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Skeletal anomalies in reared European fish larvae and juveniles. Part 1: normal and anomalous skeletogenic processes

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Abstract

This critical review summarizes the knowledge about fish skeletal tissues and inherent normal and anomalous development. Particular emphasis is given to existing literature on reared European fishes. The aim was to identify the main gaps of knowledge that require to be filled, in order to precociously identify anomalous developmental patterns that lead to skeletal anomalies in reared finfish larvae and juveniles. The review also aims to extend our knowledge about the factors that are possibly involved in the onset of skeletal anomalies. The final goal is the optimization of the morphological quality of farmed juvenile fish.

Key words: bone tissue, cartilage, juveniles, larvae, mineralization, ossification, skeletal anomaly, skeletogenesis.

Introduction

The presence of skeletal anomalies in farmed teleosts is a constant world-wide problem in aquaculture; it entails economic, biological and animal welfare issues. Deformed fishes have to be removed manually and repeatedly from production and products from these fish are often downgraded to filets or fish meal (flour) with loss of profit (Hilomen-Garcia 1997; Koumoundouros *et al.* 1997a,b; Boglione *et al.* 2001, 2003, 2009; Cahu *et al.* 2003; Matsuoka 2003; Georgakopoulou *et al.* 2007a; Lall & Lewis-McCrea 2007; Le Vay *et al.* 2007; Castro *et al.* 2008; Lijalad & Powell 2009). Even filet processing is impaired by the presence of skeletal (particularly vertebral) anomalies as machines are designed for normal shaped fish, and more manual processing and extra trimming are necessary (Branson & Turnbull 2008). The prevalence of skeletal anomalies in farmed fish suggests that we still need to improve our knowledge about genetic and epigenetic factors that can cause skeletal anomalies under rearing conditions.

The fact that an accurate study of skeletogenesis during larval development is of utmost importance for the recognition and identification of abnormalities in skeletal structures was suggested by the scientific community long ago, as can be seen in the statement of McMurrich (1883): ‘From the rather peculiar arrangement of the mandibular skeleton at this stage no little difficulty would no doubt be experienced in determining the homologies of the cartilages from a single specimen, since it is only by tracing their development that one can be certain of the signification of abnormalities.’

The first publications about skeletal anomalies in reared fishes appeared in the early 1970s. The species that have been studied and subsequent publications reflect the development of modern aquaculture. Rainbow trout (*Oncorhychus mykiss*) was the first species to be reported to show anomalies under farmed conditions (Aulstad & Kittelsen 1971). Next, skeletal anomalies were reported in gilthead seabream (*Sparus aurata*) (Paperna *et al.* 1977), European seabass (*Dicentrarchus labrax*) (Barahona-Fernandes 1978) and flatfish (*Plecoglossus altivelis*) (Komada 1980).

The first record about the cause of a specific skeletal anomaly concerned the failure of swim bladder inflation and resulting lordosis in European seabass and gilthead seabream (Chatain 1994). Since then, improvements of abiotic (Polo *et al.* 1991; Divanach *et al.* 1997; Sfakianakis *et al.* 2006; Georgakopoulou *et al.* 2007b, 2010) and nutritional (Izquierdo *et al.* 2010; Lewis-McCrea & Lall 2010) conditions have been made in order to lower the incidence of skeletal anomalies in many species. The problem is still topical and in the past decade many case studies and reviews have been published concerning skeletal anomalies in some reared species (Atlantic halibut, *Hippoglossus hippoglossus*: Hamre *et al.* 2005; Atlantic salmon, *Salmo salar*: Witten *et al.* 2005a,b, 2006, 2009; Fjelldal *et al.* 2012; red porgy, *Pagrus pagrus*: Izquierdo *et al.* 2010; European marine fishes: Koumoundouros 2010; *Sparidae*: Boglione & Costa 2011), for particular anomalies (uninflated swim bladder: Woolley & Qin 2010) and possible causative factors (Fig. 1): inflammation (Gil Martens 2010), unsaturated essential fatty acid requirements (Izquierdo 1996), inappropriate microdiet formulation (Takeuchi 2001), nutritional deficiency (Cahu *et al.* 2003; Lall & Lewis-McCrea 2007; Fjelldal *et al.* 2010; Lewis-McCrea & Lall 2010), phosphorus deficiency (Sugiura *et al.* 2004; Fjelldal *et al.* 2009, 2010), administered dietary phosphoglyceride classes (Tocher *et al.* 2008); inappropriate levels of vitamin D (Lock *et al.* 2010), vitamin A (Fernández & Gisbert 2011; Georga *et al.* 2011; Fernández *et al.* 2012), vitamin D and vitamin C (Darias *et al.* 2011); presence of toxic waterborne metals (Jezierska *et al.* 2009); inappropriate light regimes and light spectrum (Fjelldal *et al.* 2004; Blanco-Vives *et al.* 2010) and tank colour (Cobcroft & Battaglene 2009). However, the problem persists and many hypotheses for the causes of skeletal anomalies are still being discussed today, because different causative factors can have a common symptomatology and frequently act synergistically. The present difficulties in separating the causes of the many genetic and non-genetic factors that interact in aquatic organisms remain an open problem. Consequently, in marine fish farming a frequency of about 20% of severely deformed fish at the end of the hatchery phase is considered to be a good, but quite rare, result.

The scenario is complicated by the following observations: (i) different non-genetic factors can induce the same anomaly in different species; (ii) the same causative factor can induce different anomalies in different fish species (Boglione & Costa 2011); (iii) anomalies are induced by different factors in different cohorts of the same species (Kause *et al.* 2007); (iv) fish sensitivity to a causative factor may change dramatically during ontogeny (Mazurais *et al.* 2009); (v) the action of a single causative factor can be compensated by the action of a different factor (Sfakianakis *et al.* 2006); (vi) particular factors show a high correlation with anomalies in a particular body region in some species, but not in other species (Koumoundouros 2010); (vii) the same causative factor may provoke a high incidence of anomalies in some skeletal elements but not in others with the same bone type and ossification, in the same individual (Fernández & Gisbert 2011).

Mammalian skeletal tissues are often categorized as either bone or cartilage but fish skeletal tissues include

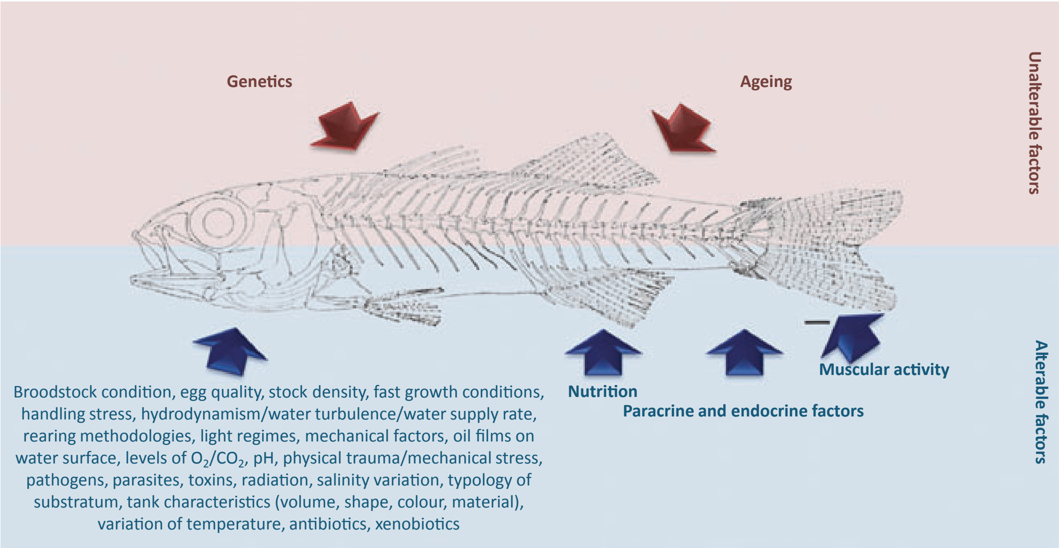

Figure 1 Diagram showing the main factors that may impact skeletal cells, cartilage or bone matrix development and bone mineralization in reared fish larvae and juveniles (modified after Waagbø 2006). Arrows do not indicate any particular body region. Bar = 1 mm.

several types of bone (Fig. 2), many different types of cartilage (Table 1) and many tissues types that are intermediate between connective tissue and bone, and between bone and cartilage (Hall & Witten 2007; Witten *et al.* 2010) (Fig. 3). Since the pioneering studies of Melvin Moss (Moss 1961a, 1962, 1965) on fish skeletal tissues, progress has been made regarding scientific knowledge on biomolecular, genetic and physiological mechanisms that underlie bone differentiation, modelling and remodelling in fish (for reviews see: Fowler 1970; Meunier 1983, 2002; Meunier & Huysseune 1992; Huysseune & Sire 1998; Huysseune 2000; Akimenko *et al.* 2003; Sire & Huysseune 2003; Sire & Akimenko 2004; Witten & Huysseune 2007, 2009; Hall & Witten 2007; Schilling *et al.* 2010; Spoorendonk *et al.* 2010; Apschner *et al.* 2011; Duran *et al.* 2011; Meunier 2011; Dean & Shahar 2012; Harris 2012; see also references in Witten *et al.* 2010; Witten *et al.* 2012).

