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Skeletal Deformity of Scoliosis in Gilthead Seabreams (*Sparus aurata*): Association with Changes to Calcium-Phosphor Hydroxyapatite Salts and Collagen Fibers

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Abstract: The development of skeletal deformities in seabream farming affects fish growth, survival, and production costs. Collagen distribution in different fish tissues might be correlated with swimming behavior. This study investigates whether scoliosis in seabreams is associated with changes to calcium-phosphor hydroxyapatite salts and collagen fibril morphology. Samples of decalcified vertebrae of scoliotic and non-scoliotic seabreams were examined with transmission electron microscopy and collagen micrographs were taken and analyzed. The mineral content, modulus of elasticity, and morphology of the vertebrae were also determined. The results indicated that fish with scoliosis had significant smaller mean vertebral collagen fibril diameters than the controls. Vertebrae in abdominal and caudal regions of the scoliotic seabreams appeared to be smaller than the respective vertebrae of the non-deformed seabreams. The calcium (Ca) and phosphorus (P) amounts of vertebrae of both scoliotic and non-scoliotic seabreams were not affected by the scoliosis deformity. The modulus of elasticity showed that the vertebrae from seabreams with scoliosis were more flexible than the vertebrae from seabreams without any skeletal deformity. The mechanical properties of bone are crucially dependent on collagen structure. Hence, how the vertebral column collagen of juvenile fish is related to the mechanism of deformities requires further investigation in order to provide a risk-reducing strategy to increase fish performance in aquaculture.

Keywords: *Sparus aurata*; scoliosis; vertebra column; collagen; calcium; phosphor

1. Introduction

Gilthead seabream (*Sparus aurata*) is the sixth most important aquaculture species produced in the European Union (EU) (13% of the total production, 548 million €) and one of the most important farmed fish species in the Mediterranean region [1,2]. In the EU markets, seabreams are commercialized as whole fish (350–500 g) and malformed fish are not accepted by the consumers. Malformations comprise a mix of different bone disorders such as spinal and vertebral deformities (scoliosis, lordosis, kyphosis, vertebral fusion, and platyspondyly). Scoliosis is the lateral bending of the vertebral column. It is easily detected, either by dorsal or ventral examination [3]. The development of skeletal malformations' is still not clear, as these conditions are developed in the early life stages (embryonic or post-embryonic). These conditions are related to nutritional, environmental, and genetic factors [4]. Studies in marine

hatcheries show that there is an average of 7—20% prevalence of skeletal deformities in the produced juveniles. However, there are cases that show that 45–100% of the total fish juveniles can be affected [5]. In aquaculture, skeletal abnormalities indicate a serious economic problem, because they reduce fish survival and lower fish price (consumers prefer not buying deformed fish) [6]. Such fish must be removed manually and repeatedly from fish cages and are often used for fish meal production with a substantial profit loss [3]. Deformed fish also raise ethical issues and concerns. The impaired swimming performance and feeding of deformed fish suggest improper welfare conditions. High stress susceptibility, sensitivity to pathogens, low feeding rates, and low growth rates are the most important consequences of skeletal deformities [3].

Fish bones may be cellular or acellular. They consist of cells (osteoblasts, osteocytes, and bone lining cells), hydroxyapatite salts (almost 65% of the dry mass of bone), and a collagen fiber matrix [7]. Although the material of fish bone (calcium phosphate hydroxyapatite) is the same as tetrapod bone, the organization is quite different in that it is often acellular [8]. The lack of Haversian systems, trabeculae, and the remodeling that accompanies cellularity imply significant differences in the ultrastructural organization of fish bone. Type I collagen is in general bone's most important organic part, but type II collagen can also be contained in teleost fish bone [9]. Collagen fibril diameter, hydroxyapatite salts, and the way that collagen fibrils are related to hydroxyapatite are very important for bone toughness, bone stiffness, and the biomechanical tissue strength [10–12]. Collagen is a naturally occurring protein that is the main protein of connective tissues, making it abundant in most invertebrates and vertebrates [13,14]. It represents approximately 25% of the total body protein content [15]. Three left-handed helix polypeptide strands—the alpha chains—form collagen, which is a long, fibrous structural protein. Its function is different from the functions of globular proteins. Collagen forms tough bundles of fibrils that can be found in the supporting extracellular matrix of most tissues. The collagen fibrils' most striking feature is the D-period (axial periodicity), which is approximately 68 nm [16].

Different connective tissues of fish show a varying complexity, with collagen fibrils forming a delicate network structure. Lee and Glimcher [17] noted that the distinctive "twisted plywood" arrangement of mineral crystals and collagen fibers found in mammalian bone is far simpler in fish bones. The fact that fish collagen contains fewer and more unstable cross-links makes it remarkably more thermolabile than the warm-blooded vertebrates' collagen. In general, its hydroxyproline content is lower than that of mammals' collagen, with values between 4.7 and 10% [18]. The amount of collagen in fish tissues depends on the fish species, leading to the theory that collagen distribution may reflect the swimming behavior of the species [19].

