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The Vertebral Column of Flatfish: A Review

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ABSTRACT

Flatfish (Pleuronectiformes) encompass fascinating fish species known for their high gastronomical value, rearing performances, and market prices, which attract both economic and scientific interest. Despite these attributes, flatfish exhibit unique morphological, developmental, and biological features, as well as distinctive swimming movements, which require special approaches in skeletal biology research, an area that remains relatively unexplored for this diverse group of fishes. These unique features emerge during metamorphosis, at the end of the larval period, when bilateral symmetric pelagic larvae gradually become asymmetric and undergo various morphological and physiological changes. Consequently, the skeletal characteristics of these species include a certain degree of asymmetry in the skeletal structures. These fish also present acellular bone (bone devoid of osteocytes). Research on flatfish skeletons is crucial for improving animal welfare and promoting sustainable farming, as the literature indicates that more than 50% of the larvae and juveniles of different cultured flatfish can be affected by skeletal deformities. This review aims to compile the available studies on the development of vertebral column deformities in flatfish in an integrative manner, addressing state-of-the-art research on the etiology, diagnostics, and innovative studies on this issue. It also covers the fundamental aspects of teleost skeletal development, particularly the flatfish vertebral column.

1 | Introduction

Flatfish (Pleuronectiformes) are a highly derived teleost order that comprises fishes characterized by flat and asymmetric body organization, with both eyes located on one side of the animal (ocular side), as a result of substantial evolutionary changes. Pleuronectiformes encompass approximately 716 recognized extant species. The animals display amazing diversity in size,

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Abbreviations: Abd v, abdominal vertebrae; Abd v, abdominal vertebrae; Afr, anal fin ray; ATP, adenosine triphosphate; Bglap, bone GLA protein; BMP, bone morphogenetic protein; Bop, basioccipital articulatory process; Ca, calcium; Cfr, caudal fin ray; dah, days after hatching; def, deformities; Dfr, dorsal fin ray; *dio 2*, deiodinases 2; DNA, deoxyribonucleic acid; E, epural; FAO, Food and Agriculture Organization; *ggcx*, γ-glutamyl carboxylase; GRP, Gla-rich protein; H, haemal arch and spine; H1–H5, hypurals 1–5; Ha, haemal arch; H–E, hematoxylin-Eosin; hh, hedgehog signaling pathway; Hprz, haemal prezigapophysis;; Hpz, haemal poszigapophysis; Hs, haemal spine; IGF, insulin-like growth factor; kt, thousands of tons; MA, methoprene acid; mah, months after hatching; Mgp, matrix-Gla protein; Mhs, modified haemal spine; miRNA, microRNA; Mns, modified neural spine; N, neural arch and spine; Na, neural arch; NGS, next-generation sequencing; Nprz, neural prezigapophysis; Npz, neural poszigapophysis; Ns, neural spine; Osx/SP7, osterix/Sp7 Transcription Factor; Ph, parhypural; PK, phylloquinone; Pp, parapophysis; Pt, pterygophore; *ptch*, patched; Pu1, preural 1; Pu2, preural 2; *pxr*, VK nuclear receptor; *pxr*, VK nuclear receptor; R, caudal fin rays; RA, retinoic acid; *rarα*, retinoic acid receptor α; *rbp*, retinol binding protein; RNA, ribonucleic acid; Runx2, Runt-related 2; *rxrα*, retinoid X receptor α; SL, standard length; Sox9, sex determining region Y-box 9; T3, triiodothyronine; T4, thyroxin; *tg*, thyroglobulin; TH, thyroid hormones; *thrab*, thyroid hormone receptor alpha b; *trαa*, thyroid hormone receptor α A; *trαb*, thyroid hormone receptor α B; *trβ*, thyroid hormone receptor β; TU, goitrogen thiourea; U, urostyle; V, vertebral centrum; VA, vitamin A; VDR, vitamin D receptor; VK, vitamin K; VKDP, vitamin K-dependent protein; vkorc, VK epoxide reductase complex.

shape, and habitats that range from the poles to the tropics in both marine and freshwater environments [1].

Flatfish have been the object of research for more than a century because of their wide distribution and use for commercial and recreational fisheries and in the aquaculture industry in many parts of the world [2]. The highly desirable attributes of some species in terms of flesh quality, rearing performance, and market price have attracted economic and scientific interest [1, 3–5].

Aquaculture production of flatfish has been slowly increasing, reaching almost 200,000 tons in 2015 and remaining stable at approximately 190,000 tons over the last years (Figure 1a) [6]. The bulk of production comes from Japanese flounder (*Paralichthys olivaceus*) and other flounder species in Japan and Korea (mostly for sea ranching and restocking), turbot (*Scophthalmus maximus*), Atlantic halibut (*Hippoglossus hippoglossus*), Senegalese sole (*Solea senegalensis*), and the common sole (*Solea solea*), which are farmed for human consumption [5, 8]. There is increasing interest in farming local species in certain regions, including summer flounder (*Paralichthys dentatus*, Northeast USA), greenback flounder (*Rhombosolea tapirina*, Australia), and winter flounder (*Pseudopleuronectes americanus*, Canada) [5], but also the European flounder (*Platichthys flesus*, Baltic Sea and Black Sea regions) [9] and the starry flounder (*Platichthys stellatus*, China, North Korea, South Korea, USA) [10]. In Europe, aquaculture production of flatfish is focused on four species (*H. hippoglossus, S. maximus*, *S. senegalensis*, and *S. solea*) and is located mostly in Spain, Portugal, Norway, Iceland, and France, depending on the cultured species (data from 2020 and 2021; Figure 1b).

From a production point of view, the quality and value of farmed flatfish can be compromised by skeletal deformities and malpigmentation [8, 11–14]. In particular, the high frequency of skeletal deformities can constitute a bottleneck for large-scale production and can lead to severe welfare concerns [15–17]. Craniofacial deformities have been frequently described [18, 19]. Vertebral column and caudal fin anomalies have been observed in farmed flatfish at variable frequencies, sometimes reaching almost 100% of the produced specimens in different species [13–15, 20, 21]. The presence of skeletal deformities has been observed in wild specimens and is suggested to be associated with water pollution [22–26].

The vertebral column, composed of the notochord and articulated vertebral bodies, plays a vital role in the support and attachment of muscles and the protection of the abdominal organs, the spinal cord, and blood vessels [27]. To assess vertebral column anomalies accurately in farmed fishes, it is crucial to improve the knowledge on the development, composition, and organization of the flatfish vertebral column and the factors that can cause malformations.

This review provides an overview of the available studies in the literature, a comprehensive view of the fundamental aspects of teleost skeletal development in general, and the development of deformities of the flatfish vertebral column. Parts of this review focus on *S. senegalensis* because of the large amount of available data about this species.

2 | The Vertebral Column and the Caudal Skeleton of Flatfish

2.1 | Evolution and Anatomy of the Skeleton

Flatfish are a monophyletic order [28, 29], but their monophyletic origin has also been questioned based on interpretations of genomic datasets. The authors suggested the independent evolution of Pleuronectoidei and Psettodoidei [30]. A more recent phylogenetic and comparative genomic analyses by Duarte-Ribeiro, Rosas-Puchuri, Friedman et al. [31], which also reanalyzed the genomic datasets, strongly supports the single evolutionary origin of flatfish. The oldest true flatfish fossil record dates back to the early Eocene, approximately 53–57 million years ago [1, 32]. Flatfish diversification took place in the middle Miocene, approximately 10–15 million years ago [33]. The discovery of

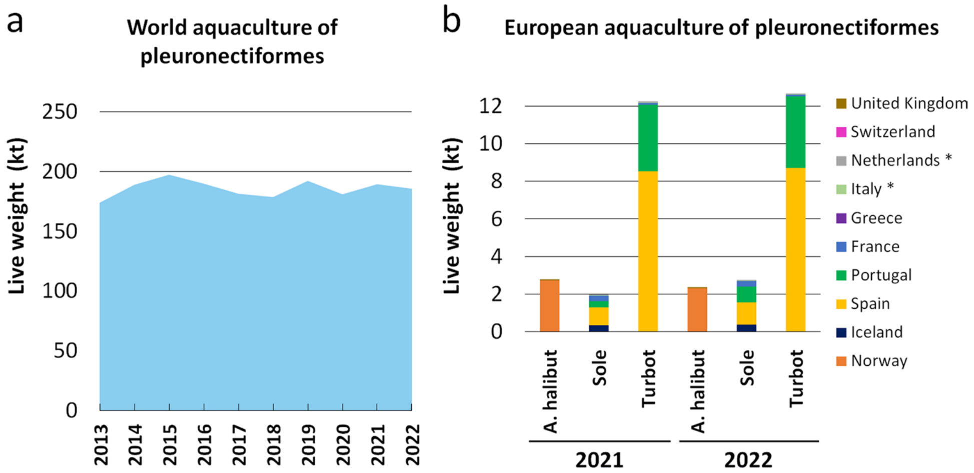

FIGURE 1 | Global flatfish production in the aquaculture industry from 2013 to 2022 (thousands of tons; kt) (a). European flatfish production in aquaculture in 2021 and 2022 by species and country (b). Atlantic halibut (*Hippoglossus hippoglossus*; A. halibut); Sole (*Solea solea* and *Solea senegalensis*); Turbot (*Scophthalmus maximus*). *Food and Agriculture Organization (FAO) estimate from available sources of information. *Source:* FAO [6] and FEAP [7]. The estimated values or those not available from FAO [6] were replaced with data from FEAP [7] whenever possible.

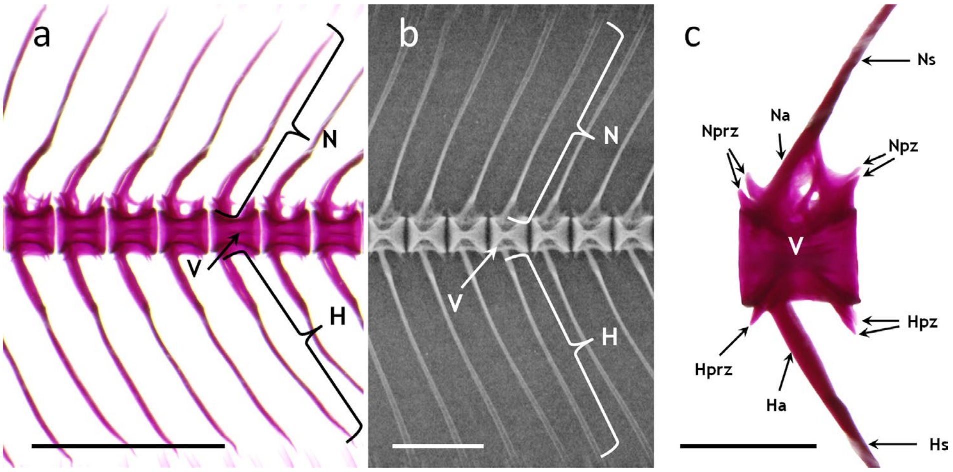

FIGURE 2 | Example of vertebrae from Senegalese sole (*Solea senegalensis*). (a) Blind side aspect of 32 days after hatching (dah) larva stained with alizarin red. (b) Latero-lateral radiography of a wild adult sole. (c) Vertebra stained with the alizarin red staining technique from a 105-dah juvenile showing the main vertebral features. 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.

extinct stem flatfish with incomplete orbital migration, such as *Amphistium* sp. and *Heteronectes* sp., may indicate that the flatfish bauplan evolved gradually into a fully asymmetrical form [28, 34]. For most living flatfish species, the post-metamorphosis position of the migrated eye (dextral vs. sinistral) appears to be genetically fixed, although some species are indeterminate with respect to the side to which the eye migrates [1].

The design and anatomy of the teleostean vertebral column have been addressed in several classical works [35–38]. Briefly, the vertebral column is composed of a sequence of bony vertebral centra with a symmetrical amphicoelous shape (resembling an horizontal hourglass) connected by the intervertebral ligaments to which the notochord contributes. Vertebrae are equipped with neural arches and neural spines (dorsal aspect of the vertebra), haemal arches and haemal spines (ventral aspect of the vertebra) or ventral apophyses of different shapes, according to the anatomic region of the vertebral column. Neural and haemal arches and ventral apophyses (Figure 2a,b) present articulating processes denominated prezygapophysis and poszygapophysis in the anteriormost and posteriormost parts, respectively (Figure 2c) [15, 37].

The teleost vertebral column can be divided into five major anatomical regions [39–41]. In some studies, the vertebrae of the anterior abdominal region, also termed postcranial [39, 41], cephalic [42], or cervical vertebrae [43, 44], are characterized by the lack of ribs. Abdominal vertebrae (also termed truncal, pre-caudal, or pre-haemal) have neural arches and spines but lack closed haemal arches and spines [27, 40]. Instead, a number of abdominal vertebrae show paired ventral parapophyses (to which the ribs can attach) [27]. The caudal vertebrae (also termed haemal) are characterized by the presence of neural arches and spines and closed haemal arches and spines, and lack ribs and parapophyses [27]. Many teleost species have a transitional region between the abdominal and caudal parts of the vertebral column [39, 45]. The transitional vertebrae show gradually shorter ribs and elongated parapophyses. Finally, the caudal-most region is characterized by the presence of elements of the caudal fin endoskeleton, termed as caudal fin complex, postcaudal, or ural region [40, 46–48]. Preural and ural centra have modified neural and haemal arch elements, which participate in supporting the dermal fin rays (lepidotrichia) of the caudal fin [15, 39]. The urostyle represents the last fused vertebra of the adult vertebral column and articulates with hypuralia [20, 48–52]. Other endoskeletal elements that are part of the caudal complex are the epurals, uroneurals, and hypurals, which represent the neural and haemal modified elements of the ural vertebrae, respectively [38, 39].

