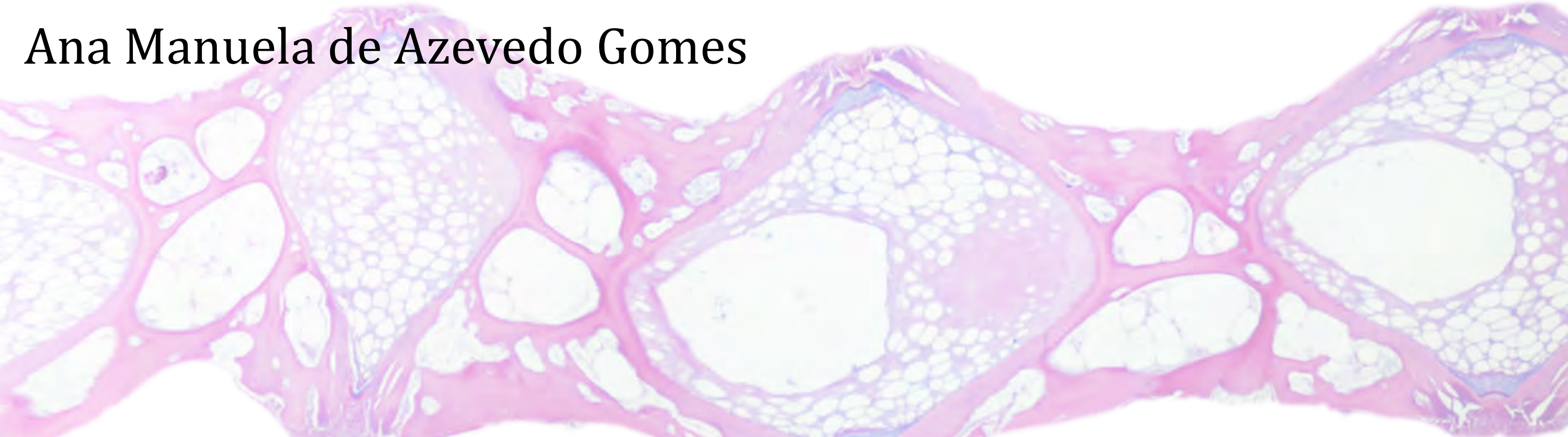
Doctoral Thesis

CHARACTERIZATION OF THE VERTEBRAL ANOMALIES IN DIFFERENT PHASES OF THE PRODUCTION OF SENEGALESE SOLE (*SOLEA SENEGALENSIS*): STEREOSCOPIC, RADIOGRAPHIC AND HISTOLOGICAL APPROACH

Ana Manuela de Azevedo Gomes



Programa de Doutoramento en Investigación Básica e Aplicada en Ciencias Veterinarias
Departamento de Anatomía, Produción Animal e Ciencias Clínicas Veterinarias
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COMPENDIUM OF ARTICLES

CHARACTERIZATION OF THE VERTEBRAL ANOMALIES IN DIFFERENT PHASES OF THE PRODUCTION OF SENEGALESE SOLE (*SOLEA SENEGALENSIS*): STEREOSCOPIC, RADIOGRAPHIC AND HISTOLOGICAL APPROACH

Caracterización de las anomalías vertebrales en distintas fases de producción del lenguado senegalés (*Solea senegalensis*): estudio estereoscópico, radiográfico e histológico

Sgd: Ana Manuela de Azevedo Gomes

DEPARTAMENTO DE ANATOMÍA, PRODUCIÓN ANIMAL E CIENCIAS CLÍNICAS VETERINARIAS

PROGRAMA DE DOUTORAMENTO EN INVESTIGACIÓN BÁSICA E APLICADA EN CIENCIAS VETERINARIAS

FACULTADE DE VETERINARIA

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Que la Tesis presentada por Dña. Ana Manuela de Azevedo Gomes es idónea para ser presentada, de acuerdo con el artículo 41 del Regulamento de Estudos de Doutoramento, por la modalidad de compendio de ARTÍCULOS, en los que la doctoranda tuvo participación en el peso de la investigación y su contribución fue decisiva para llevar a cabo este trabajo.

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"… que lo torcido se enderece…"

Is 40, 4

To my parents

(*Aos meus pais*)


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Characterization of the vertebral anomalies in different phases of the production of Senegalese sole (*Solea senegalensis*): stereoscopic, radiographic and histological approach

* * *

Caracterización de las anomalías vertebrales en distintas fases de producción del lenguado senegalés (*Solea senegalensis*): estudio estereoscópico, radiográfico e histológico

* * *

Caracterización das anomalías vertebrais en distintas fases de produción do linguado senegalés (*Solea senegalensis*): estudo estereoscópico, radiográfico e histolóxico


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ABSTRACT

Senegalese sole (*Solea senegalensis*) is one of the most promising species in the Spanish aquaculture. This Doctoral Thesis arises from the need to find solutions to prevent the high frequency of skeletal abnormalities detected in this species, coming up to 100% of deformed individuals, in some experimental studies. This supposes a major limitation for its aquaculture because they are often diagnosed using macroscopic techniques when they already have repercussions on the morphology of the fish. In consequence, besides welfare and productive inconveniences there could be rejection by consumers of a downgraded product. Currently, there is still scarce information on the anomaly profile affecting reared Senegalese sole in industrial facilities, especially, at later stages of the productive cycle. This Doctoral Thesis underlines the importance of an interdisciplinary approach to cope a multi-factorial issue in the aquaculture sector. The magnitude of the problem impelled to look for diagnostic tools to provide a detailed scan of the most common deformities at distinct farming phases in Senegalese sole.

The employment of stereomicroscopy in larvae, and radiographic techniques in juveniles, allowed the comprehensive characterization of skeletal anomalies for each developmental stage. The results showed a frequency of skeletal anomalies higher than 75% in reared larvae, early juvenile and juvenile Senegalese sole. The main differences detected in the anomaly profiles among ages lied in the incidence of deformations in the caudal complex plates or neural/haemal spines, which clearly diminished in later phases. Vertebral body anomalies and vertebral column deviations were similarly frequent at the three studied ages.

Once this was elucidated, the same systematic was used to evaluate the influence of a nutritional strategy on the skeletogenesis of reared larvae and early juveniles. In this sense, a multivariate approach was applied, revealing differences in the anomaly profile between ages rather than a clear effect of commercial enrichment products for live prey on the development of vertebral abnormalities.

The histological techniques complemented considerably the radiographic studies and allowed to deepen into the knowledge of Senegalese sole anosteocytic bone reaction during anomaly development. The presence of ectopic chondrocytes in the endplates and within the intervertebral space (IVS) was notable and it seems to be a common mechanism in different types of anomalies affecting diverse teleost species with either osteocytic or anosteocytic bone. Moreover, some histopathological changes in deformed vertebrae as flattened endplates, narrow IVS and/or abundant cartilaginous tissue in such space could be consistent with initial stages of fusion, and in one case, with the aggravation of a primary fusion process.

Keywords: Senegalese sole (*Solea senegalensis*), skeletal anomalies, skeletogenesis, radiography, histopahology.


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RESUMEN

El lenguado senegalés (*Solea senegalensis*) es una de las especies más prometedoras de la acuicultura española. Esta Tesis Doctoral surge de la necesidad de encontrar soluciones para prevenir la alta frecuencia de anomalías esqueléticas detectadas en esta especie, llegando a afectar al 100% de los individuos, en algunos estudios experimentales. Esto supone una limitación importante para su producción porque a menudo se diagnostican mediante técnicas macroscópicas, cuando ya repercuten en la morfología de los peces. En consecuencia, puede haber rechazo de los consumidores hacia un producto subóptimo, entre otros inconvenientes relacionados con el bienestar y la industria. Todavía existe poca información sobre el perfil de anomalías que afecta al lenguado senegalés cultivado en instalaciones industriales, especialmente en etapas tardías del ciclo productivo. Esta Tesis Doctoral destaca la importancia de un enfoque interdisciplinario para hacer frente a un problema multifactorial en el sector de la acuicultura. La magnitud del problema nos lleva a buscar herramientas de diagnóstico para evaluar detalladamente las anomalías más comunes en distintos momentos del cultivo del lenguado senegalés.

El empleo de la estereomicroscopía en larvas y de la radiografía, en juveniles, permitió la caracterización exhaustiva de las alteraciones esqueléticas en cada fase del desarrollo. Los resultados mostraron un porcentaje de anomalías superior al 75% en larvas y juveniles en etapas tempranas y tardías. Las principales diferencias detectadas en los perfiles de anomalías a distintas edades radicaron en la incidencia de deformaciones en los elementos del complejo caudal o en las espinas neurales/hemales, que claramente disminuyeron en fases más avanzadas del desarrollo. Las anomalías de los cuerpos vertebrales y las desviaciones de la columna vertebral obtuvieron frecuencias similares en las tres edades estudiadas.

Se utilizó la misma sistemática para evaluar la influencia de la nutrición en la esqueletogénesis de larvas y juveniles en fases tempranas de cultivo. En este sentido, se aplicó un análisis multivariante que evidenció algunas diferencias en el perfil de anomalías entre edades, más que un efecto claro de los enriquecedores comerciales para presas vivas en el desarrollo de anormalidades vertebrales.

Las técnicas histológicas complementaron considerablemente los estudios radiográficos y permitieron profundizar en el conocimiento de la respuesta del hueso anosteocítico durante el desarrollo de las anomalías en lenguado senegalés. La proliferación ectópica de cartílago en las superficies articulares y dentro del espacio intervertebral (EIV) fue notable y parece ser un mecanismo común en diferentes tipos de anomalías que afectan a diversas especies de teleósteos con hueso osteocítico o anosteocítico. Además, algunos de los cambios histopatológicos en vértebras deformadas, como las caras articulares aplanadas, EIV estrechos y/o abundante tejido cartilaginoso en dicho espacio podrían estar relacionados con etapas iniciales de fusión y, en un caso, con el agravamiento de una fusión previa.

Palabras clave: Lenguado senegalés (*Solea senegalensis*), anomalías esqueléticas, esqueletogénesis, radiografía, histopatología.


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RESUMO

O linguado senegalés (*Solea senegalensis*) é unha das especies máis prometedoras da acuicultura española. Esta Tese de Doutoramento xorde da necesidade de atopar solucións para previr a alta frecuencia de anomalías esqueléticas detectadas nesta especie, chegando a afectar ao 100% dos individuos, nalgúns estudos experimentais. Isto supón unha limitación importante para a súa produción porque a miúdo diagnostícanse mediante técnicas macroscópicas, cando xa repercuten na morfoloxía dos peixes. En consecuencia, pode haber rexeitamento dos consumidores cara a un produto subóptimo, entre outros inconvenientes relacionados co benestar e a industria. Existe pouca información sobre o perfil de anomalías que afecta ao linguado senegalés cultivado en instalacións industriais, especialmente en etapas tardías do ciclo produtivo. Esta Tese de Doutoramento destaca a importancia dun enfoque interdisciplinario para facer fronte a un problema multifactorial no sector da acuicultura. A magnitude do problema lévanos a buscar ferramentas de diagnóstico para avaliar detalladamente as anomalías máis comúns en distintos momentos do cultivo do linguado senegalés.

O emprego da estereomicroscopía en larvas, e da radiografía, en xuvenís, permitiu a caracterización exhaustiva das alteracións esqueléticas en cada etapa do desenvolvemento. Os resultados mostraron unha porcentaxe de anomalías superior ao 75% en larvas e xuvenís en etapas temperás e tardías. As principais diferenzas detectadas nos perfís de anomalías a distintas idades radicaron na incidencia de deformacións nos elementos do complexo caudal ou nas espiñas neurales/hemais, que claramente diminuíron en fases máis avanzadas do desenvolvemento. As anomalías dos corpos vertebrais e as desviacións da columna vertebral obtiveron frecuencias similares nas tres idades estudadas.

Utilizouse a mesma sistemática para avaliar a influencia da nutrición na esqueletoxénese de larvas e xuvenís en fases temperás de cultivo. Neste sentido, aplicouse unha análise multivariante que evidenciou algunhas diferenzas no perfil de anomalías entre idades, máis ca un efecto claro dos enriquecedores comerciais para presas vivas no desenvolvemento de anormalidades vertebrais.

As técnicas histolóxicas complementaron considerablemente os estudos radiográficos e permitiron afondar no coñecemento da resposta do óso anosteocítico durante o desenvolvemento das anomalías en linguado senegalés. A proliferación ectópica de cartilaxe nas superficies articulares e dentro do espazo intervertebral (EIV) foi notable e parece ser un mecanismo común en diferentes tipos de anomalías que afectan a diversas especies de teleósteos con óso osteocítico ou anosteocítico. Ademais, algúns dos cambios histopatolóxicos en vértebras deformadas, como caras articulares aplanadas, EIV estreitos e/ou abundante tecido cartilaxinoso no devandito espazo, poderían estar relacionados con etapas iniciais de fusión e, nun caso, co agravamento dunha fusión previa.

Palabras chave: Linguado senegalés (*Solea senegalensis*), anomalías esqueléticas, esqueletoxénese, radiografía, histopatoloxía.


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LIST OF PUBLICATIONS DERIVED FROM THIS THESIS

The development of this Doctoral Thesis has led to the publication of the following scientific articles:

Losada AP, **de Azevedo AM**, Barreiro A, Barreiro JD, Ferreiro I, Riaza A, Quiroga MI, Vázquez S (2014). Skeletal malformations in Senegalese sole (*Solea senegalensis* Kaup, 1858): gross morphology and radiographic correlation. *Journal of Applied Ichthyology*, 30, 804-808. Impact factor: 0.783 (2015).

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de Azevedo AM, Losada AP, Ferreiro I, Riaza A, Vázquez S, Quiroga MI (in press). New insight on vertebral anomalies in cultured Senegalese sole (*Solea senegalensis*, Kaup) at early stages of development. *Journal of Fish Diseases*, doi: 10.1111/jfd.12575. Impact factor: 2.053 (2015).

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de Azevedo AM, Losada AP, Barreiro A, Barreiro JD, Ferreiro I, Riaza A, Vázquez S, Quiroga MI (in press). Skeletal anomalies in reared Senegalese sole (*Solea senegalensis*, Kaup 1858) juveniles: a radiographic approach. *Diseases of Aquatic Organisms*, doi: 10.3354/dao03110. Impact factor: 1.77 (2015).

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LIST OF ARTICLES IN PREPARATION FOR PUBLICATION

The development of this Doctoral Thesis allowed the preparation of the following scientific articles for future publication:

de Azevedo AM, Losada AP, Ferreiro I, Riaza A, Losada V, Russo T, Boglione C, Vázquez S, Quiroga MI (manuscript in preparation). Skeletal anomalies in Senegalese sole (*Solea senegalensis*, Kaup 1858) larvae and juveniles fed with different commercial enriched *Artemia*.

de Azevedo AM, Losada AP, Barreiro A, Vázquez S, Quiroga MI (manuscript in preparation). Histopathology of different skeletal anomalies in an anosteocytic boned flatfish species.


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LIST OF ABBREVIATIONS

ABDOM: abdominal region (in Article IV it was abbreviated as “A”)

AB-H&E: modified alcian blue/haematoxylin-eosin staining

AB-PAS: alcian blue/periodic acid-Schiff staining

ABS: absence of anomalies

ACV: anomalías del cuerpo vertebral

A-D: abdominal deformation

A-F: abdominal fusion

Afr: anal fin ray

A-K: abdominal kyphosis

A-L: abdominal lordosis

A-N: abdominal neural elements

ANOVA: analysis of variance

ARA: arachidonic acid

A-S: abdominal scoliosis

BM: binary matrix

CA: correspondence analysis

CAUDAL: caudal region (in Article IV it was abbreviated as “C”)

Cb: compact bone

CC: caudal complex region (in Article IV it was abbreviated as “CC”)

CC-D: caudal complex deformation

CC-F: caudal complex fusion

CC-H: caudal complex haemal elements

CC-K: caudal complex kyphosis

CC-L: caudal complex lordosis

CC-N: caudal complex neural elements

CC-S: caudal complex scoliosis

C-D: caudal complex deformation

C-F: caudal complex fusion

Cfr: caudal fin ray

C-H: caudal haemal elements

C-K: caudal kyphosis

C-L: caudal lordosis

C-N: caudal neural elements

col1a1: *collagen type I alpha 1 chain*

col2a1: *collagen type II alpha 1 chain*

col10a1: *collagen type X alpha 1 chain*

CR: computed radiography

C-S: caudal scoliosis

dah: days after hatching

DCV: desviaciones de la columna vertebral

dde: días después de la eclosión

def: deformities

df: degrees of freedom

Dfr: dorsal fin ray

DHA: docosahexaenoic acid

DV: dorso-ventral radiographic projection

dw: dry weight

E: epural

ECM: extracellular cartilaginous matrix

EIV: espacio intervertebral

Em: elastic membrane

EPA: eicosapentaenoic acid

FAO: Food and Agriculture Organization of the United Nations

FID: flame ionization detector

FM: frequency matrix

FUS: vertebral fusions

GT: modified Gallego's trichrome staining

H: haemal arch and spine

H1-H5: hypurals 1-5

Ha: haemal arch

H&E: haematoxylin-eosin staining

HPLC: high performance liquid chromatography

Hprz: haemal prezigapophysis

Hpz: haemal poszigapophysis

Hs: haemal spine

HUFA: highly unsaturated fatty acids

HYE: hypurals, epural, parhypural

%I: percentage of individuals showing the most frequent value

ICP-OES: inductively coupled plasma optical emission spectrometer

%Im: percentage of individuals showing the median value

IV: intermediary (extra) vertebra between the two preurals (when present)

List of abbreviations

IVS: intervertebral space

KYP: kyphosis

LCD: lymphocystis disease

LL: latero-lateral radiographic projection

LOR: lordosis

mah: months after hatching

Max: maximum

***mef2c*:** *myocyte enhancer factor 2c*

Mhs: modified haemal spine

microCT: microcomputed tomography

Min: minimum

Mns: modified neural spine

N: neural arch and spine

Na: neural arch

NA: number of observed alterations

NA/I: average number of malformations per specimen

nch: notochord

nchs: notochordal sheath

NI: number of fish showing at least one skeletal anomaly

%NI: percentage of affected individuals

NPMANOVA: non-parametric multivariate analysis of variance

Nprz: neural prezigapophysis

Npz: neural poszigapophysis

Ns: neural spine

Ol: osteogenic layer

OS: osteoid staining

PDA: photodiode array

Ph: parhypural

Pp: parapophysis (or PP; in Article II it was abbreviated as “P”)

ppm: part per million

Pt: pterygophore

Pu1: preural 1

Pu2: preural 2

PUFA: polyunsaturated fatty acids

R: caudal fin rays

***runx2*:** *runt related transcription factor 2*

SCO: scoliosis

SD: standard deviation

SEM: standard error of the mean

SOM: Self-Organizing Map

StH: standard height

StL: standard length

Tb: trabecular bone

% TFA: percentage of the total of fatty acids

U: urostyle

uns: unspecified

V: vertebral centrum

VBA: vertebral body anomalies

VCD: vertebral column deviations

VER: viral encephalopathy and retinopathy

VHS: viral hemorrhagic septicaemia

ww: wet weight

Introduction


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INTRODUCTION

1. GENERAL CONSIDERATIONS ON SENEGALESE SOLE (*SOLEA SENEGALENSIS*) BIOLOGY AND AQUACULTURE

1.1. TAXONOMY AND MORPHOLOGY

Senegalese sole (*Solea senegalensis*, Kaup 1858) is a flatfish (order Pleuronectiformes) belonging to the Soleidae family. This species is characterized by having a flat asymmetric morphology with both eyes located in the right side of the fish (ocular side) and an oval and elongated body shape (Figure 1). The ocular side presents a brownish coloration interposed by small spots of diverse colour tone and a pectoral fin with a black interradial membrane (Figure 1). This feature serves as distinctive from other species such as common sole (*Solea solea*) which has a black spot on the caudal end of this fin (FAO 2016b) and can cohabit with Senegalese sole in the nature. In turn, the blind side of the fish, on which it settles to the seabed, is usually white. The lateral line describes a slight arch in the subtemporal region (FAO 2016b), although it straightens caudally. Dorsal and anal fin are different from caudal fin, extending along the border of the body to join the base of caudal fin through a short membrane (FAO 2016b). In addition, the head exhibits a rounded snout and a small, semicircular inferior mouth (Figure 1). Adults from this species measure around 45 cm of length although they can reach 60 cm in the Atlantic Ocean (females are bigger than males) and 3 kg of weight (Rodríguez & Peleteiro 2014).

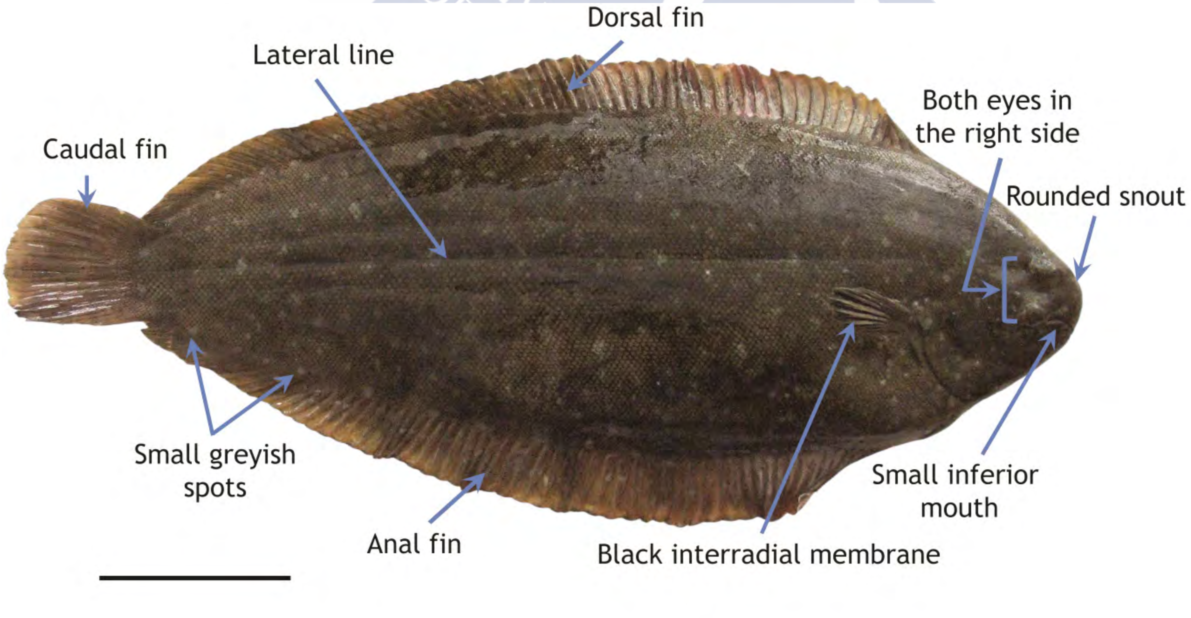


Figure 1: External morphology of the ocular side of a young adult wild Senegalese sole (*Solea senegalensis*) specimen. Bar = 5 cm.

1.2. DISTRIBUTION AND BIOLOGY

Solea senegalensis is a benthic species whose natural habitat rests on sandy or muddy bottoms up to 100 m depth and brackish areas along the Atlantic coast from the Gulf of Biscay to the Senegal coast and in a part of the West Mediterranean (Figure 2). This carnivorous fish usually feeds on small benthic invertebrates such as polychaete larvae (e.g. *Hediste diversicolor*), bivalve molluscs (e.g. *Scrobicularia plana*) and small crustaceans (e.g. *Corophium* spp.) (Cabral 2000, Rodríguez & Peleteiro 2014), with an increased activity in spring and summer (Cabral 2000).

Figure 2: Senegalese sole (*Solea senegalensis*) distribution (in red) and producer European countries (until 2014) (source: FAO, 2016a,b; FishBase, 2016).

Regarding swimming activity, flatfish generally swim in a relatively slow undulatory movement, the anguilliform mode, albeit the waves are vertical (side to side) rather than horizontal (Lindsey 1978). The synchronic movements may pass along the dorsal and anal fins, in order to attain wider amplitude in midwaters (Lindsey 1978). Senegalese sole adults present nocturnal habits while, during the day, rest half buried in the seabed by throwing up sand with undulations of the fin (Lindsey 1978, Rodríguez & Peleteiro 2014).

Individuals become reproductively mature at 3 years old with around 30 cm of length and lack differentiable external sexual characters (Rodríguez & Peleteiro 2014). In wild broodstock acclimated to captive conditions, the main spawning period occurs during spring between February and May, with a secondary spawning in autumn (Anguis & Cañavate 2005, Anguís *et al.* 2007). Higher fecundity rates were obtained at 16-22 °C (Anguis & Cañavate 2005), although short-term fluctuations in water temperature could constitute a triggering factor for spawning (Anguis & Cañavate 2005), which in turn, may be applied to reproductive management in fish farms (Anguis & Cañavate 2005, Cañavate 2013). After egg incubation, the newly hatched larvae have a pelagic lifestyle in the water column, showing bilateral symmetry (Cañavate 2013). Padrós *et al.*, 2011 provided a detailed description of morphological and histological features in larval development, including one important period, the metamorphosis. It usually takes place around 13 days after hatching (dah) and is completed at around 27 dah (Gavaia *et al.* 2002), although the pattern may vary depending on environmental conditions (Fernández-Díaz *et al.* 2001, Geffen *et al.* 2007). During this endocrine regulated process (reviewed in Fernández-Díaz *et al.*, 2001, Power *et al.*, 2008), pelagic larvae gradually become asymmetric, the left eye migrates to the right side, the urostyle bends upwards and specimens acquire a benthic lifestyle (Gavaia *et al.* 2002), settling into the bottoms. These morphological changes occur in parallel with the behavioural and physiological adaptations (Fernández-Díaz *et al.* 2001), preparing the developing fish for its nutritional, metabolic, locomotive, and environmental forthcoming demands (Power *et al.* 2008). Nevertheless, in flatfish, anomalies are relatively common to find in the skull due to abnormal ocular migration and incorrect migration of the cranial structures during metamorphosis (Boglione *et al.* 2013b). In fact, some of the abnormalities reported in flatfish become evident in this period and therefore, attention should be paid to identify markers of abnormal metamorphosis (Power *et al.* 2008).

1.3. INDUSTRIAL PRODUCTION

Senegalese sole is a promising species in aquaculture (APROMAR 2016). Its high commercial value (around 9.56 €/kg; APROMAR, 2016) and market interest, allied to the excellent flesh quality, and the completion of its production cycle show its great potential to the industry, meeting the demand for species diversification (Cañavate 2013, Rodríguez & Peleteiro 2014, Morais *et al.* 2016). In this sense, it has been included in diverse Spanish National Plans and other investigations (Secretaría General del Mar 2009). The outcome of these initiatives was reflected in new advances on Senegalese sole farming procedures, recently reviewed in Morais *et al.*, 2016. This lead to an increasing production in Europe that almost doubled in just one year, reaching 1065 t in 2014 (Figure 3; FAO, 2016a). Senegalese sole is the most cultivated species from the *Solea* genus seconded by common sole with a much lower value (88.21 t in 2014; FAO, 2016a). Apart from Europe, worldwide production is complemented with at least Chinese farms output with a rough estimation of 300–500 t (unofficial data FAO, 2016b). In Europe, *S. senegalensis* production is situated mainly in Spain and France (until 2014) (Figures 2 and 3) according to FAO, 2016a, although nowadays, Iceland and Portugal present also industrial farms (Rodríguez & Peleteiro 2014, APROMAR 2016), which produced around 290 and 175 t of sole, respectively (data from *S. senegalensis* and *S. solea* production) (FEAP 2016). Since 2007, the Autonomous Community of Galicia has been the leader in the Spanish scene, with a production of around 388 t of sole (in 2015) followed by Andalucía and Canary Islands in decreasing order of importance (APROMAR 2016). In parallel, juvenile production, which started in 1993, augmented to 7.4 million of specimens in Spain in 2015 (APROMAR 2016, FAO 2016b). Moreover, it is expected a further expansion of the industry since new projects to build several sole farms exist, opening more job and business opportunities (Rodríguez & Peleteiro 2014, APROMAR 2015).

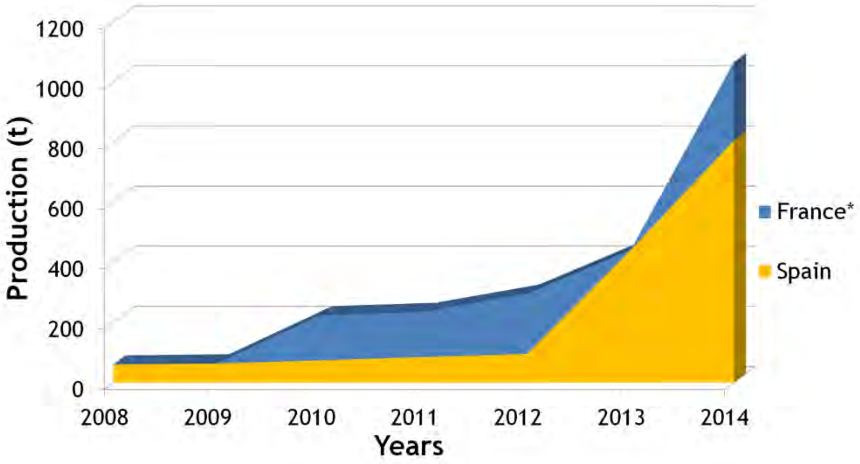


Figure 3: *Solea senegalensis* production in Europe in metric tons (t) from 2008 to 2014 (source: FAO, 2016a). *FAO estimate from available sources of information.

1.4. REARING PROCEDURES

Despite traditional extensive and semi-intensive rearing systems have been conducted in natural conditions in earthen ponds, nowadays large-scale production is performed in intensive indoor infrastructures (FAO 2016b). Moreover, the implementation of recirculation systems provided a better control of environmental conditions, which could increase growth and survival of the fish (Morais *et al.* 2016). Senegalese sole production cycle is divided into several stages from spawning to the adult phase (Figure 4). In general, it takes 20-21 months for Senegalese sole to attain commercial size (with around 300-350 g; Rodríguez and Peleteiro, 2014).

1.5. PRODUCTION PRIORITIES

From an industrial point of view, the main priorities for the sector rely on key issues as disease control, larval quality, as well as reproductive and nutritional advances (Howell *et al.* 2009, 2011). Nowadays, most detrimental diseases affecting Senegalese sole culture include tenacibaculosis, photobacteriosis (former pasteurellosis) and vibriosis (FAO 2016b, Morais *et al.* 2016). Table 1 describes briefly the main infectious diseases in this species. In general, cultured *Solea* spp. are quite susceptible to a host of diseases affecting other fish species and its severity could be linked with a further intensification of farming (FAO 2016b). Other pathological problems, more or less related with zootechnical protocols, with no infectious origin, are skeletal anomalies, cell necrosis of the adipose tissue and kidney lesions related with mineral deposits in the tubular lumen, similar to nephrocalcinosis (Padrós *et al.* 2003, Rodríguez & Peleteiro 2014). Skeletal abnormalities constitute an important limitation in Senegalese sole aquaculture (Gavaia *et al.* 2002, Fernández *et al.* 2009), which will be addressed in the next sections. Regarding the fat cell necrosis, lesions consist in yellowish patches in dorsal and/or anal fins base corresponding to necrosis of the subdermical adipose tissue (Padrós *et al*. 2003). The lipid peroxidation and/or excessive sunlight exposition could be related with the development of this disorder (Padrós *et al*. 2003).

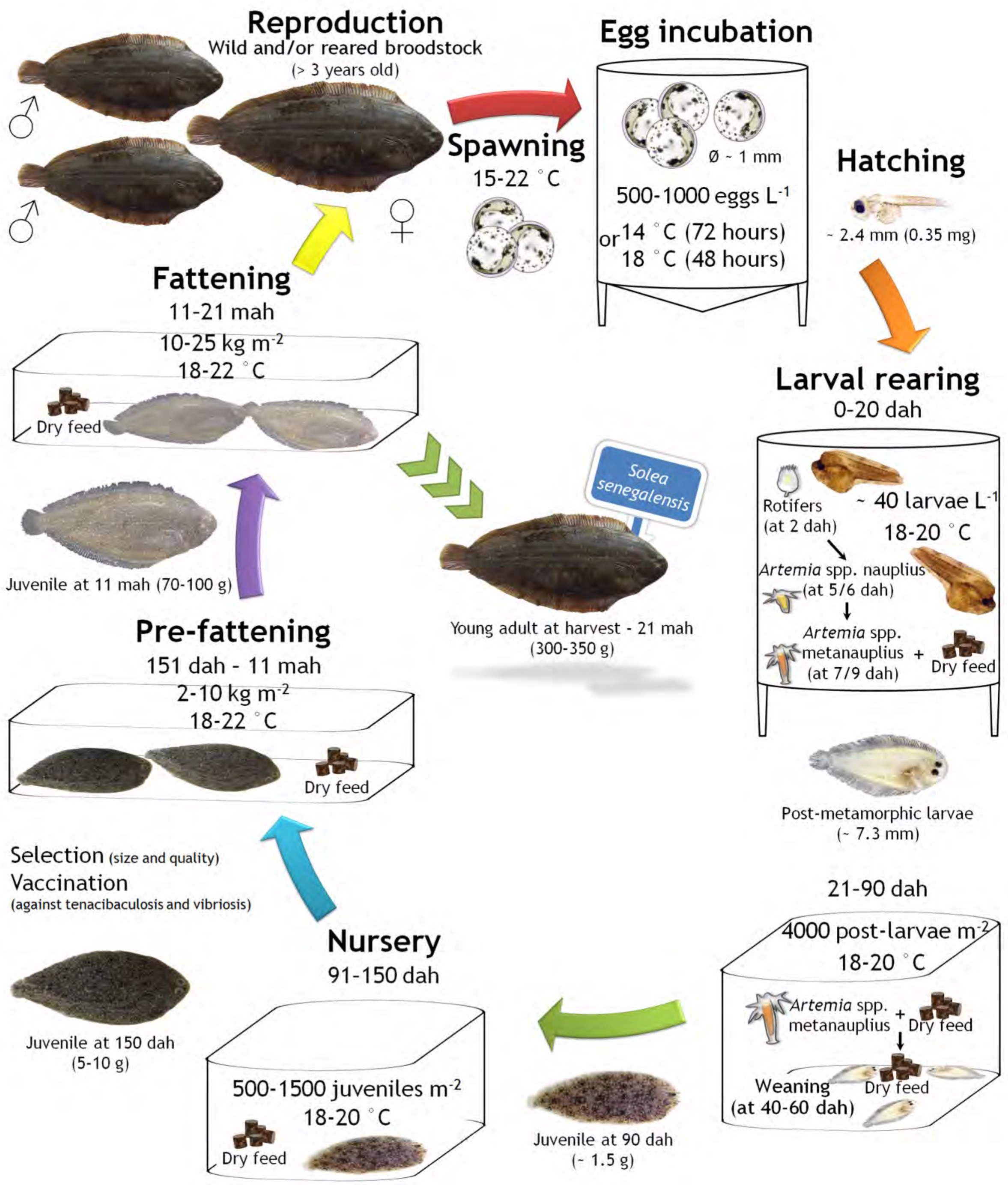


Figure 4: Rearing cycle of Senegalese sole (*Solea senegalensis*), adapted from FAO, 2016b. Production stages are briefly described in terms of the most used stocking density, water temperature, feeding regimes and types of tanks from spawning up to harvest (source: Cañavate, 2013; Rodríguez and Peleteiro, 2014; FAO, 2016b). dah: days after hatching; mah: months after hatching.

Table 1: Main infectious diseases affecting Senegalese sole (*Solea senegalensis*) culture (adapted from FAO, 2016b). References: [1]Padrós *et al.*, 2003, [2]Noga, 2010, [3]Hodneland *et al.*, 2011, [4]Toranzo, 2003, [5]Rodríguez *et al.*, 1997, [6]Faílde, 2013, [7]Zorrilla *et al.*, 2003, [8]Magariños *et al.*, 2011, [9]Castro *et al.*, 2012, [10]Constenla and Padrós, 2010, [11]Constenla *et al.*, 2014.

Disease	Agent	Type	Clinical signs and lesions
Lymphocystis disease (LCD)[1,2]	*Iridovirus* (genus *Lymphocystivirus*)	Virus	Dermal fibroblast hypertrophy forming skin nodules
Viral encephalopathy and retinopathy (VER)[3]	*Nodavirus* (genus *Betanodavirus*)	Virus	Abnormal swimming, vacuolization and necrosis of the central nervous system and retina
Viral hemorrhagic septicaemia (VHS)[2,4]	*Rhabdovirus* (genus *Novirhabdovirus*)	Virus	Ascites, haemorrhages in skin, eyes, muscle and serosal surfaces
Solevirus disease[5]	*Birnavirus* (genus *Aquabirnavirus*)	Virus	Dark coloration, hyperactivity, uncoordinated swimming and behaviour
Tenacibaculosis[6]	*Tenacibaculum maritimum* (most common), *T. soleae*	Bacterium	Whitish erosions, shortening/erosion of fin rays, ulcers in the skin and fins (some showing necrotic epidermic flaps)
Photobacteriosis[1,2]	*Photobacterium damselae* subsp. *piscicida*	Bacterium	High mortality, petechiae and/or ecchymosis on operculum and base of the fins, enlargement of the spleen and kidney, white miliary granulomas in spleen and kidney (chronic form)
Vibriosis[1,2,4,7]	*Vibrio* spp. - *Vibrio anguillarum, V. harveji, V. alginolyticus, V. pelagius, V. parahaemolyticus*	Bacterium	Anorexia, skin ulcers, haemorrhagic septicaemia (visceral petechiae, necrosis of liver, spleen, kidney and heart)
Aeromoniasis[8]	*Aeromonas salmonicida* subsp. *salmonicida*	Bacterium	Skin ulcers, haemorrhages in the base of the fins, ascites, petechiae in liver, pale liver
Edwardsiellosis[9]	*Edwardsiella tarda*	Bacterium	Periocular tumefactions, ascites, anaemic liver, petechiae in kidney
Amoebiasis[10,11]	*Endolimax piscium*	Parasite (Protozoa - Archamoebae)	Granulomatous inflammatory lesions in muscle, kidney, heart, liver and digestive tract, containing the parasite
Amyloodiniosis[1,2]	*Amyloodinium* spp.	Parasite (Protozoa - Dinophyceae)	Gill parasitism with hyperplasia, inflammation, haemorrhage and necrosis; the skin can also be affected
Cryptobiosis[1,2]	*Cryptobia* spp.	Parasite (Protozoa - Kinetoplastida)	Gill parasitism, emaciation, anorexia
Cryptocaryonosis[1,2]	*Cryptocaryon* spp.	Parasite (Protozoa - Ciliophora)	Discrete white spots in skin
Myxosporidiosis[1]	Myxosporea	Parasite (Metazoa – Myxosporea)	Presence of parasites in renal tubules
Digenean trematode infection[1,2]	Digenea	Parasite (Metazoa – Trematoda)	Metacercariae encysted in the muscle
Microsporidiosis[1,2]	*Tetramicra* spp.	Parasite (Fungi – Microsporidia)	Formation of cyst-like structures with sequential developmental phases of the parasite (xenoma) in the muscle; degeneration of the muscle fibres

To cope some of these health problems, therapeutic strategies have been developed involving vaccination programs (against photobacteriosis, tenacibaculosis and vibriosis), although conventional methods, as formalin baths and antibiotic administration are also used (Howell *et al.* 2009, Rodríguez & Peleteiro 2014). However, the appliance of such approaches was not sufficient to solve the pathologic issues. Research efforts should be focused on the development of more specific and efficient vaccines (Padrós *et al.* 2003) and also nutritional and zootechnical improvements to promote sole immune-competence (Howell *et al.* 2009, Cañavate 2013).

Larval quality and, in general, product quality is an important priority in Senegalese sole farming (Howell *et al.* 2009). Particularly, the high incidence of skeletal anomalies constitutes one of the bottlenecks for this species industrial production (Gavaia *et al.* 2002, Fernández *et al.* 2009, Riaza personal communication), which will be addressed in the following sections of the present Thesis.

Senegalese sole culture can occasionally be affected by pigmentary disorders under current production protocols (Cañavate 2013, Morais *et al.* 2016). These tend to be associated with nutritional imbalances, such as a high arachidonic acid (ARA) dietary content and a suboptimal ratio between ARA and eicosapentaenoic acid (EPA) (ARA/EPA) (Villalta *et al.* 2005). In this sense, a better understanding of sole feeding requirements and strategies at larval stages is highly requested at industrial level in order to modulate the occurrence of disorders as vertebral anomalies, susceptibility to diseases, stress and malpigmentation (Howell *et al.* 2009, Morais *et al.* 2016). Other challenge, specially related with the rearing process, is the low reproductive performance of first generation reared broodstocks, concerning natural fertilization and reproductive behaviour, among other problems (Howell *et al.* 2011, Morais *et al.* 2016).

