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REVIEWS IN Aquaculture

REVIEW

The problems with pin bones: Intermuscular bone development and function in salmonids and their implications for aquaculture

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Abstract

A healthy skeletal system is fundamental to fish welfare and performance and a key physiological feature of a robust fish. The presence of skeletal deformities in farmed salmonids is a persistent welfare problem in aquaculture, and one which threatens to impede industry growth. Deformities of the fine bones of fish, such as ribs and intermuscular bones (IBs), have received less attention than vertebral body deformities, despite their potential to compromise fish welfare and product quality. IBs, commercially known as pin bones, are small spicule-like bones embedded in the muscle fillets of salmonids, cyprinids and other basal teleost species. In farmed basal teleosts, they impact fish processing, have negative effects on the economic value of fish and present a potential consumer health concern if ingested. Current understanding of IB development and function in teleosts has mostly relied on morphological research. More recently, advances in our understanding of molecular mechanisms of IB development in cyprinids have been made, largely due to the exploration of breeding IB-free fish for use in aquaculture. In this review, we explore the existing literature on IBs in teleosts, highlight the points of contention within this field of research and identify the significant knowledge gaps about the development and function of salmonid IBs. To our knowledge, there is no available research on the function of IBs and scarce research concerning IB development in salmonids. Future research on teleost IBs would benefit from the use of consistent terminology to facilitate interdisciplinary collaboration and identify research outputs in this field.

KEYWORDS
intermuscular bones, pin bones, salmon, skeletal deformities, welfare

1 | INTRODUCTION

Aquatic animal production is rapidly expanding and in an increasing number of countries.[1–3] Global salmonid production grew to 3.8 million metric tons in 2020, which was mainly attributed to farmed Atlantic salmon (*Salmo salar*).[4] The salmon farming industry is meeting the increasing market demand with transformation in production technologies, feeds and farm management.[5–7]

Skeletal deformities are a persistent problem in salmon aquaculture which impact on fish welfare and result in financial losses to farmers throughout the value chain.[8–10] Generally, bone deformities occur long before they become visible externally.[11] Additionally, certain skeletal deformities can be present in high frequencies in externally normal fish, and are therefore only discovered at processing, which may cause technical difficulties during filleting[8] or when removing malformed intermuscular bones (IBs). IBs, commonly known as pin bones, are small spicule-like bones embedded in the muscle fillets of salmon, carp, and other basal teleosts.[12] Historically, IBs and ligaments of teleosts are the more overlooked elements of the musculoskeletal system.[12] The functions of IBs are still predominantly unknown, however several hypotheses exist including their proposed role in mechanical force transmission between axial muscle segments during swimming.[13–15] In farmed fish IBs are mostly recognised for their impact on fish processing, negative effect on the economic value of fish and as a consumer health concern if ingested.[16–18]

Our understanding of the homology and origins of IBs is primarily based on morphological data. Past studies focused on the morphology, number and distribution of IBs.[12,19–21] However, from early 2000, there was a shift in attention towards the development of IBs, which included technologies such as high-throughput gene sequencing and other molecular techniques.[18,22,23] Interest in identifying the genes and proteins involved in IB development has grown significantly. Breeding an IB-free strain of cyprinid for use in aquaculture has become a topical issue in fish selective breeding.[22–25] However, despite the progress made to date in cyprinid IB development, knowledge about IB development and function is still lacking, particularly for Atlantic salmon and other salmonid species. This knowledge gap should be closed as artificial selection for production-related traits in farmed fish may have unknown effects on welfare-related traits.[26,27]

The number, morphology and ossification of IBs and, more recently, molecular mechanisms of IB development have been well documented in some teleost species, predominantly cyprinids, as evidenced by three recent reviews on teleost IBs.[17,18,22] Asian carp species, from the family Cyprinidae, account for almost half of global finfish production and possess a considerable number of IBs, which pose a significant concern among consumers.[18] In contrast, salmonid IBs have received little attention, despite their significance for global aquaculture production, and similar processing issues with regard to IB removal. That is evidenced by the few existing studies on IBs in salmonids, which predominantly focus on their removal from fillets.[16,28–31]

This review highlights the knowledge and knowledge gaps in IB development and function in salmonids and potential implications for aquaculture production and farmed salmonid health and welfare.

2 | DEFINITION AND ORIGIN OF IBs

The main function of the musculoskeletal system in fish is locomotion. The myotomal architecture and muscle fibre organisation are interrelated with the swimming modes of the fish.[32,33] The trunk of bony fishes is divided into two lateral halves, separated by a thick sheet of collagenous connective tissue (i.e., the median septum), with blocks of myotomal muscles arranged on either side.[33] As in all gnathostomes, the skeletal musculature is further divided by the horizontal septum, separating the dorsal (epaxial) and ventral (hypaxial) muscle sections. Myotomal muscles consist of a series of myomeres, which are W-shaped units separated by collagenous sheets called myosepta. The median and horizontal septa and the myosepta are involved in facilitating transmission of muscular force to the axial skeleton. The skeletal musculature is supported by the vertebral column, and the myosepta are further strengthened by IBs, which are in close contact with the muscle fibres.[33]

IBs, also referred to in the literature as false ribs, pin bones, fillet bones, intramyoseptal bones, mineralised spicules, myoseptal spines or fish bones, are present only in the myosepta of teleosts.[12] They are small spicule-like bones, which form from ossified myoseptal tendons.[22,23,34]

The formation of IBs has been highly contested among researchers, which is also reflected in the lack of a consistent terminology for these bones. For example, IBs have previously been described as ossified connective tissues,[35] calcified ligaments[12] and only more recently has consensus been achieved that they are ossified tendons.[13–15,19,22] Gemballa and Britz classified IBs as ossified tendons, because myoseptal tendons connect muscle to axial skeletal bones, while ligaments are attachments between bone and bone.[19] Intermuscular tendons transfer muscular forces from myomeres to the vertebral column, and thus, when parts of the tendon ossify, one could speculate the IBs maintain a similar function to the tendons. When the tendons ossify, the resulting IBs may be attached to the vertebral column via a short bundle of non-mineralised fibres.[19] Patterson and Johnson argued that this proximal part should thus be termed a ligament due to the bone-to-bone connection,[12] however Gemballa and Britz saw no need to apply a different term to the same structure at a later point in ontogeny. The development of IBs is discussed further in Section 4.

