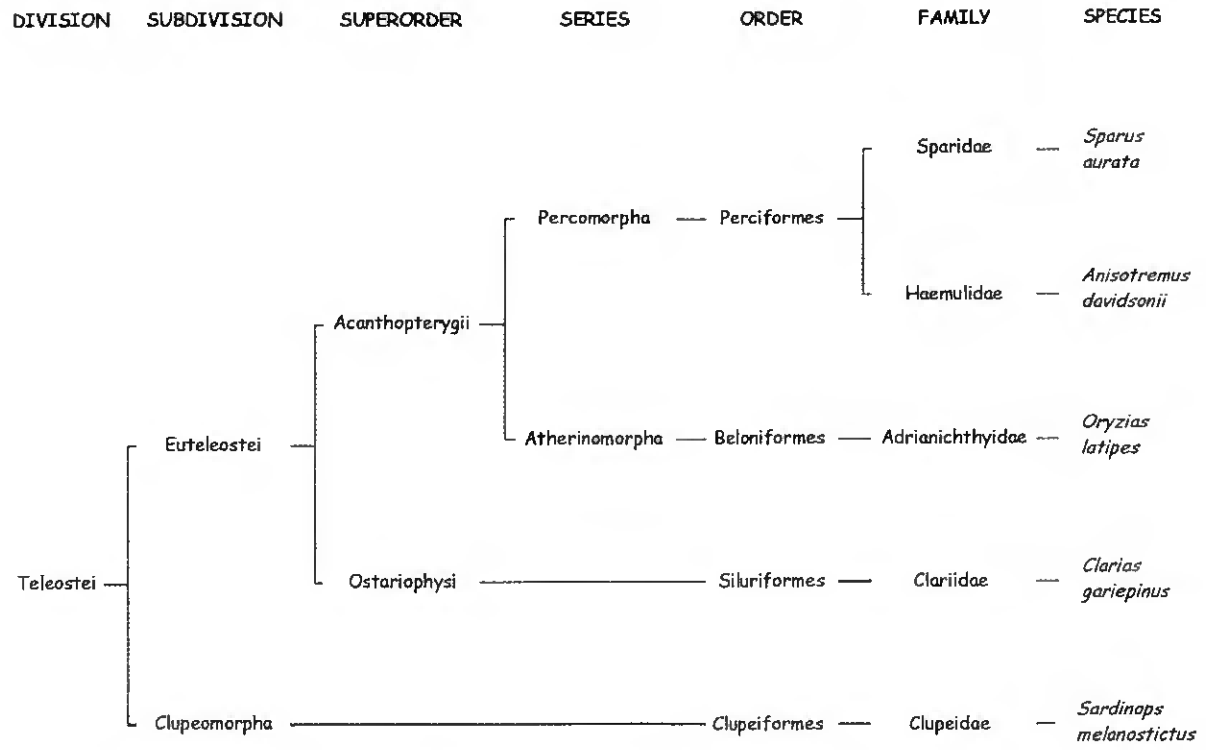


Figure 7. Cladogram showing the taxonomic status of each species used in the study (based upon Nelson, 1994).

For example, generally the ossification of the ethmoid region is conserved poorly between phylogenetically distant species, such as, sea bream and *S. melanostictus* [(Fig. 8 (a)], although all the elements of the ethmoid region have been conserved, with the exception of the vomer which is absent in *O. latipes*. The sequence of ossification of this region in sea bream is: vomer → lateral ethmoid → nasal → ethmoid, and in *A. davidsonii* the same sequence occurs with the exception that the lateral ethmoid is the first structure to ossify. In *O. latipes*, *C. gariepinus* and *S. melanostictus*, which are phylogenetically distant from sea bream, the first structure to ossify is the ethmoid and the subsequent sequence is entirely different [Fig 8 (a)]. In *S. melanostictus* it is notable that the onset of ossification of this region is delayed considerably compared with the other species. For example, the ethmoid region in *A. davidsonii* starts to ossify at 5.5 mm L_S and ossification is complete at 12.4 mm L_S while in *S.*

melanostictus this region only starts to ossify at 20.0 mm L_S and completes ossification at 25.05 mm L_S [Fig. 9 (a)].

In the different species considered the sequence of ossification of the hyoid arch [Fig. 8 (h)] and branchial arches [Fig. 8 (i)], which are composed of an elevated number of structures, is poorly conserved.

In contrast, ossification of some regions of the viscerocranial skeleton is highly conserved. For example, ossification of the basicranial region is fully conserved between the species studied [Fig. 8 (d)]. The orbital region [Fig. 8 (b)], otic region [Fig. 8 (c)], opercular series [Fig. 8 (f)] and suspensorium [Fig. 8 (g)], of the viscerocranial skeleton show partial conservation. For example, in the orbital region the frontal is the first structure ossified in all five species, followed by the lacrimal (*S. aurata*, *A. davidsonii*, *O. latipes* and *C. gariepinus*) or lacrimal and infraorbitals 2-5 (*S. melanostictus*). The sequence of ossification of the first five structures in the orbital region is identical in sea bream and *A. davidsonii* and the sequence of the first three structures in *O. latipes* is conserved.

Figure 8. (next pages). Comparative diagram of the ossification sequence in each region of the species studied; *Sparus aurata* (present study), *Anisotremus davidsonii* (Watson & Walker, 1992), *Clarias gariepinus* (Adriaens & Verraes, 1998), *Oryzias latipes* (Langille & Hall, 1987) and *Sardinops melanostictus* (Matsuoka, 1997). Arrows represent the progression of ossification and when more than one bone ossifies at the same time they are listed in alphabetic order and separated by (+). Dermal bones are in bold; cartilage replacement bones are in normal text. An, angular; Bas, basisphenoid; Bb, basibranchial; Bh, basihyal; Bo, basioccipital; Br, branchiostegal rays; Cb, ceratobranchial; Ch, ceratohyal; De, dentary; Eb, epibranchial; Ecp, ectopterygoid; Eh, epihyal; Enp, endopterygoid; Ep, epiotic; Eth, ethmoid; Exo, exoccipital; Fr, frontal; Hb, hypobranchials; Hh, hypohyal; Hh Lo, lower hypohyal; Hh Up, upper hypohyal; Hm, hyomandibular; Ih, interhyal; Ino, infraorbitals; Int, intercalar; Io, interopercle; Lac, lacrimal; Le, lateral ethmoid; Mp, metapterygoid; Mx, maxillary; Nas, nasal; Ope, opercle; Pa, parietal; Pal, palatine; Pb, pharyngobranchials; Pm, premaxillary; Po, prootic; Pro, preopercle; Ps, parasphenoid; Pto, pterotic; Pts, pterosphenoid; Qu, quadrate; Ra, retroarticular; Sc, sclerotic; Sca, anterior sclerotic; So, sphenotic; Soc, supraoccipital; Sub, subopercle; Sy, symplectic; Uh, urohyal; Vo, vomer.

a)

ETHMOID REGION

S. aurata	**Vo** → Le → **Nas** → Eth
A. davidsonii	Le → **Vo** → **Nas** → Eth
O. latipes[1]	Eth → **Nas** → Le
C. gariepinus	Eth → Le + **Nas** → **Vo**
S. melanostictus	Eth → **Vo** → Le + **Nas**

b)

ORBITAL REGION

S. aurata	**Fr** → **Lac** → Pts → **Ino 3-5** → Bas → Sca → **Ino 2, 6**
A. davidsonii	**Fr** → **Lac** → Pts → **Ino 3-5** → Bas + **Ino 2, 6**
O. latipes	**Fr** + **Lac** → Pts + **Ino 6**
C. gariepinus	**Fr** → Bas + **Lac** → **Ino 2** + Pts → **Ino 3, 4**
S. melanostictus	**Fr** + **Ino 2-5** + **Lac** → Bas + **Ino 6** + Pts + Sc

c)

OTIC REGION

S. aurata	Po → Pto → Ep + **Pa** → So + Soc → Int
A. davidsonii	Po → Pto → Ep → **Pa** + So + Soc
O. latipes	Soc → Po + Pto + So → Ep
C. gariepinus	Po → **Pa** + Pto + Soc → So → Ep
S. melanostictus	Pto → **Pa** + Po + Soc → Ep + Int + So

Figure 8. (a-c).

[1] The vomer is absent in *O. latipes*, a feature caracteristic of adrianichthydid fish (Nelson, 1994).

d)

BASICRANIAL REGION

S. aurata	**Ps** → Bo + Exo
A. davidsonii	**Ps** → Bo + Exo
O. latipes	**Ps** → Bo + Exo
C. gariepinus	**Ps** → Bo + Exo
S. melanostictus	**Ps** → Bo + Exo

e)

JAWS

S. aurata	**Mx** → **De** → An + Ra → **Pm**
A. davidsonii	**De** + **Mx** + **Pm** → An + Ra
O. latipes	**Pm** + **Mx** + **De** → An → Ra
C. gariepinus	**De** → **Mx** + **Pm** → An + Ra
S. melanostictus	**Mx** → **De** → An + Ra → **Pm**

f)

OPERCULAR SERIES

S. aurata	**Ope** + **Pro** → **Io** + **Sub**
A. davidsonii	**Ope** + **Pro** → **Io** + **Sub**
O. latipes	**Io** + **Ope** + **Pro** + **Sub**
C. gariepinus[2]	**Ope** → **Pro** → **Io**
S. melanostictus	**Ope** → **Io** + **Pro** + **Sub**

Figure 8. (d-f).

[2] Lack the subopercle which is a characteristic of Siluriformes (Nelson, 1994; Fink & Fink, 1996).

g)

SUSPENSORIUM

S. aurata	Hm + Sy → **Enp** + Qu → **Ecp** + Pal → Mp
A. davidsonii	**Enp** + Hm + Qu + Sy → **Ecp** → Pal → Mp
O. latipes	Hm + Sy → Qu + Pal → **Enp**
C. gariepinus	Hm + Qu → **Enp** → Mp + Pal
S. melanostictus	Qu → Hm + Sy → Mp → **Enp** + **Ecp** → Pal

h)

HYOID ARCH

S. aurata	**Br 3-6** → Ch → **Br 1, 2** → Bh → Hh Lo + **Uh** → Eh → Ih + Hh Up
A. davidsonii	Hh Lo + Hh Up → **Br** + Ch → Eh + Ih + **Uh** → Bh
O. latipes	**Br** + **Uh** → Bh + Ch → Eh → Hh Lo + Hh Up
C. gariepinus	**Br 7-10** → **Br 5, 6** → Ch + Hh Lo → **Br 2-4** → Eh → **Br 1** →Hh Up
S. melanostictus	Ch → Eh + Ih + Hh Lo → **Br** → **Uh** → Hh Up → Bh

i)

BRANCHIAL ARCHES

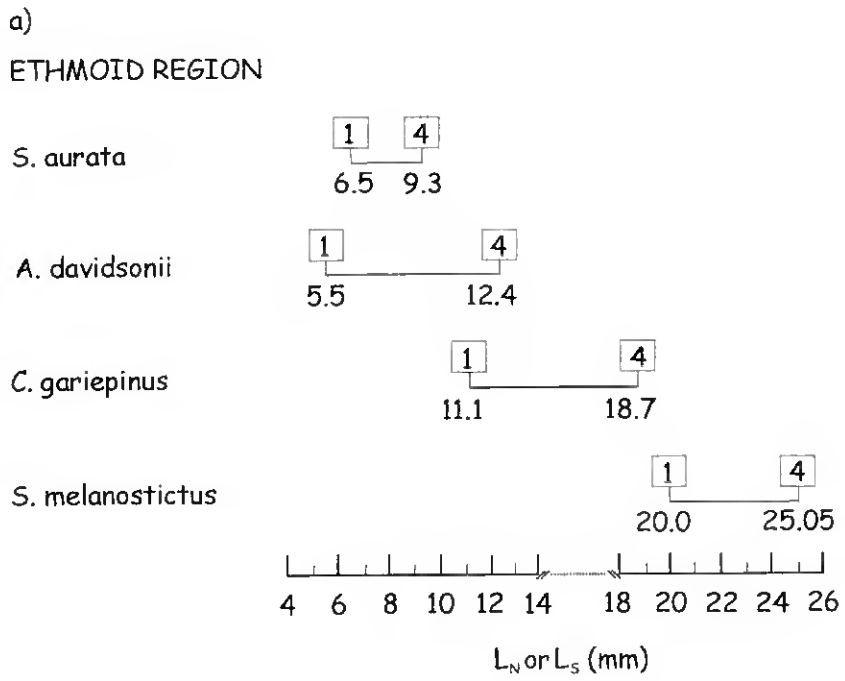
S. aurata	Cb5 → Cb3,4 → Cb1,2 →Eb1-4 + Pb3 → Bb1-3 → Hb3 + Pb2-4 → Pb1 → Hb1, 2
A. davidsonii	Cb1-5 + Eb1-4 → Bb1-3 + Hb1-3 → Pb1-4
O. latipes	Cb5 + Eb4 + Pb4 → Bb1-3 → Cb1-4 → Eb1-3 + Hb1-3 + Pb2
C. gariepinus	Cb1-5 + Eb1-4 → Bb2, 3 + Hb1, 2 → Pb3 → Pb4
S. melanostictus	Bb1+Cb1, 2 → Hb1 → Bb2-4 + Cb3-5+ Eb1-4 + Hb2-4 + Pb2-4 → Pb1

Figure 8. (g-i).