This study aims to determine whether scoliosis is associated with changes to calcium-phosphor hydroxyapatite salts and the matrix of collagen fibers. To the authors' knowledge, there is no published study addressing this growing field of research in order to provide a risk reduction strategy approach to increase fish performance in aquaculture. The vertebrae morphology and mechanical properties were also determined. The results will help us to better understand the mechanism of skeletal malformations, one of the major problems in aquaculture.

2. Materials and Methods

2.1. Fish Sampling

In this study, 20 individual adult seabreams (fourth generation hatchery fish) were provided by a Greek fish farm. Larvae were obtained from a central Greece hatchery and were from the same egg batch. Sea cages of 8 kg/m^3 fish density were used for fish farming and fish were fed to satiation with a commercial diet (46% crude protein, 17% fat). Fish were euthanized immediately after sampling by ice immersion. The wet weight of each fish was measured. Average water temperature at the sampling period was 22.6 °C and the average water dissolved oxygen was 8.6 mg/L. Temperature and oxygen measurements were conducted by using a portable polymeter (Hach Lange HQ40D,

Düsseldorf, Germany). This research does not fall under the regime of animal experimentation. Fish were divided into two groups. The first group contained fish with scoliosis (10 fish, mean weight 336.69 ± 26.48 g, mean length 25.11 ± 0.91 cm), while the second group contained non-deformed fish (10 fish, mean weight 352.64 ± 39.18 g, mean length 27.39 ± 0.96 cm).

2.2. X-ray

Each individual fish was examined by X-ray (50 kV) (Figure 1). Three regions were identified in the vertebral column of each *S. aurata*: the cervical region (4 vertebrae), the abdominal region (12 vertebrae), and the caudal region (8 vertebrae). K-PACS V1.6.0 software was used to measure the vertebrae length of each region.

2.3. Ca and P Level Measurement

Because the scoliosis in the first group fish occurred in the caudal region, vertebrae sampling of both groups occurred in this region. Two vertebrae were taken from the caudal region where scoliosis occurred. The first vertebra was incinerated to measure calcium (Ca) and phosphorus (P) levels. Three measurements were taken per vertebra. A scanning electron microscope (Jeol JSM-6510 LV, Ltd, Tokyo, Japan) equipped with an X-ray analyzer (x-act Oxford, Abingdon, United Kingdom) was used for the stoichiometric analysis of the vertebrae by energy dispersive spectroscopy (EDS).

2.4. Compressive Strength and Modulus of Elasticity

The second vertebra was used to measure the compressive strength and modulus of elasticity. An INSTRON 3382 Universal Testing Machine (100 KN capacity) (Innovatest, Maastricht, The Netherlands) was used for compressive tests with compression and applied force measurements, which were analyzed by LabView software (version 2014). For the estimation of the vertebral modulus of elasticity and the vertebral compressive strength, a 2mm/min displacement crosshead speed rate was used. The modulus of elasticity was defined from Hook's law as a stress to strain fraction. Stress is defined as the fraction of the force to the force-applied area and strain is the ratio of the vertebra length change.

2.5. Electron Microscopy

A third vertebra was taken from three fish with and without scoliosis (caudal region). The vertebra was fixed with glutaraldehyde, decalcified, dehydrated, and embedded in resin. Ultrathin sections (60–80 nm) were taken, double-stained with uranyl acetate (UA) and phosphotungstic acid (PTA) and examined with a Philips CM-10 transmission electron microscope equipped with a digital camera (Veleta, Olympus, Tokyo, Japan). The collagen fibril diameter and D-period were measured using a special algorithm [20].

2.6. Statistical Analysis

All values are given as the means ± standard errors or medians ± interquartile ranges. All the differences presented at the 5% level were considered significant. The Kolmogorov–Smirnov test or the Shapiro–Wilk test (depending on the data number) was used to test for normality checking. The *t*-test was used for statistical comparisons among collagen fibril diameters, collagen D-period, P and Ca levels, and the vertebrae lengths. The Mann–Whitney test was used for statistical comparisons between inter-vertebrae spaces [21].