Several forms of tendons exist in teleosts including: myoseptal tendons, that is, epineural (ENT), epipleural (EPT), myorhabdoid (MT), epaxial lateral tendon (eLT) and hypaxial lateral tendons (hLT); and horizontal septum tendons, that is, posteriorly oriented tendon (POT) and anteriorly oriented tendon (AOT), also known as epicentral tendons.[13,32] IBs originate from myoseptal and horizontal septum tendons.[13,19,36] Since Richard Owen's initial studies,[37,38] three categories of IBs have been recognised, that is, epineurals, epicentrals and epipleurals, which are classified based on area of attachment or placement, that is, neural arches, horizontal septum, and haemal arches or ribs respectively (Figure 1).[12,13,17,19] Additionally, there is one or two extra series of IBs which do not attach to the axial skeleton, called myorhabdoi, which have been confirmed in a few teleost species, mainly Clupeiformes.[12,13,17,39]

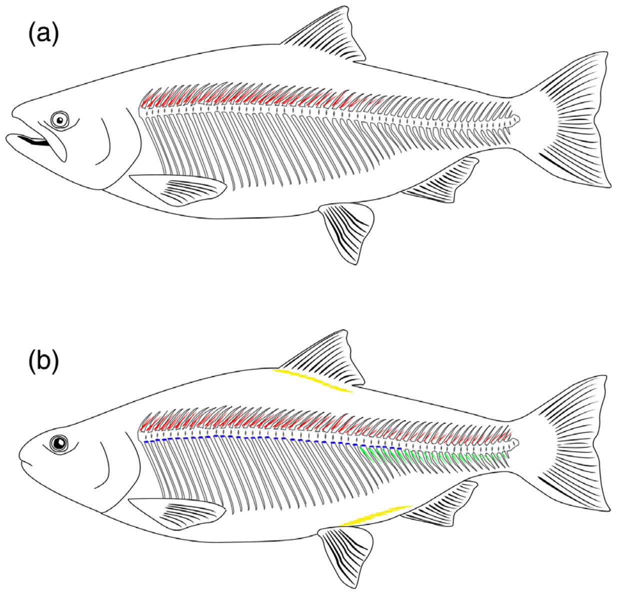

FIGURE 1 Types and locations of intermuscular bones (IBs): epineurals (red lines), epipleurals (green lines), epicentrals (blue lines) and myorhabdoi (yellow lines) in (a) Chinook salmon and (b) generalised diagram of a teleost. Number of IBs is for visual reference only and does not represent actual counts. Figure adapted from Nie et al.[17] (2020).

The types of IBs teleosts possess vary by species (Table 1). For example, some teleost species, including the Japanese eel (*Anguilla japonica*), have all three types of IBs; cyprinids, such as zebrafish (*Danio rerio*), gibel carp (*Carassius gibelio*), and blunt snout bream (*Megalobrama amblycephala*) have only epineurals and epipleurals (Figure 2), which is the same for characiformes such as tambaqui (*Colossoma macropomum*); Salmonids, such as Atlantic salmon (*Salmo salar*) have only epineurals and in some perciformes and beryciformes, IBs have disappeared completely.[12,13,15,17,25,40–43]

Significant changes in the types, number, shape and complexity of IBs has accompanied the evolution of teleosts (Table 1).[12,17,20,23] Primitive teleosts are said to have only epineurals, with epipleurals appearing later in the fossil record. Patterson and Johnson described basal actinopterygian epineurals as short broad outgrowths of the anterior neural arches.[12] The authors added that primitive epineurals developed via perichondral bone formation, and had a cartilaginous core and cap, so that in fossil actinopterygians the epineurals were hollow.[12] Epineurals and epipleurals were found in a fossil of an extinct cyprinid fish, *Hsianwenia wui*, from the Pliocene lake deposits of the Qaidam Basin (Tibet).[44] The fish was characterised by an extraordinarily thick skeleton, including the vertebrae, arches, spines and IBs. It was proposed that this skeletal thickening was most likely formed through overdeposition of calcium in their bones, from their environment, which was rich in carbonates ($CaCO_3$) and sulphates ($CaSO_4$).

With the evolution of teleosts, IB morphology appears to have evolved from simple to complex shapes and then regressed to simple again,[17,20,23] and the number of IBs gradually increased, then decreased gradually in more advanced teleost species.[22] It is very plausible that IB presence and morphology relate to the evolution of actinopterygian fish species.[17,20] Changes in complexity could be viewed as adaptations to changes in locomotion type and use of musculature.[17] Yang et al. proposed that adaptation to various epigenetic factors, including diet, water depth and water velocity, could be affecting the evolution of IBs.[15] The study revealed that some carnivorous species have more IBs, based on a comparison between carnivorous and non-carnivorous species in the subfamily Cultrinae and in Danioninae. The authors suggested that this could be due to the rapid acceleration of carnivorous fish when catching prey, which would require more IBs in the posterior region of the body, assuming that IBs are involved in transmitting and enhancing muscle strength.

3 | NUMBER, DISTRIBUTION AND MORPHOLOGY OF IBs

IBs are often used for taxonomic classification due to the variation in their morphology and number between species.[12,15,45] The number of IBs varies greatly among different species and even subspecies from different populations (Table 1).[17] The total number of IBs in Cyprinidae species ranges between 99 and 133.[17,20] However, Yang et al. reported a larger range of IB number among cyprinids (73–169).[15] The authors also found a positive correlation between the number of IBs and the number of vertebrae in cyprinids, thus vertebrae number could be used to predict approximate number of IBs.[15] IB number can vary between different strains of the same species. Cao et al. reported between 55 and 110 IBs among three strains of common carp (*Cyprinus carpio*).[45] When comparing species from different orders, IB numbers can range from 409 in the Daggertooth pike conger (*Muraenesox cinereus*) to 8 in the yellow catfish (*Pelteobagrus fulvidraco*).[20,22] Most studies reporting IB numbers have shown no significant difference between left and right sides of the body, however several studies have reported a significant difference in number between the dorsal and ventral sections.[15,20,21,45–48] Therefore, it has been speculated that IBs in the dorsal and ventral sections, for example, epineurals and epipleurals, respectively, may have different developmental patterns.[48] Differences in number and morphology of IBs between the anterior and posterior regions of the body have also been reported in several species.[46,49–52] Other factors that can cause different numbers of IBs between individuals within a population are discussed below.

Salmonids have relatively fewer IBs than cyprinids. Balaban et al. observed 30 IBs per fillet in Chinook salmon (*Oncorhynchus tshawytscha*) (i.e., 60 IBs in the whole body).[28] Skjelvareid et al. reported approximately 30 IBs per fillet in Atlantic salmon.[29] Schroeder et al. recorded 29 IBs per fillet of Atlantic salmon and 31 for rainbow trout (*Oncorhynchus mykiss*),[31] which was consistent with the author's previous findings.[30] The authors noted the range of IB lengths for a 3–4 kg salmon was 14–50 mm, and 18–50 mm for 4–5 kg salmon. For 3–4 kg trout, IB lengths were 15–50 mm and 20–55 mm for 4–5 kg trout.[31] The IB lengths could not be directly compared between

TABLE 1 Variations in IB presence and development in teleost species.