Unfortunately, and despite the many publications about special characters of the fish skeleton, it is still common to view fish skeletal tissues from a 'human textbook perspective' (Witten & Huysseune 2010). As a consequence, teleost bone metabolism is often still misunderstood as being primarily calcium driven (as is the case in humans but not in fish) and the specific changes in vertebral bone tissues in fishes reared under different conditions are often misinterpreted.

The presence of deformed fish concerns also ethical issues: fish with a deformed mouth, fins or vertebral axis show impaired feeding and swimming performances, with consequent lower feeding rates, slower growth rates and a higher susceptibility to stress and pathogens than healthy nondeformed individuals. These deformed fish cannot be considered to be in a proper welfare condition.

The aim of this review is to provide a synthetic but comprehensive picture of the actual knowledge on bone and cartilage development in larvae and juveniles of European farmed fish; to identify the main gaps of knowledge that require to be filled, in order to identify anomalous developmental pattern leading to skeletal anomalies in reared finfish larvae and juveniles. Moreover, we aim to extend knowledge on the factors that are possibly involved in the onset of skeletal anomalies. The long-term goal is the optimization of the morphological quality, welfare and health status of farmed juvenile fish.

In the present review, all the information on skeletal tissues, cells and processes are drawn from the comparative analysis of the available literature about vertebrates,

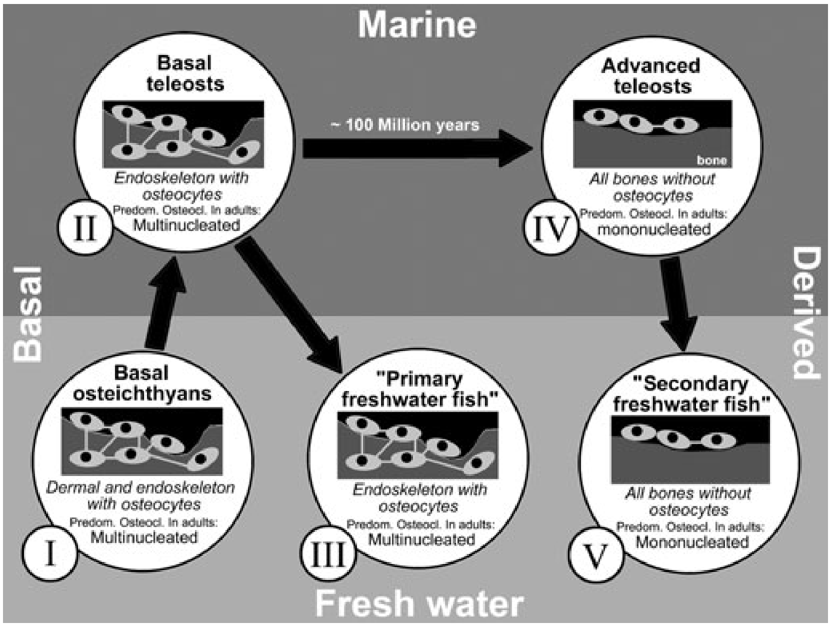

Figure 2 Relationships between phylogeny, environment, the presence of osteocytes, and the predominant (predom.) type of osteoclasts (osteocl.) in teleost fish. (II–V) Basal Osteichthyans (I), which also gave rise to tetrapods, and Basal Teleosts (II) have bone that contains osteocytes. These fish have mononucleated and many multinucleated osteoclasts. Osteocytes and multinucleated osteoclasts have been preserved during 'a first' wave of freshwater reinvasion by teleost fish (III); 'primary freshwater fish': it refers to fish such as cyprinids and salmonids. During a long evolutionary period in the marine environment, osteocytes disappeared (acellular bone) in almost all advanced marine teleosts groups (IV). The predominant osteoclast type of 'Advanced Teleosts' is mononucleated. This character was maintained when advanced Teleosts reinvaded the fresh waters (V); 'secondary freshwater fish': e.g. chichlids. Consequently, Teleosts that live in fresh water (c.f. III and V) or in the marine environment (c.f. II and IV) can have different bone types and different predominant types of bone resorbing cells. Modified after Witten and Huysseune (2009).

Table 1 Main type of cartilage tissues in fish, reviewed by Witten *et al.* 2010. Data from Benjamin 1988a,b, 1990; Benjamin & Ralphs 1991; Beresford 1993; Huysseune 1990; Huysseune and Verras 1986; Huysseune & Sire 1992; Witten & Hall 2002

Category	Sub-category	Intermediate sub-category	Localization
1. Hyaline cartilages – amount of extracellular matrix	Matrix-rich hyaline cartilage (MRHC): cartilage that largely resembles mammalian textbook hyaline cartilage		Neurocranium and gill arches
	Cell-rich hyaline cartilage (CRHC): contains more cells and less matrix compared with matrix-rich hyaline cartilage – it is susceptible to calcification and can permanently mineralize		Neurocranium and gill arches
		Scleral cartilage (SK): tissue with a central cell-rich cartilage surrounded by a matrix-rich zone	Surrounding the eyeball (sclera)
	Hyaline-cell cartilage (HCC): chondrocytes are surrounded by a very narrow rim of weakly staining cartilage matrix – does not usually calcify		Lips; expansions of periosteum of the hypurals in some species; developing as a secondary cartilage
		Zellknorpel: strongly resembling HCC but the cells shrunken in their lacunae and a matrix stains more intensely with Alcian blue – does not usually calcify	Barbels
	Lipohyaline-cell cartilage (LCC): contains a mixture of hyaline cartilage and adipocytes		In the oromandibular region of the cypriniform species *Pseudogastromyzon myersi*
2. Cartilages with additional fibres – presence of extra collagen and/or elastic fibres	Elastic/cell-rich cartilage (ECRC): share properties with CRHC but the narrow rim of cartilage matrix that surrounds the cells contains elastin fibres		In cypriniform species: in barbels, in sucking disks or it may function as a semi-flexible syndesmotic connection between different cartilage elements
	Elastic hyaline cell cartilage (ECC): share properties with CRHC but the cartilage matrix is rich in elastin fibres		
	Fibro/cell-rich cartilage (FCRC): matrix rich in collagen, as described in classic (i.e. mammalian-focused) textbooks, would substitute fibrocartilage		Articular surface between dorsal and caudal pterygiophores and rays or attached to the lepidotrichia; symphyseal tissue
	Fibrohyaline-cell cartilage (FCC)		Suspensorium

Table 1 (continued)

