

## Microstructure, Growth and Remodeling of the Non-deformed and Deformed Vertebrae of the Red Porgy (*Pagrus pagrus*)

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## **Dedication**

To my pillars, My mother, My father, Sister, brothers and nephews

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### List of Abbreviation

APROMAR: Asociación Empresarial de Productores de Cultivos Marinos de España.

BW: body weight

Ca: calcium

cf: Condition factor

dpi: Dots per inch

**DPX:** Digital Picture Exchange

EDTA: ethylene-diamin-etetr-acetate

EU: European Union

ICCM: Instituto Canarias de Ciencias Marinas

K: condition factor

Kv: kilovolts

m/s: Meter per second

mAs: milliampere second

MilliQ water: Millipore Corporation Milli-Q refers to water that has been purified and deionized to a high degree by a water purification systems manufactured by Millipore Corporation. It is also a registered trademark of Millipore.

nm: nanometer

NMFS: National Marine Fisheries Service

°C: degree Celsius

OsO4: Osmium

P: Phosphorus

PC: phosphatidylcholine

PI: phosphatidylinositol

Ppm: parts-per-million

SGR: Specific growth rate

STR dev.: Standard deviation

t: tonne TL: total length Tm: Metric Tons TRAP: Tartrate-resistant acid phosphatase T-test: statistical hypothesis test μm: micrometer

## ABSTRACT

In the present work, we investigate the skeletal deformities occurrence in a stock of red porgy (*Pagrus pagrus*) reared in two different water current protocols, high and normal, in tanks specifically designed for this study. Deformities were determined by xraying all fish three times during the trial period. The results showed that the degree of deformation can be aggravated by the higher water current. Whereas postcranial lordosis may become worse under high water current, the vertebral fusion did not seem affected. Normal deformed and fused vertebral bodies fish were selected for histological studies. The results of the histological studies confirmed the x-ray tracing, the comparison with normal vertebral bodies allowed to understand the deformed vertebral body. In the case of the fused vertebral body we could observe containment, when two vertebral bodies could fuse to one.

# **INTRODUCTION**

#### I. INTRODUCTION

#### I.1. Aquaculture development

Aquaculture production has rapidly increased during the last decades in order to provide protein rich fish to the growing human population. European aquaculture production comprises more than 16.3% of the total aquatic production. Particularly, Spain is the second most important EU aquaculture producer with 281266 tonnes in 2007 (21.6% of total EU), from which marine fish for 46272 Tm in 2009, being mainly constituted by gilthead sea bream (Sparus aurata) with 23930 Tm and sea bass (Dicentrarchus labrax) with 11.630 Tm (APROMAR, 2009). More than 20% of the Spanish production of sea bream and 41% of sea bass is produced at the Canary Islands, being one of the leading regions in marine fish production in Spain. Nevertheless, the Canary aquaculture production is constituted mainly by these two species leading to a high dependence of this sector on their market. Prices variations of these two species are a risk for the further aquaculture business development in this region. In addition, the new world and Spanish economic crisis has affected seriously the aquaculture competitiveness causing a collapse of many canaries aquaculture companies. Therefore, new strategies have been proposed to guarantee the sustainable development of the sector including species diversification to offer new aquaculture products to the consumers and avoid a price decrease.

Red porgy (*Pagrus pagrus*) has emerged as one of the most attractive potential aquaculture species during the last decade due to its high market value, while the supply of this species by fisheries to the market decreased significantly to about 30% (NMFS, 2007b) due to a decline in the wild stock abundance during the last decade (Vaughn and Prager, 2002; SAFMC, 2006). The nutritional and physiological requirements of this species are similar to already cultivated Sparidae species. Moreover, still, there are

some challenges related to the rearing techniques need to be improved before its complete introduction into the aquaculture market in order to provide the same characteristics as the wild fish.

The increase in the aquaculture production has been accompanied by the development of new technologies and intensive production systems in order to reach higher profitability. However, production systems diversification has been associated with some negative challenges to the fish physiology affecting fish welfare and health. These abnormalities are considered to be a big challenge to the intensive aquaculture systems causing lowered growth, high mortalities, reduces market value of the product, and consequently big losses for the farmer.

#### I.2. Bone deformities in fish

Deformities are considered as one of the most important problems occurring in intensive aquaculture and for example in reared red porgy its represents about 40 % in intensively production system (Izquierdo *et al.*, 2010). Studies on the mechanisms causing skeletal deformities in fish are considered of great importance in order to establish technical and scientific solutions to prevent the occurrence of malformations. Thus, many studies reported and discussed various possible causes of deformities in the marine fish (Andrades *et al.*, 1996; Koumoundourous *et al.*, 1997b, 2001a, b; Afonso *et al.*, 2000; Boglione *et al.*, 2003; Roo *et al.*, 2005b; Gjerbe *et al.*, 2005; Lewis and Lall, 2006) but no study has definitely documented a single specific cause (Eissa and Moustafa, 2008).

Bone deformities are usually diagnosed late, making its prediction difficult due to the lack of sufficient information about the occurrence and cause of the malformations (Witten *et al.*, 2005a); because the changes of the internal bone structure occur before the deformities become externally visible (Witten *et al.*, 2006). Different malformations have been identified, among those spinal malformations like lordosis (ventral curvature of the vertebral column and an abnormal extra-calcification of the affected vertebrae) (Chatain, 1994), scoliosis (lateral curvature), kyphosis (dorsal curvature) observed in juvenile sea bass with functional swimbladder, and vertebral fusion (fusion of two or more vertebral bodies in one), are the most common skeletal deformities (Afonso et al., 2000; Boglione et al., 2001; Gjerde et al., 2005; Roo et al., 2005b; Eissa and Moustafa, 2008). Furthermore, deformed opercula can occur at one or both sides of the fish head and it was associated with malformations of the bronchial arches (Sadler et al., 2001), and jaw deformities are expressed by prolongation or torsion of the lower or upper jaw. The deformities associated with the neurocranium (opercula complex and jaws) are often found in commercial hatcheries, and they occur from early ages of development, without association with a particular side of the body, and could affect up to 98.3% of the population (Beraldo et al., 2003). Besides, it is also reported that several types of malformations could occurs in the same fish (Afonso et al., 2000; Sfakianakis et al., 2003). In addition, other deformities could affect flat fish such as turbot, halibut, or sole concerning the pigmentation, the incomplete eye migration and/or mandibular anomalies (Lewis and Lall 2006). Notochord distortions caused by unfavorable environmental conditions or by unidentified factors have been frequently implicated in the induction of axis deformities (Van Leeuwen *et al.*, 1986; Kiriakos et al., 1994; Andrades et al., 1996). Elsewhere, skeletal abnormalities could affect the swimming performance in marine fish constituting a big challenge for their survival (Plaut, 2001). Basaran et al. (2007) reported that there were significant differences between the swimming performances of normal and deformed fish.

#### I.3. Factors causing deformities in fish

#### I.3.1. Abiotic factors

#### I.3.1.1. Temperature

Different studies have considered the effect of different causative factors for fish deformities. Among them, temperature is one of the most important factors (Sfakianakis *et al.*, 2006a; Georgakopoulou *et al.*, 2007). For instance, some reports have indicated that during larval rearing, increasing water temperature from 15 up to 20 °C significantly increased the occurrence of lordosis in European sea bass larvae. Interestingly, lordosis is considered to be the most severe and extensively studied vertebral deformity. It particularly affects the vertebral bodies' number V14 and V15 (Sfakianakis *et al.*, 2005). The authors suggested that both lordosis angle and number of affected vertebrae are affected by water temperature.

#### I.3.1.2. Salinity

Salinity has been reported to affect deformity occurrence in some species. Thus, in a study made by Mihelakakis and Yoshimatsu, 1998, in red sea bream, testing the effect of the salinity and temperature on hatching and deformities in larval stage, 12‰ salinity has been reported like the lower tolerance limit where body curvature and tail flexure were the most abundant deformities, and the 18‰ and 36‰ salinity are the salinities where the lowest deformity occurs.

