New insight on vertebral anomalies in cultured Senegalese sole (*Solea senegalensis*, Kaup) at early stages of development

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Short running title: Vertebral anomalies in cultured Senegalese sole

Abstract

Senegalese sole (*Solea senegalensis*, Kaup) is a promising flatfish species in aquaculture. However, skeletal anomalies are still a great concern in sole farming. Investigation of this issue is crucial to improving larval quality and optimizing production. The aim of this study was to thoroughly assess anomalies in the rachis of reared sole at early developmental stages. Sole (n = 507) were sampled at 31 or 32 days after hatching (dah). The specimens were stained with alcian blue and alizarin red, and evaluated for the detection of vertebral deformities. Most fish presented 9:34:3 vertebrae in abdominal, caudal, and caudal complex regions, respectively. Remarkably, all specimens showed at least one spinal anomaly. Alterations of neural/haemal elements, as well as deformities of hypurals, parhypural, and epural, were recurrent. Vertebral body anomalies (VBA) and/or vertebral column deviations (VCD) were identified in 52% of the individuals. Vertebral deformations and fusions were common, especially in caudal complex. "Minor" anomalies were predominant and some of the detected disorders might be a result of non/low-pathological processes. These results contribute a new insight into the main skeletal anomalies affecting cultured sole larvae. Further research is required to determine their impact on fish welfare and external appearances at commercial stages.

Keywords: Skeletal anomalies; Senegalese sole (*Solea senegalensis*); larval quality; vertebrae; skeletogenesis

Introduction

Senegalese sole (*Solea senegalensis*, Kaup) is a flatfish upon which Iberian aquaculture has placed great expectations (Howell, Conceição, Prickett, Cañavate & Mañanos 2009; Imsland, Foss, Conceição, Dinis, Delbare, Schram, Kamstra, Rema & White 2003; APROMAR 2015). Currently, fish farms are able to complete the production cycle and commercialize sole. However, its intensive culture is limited by the incidence of skeletal anomalies (Fernández, Pimentel, Ortiz-Delgado, Hontoria, Sarasquete, Estévez, Zambonino-Infante & Gisbert 2009; Gavaia, Dinis & Cancela 2002; Boglino, Darias, Ortiz-Delgado, Özcan, Estevez, Andree, Hontoria, Sarasquete & Gisbert 2012b). Approximately, 40% of juveniles present vertebral deformities in some Senegalese sole hatcheries, at the grading point for transfer to ongrowing farms (Riaza, personal communication). Similarly, in a number of scientific investigations, skeletal malformations have ranged from 44% (Gavaia *et al.* 2002) to around 80% (100% in some cases) in Senegalese sole larvae (Engrola, Conceição, Gavaia, Cancela & Dinis 2005; Gavaia, Domingues, Engrola, Drake, Sarasquete, Dinis & Cancela 2009; Fernández *et al.* 2009).

In other fish species, the incidence of skeletal anomalies can be also elevated, as in gilthead sea bream (*Sparus aurata*, L.), with frequencies of 100% of fish affected in some cases (Boglione, Gagliardi, Scardi & Cataudella 2001; Fernández, Hontoria, Ortiz-Delgado, Kotzamanis, Estévez, Zambonino-Infante & Gisbert 2008). In European sea bass larvae (*Dicentrarchus labrax*, L.), from 10% to 30% of the specimens showed vertebral column deformities (Mazurais, Darias, Gouillou-Coustans, Le Gall, Huelvan, Desbruyeres, Quazuguel, Cahu & Zambonino-Infante 2008), whereas a range of 4-9%

vertebral anomalies was seen in Atlantic salmon juveniles (*Salmo salar*, L.) (Fjelldal, Hansen & Albrektsen 2012b). Moreover, studies on other flatfish species have reported skeletal abnormalities, with halibut (*Hippoglossus hippoglossus*, L.) at an incidence of 41% to 89% of specimens showing at least one malformation (Lewis, Lall & Witten 2004); Japanese flounder (*Paralichthys olivaceus*, Temminck & Schlegel), with a range of 41-53% (Haga, Takeuchi, Murayama, Ohta & Fukunaga 2004), and turbot (*Scophthalmus maximus*, L.), where 51% of individual fish were deformed (Tong, Liu, Xu, Ma, Xiao, Xiao & Li 2012).

Skeletal deformities seriously hinder farming of many species since they affect growth, food efficiency level, susceptibility to diseases, and welfare of affected individuals (Boglione, Gavaia, Koumoundouros, Gisbert, Moren, Fontagné & Witten 2013a; Fjelldal, Hansen, Breck, Ørnsrud, Lock, Waagbø, Wargelius & Witten 2012a), leading to economic repercussions (Koumoundouros, Oran, Divanach, Stefanakis & Kentouri 1997b). Due to the significance of this problem, skeletal anomalies have been studied in numerous aquaculture species (for review, see Boglione *et al.* 2013a; Boglione, Gisbert, Gavaia, Witten, Moren, Fontagné & Koumoundouros 2013b). In some species, the development of skeletal malformations was associated with nutritional (Cahu, Zambonino Infante & Takeuchi 2003; Fernández *et al.* 2008; Fernández *et al.* 2009; Darias, Mazurais, Koumoundouros, Glynatsi, Christodoulopoulou, Huelvan, Desbruyeres, Le Gall, Quazuguel, Cahu & Zambonino-Infante 2010b), environmental, and genetic factors (Lall & Lewis-McCrea 2007; Mazurais, Glynatsi, Darias, Christodoulopoulou, Cahu, Zambonino-Infante & Koumoundouros 2009; Georgakopoulou, Katharios, Divanach & Koumoundouros 2010; Gjerde, Pante & Baeverfjord 2005), although parasites and bacteria could also cause these anomalies (Roberts & Rodger 2001; Madsen, Arnbjerg &

Dalsgaard 2001). In particular, intensive rearing protocols could be characterized by high incidences of skeletal abnormalities (Russo, Prestinicola, Scardi, Palamara, Cataudella & Boglione 2010). Therefore, research on production conditions at the first stages of development is important in order to improve larval quality and hone Senegalese sole farming practices. Former descriptions of skeletal deformities in this species were conducted mainly in research facilities (Fernández *et al.* 2009; Gavaia *et al.* 2009; Gavaia *et al.* 2002). Still, some gaps persist on the main typologies of vertebral anomalies affecting sole reared in fish farms, where multilevel factors may interact on fish in unknown manners, which experimental conditions are unable to mimic (Boglione *et al.* 2013b). Moreover, from a commercial point of view, the establishment of bench marks to compare levels of deformity is highly required (Howell *et al.* 2009).

The aim of this study was to provide a thorough description and quantification of the most common anomalies in the vertebral column of reared Senegalese sole at early stages of development. This work also sought to provide simple definitions of some criteria to meet the demand for a uniform categorization of anomaly typologies (Boglione *et al.* 2013b) affecting Senegalese sole.