Superorder	Order; Family	Species; Common name	Total IB number	Morphology of IBs	Type of IBs	Ossification pattern	Swimming mode	Initial IB ossification	References
Osteoglossomorpha	Osteoglossiformes Osteoglossidae	*Osteoglossum bicirrhosum* Silver Arowanas	70	1	Epineurals	-	-	-	20
Elopomorpha	Anguilliformes Muraenesocidae	*Muraenesox cinereus* Daggertooth pike conger	409 (405–413)	1, 2, 3	Epineurals, Epipleurals, Epicentrals	-	-	-	20,40
	Anguilliformes anguillidae	*Anguilla japonica* Japanese eel	351–360	1	Epineural, Epipleurals, Epicentrals	Anterior to posterior	Anguilliform	‘Elvers Period’	40,41,56
Clupeomorpha	Clupeiformes Dorosomatidae	*Tenualosa reevesii* Reeves shad	143 (133–152)	1, 2, 3, 4	Epineurals, Epipleurals, Epicentrals	-	-	-	20,40
	Cluipeiformes Engraulidae	*Coilia ectenes* Japanese grenadier anchovy	221	1, 2, 3, 4	Epineurals, Epipleurals, Epicentrals	-	-	-	20,40
Ostariophysi	Cypriniformes Cyprinidae	*Cyprinus carpio var. color* Common carp	99 (86–90)	1, 2, 3, 4, 5, 6, 7	Epineurals, Epipleurals	Posterior to anterior	Carangiform	32 (DPF)	20,56
	Cypriniformes Cyprinidae	*Cyprinus carpio var. haematopterus* Common carp	93–104	1, 2, 3, 4, 5, 6, 7	Epineurals, Epipleurals	Posterior to anterior	Carangiform	14 (DPF)	46
	Cypriniformes Cyprinidae	*Hypopthalmichthys molitrix* Silver Carp	117–124	1, 2, 3, 4, 6, 7	Epineurals, Epipleurals	Posterior to anterior	Carangiform	43 (DPF)	20,49,50
	Cypriniformes Cyprinidae	*Hemibarbus labeo* Barbel Steed	124 (122–128)	1, 2, 3, 4, 5	Epineurals, Epipleurals	Posterior to anterior	-	35 (DPF)	20,55
	Cypriniformes Cyprinidae	*Megalobrama amblycephala* Blunt snout bream	84–146	1, 2, 3, 4, 6, 7	Epineurals, Epipleurals	Posterior to anterior	Carangiform	20–24 (DPH)	34,47,49,51,63
	Cypriniformes Cyprinidae	*Carassius gibelio* Gibel Carp	79–87	1, 2, 3, 4, 5, 6, 7	Epineurals, Epipleurals	Posterior to anterior	-	14 (DPH)	25,49
	Cypriniformes Cyprinidae	*Carassius auratus* Crucian carp	78–83	1, 2, 3, 4, 5, 6, 7	Epineurals, Epipleurals	-	-	-	21
	Cypriniformes Cyprinidae	*Danio rerio* Zebrafish	83–86	1, 2, 3, 4	Epineurals, Epipleurals	Posterior to anterior	Sub-carangiform	20 (DPH)	41,57,62,80
	Cypriniformes Cobitidae	*Misgurnus anguillicaudatus* Pond loach	108 (99–113)	1, 2, 3	Epineurals, Epipleurals	Posterior to anterior	Carangiform	27 (DPH)	20,57
	Siluriformes Bagridae	*Pelteobagrus fulvidraco* Yellow catfish	8	1	Epipleurals	-	-	-	20
	Characiformes Characidae	*Salminus brasiliensis* Dorado	-	-	Epineurals, Epipleurals	Posterior to anterior	Carangiform	<30 (DPH)	67
	Characiformes Serrasalmidae	*Colossoma macropomum* Tambaqui	53.9 (mean)	1, 2, 3, 4, 5, 6	Epineurals, Epipleurals	-	-	-	43

TABLE 1 (Continued)

Superorder	Order; Family	Species; Common name	Total IB number	Morphology of IBs	Type of IBs	Ossification pattern	Swimming mode	Initial IB ossification	References
Protacanthopterygii	Salmoniformes Salmonidae	*Salmo salar* Atlantic Salmon	58–60	1	Epineurals	-	Sub-carangiform	-	29–31
	Salmoniformes Salmonidae	*Oncorhynchus tshawytscha* Chinook Salmon	60	1	Epineurals	-	Sub-carangiform	-	28
	Salmoniformes Salmonidae	*Oncorhynchus mykiss* Rainbow Trout	62	1	-	-	Sub-carangiform	-	30,31
Acanthopterygii	Synbranchiformes Synbranchidae	*Monopterus albus* Asian swamp eel	180–188	1	Epicentrals	Anterior to posterior	Anguilliform	30 (DPH)	57

Note: Morphology of IBs: (1) non-forked, (2) one-end-unequal-bi-forked, (3) one-end-equal-bi-forked, (4) one-end-multi-forked, (5) two-end-bi-forked, (6) two-end-multi-forked and (7) tree-branch. Initial IB ossification: days post fertilisation (DPF), days post hatch (DPH).

Atlantic salmon and rainbow trout, because the length of the bones varied with their position along the body, that is, salmon had longer IBs between vertebral centra 5 and 12 (anterior abdominal vertebral column) and trout had longer IBs more posterior, at positions 14–31. Schroeder et al. also found that trout had a lower maximum IB volume (11.07 mm^3) than salmon (16.85 mm^3),[31] which was measured from CT scans, albeit no voxel size was reported. Thus, how much of the thin bone volume remained undetected is unknown. Balaban et al. noted Chinook salmon IB lengths ranged from 30 to 55 mm.[28]

Contrary to cyprinids, which have epineurals all along the vertebral column including the caudal region (Figure 2), IBs in salmonids, where described, are found only in the abdominal region of the vertebral column embedded in the epaxial muscles (Figure 3). De Clercq et al. refined the identification of regionalisation in the vertebral column of juvenile Chinook salmon and reported the presence of IBs, specifically epineurals, in the postcranial and abdominal regions of the vertebral column (Figure 4).[53] A definition of vertebral column regions is useful when investigating the development of skeletal elements and the progression of deformities through freshwater and marine life stages of farmed salmon.