#### **I.3.1.3.** Hydrodynamic of the tank

Tank current disturbances have been reported to be causative factor that leads to skeletal malformation in different species (Chatain, 1994; Divanach *et al.*, 1997; Koumoundourous *et al.*, 1997 and Kihara *et al.*, 2002). Divanach et al., 1997 reported that in sea bass fry (*Dicentrarchus labrax*) with mean weight of 1 to 2g reared in normal water current, 93% of the fry had a normal abdominal bodies compared with the wild

standard, and 7% of the animals showed lordotic vertebrae; however in fish reared with high water current, 33% of fry conformed to the wild standard but 47% showed mild deformation, and 20% of the animals were seriously deformed.

Another study reported that in sea bass fry (*Dicentrarchus labrax*) and sea bream (*Sparus auratus*), of mean weight 0.7 to 4g that were forced to swim against water current of (0.2 m/s), all animals suffered from lordosis whereas only 20 to 30% of fish in stagnant water were affected, the curvature of the lordotic region (between the vertebrae V14 and V15) changed with weight from 10° C in 0.7g fish to 120° C in 150 g fish (Chatain, 1994).

#### I.3.2. Biotic factors

Fish deformities are also related to several biotic factors. Diseases, particularly caused by bacterial origin, can cause deformities (Madsen and Dalsgaard, 1999) affecting the elastin content; virus, fungi and parasites have also been reported to cause deformities.

Some anatomical disorders could also affect the morpho-anatomical structure of the fish, for example the absence, the late or the wrong inflation of the swim bladder would cause lordosis in sea bream larvae (Divanach *et al.*, 1997). In addition some other disorders are reported such as swim bladder over-inflation (Grotmol *et al.*, 2005) or digestive tract overfilling (Roo *et al.*, 2010; Izquierdo *et al.*, 2010) which induces a dorsal curvature of the notochord related to abdominal pressure.

#### I.3.3. Xenobiotic factors

Different xenobiotic factors like algaecides, fungicides, insecticides, heavy metals and industrial effluents are causative of abdominal deformities.

Heavy metals like have been reported to cause scoliosis (lateral ventral curvature) in common carp (*Cyprinus carpio*) (Slominska and Jezierska 2000) where

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the authors observed a diminution of the skeletal ossification in fish exposed to copper, with a cartilaginous skeleton, comparing to the control. Furthermore, industrial effluents like Pulp mill effluent caused abnormalities in the operculum and the cratenuous formation of perch (*Perca fluviatilis*), in the area affected by this pollutant, 34% of the fish were affected by cratenuous formation and 20% with operculum deformities, these values were decreased to 1 and 1.4% respectively when the pollution level was reduced (Lindesjoo *et al.*, 1994). In addition, Sheepshead minnows (*Cyprinodon variegates*) exposed to herbicides trifluralin, developed a vertebral dysplasia that consist of semi-symmetrical hypertrophy of vertebrae, also characterized by foci of osteoblasts and fibroblasts actively laying down bone and bone precursors (Couch *et al.*, 1979).

#### I.3.3.1. Rearing system

In intensive commercial hatcheries, about 10 to 30% of the marine fish larvae showed some type of spinal column deviations (Andrades *et al.*, 1996). However, extensive and semi-intensive systems cause less deformity than intensive systems (Roo *et al.*, 2005b). This likely relates to the different biotic and abiotic conditions of each culture system, such as tank hydrodynamic, live preys' nutritional quality, food availability ...etc. For example, the tank hydrodynamics, and particularly water current intensity has been related to the occurrence of lordosis (Chatain, 1994). Recent studies have shown that in red porgy there is also a higher incidence of skeletal deformities that occur under intensive rearing conditions and a lower under semi-intensive conditions, Intensive rearing conditions particularly promote the occurrence of kyphosis and cranial deformities (Roo *et al.*, 2010). In those studies, system intensive rearing conditions increased upper-jaw shortening and cross bite jaw, related to a nose-walling effect in the intensive system. At the same time, kyphosis, identified in the transition between cephalic and pre-haemal region, was associated with a higher prey density in the

intensive system which would cause over-predation and abdominal distension in the voracious red porgy larvae (Izquierdo *et al.*, 2010).

The rearing system has also a significant effect on the regionalisation of the vertebral fusion. In the semi-intensive system, vertebral bodies fused in the pre-haemal region and being the  $3^{rd}$  to the  $5^{th}$  vertebral bodies, the most frequently affected. In the intensive system the fused vertebral bodies were mostly located in the caudal region. In both rearing systems the most common skeletal abnormalities were the vertebral fusion and lordosis (Izquierdo *et al.*, 2010, Roo *et al.*, 2010).

#### **I.3.4. Nutritional factors**

Among nutritional factors, essential fatty acids, phospholipids, minerals, amino acids, proteins and vitamins have been documented to affect skeletal deformities (Watkins and Seifert, 2000; Geurden *et al.*, 1998; Lall, 2002; Cahu *et al.*, 2003). Particularly, increasing dietary phospholipids contents from 2.7% to 11.7% in diet for sea bream decreased significantly the malformation in larvae from 30% to only 2% (Cahu *et al.*, 2003), indicating the structural role of phospholipids as membranes components necessary for larval growth. Thereby, Geurden *et al.* (1998) has shown that phosphatidylcholine (PC) in Carp (*Cyprinus carpio*) improves growth while Phosphatidylinositol (PI) positively regulates carp morphogenesis. In addition, phospholipids such as Phosphatidylinositol (PI) are important for bone formation since they are precursors of second messengers regulating calcium entry in the cell, being the high calcium absorption a cause of bone resorption decrease (Cahu *et al.*, 2003). Moreover, other study showed that increased dietary protein hydrolysate is associated with decreased malformation rated in European sea bass (Cahu *et al.*, 1999).

#### I.3.4.1. Minerals

Micro or trace elements such as zinc, manganese and copper are required for growth and development of healthy bones of fish (Lall, 2002). Bone matrix is constituted from organic part (proteins) and inorganic part (minerals like calcium and phosphate) (Meunier, 1983; Roy and Lall, 2007).

Phosphorus (P) content is essential for freshwater and seawater fish. This essential nutrient must be provided in sufficient amounts by the diet in order to guarantee normal growth (Lall, 2002). In contrast to phosphorus all fish can absorb calcium (Ca) from the water in addition to dietary calcium intake. Calcium is deposited in bone, scales teeth and otoliths. The ratio of Ca to P in the whole body of several fish species ranges from 0.7 to 1.6%; however the level of P in the whole body is about 0.4 to 0.5% (Lall, 2002). Ca deficiency is not common in bony fish but P deficiency has been found to inhibit growth, decrease feed efficiency, reducing bone mineralization or trigger bone resorption (Lall, 2002; Lall and Lewis-McCrea, 2007; Witten and Huysseune, 2009), leading to skeletal abnormalities. Histological and histochemical examination of P deficient in haddock (Melanogrammus aeglefinus) showed an initial increase in bone resorption, decreased bone mineralization and reduced bone formation (Roy and Lall, 2003). These observations were based on the increase in the number of osteoclasts, suggesting their implication in P homeostasis. Moreover, the occurrence of bone deformities was associated with an increased amount of non-mineralised newly formed bone (osteoid) and a decreased bone mineral content (Roy and Lall, 2007).

#### I.3.4.2. Peptides

Incorporation of peptides in the diet for young freshwater and marine fish promotes growth, and replacement of dietary whole proteins by protein hydrolysate improved larval growth and survival (Cahu *et al.*, 2003). The incidence of skeletal

malformations after feeding compound diets for 31 days and assessed by examining larvae at day 40, significantly decreased with increasing levels of hydrolyzed squid meat in the diet.