Materials and methods

Senegalese sole, reared in a fish farm in Northwest Spain, were randomly sampled (n = 507). Fish were taken at 31 or 32 days after hatching (dah), humanely euthanized with an overdose of Tricaine methanesulfonate (MS-222, Sigma), and fixed in 10% buffered formalin. The specimens were stained for cartilage and bone, using a modified double staining technique, with alcian blue and alizarin red (Gavaia, Sarasquete & Cancela 2000; Darias, Lan Chow Wing, Cahu, Zambonino-Infante & Mazurais 2010a).

After staining, fish were measured, taking into account the standard length (StL; cm) from the rostral end of the skull to the end of hypurals; the standard height (StH; cm), which comprised the highest distance between the ventral and dorsal aspect of the fish, without taking into account fin rays. The StL/StH ratio was then calculated.

All specimens were observed from both sides using a binocular model (Olympus®) SZX16 to count the number of vertebrae and caudal fin rays, and to evaluate anomalies. The vertebral column was divided into three anatomical regions: abdominal, caudal, and caudal complex, according to Gavaia *et al.* (2002) (Figure 1). Each vertebra was assessed by considering anomalies in the neural arch and spine (dorsal aspect of the vertebra), in the parapophysis/haemal arch and spine (ventral aspect of the vertebra), and in the vertebral centrum. The terms for anatomical regions and anomalies used in this study were adapted from previous descriptions (Boglione *et al.* 2001; Gavaia *et al.* 2002; Lewis *et al.* 2004; Boglione *et al.* 2013a; Boglione *et al.* 2013b). Alterations in the arches/spines and parapophysis were categorized as bifurcation, number alteration, insertion alteration, fusion, incomplete arch, and deformation (alteration of the shape). Vertebral body anomalies (VBA) were considered as fusion or deformation, and three types of axis deviations (VCD) were assessed: kyphosis, lordosis, and scoliosis. Kyphosis and lordosis corresponded to the curvature of the axis towards, respectively, the dorsal and ventral aspects of the fish. Scoliosis was the bending of the rachis in the ocular/blind side of the body. Disorders in the caudal complex plates (parhypural, epural and hypurals) were also evaluated (bifurcation, number alteration, insertion alteration, fusion, and deformation).

During the development of this study, some considerations were made regarding types of anomalies:

1) Alterations in the number of parapophysis comprised the presence of one, or more than two parapophysis in each vertebral body, or the absence of these elements in a vertebra located between two vertebrae exhibiting parapophysis. 2) The presence of two complete and parallel neural/haemal spines (without forming an arch) was regarded as an alteration of the number of spines and an incomplete arch. 3) Partial fusions were considered as fusions. 4) Evident misalignments/subluxations of a vertebra were also considered as deviations. When a deviation towards more than one plane was present, the lesion was classified according to the deviation exhibiting the most obvious curvature. 5) When vertebral fusions or axis deviations affected more than one region in the same anomaly, they were registered in the anatomical area with the highest number of implicated vertebrae. 6) Hypural, epural, and parhypural fusions were regarded as anomalies, as were, also, the fusions between these elements and modified neural/haemal arches of the caudal complex.

Results

1. Measurements and number of vertebrae

Descriptive statistics on StL, StH, and the ratio StL/StH are displayed in Table 1 (mean, standard deviation (SD), mode, median, minimum and maximum values). The number of vertebral centra for each anatomical region and the number of caudal fin rays are shown in Table 2 (mean, SD, mode and percentage of individuals that showed this rate (%I), as well as minimum and maximum). The majority of the individuals presented 46 vertebral centra distributed in 9:34:3 vertebrae in the abdominal region, caudal area, and caudal complex, respectively. Just under 5% of specimens presented 4 vertebrae in the caudal complex, including the urostyle.

2. Skeletal anomalies

A summary of the results regarding skeletal anomalies is displayed in Table 3. All specimens presented at least one skeletal alteration, and around 52% had at least one anomaly in the vertebral centra or one rachis deviation. The number of observed skeletal anomalies reached 5,654 alterations, and the 16.1% of them corresponded to VBA and VCD.

The most frequent alterations of the vertebral column are illustrated in Figure 2 (A-E) and Figure 3 (A-E). Table 4 shows the number of deformed individuals (N.I.), the percentage they represent (%N.I.), the number of anomalies (N.A.), and the average number of anomalies per affected specimen (N.A./I.), for each anatomical region and malformation type.

In this investigation, the first abdominal vertebra presented two short neural spines; all individuals were lacking the neural arch (Figure 2 A). These data are not included in Table 4. A high percentage of fish (83.2%) had alterations of the shape (deformations) of neural and/or haemal arches and spines in other vertebrae, with multiple deformations in the same specimen (Table 4) (Figure 2 A and B, and Figure 3 A). One typical modification in the morphology of these elements was the twisted tip of the haemal spines, especially in the front caudal vertebrae (Figure 2 B and Figure 3 A). Incomplete arches and alterations of the insertion of the spines were also common in caudal region (Figure 2 C and D and Figure 3 B), and were sometimes associated with mild abnormalities of the centra (Figure 2 D, and Figure 3 A and B). In the caudal complex, neural and haemal arches and spines were also affected by deformations (Figure 3 C). Fusions between these structures were primarily related to fusions among vertebral bodies (Figure 3 D).

The main anomalies in the caudal complex consisted in minimal changes in the shape of hypurals, epural, and parhypural and/or fusions between these elements (Table 4). In particular, the epural, the hypural 5, and the parhypural were often slightly bent (Figure 2 E and Figure 3 A, C and D). Fusions between hypurals were frequent, involving hypurals 3 and 4 (Figure 2 E and Figure 3 C) in 84% of the affected individuals.

A number of alterations were observed throughout the vertebral column, especially affecting the parapophysis (Table 4). In some cases, these were associated with other alterations in the vertebra, whereas, in other cases they appeared in otherwise normal abdominal vertebrae as thin single, bony spicules (Figure 2 A). Other types of anomalies in the parapophysis were scarce. In addition, all specimens presented a variable number of abdominal vertebrae with two fused parapophysis, forming an arch (Figure 2 A). This datum is not reflected in Tables 3 and 4.

Regarding VBA and VCD, there was a high incidence of affected individuals (52%), as well as an elevated number of these anomalies. Approximately 70% of affected fish showed up to three alterations in these elements (with an average of 3.5 deformities per specimen), although ten fish presented more than ten anomalies (Table 3, Figure 3 A, and Figure 4). In general, the caudal region and the caudal complex showed a higher number of affected individuals and an increased number of VBA and/or VCD (Table 4). Figure 5 (A-B) and Figure 6 (A-C) show the number of sole affected by VBA and VCD along the vertebral axis, respectively. For every type of VBA, the critical location was preurals, followed by the first two abdominal vertebrae (affected mainly by fusions) and the intermediary area between abdominal and caudal regions, in decreasing order of occurrence (Figure 5 A and B). Fusions and deformations were more frequent than VCD in every anatomical region (Table 4). In the abdominal region, 7.3% of the specimens showed partial fusions involving the first and the second centra. The two vertebral bodies were commonly bound in the dorsal or ventral portion of the endplates due to rotation or deformation of the centra. Fusions among vertebral bodies were distinguished in a certain frequency along the column (Figure 3 A-D and Figure 5 A) and involved a different number of vertebrae, with an average of 2.3. In some cases, fused vertebrae were almost the same length and shape as those around them, being almost totally reshaped into a single vertebra, although the line of fusion remained (Figure 3 B and C). In severe cases (more common in the caudal region), several vertebrae were deformed and fused (Figure 3 A). In the caudal complex, fusions comprising preurals 1 and 2 were the most common (Figure 3 C and Figure 5 A). Curiously, in the same area, certain individuals showed four vertebrae, in which the intermediary (extra) vertebra (I.V.) was located between the two preurals and fused with preural 1 (Figure 3 D and Figure 5 A) in different degrees of severity.