Several reports have described the vertebral column of different flatfish species [9, 10, 14, 15, 20, 25, 35, 47, 53–59]. The anatomical regions that may divert from the general anatomy of the teleostean vertebral column are the abdominal and the caudal complex regions (Figures 3a–c and 4a–k). The abdominal region, which is commonly referred to in the literature as post-cephalic and abdominal together, is usually short (Figure 4a–k), for example, in *S. senegalensis*, it presents only 8–9 vertebral centra (Figures 3a,b and 5a,b) [15, 35]. Curiously, two characters in this region can be used to distinguish different groups within the Pleuronectiformes: the length of the first neural spine and the nature of the parapophyses [35]. Specific features of the first vertebra also distinguish the European plaice (*Pleuronectes platessa*) from the European flounder (*Platichthys flesus*) and the common dab (*Limanda limanda*) [60]. In *S. senegalensis* and *S. solea*, the first vertebral centra carry two separate short neural spines (Figure 5a,b) instead of a complete neural arch and spine. Moreover, the first vertebral body is compressed [12, 13, 35, 53]. Members of Bothinae and Soleidae subfamilies display a number of vertebrae in the posterior abdominal region with fused parapophyses, which form a haemal canal. In pleuronectids, for example, parapophyses normally remain unfused [35]. *S. senegalensis* carries a variable number of this type of transitional vertebrae with two fused parapophyses in the same vertebra, forming an arch [13], which is consistent with the findings of Ford [35] for *S. solea*. Ventral parapophyses also present

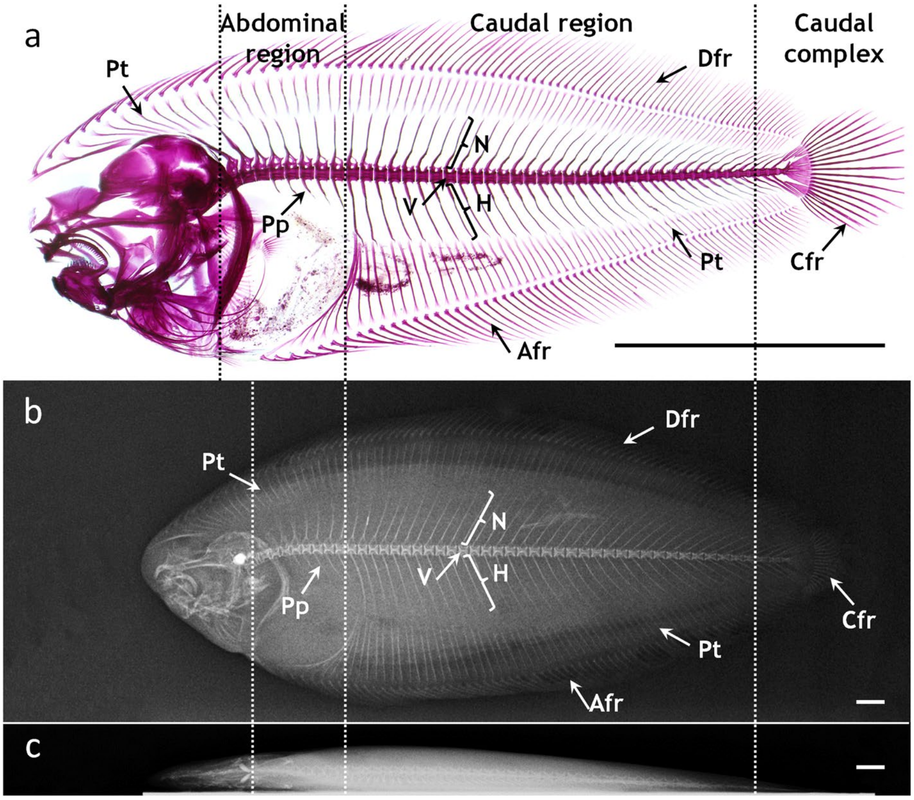

FIGURE 3 | Main anatomical regions of the body in Senegalese sole (*Solea senegalensis*) larvae (a) and juveniles (b, c) showing the features of the vertebral column and caudal fin skeleton and of the dorsal and anal fins (adapted from de Azevedo, Losada, Barreiro et al. [12]). (a) Blind side aspect of 32 days after hatching (dah) larva stained with alizarin red. (b) Latero-lateral radiographic projection of a juvenile (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.

articulating processes denominated prezygapophysis and postzygapophysis in the anteriormost and posteriormost parts, respectively (Figure 5c).

The fossil record shows that the evolutionary origin of flatfish asymmetry took place with gradual orbital migration [34]. The rest of the skeleton can present a certain degree of asymmetry as well [61]. In flatfish species, except for *Psettodes*, individual vertebrae exhibit a greater or lesser degree of asymmetry, with fewer alterations in caudal elements [54, 62]. In the genus *Solea*, the first three to four vertebrae tend to curve towards the eyed side [61]. In *P. platessa* and *H. hippoglossus*, the mesial vertical plane is curved towards the eyed side, but the most anterior neural spines usually bend towards the blind side [54, 63, 64]. More asymmetry is observed in different Scophtalmidae species. The lateral apophyses of the caudal vertebral centra are more strongly developed on the ocular side than on the blind side [35, 64].

The caudal complex (Figures 3 and 6) of *S. senegalensis* can be used as an example of the basic arrangement of the flatfish caudal fin endoskeleton. It is usually composed of two preural vertebrae and the urostyle, which is fused to the base of the first four hypurals (Figure 6a). The hypurals and the parhypural are branched in the adult specimens (Figure 6b,c) [15]. In *S. senegalensis*, only a single epural and no free uroneural processes have been reported [15]. The caudal fin endoskeleton supports the 20 caudal dermal fin rays (lepidotrichia), which are composed of branched and segmented hemirays [15]. The characters of the caudal fin endoskeleton can vary among the different families of pleuronectiforms in terms of the number of epurals/ossicles, the number and branching of hypurals, the fusion between the hypurals, and the fusion of the hypurals with the urostyle [47, 55]. These main differences are thoroughly described for pleuronectids by Sakamoto [55], although the nomenclature of certain bones can be controversial [47]. The number and degree of segmentation and branching of caudal dermal fin rays are also features that differ among flatfishes [55] and are summarized in Hoshino [65].

The distribution of vertebral and other meristic counts for members of the Pleuronectidae family and some Cynoglossidae species are depicted in Sakamoto [55], Fitch [66], Munroe and McCosker [67], Lee, Munroe, and Shao [68], and Lee, Munroe, and Shao [69]. In *S. senegalensis*, the number of vertebrae can range between 8 and 11, 30–35, and 2–4 in the abdominal, caudal (usually 34), and caudal complex regions, respectively [12, 13, 15]. There is a certain degree of variation in the number of vertebrae in individual fish in every species, but in flatfish, the number of vertebrae in the abdominal region is always comparatively low in contrast with the number of vertebral centra in the caudal region [35]. Vertebral counts may be affected by environmental conditions during early development, such as water temperature, salinity, light, rearing density, and dietary factors such as vitamin A (VA) and

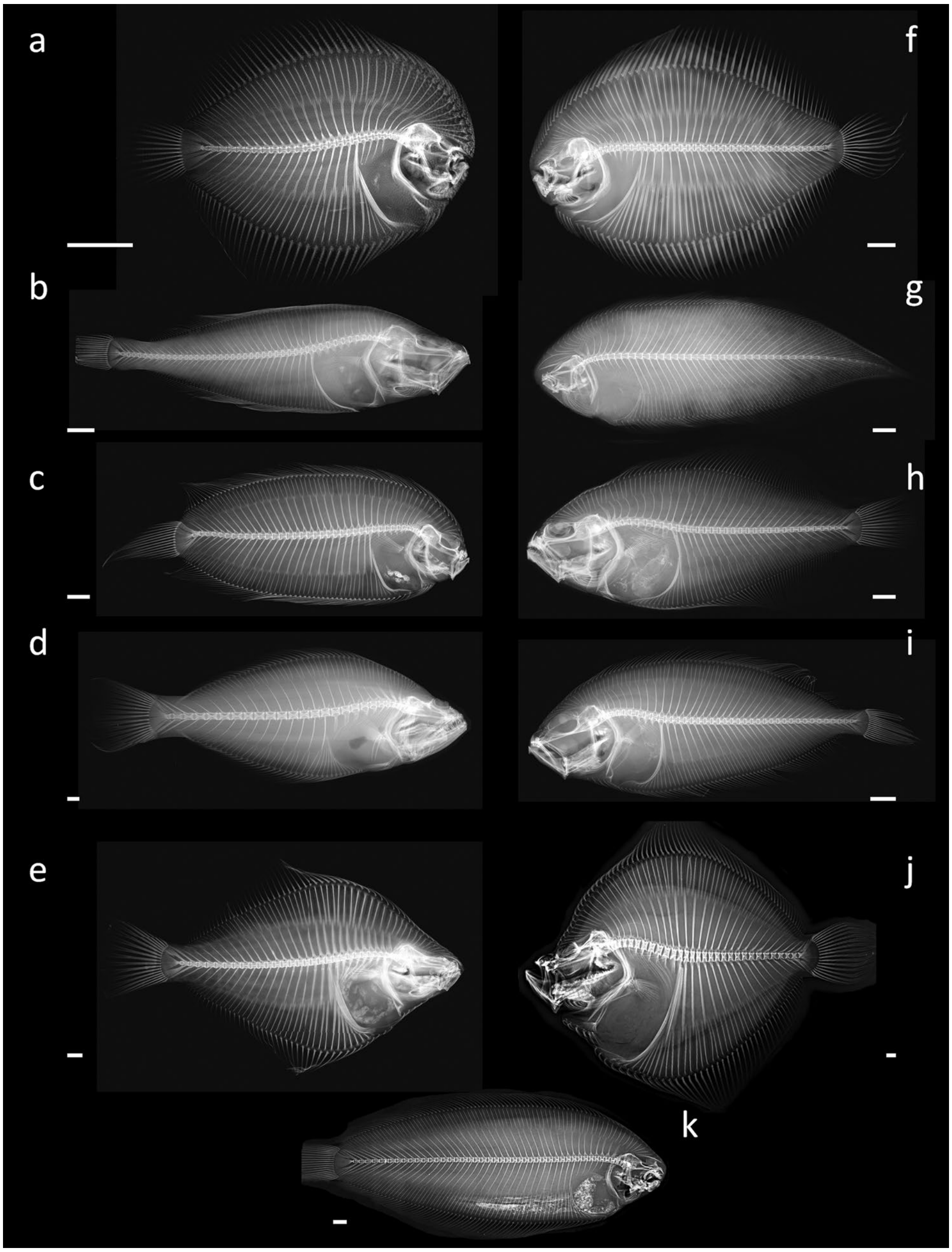

FIGURE 4 | Skeletal anatomy of different flatfish species (radiographic images), modified from Black and Berendzen [33]. (a) *Achirus lineatus* (Achiridae); (b) *Lyopsetta exilis* (Pleuronectidae); (c) *Plagiopsetta glossa* (Samaridae); (d) *Psettodes belcheri* (Psettodidae); (e) *Rhombosolea plebeia* (Pleuronectidae); (f) *Gymnachirus melas* (Achiridae); (g) *Symphurus plagiusa* (Cynoglossidae); (h) *Syacium micrurum* (Paralichthyidae); (i) *Lepidorhombus boscii* (Scophthalmidae); (j) *Scophthalmus maximus* (Scophthalmidae); (k) *Solea senegalensis* (Soleidae). Bars = 1 cm.

polyunsaturated fatty acids [70–78]. Studies in *S. senegalensis* and *P. olivaceus* reported a greater incidence of supernumerary vertebrae in fish fed higher VA levels at early stages of development [79, 80].

2.2 | Microanatomical Structure of the Vertebral Bodies and Intervertebral Space

There are few studies in the literature focusing on the histological organization of the vertebral column in flatfish. Here, we take the *S. senegalensis* vertebral structure as an example of flatfish vertebral microanatomy, with vertebrae composed of acellular bone (i.e., bone devoid of osteocytes), a characteristic of advanced teleosts and thus of all flatfish species [81–83]. Like in other teleosts, bone trabeculae support the vertebral body endplates (Figure 7a,b) [84, 85].

Vertebral trabecular arrangement has been analyzed in different fishes, including flatfish [86–88]. The growth zone of the vertebral body endplates is characterized by osteoid-secreting osteoblasts. Opposing vertebral body endplates are separated by the intervertebral space and connected by an intervertebral ligament (Figure 7b) [84, 89, 90]. In the intervertebral space, the notochord expands [91] with vacuolated chordocytes [92], and the chordoblasts define a notochordal epithelial layer (Figure 7b)

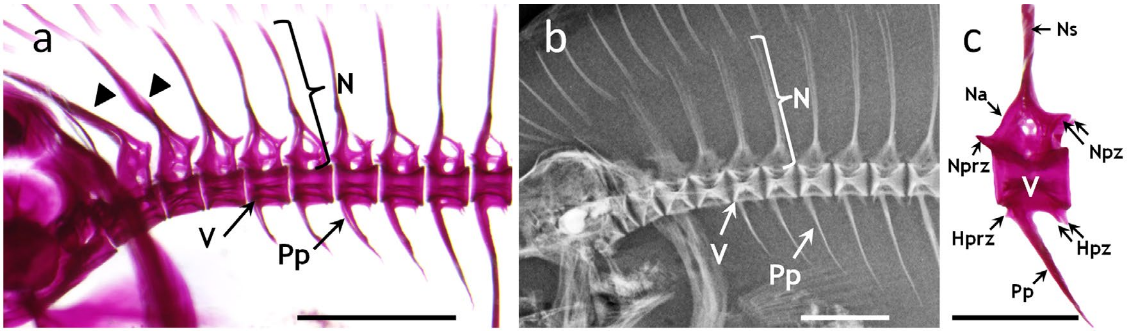

FIGURE 5 | Abdominal region of Senegalese sole (*Solea senegalensis*) with cephalic, abdominal, and transitional vertebrae. (a) Blind side aspect of 32 days after hatching (dah) larva stained with alizarin red. Note the thicker cranial neural spines (arrowheads in vertebrae 2 and 3). (b) Latero-lateral radiography of a wild adult sole. (c) Abdominal vertebra of a 105-dah juvenile stained with alizarin red. 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.

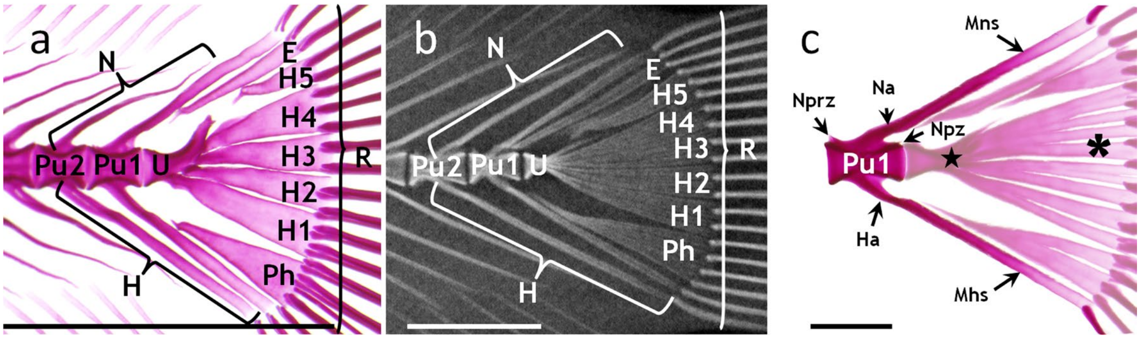

FIGURE 6 | Caudal complex of Senegalese sole (*Solea senegalensis*). (a) Blind side aspect of 32 days after hatching (dah) larva stained with alizarin red. (b) Latero-lateral radiography of a wild adult. (c) Preural 1, urostyle, and caudal complex elements of a 105 dah juvenile stained with alizarin red. Note the elongated amphicoelus shape of the vertebral centrum (Pu1), the fusion of urostyle with hypurals 1–4 (star), and branching of hypurals 1–4 and parhypural (asterisk). Hypurals 3 and 4 are partially fused on the cranial-most part. 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.

[93]. Depending on the parasagittal section, some chordocytes appear less vacuolated and more compact in the notochordal septa and longitudinal strand [84, 92], delimitating vacuolated spaces filled with extracellular fluid [89, 92].

The intervertebral ligament is composed, from inside to outside, of the collagen type II-based extended notochord sheath, an extended elastic layer and a layer of collagen type I fibre bundles, and a second layer of collagen type I fibres that continue inside the vertebral body endplates as Sharpey fibres (Figure 7b) [89, 94, 95]. The organization of notochord structures in the intervertebral space undergoes profound changes, as it has been demonstrated throughout Atlantic salmon (*Salmo salar*) development [92]. In this sense, the biology of the notochord is extensively reviewed in Witten and Hall [95].

Unlike tetrapods, teleost bone medullary cavities are never hematopoietic and, in many cases, are completely filled with adipose tissue [96]. A scheme of the main histological features of the juvenile flatfish vertebral column is illustrated in Figure 7c.