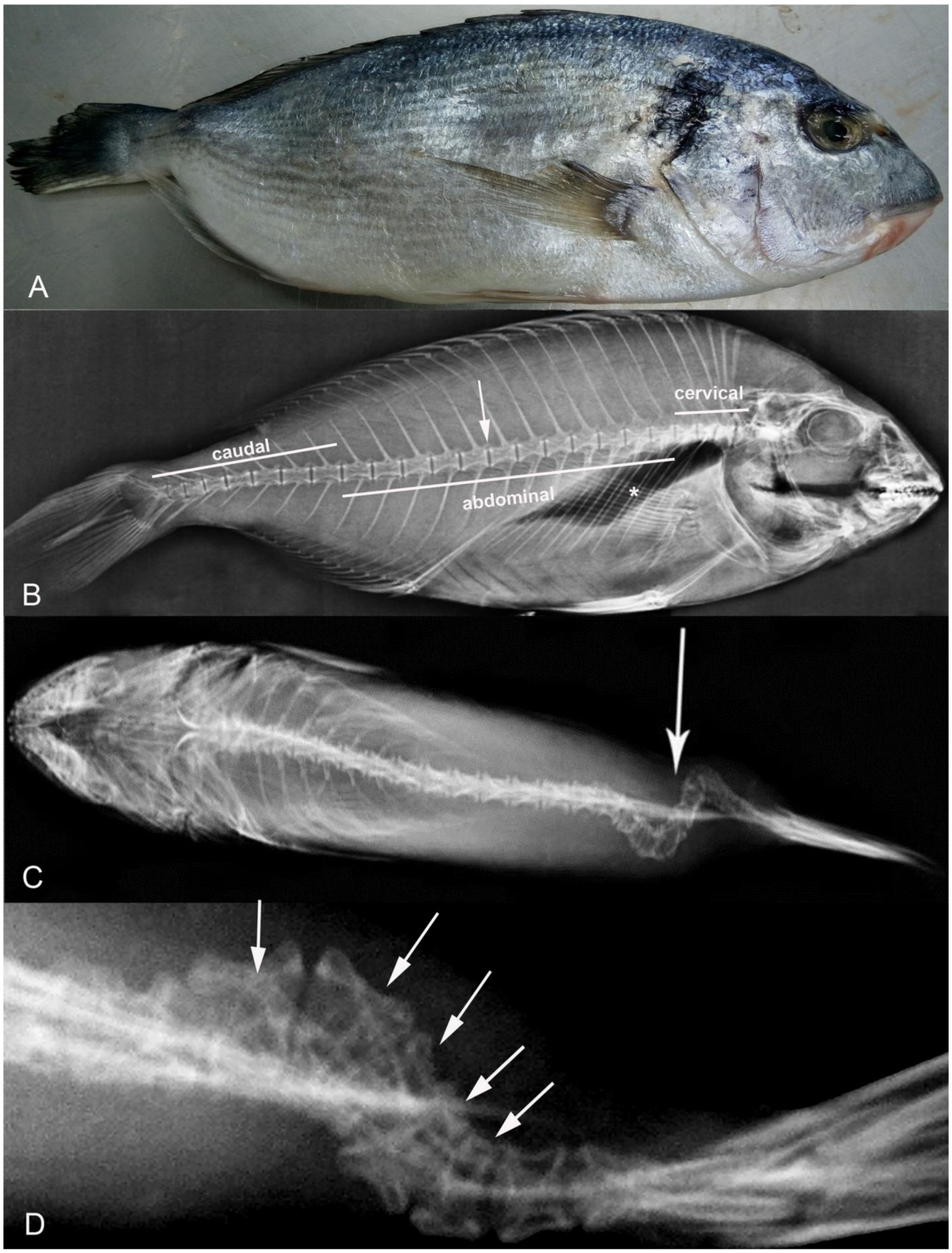

Figure 1. (**A**) Seabream with scoliosis at the caudal region. (**B**) X-ray of seabream without scoliosis. The cervical, abdominal, and caudal regions are shown. Inter-vertebrae space is highlighted with an arrow, whereas swim bladder is indicated with a star. (**C**) Apical X-ray of seabream with scoliosis. The scoliosis area is highlighted with an arrow. (**D**) Magnified apical X-ray of seabream caudal region with scoliosis. Deformed vertebrae with trapezoid shape are highlighted with arrows.

3. Results

A functional swim bladder was detected in all of the examined fish. Smaller vertebral collagen fibril diameters were measured in scoliotic seabreams compared with non-deformed seabreams (Figure 2; Table 1). Longitudinal sections from the vertebrae of both groups (scoliotic and non-deformed seabreams) (Figure 3) show that the D-period of collagen is not affected (Table 1). Seabreams with scoliosis had a collagen periodicity of 55.69 ± 0.60 nm, whereas in the non-deformed ones the collagen periodicity was 55.95 ± 1.12 nm (Table 1).