Category	Sub-category	Intermediate sub-category	Localization
3. Calcified cartilaginous tissues – permanently calcified cartilages and chondroid bone	Chondroid bone (CB): tissue composed of chondrocyte-like cells embedded in a bone-like matrix		On the lower jaw of Atlantic salmon, with both chondrocytes and osteocytes present within the bone matrix
		CB I: derived by transdifferentiation of skeletal cells (from osteoblast into chondroblast) within multipotential periostea	In acellular-boned cichlids
		CB II: permanently calcified hyaline cartilage derived by the incomplete ossification of calcified cartilage	
4. Degrading cartilages – based on modes of transformation and resorption and associated with endochondral bone formation	Cartilage replacement	In smaller Teleosts species (zebrafish and medaka): cartilage is enclosed by perichondral bone and chondrocytes hypertrophy, but the cartilage is removed without being replaced by bone but by adipose tissue	Splanchno-cranium
		In larger Teleosts species (salmon and carp) cartilage undergoes endochondral bone formation and replaces cartilage with spongiosa	
	Subdivision of cartilaginous anlage into elements	Cells are not degraded but undergo metaplasia and the surrounding matrix loses its cartilaginous character	During the development of the endoskeleton of Teleost pectoral, dorsal and anal fins
5. Cartilage-related tissues – tissues intermediate between cartilage and connective tissue	Mucochondroid or mucus connective tissue: heterogeneous group of tissues containing either fibroblasts or hyaline cells in a pale staining matrix		Skull of Teleosts
	Notochord: notochord cells produce the same matrix components as chondrocytes, but the intercellular matrix is sparse, the cells are connected via cell processes, and contain vacuoles		Notochord

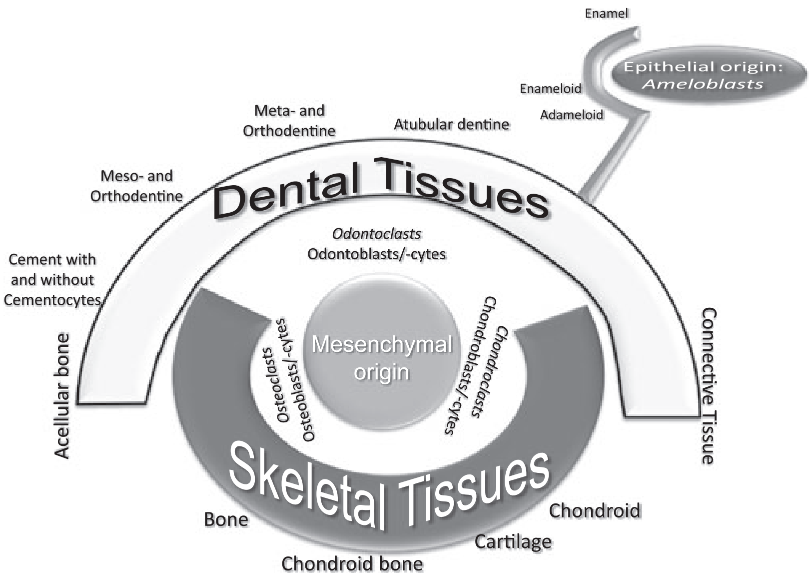

Figure 3 Diagram with the main subcategories of skeleton tissues and cells and origin in fish. Overlapping semicircles indicate the continuum between the different tissues. Resorbing cells are indicated in italics. Redrawn by Hall & Witten 2007.

integrated with recent information that can be ascertained about reared finfish species, where available.

Plasticity, ontogenesis, remodelling and resorption of skeletal elements in teleost fish

There are several differences between mammals and teleosts regarding skeletal tissue types and their differentiation, as well as remodelling and resorption of skeletal tissues (Huxley 1859; Huysseune & Sire 1992; Gillis *et al.* 2006; Witten & Huysseune 2009; Dewit *et al.* 2010, 2011; Renn & Winkler 2010; Apschner *et al.* 2011). In tetrapods, the skeleton is tightly integrated into the animals' daily calcium homeostasis, whereas in most teleosts, calcium from the skeleton is only mobilized in the case of extreme calcium deficiency. This is because fish can obtain and release calcium from and into the water *via* their gills (Lewis-McCrea & Lall 2010). The bone marrow in tetrapods contains haematopoietic tissue, from where the cells of the osteoclastic lineage differentiate. Osteoclasts are bone resorbing, multinucleated giant cells. In teleosts, the bone marrow is filled with adipose tissue, besides nerves and blood vessels and some connective tissue cells, and the head kidney is the haematopoietic organ (Witten & Huysseune 2009). Early stages of all teleosts and also later stages of advanced teleosts typically do not show multinucleated osteoclasts. The majority of osteoclasts are small and mononucleated. These cells can perform an alternative mode of bone resorption, without generating typical resorption lacunae (Witten & Huysseune 2010). Another characteristic of bone in advanced teleosts is the lack of osteocytes (cells inside the bone) (Moss 1961a,b; Parenti 1986; Meunier & Huysseune 1992). In mammals, osteocytes are responsible for the maintenance of the bone matrix and serve as receptors for mechanical loads, transducing the physiological responses to these forces (Bonucci 2009). The lack of osteocytes (acellular bone) in teleosts implies that bone remodelling in response to mechanical load, as shown by Huysseune *et al.* (1994), is triggered by cell types other than osteocytes.

Many teleost species never stop growing and therefore growth-related (and not only metabolism-related) skeletal modelling continues throughout life (Witten & Huysseune 2009). Unlike sharks, that are not able to repair their endoskeleton (Ashhurst 2004), teleosts can repair their skeleton (e.g. fracture repair) (Moss 1962; Dean & Shahar 2012). Dermal skeletal elements (teeth, scales, fin rays) have a large regenerative capacity (Akimenko *et al.* 2003; Huysseune *et al.* 2007), whereas in most cases, and in contrast to amphibians and basal actinopterygian fish, the endoskeleton can only be repaired but cannot regenerate (Kirschbaum & Meunier 1981; Cuervo *et al.* 2012). Other differences concerning the skeletal system among terrestrial vertebrate and fish are furnished below.

Teleost skeletal tissues

It is generally considered that four classes of mineralized tissues can be identified in vertebrates: bone, cartilage, dentine and enamel/enameloid. These main categories and the tissue-related cells (chondroblasts, chondrocytes, osteoblasts, bone lining cells, osteocytes, osteoclasts, odontoblasts, ameloblasts; Fig. 3) are conserved in teleosts, but different cell morphologies and intermediate tissue types occur. Teleost fish display a large range of intermediate skeletal tissues as part of their mature – non-pathological, non-regenerating – skeleton (Beresford 1981; Benjamin 1990; Hall & Witten 2007) (Figs 2,3, Table 1). As many as

15 diverse types and subtypes of cartilage have been identified in teleosts (Benjamin 1988a,b, 1989, 1990; Benjamin *et al.* 1992). In addition, many permanent skeletal tissues found in teleost are *intermediate forms* between any of the above mentioned tissues (Beresford 1981) representing a *continuum* (or perhaps *continua*), and not discrete skeletal categories (Hall & Witten 2007). Some intermediate tissues are characteristic for pathological alterations of the teleost skeleton, as described below. Skeletal anomalies in reared fish can affect all skeletal tissues, but from a production related viewpoint, alterations of the notochord, cartilage and bone abnormalities are the most important. Anomalies of dermal skeletal elements, such as teeth, scales (and fin rays), are possibly indicative for the skeletal heath status of the animal (Persson *et al.* 1997, 2000); however, anomalies affecting teeth and scales are rarely studied.

Bone is a specialized mesenchymal tissue, an aerobic vascularized tissue with high oxygen consumption, supporting skeletal tissue. Bone tissue consists of cells (osteoblasts, osteocytes and bone lining cells), a mineral phase (mainly composed of calcium phosphate forming hydroxyapatite crystals) and an organic, mineralized, extracellular matrix. Collagen type I is referred to as the major organic component of bone but in teleost fish bone can also contain collagen type II (Benjamin & Ralphs 1991). The degree of bone matrix mineralization is variable and seems to depend on the type of bone (acellular vs. cellular bone), life style (active swimmers vs. poor swimmers) and on the nature of the aquatic environment that the fish inhabit (seawater vs freshwater) (Meunier & Huysseune 1992; Danos & Staab 2010; Sfakianakis *et al.* 2011; Dean & Shahar 2012; Fiaz *et al.* 2012). As fish have no haematopoietic tissue inside the bone marrow, bone marrow spaces are filled with adipose tissue; blood vessels can be also present (Huysseune 2000; Witten *et al.* 2001; Witten & Huysseune 2009; Apschner *et al.* 2011).