IBs are classified into seven different morphological shapes (Figure 5).[15,17,18,20,21] The morphology of IBs in cyprinids are more complex than in other species and with all seven morphological types present.[20,21,46,54,55] Epicentrals are quite distinct from epineurals and epipleurals, as they are simple, straight/rod-like and lack any forks or branches.[12,18] Epipleurals also tend to be simpler than epineurals, with the majority being non-forked.[17,20] Differences in IB morphology may be related to fish swimming mode and body shape,[13,41,56,57] which will be explained further in Section 4.3. Danos and Ward investigated the ossification of myoseptal tendons in teleosts and noted that the ossification of the epipleural tendons (EPT) and hypaxial lateral tendons (hLT) in an adult zebrafish gave the resulting IB a Y-shaped appearance.[13]

Currently, four techniques have been applied to study the morphology and number of IBs; (1) whole-mount staining, (2) X-ray imaging, (3) ultra-sound imaging and (4) anatomical analysis.[15,17,18,22,58] Staining fish bones with Alizarin red S is a good method for observing IBs and for investigating ossification patterns. Small, mineralised structures, for example, IBs, or the initial mineralisations in early life stages can be visualised by Alizarin red staining with high fidelity, especially when using fluorescent light which greatly enhances their visibility.[59,60] However, whole-mount staining, although not labour intensive can take a long time, particularly for large individuals. Radiography is a relatively quicker technique, which can be non-lethal and does not destroy the samples.[17] MicroCT data can also be used to obtain quantitative data of bone measurements, such as bone mineral content, bone mineral density, size, volume and thickness.[42,59,61] However, x-ray imaging and thus also MicroCT is limited by fish size and the degree of mineralisation of the thin IBs. Depending on the equipment used, the resolution/contrast of the image may be too low to detect IBs. Therefore, thin, or bones with low mineral content can remain undetected and generate a false negative result. Ultrasound is another non-invasive digital device, which is a relatively new

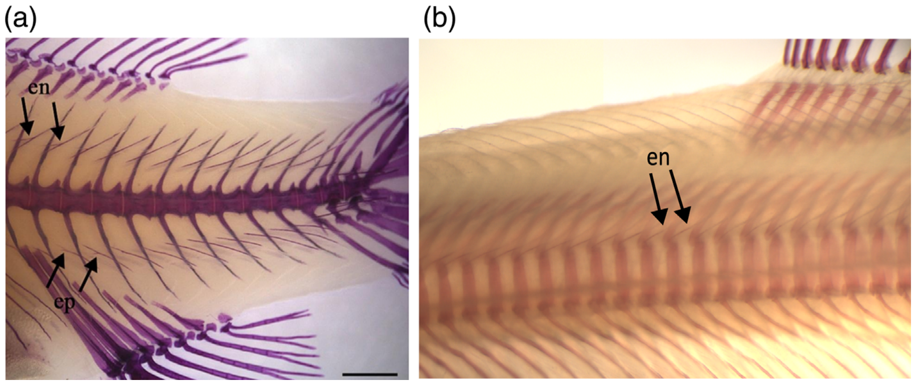

FIGURE 2 The intermuscular bones (epineurals [en] and epipleurals [ep]) of a gibel carp 26 DPH (Gan et al.[25] 2023) (a) and a Chinook salmon ~37 DPH (b).

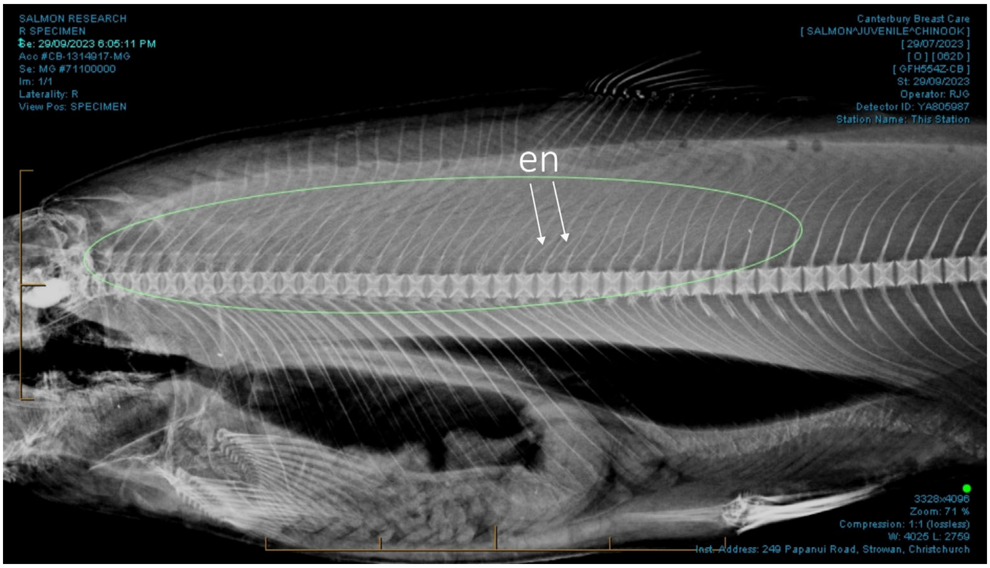

FIGURE 3 Mammography X-ray of a 60 g (~7 months post hatch) Chinook salmon smolt. Location of epineural (en) intermuscular bones circled in green.

technique for identifying the presence or absence of IBs on live fish.[58] Although ultrasound has a low resolution compared to x-rays, it offers a relatively simple and efficient way to detect IBs in live fish. Anatomical analysis can be used for accurate counts of IBs and does not require the use of expensive equipment, however it is very labour intensive and lethal for fish.[17,18,20,50]

4 | IB DEVELOPMENT

4.1 | Ossification process

IB development has been identified as a type of intramembranous bone formation.[13,18,34,62,63] There are three types of bone formation in teleosts: (1) intramembranous, (2) perichondral and (3) endochondral. These types of bone formation largely differ based on whether the bone has a cartilaginous precursor and where the bone matrix is deposited. Intramembranous bones form directly and without a cartilage anlage. For perichondral formation, the bone matrix is deposited around a preformed cartilaginous skeletal element. Endochondral bone formation follows perichondral bone formation and involves the mineralisation of hypertrophic cartilage and eventually its replacement by bone.[64]

In all types of bone formation previously dispersed mesenchymal cells form a condensation at the site of the future skeletal element, which is the primary resource from which the skeleton is built.[65] With perichondral and endochondral bone formation, the mesenchymal cell condensation differentiates into chondrocytes, that form the cartilaginous template that is eventually surrounded and/or replaced by bone. With intramembranous bone formation, mesenchymal cells differentiate directly into osteoblasts, that is, bone precursor cells.[65] Intramembranous bone formation is more common in teleosts than in mammals.[17] For example, in contrast to their osteichthyan ancestors and to tetrapods, teleost vertebral body centra develop through