2. THE VERTEBRAL COLUMN AND THE CAUDAL SKELETON OF SENEGALESE SOLE

2.1. ANATOMIC FEATURES

According to Gavaia *et al.*, 2002, the vertebral column of Senegalese sole can be divided into three major regions: abdominal, caudal and caudal complex (Figure 5). The rachis is composed by a sequence of 45 vertebrae, each one (excluding the urostyle) is equipped with a neural arch and spine (dorsal aspect of the vertebra), parapophysis/haemal arch and spine (ventral aspect of the vertebra), and the vertebral centrum (Figure 5). Teleostean vertebral bodies usually exhibit amphicoelous morphology (similar to an hour-glass shape) (Figures 6-8), and are connected by the intervertebral notochordal tissue which can cushion eventual shock (Stiassny 2000). This configuration confers a substantial lateral flexibility to the rachis and, at the same time, some resistance to compression (Stiassny 2000). The vertebral centra can show different shape according to the anatomic region in which it is situated, and, in general, the caudal-most vertebrae are commonly elongated (Figure 8) (Harder 1975). The number of vertebrae can be characteristic of a given species, within certain variation (Harder

1975). In this sense, vertebral counts tend to be higher in fish populations from cooler waters or from higher latitudes (Lindsey 1978) and they can be affected by environmental conditions during early development (Roberts & Ellis 2001). The Senegalese sole abdominal region usually shows eight vertebrae (Gavaia *et al.* 2002). The first five neural spines are generally thicker than the others, while ventrally, abdominal vertebrae display paired parapophysis from the fourth to the eighth centra (Figures 5 and 6) (Gavaia *et al.* 2002). In turn, caudal vertebrae (around 34) (Figure 7) bear an haemal arch and spine (Gavaia *et al.* 2002). In both regions, neural arches present a neural prezygapophysis (cranial aspect) and a neural poszygapophysis (caudal aspect) (Figures 6 and 7) (Gavaia *et al.* 2002). Similarly, the haemal arches show haemal prezygapophysis and haemal poszygapophysis in the anteriormost and posteriormost aspects, respectively (Figures 6 and 7) (Gavaia *et al.* 2002). These overlapping structures (zygapophysis) maintain the vertebral alignment, reducing dorso-ventral curving while lateral flexibility is preserved (Lindsey 1978). Arches and spines provide insertion to the musculature and have an important role in protecting vital organs as the spinal cord (dorsally to vertebral centra) and caudal artery and vein (ventrally to the vertebrae) (Stiassny 2000, Boglione *et al.* 2013b). Moreover, the length and the angle of projection of neural and haemal processes from the axis may influence the shape of the fish body (Harder 1975).

The caudal complex (Figures 5 and 8) is usually composed by three vertebrae: preural 2, preural 1 and the urostyle, caudally. As in previous regions, preural vertebrae present also neural and haemal arches and spines. These cases are modified, extending more caudally to articulate with the bones of the caudal fin, except the neural spine of preural 2. The urostyle is the last vertebra without arches or spines but instead, is fused to the base of the first four hypurals (Figure 8). These structures are like small triangular plates flattened and branched in the adult specimens (Figure 8) (Gavaia *et al.* 2002). Other small bony elements located in the caudal complex consist in the epural, located between the neural spine of preural 1 and the hypural 5, and ventrally the parhypural, between the haemal spine of preural 1 and hypural 1, which could also show a slight branching (Figure 8) (Gavaia *et al.* 2002). In this species there is only a single epural and no free uroneurals processes were appreciated (Gavaia *et al.* 2002). The caudal complex structures support the caudal fin, articulating with the fin rays: the first four hypurals are connected with 10 rays, the modified haemal spines and the parahypural with 5, the modified neural spine and the epural with 3 and finally, the hypural 5 with 2 rays. Generally, the caudal fin is composed by 20 paired, branched and segmented hemirays (lepidotrichia), symmetrically distributed regarding the lateral line (Gavaia *et al*. 2002). Considering other unpaired fins, the dorsal and anal fins (Figure 5) are equipped with 73 to 86 and 61 to 74 soft rays respectively (FAO 2016b), supported by corresponding pterygophores (proximal, middle and distal). In particular, the pterygophores can determine the characteristic shape of the skeleton of some flatfish species (Harder 1975).

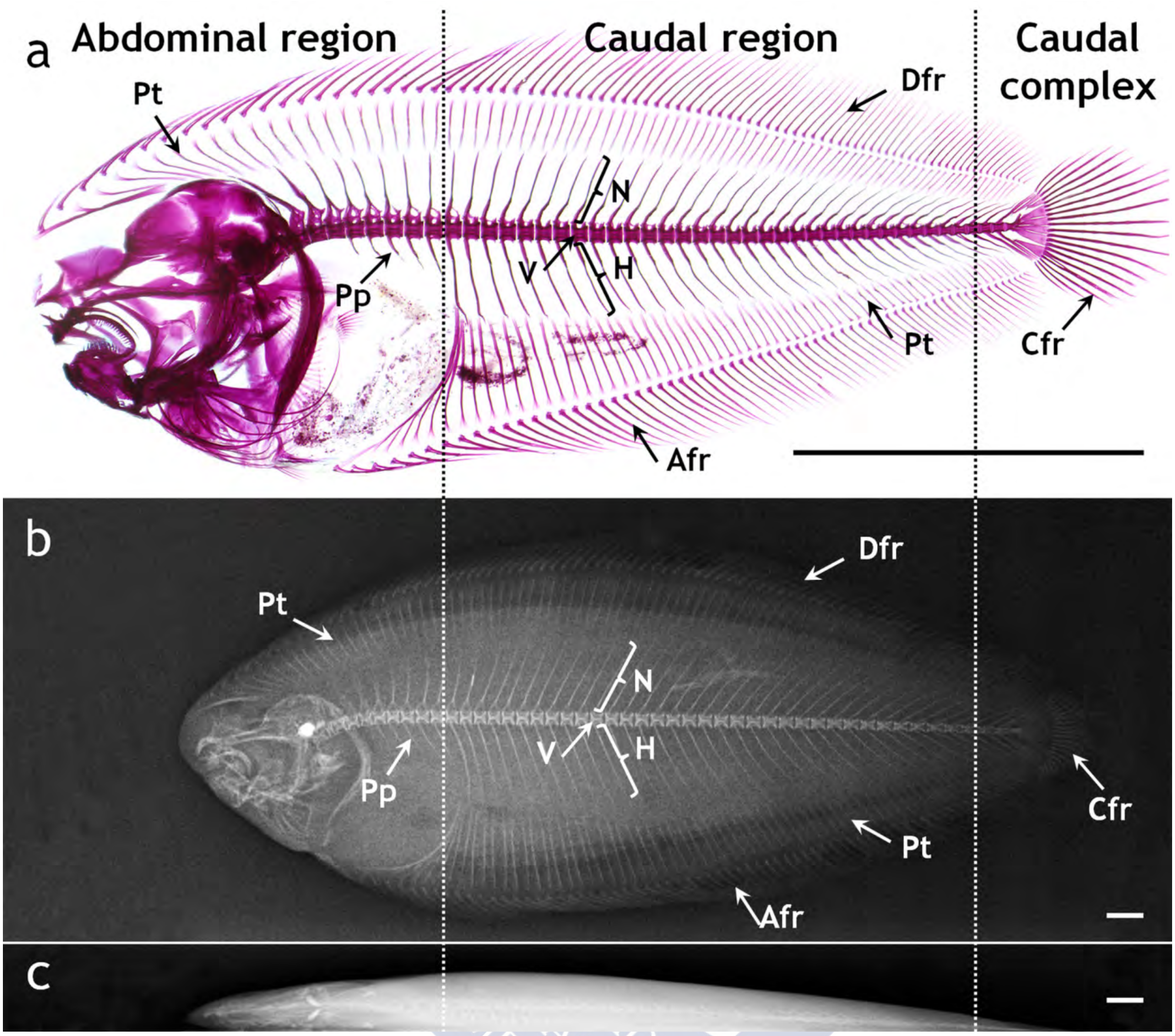


Figure 5: Main anatomical features of vertebral column and caudal skeleton in Senegalese sole (*Solea senegalensis*) larva (a) and juvenile (b, c) (adapted from de Azevedo *et al.*, in press). (a) Blind side aspect of a 32 days after hatching (dah) larva stained with double staining technique with alcian blue and alizarin red. (b) Latero-lateral radiographic projection of a juvenile sole (342 dah). (c) Dorso-ventral view of the same specimen as in (b). Afr: anal fin ray; Cfr: caudal fin ray; Dfr: dorsal fin ray; H: haemal arch and spine; N: neural arch and spine; Pp: parapophysis; Pt: pterygophore; V: vertebral centrum. Bars = 0.5 cm.

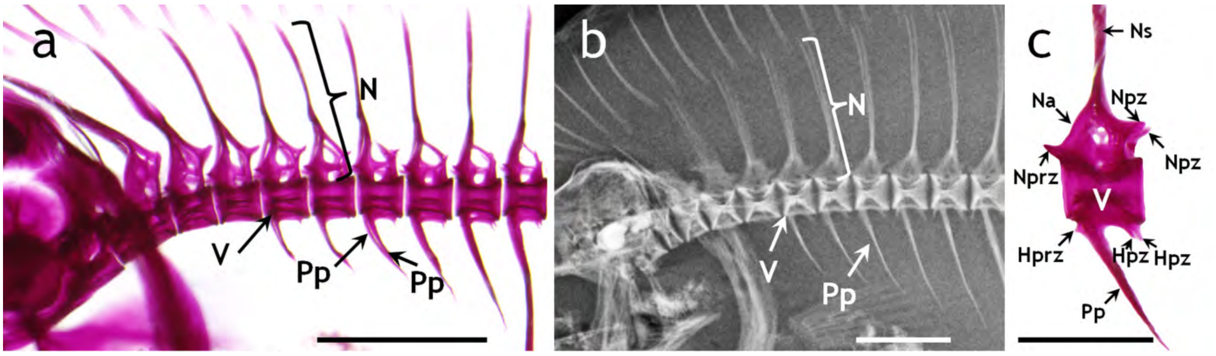


Figure 6: Abdominal region of Senegalese sole (*Solea senegalensis*). (a) Blind side aspect of a 32 days after hatching (dah) larva stained with double staining technique with alcian blue and alizarin red. Note the thicker

cranial neural spines. (b) Latero-lateral radiography of a wild adult sole. (c) Abdominal vertebra stained with double staining technique of a 105 dah juvenile. Note the amphicoelus shape of the vertebral centrum (V). Hpz: haemal poszigapophysis; Hprz: haemal prezigapophysis; N: neural arch and spine; Na: neural arch; Npz: neural poszigapophysis; Nprz: neural prezigapophysis; Ns: neural spine; Pp: parapophysis; V: vertebral centrum. Black bars = 1 mm; White bar = 1 cm.

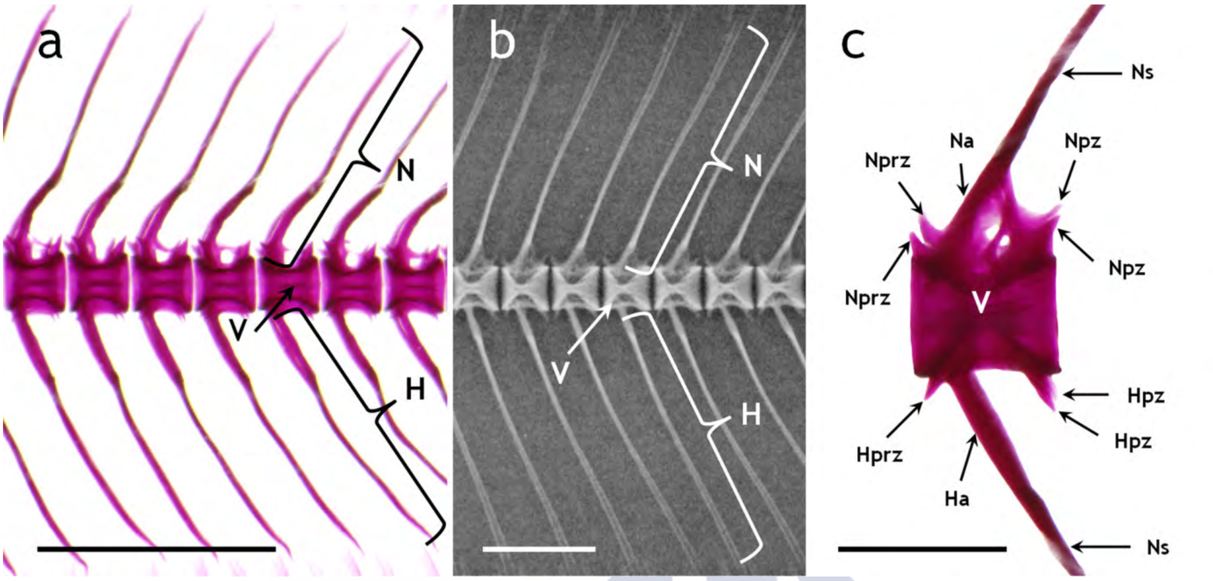


Figure 7: Vertebrae from caudal region of Senegalese sole (*Solea senegalensis*). (a) Blind side aspect of a 32 days after hatching (dah) larva stained with double staining technique with alcian blue and alizarin red. (b) Latero-lateral radiography of a wild adult sole. (c) Caudal vertebra stained with double staining technique of a 105 dah juvenile. Note the amphicoelus shape of the vertebral centrum (V). H: haemal arch and spine; Ha: haemal arch; Hpz: haemal poszigapophysis; Hprz: haemal prezigapophysis; Hs: haemal spine; N: neural arch and spine; Na: neural arch; Npz: neural poszigapophysis; Nprz: neural prezigapophysis; Ns: neural spine; V: vertebral centrum. Black bars = 1 mm; White bar = 1 cm.

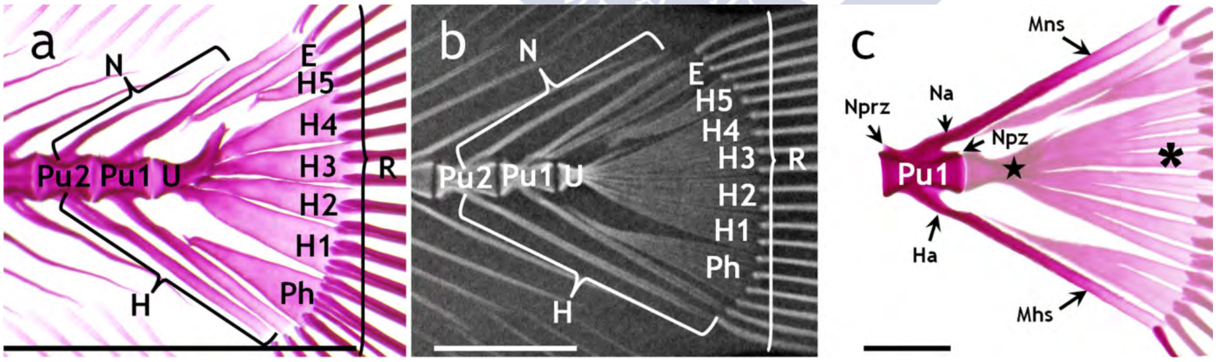


Figure 8: Caudal complex of Senegalese sole (*Solea senegalensis*). (a) Blind side aspect of a 32 days after hatching (dah) larva stained with double staining technique with alcian blue and alizarin red. (b) Latero-lateral radiography of a wild adult. (c) Preural 1, urostyle and caudal complex elements stained with double staining of a 105 dah juvenile. Note the elongated amphicoelus shape of vertebral centrum (Pu1), the fusion of urostyle with hypurals 1-4 (star) and branching of hypurals 1-4 and parhypural (asterisk). Neural and haemal spines are modified (Mns and Mhs, respectively) to support fin rays. E: epural; H: haemal arch and spine; H1-H5: hypurals 1-5; Ha: haemal arch; Mhs: modified haemal spine; Mns: modified neural spine; N: neural arch and spine; Na: neural arch; Npz: neural poszigapophysis; Nprz: neural prezigapophysis; Ph: parhypural; Pu1: preural 1; Pu2: preural 2; R: caudal fin rays; U: urostyle. Black bars = 1 mm; White bar = 1 cm.

At microscopic level, little is known on the architecture and material properties of fish bone, particularly anosteocytic bone (i.e. bone devoid of osteocytes) (Dean & Shahar 2012), as it is the Senegalese sole bone (Cardeira *et al.* 2012). In an anosteocytic (or acellular) bone fish species as gilthead seabream (*Sparus aurata*) the normal vertebral histology was described by Ortiz-Delgado *et al.*, 2014 (Figure 9). However, studies are scarce on the microscopical organization of normal and deformed bone of different fish species, including Senegalese sole. In gilthead seabream, the mediosagittal section of the vertebral centrum show a central part of cancellous bone with longitudinal and transverse trabecula and funnel-shaped vertebral endplates (Ortiz-Delgado *et al.* 2014). An osteogeneous tissue layer with osteoblasts and the connective tissue from periosteum complete the vertebral structure (Ortiz-Delgado *et al.* 2014). Unlike tetrapods, teleost bone medullary cavity is never hematopoietic and in many cases is completely filled with fat (Huysseune 2000). Between opposing vertebrae, the vacuolated notochordal tissue is present, surrounded by the notochordal sheath and the elastic membrane (Figure 9) (Ortiz-Delgado *et al.* 2014). Bone as a tissue is composed by three main constituents: cells (osteocytes, osteoblasts and osteoclasts), extracellular matrix and a mineral phase (Witten & Huysseune 2007). In anosteocytic bone, the osteoblasts produce the unmineralised bone extracellular matrix (osteoid) and retract from the surface, so they do not became encapsulated in osteoid as osteocytes (Huysseune 2000). Bone matrix is predominately composed by type I collagen fibrils, less abundant non-collagenous proteins (osteocalcin, osteopontin, osteonectin) and when it is mineralised, by hydroxyapatite crystals (major component of mineral phase) (Witten & Huysseune 2007, Hall 2015). The predominating bone resorption cells (osteoclasts) in advanced teleost are often small, mononucleated cells, performing a smooth bone resorption (Witten & Huysseune 2009, 2010). The prevalence of these cells appears to coincide with the evolutionary disappearance of osteocytes (Witten & Huysseune 2009, 2010).

2.2. SKELETAL DEVELOPMENT

Three main processes regarding bone formation have been described in teleosts, depending on the species and skeletal structures: intramembranous, perichondral and endochondral (Boglione *et al.* 2013a). During intramembranous ossification, osteoblasts directly differentiate from mesenchymal osteoprogenitor cells (Witten & Huysseune 2007), while perichondral and endochondral ossification involve an intermediate cartilaginous template (Huysseune 2000, Boglione *et al.* 2013a). In particular, in perichondral ossification, a common process in fish, the bone matrix is deposited in contact with the outer layers of cartilage, and both matrices can combine (Huysseune 2000). For Senegalese sole, elements that undergo intramembranous ossification are: the vertebral centra, the neural and haemal arches and spines, the urostyle and the caudal fin rays (Gavaia *et al.* 2002). At the same time, the endochondral ossification occurs in the hypurals, the epural and the neural and haemal arches and spines of the vertebrae 41, 42, preural 1 and preural 2 (Gavaia *et al.* 2002).

In this species, vertebral development was enlightened in the study of Gavaia *et al.*, 2002. Briefly, at 13 dah, the neural arches of the abdominal vertebrae 2-6 could already be observed along with the caudal fin elements endochondral differentiation and eye migration (Gavaia *et al.* 2002). Arch development initiated with two latero-dorsal intramembranous buds which elongate and join together to form the arch and later the spine by intramembranous ossification (Gavaia *et al.* 2002), although Padrós *et al.*, 2011 observed cartilaginous structures in this location. The process continued caudally for all the remaining neural and haemal arches except for the first, which formed cephalad. At 18 dah, the cranial vertebrae were almost formed, showing the notochord surrounded by calcified tissue, starting from the base of the arches (Gavaia *et al.* 2002). At this stage, the urostyle started to bend upwards (Gavaia *et al.* 2002). Finally, at 45 dah, metamorphosis was completed and all vertebral structures were developed and calcified (Gavaia *et al.* 2002). Later, juvenile specimens showed branched hypurals 1 to 4 which were fused with the urostyle (Gavaia *et al.* 2002).

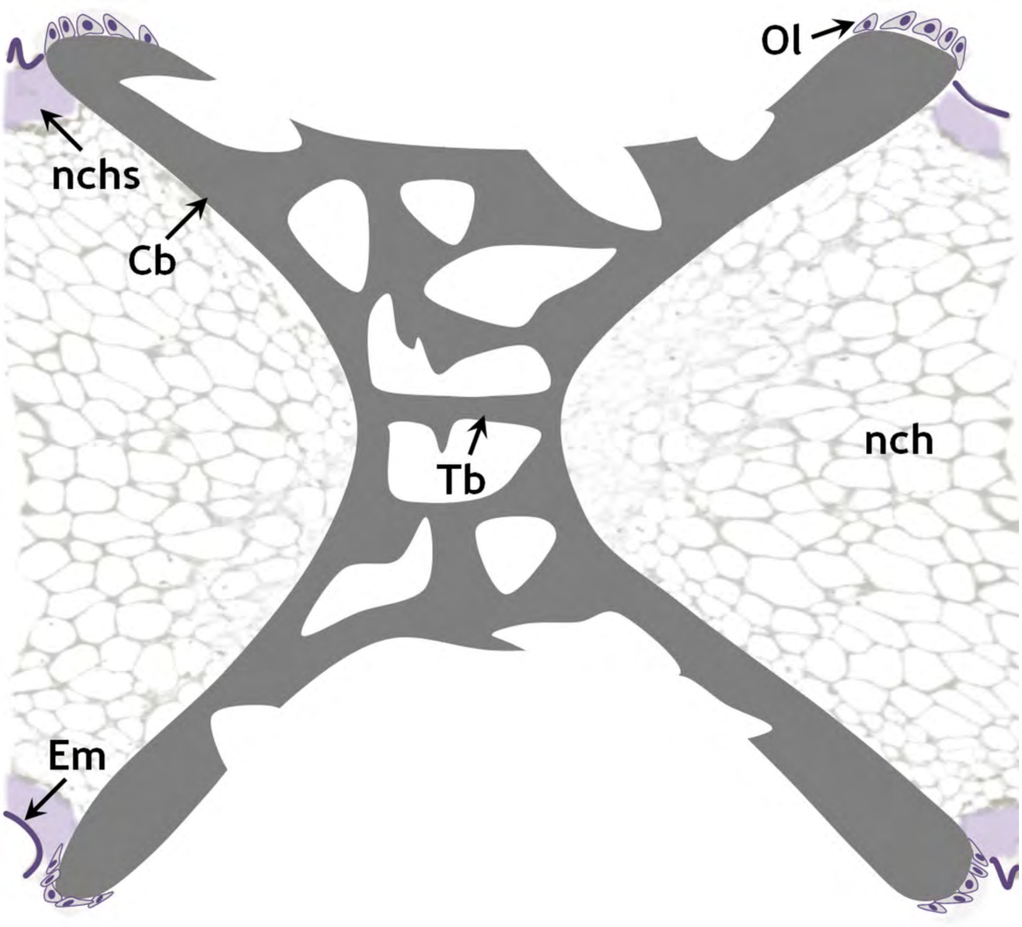


Figure 9: Schematic representation of a histological section of a vertebral centrum and part of the adjacent intervertebral spaces (adapted from Fernández *et al.*, 2012 and Ortiz-Delgado *et al.*, 2014). Cb: compact bone; Em: elastic membrane; nch: notochord; nchs: notochordal sheath; Ol: osteogenic layer; Tb: trabecular bone.

3. SKELETAL ANOMALIES IN TELEOSTS: THE CASE OF SENEGALESE SOLE

3.1. DEFINITION AND IMPACT

Diverse studies, using different methodologies and focus, have been conducted on skeletal anomalies in teleosts and were recently reviewed (Boglione *et al.* 2013a,b). Some terminological discrepancies were found regarding the literature on skeletal anomalies in fish (Boglione *et al.* 2013b), although in general, these consist in abnormalities concerning fish skeletal elements. This world-wide problem can affect numerous fish species aquaculture showing a large range of incidence varying between farms, fish species, batches from the same hatchery and even between fry from the same batch of eggs (Boglione *et al.* 2013b, Chin *et al.* 2017). Moreover, the use of different diagnostic tools may provide different levels of accuracy (varying according to the life stage considered) (Boglione *et al.* 2013b), which should be taken into consideration when comparing anomaly rate. The incidence of skeletal anomalies in the main European cultured fish species is shown in Table 2, although other fish species have been studied on disorders affecting the skeleton (Sfakianakis *et al.* 2004, Giménez & Estévez 2008, Roo *et al.* 2010, Cobcroft & Battaglene 2013, Chin *et al.* 2017). Generally, in marine aquaculture, a frequency of 20% severely deformed fish could constitute a good outcome at the end of the hatchery stage (Boglione *et al.* 2013a). Studies on laboratory fish also detected a high number of skeletal anomalies in around 93% of reared zebrafish (*Danio rerio*) (Ferreri *et al.* 2000).

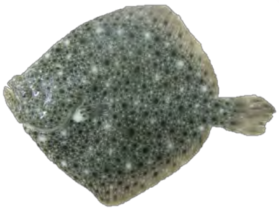
Regarding flatfish, the skeletal anomaly incidence is briefly summarized for the main cultured species in Table 3, including Senegalese sole. Notably, in a number of scientific investigations, skeletal deformities have ranged from 44% (Gavaia *et al.* 2002) to around 80% (100% in some cases) in this species larvae (Fernández *et al.* 2009, Gavaia *et al.* 2009). Among the different anatomical regions, the head is the area less affected by skeletal disorders (Morais *et al.* 2016). Moreover, in some hatcheries, around 40% of Senegalese sole juveniles present vertebral anomalies, at the grading point before transfer to ongrowing farms (Riaza personal communication). However, little is known on the incidence and main typologies of vertebral anomalies affecting reared sole in fish farms, where multilevel factors may interact on fish in unknown manners, which experimental conditions are unable to mimic (Boglione *et al.* 2013b). Also, information is still lacking on the frequency of skeletal abnormalities at later phases of productive cycle for sole. In addition, from a commercial point of view, the establishment of bench marks to compare levels of deformity is highly required (Howell *et al.* 2009). In order to explain the high incidence of deformities in cultured Senegalese sole, Fernández *et al.*, 2009 suggested that this species could be more prone to develop bone deformities than other species; and that deformed animals can survive and be detected later, since such anomalies are non-lethal.

Table 2: Skeletal anomaly incidence for the main European reared fish species. def: deformities. Modified from Boglino, 2013. Image source: FEAP, 2015 and FishBase, 2016.

Species	**Anomaly incidence**	**Developmental stage**	**References**
Atlantic salmon (*Salmo salar*) 	4-9% 12%	Parr Harvest	Fjelldal *et al.*, 2007 Sullivan *et al.*, 2007
Gilthead seabream (*Sparus aurata*) 	87-100% 23.6%	Juvenile Harvest	Prestinicola *et al.*, 2013 Lee-Montero *et al.*, 2015
European seabass (*Dicentrarchus labrax*) 	10-30% (vertebral def) 35-55% (head def)	Larvae	Mazurais *et al.*, 2008
Atlantic cod (*Gadus morhua*) 	20-75%	0-2 years after hatching	Fjelldal *et al.*, 2009b
Rainbow trout (*Oncorhynchus mykiss*) 	0-55% (of normal-shaped specimens)	Ongrowing juvenile	Deschamps *et al.*, 2008

In contrast, disorders affecting the skeleton of wild *S. senegalensis* specimens are less frequent (19%), suggesting the presence of selective forces that tend to eliminate affected individuals from natural populations and/or an effect of culture conditions on skeletal development (Hosoya & Kawamura 1997, Gavaia *et al.* 2009). Similarly, anomaly incidence differences between reared and wild specimens were also observed in other species (Hosoya & Kawamura 1997, Boglione *et al.* 2001, 2003, Fjelldal *et al.* 2009b).

Table 3: Skeletal anomaly incidence for the main reared flatfish fish species. dah: days after hatching; def: deformities; mah: months after hatching. Modified from Boglino, 2013. Image source: FishBase, 2016.

Species	Anomaly incidence	Developmental stage	References
Bastard halibut (*Paralichthys olivaceus*) 	13% (vertebral def)	Juvenile	Lü *et al.*, 2015
Atlantic halibut (*Hippoglossus hippoglossus*) 	83-92%	Larvae Juvenile	Lewis and Lall, 2006
Senegalese sole (*Solea senegalensis*) 	44%	0-75 dah	Gavaia *et al.*, 2002
	79%	40 dah	Gavaia *et al.*, 2009
	80-100%	48 dah	Fernández *et al.*, 2009
Turbot (*Scophthalmus maximus*) 	51%	Larvae	Tong *et al.*, 2012
	30-38%	50-80 dah	Torres-Núñez *et al.*, 2014
	30%	6-12 mah	Hernández-Urcera *et al.*, 2012

Skeletal deformities seriously hinder the industrial production of many fish species since they may affect proper feeding and swimming performances (Boglione *et al.* 2013a). As consequence, affected specimens may present discomfort, slower growth, low food efficiency level and higher susceptibility to diseases than non-deformed fish (Figure 10) (Fjelldal *et al.* 2012b, Boglione *et al.* 2013a). Moreover, some severe lesions in the rachis may have repercussions on the external appearance of the fish (Figure 10) (Fjelldal *et al.* 2012a, Cardeira *et al.* 2015) and be less attractive for the consumer (Gavaia *et al.* 2002, Boglione *et al.* 2013b), especially when sold as a whole-fish (e.g. Senegalese sole, Rodríguez and Peleteiro, 2014) (Boglione *et al.* 2013b). Even for fillets processing, severely deformed fish may be harder to process and unable to be machine filleted (Sullivan *et al.* 2007). To confront this problem, on one hand, producers have to discard abnormal specimens, usually by manual selection (Koumoundouros 2010), before they reach the market or at juvenile grow-out farms transfer (Morais *et al.* 2016), which also increases personnel costs and delays the productive cycle (Koumoundouros 2010). However, the anomaly detection based on external criteria may underestimate some minor vertebral deformities (Sullivan *et al.* 2007). In short term, these undetected alterations could not represent a direct market problem (Deschamps *et al.* 2008), but later, these might aggravate during growth (Witten *et al.* 2006, Fjelldal *et al.* 2007) and can become externally apparent (Deschamps *et al.* 2008). On the other hand, fish displaying skeletal anomalies result in a suboptimum product (Boglione *et al.* 2013b) driving the producers to decrease its market value (Fernández *et al.* 2008, Morais *et al.* 2016). All these inconveniences represent a burden to the aquaculture sector and can originate serious economic losses (Fernández *et al.* 2008, Morais *et al.* 2016). Therefore, the high incidence of skeletal anomalies represents an important limitation to the industrial culture of Senegalese sole (Gavaia *et al.* 2002, Fernández *et al.* 2009, Riaza personal communication).

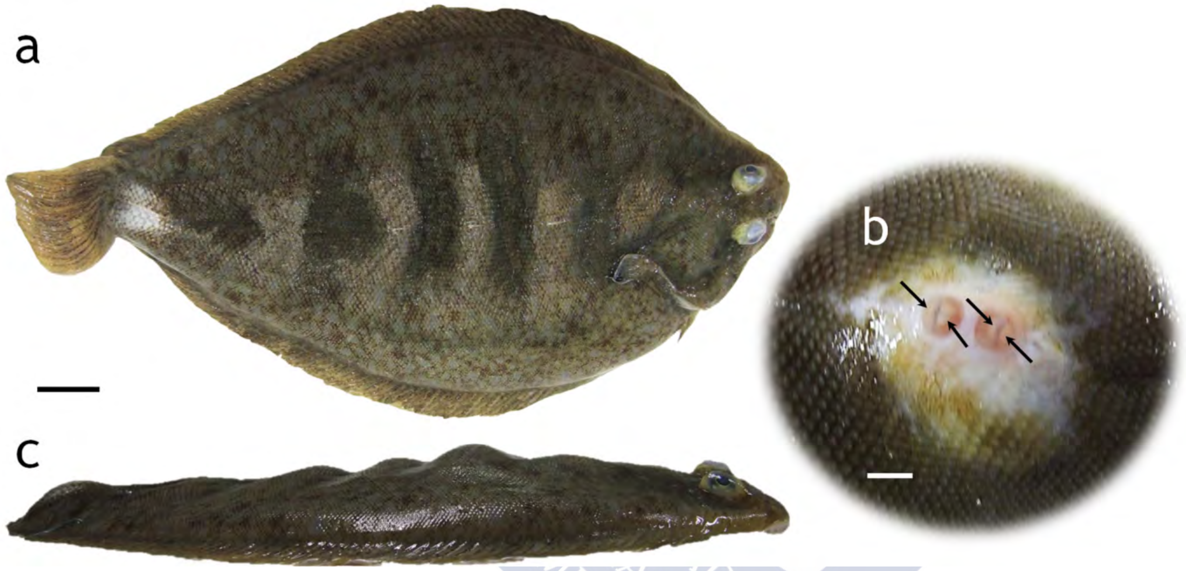


Figure 10: Photographs of a juvenile Senegalese sole (*Solea senegalensis*) (741 days after hatching) displaying severe skeletal abnormalities, especially scoliosis. (a) Ocular side aspect. (b) Small detail of the blind side of the fish, showing pigmentation and two deep ulcers in the skin in a convex area of the curvature which exposed the vertebral bodies (black arrows). (c) Ventral perspective. Note the bulge in the surface of the body. Bar = 2 cm; Insert bar = 0.5 cm.

In the literature, anomalies affecting fish skeleton are often addressed by different terminology, e.g. deformation, malformation, anomaly, and sometimes similar terminology refer to unrelated anomalies (Boglione *et al.* 2013b). However, considering the semiology, especially in the human medicine context, each one of the words refers to a specific disorder (Boglione *et al.* 2013b). In the following lines it is described the most common terms. Malformation is a primary defect in morphogenesis; syndrome represent several defects usually caused by only one factor; deformity or deformation is the alteration of the shape or structure after fetal stage, as a result of mechanical factors (Kumar *et al.* 1999); disorder is the same as alteration; abnormality or anomaly are particularities that an individual present compared to the majority of the individuals of its species; fusion is the abnormal coalescence of two adjacent parts; bone ankylosis refers to the abnormal union of the bones of a joint;

kyphosis is the abnormal curvature with a dorsal prominence of the vertebral column; lordosis refers to the vertebral column curvature with anterior convexity, opposed to kyphosis; scoliosis mean the lateral deviation of the rachis (Iracet 2003). A standardized classification of skeletal anomalies is still lacking in the literature for some fish species (Boglione *et al.* 2013b). In Atlantic salmon (*Salmo salar*), Witten *et al.*, 2009 proposed an extensive classification for vertebral body anomalies using radiography and a general glossary including skeletal disorders was also elaborated for some European reared fish species (Baeverfjord *et al.* 2009). Still, some gaps persist on the terminology and unification of criteria to describe anomaly typology affecting the vertebral column of Senegalese sole.

3.2. AETIOLOGY

Due to the significance of this problem, several studies have been conducted on causative factors (reviewed in Boglione *et al.*, 2013a,b). In fact, the cause of skeletal anomalies has a multi-factorial nature (Boglione *et al.* 2013b), where environmental (Georgakopoulou *et al.* 2010, Prestinicola *et al.* 2013), genetic (Gjerde *et al.* 2005, Opstad *et al.* 2013) and nutritional factors may be involved (Cahu *et al.* 2003b, Lall & Lewis-McCrea 2007, Fernández *et al.* 2008). Moreover, parasites, bacteria and pollutants could also influence the development of deformities although they are usually well controlled under industrial settings (Madsen *et al.* 2001, Gisbert *et al.* 2008, Boglione *et al.* 2013b).

The importance of environmental factors is indubitable since intensive rearing protocols might be characterized by high incidences of skeletal abnormalities (Russo *et al.* 2010). Moreover, in gilthead seabream it is possible to ameliorate the morphological quality of reared juveniles by acting on water volumes, hydrodynamics and administered food preys (rearing under semi-intensive conditions) (Prestinicola *et al.* 2013). Elevated water temperatures are commonly applied for fast-growing Atlantic salmon (Ytteborg *et al.* 2010a, Boglione *et al.* 2013b). However, thermic conditions may influence the development of deformities, at egg incubation as well as during larval rearing in this and other species (Sfakianakis *et al.* 2004, Georgakopoulou *et al.* 2010, Ytteborg *et al.* 2010a, Dionísio *et al.* 2012). Moreover, the frequency of skeletal deformities in Senegalese sole larvae augmented due to the combined effect of warming and hypercapnia (Pimentel *et al.* 2014). In turn, salmonid vertebrae under hypercapnia showed an increased vertebral trabecular volume and a higher rate of bone remodelling (Gil Martens *et al.* 2006). Furthermore, there could be an association between the presence of vertebral column anomalies and the time of exposure to hypoxia (Sánchez *et al.* 2011). Artificial light conditions, including photo-cycle, light intensity and spectrum, could have a negative effect on the welfare and development of fish (Blanco-Vives *et al.* 2010, Villamizar *et al.* 2011, Handeland *et al.* 2013). In this sense, for Senegalese sole, the optimal light conditions are those closer to its natural environment, namely, natural light-cycle with blue wavelength spectrum (Blanco-Vives *et al.* 2010). In European seabass (*Dicentrarchus labrax*), the development of lordosis was related with hydrodynamic conditions endorsed to increased muscular activity in the caudal fin and/or the action of the muscles on the vertebral axis (Kihara *et al.* 2002, Kranenbarg *et al.* 2005b, Sfakianakis *et al.* 2006). In this sense, a higher skeletal anomaly load was registered by Prestinicola *et al.*, 2014 in gilthead seabream displaying non-inflated swim bladder, particularly affecting the pectoral fin. The overuse of the pectoral fin flapping, to maintain buoyancy in these fish, possibly augment the muscular activity and the mechanical load in the surrounding muscles and bones (Prestinicola *et al.* 2014). High water current exercised rainbow trout (*Oncorhynchus mykiss*) showed some micro-morphological changes in the vertebral centra trabeculae (Owen *et al.* 2012). Even the use of certain vaccines can constitute an important risk factor linked to the development of skeletal abnormalities (Aunsmo *et al.* 2008). However, some controversy persists on the causes underneath such association affecting bone development: the mechanical trauma of the injection, the inflammation or the systemic reaction produced (Gil-Martens 2010, Berg *et al.* 2012, Boglione *et al.* 2013b).

Some vertebral deformities can be influenced by a genetic component (Gjerde *et al.* 2005, Garcia-Celdrán *et al.* 2015, Lee-Montero *et al.* 2015) and show a certain heritability, or a familiar association (Afonso *et al.* 2000, Castro *et al.* 2008, Bardon *et al.* 2009). In addition, the application of polyploidy in some reared fish species resulted in a higher incidence of skeletal deformities (Opstad *et al.* 2013, Fraser *et al.* 2015, Amoroso *et al.* 2016). However, it remains unclear if the anomalies are originated by the triploidy itself or by the induction shock applied to the eggs (Boglione *et al.* 2013b). In this sense, cold shock induced triploid turbot (*Scophthalmus maximus*) showed no significant variation in the percentage of deformities compared to diploid group (Hernández-Urcera *et al.* 2012).

Nutrition plays an important role during fish early stages development (Cahu *et al.* 2003b). In particular, dietary imbalances can provoke impairments in skeletogenesis and bone metabolism, resulting in deformities (Conceição *et al.* 2007, Lall & Lewis-McCrea 2007). In juvenile Atlantic halibut (*Hippoglossus hippoglossus*), different diets containing high vitamin A content, oxidized oil, low vitamin C or low phosphorous (P) produced specific patterns of skeletal anomalies (Lewis-McCrea & Lall 2010). Regarding vitamins, these are involved in skeletal tissue formation, especially vitamins A, C, D, K and E (Gisbert *et al.* 2008). In Senegalese sole and gilthead seabream larvae, high amounts of dietary vitamin A can lead to skeletal abnormalities, affecting mainly the cranial skeleton, vertebral centra and caudal fin complex (Fernández *et al.* 2008, 2009). An excess of this vitamin in diet accelerated the intramembranous ossification process of vertebral centrums (Fernández *et al.* 2008, 2009), although skeletal elements with endochondral ossification may present a higher sensitivity to this nutrient imbalance than intramembranous bones (Fernández & Gisbert 2010). At microscopic level, Fernández *et al.*, 2012 suggested that hypervitaminosis A accelerate bone mineralization which might increase mechanical load at vertebral end-plates, inducing vertebral deformities in gilthead seabream juveniles through a bone remodelling process. In addition, studies suggest that retinol dietary levels for an optimal larval development depend on the skeletal structures under consideration (Mazurais *et al.* 2009) and on larval developmental stage (Fernández & Gisbert 2011).