2.3 | Acellular Bone

The bony tissue of basal teleosts and tetrapods is a living, active connective tissue composed of a mineralized extracellular matrix predominantly containing type I collagen and less abundant non-collagenous proteins (osteocalcin, osteopontin, and osteonectin) and cells embedded in the matrix, osteocytes [97, 98]. In mammals, osteocytes represent 95% of all bone cells [99]. Osteocytes are mechanosensors, the cells that regulate bone metabolism, and to control the function of the cells on the bone surface: osteoblasts (bone forming cells), bone lining cells (quiescent osteoblasts), and osteoclasts (bone resorbing cells) [100, 101]. Bone is constantly modelled and remodeled to ensure correct growth and homeostasis, fracture repair, and mechanical adaptation. The latter function is extremely important, as large parts of the skeleton only form in response to the trajectories of mechanical loads [102–105].

Not all teleosts have osteocytes, and in those that have osteocytes, these cells are not present in all life stages and not in all skeletal elements. Bone without osteocytes is designated anosteocytic or acellular bone. The bone from early stages of

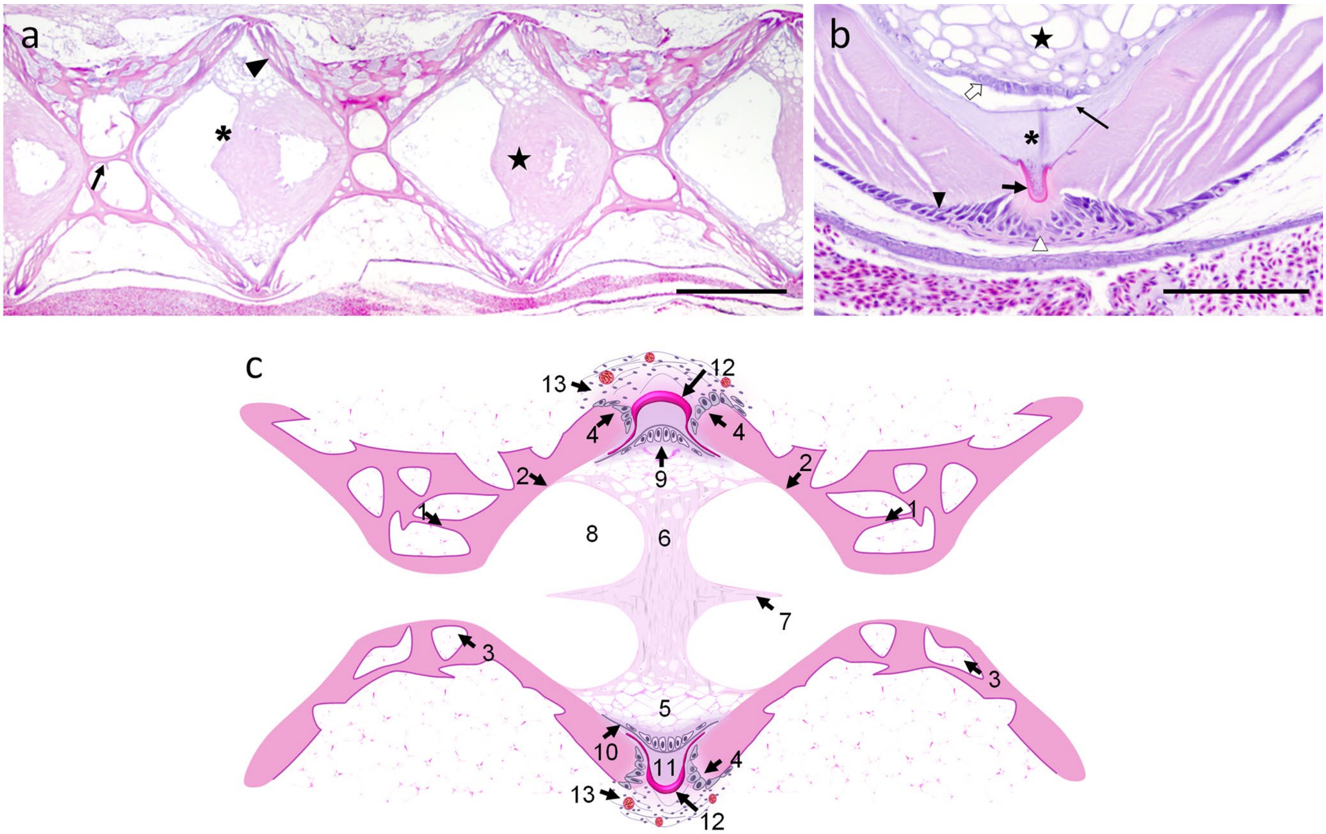

FIGURE 7 | Microarchitecture of the vertebral column in juvenile Senegalese sole (adapted from de Azevedo, Losada, Barreiro et al. [84]). (a) Histological section of a vertebral segment. Osseous tissue is arranged in bony trabeculae (arrow) and laminar bone in the endplates (arrowhead). The intervertebral space (IVS) (asterisk) is occupied by notochordal structures. Note the denser central area of the IVS (star). Alcian blue/hematoxylin and eosin staining. Bar = 500 µm (b) Growth zone of the endplates showing a line of osteoblasts (black arrowhead) and the intervertebral connective tissue (white arrowhead). Note the lack of osteocytes in the endplate. Notochordal structures in the IVS: Central vacuolated chordocytes (star), the layer of chordoblasts (white arrow), thin inner elastic membrane (long arrow), the collagenous notochordal sheath (asterisk), and the external elastic membrane (black arrow) displaying an intense eosinophilic color. Hematoxylin and eosin. Bar = 100 µm (c) Schematic representation of a histological sagittal section of two vertebral centra and part of the adjacent intervertebral spaces. 1. Trabeculae. 2. Concave endplates. 3. Trabecula lacunae. 4. Osteoblast layer. 5. Vacuolated chordocytes. 6. Notochordal septum. 7. Notochord longitudinal strand. 8. Extracellular vacuole. 9. Layer of chordoblasts. 10. Inner elastic membrane. 11. Collagenous notochordal sheath. 12. External elastic membrane. 13. Connective tissue.

zebrafish (*Danio rerio*), for example, has no osteocytes. Teleost scales and fin rays, which are more related to dentine than to true bone tissue, remain acellular throughout life [106–108].

Osteocytes are present in the endoskeleton of basal osteichthyans and teleosts, which include Salmoniformes and Cypriniformes. Advanced teleosts, such as Paracanthopterygii and Percomorpha, thus including flatfish, have completely lost osteocytes in the endoskeleton after a long marine evolutionary history [82, 104, 109, 110]. As osteocytes are absent from bone, alternative regulatory pathways must have evolved that compensate for the absence of the cells that regulate bone remodeling in tetrapods, basal osteichthyans, and basal teleosts [104, 111].

Despite the lack of osteocytes, bone resorption, modeling, and remodeling in response to mechanical loads are well documented for teleosts with acellular bone [105, 108, 112–114]. Unlike the bone of tetrapods and basal teleosts, bone resorbing cells (osteoclasts) in species with acellular bone are typically small, mononucleated cells. These osteoclastic cells can resorb bone without generating prominent resorption lacunae [104], as also reported by Parfitt [115] for human bone. For this reason, bone surfaces in anosteocytic teleosts, and thus also in flatfish, may look smooth and without signs of bone resorption. Without special labeling, osteoclasts of advanced teleosts are easily overlooked if investigators only search for typical multinucleated giant textbook osteoclasts [104, 112, 116].

The dominant factors that trigger bone resorption in teleosts with acellular bone are lifelong growth and continuous tooth replacement. Bone resorption related to these processes should not be mistaken for metabolism-triggered resorption [104, 117]. Recent studies on transgenic medaka (anosteocytic) with defective osteoclasts have shown dramatic consequences for skeletal development. Without functional osteoclasts, the lumen of neural and haemal arches fails to increase, resulting in constriction of the neural tube and interruption of blood vessels [118]. Other factors that trigger teleost bone resorption by mono- and multinucleated osteoclasts are inflammation, fracture repair, or extreme mineral-deficient conditions. Like in mammals, fish osteoclasts express tartrate-resistant acid phosphatase (TRAP), a vacuolar proton pump (V-ATPase), and cathepsin K [104, 112, 113, 116, 119]. Like mammalian osteoclasts, mononucleated osteoclasts in medaka are activated by the receptor activator of NF-kB ligand (Rankl) [119].

2.4 | Mineral Metabolism

The dietary mineral supply and mineral uptake from the water play an important role in the skeletal development of teleosts. Calcium and phosphorus are the main constituents of mineralized vertebrate skeletal tissues and are also referred to as main minerals. Other minerals that are important for developmental and metabolic processes in low quantities, such as zinc, selenium, and manganese, are summarized as trace or microminerals. In particular, early skeletal development depends on a precise supply of microminerals. Reviews and original articles on the functions and dietary requirements of microminerals have been published by Hamre, Yúfera, Rønnestad et al. [120], Prabhu, Schrama, and Kaushik [121], Terova, Rimoldi, Izquierdo et al. [122], Lall and Kaushik [123], Domínguez, Robaina, Zamorano et al. [124], Eryalçın, Domínguez, Roo et al. [125], Viegas, Salgado, Aguiar et al. [126], Viegas, Salgado, Aguiar et al. [127], and Tseng, Eryalçın, Sivagurunathan et al. [128]. Here, we will focus on the macrominerals that are deposited on the skeletal matrix.

2.4.1 | Calcium

Vertebrates mineralize their type I collagen-based bone matrix primarily with calcium (Ca) and phosphorus (P) as carbonated apatite [129, 130]. Consequently, bone mineralization depends on sufficient Ca and P intake. The primary source for these minerals in tetrapods is intestinal uptake. For tetrapods, Ca uptake from the diet is particularly important since vertebrates must regulate the plasma Ca level within a narrow range. In mammals, total plasma Ca levels range from 2.3 to 2.7 mmol/L [131, 132]. Marine and freshwater teleosts maintain total plasma Ca levels within similar limits: typically, 2.5 mmol/L to 3.0 mmol/L. Marine species appear to maintain slightly higher plasma Ca values than freshwater species do [133, 134], and the same occurs for seawater and freshwater stages in the euryhaline fish such as *S. salar* [135, 136].

In the control of plasma Ca levels, the bone of tetrapods functions as Ca storage and reservoir. Plasma Ca levels are maintained by osteocytes [100]. In the case of increased Ca demand, minerals are mobilized through bone resorption by osteoclasts. In teleosts, gonad maturation in female *S. salar* is a reported example of a process that triggers scale resorption by osteoclasts [123, 137].

Unlike mammals, teleosts depend little, or even not at all, on dietary calcium. This is because teleost fish can, in addition to intestinal uptake, obtain Ca from the water via the gills [104, 123, 138]. Likewise, in addition to renal excretion, Ca can also be released via the gills. This is true for marine and freshwater species. As Ca is exchanged across the sensitive gill epithelia, gill health should be an important factor for Ca metabolism. ATP-driven active Ca intake over the gill epithelium is effective even at low environmental Ca levels [139]. The activity of ATP-driven exchangers in the gills does not change with acclimation to different salinities; only the Na^+/Ca^{2+} exchanger activity in the intestine increases in freshwater species. Moreover, Ca^{2+} ATPase activity increases in the kidney [123, 140].

In the case of severe dietary deficiency, primary freshwater teleosts, such as salmonids and cyprinids, can obtain 100% of the required Ca from water. Marine teleosts are also capable of up-taking calcium via the gills, but not all marine teleosts are able to compensate for a complete lack of dietary Ca via gill intake. Seawater contains approximately 10 mmol/L Ca, whereas plasma contains 2–3 mmol/L Ca. The excess Ca in seawater liberated marine teleosts from the need to evolve the same level of gill Ca uptake capacity as in freshwater species. Some marine species can obtain only approximately 50% of the required Ca via their gills, as is the case of *P. olivaceus* [141]. This is also true for freshwater fish of marine descent (secondary freshwater fish) [142]. For example, cichlids cannot completely replace dietary calcium with gill intake. However, a large amount of Ca gill intake is possible for all teleosts; thus, dietary Ca levels are usually not critical for all famed fish species. However, high levels of indigestible calcium can also interfere with the intestinal absorption of phosphorus [143, 144].

2.4.2 | Phosphorus

Phosphorus is the second main skeletal mineral, and without it, the skeleton does not become mineralized [145–148]. Moreover, P is of utmost importance for the structure of soft tissues (e.g., cell membranes) and metabolic processes (e.g., ATP, phosphorylation). In contrast to Ca, P cannot be obtained from the water via the gills in sufficient amounts. All teleosts, especially fast-growing animals under farming conditions, depend on sufficient dietary P intake for healthy skeletal development [135, 149, 150]. As a minimum factor in ecosystems [150], it has been suggested that the dermal skeleton of ancient jawless vertebrates did not evolve as a dermal armor but rather as a storage for phosphorus [151–154]. The intestinal uptake of phosphate is vitamin D dependent, while renal excretion of P is stimulated by fibroblast growth factor 23 [136, 155, 156].

The primary source of vitamin D in the aquatic food chain is the vitamin D-rich plankton. Teleosts store vitamin D in the liver, and under natural conditions, they do not suffer from vitamin D deficiency. To ensure sufficient P uptake and healthy skeletal development, diets for farmed fish must be enriched with vitamin D [157, 158]. Farmed teleosts can also suffer from insufficient P intake if P-rich diets are low in available P. This can be the case if the main dietary P sources are indigestible tricalcium phosphate and apatite from fish meal ingredients [142, 159]. Additionally, the transition in aquaculture from marine-based ingredients rich in phosphorus, such as fish meal, to plant-based alternatives, which often store phosphorus in an unavailable form such as phytate, implies the need for alternative strategies, such as incorporating phytases into the diet, as well as a precise understanding of the species-specific phosphorus requirements [123, 160, 161].

2.5 | Skeletal Development

Three main processes related to bone formation have been described in teleosts, depending on the species and skeletal structures: intramembranous, perichondral, and endochondral ossification [162]. During intramembranous ossification, osteoblasts directly differentiate from mesenchymal osteoprogenitor cells [98], whereas perichondral and endochondral ossification involve a cartilaginous template [96, 162], and these two types of ossification processes can frequently be confusing to determine in fish. 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 [96].

In *S. senegalensis*, the skeletal elements that compose the axial skeleton are formed by both endochondral and intramembranous ossification. The vertebral centra, like in other teleosts, are formed first by the segmented mineralization of the notochord sheet (chordacentrum), followed by intramembranous bone formation around the notochord sheath (autocentrum) [49, 163, 164]. The neural and haemal arches and spines form first as cartilaginous structures that later undergo peri-/endochondral ossification [165]. At the same time, endochondral bone formation occurs in the hypurals, the epural, and the neural and haemal arches and spines of the last two caudal vertebrae, preural 1 and preural 2 [15]. The formation of caudal dermal fin rays occurs during inflexion of the urostyle [15]. Similarly, the neural and haemal arches and spines in *S. maximus* and *P. olivaceus* present a cartilaginous precursor as well as prehaemal neural arches in *H. hippoglossus* [20, 25, 56, 166].