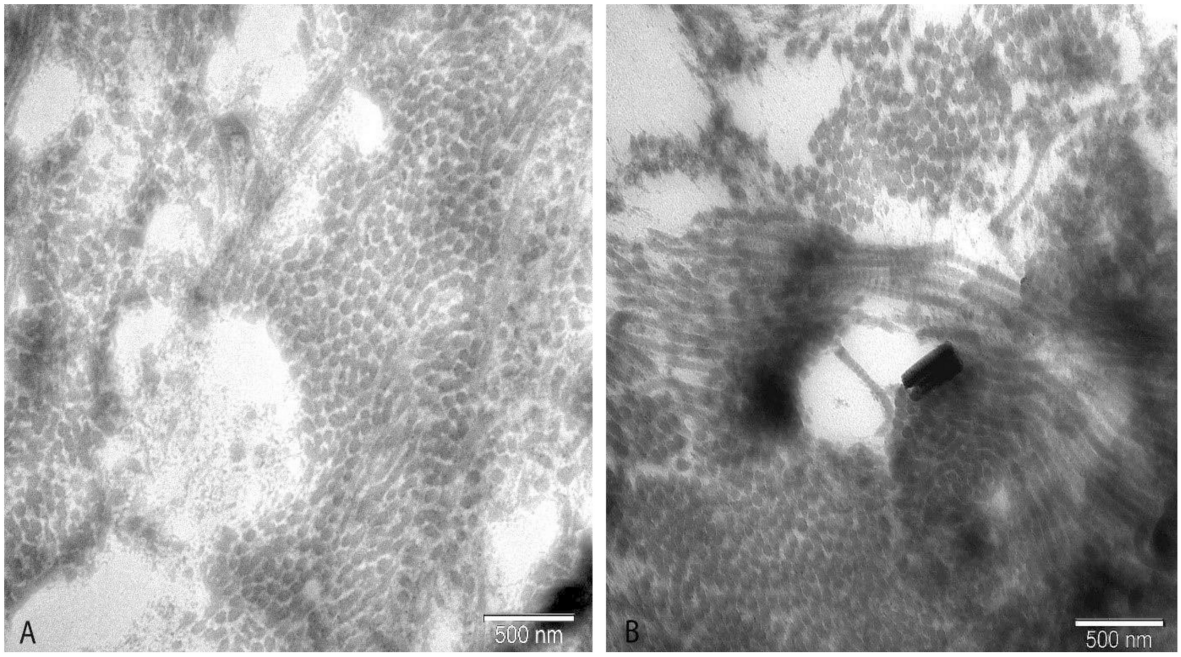

Figure 2. (**A**) Vertebral collagen fibrils from a non-deformed seabream. (**B**) Vertebral collagen fibrils from a seabream with scoliosis. In the second image, the fibrils have a significantly smaller diameter. The Kolmogorov-Smirnov test was used for normality checking of the collagen fibrils' diameter.

Table 1. Scoliotic and non-deformed seabreams' vertebral collagen fibril measurements.

	Diameter (nm)	D-Period (nm)
Scoliotic seabreams	43.18 ^{a} ± 0.28 (810)	55.69 ^{a} ± 0.60 (80)
Non-deformed seabreams	46.68 ^{b} ± 0.44 (539)	55.95 ^{a} ± 1.12 (53)

Note: Results are means ± SE. The numbers of fibrils sampled are given in parenthesis. Means in columns with same superscript are not significantly different ($p > 0.05$). The Kolmogorov–Smirnov test was used for normality checking of the collagen fibrils' diameter and period.

The X-ray examination revealed that scoliosis occurred at the caudal region, between the 17th and 23rd vertebrae (where the biggest angle of the scoliosis was estimated). A scoliosis frequency per vertebra diagram is shown in Figure 4. Vertebrae from the regions where scoliosis occurred had a deformed shape (in the X-rays these vertebrae seemed to be trapezoid in shape, Figure 1D). The mean vertebral length in the cervical region where scoliosis occurred was similar to the mean vertebral length of the respective region of the non-deformed individuals (4.92 ± 0.16 mm and 4.73 ± 0.19 mm, respectively) (Table 2). The mean abdominal vertebra length where scoliosis occurred was significantly smaller than the mean length of the respective regions of the non-deformed individuals (6.95 ± 0.08 mm and 7.47 ± 0.10 mm, respectively) (Table 2). Additionally, non-deformed seabreams had a mean caudal vertebral length of 6.43 ± 0.20 mm, which is larger than the mean caudal vertebra length (5.36 ± 0.18 mm) of the scoliotic seabreams (Table 2).