In general, fish are unable to synthetize vitamin D_3 in skin or vitamin C, and therefore they obtain these nutrients from food (Darias *et al.* 2011a). Vitamin D_3 deficiency has an impact on digestive system ontogenesis with consequences on minerals absorption and bone mineralization, leading to the appearance of skeletal anomalies (Darias *et al.* 2010b). Both vitamins influence bone ossification degree by regulating osteoblast differentiation and bone mineralization (Darias *et al.* 2011a). Therefore, an interruption of these processes might induce skeletal deformities (Darias *et al.* 2011a) particularly; vitamin D_3 especially affected intramembranous bones, whereas vitamin C influenced mainly the skeletal structures undergoing chondral ossification (Darias *et al.* 2010b, 2011a,b). Moreover, in haddock (*Melanogrammus aeglefinus*), vitamin K deficiency in diet may primarily decrease bone mineralization, although with time, it could increase the susceptibility to develop skeletal anomalies (Roy & Lall 2007). In this sense, the use of vitamin K supplemented diets decreased the incidence of bone deformities in Senegalese sole larvae, in a significant manner of vertebral fusions and malformed haemal and neural arches or spines in the caudal region (Richard *et al.* 2014). Studies using vitamin mix reported a lower rate of vertebral column deformities and a higher ossification status at higher dietary concentrations of the mix (Mazurais *et al.* 2008).

Regarding other nutrients as minerals, calcium (Ca) and P are closely related with skeletal development and maintenance. Fish are able to absorb these elements directly from water (Lall & Lewis-McCrea 2007). Suboptimal P levels and impaired bone mineralization can predispose juvenile Atlantic salmon to develop long-term skeletal disorders (Fjelldal *et al.* 2012a). However, a primary P-deficient vertebral phenotype in Atlantic salmon juveniles was characterized by homogenous osteomalacia, with continuous bone formation, albeit without mineralization or skeletal anomalies. Thus, the deficit in mineralization must not be the only cause of the alterations of the vertebral bone in this species (Witten *et al.* 2015). Studies indicate that a supplementation with dietary minerals may reduce the incidence of vertebral anomalies in Atlantic salmon smolts (Fjelldal *et al.* 2009a), while triploid salmon seems to require a higher P dietary amount than diploid fish regarding deformities rate (Fjelldal *et al.* 2016). In rainbow trout, P deficiency also impairs bone mineralization, whereas an excess of this nutrient affects survival. In turn, Ca dietary deficiency induces a delay on ossification, without affecting bone calcification (Fontagné *et al.* 2009).

In European seabass larvae, it appears that the ratio phospholipid/neutral lipid is more related to the frequency of deformities than the overall lipid content in the diet (Cahu *et al.* 2003a). In addition, the nutritional quality of live preys during early feeding could be a determinant factor in the appearance of anomalies in red porgy larvae (*Pagrus pagrus*) (Izquierdo *et al.* 2010, Boglione *et al.* 2013b). Particularly, higher docosahexaenoic acid (DHA) amounts in rotifers significantly improved larval quality (Roo *et al.* 2009). On the other hand, arachidonic acid dietary levels showed no influence on skeletal anomalies in Senegalese sole (Boglino *et al.* 2012a).

Enriched *Artemia* spp. metanauplii are commonly incorporated to sole diet during larval rearing (Conceição *et al.* 2007, Boglino *et al.* 2012b, Rodríguez & Peleteiro 2014), although they may present some dietary disbalances, requiring supplements in order to improve larval performance (Richard *et al.* 2014). Therefore, the nutritional value of live prey is important to the success of larval rearing (Morais *et al.* 2004), but it can vary according to culture/enrichment conditions (Conceição *et al.* 2010). Nowadays, there are diverse commercial products for the enrichment of *Artemia* spp., dislaying differencies in the physical form, nutritional content or ingredients (Boglino *et al.* 2012b). In this sense, there are scarce reports about the influence of the enrichments on the development of bone deformities in reared Senegalese sole, and mostly focused on lipidic profile (Dâmaso-Rodrigues *et al.* 2010, Boglino *et al.* 2012b). Hence, it is essential to determine their role in the development of skeletal abnormalities since they could present nutritional levels considered as non optimal for skeletogenesis in this species.

The response of anomalies development to environmental or nutritional conditions could be dependent of the affected anatomical region, due to different ontogenic stages, which present distinct nutritional requirements (Mazurais *et al.* 2009, Georgakopoulou *et al.* 2010). Table 4 briefly summarizes some of the factors affecting skeletal anomalies in flatfish, including Senegalese sole. Studies on this species are scarce and especially focused on nutrition at early stages of development (Morais *et al.* 2016).

Table 4: Main factors affecting skeletal anomalies in flatfish species. Image source: FishBase, 2016.

Species	Agent	Main anomalies	References
Bastard halibut (*Paralichthys olivaceus*) 	Vitamin D_3 and $1{,}25(OH)_2D_3$	Deviations in abdominal region	Haga *et al.*, 2004
Atlantic halibut (*Hippoglossus hippoglossus*) 	Phosphorous	Deformed neural and haemal elements	Lewis-McCrea and Lall, 2010
	Ascorbic acid	Scoliosis and bifurcated spines	
	Vitamin A	Scoliosis	
	Oxidized oil	Scoliosis	
Senegalese sole (*Solea senegalensis*) 	Temperature	Skeletal anomalies	Dionísio *et al.*, 2012
	Vitamin A	Skeletal anomalies	Fernández *et al.*, 2009, Fernández and Gisbert, 2010
	Vitamin K	Vertebral fusions Deformed neural spines	Richard *et al.*, 2014
	Hypercapnia	Severe deformities	Pimentel *et al.*, 2014
	Thermo-cycle	Jaw deformities	Blanco-Vives *et al.*, 2010
	Light	Jaw deformities	
	Photoperiod	Jaw deformities	

According to Boglione *et al.*, 2013a, the deformity aetiology scenario is complicated by the following observations: (i) different non-genetic factors can induce the same anomaly in different species; (ii) the same causative agent can originate different anomalies in different fish species (Boglione & Costa 2011); (iii) fish sensitivity to a causative factor may change during ontogeny (Mazurais *et al.* 2009, Georgakopoulou *et al.* 2010); (iv) the action of a single factor can be compensated by the action of another factor (Kihara *et al.* 2002, Sfakianakis *et al.* 2006); (v) the same cause may provoke a high incidence of anomalies in some skeletal elements but not in others with the same bone type and ossification, in the same individual (Fernández & Gisbert 2011).

3.3. MONITORING AND DIAGNOSTIC TECHNIQUES

Even when optimal conditions for normal development of fish species have been standardized, fish quality has to be routinely monitored and continuously verified in order to either guarantee the conformity of the protocols, or generate an early warning for fault/accidental alterations of the complex hatchery process (Koumoundouros 2010). Nowadays, the traditional procedure to control and monitor spinal abnormalities during the productive cycle in Senegalese sole farms still relies on palpation and visualization of specimens with an appropriate size (Figure 11a) (Rodríguez & Peleteiro 2014). Future research efforts should be focused on the development of automated processes for the mass removal of the deformed fish from the cultured stocks, on the establishment of methods for the early quality assessment, control and enhancement, as well as on the development of a scale of quality for the precise distinction of commercially severe deformities from those of scientific interest only (Koumoundouros 2010).

In Senegalese sole larvae, skeletal deformities were described using double staining technique for cartilage and bone (Gavaia *et al.* 2002, 2009). The procedure involves a diaphanization process and staining with alcian blue (with affinity for cartilage acid mucopolysaccharides) and the alizarin red (a fluorescent calcium dye, Bensimon-Brito *et al.*, 2016) for calcified structures (Gavaia *et al.* 2000). This staining is particularly interesting for ontogenic studies and skeletal anomaly detection as well to evaluate the ossification level in fish larvae and small juveniles (Figure 11b) (Gavaia *et al.* 2002, Darias *et al.* 2010a). Nevertheless, in larger juveniles and adults, the specimens' size limits the technique (Gisbert *et al.* 2008), which becomes time-consuming and laborious as they need more time for processing and stronger reagents to promote transparentation (Helland 2009). Radiographic procedures are suitable as well for the integral study of the skeletal system and it is the preferred technique for whole-body diagnosis of vertebral deformities in teleosts (Hjelde & Bæverfjord 2009), especially in medium-sized and large fish (Figure 11c,c') (Gisbert *et al.* 2012, de Azevedo *et al.* 2013). In addition, this non-invasive method can be utilized to estimate the degree of ossification/compactness of the bony elements (Deschamps *et al.* 2008, Fontagné *et al.* 2009) and to *in vivo* diagnose other disorders, namely involving the skeletal and urogenital systems, swim bladder and otholits (Ortega 2008, Macrì *et al.* 2012). Moreover, it is being routinely applied in Sparidae fish farms in order to prove the absence of skeletal anomalies at juvenile commercialization to ongrowing farms (Boglione & Costa 2011). However, the use of X-ray techniques at an industrial setting or for scientific research is very scarce in Senegalese sole. Cardeira *et al.*, 2015 employed this approach to describe the morphological changes associated to lordotic and kyphotic vertebral curvatures in this species. Computed tomography images scan can provide significant information on vertebral structures and deformities (Figure 11d), as so as, differentiate tissues of density (Gisbert *et al.* 2012, de Azevedo *et al.* 2013). However, as conventional equipment is designed for medical applications, the size of the specimens is the main limitation for the application of this imaging technique (Gisbert *et al.* 2012, de Azevedo *et al.* 2013). In this sense, the use of microcomputed tomography (microCT) could be helpful in order to assess in detail the skeleton of small-size fish (Neues *et al.* 2007, Epple & Neues 2010, Losada 2016).

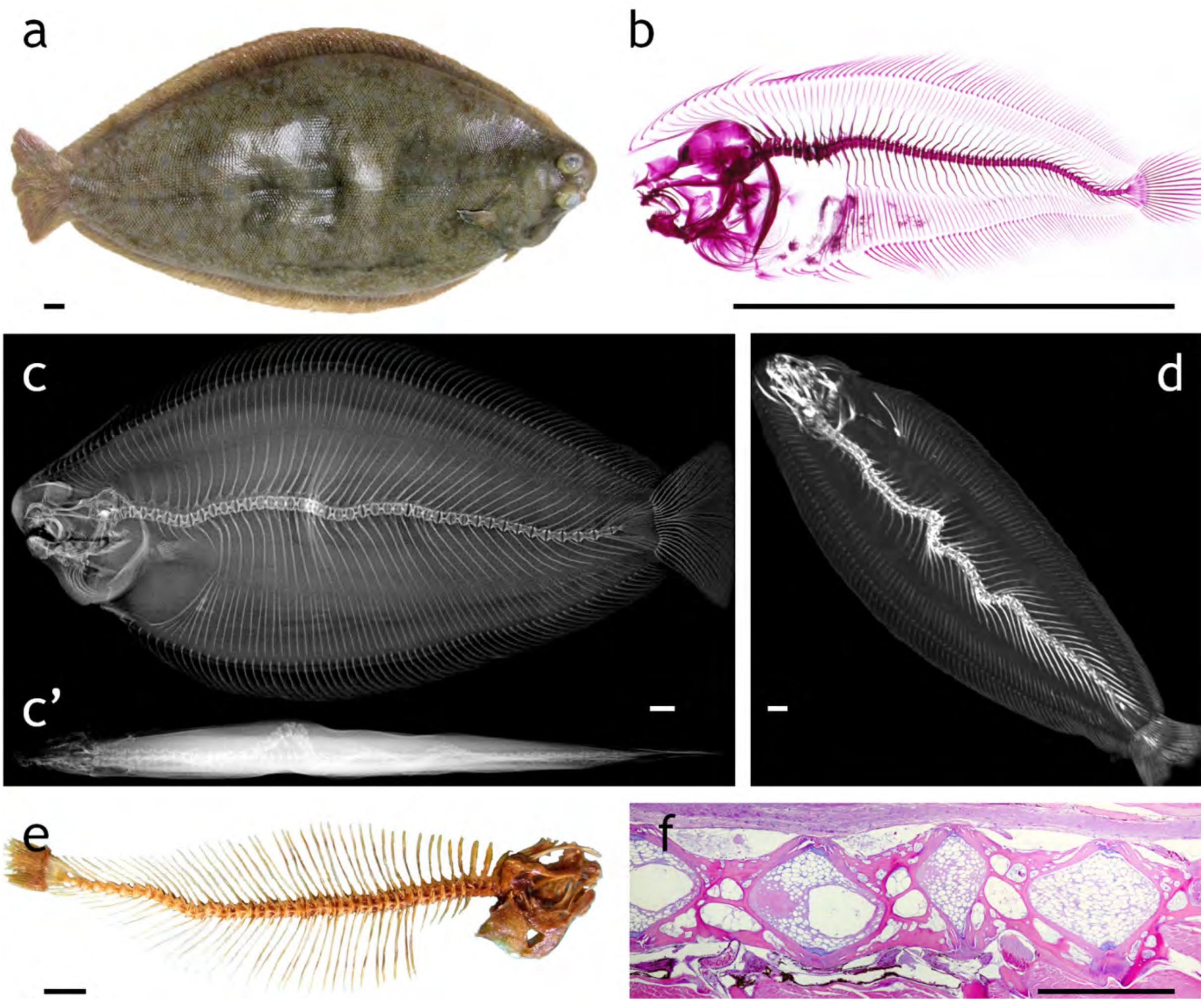


Figure 11: Different diagnostic methods for the assessment of skeletal anomalies in Senegalese sole (*Solea senegalensis*). (a) Macroscopic visualization. (b) Double staining technique for bone and cartilage. (c) and (c') Computed radiography: latero-lateral (c) and dorso-ventral projection (c') of the same specimen as (a). (d) Oblique image of computed tomography of the same specimen as (a), (c) and (c'). (e) Dissected skeleton of a juvenile Senegalese sole after maceration in KOH solution. (f) Histological section of a deformed segment of the vertebral column in a 105 days after hatching Senegalese sole (Bar = 1 mm). Bars = 1 cm.

Different methods can be used to obtain dissected fish skeleton in order to detect skeletal deformities, mainly through trypsin digestion, KOH solution maceration or even with dermestid beetles (*Dermestes maculatus*) (Figure 11e) (Witten *et al.* 2005, Yurtseva *et al.* 2014, Chin *et al.* 2017). Furthermore, non-toxic *in vivo* diagnostic techniques as alizarin red S *in vivo* staining permit the continuous monitoring of skeletal development and tracking of deformities in living individual zebrafish (Bensimon-Brito *et al.* 2016). The application of this protocol with a green fluorescent protein reporter transgenic line may be relevant for the study of bone pathologies (Bensimon-Brito *et al.* 2016).

The assessment of bone status and performance regarding mineral content, vertebral biomechanical properties, bone area and vertebral bone profile (among others) was also conducted majorly in salmonids, using distinct combined techniques such as biochemistry, texture analysis, radiography, microCT, and histomorphometry and/or mathematical and computer modelling (Kranenbarg *et al.* 2005a, Deschamps *et al.* 2009, Fjelldal *et al.* 2012c, Owen *et al.* 2012).

Other methods were employed in order to study bone deformities, especially when considering microscopic and molecular changes. Namely, histological, histochemical and immunohistochemical approaches are valuable tools for providing basic knowledge on bone formation and organization (Ytteborg *et al.* 2012, Ortiz-Delgado *et al.* 2014). These techniques have been applied to study the structural changes occurring in deformed skeletal elements and the possible mechanisms by which they develop (Witten *et al.* 2005, Ytteborg *et al.* 2012, Ortiz-Delgado *et al.* 2014). Nevertheless, there are scarce information characterizing the architecture and response of fish bone, particularly anosteocytic bone, to mechanical load (Dean & Shahar 2012). In few studies, vertebrae were assessed at a tissue level in anosteocytic bone fish as gilthead seabream (Fernández *et al.* 2012, Ortiz-Delgado *et al.* 2014) and European seabass (Kranenbarg *et al.* 2005a). However, in Senegalese sole, scarce reports exist on the histological features of normal and deformed bone (Figure 11f). In this sense, Cardeira *et al.*, 2012, 2015 characterized the micro-anatomical changes in lordotic and kyphotic vertebral curvatures for this species using histologic and histochemical procedures. Therefore, investigation with techniques that illustrate vertebral column disorders at tissue level could contribute to increase the understanding of osseous reaction underneath the development of different vertebral anomalies in this flatfish species.

One of the challenges for fish skeletal anomaly studies has been the lack of information on bone formation during development and reliable biochemical markers to characterize specific changes (Ytteborg *et al.* 2012). Few studies have been reported on the molecular events during abnormal bone development in fish species (Wargelius *et al.*, 2010, Ytteborg *et al.*, 2010a,b,c, 2012). Further analyses identified 20 genes involved in the skeletal development of the Atlantic salmon vertebrae (Ytteborg *et al.* 2010a) using real time PCR gene expression and *in situ* hybridization. Some of these markers were analysed in latter studies in order to detect cellular activity in vertebrae, namely *osteocalcin*, *osteonectin*, *col1a1*, *col2a1*, *col10a1*, *runx2* and *mef2c* (Ytteborg *et al.* 2016). In Senegalese sole, studies on genes encoding osteocalcin and matrix Gla protein provided a comprehensive description of the onset of their expression and sites of protein accumulation regarding skeletal structures throughout larval development (Gavaia *et al.* 2006). Recently, the analysis of their expression (among others) has been employed to investigate the possible mechanisms of vitamin K action in sole skeleton (Richard *et al.* 2014).

3.4. MAJOR CONCERNS AND KNOWLEDGE REQUIREMENTS

Despite the new impetus of Senegalese sole industrial rearing driven by recent advances on zootechnical, biological and nutritional features (Morais *et al.* 2016), further knowledge is needed on skeletal anomalies affecting seriously this species in order to optimize this flatfish production. The main gaps of knowledge concerning skeletal deformities can be divided mainly into diagnostic, causative factors and skeletogenic features (Boglione *et al.* 2013b). As mentioned previously, Senegalese sole fish farms still rely on macroscopic manual sorting of deformed fish, especially at the time of juvenile transfer to ongrowing farms (Rodríguez & Peleteiro 2014), and therefore lack systematic deformity monitoring routines as implemented for other species (European seabass, gilthead seabream) (Boglione *et al.* 2013b). The precise control of all stages of the rearing process would benefit the identification of the responsible anomaly causative factors and would guarantee the conformity of the applied rearing protocols for normal skeletal development in fish batches (Koumoundouros 2010). In order to monitor skeletal defects in different stages of industrial production of Senegalese sole, further attention should be paid on accurate diagnostic techniques that could be used in a massive scale and applied in distinct fish rearing phases. Nowadays, bone deformities on Senegalese sole have been described mainly on experimentally reared larvae and juveniles (Gavaia *et al.* 2002, 2009). The information regarding the incidence of skeletal abnormalities on this species aquaculture is scarce and lacks an exhaustive screening of the skeletal anomaly profile for the main stages of production, to establish benchmarks to compare levels of deformity (Howell *et al.* 2009). In addition, standardized terminology, gathering industrial and scientific criteria is missing for the different skeletal anomalies (Boglione *et al.* 2013b), particularly in Senegalese sole. This systematized classification could provide a pattern to improve diagnosis, connect particular types of anomalies to particular causal factors and to better discern deformity transitory stages (Witten *et al.* 2009, Boglione *et al.* 2013b). In this regard, more investigation is required on the progression of deformities during life and their impact on external morphology at the end of the hatchery and ongrowing phases (Koumoundouros 2010, Boglione *et al.* 2013b), on fish rearing performance and welfare.

Deformity causative factors constitute a major gap of knowledge in many fish species (Boglione *et al.* 2013b). Studies on Senegalese sole skeletal anomaly aetiology have been conducted mainly on experimental research centres (Fernández *et al.* 2009, Dionísio *et al.* 2012, Pimentel *et al.* 2014). Investigation in commercially reared fish is important in order to incorporate multilevel factors interacting with developing fish in an unknown way, that are not reproducible under experimental conditions (Boglione *et al.* 2013b, Prestinicola *et al.* 2013). Furthermore, to address this multi-factorial problem, multilevel investigations should be carried out, integrating environmental, genetic, biomolecular, deformity frequency and production data, among others (Boglione *et al.* 2013b), for which the use of multilevel mathematical models and neural networks would be advantageous (Aunsmo *et al.* 2009, Russo *et al.* 2010, 2011).

Few studies have been conducted on the aetiology of skeletal abnormalities in Senegalese sole (Morais *et al.* 2016). Further research would be required on environmental and nutritional factors and their interactions affecting deformity development in this species. Moreover, it would be also interesting to corroborate the nutrients role on skeletal development under the diverse environmental conditions of fish hatcheries and farms (Lall & Lewis-McCrea 2007).

As for skeletogenesis, the literature is scarce on the organization and material properties of fish anosteocytic bone (Dean & Shahar 2012). Studies unveiling bone response to distinct causative factors of deformities are lacking for this species. Therefore it would be interesting to investigate on molecular pathways linking mechanical load and bone development (Boglione *et al.* 2013a), as well as on the pathogenic mechanisms underlying different types of bone deformities in Senegalese sole. In this regard, the use of histopathological techniques could be helpful to establish a connection between molecular and tissue events occurring during the onset of skeletal anomalies. Moreover, new insights are required on the characterization of the diverse types of ossification in fish skeletal elements (Boglione *et al.* 2013a).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Objectives


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OBJECTIVES

The present Doctoral Thesis is part of a wider research project entitled "Influence of environmental and nutritional factors on Senegalese sole (*Solea senegalensis*) skeletogenesis" (10MMA020E) from Xunta de Galicia, Spain. This project was conducted in a close collaboration with a Senegalese sole aquaculture industry. The work intends to deepen into the study of skeletal anomalies occurring in diverse stages of development of reared Senegalese sole, focusing in different levels. On one hand, it aims to establish a profile of most common deformities at distinct farming phases, using different diagnostic techniques. This study also pursues to evaluate the influence of dietary component on the skeletogenesis of larvae and juvenile Senegalese sole.

Therefore, the specific objectives for this Doctoral Thesis are:

- To assess the suitability of traditional (observation and palpation) and radiographic techniques for the proper detection of anomalies affecting the skeleton of farmed juvenile Senegalese sole and subsequently define a straightforward method for their evaluation using X-rays.
- To comprehensively characterize the main skeletal anomalies in this species at larval and ongrowing juvenile by means of stereoscopy and computed radiography, providing some criteria to meet the demand for a uniform categorization of anomaly typologies.
- To compare the anomaly pattern among developmental stages, taking into consideration the impact of each deformity on the respective anomaly profile.
- To evaluate the presence of vertebral anomalies in larvae and juveniles fed with *Artemia* spp. metanauplii enriched with different commercial products by means of multivariate approaches.
- To study normal and deformed vertebral segments of juvenile Senegalese sole by means of radiographic and histological procedures in order to illustrate the histopathological changes associated to different types of vertebral body anomalies.
- To deepen into the knowledge on the possible pathogenic mechanisms inherent to the development of vertebral anomalies.


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Article I

ARTICLE I: SKELETAL MALFORMATIONS IN SENEGALESE SOLE (*SOLEA SENEGALENSIS*, KAUP 1858): GROSS MORPHOLOGY AND RADIOGRAPHIC CORRELATION

AP Losada[1], AM de Azevedo[1], A Barreiro[1,2], JD Barreiro[1,2], I Ferreiro[3], A Riaza[3], MI Quiroga[1] and S Vázquez[1]

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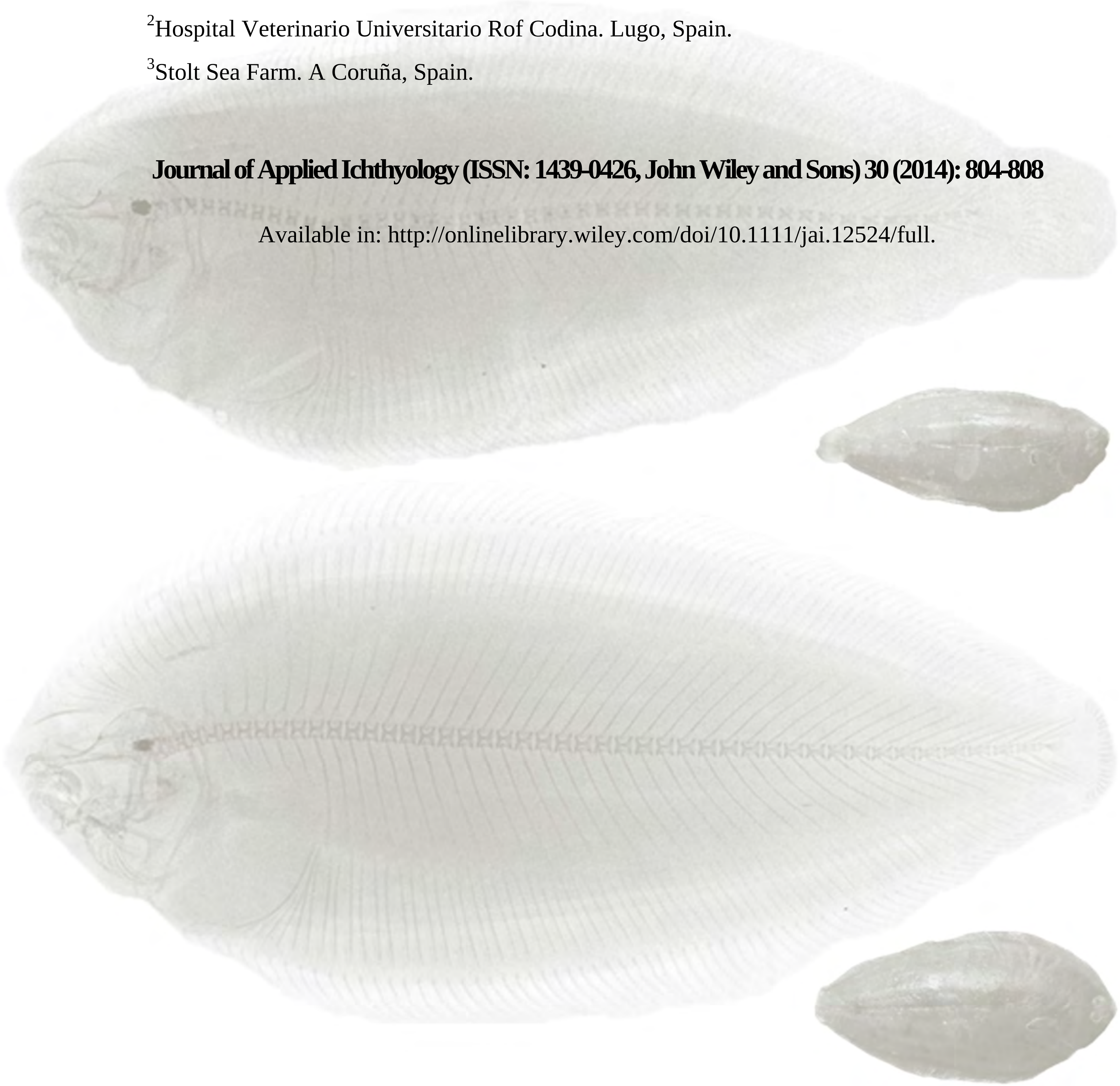


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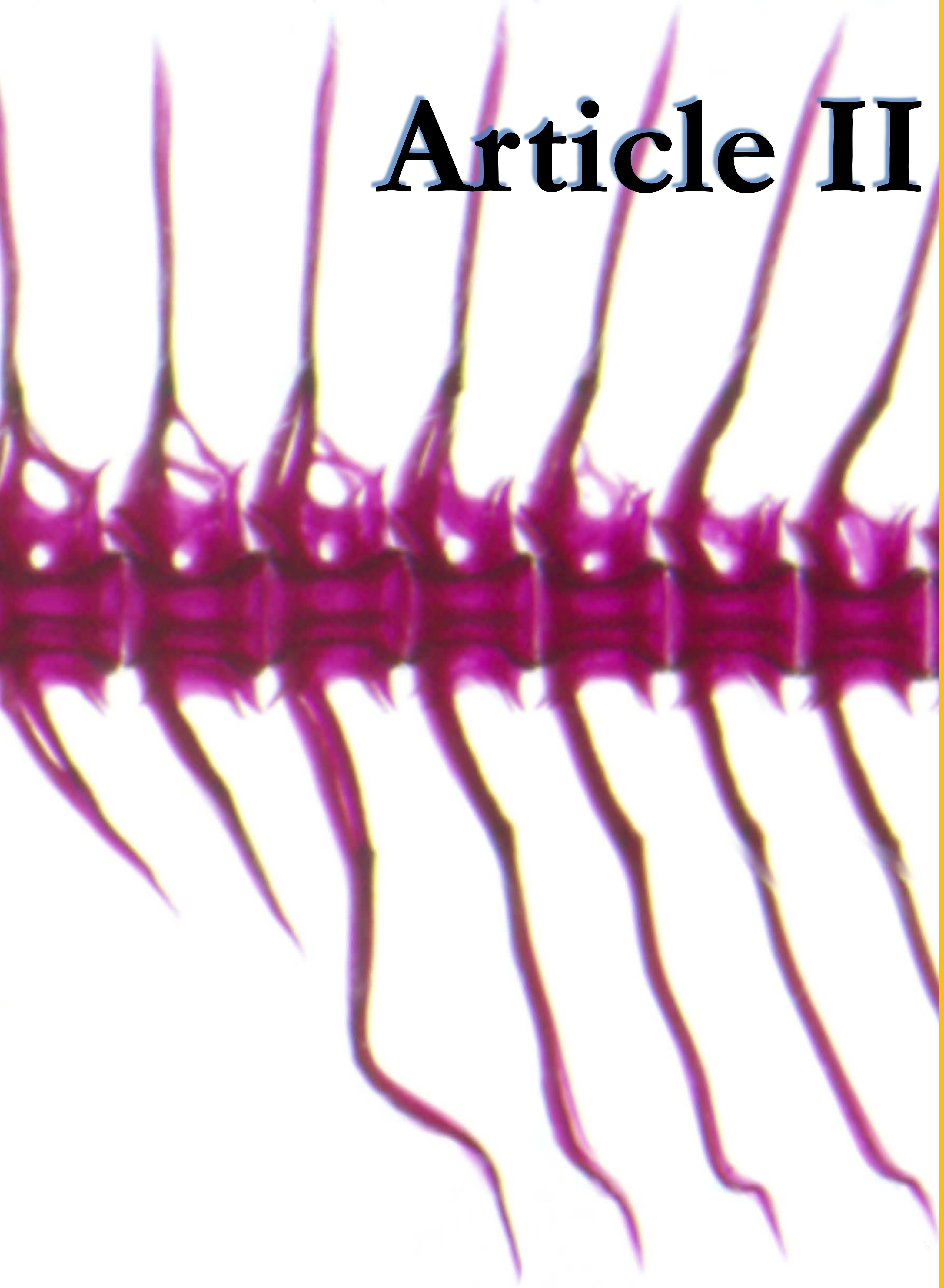
Article II

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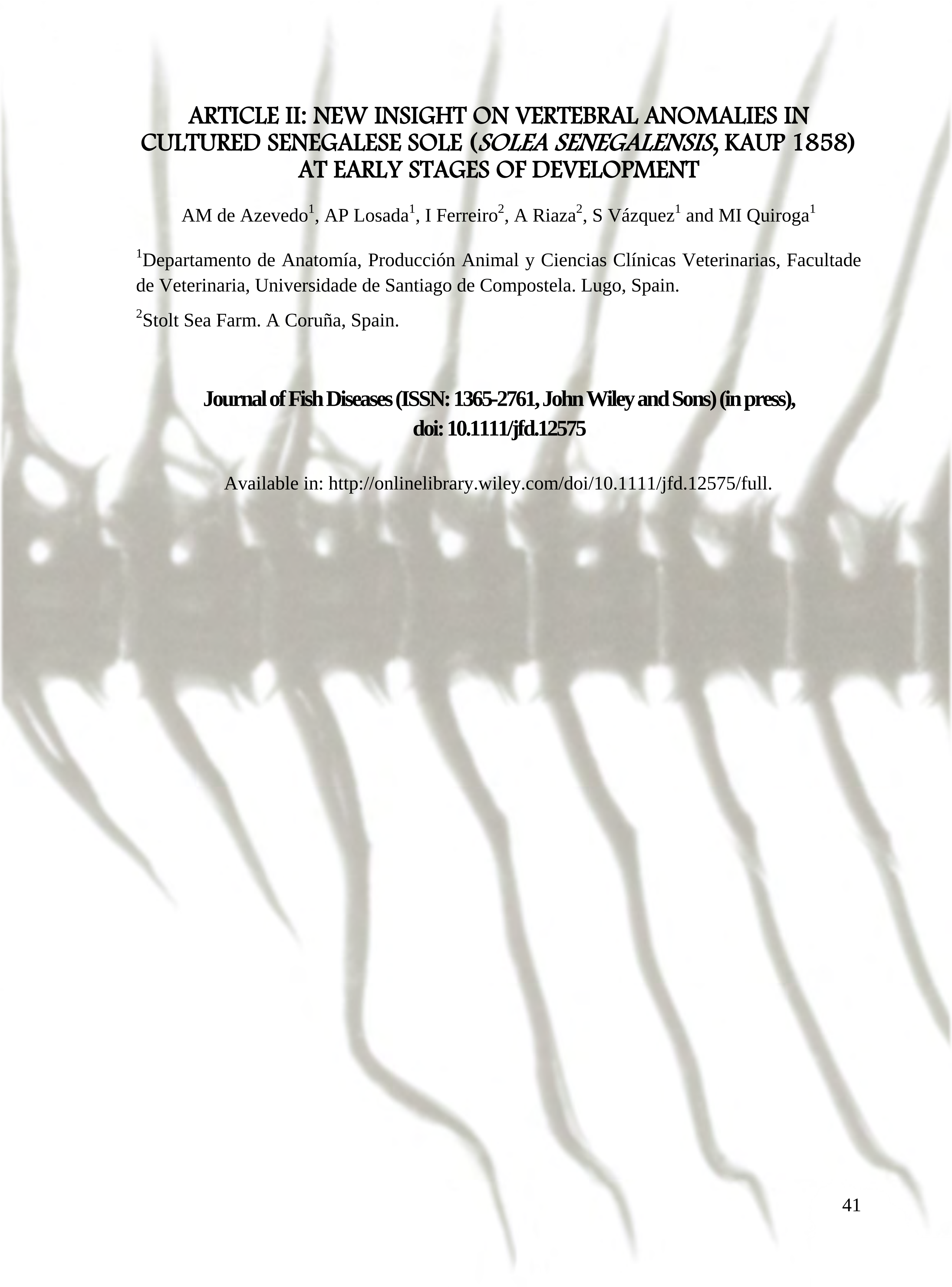

ARTICLE II: NEW INSIGHT ON VERTEBRAL ANOMALIES IN CULTURED SENEGALESE SOLE (*SOLEA SENEGALENSIS*, KAUP 1858) AT EARLY STAGES OF DEVELOPMENT

AM de Azevedo[1], AP Losada[1], I Ferreiro[2], A Riaza[2], S Vázquez[1] and MI Quiroga[1]

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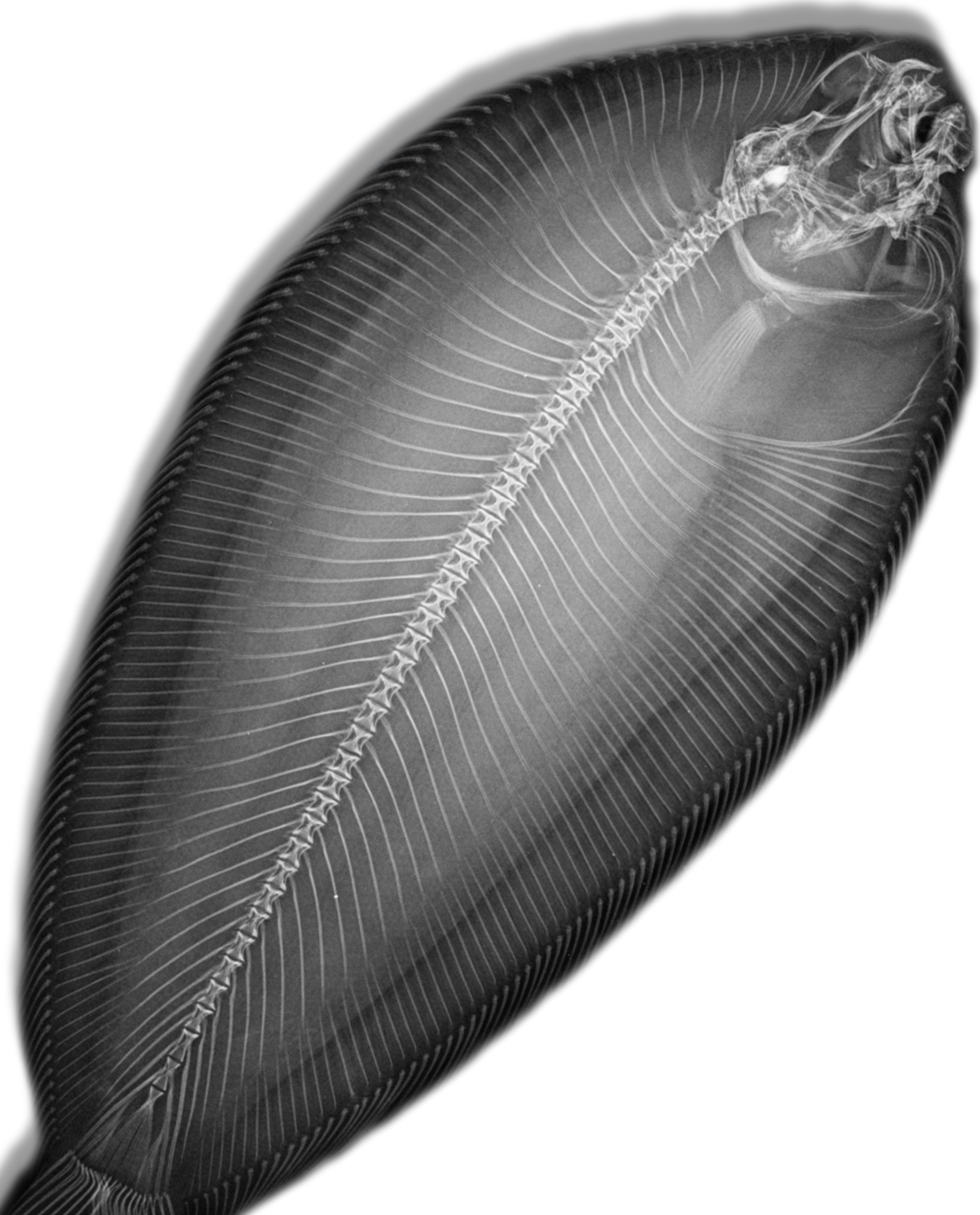
Article III

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ARTICLE III: SKELETAL ANOMALIES IN REARED SENEGALESE SOLE (*SOLEA SENEGALENSIS*, KAUP 1858) JUVENILES: A RADIOGRAPHIC APPROACH