#### I.3.4.3. Vitamins

#### 1) Vitamin A

Vitamin A and its derivatives retinols, especially retionoic acid regulate skeletogenesis and cartilage development by regulating chondrocytes functions, maturation and proliferation of cells in vertebrates (Kochhar, 1973; Harada *et al.*, 1995; Koyama *et al.*, 1999). In the mammalian skeleton retinoid toxicity reduces collagen synthesis (Dickson and Walls, 1985), and bone formation (Frankel *et al.*, 1986) and also increases the number osteoclasts causing a net bone loss and increased skeletal turnover (Hough *et al.*, 1988). Moreover, osteoblasts are target cells for retinoids (Kindmark *et al.*, 1992, 1993).

#### 2) Vitamin E

Fish skeleton can suffer damages by endogenous and exogenous free radicals. Vitamin E has been considered as a first line of defence against free radicals (Arjmandi *et al.*, 2002), and free radical stimulates osteoclastic differentiation and may cause deformities (Tintut *et al.*, 2002).

#### 3) Vitamin K

In most vertebrates, the classic role of vitamin K is to regulate and prevent ectopic mineralisation through osteocalcin synthesis by osteoblasts (Gundberg and Nishimoto, 2006) as well as to inhibit bone resorption, which decreases the loss of bone (Knapen *et al.*, 1993). Vitamin K plays an important role as a cofactor in vitamin K-dependent protein gamma-carboxylation (Pinto *et al.*, 2001).

#### 4) Vitamin C

Deficiency of vitamin C has been reported to be implicated in the development of skeletal deformities (Gapasin *et al.*, 1998; Cahu *et al.*, 2003), through impaired collagen formation and delayed bone mineralization (Tucker and Halver, 1984; Dabrowski *et al.*, 1990).

#### I.4. Understanding of the bone structure

#### I.4.1. Bone structure

Various observations initially led to the view that the teleost skeleton is metabolically inactive and not subject to resorption and remodelling (Carroll, 1988) being considered as a dead tissue for some teleosts (Moss, 1962). However, at present, bone is considered not just as an inert mineralized hard structure, but as a metabolically active tissue (Lall and Lewis-Mc-Crea, 2007).

Skeletal cells include chondroblasts, osteoblasts, bone lining cells, osteocytes, osteoclasts, odontoblasts and ameloblasts (Huysseune, 2000). During endochondral bone formation mesenchymal cells turn into packed prechondroblasts before forming chondroblasts and then form the extracellular cartilage matrix. Bone is finally formed around the cartilage. In early life stage bone develops as woven bone and parallel-fibered, while in mature individuals bone develops as lamellar bone (Witten and Huysseune, 2009). During intra-membranous bone formation, cell condensations are being directly transformed into bone without any cartilaginous interface.

Bone tissue contains tree major type of cells: osteoblasts, osteocytes and osteoclasts. Osteoblasts (bone-forming cells) are of mesenchymal origin, secrete nonmineralized bone matrix (osteoid), and are finally entrapped in mineralized bone matrix as osteocytes. Osteoclasts, called also bone resorbing cells, dominate the early skeletal development in all teleosts. Depending on the cell content in bone matrix, two types of bone are distinguished, acellular and cellular bone.

Cellular bone biology and composition has been previously studied in teleost fish like Salmonids and cyprinids (Meunier, 1989; Sire *et al.*, 1990; Meunier and Balinière, 1998; Person *et al.*, 1999; Witten *et al.*, 2001; Witten and Hall, 2002, 2003; Dommon *et al.*, 2004; Hall, 2005), and the authors observed that this type of bone is specific to the basal bony fishes, basal teleost species and tetrapods including mammals. Bone of basal teleosts contains encapsulated osteocytes enclosed in lacunar spaces in the bone matrix, as in the case of tetrapod bone called cellular or osteocytic bone (Moss, 1961b; Meunier and Huysseune, 1992; Witten *et al.*, 2004, Witten and Huysseune, 2009).

By contrast, acellular bone is characteristic for a more advanced teleost species, in bone of *Atherinomorpha, Paracanthopterygii* or *Acanthopterygii*, where the osteocytes are not present in bone matrix (Witten *et al.*, 2001; Witten and Huysseune, 2009) and no cell process appears inside the bone (Witten and Huysseune, 2009).

Osteocytes play an important role in strain detection and in the regulation of bone metabolism (Burger *et al.*, 2003). Whereas, the lack of the strain cells detectors in the matrix of acellular bone and the resorption of the bone by mononucleated osteoclasts typifies the bone of more advanced teleosts (Weiss and Watabe, 1979; Glowacki *et al.*, 1986; Sire *et al.*, 1990; Witten and Villwock, 1997; Witten *et al.*, 2001; Witten *et al.*, 2004; Witten *et al.*, 2005a; Witten and Huysseune, 2009).

Osteoclasts can be identified by visualising Tartrate-resistant acid phosphataseactivity (TRAP) in enzyme histochemical studies. Witten and Willwock reported in 1997 that in teleost with acellular bone, bone resorbing cells are mononucleated (Witten and Huysseune, 2009) and located at the growth zones that required resorption for growth. It has been discussed if the type and presence of osteoclasts has been relates to the presence or absence of osteocytes, which activate the proliferation of the osteoclasts when bone has to be remodelled or when it is subject to mechanical forces (Witten and Huysseune, 2009).

Fish growth and development is a result of bone resorption and remodelling (Witten and Huysseune, 2009). Bone turnover, consists of cycles of resorption that remove discrete packets of bone in the skeleton followed by bone formation. As bone deposition is not continuous inside the bone matrix, growth lines (or growth arrest, or cement lines of resorption) are visible, which indicate reduced and accelerated growth of the animal, these lines provide valuable information about the life history of the animal (Castanet *et al.*, 1993).

#### **I.5.** Development of the bone of red porgy

Previous study made by Roo *et al.*, (2009) concerning the osteological development and occurrence of skeletal deformities in red porgy, the authors found that the ontogeny of the head region was characterized by the apparition of some skeletal elements in relation to the necessity of feeding and respiration activities. Thereby, the authors have found that in the intensive and the extensive rearing systems, the first sign of ossification was found in the pre-opercula spines in larvae from 4.8mm TL onwards regardless of the larval age. At the  $10^{th}$  day, the upper jaw, the square and the hyomandibular cartilage were formed and started to ossify by the day  $16^{th}$  to  $20^{th}$  where they are completely ossified. The ossification of the jaws occurred on the  $25^{th}$  day and the teeth became formed in the day  $15^{th}$ .

The cartilage began to ossify from the most dorsal region, and the ossification was intensified between days 20 and 25 being completed at the day 30. The preopercula ossified by the 16<sup>th</sup> day. Some of the vertebral bodies began to ossify before the day 25, being the two thirds of the vertebrates completely ossified by the day 30 starting from the cranial to caudal sequence. In parallel, the ossification of the first anal fin rays in the red porgy larvae started on the  $30^{\text{th}}$  day.

Generally, there is a lack of information concerning the mechanisms implicated in deformities occurrence in fish and the low number of published reports related to this issue has been more related to the effect and consequences of deformities in fish. But no study have been focused in investigating the histological evolution of fish bone tissue in reared red porgy in order to find out the histological changes and the age of their occurrence.

#### I.6 Objectives

In view of the high incidence of deformities appearing in farmed red porgy, particularly in relation to axial skeletal malformations, and the lack of studies describing in detail the type and progression of these malformations, the main aim of this thesis was to further investigate the deformities occurrence in red porgy using radiographies and histological techniques. The specific objectives to achieve this goal were:

- 1. To study the external and internal appearance and progression of vertebral malformations in red porgy, by visual and x-ray studies.
- 2. To determine the influence of tank hydrodynamics on vertebral malformations progression.
- 3. To describe at a histological level normal and fused deformations occurring in red porgy.