The time course of the axial skeleton development has been described for several flatfish species, such as *S. senegalensis* [15, 167], *S. maximus* [56], *H. hippoglossus* [20, 42], *P. olivaceus* [25, 47], *P. flesus* [9], *P. stellatus* [10], and Sakhalin sole (*Limanda sakhalinensis*) [57]. Research has also been conducted on the development of different organ systems in some Soleidae species [168–170]. Briefly, the first vertebral elements to be observed are the abdominal neural arches, followed by the haemal arches and parapophysis (Figure 8a,b). Arch development starts from two latero-dorsal buds, which elongate and join to form the arch and, later, the spine [15]. The cephalic, abdominal, and caudal chordacentra start to mineralize [167], showing the notochord surrounded by calcified tissue, starting from the base of the arches (Figure 8c) [15]. In parallel, the caudal fin endoskeleton elements (hypurals 1–4 and parhypural) begin endochondral ossification (Figure 8a) [15, 56, 167]. The process continues caudally, and most caudal chordacentra start to form, along with their respective neural and haemal arches. The modified neural and haemal arches and spines of the preurals, the epural, and the fifth hypural start to form their cartilage anlage (Figure 8c) [167]. Finally, the preural vertebrae become ossified (Figure 8d).

Later, in juvenile *S. senegalensis* specimens, hypurals 1 to 4 branch distally and fuse with the urostyle (Figure 6c) [15]. Specific variations in this pattern in other flatfish species consist essentially of the timing and direction of ossification, for example, in *H. hippoglossus*, the chordacentrum starts to mineralize in the posterior haemal region and continues anteriorly and caudally [20]. Moreover, fish size/age in each of the ossification stages varies among flatfish species. Furthermore, developmental and ossification stages can be influenced by rearing conditions [79, 171] and are usually better correlated with fish size than with fish age [172, 173].

2.6 | Metamorphosis

The unique features of flatfish appear during metamorphosis, at the end of the larval period [174]. During this endocrine-regulated process (reviewed in Power, Einarsdóttir, Pittman et al. [175], Geffen, van der Veer and Nash [174], and Campinho [176]), symmetric pelagic larvae gradually become asymmetric and undergo a series of changes. These include eye migration (Figure 9) with the torsion of internal organs and pigmentation of the ocular side of the fish. Furthermore, the distal notochord bends upwards, and specimens acquire a benthic lifestyle [15, 174, 175], settling into the bottom. These morphological changes occur in parallel with behavioral and physiological adaptations [177], which prepare the developing fish for the forthcoming nutritional, metabolic, locomotive, and environmental demands [175].

The transition from pelagic to benthic behavior supposes a stressful condition that might lead flatfish to be more prone to abnormal skeletogenesis [167]. Numerous staging systems have been published to categorize the development of flatfish larvae [14, 174, 177–179]. The size at metamorphosis, duration of metamorphosis, order of ontogenetic events, and synchrony of metamorphosis with settlement vary among species [174]. In *S. senegalensis*, Fernández-Díaz, Yúfera, Cañavate et al. [177] established five substages for metamorphosis, according to the position of the left eye, and Seikai, Tanangonan, and Tanaka [180] proposed a classification of the developmental stages from larval up to juvenile stage in *P. olivaceus* that encompass metamorphosis. These stages were reported to be influenced by nutritional and environmental conditions, such as temperature [8, 174, 177, 180].

As reported for different flatfishes, the oral and oro/pharynx skeletal elements involved in breathing and feeding processes begin to form before metamorphosis, whereas axial skeleton mineralization begins at the onset of metamorphosis and is completed at its end [20, 56, 167, 179, 181]. The development and ossification of the vertebral bodies are especially notable during metamorphosis [176]. Gavaia, Simes, Ortiz-Delgado et al. [182] reported a dramatic increase in whole-body bone osteocalcin (bone Gla protein, *bglap*) and matrix Gla protein (*mgp*) gene expression at the beginning of metamorphosis, which can be related to the formation and mineralization of the developing skeleton. In *S. senegalensis*, some events still occur after eye migration, such as the completion of cranial ossification [167] and the completion of digestive system development [183].

Simultaneous with the morphological changes associated with metamorphosis, complex hormonal regulation and molecular signaling processes occur [184]. Thyroid hormones (THs) are necessary factors that orchestrate flatfish eye migration.

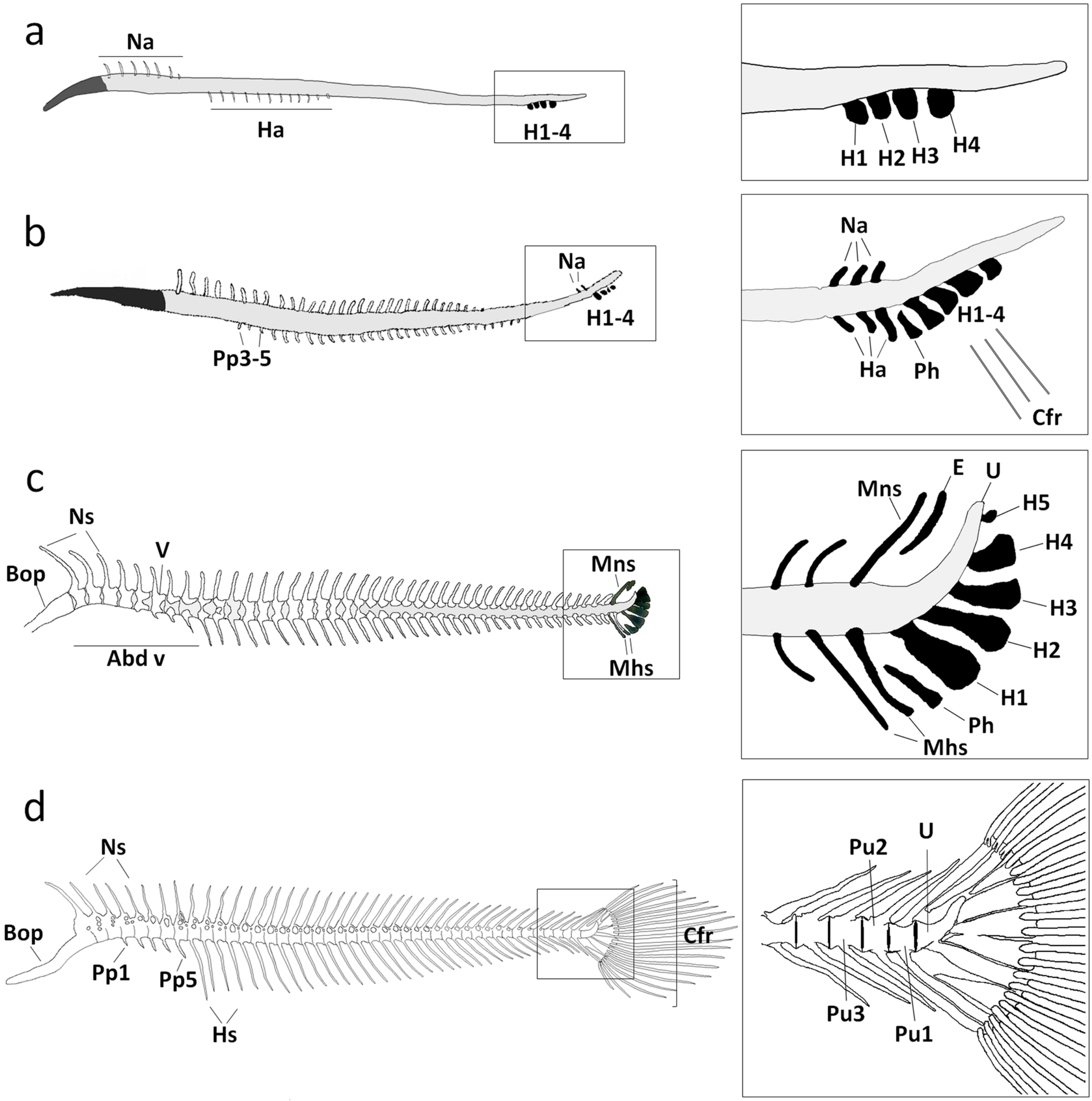

FIGURE 8 | Schematic representation of the vertebral column development in Senegalese sole (*Solea senegalensis*) (modified from Gavaia, Dinis, and Cancela [15]). Insets represent the caudal complex region. Calcified structures are presented in white, and cartilaginous structures in black. Notochord is shown in gray. (a) 15 days after hatching (dah) specimen [4.55 mm standard length (SL)]. (b) 18 dah specimen (4.7 mm SL). (c) 18 dah specimen (5.65 mm SL). (d) 45 dah specimen (8.3 mm SL). Inset corresponds to 34 dah specimen (7.8 mm SL). Abd v, abdominal vertebrae; Bop, basioccipital articulatory process; Cfr, caudal fin rays; E, epural; H1–H5, hypurals 1-5; Ha, haemal arch; Hs, haemal spine; Mhs, modified haemal spine; Mns, modified neural spine; Na, neural arch; Ns, neural spine; Ph, parhypural; Pp, parapophysis; Pu1, preural 1; Pu2, preural 2; U, urostyle; V, Vertebral centra.

Subocular asymmetric ossification and subdermal proliferation contribute to the movement of the eye [176, 185, 186]. At the molecular level, Campinho, Silva, Martins et al. [185] reported a left–right (blind-ocular) asymmetric expression of the TH signaling genes deiodinase 2 (*dio2*) and thyroid hormone receptor alpha b (*thrb*) in the head of *S. senegalensis*, determining a TH-responsive asymmetric center located just ventral to the migrating eye. This asymmetric expression was correlated in time and space with the asymmetric ossification of the ethmoid and pseudomesial bones, which ultimately could lead to eye migration [185]. In *P. olivaceus*, it was shown that the control and progression of metamorphosis are dependent on strict regulation of TH and on the coordinated expression of deiodinases and thyroid hormone receptors [187]. It has been demonstrated that thyroxine (T4) is a critical regulator of metamorphosis in *S. senegalensis*, with larvae showing a peak in T4 content and

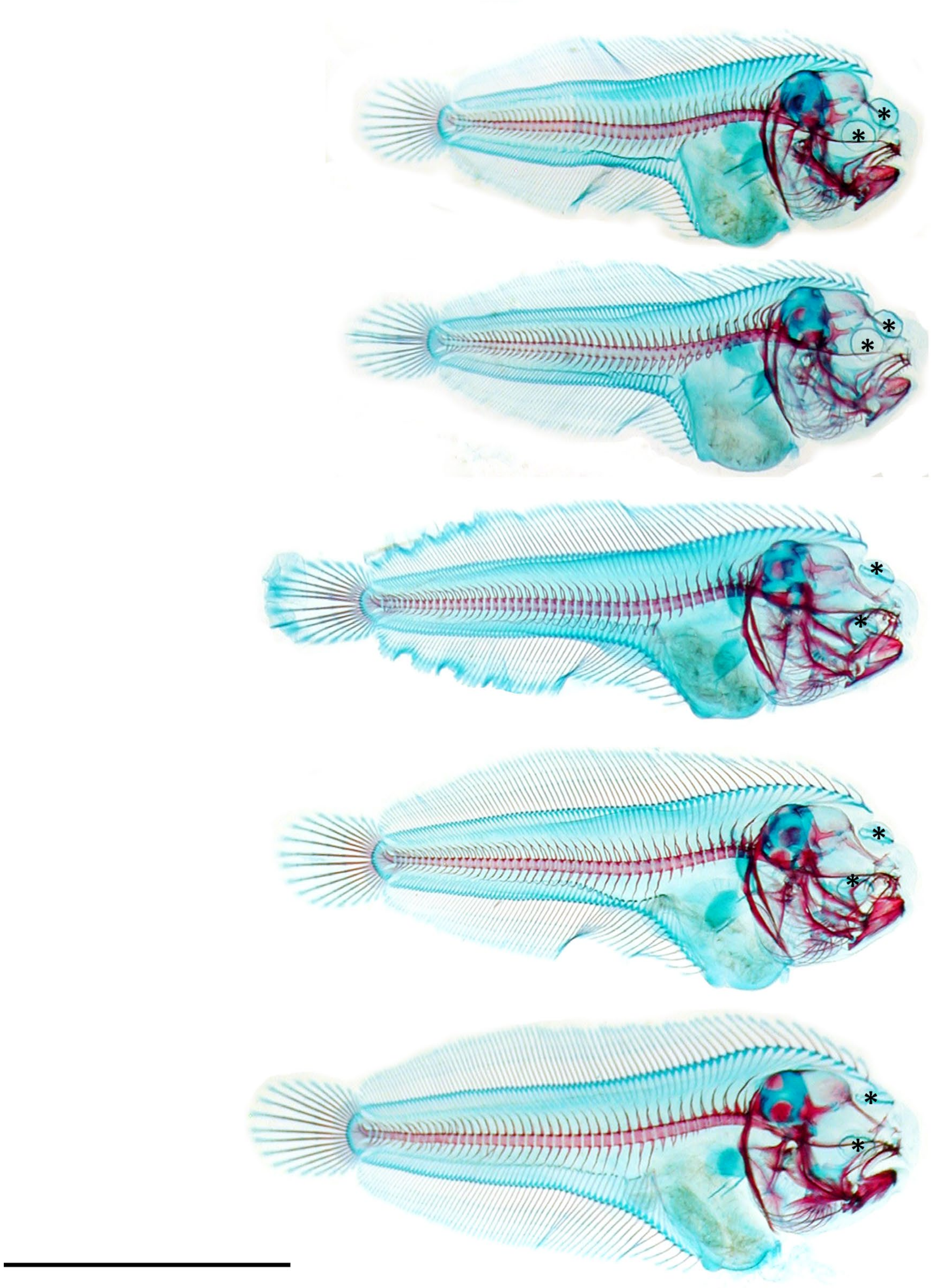

FIGURE 9 | Eye migration during metamorphosis in Senegalese sole (*Solea senegalensis*) from top to bottom (alcian blue-alizarin red staining). Top images show the left eye on the left side of the fish. On the bottom image, the left eye is on the right side of the specimen; note the increasing mineralization of the preural chordacentra. Asterisks denote the eyes of the fish. Bar = 0.5 cm.

thyroglobulin overexpression during metamorphosis [184]. This was further confirmed by treatments with the goitrogen thiourea (TU), which induced a marked decrease in T4 levels, causing a delay in metamorphosis and a marked reduction in the metamorphic index, with no larvae reaching stage S4 at 30 dph [184]. However, no regulatory function was established between the hypothalamic–pituitary–thyroid axis regulator thyrotropin-releasing hormone and metamorphosis, with no alterations in gene expression levels during metamorphosis caused by treatments with T4 or TU [188].

Control of gene expression during metamorphosis mediated by microRNAs (miRNAs) has been reported in *P. olivaceus*, where 66 differentially expressed miRNAs were found between two different metamorphic stages at 17 and 29 dph [189]. The identified miRNAs regulate multiple metabolic and developmental processes, such as myoblast proliferation and muscle development and skin and pigmentation development, that are critical for a successful metamorphosis. Furthermore, it has been suggested that miRNA-92 regulates early development and metamorphosis by regulating *gata5* and *sox17* expression [190]. The importance of miRNAs in the regulation of metamorphosis was further confirmed by [191], which suggested that the miRNA processing gene *drosha* has a regulatory role in development and metamorphosis since this gene responds to thyroid hormone levels and is upregulated during *P. olivaceus* metamorphosis.