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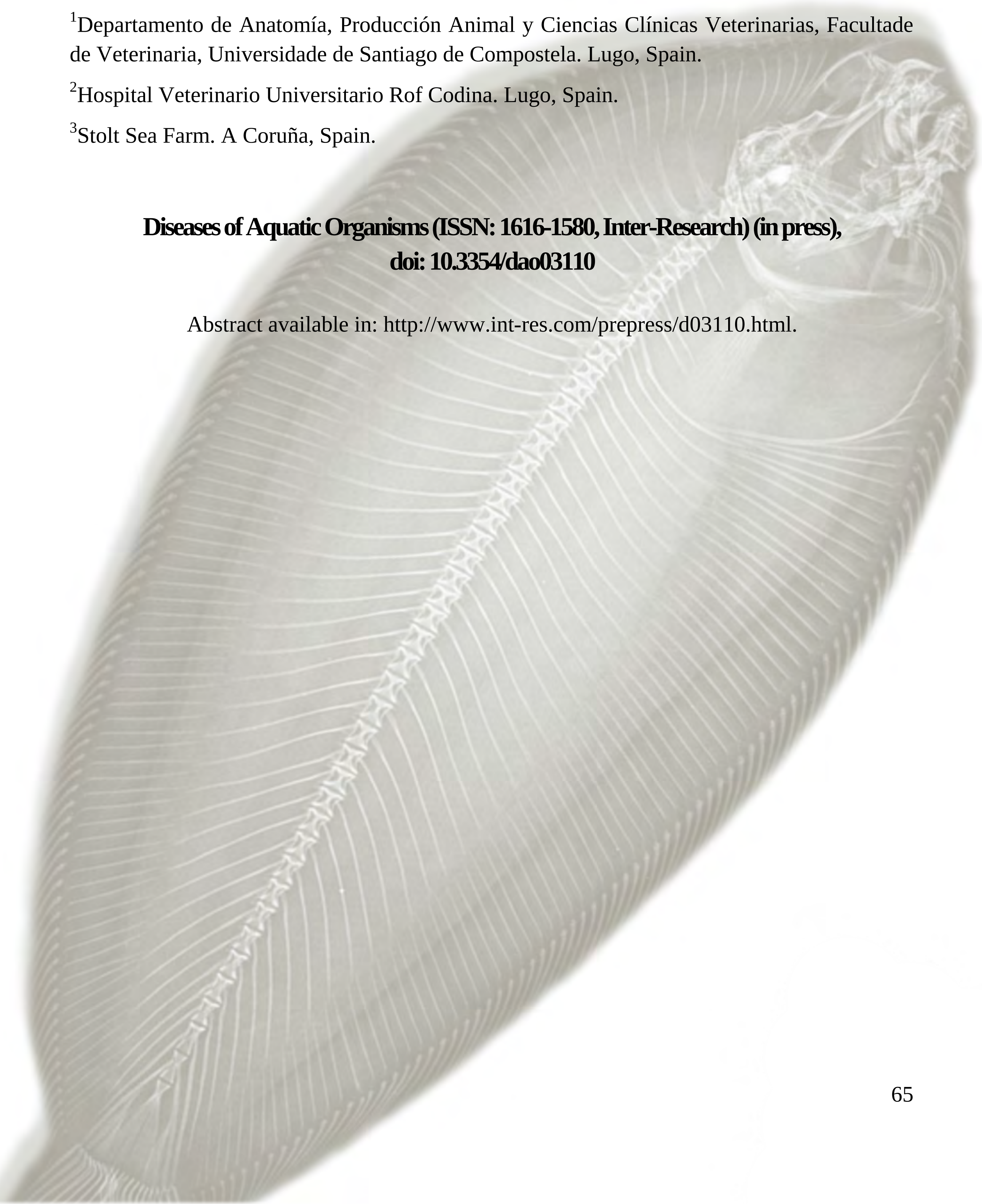


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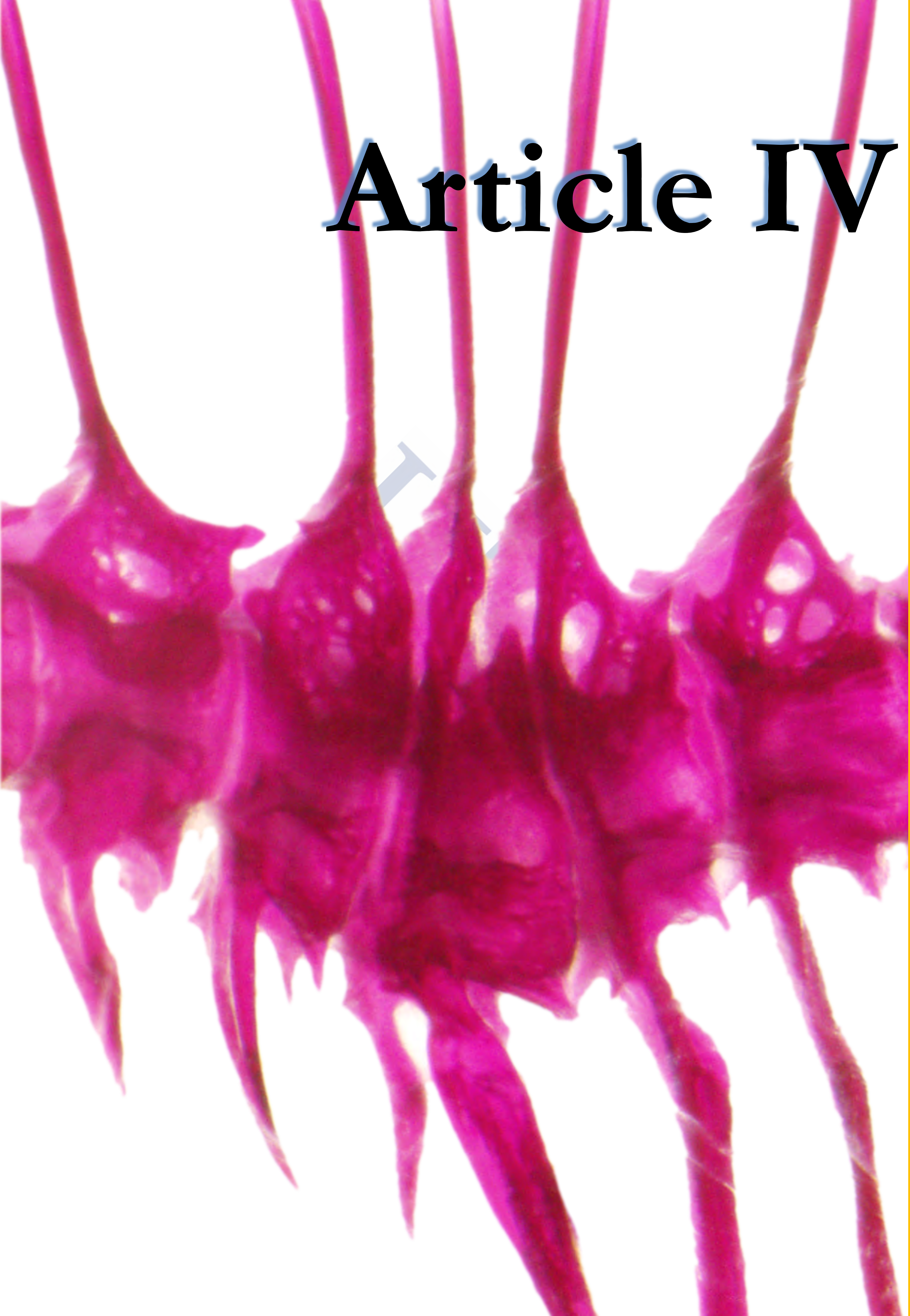
Article IV

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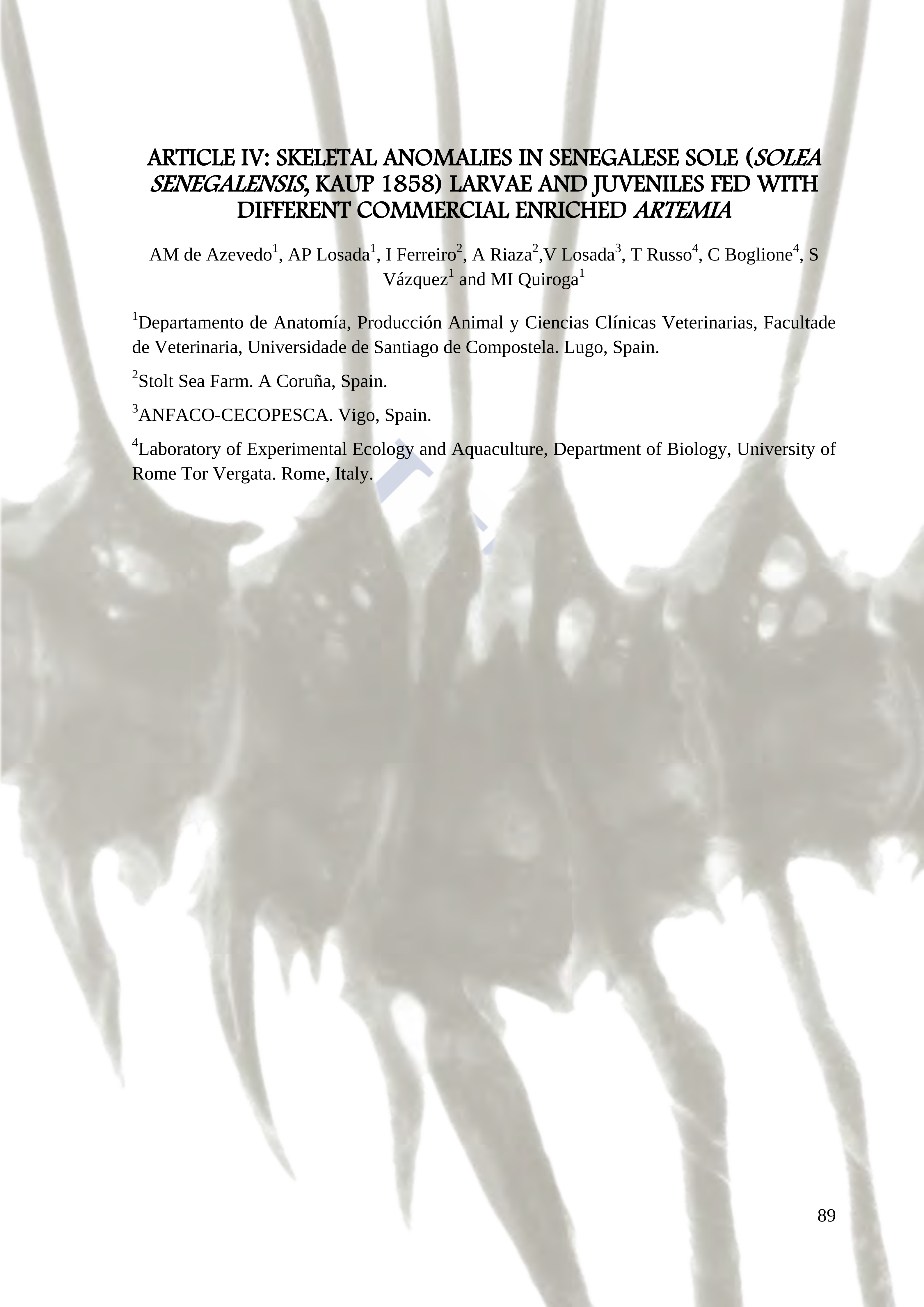

ARTICLE IV: SKELETAL ANOMALIES IN SENEGALESE SOLE (*SOLEA SENEGALENSIS*, KAUP 1858) LARVAE AND JUVENILES FED WITH DIFFERENT COMMERCIAL ENRICHED *ARTEMIA*

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ABSTRACT

Senegalese sole (*Solea senegalensis*) is a marine flatfish species with a great potential for aquaculture. However, the high incidence of skeletal anomalies still constitutes one of the bottlenecks constraining its production. The correct skeletal development could be impaired by nutritional imbalances provoking vertebral deformities. Nowadays, there are diverse commercially available products for the enrichment of live preys. However, there are few reports of the influence of the enrichments on the development of bone anomalies in reared Senegalese sole. The aim of this study was to evaluate the presence of vertebral anomalies in larvae and juvenile Senegalese sole fed with *Artemia* spp. metanauplii enriched with four different commercial products (EA, EB, EC and ED) by means of multivariate approaches. Fish were reared in a fish farm in two separated tanks for each diet and sampled at 31 and 105 days after hatching (dah) comprising a total of 407 individuals. For anomaly detection and meristic counts, double staining technique for cartilage and bone was performed. Statistical study involved NPMANOVA tests and correspondence analysis (CA). In this study the incidence of skeletal anomalies was very high (100%), since every specimen showed at least one alteration at both stages. The most frequent alterations consisted in deformations of the neural/haemal arches and spines, fusions and deformations of hypurals, epural or parhypural. Vertebral body anomalies (VBA) and vertebral column deviations (VCD) were less common, prevailing fusions and/or alterations of the shape of preurals. CA output ordered fish from each age in separated semiaxis indicating a different anomaly pattern for each stage. The results showed some variations in the frequency of anomalies among diets, although the influence of enrichment products on the development of vertebral abnormalities at both sampling points was not clear. Nevertheless, individuals from EC and EA lots displayed less VBA and/or VCD at 31 and 105 dah, respectively. The existence of very common anomalies to all diets in both stages leads to deepen into the impact of rearing conditions underneath the onset of such group of anomalies. Remodelling and modelling processes during development should also be investigated.

Keywords: Skeletal anomalies, Senegalese sole (*Solea senegalensis*), enrichment products, multivariate analysis

1. INTRODUCTION

Senegalese sole (*Solea senegalensis*) is a marine flatfish species with an increasing production over the last few years in Europe (FAO 2016). However, skeletal anomalies still represent one of the major limitations in its aquaculture (Gavaia *et al.* 2002, Fernández *et al.* 2009, Boglino *et al.* 2012b). Several reports, both in experimental as in industrial conditions, showed an incidence from 44% to 100% of deformed fish (Gavaia *et al.* 2002, 2009, Losada *et al.* 2014, de Azevedo *et al.* in press b). Studies in diverse cultured fish species showed the influence of environmental, nutritional, genetic and many other factors (Boglione *et al.* 2013a,b). Nutrition plays an important role during early stages of development (Cahu *et al.* 2003). In particular, dietary imbalances can provoke impairments in skeletogenesis and bone metabolism, resulting in skeletal abnormalities (Conceição *et al.* 2007, Lall & Lewis-McCrea 2007). Moreover, nutrients like vitamins A, C, D and K, minerals as calcium and phosphorus, and other elements as some fatty acids are related to the development of bone disorders (Lall & Lewis-McCrea 2007, Fernández *et al.* 2009, Fontagné *et al.* 2009, Darias *et al.* 2011, Richard *et al.* 2014).

Nowadays, there are diverse commercial products for the enrichment of *Artemia* spp. (Boglino *et al.* 2012b). These differences consist in the physical form, nutritional content or ingredients (Boglino *et al.* 2012b), although the composition of live prey can also vary according to culture/enrichment conditions (Conceição *et al.* 2010). Enriched *Artemia* spp. metanauplii are commonly incorporated to sole diet during larval rearing (Conceição *et al.* 2007, Boglino *et al.* 2012b, Rodríguez & Peleteiro 2014). However, there are scarce reports of the influence of the enrichments on the development of bone deformities in reared Senegalese sole. In this sense, the study of Boglino *et al.*, 2012b showed no significant effect of the analysed commercial enriching products in the total incidence of skeletal anomalies in experimentally reared larvae.

The aim of this study was to evaluate the presence of vertebral anomalies in larvae and juvenile Senegalese sole fed with *Artemia* spp. metanauplii enriched with different commercial products by means of multivariate approaches. In particular, we applied to our data the correspondence analysis (CA) and/or statistical test NPMANOVA as used in other studies on fish skeletal anomalies (Prestinicola *et al.* 2013). These tools could be effective in determining some possible pattern or relationship among lots and deformities (Boglione *et al.* 2003, 2009).

2. MATERIALS AND METHODS

2.1. REARING PROTOCOL

For this experiment, around 25000 Senegalese sole larvae from a single egg batch were reared in a fish farm in the northwest of Spain. Genetic test demonstrated that the offspring came from a single female and male. At one day after hatching (dah), larvae were stocked in eight cylindrical tanks of 155 L with an open flow system, at an initial density of 20 larvae L^{-1}. The number of fish in each tank was estimated by volumetric counting. The rearing procedure was identical for every tank. Water was daily renewed, starting from a 4% per hour to a 50% at the end of larval rearing in each tank; pH was the same as the sea water pH in the Atlantic Ocean (8.0-8.2) and photoperiod was 12 h light:12 h dark. At day 15, each batch of Senegalese sole was transferred to eight 25 L cylindrical tanks (EA-1, EA-2, EB-1, EB-2, EC-1, EC-2, ED-1, ED-2). A daily individual counting for dead fish was initiated at 15 dah. Table 1 illustrates the number of individuals in each tank at this point. At 28 dah, specimens were relocated at eight squared tanks of 130 L. During the experiment, water temperature and dissolved oxygen were respectively 19.2±0.3 °C and 6.9 ± 0.8 ppm: mean ± standard deviation (SD).

Table 1: Number of individuals in each tank (EA-1, EA-2, EB-1, EB-2, EC-1, EC-2, ED-1, ED-2) at 15 days after hatching.

Dietary Replicates	EA-1	EA-2	EB-1	EB-2	EC-1	EC-2	ED-1	ED-2
Number of individuals	2705	2568	2367	2354	2323	2108	1728	2366

Larvae were fed twice a day, from 2 dah to 8 dah, with rotifers (*Brachionus plicatilis*) enriched with microalgae (*Isochrysis galbana*), at a density of 6-7 rotifers mL^{-1} from 2 to 4 dah and of 2-3 rotifers mL^{-1} from 5 to 8 dah, respectively. Newly hatched *Artemia* spp. nauplii were supplied to larvae from 5 to 8 dah twice a day, at increasing density from 1 to 3 nauplii per 10 mL. Four different commercial products (EA, EB, EC, ED) (manufacture's labelled nutritional composition for each enrichment is provided in Table 2) were used to the enrichment of *Artemia* spp. metanauplii, according to the fish farm protocol. Each enriched live prey diet was used to feed fish from two separated tanks (EA-1, EA-2; EB-1, EB-2; EC-1, EC-2; ED-1, ED-2, respectively). Enriched *Artemia* metanauplii were supplied to larvae from 8 to 14 dah twice a day, at increasing density from 6 to 24 metanauplii per 10 mL, adjusted according to the increase of weight of the larvae. From 15 to 30 dah live prey diets were fed four times a day at increasing density from 40 to 80 metanauplii mL^{-1}. Commercial inert weaning diet was offered at regular intervals 10 to 20 times a day with automatic feeders from day 31 to 38, until the total withdrawal of live feed. Fish were fed commercial inert feed until the end of the experiment at 105 dah.

Table 2: Nutritional composition for each enrichment (EA, EB, EC, ED), according to the manufacture's label. DHA: docosahexaenoic acid; dw: dry weight; EPA: eicosapentaenoic acid; HUFA: highly unsaturated fatty acids; uns: unspecified.

	EA	EB	EC	ED
Vitamin A (mg kg^{-1} dw)	45	uns	43	47
Vitamin C (mg kg^{-1} dw)	33000	uns	38571	21053
Vitamin D_3 (µg kg^{-1} dw)	1250	uns	714	395
Phosphorous (g kg^{-1} dw)	uns	uns	28.571	6.316
Total n-3 HUFA (mg g^{-1} dw)	150	105	186	150
20:5n-3 (EPA) (mg g^{-1} dw)	10	uns	14	uns
22:6n-3 (DHA) (mg g^{-1} dw)	130	uns	157	uns
DHA/EPA	13	4	11	9

2.2. BIOCHEMICAL ANALYSIS

Basic biochemical analysis was performed for guidance only. For each enrichment diet, 15 g of *Artemia* metanauplii were collected at day 13 of larval rearing. Later, at 29 dah, 300 larvae per experimental tank were sampled. All samples were cryopreserved in liquid nitrogen at -80 °C until posterior analysis at ANFACO-CECOPESCA (Vigo, Spain). The wet weight (ww) content of vitamins A (retinol) and C (mg kg^{-1}), calcium and phosphorous (g kg^{-1}) was determined in live prey and larvae. Vitamin D_3 (cholecalciferol) was quantified in µg kg^{-1} ww in every sample. Ratio Ca/P was then calculated. The fatty acid determination was performed in terms of percentage of the total of fatty acids (% TFA), focusing in polyunsaturated fatty acids (PUFA), in particular n-3 and n-6 PUFA, eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). DHA/EPA and (n-3)/(n-6) ratios were computed.

Fat-soluble vitamins (A and D) were analysed by HPLC after prior alkaline hydrolysis. Saponification and vitamin extraction procedures were adapted from Salo-Väänänen *et al.*, 2000. The residue was filtered in a 0.45 µm filter prior to HPLC analysis. A HPLC Waters® 2695 system was used equipped with a Sunfire Pro Silica column (Waters®), a photodiode array (PDA) detector (Waters®) and a fluorescence detector (Waters®). The analysis ran at a flow rate of 1.5 mL min^{-1}, showing a retention time of 37.77 min. For vitamin D_3 (cholecalciferol) detection, the flow rate was 1 mL min^{-1}, and the obtained retention time was 38.17 min. Regarding vitamin C analysis, each sample was prepared according to Oruña-Concha *et al.*, 1998. The supernatant was filtered by a porous filter of 0.2 µm and then by a syringe filter of 0.22 µm previously to HPLC analysis. A HPLC Waters® 2695 system was used equipped with an YMC-Pack Pro C18 column (YMC™) and a PDA detector (Waters®). The HPLC ran at a flow rate of 0.5 mL min^{-1} and the retention time was 30 min. The concentration of the vitamins A, C and D_3 in each sample was then calculated taking into account the calibration curve with standard solutions of retinol, L-ascorbic acid and cholecalciferol, respectively. The techniques were validated taking into account the accuracy, precision, quantification limit, selectivity, specificity and linearity of the method.

For calcium and phosphorous determination, a quantity of each sample between 0.5 g and 1 g was used and digested with HNO_3 and H_2O_2 and then dissolved with mili-q water. The identification and quantification of Ca and P was conducted in an inductively coupled plasma optical emission spectrometer (ICP-OES) VISTA MPX (Agilent®).

For fatty acid determination, 1 g of each sample was used. Total lipids were extracted in chloroform:methanol (1:2, v:v) using the method of Bligh and Dyer, 1959. The chloroform layer was separated and dissolved in hexane. Later, esters methylation was performed by adding KOH solution (2N) in methanol. Chloroform was evaporated under N_2 flow. Fatty acid separation and identification was performed using the method of Sato and Murata, 1988 by means of gas-liquid chromatography using a gas chromatograph equipped with a flame ionization detector (FID) 6890 N (Agilent®).

2.3. STAINING AND SKELETAL ANOMALY EVALUATION

Senegalese sole from each tank were randomly sampled at 31 dah and 105 dah (Table 3) for meristic counts and skeletal anomaly detection. All sampled fish were sacrificed using an overdose of Tricaine methanesulfonate (MS-222, Sigma-Aldrich). Specimens were fixed in 10% buffered formalin and stained using a modified double staining technique for cartilage and bone (Gavaia *et al.* 2000, Darias *et al.* 2010). Standard length (StL, cm) and standard height (StH, cm) were measured at the end of the experiment. StL is the distance between the rostral end of the skull and the end of hypurals. StH comprised the higher length between the ventral and dorsal aspect of the fish, without taking into account the fins rays. The ratio StL/StH was also computed.

Table 3: Number of sampled specimens of each tank (EA-1, EA-2, EB-1, EB-2, EC-1, EC-2, ED-1, ED-2) at 31 and 105 days after hatching for meristic counts and skeletal anomalies detection. dah: days after hatching.

Dietary Replicates	EA-1	EA-2	EB-1	EB-2	EC-1	EC-2	ED-1	ED-2
Sampled individuals at 31 dah	26	25	25	25	25	20	35	24
Sampled individuals at 105 dah	26	24	26	25	26	25	25	25

Stained individuals were observed by both sides using a binocular model Olympus® SZX16. In the vertebral column, three anatomical regions were considered: abdominal vertebrae, caudal vertebrae and caudal complex, according to Gavaia *et al.*, 2002. For every region, the number of vertebrae was counted. The number of caudal fin rays was also assessed. Each vertebra was evaluated for anomaly detection taking into account the alterations of the parapophyses, haemal arches and spines, neural arches and spines and vertebral bodies, as described in previous studies (de Azevedo *et al.* in press b). Anomalies in caudal complex elements were also assessed. Some considerations were made regarding completely fused vertebrae which were considered as one single vertebra in meristics counts if the union presented the same appearance of one single vertebra. However, these were registered as fusions in anomalies assessment. For every individual, it was registered the number of anomalies of each type. Table 4 shows the typology of anomalies which was taken into account for this study, according to the anatomical region. In this paper, we grouped the different anomaly typologies under 22 categories, one for each of the considered skeletal elements and region. I.e., category C-H consists of 6 anomaly typologies. General categories were also considered to assess anomalies without taking into account the affected region. In particular, vertebral body anomalies (VBA) (fusions or deformations) and vertebral column deviations (VCD) (kyphosis, lordosis and scoliosis) were specially considered, since they could originate the most important losses to industrial farming (Gavaia *et al.* 2002, de Azevedo *et al.* in press b).

	Anomaly typology		Anomaly categories
Abdominal region	Parapophysis	Bifurcation Number alteration Insertion alteration Fusion Deformation	Parapophysis (PP)
	Neural arches and spines	Bifurcation Number alteration Insertion alteration Fusion Incomplete arch Deformation	A Neural elements (A-N)
	VBA	Fusion Deformation	A Fusion (A-F) A Deform (A-D)
	VCD	Kyphosis Lordosis Scoliosis	A Kyphosis (A-K) A Lordosis (A-L) A Scoliosis (A-S)
Caudal region	Neural arches and spines	Bifurcation Number alteration Insertion alteration Fusion Incomplete arch Deformation	C Neural elements (C-N)
	Haemal arches and spines	Bifurcation Number alteration Insertion alteration Fusion Incomplete arch Deformation	C Haemal elements (C-H)
	VBA	Fusion Deformation	C Fusion (C-F) C Deform (C-D)
	VCD	Kyphosis Lordosis Scoliosis	C Kyphosis (C-K) C Lordosis (C-L) C Scoliosis (C-S)
Caudal complex	Neural arches and spines	Bifurcation Number alteration Insertion alteration Fusion Incomplete arch Deformation	CC Neural elements (CC-N)
	Haemal arches and spines	Bifurcation Number alteration Insertion alteration Fusion Incomplete arch Deformation	CC Haemal elements (CC-H)
	Hypurals epural parhypural	Bifurcation Number alteration Insertion alteration Fusion Deformation	Hypurals, epural and parhypural (HYE)
	VBA	Fusion Deformation	CC Fusion (CC-F) CC Deform (CC-D)
	VCD	Kyphosis Lordosis Scoliosis	CC Kyphosis (CC-K) CC Lordosis (CC-L) CC Scoliosis (CC-S)

Table 4: Typology of skeletal anomalies that were considered in this study for each anatomical region. The anomaly types were merged in anomaly categories according to the body region and the affected bony elements (code name is between parentheses). A: abdominal region; C: caudal region; CC: caudal complex region; VBA: vertebral body anomalies; VCD: vertebral column deviations.

2.4. STATISTICS

For each tank replicate, the daily survival rate was calculated from 15 dah to the second sampling stage. A Kolmogorov-Smirnov test was performed among the two replicates corresponding to the same dietary treatment in order to evaluate similarity among distributions respect to survival rate and measurements (StL, StH and StL/StH) at 105 dah. Main descriptive statistical evaluation was performed on survival rate and biometric data: mean, standard error of the mean (SEM), median, minimum and maximum. Survival rate and biometric data were checked for normality (Shapiro-Wilk test) and, in case of compliance, for homogeneity of variance (Levene's test). A one-Way ANOVA was performed among dietary groups followed by Tukey's post-hoc test. In non-normal distributions, Kruskal-Wallis tests were applied in order to detect significant differences in the median among enrichments. In this case, pairwise Mann-Whitney comparison tests (with Bonferroni corrections) were performed in case of finding significant differences ($P < 0.05$). Meristic counts were expressed by means of percentage of fish displaying a given number of vertebrae or caudal fin rays. The Pearson's chi-square independence test was used to assess differences in the proportion of fish with a certain number of vertebrae/caudal fin rays between diets. If observed frequencies were less than five, the Fisher exact test was performed instead. When significant differences were found ($P < 0.05$), pairwise comparison tests were computed.

For the two sampling points and for each dietary treatment it was calculated: the incidence of individuals displaying at least one anomaly and the percentage of individuals with VBA and/or VCD; the frequency of skeletal abnormalities; the percentage of VBA and/or VCD in the total number of anomalies of each lot and the average number of anomalies per affected fish (median ± range). Differences in the frequencies of specimens showing VBA and/or VCD among diet groups were assessed by means of an independence chi-square test. Regarding the incidence of each general category, the Pearson's chi-square test was used to check for differences in the frequencies of individuals with or without anomalies and dietary group. In case of frequencies under five observations, the Fisher exact test was performed in turn of chi-square test. When significant comparisons were found ($P < 0.05$), pairwise comparison tests were computed.

Similarly to the procedure described in Prestinicola *et al.*, 2013, two different matrices were elaborated. The first one was a binary matrix (BM) regarding the presence or absence of each anomaly category for each diet. Therefore, in this matrix, the zero corresponded to the absence of anomalies and one, to the presence of at least one skeletal alteration. A binary variable was added in order to represent absence of anomalies (ABS) (1 = absence of anomalies; 0 = presence of at least one anomaly). The second matrix (frequency matrix, FM) was made on calculating the relative frequency of individuals displaying each anomaly category (and ABS) for each dietary treatment. CA of FM was performed involving both developmental stages in order to organize data seeking for any relationship between anomaly categories, diet protocols and age. Moreover, a simple matching similarity matrix was calculated from BM and subjected to Two-way NPMANOVA test to detect significant differences in the incidence of anomaly category among different dietary groups and stages. When significant comparisons were found ($P < 0.05$), pairwise comparison tests were computed; *P* values were corrected using Bonferroni correction. CA was performed using RStudio® version 1.0.44 software (RStudio Team 2016) with R version 3.3.2 (R Core Team 2016). Descriptive statistics were calculated with Microsoft Office Excel® 2007. All the other statistical analyses were performed with Past version 2.17 (Hammer *et al.* 2001).

3. RESULTS

3.1. BIOCHEMICAL ANALYSIS

Metanauplii nutritional composition is summarized in Table 5. For Senegalese sole at 30 dah, biochemical results are shown in Table 6 for each tank.

Table 5: Nutritional composition of *Artemia* spp. metanauplii enriched with four different products (EA, EB, EC, ED). Asterisks represent values below the equipment detection limit (< 0.5). DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; PUFA: polyunsaturated fatty acids; % TFA: percentage of the total fatty acids; ww: wet weight.

	EA	EB	EC	ED
Vitamin A (mg kg^{-1} ww)	*	*	*	*
Vitamin C (mg kg^{-1} ww)	957	1113	1007	1066
Vitamin D$_3$ (µg kg^{-1} ww)	15	28	10	12
Calcium (Ca) (g kg^{-1} ww)	0.195	0.155	0.141	0.229
Phosphorous (P) (g kg^{-1} ww)	1.085	0.879	1.154	0.980
Ratio Ca/P	0.18	0.18	0.12	0.23
Total n-3 PUFA (% TFA)	34.34	32.98	30.32	34.96
Total n-6 PUFA (% TFA)	5.87	8.97	6.83	6.70
20:5n-3 (EPA) (% TFA)	8.09	5.85	7.17	8.17
22:6n-3 (DHA) (% TFA)	1.81	1.82	2.52	1.91
DHA/EPA	0.22	0.31	0.35	0.23
(n-3)/(n-6)	5.85	3.68	4.44	5.22

Table 6: Basic nutritional composition of Senegalese sole (*Solea senegalensis*) at 30 days after hatching for each tank (EA-1, EA-2, EB-1, EB-2, EC-1, EC-2, ED-1, ED-2). Asterisks represent values below the equipment detection limit (< 5). DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; PUFA: polyunsaturated fatty acids; % TFA: percentage of the total fatty acids; ww: wet weight.

	EA-1	**EA-2**	**EB-1**	**EB-2**	**EC-1**	**EC-2**	**ED-1**	**ED-2**
Vitamin A (mg kg^{-1} ww)	0.7	0.6	0.6	0.7	0.8	0.7	0.9	1
Vitamin C (mg kg^{-1} ww)	323	272	278	270	320	325	316	332
Vitamin D$_3$ (µg kg^{-1} ww)	5	6	13	14	6	*	*	*
Calcium (Ca) (g kg^{-1} ww)	5.502	6.309	6.000	5.752	5.472	5.370	5.057	5.169
Phosphorous (P) (g kg^{-1} ww)	3.069	2.415	4.939	4.627	4.435	4.415	4.183	4.315
Ratio Ca/P	1.79	2.61	1.21	1.24	1.23	1.22	1.21	1.20
Total n-3 PUFA (% TFA)	22.10	28.83	23.44	26.42	25.49	28.05	26.02	31.79
Total n-6 PUFA (% TFA)	8.49	7.81	9.76	10.05	8.35	9.40	6.70	7.73
20:5n-3 (EPA) (% TFA)	2.23	2.38	3.13	2.98	2.46	3.34	3.07	3.05
22:6n-3 (DHA) (% TFA)	9.22	7.23	9.29	5.88	9.05	12.96	9.00	8.36
DHA/EPA	4.13	3.04	2.97	1.97	3.68	3.88	2.93	2.74
(n-3)/(n-6)	2.60	3.69	2.40	2.63	3.05	2.98	3.88	4.11

3.2. SURVIVAL RATE AND MEASUREMENTS

Daily survival rate (%) from 15 dah to 105 dah (Figure 1 and Table 7) showed no statistically significant differences among replicates with the Kolmogorov-Smirnov test. Similarly, StL, StH and StL/StH did not presented different distributions between replicates using the same test at the end of the experiment (105 dah). Therefore, replicates were merged into the respective four dietary groups. Table 7 display descriptive statistics for daily survival rate, StL, StH and StL/StH among enrichment treatment replicates. Survival rate, StL and StH values were very comparable among diets and no statistically significant differences were found among dietary groups. However, the ratio StL/StH, was significantly lower in fish fed with ED enriched *Artemia*, compared with EA, EB and EC groups using One-Way ANOVA and Tukey's pairwise comparison tests (P = 7.72E-06, P = 9.08E-06, P = 0.01, respectively).

3.3. NUMBER OF VERTEBRAE AND CAUDAL FIN RAYS

For each diet and age group, the number of vertebral centra and the number of caudal fin rays are shown in Table 8: median, minimum, maximum and percentage of individuals showing the median value (%Im).

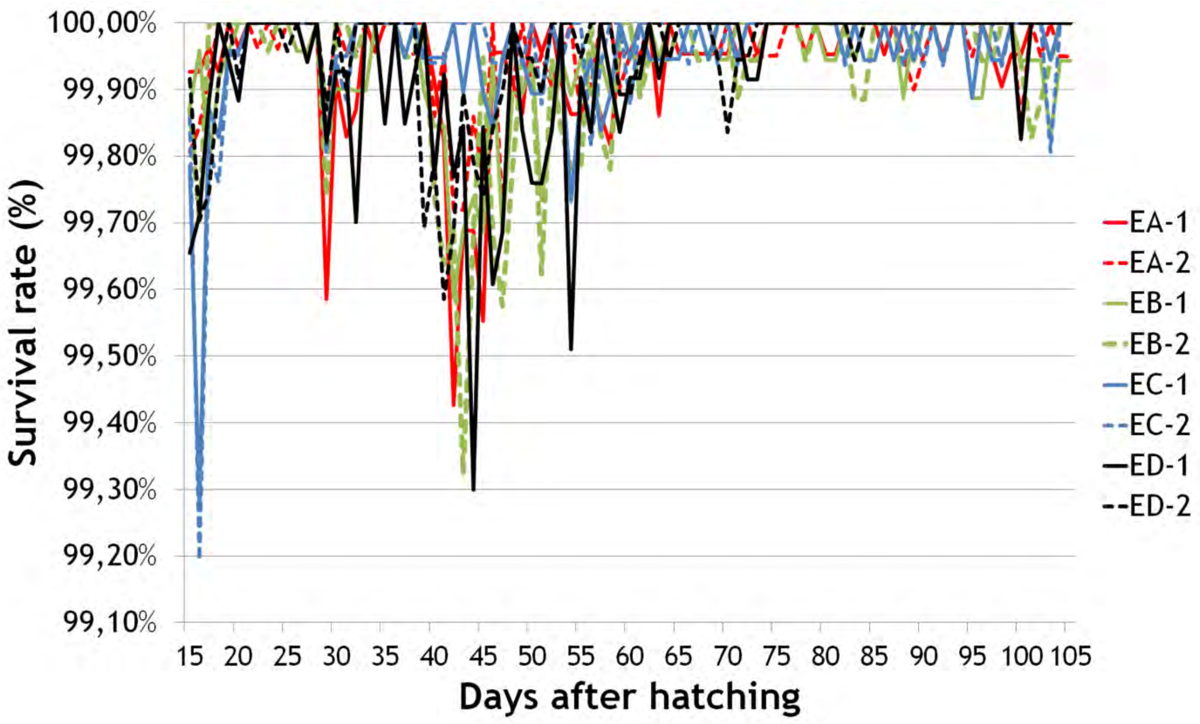

Figure 1: Survival rate (%) for each replicate (EA-1, EA-2, EB-1, EB-2, EC-1, EC-2, ED-1, ED-2) from 15 to 105 days after hatching.

		EA-1	EA-2	EB-1	EB-2	EC-1	EC-2	ED-1	ED-2
Survival rate (%)	Mean ± SEM	99.94 ± 0.01	99.96 ± 0.01	99.94 ± 0.01	99.92 ± 0.01	99.95 ± 0.01	99.96 ± 0.01	99.93 ± 0.01	99.95 ± 0.01
	Median	100.00	100.00	99.96	100.00	100.00	100.00	100.00	100.00
	Minimum	99.43	99.72	99.59	99.32	99.28	99.19	99.30	99.59
	Maximum	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
StL (cm)	Mean ± SEM	3.71 ± 0.15	4.18 ± 0.22	3.66 ± 0.14	3.80 ± 0.17	3.82 ± 0.15	4.02 ± 0.14	3.92 ± 0.15	3.67 ± 0.15
	Median	3.7	3.95	3.7	3.6	3.8	4	3.9	3.6
	Minimum	2.6	2.5	2.3	2.8	2.8	2.8	2.7	2.5
	Maximum	5.6	6.7	5.7	5.9	6.4	5.4	5.8	5.5
StH (cm)	Mean ± SEM	1.13 ± 0.05	1.32 ± 0.22	1.14 ± 0.05	1.19 ± 0.06	1.20 ± 0.05	1.31 ± 0.05	1.31 ± 0.06	1.20 ± 0.05
	Median	1.1	1.2	1.1	1.1	1.2	1.3	1.3	1.1
	Minimum	0.8	0.7	0.8	0.9	0.8	0.9	0.8	0.8
	Maximum	1.8	2.1	2	1.9	2.1	1.9	2.1	1.8
StL/StH	Mean ± SEM	3.31^{a*} ± 0.04	3.21^{a*} ± 0.22	3.23^{ab*} ± 0.04	3.20^{ab*} ± 0.02	3.19^{b} ± 0.03	3.10^{b} ± 0.04	3.01^{c*} ± 0.03	3.06^{c*} ± 0.03
	Median	3.3	3.2	3.2	3.2	3.2	3.0	3.0	3.1
	Minimum	3.0	2.8	2.9	3.0	2.9	2.8	2.6	2.7
	Maximum	3.7	3.6	3.7	3.5	3.5	3.6	3.4	3.5

Table 7: Daily survival rate (%) from 15 to 105 days after hatching (dah) and biometric results for standard length (StL, cm), standard height (StH ,cm) and the ratio StL/StH (cm) at the end of the experiment (105 dah) for each dietary group replicate (EA-1, EA-2, EB-1, EB-2, EC-1, EC-2, ED-1, ED-2): mean ± standard error of the mean, median, minimum and maximum. Statistically significant differences were not found in the distribution of survival rate, StL, StH and StL/StH among replicates ($P > 0.05$). Different superscript letters denote significant differences among dietary groups ($P < 0.05$). Asterisks indicate differences among diets with $P < 0.001$. SEM: standard error of the mean.

At both sampling points, most fish presented the following vertebral formula in abdominal, caudal and caudal complex regions, respectively: 9:34:3 (Table 8). Fish displayed an analogous number of vertebrae in every anatomic region among dietary treatments with the only exception of the number of caudal vertebrae that was more variable. Fisher exact test revealed significant differences in the distribution of the frequencies of individuals regarding the number of caudal vertebrae at 31 dah between EA lot respect to EB and EC (Fisher exact $P = 0.003$ and Fisher exact $P = 0.007$, respectively) (Table 8). In particular, there were a lesser number of specimens of EA diet with 34 vertebrae (56.9%) with a consequent higher number of individuals with 32, 33 and 35 vertebrae.

Table 8: Number of vertebrae in abdominal, caudal and caudal complex regions, as well as, number of caudal fin rays for each dietary group (EA, EB, EC, ED) and age: median, minimum and maximum values and occurrences of individuals showing the median values (%). Different superscript letters denote significant differences in the distribution of the number of specimens with a certain number of vertebrae among diets for each age (Fisher exact $P < 0.05$). Due to technical problems, the caudal fin rays of two specimens of 105 days after hatching could not be counted; one from EC diet and another from ED group. dah: days after hatching. %Im: percentage of individuals showing the median value.