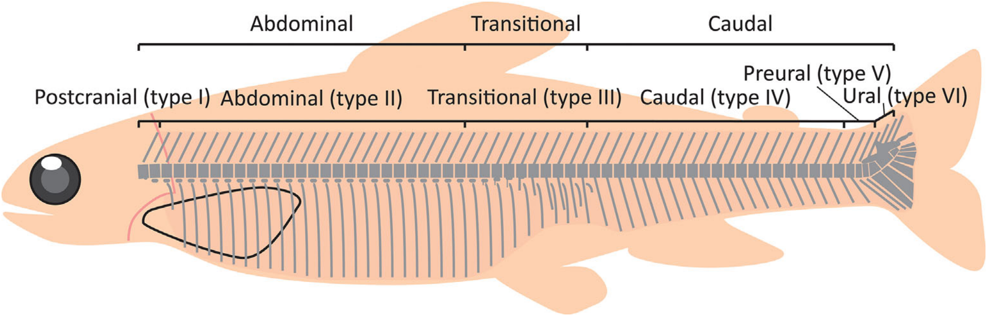

FIGURE 4 Definitions of the vertebral column regions and types of vertebrae in juvenile Chinook salmon, from De Clercq et al.[53] (2017). (Type I) Postcranial vertebrae: vertebral centrum, rudimentary or complete parapophyses and epineurals. (Type II) Abdominal vertebrae: vertebral centrum, parapophyses, ribs and epineurals. (Type III) Transitional vertebrae: four distinct types with variations of haemal arches, haemal spines and ribs. (Type IV) Caudal vertebrae: vertebral centrum, neural arch, neural spine, haemal arch and haemal spine. (Type V) Preural vertebrae: vertebral centrum, neural arch, modified neural spine, haemal arch and modified haemal spine. (Type VI) Ural vertebrae: vertebral centrum, stegural, epurals, uronurals and hypurals. Figure by Adelbert De Clercq.

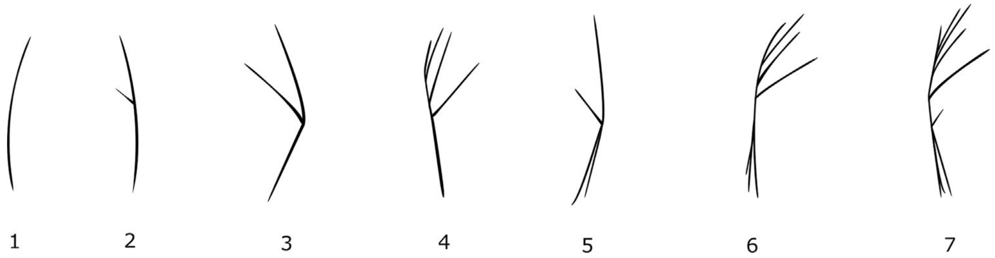

FIGURE 5 Types of intermuscular bone morphologies: (1) Non-forked, (2) one-end-unequal-bi-forked, (3) one-end-equal-bi-forked, (4) one-end-multi-forked, (5) two-end-bi-forked, (6) two-end-multi-forked and (7) tree-branch. Figure adapted from Li et al.[21] (2013).

intramembranous bone formation and without a cartilaginous precursor.[66] Morphological analyses of IBs in Atlantic salmon and Atlantic cod (*Gadus morhua*) revealed an active growth zone at the tip of the IB, consisting of a dense layer of osteoblasts surrounding the IB in both salmon and cod.[16] Osteocytes within the IBs were also observed. These results suggest that the extracellular matrix of the bone is synthesised and secreted by these osteoblasts in salmon and cod.[16]

It was raised in a recent review that although the formation of epineurals and epipleurals is believed to take place via intramembranous ossification, that is, without a cartilaginous phase, it is yet to be determined if epicentrals also form via intramembranous ossification.[17]

Two types of ossification patterns have been identified during IB development. Ossification from posterior to anterior regions of the body, which, for example, occurs in zebrafish, silver carp (*Hypophthalmichthys molitrix*), gibel carp, blunt snout bream and dorado (*Salminus brasiliensis*); and from anterior to posterior, which occurs in Japanese eel and Asian swamp eel (*Monopterus albus*; Table 1).[22,25,41,51,57,62,67] In cyprinids, IBs begin to ossify at approximately 14–24 days post hatch (DPH) and are present throughout the body within 2 months (Table 1).[25,36,46,51,57,63] Nie et al. reported that initial IBs were ossified in the anterior region at 30 DPH in the Asian swamp eel and in the posterior region at 27 DPH in the pond loach (*Misgurnus anguillicaudatus*) (Table 1).[57]

When investigating the development of the notochord in Atlantic salmon, Kryvi et al. noted the lateral ribs and IBs first appeared cranially at 560 Degree Days (d°; embryos hatched at approximately 500 d°) and completed development by 640 d°.[68] At 640 d° vertebral chordacentra were the last skeletal elements to appear, forming intervertebral joints, which were present throughout most of the vertebral column by 750 d°.[68] Conversely, Mattox et al. reported the sequence of ossification of the axial skeleton in *S. brasiliensis* to be: vertebral centra-neural arches-haemal arches-haemal spines-ribs-neural spines-parapophyses-supraneurals-IBs.[67] Bird and Mabee also reported IBs among the last to ossify within the axial skeleton of zebrafish, after centra, neural and haemal spines and arches, and ribs.[62]

Teleost ribs display both flexible and tough mechanical properties much like IBs,[69–75] however IBs and ribs have different developmental processes.[75] Ribs have been reported to develop via perichondral bone formation in silver carp[74] and *S. brasiliensis*.[67] Furthermore, comparative proteomic analysis shows that different key proteins are involved in the composition and development of ribs and IBs in blunt snout bream.[75] Similar to IBs, the neural and haemal spines of Chinook salmon were identified as intramembranous ossifications, except for the modified neural and haemal spines of the preural vertebrae, which were identified as perichondral ossifications.[53] Scarce information is available on the ossification process and pattern of development of salmonid IBs. Further research is essential on the mechanisms of IB development in salmonids, and the role IBs play in the musculoskeletal system of fish. Particularly, in the face of existing and novel bone deformities in farmed salmonids.

4.2 | Molecular mechanisms

A variety of techniques, including genomics, transcriptomics and proteomics, have been used to explore differentiation and development of IBs in teleosts.[22,42,48,63,75–77] Technological advances over the past 10 years have facilitated an increasing number of studies on the molecular mechanisms of IB development, particularly in cyprinids, which is covered in a recent review by Li et al.[22] The review highlighted the strong research focus towards cyprinids, with no mention of salmonids.

Key genes involved in tendon development, osteoblast differentiation and bone formation, have been shown to play a role in the development of IBs, for example *scleraxis* (*scx*) which is involved in tendon cell differentiation and maturation, muscle segment homeobox C (*MsxC*) which plays a role in the regulation of mesenchymal cell differentiation during bone formation, and runt-related transcription factor 2 (*runx2*), which is required for early bone cell differentiation.[17,22,36,42,77–79] Using in situ hybridization analysis, Lv et al. found that *MsxC* was expressed in the myosepta of Barbel steed (*Hemibarbus Labeo*) from 26 to 41 days after hatching, which coincides with the onset of IB ossification.[78] The authors concluded that *MsxC* may have a role in epithelium-mesenchyme interactions during IB formation.