# MATERIALS AND MHETODS

#### **II. MATERIALS AND METHODS**

#### **II.1. Experimental conditions**

#### **II.1.1.** Treatment tanks

The set up consisted of a closed circular PVC wall threat, divided around cylindrical conical tanks of 1000L into two concentrically compartments. It was designed to prevent animal contact with the divider in order to avoid any fish injuries. Openings were perforated on the bottom to facilitate the evacuation of solid wastes (4 openings in each tank). In order to eliminate turbulences the air pipes were placed in the centre of the tank. The total water in the tank was 756 and the swimming water space was 451 liters (A) (Figure 1).

These tanks were designed and set up to create a swimming corridor for the fish instead of providing them with the entire tank volume. Pipes of the tank's outflow were perforated vertically to create laminar current and flow. This set up assured a unidirectional current in the tank and enabled counter current fish husbandry and forced swimming. The water current did not interfere with the feed availability.



Figure 1: Design of the experimental tanks for studies at different water currents.



Figure 2: Drawing of the experimental tanks for studies at different water currents. Vertical tube having four holes to allow water exchange.

#### **II.1.2 Treatments**

Two treatments were applied in the present work, one low water current treatment and high water current treatment. The water velocity was recorded manually by measuring the time it takes for the buoyant (Figure 3) object to move between the water entrance point, and the point before the entrance (one complete circle).



Figure 3: Buoyant used for water speed measurements.

The buoyant has a part that floats above the water level, a central part with a PVC pipe and a lower part with a plastic material in a cross form to allow the circular movement of the float inside the water column, a lead weight was placed at its final point.

The buoyant circulated in the middle of the fish swimming area, the distance between the position of the buoyant (center of the swimming area), and the center of the tank (60.5 cm)( red point in the figure 2) was measured to calculate the full circle, the velocity of the buoyant was calculated following the equation of Circular circumference ( $2\pi$ \*R), the full circle of the tank is 3.80m.

• In the high current water velocity tanks, the buoyant completed a full circle (3.80m) in 10.9s, making the velocity **5.31 circulation /min** 

• In the low current water velocity the buoyant completed a full circle (3.80m) in 49.05s, the velocity was then **1.22 circulation** /min.

The trial was conducted in a natural photoperiod, the temperature in the tanks varied in all tanks in parallel from 20.2 to 24.5°C, oxygen varied from 4.7 to 7mg/l, salinity was constant at 37ppm, the water exchange (1.32% renovation of total water volume/hour) was maintained equal in the both treatments tanks.

#### II.1.3 Fish

On July 2009, 360 juveniles red porgy (*Pagrus pagrus*), were obtained from a natural spawning at the ICCM (Instituto Canario de Ciencias Marinas, Telde Spain) facilities (without any previous selection based on the vertebral bodies deformities). After a period of acclimatizing to the designed trials conditions, the trial was carried out from October 2009 to April 2010 (190 days).

Fish were tagged in the right side of the muscle, using a Microchip (11 mm length, weight of 0.096g) (Ref. ID-162-A). The system consists of an integrated circuit and an antenna which transmits identification information that is encrypted, providing a number, induced by a reading unit ARE H5 (providing by TROVAN Ltd). One hundred and sixty one tagged fish (13.09±2.25g) were randomly distributed in 6 treatment tanks. All the fish were fed to *ad libitum* three times a day (at 9h, 12h and 15h) with a commercial diet, mortalities were recorded. The tanks were cleaned three times a week until the total recuperation and recovery of any skin damages (20 days), and then the juveniles were subject to X-rays to identify vertebral column deformities.

All the fish were weighed at the beginning in the middle and after 190 days of treatment. The specific growth rates (SGR) and the condition factor, of the different treatments were calculated according to the following equation:

#### SGR= (ln N2 -ln N1) \* 100 / t

Were N2=average weight after 190 days of the treatment, N1=average weight at the beginning of the trial.

Condition factor (cf) was calculated according to Fulton's formula:  $cf = (weight in g X 100) / (length in cm)^3$ .

#### **II.1.4 Sampling**

At the beginning of the trial on October 2009, after taking the X-rays, samples of nondeformed fish were selected following the X-ray tracing to make histological study Haematoxylin and Eosin (H&E) staining in paraffin sections.

On December 2009 non deformed fish were selected, depending on the tracing of October to make the Masson Trichromic staining in paraffin sections.

On February 2010, X-rays were made and after the revelation four fish with the most common deformities (postcranial lordosis and vertebral fusion) were selected to make the resin embedding and semithin sections.

On April 2010, X-rays were made; six fish were selected with postcranial lordosis and vertebral fusion to make also semithin sections.

#### **II.2. X-raying of the fish**

Radiographic imaging was done on live anesthetized fish.

The X-rays were made using a portable X-ray: Orange 8674 (Figure 4) in the ICCM facilities according and under the assistance of "Clínica Veterinaria Albea" (Las Palmas, Spain). Fish were anesthetized using 4 ml of clove oil per 10 liter of seawater, in order to tranquilize the fish and avoid any movement that could cause the loose of the projection quality on the vertebral column. Fish were kept in the clove oil solution for 1 min, and then placed on the radiographs (AGFA Structurix, Daylight Packing, D4. 30x40 cm). Up to 8 fish could be radiographed on one sheath of x-ray film (Figures 5 and 6). The distance and the parameters of the instrument were fixed after trials have been made to establish parameters that provide the best X-rays quality (Figure 7).


Figure 4: X-ray machine Orange.

The parameters chosen were:

- Distance between the X-ray machine and the Plaque: 71.5 cm
- 72 Kv
- 50 mAs
- With one exposure time

After X-raying process, the microchips in the fish were read from the two lines A and B using the PIT reader ARE H5 provided by TROVAN Ltd. Finally, fish were weighed (BW) and size measured (TL) and placed to the recovery tanks with clean sea water with high water exchange rate and high aeration (Figure 8).



Figure 5: Placing the fish in column.



Figure 6: Fish in the x-ray plaque.



Figure 7: X-raying the fish.



Figure 8: Reading of the microchip.

## II.2.1. X-rays plaques development

The X-rays film development was done in a dark room in the ICCM (Figure 9), using Structurix G128 developer (Art code 35 TAL) for 8 min with continuous agitation.



Figure 9: Dark room for X-rays development.

After 5 min, the film was moved to the acetic acid for 5 min, and subsequently treated with fixative (Structurix G328 Art Code 35 TAL) for 5 min. Films were rinsed in tap water for a minimum of minimum of 20, dried over night in room temperature (Figure 10). The radiographs were then scanned using an EPSON perfection V750 PRO scanner with the software EPSON Scan in professional mode with the following parameters (Table 1):

Film type	Positive film
Document type	film( with area guide holder)
Image type	8-bit Grayscale
Scanning Quality	Best
Resolution	1200 dpi
Document size	W: 149.9mm ; H: 246.4mm

Table 1: Parameters used for the scanning of the X-rays plates



Figure 10 : X-rays plaque drying.

Selection of the deformities were done following the X-ray tracing, the histological samples were than defined.

## **II.3.** Histological study

The musculature on both sides of the fish was removed from the selected part of the vertebral column of undeformed or deformed fish was sectioned following the X-ray tracing.

## **II.3.1.** Paraffin inclusion

A part of the vertebral column of a normal (undeformed) fish was fixed in 10% buffer formalin, and decalcified for 24 hours in HCl 10%. After decalcification, fish samples were then dehydrated in a series of graded alcohol in a tissues processor (Histokinette 2000; Leica, Germany), according to Socorro (2006).

## 1) Dehydration

In the processor the samples were first dehydrated in graded Alcohol (Table 2),

Product	Time (min)
Alcohol 70%	60
Alcohol 80%	90
Alcohol 90%	90
Alcohol 96%	90
Alcohol 100%	90
Alcohol 100%	120
Alcohol 100%	120

Table 2: Stage of deshydratation

## 2) Clarifying

Then the samples were clarified in Xylol (Table 3).