Structurally, teleost bone first develops as woven bone, followed by parallel-fibred and lamellar bone in more mature individuals. In larger fish, lamellar bone can also form osteons (Moss 1961a; Smith-Vaniz *et al.* 1995; Meunier 2002; Witten & Hall 2002, 2003). The various terms that are used to describe the same bone element can be confusing. Different terms are, however, not synonymous. They relate to the location of the bone (a), to the origin of the bone (b), to its mode of development (c), to its structural properties (d) or to phylogenetic assets (e). The terms are sometimes mixed up in the literature. Terms such as endo- and exoskeleton refer to the location (a) of the skeleton. Using the term dermal skeleton instead of exoskeleton, refers to the fact that these exoskeletal elements (teeth, scales, dermal bones, fin rays) originate (b) from the interaction between the epidermis (ectoderm) and the underlying mesenchyme. The endoskeleton originates (b) from sclerotome-derived mesenchyme. The mode of development (c) of the dermal skeleton is usually intramembranous, mesenchymal precursor cells developing directly into bone. Cartilage and chondroid bone as part of the dermal skeleton are secondary tissues (e). The default mode of development (c) of endoskeletal bone elements is trough endochondral ossification, where a cartilaginous scaffold is replaced by bone. However, several endoskeletal elements develop through intramembranous bone formation. Structurally (d) both, dermal skeletal elements and endoskeletal elements, can consist of woven or of lamellar bone. Phylogenetically (e), the bony elements of the dermal skeleton are older than bone of the endoskeleton (for reviews see Smith & Hall 1990; Huysseune & Sire 1998; Donoghue & Sansom 2002; Sire & Huysseune 2003; Hall & Witten 2007; Witten & Huysseune 2007).

The different skeletal tissues, cells and extracellular matrices in fish have been described in detail (Kölliker von 1859, 1873; Moss 1961a,b, 1962, 1964, 1965; Meunier 1983; Huysseune 1985, 1989; Benjamin 1989, 1990; Huysseune *et al.* 1989; Francillon-Vieillot *et al.* 1990; Benjamin & Ralphs 1991; Takagi & Yamada 1991, 1992; Meunier & Huysseune 1992; Nishimoto *et al.* 1992; Takagi & Yamada 1992, 1993; Beresford 1993; Hamada *et al.* 1995; Persson *et al.* 1995; Smith-Vaniz *et al.* 1995; Witten 1997; Witten & Villwock 1997; Ramzu 1998; Lehane *et al.* 1999; Witten *et al.* 1999, 2000, 2001, 2005a,b, 2006, 2009, 2010; Huysseune 2000; Pinto *et al.* 2001; Weiss Sachdev *et al.* 2001; Diekwisch *et al.* 2002; Kemp 2002; Witten & Hall 2002, 2003; Pinto *et al.* 2003; Smits & Lefebvre 2003; Takenaka *et al.* 2003; Cole & Hall 2004; Kang *et al.* 2004; Kawasaki *et al.* 2004; Gil Martens *et al.* 2005; Hall 2005; Kranenbarg *et al.* 2005b; Nordvik *et al.* 2005; Redruello *et al.* 2005; Franz-Odendaal *et al.* 2006; Gavaia *et al.* 2006; Gillis *et al.* 2006; Hall & Witten 2007; Roy & Lall 2007; Witten & Huysseune 2007, 2009, 2010; Kang *et al.* 2008; Rotllant *et al.* 2008; Zylberberg & Meunier 2008; Horton & Summers 2009; Renn & Winkler 2010; Apschner *et al.* 2011; Estêvão *et al.* 2011; Meunier 2011; Lie & Moren 2012; To *et al.* 2012).

The notochord

The notochord is an essential tissue that plays both structural and patterning roles: early notochord signals influence the cell-fate and patterning in the spinal cord and in somites (Fleming *et al.* 2001; Anderson *et al.* 2007; de Azevedo *et al.* 2012). Structurally, the notochord is the sole skeletal support tissue in the embryo and in early life stages post-hatching, a stiffened rod against which muscular contraction can drive motility.

Along with development, the teleost notochord stiffens throughout secretion of fibrous collagens (mostly collagen

II) and cells then vacuolate and differentiate into epithelial cells, called notochordoblasts (Fishelson 1966; Yan *et al.* 1995; Nordvik *et al.* 2005). These produce a fibrous sheath surrounding the notochord. New evidence allows the hypothesis that the rigidity given by the collagen encasing sheath constrains the expanding notochord cells from inflation of their vacuoles, thus generating a hydrostatic pressure that drives the elongation, stiffening and straightening of the notochord (Anderson *et al.* 2007). Differing from other vertebrates, the teleost vertebral centra form without a cartilaginous anlage (primordium). In teleosts, the mineralization of the notochord sheath, not cartilage and not bone formation, establishes the identity of vertebral bodies. Formation of schelerotome-derived bone is only the second step of teleost vertebral body formation (Huxley 1859; Kölliker von 1859, 1873; de Azevedo *et al.* 2012; Bensimon-Brito *et al.* 2012). Consequently, anomalies/mutations affecting specification of the early notochord cells provoke profound defects on vertebral body patterning in teleost fish (Fleming *et al.* 2001, 2004; Morin-Kensicki *et al.* 2002; Crotwell & Mabee 2007; Willems *et al.* 2012). After the vertebral bodies have developed fully, notochord tissue in the intervertebral spaces can transform into cartilage under pathological conditions (Witten *et al.* 2005a). Studies on the structure of the notochord in basal actinopterygians and in teleost fish (Fig. 4) are available for the short-nose sturgeon, *Acipenser brevirostratus* (Schmitz 1998), medaka *Oryzia latipes* (Ekanayake & Hall 1991), yellow perch *Perca flavescens* (Schmitz 1995), Atlantic salmon (Grotmol *et al.* 2006) and zebrafish *Danio rerio* (Inohaya *et al.* 2007).

Regulatory mechanisms of skeletal tissues in fish

The assembly of the structures composing the skeleton is the net result of two processes acting at two different time scales: a phylogenetic process (over millions of years) and an ontogenetic process (over the life span of the individual) (Prendergast 2002). In general, according to Hall (2005), the skeletal development is modular at levels of: (i) individual skeletal systems (axial, appendicular, cranio-facial, 'extraskeletal' elements); (ii) individual skeletal elements; (iii) cellular condensations; (iv) gene networks; (v) epigenetic control.

As far as epigenetic (i.e. non-genetic) control is concerned, mechanical forces are by far the main studied factor, due to their implication for human health (e.g. osteoporosis, fracture healing). External mechanical forces are recognized to regulate genetic pathways of both cartilage and bone development in all vertebrates (Danos & Staab 2010). This gives skeletal tissues the capability to adapt their structures, shape and mechanical features in response to altered loading conditions in teleost fish (Huysseune *et al.* 1994; Kranenbarg *et al.* 2005a,b; Fiaz *et al.* 2012) and in mammals (Vahdati & Rouhi 2009). At present, a large body of studies deals with mechano-regulated tissue differentiation models developed with Pauwels's theory (Pauwels 1980) that the mechanical environment in the medium determines tissue phenotypes. It is well known from studies on mammals that bone responds preferentially to dynamic stimuli rather than static and that only short loading durations are necessary to induce an adaptive response,