Figure 9. (next pages). Graphical representation of onset and completion of ossification in each of the regions constituting the viscerocranial skeleton of *Sparus aurata* (present study), *Anisotremus davidsonii* (Watson & Walker, 1992), *Clarias gariepinus* (Adriaens & Verraes, 1998) and *Sardinops melanostictus* (Matsuoka, 1997). The horizontal solid bars on the figure indicate the duration of the process and values within the squares associated with this bar indicate the number of ossified structures at the start and on completion of development. The length of specimens at the start and completion of development is indicated below the solid bar. Length: *S. aurata* - $L_N \leq 5.6$ mm, $L_S \geq 5.7$ mm; *A. davidsonii* and *C. gariepinus* - all the values are L_S; - $L_N \leq 16.55$ mm, $L_S \geq 17.0$ mm. Note, in *C. gariepinus*, with the exception of the epiotic (140.1 mm L_S) and upper hypohyal and pharyngobranchial 4 (46.8 mm L_S) all other strucutres complete ossification (18.7 mm L_S) well before that observed in *S. melanostictus*.

Interestingly, the sequence of ossification of the jaws is highly conserved between *S. melanostictus* and sea bream despite their phylogenetic distance [Fig. 8 (e)]. The ossification of this region is also highly conserved between the Sparidae, sea bream, *D. dentex* and *P. major* (Matsuoka, 1985, Koumoundouros *et al.*, 2000). The variations encountered in the ossification of this region between species may be a consequence of different functional requirements.

The most striking difference in the ontogeny of the viscerocranial skeleton of phylogenetically distant species (Fig. 7) is the time of onset and completion of ossification [Fig. 9 (a)-(j)]. Moreover, several different ossification strategies exist which raise intriguing questions about the evolution of the two principal types of endoskeletons encountered in fish. In Chondrichthyes, an embryonic cartilaginous endoskeleton is retained and in Osteichthyes, the embryonic cartilaginous skeleton is replaced by bone. The developmental differences which exist between these two classes of fish responsible for the persistence of cartilage in one class and its conversion into bone in the other, not to mention the evolution of dermal bone, is a largely unexplored area. However, the observations presented here suggest a comparative approach will be important for the elucidation of this complex process. In spite of the relatively limited number of species studied, there is evidence that ossification of all regions of the viscerocranial skeleton described


a)
ETHMOID REGION
S. aurata
1 4
6.5 9.3
A. davidsonii
1 4
5.5 12.4
C. gariepinus
1 4
11.1 18.7
S. melanostictus
1 4
20.0 25.05
4 6 8 10 12 14 18 20 22 24 26
L_N or L_S (mm)

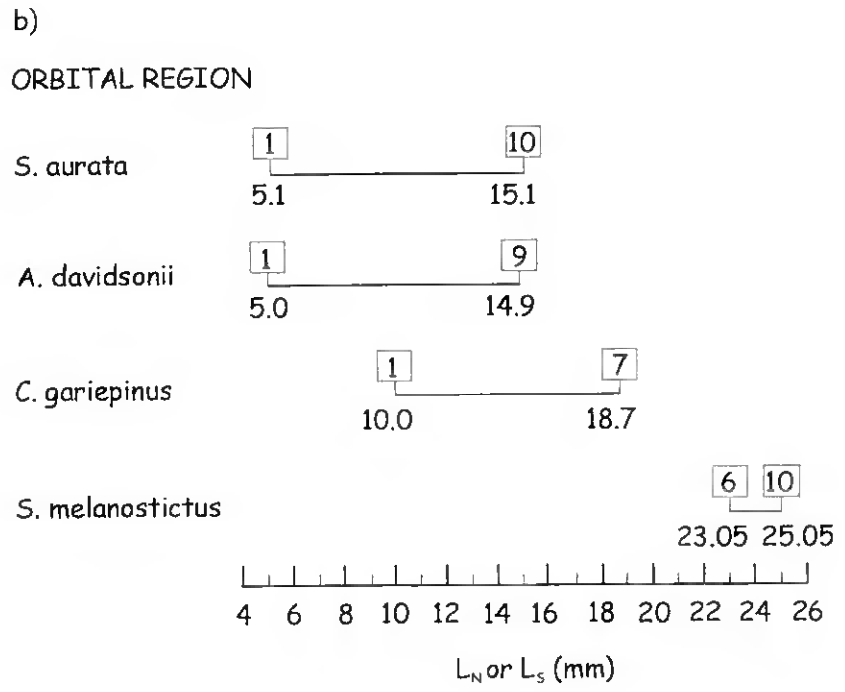
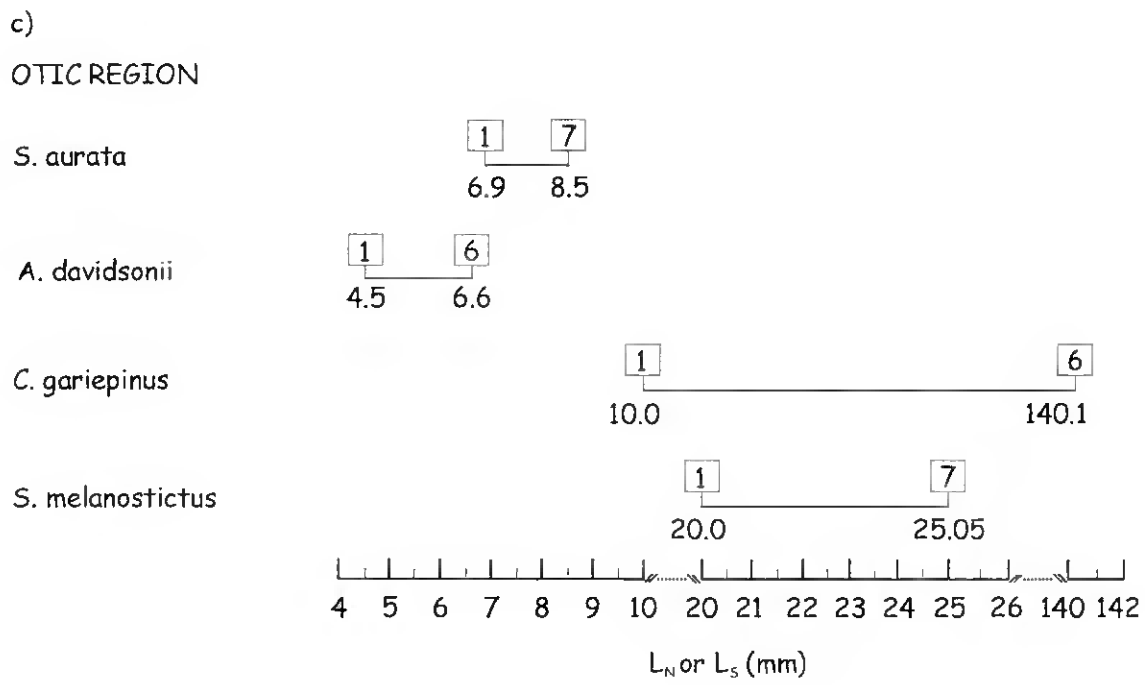

b)
ORBITAL REGION
S. aurata
1 10
5.1 15.1
A. davidsonii
1 9
5.0 14.9
C. gariepinus
1 7
10.0 18.7
S. melanostictus
6 10
23.05 25.05
4 6 8 10 12 14 16 18 20 22 24 26
L_N or L_S (mm)

Figure 9. (a-b).


c)
OTIC REGION
S. aurata
1
7
6.9
8.5
A. davidsonii
1
6
4.5
6.6
C. gariepinus
1
6
10.0
140.1
S. melanostictus
1
7
20.0
25.05
4 5 6 7 8 9 10 20 21 22 23 24 25 26 140 142
L_N or L_S (mm)

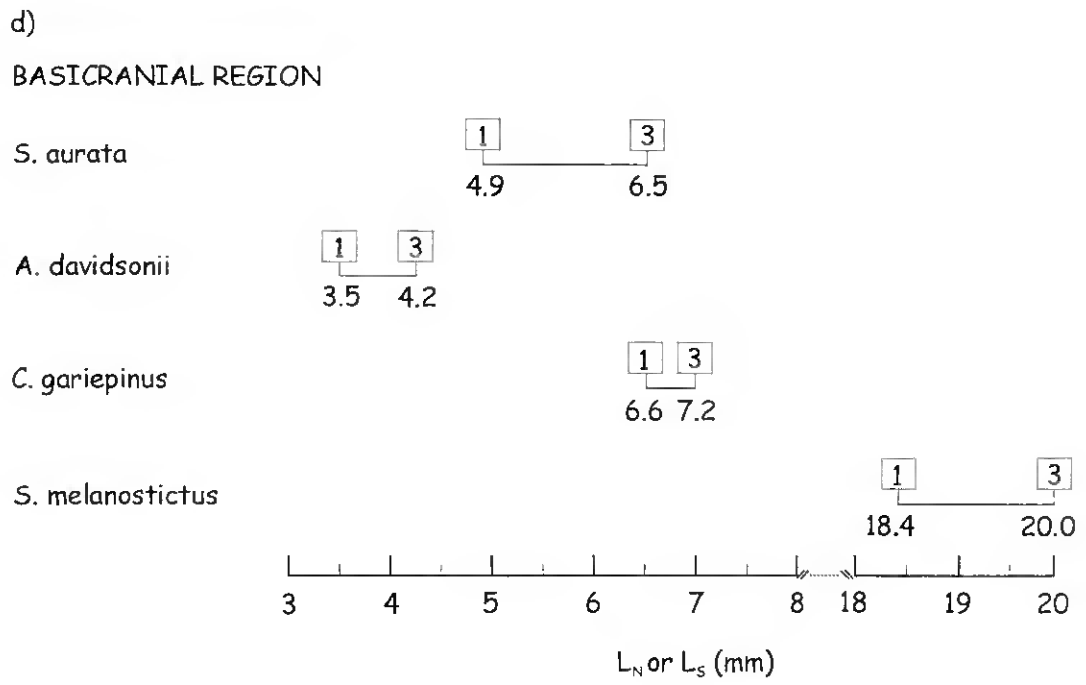
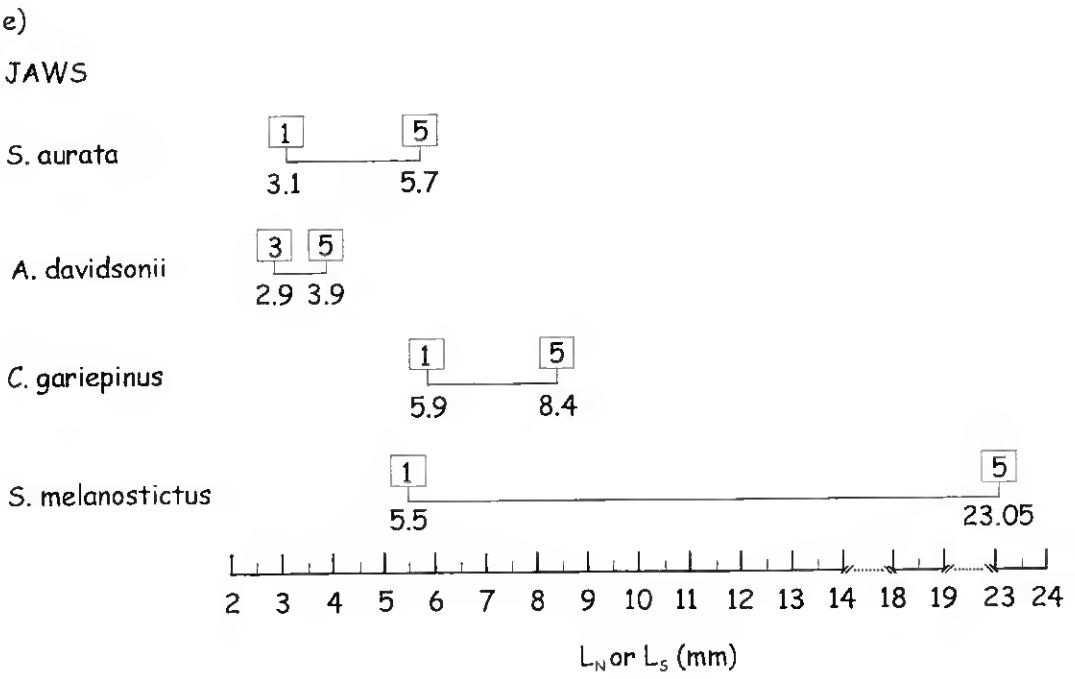

d)
BASICRANIAL REGION
S. aurata
1
3
4.9
6.5
A. davidsonii
1
3
3.5
4.2
C. gariepinus
1
3
6.6
7.2
S. melanostictus
1
3
18.4
20.0
3 4 5 6 7 8 18 19 20
L_N or L_S (mm)

Figure 9. (c-d).


e)
JAWS
S. aurata
1
5
3.1
5.7
A. davidsonii
3
5
2.9 3.9
C. gariepinus
1
5
5.9
8.4
S. melanostictus
1
5
5.5
23.05
2 3 4 5 6 7 8 9 10 11 12 13 14 18 19 23 24
L_N or L_S (mm)