### **Table 3: Clarification process**

Product	Time (min)
Xylol I	30
Xylol II	30
Xylol III	60

The samples were moved from the processor and embedded in paraffin using the paraffinic dispenser (Jung Histoembedder; Leica, Germany).

Paraffin blocks were cut in the microtome (Jung Autocut 2055; Leica, Nussloch, Germany). Initially sections of 20-25  $\mu$ m were done to eliminate the muscle that is surrounding the bone tissue. Finally, sections of 4-5  $\mu$ m were taken of bone tissue.

## **II.3.1.1.** Massons Trichromic staining

Massons trichrome staining was prepared according to the protocol used by

Witten and Hall (2003) for salmon bone. Staining time is listed below (Table 4).

Product	Time (min)
Xylol 100%	5
Histoclear	5
Ethanol 100%	1
Ethanol 80%	1
Demineralised water	1
Mayers Haematoxylin	10
Ranning Tap water	10
Demineralized water	Short bath
Phosphomolybdic acid	5
Demineralised water	Short bath
Light green	2
Demineralised water	Short bath
Demineralised water	Short bath
Ethanol 96 %	1
Ethanol 100%	1
Histoclear	5
Xylol 100%	5

Table 4: Masson Trichromic staining time

Slides were mounted with DPX and observed and photographed with a Microscope (DMBE, Leica, Germany) and photographs were taking.

### **II.3.2.** Resin sections

### **II.3.2.1.** Semithin sections

### 1) Decalcification and fixation

Samples were fixed in formaldehyde buffer at 10% during 48 hours at room temperature ( $\approx 22^{\circ}$ C); fish samples were decalcified for 8 days in room temperature with 10% EDTA (ethylenediaminetetracetate) pH 7. Before starting the resin embedding, samples were then rinsed in sodium cacodylate buffer with sucrose for three days at 4°C.

### 2) Post fixation

Post fixation for visualizing lipids and cell membranes was carried out with 1% Osmium ( $O_sO_4$ ), for 8 hours in room temperature.

### 3) Dehydration and Inclusion

Dehydration was done in ascending acetone series (30, 50, 70, 80, 90, 100%), each step lasts 15 min, taking account that the Epoxy resin is hygroscopic (able to attack water molecule) and that proper dehydration is fundamental.

Table 5:	Inclusion	stapes
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Product
1 :2 acetone : resin
1 :1 acetone : resin
2 :1 acetone: resin
Pure resin

The inclusions stapes (Table 5 and Figure 11) were maintained during 24hours, epoxy resin range from viscous liquid to fusible solids with the high temperature. After the embedding in resin and acetone, the samples were placed in the mold (Figure 11), and identify with small numbers putted inside the mold with the resin, and then putted in an oven at 60°C overnight.

### 4) Preparing the molds for placing in the specimen holder

The mold with the samples (Figure 11) were adjusted to microtome knife size (maximum of 5 mm) and orientated for cutting of parasagital sections from the vertebral bodies.

### 5) **Preparation for the sectioning**

- Clamp specimen into the specimen holder. Insert specimen holder into segment arc and tighten.
- Mount the block onto the knife stage.
- Trim the block with the razor blade to desired size, in pyramid form.
- Remove the trimming block and place the knife holder back to the ultramicrotome.
- Approach the specimen with the knife, align the bottom of the block parallel to the knife edge and make sure the sample is vertical orientated.



B



Figure 11: A bone in resin embedding, B placing bone samples in the mold, C bone samples in the mold, D Bone sample after the complete embedding and drying, placed in the mold holder.

Samples were first sectioned at 5µm with a speed of 2.5 mm/s to eliminate the muscle tissue. Finally, sections of a 1µm were obtained.

A slide was prepared with drops of MilliQ water, in each slide over 10 sequential sections were collected from the most superficial to more profound planes of the vertebral body.

And thus, the slides were heated using at 50°C for 20s, then stained with Toluidine blue during 20s, rinsed with demineralized water and dried before being mounted with DPX and cover slip.

Slides were observed using the microscope (Mod. DMBE, Leica, Nussloch, Germany) and pictures were taking.

## RESULTS

### III. RESULTS

### **III.1. Fish growth**

All fish fed equally well; despite during the first part of the study they were weak and took some time to adapt. Indeed, all fish from tank  $n^{\circ}2$  (normal water current) died and were replaced by fish from the same original stock after 12 weeks, despite their weight and length were not considered in order to avoid external influences on those parameters.

After 4 months of trial, fish submitted to higher water current showed a slightly lower increase in weight (Figure 12). This difference was even higher after 6 months of study, although the high standard deviations in weight among fish did not allow observing significant differences. Comparison of final fish body weight from the different tanks showed that there were no significant differences among tanks of the same water current treatment (Figure 13). However, body weight of fish from tanks in high water current tend to have lower body weight and in the case of tank n°1 held under high current growth was significantly (P< 0.05) lower than tank n°6 (Figure 13 and table 6) held under low current.

A similar tendency was also observed in fish total standard length, where fish held under higher water current were slightly smaller (Figure 14), but no significant differences were obtained (Figure 14 and table 7).



Figure 12: Average development of body weight (g) in red porgy submitted to two different water current rates.



Figure 13: Final weight of the fish from each experimental tank at the end of the study in the different treatment tanks H1, H3 and H5 held under high water current, N4 and N6 held under normal water current.

	H1	H2	N2	Н3	N3
Final weight (g)	82.19±24.6 <sup>b</sup>	87.05±24.5 <sup>ab</sup>	91.14±27.1 <sup>ab</sup>	97.13±27 <sup>ab</sup>	105.1±26.6 <sup>a</sup>

Table 6: Body weight of the fish fee	commercial diet at the end	of the experimental period.
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Values are means  $\pm$  standard deviation of the fish in the same treatment. Values in the same row with different superscript letter are significantly different (p<0.05).



Figure 14: Final total length of fish from each experimental tank at the end of the study in the different treatment tanks H1, H2 and H3 held under high water current, N2 and N3 held under normal water current.

	H1	H2	N2	Н3	N3
Final length (cm)	14.86±1.5 <sup>ab</sup>	15.09±1.5 <sup>ab</sup>	15.41±1.4 <sup>ab</sup>	15.63±1.7 <sup>ab</sup>	15.57±1.46 <sup>ab</sup>

Table	7.	Total	length	of th	e fich	at	the	end	of t	he evi	nerimenta	l nerio	h
rable	1.	Total	length	or un	e nsn	aı	une	enu	or u	ne ex	регипениа	i perio	u.

Values are means  $\pm$  standard deviation of the fish in the same treatment. Values in the same row with different superscript letter are significantly different (p<0.05).

SGR registered no significant differences among the treatments and was around 0.99. The short period of the trial did not allow to see significant differences among the treatments in terms of growth and feed utilisation; this may be explained by the short time period of the trial and/or by the adaptation of the fish to both rearing conditions without affecting growth.

### **III.2. X-rays studies**

#### III.2.1. Normal and deformed fish X-rays

The quality of X-rays taken at the first sampling point in October, resulted in a low quality and only allowed to identify gross skeletal deformities (Figure 15A). It was necessary to improve X-ray methodology used to allow obtaining a better quality and to identify skeletal anomalies in the second sampling in February and trace back some of those anomalies also present in fish X-rayed in October (Figure 18B).

These modifications were based on Witten (personal communication) such as;

- changing of the plaque radiographs
- adjusting the distance between the x-ray machine and the plaque
- changing of the machine parameters (Kv, mAs).

All fish were X-rayed in February, skeletal anomalies identified and their incidence was determine in each tank. From all fish X-rayed in February had normal shape vertebral body, 4.96% showed postcranial lordosis, 4.34% abdominal vertebral fusion and 1.24% a double deformity which included postcranial lordosis and abdominal vertebral fusion and other deformities were present in the group (Figure 16).