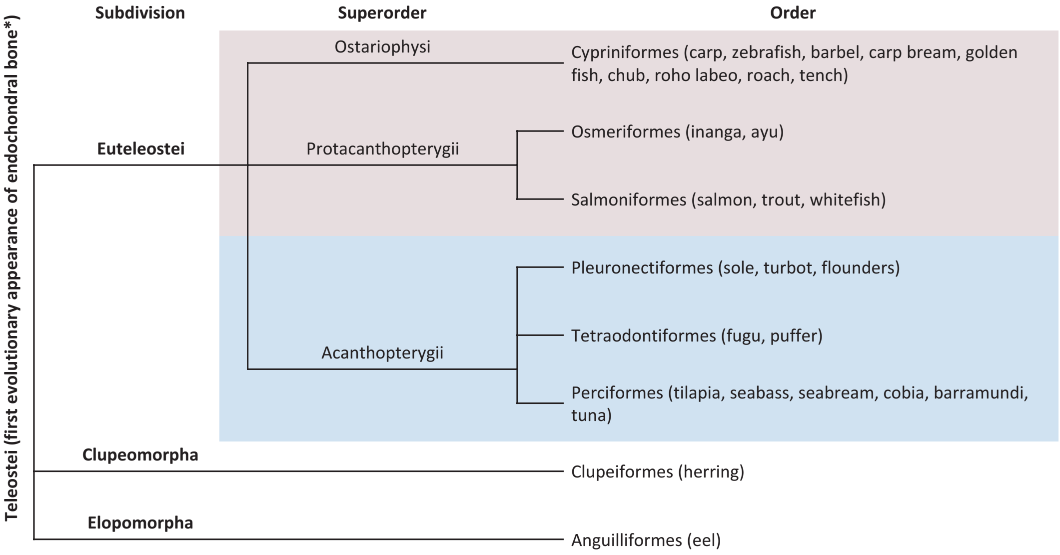

Figure 4 Phylogenetic tree, based on morphological characters, showing evolutionary relationships among some commonly reared teleosts (redrawn by Rise *et al.* 2004). Red shadow highlights the basal and the blue one the advanced teleosts. *Carter & Beaupré 2001.

allowing bone cells to adapt to specific mechanical loading environments (Warden 2006). A series of different mechanobiological models introduced a growing number of mechanical and biological theoretical factors acting and interacting during mesenchymal cell differentiation, fracture healing, intramembraneous bone formation, distraction osteogenesis, bone-implant reaction, osteochondral defect repair in tetrapods and then tested *in vivo*. Deeper insights on different mechano-biological modelling are achievable in Gómez-Benito *et al.* (2005) (strain-based), Carter and Beaupré (2001) (hydrostatic stress/deformation), Prendergast (2002), Kelly and Prendergast (2005) (biophysical) and Vahdati and Rouhi (2009) (semi-mechanistic).

Bone formation and the replacement of the cartilaginous anlage

Bone in mammals is formed by replacement of a cartilaginous template (Fig. 5) by bone (endochondral bone) or by intramembranous ossification, originating as dermal bones. In fish, three bone formation mechanisms, according to the considered species and skeletal elements, have been described: endochondral, perichondral and intramembranous (or direct or dermal) ossification. Below, a synthetic characterization of bone formation processes in vertebrate is provided: some highlights from the literature on reared teleost species are given, if available.

Endochondral ossification

According to Hall and Witten (2007), not all the bony material of endochondral bone originates by endochondral ossification in vertebrates, but (i) for primary ossification of the cartilaginous anlage occurring subperiosteally around the shaft by perichondral bone apposition; (ii) for a secondary endochondral replacement of cartilage of the shaft metaphysis; (iii) for the later extension of the skeletal element by appositional bone (*Zuwachsknochen*), in a process similar to intramembranous bone formation. Endochondral ossification can replace marrow, tendon or ligament tissue, without any cartilaginous template. Thus, the use of the terms replacement bone and indirect ossification should be restricted to endochondral bone and endochondral ossification, respectively (Hall & Witten 2007).

Most of the bones that ossify endochondrally originate from embryonic mesoderm (Hall 2005). This involves a cartilaginous template, which is replaced by, or remodelled into, bone by several coordinated sequential steps. Endochondral bone formation is one of the main ossification processes in mammals but the typical process is often lacking in teleosts, especially in small size species: replacement of cartilage by *spongiosa* (endochondral bone formation) can be observed in large (e.g. carp *Cyprinus carpio*, Atlantic salmon) but not in small (medaka and zebrafish) teleost species (Witten *et al.* 2000, 2001, 2010; Verreijdt *et al.* 2002; Witten & Huysseune 2007, 2009; Zylberberg &

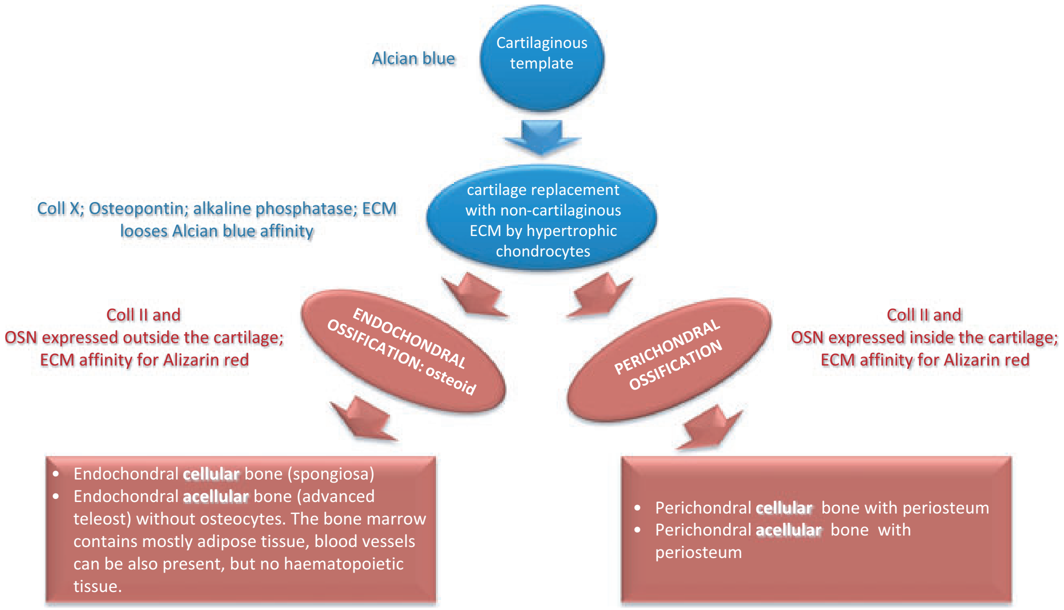

Figure 5 Possible fate of cartilage templates in vertebrates. For each step, some features (expressed molecules, staining) are indicated. It should be emphasized that spongiosa (spongy or trabecular bone) is rather uncommon in fish: larvae essentially do not have spongiosa and no endochondral bone formation, but perichondral bone formation. Coll, collagen; ECM, extracellular matrix; OSN, osteonectin.

Meunier 2008; Apschner *et al.* 2011). In smaller teleosts, such as medaka and zebrafish, where endochondral bone formation is uncommon typically a persisting cartilage rod remains inside the bone shaft and if cartilage is removed, it is replaced by adipose tissue. Young fish (larvae) generally are considered not to have endochondral bone formation (Witten *et al.* 2001, 2010), but Estêvão *et al.* (2011) described it as occurring in hypurals, vertebral arches, frontal bone, coracoid, sclera and dentary during gilthead seabream osteogenesis. Whilst vertebrae bodies are formed through endochondral ossification of cartilage templates in tetrapods, vertebral centra ossify without any cartilaginous anlage in all teleosts. Although no cartilage contributes to the initial formation of teleost vertebral bodies (Huxley 1859; Witten & Villwock 1997; Nordvik *et al.* 2005), in older individuals cartilage at the base of the arches undergoes endochondral ossification, and bone that derives from this process becomes part of the vertebral body (Zylberberg & Meunier 2008; Apschner *et al.* 2011).

The growth of endochondral bone depends on maintaining the growth of a primary cartilaginous model that, in its turn, requires functional stimuli, such as mechanical stress. Consequently, the continuation of the deposition of endochondral bone depends secondarily upon biomechanical stimuli (Hall 2005).