Three genes involved in IB development, that is, *runx2b*, *scxa*, *bmp6*, have been identified using CRISPR-Cas9 zebrafish mutants.[36,76,80] Nie et al. successfully created Zebrafish *runx2b* mutants, that completely lacked IBs, while the growth and mineralisation of other bones were not obviously affected.[36] The authors reported that *runx2b* deletion did not have a significant effect on the average swimming velocity (as a measure of swimming performance) of zebrafish up to 90 days post fertilisation (DPF). *Runx2* is a well-known transcription factor, which is essential for osteoblast differentiation. Zebrafish have two orthologs of the *runx2* gene, that is, *runx2a* and *runx2b*. They found that deletion of *runx2a* had no effect on IB formation, whereas deletion of the *runx2b* gene completely inhibited IB formation. Additionally, *runx2* double mutants, that is, deletion of *runx2a* and *runx2b*, completely lacked IBs, but the animals' vertebral columns showed severe curvatures. The same research group recently successfully obtained *runx2b* mutants without IBs in blunt snout bream, an important aquaculture species in China.[42] Gan et al. investigated the role of *runx2b* in IB formation of amphitriploid gibel carp.[25] The study confirmed the decisive function of the transcription factor through the successful creation of IB-free and IB-less carp by disrupting the function of *cgrunx2b-A* and *cgrunx2b-B* simultaneously. *Cgrunx2b-A* and *cgrunx2b-B are* homeologs of *runx2b* in gibel carp. They found that the loss of either *cgrunx2b-A* or *cgrunx2b-B* alone did not affect normal IB development, but simultaneous disruption led to complete loss of IBs. The results suggest that *cgRunx2b-A* and *cgRunx2b-B* cooperatively regulate IB development and ossification in gibel carp. Interestingly, they reported that half of the IB-free individuals had subtle rib defects, for example, bifurcate and curved ribs. Similarly, Nie et al. found that the number of IBs in zebrafish *scxa* mutants was reduced by 70% compared with wild type, however ribs of the *scxa* mutants were seriously defected.[76] Scleraxis (*scx*) is a transcription factor involved in tendon cell differentiation by promoting type 1 collagen expression.[76] There are two *scx* orthologue genes in zebrafish, that is, *scxa* and *scxb*. It was reported that the *scxb* mutants had normal IBs and ribs, which revealed a functional differentiation between the two *scx* orthologues. These results are consistent with a study by Kague et al. who showed the importance of scleraxis genes in the growth and mineralisation of ribs in zebrafish.[70] The *scxb* mutants showed no obvious phenotype, whereas *scxa* mutants showed cranial tendon defects, abnormal rib mineralisation and/or fractures, reduced body size and muscle volume, and abnormal swim movement. It is not currently known why different rib phenotypes are produced, but these studies suggest that genes involved in IB development may also play some role in the development of ribs.

Two zebrafish mutants (IB-free and IB-less) have also been created by knocking out bone morphogenetic protein 6 (*bmp6*).[80] The knockout of *bmp6* caused the complete absence of IBs in zebrafish mutants, without affecting growth, muscle, or skeletal development.[54,80,81] In a recent study by the same research group, a new strain of crucian carp (*Carassius auratus*) without IBs was generated, by knocking out the two *bmp6* orthologs, that is, *bmp6a* and *bmp6b*.[82] Similar to the study by Gan et al.,[25] the authors found that the double knockout of *bmp6a* and *bmp6b* caused the complete elimination of IBs, whereas knockout of either *bmp6a* or *bmp6b* caused only a partial deletion of IBs. *Bmp6* is an important gene involved in vertebrate skeletal development.[82,83] The study showed that the knockout of *bmp6a* and *bmp6b* did not affect the growth and reproductive performance of the IB-free strain of crucian carp, which was consistent with the group's previous studies on zebrafish.[80,84] These studies have revealed important insights into the molecular mechanisms and essential genes involved in the development of IBs in cyprinids.

The production of IB-free fish has become of interest to breeders, due to the potential benefit for fish processing and consumer health.[25] Increasing research effort is being placed on breeding farmed cyprinid species without IBs via selective breeding and genome editing.[25,42,48,82] Selectively breeding cyprinids to reduce or eliminate IBs has been explored since the 1960s.[18,85–87] Cao et al. suggested Mirror carp as a candidate for selective breeding as they showed a relatively high coefficient of variation (CV) of number of IBs compared to the other two strains of common carp investigated.[45] Xiong et al. reported that the number of IBs in grass carp (*Ctenopharyngodon idella*)[48] and blunt snout bream[47] showed low level heritability, highlighting the difficulty of traditional selective breeding for reducing the number of IBs. However, their results revealed a high genetic correlation between body height and number of IBs in grass carp.[48] This is a growing area of study, which could be of benefit to the aquaculture industry. However, further exploration of IB-free fish should be accompanied by rigorous testing of potential impacts on health and welfare, for example, swimming performance, bone mineral density, volume and biomechanics, growth, and skeletal malformations. In addition, further research effort should be placed on filling the knowledge gaps of IB function in a range of teleost species, including salmonids.

4.3 | IB plasticity

The teleost skeleton is a plastic, dynamic organ which responds to its internal and external environment throughout all life stages.[88] Skeletal bones can remodel, reshape, resorb, and re-mineralise, compress and deviate, react to the availability of minerals and vitamins, and respond to the stress of inflammation, high temperatures, fast growth and increased mechanical loads.[11,89–96]

External mechanical forces, that is, locomotion and feeding, affect bone growth and shape in teleosts and can affect early bone development.[88] The ossification of tendons as a response to tensile load is a good example of how epigenetic factors can initiate, as well as accelerate and delay bone development.[88] A review by Danos and Staab[97] explored the idea that mechanical forces can lead to the formation of novel skeletal tissues, such as IBs and the kinethmoid (a median bone in the rostral skeleton that enhances the protractability of the upper jaw and is seen only in Cypriniformes). The authors hypothesized that mechanical forces play a role in kinethmoid chondrogenesis and ossification, given that the kinethmoid only develops once forces are present on the maxillae and that ossification occurs at ligamentous attachment sites.[97] They explored this idea further by highlighting that zebrafish IBs are homologous to the epineural and lateral tendons and proposed that when myoseptal tendons experience higher mechanical forces, due to either changes in body form or functional changes related to ecology, the tendons may ossify. This is in line with the observations of Wake[98] who emphasised that developmental processes are sufficiently plastic that bone will be produced where required for functional reasons.