Retinoic acid may play an important role in modulating eye migration in flatfish metamorphosis via crosstalk with TH [192]. Fernández, Granadeiro, Darias et al. [167] reported a wave-like expression pattern of genes related to vitamin A and TH signaling pathways (retinoic acid receptor α (*rarα*), retinoid X receptor α (*rxrα*), retinol binding protein (*rbp*), thyroid hormone receptor α A (*trαa*), thyroid hormone receptor α B (*trαb*), thyroid hormone receptor β (trβ), and thyroglobulin (*tg*)) in whole-body samples of metamorphosing *S. senegalensis*.

Conversely, in *H. hippoglossus*, most of the differentially expressed TH-responsive genes were downregulated during metamorphosis [193]. However, the large majority of the differentially expressed transcripts presumably represented transcripts underlying changes that accompany metamorphosis [193]. In this sense, Shao, Bao, Xie et al. [192] proposed the role of light-sensing opsins in generating retinoic acid gradients in the skin, which may be crucial for the establishment of asymmetric pigmentation during metamorphosis. Some studies have also suggested that TH is involved in developmental events in the skin, axial muscle, and gastrointestinal tract that accompany metamorphosis (reviewed in Campinho [176] and Power, Einarsdóttir, Pittman et al. [175]). Flatfish metamorphosis remains an understudied and complex event.

3 | Skeletal Anomalies in Flatfish

3.1 | Definition, Classification, and Impact

In teleosts, skeletal elements can show a certain degree of variation in number, shape, and architecture within the population average [194]. However, establishing the limits for deviation of "normal variation" is not simple [194], and as mentioned in Boglione, Gisbert, Gavaia et al. [16], different terminology is often used in the literature. In this sense, Martini, Sahd, Rücklin et al. [194] and Boglione, Gisbert, Gavaia et al. [16] have described a comprehensive summary of the most common nomenclature used for skeletal variation. A definition of terms is provided in Table 1, adapted from Martini, Sahd, Rücklin et al. [194], and Boglione, Gisbert, Gavaia et al. [16]. In many cases, the lack of knowledge about the onset and etiology of skeletal deformities in fish prevents the use of more specific vocabulary [16], and authors usually address them using a generic term such as malformations. However, in these cases, terms such as anomalies and deformities are used hereafter, when possible.

TABLE 1 | Definition of terms related to skeletal health, adapted from Boglione et al. [16] and Martini et al. [194].

Term	Definition	References
Anomaly abnormality	An anatomical phenotype whose frequency is substantially lower than the population average. Anomalies can be major, with significant consequences for health or appearance, or minor, with minimal or devoid of health consequences but may have a modest impact on appearance	[195, 196]
Deformation	Alteration of shape or structure of a body part from a previously normal structure, due to extrinsic or intrinsic aberrant mechanical force(s)	[195, 196]
Fusion	Abnormal coalescence of two adjacent parts	[197]
Kyphosis	Abnormal curvature with a dorsal prominence of the vertebral column	[197]
Lordosis	Vertebral column curvature with ventral convexity	[197]
Malformation deformity	Morphological anomaly of shape or structure of a body part due to an alteration of the primary developmental process	[195]
Pathological condition	Disease of an organ, or a system resulting from various causes, such as infection, genetic defect, or environmental stress	[196]
Scoliosis	Lateral deviation of the vertebral column	[197]
Variation	The observed differences or dissimilarities between individual units. Variation can be the result of environmental conditions (e.g., Wolff's law), or it may seem to appear randomly In this work, the use of the term 'variation' is intended irrespective of the presence or absence of a pathological condition. The literature parallels natural and normal variation. Here, the term normal variation is used for variants that occur regularly, under natural conditions, or in ancestral species	[97, 198]

Classifications for skeletal deformities have been suggested for some teleost species. For *S. salar*, Witten, Gil-Martens, Huysseune et al. [199] proposed an X-ray-based classification system for vertebral body anomalies. In the literature, many of the reported categories of deformities include the type of deformity, the skeletal structures affected, and the location [13, 200]. For paired elements, such as neural and haemal vertebral arches, left–right anomalies, including the absence of arches, have also been recorded [194, 201, 202]. Such detailed evaluations, such as those published by Martini, Sahd, Rücklin et al. [194], Martini, Huysseune, Witten et al. [201], and by Ferreri, Nicolais, Boglione et al. [202], can help in understanding the factors that lead to specific types of anomalies. Nevertheless, adapting the deformity categories developed for salmon and zebrafish to flatfish anatomical characters remains a challenge.

On the other hand, the development of a quality scale is required at the industrial level, since not all the deformities present downgrade the quality of the reared fish [203]. Considering the profile of some types of anomalies at later rearing stages [12], *S. senegalensis* farms should focus mainly on preventing the anomalies affecting the vertebral bodies (VBA) and column curvatures (VCD). In this sense, the scoring method provided in Losada, de Azevedo, Barreiro et al. [204] represents a tool applicable in aquaculture and research settings [126].

In flatfish, the frequency of anomalies in the postcranial axial skeleton is briefly summarized for the main cultured species in Table 2. However, the values should be interpreted with care because different studies used different methods and recorded deformities with different levels of accuracy. Frequent types of anomalies affecting *S. senegalensis* are depicted in Figure 10. Notably, according to the literature, it is common that more than 50% of the larvae and juveniles of different flatfish can be affected by skeletal deformities [10, 12–14, 20, 22, 25, 42, 79, 204]. The frequency of deformities in farmed *S. senegalensis* can be particularly high (Table 2). Deformities of the vertebral bodies or vertebral column curvatures were reported to affect 50% of postlarvae and juveniles [12, 13]. In some hatcheries, approximately 40% of juveniles might present vertebral deformities at the grading point before transfer to ongrowing farms [209]. In contrast, vertebral column disorders also occur in wild specimens but are less frequent [22, 24–26, 210–212].

TABLE 2 | Anomaly frequency in the post-cranial skeleton for the main farmed flatfish species (under conventional rearing conditions).

Species	Anomaly frequency	Developmental stage	References
Japanese flounder (*Paralichthys olivaceus*)	13%	Juvenile	[21, 25, 206]
	63%–74% (vertebral fusion and shortening)	Juvenile	
	33.3%	Juvenile	
By CAFS. Fishbase (2024) [205]			
Atlantic halibut (*Hippoglossus hippoglossus*)	83%–92%	Larvae and juvenile	[20, 42]
	41%–89%	Juvenile	
By Hendron, C. Fishbase (2024) [205]			
Senegalese sole (*Solea senegalensis*)	44%	0–75 dah	[12, 13, 15, 22, 204]
	100%	31–31 dah	
	79%	40 dah	
	75%	297–342 dah	
	78%	255–352 dah	
Turbot (*Scophthalmus maximus*)	2.3% (spinal)	0–45 dah	[14, 56, 207, 208]
	51%	0–60 dah	
	87%	0–60 dah	
	30%	6–12 mah	

Note: dah, days after hatching; mah, months after hatching. Modified from Boglino et al. [17].

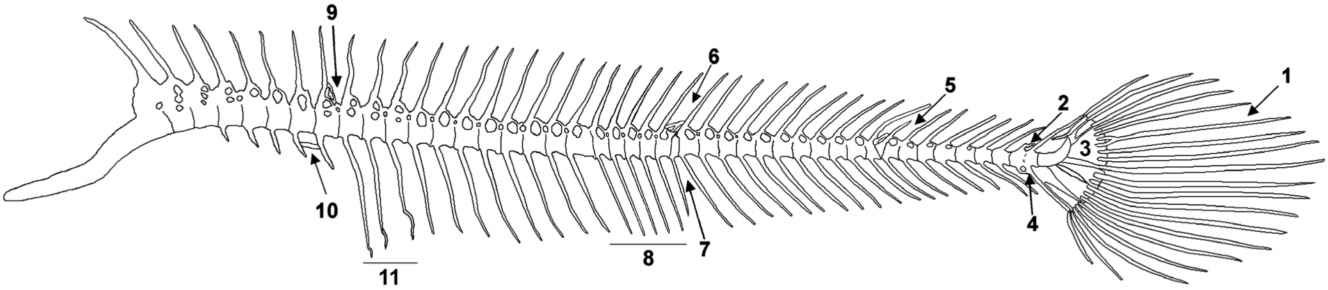

FIGURE 10 | Schematic representation of the axial skeleton of a postlarva, presenting the major types of deformities according to Gavaia, Dinis, and Cancela [15] and to de Azevedo, Losada, Ferreiro et al. [13]. (1) Fusion of the proximal portion of the caudal fin rays. (2) Fusion of preurals 1 and 2 with fusion of their neural arches. (3) Fusion of hypurals 3–4. (4) Fusion of the haemal arches of preurals 1–2, resulting in only one spine, which is associated with fusion among the preural vertebrae. (5) Anomaly in the caudal vertebra associated with partial fusion with adjacent vertebrae, resulting in the absence of the corresponding haemal arch and spine. The corresponding neural arch is deformed and fused with the arch of the following vertebra, exhibiting an atrophied neural spine. (6) Fusion of the neural arches of two adjacent caudal vertebrae. (7) Abnormal caudal vertebra with an atrophied haemal spine. (8) Compression in caudal vertebrae and corresponding processes. (9) Deformity in the neural arch of the last abdominal vertebra. (10) Abnormal presence of ectopic bony elements connecting parapophysis. (11) Twisted tip of haemal spines.

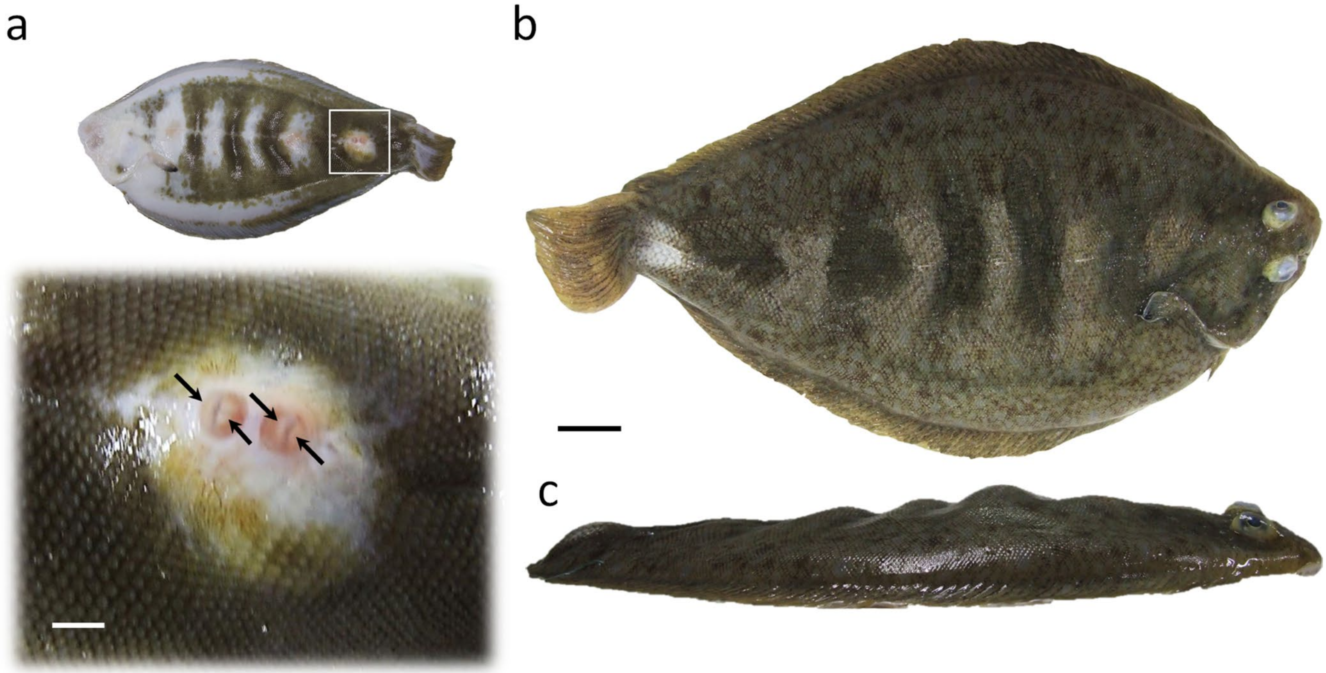

FIGURE 11 | Photographs of a juvenile Senegalese sole (*Solea senegalensis*) (741 days after hatching) displaying severe skeletal abnormalities, especially scoliosis. (a) Detailed view of the blind side of the fish (white square). Note the pigmentation and two deep skin ulcers present in a convex area of the curvature, probably caused by friction against the surface of the tank. These lesions exposed the vertebral bodies (black arrows) and can be a direct entry route for pathogens. (b) Ocular and ventral (c) views of the fish. Note the bulge in the surface of the body. Bar = 2 cm; Insert bar = 0.5 cm.

Possibly, selection eliminates affected individuals from natural populations, whereas aquaculture rearing conditions reduce natural selection pressure and might promote the development of skeletal anomalies [22, 25].

Skeletal deformities impact the production of many fish species. Skeletal deformities affect animal welfare, growth, food intake, swimming performance, and susceptibility to diseases and, eventually, product quality (Figure 11a) [162, 213, 214]. Some severe vertebral column lesions significantly alter the external appearance of the fish [215] (Figure 11b,c). Moreover, severely deformed fish may be harder to process and unable to be machine filleted [216]. Removing deformed specimens from production increases labor costs and reduces the revenue of fish farms [203]. However, the detection of deformities based on external criteria may underestimate the number of vertebral deformities [12, 204, 216, 217]. In the short term, undetected alterations can further develop and aggravate during growth [218, 219] and eventually become externally apparent [41].

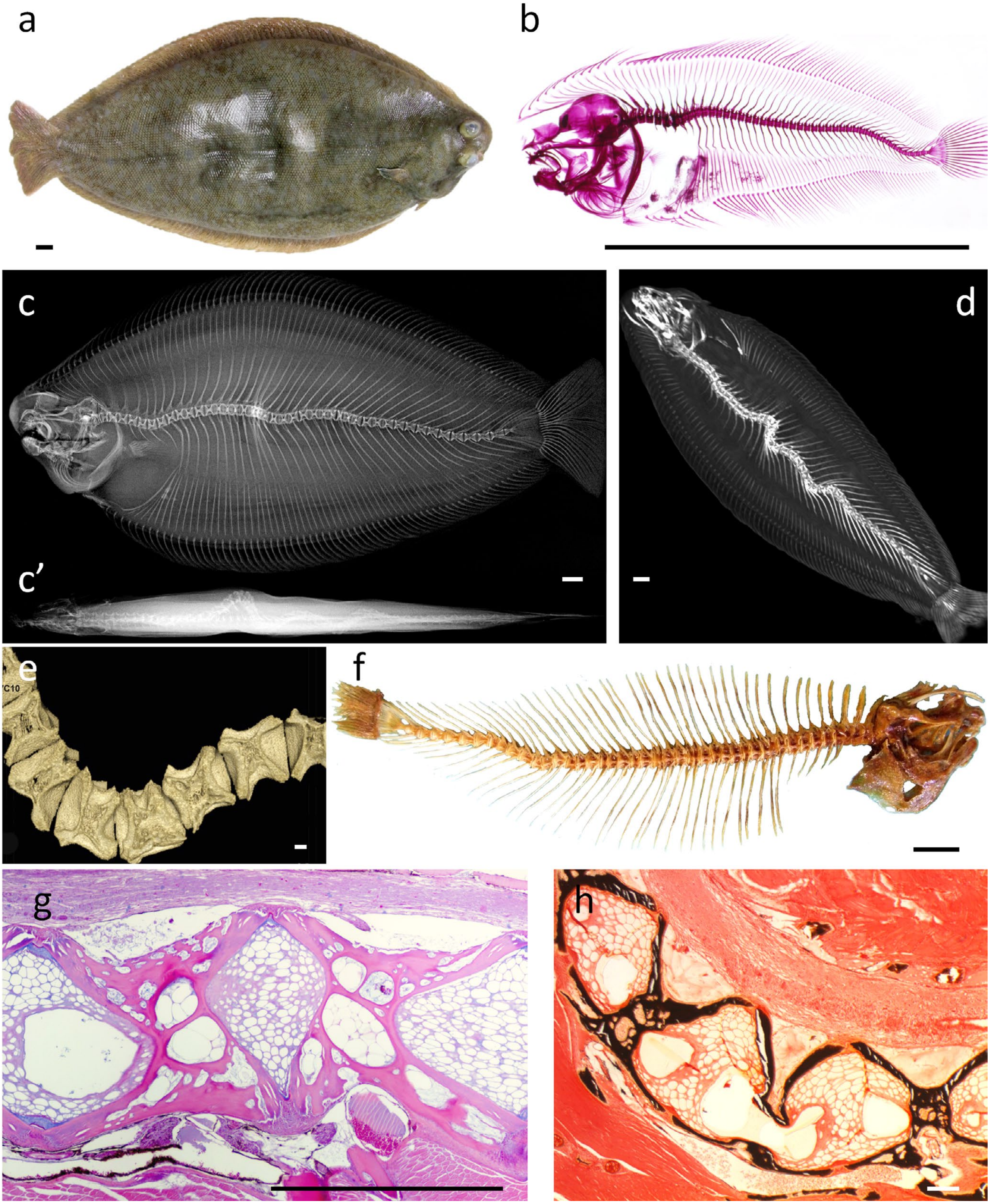

FIGURE 12 | Legend on next page.