Modifications of the shape (deformations) of vertebral bodies were frequently associated with fusions and/or axis deviations (Figure 2 E and Figure 3 A, D and E). Therefore, the distribution of deformations along the rachis was especially similar to the fusion's main location (Figure 5 B). Vertebral shortening (compressions) and lack of symmetry of the vertebral structure (Figure 2 D) were predominant. These modifications in the shape of the centra were pronounced in vertebrae with a "k" figure or a trapezoidal or triangular form (Figure 3 D). Some fish presented more severe deformations, as incomplete vertebrae (vertebrae lacking a part of the centra) (Figure 2 E) or vertebral bodies were aberrantly enlarged, some exhibiting also hyperostosis on the surface (Figure 3 E).

Axis deviations were a less frequent alteration of the vertebral column. The studied alterations of the rachis (kyphosis, lordosis, and scoliosis) displayed different bending angles and in certain cases, distinct types of curvatures were overlapped in the same vertebral segment (Figure 3 A and E). The caudal complex region was more affected by kyphosis (Figure 3 D and Figure 6 A). At the same time, lordosis was the most common deviation in the abdominal and caudal regions (Figure 3 E and Figure 6 B). In this investigation, there were few individuals (4%) showing a curved spine to the ocular/blind side of the body (scoliosis) (Figure 6 C). Moreover, axis deviations usually comprised diverse vertebrae (mean values are3.0, 3.2, and 4.3 vertebrae for kyphosis, lordosis, and scoliosis, respectively) although four specimens presented a dorsal/ventral subluxated vertebra.

Discussion

In this study, skeletal anomalies were thoroughly assessed in reared Senegalese sole. The present work complements former descriptions of vertebral disorders (Fernández *et al.* 2009, Gavaia *et al.* 2002) and provides a new insight into the deformities profile in reared larvae of this species. A double staining technique for cartilage and bone was convenient for the accurate identification of skeletal features at early stages of development. Moreover, it can also be used for the detection of vertebral deformities and in ontogenetic studies (Gavaia *et al.* 2002; Darias *et al.* 2010a). The observation of both sides of the specimens was useful to confirm alterations that occur on only one aspect of the fish, and it had special application in distinguishing the direction of axis deviations. In addition, since a standardized classification of skeletal anomalies is still lacking in the literature for some fish species (Boglione *et al.* 2013b), the terminology used in the present study was adapted from several reports to unify some criteria on anomaly categories affecting Senegalese sole. The comprehensive anomaly typologies evaluated in this work can serve as a basis for comparison for forthcoming research and industrial monitoring purposes.

In general, a very high frequency of anomalies was observed, given that all individuals presented at least one lesion. The most often repeated deformities concerned mainly the neural/haemal arches and spines and caudal complex elements. Curiously, the first abdominal vertebra presented two separated neural spines in all specimens. To our knowledge, this is the first description of such an abnormality, which is characterized by its specific setting in the neural aspect of the first abdominal vertebra and its very high incidence. A similar absence of fusion in the arch, affecting different locations, was reported in the present work, which is consistent with other studies in Senegalese sole (Boglino *et al.* 2012b). During arch development, two latero-dorsal intramembranous buds elongate and join together to form the arch (Gavaia *et al.* 2002). Sole affected by the former alteration probably present an impairment of this junction, although factors such as genetics should not be disregarded. Hence, it would be interesting to investigate the onset of this specific type of anomaly to deepen our understanding of its aetiology. Other types of skeletal disorders have contributed to the high incidence observed in this study, especially caudal haemal spine deformations, as well as anomalies in the caudal complex plates (deformations and fusions). Despite the scarcity of literature on the causes of such abnormalities, some nutritional factors have been reported, as they could influence appearances, namely, high vitamin A content (Fernández *et al.* 2009) and low levels of phosphorous in the diet (Lewis-McCrea & Lall 2010; Fontagné, Silva, Bazin, Ramos, Aguirre, Surget, Abrantes, Kaushik & Power 2009). Fusions between hypurals were also frequent, which is in agreement with other studies on reared Senegalese sole (Gavaia *et al.* 2002; Gavaia *et al.* 2009), Japanese flounder (Hosoya & Kawamura 1998), and hatchery-reared and wild juveniles of gilthead sea bream (Boglione *et al.* 2001). Nevertheless, some authors have considered this particular alteration as a common and normal event in caudal complex development and ossification, rather than a skeletal deformity (Boglino, Darias, Estévez, Andree & Gisbert 2012a).

Another typical feature observed in this work was the presence of a variable number of vertebrae displaying fused parapophysis, forming an arch. These structures could be consistent with haemapophyses, occurring in several Pleuronectides genera (Sakamoto 1984). It remains uncertain if fused parapophysis should be regarded as a disorder or a simple tendency of these skeletal elements.

The high incidence of anomalies observed in the present work is consistent with other studies that have reported broad ranges in the incidence of anomalies in reared sole, from 44% up to 100% in some cases (Gavaia *et al.* 2002; Gavaia *et al.* 2009; Fernández *et al.* 2009). In contrast with reared fish, wild Senegalese sole showed a lower incidence of skeletal anomalies (Gavaia *et al.* 2009), and these differences could indicate the presence of a selective mortality of the affected wild specimens and/or an effect of culture conditions on the development of skeletal malformations (Gavaia *et al.* 2009). In this regard, studies in Senegalese sole have reported an influence of environmental conditions like temperature, light, hypercapnia (Dionísio, Campos, Valente, Conceição, Cancela & Gavaia 2012; Blanco-Vives, Villamizar, Ramos, Bayarri, Chereguini & Sánchez-Vázquez 2010; Pimentel, Faleiro, Dionísio, Repolho, Pousão-Ferreira, Machado & Rosa 2014), and nutritional factors, such as the vitamin A dietary content (Fernández *et al.* 2009). Two hypotheses were suggested by Fernández *et al.* (2009) in order to explain the high incidence of deformities in reared Senegalese sole: this species is more prone to the development of malformations than other species; and these anomalies are non-lethal, so deformed animals can survive and be detected. In this sense, as previously mentioned, the majority of the observed lesions concerned neural/haemal spines and caudal complex elements. This is in line with other studies in reared Senegalese sole (Gavaia *et al.* 2009) and could constitute a baseline level of deformities, intrinsic to the rearing process at research and production centres. Moreover, from an industrial approach, the slight defect of spines and caudal complex structures could eventually be considered as "minor" anomalies. However, the chondral bones as caudal complex plates present a higher sensitivity to high vitamin A and to the amount of ascorbic acid in the diet (Fernández & Gisbert 2010; Darias, Mazurais, Koumoundouros, Le Gall, Huelvan, Desbruyeres, Quazuguel, Cahu & Zambonino-Infante 2011). Therefore, alterations in their skeletogenesis may provide clues towards factors affecting other skeletal elements. Also, arches and spines have an important role in protecting vital organs, which should not be ignored (Boglione *et al.* 2013b; Stiassny 2000). In addition, neural and haemal process length may influence the fish shape (Harder 1975), and serious bone alterations in several of these elements could harm fish performance (Boglione *et al.* 2013b). On the other hand, resorption and remodelling are crucial processes for bone development, growth, and mechanical adaptation of the fish (Witten & Huysseune 2009). Likewise, as reported by Witten, Obach, Huysseune & Baeverfjord