The leading hypothesis is that the ossification of myoseptal tendons, connecting the muscles to the axial skeleton, that results in IB formation, is triggered by mechanical loading while swimming.[17,22,99] Gemballa and Treiber[32] revealed different functional adaptations of myoseptal tendons between teleosts with different swimming modes. The authors identified derived characteristics of the cruising specialist, the mackerel (*Scomber scombrus*), such as elongated myorhabdoid and lateral tendons and lack of epineural and epipleural tendons, which differed from the plesiomorphic characteristics of an accelerator specialist, the snakehead (*Channa obscura*). They speculated that elongated myoseptal tendons facilitate posterior force transmission and may be correlated with the long propulsive wavelengths of cruises.[32] Similarly, it has been proposed that different ossification patterns of IBs may be associated with the animals swimming mode.[41,56,57] For example, ossification of IBs from anterior to posterior in anguilliform swimmers and posterior to anterior in carangiform swimmers (Table 1). Furthermore, body shape, fin position, vertebral number and aspect ratio could be used as predictors of myoseptal ossification, as these morphometrics likely affect the distribution of stresses along myoseptal tendons.[13] Yao et al. investigated the difference in ossification patterns between zebrafish and Japanese eels.[41] Zebrafish are sub-carangiform swimmers (as are salmonids[100]), and thus, during swimming the amplitude of the undulations is predominately in the posterior half of their body. The thrust generated is mainly reliant on the posterior part of the body. On the other hand, Japanese eels are anguilliform swimmers, and therefore, during swimming at high speeds and acceleration, the majority of the body contributes to large amplitude undulations. Yao et al. showed that tail amputation of zebrafish and Japanese eels affected the ossification of IBs.[41] The length of IBs in the posterior area of both zebrafish and Japanese eels became significantly shorter in tail-amputated individuals, which consequentially had slower swimming speeds and were less active. IB lengths also became shorter in the anterior area, near the head, of zebrafish, but not Japanese eels. Furthermore, a positive correlation was found between the length of the tail amputated and the shortening of the IBs in zebrafish, that is, when a longer portion of the tail was amputated the IBs became even shorter. These results further indicate that the ossification of IBs has a relationship with mechanical force loadings that are induced by swimming.[13,41]

It is widely accepted that epigenetic factors elicit phenotypic plasticity in skeletal structures and that mechanical load induces bone formation.[88,101–109] Remodelling, which is the resorption of a skeletal tissue and replacement by the same or another tissue type, can turn bones from mineralised supporting tissue to a vital and metabolic organ.[108] Osteoclasts are the cells responsible for breaking down the mineral and matrix component of bone, that is, resorption. Bone remodelling occurs as part of normal and pathological processes. In teleosts the first main function of bone resorption, and only function in early development, is to facilitate bone growth.[110] Bone resorption is also required for tooth replacement and removing temporary skeletal elements such as the kype in male Atlantic salmon. Bone resorption also facilitates bone remodelling as an adaptation to increased mechanical load, or to remodel fused vertebral bodies.[11,111,112] A recent analysis using Nuclear Magnetic Resonance spectroscopy (NMR) found evidence of bone resorption in herring IBs.[99] In contrast, Nie et al. identified osteoclasts in the internal tissues of ribs but not IBs of Blunt snout bream and concluded that there is less bone remodelling and resorption in IBs compared to ribs.[75] Thus, remodelling in teleost IBs warrants further investigation.

5 | FURTHER IB INVESTIGATIONS

The unusual mechanical behaviour and developmental origin of IBs have gained recent research interest, particularly in regard to the biomimetic potential for inspiring innovative biomaterials with specific structure–function relationships.[14,99,113] One research group has focused on the mechanical properties of IBs in North Atlantic herring (*Clupea harengus*).[14,113] They showed that herring IBs displayed hard tissue characteristics including fracture strains reaching up to 22%, which is much higher than mammalian cortical bone, and soft tissue characteristics, such as a lower elasticity and high deformability, with a Young's modulus (a measure of relative stiffness) in the magnitude of light, non-mineralised tendons.[14] The authors suggested that this combination of soft and hard tissue characteristics was specific to IBs due to their development as ossified tendons. However, further investigation by the group revealed that the ribs of herring showed similar fracture strains. Additionally, they found that the ribs were stronger and more elastic, but had less surface mineralisation than IBs.[113] Their results highlighted the rare combination of stiffness, strength, and toughness of IBs, and ribs compared to other bone types.

Unsurprisingly, studies on IBs in aquaculture species have focused on their removal from the fillets.[16,28,30,31,114,115] The process of deboning IBs during processing involves pulling the bones, manually or automated, to remove them from the fillet.[18] Schroeder et al. found that removing Atlantic salmon IBs from the fillets required a significantly higher pulling force than removing rainbow trout IBs.[31] Balaban et al. found that pulling force of IBs depended on the size of the fish and length of the IBs in Chinook salmon, snapper (*Pagrus auratus*), and kahawai (*Arripis trutta*).[28] Suárez et al. conducted a microstructural and physicochemical analysis of collagen in IBs of bocachico fish (*Prochilodus magdalenae*), to investigate the effect of thermal treatments as a method of degrading IBs in fillets.[114] Like other bones, IBs consist of a collagen matrix, which eventually becomes mineralised with carbonated hydroxyapatite (HAP).[116] It is estimated that collagen represents approximately 30% of IB components and the rest are inorganic substances, for example, calcium and phosphorus.[114] When collagen degradation occurs, as a result of the thermal treatment, bone strength decreases. The authors found that bocachico IBs contain type I collagen, and collagen degradation initiates in the internal structure of IBs and then moves towards the external layer. The external layer showed no effects of the thermal treatment, and thus the authors concluded that it is necessary to fracture the IBs to promote heat degradation. Studies have also investigated the degradation of the connective tissue surrounding IBs in Atlantic salmon and cod, including enzyme activity and protein composition.[16,115] These studies highlighted the differences in bone attachment between Atlantic salmon and cod (i.e., connective tissue attaches the IBs to the muscle in Atlantic cod, while Atlantic salmon IBs are additionally embedded in adipose tissue), and the differences in enzyme profile and distribution in the connective tissue. The authors therefore suggested that IB removal should be tailored to the individual species.

6 | THE FUNCTION OF IBs

As indicated throughout this review, there is a significant lack of knowledge around the function of IBs in salmonids and other teleosts. On the one hand, we have studies showing that IBs play a significant role for muscle attachment and swimming, and there is active remodelling at the bone-muscle interfaces.[63] On the other hand, the discovery of IB-free individuals among tambaqui broodstock in a hatchery in Brazil,[58] and the evolutionary loss of IBs in more advanced teleost species, have raised questions regarding their functional significance.[36]

As discussed earlier in this review, there is ample evidence that IBs are ossified tendons, and thus their function is thought to be similar to myoseptal tendons, for example, transmitting force between muscle segments that allows for body movements, increasing body stiffness and constraining myomere deformation during contractions.[13,14,41] Other proposed roles of IBs include bilateral support for the muscles, enhancing body stiffness, propulsion during locomotion, storing and releasing energy, and strengthening connections between sarcomeres.[13,15,17,21,22,79] Tendon ossification in any animal is likely to have functional effects, because changing the tissue character from collagenous tendons to bone signifies a change in the material properties of the structure.[13] The unique soft and hard tissue characteristics of IBs described earlier further highlight their origins as tendons and likely functional role in the musculoskeletal system of fish, for example, changing the body's flexural stiffness. Furthermore, the plausible role that IBs have in swimming efficiency emphasises the potential impact of IB malformations on the health and welfare of farmed salmonids.