FIGURE 12 | Different diagnostic methods for the assessment of skeletal anomalies in Senegalese sole (*Solea senegalensis*). (a) Macroscopic visualization. (b) Alizarin red staining technique for bone. (c) and (c') Computed radiography: Latero-lateral (c) and dorso-ventral projection (c') of the same specimen as in (a). (d) Oblique image of computed tomography of the same specimen as in (a), (c), and (c′). (e) Dorso-ventral micro-CT virtual section of a scoliotic vertebral segment in a commercial size fish (Bar = 1 mm). (f) Dissected axial skeleton of a juvenile Senegalese sole after maceration in sodium hypochlorite solution. (g) Histological parasagittal section of a deformed segment of the vertebral column from 105 days after hatching (dah) Senegalese sole (alcian blue-H&E staining; Bar = 1 mm). (h) Histological parasagittal section of a lordotic segment of the vertebral column in a 105 dah Senegalese sole (von Kossa's staining). Bar = 50 μm.

3.2 | Monitoring and Diagnostic Techniques

Even when best farming practices for the normal development of fish species have been standardized, fish quality must be routinely monitored to guarantee the consistency of rearing conditions. In addition, constant monitoring of animal health can also help to obtain early warnings for accidental alterations in complex hatchery processes [203]. Nowadays, the traditional procedures used to control and monitor vertebral deformities during the production cycle of *S. senegalensis* at farms still rely on palpation and external inspection of the specimens. This straightforward procedure is restricted to juveniles and excludes earlier developmental stages (Figure 12a) [220], and has been used for the detection of externally visible deformities in other flatfish species [212]. However, it has been shown that in aquaculture-produced *S. senegalensis*, this conventional technique fails to detect 72%–75% of individuals with skeletal deformities [12, 204]. Nearly 46% of the "normal" specimens displayed vertebral body anomalies (VBA) and/or vertebral column deviations (VCD) [12], which in turn may be aggravated with time [218, 219]. Therefore, the high percentage of undetected anomalies by external observation likely explains the high incidence of skeletal deformities radiographically registered at later stages [12, 204]. Hence, the application of more sensitive methods to detect deformities of the vertebral column in farmed flatfish lots could result in increased animal welfare and fish performance.

For many flatfish larvae and small juveniles, skeletal deformities have been described using whole-mount double-staining techniques for cartilage and bone [14, 15, 20, 22, 25, 56]. The procedures of whole-mount clearing and staining show cartilaginous tissues in blue (based on the high affinity of alcian blue for acid mucopolysaccharides) and mineralized bone in red (based on the affinity of alizarin red for calcium) [221, 222]. These staining techniques display the skeleton of early developmental stages in high detail and thus enable the detection of skeletal anomalies as well as the evaluation of the mineralization level in fish larvae and small juveniles (Figure 12b) [15, 222, 223]. Nevertheless, in larger juveniles and adults, the dimension of the specimens limits the use of these techniques [224], which become time-consuming and laborious, as more time is needed for processing and stronger reagents are required to promote clearing [225].

Radiographic procedures are also suitable for the integral study of the skeletal system and are the preferred technique for whole-body diagnosis of vertebral deformities in teleosts [199], especially in medium-sized and large fish (Figure 12c,c′) [206, 207, 211, 226, 227]. However, radiography represents a two-dimensional image of a three-dimensional structure; thus, the overlapping of certain bilateral skeletal elements can hinder the detection of some deformities in paired structures, for example, incomplete arches or the lack of one parapophysis in a vertebra.

Although rarely applied in other teleost species, the use of two orthogonal radiographic projections offers a three-dimensional perspective of the skeleton since dorso-ventral projection greatly complements the latero-lateral view and allows the assessment of any vertebral misalignment in the blind/ocular plane of flatfishes such as *S. senegalensis* [12, 84, 204]. For example, the one-sided occurrence of hyperdense vertebrae reported in *S. salar* also requires a dorso-ventral assessment [228]. In addition, this non-invasive method can be utilized to estimate the relative bone mineral content [126] and to diagnose other disorders in vivo, namely, those involving the skeletal and urogenital systems, swim bladder, and otoliths [229, 230].

The main advantages and limitations of radiographic techniques have been reviewed by Bruneel and Witten [231]. At the commercial level, samples of juvenile fishes, such as *Sparus aurata* and other sparids, are examined for skeletal malformations with X-rays before the animals are transferred to fish farms [232]. Implementing regular X-ray monitoring of skeletal health on flatfish farms can be challenging and costly, due to the need for trained personnel, compliance with legal requirements for X-ray safety, and the procurement of radiographic equipment. Future research on the development of automated processes for mass detection and removal of the deformed fish from farmed stocks is important for reducing production costs [203].

The bone elements of teleosts can be very small and may thus require special radiographic techniques, such as 2-D X-rays or 3-D microcomputed tomography (microCT), as reviewed by Bruneel and Witten [231]. The use of advanced imaging techniques, like conventional computed tomography scans, as well as microCT (Figure 12d,e) and Synchrotron X-ray tomographic microscopy, can provide detailed information on vertebral structure and deformities in large and small-size samples, respectively [148, 227, 233–236]. However, with the exception of Synchrotron X-ray tomographic microscopy, none of the above-listed new radiographic 3-D techniques reach the high resolution of conventional whole-mount double-staining and clearing [231].

A traditional, but still very useful method, is the direct dissection of fish bones using trypsin digestion, heat, and maceration in alkaline solutions or even the use of dermestid beetles (*Dermestes maculatus*) (Figure 12f) [21, 237–239]. These techniques allow

TABLE 3 | Known biotic and dietary factors affecting skeletal anomalies in flatfish species.

Species	Agent	Main anomalies	References
Japanese flounder (*Paralichthys olivaceus*)  By CAFS. Fishbase (2024) [205]	Hypervitaminosis D_3 Hypervitaminosis A	Deviations in abdominal region Fusion of caudal complex elements Vertebral fusion Hypertrophy of centra	[257–260]
Atlantic halibut (*Hippoglossus hippoglossus*)  By Hendron, C. Fishbase (2024) [205]	Phosphorus deficiency Ascorbic acid deficiency Hypervitaminosis A Oxidized oil	Deformed neural and haemal elements Scoliosis and bifurcated spines Scoliosis Scoliosis	[261]
Senegalese sole  (*Solea senegalensis*)	High incubation temperature Hypervitaminosis A Vitamin C deficiency Vitamin K deficiency Zn deficiency Hypercapnia Altered thermo-cycle, light intensity, and/or photoperiod	Skeletal anomalies Skeletal anomalies Vertebral compression, fusions, and deformed neural spines Vertebral fusions, deformed neural spines Caudal complex anomalies Severe deformities Jaw deformities	[79, 126, 165, 262–266]
European plaice (*Pleuronectes platessa*)  By Østergaard, T. Fishbase (2024) [205]	High rearing temperature	Severe deformities	[267]

3-D evaluation of skeletal gross morphology and deformities, as an alternative to staining–clearing techniques and radiologic imaging. Furthermore, for early developmental stages, non-toxic in vivo diagnostic techniques such as alizarin red S or calcein staining permit continuous monitoring of skeletal development, calculation of mineral apposition rate in vertebrae [126, 127], and tracking of deformities in live animals [222].

Other methodologies are available to assess skeletal tissues at a deeper level, although they are more commonly used in laboratory conditions. Biochemical analysis and histological, histochemical, and immunohistochemical approaches are valuable tools for providing basic knowledge on tissue organization and mineral content (Figure 12g,h) [84, 90, 240–243].

Assessments of vertebral biomechanical properties and bone volume/area profiles have been performed using texture/tension analysis and nanoindentation, as well as a combination of imaging techniques with histomorphometry and/or computer modeling [148, 241, 244–248]. At the ultrastructural level, transmission electron microscopy (TEM) provides a detailed visualization of cellular and matrix structures in bone and surrounding tissues [110, 119, 236], also allowing the measurement of the collagen fibril diameter [148, 247, 249]. Confocal Raman microspectroscopy, combined with fluorescence imaging techniques, is also used to investigate skeletal mineralization in living fish, such as *D. rerio* [129, 250]. The above-mentioned techniques were applied in model and farmed fishes and can be adapted to flatfish to strengthen the study and characterization of bone deformities.

3.3 | Etiology

Several studies have been conducted on the causative factors of skeletal deformities in teleosts (reviewed in Boglione, Gavaia, Koumoundouros et al. [162], Boglione, Gisbert, Gavaia et al. [16]). In fact, several factors were associated with this problem [16], including environmental [251, 252], genetic [253, 254], and nutritional factors [71, 138, 255], and also pathogens such as parasites and bacteria [224, 256]. Some factors, such as pathogens and pollutants, are strictly controlled in industrial settings [16]. Table 3 briefly summarizes the known factors affecting skeletal anomalies in flatfish.

3.3.1 | Environmental Factors

The importance of the environment is evident since fish from intensive rearing conditions are characterized by high incidences of skeletal deformities [201]. Results obtained in *S. senegalensis* suggest that the moment of contact of the embryo with the surrounding water at hatching is a crucial moment for DNA methyltransferases regulation and the modulation of larval plasticity [268], which allows the animals to adapt to rearing conditions. The temperature during egg incubation, as well as during the larval rearing and on-growth stages, strongly influences the development of skeletal deformities in different species, including flatfish [252, 262, 267, 269, 270]. The frequency of skeletal deformities in *S. senegalensis* larvae is increased due to the combined effects of high temperature and hypercapnia [263].

Artificial light conditions, including photo-cycle, light intensities, and spectra, also have an effect on the welfare and development of fish [264, 271, 272]. For *S. senegalensis*, the optimal light conditions are those close to those of its natural environment, namely, a natural light-cycle with a blue wavelength spectrum (435–500 nm) [264]. Fifteen percent of the larvae exposed to high doses of ultraviolet radiation displayed curvatures of the notochord (prior to vertebral centrum formation) [273]. In other fishes, increased water velocity has been associated with increased muscular activity in the caudal fin, leading to lordosis, probably due to the pathological increase in mechanical load in the surrounding muscles and bones [274–276]. Other features related to the adaptive response of the teleost skeleton to mechanical loads include the deposition of more bone, greater bone mineralization and stiffness, and the development of chondroid and cartilaginous tissue in the affected areas [85, 104, 228, 241, 276]. Nevertheless, there is scarce information on the effects of increasing the mechanical load in flatfish, which swim with relatively slow undulatory vertical movements (side to side of the fish) rather than horizontal movements [77].

3.3.2 | Genetic and Nutritional Factors

Some vertebral deformities can be influenced by a genetic component [253, 277, 278] and show a certain heritability or a family association [279–281]. In addition, the generation of polyploid animals to prevent maturation and increase growth resulted in a higher incidence of skeletal deformities in some reared fishes [254, 282, 283]. However, it remains unclear whether these anomalies are caused by triploidy itself, the pressure or thermal shock applied to the eggs, or the extremely fast growth of the animals [16, 207]. Still, cold shock-induced triploid *S. maximus* presented no significant variation in the percentage of deformities compared with the diploid group at different stages of development [207, 208].

Nutrition plays an important role during the early development of teleost fish [255]. Dietary imbalances can affect skeletogenesis and cause deformities [138, 284]. In juvenile *H. hippoglossus*, different diets containing high levels of VA, oxidized oils, low vitamin C, or low P produced specific patterns of skeletal deformities [261, 285].

Several vitamins are important for skeletal tissue formation, particularly vitamins A, C, D, K, and E [224]. In *S. senegalensis* and *P. olivaceus* postlarvae and in juvenile *H. hippoglossus*, high amounts of dietary VA (offered as different vitamin A compounds) can lead to skeletal deformities, including vertebral compression, vertebral fusion, scoliosis, and vertebral hypertrophy [79, 80, 257, 258, 261, 286].

Early hypervitaminosis A increases the number of vertebral column deformities [287]. An excess of VA in the diet accelerated the intramembranous ossification process of vertebral centra [71, 79], although skeletal elements with chondral ossification may present a greater sensitivity to this nutrient imbalance than intramembranous bones [165]. The increased skeletal mineralization may be related to developmental stage and dependent on the levels of vitamin A in the diet [167, 287]. In addition, studies suggest that the optimal levels of the VA vitamer, retinol, are different for different skeletal structures [288] and for different larval stages [80, 287, 289]. The disruption of the VA and thyroid hormone crosstalk signaling pathways at specific developmental stages was correlated with skeletal deformities in the caudal fin vertebrae of *S. senegalensis* [167]. *P. olivaceus* larvae also displayed a loss of dorsal fin proximal radials when exposed to retinoic acid [166].

In general, fish are unable to synthesize vitamin C or vitamin D_3, and therefore, they obtain these nutrients from the diet [158, 290]. Both vitamins are vital for bone formation [158]. Vitamin C is important for the synthesis of collagen type I, the main constituent of the bone matrix. Vitamin D_3 influences Ca absorption in the gut and is of great importance for bone mineralization [158]. European seabass (*Dicentrarchus labrax*) has a narrow range between requirements and tolerance for dietary vitamin C, since both deficiency and dietary excess of vitamin C can cause skeletal deformities [291]. Additionally, *S. senegalensis* and *H. hippoglossus* presented a greater frequency of vertebral malformations when fed a diet not supplemented with vitamin C [261, 265].

Vitamin D_3 deficiency has an impact on the functional maturation of the digestive system, with consequences for mineral absorption and thus bone mineralization, leading to lower vertebral mineralization and the appearance of skeletal deformities [292, 293]. Furthermore, excess supplementation of the diet with vitamin D_3 (cholecalciferol) or $1{,}25(OH)_2D_3$ (calcitriol) resulted in an increase in vertebral column curvatures in the abdominal region of *P. olivaceus* [259], as well as cranial deformities in other fish species [293].