(2006), some vertebral fusions seemed to start bone re/modelling, in which the outcome was a structure with almost the same shape and size of a single non-deformed vertebra. Hence, extrapolating this reshaping process to the mildly bent elements or fused caudal plates, it could be hypothesised that they could acquire a normal final shape as they develop. Since this study is limited to the larval period, further research is required to investigate and monitor the evolution of these anomalies and their repercussions on fish welfare and external appearances, up to commercial stages.

The results also highlighted a high incidence of sole showing VBA and/or VCD that was far greater than the 20% indicated as a good value at the end of the hatchery stage by Boglione *et al.* (2013a). VBA and VCD could be considered the most deleterious anomalies in intensive production, because some severe lesions in centra and the rachis may have repercussions on the external morphology of the fish and their welfare (Boglione *et al.* 2001; Fjelldal *et al.* 2012a; Cardeira, Mendes, Pousão-Ferreira, Cancela & Gavaia 2015), and lead to subsequent rejection by the consumer (Gavaia *et al.* 2002; Boglione *et al.* 2001; Ambrosio, Costa, Sanchez & Flos 2008). This supposes an added expense due to manual selection of malformed individuals (Koumoundouros *et al.* 1997b; Gavaia *et al.* 2009). In Senegalese sole farms, sorting is usually performed by observing and palpating each fish (Rodríguez & Peleteiro 2014). Curiously, around 6% of sole categorized as “normal” by this method present severe skeletal deformities (Losada, de Azevedo, Barreiro, Barreiro, Ferreiro, Riaza, Quiroga & Vázquez 2014). This can lead to a decreased market value of a suboptimum product (Fernández *et al.* 2008; Koumoundouros, Gagliardi, Divanach, Boglione, Cataudella & Kentouri 1997a; Koumoundouros 2010), which, combined with other known issues, can cause serious economic losses (Fernández *et al.* 2008).

VBA and VCD appeared mainly in the caudal region and caudal complex area, which is in agreement with other studies in Senegalese sole (Gavaia *et al.* 2009; Gavaia *et al.* 2002). The same types of anomalies described here have also been reported in other fish species at different stages of development (Fernández *et al.* 2008; Dionísio *et al.* 2012; Fjelldal, Hansen & Berg 2007). Furthermore, Witten, Gil-Martens, Huysseune, Takle, & Hjelde (2009) have extensively characterized the main vertebral body deformities in Atlantic salmon by radiographic analysis. In the present study, the vertebrae most affected by VBA and/or VCD were the preurals, the first two abdominal vertebrae, and the centra in the intermediary area between the abdominal and caudal regions. Preural fusions were very common, as in other studies, in wild and reared Senegalese sole (Gavaia *et al.* 2009; Dionísio *et al.* 2012). In zebrafish (*Danio rerio*, Hamilton), the two vertebrae adjacent to the urostyle were often fused and could be more susceptible to this anomaly (Bensimon-Brito, Cancela, Huysseune, & Witten 2010). In the abdominal region, the most frequent alteration consisted in fusions between the first pair of vertebrae. It is known that fusions of one or two vertebral bodies to the occipital region of the skull could be considered as frequent non-pathological phenomena in osteichthyans (Witten *et al.* 2009; Boglione *et al.* 2013b). Still, different grades of fusion and deformity of the vertebral bodies were also distinguished along the rachis. In various situations, deformations were associated with other types of anomalies, such as fusions and VCD. Similarly, other studies reported some deformations concomitant with fusions or deviations affecting the same vertebral centra (Fjelldal *et al.* 2007; Cardeira *et al.* 2015).

Few sole showed axis deviations and some presented several types of curvatures in the same lesion, presenting a 3-D configuration. Kyphosis, lordosis, and scoliosis were observed in a diversity of fish species, including flatfish (Nagano, Hozawa, Fujiki, Yamada, Miyaki, Sakakura & Hagiwara 2007; Cardeira, Bensimon-Brito, Pousão-Ferreira, Cancela & Gavaia 2012; Lewis *et al.* 2004; Boglione *et al.* 2001). Lordosis was mainly located in the intermediary vertebral segment between the abdominal and caudal regions, as pointed out by Sfakianakis, Georgakopoulou, Papadakis, Divanach, Kentouri & Koumoundouros (2006) in European sea bass. This deviation was related to temperature and hydrodynamics due to increased muscular activity in the caudal fin and/or the action of the muscles on the vertebral axis (Kihara, Ogata, Kawano, Kubota & Yamaguchi 2002; Kranenbarg, Waarsing, Muller, Weinans & van Leeuwen 2005; Sfakianakis *et al.* 2006). Further research would be required in order to determine the effects of nutritional and environmental causes of vertebral centre deformities and deviations in Senegalese sole larvae.

In conclusion, these data complement former studies, and provide new insights into the main skeletal anomalies that affect early stages of farmed Senegalese sole. This thorough evaluation constitutes a baseline and serves as a tool with which to compare future investigations. The results highlighted a high incidence of skeletal anomalies, affecting mainly the neural/haemal arches and spines and caudal complex elements. On the other hand, a considerable number of specimens presented VBA and VCD, especially deformations and fusions in caudal complex region. However, some of the detected disorders might be a result of non/low-pathological significance processes. Further research is required to determine the impact of such anomalies on the physiology and external appearance of the adult fish, as well as on innovative rearing protocols in order to optimize larval quality.

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Tables:

Measurements	Mean		SD	Mode	Median	Min	Max
StL (cm)	1.44	±	0.14	1.5	1.4	0.7	1.8
StH (cm)	0.46	±	0.05	0.5	0.5	0.3	0.5
Ratio StL/StH	3.29	±	0.42	3.0	3.2	2.0	5.3

Table 1. Specimen measurements (StL, StH, and Ratio StL/StH): mean ± standard deviation (SD), mode, median, minimum (Min), and maximum (Max).