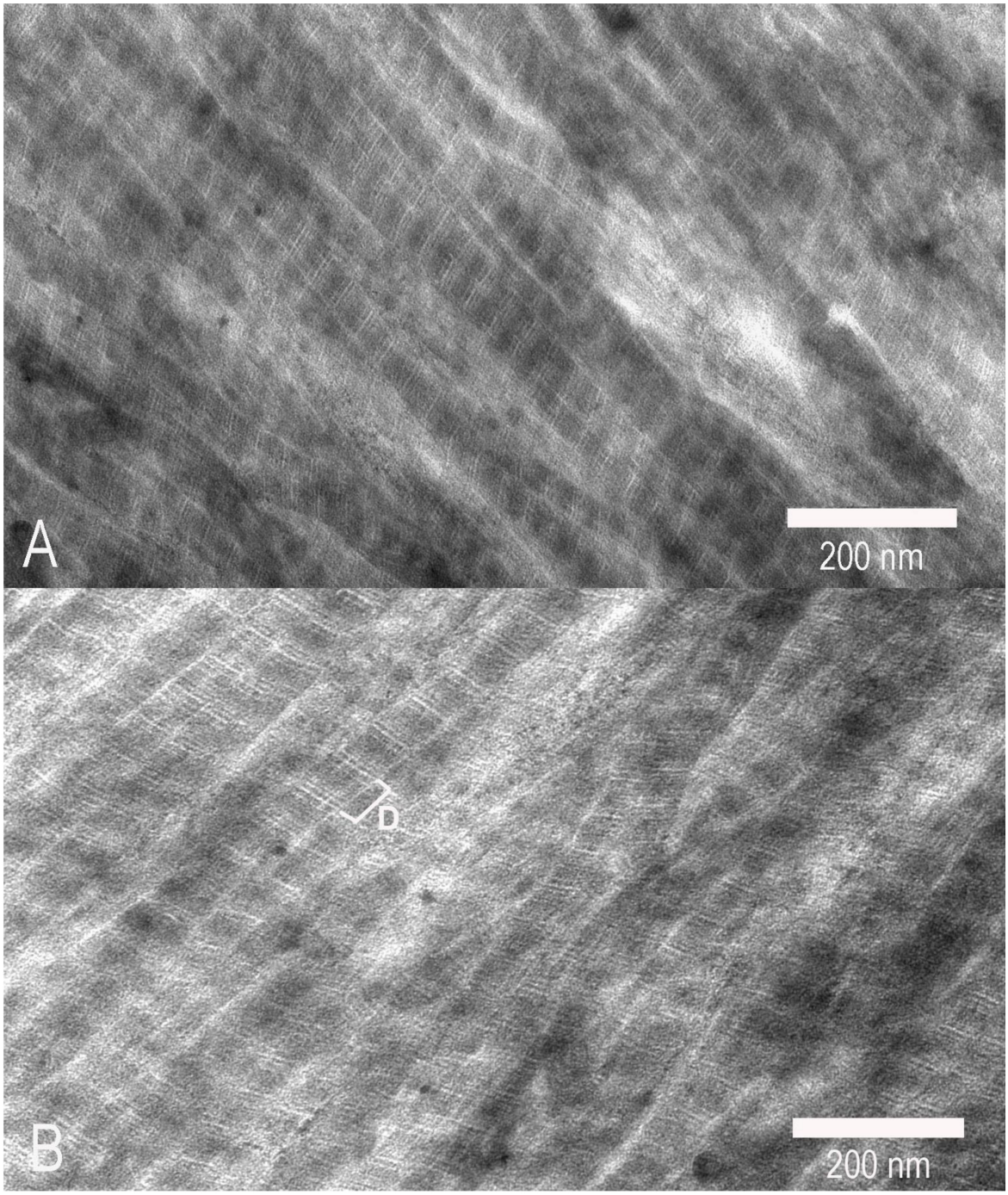

Figure 3. Longitudinal sections of vertebral collagen fibrils (**A**) from a scoliotic seabream and (**B**) from a non-deformed seabream. Vertebrae collagen fibrils' periodicity (D) did not differ between seabream without any skeletal deformity and seabream with scoliosis. The Kolmogorov–Smirnov test was used for normality checking of the collagen fibrils' period.

Table 2. Seabreams' vertebrae mean lengths.

	Cervical (mm)	Abdominal (mm)	Caudal (mm)
Non-deformed seabreams	4.73 ^{a} ± 0.19 (n = 40)	7.47 ^{b} ± 0.10 (n = 120)	6.43 ^{d} ± 0.20 (n = 80)
Scoliotic seabreams	4.92 ^{a} ± 0.16 (n = 40)	6.95 ^{c} ± 0.08 (n = 120)	5.36 ^{e} ± 0.18 (n = 80)

Note: Results are means ± SE. Means in columns with same superscript are not significantly different ($p > 0.05$). The Shapiro–Wilk test was used for normality checking of the cervical region's vertebrae length. The Kolmogorov–Smirnov test was used for normality checking of the abdominal and caudal regions' vertebrae length.

The data frequency counts for the non-deformed seabreams showed a diameter range of 25–45 nm for 44% of the collagen fibrils, whereas the respective data frequency count for scoliotic seabreams was 59% of the collagen fibrils (Figure 5).

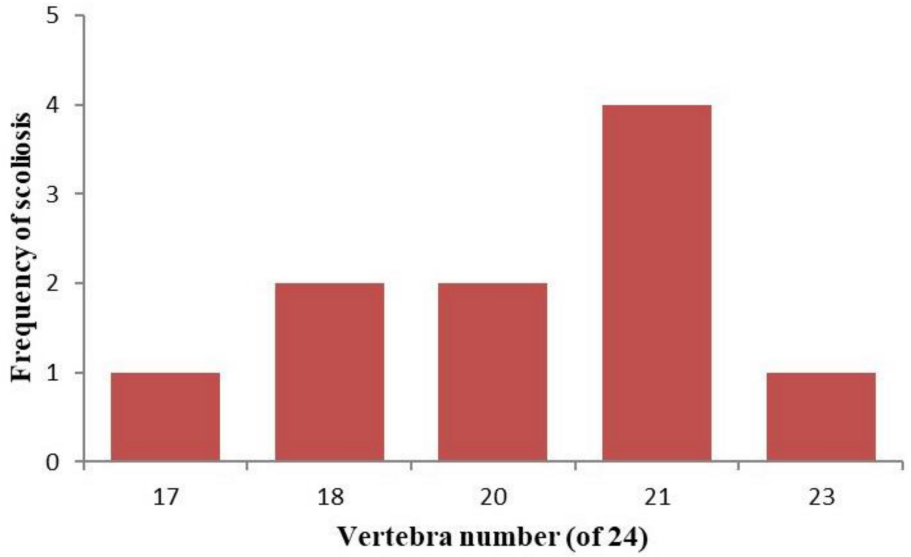

Figure 4. Vertebra number and scoliosis frequency of examined seabreams.