Meristic counts		31 dah				105 dah			
		EA	EB	EC	ED	EA	EB	EC	ED
Abdominal vertebrae	Median	9	9	9	9	9	9	9	9
	Minimum	8	8	8	8	8	8	8	8
	Maximum	9	9	9	9	9	9	9	9
	%Im	98.0	98.0	97.8	98.3	98.0	98.0	98.0	94.0
Caudal vertebrae	Median	34	34	34	34	34	34	34	34
	Minimum	32	33	33	33	33	33	33	33
	Maximum	35	35	35	35	35	35	35	35
	%Im	56.9^{a}	88.0^{b}	86.7^{b}	72.9^{ab}	86.0	88.2	88.2	78.0
Caudal complex vertebrae	Median	3	3	3	3	3	3	3	3
	Minimum	2	2	2	2	2	2	2	2
	Maximum	3	3	3	4	3	3	3	3
	%Im	90.2	90.0	93.3	89.8	88.0	90.2	84.3	84.0
Caudal fin rays	Median	20	20	20	20	20	20	20	20
	Minimum	20	20	20	20	20	20	20	20
	Maximum	21	20	20	20	21	20	20	21
	%Im	88.2^{a}	100.0^{b}	100.0^{b}	100.0^{b}	90.0^{a}	100.0^{b}	98.0^{ab}	96.0^{ab}

Regarding caudal fin rays counts, EA group presented higher number of individuals with 21 of these elements (11.8% and 10.0% at 31 and 105 dah, respectively) with respect to other treatments that resulted significantly differing *vs* all the other diet groups at 31 dah (Fisher exact *P* = 0.03; Fisher exact *P* = 0.03 and Fisher exact *P* = 0.008, for EB, EC and ED lots, respectively), and only to EB batch at 105 dah (Fisher exact *P* = 0.03) (Table 8).

3.4. VERTEBRAL ANOMALIES

In this study, all individuals presented skeletal anomalies (Table 9). The most common lesions affected neural/haemal arches and spines as well as caudal complex elements (Table 10 and Figure 2a-d). From the 55 considered types of anomalies, 15 of them were not detected, i.e., bifurcation, alteration of number and insertion of arches and caudal elements, kyphosis and scoliosis mainly in the abdominal region (Table 10). In general, every specimen showed two short neural spines without neural arch in the first abdominal vertebra (Figure 2a). This highly frequent alteration on the same vertebra was not considered in the remaining results as anomaly but considered as a species-specific plasticity feature. At 31 dah, typologies like twisted caudal haemal spines (Figure 2a,b), fusion and/or bending of hypurals, epural or parhypural (Figure 2c) were highly recurrent (Table 10). Alterations in the shape of caudal complex plates (Figure 2d) were predominant at 105 dah, with incidences that ranged from 96.0% to 100.0% (Table 10). The frequency of some other types of anomalies as for instance, incomplete arches (Figure 2b) and alterations in the number or insertion of the neural/haemal spines (Figure 2b) was reduced, or in some cases absent (Table 10). Specimens showing VBA and/or VCD ranged from 28.0% to 44.0% (Table 9). The most common VBA was the deformation of caudal complex vertebral bodies at both stages (22.2-26.0%; 18.0-36.0%; Table 10), although some could be associated with preural fusions. Fusions among caudal complex vertebrae were very frequent as well (Figure 2c,d). Alterations of the shape (Figure 2a) or shortening (Figure 2a,b) of the vertebral centra were also observed in other regions and sometimes concurrently with vertebral fusions (Figure 2e) or deviations of the rachis (Figure 2f). In every anatomic area, VCD, namely lordosis (Figure 2f) and scoliosis, were more frequent in the caudal complex at 105 dah, although did not reach more than 10.0% of the individuals of each diet group (Table 10).

Table 9: General results on the incidence of skeletal anomalies for each diet (EA, EB, EC, ED) and age: frequency of individuals displaying at least one anomaly (%); frequency of skeletal abnormalities with respect to the total number of anomalies observed in each age group; frequency of individuals with vertebral body anomalies (VBA) and/or vertebral column deviations (VCD) (%); percentage of VBA and/or VCD in the total number of anomalies of that diet-age group. No differences were found in the number of individuals with VBA and/or VCD among dietary groups for each age (*P* > 0.05). dah: days after hatching.

	31 dah				**105 dah**			
	EA	**EB**	**EC**	**ED**	**EA**	**EB**	**EC**	**ED**
Frequency of individuals with at least one anomaly (%)	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Frequency of anomalies (%)	26.5	24.3	17.0	32.2	24.0	27.0	24.6	24.4
Frequency of individuals with VBA and/or VCD (%)	41.2	32.0	28.9	39.0	28.0	39.2	41.2	44.0
Frequency of VBA and/or VCD (%)	11.1	6.4	8.1	7.7	13.2	14.8	18.2	19.8

At the first sampling point, VBA and/or VCD were less frequent in fish fed with EC enriched metanauplii (28.9%) and more frequent in EA diet (41.2%) (Table 9). In turn, at 105 dah, EA lot displayed the lower percentage of individuals with these anomalies (28.0%). However, these differences among diets were not significant at both ages with the independence chi-square test ($\chi^2 = 2.1527$, df = 3, $P > 0.05$; $\chi^2 = 3.1317$, df = 3, $P > 0.05$, respectivelly). EB, EC and ED diets presented a higher frequency of individuals with VBA and/or VCD at 105 dah than the respective groups at 31 dah (Table 9). Senegalese sole fed with EA enriched metanauplii presented a lower percentage of the number of anomalies and of VBA and/or VCD (24.0% and 13.2%, respectively) at 105 dah (Table 9). In this sense, the frequency VBA and/or VCD kept some correspondence with the number of individuals showing these anomalies, since EA and ED diets presented the highest values at 31 and 105 dah, 11.1% and 19.8% respectively.

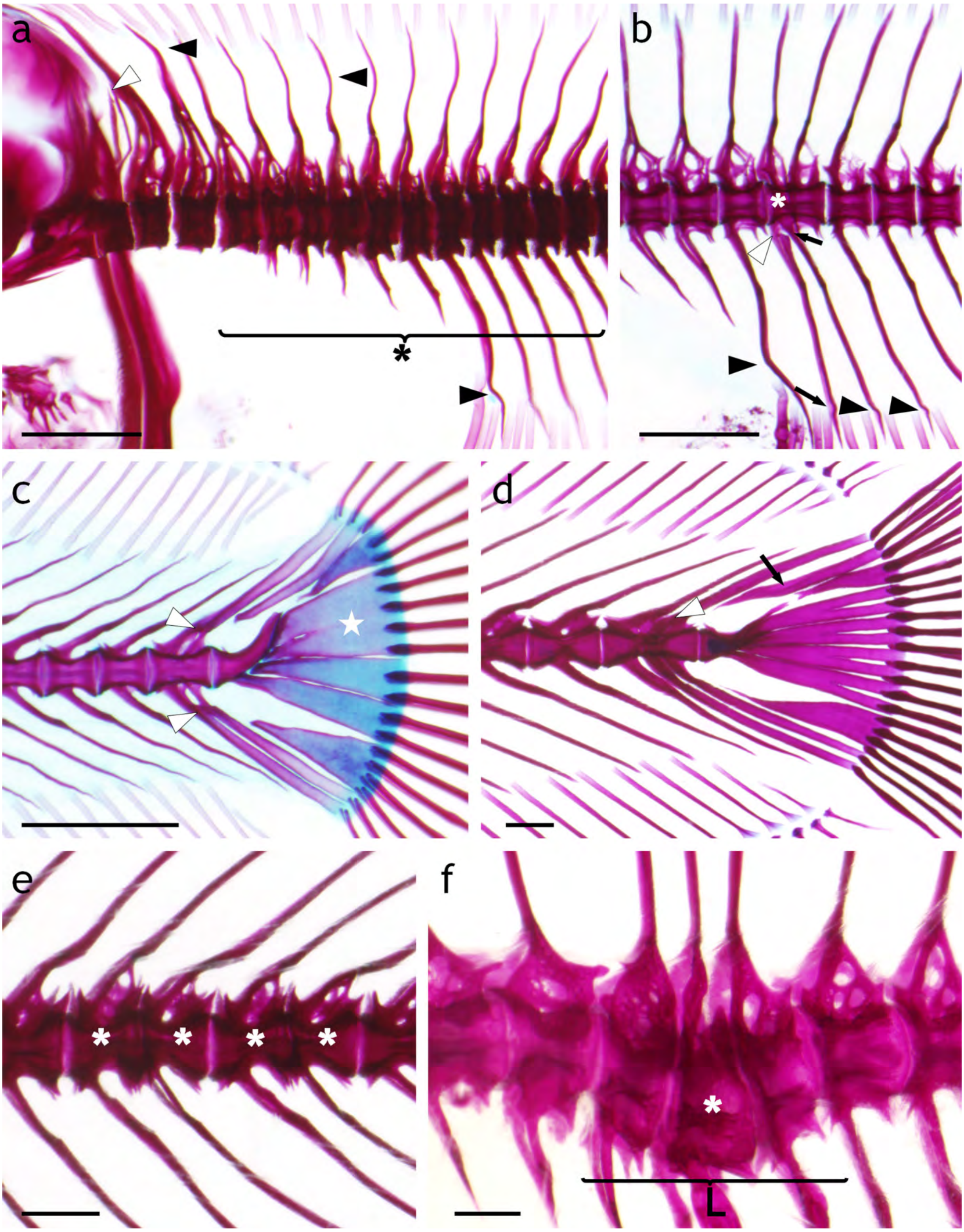
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Figure 2: More common anomalies in 31 days after hatching (dah) (a-c) and 105 dah (d-f) Senegalese sole (*Solea senegalensis*). Double staining technique with alcian blue and alizarin red. (a) Severe deformations affecting both abdominal and caudal vertebrae exhibiting evident flattening of centra and irregularity of vertebral surface and central fusion among the affected elements (asterisk); note also the two short neural spines with absence of the neural arch of the first abdominal vertebra (white arrowhead); slight deformities in abdominal neural spines and first caudal haemal spine (black arrowheads). (b) Fusion between the second and third caudal vertebrae with shortening of the first vertebral body (asterisk); incomplete arch (white arrowhead) and alteration of the insertion (short arrow) of the caudal haemal arches; twisted haemal spines (black arrowheads), with a thin layer of bone and slight torsion at the tip of the haemal spine (black arrow). (c) Fusion between preurals with fusion of neural and haemal arches (white arrowheads), the union has the appearance of one elongated vertebra; alterations of the shape of epural and hypural 5; fusion among hypurals (white star). (d) Vertebral fusion between preurals and the last caudal centra, displaying also fusion of preurals neural arches (white arrowhead); deformities of epural, hypural 5 and parhypural, denote the thin layer of bony tissue in epural shaping the element (black arrow). (e) Fusions among caudal vertebrae exhibiting an altered orientation of the central structure in opposite directions (asterisks). (f) Lordosis in the transition area between abdominal and caudal regions (L); the affected vertebrae are also shorter and deformed, highlights the central vertebra which shows a trapezoidal shape (asterisk). Bars = 500 μm.

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The percentage of individuals with at least one anomaly category is represented in Figure 3a,b, considering groups and age. These values corresponded to the FM matrix used to the CA analysis. In general, the incidence of each anomaly category varied among lots, although it was not possible to directly visualise a pattern towards a diet. At 31 dah, only twelve of the 33 observed typologies presented an incidence equal or superior to 10.0% in almost every group. These typologies belong to categories PP, C-N, C-H, CC-N, CC-H, HYE, CC-F, CC-D (Table 10 and Figure 3a). These were mainly located in caudal complex region. Some of the other anomaly categories were only observed in a few individuals (Figure 3a). Moreover, A-D was only detected in EA reared fish (Figure 3a). Some of the predominant anomaly typologies at 31 dah were markedly less common at 105 dah, mostly deformations on caudal neural and haemal elements (C-N and C-H categories, respectively) and fusions in among caudal plates (HYE category) (Table 10 and Figure 3b). Alterations in parapophyses (PP) were more frequent in this stage. A-D and A-L categories were only present in ED group as well as C-L in EA lot at this age (Figure 3b).

Anomalies typology				31 dah				105 dah			
				EA	EB	EC	ED	EA	EB	EC	ED
Abdominal region	Parapophysis (PP)		Bifurcation	2.0				2.0			
			Number alteration	9.8	18.0	13.3	16.9	22.0	27.5	21.6	30.0
			Insertion alteration						11.8		10.0
			Fusion								
			Deformation								
	Neural arches and spines (A-N)		Bifurcation								
			Number alteration					2.0			
			Insertion alteration								
			Fusion								
			Incomplete arch								
			Deformation	3.9		2.2	3.4				2.0
	VBA	(A-F)	Fusion	5.9			8.5				
		(A-D)	Deformation	3.9							2.0
	VCD	(A-K)	Kyphosis								
		(A-L)	Lordosis								2.0
		(A-S)	Scoliosis								
Caudal region	Neural arches and spines (C-N)		Bifurcation								
			Number alteration	2.0							
			Insertion alteration								
			Fusion	2.0			1.7	2.0			4.0
			Incomplete arch	2.0		2.2		2.0	2.0		
			Deformation	39.2	28.0	6.7	13.6	6.0	3.9	3.9	4.0
	Haemal arches and spines (C-H)		Bifurcation	2.0	2.0						2.0
			Number alteration						2.0		4.0
			Insertion alteration	2.0	4.0	6.7	10.2	4.0		3.9	2.0
			Fusion				5.1		2.0		2.0
			Incomplete arch	7.8	6.0	2.2	8.5	2.0			4.0
			Deformation	94.1	96.0	53.3	100.0	10.0	9.8	5.9	8.0
	VBA	(C-F)	Fusion	5.9	2.0	2.2	6.8	2.0	2.0	3.9	4.0
		(C-D)	Deformation	5.9	4.0	2.2	5.1	6.0	3.9	5.9	8.0
	VCD	(C-K)	Kyphosis			2.2	3.4				
		(C-L)	Lordosis	5.9	4.0		1.7	6.0			
		(C-S)	Scoliosis	2.0	2.0	2.2	5.1				
Caudal complex	Neural arches and spines (CC-N)		Bifurcation								2.0
			Number alteration	9.8	4.0	6.7	6.8	2.0	7.8	2.0	4.0
			Insertion alteration								
			Fusion	7.8	10.0	6.7	6.8	10.0	7.8	9.8	4.0
			Incomplete arch	2.0	2.0						
			Deformation	3.9	2.0	2.2	10.2	10.0	7.8	2.0	2.0
	Haemal arches and spines (CC-H)		Bifurcation								
			Number alteration	2.0		2.2			2.0	3.9	
			Insertion alteration			2.2			2.0	5.9	
			Fusion	9.8	6.0	6.7	10.2	6.0	9.8	7.8	6.0
			Incomplete arch	5.9	4.0		3.4			2.0	
			Deformation	5.9	2.0	6.7	11.9	2.0	7.8	5.9	2.0
	Hypurals epural parhypural (HYE)		Bifurcation								
			Number alteration								
			Insertion alteration								
			Fusion	84.3	88.0	86.7	94.9	30.0	25.5	11.8	12.0
			Deformation	90.2	92.0	95.6	94.9	96.0	96.1	96.1	100.0
	VBA	(CC-F)	Fusion	17.6	18.0	17.8	11.9	12.0	19.6	21.6	22.0
		(CC-D)	Deformation	23.5	26.0	22.2	23.7	18.0	27.4	35.3	36.0
	VCD	(CC-K)	Kyphosis	3.9		4.4	1.7	4.0	5.9	2.0	10.0
		(CC-L)	Lordosis					6.0	7.8	9.8	4.0
		(CC-S)	Scoliosis						5.9	2.0	2.0

Table 10: Relative frequency (%) of affected individuals by each anomaly type (code name is between parenthesis) for each dietary treatment (EA, EB, EC, ED) at 31 and 105 days after hatching. Empty cells denote a frequency equal to zero (0). dah: days after hatching; VBA: vertebral body anomalies; VCD: vertebral column deviations.

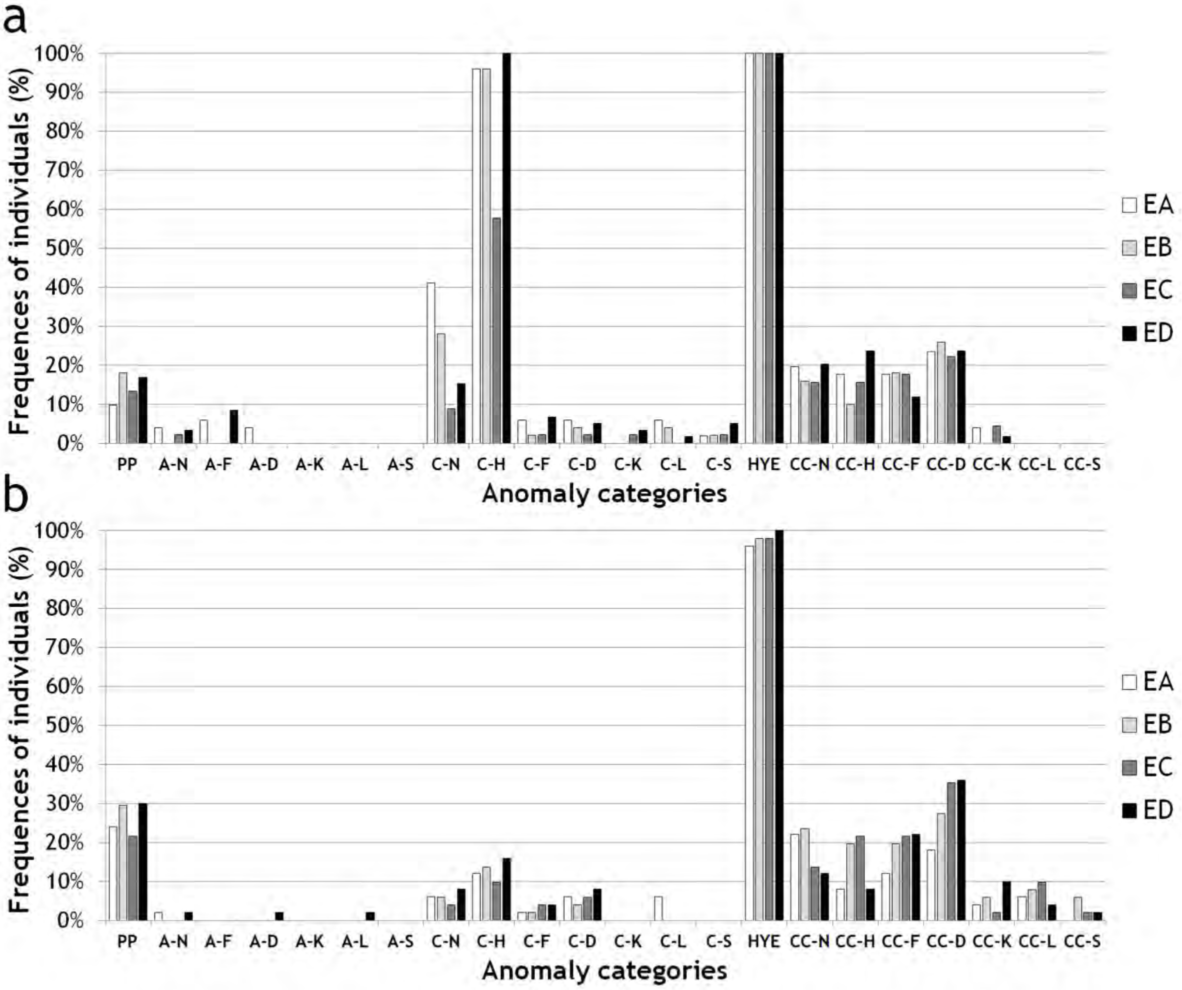
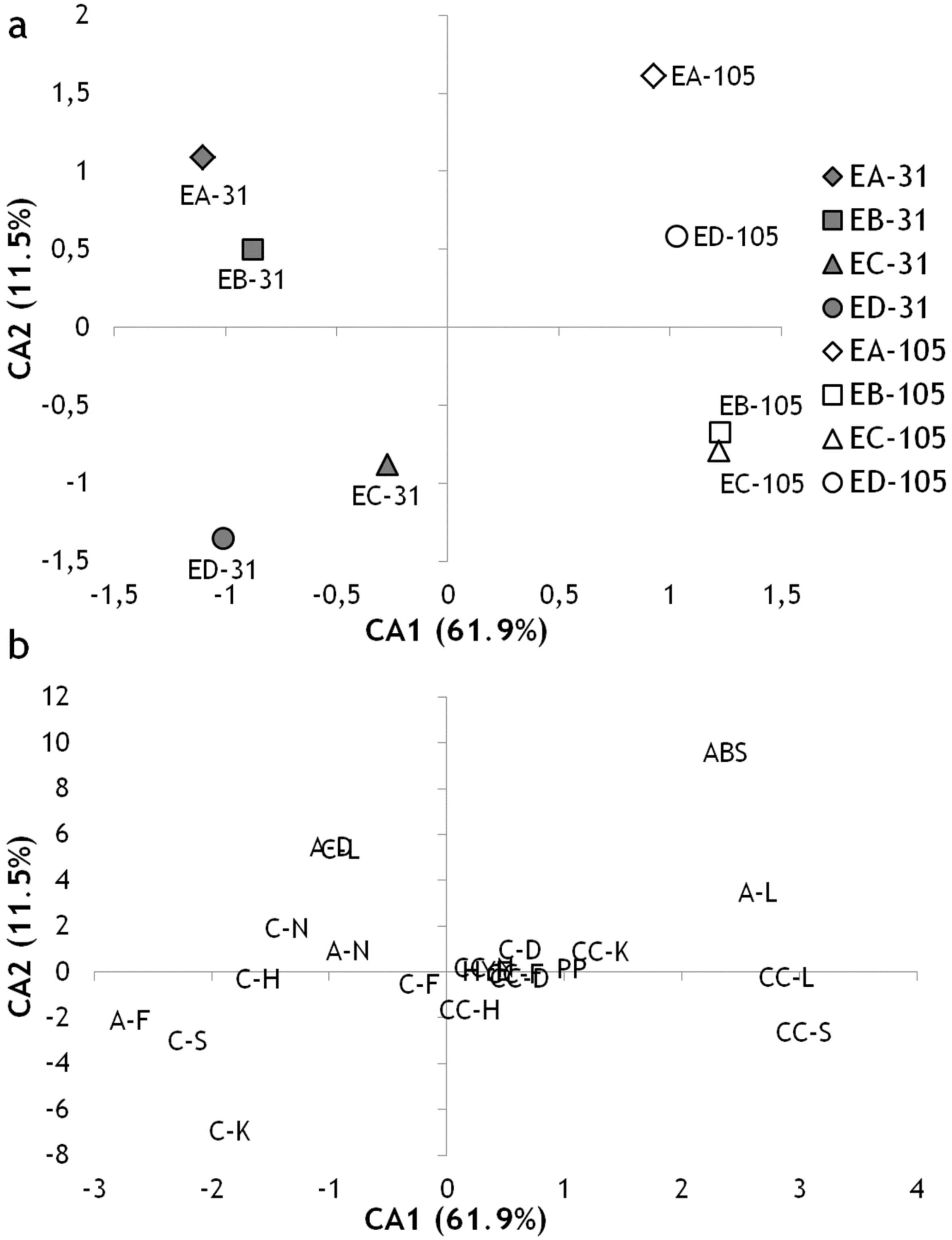


Figure 3: Relative frequency (%) of individuals with at least one anomaly in each dietary group (EA, EB, EC, ED). (a) 31 days after hatching (dah). Haemal caudal elements and the structures of caudal complex were the most affected. (b) 105 dah. Caudal complex region presented the highest incidence of anomalies. A-D: abdominal (A) deformation; A-F: A fusion; A-K: A kyphosis; A-L: A lordosis; A-N: A neural elements; A-S: A scoliosis; CC-D: caudal complex (CC) deformation; CC-F: CC fusion; CC-H: CC haemal elements; CC-K: CC kyphosis; CC-L: CC lordosis; CC-N: CC neural elements; CC-S: CC scoliosis; C-D: caudal (C) deformation; C-F: C fusion; C-H: C haemal elements; C-K: C kyphosis; C-L: C lordosis; C-N: C neural elements; C-S: C scoliosis; HYE: hypurals, epural, parhypural; PP: parapophysis.

Regarding CA results, the overall variance for the first two correspondence axes (CA1, CA2) was 73.4% (Figure 4a,b). CA1 *versus* CA2 output ordered fish from each age in separated semiaxis: fish at 105 dah occupied the right side of CA1, whereas larvae at 31 dah were located in the negative side of CA1. (Figure 4a). Dietary groups did not conserve a similar distribution among ages, for instance, EB and ED fish at 31 dah were situated in completely opposed quadrants regarding their location at 105 dah (Figure 4a). Moreover, the distribution among the second and the third correspondence axes (CA2, CA3) presented a distinct organization, with diet-age groups located very close to the CA2 axis, except EA and ED lots at 105 dah (data not shown). In CA1 *vs* CA2 output, most anomaly categories were distributed near axis centres. However A-F, C-S and C-K were slightly further at the left of CA1, while ABS, A-L, CC-L and CC-S were the farthest in the positive side of CA1 (Figure 4b). Furthermore, analysis of BM matrix evidenced significant differences on anomaly categories regarding age and diet (P = 0.0001, P = 0.0026, respectively), although the interaction among them was not statistically significant ($P > 0.05$). Regarding dietary groups, pairwise comparisons showed that fish from EC group were statistically different from EA and ED lots using NPMANOVA test (P = 0.0008, P = 0.0058, respectively). EA, EB, and ED groups were not statistically distinct among each other respect to the incidence of anomaly categories.

Regarding the frequency of anomalies per category (anomaly charge), the majority of individuals displayed each anomaly once (Figure 5a,b). The anomalies affecting the haemal arch of caudal vertebrae (C-H) was the one with the higher recurrent anomalies (1-12 anomalous haemal arches in 31 dah and 1-9 in 105 dah affected samples) in all the diet groups (Figure 5a,b), although one specimen presented 18 altered neural elements at 31 dah. Anomalies affecting hypurals, epural and parhypural (category HYE) presented a mean charge of at least two anomalies per affected fish in every diet or stage group (Figure 5a,b). At 31 dah, specimens from EA diet showed the higher charge of anomalies with respect to the same age-diet group, except in C-H, C-K and HYE categories (Figure 5a). As in the incidence of anomalies, category charge was slightly lower at 105 dah, especially in C-N and C-H, but no clear dietary trend was detectable (Figure 5b).

Figure 4: Correspondence analysis results on the frequency matrix (FM) at 31 and 105 days after hatching: ordination model of age-diet groups (EA-31, EB-31, EC-31, ED-31, EA-105, EB-105, EC-105, ED-105) (a) and descriptor points (b) in axis 1 and axis 2. A-D: abdominal (A) deformation; A-F: A fusion; A-K: A kyphosis; A-L: A lordosis; A-N: A neural elements; A-S: A scoliosis; CC-D: caudal complex (CC) deformation; CC-F: CC fusion; CC-H: CC haemal elements; CC-K: CC kyphosis; CC-L: CC lordosis; CC-N: CC neural elements; CC-S: CC scoliosis; C-D: caudal (C) deformation; C-F: C fusion; C-H: C haemal elements; C-K: C kyphosis; C-L: C lordosis; C-N: C neural elements; C-S: C scoliosis; HYE: hypurals, epural, parhypural; PP: parapophysis.


a
2
1,5
1
0,5
0
-0,5
-1
-1,5
-1,5
-1
-0,5
0
0,5
1
1,5
CA2 (11.5%)
CA1 (61.9%)
EA-105
EA-31
EB-31
ED-105
EB-105
EC-105
EC-31
ED-31
◆ EA-31
■ EB-31
▲ EC-31
● ED-31
◇ EA-105
□ EB-105
△ EC-105
○ ED-105
b
12
10
8
6
4
2
0
-2
-4
-6
-8
-3
-2
-1
0
1
2
3
4
CA2 (11.5%)
CA1 (61.9%)
ABS
A-D
C-L
A-L
C-N
A-N
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PP
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C-H
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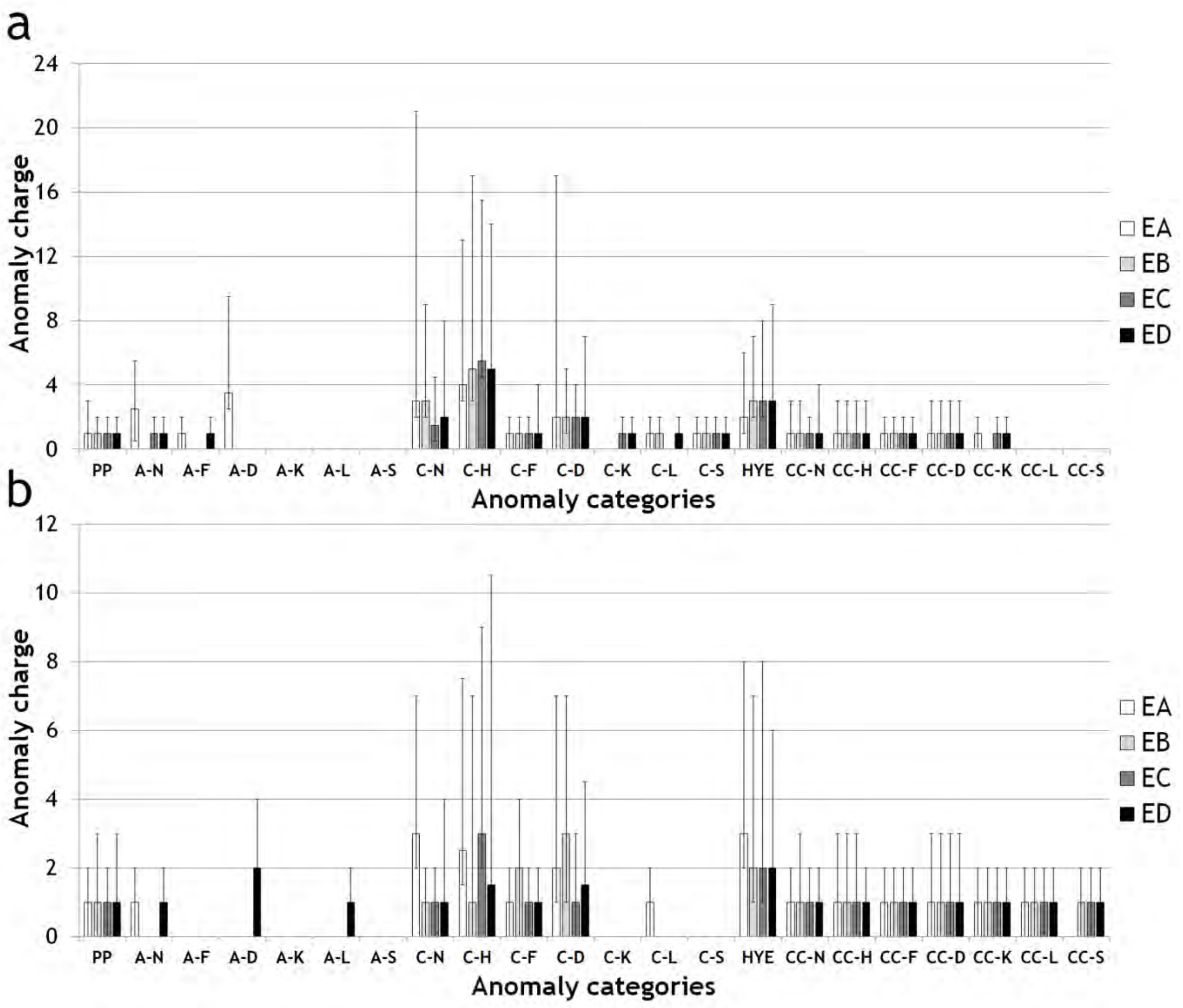

Figure 5: Anomaly charge (average number of anomalies per affected specimen) (median ± range) for each dietary group (EA, EB, EC, ED). (a) 31 days after hatching (dah). (b) 105 dah. Note that y-axis scaling is different in the two graphs. A-D: abdominal (A) deformation; A-F: A fusion; A-K: A kyphosis; A-L: A lordosis; A-N: A neural elements; A-S: A scoliosis; CC-D: caudal complex (CC) deformation; CC-F: CC fusion; CC-H: CC haemal elements; CC-K: CC kyphosis; CC-L: CC lordosis; CC-N: CC neural elements; CC-S: CC scoliosis; C-D: caudal (C) deformation; C-F: C fusion; C-H: C haemal elements; C-K: C kyphosis; C-L: C lordosis; C-N: C neural elements; C-S: C scoliosis; HYE: hypurals, epural, parhypural; PP: parapophysis.

In Figure 6a,b the incidence (frequency of individuals) of general categories is shown for each of 31 and 105 dah diet-groups. Hypurals, epural and parhypural (HYE) were the most affected elements at both stages and in all diet groups. At 31 dah, 100.0% of the specimens showed at least one anomaly in these structures (Figure 6a). High occurrences (64.4-100.0%) of individuals were also affected by deformities of haemal elements in 31 dah groups. EC diet displayed the lowest percentage of abnormalities in neural and haemal structures, fusions (together with EB group), vertebral body deformations and lordosis, but some higher percentage of kyphosis (Figure 6a). Fish fed with EA diet showed more anomalies in neural elements, deformities of vertebral centra and lordosis, whereas parapophysis were more often affected in specimens from EB group (Figure 6a). ED diet showed the highest incidences of anomalies in haemal arches and spines, fusions and scoliosis (Figure 6a). The lower frequency of anomalies of diet EC group in haemal elements was statistically significant compared with EA, EB and ED lots (Fisher exact *P* values of 0.001, 1.69E-05 and 2.44E-07, respectively) (Figure 6a). However, these differences were disregarded as replicates presented differences between them (Fisher exact *P* = 3.77E-06). At 105 dah, EA group showed the highest value for anomalies in neural elements and lordosis, whereas, a larger amount of specimens of ED dietary treatment displayed alterations in parapophysis, fusions, deformations, kyphosis and lesions in the caudal complex structures (Figure 6b). EB and EC groups presented the highest percentage of haemal elements anomalies and scoliosis was more frequent in EB diet (Figure 6b). The incidence of general categories slightly decreased at 105 dah compared to 31 dah and showed some variations among dietary groups (Figure 6b), albeit no pattern or statistically significant differences have been identified for each general category.

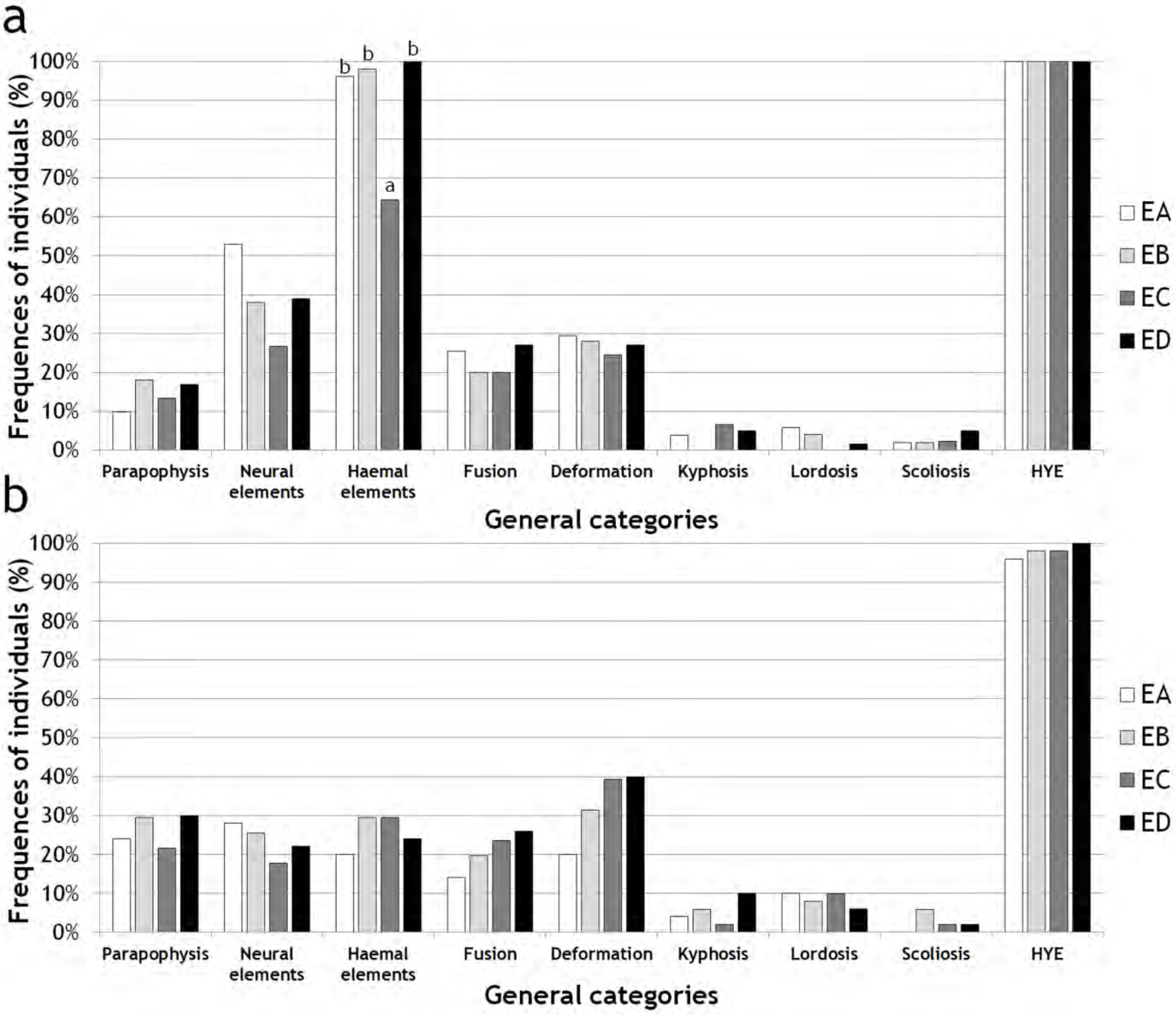

Figure 6: Relative frequency (%) of individuals with at least one anomaly for each dietary group (EA, EB, EC, ED), considering general categories of anomalies. (a) 31 days after hatching (dah). (b) 105 dah. Different letters indicate significant differences in the number of specimens with at least one anomaly among dietary treatments considering each general category (Fisher exact *P* < 0.05). HYE: hypurals, epural, parhypural.

4. DISCUSSION

During this experiment, the survival rate (from 15 dah to 105 dah) remained very high comparing with other studies in Senegalese sole (Dâmaso-Rodrigues *et al.* 2010, Boglino *et al.* 2012b). Growth variations were subtle at the end of the experiment, where no effect of the enrichment products was observed over StL and StH. On the other hand, at 105 dah, the ratio StL/StH was significantly inferior in ED fish than EA, EB and EC groups. This indicates that Senegalese sole fed with ED enriched metanauplii, presented a less elongated profile than other groups. In this sense, at industrial level, the elongated external profile of the sole is preferred than a "plaice-like" form (Riaza personal communication). Interestingly, in this sampling point, individuals from ED diet presented a higher incidence of VBA and/or VCD with respect to other groups. Vertebral anomalies as fusions and rachis deviations can alter the shape and the length of the affected fish (Gavaia *et al.* 2002, Boglione *et al.* 2013b, Cardeira *et al.* 2015), which might partially explain the lower ratio StL/StH observed for ED specimens.

Under these experimental conditions, the majority of fish from all lots presented 9:34:3 vertebrae in abdominal, caudal and caudal complex regions, respectively. These meristic counts are in agreement to those reported by Gavaia *et al.*, 2002 in reared Senegalese sole. However, EA group showed a significant different distribution of the number of caudal vertebrae at 31 dah. These variations could suggest individual variability or the presence of completely fused vertebrae in caudal area, considered as one vertebra. Since no complete fusions were observed in the caudal region of EA group specimens at 31 dah, the last hypothesis should be discarded. The number of vertebrae can be affected by environmental conditions during early development (Roberts & Ellis 2001). Also, some studies have related meristic count variability with the frequency of skeletal anomalies (Prestinicola *et al.* 2013). In addition, EA fish presented more frequently 21 caudal fin rays than the other treatments. Factors as temperature, intensive rearing systems, dietary vitamin A and polyunsaturated fatty acids can affect meristic counts (Villeneuve *et al.* 2006, Fernández *et al.* 2008, Fernández *et al.* 2009, Roo *et al.* 2010, Sfakianakis *et al.* 2011), although the present data is insufficient in order to deduce some effect of dietary enrichment products.