Vitamin K is required for the synthesis and function of matrix-Gla protein (Mgp) and osteocalcin (bone Gla protein, Bglap). Mgp is a potent inhibitor of connective tissue mineralization, and a deficiency in this protein causes ectopic calcification [294, 295]. The use of vitamin K supplemented diets significantly decreased the incidence of certain skeletal deformities in *S. senegalensis* larvae, such as vertebral centra fusions and anomalies of haemal and neural arches and spines in the caudal region [266]. In response to dietary vitamin K supplementation, the expression patterns of genes encoding the vitamin K epoxide reductase complex subunits involved in the vitamin K cycle and vitamin K-dependent proteins that regulate mineral deposition were modulated [266, 296]. However, high levels of vitamin K3 increased the incidence of abdominal kyphosis and reduced bone mineralization in larvae of other species [297].

Regarding minerals, Ca and P are closely related to skeletal development and maintenance. Teleosts can obtain calcium from different sources, such as the diet, via intestinal uptake, and from the water through gill intake [104, 123, 138]; thus, calcium deficiency is uncommon. Nonetheless, it appears that some flatfish species, such as *P. olivaceus*, may require dietary Ca supplementation [142, 298, 299].

Only a minimal amount of P can be obtained through the gills, and therefore, it remains a crucial component of the diet [135, 145, 149, 150, 199, 300]. Accordingly, skeletal development in the fast-growing farmed fish requires a sufficient dietary P supply [145, 301–303].

The literature relates many types of skeletal deformities to an insufficient dietary phosphorus supply. Among those are vertebral body compression, vertebral body fusion, undersized vertebral bodies, hyper radio-dense vertebral bodies, abnormal bone softness, and structural distortions such as curled ribs and spines [41, 150, 156, 216, 228, 242, 301, 302, 304, 305]. Witten, Owen, Fontanillas et al. [145] investigated the primary bone pathology caused by a P-deficient diet in *S. salar* in the early seawater phase (post-smolts). These authors observed severe osteomalacia related to P deficiency but no evidence of bone deformities, structural changes in the bone matrix, or aberrations in bone cells. Bone growth continued normally, although the new bone matrix was completely devoid of minerals, demonstrating the uncoupling of bone formation and bone mineralization. The same results were also obtained for freshwater stages in this species by Drábiková, Fjelldal, De Clercq et al. [242]. Other studies have demonstrated that it is possible to rescue the P-deficient bone phenotype with a P-sufficient diet that restores the mineral composition and mechanical properties of previously nonmineralized bone [146, 228].

Interestingly, studies on *S. salar* have confirmed the results from studies on *S. senegalensis*. Salas-Leiton, Dias, Gavaia et al. [306] reported that low dietary P intake indeed lowered the bone mineral content. However, the low dietary bone mineral content did not negatively affect the occurrence of deformities and had no effect on production parameters. The study concluded that *S. senegalensis* is highly tolerant to diets with low P contents. Studies on other flatfish species have shown a lower resistance to low dietary phosphorus intake. Like in *S. senegalensis*, a diet with low P content reduced the bone mineral content in juvenile *H. hippoglossus*; however, this caused an increase in abnormalities of haemal and neural spines as well as deformities in the postcranial and caudal regions of the vertebral column [261]. In *P. olivaceus*, low dietary P intake resulted in poorly mineralized bone and growth reduction; however, deformities were observed only at the operculum [141]. Uyan, Koshio, Ishikawa et al. [141] observed an increase in whole-body lipid content as a reaction of *P. olivaceus* to reduced dietary P, an observation that was confirmed by studies on *S. salar* and on several other advanced teleost species, see Albrektsen, Hope, and Aksnes [159] and references therein. Conversely, low-phosphorus diets did not significantly affect the bone mineral density of acellular turbot bone or the whole-body phosphorus/calcium concentrations [161].

Trace minerals, such as Zn and Mn, are critical nutrients for skeletal development, and their role in bone health was reviewed in Baeverfjord, Antony Jesu Prabhu, Fjelldal et al. [307]. In *S. senegalensis* postlarvae, the supplementation of diets with organic Mn and Zn resulted in a decrease in the severity of skeletal deformities [126]. Diets rich in organic Zn significantly decreased the deformity score in the caudal complex [126]. However, the supplementation of diets with organic glycinate-chelated forms of microminerals (Cu, Fe, Mn, and Zn) had no significant impact on bone mineralization or on the incidence of skeletal deformities [127]. Different levels of dietary supplementation with Zn and Mn also promoted bone mineralization and prevented skeletal anomalies in larvae of other marine species [308, 309].

Enriched *Artemia* spp. metanauplii and rotifers are common components of flatfish diets during larval rearing to improve larval performance [220, 223, 284, 310–312]. Therefore, the nutritional value of live preys is important for the success of larval rearing [313]. However, the nutritional composition of live preys varies greatly according to the specific conditions of *Artemia* spp. culture and enrichment conditions, as reviewed by Conceição, Yúfera, Makridis et al. [314]. Moreover, several commercial products for *Artemia* spp. enrichment are available, displaying differences in the physical form, nutritional content, and ingredients [223, 314]. The same occurs with commercial diets and enrichment products for rotifers, which exhibit considerable variation in nutrient composition and form, which not only impacts the quality and quantity of rotifers produced but also may have an effect on fish larvae [315, 316]. Although these products are efficient in rotifer culture, further research is needed on the vitamin and mineral contents of rotifers fed with these products [317]. Studies addressing the nutritional composition of rotifers fed different enriched diets revealed that although all the rotifers contained adequate levels of Ca, P, Mg, and Co for marine larval requirements, they had very low contents of vitamin E, I, and Zn [317]. Specific adjustments for microminerals or vitamins are thus required in these feeds for rotifers to adequate their nutritional value for marine larvae [317], particularly considering the importance of Zn for skeletal health and development in flatfish [126]. With respect to skeletal deformities, studies in *S. senegalensis* did not find a clear influence of *Artemia* spp. enrichment products on the development of overall skeletal deformities in different rearing stages [223, 318, 319]. However, *S. senegalensis* larvae fed certain dietary enrichment products showed differences in terms of the degree of ossification, the frequency of vertebral fusions, and the frequency of caudal complex deformities, especially in modified neural and haemal spines [223]. Moreover, regarding development, both *Artemia* nauplii and on-grown *Artemia* were fed to *H. hippoglossus*, and similar efficiency rates of eye migration were observed in *H. hippoglossus* [312].

The diversity of commercially available enrichment products and their nutritional variability should be considered when comparing results from different studies. The high variety of dietary composition of the enrichment products used to feed the different flatfish species opens a vast field for further research on the role of nutrients in skeletal development.

3.3.3 | Other Factors

Water pollutants are other factors that play a role in early development in flatfish. Most toxicological studies on fish skeletal deformities examine water samples and specimens close to the hatching period [320]. Therefore, the deformities reported in such studies are related to the notochord. In the long term, notochord malformations can result in permanent vertebral column deformities, muscle abnormalities, and neurological dysfunction [320, 321]. Abnormal curvature of the notochord was observed in *S. senegalensis* larvae after exposure to 4-methylbenzylidene camphor, a UV filter for personal care products [322]. Additionally, polybrominated diphenyl ethers may cause non-lethal deformities in the tail region of *S. maximus* larvae [323].

Certain parasitic protozoans, such as *histophagous scuticociliates*, were observed in the vicinity of the vertebral bone in *P. olivaceus* [324], and perichondral ossification was altered in association with the presence of these parasites in *S. maximus* [324, 325]. In addition, ectopic cartilaginous cylindrical structures were observed in the vicinity of proximal dorsal and anal fin pterygiophores, associated with the presence of *Myxobolus groenlandicus* in Greenland halibut (*Reinhardtius hippoglossoides*) [326]. However, vertebral deformities were not specifically investigated in previous studies.

3.4 | Anomalies, Development, and Growth

The onset of skeletal deformities can occur during the early stages of skeletal development and during later stages [167]. Disparities among deformity patterns with age were detected in *S. senegalensis* [12, 318]. While anomalies, especially deformations in the caudal complex plates or caudal haemal spines, predominated in postlarvae, they were clearly reduced in later stages, whereas the percentage of specimens with caudal scoliosis considerably increased [12, 318]. Certainly, the utilization of distinct diagnostic techniques should be considered when assessing differences in the anomaly profile.

In *H. hippoglossus*, Lewis, Lall, and Witten [42] observed an increasing number of the most serious anomalies and axis curvatures with increasing length of the fish. However, the frequency of hypertrophic vertebrae (vertebrae with increased diameters that form around an enlarged notochord) was decreased in larger fish, possibly due to compensatory growth of the nonhypertrophied vertebrae, resulting in uniformly sized vertebrae later in development [20, 42].

The different frequencies of deformities observed at different developmental stages may be due to an adaptive response. The skeleton of teleosts can show considerable plasticity and an adaptive response to altered mechanical loads [104, 111, 117, 194, 327]. Moreover, fish bone has the ability to remodel and model some vertebral fusions and repair fractures [105, 218, 328].

As mentioned earlier, several fish species affected by lordosis or alterations associated with a low-phosphorus diet, such as low-mineralised vertebrae and hyperdense vertebrae, recover with time under reverse causal conditions [228, 329, 330]. Hence, the deformed spines and caudal complex plates observed in early *S. senegalensis* might be undergoing remodeling and modeling processes during its life cycle, leading to apparently nondeformed elements and to the attenuation of deformities at later juvenile stages. The aggravation of incipient lesions at early stages and/or the emergence of *de novo* anomalies due to different causative factors over time [42, 105, 218, 261] might explain the increase in the frequency of scoliosis in flatfish juveniles. Studies tracking individual fish should be conducted to study the progression or possible regression of each type of deformity.

The *Danio rerio* vertebral column elements show a certain degree of variation, although it can occur in natural populations of this and other teleost species. Thus, distinguishing normal deformities from nonpathological or pathological deformities can be difficult [35, 49, 194]. Similarly, it should be considered that some of the frequently observed minor skeletal alterations in flatfish possibly represent natural variation in skeletal anatomy, as is the case for all vertebrates [194, 331]. Some examples of these minor alterations are provided in the following paragraphs.

Studies in juvenile *S. senegalensis* reported a relatively shortening of the first abdominal centrum compared with the same vertebra in postlarvae [12, 13]. As this feature was inconspicuous and present in every individual, it could be regarded as a normal event, as also observed in adult *S. solea* [12, 53].

Fusions between hypurals are also frequently observed in *S. senegalensis* postlarvae, and are also observed in several reared and wild fish species [22, 47, 200]. Nevertheless, some authors have considered this particular feature as a common and normal event in caudal complex development and ossification rather than a skeletal deformity [332]. In fact, for some flatfish species, such as *S. maximus*, the normal development of the hypural plate is a result of fusion between two hypurals, which occurs before eye migration [333].

Other commonly observed fusions affect the preurals of wild and reared *S. senegalensis* [12, 13, 22, 262, 318] and other Pleuronectiformes (reviewed in Chanet and Wagemans [334]). Most of these were stable or contained fusions [228], as evidenced by the presence of double neural and haemal spines in the last preural vertebra [334]. Since these vertebrae fully mineralize at the end of prometamorphosis (approximately 9.14 mm; 21 dah) in *S. senegalensis*, this may be a critical stage for the establishment of caudal fin vertebral fusions [167]. The frequency of this event in numerous flatfish species raises the question of whether they should be considered as deformities or not [334]. Additional studies on wild specimens from larvae up to juveniles and adults could elucidate some of these aspects of vertebral column development.

3.5 | Micro-Anatomical Changes Associated With Skeletal Anomalies

Few reports exist on the histological features of normal and deformed bone in flatfish. In *S. senegalensis*, Cardeira, Bensimon-Brito, Pousão-Ferreira et al. [85], and Cardeira, Mendes, Pousão-Ferreira et al. [215] characterized the micro-anatomical changes related to lordotic and kyphotic vertebral curvatures. De Azevedo, Losada, Barreiro et al. [84] provided a correspondence between radiographic and histopathological features of deformed and fused vertebrae unrelated to axis curvatures.

In addition to structural alterations of the bony trabeculae, most of the deformed vertebrae (with or without curvatures) presented a shift in mesenchymal cells, such as osteoblasts and chondrocytes, towards a chondrogenic phenotype, mainly in the endplates and intervertebral spaces (IVS). The chondrogenic transdifferentiation is possibly related to increased pressure on the cells located in the vicinity of intervertebral spaces [84, 85, 215, 238, 335]. Cardeira, Mendes, Pousão-Ferreira et al. [215] suggested that the chondrocyte distribution in the endplates and in the growth zone could be related to the reduced availability of oxygen and nutrients in the peripheral areas to be able to survive.

Some of the alterations in deformed vertebrae, such as flattened endplates, narrow IVS, and/or abundant cartilaginous tissue in IVS, could be consistent with the initial stages of vertebral body fusion [218, 219]. Additionally, de Azevedo, Losada, Barreiro et al. [84] noticed an irregular and scattered external elastic membrane of the notochord, close to opposing endplates with chondrocytes, similar to what was observed in other studies in *S. salar* [336–338].

Given that, in mammalian bone, osteocytes are the main receptors of mechanical load and that these are absent in advanced teleosts and thus also absent in flatfish, we still need to uncover which cells are responsible for mechanical load reception in acellular (anosteocytic) bone. However, osteoblasts and bone lining cells on the bone surface are possible candidate cells for sensing mechanical load [104, 105, 111]. Additionally, chondrocytes in most compressed areas could serve as mechanosensors themselves, as proposed by Cardeira, Mendes, Pousão-Ferreira et al. [215] and Ofer, Dean, Zaslansky et al. [111]. Therefore, further investigation is required, focusing on the signaling pathways involved in mechanosensory and local effector mechanisms underlying the development of cartilaginous tissue in vertebral anomalies in species with acellular bone.

Histopathological features of reshaped vertebral fusions suggested remodeling and/or modeling processes. Remnants of intervertebral notochord and cartilaginous residue persisted in the center of the fused elements; although later, they appear to be replaced by osseous tissue [84]. This was also observed in the intermediate and final stages of fusion in salmonids [218, 336].

3.6 | Molecular Insights Into Normal and Deformed Bone

The processes of skeletogenesis and the paths that lead to the process of bone by the osteoblastic lineage and the regulation of its functions involve multiple regulators, like morphogens and developmental signals, including bone morphogenetic proteins (BMP), Wnt ligands, hormones and vitamins, and growth factors, such as Sox9, Runx2, and Osx/SP7, and cytokines, which exert their action at multiple stages for the control of osteogenesis and chondrogenesis [339, 340]. The maintenance and homeostasis of the extracellular matrix are ensured by highly conserved noncollagenous proteins such as osteocalcin (also known as bone Gla protein, Bglap). Bglap is a vitamin K-dependent protein (VKDP) with several physiological functions, such as endocrine glucose regulation or a role in osteoclast recruitment [341]. However, its main role is as a regulator of mineral deposition, and it is a well-known marker of mature osteoblasts that appear after the onset of mineralization, as observed in *S. senegalensis* at pre-metamorphosis [182]. Recent studies in Bglap-deficient (Bglap−/−) mice revealed the specific role of Bglap in the alignment of apatite crystals along the collagen fibrils and thus in regulating adequate mineralization of bone [342]. The expression of *bglap* was increased at later stages of *S. senegalensis* development, concomitant with the advancement in mineralization of the skeleton [182]. However, studies in *S. salar* have shown comparable *bglap* expression in vertebrae with low and normal mineralization, associated with different dietary phosphorus contents or with levels of CO_2 [136].