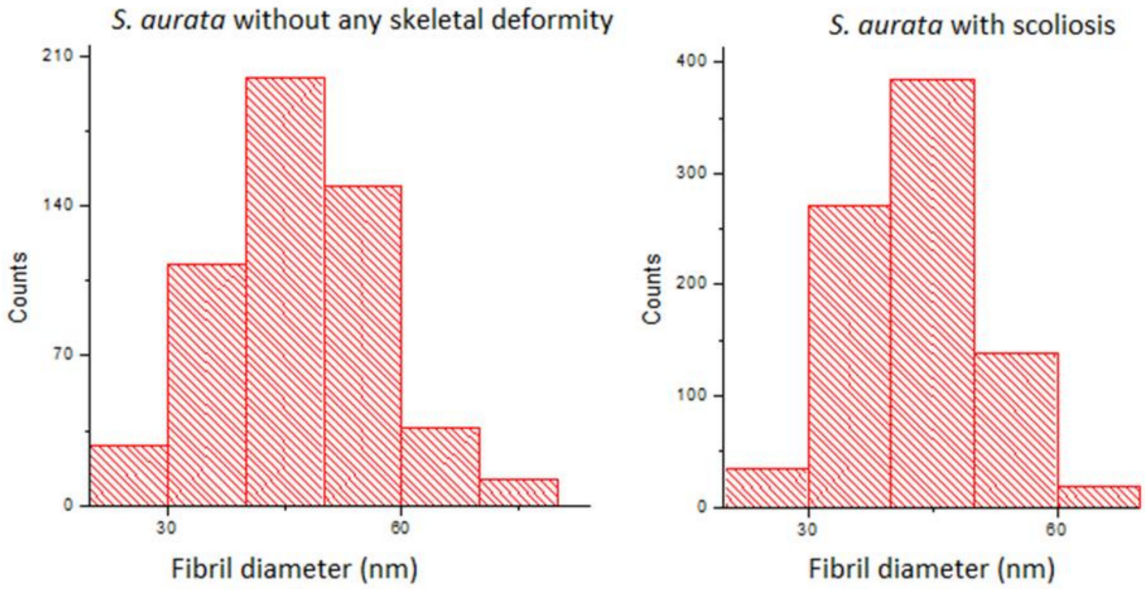

Figure 5. Histogram of vertebral collagen fibril diameter (nm) of the non-deformed seabreams and the scoliotic ones. In the scoliotic seabreams, the collagen fibrils with a diameter smaller than 50 nm are much more than the non-deformed ones, leading to a significantly decreased mean collagen fibril diameter.

The mean length of the cervical inter-vertebrae space in the scoliotic area was similar to the mean length of the respective area of the non-deformed individuals (0.60 ± 0.20 mm and 0.65 ± 0.30 mm, respectively) (Table 3). The mean abdominal inter-vertebra space for scoliotic seabreams was 0.60 ± 0.35 mm, which was similar to the mean space of the respective regions of the non-deformed individuals (0.70 ± 0.20 mm, Table 3). The mean caudal inter-vertebra space for scoliotic seabreams was smaller than the same space of the non-scoliotic seabreams (0.50 ± 0.20 mm and 0.70 ± 0.30 mm, respectively, Table 3).

Table 3. Seabreams' inter-vertebrae space mean lengths.

	Cervical (mm)	Abdominal (mm)	Caudal (mm)
Non-deformed seabreams	0.65 ^{a} ± 0.30 (n = 40)	0.70 ^{b} ± 0.20 (n = 120)	0.70 ^{c} ± 0.30 (n = 70)
Seabreams with scoliosis	0.60 ^{a} ± 0.20 (n = 40)	0.60 ^{b} ± 0.35 (n = 120)	0.50 ^{d} ± 0.20 (n = 70)

Note: Results are median ± interquartile range. Medians in columns with same superscript are not significantly different ($p > 0.05$). The Shapiro–Wilk test was used for normality checking of the cervical region's inter-vertebrae space mean lengths. The Kolmogorov–Smirnov test was used for normality checking of the abdominal and caudal regions' inter-vertebrae space mean lengths.

The EDS atomic percentage (t %) analysis revealed that the Ca and P contents of the seabreams' vertebrae are not associated with scoliosis. Ca levels were 57.76 ± 0.70 at % and 58.18 ± 0.92 at % for the scoliotic seabreams and for the non-deformed, respectively, whereas the P levels were 32.45 ± 0.39 at % and 32.80 ± 0.65 at %, respectively (Table 3). The measurement of the modulus of elasticity showed that vertebrae from seabreams with scoliosis were more flexible than the vertebrae from seabreams without any skeletal deformity (Table 4, Figure 6).

Table 4. Ca/P levels and modulus of elasticity (M.E.) of seabreams' vertebrae.

	Ca (at %)	P (at %)	Ca/P (Level)	M.E. (MPa)
Non-deformed seabreams	58.18 ^{a} ± 0.92 (n = 30)	32.80 ^{b} ± 0.64 (n = 30)	1.79 ^{c} ± 0.07 (n = 30)	20.79 ^{d} ± 2.32 (n = 10)
Seabreams with scoliosis	57.76 ^{a} ± 0.70 (n = 30)	32.45 ^{b} ± 0.39 (n = 30)	1.78 ^{c} ± 0.04 (n = 30)	6.81 ^{e} ± 1.52 (n = 10)

Data are presented as means ± S.E. Means in columns with same superscript are not significantly different ($p > 0.05$). The Shapiro–Wilk test was used for normality checking of the Ca/P levels and of the M.E of the vertebrae.