Meristic counts	Mean		SD	Mode	(%I)	Median	Min	Max
Abdominal vertebrae	8.93	±	0.28	9	92.11	9	8	11
Caudal vertebrae	33.57	±	0.68	34	51.48	34	30	35
Caudal complex vertebrae	3.05	±	0.23	3	94.67	3	2	4
Caudal fin rays	20.25	±	0.54	20	79.09	20	20	24

Table 2. Number of vertebrae in abdominal, caudal, and caudal complex regions, as well as caudal fin ray counts: mean ± standard deviation (SD), mode, percentage of individuals exhibiting the most frequent value (%I), median, minimum (Min), and maximum (Max).

Number of individuals with at least one anomaly (%)	100.0
Total number of anomalies	5,654
Number of anomalies/number of affected individuals	11.2
Number of individuals with VBA and/or VCD (%)	51.7
Total number of VBA and VCD	913
Number of VBA and VCD/number of individuals with VBA and/or VCD	3.5
Number of VBA and VCD/total number of anomalies (%)	16.1

Table 3. General results on the incidence of skeletal anomalies in the studied fish: number of individuals displaying at least one anomaly (%); number of individuals with vertebral body anomalies (VBA) and/or rachis deviations (VCD) (%); total number of skeletal abnormalities; number of VBA and VCD and the percentage of VBA and VCD in the total number of anomalies; anomalies and VBA and/or VCD loads.

	Anomaly typology		N.I.	%N.I.	N.A.	N.A./I.
Abdominal region	Parapophysis	Bifurcation	1	0.20	1	1.00
		Number alteration	65	12.82	71	1.09
		Insertion alteration	3	0.59	3	1.00
		Fusion	8	1.58	9	1.13
		Deformation	4	0.79	4	1.00
	Neural arches and spines	Bifurcation	3	0.59	3	1.00
		Number alteration	12	2.37	24	2.00
		Insertion alteration	8	1.58	13	1.63
		Fusion	8	1.58	9	1.13
		Incomplete arch	16	3.16	36	2.25
		Deformation	8	1.58	16	2.00
	VBA	Fusion	55	10.85	62	1.13
		Deformation	28	5.52	65	2.32
	VCD	Kyphosis	4	0.79	4	1.00
		Lordosis	10	1.97	10	1.00
		Scoliosis	3	0.59	3	1.00
Caudal region	Neural arches and spines	Bifurcation	2	0.39	2	1.00
		Number alteration	31	6.11	46	1.48
		Insertion alteration	30	5.92	58	1.93
		Fusion	14	2.76	15	1.07
		Incomplete arch	61	12.03	101	1.66
		Deformation	79	15.58	224	2.84
	Haemal arches and spines	Bifurcation	3	0.59	3	1.00
		Number alteration	21	4.14	30	1.43
		Insertion alteration	53	10.45	71	1.34
		Fusion	22	4.34	30	1.36
		Incomplete arch	75	14.79	111	1.48
		Deformation	394	77.71	1499	3.80
	VBA	Fusion	81	15.98	110	1.36
		Deformation	83	16.37	252	3.04
	VCD	Kyphosis	28	5.52	30	1.07
		Lordosis	36	7.10	43	1.19
		Scoliosis	15	2.96	16	1.07
Caudal complex	Neural arches and spines	Bifurcation	1	0.20	1	1.00
		Number alteration	32	6.31	32	1.00
		Insertion alteration	4	0.79	4	1.00
		Fusion	31	6.11	31	1.00
		Incomplete arch	13	2.56	13	1.00
		Deformation	56	11.05	59	1.05
	Haemal arches and spines	Bifurcation	2	0.39	2	1.00
		Number alteration	13	2.56	13	1.00
		Insertion alteration	6	1.18	8	1.33
		Fusion	45	8.88	45	1.00
		Incomplete arch	24	4.74	26	1.08
		Deformation	51	10.06	55	1.08
	Hypurals epural parhypural	Bifurcation	0	0.00	0	0.00
		Number alteration	0	0.00	0	0.00
		Insertion alteration	0	0.00	0	0.00
		Fusion	231	45.56	282	1.22
		Deformation	471	92.90	777	1.65
	VBA	Fusion	95	18.74	95	1.00
		Deformation	127	25.05	206	1.62
	VCD	Kyphosis	13	2.56	13	1.00
		Lordosis	1	0.20	1	1.00
		Scoliosis	3	0.59	3	1.00

Table 4. Frequencies of skeletal alterations by location, affected skeletal structure, and type: number of fish showing at least one skeletal anomaly (N.I.), percentage of affected

individuals (%N.I.), number of observed alterations (N.A.), and average number of malformations per specimen (N.A./I.). VBA: Vertebral body anomalies. VCD: Vertebral column deviations.

Figure legends:

Figure 1. Sole (*Solea senegalensis*) larva stained with alcian blue and alizarin red, showing anatomical regions considered: abdominal, caudal, and caudal complex. Note the slightly twisted front caudal haemal spines and bending of caudal plates. P: parapophysis; N: neural arch and spine; H: haemal arch and spine; V: vertebral centrum; Pu1: preural 1; Pu2: preural 2; U: urostyle; H1-H5: hypurals 1-5; E: epural; Ph: parhypural; R: caudal fin rays. Bar = 5 mm. Insert bar = 500 µm.

Figure 2. Main skeletal alterations in 31/32 dah Senegalese sole (*Solea senegalensis*). A. Absence of the neural arch of the first abdominal vertebra (white arrowhead); slight deformities in neural arches (black arrowheads) and alteration in the number of parapophyses (black arrow); note that some parapophyses were fused, forming an arch (asterisk). B. Twisted haemal elements in the caudal region (black arrowheads). C. Slightly incomplete neural and haemal arches (white arrowheads). D. Alteration of the haemal spine insertion, note the lack of symmetry of vertebral bodies (white asterisks). E. Slight bending of all caudal plates; hypurals 3 and 4 are fused (white star); fusion between the last caudal vertebra and preural 2, with severe deformations of the vertebral bodies (white asterisks) and fusion among neural arches (white arrowhead), preural 2 showed an incomplete vertebral centrum (lacked a part of the vertebra); twisted haemal spines (black arrowhead) associated to this lesion. Bars = 500 µm.