Perichondral ossification

Perichondral bone formation is the most common mode of ossification in fish. If a cartilaginous precursor is present, it usually starts with the transformation of a perichondrium into a periosteum. It typically occurs in the teleost fin endoskeleton (Fig. 6). Young fish (larvae) essentially only have perichondral bone formation and no endochondral bone formation. Differently from mammals, it is often not linked to endochondral bone formation (Witten & Villwock 1997; Hall 1998; Huysseune 2000; Apschner *et al.* 2011). Perichondral ossification produces the bone that surrounds the cartilage of the gill arches in mosquitofish *Gambusia affinis*, the pharyngeal jaws of *Astatotilapia elegans* (now *Haplochromis elegans*) (Benjamin 1989), the hypural cartilage in Nile tilapia (*Oreochromis niloticus*) and desert pupfish (*Cyprinodon macularis*) (Witten & Huysseune 2007), in dorsal and anal proximal pterygiophores in desert pupfish (Witten & Huysseune 2007), in pectoral, pelvic and caudal fins of rainbow trout (Ferreira *et al.* 1999), in dorsal and anal proximal pterygiophores, initial pectoral girdle ossification, secondary gill arches ossification and pectoral radials in teleost (Witten & Huysseune 2007). Parahypural (a ventral support of caudal fin) is reported by Witten and Huysseune (2007) as undergoing perichondral ossification, whilst Fernández *et al.* (2008) report that it is formed by endochondral ossification. The hyaline-cell cartilage (HCC) attached to the basioccipital in adult of black molly (*Poecilia sphenops*) only develops after perichondral bone has appeared (Benjamin 1989). As reviewed by Witten and Huysseune (2007) in teleost fish, perichondral bone formation is the basic process of ossification of the fin endoskeleton. Perichondral bone is laid down at the immediate contact of the cartilaginous template by cells that were formerly part of the perichondrium, but have now taken up the characteristics of osteoblasts and secrete the bone matrix. Nevertheless, an admixture of cartilage and bone matrix is not excluded (Huysseune & Sire 1992; Huysseune 2000; Verreijdt *et al.* 2002).

In smaller species, such as zebrafish and medaka (*Oryzias latipes*), cartilage that is enclosed by perichondral bone and chondrocytes can hypertrophy, but the cartilage is removed without being replaced by bone (for example, in the splanchnocranium). The result is a bone collar with cartilage at one or both ends (enabling further growth) and adipose tissue inside (Witten *et al.* 2010).

In the endoskeleton, membranous apolamellae can form from osteoblasts of perichondral bone, a process that resembles intramembranous bone formation (Witten & Huysseune 2007).

Parachondral ossification

Blanc (1953) cited the formation of bone around Meckel's cartilage in Atlantic salmon as an example of parachondral ossification. Such bone develops around cartilage, but is separated from it by a layer of ordinary connective tissue (Benjamin 1989).

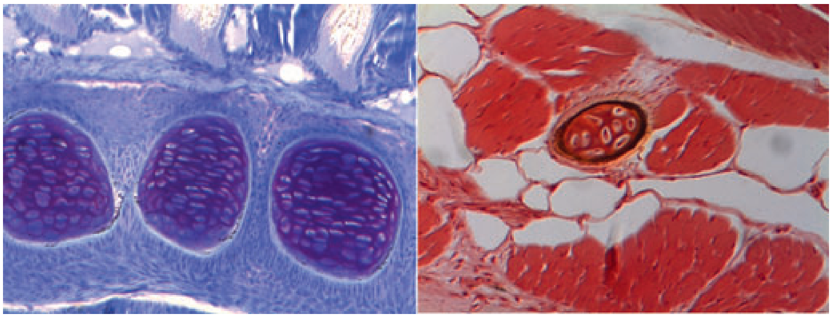

Figure 6 Perichondral ossification in dorsal pterygiophores of Senegalese sole (*Solea senegalensis*) (on the left: Toluidine blue; on the right: von Kossa's). Photo by P. Gavaia.

Intramembranous ossification

Cranial bones that have undergone intramembranous ossification have a neural crest origin (Hall 2005). This mode of bone development has been described in many teleosts. Mesenchymal cells differentiate into osteoblasts and form bone without a cartilaginous template (Franz-Odendaal *et al.* 2006). The bones formed in this way are designated dermal or membrane bones. Sire and Huysseune (2003) described categories of odontode derived dermal skeletal elements in vertebrates: teeth, denticles, cranial dermal bones, scutes, postcranial dermal plates, ganoid scales of polypterids, ganoid scales of lepisosteids, elasmoid scales and fin rays. With the exception of ganoid scales of polypterids and of lepisosteids, these elements are present in teleosts. Cranial dermal bones are frontal, infraorbitals, lacrimal, nasal, parasphenoid, parietal, vomer, ectopterygoid, endopterygoid, dentary, maxillary, premaxillary, interopercle, opercle, preopercle, subopercle, branchiostegal rays and urohyal. All these dermal skeleton elements form in the mesenchyme or in the dermis, without a cartilaginous precursor and below a multilayered epithelium or epidermis (Sire & Huysseune 2003). During the early development of vertebral bodies in salmon, Nordvik *et al.* (2005) described the importance of the notochord sheath tissue for the initial mineralization of the vertebral body. These authors described how, after the initial notochord segmentation and expression of segmented alkaline phosphatase activity by the chordoblast layer (Grotmol *et al.* 2005), the formation of a chordacentrum by direct mineralization of the external layers of the notochord occurs.

Prior to the beginning of dermal ossification, the dentary anlage consists of densely packed mesenchymal cells with a barely visible extracellular matrix (hereafter called ECM), while in the frontal bone, that develops later in ontogeny than the dentary, cells are sparse amidst a collagenous matrix (Sire & Huysseune 2003).

In fin development, mesenchymal cells populate the area between the two epithelial layers that constitute the embryonic fin fold. They progressively increase their number, so forming a dense core separated by the basal lamina, an acellular layer with randomly arranged collagen (Sire & Huysseune 2003).

The anlagen of cranial dermal bones and fin rays in teleosts are poorly defined papillae (or osteogenic papillae), located deep in the mesenchyme or close to a cartilage anlage. No indication of epithelial–mesenchymal interactions has been shown and matrix deposition is not polarized, it occurs on both surfaces (Sire & Huysseune 2003). The resulting tissue is fairly identifiable as bone, with an inner part composed of woven-fibred matrix and a peripheral part composed of parallel-fibred matrix, deposited after the initial fast growth of the inner part.

In dentary and frontal bones, the papillae merge with the perichondral cell population very close to the cartilage of *taenia marginalis* (frontal) or Meckel's cartilage (dentary). The start of ossification is characterized by a local thickening of the *stratum compactum* along with an accumulation of flattened fibroblast-like cells and tubby pre-osteoblasts (mesenchymal cells agglomeration and condensation). Chondroblasts/cytes are at this stage PCNA (proliferating cell nuclear antigen)-positive in gilthead seabream (Estêvão *et al.* 2011).

The subsequent phase is characterized by the synthesis of the different matrices constituting the different skeletal dermal elements and, so, it differs in different skeletal elements: odontodes, teeth, denticles and fin rays maintain a close relation with the epithelial cells, whilst dermal cranial bones do not maintain, or establish no, relation with epithelial cells.

Fin ray dermal ossification has some peculiar features: the anlagen are osteogenetic papillae but differentiation takes place immediately below the epithelial–mesenchymal boundary, thus suggesting the presence of some relationship with the epithelial cover. At the beginning of fin ray ossification, a multilayered epithelium thickens in the vicinity of the developing lepidotrichium. The epithelial cells show features of differentiation, indicative of epithelial–mesenchymal interactions, but they do not directly participate in matrix production (Sire & Huysseune 2003). Fin ray anlagen are composed of a woven-fibred matrix, acellular and not penetrated by cellular processes. The anlagen form a continuous sub epidermal sheet located between the epithelial basal cells and the mesenchymal cells. Collagen type I and osteonectin (here after called OSN) are secreted and mesenchymal PCNA-positive cells are detectable around the bony structures in gilthead seabream (Estêvão *et al.* 2011). The matrix mineralizes rapidly, from the central region of the subepidermal sheet toward the exterior. With growth, a single layer of mesenchymal cells infiltrates the epithelial–mesenchymal interface and progressively separates the ray matrix from the epithelial surface.

Different from fin rays, the cranial dermal bone ossification process has no relation with epithelial cells, and their anlagen are initially not sharply defined from the surrounding tissues. The anlage is covered by osteoblasts in a single layer, surmounted by a mesenchymal space, at the side facing the epithelium. At the opposite side, the anlage is separated from the underlying cartilage by not clearly delimited by osteoblasts. In jewelfish (*Hemichromis bimaculatus*), the space between the osteoblast monolayer and the epithelium is filled with a woven-fibred, acellular network, embedded in a fine granular, electron-dense, background substance. This substance mineralizes soon after its deposition (*periosteal ossification*). Later, a parallel-fibred matrix is deposited on both bone surfaces (Sire & Huysseune 2003).