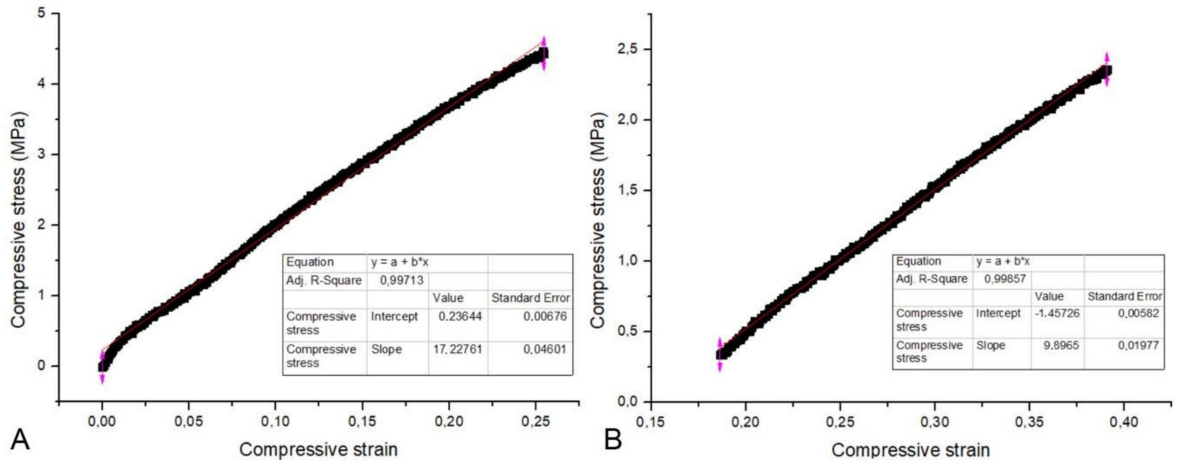

Figure 6. Estimation of modulus of elasticity from (**A**) seabreams without any skeletal deformity and (**B**) seabreams with scoliosis.

4. Discussion

Gilthead seabream is a well-known large-scale farmed fish species in the European Union. Fish with skeletal abnormalities are not preferred by consumers, have higher mortality rates, and cause a substantial increase in the production costs. As it has already been reported, the development of skeletal malformations is not well understood. Many factors, such as nutritional, environmental, and genetic factors, could be the reason for the development of these deformities [4]. Animals with fast growth rates have more probabilities of developing skeletal pathology [22,23]. Collagen represents approximately 90% of the bone's organic content, conferring stability and establishing the bone's mechanical properties [24]. Lim and Lowell [25] showed that channel catfish (*Ictalurus punctatus*) with vitamin C deficiency had a decreased bone collagen content and developed vertebral column malformations (kyphosis, scoliosis, lordosis). According to Santamaría et al. [26], lordosis in seabreams established during the embryonic stage was characterized by disorganized connective tissue and

muscle bundles. Berillis et al. [27] showed that lordosis deformity in adult seabreams was associated with collagen fibrils with a significantly decreased diameter. Our results indicate a correlation between scoliosis and the diameter of vertebral collagen fibrils. The formation of collagen fibrils depends on many secondary and post-translational changes in the synthesis of collagenous precursors [28]. The possibility that fibril diameters could be affected by crosslinking formation during assembly should be considered [29]. Collagen abnormalities have been suggested as the reason for adolescent idiopathic scoliosis (AIS) in humans. Pedrini et al. [30] detected an abnormal collagen content and glycosaminoglycan proportion in the nucleus pulposus of intervertebral discs in patients with AIS. Roberts et al. [31] performed histological and biochemical examinations of vertebrae and intervertebral discs in teenagers with AIS. The arrangement of collagen fibers and annular lamellae was often different in the specimens from patients with scoliosis compared with patients without scoliosis. The development of scoliosis leads to collagen and elastin architectural distortion, lower contents of elastin, and collagen type I in the annulus fibrosus and an increment of minor collagens [32].

The scoliosis deformity seems to have no effect on the vertebrae's collagen fibril period, as the D-period values of the scoliotic seabreams and the non-deformed seabreams were similar ($p > 0.05$). This fact suggests that there is no relationship between collagen molecule arrangement and their in-between, covalent crosslinking. In order for collagen fibrils to be formed with the D-period characteristics, collagen molecules must be self-assembled in a quarter-staggered array. Our results suggest that there is no distribution of the axial relationship between collagen molecules in scoliotic seabreams' vertebrae. The fact that the mean value of the collagen D-period that we measured was smaller than 68 nm is expected; as indicated in the electron microscopy analysis, dehydration can occur and the examined specimens were shrunken. Therefore, the D-period values obtained by electron microscopy were smaller than those obtained with atomic force microscopy or low-angle X-ray diffraction examination [33].