Figure 3. Distinct abnormalities observed in the vertebral column. A. Multiple severe VBA as fusions (black arrows) and deformations of the affected vertebrae, mostly in abdominal and caudal regions. Misaligned vertebrae (black asterisk), kyphosis (K), and lordosis (L) were also present. In the lordotic segment, vertebrae were also slightly

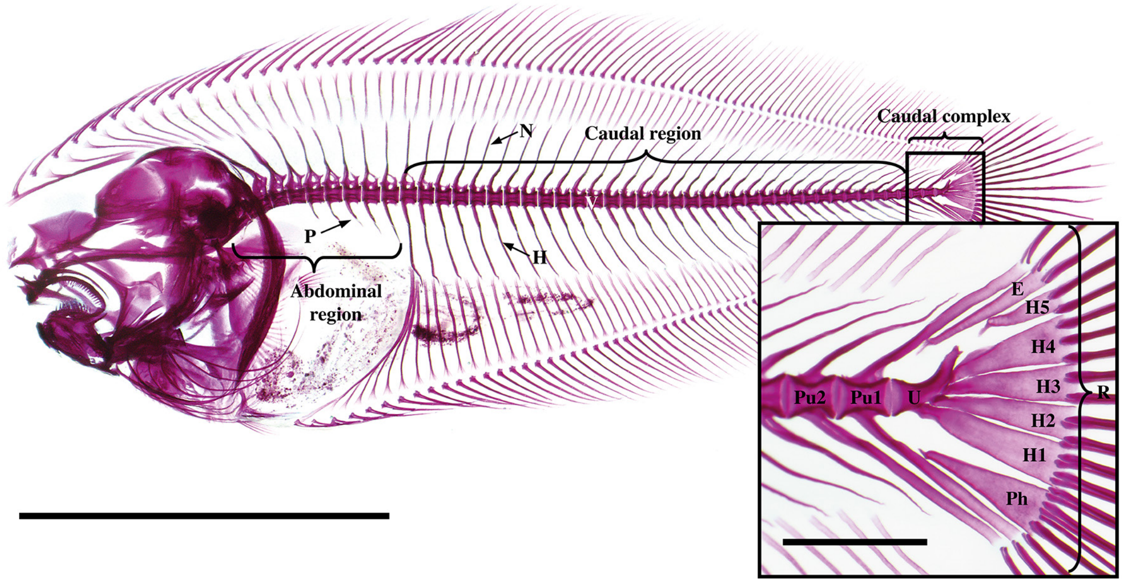
deviated to the blind side of the fish. Note the alterations in the insertion of neural and haemal arches in the affected segments, and the slight bending of some neural and haemal spines and caudal complex plates. B. Caudal vertebral fusion and incomplete neural arch (white arrowhead). C. Fusions among preurals, note the remaining line of fusion (arrow) and mild deformations of neural and haemal spines (black arrowheads). Fusion of hypurals 3-4 (white star), and slight bending of the caudal complex plates. D. Presence of an extra element between the preurals (arrow), fused with preural 1, with kyphosis (K) and deformations in the caudal complex vertebrae and fusion among the haemal arches. Note the trapezoidal vertebra (white asterisk). Alterations of the shape of haemal spines (black arrowhead) and in caudal complex plates were observed. E. Gross deformations of abdominal vertebral centres (white asterisks) and lordosis (L), with slight deviation towards the blind aspect of the individual. Note the enlargement and the darker and irregular articular surface in the affected abdominal vertebrae. The first abdominal vertebra lacked a neural arch (white arrowhead). Deformations of neural spines were also present (black arrowheads). VBA: Vertebral body anomalies. Bars = 500 µm.

Figure 4. Frequency of vertebral anomalies (VBA and VCD) per specimen. Most affected fish presented one or three alterations (90 and 61 *Solea senegalensis*, respectively), although ten of them showed more than 10 VBA or VCD. VBA: Vertebral body anomalies. VCD: Vertebral column deviations.

Figure 5. Distribution of the vertebral body anomalies (VBA) throughout the spinal column. A. Fusions. The first two abdominal vertebrae and preurals were the most affected. B. Deformations. The caudal complex vertebrae showed more alterations of

the shape, in some cases, associated to fusions. Pu1: preural 1; Pu2: preural 2; U: urostyle; I.V.: intermediary (extra) vertebra between the two preurals (when present).

Figure 6. Distribution of the vertebral column deviations (VCD) throughout the spinal column. A. Kyphosis. These deviations were more frequent in the caudal complex region. B. Lordosis. The intermediary vertebral segment between abdominal and caudal regions was the most affected by lordotic curvatures. C. Scoliosis. Scoliotic lesions were observed throughout the vertebral column, in a small number of cases. Pu1: preural 1; Pu2: preural 2; U: urostyle; I. V.: intermediary (extra) vertebra between the two preurals (when present).



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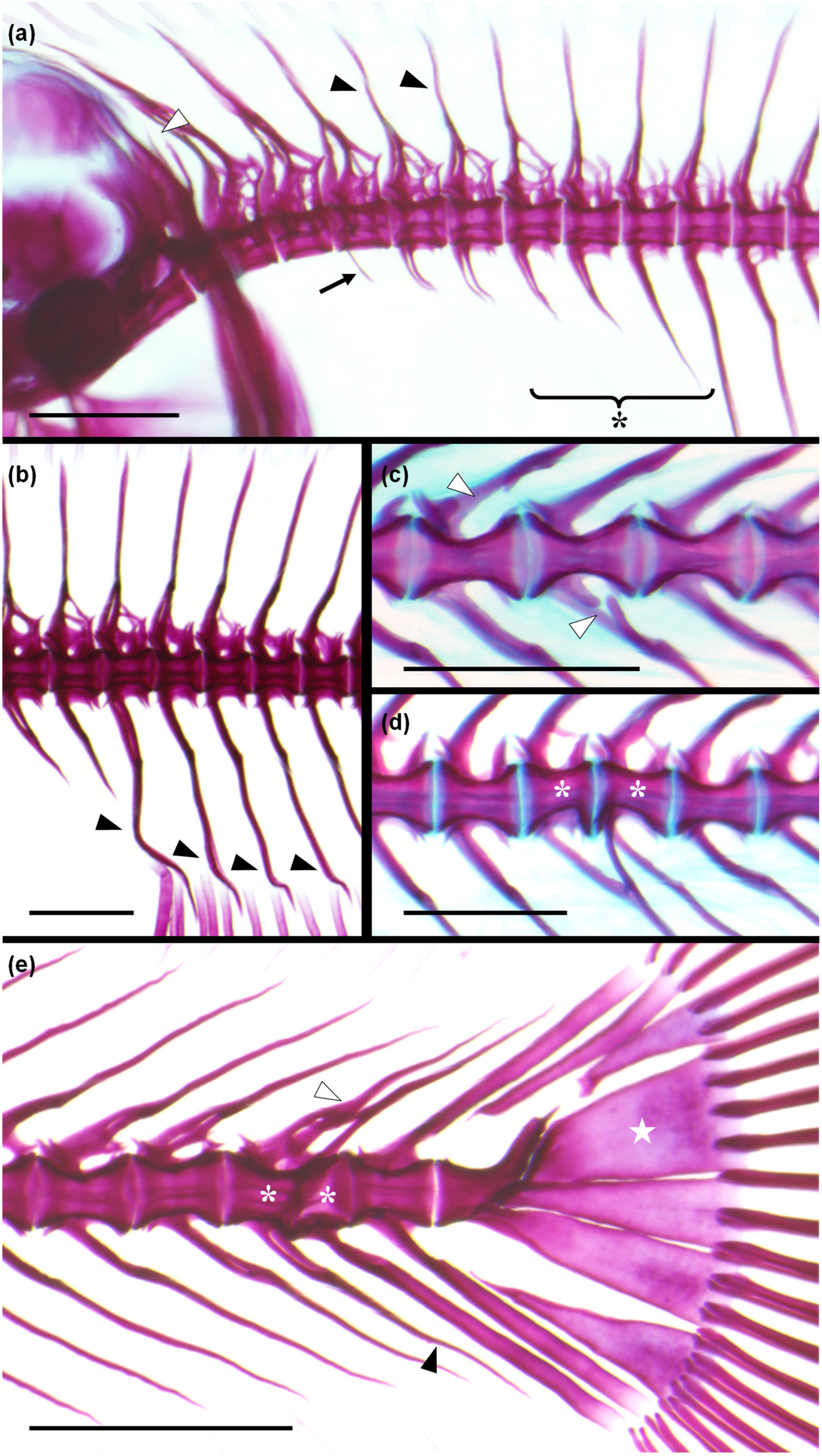