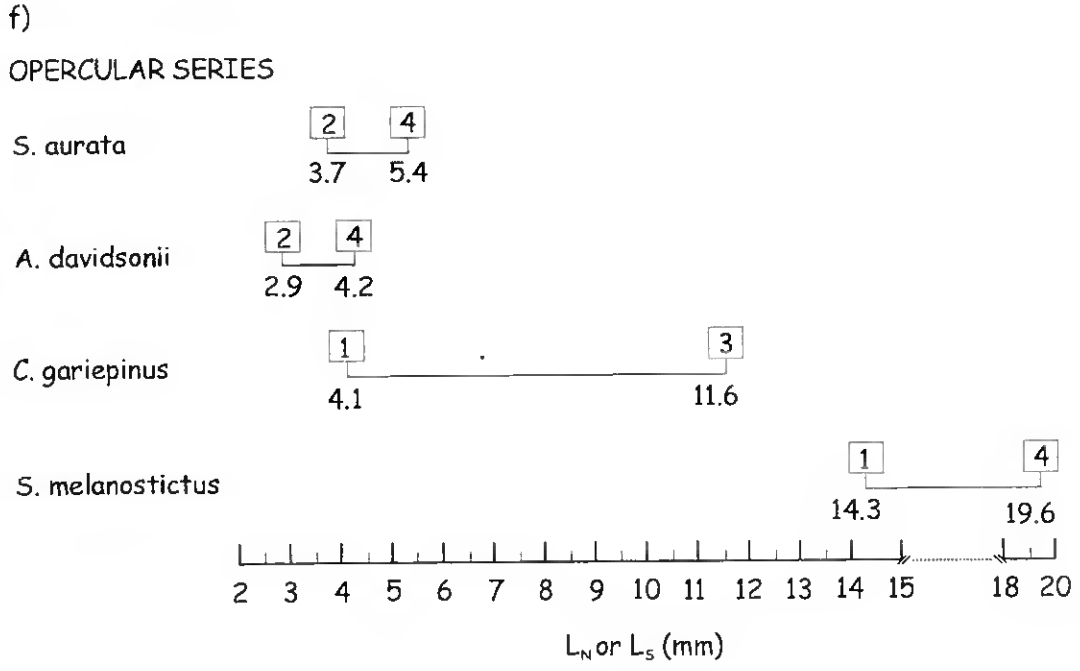
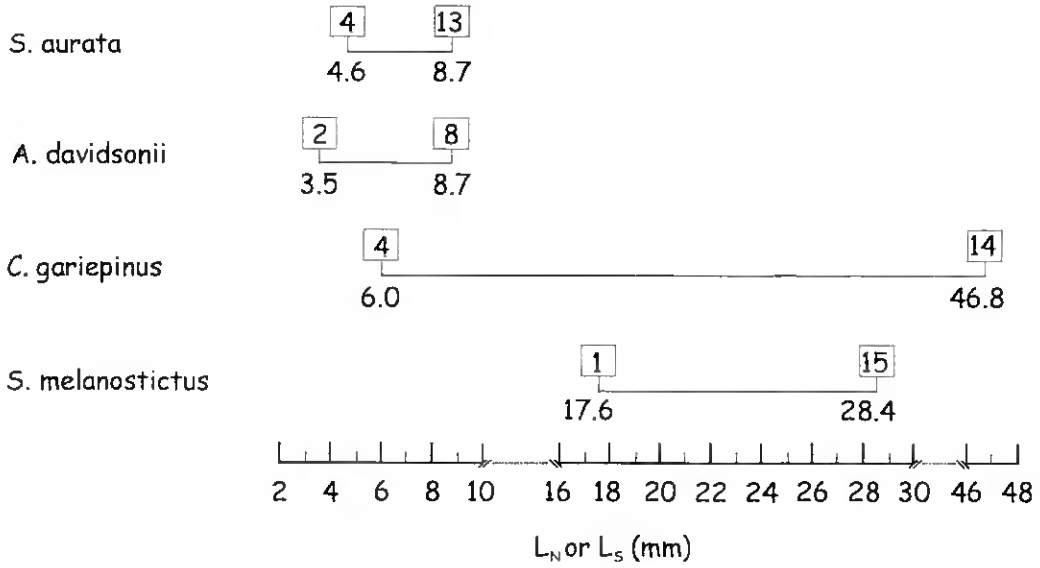

f)
OPERCULAR SERIES
S. aurata
2
4
3.7
5.4
A. davidsonii
2
4
2.9
4.2
C. gariepinus
1
3
4.1
11.6
S. melanostictus
1
4
14.3
19.6
2 3 4 5 6 7 8 9 10 11 12 13 14 15 18 20
L_N or L_S (mm)

Figure 9. (e-f).

g)

HYOID ARCH



h)

BRANCHIAL ARCHES

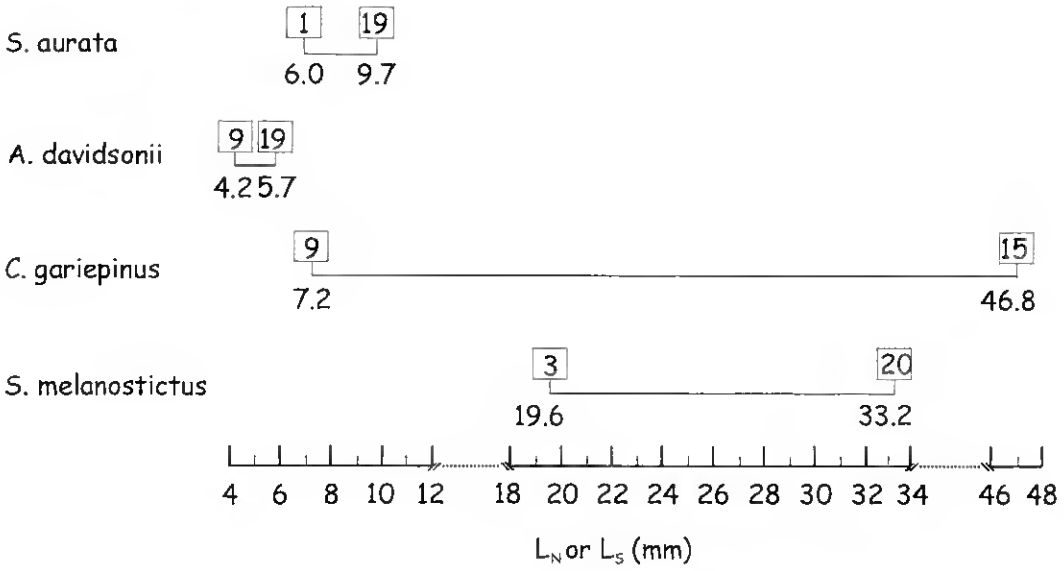
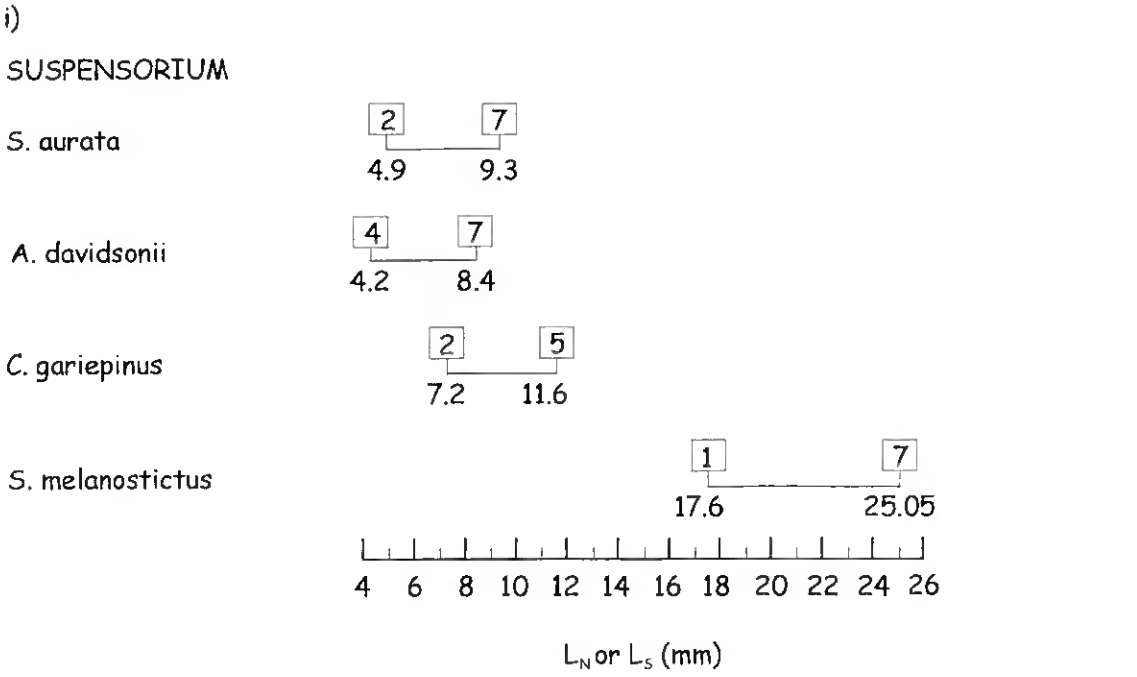


Figure 9. (g-h).


i)
SUSPENSORIUM
S. aurata
2
7
4.9
9.3
A. davidsonii
4
7
4.2
8.4
C. gariepinus
2
5
7.2
11.6
S. melanostictus
1
7
17.6
25.05
4 6 8 10 12 14 16 18 20 22 24 26
L_N or L_S (mm)

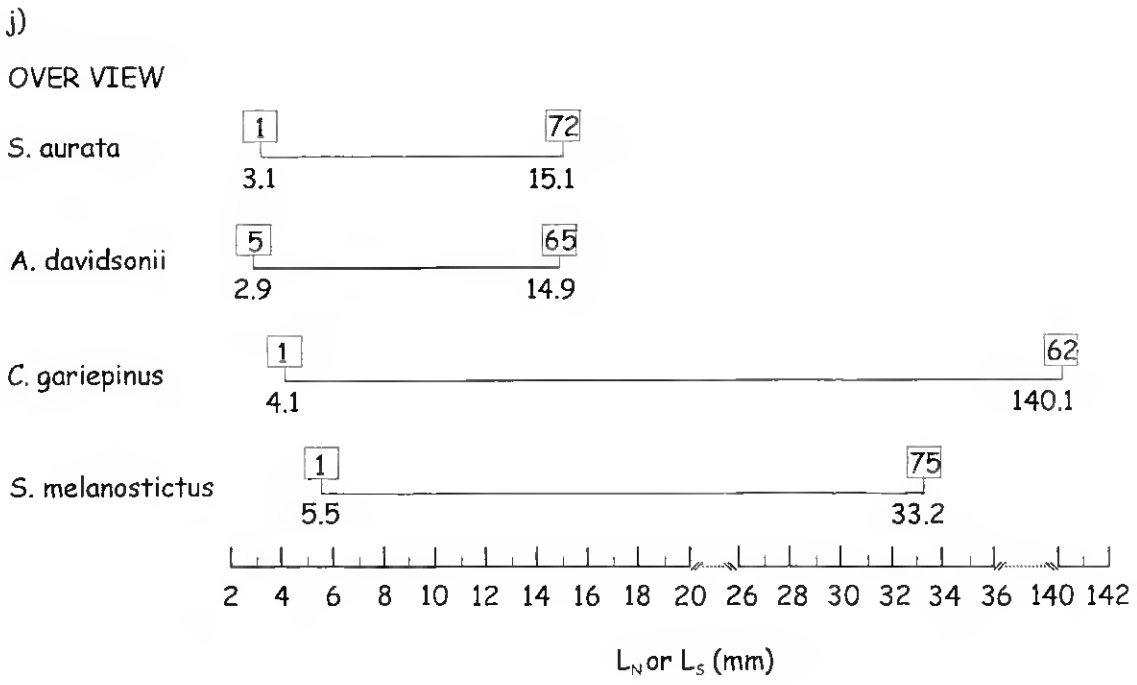

j)
OVER VIEW
S. aurata
1
72
3.1
15.1
A. davidsonii
5
65
2.9
14.9
C. gariepinus
1
62
4.1
140.1
S. melanostictus
1
75
5.5
33.2
2 4 6 8 10 12 14 16 18 20 26 28 30 32 34 36 140 142
L_N or L_S (mm)

Figure 9. (i-j).

in the Clupeomorpha, *S. melanostictus* and Japanese anchovy *Engraulis japonicus* (Schlegel) (Balart, 1985) was delayed compared with higher teleosts (e.g. *A. davidsonii*, sea bream). These observations in Clupeomorpha, the group in the present comparison closest to the cartilaginous fishes, raise interesting questions about the evolution of the mechanisms that give rise to bone in teleosts and suggests that studies of Clupeomorpha may provide some important clues about this process. The ossification of the viscerocranial skeleton in *S. melanostictus* also generally takes longer than that observed in advanced teleosts [Fig. 9 (a)-(j)]. Interestingly *C. gariepinus*, which lies between *S. melanostictus* and *A. davidsoni* and sea bream (Fig. 7), has a pattern of ossification intermediate between the two groups, the onset is not so delayed as *S. melanostictus* but the process is generally prolonged [Fig. 9 (c), (f), (g), (h), (j)].

The high degree of conservation observed in the number of structures and their disposition in each region of the viscerocranial skeleton in different species of Teleostei, suggests that genetic factors play an important and determinant role. However, the detailed study made of the viscerocranial skeleton in sea bream demonstrated that the sequence, and timing of onset and completion of ossification, is plastic within the different regions considered. The latter may explain partially how environmental factors can influence the ontogeny and final appearance of different structures in the viscerocranial skeleton. It is tempting to speculate that the plasticity of viscerocranial skeleton development observed in sea bream is a common feature of most species and may be an important characteristic for their adaptation to different environments. The importance of functional demands on the evolution of the viscerocranial skeleton in fishes is apparent, and in all species studied the first

structures to appear are essential for survival and are those associated with feeding and breathing.

Clearly many more studies will be required at both a basic and applied level to determine how the development of the viscerocranial skeleton is regulated in fish and the role of environmental factors in this process.