A study on the response of *S. senegalensis* to different levels of vitamin K (VK), specifically phylloquinone (PK), was performed by Richard, Fernandez, Wulff et al. [266] to elucidate the effects on the skeleton and expression of genes and proteins. The dietary supplementation with PK was shown to modulate the expression of proteins involved in several biological processes relevant for skeletal maintenance, such as muscle contraction and development, the cytoskeletal network, skin development, energy metabolism, protein chaperoning and folding, and bone development. It has been observed that some key genes regulating the VK cycle (*ggcx*, *vkorc1l1*, and *vkorc1*) and the VK nuclear receptor that mediates VK-dependent gene regulation (*pxr*) were responsive to increasing VK levels, with their expression being downregulated at a dietary level of 250 mg/kg of PK. Also, the expression of some of the VK-dependent proteins (VKDP) (*bglap1*, *bglap2*, *mgp*, and *grp*) involved in bone metabolism and development was evaluated. The *bglap1* mRNA was downregulated with PK supplementation, and *grp* was upregulated, whereas *bglap2* and *mgp* were not affected [266]. The authors concluded that the downregulation observed in most of the genes analyzed in the treatment with the highest VK1 level could be due to a feedback mechanism by which the organism tries to compensate for a deficient γ-carboxylation of VKDPs caused by VK deficiency in the lower VK levels tested, therefore increasing the level of mRNA transcription that can translate into more protein available for recycling VK and more gamma-carboxylation occurring for VKDPs to perform their biological functions. In fact, VK treatment resulted in lower levels of skeletal deformities, which could be correlated with better VK status and a more efficient regulation of skeletal formation by VKDPs

[266] and possibly other skeletogenic genes downstream of the VK receptor Pxr [296].

In *P. olivaceus*, some studies have focused on the influence that dietary VA imbalance has on skeletal deformities, although the molecular pathways involved in the increase in deformities are still not clear. It has been reported that the involvement of retinoic acid receptors (RARs) and retinoid X receptors (RXRs) and several transcription factors (e.g., insulin-like growth factor-IGF, BMPs) in the regulation of cell proliferation and differentiation processes, in addition to their known roles in modulating the expression of genes related to morphogenesis and remodeling of skeletal tissues [72, 343, 344]. As previously reported, the effects of retinoic acid (RA) on early development are mediated through the modulation of the expression of master regulatory genes, such as the Hox gene family, which act as regulators of differentiation and patterning, and are involved in segmentation and anterior–posterior axis determination [345, 346]. Hox genes are key effectors of retinoid signaling during embryogenesis; hence, disruption of the normal RA signaling will affect the expression of Hox genes and lead to defects in embryogenesis [346].

The alterations in skeletal formation can differ according to the RA isomers and life stage. Haga, Suzuki, and Takeuchi [258] and Haga, Suzuki, Kagechika et al. [343] have reported that RA induces skeletal deformities through the RAR/RXR signaling pathway during later larval stages, with differences in the incidence and severity of skeletal deformities depending on the RA isomer used to treat the larvae. These authors concluded that all-trans RA activated the expression of *rar* while inhibiting vitamin D receptor (VDR) expression, and that 9-cis-RA activated the expression of *rar* and *rxr*. In contrast, 13-cis-RA had no effect on the gene expression of *rar* or *vdr*.

When analyzing the expression of the *pitx2* gene, it was shown that all-trans RA and 9-cis-RA inhibited *pitx2* expression in the lower jaw and induced a greater incidence of jaw deformities [258]. By exposing *P. olivaceus* larvae from 6 to 9 dah to the synthetic retinoid compounds Am80 and methoprene acid (MA), it was observed that Am80 treatment at 25 nM was able to induce the expression of RAR in the lower jaw. However, neither Am80 nor MA affected RXR gene expression. Interestingly, both Am80 and MA treatments inhibited the gene expression of patched (*ptch*) in the lower jaw, causing a disruption of the hedgehog (*hh*) signaling pathway, which may be the cause of these jaw deformities [343]. Ptch is a transmembrane receptor of the *hh* family that plays a negative regulatory role in its signaling pathways and is essential for the differentiation of mesenchymal cells into chondrocytes and osteoblasts [347].

In *S. senegalensis*, it was shown that dietary imbalance in VA causes disruption of RARα signaling, which is thought to be the main regulator of VA effects on skeletal formation in this species. Additionally, it was shown that formation of thyroid hormone follicles is affected, with an increased size induced by high VA, leading to imbalances in thyroxine (T4) and triiodothyronine (T3) levels, and to an upregulation of the thyroid hormone receptors trαa, trαb, and trß. Therefore, it was suggested that these alterations in the expression of RA receptors and a disruption in thyroid signaling were possible causes for the negative repercussions on skeletogenesis and the increase in skeletal deformities observed in larval sole under high VA levels [79, 287, 348]. Additionally, excess dietary VA can lead to increased expression of osteocalcin [167].

With the rise of Next-Generation Sequencing (NGS), the transcriptomes and genomes of several flatfish species, including *S. senegalensis*, *H. hippoglossus*, *S. maximus*, and *P. olivaceus*, have been sequenced, and a comparative analysis of these resources has been revised and integrated [349]. In *S. senegalensis*, transcriptomic data were obtained from adult and larval tissues and from several developmental stages [350]. The many libraries that were available have been *de novo* assembled to produce a more robust transcriptome [351, 352]. Specifically, comprehensive transcriptomics of the vertebral bone has been recently performed [353]. The results revealed different profiles among postlarvae and juveniles and unraveled candidate genes related to bone cell processes, which can serve as a reference for functional studies comparing normal and deformed vertebral columns and for exploring the related cell lineages in this and other fish species [353]. In *P. olivaceus*, both the genome and transcriptome have been sequenced. The genome of *P. olivaceus* has been compared with that of the Chinese tongue sole (*Cynoglossus semilaevis*) to identify differentially expressed genes that are exclusive to flatfish [192, 354]. The sequencing data generated by the different projects are available in public databases (NCBI-Sequence Read Archive (SRA) database https://www.ncbi.nlm.nih.gov/sra/; NCBI-Reference Sequence https://www.ncbi.nlm.nih.gov/refseq/; NCBI-BioProject https://www.ncbi.nlm.nih.gov/bioproject/; NCBI's Gene Expression Omnibus (GEO) http://www.ncbi.nlm.nih.gov/), and information on relevant genes for skeletal development, homeostasis, and remodeling can also be obtained by data mining.

3.7 | Major Concerns and Knowledge Requirements

Despite the new impetus of flatfish industrial rearing driven by recent advances in zootechnical, biological, and nutritional features [3], further knowledge is needed on skeletal anomalies to optimize production. In this sense, the numerous scientific articles concerning the big money production species, such as *S. salar*, and the new molecular insights gained from zebrafish studies may be useful for applying to flatfish research. A brief meta-analysis of the available literature on flatfish vertebral structures was performed in April 2025. This study used the research database Scopus and included the documents that presented in the title, abstract, or keywords the words "vertebr*" and "flatfish," "halibut," "plaice," "solea," "turbot," or "flounder" for the period between 2000 and April 2025. Documents on "skeletal muscle," "bone marrow," or "vertebrates" were disregarded. This analysis revealed 213 documents, of which 32, 18, and 8 presented in the title the names of the most farmed flatfish species: *Solea* sp., turbot, and Atlantic halibut, respectively. Studies on the vertebral elements of flatfish were more frequent in the first decade of 2000, with an average of four articles per year in the following years (Figure 13a). Regarding publishing countries, Japan, the United States of America, and France were the countries that published the most in this period (Figure 13b). Agricultural and biological sciences were by far the most explored subject area (Figure 13c).

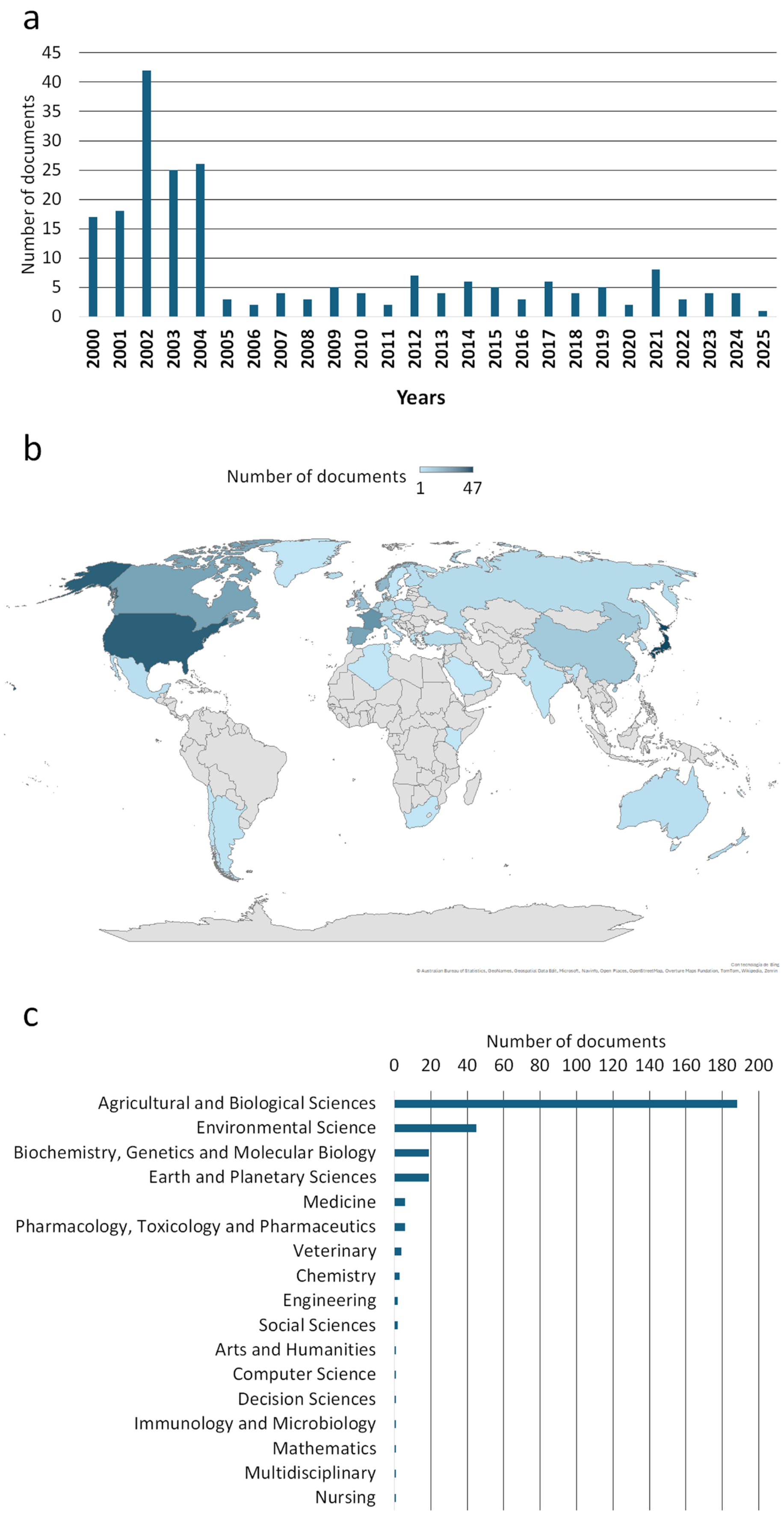

FIGURE 13 | Legend on next page.

FIGURE 13 | Brief meta-analysis of the available literature on flatfish vertebral structures performed in April 2025, using the Scopus research database. The search terms included "vertebr*" and "flatfish," "halibut," "plaice," "solea," "turbot," or "flounder" in the title, abstract, or keywords for the period between 2000 and April 2025. Documents on "skeletal muscle," "bone marrow," or "vertebrates" were excluded. (a) Published documents per year. (b) Published documents per country. (c) Results considering the subject area.

The main gaps in knowledge concerning flatfish skeletal deformities can be divided mainly into diagnostic factors, causative factors, and skeletogenic features [16]. As mentioned previously, fish farms still rely on gross observation and manual sorting of deformed fish, especially at the time of juvenile transfer to ongrowing farms [220], and therefore lack systematic monitoring of deformities as routine procedures, like it has been implemented for other species (*D. labrax*, *S. aurata*) [16]. The precise control of all stages of the production process would benefit from the identification of the causative factors of malformations and would facilitate the conformity of the rearing protocols for normal skeletal development in fish batches [203].

In order to monitor skeletal defects in different stages of industrial production, further attention should be given to accurate diagnostic techniques that can be used on a large scale and applied in different phases of flatfish rearing. In addition, standardized terminology that gathers industrial and scientific criteria is missing for different skeletal anomalies [16], particularly in flatfish. This systematized classification could provide a pattern to improve diagnosis, connect particular types of anomalies to specific causal factors, and better discern deformity transitory stages, similar to what was suggested for *S. salar* by Witten, Gil-Martens, Huysseune et al. [199]. In this context, a preliminary proposal has been presented, adapting the vertebral body malformation classification used for salmonids [199] to *S. senegalensis* [355]. More research is needed on the progression of deformities during life and their impact on external morphology at the end of the hatchery and ongrowing stages [16, 203] and on the rearing performance and welfare of fish. Such studies were recently performed for *S. salar*, *D. labrax*, and *S. aurata*, which revealed that some malformations detected in earlier stages had disappeared at later stages and at harvest [228, 329, 330]. However, for farmed rainbow trout (*Oncorhynchus mykiss*), the frequency and severity of vertebral deformities have increased over time, especially in the ural region [356].

Studies on the etiology of flatfish skeletal anomalies have not been conducted at farms but at experimental research centers [79, 262, 263]. Studies under commercial farming conditions are important to incorporate the multiple undocumented factors that interact with developing fish and factors that are not reproducible under experimental conditions [251]. This knowledge is critical for improving animal welfare and promoting sustainable farming. Furthermore, to address this multifactorial problem, multilevel research should be conducted, integrating environmental, genetic, biomolecular, deformity frequency, and production data, among other methods, for which the use of multilevel mathematical models and artificial intelligence would be advantageous [357–359]. In this sense, some of the major points that could be addressed in future research include the optimization of nutritional requirements regarding bone-related minerals and vitamins. Additionally, the enforcement of selection programs with refinement of the breeding stocks might improve the skeletal quality of the offspring. Finally, the literature revealing the mechanisms underlying the response and plasticity of bone to the distinct causative factors of deformities is still scarce for Pleuronectiformes. Such studies would also complement the characterization of osteogenesis in anosteocytic fishes [360].

Author Contributions

A. M. de Azevedo: conceptualization, formal analysis, data curation, investigation; writing – original draft preparation, writing – review and editing. **A. P. Losada:** investigation, writing – review and editing. **S. Vázquez:** project administration, writing – review and editing. **P. E. Witten:** conceptualization, validation, writing – original draft preparation, writing – review and editing. **M. I. Quiroga:** conceptualization, visualization, writing – review and editing. **P. J. Gavaia:** conceptualization, supervision, writing – original draft preparation, writing – review and editing

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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