Deposition of membrane bones is far less dependent on mechanical factors than endochondral bone growth (Hall 2005) but transdifferentiation in chondroid bone is reported to occur in compressed and fused salmon vertebrae (Witten *et al.* 2005a, 2006).

In conclusion, intramembranous ossification has features that are different for different dermal skeleton elements: however, all need the presence of a support, a well-structured mesenchyme or a bone, cartilage or another support, but epithelial–mesenchymal interactions are not always necessary. The fulfilment of this need modulates the timing of ossification of different dermal bones.

Modulation and transformation

The differentiation and ossification processes of different skeletal elements can be altered by microenvironment conditions (Hall 2005). Modulation (of cellular activity) is a physiological response to altered environmental conditions: it is characterized by a temporary change in cell behaviour, structure and/or the type of extracellular matrix products. The maintenance of the 'new' state is depending on the enduring of the environmental stimulus, so it is reversible. An example is given by the cell switching from the synthesis of collagen type I to the synthesis of collagen type II.

Transformation (of cell identity) is generally a permanent change, even when the stimulus is not present anymore. It creates a permanent intermediate tissue (chondroid or chondroid bone) (Hall & Witten 2007).

Dedifferentiation, transdifferentiation and metaplasia

In a dedifferentiation process, a differentiated cell loses its specific phenotypic characteristics and transforms into an undifferentiated mesenchymal cell.

Transdifferentiation is a transformation of one differentiated cell type into another cell type (Okada 1991): i.e. chondroid bone type I can arise from incomplete endochondral ossification (forming chondroid bone II) or transdifferentiation of skeletal cells (from osteoblast to chondroblast) within multipotential periostea (thus forming chondroid bone type I as in the dentary tip of salmon) (Witten & Hall 2002; Gillis *et al.* 2006). It can be either a pathological or a normal process: the transdifferentiation from osteogenic to chondrogenic cells of the vertebral growth zone in compressed and fused vertebrae of Atlantic salmon, Senegalese sole (*Solea senegalensis*), European seabass and gilthead seabream is a pathological condition in response to a compressive mechanical environment (Beresford 1981; Hall 2005; Kranenbarg *et al.* 2005b; Witten *et al.* 2005a, 2009; Roberto 2006; Fiaz *et al.* 2010; Cardeira *et al.* 2012). This is in accordance with Pauwels's mechano-regulated theory of tissue differentiation, which states that compression is the specific stimulus for the development of cartilaginous tissue (Prendergast 1997). Transdifferentiation, however, occurs also during normal development in other skeletal elements of Atlantic salmon and in other teleost species (Witten & Hall 2002, 2003; Kranenbarg *et al.* 2005a; Gillis *et al.* 2006; Hall & Witten 2007; Witten & Huysseune 2007; Fiaz *et al.* 2010).

Chordoblasts and intervertebral ligament cells likely show a mechanically induced transdifferentiation into a cartilaginous phenotype (Fiaz *et al.* 2010). In some cases, transdifferentiation can be accompanied by cell division, whereas in others it is not (Beresford 1990). Transdifferentiation can occur either directly (the cells possess characteristics of both cell types simultaneously during the transition period) or indirectly (implies a dedifferentiation phase in which the phenotypic characteristics of the cell first disappear before a new phenotype is established) with respect to the timeframe of the phenotypic transformation (Dewit *et al.* 2011).

Although transdifferentiation of cartilage into fibrous tissue may be rare, many other types of transdifferentiation in connective tissues have been described: cartilage and chondroid bone into bone, bone into chondroid bone, bone into cartilage, fibroblastic tissue into bone or cartilage, hyaline- into fibro-cartilage, periosteal chondrogenesis, perichondral osteogenesis, fat into bone, and muscle into bone (see Dewit *et al.* 2011 for details). This scenario evidences that the different phenotypes of connective tissues are neither fixed nor terminal, but rather form a continuous spectrum in which differentiation can be modulated by a variety of factors, and that phenotypic plasticity can play an important role in various developmental and homeostatic processes (Hall & Witten 2007).

Metaplasia is the normal transformation of tissue from one type to another, as in the ossification of cartilage to form bone. Differing from mammals, in teleost normal skeletogenic processes, cartilage elements may arise also by multiple subdivisions of an existing cartilage, through dedifferentiation of chondrocytes into fibroblasts. During this process, the surrounding matrix loses its cartilaginous character (Witten *et al.* 2010). This kind of metaplasia occurs during the development of the endoskeleton of teleost pectoral, dorsal and anal fins (Grandel & Schulte-Merker 1998; Witten & Huysseune 2007; Dewit *et al.* 2010; Witten *et al.* 2010). The absence of signs of apoptosis or resorption during subdivision of the cartilage larval pectoral plate into radials in zebrafish suggests that the separation of cartilage elements may involve metaplasia that occurs through dedifferentiation of cartilage cells and their redifferentiation into noncartilaginous connective tissue (Witten & Huysseune 2009; Dewit *et al.* 2011). So bone elements can arise via metaplasia, a process that intervenes when matrix changes as the result of trapped chondroblasts and chondrocytes assume osteoblastic activity, and modify the ECM toward an osseous tissue (Hall 2005).

Unlike in intramembranous ossification, cells resembling osteoblasts are generally absent during metaplasic ossification (Sire *et al.* 2009).

Chondro-bone metaplasia is a progressive transformation of cartilage into bone, without any previous destruction of pre-existing tissue, in contrast to neoplasia which is the substitution of a tissue (i.e. cartilage) with another (i.e. bone) (Meunier *et al.* 2008). In fish, it may occur in both normal and in pathological development: from a histopathological point of view, both vertebrae compression and fusions involve metaplasic changes of bone forming cells (osteoblasts) that differentiate into cartilage forming cells (chondroblasts); then, in the growth zone of vertebral bodies, developing heterotopic cartilage mineralizes and it is remodelled into bone tissue (Witten *et al.* 2005b, 2006; Gil Martens 2010). Compressed vertebrae in short-tail Atlantic salmon, for example, are the result of metaplasic chondrogenesis (metaplasic synchondrosis) as a skeletogenic response late in ontogeny (Witten *et al.* 2005a).

Late events in teleost skeletal tissue modelling and remodelling

Many teleost species never stop growing and thus all skeletal tissues may continue to differentiate and to transform through metaplasia, mineralization or remodelling throughout life (Witten & Huysseune 2009). Modelling occurs when the shape of a bone needs to be altered. In bone remodelling (bone turnover) resorption is followed by new bone formation (either through intramembranous or endochondral ossification), without any change in shape. Osteoblasts and osteoclasts are the bone remodelling units. Remodelling in teleosts occurs by resorption and *de novo* formation, but also by transdifferentiation (metaplasia) of skeletal tissues (Beresford 1981; Witten & Hall 2002; Hall 2005; Gillis *et al.* 2006; Hall & Witten 2007; Witten & Huysseune 2009). So the replacing one tissue type by another could be included also in skeletal remodelling.

Some mechanisms of skeletal remodelling are more prominent and/or more commonly observed in teleosts than in mammals and are thus recognized as regular processes that shape the skeleton in the course of development and growth (Sire *et al.* 1990; Huysseune 2000; Witten *et al.* 2001; Hall & Witten 2007; Witten & Huysseune 2007), bone repair processes (Gil Martens 2012) and the growth of the kype in mature male salmon (Witten & Hall 2003; Witten & Huysseune 2009). Furthermore, lordosis, scoliosis, kyphosis and fusion of vertebral bodies must involve bone resorption and bone remodelling, as a primary pathology or in response to altered mechanical load (Kranenbarg *et al.* 2005b; Witten *et al.* 2006). There are some reports of bone resorption connected to bone anomalies, mainly caused by phosphorus deficiency (in haddock *Melanogrammus aeglefinus*: Roy *et al.* 2002; in farmed Atlantic salmon: Roberts 2001).