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CHAPTER 5

CHARACTERIZATION AND FREQUENCY OF THE PRINCIPAL SKELETAL ALTERATIONS IN CULTURED SEA BREAM (*Sparus aurata*)

Co-author

Deborah M. Power

(*in preparation*)

CHARACTERIZATION AND FREQUENCY OF THE PRINCIPAL SKELETAL ALTERATIONS IN CULTURED SEA BREAM (*Sparus aurata*)

ABSTRACT

The frequency and nature of anomalies and meristic variations in a population of 80 reared *Sparus aurata* larvae and lordosis in a group of 20 hatchery-reared *S. aurata* juvenile is characterized. In larvae the most frequent anomalies are associated with the caudal complex and up to 60% of specimens are affected. Other anomalies are localized in jaws or along the vertebral column. A strong correlation is observed between the origin of bone structures and the frequency of anomalies and over 77% arise in cartilage replacement bone. The lordosis present in the juveniles is concentrated in the posterior half of the vertebral column (22% in vertebrae 16 and 25% in vertebrae 17) and is characterized by a marked change in external morphology evident as a deviation in the body axis and generally involving two vertebrae.

Key words: *Sparus aurata*, osteology, anomalies, meristic variations, dermal bone, endochondral bone.

INTRODUCTION

The importance of morphology, both external and internal, for the taxonomic classification of fish has led to a range of studies aimed at determining typical meristic variation and the frequency with which they normally occur in different species. Recently, considerable attention has been focussed on the identification of anomalies in both wild and hatchery-reared fish, in the former case as an indicator of environmental quality (Baumann & Hamilton, 1984; Bengtsson *et al.*, 1988; Sherwood & Mearns, 1977; Tutman *et al.*, 2000) and in the latter case as a consequence of their negative impact on aquaculture production (Houde, 1971; Paperna, 1978; Barahona-Fernandes, 1982; Matsuoka, 1987; Poyton, 1987; Francescon *et al.*, 1988; Santamaría *et al.*, 1994; Ellis *et al.*, 1997). Interpretation of results from studies of morphology in wild fish is complicated by numerous factors and frequently only anomalies resulting in the alteration of external morphology are recorded and more subtle anomalies that require application of methodologies to examine internal structures, such as the skeleton, are overlooked.

The need to control the quality of aquaculture fish, has meant, that unlike wild fish, they have been subject to more detailed studies, although in hatchery reared fish relatively few studies defining the limits of normal and abnormal internal morphology exist. The most frequently reported internal anomalies in aquaculture species are those that affect the skeleton (Gill & Fisk, 1966; Barahona-Fernandes, 1982; Andrades *et al.*, 1996; Ellis *et al.*, 1997; Koumoundouros *et al.*, 1997a) and swim bladder (Paperna, 1978; Kitajima *et al.*, 1981; Chatain, 1987; Daoulas *et al.*, 1991; Chatain, 1994; Kitajima *et al.*, 1994). Opercular abnormalities, clearly visible on external examination, are also frequent in intensively cultured sea bream (*Sparus aurata*) and sea bass

(*Dicentrarachus labrax*) (Paperna, 1978; Barahona-Fernandes, 1982; Francescon *et al.*, 1988; Chatain, 1994; Koumoundouros *et al.*, 1997b; Galeotti *et al.*, 2000). It is difficult to estimate the incidence of skeletal anomalies and their cost to the aquaculture industry, as there is an absence of published data about this phenomenon. The cause of anomalies in a hatchery situation is unclear, but given the ample food supply and absence of predators in hatcheries, both factors that favour survival of abnormal larvae, a relatively higher proportion of abnormal larvae might be expected compared with larvae in the wild. The importance of genetic, environmental or dietary factors on this phenomenon remains to be characterized, but the recent advances in developmental studies may provide important clues about the genes implicated in anomalous development.

In the last decade numerous genes and their products involved in vertebrate development have been characterized. The Tübingen mutants database that consists of zebrafish (*Danio rerio*) mutants displaying a visible phenotype, has played a central role in the identification of genes involved in normal developmental processes of a range of structures, such as notochord, brain, spinal cord, somites and musculature, cardiovascular system, gastrointestinal system, sensory system, skin, fin, jaw and branchial arches, pigment pattern and formation (Haffter *et al.*, 1996). Some of these mutants bear a remarkable resemblance to anomalous hatchery-bred sea bream. Application of results from model organisms, such as the zebrafish, to aquaculture species may provide important clues for identifying genetic factors that contribute to the development of anomalies in a commercial situation. This is the first step in the development of a rational management program for the elimination or minimization of the problem.

The extensive characterization in the preceding chapters (2, 3 and 4) of the normal skeletal ontogeny of sea bream forms the basis for distinguishing

normal meristic variations from anomalous development. The frequency and nature of anomalies are characterized in larvae and in a group of hatchery-reared fish (6 month post hatch) that showed a clear external phenotype (lordosis).

MATERIAL AND METHODS

Collection of material

Larval samples of sea bream (*Sparus aurata*) were collected from a standard culture, in which eggs were hatched and cultured in 0.2m^3 conical tanks supplied with a continuous flow of gently aerated sea water (19 $\pm$ 1 $^{\circ}$C) under a photoperiod cycle of 12h light:12h dark. The feeding regime has previously been reported, in brief, after yolk sac depletion (4 days post hatch, DPH) larvae were fed on *Brachionus plicatilis*, followed by *Artemia* sp. (15 DPH) and weaned onto dry food from 40 DPH (Chapter 2).

Larvae were anaesthetized in MS-222 (Sigma, Madrid) and fixed overnight at 4 $^{\circ}$C in 2% paraformaldehyde (PFA) solution, washed and stored in 70% methanol. Experiments have previously been carried out to confirm that morphology was not altered as consequence of fixation and subsequent staining methods (Chapter 2).

Sea bream juveniles were sampled from a stock reared on a Portuguese fish farm in which up to 30% had visible lordosis. Fish received an overdose of anaesthetic (MS-222) and were frozen at -20 $^{\circ}$C. To analyse the skeleton, specimens were thawed, lightly cooked and the flesh dissected away. Samples

were then fixed in 4% PFA overnight at 4 °C washed and stored in 70% methanol until whole-mount staining of bone and cartilage.

Classification of skeletal deffects

The skeleton of larvae (n=80, Table I) was inspected after whole-mount staining of bone and cartilage using a modification of the alcian blue/alizarin red method and the alkaline phosphatase method (Chapter 2). Standard length, L_S (from anterior tip of snout to base of the caudal complex) of each larvae (Table I) was measured with the aid of an ocular micrometer in a stereoscopic microscope (Wild M8).

Table I. Number of sea bream larvae and juveniles studied in each size range.

Length (mm, L_S)	Number of specimens examined
9.9 - 10.5	15
10.9 - 11.5	15
12.0 - 13.6	15
14.6 - 16.0	13
16.5 - 17.6	10
18.3 - 20.4	12
63,5 - 78,6 (juveniles)	20

The status of cartilage and bone ontogeny was determined and the incidence and nature of anomalies was considered in 3 principal regions: head, vertebral column and fins. Some of the juvenile specimens (n=20, Table I) collected were subject to the same whole-mount staining procedure as described

for larvae. Terminology used to describe cartilage and skeletal structures is based upon Houde & Potthoff (1976), Matsuoka (1987) and Balart (1995).

RESULTS

The principal objective of the present study was to characterize the nature and frequency of anomalies and not the stage at which they appeared, for this reason only specimens that had the full cartilage complement (9.6 mm L_S) were considered (Chapters 2, 3 and 4). Bilateral assessment of specimens did not result in the detection of any significant morphological differences.

Meristic variations: deviations that do not alter body outline

Table II presents the variations identified and classified as meristic variations in the present study on sea bream and compares them to those identified in wild sea bream by Albuquerque (1956), Whitehead *et al.* (1986) and Fisher *et al.* (1987) and hatchery-reared sea bream (Boglione *et al.*, 2001).

In the present study, in addition to variations previously identified in sea bream that are presented in Table II, additional variations that do not alter the body plan were also identified. These alterations were associated with accessory cartilages and the predorsals and in the former structures included location in an alternative position, fusion of two adjacent elements, separation and subsequent formation of independent elements and in the latter structure included shortening and/or fusion between adjacent elements.

Interestingly, in the present study the pelvic fins were unique in that they did not present any variation in any of the specimens examined.

Table II. Quantitative analysis of the meristic characters.

	Our study	Albuquerque, 1956	Whitehead *et al.*, 1986	Fisher *et al.*, 1987	Boglione *et al.*, 2001
N° of vertebrae (V)	24 V - 76 larvae 25 V - 4 larvae	24 V	lacking data (-)	(-)	18-26 V
Spines (S) and rays (R) of the dorsal fin	XI S, 13 R - 73 larvae XI S, 12 R - 6 larvae XI S, 14 R - 1 larvae	XI S 13 R	XI S 13-14 R	XI S 12-13 R	VII-XII S 11-15 R
Spines (S) and rays (R) of the anal fin	III S, 11 R - 75 larvae III S, 12 R - 5 larvae	III S 11-12 R	III S 11-12 R	III S 11-12 R	12-17 R
Rays (R) of the pectoral fin (in each one)	15 R - 74 larvae 14 R - 5 larvae 16 R - 1 larvae	16 R	(-)	(-)	8-17 R
Spine (S) and rays (R) of the pelvic fin (in each one)	I S, 5 R - 80 larvae	I S 5 R	(-)	(-)	(-)
Upper rays (UR) and lower rays (LR) of the caudal fin	9 UR + 8 LR - 72 larvae 9 UR + 7 LR - 1 larvae 9 UR + 9 LR - 2 larvae 8 UR + 8 LR - 4 larvae 8 UR + 7 LR - 1 larvae	(-)	(-)	(-)	7-10 UR + 3-10 LR
Dorsal pterygiophores (DP)	22 DP - 73 larvae 21 DP - 6 larvae 23 DP - 1 larvae	(-)	(-)	(-)	(-)
Anal pterygiophores (AP)	12 AP - 75 larvae 13 AP - 5 larvae	(-)	(-)	(-)	(-)
Distal radials (DR) of pectoral fins (in each one)	15 DR - 74 larvae 14 DR - 5 larvae 16 DR - 1 larvae	(-)	(-)	(-)	(-)

Anomalies: result in deviations from the normal body plan

The skeletal abnormalities identified in the sea bream larvae analysed were predominantly localized in the head, vertebral column and caudal fin complex. The most common types of abnormalities were:

Upper/lower jaws - shortening of both upper and lower jaws or shortening of upper or lower jaw only (Fig. 1b); shorter upper jaw with reduction of premaxillary, maxillary and of frontal (called pugheadness) (Fig. 1c); various degrees of severity of these alterations are generally detected.

Neural arches and haemal arches and spines - fusion of adjacent neural and/or haemal arches (Fig. 2b, d); junction of neural or haemal spines.

Centrum - fused centra especially at the posterior end of the vertebral column (Fig. 2d, e); deformation of two/three successive centra.

Haemal arches and parhypural - fusion between the last haemal arch and parhypural (Fig. 2b).

Parhypural and hypurals - fusion of parhypural and hypural 1 or parhypural and hypurals 1 and 2 (Fig. 2c, d).

Epurals - variable number and shape (shortening, branching), fusion, over-formed (Fig. 2c, e).

As discussed in Chapter 2, the normal centra is cuboid constricted at its center and separated from adjacent centra by an intervertebral region. The caudal fin supports, the epurals, hypurals, parhypural and the last haemal arch always appear as separate structures in normal specimens (Fig. 2a).

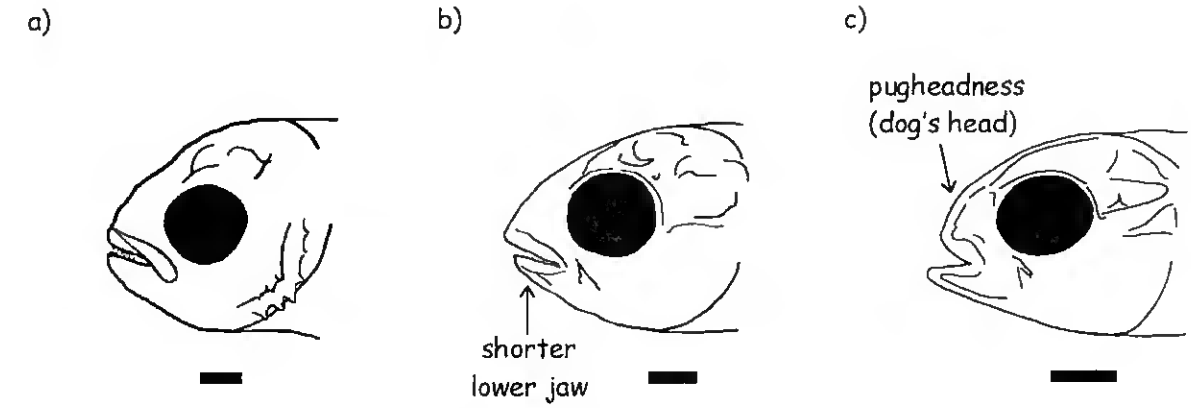


Figure 1. Camera lucida drawings of the head of sea bream larvae. a) normal specimen, 20.4 mm L_S; b) deformation of lower jaw, 15.7 mm L_S; c) deformation of frontal and upper jaw, 11.6 mm L_S. Scale bar correspond to 1.0 mm.