Figure 15: Radiographs on the top fish x-rayed in October(A) and (B) fish x-rayed in February



Figure 16: General deformities incidence in the red porgy stock in high (H) and normal (N) water current tanks treatments, (T=total, Pc=postcranial, Ab=abdominal, PcL= postcranial lordosis).

Average total number of vertebrae per individual was 24, ranging between 23 and 25 (Figure 17) vertebrae including the urostyle. The vertebral column is composed of adjacent vertebral bodies separated by the intervertebral space. The three regions: (postcranial, abdominal and caudal) were analysed.

In the postcranial region, the first three vertebrae were closer one to another and the space between two vertebral bodies was restricted (vertebrae V1, V2 and V3 in the Figure 18), all remaining vertebrae are separated by a clear intervertebral space.

In the abdominal part of the vertebral body one neural arch and one haemal arch are present; the intervertebral space is well distinguished in this part (Figure 19). The caudal region has the same components as the rest of the column with one haemal and neural arches.



Figure 17: Radiograph of a fish with a normal vertebral column (Fish characteristics: TL= 14.5 cm, BW=48g, K=1.57)



Figure 18: Radiograph of normal postcranial region (Fish characteristics: TL= 14.5 cm, BW=48g, K=1.57)



Figure 19: Radiograph of normal abdominal and caudal region (Fish characteristics: TL= 14 cm, BW=33g, K=1.2)

Figure 20 shows an example of radiograph from a lordotic fish. In this fish, the vertebral column suffered a ventral curvature (lordosis) between the vertebrae V4 and V6, causing an angle in the vertebral column between the anterior and posterior part (Figure 21) which affected normal shape vertebral body. The rest of the osseous components of the vertebral column had no visible deformities.



Figure 20: Radiograph of a fish with lordosis (Vertebrae V4, V6 and V6) (Fish characteristics: TL= 16.5, BW=60g, K=1.3)

The vertebrae number 5 (V5) was in the middle of the lordotic region on the vertex of the curvature point (Figure 21, V5). This vertebra was deformed and adapted to the angle formed by the vertebral column, being shorter in its dorsal part and larger in its ventral part. Its neural arch was very close to that of V6. V6 and V4 were both

displaced in opposite directions, forming the two main directions of the vertebral column.

Both vertebras were very close to V5 forming very narrow inter-vertebral spaces. V3 and V2 were aligned with V4 adopting an abnormal position in comparison to a normal fish. Finally, V2 was completely displaced in comparison to vertebra 1 which was in its right place. The rest of the vertebra in general seemed less dense to the X-rays than those in the normal fish. The conformation of the swimbladder was also altered by the ventral curvature.



Figure 21: Detail of the lordotic area in the same fish with lordosis (vertebrae V4, V5 and V6) (Fish characteristics: TL= 16.5 cm, BW=60g, K=1.3).

Figure 22 shows an example of a radiograph image from a fish bearing a vertebral fusion. In this fish, 6 vertebral bodies were affected (V9-V14), whereas the rest of the osseous components of the vertebral column had no visible deformities.

V9 was only slightly affected and showed a light bending forward of the neural spine together with a shortening of the ventral part of the vertebral body. V10 axis was displaced downwards from that of previous vertebral bodies, forming a 150° angle with V9 axis, rather than 180°. V11 axis was displaced upwards respect V10, both vertebra adopting a V disposition with their dorsal regions approaching closer than the ventral ones. Accordingly their neural spines were closer whereas the haemal ones were further

apart among them than in other vertebrae. V12 and V13 were fused and no intervertebral space was found between them, their neural and haemal spines were close. Finally, the dorsal part of the vertebral body from V14 was shortened and both neural and haemal spines slightly deformed (Figure 23).



Figure 22: Radiograph of the fused vertebral bodies. (Fish characteristics: TL=14cm, BW=30.4g, K=1.1)



Figure 23: Detail of the fused part of the vertebral body (Fish characteristics: TL=14cm, BW=30.4g, K=1.1)

In the case of severe vertebral fusions, several vertebral bodies were completely fused and no intervertebral spaces were found among them. Nevertheless, they still beard the neural and haemal spines what allowed the identification of the number of cells involved. For instance in the case showed in Figure 24, vertebral fusion affected up to 9 (Figure 25) abdominal vertebrae, the intervertebral space being completely missing. A complete restructure of the implied vertebra seemed to occur and, as a consequence, there was only a slight bending of the posterior part of the vertebral column. Nevertheless, the total length of the vertebral column was shortened and fish total length accordingly reduced.



Figure 24: Radiograph of a fish with severe vertebral fusion affecting up to 9 vertebral bodies (Fish characteristics: TL=12cm, BW=56g, K=3.24)



Figure 25: Detail of the fused area (Fish characteristics: TL= 12cm, BW=56g, K=3.24)

Thus, comparison of the growth of this fish from the beginning of the study with normal fish of the same size class showed a 16.41% reduction in body high, a 24.70% in total length and 40% in the length of the vertebral column.

Finally, in certain fish both postcranial lordosis and vertebral fusion occurred (Figure 26). Lordosis affected vertebrae V1 to V9 (Figure 27), deforming their vertebral bodies and spines and causing a fusion between V4 and V5 (Figure 27). Besides an advanced fusion was observed among V13-V16 (Figure 27), with their vertebral bodies

fused and the neural and haemal spines deformed. No inter-vertebral space could be observed among those vertebrae and the fused vertebral bodies tend to adopt the form of a single vertebral body.



Figure 26: Radiograph of a fish with co-occurrence of postcranial lordosis and abdominal fusion (Fish characteristics: TL= 11.5 cm, BW=17.3g, K=1.13)



Figure 27: Detail of the lordotic region and the fusion of 4 vertebral bodies, and the over ossification of the haemal arch ( in the right) (Fish characteristics: TL= 11.5 cm, BW=17.3g, K=1.13)

Haemal spines also showed round engrossments of over ossification and distortions. The vertebral bodies of adjacent vertebrae (V10, V11, V17 and V18) seemed to be enlarged although they kept the same height. The swim-bladder was forced to adopt the curvature of the deformed vertebral column and abdominal cavity. The shape of the whole fish was more elongated than in a normal fish and even the cranium seemed to be affected.

### III.4.1. Effect of high water current on anomalies development

Three fish from the 161 fishes of the experiment (Figure 28). Fish **117758**, which presented a normal morphology in October, started to show a slightly higher density in post-cranial vertebra and a much rounded body shape, with a higher high in relation to its length in comparison to its previous shape. In April this fish showed a clear lordosis and a much rounded form. Accordingly the shape of the swim-bladder was changed adopting the new form of the vertebral axis.



Figure 28: Radiographs of changed fish. Normal fish (Fish characteristics: TL=10.4 cm, BW=11.2g, K=0.99) in October 2009; Radiograph of the same fish en (A) in February, (Fish characteristics :TL :, BW=25.2 g, K=1.02); Radiograph of the same fish en (A) and (B) en April (Fish characteristics :TL=12.7cm, BW=74g, K=3.61)

Another two fish submitted to high water current showed a worsening of the lordosis that became acute and caused a stronger deviation angle in the vertebral axis. thus, fish with the pit tag number **120722** showed a mild lordosis in October with lordotic angle of 20° (Figure 29 A), that was worse in February forming an angle of 22° (Figure 29 B), and it was acute in April with an angle of 40° (Figure 29 C).



Figure 29: radiographs of the same fish during 6 mounts of the trial;(A) Fish with postcranial lordosis 20° (Fish characteristics TL=10.8, BW=12.2, K=0.96); (B) Fish with postcranial lordosis 22° (Fish characteristics TL=11.7, BW=26.4, K=1.64); (C) Fish with postcranial lordosis 40° (Fish characteristics TL=13.2cm , BW=48 g, K=2.08)

Similarly, in the case of a fish **120261** submitted to high water current and suffering a postcranial lordosis and fused vertebrae, the lordosis was worsened along the rearing in high water current and the lordotic angle changed from  $37^{\circ}$  in October, to an  $50^{\circ}$  in February and  $60^{\circ}$  in April (Figure 30).