An extreme case of remodelling is the complete pathological fusion of vertebral bodies in Atlantic salmon (Witten *et al.* 2006) and in advanced teleosts with acellular bone (Sawada *et al.* 2006). So compression and fusion of vertebrae bodies involve the metaplasic transformation of bone-forming cells in the vertebral growth zone into cells that produce cartilaginous tissue instead of bone. Later this cartilage is mineralized and remodelled into bone (Witten *et al.* 2005a, 2006). Remnants of notochord tissue in the intervertebral space are also remodelled.

Hypermineralized vertebral bodies constitute another pathology that involves modelling and can be caused by heterotopic cartilage occupying bone marrow spaces (Helland *et al.* 2006). The observed resorption of such cartilage suggests that it is not a permanent structure.

Also infectious diseases can trigger skeletal resorption in teleosts: the parasite *Myxobolus cerebralis* causes skeletal deformities and lysis of cranial cartilage (Halliday 1973; Garden 1992; Kelley *et al.* 2004).

Bone resorption and remodelling

Differing from cartilage that is reshaped by chondroclasts, changes in bone structure can only occur through remodelling, a process in which resorbing cells (osteoclasts) remove existing bone and osteoblasts form new bone.

Basically, bone remodelling in fish is required for tooth replacement, allometric growth and for removing temporary skeletal elements (e.g. kype in male Atlantic salmon), or it occurs as adaptation to mechanical load (Hall & Witten 2007). Bone remodelling intervenes also in bone repair processes in teleosts that, however, evidence a higher regenerative capacity of dermal (rays and scales) than endoskeleton (Witten & Huysseune 2009).

Differences between aquatic and terrestrial vertebrates have been outlined by Witten and Huysseune (2009). In particular, some differences other than morphological have been found between small, mononucleated osteoclast-like cells in advanced teleosts, and giant, multinucleated osteoclast of tetrapods and basal teleosts: fish do not show any intimate spatial relationship between bone resorbing cells and haematopoietic cells (as in teleosts the bone marrow is filled with adipose tissue); in advanced teleosts osteoclast-like cells can perform resorption without generating typical resorption Howships's lacunae.

All teleosts have mono- and multinucleated osteoclasts, but the first are the main bone-resorbing cells observed in advanced teleosts. Remodelling processes with intervention of osteoclasts are prominent in basal teleosts, e.g. cyprinids and salmon that have cellular bone, where it starts only late in development (around 60 DPH in zebrafish) (Witten *et al.* 2001; Witten & Huysseune 2010). In advanced teleosts, with acellular bone, metabolically driven bone remodelling is considered to be limited and there are reports in the literature on the absence of the process (see Witten & Huysseune 2009 for references). Regular resorption and rebuilding of scales and bony skeletal elements is well documented for Atlantic salmon (Kacem *et al.* 1998; Persson *et al.* 2000, 2007; Witten & Hall 2002, 2003). A comparative review on skeletal remodelling in teleostean fish has been published recently (see Witten & Huysseune 2009).

Studies carried out on zebrafish (Witten *et al.* 2001) evidenced heterochronic shifts in the appearance of bone-resorbing cells: in early stages, bone is resorbed by mononucleated osteoclasts, while multinucleated osteoclasts (as in mammals) appear only later, when the bone switches from acellular to cellular. Bone resorption by mononucleated osteoclasts was found to coincide with the dominance of acellular bone (Witten & Huysseune 2010) but in all teleosts that have been studied so far, mononucleated are the dominating resorbing cells in the early stages, when the bone is still acellular. The presence of osteoclast key enzymes, transcription factors and receptors (H^+-ATPase, TRAP, RANK, RANKL, Cathepsin K) in mononucleated osteoblasts prove their capability for bone resorption as shown in the advanced teleosts such as Nile tilapia (*Oreochromis niloticus*) and medaka (Witten 1997; Witten & Villwock 1997; To *et al.* 2012). Lysis of cranial cartilage and increased skeletal resorption resulting from bacterial infection has been reported for salmonids infected with *Flexibacter psychrophilus* (Ostland *et al.* 1997; Witten & Huysseune 2009).

Despite the many morphological differences between mammalian multinucleated osteoclasts and teleost mononucleated osteoclasts, the molecular mechanisms underlying bone resorption regulation are considered to be identical (consult Witten & Huysseune 2009; Gil Martens 2012; To *et al.* 2012).

Osteocytic osteolysis

Osteocyte interconnected cell processes function as stress sensors in cellular bone, so activating bone deposition and resorption processes carried out by the osteocytes themselves (Witten & Huysseune 2010): e.g. the enhanced demand for calcium in pregnant and lactating bats is satisfied by osteocytic osteolysis and not by osteoclasts resorption. Osteocytic osteolysis has been described only in teleosts from different groups, such as eels (*Anguilla anguilla*) (Lopez *et al.* 1980; Sbaihi *et al.* 2007), salmonids (Hughes *et al.* 1994; Kacem & Meunier 2009) and cyprinids (*Cyprinus carpio*) (Witten *et al.* 2000).

Main gaps in the scientific knowledge and further research needs

Studying the available knowledge on amniotes (i.e. birds and mammals, including humans) skeletogenic processes can be a promising tool for deeper insight in what happens in the same processes occurring in teleost fishes. However, data from the amniote skeleton are not always applicable to the fish skeleton. Gaps in knowledge are evident concerning endochondral ossification in different fish species and different life stages of fish. For example, studies by Benjamin (1989), Benjamin *et al.* (1992) and Witten *et al.* (2010) have described the gill arches as being composed of hyaline cell cartilage (HCC) or chondroid but not as chondroid bone (for review see Witten *et al.* 2010), while another specific type of cartilage, Zellknorpel, is found supporting the gill filaments. Some controversy still persists in the classification of the skeletal tissues of teleosts, particularly the many forms of cartilage and in the transitory forms between cartilage and bone that coexist in teleost skeletons. Even the same ossification modality (e.g. intramembranous ossification) may show differences among the considered skeletal elements in fish. Consequently, a deeper insight into the current molecular literature achieved on model fishes (zebrafish and medaka, for example) and comparison with the mammalian literature is highly recommended in order to fully establish what is specific for fish, what is specific to mammals and what is common in fish and mammals. In this scenario, this review represents a starting basis for further studies.

Other more specific gaps and needs in scientific knowledge are as follows:

- cartilage development is a tightly regulated morphogenetic event where much has been studied on gene regulation by different types of signalling molecules, but less is known of their downstream regulation, even at the mammalian level. In particular, the regulatory mechanisms that control the synthesis of the noncollagenous elements of the cartilage remain unknown. Also, the role of ECM during mesenchymal condensation still remains unclear and needs further investigation (Quintana *et al.* 2009). Time- and space-dependent expression of transcription factors that regulate the first step of chondrogenesis are quite common to all vertebrates but the large variety of cartilaginous tissues found in fish requires deeper study of the regulatory and differentiation processes. Further, according to Hall (2005) the most critical event in skeletal patterning is arguably the formation of the condensations that prefigure skeletal elements but too little is known about what determines their size, shape and number, particularly in fish;
- perichondral ossification and chondral bone need further characterization to enhance our understanding about the

mechanisms, (ultra) structural and chemical processes of what happens in different fish species;

- further insights into the external (non-genetic factors) and internal (genetic, microenvironmental) factors that modulate morphogenesis of different skeletal tissues and mineralization in phylogenetic basal and more advanced teleosts are required. Also, the ontogenetic steps of different skeletal anomalies (with the identification of timing windows) should be studied in a comprehensive and comparative context, in order to be able to manipulate biotic and abiotic factors for improving larval development and promoting a 'better' skeletal quality. For instance, the ontogenic pathway of many skeletal anomalies is rather unclear as well the changes in the vertebral architecture during growth in different reared species; mechanical load significantly affects bone formation, but the molecular pathways linking mechanics and bone development are largely unknown;
- further work is needed to study the relationships between rearing conditions, bone condition and vertebral abnormalities. Bone parameters appear to have a wide range of responses. This requires the consistent use of the existing scientific terminology and for observations made through well-defined experimental studies. Studies related to genetics, nutrition, mineral balance and/or biomechanics, cellular features related to bone modelling and hormonal regulation of bone metabolism should be performed. Fish skeletal biology will further incorporate available knowledge about human bone pathology but it must consider the differences between the mammalian and the teleost skeleton, as well as the differences between basal osteichthyans and advanced teleosts.

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