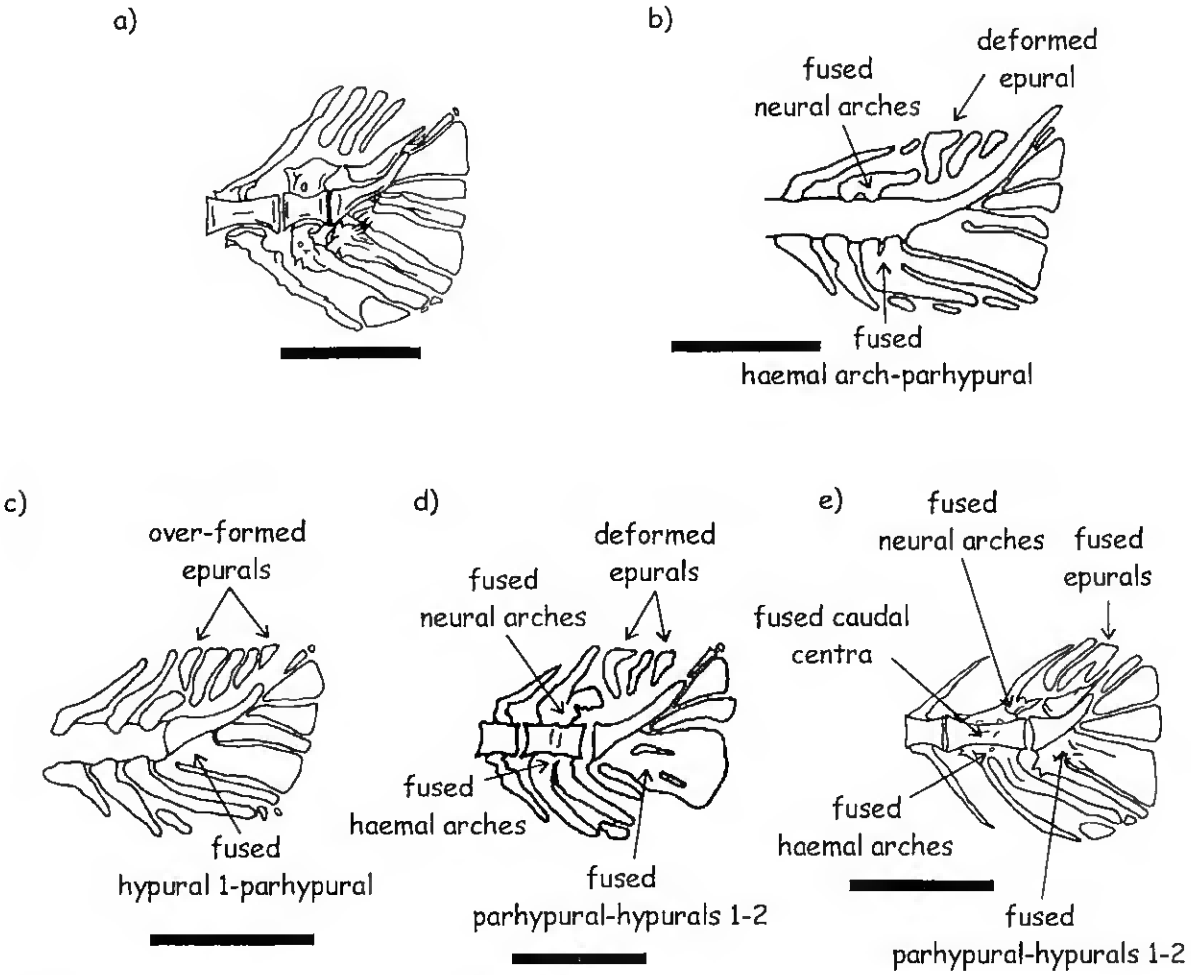


Figure 2. Camera lucida drawings showing the most frequent anomalies in the posterior region of sea bream larvae. a) normal specimen; b) to e) abnormal specimens, the principal anomalies are indicated; a) 16.0 mm L_S; b) 7.7 mm L_S; c) 8.7 mm L_S; d) 10.8 mm L_S; e) 18.7 mm L_S. Scale bar indicates 1.0 mm.

Larval sea bream anomalies: frequency and association

A large proportion (60%) of all the anomalies detected in larval sea bream were found to be associated with structures of the caudal complex (Fig. 3). The most frequently occurring anomalies of the caudal complex were fusion of structures and in 28% of the larvae the parhypural and hypural(s) were fused and in several larvae the parhypural fused with more than one hypural, in 17% of the larvae the epurals were fused and the accessory cartilages were fused in 15% of larvae (Fig. 3).

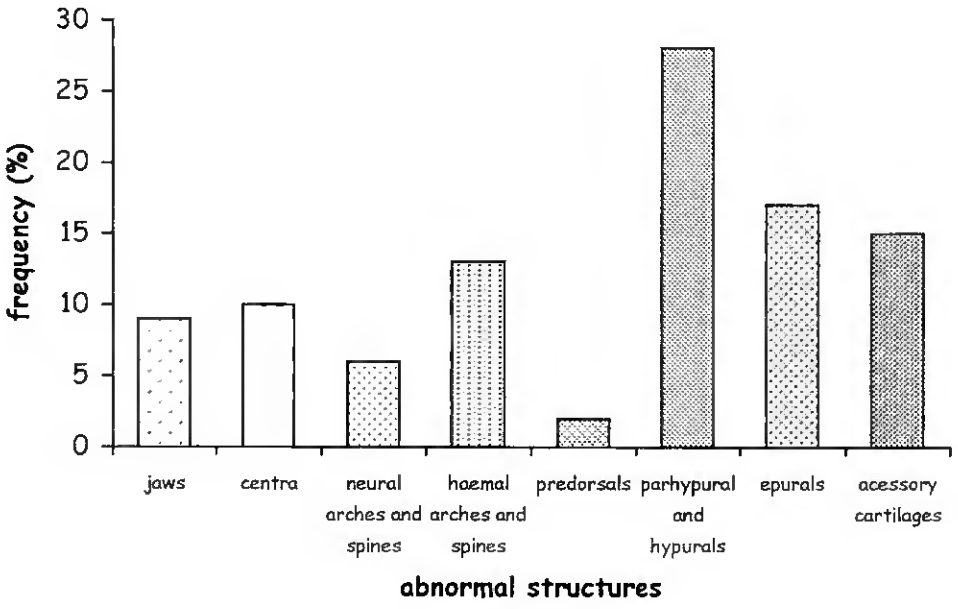


Figure 3. Relative frequency of anomalies between the different structures of sea bream larvae.

The most frequently observed vertebral anomalies in the sea bream larvae were localized at the posterior region. These anomalies generally involved fusion between adjacent centra and also with the respective neural and/or haemal arches and spines.

The skeleton in sea bream, in common with most other teleosts, arises from two principal processes, direct deposition of bone (dermal bone) and

deposition of bone in cartilage (cartilage replacement bone). The relative importance of the origin of bony structures in the development of anomalies is uncertain and has not previously been analysed. In the present study, the origin of bony structures appeared to be associated with the frequency at which anomalies occur, so that only 22.4% of bone anomalies were associated with dermal bone and the large majority (77.6%) arose in cartilage replacement bones (Table III, Fig. 4).

TABLE III. Indication of region of the skeleton in which anomalies are observed and bone type in sea bream larvae (D, dermal bone; C, cartilage replacement bone).

STRUCTURE	BONE TYPE
Jaws	D
Centra	D
Neural arches and spines	C
Haemal arches and spines	C
Predorsals	C
Parhypural and hypurals	C
Epurals	C

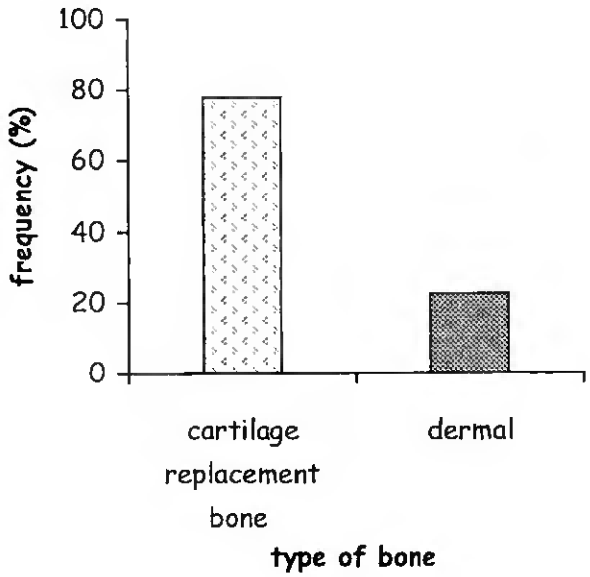


Figure 4. Relative frequency of anomalies encountered in dermal and cartilage replacement bones in sea bream larvae.

Despite the relatively limited number of samples analysed in the present study there was a very strong association between bone origin and susceptibility to anomalies suggesting that this is an aspect that deserves more careful analysis in the future.

Anomalies associated with the vertebral column in juvenile sea bream

The vertebral column tipically consists of a straight row of vertebrae along the body that begins with the 1st vertebrae in the head region and finishes with the 24th vertebrae (urostyle) in the caudal region.

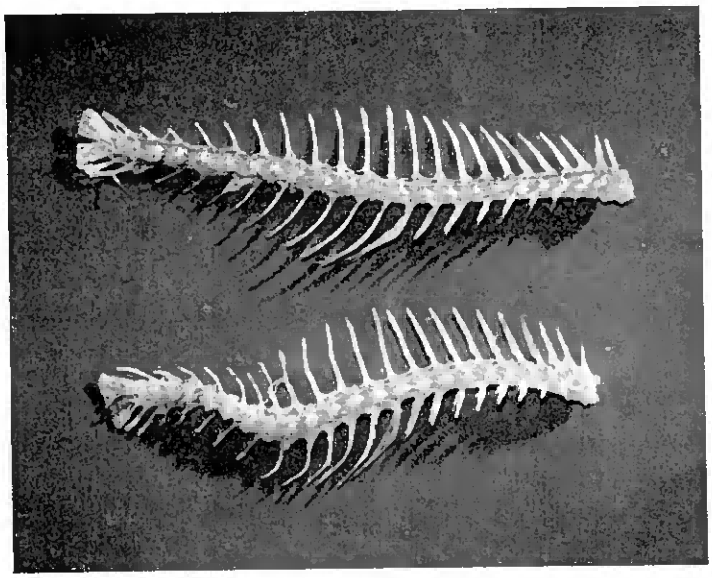
In juveniles, abnormalities were observed predominantly in the vertebral column with serious deviation of the normal body plan (Table IV, Fig. 5a, b). The unusual alignment of the vertebral column and spinal cord is characterized by an abnormal and more or less acute V-shaped dorsoventral curvature.

Table IV. Localisation and incidence of vertebral anomalies giving rise to deviation in the body axis of juvenile sea bream.

Number of vertebrae	13	14	15	16	17	18	19	20	21	22
Incidence of anomaly (%)	3.0	6.0	12.5	22.0	25.0	12.5	9.0	3.0	3.0	3.0

The external morphology of the juvenile specimens of sea bream was very striking as the body axis deviated substantially from the normal pattern and a large proportion of fish examined (83.3%) were lordotic. For this reason a detailed analysis was made of the vertebral column in order to determine the nature of the skeletal abnormality responsible for the condition. A large

a)



b)

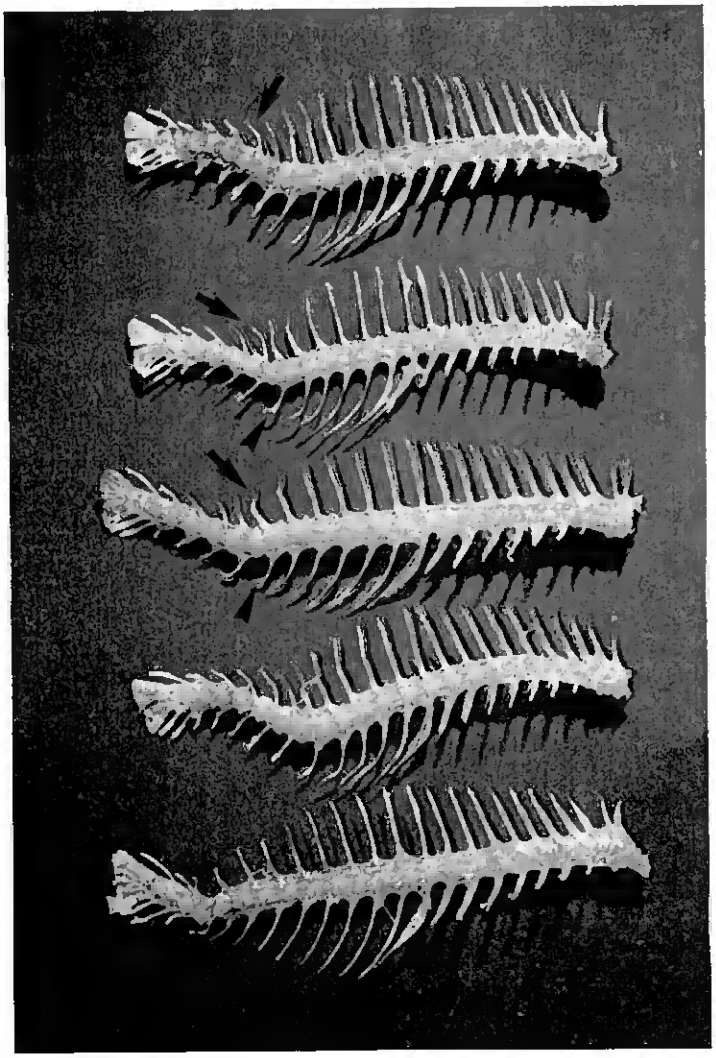


Figure 5. Photographs with dissected vertebral column of juvenile sea bream analysed. a) comparative approach between normal (upper) and lordotic (bottom) vertebral column; b) some examples of lordotic vertebral column; note the serious deviation of the vertebral column. Altered neural and haemal spines are indicated, respectively, by an arrow and an arrowhead.

proportion of the fish analysed had only 23 vertebrae (63.6%) which is 1 fewer than the most frequently occurring number, 24 (Chapter 2). The reduction in the number of the vertebrae by 1 could not be classified only as a meristic variation, as it was associated with a dramatic change in external morphology, in which the body axis appeared truncated. The most severe change was an associated lordosis.