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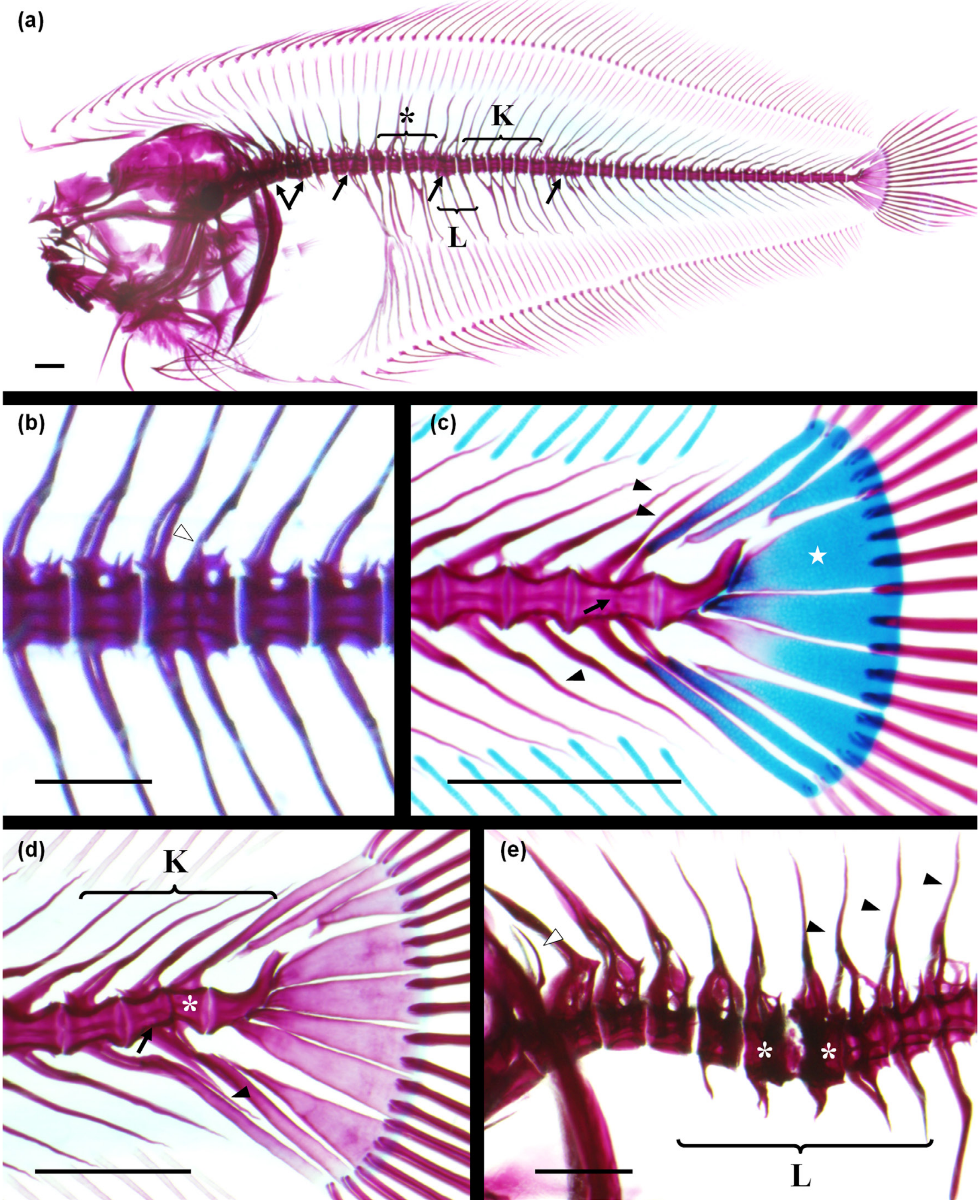

Figure 3. Distinct abnormalities observed in the vertebral column. A. Multiple severe VBA as fusions (black arrows) and deformations of the affected vertebrae, mostly in abdominal and caudal regions. Misaligned vertebrae (black asterisk), kyphosis (K), and lordosis (L) were also present. In the lordotic segment, vertebrae were also slightly deviated to the blind side of the fish. Note the alterations in the insertion of neural and

haemal arches in the affected segments, and the slight bending of some neural and haemal spines and caudal complex plates. B. Caudal vertebral fusion and incomplete neural arch (white arrowhead). C. Fusions among preurals, note the remaining line of fusion (arrow) and mild deformations of neural and haemal spines (black arrowheads). Fusion of hypurals 3-4 (white star), and slight bending of the caudal complex plates. D. Presence of an extra element between the preurals (arrow), fused with preural 1, with kyphosis (K) and deformations in the caudal complex vertebrae and fusion among the haemal arches. Note the trapezoidal vertebra (white asterisk). Alterations of the shape of haemal spines (black arrowhead) and in caudal complex plates were observed. E. Gross deformations of abdominal vertebral centres (white asterisks) and lordosis (L), with slight deviation towards the blind aspect of the individual. Note the enlargement and the darker and irregular articular surface in the affected abdominal vertebrae. The first abdominal vertebra lacked a neural arch (white arrowhead). Deformations of neural spines were also present (black arrowheads). VBA: Vertebral body anomalies. Bars = 500 µm.

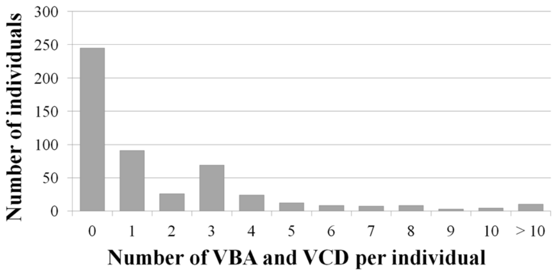

Figure 4. Frequency of vertebral anomalies (VBA and VCD) per specimen. Most affected fish presented one or three alterations (90 and 61 *Solea senegalensis*, respectively), although ten of them showed more than 10 VBA or VCD. VBA: Vertebral body anomalies. VCD: Vertebral column deviations.