The skeleton is the calcium and the phosphate reservoir in most vertebrates. Many techniques can be used to measure the inorganic components of the bones (EDS, Auger electron spectroscopy, high photon flux X-ray beam) [27,33]. Skeletal system development and vertebrae stability are related to calcium and phosphorus levels as well as the solid phase of calcium phosphate [33]. Fish can absorb various elements from water. Ca can be absorbed directly from water, so calcium deficiency is uncommon in fish. Phosphorus is mainly absorbed by food and low phosphorus concision results in reduced bone mineralization, skeletal abnormalities, and reduced growth [34,35]. Lordosis deformity in adult seabreams does not seem to be linked with Ca and P vertebral deficiency [27]. Our results show that scoliosis also does not seem to be linked with Ca and P vertebral deficiency. The whole-body Ca/P ratio of many fish ranges between 0.7 and 1.6 [34]. According to Ozawa and Suzuki [36], the Ca/P ratio of 800 °C heat-treated bones of Japanese seabreams was 2.02, whereas Berillis et al. [27] found that the Ca/P ratio in gilthead seabream vertebrae was 2.13 for lordotic ones and 2.36 for the non-deformed ones. These values are close to the results of the present study. The Ca/P ratio that we determined in this study compared with the results of Berillis et al. [27] is 16.0% smaller for the scoliotic seabreams and 24.6% smaller for the non-deformed seabreams. These differences can be explained easily because bones, as biomaterials, are adapted to different loading situations and functions. Consequently, their composition may vary.

Two bone types can be identified in fish—the cellular and the acellular bones. For example, Salmonidae have cellular bones and Sparidae have acellular bones [37,38]. In cellular bone osteocytes can be detected in the matrix, whereas in the acellular bones there is a lack of osteocytes as the osteoblasts move away from the mineralization area [37]. Osteocytes can be considered as the strain sensors recruiting new osteoblasts for bone matrix deposition [39]. Acellular bone, despite the lack of osteocytes, shows modulation changes under increased pressure or tensile stress [37,40]. Gilthead seabream vertebrae are formed by acellular bone (a feature of the Sparidae). During mechanical loading, bone remodeling is performed by cells other than osteocytes [38]. The mechanical properties of bone change with the age of the fish and differ among fish species. The vertebrae of

brook trout (cellular bone), channel catfish (cellular bone), and bluegill (acellular bone) have been shown to depend on age, resulting in different moduli of elasticity, namely, 49–68 MPa, 79–158 MPa, and 59–112 MPa, respectively [37,41–43]. In grey smooth-hound sharks (*Mustelus californicus*), the modulus of elasticity of the vertebrae was between 100 and 150 MPa [44], whereas in the torpedo ray *Torpedo californica* the modulus of elasticity was 25.5 MPa [45]. Stiff skeletons are more capable of efficient energy transportation at all speeds of swimming [46]. Fish with stiff bodies can more easily resist skeletal deformation by the swimming forces [45]. In our results. vertebrae from the seabreams with scoliosis showed a smaller modulus of elasticity than vertebrae from seabreams without any skeletal deformity. This result may be explained by the hypothesis that vertebrae from the scoliosis group have fewer or smaller collagen fibrils than the vertebrae from the non-deformed group. Ortiz-Delgado et al. [38] found that deformed seabreams' vertebrae pathologically formed a fibro/cell-rich cartilage in order to replace the notochordal and the cancellous acellular bone in the haemal or neural sides. This could be another explanation for why in our case, vertebrae from scoliotic seabreams had a significantly lower modulus of elasticity. In general, cartilaginous skeletons have a lower modulus of elasticity than bony skeletons from animals of the same size [44]. Vertebrae must be stiff and strong. Stiff materials have a high modulus of elasticity and slightly change their shape under elastic loads (e.g., diamonds). Flexible materials have a low modulus of elasticity and change their shape considerably. Scoliotic seabreams had significantly shorter vertebrae in the abdominal and caudal regions in comparison to the non-deformed seabreams. The inter-vertebrate spaces in the caudal region of the scoliotic individuals were also significantly smaller than those of the non-deformed ones. As the vertebrae of the scoliotic seabreams had smaller moduli of elasticity, they were more prone to changing their shape and eventually to decreasing their size.

Dietary factors such as phosphorus deficiency, vitamin C deficiency, vitamin K deficiency, and hypervitaminosis A, play a very important role in the formation of skeletal abnormalities [47]. In particular, vitamin C deficiency can lead to a decrease in the bone collagen content of bone and therefore to the formation of spinal kyphosis, scoliosis. and lordosis [25]. In our results, no differences in the vertebral phosphorus levels were detected in contrast to the smaller vertebral collagen fibrils that were measured in scoliotic seabreams. An increase in vitamin C levels in the seabream diet could help to improve collagen fibril formation and decrease skeletal deformity formation.

5. Conclusions

The present study shows for the first time that Ca and P vertebral deficiency is not associated with the scoliosis of adult gilthead seabreams. In contrast, abnormalities in the collagen fibril diameter seem to be an important factor. The modulus of elasticity of the scoliotic vertebrae also appeared to be decreased, making them more prone to changes in their shape and to decreases in their size. Collagen is crucial for the mechanical properties of bone, and its relationship with malformation development in the vertebral column of juvenile fish is still unclear. Additionally, further research is necessary in order to understand the exact relationship between hydroxyapatite and collagen in the vertebral column of juvenile fish.

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