The localisation of the deformation associated with the vertebral column that led to the observed lordosis (Fig. 5a, b) was concentrated in the posterior half of the vertebral column. Generally, in all the fish examined only two vertebrae were altered in the affected region. The greatest incidence of deformation occurred around vertebrae 16 (22% of fish examined) and 17 (25% of fish examined) and the remaining fish had deformation in other vertebrae in the posterior half of the column (Table IV, Fig. 5a, b). In the region of curvature of the lordotic specimens, vertebrae had an altered shape (compression or elongation of the vertebral body, triangular shaped), were frequently hypercalcified and had fused, shorted and/or bent neural spines and bent haemal spines (Fig. 5b).

DISCUSSION

One of the major problems in aquaculture is the production of high quality larvae that will develop into healthy and vigorous juveniles and adults. The hatchery represents one of the high-risk areas for successful aquaculture and good survival rates and growth and reduced rates of anomalies are the main challenges to be met. The appearance of anomalies of the skeleton is a considerable problem for the aquaculturists, because they reduce the product

quality, decrease final price and this results in a negative economic impact. Several studies have shown that hatchery produced fish are less vigorous than wild fish (Blaxter, 1969; Paperna, 1978; Komada, 1980; Matsuoka, 1987; Daoulas *et al.*, 1991; Boglione *et al.*, 2001). The artificial environment that exists in the hatchery in which there is an abundance of food and lack of predators, leads to survival of less fit larvae and this has a series of consequences that include survival of abnormal larvae.

The main objective of hatcheries is to ensure maximum production of larvae and this has meant that most attention has been given to attaining high larval growth rates and survival. This has generally led to excessive feeding levels, although it is now appreciated that such approaches do not always have a positive effect. A balance needs to be achieved in the hatchery between overfeeding, quality of diets and the consequent changes in larval environmental conditions.

Anomalies that cause deformation of the body, such as lordosis or slight abnormalities causing little deformation of the body can certainly diminish the swimming capacity of specimens. For example, fusion of adjacent centra seems to reduce the flexibility of the fish body and fusion of the hipurals may impede differential movement of the caudal fin rays (Matsuoka, 1987). Reduced swimming capacity might be expected to reduce survival rate in the wild as a consequence of predation and reduced feeding ability. According to Matsuoka (1987) the high incidence of jaws abnormalities in cultured *Pagrus major*, probably hinder capture of live prey and reduce the viability of larvae.

Skeletal deformations, including meristic counts, may arise as a result of genetic (Barahona-Fernandes, 1982; Poynton, 1987; Ishikawa, 1990; Campbell, 1995) or/and epigenetic (Seymour, 1959; Lee & Menu, 1981; Newsome & Piron, 1982; Wiegand *et al.*, 1989; Polo *et al.*, 1991; Wimberger, 1993; Chatain, 1994;

Tutman *et al.*, 2000) factors. Anomalies due to genetic alterations result from mutation or recombination of DNA and unless lethal are heritable. Anomalies caused by epigenetic factors such as temperature, salinity, dissolved oxygen, handling, diet, radiation and chemical pollution arise during the formation of the embryo. The critical period for the induction of abnormalities is not very well characterized, but it is known that the alteration in the number of vertebrae can only occur during early embryogenesis (Lindsey & Ali, 1965) while the number of fin elements can be modified after hatching (Fowler, 1970).

Several morphological abnormalities affecting fins, body axis, head and jaws and the operculum have been described during development of reared larvae. Abnormalities of the operculum are evident as severe folding and twisting of the operculum and/or suboperculum and may affect up to 80% of individuals in some batches (Paperna, 1978; Barahona-Fernandes, 1982; Francescon *et al.*, 1988; Daoulas *et al.*, 1991; Chatain, 1994; Andrades *et al.*, 1996; Hilomen-Garcia, 1997; Koumoundouros *et al.*, 1997b). In the present study no opercular abnormalities were recorded. It has been suggested that epigenetic factors may be most important cause of anomalies in hatchery-produced fish. This would appear to be substantiated by the high variability of abnormalities detected in different batches of eggs from the same broodstock. Factors in hatcheries that may contribute to this problem include excessive manipulation, stocking density, intensity and quality of light, photoperiod, tank colour and water quality.

Interestingly the meristic counts characterized in the present study are in general agreement with previously published data in wild species (Albuquerque, 1956; Whitehead *et al.*, 1986; Fisher *et al.*, 1987) suggesting that there are biological limits for variation and that outside such limits normal development is compromised and anomalies arise. The meristic variations identified in the sea

bream with the exception of those reported by Boglione *et al.* (2001), appear to be remarkably stable, particularly considering the very different stocks studied and the lapse in time (over 45 years), that correspond to over 15 generation times for this species, between the different studies.

Surprisingly, the study of Boglione *et al.* (2001) details an unusually wide range in the meristic counts (number of vertebrae, counts of dorsal spines and rays, anal rays, pectoral rays and caudal upper and lower rays) (see Table II). The data they present is not comparable with any other known studies in reared or wild sea bream or even in other reared teleosts. The basis for classification of variations as meristic or anomalous is not defined by the authors and the meristic variations indicated for vertebrae, 18-26, would be expected to have a consequence for the external morphology of specimens examined. The extremely low number of fish analysed by Boglione *et al.* (2001) from some fish farms (in several cases <30 specimens), the lack of information about how fish were captured and the exceedingly high incidence of anomalies, over 70%, in the majority of samples, suggests that the data may be biased by inadequate sampling methodology.

Relatively few studies report abnormalities of the mouth and/or cephalic region and this may be a consequence of compromised feeding of such larvae and a consequent high mortality rate. Furthermore, since development of the pharyngeal apparatus is closely related with brain development, this might also be expected to severely compromise larval survival. In *D. labrax* (Barahona-Fernandes, 1982), spinal cord anomalies are present at hatching onwards, however, larval mouth abnormalities have been proposed to have a strongly lethal character because there is a progressive reduction in the prevalence of such abnormalities from 6 to 40 DPH and an associated decrease in the variety of mouth abnormalities identified.

In the present study the highest incidence of anomalies are related with the caudal complex. These deformities probably reduce flexibility and have a negative impact on swimming capacity, but as hatchery-reared larvae have an almost endless supply of food they survive. Anomalies of the caudal complex have also been identified in *S. aurata* (Koumoundouros *et al.*, 1997a), *P. major* (Matsuoka, 1987) and *D. labrax* (Daoulas *et al.*, 1991). However the caudal anomalies described by Koumoundouros *et al.* (1997a), a "double" caudal fin and a laterally twisted caudal fin, was not observed in the present study. It remains to be established if, in the wild, caudal fin abnormalities are detrimental for survival. In the present study larvae with abnormalities of the head and jaws were detected. However, even in a favourable hatchery environment, severe jaw abnormalities (for example pugheadness) seems to be deleterious and lethal for specimens as has been reported in sea bass and red sea bream (Barahona-Fernandes, 1982; Matsuoka, 1987). In fact, in the present study, no severe jaws deformations were detected in the older specimens analysed.

The more frequent occurrence of anomalies in cartilage replacement bones suggests that a new approach is required when anomalies are being considered in fish. In fact the incidence of anomalies associated with cartilage replacement bone is remarkable and it is surprising that this association has not previously been recorded. Clearly it is essential to look at other published studies and see if a similar relationship holds true. The reason for the higher incidence of anomalies associated with cartilage replacement bone is not immediately evident and requires further investigation.

The occurrence of lordosis in aquaculture species has most frequently been associated with anomalies that arise during development as a consequence of the extreme sensitivity of the larvae at this stage. Relatively few studies have reported the spontaneous appearance of lordosis in adults, and where it has

been experimentally induced it has been associated with the presence of excessive levels of heavy metals or excessive use of the antibiotic oxytetracyclin (Hodson *et al.*, 1980; Toften & Joblin, 1996). The lordosis observed in the juvenile sea bream in the present study was predominantly associated with the posterior half of the vertebral column. This part of the skeleton in sea bream develops initially as cartilage, between 4.1 mm notochord length (L_N) and 5.7 mm standard length (L_S) and subsequently ossifies and development is complete by 9.6 L_S (Chapter 2). Thus it seems most likely that the lordosis evident in the juvenile fish examined arose between 11-26 DPH. The cause, in sea bream, of this abnormality remains to be established, but there was no indication in the present study that it was associated with functional problems at the level of the swim bladder (Chatain, 1994; Andrade *et al.*, 1996). It seems most likely that anomalies arose as a result of environmental factors, the identity of which remain to ascertained.

Santamaría *et al.* (1994) proposed that the lordotic sea bream larvae are linked with dysfunctions in collagen metabolism and that this axis deviation may occur very early in development, prior to the appearance and development of a vertebral column. Histological observations have show irregularities of the notochord and perinotochordal collagen sheet and associated muscle bundles in the region of curvature (Santamaría *et al.*, 1994). It is proposed that the vertebral column probably collapses in zones that are most affected by muscle pressure, particularly during active swimming. Chatain (1994) also suggests that lordotic spinal collapse is mechanically induced since deformities primarly occurred in the haemal spine region (vertebrae 14-15), where the junction of the renal bladder is located and where the ribs are most reduced. However, in the present study, spinal curvature occurred with greatest frequency at vertebrae 16 and 17 and there was an associated shortening of the body axis suggesting

that the latter explanation is rather simplistic and does not account for the wide variability of observations in lordotic sea bream. For example, in other studies of sea bream, vertebrae 10-16 (Andrades *et al.*, 1996) and 8-11 (Paperna, 1978) were found to be most frequently involved in lordosis. The number of vertebrae implicated in the process is also variable and may involve between 2 and 6 vertebrae although the former is most common (Paperna, 1978; Chatain, 1994; Andrades *et al.*, 1996; our observations). The causes of lordosis are clearly complex and Kitajima *et al.* (1994) has suggested that in red sea bream (*P. major*), Japanese sea bass (*Lateolabrax japonicus*) and amberjack (*Seriola aureovittata*) unnatural upward swimming make a contributory factor.

In the hatchery situation a key difficulty in identifying and classifying anomalies in larval sea bream is their small dimensions and the failure so far by the industry to establish clear quality related indices. This is an issue that has yet to be seriously addressed but recently association between shape and internal anatomical characters has been studied and may be of importance as a measure of the typical shape of a normal specimen in comparison with the shape of malformed individuals. Such association concerns the link between the shape of the head and anomalies in the cephalic region; the association between the shape of the trunk (linked to swimming and maneuverability) and lordosis and vertebral anomalies of haemal and prehaemal regions; and caudal lordosis and the downward bending of the caudal region (Loy *et al.*, 2000).

Improved husbandry will contribute to controlling abnormalities that arise from epigenetic factors, but clearly a range of abnormalities arise as a consequence of molecular changes. The recent explosion in information about zebrafish development and the extensive Tübingen mutants database may be expected to contribute to our understanding of the underlying molecular basis of anomalies in larvae. The importance of the *Hox* genes in regulating the

development of bone and cartilage represent important target molecules for future studies (Goff & Tabin, 1997). The work necessary to understand of how and why abnormalities arise has only just begun and will certainly require investigation of genetic factors and a study of the ontogenic steps of some typical anomalies during embryogenesis. It will be fundamental to identify not only the genes but the products that they encode and where, when and how they are involved in development of normal and abnormal structures.

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CHAPTER 6

FINAL DISCUSSION

FINAL DISCUSSION

The present thesis set out to characterise in detail the skeleton and its ontogeny in the sea bream (*Sparus aurata*), the latter is an essential tool for future comparative morphogenetic and phylogenetic studies. In addition, it is expected that this work will be useful in defining indices of quality for sea bream larvae and contribute in this way to the further improvement of the production cycle in aquaculture.

Fish ontogeny is a complex phenomenon that involves both growth and differentiation. Some parameters, such as body shape, fin formation and pigmentation have a close relationship with feeding and locomotive capacity of the larvae (Koumoundouros *et al.*, 1999). Besides fish ontogeny, fish classification is an exciting and dynamic process that is under constant modification as new tools and methods become available. The theory of classification of animals (in which fishes comprise more than one-half of the vertebrates) is a permanent challenge that is played according to the available biological information and where many controversies and problems exist. The data of this study contributes to the establishement of a general pattern of ossification among teleosts and also specifically increases the information available about Sparidae. In this field the data are particularly important for a better identification of larval and/or juvenile wild specimens of sea bream and more effective future comparative studies with related species. Figure 1 shows the external morphology of the principal stages of sea bream. Note the

elongated shape of the larvae and the adoption of the characteristic adult shape of a juvenile post-metamorphosis.