Figure 30: Radiographs of changed fish during the 6 months of the experiment;(A), Fish with postcranial lordosis and vertebral fusion 37° (Fish characteristics TL=9.5cm, BW=13.8g, K=1.6);(B) same fish like (A) with a lordotic angle of 50° (Fish characteristics TL=13.5cm, BW=32.9g, K=1.33);(C) Same fish like (A) and (B) with a lordosis angle of 60° (Fish characteristics TL=14.3cm, BW=70g, K=2.39)

## **III.3. Histology**

The acellular bone of the red porgy is a bone that no osteocytes inside the bone matrix. The growth and the remodelling of this bone will be describe in the normal shape vertebral body and the fused vertebrae will be described based on semithin sections stained with the Toluidine blue.

All the fish were examined with radiography or with radiography and histological sectioning with Masson Trichromic staining or with resin embedding stained with the Toluidine blue.

### III.3.1. Normal vertebral body

Growth and remodelling of vertebral body follows the growth of the fish during his life cycle, the growth of the vertebral body in length and diameter takes place in the articulation area between two vertebral bodies, this zone is called bone growth zone (Figure 31), this junction between to vertebral bodies contains three flexible components: a thickening of the notochord sheath, an elastic membrane, and a ligament made from curled collagen fibre bundles (Figure 32).



Figure 31: Radiograph of normal fish proceeds for histology study (Toluidine Blue staining), (Fish characteristics: TL=7.2cm, BW=13.5 g, K=0.78)



Figure 32: Enlarged view of the space between two vertebral bodies' endplates of a normal abdominal vertebral body, white asterisk labels the notochord sheath, white arrowheads points the bone of the vertebral body and the white arrows point the bone growth zone. n: notochord cells. (In the left) Toluidine blue staining x100.(in the right) Masson Trichromic staining x200

Continuous layers "ventro-dorseally" orientated collagen fibres surrounded the notochord, and extended inside the bone matrix with collagen fibres bundles (Figure 33).



Figure 33: Vertebral body growth zone with collagen fibre bundles that continue from the surrounding connective tissue into to bone (Sharpey's fibres) Black arrow head points to a collagen fibre bundle; the black arrows point the osteoid, the new formed non-mineralized bone. Toluidine blue staining X630.

The notochord consists of a stratified epithelial tissue that is enclosed by a thick acellular fibrous sheath. The sheath consists of a thin external elastic membrane with a high content of elastin that permits the stretch and retracts movement of the vertebrae, covering a thicker collagenous layer.

The vertebral body consists of four layers called also compartments, one formed through mineralization of notochord sheath and three layers from an osseous origin (Figures 34 and 35).

The orientation of the collagen, forming the layers, differs from each layer. Thus, the continuous collagen that forms the layer and surrounds the notochord is oriented cranio-caudally (in both the vertebral and the intervertebral regions). The collagen of the layer 3 is oriented dorsa-ventrally (Figure 34).



Figure 34: layers (L1, L2, L3 and L4) surrounding the notochord. At left Masson Trichromic staining x630. At right Toluidine blue staining x630



Figure 35: Representation of bone layers in red porgy (Pagrus pagrus). courtesy of Pr.P.E.Witten.

Bone contains growth lines that indicate the different stage of the growth of the animal, these lines have an accentuated coloration compared with the bone matrix (Figure 36).



Figure 36: The surrounding bone shows growth lines (black and white arrows) indicating intermitted bone growth and remodelling Adipose tissue (black asterisk), and red blood cells (black arrowhead) inside bone marrow space. (A) Toluidine blue staining x630, (B) Masson trichromic staining x200.

### III.3.2. Deformed vertebral body

### Fused vertebral bodies

Fused vertebral bodies were detected with the radiographs (Figures 37 and 38),

and then histological study was done from a fish with 37.2g and 14 cm.



Figure 37: Radiograph of deformed fish proceeds for histology study Fish characteristics: TL= 14cm, BW=37.2g, K=1.35).



Figure 38: Detail of the fused area (Fish characteristics: TL= 14cm, BW=37.2g, K=1.35).

Advanced stage of vertebral fusion, maybe later two vertebral bodies will fuse into a single vertebral body but the neural and haemal arch will stay separated.

The spinal nerve that we see between the two notochords, prove that this is a fusion process and that one notochord is missing.



Figure 39: Advanced stage of vertebral fusion, the notochord is replaced with bone spongiosa, the bone spongiosa is full of adipose tissue (black asterisks), sn: spinal nerve, n: notochord. Toluidine blue staining x40

Histological sections revealed a vertebral fusion between two adjacent vertebral bodies, let to that one complete notochord and let two distinguishable notochord sheaths and a bone tissue inside the two notochords (Figure 39).

The presence of spinal nerve between the two notochords in the left and the bone tissue (Figure 40) confirm the occurrence of the vertebral fusion and the resorption of the deformity.



Figure 40: Advanced stage of vertebral fusion. Intervertebral bone spongiosa (white asterisk), blood vessel (black arrowhead) and spinal nerve. The intervertebral space is full of adipose tissue (black asterisks). sn: spinal nerve. Toluidine blue staining x100

# DISCUSSIONS

### **IV. DISCUSSION**

There is a strong interest of scientists and professional fish farmers to reduce skeletal deformities that affect up to 40% of farmed fish (Izquierdo *et al.*, 2010) because deformities downgrade the product and cause a big loss at the economical level.

Deformities impact negatively fish welfare, growth and swimming performance. Nevertheless external recording may underestimate the true deformity rate occurring in the vertebral column. A multitude of deformities are only modest and the fish look externally normal. The fish can then be affected by unseen deformities and at production level, malformations cause technical problems to automated filleting equipment (Grejde *et al.*, 2005). In our study, in the aim to define the deformities occurrence in a group of animals of red porgy that have a normal external shape, we examined the animals with radiological and/or histology techniques, permitting the detection and description of abnormalities that are not visible externally.

The present study emphasizes that, in red porgy, external recording underestimates the true deformity rate. Less severe deformities like the postcranial lordosis or the fusion of two vertebrae remain unseen. This has already been reported for sea bass (Bardon *et al.*, 2009), Atlantic cod (Kolstad *et al.*, 2006b), and in Atlantic salmon (McKay and Grejde, 1986; Witten *et al.*, 2006; Fjelldal *et al.*, 2007a), where after x-raying the animals the incidence of deformities was higher than after external inspection.

Many different types of deformities can affect the vertebral column, ranging from slight abdominal malformation to significant shape alterations of single or several vertebral bodies (Divanach *et al.*, 1997; Grajerde *et al.*, 2005, Witten et al. 2009). Thus, x-raying the fish is the only way to detect most deformities. Concerning the growth rate of the fish in the last trial period, no difference has been seen in the growth rate between the two treatments. The growth in weight in normal water current was higher compared to the fish that were subjected to high water current, as it could be expected from the higher energy expenditure of the later. Nevertheless the water current influence was not so strong as to cause significant differences in the period of study or with this fish size, despite inducing skeletal deformities.

Multiple factors have been reported to cause deformities. Water speed has been reported to cause lordotic alterations by Divanach *et al.*, 1997, based on studies in sea bass (*Dicentrarchus labrax*). In fish without swimbladder, permanent swimming, induced lordotic of the vertebral column as a result from the forced swimming and to avoid the sinking tendency. In fish with swimbladder, the lordosis has been hypothesizesed to be caused by both rhetrotactism and schooling, deformity being the result from an "extended local dysfunction" due to the effort made by swimming activity (Chatain, 1994; Kihara *et al.*, 2002).