In this experiment, anomalies of the vertebral column in Senegalese sole were very common, reaching 100% incidences. This value is consistent with other reports in this species where the frequency of deformed individuals reached 44% (Gavaia *et al.* 2002, Boglino *et al.* 2012a), 80% (Engrola *et al.* 2005, Gavaia *et al.* 2009, Boglino *et al.* 2012b) and up to 100% in some cases (Fernández *et al.* 2009, de Azevedo *et al.* in press b). The incidence of anomaly categories showed some variations regarding dietary treatments. In particular, only EA individuals showed A-D at 31 dah, as well as C-L and completely normal specimens (ABS) at 105 dah. ED group displayed a higher number of individuals with A-D and A-L at the end of the experiment, although these percentages were below 10%. Therefore the hypothetical association of some anomalies with one diet at 31 dah was not maintained at 105 dah. Moreover, dietary groups did not conserve a similar distribution among ages, for instance, EB and ED fish at 31 dah were situated in completely opposed quadrants regarding their location at 105 dah. The differences among dietary groups were statistically significant using NPMANOVA test where EC group was different from EA and ED. Some studies in Senegalese sole at larval stage showed a higher percentage of specimens with alterations of neural and haemal elements of the caudal complex region with respect to dietary enrichment products, although these variations were minor (Boglino *et al.* 2012b). Since in the present work, fish fed EC diet presented a replicate variation in the incidence of deformities in the haemal structures, Two-Way NPMANOVA was repeated using a modified BM matrix without C-H data. This new test did not show any statistical difference among diets, but differences persisted for age (data not shown). Furthermore, the frequency of C-H among replicates of EC presented variations and the lower number of individuals with C-H in EC lot could be due only to a replicate (data not shown). Hence, significant differences that were found could be driven by the incidence of C-H in one replicate of this batch. This might be due to individual variability or other factors apart from feeding regime. Consequently, the possible effect of EC diet on the incidence of the studied anomalies categories was disparaged. Also chi-square test and Fisher exact test failed to establish a marked influence of enrichments for each general category except haemal elements anomalies, which was not considered due to the previous motive. In addition, other studies did not found a clear influence of *Artemia* spp. enrichment products on the incidence of the overall anomalies (Dâmaso-Rodrigues *et al.* 2010, Boglino *et al.* 2012b).

Concurrently, CA results ordered fish according to the age groups (31 and 105 dah) in separate CA1 semiaxes, establishing different anomaly patterns for each age. Accordingly, Two-Way NPMANOVA found significant differences in anomaly category incidence between both stages. As far as we know, this is the first study that analyses vertebral anomalies in 105 dah Senegalese sole juveniles, using a multivariate exploratory technique. At this age, there was a reduction in the number of fish displaying certain anomalies as A-N, A-F, A-D, C-K, C-L, C-S, but markedly C-N and C-H. The substantial diminution of alterations of caudal neural and haemal elements might be due to an adaptive response. Teleost skeleton can show a considerable plasticity and adaptive response to loading conditions (Witten & Huysseune 2009, Fiaz *et al.* 2010, Dean & Shahar 2012). Fish bone has the ability to remodel and model some vertebral fusions and repair fractures (Witten *et al.* 2006, Takeyama *et al.* 2014, Witten & Hall 2015). In this sense, the presence of a thin layer of bony tissue in some deformed structures at both stages could indicate bone formation, helping to strengthen the concave side of the spines or plates. These altered elements might be undergoing remodelling and modelling processes and perhaps afterwards acquire a non-deformed shape, leading to the attenuation of the incidence of spine deformities at later stages (de Azevedo *et al.* in press a). Further investigation is needed to deepen in the histopathologic features of Senegalese sole's bone adaptive processes.

Some anomaly categories were very frequent in all groups indicating the presence of a common trend or mainstay of vertebral deformities. In particular, the absence of the neural arch in the first abdominal vertebra was recurrent in all specimens, agreeing with a previous work of our research group (de Azevedo *et al.* in press b). The elevated incidence of lesions in caudal complex elements and fusions and deformations of caudal complex vertebrae are comparable with other reports for this species (Fernández *et al.* 2009, Gavaia *et al.* 2009, Losada *et al.* 2014, de Azevedo *et al.* in press b) and were repeatedly common at all diet and age groups. In all groups, VBA and/or VCD frequencies exceeded the 20% reference value for severe deformed fish in intensive production (Boglione *et al.* 2013a), a relevant and sensible value in the industry (Fjelldal *et al.* 2012b).

Comparing the incidence of deformities, VBA and VCD between both stages, some discrepancies can be described among dietary treatments. As for instance, at 31 dah, VBA and/or VCD were more frequent in Senegalese sole fed with EA enriched metanauplii, while, at 105 dah, EA lot displayed the lowest percentage of individuals with these anomalies. Moreover, EB, EC and ED diets presented a higher frequency of individuals with VBA and/or VCD at 105 dah than the respective groups at 31 dah. In fact, the association of some anomalies with one diet at 31 dah changes at 105 dah. These variations in the profile of the abnormalities with age might be due to certain nutrients acting later in development or predispose sole to vertebral deformities, as in Atlantic salmon (*Salmo salar*) juveniles fed with inadequate phosphorus diet (Fjelldal *et al.* 2012a). Also, the existence of common anomalies to all diets in both stages suggests that other causes could contribute to the onset of such alterations. Skeletal malformations are a multi-factorial problem related with several causes mainly environmental and nutritional factors (Boglione *et al.* 2013a,b). In particular, Senegalese sole is influenced by temperature, hypercapnia, photoperiod, vitamin A and vitamin K (Fernández *et al.* 2009, Blanco-Vives *et al.* 2010, Fernández & Gisbert 2010, Dionísio *et al.* 2012, Pimentel *et al.* 2014, Richard *et al.* 2014). Thus, distinct factors, such as rearing conditions, may be exerting their influence on the development of vertebral anomalies in this study. Although no clear dietary influence was detected, other factors may be masking a potential effect of enrichment products. Therefore, further research is required in order to assess the role of zootechnical conditions over the development of skeletal deformities.

At 31 dah, despite a direct association was not found in the number of specimens showing VBA and/or VCD and the incidence of general categories among diets, EC group showed slightly better performances in terms of larval quality, with lower incidences related to VBA and VCD (namely fusions, deformations, lordosis and scoliosis) and neural elements. Studies at the same stage in Senegalese sole showed no effect of *Artemia* spp. enrichment products on major deformities of the vertebral centre except for vertebral fusions (Boglino *et al.* 2012b). Considering the nutritional composition of Senegalese sole larvae, EC group showed intermediate mean values of all analysed nutrients, except for DHA and DHA/EPA ratio, where EC fish presented higher values (9.05-12.96% TFA, 3.58-3.88, respectively). Curiously, nutritional composition of *Artemia* metanauplii enriched with EC diet displayed also an elevated DHA content and ratio DHA/EPA (2.52% TFA, 0.35, respectively), suggesting a good retention of these nutrients in fish. In red porgy (*Pagrus pagrus*), a reduction in the incidence of skeletal deformities was achieved in larvae fed with higher DHA content in rotifers (Roo *et al.* 2009). Nevertheless, in present study, DHA and DHA/EPA content are greatly diminished compared to the recommended dietary levels (9.5% TFA and

3.0) by Boglino *et al.*, 2012b. Still, fatty acid content in Senegalese sole regarding (n-3) PUFA, (n-6) PUFA, DHA and the ratio DHA/EPA were slightly inferior to those reported in Boglino *et al.*, 2012b in the same developmental stage. In turn, DHA and the ratio DHA/EPA dietary amounts were reduced in 40 dah Senegalese sole, in comparison to the present study (Dâmaso-Rodrigues *et al.* 2010). At nutritional level, some molecules can affect the anomaly development (Cahu *et al.* 2003). In particular, reports in Senegalese sole studied the role of vitamin A (Fernández *et al.* 2009, Fernández & Gisbert 2010) and K (Richard *et al.* 2014) as well as arachidonic acid (Boglino *et al.* 2012a) on skeletal deformities. In order to optimize sole's dietary nourishment it should be consider the balance of among nutrients and its interactions (Boglino *et al.* 2012b). Moreover, the final nutritional profile of live preys can change according to culture/enrichment conditions (Conceição *et al.* 2010) and could be affected by certain *Artemia* spp. metabolic pathways (Boglino *et al.* 2012b). Therefore, with the present data it is greatly difficult to detach a single enriching nutrient that could be having an effect in the development of vertebral anomalies.

Considering the overall quality of 105 dah juveniles, the EA diet presented lower incidences of VBA and/or VCD and alterations in the caudal complex elements. The nutritional composition of Senegalese sole fed with EA enriched metanauplii stands out a reduced phosphorous content resulting in a higher ratio Ca/P respect to the other groups, whereas EA metanauplii showed an unexpected intermediate/high P content. However, studies in rainbow trout (*Oncorhynchus mykiss*) showed that the whole body phosphorous concentration responded to its dietary intake (Fontagné *et al.* 2009). In Atlantic salmon, an inadequate phosphorus content in the diet at the juvenile stage could predispose to develop compressed vertebral bodies as postsmolts (Fjelldal *et al.* 2012a). Nevertheless, primary phosphorous deficiency should not be considered the only cause of bone disorders observed in *Salmo salar* since the resulting defect in mineralization was not consistent with the appearance of skeletal anomalies (Witten *et al.* 2015).

In the present study a high percentage of individuals exhibited skeletal anomalies in every dietary group. The main anomalies consisted in alterations of neural/haemal elements, as well as caudal complex plates. Despite some variations in the incidence of anomaly categories among diets, it was not possible to establish a clear effect of enrichment products on the development of vertebral abnormalities at both sampling points. CA results indicated a different anomaly pattern among stages. Regarding VBA and VCD overall incidence, EC and EA groups showed a better performance at 31 and 105 dah, respectively. The existence of very frequent anomalies points to the presence of a common mainstay of vertebral deformities and the influence of other factors like rearing conditions, underneath the onset and progression of such alterations.

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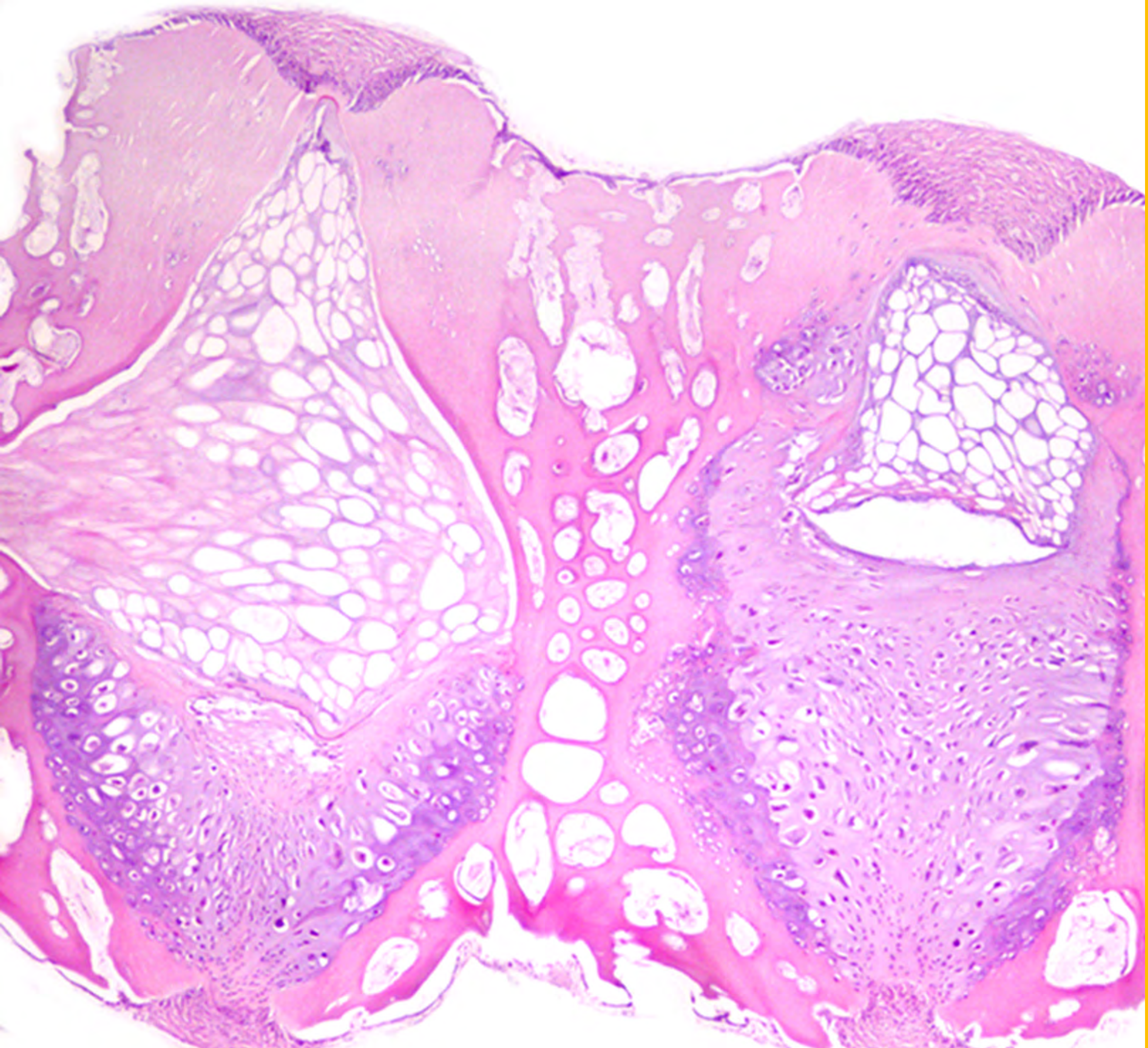
Article V



ARTICLE V: HISTOPATHOLOGY OF DIFFERENT SKELETAL ANOMALIES IN AN ANOSTEOCYTIC BONED FLATFISH SPECIES

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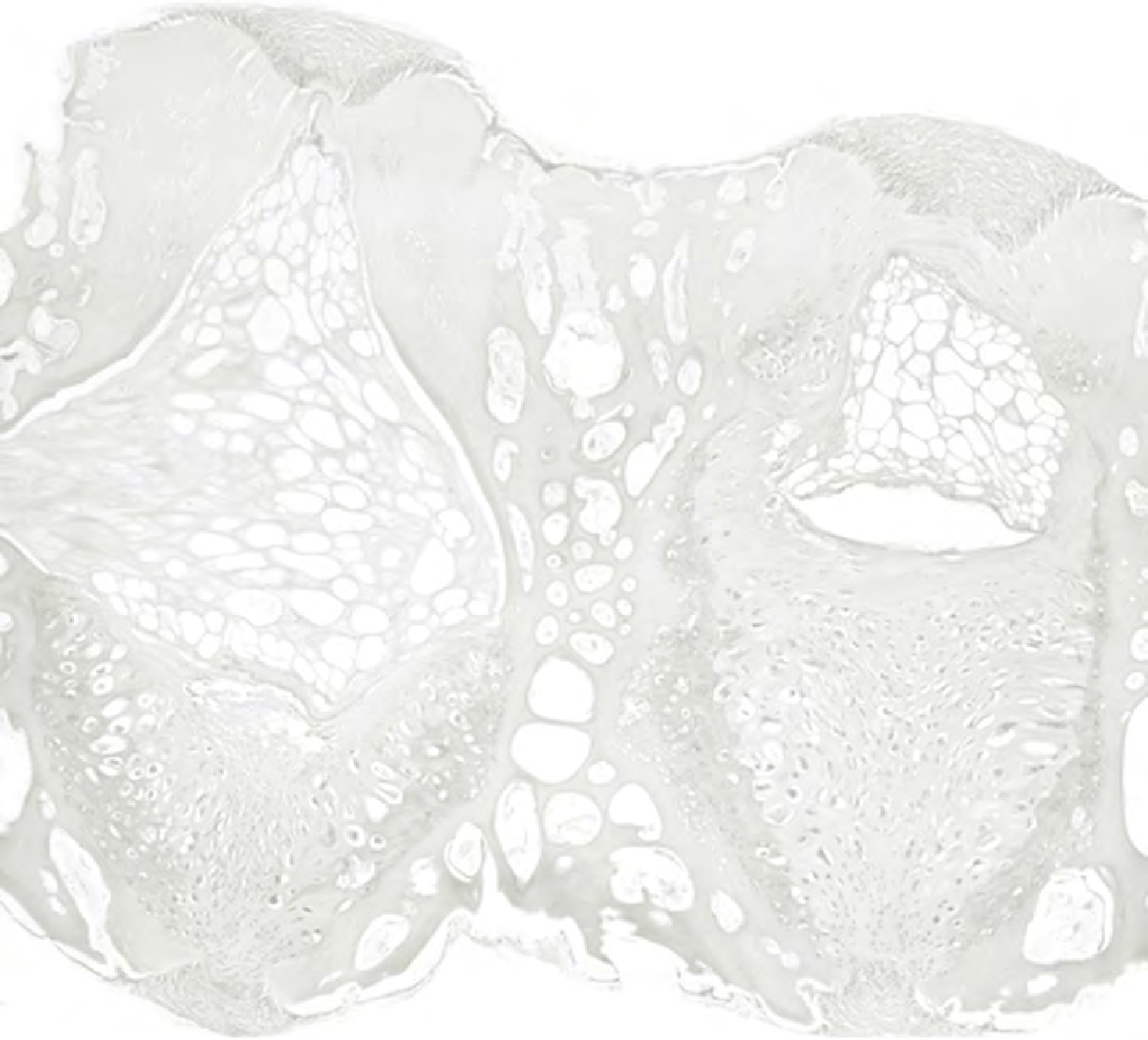


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ABSTRACT

Despite the high incidence of vertebral anomalies in reared Senegalese sole (*Solea senegalensis*), the literature is scarce on the histological features of this flatfish normal and deformed vertebrae. The aim of this study was to evaluate vertebral segments of juvenile Senegalese sole by means of radiographic and microscopical techniques, in order to illustrate the histopathological changes associated to different types of vertebral body anomalies. Senegalese sole from 104 or 105 days after hatching were sampled and radiographed. Based in radiographic images, normal and altered vertebral segments were collected and processed for histopathologic studies. Six individuals presented deformed vertebrae whereas three specimens displayed vertebral fusions. Vertebrae with alterations of the morphology showed narrowing of the intervertebral space (IVS) as well as flattening of the endplates in the radiographic images. These findings were consistent with the histopathological approach which also detected irregular articular surfaces and changes in trabecular bone, sometimes in opposing vertebrae. The presence of radiolucent ectopic cartilaginous tissue was obvious mainly in the endplates of most of the deformed vertebra and occasionally from the growth zone into the IVS. Some of these lesions could be associated to an initial step of fusion. The majority of the fused vertebrae were reshaped, showing reorganization of the trabeculae, although in one case, the proliferation of cartilage in the adjacent IVS could indicate the aggravation of the primary fusion process. Moreover, sporadic notochordal alterations were observed mainly in the external elastic membrane and the collagenous layer, which could be related with the development of certain vertebral anomalies. The histological techniques greatly complemented the radiographic approach and contributed to increase the understanding of some possible mechanisms inherent to the onset of vertebral deformities in Senegalese sole. Further research would be required on sensing and local effector pathways underneath the development of cartilaginous tissue related with vertebral disorders in anosteocytic bone.

Keywords: Skeletal anomalies, Senegalese sole (*Solea senegalensis*), histopathology, vertebrae, anosteocytic bone

1. INTRODUCTION

Skeletal anomalies still constitute a great concern in many species aquaculture (Boglione *et al.* 2013a), and Senegalese sole (*Solea senegalensis*) is no exception (Howell *et al.* 2009, Boglino *et al.* 2012). This flatfish species shows a great potential to the sector, as its European production has almost doubled in just one year (FAO 2016). However, it is affected by a high incidence of vertebral abnormalities both in experimental conditions, with 44-100% of deformed individuals in some lots (Gavaia *et al.* 2002, Fernández *et al.* 2009, Gavaia *et al.* 2009), as well as, in farming settings with an incidence ranging from near 80% up to 100% (Losada *et al.* 2014, de Azevedo *et al.* in press). Like in other fish species, the problem affects animal welfare (Fjelldal *et al.* 2012, Boglione *et al.* 2013a) but also leads to serious economic losses as it decreases market value (Fernández *et al.* 2008) and downgrades the final product (Fjelldal *et al.* 2012, Boglione *et al.* 2013b).

Some causative factors have been associated with the development of skeletal deformities in Senegalese sole, especially those involving environmental (Blanco-Vives *et al.* 2010, Dionísio *et al.* 2012, Pimentel *et al.* 2014) and nutritional conditions (Fernández *et al.* 2009, Fernández & Gisbert 2010, Richard *et al.* 2014). Boglione *et al.*, 2013b reviewed some of the main skeletal anomaly causes. Nevertheless, there are still some gaps in the knowledge to identify all the causal agents at industrial rearing where multilevel factors interact on the developing organisms (Boglione *et al.* 2013b).

Despite the fact that macroscopic monitoring of malformations in Senegalese sole farms is based in palpation and observation of the fish (Rodríguez & Peleteiro 2014), nowadays other techniques are available to address the problem. Studies in this species were conducted mainly using double staining technique (Gavaia *et al.* 2000, 2002, de Azevedo *et al.* in press), computerized radiography (Losada *et al.* 2014, Cardeira *et al.* 2015) and even computed tomography (Losada 2016) in order to accurately detect spinal alterations in whole-body samples. Nevertheless, little is known on the architecture and mechanical properties of fish bone, particularly anosteocytic bone (i.e. bone without osteocytes) (Dean & Shahar 2012). Considering the histopathological description of skeletal anomalies, vertebral compression and fusion were characterized in an osteocytic bone species as Atlantic salmon (*Salmo salar*) (Kvellestad *et al.* 2000, Witten *et al.* 2005, 2006). Also a molecular approach was used in developing vertebral deformities in this species (Ytteborg *et al.* 2010a,b). On the other hand, vertebrae were also assessed at a tissue level in anosteocytic bone fish as gilthead seabream (*Sparus aurata*) (Fernández *et al.* 2012, Ortiz-Delgado *et al.* 2014) and European seabass (*Dicentrarchus labrax*) (Kranenbarg *et al.* 2005a). However, specifically for the species under study, scarce reports exist on the histological features of bone lesions. In this sense, Cardeira *et al.*, 2015 characterized the micro-anatomical changes in lordotic and kyphotic vertebral curvatures of Senegalese sole, a species with intramembranous anosteocytic-boned vertebrae (Cardeira *et al.* 2012). Therefore, investigation with techniques that elucidate vertebral column disorders at tissue level could contribute to increase the understanding of osseous reaction underneath the development of different vertebral anomalies in this flatfish.

The aim of this work was to evaluate normal and deformed vertebral segments of juvenile Senegalese sole by means of radiographic and microscopical techniques, in order to illustrate the histopathological changes associated to different types of vertebral body anomalies. In a wider scope, this study also pursued to deepen into the knowledge on the possible pathogenic mechanisms underlying their development.

2. MATERIALS AND METHODS

A total of 67 juvenile Senegalese sole from 104 or 105 days after hatching, ranging from 5.9 cm to 9.8 cm (standard length) were sampled. Individuals came from a fish farm in Northwest of Spain showing an incidence of skeletal anomalies around 40% at grading point

(Riaza personal communication). Specimens were then euthanized with an overdose of Tricaine methanesulfonate (MS-222, Sigma-Aldrich) and fixed in 10% buffered formalin. Later, computed radiography was performed in two orthogonal projections according to Losada *et al.*, 2014 and using methacrylate plates in order to position the fish. Radiographic images were then evaluated with RadiAnt DICOM Viewer 1.9.16.7446 software to detect skeletal deformities. Assessment was focused exclusively on vertebral body anomalies (VBA) (fusions and deformations) and vertebral column deviations (VCD) (kyphosis, lordosis and scoliosis). Seven fish showing radiographically evident deformities were selected, as well as, seven non-deformed specimens which served as control. Vertebral segment sampling was performed taking into account all affected vertebrae and normal vertebrae from control fish corresponding with similar anatomic areas. These portions were decalcified for 48 h in Osteodec® (Bio-Optica, Milano, Italy) and embedded in paraffin. Parasagittal sections of 3 µm were obtained and stained with general procedures as haematoxylin-eosin (H&E). A modified alcian blue/haematoxylin-eosin staining (AB-H&E) and alcian blue/periodic acid-Schiff technique (AB-PAS) were performed for the identification of acidic mucins in cartilaginous matrix. Also, a modified Gallego's trichrome (GT) (Ortiz-Hidalgo 2011) was used for the differentiation of collagen fibres, muscle and cartilage. To demonstrate osteoid, mature bone and cartilage, an osteoid staining (OS) was applied using a modification of Ralis and Watkins, 1992 method. Histological slides were mounted using Coverquick 2000 mounting medium (Prolabo®) and observed using an Olympus® BX51 microscope equipped with a digital camera Olympus® DP72. A comparative study was performed between affected areas and non-deformed vertebrae both from the same individual and from control segments.

3. RESULTS

The radiographic approach of sole juveniles allowed an adequate visualization of the vertebral column, although in smaller specimens, the definition of the preurals and the urostyle was impaired. Radiographic images of non-deformed individuals showed an aligned rachis, composed by subsequent vertebrae along the abdominal, caudal, and caudal complex region (Figure 1a,b). Vertebrae showed an internal structure similar to an "X" interleaved by radiolucent intervertebral spaces (IVS) (Figure 1c). Histologically, vertebral bodies presented a symmetrical amphicoelous form and followed a line interposed by IVS filled with notochordal tissue (Figure 1d). Table 1 displays the main staining characteristics of the tissues of the vertebral column. Vertebrae composed by anosteocytic osseous tissue displayed trabecular bone situated between the two articular surfaces of laminar bone (Figure 1d,e). A layer of osteoblasts secreting bone extracellular matrix (osteoid) was observed in the growth zone of the endplates covered by external intervertebral connective tissue (Figure 1e). In the intervertebral space, the notochord was evident with vacuolated chordocytes in the centre and chordoblasts in the notochordal epithelial layer (Figure 1d). In some cases, chordocytes were less vacuolated in a denser area in the notochord centre, depending on the parasagittal section (Figure 1d). In IVS, notochordal cells were encased by a collagenous layer (notochordal sheath) and the external elastic membrane (Figure 1e). Osteoid staining revealed the presence of mature bone in the vertebral centrum, except the endplates where osteoid was the major component (Figure 1f).

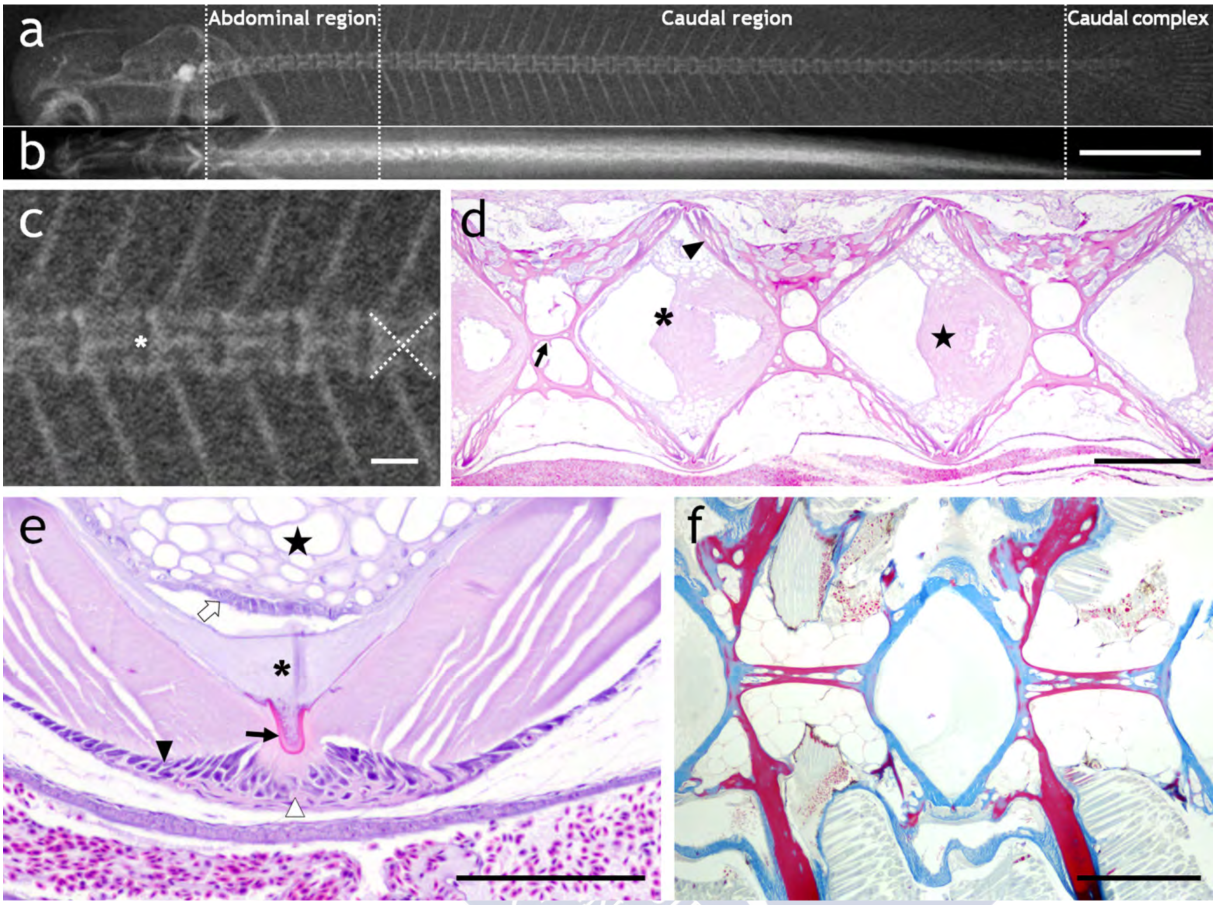

Figure 1: *Solea senegalensis*, control fish vertebrae visualised by radiography (a-c) and histological procedures: modified alcian blue/haematoxylin-eosin staining (AB-H&E) (d), haematoxylin-eosin (H&E) (e) and osteoid staining (f). (a) and (b): Latero-lateral (LL) (a) and dorso-ventral (b) radiographic projections of the vertebral column. Vertebrae were aligned and interposed by the intervertebral space (IVS) along the abdominal, caudal and caudal complex regions. Bar = 1 cm. (c) LL detail of an undeformed vertebral segment. Radiographically, vertebral internal structure was symmetric and similar to an "X" (dashed line). Radiolucent and uniform IVS (asterisk) were located between vertebral centra. Bar = 1 mm. (d) Histological section of a vertebral segment displaying symmetrical biconcave vertebral bodies (AB-H&E). Osseous tissue was arranged in bony trabeculae (arrow) in the main centra whereas lamilar bone was present in the articular surfaces (arrowhead). The IVS (asterisk) was occupied by the notochordal structures. Note the less vacuolated chordocytes in a central area of the IVS (star). Bar = 500 µm. (e) Growth zone of the endplates showing a line of osteoblats (black arrowhead) secreting osteoid surrounded by the intervertebral connective tissue (white arrowhead) (H&E). Note the lack of osteocytes in the laminar bone of the articular surface. Notochordal structures were composed by central vacuolated chordocytes (star) surrounded by a layer of chordoblasts (white arrow). The collagenous notochordal sheath (asterisk) and the external elastic membrane (black arrow), displaying an exuberant fuchsia colour, can be also observed. Bar = 100 µm. (f) Osteoid staining displaying mature bone (bright red) in almost the entire vertebral centra. Note the blue staining of the endplates, revealing osteoid. Bar = 500 µm.

Table 1: Summary of staining characteristics of some tissue components of the normal/deformed vertebral column (modified from Ralis and Watkins, 1992). AB-H&E: modified alcian blue/haematoxylin-eosin staining; AB-PAS: alcian blue/periodic acid-Schiff staining; GT: modified Gallego's trichrome; H&E: haematoxylin-eosin; OS: osteoid staining.

	H&E	AB-H&E	AB-PAS	GT	OS
Osteoid	Pink	Pink	Pink	Cyan	Deep blue
Mature bone	Pink	Pink	Pink	Cyan	Red
Notochordal external elastic membrane	Fuchsia	Fuchsia	Fuchsia	Dark magenta	Yellow/orange
Notochordal collagenous layer	Light purple	Bluish purple	Bluish purple	Light magenta	Light blue
Cell nuclei	Purple	Purple	Pink	Magenta	Brown
Cartilage	Purple	Alcian blue	Alcian blue	Dark magenta	Pale blue

In this study, deformations and fusions were the two major types of anomalies found in radiographic images. These lesions were mainly located between the last three abdominal vertebrae and the first ten caudal centra. Six individuals presented 23 deformed vertebrae in total, and 3 fish displayed 4 fusions; three involving two vertebral bodies, whereas one comprised three centra. Alterations of the shape of the vertebral bodies (deformations) showed distinct degrees of severity. The most common features consisted in flattened and irregular articular surfaces often more radiodense (Figure 2a). Generally, IVS between deformed vertebrae was narrowed (Figure 2a,b), and, occasionally, affected centra connected in an aspect of the articular surface (Figure 2a). Micro-alterations of the articular bone consisted in loss of symmetry and/or flattening of one part or the whole endplate as well as irregular articular surfaces (Figure 2b). The deformed vertebral bodies showed an alteration of the bony internal trabeculae in all specimens (especially longitudinal ones). In most cases, the main trabeculae were obliquely, sometimes in an opposed manner similar to a convex (Figure 2b) or concave structure without affecting the vertebral axis. Around 70% of the deformed vertebrae showed a variable number of cells resembling chondrocytes located majorly in the articular surfaces, towards the IVS. These cells were especially located in dorsal or ventral aspect of the endplate, near the growth zone (Figure 2c). Usually, when opposed centra trabeculae formed a convex structure, chondrocytes were located ventrally (Figure 2b). In several vertebrae, the number of chondrocytes was high, as well as the amount of extracellular cartilaginous matrix (ECM) between these cells. Chondrocytes were placed in lacunae bordered by a halo of ECM (Figure 2c).

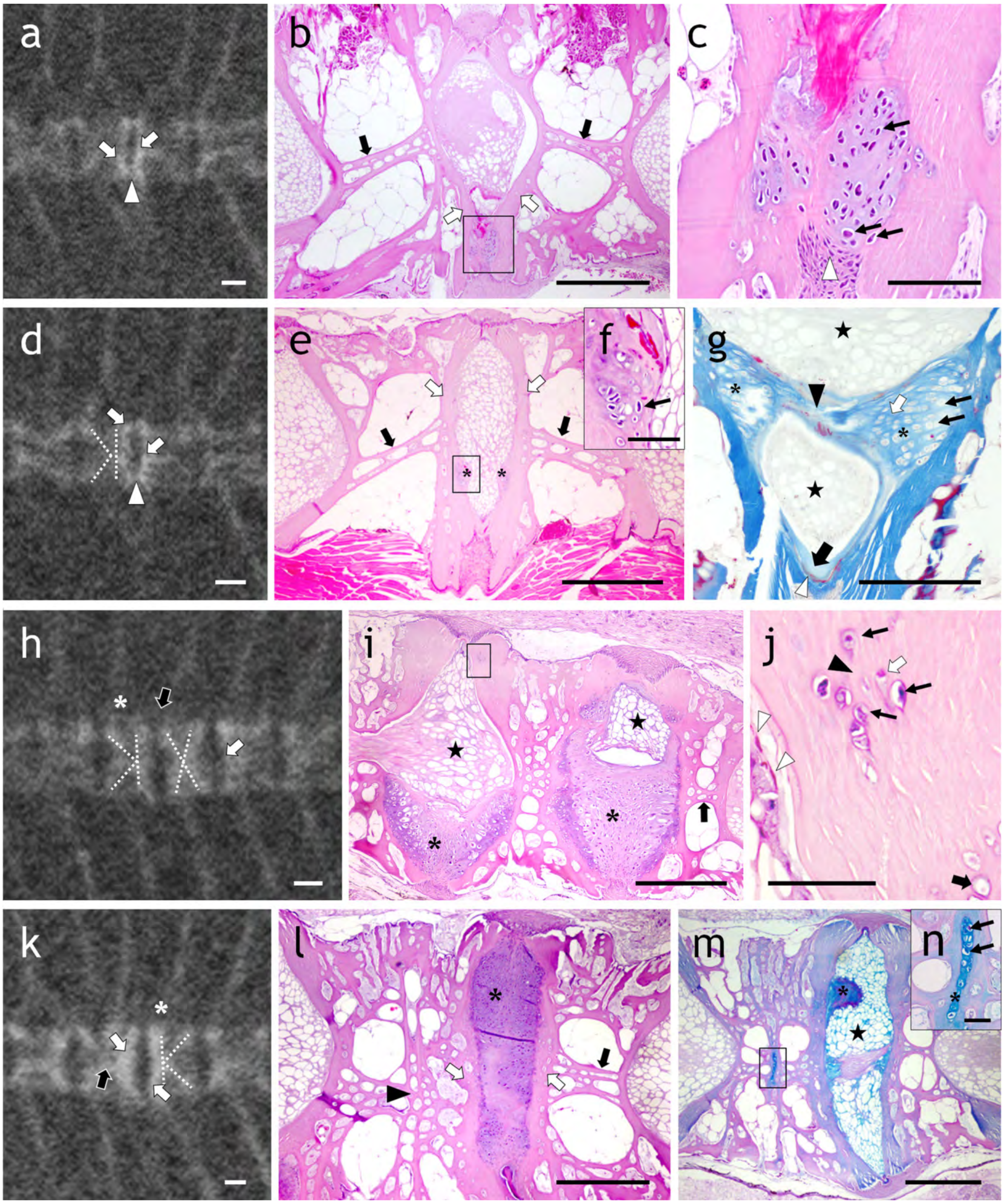
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Figure 2: *Solea senegalensis*, deformed vertebrae visualised by radiography (a,d,h,k) and histological procedures: haematoxylin-eosin (H&E) (b,c,e,f,i,j,l,), osteoid staining (OS) (g) and alcian blue/periodic acid-Schiff staining (AB-PAS) (m,n). (a) Latero-lateral radiographic projection (LL) of deformed centra displaying flattened endplates (white arrows) which were more radiodense and seemed to contact in one point (white arrowhead). These lesions were distributed as a "mirror" image. The intervertebral space (IVS) between the affected vertebrae was narrower than the neighbouring IVS. Bar = 500 µm. (b) Histological evaluation of the same segment as (a) (H&E). Longitudinal trabeculae were altered and obliquely orientated (black arrows) in a "mirror" image, forming a convex structure. The articular surfaces showed loss of symmetry both in cranial-caudal axis as well as in dorsal-ventral axis (white arrows). Note the presence of cartilaginous tissue in the proximity of the growth zone (rectangle), in the ventral aspect of the vertebral column. Bar = 500 µm. (c) Magnification of the rectangle from (b) (H&E). Cartilage was observed in straight contact with the growth zone (white arrowhead) and the endplates. Chondrocytes were evident (black arrows) in lacunae, surrounded by abundant purple cartilaginous extracellular matrix (ECM). Bar = 100 µm. (d) LL showing alterations of the vertebral shape and internal structures as "K"-like vertebrae (dashed lines) and opposing flattened and radiodenser articular surfaces (white arrows). The IVS was reduced and, in one point, the endplates connected with each other (white arrowhead). Bar = 500 µm. (e) Section from the same segment as (d) (H&E). Longitudinal trabeculae were oblique (black arrows) configuring a convex structure. Articular bone was flattened in facing vertebrae (white arrows) and showed bulges of chondrocytes towards the narrower IVS (asterisks) in a "mirror" image. These protuberances added an irregular appearance to middle ventral articular surfaces. Bar = 500 µm. (f) Higher magnification of one of the irregular prominences displaying chondrocytes (black arrow) embedded in cartilaginous ECM (H&E). Bar = 50 µm. (g) Higher magnification of (e) in a deeper section (OS). The cartilaginous prominences (asterisks) were composed by chondrocytes (black arrows) which were surrounded by a deep blue matrix compatible with osteoid (white arrow). This matrix connected both protuberances (black arrowhead), sectioning the notochord in two parts (stars). Note the alteration of some notochordal structures as the irregular external elastic membrane (white arrowhead) and the slightly thinner collagenous layer (thick black arrow). Bar = 200 µm. (h) LL of a deformed vertebral segment. Centra displayed alterations of the morphology, such as "K"-like shape (inversed "K" dashed line) and both sides compression ("X" dashed line). Flattened endplates were also observed (white arrow). One vertebra (asterisk) seemed slightly enlarged respect to normal vertebrae around the lesion. A minimum misalignment of the rachis was observed in the same region towards the ventral aspect of the fish (black arrow). The IVS between affected centra were irregular and reduced. Bar = 500 µm. (i) Histopathologic section of the segment from (h) (H&E). Alterations of the orientation of the bony trabeculae were observed (black arrow). Two of the IVS were filled with cartilaginous tissue (asterisks), at least in one part of the space. Cartilage was quite symmetrically distributed in cranio-caudal axis, associated with the articular surfaces which were irregular. In consequence of the IVS invasion by cartilaginous tissue, the notochord was condensed, occupying the remaining part of the IVS (stars). Bar = 500 µm. (j) Magnification of the rectangle in (i) (H&E). Chondrocytes (black arrows) in this zone of the articular surface were few, showing scarce amount of ECM around lacunae, as a halo. Surrounding these cells, a pink matrix compatible with osteoid was observed (black arrowhead). As chondrocytes went deeper into the endplate, they appeared smaller (white arrow). Round empty lacunae were also observed, presenting the same halo, disclosing the nature of the cell (thick black arrow). Note the fragmentation of the notochord external elastic membrane (white arrowheads). Bar = 50 µm. (k) LL of fused (black arrow) and deformed centra which displayed facing flattened endplates (white arrows). One centra showed a "K"-like structure (dashed lines) as well as a mild enlargement respect to the adjacent caudal vertebra (asterisk). Fused vertebrae were also shorter in cranial-caudal axis. The IVS between fused and deformed centra was narrower although radiolucent. Bar = 500 µm. (l) Histological section of the same sample of (k) (H&E). On one hand, vertebral fusion was observed among two centra, displaying reorganized trabeculae and the line of fusion which was merged to longitudinal trabeculae (black arrowhead). Adjacent vertebra showed also deformations with alterations of longitudinal trabeculae (black arrow). The opposing articular surfaces from caudal fused vertebra and the adjacent centre were flattened (white arrows) and surrounded the IVS, completely occupied by cartilage which blended with the endplates (asterisk). Bar = 500 µm. (m) Serial section from the same segment as (l) (AB-PAS). The IVS is occupied by notochordal structures (star). A matrix compatible with ECM was observed in a small area (asterisk). Bar = 500 µm. (n) Magnification of the rectangle in (m) (AB-PAS). Remnants of chondrocytes (black arrows) surrounded by alcian blue stained ECM (asterisk) were present in the vertebral fusion centre, in the intersection of perpendicular trabeculae. Bar = 50 µm.