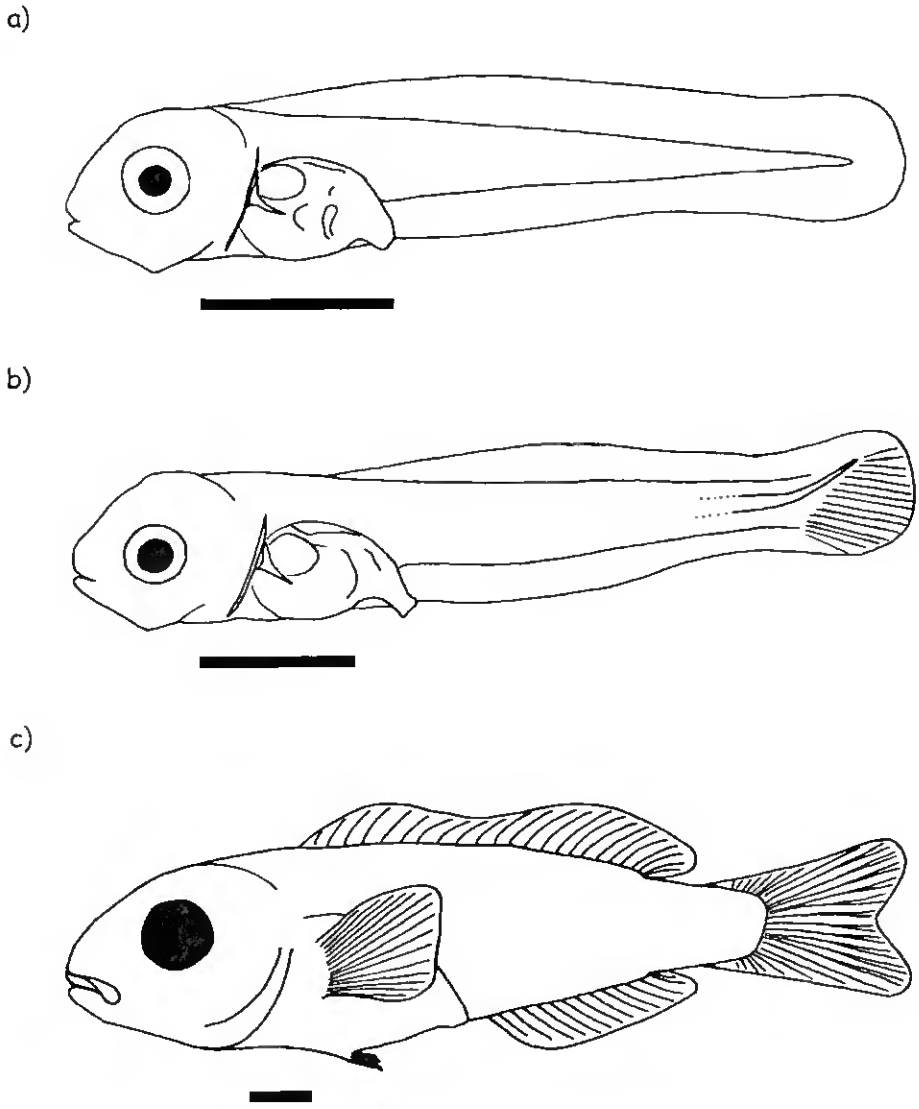

Figure 1. External morphology of main developmental stages in sea bream. (a) 4.1 mm L_N, first-feeding; (b) 5.9 mm L_S, early flexion; (c) 14.7 mm L_S, juvenile. Note that in juvenile the scales are not draw. Scale bars indicate 1.0 mm.

Our study of sea bream revealed that the sequence of ossification of the different regions is a process more or less well conserved within this species. The main variability in skeletal ontogenesis was observed in the viscerocranial skeleton and, for example, the prootic was observed in various specimens to

develop before and after the basioccipital and pharyngobranchial 3. It seems probable that further variability may exist as accurate observation of development of specific viscerocranial bones was frequently complicated by the close proximity of numerous bones in this region and in particular between dermal bones and cartilage replacement bones. In the sea bream some variability was encountered in the development of all regions of the skeleton and appeared to have a complex association with age and length. In order to evaluate and characterize the degree of variability detailed studies must be developed, not only in reared but also in wild specimens.

At hatching sea bream have relatively few cartilaginous structures and no bone and possess a yolk sac located on the ventral axis in the putative abdominal region, similar to that detailed in the teleosts sea bass *Dicentrarchus labrax* and pike-perch *Stizostedion lucioperca* (Mani-Ponset *et al.*, 1996), rockfish *Sebastes schlegeli* (Shimizu & Yamada, 1980) and the trout *Salmo fario* (Walzer & Schonenberger, 1979). From hatching to the first feeding, the nutritional reserves of the yolk sac are responsible for the continuation of organogenesis and mouth opening is the event associated with the transition from the endotrophic to the exotrophic phase.

First-feeding is a critical period and larval mortality is often high at this stage because many larvae are unable to ingest external food or to capture sufficient prey to survive (Mani-Ponset *et al.*, 1996). This means that from hatching to mouth opening (at about 4 days post hatch in sea bream), it is essential that a range of cartilaginous or bony structures develop in a coordinated manner so that larvae are able to swim and feed sufficiently for survival. Typically the structures involved in essential early functions, such as feeding and breathing, appear and ossify before other structures of the skeleton. The sequence of ontogenic priorities is clearly reflected in the

differential between growth rates of the various body parts. In many fish larvae, the head and caudal regions grow faster at the start of development than the mid-region in order that swimming, feeding, respiration and sense organs are functional (Fuiman, 1983). Rapid growth of the posterior region of the larvae increases the propulsive surface area of the caudal complex and ensures the locomotive ability in the early larvae. Mobility in early larval stages has obvious advantage for survival, such as, avoidance of predators and more efficient capture of prey (Fuiman, 1983).

The ontogeny of cartilaginous and bony structures in sea bream does not occur in a continuous manner, but is saltatory and is in accordance with the developmental models proposed for fish by Balon (1979, 1981, 1984, 1985, 1986). In the present study of sea bream three principal phases were clearly identified: the yolk-sac stage (or free embryo) ($\leq$3.1 mm L_N), the larval phase (3.1 mm L_N - 11.6 mm L_S) and the juvenile phase (>11.6 mm L_S). The first phase is characterised by development of the cartilaginous and bony structures that are necessary for exogenous feeding, vision, opening and closing of the mouth, expanding and narrowing the oral and branchial areas and propulsion. This is clearly evident upon analysis of Figure 2, that presents schematically the stage of development of the various structures that make up the skeleton in sea bream. In the first phase the first structures visible are the sclerotic cartilage, jaws (angular, maxillary, retroarticular), cartilaginous suspensorium (hyomandibular, quadrate, symplectic), cartilaginous hyoid arch (ceratohyal, epihyal, hypohyals, interhyal), cartilaginous branchial arches (ceratobranchials, hypobranchials) and pectoral fin supports (cleithrum, cartilaginous coracoid-scapula). In the second phase the remaining structures form and ossify and this leads naturally to the transition to the third phase, the juvenile phase.

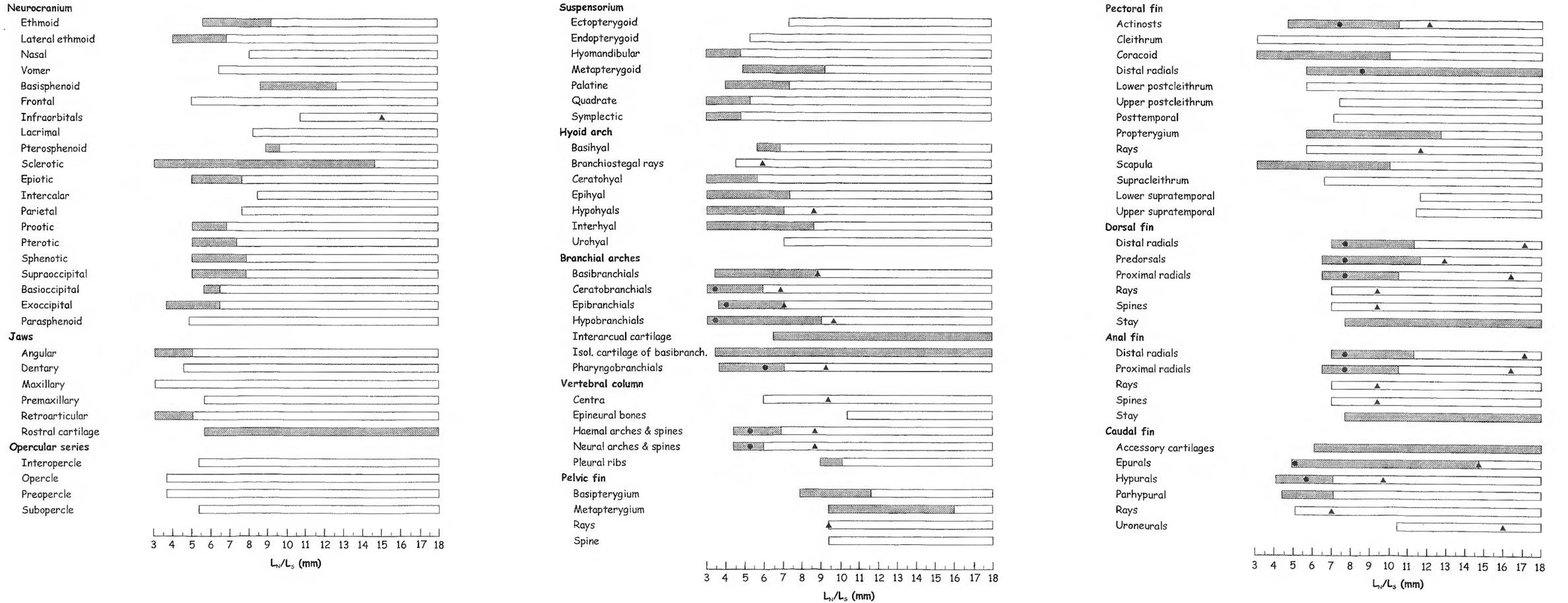

Figure 2. Summary of the ontogeny of the skeletal structures in reared sea bream. Stippled areas, cartilage; Open areas, bone; ●, when the full complement of cartilage structures has developed; ▲, when structures are fully ossified.

The overall development of the skeleton in sea bream is strongly conserved when compared with other teleost species. This is evident by the remarkably similar number of structures and their disposition in the skeleton of nearly all teleosts analysed (Gwyn, 1940; Houde *et al.*, 1974; Houde & Potthoff, 1976; Mook, 1977; Johnson & Loesch, 1983; Potthoff *et al.*, 1984; Potthoff *et al.*, 1986; Langille & Hall, 1987; Matsuoka, 1987; Watson & Walker, 1992; Balart, 1995; Adriens & Verraes, 1998; Koumoundouros *et al.*, 2000). Moreover, the sequence of ossification in different regions of the skeleton is frequently conserved. This may suggest that functional constraints are important and, for example, comparison of the sequence of ossification of the jaws between two phylogeneticaly distant species, *Sardinops melanostictus* and sea bream, shows that the process is highly conserved. A similar situation is observed if the development of the pectoral, dorsal, anal and pelvic fins is compared between sea bream and *Engraulis japonicus* (Engraulidae) (Balart, 1995). However, in spite of the conservation mentioned above, the timing is very variable and in some species, such as sea trout *Salmo trutta* (L.) (de Beer, 1937), *Barbus barbus* (Vandewalle *et al.*, 1992), fork-tailed catfish *Arius graeffei* (Kner & Steindachner) (Rimmer, 1985) or sea catfish *Galeichthys feliceps* (Valenciennes) (Tilney & Hecht, 1993), skeletal development initiates before hatching, whereas in other species such as sea bream, *Anisotremus davidsonii* and *Xenistius californiensis* (Watson & Walker, 1992), African catfish *Heterobranchus longifilis* (Valenciennes) (Vandewalle *et al.*, 1997), sea bass *Lates calcarifer* (Bloch) (Kohno *et al.*, 1996*a*), milkfish *Chanos chanos* (Forsskål) (Kohno *et al.*, 1996*b*), African catfish *Clarias gariepinus* (Burchell) (Adriaens & Verraes, 1997), *Chrysichthys auratus* (Vandewalle *et al.*, 1999), Japanese medaka *Oryzias latipes* (Jordan & Snyder) (Langille & Hall, 1987), skeletal development begins after hatching.

The factors controlling stage of osteogenesis in sea bream are complex and appear to be related to both age and length; the relative importance of each factor is variable and dependent on cirscumstances. For example, in larvae that are of the same age, but in which one larvae is larger, the latter is found to be at a more advanced stage of skeletal development. However, on comparison of specimens of the same length but of different ages, the oldest larvae generally have the greatest number of ossified or ossifying structures. Considerable work is still required to understand the interplay between age, length and skeletal development, but in red sea bream *Pagrus major* and common carp *Cyprinus carpio* (Osse & van den Boogaart, 1995), in common with the observations made in the present study on sea bream, length is a better measure of morphological development than age. Also in starry flounder *Platichthys stellatus* (Policansky, 1982) and in *P. major* (Fukuhara, 1991), it has been demonstrated that metamorphosis is influenced more strongly by length than by age.

The process of ossification is different according to the structures considered and in the sea bream two principal types of bones were observed: cartilage replacement bones, in which bone develops within a cartilage matrix and dermal bones which develops in the absence of a cartilaginous precursor (Fig. 2). As has been observed in other fishes (Jollie, 1975; Langille & Hall, 1987; Matsuoka, 1987; Arratia & Schultze, 1990; Vandewalle *et al.*, 1992; Watson & Walker, 1992; Adriens & Verraes, 1998; Wagemans *et al.*, 1998), in sea bream dermal bones develops before cartilage replacement bones. This is true for almost all regions of the skeleton (neurocranium, jaws, opercular series, suspensorium, hyoid arch, branchial arches, vertebral column and pelvic, pectoral, dorsal, anal and caudal fins) and the only exceptions are the elements of the suspensorium and branchial arches (Fig. 2); (Chapters 2, 3 and 4). Overall the skeleton of sea bream is composed of 83 different groups of bones (see Fig.

2) of which 37 are dermal bones and 46 are cartilage replacement bones. Several structures are considered as a single unit in this representation and, for example, all the 24 centra of vertebral column are included in one group (the centra), the 2 cleithrum correspond to one group (the cleithrum), the unique parasphenoid correspond to the group of parasphenoid and so on. If the skeleton of sea bream is divided into three regions: anterior (viscerocranial and pectoral fins), middle (vertebral column, neural and haemal arches and pelvic, dorsal and anal fins) and posterior (caudal complex) it is notable that the first 12 groups to ossify (8 dermal bones and 4 cartilage replacement bones) are localised in the anterior region and of the first 22 ossified groups (15 dermal bones and 7 cartilage replacement bones) only one is localised in a posterior region. Assessment of the localization of ossified groups at mid-point of skeletal ossification (50% structures ossified) shows that 33 of the groups are localised in anterior region of the specimens, 7 are in the mid-region and 2 are located in the posterior region (Fig.2).