In the present study, faster water current and, therefore faster swimming speed not only worsened the appearance of light lordosis, but even induced the origin of new lordosis in fish with a normal appearance. Lordosis has been claimed to be caused by forced swimming in fish that had not completed mineralization (Chatain *et al.*, 1994; Bardon *et al.*, 2009), whereas in our study faster swimming induced lordosis appearance despite the fish bones were completely and well mineralized.

Our radiographic images showed that postcranial lordosis was the most common deformation in the trial group. In the present work, 4.96% of the studied animals suffered from lordosis in the postcranial part of the vertebral column. In two animals lordosis changed the angle from  $37^{\circ}$  in October to  $60^{\circ}$  in April and from  $20^{\circ}$  to  $40^{\circ}$ ,

respectively. Mediation of the angle was mentioned by Sfakianakis *et al.*, 2006 and by Fjelldal *et al.*, 2009 when the authors measured the angle between the affect zone of the vertebrae and the rest of the vertebral column, of deformed fish to find out how the lordosis can increase with time. Appearance or worsening of the lordosis by increased water current and higher swimming speed could be related to the higher compression forces exert by lateral swimming musculature in the postcranial region as suggested by Fjelldal *et al.* (2007b, 2009).

In one fish the vertebral body fusion involved 9 adjacent vertebral bodies, similar to a case describe by Witten *et al.*, 2006 for Atlantic salmon. This fusion caused a clear spine shortening and the animal had a higher K value compared to non-deformed animals that start with the same weight. In the deformed individual the the K value was 3.2 compared to normal fish that had a K-value of 2.1. Similar observations have been published for Atlantic salmon (*Salmo salar*) by Witten *et al.* (2005) where the K factor was higher in the deformed fish compared to the normal shaped fish. This type of malformation is not only well studied in Salmon but also in sea bream (Afonso *et al.*, 2000) where a similar vertebral compression has been described.

Measurement of vertebra from multiple fused vertebra individuals revealed reductions of 24.70% in vertebra length and 16.4% in vertebra height and 40% in the vertebral column length in comparison to the vertebra of a non-deformed fish. These reductions were lower than those found in Atlantic salmon were studies have shown up to 47% reduction in length and 17% of reduction in height of vertebral bodies (Witten *et al.*, 2005).

Neural and haemal arches and a vertebral Centrum, as describe by Inohaya *et al.*, 2007 for medaka. Vertebral bodies studied by histology were composed of notochord tissue, bony endplates, intravertebral bone spongiosa and intervertebral tissue as
describe for Atlantic salmon (Witten *et al.*, 2005, 2006) and for medaka (Inohaya *et al.*, 2007).

In the normal shaped vertebral bodies the notochord is symmetrical like it is described in Atlantic salmon. The notochord sheath and the notochord cells in red porgy are arranged as in Atlantic salmon. Also the bone growth zone is composed like in Atlantic salmon (Witten *et al.*, 2005, 2006) and medaka (Inohaya *et al.*, 2007). Like in other teleost species in red porgy collagen fibers continue from the surrounding connective tissue into the bone (Nordvik *et al.*, 2005).

The mineralized layers observed in red porgy bone are the same that have been described for Atlantic salmon (Nordvik *et al.*, 2005) (Figure 41). They consist of four compartments one mineralized collagenous layer and three ossified layers.



Figure 41: Shematique representation of vertebrae structure following the nomenclature of Nordvik et al. (2005) (Courtesy of Prof. E.P. Witten).

The orientation of the layers described in red porgy was in accordance with the orientation of these layers in Atlantic Salmon. The first layer is composed from craniocaudally oriented collagen fibers. A second layer surrounded the notochord and is devoid of cells. The third layer was described by the same authors to be circular, and the forth layer is a part of the arcocentrum that surrounds the entire vertebral body.

Nordvik *et al.*, 2005 demonstrated that the first structure of the vertebral column that forms in Atlantic salmon is the cartilage of the neural and haemal arches. Nevertheless, the authors conclude that the development of the vertebral body is initiated by the notochord, which is encased by a layer of longitudinally oriented collagen fibers.

The bone of red porgy contains growth lines that indicate points at which the bone growth has resumed after it had stopped. A line forms as the bone forming cells start to lay down again bone matrix. This represents bone re-growth after temporary cessation. The same phenomenon was described in tetrapods including mammals and in other teleost species. Many authors conclude that the cessation of bone growth is partly caused by a stop of the activity of the osteoblasts during periods of malnutrition.

The bone of red porgy contained osteoclasts at sites of bone resorption. Two types of osteoclasts are known, one type is multinucleated. Multinucleated osteoclasts are typically found in mammals, birds, and basal teleosts. The other type of osteoclasts is mononucleated, present in all vertebrates but dominating in advanced teleosts (Witten and Huysseune, 2009). In the bone matrix of red porgy we did observed resorption lacunae with mononucleated. In *Danio rerio*, osteoclasts are involved in the enlargement of bone marrow space and in bone remodeling (Witten *et al.*, 2001). As it is the case in all teleost species that have been studied so far, bone resorption in *Danio* 

*rerio* starts with mononucleated osteoclasts, whereas multinucleated osteoclasts appears later (Witten 1997).

Salmonids and cyprinids as basal teleosts have cellular bone (osteocytes enclosed in the bone matrix). Although the endoskeleton of these fish is acellular the dermal skeletal elements such as scales and fin rays are acellular. This is also the case for the advanced teleost red porgy. Our histological studies have confirmed that in red porgy all osseous elements are composed from acellular bone, no cells are present inside the bone matrix (see Moss, 1961; Meunier and Huysseune, 1992; Witten *et al.*, 2004, Witten and Huysseune, 2009). This work concerned the study of an advanced teleost, red porgy (*Pagrus pagrus*) with an acellular bone (no osteocytes are present inside the bone)

In red porgy's bone no cells have detected in the bone matrix. This is in accordance with the works done on other teleost species with acellular bones (Moss, 1961). Acellular bone may have a woven bone structure, and can contain vascular channel (Moss, 1961).

In red porgy, similar to salmon and reported by Witten *et al.*, 2006, vertebral bodies involved in the fusion, go through a phase of replacing intervertebral notochord tissue by cartilage. In advanced teleosts like red porgy, vertebral fusion involves rather the formation of chondroid bone than of cartilage (Kranenbarg et al., 2005) Based on x-ray imaging we could only establish the present anomalies and we could count supernumerary haemal and neural arches. If only two or three vertebral centra are involved in vertebral body fusion, Witten et al., 2006 described cases in which fusion could be contained and no additional changes of neighboring vertebrae have been inflicted. These results have been confirmed for farmed cod (*Gadus morhua*) by Fjelldal *et al.*, 2009.

In the same study authors describe an aggravation of the fused part of the vertebral body. Nevertheless in our trial no fish with abdominal fusion has changed the conformation and no other vertebral body was involved after 6 mounts of the trial.

## CONCLUSIONS

## **V. CONCLUSIONS**

- 1. Appropriate techniques to X-ray with high quality red porgy of different size were developed in the present study and will be available to be used in the GIA laboratory in this another species.
- 2. Masson Trichromic staining and other fish bone staining specific histology techniques were also up dated in GIA lab and will later allow conducting more specific studies on bone structure of normal and deformed fish.
- 3. The combination of both techniques in the same fish proved to be useful to better identify deformities that can not be detected "de visu" and understand the causes of the deformities.
- 4. The most common deformities found in red porgy were postcranial lordosis, abdominal vertebral fusion and the combination of both types of deformities.
- 5. The histological study confirmed that fused vertebra in red porgy are able to become completely remodeled.
- 6. Increased water current, despite not causing a significant reduction if fish growth rate, may increase the acuteness of vertebral deformities, particularly causing or worsening postcranial lordosis.
- 7. Being the protocol of x-raying the fish established and the technique of making histological slides of bone well known, future works can focus more in specific parts of deformities of vertebral bodies, performed with electron microscopy. Different staining methods can be of a grateful help in understanding the occurrence the composition of the bony parts and the occurrence of abdominal deformities.

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