In one side compressions, the radiological image of the centrum presented a "K"-like shape in some vertebrae (Figure 2d), which microscopically corresponded with a flattened endplate from one side and oblique trabeculae (Figure 2e). Chondrocytes were also situated in other regions of the articular bone than the growth zone. Sometimes, the numerous chondrocytes formed a bulge which protruded towards the notochord in the IVS in a specular manner in the opposing vertebra (Figure 2e,f). In two individuals, the bulges joined together, forming a bridge among the vertebral bodies. This union was mediated by the presence of osteoid material in one case (Figure 2g). A vertebra showing compression from both sides in the cranial-caudal axis was observed in one sole (Figure 2h). Moreover, the thickness and height of some vertebral bodies were enlarged respect to the normal vertebrae in the proximity (Figure 2h). These lesions were concomitant with a mild misalignment of the rachis in that area in one individual (Figure 2h). At microscopical level, the presence of cartilaginous tissue was evident invading the IVS bounded by the enlarged and deformed vertebrae (Figure 2i). The extension of this material ranged from 30% to 100% of the IVS, depending on the depth of the cut section.

It should be noted that almost all the trabecular and articular lesions were situated as a mirror image between two consecutive vertebrae (Figure 2a,b,d,e,g-i). Respect to the presence of chondrocytes, when the affected area showed few chondrocytes, those were isolated and embedded in bone matrix (osteoid), forming a tissue compatible with chondroid (Figure 2j).

More severe anomalies were observed, combining fusions and deformations (Figure 2k). In this case, a huge amount of cartilaginous tissue was perceived in the IVS between fused vertebrae and the opposing centra in histological sections (Figure 2l). However, in a serial section, chondrocytes disappeared and the IVS was occupied by notochordal structures with a small amount of ECM (Figure 2m) related with the presence of chondrocytes in the articular bone.

Cartilaginous tissue within the IVS was rich in chondrocytes surrounded by abundant ECM (Figure 3a,b), compatible with cell-rich hyaline cartilage. Chondrocytes presented different degrees of maturation according with their location in the IVS (Figure 3a). The central region was usually occupied by small slightly elongated chondrocytes (Figure 3a,b). At both sides of this area, hypertrophied chondrocytes were present and finally, there was a zone where cartilaginous tissue was associated with articular surfaces (Figure 3a,b). In the former region, the matrix involving some groups of chondrocytes changed its affinity for the dye (Figure 3b,c).

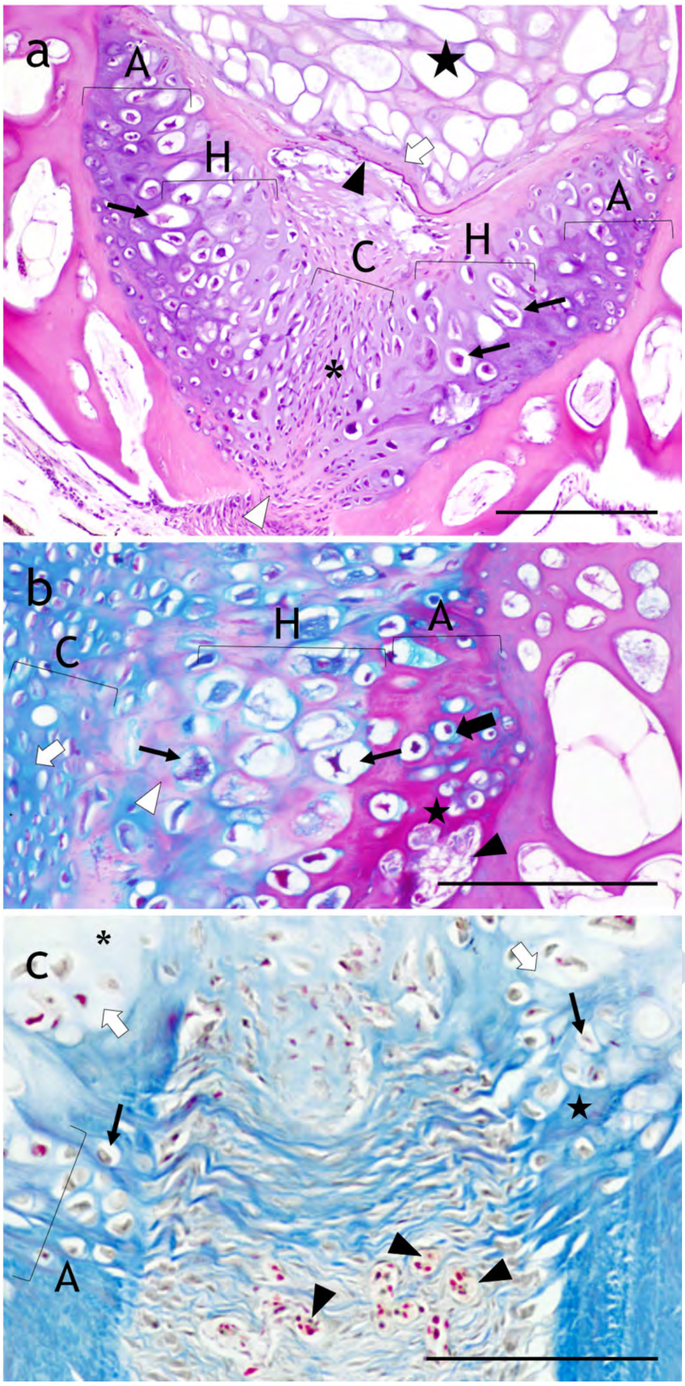

Figure 3: Detail of the intervertebral space (IVS) area from Figure 2h stained with haematoxylin-eosin (H&E) (a), alcian blue/periodic acid-Schiff staining (AB-PAS) (b) and osteoid staining (OS) (c). (a) Cartilage proliferating in the IVS (asterisk) from the growth zone (white arrowhead) that seems to push the notochord (star) (H&E). The tissue was composed by a central area (C) mainly with elongated small chondrocytes. At both sides (H), cartilaginous cells were rounded and hypertrophied (black arrows). In the part close to the articular surfaces (A) chondrocytes were smaller. A purple stained cartilaginous extracellular matrix (ECM) surrounded the cartilage cells, although acquired a darker colour in the area in contact with the endplates (A). Note the presence of lesions in the notochordal structures as the irregular external elastic membrane (black arrowhead) and the thinner collagenous sheath (white arrow). Bar = 200 µm. (b) AB-PAS staining showing a high number of chondrocytes embedded in an abundant alcian blue stained ECM (white arrow), especially in the central area (C) (AB-PAS). Note the slight change in the colour of the matrix around the

hypertrophic chondrocytes (black arrows) in the intermediate zone (H) to pink (white arrowhead). In the area related with the articular bone (A) the territorial ECM (black thick arrow) around the chondrocytes was blue stained although the interterritorial matrix (star) was almost the same colour from the adjacent bone. Occasionally, it was difficult to distinguish the boundaries between the cartilage in the IVS and the articular surface since both presented the same aspect. Some trabecular lacunae were located in the endplates of altered vertebrae near areas related with the presence of chondrocytes (black arrowhead). Bar = 200 µm. (c) Osteoid staining showing cartilaginous tissue associated with the articular bone near the growth zone (A) (OS). Chondrocytes in this area (black arrows) were mainly embedded in deep blue osteoid matrix (star). However, in the furthest parts from the endplate, a pale blue cartilaginous ECM was present around the chondrocytes (white arrows) and in the IVS (asterisk). In the growth zone, adjacent to the osteoblast layer, a considerable number of blood capillaries was observed (black arrowheads). Bar = 100 µm.

Deformed vertebral segments showed also alterations in notochordal structures. The external elastic membrane was usually scattered and irregular in the convergent area of two articular surfaces showing chondrocytes (dorsal or ventral) (Figures 2g,j and 3a). The collagenous layer associated to the damaged membrane was occasionally less evident or thinner (Figures 2g and 3a). A high number of blood capillaries was occasionally present in the connective tissue, externally to the layer of osteoblasts (Figure 3c). A schematic summary of the histological changes in deformed vertebrae is represented in Figure 4.

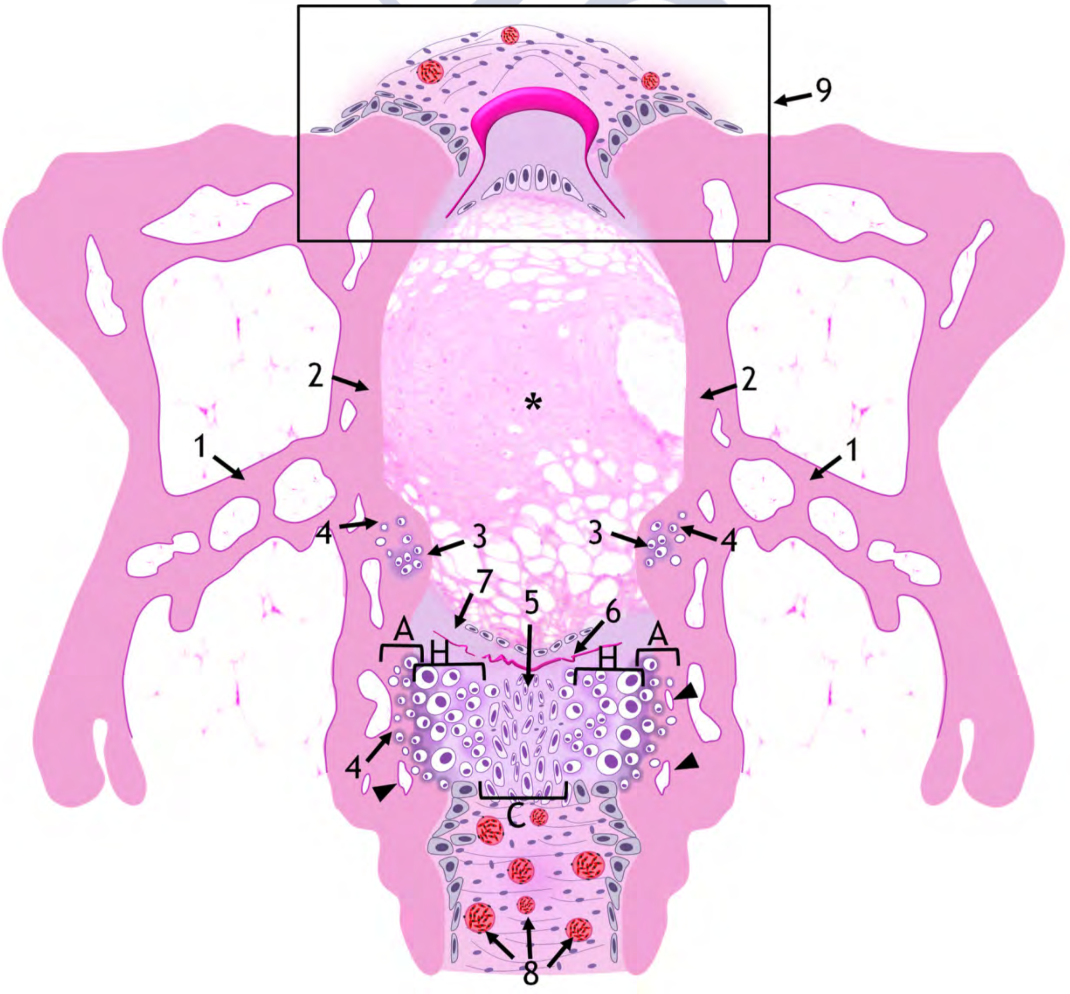

Figure 4: Schematic illustration of the most representative histological changes observed in deformed vertebrae. 1. Alteration of the bony internal trabeculae in a specular oblique manner. 2. Loss of concavity of one part as well as irregular articular surfaces. 3. Presence of a variable number of chondrocytes located in the articular surfaces, occasionally forming protuberances towards the notochord (asterisk) in the intervertebral space (IVS) in opposing vertebrae. 4. Chondrocytes isolated and embedded in bone matrix. 5. Proliferation of cartilaginous tissue invading the IVS showing some organization: central area (C) with small chondrocytes; hypertrophied chondrocytes (H) and chondrocytes associated with the articular bone (A) showing the presence of some trabecula lacunae (arrowheads). 6. External elastic membrane was scattered and irregular. 7. Thinner collagenous layer. 8. High number of blood capillaries in the connective tissue near the growth zone. 9. Unaffected area.

In relation to fused vertebrae, these were radiographically evident displaying an extra number of neural and haemal elements (Figure 5a), although in many cases the union was reshaped, showing almost the same length as a normal vertebra (Figure 5a). Histopathologic findings in fused vertebrae consisted mainly in the reorganization of the internal longitudinal trabeculae which were cranio-caudally linked (Figure 5b). The fusion line persisted perpendicular to the longitudinal axis (Figure 5b). Generally, in serial sections, remnants of the notochord and the external elastic membrane were detected in the centre of the anomaly (Figure 5c). In one case, a small portion of cartilaginous tissue was observed in the same location (Figure 2n). Osteoid staining showed mature bone in the central region in most of the fused vertebrae (Figure 5d). Fusions among three vertebral bodies were also detected (Figure 5e). In this case, bone trabeculae were not completely reshaped and two small notochord portions were observed in the corresponding location of the former IVS (Figure 5f).

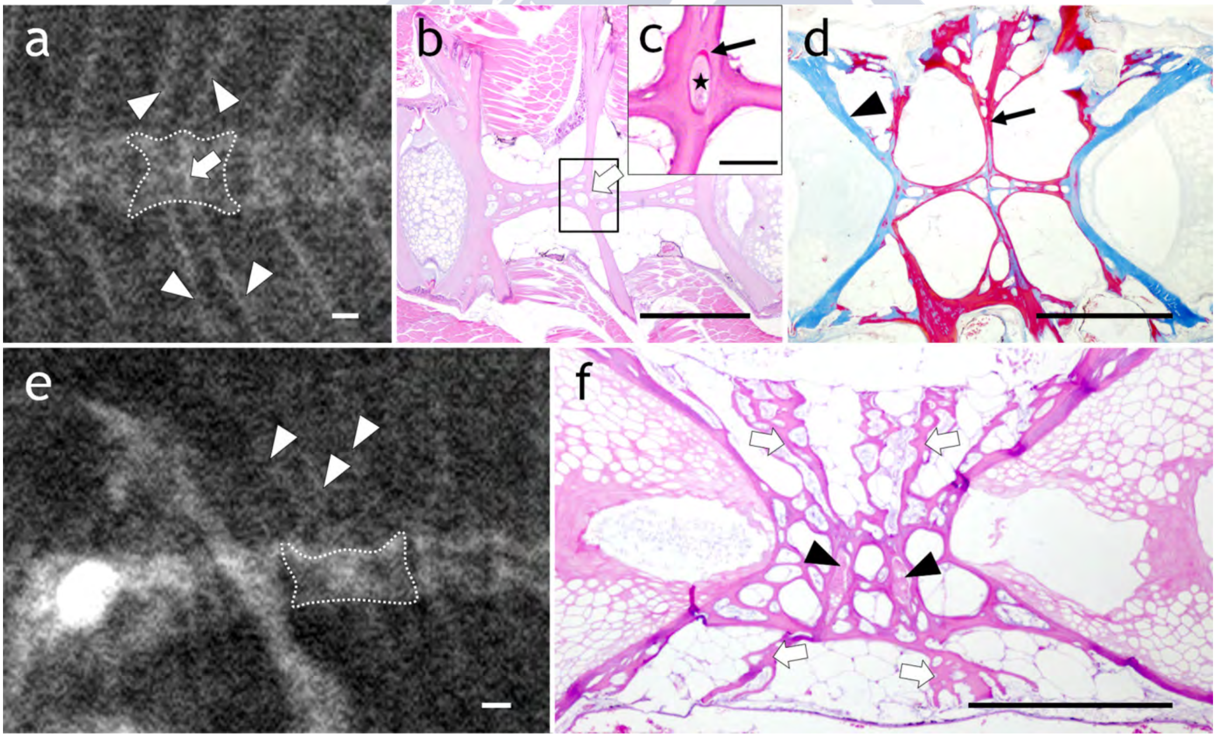

Figure 5: *Solea senegalensis*, fused vertebrae visualised by radiography (a,e) and histological procedures: haematoxylin-eosin (H&E) (b,c), osteoid staining (OS) (d) and a modified alcian blue/haematoxylin-eosin staining (AB-H&E) (f). (a) Latero-lateral radiographic projection (LL) of a complete fusion between two vertebrae (dashed line) exhibiting two neural and haemal elements (white arrowheads). The union was reshaped although the line of fusion remained (white arrow). The affected vertebral bodies were shortened and the fusion displayed almost the same length as the adjacent non-deformed vertebrae. Bar = 500 µm. (b) Histopathologic assessment from the same vertebral segment as (a). (H&E). Bone internal longitudinal trabeculae from both centra were reoriented and connected in the cranio-caudal direction. In the middle point, the perpendicular line of fusion remained (white arrow). Bar = 500 µm. (c) Higher magnification of the rectangle area from (b) in a serial section (H&E). A remnant of the notochordal structures (star) persisted in the intersection of the trabeculae. Note the presence of the fuchsia external elastic membrane (black arrow). Bar = 50 µm. (d) Osteoid staining from a serial section from the same sample as (b). Note the presence of mature osseous tissue in the reorganized trabeculae in the fused centra and in the fusion line (black arrow). The endplates still displayed a deep blue staining compatible with inmature osteoid (black arrowhed). Moreover, the intervertebral space (IVS) at both sides of the fusion lacked significant alterations. Bar = 500 µm. (e) LL of a specimen showing a fusion among three vertebral centra (dashed line) in the abdominal region. The correspondent neural spines are visible (white arrowheads). Bar = 500 µm. (f) Histological evaluation from the same sample of (e) (AB-H&E). The vertebral trabeculae were almost reshaped in this three centra fusion. However, some osseous trabeculae, resembling the fusion lines, were present (white arrows), delimiting the affected vertebrae. In the centre of the fusion, two ovoid areas were observed containing notochordal structures (black arrowheads) in the corresponding area of the former IVS. Bar = 500 µm.

4. DISCUSSION

The present study enlightens some of the main vertebral body anomalies affecting Senegalese sole by means of radiographic and histopathologic approaches. Radiographic technique was useful and convenient to the proper detection of the alterations in vertebral centra at this stage of development. This method allowed a guided approximation of the main lesions in order to establish an exact correspondence with histologic sections. However, there existed some difficulties to assess vertebral anomalies in caudal complex region, especially in smaller juveniles, which constitutes some of the most affected area in Senegalese sole (Losada *et al.* 2014). Decreased bone definition was observed in these situations and could be due to reduced bone density at juvenile stages (Hjelde & Bæverfjord 2009) as well as to a low amount of soft tissue (Witten *et al.* 2009) in the caudal complex area. So, other diagnostic techniques compatible with histopathologic procedures should be addressed in such circumstances, as for instance, the micro-computed tomography (Epple & Neues 2010, Losada 2016).

Two major types of vertebral abnormalities, deformed and fused vertebrae were observed. Similar alterations were previously described in other studies in other stages of development, using double staining technique for cartilage and bone (Gavaia *et al.* 2009, de Azevedo *et al.* in press). Deformed vertebral segments showed alterations of longitudinal bony trabeculae and flattened articular surfaces both in radiographic images as at tissue level. These lesions were also detected in distinct fish species (Witten *et al.* 2005, Ortiz-Delgado *et al.* 2014, Cardeira *et al.* 2015). Similarly to Kvellestad *et al.*, 2000, some vertebrae presented an increased radiodensity and/or a higher diameter, compared to the neighbouring centra.

Delving into histopathologic sections, the majority of the affected vertebral centra responded similarly by displaying ectopic cartilaginous components either in the endplates as in IVS, although the cartilaginous tissue was not appreciable in the radiographic images. In this sense, histopathologic approach contributed with new information on pathogenic mechanisms underneath such disorders.

Microscopically, trabecular bone was altered in the deformed vertebrae, displaying an oblique specular orientation. This change could be connected with the distribution of the chondrocytes in the concave aspect of the articular surfaces of opposing centra trabeculae. Such findings are in some way likewise to primary shape distortions observed in the concave side of lordotic and kyphotic vertebrae in *curveback* lineage of guppies (*Poecilia reticulata*) (Gorman *et al.* 2010). As Kvellestad *et al.*, 2000 observed at macroscopic level, in the present study, most of the lesions affecting deformed vertebra displayed a specular behaviour regarding facing vertebrae. Such phenomenon might indicate that changes are initiated in the region comprising intervertebral tissue and opposing end plates (Kvellestad *et al.* 2000).

Regarding vertebral fusions, reshaped fused centra were radiographically detected by the presence of two or more neural and haemal structures. Histopathologic evaluation suggested remodelling or modelling processes and remnants of intervertebral notochord persisted, bounded by fused vertebral bodies. In one specimen, a cartilaginous residue was found in the fusion line (just in the intersection of perpendicular trabeculae), corresponding to the location of the IVS. Similar findings were observed in Atlantic salmon intermediate and final stages of fusion (Witten *et al.* 2006, Ytteborg *et al.* 2010b). In other specimen, two fused centra showed also abundant cartilaginous tissue in the adjacent IVS, indicating the progression into a three vertebrae fusion (Witten *et al.* 2006). In addition, some deformities may constitute different steps of fusion development as proposed Fjelldal *et al.*, 2007. In this regard, those segments showing flattened endplates, narrow IVS and/or abundant cartilaginous tissue in such space could be already initiating a fusion process (Witten *et al.* 2006). These vertebrae were considered as deformed in order to specifically detail the current stage of the anomalies, although they might show early signs of fusion as reported by Witten *et al.*, 2006. Other cases of deformed vertebrae without chondrocytic cells may possibly constitute a point of instability in the vertebral column, promoting the aggravation of the lesion, albeit the development of a fusion cannot be predicted. Witten *et al.*, 2009 suggested that adjacent asymmetrically compressed vertebrae would fuse, while homogenously compressed vertebrae might represent a more "stable alteration" (Witten *et al.* 2005, 2006, Sullivan *et al.* 2007). It seems that the formation of cartilage can be present in both anomaly types, deformations and fusion. Or even these may constitute an ongoing process of aggravation of the lesions in which some of the deformed vertebral bodies fuse together. Therefore, it would be interesting to deepen on vertebral anomaly signalling pathways as well as on environmental factors involved in the containment and aggravation of the problem (Witten *et al.* 2006).

Anosteocytic teleost bone is able to perform adaptive, plastic responses to loading conditions (Kranenbarg *et al.* 2005a,b, Dean & Shahar 2012). At the cellular level, the presence of ectopic cartilaginous tissue was detected in the endplates, possibly attenuating mechanical stress at the autocentrum (Cardeira *et al.* 2015). Cartilage constituents were reported related with deformed centra (Witten *et al.* 2005, Cardeira *et al.* 2012) and vertebral fusions (Witten *et al.* 2006), and might appear in several parts of the vertebral centra. Compressed (platyspondylic) vertebrae displayed alterations in the endplates and presented chondrocytes surrounded by ECM towards the IVS (Kvellestad *et al.* 2000, Witten *et al.* 2005). Deformed vertebrae associated with spinal curvatures as lordosis and lordo-kyphosis presented cartilage or isolated chondrocytes embedded in osteoid matrix, mostly in the endplates and in some cases, near the periosteum (Kranenbarg *et al.* 2005a, Ortiz-Delgado *et al.* 2014, Cardeira *et al.* 2015). This chondrocyte distribution can be based on the availability of oxygen and nutrients in the peripheral areas as the endplates and the growth zone to be able to survive (Cardeira *et al.* 2015).

The ectopic development of chondroid and cartilaginous tissue related with skeletal anomalies is not quite understood in fish. Chondroid tissue seems to meet the demand for an accelerated local growth rate and the need for a shear-resistant support (Huysseune 2000). Our results showed, in some cases, the proliferation of exuberant cartilaginous tissue visibly from the growth zones into the IVS. Also, chondrocytes in the endplate were located especially near these areas constituted by osteoblasts and mesenchymal cells. These findings may indicate that chondrocytes could arise from the same population of mesenchymal cells from which osteoblasts differentiate in the growth plate (Cardeira *et al.* 2015), as a result of altered mechanical load, such as suggested for vertebral curvatures (Cardeira *et al.* 2012). In this line of thought, mesenchyme cells may differentiate into chondroblasts rather than osteoblasts in response to high rates of change in compression (Hall 2015) or in a low oxygen environment during fracture repair (Carlson & Weisbrode 2012). Despite the mesenchymal source of osteoprogenitor cells has not been clearly identified in fish (Witten & Huysseune 2007), co-transcription of some osteogenic and chondrogenic markers may occur in the osteoblasts in the growth zone during fusion process (Ytteborg *et al.* 2010b). Moreover, osteogenic cells can develop into osteocytes or into chondrocytes in the Atlantic salmon kype skeleton as they become entrapped in the bone matrix (Witten & Hall 2002). Therefore the possible involvement of osteoblasts should not be disregarded as well as a metaplastic transformation of the notochord area into cartilage, as a consequence of mechanical load (Witten *et al.* 2005).

Once the chondrocytes differentiate in the growth zone, they can be entrapped by the newly deposited osteoid matrix and eventually die due to lack of nutrients and hypoxia, remaining only those near the articular surface (Cardeira *et al.* 2015). Another hypothesis is that cartilage derived from the growth zone could be developing into the IVS, stuck on the articular surface in a specular way, causing alterations in the endplate shape and compressing the notochord. The former hypotheses are not mutually exclusive and perhaps might present one same subsequent process whereby cartilage is replaced by osseous tissue, as occurring in later steps of fusion (Witten *et al.* 2006).

Three main bone formation mechanisms have been described in teleosts, depending on the species and skeletal structures: intramembranous, perichondral and endochondral (Boglione *et* *al.* 2013a). Of these, the latter two usually involve a cartilaginous template (Boglione *et al.* 2013a). In this regard, and focusing in the IVS cartilage organization, chondrocytes showed distinct phases of development arranged in different sections. Hypertrophic chondrocytes were located proximate to cartilaginous tissue in articular bone and proliferating cells in the middle region of IVS. An analogous organization was also reported in lordo-kyphotic deformed vertebrae (Cardeira *et al.* 2012) and, in an identical way during arch centra endochondral ossification in low temperature reared Atlantic salmon (Ytteborg *et al.* 2010a). Hypertrophied chondrocytes secrete collagen type X and other macromolecules that modify ECM, allowing matrix mineralization, vascular invasion and posterior bone formation by osteoblasts at final phases of higher vertebrates endochondral ossification (Safadi *et al.* 2009, Carlson & Weisbrode 2012). In parallel, the present results showed peripheral hypertrophic chondrocytes embedded in a more basophilic matrix (H&E). Moreover, even in osteoid staining, cartilage mineralization was not detected, albeit sample decalcification should be considered. Instead, a matrix compatible with osteoid was detected in the corresponding areas, scattered between chondrocytes. However, clear evidences of ECM resorption and osteoblast activity were not observed in direct association with cartilaginous tissue. The histopathological evaluation could not provide conclusive evidence that a chondroidal mechanism of ossification perhaps could be involved during vertebral fusions and deformations as suggested for Atlantic salmon (Ytteborg *et al.* 2010b) and European seabass (Kranenbarg *et al.* 2005a). However, it should not be ruled out other pathways of ossification that could be taking part in these processes. In this regard, endochondral and perichondral ossification as well as chondral bone need further characterization in order to better understand the structural and chemical processes occurring in different fish species (Boglione *et al.* 2013a). Moreover, the function of some trabeculae lacunae detected in the vicinity of chondroid and chondrocytic areas in the endplates of altered vertebrae should be investigated.

Other interesting findings were noticed affecting the IVS and its constituents. Besides IVS narrowing, the external elastic membrane of the notochord was irregular and scattered nearby opposing endplates with chondrocytes. These observations are in line with other studies in Atlantic salmon with a reduced IVS (Ytteborg *et al.* 2010b) and a thinner elastic membrane around the collagenous layer in developing vertebral fusions (Ytteborg *et al.* 2010c). Also, a fragmented elastic membrane was detected in severely fused (Ytteborg *et al.* 2010c) and compressed vertebrae (Kvellestad *et al.* 2000). The rupture of this structure may be involved in the progression of spinal anomalies and likely co-evolve with structural changes of the notochordal sheath (Ytteborg *et al.* 2010c). In this sense, the collagenous layer associated to the fragmented membrane was occasionally less evident or thinner. The thinning of the elastic membrane and alterations in the notochordal sheath decreases flexibility and possibly nutritional transportation across the notochord and could be related with the development of spinal fusions (Ytteborg *et al.* 2010c). Moreover, a high number of blood capillaries was occasionally present in the connective tissue externally to the layer of osteoblasts in the growth zone. This perhaps could be related with ossification process, since vascular invasion is a critical step at final phases of higher vertebrates endochondral ossification (Carlson & Weisbrode 2012).

Nowadays, many concerns still persist in order to identify all the pathogenic mechanisms involving the onset of skeletal deformities. Especially, the presence of ectopic cartilaginous tissue in deformed vertebrae raises great interest in detecting critical cells involved in the reception and response against stimuli from causative conditions. Witten and Huysseune, 2009 and Witten and Hall, 2015 suggested osteoblasts and bone lining cells on the bone surface as candidate cells for sensing mechanical load in anosteocytic bone. The particular location of the ectopic cartilage in the growth zones of the articular surface near osteoblasts and mesenchymal cells observed in this study may favour this hypothesis, although it requires further investigation. Moreover, Cardeira *et al.*, 2015 proposed that chondrocytes could serve as mechanosensors themselves, within the ectopic cartilage in most compressed areas.

As far as we know, this is the first histopathologic description of namely fused and deformed vertebrae in Senegalese sole. The histological techniques complemented considerably the radiographic studies and enlightened some of the possible pathogenic mechanisms associated with vertebral deformities. In this regard, lesions consisted in alterations of the shape and orientation of bony trabeculae and vertebral endplates. In parallel, the presence of ectopic chondrocytes in the articular surfaces and within the IVS was notable and it seems to be a common response mechanism in different types of anomalies affecting diverse teleosts species with either osteocytic or anosteocytic bone. Moreover, some of the alterations in deformed vertebrae as flattened endplates, narrow IVS and/or abundant cartilaginous tissue in such space could be consistent with initial stages of fusion, and in one case, with the aggravation of a primary fusion process. This cartilage proliferation from the growth zones into the IVS, pushing the notochord structures, seems to indicate that the chondrocyte mesenchymal origin could arise from cells in the growth area, as a result of altered mechanical load. Later, the hyaline cartilage appears to be replaced by osseous tissue in which chondroidal ossification or other types of ossification processes may be involved. Attention should also be paid regarding notochordal alterations, mainly in the external elastic membrane and the collagenous layer since they could be related with the development of some vertebral anomalies. Further research would be needed on molecular pathways involved in mechanosensor and local effector mechanisms promoting the development of cartilaginous tissue related with vertebral anomalies in anosteocytic bone.

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Discussion


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DISCUSSION

Skeletal anomalies are a serious problem affecting fish farming (Boglione *et al.* 2013a), requiring by producers a continuous awareness to the whole rearing process and control of the produced fish quality (Koumoundouros 2010). This Doctoral Thesis arises from the need to find solutions to prevent the high incidence of skeletal abnormalities detected in Senegalese sole (*Solea senegalensis*) aquaculture. In order to proper monitoring deformities in distinct stages of this species industrial production, it is necessary to have accurate diagnostic techniques, tailored for each culture phase. A comprehensive study of skeletal anomalies affecting the vertebral column of Senegalese sole was performed from macroscopic through histological evaluation and integrating complementary diagnostic methodologies at different rearing stages and feeding regimes. In this sense, Articles I and III of this Thesis showed that a conventional monitoring technique in this species farms, as external inspection and palpation, (Rodríguez & Peleteiro 2014) unnoticed the presence of skeletal defects in around 72-75% of the individuals considered as “normal”. Despite the high relationship observed between external evaluation and radiography, some deformities, mainly “minor”, were undetected by the first approach, as also reported in other fish species (Sullivan *et al.* 2007). However, along with these, around 46% of the “normal” specimens displayed vertebral body anomalies (VBA) and/or vertebral column deviations (VCD) which in turn may aggravate with time (Witten *et al.* 2006, Fjelldal *et al.* 2007). Therefore, the relevant percentage of underestimated anomalies by external inspection, allied with the high incidence of skeletal deformities radiographically registered at later stages (Articles I and III), may be translated into an important rate of suboptimum quality fish (Boglione *et al.* 2013b). This forces the producers to downgrade its market value (Fernández *et al.* 2008, Morais *et al.* 2016), leading to economic repercussions (Fernández *et al.* 2008, Morais *et al.* 2016). Hence, the application of more sensitive methods to detect abnormalities of the spinal column in farmed Senegalese sole lots, like radiography, could result in an increased quality of the fish that hit the market.

The radiographic approach used in the present Thesis allowed the scored evaluation of main deformities affecting reared juvenile Senegalese sole in Article I and its subsequent detailed characterization and quantification in Article III. This non-invasive method is the preferred technique for skeletal anomaly diagnostics in teleosts and it can be used in live fish (Hjelde & Bæverfjord 2009). Although scarcely applied in other reared species, the two orthogonal radiographic projections in Articles I, III and V offered a three-dimensional perspective of the skeleton since dorso-ventral projection greatly complemented the latero-lateral view and allowed the assessment of any vertebral misalignment in the blind/ocular plane of the fish. Therefore, we recommend the integration of these two orthogonal projections in routine radiographic evaluation of skeletal anomalies in Senegalese sole and other teleost species. At industrial level, the commercialization of Sparidae juveniles for ongrowing farms involves the use of radiography to attest fish quality (Boglione & Costa 2011). The applicability of such technique to Senegalese sole farming as a sorting method is in some way relative, requiring a cost-benefit analysis considering radiographic equipment price. As demonstrated in Articles I, III and V, X-ray can be employed to evaluate skeletal anomalies in large juveniles as well as to identify at least VBA and VCD in abdominal and caudal regions of smaller specimens, from 105 days after hatching (dah) onwards. In this last case, the definition and contrast of skeletal elements was severely reduced, mainly in caudal complex and occasionally in the tip of the spines. This limitation may occur because the thickness of the soft tissue is particularly diminished in the caudal complex area (Witten *et al.* 2009) and bone density could be relatively low (Hjelde & Bæverfjord 2009) at juvenile stage in this flatfish species. The radiographic technique showed also some inconveniences in detecting deformities in paired structures as a result of overlapped parallel elements, e. g. incomplete arches or lack of one parapophysis in a vertebra. In order to perform a precise monitoring of skeletal defects at all rearing stages it is necessary to dispose of procedures also suitable in smaller specimens at early stages of development. In this sense, double staining technique for cartilage and bone is suitable for the detection of skeletal disorders and ossification degree in Senegalese sole larvae and small juveniles (Gavaia *et al.* 2002, Boglino *et al.* 2012b), as well as for ontogenic studies (Gavaia *et al.* 2002, Darias *et al.* 2010a). The application of accurate methods for deformity diagnosis as double staining technique and radiography allowed the characterization of the anomaly profile in different culture stages, namely in Senegalese sole larvae (Article II) and juvenile (Article III), respectively.

The thorough anomaly characterization and quantification performed in Articles II and III considered distinct types of anomalies affecting the vertebral centra and rachis alignment (VBA and VCD) as well as the neural and haemal/parapophysis structures and caudal complex elements (parhypural, epural and hypurals). This detailed assessment was supported by the use of a terminology and anomaly categorization adapted from several reports to unify some criteria and considering Senegalese sole particular physiognomy. The comprehensive anomaly typologies described in Article II and applied in Articles III and IV served as a framework to compare anomaly incidences between ages and nutritional factors. Thus, it could constitute a basis for collating of forthcoming research and industrial monitoring records. Moreover, the study of Article II complemented former descriptions of vertebral anomalies (Gavaia *et al.* 2002, 2009) and provided a new insight into the deformities profile in Senegalese sole reared larvae. In this regard, some repeated anomalies were present as for instance, two separated neural spines in the first abdominal vertebra were observed in all the specimens studied in Article II and IV. Such abnormality was characterized by its specific setting in the neural aspect of the first abdominal vertebra and its very high frequency. However, it was not considered as anomaly in some of the results, but as a species-specific plasticity feature. A similar absence of fusion in the arch, affecting different locations, was also observed, and is consistent with other studies in Senegalese sole (Boglino *et al.* 2012b). During ontogeny, two latero-dorsal intramembranous buds elongate and join together to form the arch (Gavaia *et al.* 2002). Probably, this junction process is impaired in the affected sole, although factors such as genetics should not be disregarded. Articles II and IV underlined a high incidence of skeletal anomalies, especially caudal haemal spine deformations, as well as anomalies in the caudal complex plates (deformations and fusions) at larval stages. Despite the scarcity of literature on the causes of such abnormalities, some nutritional factors, namely, high vitamin A content and low levels of phosphorous in the diet could be involved (Fernández *et al.* 2009, Fontagné *et al.* 2009, Lewis-McCrea & Lall 2010). Fusions between hypurals were also frequent in 31 or 32 dah larvae (Articles II and IV), which is in agreement with other studies on diverse reared and wild fish species (Hosoya & Kawamura 1998, Boglione *et al.* 2001, Gavaia *et al.* 2009). Nevertheless, some authors have considered this particular defect as a common and normal event in caudal complex development and ossification, rather than a skeletal deformity (Boglino *et al.* 2012a). Another typical feature observed especially in Article II was the presence of a variable number of vertebrae displaying fused parapophysis, forming an arch. However, as in the previous case, it remains uncertain if fused parapophysis should be regarded as a disorder or a simple tendency of these skeletal elements. At larval stages, the vertebrae most affected by VBA and/or VCD were the preurals, displaying mainly fusions and deformation. In this regard, preural fusions were also commonly observed in other studies, in wild and reared Senegalese sole (Gavaia *et al.* 2009, Dionísio *et al.* 2012). On the other hand, radiographed specimens showed shortening of the first abdominal vertebra (Article III).

Comparing skeletal anomaly profile between developmental stages (larvae, early juveniles and juveniles) (Articles II, IV and III, respectively), results highlighted a high incidence of skeletal disorders in all ages (100%, 100% and 75%, respectively). While anomalies in the caudal complex plates or affecting neural/haemal spines predominated in larvae, they clearly reduced in later phases. These variations were more pronounced especially regarding deformations of caudal haemal spines and caudal complex plates, which showed a very high frequency at early phases. As far as we know, Article IV is the first study that analyses vertebral anomalies in 105 dah Senegalese sole early juveniles, using a multivariate exploratory technique. Disparities among anomaly patterns were statistically detected in Article IV, at 31 and 105 dah. In this study, the marked reduction in the number of fish displaying alterations of caudal neural and haemal elements observed in X-rayed juveniles (Article III) was already perceived at 105 dah. Regarding the caudal complex region, despite 105 dah individuals showed fewer fusions in the caudal complex plates, deformations of these elements were equally very common respect to larval stages (Article IV). Nevertheless, X-rayed juvenile displayed a marked decrease in both fusions and deformations of the caudal complex plates (Article III). The distinct features characterizing the three anomaly profiles may be due to an adaptive response. Teleost skeleton can show a considerable plasticity and adaptive response to loading conditions (Witten & Huysseune 2009, Fiaz *et al.* 2010, Dean & Shahar 2012). Moreover, fish bone has the ability to remodel and model some vertebral fusions and repair fractures (Witten *et al.* 2006, Takeyama *et al.* 2014, Witten & Hall 2015). Hence, deformed spines and caudal complex plates might be undergoing remodelling and modelling processes along sole life cycle, towards apparently non-deformed elements, leading to the attenuation of the incidence of such anomalies at later juvenile stages.