It has frequently been noted that the regions of the skeleton in fish larvae subject to mechanical stress associated with movement are the first to undergo ossification (Mook, 1977; Vandewalle *et al.*, 1992; Adriens & Verraes, 1998. The observations made of the skeletal ontogeny in sea bream are in accordance with this suggestion that arose from observations in sheepshead *Archosargus probatocephalus* (Mook, 1977), *Barbus barbus* (Vandewalle *et al.*, 1992), African catfish *Clarias gariepinus* (Adriens & Verraes, 1998) and in pumpkinseed *Lepomis gibbosus* (Arendt & Wilson, 2000). Ossification in sea bream starts in the anterior region, which re-enforces the idea that the process of ossification seems to be related with the important functions of locomotion, breathing and feeding.

The saltatory process (Balon, 1979, 1981, 1984, 1985, 1986) is clear evident when the skeleton develops and three principal "waves" of ossification occurs. The first "wave" occur between 3.1 mm L_N and 6.5 mm L_S in which 27 groups of structures ossify (one third of all groups of structures); the second between 6.6 mm L_S and 7.4 mm L_S in which 48 groups ossify (more than a half of total) and a third slower and more extended "wave", between 7.7 mm L_S and 16.0 mm L_S in which the remaining 35 groups of structures ossify (Fig. 3).

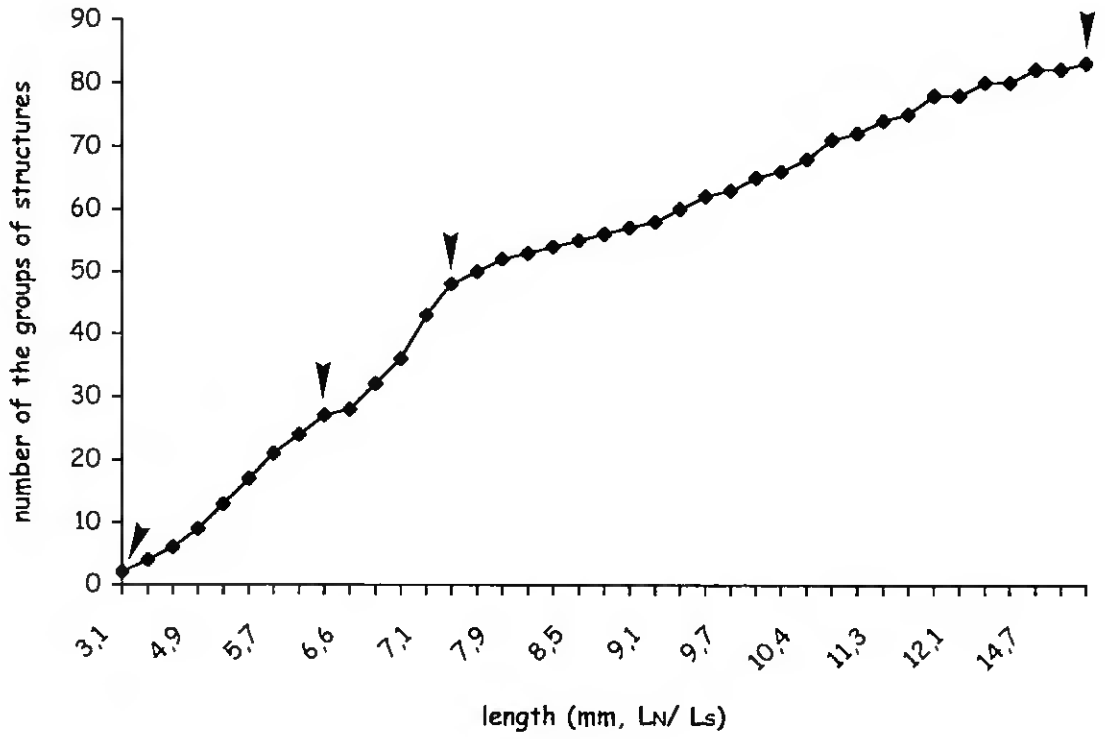

Figure 3. Ossification in sea bream is a saltatory process and it is possible to observe three different "waves" of ossification. These "waves" are bordered by arrowheads.

The completed skeleton of the sea bream with 17.9 mm L_S is presented in Figure 4.

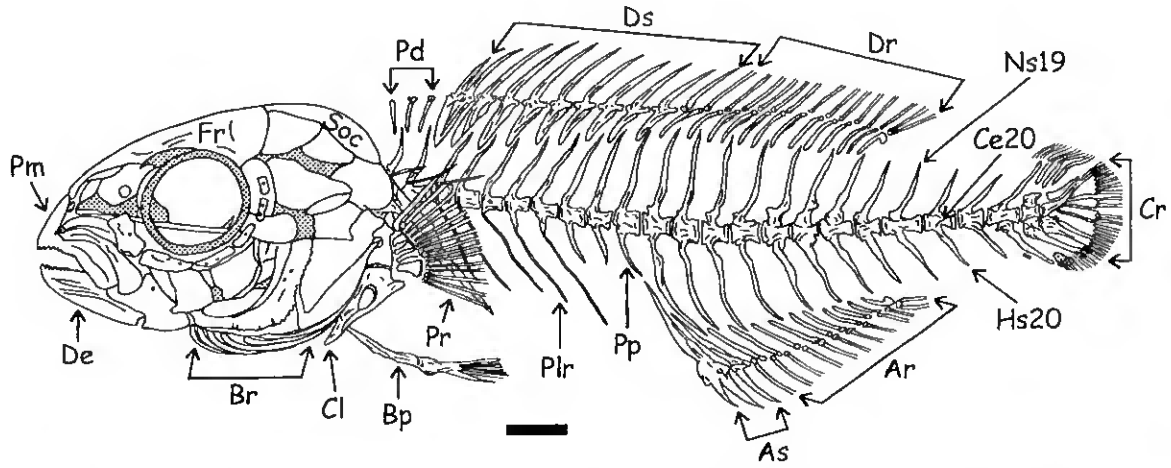

Figure 4. General arrangement of the skeletal structures in a juvenile specimen of sea bream with 17.9 mm L_S. Ar, soft rays of anal fin, As, spines of anal fin, Bp, basipterygium; Br, branchiostegal rays; Ce, centrum; Cl, cleithrum; Cr, principal rays of caudal fin; De, dentary; Dr, soft rays of dorsal fin; Ds, spines of dorsal fin; Fr, frontal; Hs, haemal spine; Ns, neural spine; Pd, predorsals; Plr, pleural rib; Pm, premaxillary; Pp, parapophyses; Pr, ray of pectoral fin; Soc, supraoccipital. Scale bar indicate 1.0 mm.

Morphological anomalies that affect the skeleton in reared or wild fishes have been described (Paperna, 1978; Barahona-Fernandes, 1982; Francescon *et al.*, 1988; Chatain, 1994; Andrades *et al.*, 1996; Ellis *et al.*, 1997; Koumoundouros *et al.*, 1997a; Koumoundouros *et al.*, 1997b; Galeotti *et al.*, 2000). In our study most of the anomalies observed in sea bream larvae occurred in the caudal complex. It seems unlikely that in a hatchery environment where an abundant food source is present that minor caudal anomalies affect survival.

If the data of meristic counts in this study of sea bream are compared with data previously published for wild species (Albuquerque, 1956; Whitehead *et al.*, 1986; Fisher *et al.*, 1987) remarkably similar results were obtained suggesting that skeletal development is a highly conserved process. However, a recent controversial report on hatchery-reared sea bream in which over 70% of fish, in the majority of samples, are deformed presents unusual data for meristic counts (Boglione *et al.*, 2001). The reliability of the data is uncertain as

insufficient methodological detail is provided and a series of questions about the study exist. For example, the stage of development of the specimens examined is not evident; was the reduced number of vertebrae reported identified in specimens with the vertebral column fully ossified? Was the reduction in vertebrae counts a result of fusion? Does any direct relationship exist between the meristic variations in vertebrae counts and anomalies in associated structures, such as haemal and/or neural arches and/or spines? It is unclear if the high variability of caudal ray counts observed by the authors were related to anomalies in the hypurals, epurals, parhypural, haemal and neural arches and/or spines. However, in spite of the incompleteness of the study the report of Boglione *et al.* (2001) describes some common anomalies present in sea bream and as such is useful.

FUTURE PERSPECTIVES

Quality indices in aquaculture

In the last two decades considerable improvements have been made in fish-husbandry. The majority of fishes present critical developmental periods during early life stages in which behavioural, morphological, physiological and biochemical modifications occur and that may affect the survival rate. Critical periods are hatching, for example lack of enzymes to break down the chorion will reduce viability; first-feeding when larval mortality is often high because many larvae either fail to ingest external food or are unable to capture sufficient prey to ensure continued growth and survival and metamorphosis (Blaxter, 1988; Benoît & Pepin, 1999).

The qualitative evaluation of reared larvae and/or juveniles would allow an analysis of the growth and survival potential of different cultures and this could have substancial economic advantages. This would permit early intervention to eliminate poor cultures or those that have a high number of abnormal larvae and minimize in this way financial losses incurred by their cultivation until juveniles when abnormalities become generally more evident. Therefore knowledge of normal larval ontogeny of the skeletal framework (Chapters 2, 3 and 4) and subsequent morphology is essential to determine the quality of reared specimens. Any alteration from this normal skeletal development must be considered as an anomaly. External morphological observations can be usefull for detection of some anomalies such as lordosis, scoliosis, kyphosis, opercular and jaws deformations or malpigmentation and may be utilised as a simple index for qualitative evaluation. However, a more detailed index is needed for internal anomalies that may compromise fish quality and are not evident during external observation. In this case a detailed study and a new approach is needed and whole mount staining may be a usefull tool. It seems likely that several anomalies encountered in reared specimens are induced directly by deficiencies in rearing conditions. Defective and/or excessive handling, high stock density, intensity and quality of light, photoperiod, food conditions, tank colour and water quality (temperature, salinity and dissolved oxygen) seem to be factors that contribute to increased rates of anomalies in hatcheries. In aquaculture the main objective is to produce specimens similar, in morphological, physiological and biochemical aspects, to wild fish. However, the interdependence of several factors in the rearing environment and larval nutritional requirements have complicated the optimisation of culture regimes and advances have been much slower than desired.

The endocrine system

The role of the endocrine system on development and survival of fish embryos and eggs has still to be clarified.

It is well know that thyroid hormones (THs), thyroxine (T_4) and triiodothyronine (T_3) have numerous functions, which include regulation of metabolism, growth, metamorphosis, reproduction and development of skin, bones and scales in vertebrates. Recently several studies in fish have established the importance of THs during flatfish metamorphosis (Yamano *et al.*, 1991; Miwa *et al.*, 1992; de Jesus *et al.*, 1993; Huang *et al.*, 1998). However, the role of the THs in sea bream is unknown. Previous experiments show that T_3 has a significant effect on the growth, survival and early skeletal development of sea bream and suggest it may normally be involved in skeletal development in fish larvae (our observation). Nevertheless, the mode of action and the way in which T_3 functions in early fish larvae is unclear and will require much more work.

The role of other hormones, such as those involved in calcium regulation, in the formation of and metabolism of bone has still to be clarified. The mode of action of these hormones at the level of bone is almost enterily unstudied.

Cellular or acellular bone?

Typically the skeleton of vertebrates are characterized by cellular bone. However, some species of teleosts appear to possess acelular bone (Moss, 1961; Ekanayake & Hall, 1987; Stibane, 1992; Sire & Huysseune, 1993; Takagi & Kaneko, 1995; Witten, 1997).

Preliminary studies with sea bream larvae, using histological sections of vertebral column bone suggests that it is acellular (our observation). However, more detailed and exhaustive experiments will be required particularly at the ultrastructural level using electronic microscopy to resolve this question. Also

little is know about the way in which tissue that was initially cellular become acellular bone during ontogeny and the mechanisms underlying this process have yet to be studied.

Incidence of anomalies and type of bone

The present study relates for the first time the incidence of anomalies and bone type. There is a higher frequency of bone anomalies in endochondral bone than in dermal bone. The reason for the predominance of abnormalities in endochondral bone remains to be clarified and it is unclear if it is a consequence of molecular mechanisms or merely that alterations in endochondral bone structures do not compromise survival and these larvae persist in cultures. Clearly further work will be required to better understand this phenomenon.

Morphometrics

The study of morphometrics and growth trajectories of fish larvae is also a poorly exploited field. Geometric morphometrics may be useful and important tool for dividing the developmental process into distinct phases, such as the boundary between larvae and juvenile, or metamorphosis in sea bream and other related fishes.

Phylogeny

Comparative studies of osteology in fish have markedly advanced the understanding of phylogenetic relationships between teleosts, particularly Perciformes, which represents the largest and most diverse group of fishes (Nelson, 1994). The number and disposition of skeletal elements and the pattern and sequence of chondrification and ossification has been of great importance for the establishement of phylogenetic relationships among different taxa. For

example, Johnson (1984) using the specialization of several structures, including the maxillary-premaxillary articulation, suspensorium and meristic variation was able to reorganise families closely related to the Sparidae.

In addition to the intermuscular bones (Patterson & Johnson, 1995), other characters such as the variations in the morphology of premaxilla, bones of the neurocranium or in pharyngeal jaws, dentition and acessory cartilages have been considered as taxonomically valuable diagnostic characters in several groups of teleosts (Johnson, 1983; Matsuoka, 1987; Tigano & Parenti, 1988; Parenti & Tigano, 1993; Vandewalle *et al.*, 1995; Tigano *et al.*, 1999) such as serranids, sparids, cyprinids and atherinomorphs.

It seems likely that the combination of conventional studies of the skeleton, coupled to complementary molecular studies will help resolve some of the existing classification problems.

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