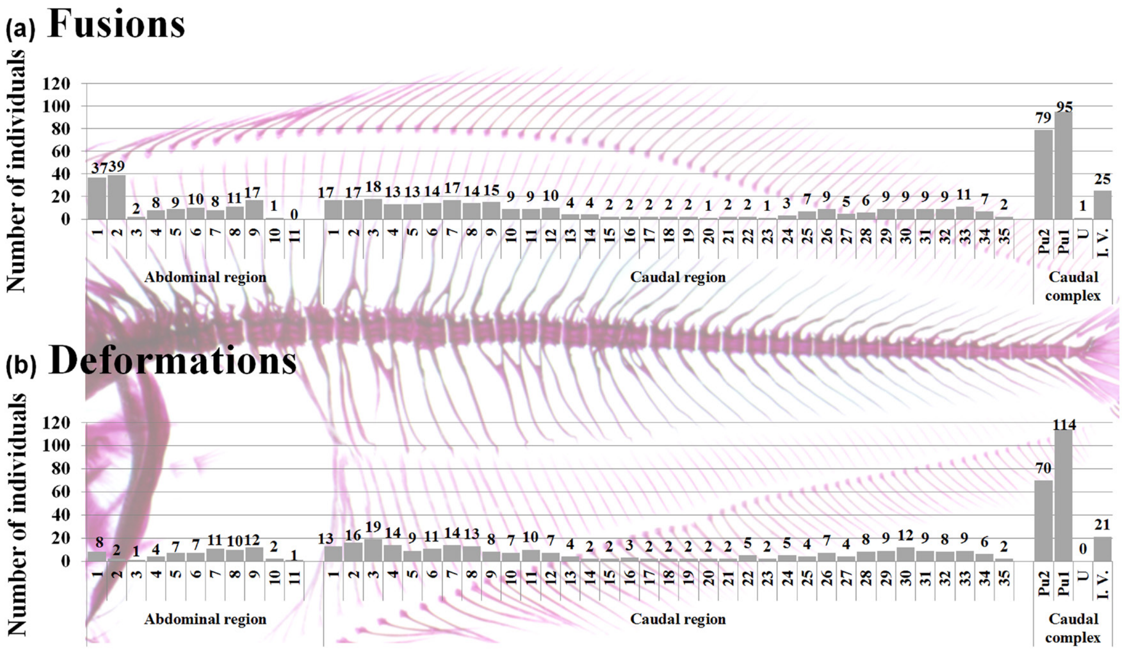

Figure 5. Distribution of the vertebral body anomalies (VBA) throughout the spinal column. A. Fusions. The first two abdominal vertebrae and preurals were the most affected. B. Deformations. The caudal complex vertebrae showed more alterations of

the shape, in some cases, associated to fusions. Pu1: preural 1; Pu2: preural 2; U: urostyle; I.V.: intermediary (extra) vertebra between the two preurals (when present).

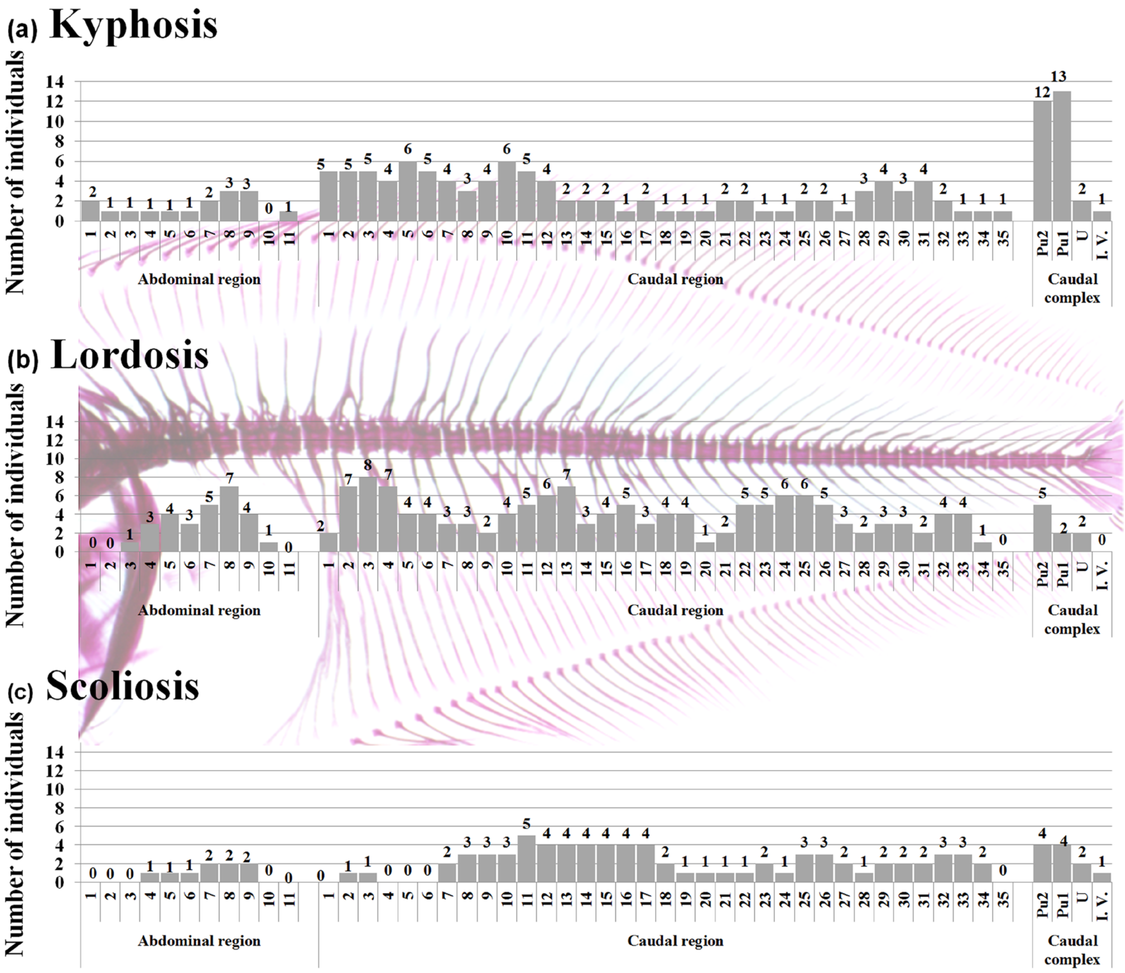

Figure 6. Distribution of the vertebral column deviations (VCD) throughout the spinal column. A. Kyphosis. These deviations were more frequent in the caudal complex region. B. Lordosis. The intermediary vertebral segment between abdominal and caudal regions was the most affected by lordotic curvatures. C. Scoliosis. Scoliotic lesions were observed throughout the vertebral column, in a small number of cases. Pu1: preural 1; Pu2: preural 2; U: urostyle; I. V.: intermediary (extra) vertebra between the two preurals (when present).