Considering the number of individuals displaying VBA and VCD, it was more or less similar in the three stages (larvae, early juveniles and juveniles) (Articles II, IV and III, respectively), exceeding the 20% reference value for severe deformed fish in intensive production (Boglione *et al.* 2013a), a relevant and sensible value in the industry (Fjelldal *et al.* 2012b). Moreover, these were more representative in juveniles regarding the number of anomalies since they presented a higher percentage of VBA and/or VCD in the total number of anomalies. VBA were mostly concentrated in preural vertebrae in the three stages, although caudal centra were also frequently affected. VCD did not show a marked incidence in all studies. However, the percentage of specimens with caudal scoliosis was considerably increased at later stages of development respect to larvae. The aggravation of incipient scoliotic lesions at early phases and/or the emergence of *de novo* anomalies with time as occurring in other types of deformities in distinct fish species (Witten *et al.* 2006, Lewis-McCrea & Lall 2010, Witten & Hall 2015), might explain the raise of caudal scoliosis incidence in juvenile sole. Relative to the second motive, in juvenile Atlantic halibut (*Hippoglossus hippoglossus*), scoliosis was largely detected in fish fed with diets containing high vitamin A content or oxidized oil, low vitamin C or low P amounts, in decreasing order of frequency, although it was not found at the beginning of the experiment (Lewis-McCrea & Lall 2010). Suboptimal P nutrition can also predispose juvenile Atlantic salmon (*Salmo salar*) to develop skeletal disorders with time (Fjelldal *et al.* 2012a). Moreover, nutritional quality of live preys during early feeding could be a determinant factor in the appearance of anomalies such as scoliosis and other abnormalities as compressed vertebrae in red porgy (*Pagrus pagrus*) larvae (Izquierdo *et al.* 2010, Boglione *et al.* 2013b).

Certainly, the utilization of distinct diagnostic techniques should be taken into account when assessing differences in the anomaly profile. In this sense, radiography prevented the proper assessment of the lack of neural arch in the first abdominal vertebra. In addition, it remains unclear if some of the frequently detected skeletal alterations should be regarded as a non/low-pathological alteration or a simple tendency of the bony structure during lifetime, as for instance, fusions among hypurals, fused paired parapophysis and shortened first abdominal centrum. Additional data on "wild type" specimens at different age could enlighten some of these aspects of the vertebral column of larvae up to juvenile sole. Investigation should be addressed on the progression of each type of anomaly and its precise impact on the physiology and external morphology of the adult fish.

As previously mentioned, in Article IV, the multivariate approach established different anomaly patterns regarding age (31 and 105 dah), and dietary enrichments (EA, EB, EC, ED). The incidence of anomaly categories varied among diets, although the hypothetical association of some anomalies with one diet at 31 dah was not maintained at 105 dah. Respect to the correspondence analysis, dietary groups did not conserve a similar distribution among ages, for instance, EB and ED fish at 31 dah were situated in completely opposed quadrants regarding their location at 105 dah. Despite the significant differences that were found in EC group, it was not possible to establish a clear effect of enrichment products on the development of vertebral abnormalities at both sampling points. Moreover, variations in the number of specimens showing VBA and/or VCD were not significant among diets at both ages. Nevertheless regarding VBA and VCD overall incidence, EC and EA groups showed a better performance at 31 and 105 dah, respectively. Studies at larval stage in Senegalese sole showed no effect of *Artemia* spp. enrichment products on major deformities of the vertebral centre except for vertebral fusions (Boglino *et al.* 2012b). Despite the diversity observed in the composition of enriched *Artemia*, the final nutritional profile of live preys may change according to culture/enrichment conditions (Conceição *et al.* 2010) and could be affected by certain metabolic pathways (Boglino *et al.* 2012b). Therefore, the balance among nutrients and its interactions should be considered in order to optimize sole's dietary nourishment (Boglino *et al.* 2012b). The existence of some common anomalies to all diets in both stages might indicate that the variations on the composition of live preys were not enough to produce an direct effect at skeletal level and/or that other causes could contribute to the onset of such alterations, masking a potential influence of enrichment products. In this sense, some non-nutritional and environmental factors were related with the development of skeletal anomalies in Senegalese sole like the temperature, hypercapnia and light conditions (Blanco-Vives *et al.* 2010, Dionísio *et al.* 2012, Pimentel *et al.* 2014). Therefore, attention should be paid to causative factors of skeletal deformities in early stages of reared Senegalese sole, as well as to other factors that could potentiate or have an effect on the development and aggravation of skeletal disorders at later stages of the lifecycle.

From consumer’s point of view, the body shape is the first quality criterion (Koumoundouros 2010) which, in the case of Senegalese sole, should present an elongated external profile (Riaza personal communication). As mentioned previously, the external morphology of the fish could be affected by skeletal deformities (Cardeira *et al.* 2015), although other factors could contribute to deviations from the normal pattern, as individual conformation and rearing conditions (Ambrosio *et al.* 2008). However, not all of the deformities downgrade the quality of the reared fish: i.e. slight deviations of skeletal elements from the normal do not necessarily affect the external appearance of the fish (Koumoundouros 2010). In this sense, the development of a scale of quality is required at industrial level for the precise distinction of commercially severe deformities from those of scientific interest only (Koumoundouros 2010). From this Thesis results and in light of the profile of some types of anomalies at later stages (Article III), it would be suggested that Senegalese sole farms should focus in preventing mainly the anomalies affecting the vertebral bodies (VBA) and column alignment (VCD). In this sense, the straightforward scoring method provided in Article I represent a practical and useful tool, applicable in aquaculture settings for the detection of vertebral anomalies with diverse severity degrees. Nevertheless, further research is required to determine the correlation between skeletal development and the external morphology of the fish at the end of hatchery and ongrowing phases (Koumoundouros 2010, Boglione *et al.* 2013b). For the early assessment of anomalies affecting the vertebral centra in Senegalese sole, Article V showed that radiography is an adequate method (from 105 dah onwards) and could be complemented by the detailed analysis addressed by double staining technique (Article IV). Therefore, at phases close to 105 dah, two different techniques may be selected for quality monitoring of a sample of fish lots, according to the intended level of accuracy. In any case, the statements above do not detract importance to other type of skeletal deformities like those involving neural/haemal elements or caudal complex structures. In fact, research on alterations in their skeletogenesis may provide clues towards factors affecting other skeletal elements. In this regard, chondral bones as caudal complex plates present a higher sensitivity to dietary vitamin A and ascorbic acid content (Fernández & Gisbert 2010, Darias *et al.* 2011b). Also, neural and haemal processes should not be ignored since they perform an important role protecting vital organs (Stiassny 2000, Boglione *et al.* 2013b) and may influence the fish shape (Harder 1975).

Regarding histological evaluation, Article V elucidated the main microscopical organization of non-deformed vertebral bodies as well as provided a correspondence between radiographic and histopathologic features of deformed and fused vertebrae. Vertebral centra with morphological anomalies showed mainly alterations of longitudinal bony trabeculae which displayed an oblique specular orientation. This specular behaviour of the lesions regarding opposing vertebrae might indicate that changes are initiated in the region comprising intervertebral tissue and opposing end plates (Kvellestad *et al.* 2000). Flattened articular surfaces were also observed both in radiographic images as at tissue level. The detected lesions in deformed vertebrae are in line with other studies in different fish species (Witten *et al.* 2005, Cardeira *et al.* 2015). In the histopathologic evaluation, the majority of the affected vertebral centra behaved similarly by displaying ectopic cartilaginous components either in the endplates as in the intervertebral spaces (IVS), although the cartilaginous tissue was not appreciable in the radiographic images. Cardeira *et al.*, 2015 suggested that this chondrocyte distribution in the endplates and the growth zone could be based on the availability of oxygen and nutrients in the peripheral areas to be able to survive. Regarding vertebral fusions, results revealed reshaped fused centra, with histopathological features suggesting remodelling and/or modelling processes. Remnants of intervertebral notochord and cartilaginous residue persisted bounded by fused vertebral bodies in the centre of the fusion, similarly as observed in Atlantic salmon intermediate and final stages of fusion (Witten *et al.* 2006, Ytteborg *et al.* 2010b).

The histopathologic approach contributed with new information on pathogenic mechanisms underneath fusions and alterations of the vertebra shape, non-related with axis deviation. It seems that the formation of cartilaginous tissue could be a common response mechanism in different types of anomalies affecting diverse teleosts species with either osteocytic or anosteocytic bone (Witten *et al.* 2005, 2006, Ortiz-Delgado *et al.* 2014, Cardeira *et al.* 2015). Moreover, some of the alterations in deformed vertebrae as flattened endplates, narrow IVS and/or abundant cartilaginous tissue in such space could be consistent with initial stages of fusion (Witten *et al.* 2006, Fjelldal *et al.* 2007), in which certain deformed vertebral bodies could fuse together. In one case, these lesions also suggested an ongoing process of aggravation of a primary fusion process (Witten *et al.* 2006).

The ectopic development of chondroid and cartilaginous tissue in fish skeletal anomalies is still not quite understood. The location of exuberant cartilaginous tissue from the growth zones into the IVS and the chondrocytes near the osteoblasts layer might indicate that chondrocytes may arise from the population of mesenchymal cells in the growth plate, from which osteoblasts differentiate (Cardeira *et al.* 2015), as a result of altered mechanical load, such as suggested for vertebral curvatures (Cardeira *et al.* 2012). However, the possible involvement of osteoblasts should not be disregarded as well as a metaplastic conversion of the notochord into cartilage (Witten *et al.* 2005).

Great interest still persists in detecting critical cells involved in the reception and response against the stimuli from deformities causative factors. This is especially intriguing regarding anosteocytic bone where osteoblasts and bone lining cells on the bone surface could be candidate cells for sensing mechanical load (Witten & Huysseune 2009, Witten & Hall 2015). The particular location of the ectopic cartilage in the growth zones of the articular surface near osteoblasts and mesenchymal cells observed in Article V may favour this hypothesis, although it requires further investigation. On the other hand, chondrocytes within the ectopic cartilage in most compressed areas could serve as mechanosensors themselves, as proposed Cardeira *et al.*, 2015. Therefore, further investigation is required on signalling pathways involved in mechanosensor and local effector mechanisms underneath the development of cartilaginous tissue regarding vertebral anomalies in species with anosteocytic bone.

In later stages of the observed lesions, hyaline cartilage appears to be replaced by osseous tissue. Results could not provide conclusive evidence that a chondroidal mechanism of ossification could be involved during vertebral fusions and deformations as suggested for other fish species (Kranenbarg *et al.* 2005a, Ytteborg *et al.* 2010b), although other types of bone formation might be taking part in these processes. Additional findings were noticed involving IVS narrowing or an irregular and scattered external elastic membrane of the notochord, nearby opposing endplates with chondrocytes, similarly to other studies in Atlantic salmon (Kvellestad *et al.* 2000, Ytteborg *et al.* 2010b,c). Further investigation is needed to deepen into the histopathologic features of Senegalese sole's bone adaptive processes and on the types of ossification that could occur in the different elements of the skeleton.

The present Thesis contributed with a new insight on the skeletal anomaly problematic affecting Senegalese sole. This work underlines the importance of an interdisciplinary approach to cope a multi-factorial issue in the aquaculture sector. As far as the industry is concerned, further research is required on innovative rearing protocols and nourishment strategies in order to optimize morphological quality and welfare of the fish. Furthermore, it would be interesting to dispose of quick diagnostic tests, suitable and accurate, in order to facilitate monitoring of the lots and detect deformed specimens in every step of the rearing process. In this regard, is vital to deepen the knowledge on factors that originate and/or may predispose to the development of skeletal anomalies as well as on anosteocytic bone physiology. The availability of recent genomic resources for Senegalese sole (Benzekri *et al.* 2014, Manchado *et al.* 2016) could be useful to provide new perspectives on bone homeostasis as well as to unveil the molecular basis of its response to different stimuli regarding the development of skeletal deformities. Multilevel data analysis (e. g. artificial neural networks) may also be helpful in order to integrate multidisciplinary data towards pattern recognition and empirical modelling (Russo *et al.* 2010, 2011, Boglione *et al.* 2013b).

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Conclusions


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CONCLUSIONS

❖ Computed radiography, with two orthogonal projections, is suitable for the adequate visualization of the main vertebral anomalies in juvenile Senegalese sole (*Solea senegalensis*) from 105 days after hatching onwards, including macroscopically undetected deformities.

❖ Stereoscopic and radiographic techniques allowed the characterization of the anomaly profile in different rearing stages, revealing an incidence of skeletal anomalies higher than 75% in larvae and juvenile Senegalese sole.

❖ The two methods described in this Thesis for the evaluation of vertebral anomalies are applicable in aquaculture: the straightforward scoring can be employed in industrial settings, whereas the comprehensive anomaly typologies serve as a framework to investigate anomaly incidences between different factors.

❖ The anomaly profile in late juveniles was different with respect to larval period, prevailing vertebral body anomalies and vertebral column deviations. The incidence of "minor" deformities, as those affecting mainly the neural/haemal arches and spines and caudal complex elements diminished considerably at this stage.

❖ It was not possible to establish a clear effect of enrichment products for *Artemia* spp. on the development of vertebral abnormalities in Senegalese sole in larvae and early juveniles.

❖ The histopathological study complemented considerably the radiographic evaluation. The most remarkable finding was the presence of ectopic cartilage in the endplates of deformed vertebrae and from the growth zone into the intervertebral space, suggesting that the chondrocyte origin arise from mesenchymal cells in the growth area.

❖ Alterations in deformed vertebrae as flattened endplates, narrow intervertebral space and/or abundant cartilaginous tissue in such space indicate an initial fusion process. At later phases, fused vertebrae were reshaped and showed remnants of the notochord in the corresponding intervertebral space.


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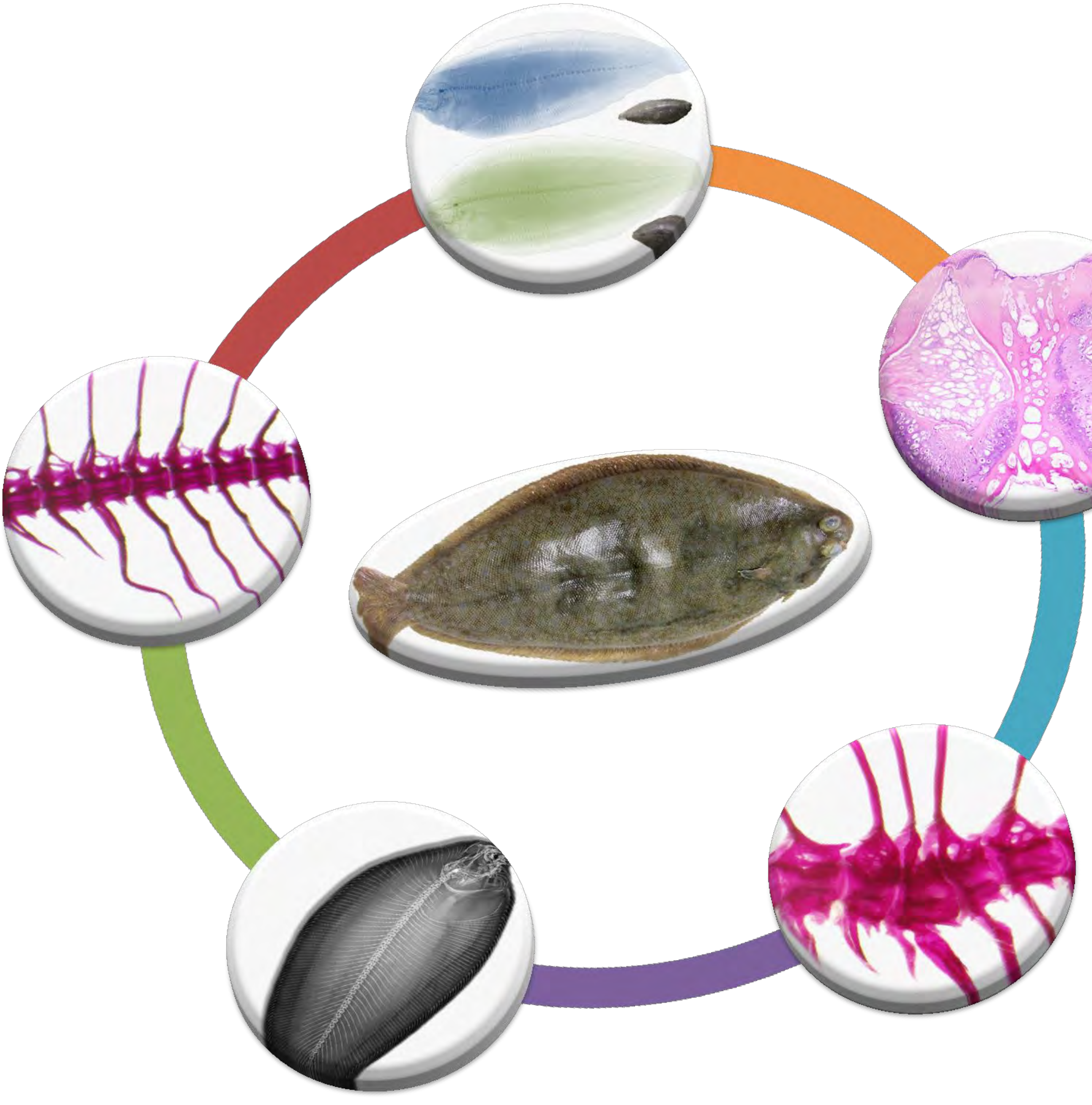
Appendix

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APPENDIX: RESUMEN

La presente Tesis Doctoral surge de la necesidad de encontrar soluciones eficaces para reducir la alta incidencia de anomalías esqueléticas detectada en la producción del lenguado senegalés (*Solea senegalensis*). Se trata de una de las especies más prometedoras en la acuicultura española, ya que alcanza un elevado valor económico en el mercado. Sin embargo, su cultivo se ve limitado por el alto porcentaje de individuos que presentan anomalías vertebrales, observándose incluso el 100% de individuos con deformidades, en algunos estudios experimentales. Las anomalías vertebrales pueden afectar el crecimiento, el bienestar de los animales y aumentar la susceptibilidad a las enfermedades en diferentes especies de teleósteos. Además, estas deformaciones repercuten en la calidad del producto y algunas en la morfología externa del pez, provocando rechazo por parte del consumidor y dificultando el procesado de las piezas. Esto supone un aumento de los gastos de producción a nivel industrial, porque muchas veces sólo se consiguen diagnosticar en etapas muy avanzadas del desarrollo. Para contrarrestar esta situación, las empresas se ven obligadas a disminuir el precio de mercado y a establecer un sistema exhaustivo de monitorización de las anomalías vertebrales, lo que implica un aumento de la carga de trabajo y de la tasa de sacrificio de los individuos afectados, y por lo tanto, una considerable merma económica en las empresas. Por este motivo es importante investigar en protocolos que persigan minimizar la incidencia de deformidades y optimizar la producción de lenguado senegalés.

El desarrollo de anomalías esqueléticas se ha relacionado principalmente con factores ambientales, genéticos y nutricionales, aunque otras causas pueden estar implicadas en este problema multifactorial. La importancia de los componentes ambientales es incuestionable ya que los protocolos de cultivo intensivos suelen presentar una alta frecuencia de anomalías esqueléticas. La literatura destaca algunos parámetros como la temperatura, las condiciones lumínicas, fotoperiodo, concentraciones de dióxido de carbono y la velocidad del flujo de agua que pueden afectar al desarrollo larvario y juvenil y originar deformidades. En el lenguado senegalés, se ha observado el efecto de la luz, el foto- y el termoperiodo, la temperatura del agua durante la incubación de los huevos y la hipercapnia. La nutrición desempeña un papel importante en etapas tempranas del desarrollo de los peces. Se ha comprobado que valores subóptimos de algunas vitaminas (A, C, D_3, K) alteran el desarrollo y mineralización del hueso, provocando anomalías. Con respecto a otros nutrientes como los minerales, se observó que el calcio y el fósforo están estrechamente asociados al desarrollo y mantenimiento óseos. Por ello, la deficiencia primaria de P, descrita en salmónidos, se caracteriza por una osteomalacia homogénea del esqueleto, mientras que el aporte deficitario de Ca retrasa el proceso de osificación. En el lenguado senegalés, la hipervitaminosis A puede conllevar el desarrollo de anormalidades principalmente en el cráneo, los cuerpos vertebrales y en el complejo caudal. En cambio, se ha observado que la suplementación de las dietas con vitamina K puede disminuir la incidencia de las alteraciones esqueléticas.

Los metanauplios de *Artemia* spp. enriquecidos se incorporan habitualmente en la dieta del lenguado durante el cultivo larvario, aunque pueden presentar algunos desequilibrios nutricionales, requiriendo suplementos para mejorar el rendimiento. Por lo tanto, el valor nutritivo de las presas vivas es importante para el éxito de la producción larvaria, pero puede variar según las condiciones de cultivo/enriquecimiento. Actualmente existen diversos productos para el enriquecimiento de *Artemia* spp. disponibles en el mercado, que muestran diferencias en la forma física, el contenido nutricional o los ingredientes. En este sentido, existen pocos estudios sobre la influencia de los enriquecedores en el desarrollo de deformidades óseas en lenguado senegalés, y sobre todo centrados en el perfil lipídico. Por ello, resulta imprescindible determinar su papel en el desarrollo de las anomalías esqueléticas ya que podrían presentar niveles nutricionales considerados como no óptimos para la esqueletogénesis en esta especie.

Actualmente, en la literatura se dispone de escasa información sobre las anomalías que afectan al lenguado producido a nivel industrial, especialmente en las etapas más avanzadas del ciclo productivo. La magnitud del problema, precisa la búsqueda de técnicas de diagnóstico que proporcionen una caracterización detallada de las alteraciones de la columna vertebral en distintas fases del cultivo de esta especie. En la presente Tesis se estudiaron las anomalías esqueléticas en diferentes etapas del desarrollo del lenguado senegalés de cultivo, con el propósito de establecer y comparar el perfil de las alteraciones más comunes en cada fase del crecimiento. Se empleó la misma sistemática para evaluar la influencia de diferentes enriquecedores comerciales para presas vivas sobre el desarrollo de anomalías vertebrales en larvas y juveniles de lenguado, utilizando técnicas de análisis multivariante. Por último, se caracterizaron los principales cambios histopatológicos que contribuyeron a elucidar la patogénesis de diferentes tipos de anomalías vertebrales.

Para monitorizar los lotes en distintas etapas de la producción industrial del lenguado senegalés, es necesario contar con técnicas diagnósticas adecuadas y precisas, adaptadas para cada fase de cultivo. Hasta el momento, el control rutinario de las malformaciones en las granjas de esta especie se ha basado en la palpación y la observación macroscópica de los peces. Los resultados de este trabajo demostraron que esta técnica tradicional no detectó la presencia de alteraciones esqueléticas en alrededor del 72-75% de los individuos considerados como “normales". A pesar de que la evaluación externa y la radiografía mostraron estar altamente relacionadas, algunas anomalías, principalmente "menores", no fueron detectadas con la primera técnica, como se ha descrito en otras especies de peces. Además, alrededor del 46% de los especímenes "normales" presentaron anomalías del cuerpo vertebral (ACV) y/o desviaciones de la columna vertebral (DCV). Por lo tanto, el importante porcentaje de deformidades subestimadas en la inspección externa, asociado con la alta incidencia de anomalías esqueléticas registradas radiográficamente en etapas posteriores, puede traducirse en una tasa importante de lenguados de calidad subóptima. La aplicación de métodos más sensibles, como la radiografía, para detectar anomalías en la columna vertebral en lotes de cultivo de lenguado senegalés, mejoraría la calidad de los peces que llegan al mercado.

El abordaje radiográfico utilizado permitió la evaluación categorizada de las principales anomalías que afectaban a los juveniles de lenguado y su posterior caracterización y cuantificación. Las dos proyecciones ortogonales ofrecieron una perspectiva tridimensional del esqueleto, ya que la proyección dorso-ventral complementó en gran medida la latero-lateral y permitió detectar malas alineaciones del raquis en el plano entre las caras ocular y ciega de los peces. Por ello, se recomienda la integración de estas dos proyecciones ortogonales en la evaluación radiográfica rutinaria de las anomalías esqueléticas en el lenguado senegalés y otras especies de teleósteos. A nivel industrial, la radiografía se está empleando para certificar la calidad de los juveniles de espáridos, en el proceso de comercialización para las granjas de engorde. La aplicabilidad de esta técnica en el cultivo industrial de lenguado senegalés como método de clasificación, es en cierto modo relativa, requiriendo un análisis coste-beneficio, considerando el precio del equipo radiográfico. Como se desprende de esta Tesis, la radiografía es un procedimiento práctico para la visualización integral de las anomalías esqueléticas y se podrá emplear en su diagnóstico en juveniles, así como para identificar, al menos, las ACV y DCV en las regiones abdominales y caudales de especímenes más pequeños, alrededor de los 105 días después de la eclosión (dde). En este último caso, la definición y el contraste de los elementos esqueléticos se redujo drásticamente, principalmente en el complejo caudal y ocasionalmente en la punta de las espinas. Esta limitación puede ser debida a la reducida cantidad de tejido blando en el área del complejo caudal y a la relativamente baja densidad ósea en los juveniles de esta especie de peces planos. La técnica radiográfica mostró algunas dificultades para detectar anomalías en estructuras pares, como por ejemplo arcos incompletos o falta de una parapófisis en una vértebra, debido a la superposición de estos elementos.

La técnica de tinción doble para cartílago y hueso es adecuada para la detección de alteraciones esqueléticas y para estudios ontogénicos en larvas y lenguados juveniles en etapas tempranas. El empleo de las técnicas estereomicroscópicas en larvas, y radiográficas en juveniles, ha permitido la caracterización exhaustiva de las anomalías esqueléticas para cada etapa del desarrollo. Para la evaluación y cuantificación de las anomalías se consideraron distintos tipos de alteraciones que afectaban a los cuerpos vertebrales y a la alineación del raquis (ACV y DCV), así como a las estructuras neurales y haemales/parapófisis y a los elementos del complejo caudal (parhipurales, epurales y hipurales). Este estudio detallado se apoyó en el uso de una terminología y categorización de anomalías adaptados de trabajos previos para unificar criterios y teniendo en cuenta la fisionomía particular de la especie. La tipología de las anomalías descritas en este trabajo sirvió de modelo para comparar la incidencia de alteraciones entre edades y factores nutricionales. Estos perfiles de anomalías delineados a distintas edades constituyen una base valiosa para futuras investigaciones o para el su aplicación a nivel industrial. Por otra parte, los resultados de la Tesis complementaron descripciones anteriores en relación a las anomalías vertebrales y proporcionaron una nueva perspectiva de su perfil en larvas de lenguado senegalés. De manera general, algunas anomalías se repitieron en los diferentes artículos presentados en esta Tesis. Por ejemplo, se detectó una alta incidencia de deformaciones de las espinas hemales caudales, así como de las estructuras del complejo caudal. Las fusiones entre hipurales también fueron frecuentes en larvas de 31 ó 32 dde, lo cual coincide con otros estudios en diversas especies de peces de cultivo y salvajes. Un resultado de interés, no descrito hasta el momento, es la presencia de dos espinas neurales separadas en la primera vértebra abdominal en todos los especímenes estudiados mediante la técnica de doble tinción. Tal hallazgo se caracterizó por su configuración específica en el aspecto neural de la primera vértebra abdominal y por su elevada frecuencia y podría deberse a una alteración en la unión de las dos yemas precursoras de los arcos neurales durante la ontogenia. Otra característica típica fue la presencia de un número variable de vértebras que mostraban parapófisis fusionadas, formando un arco. Por otro lado, los juveniles radiografiados presentaban un acortamiento de la primera vértebra abdominal. Sin embargo, todavía no está claro si algunas de las frequentes alteraciones esqueléticas, como por ejemplo, las fusiones entre hipurales, entre pares de parapófisis o el acortamiento del primer cuerpo vertebral abdominal, deben considerarse cambios con poco significado patológico o una simple tendencia de la estructura ósea. En este sentido, el conocimiento acerca del patrón "salvaje" a diferentes edades del ciclo de vida podría esclarecer algunas de estas peculiaridades de la columna vertebral del lenguado. Serían necesarios más estudios para evaluar la progresión de cada tipo de anomalía y su impacto específico sobre la fisiología y la morfología externa en los peces adultos.

Comparando el perfil de anomalías esqueléticas entre los distintos estadios de desarrollo (31-32, 105 y 297-342 dde), los resultados resaltaron una alta incidencia de alteraciones en todas las edades (100%, 100% y 75%, respectivamente). En larvas, las anomalías predominantes se localizaban en los elementos del complejo caudal y en las espinas neurales/hemales, aunque su porcentaje se redujo claramente en fases posteriores. Estas diferencias fueron más pronunciadas con respecto a las deformaciones de las espinas hemales caudales y de las estructuras del complejo caudal, las cuales mostraron una frecuencia muy alta en los primeros estadíos. Por lo que sabemos hasta el momento, esta Tesis ha contribuido con el primer estudio estereomicroscópico de las anomalías vertebrales en juveniles de lenguado senegalés de 105 dde, utilizando una técnica de análisis multivariante. Se detectaron diferencias estadísticas entre los patrones de anomalías de los peces con 31 y 105 dde. En el segundo punto de muestreo, se observó una reducción del número de peces con alteraciones de los elementos neurales y hemales caudales que se acentuó en los juveniles radiografiados en estudios posteriores. En cuanto a la región del complejo caudal, a pesar de que los individuos de 105 dde mostraron menos fusiones en sus estructuras, las deformaciones de estos elementos fueron igualmente comunes respecto a las larvas. Sin embargo, los juveniles de etapas más avanzadas mostraron una marcada disminución en las fusiones y deformaciones de los elementos del complejo caudal. Las distintas particularidades que caracterizan los tres perfiles de anomalías se deberían a una respuesta adaptativa a lo largo del ciclo de vida del lenguado. En este sentido, las espinas y los elementos del complejo caudal alterados podrían estar sometidos a procesos de remodelación y/o modelación hacia elementos aparentemente no deformados, llevando a la atenuación de la incidencia de estas anomalías en estadios juveniles más tardíos.

Aproximadamente la mitad de los individuos presentaron ACV y DCV en las tres etapas estudiadas, superando el valor de referencia del 20% para peces severamente deformados en producciones intensivas. Por otra parte, estas alteraciones fueron más representativas en los juveniles, ya que presentaron un mayor porcentaje de ACV y/o DCV con respecto al número total de anomalías. Las ACV se concentraron principalmente en las vértebras preurales en las tres etapas, aunque también afectaron frecuentemente a los cuerpos vertebrales caudales. Las DCV no mostraron una incidencia marcada en todos los estudios, aunque, la frecuencia de individuos con escoliosis caudal se incrementó considerablemente en etapas más tardías respecto a las larvas. El agravamiento de lesiones escolióticas incipientes en las primeras fases del desarrollo y/o la aparición de nuevas anomalías con el tiempo podrían explicar este aumento del porcentaje de escoliosis caudal en los lenguados juveniles.

El análisis multivariante mostró diferentes patrones de anomalías con respecto a la edad (31 y 105 dde) y a los enriquecedores (EA, EB, EC, ED). La incidencia de las categorías de anomalías varió entre las dietas, aunque la posible asociación de algunas anomalías con un enriquecedor a 31 dde no se mantuvo a los 105 dde. Respecto al análisis de correspondencia, los grupos no conservaron una distribución similar entre las edades. Por ejemplo, los peces EB y ED a 31 dde estaban situados en cuadrantes completamente opuestos respecto a su ubicación a 105 dde. A pesar de las diferencias significativas que se encontraron en relación al grupo EC, no fue posible establecer un efecto claro de los productos de enriquecimiento sobre el desarrollo de anomalías vertebrales en ambos puntos de muestreo. Por otra parte, las diferencias en el número de especímenes con ACV y/o DCV no fueron significativas entre las dietas en ambas edades. Sin embargo, en relación a la incidencia global de ACV y DCV, los grupos EC y EA mostraron una mejor performance a los 31 y 105 dde, respectivamente. En estudios previos en fases larvarias de lenguado senegalés, no se detectó una influencia de los productos para el enriquecimiento de la *Artemia* spp. sobre las principales anomalías del cuerpo vertebral, excepto sobre las fusiones vertebrales. La existencia de algunas anomalías comunes a todas las dietas en ambas edades indica que las oscilaciones en la composición de las presas vivas no fueron suficientes para producir un efecto directo a nivel esquelético y/o que otras causas pudieran intervenir en la aparición de esas alteraciones, enmascarando una posible influencia de los enriquecedores. Además, el perfil nutricional final de las presas vivas puede variar en función de las condiciones de cultivo/enriquecimiento y verse afectado por ciertas rutas metabólicas. Por lo tanto, el equilibrio entre los nutrientes y sus interacciones deben ser considerados a la hora de optimizar la alimentación de los lenguados. Las futuras investigaciones deberían abarcar otras causas de anomalías esqueléticas en fases larvarias, así como factores implicados en su desarrollo y/o agravamiento en etapas posteriores de su ciclo de vida.

Desde el punto de vista del consumidor, el primer criterio de calidad es la forma del cuerpo, que en el caso del lenguado senegalés, debe presentar un perfil externo alargado. Como se mencionó anteriormente, la morfología externa del pez puede verse afectada por las anomalías esqueléticas, aunque otros factores, como la conformación individual y las condiciones de cultivo, podrían contribuir al alejamiento del patrón normal. Sin embargo, no todas las alteraciones disminuyen la calidad de los peces cultivados: es decir, ligeras irregularidades de la morfología normal de los elementos esqueléticos no afectan necesariamente a la apariencia externa del pez. En este sentido, se requiere el desarrollo de una escala de calidad a nivel industrial para la separación precisa de las deformidades comercialmente severas de aquellas de exclusivo interés científico. A partir de los resultados de esta Tesis y teniendo en cuenta el perfil de algunos tipos de anomalías en las etapas más tardías, se sugiere que las granjas productoras de lenguado senegalés enfoquen sus esfuerzos en prevenir principalmente las anomalías que afectan a los cuerpos vertebrales (ACV) y a la alineación de la columna (DCV). Por ello, el método sencillo de clasificación propuesto representa una herramienta práctica y útil para la detección de anomalías vertebrales con diversos grados de gravedad y aplicable en el contexto de la acuicultura. Sin embargo, se requiere más investigación para determinar la correlación entre el desarrollo esquelético y la morfología externa de los peces al final de las fases larvarias y en etapas más tardías. Los resultados muestran que la radiografía es un método adecuado para la evaluación precoz de las anomalías que afectan a los cuerpos vertebrales en el lenguado senegalés (a partir de 105 dde) y que se complementa con el análisis minucioso proporcionado por la tinción doble. Por lo tanto, en fases cercanas a los 105 dde, se pueden elegir dos técnicas diferentes para el control de la calidad de los lotes, de acuerdo con el nivel de precisión deseado. En cualquier caso, no hay que restar importancia a otro tipo de alteraciones esqueléticas como las que conciernen a los elementos neurales/hemales o a las estructuras del complejo caudal. De hecho, la investigación sobre alteraciones de su esqueletogénesis puede proporcionar pistas sobre los factores que afectan a otras estructuras esqueléticas. En este sentido, se ha visto que los huesos endocondrales, como los elementos del complejo caudal, presentan una mayor sensibilidad al aporte dietético de vitamina A y ácido ascórbico. Además, los procesos neurales y hemales no deben de ser ignorados ya que desempeñan un papel importante en la protección de los órganos vitales e influyen en la morfología de los peces.

Con respecto a la evaluación histológica, la presente Tesis mostró la organización microscópica básica de los cuerpos vertebrales no alterados. Por otra parte, proporcionó una correspondencia entre las características radiográficas e histopatológicas de las vértebras deformadas y fusionadas. Los cuerpos vertebrales que presentaban anomalías de la forma mostraron principalmente alteraciones de las trabéculas óseas longitudinales, las cuales tenían una orientación especular oblicua. También se observaron superficies articulares aplanadas tanto en las imágenes radiográficas como a nivel de los tejidos. El comportamiento especular de las lesiones con respecto a vértebras opuestas indica que probablemente las anomalías se iniciaron en la región intervertebral y en las superficies articulares. Las lesiones detectadas en las vértebras deformadas están en línea con estudios realizados en otras especies de peces. En el análisis histopatológico, la mayoría de los cuerpos vertebrales afectados se comportaron de forma similar al mostrar un tejido cartilaginoso ectópico tanto en las caras articulares como en los espacios intervertebrales (EIV). Este tejido, sin embargo, no se apreció en las imágenes radiográficas. En trabajos previos se ha sugerido que esta distribución de los condrocitos se debería a una mayor disponibilidad de oxígeno y nutrientes en las áreas periféricas para poder sobrevivir. Respecto a las fusiones vertebrales, los resultados revelaron una reorganización de los centros vertebrales fusionados, mostrando características histopatológicas que sugieren procesos de remodelación y/o modelación. Adicionalmente, se detectaron remanentes de la notocorda intervertebral y algún residuo cartilaginoso en el centro de la fusión, de manera similar a la observada en el salmón del Atlántico (*Salmo salar*) en las etapas intermedias y finales de fusión.

El abordaje histopatológico contribuyó con nueva información sobre los mecanismos patogénicos inherentes a las fusiones y alteraciones de la forma de las vértebras. Parece ser que la formación de tejido cartilaginoso podría ser un mecanismo de respuesta común en diferentes tipos de anomalías que afectan a diversas especies de teleósteos ya sean de hueso osteocítico o anosteocítico. Además, algunas de las lesiones en las vértebras deformadas como el aplanamiento de las caras articulares, el estrechamiento de los EIV y/o el abundante tejido cartilaginoso localizado en los EIV sugieren un proceso inicial de fusión, en el que ciertos cuerpos vertebrales deformados podrían fusionarse. El desarrollo ectópico del tejido condroide y cartilaginoso relacionado con las anomalías esqueléticas de los peces todavía no se conoce en su totalidad. La proliferación del tejido cartilaginoso desde las zonas de crecimiento hacia el EIV y la localización de los condrocitos cerca de la capa de osteoblastos parece indicar que los condrocitos puedan tener origen en la población de células mesenquimales de la zona de crecimiento.

En lesiones más avanzadas, el cartílago hialino parece ser reemplazado por tejido óseo. Los resultados no permitieron demostrar de forma concluyente que algún mecanismo de osificación condroide pudiera estar involucrado en las fusiones y deformaciones vertebrales como se ha sugerido para otras especies de peces, aunque otros tipos de formación ósea podrían estar participando en estos procesos. Otros hallazgos interesantes consistieron en la irregularidad o fragmentación de la membrana elástica externa de la notocorda, asociadas con la presencia de condrocitos en las caras articulares, al igual que en otros estudios en salmón del Atlántico. La reciente disponibilidad de recursos genómicos en el lenguado senegalés puede aportar nuevas perspectivas sobre la homeostasis ósea, así como desvelar la base molecular de su respuesta a diferentes estímulos relacionados con el desarrollo de anomalías esqueléticas en esta especie.


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Summary

This Doctoral Thesis arises from the need to find solutions to prevent the high incidence of skeletal abnormalities detected in Senegalese sole (*Solea senegalensis*) aquaculture. A comprehensive study of skeletal anomalies affecting the vertebral column of Senegalese sole was performed at different rearing stages and feeding regimes. Complementary diagnostic methodologies were integrated, from the macroscopic, stereoscopic, radiographic and histologic perspective. The present Thesis contributed with a new insight on the skeletal anomaly problematic affecting cultured Senegalese sole throughout the productive cycle, underlining the importance of an interdisciplinary approach to cope a multi-factorial issue in the aquaculture sector.

Resumen

Esta Tesis Doctoral surge de la necesidad de encontrar soluciones eficaces para reducir la alta incidencia de anomalías esqueléticas detectada en la producción de lenguado senegalés (*Solea senegalensis*). Se realizó un estudio exhaustivo de las anomalías esqueléticas que afectaban la columna vertebral del lenguado senegalés en diferentes etapas de cultivo y sometidos a distintas dietas. Para ello se han integrado metodologías de diagnóstico complementarias, desde el punto de vista macroscópico, estereoscópico, radiográfico e histológico. La presente Tesis contribuyó con una nueva visión de la problemática de las anomalías esqueléticas que afectan al lenguado senegalés a lo largo del ciclo productivo. Este trabajo subraya la importancia de un abordaje interdisciplinario para hacer frente a un problema multifactorial en el sector de la acuicultura.