IBs have been gained and lost throughout teleost evolution, which shows that certain species can thrive without them. However, as we are currently not certain of IBs role in the skeletal system of the species in which they are present, it would be negligent to assume that they do not have a function, particularly considering the potential for direct and indirect fish health and welfare impacts of breeding species to be IB-free.

7 | SKELETAL HEALTH AND WELFARE OF SALMONIDS

A healthy skeletal system is fundamental to fish welfare and performance. Bones provide a site for muscle attachment, protect vital organs and provide support to the structural integrity of the body for normal development and locomotion.[93] Skeletal deformities, such as spinal scoliosis, can negatively impact swimming performance and recovery from exhaustive exercise in salmonids by imposing a significant metabolic cost.[10,117,118] Thus, poor skeletal health poses a significant risk to the ability of salmonids to survive and thrive in their environment, particularly in physically demanding offshore and higher energy farming environments.[119] Studies contributing to our understanding of the development and function of different skeletal structures and how production strategies influence their development are paramount to ensure skeletal health in aquaculture.

Vertebral column deformities also occur in wild populations of Atlantic and Pacific salmon, but the prevalence and/or severity can be higher under commercial farming conditions.[89,120,121] Fjelldal et al. reported compression, fusion, and dislocation as the most frequent types of vertebrae deformities reported in Atlantic salmon.[120,122] Compressions and fusions of vertebral bodies are also frequently observed in rainbow trout, animals that are commonly farmed as triploids. One study revealed that 22% of externally normal shaped individuals had discreet abnormalities in their vertebrae.[123,124] In contrast to Atlantic salmon, spinal curvatures (Lordosis, Kyphosis, Scoliosis [LKS]), which are rarely observed in Atlantic salmon, are prevalent in New Zealand farmed Chinook salmon, with up to 30% of harvest size fish affected in some farms.[125] Munday et al. showed that LKS was not present to any significant extent in the freshwater rearing phase and was only detected in <0.2% of smolt radiographed prior to sea transfer.[126] Both these reports suggested that LKS developed later in the production cycle. This was confirmed by Lovett et al. who found that the onset of curvature varied between individuals but initially occurred 8 months post-seawater transfer.[127] Skeletal deformities in fish have been extensively reviewed by Boglione et al.,[9,92] Baeverfjord et al.,[93] Fjelldal et al.[122] and Witten et al.[128]

Research into the skeletal deformities of salmonids has focused mostly on vertebral body and vertebral axis deformities. Nevertheless, lower jaw deformities have received some attention although mostly occurring in triploid Atlantic salmon.[129–131] Deformities of vertebral column associated elements, such as ribs and IBs, have received much less attention, despite their potential to cause animal welfare and product quality issues. For example, externally visible rib deformities were found in a broodstock population of rainbow trout in the Faroe Islands.[132] The deformed ribs were observed as small, thin bones, located inside the abdominal wall, detached from the vertebrae and in some severe cases the ribs would perforate the skin. Results from the genetic analysis and mutation modelling suggested that a dominant-mutation mechanism was the most likely cause of the observed deformities. The authors raised that they had difficulty in naming the observed bone deformities, and suggested that they could possibly be myorhabdoi IBs; however, there is no available literature on myorhabdoi in rainbow trout to support this idea.[132] Recent studies have also found an association between rib abnormalities and fractures and focal dark spots in Atlantic salmon fillets.[71,73] IB abnormalities have been detected at processing in Chinook salmon in New Zealand. The IBs presented as incompletely mineralised on both the proximal and distal ends and therefore the mineralised section of the bone was too short to emerge from the surface of the fillet after being filleted, which caused difficulties in IB extraction (Dr. Waddington, NZKS, personal communications). The aetiology of this abnormality is not yet understood. To our knowledge IB abnormalities have not been described in the literature, apart from a brief mention of a distinct ‘curly’ deformation of IBs in deformed Atlantic salmon in a study by Sullivan et al.[133] This curled deformation has also been described in the pleural ribs of brown trout (*Salmo trutta*) and the vertebral spines of Atlantic salmon with jaw deformities.[134,135] It is very plausible that the underlying mechanisms resulting in abnormalities of associative elements, such as IBs, could compromise skeletal health more broadly. More information is needed on the effect of skeletal deformities on salmon growth rates, survival, susceptibility to pathogens, social interactions, swimming and feeding behaviours to better understand the magnitude of the impact different deformity types have on salmon welfare and performance in different production environments.

8 | FUTURE RESEARCH REQUIREMENTS

Historically, the fine bones of fish, such as the IBs, ribs, spines and fin rays, have been overshadowed by larger skeletal elements in salmon musculoskeletal research. This may be attributed to limitations in available technology as the bones are very small and, if weakly mineralised, techniques such as standard x-rays or micro-CT may fail to visualise bones such as IBs. Patterson and Johnson believed that the IBs and ligaments of teleosts had been neglected.[12] The authors suggested that the major obstacle contributing to this neglect was above all the lack of a coherent and homogenous terminology. Although the terminology and information they pioneered, including the variation in IBs among teleosts, provided significant progress in this field, there is still much to discover. Achieving a consensus of terminology, for example, using the term ‘intermuscular bones’ and clarifying types of IBs in the literature and key search terms, following Patterson and Johnson,[12] will help the research community, aquaculture industry, and wider communities identify research outputs in this field which, as shown in this review, span across a range of different scientific disciplines.

The growing number of studies investigating the potential of eliminating or reducing the number of IBs could be of great interest to producers, as it may reduce processing costs, for example, specialised equipment and/or manual labour associated with IB removal. However, gaining a solid understanding of the function of IBs and thoroughly assessing the impacts of reducing or eliminating IBs on the fish's ability to thrive in their environment is critical before any such breeding strategies could be realised.

To effectively monitor and assess the skeletal health of farmed fish, we must understand how the bones form, mineralise, and remodel in the specific species, taking into account all parts of the musculoskeletal system, including the IBs, tendons and ligaments. While it is valuable to investigate these parts in isolation, it is not until we examine the body and its environment holistically that we can find comprehensive and sustainable solutions for issues such as skeletal deformities in farmed fish production. Embracing an interdisciplinary approach, such as the One-Welfare framework that recognises the interrelationships between animal welfare, human wellbeing, and the physical and social environment,[136,137] will advance future research, especially in the face of increased production, and rising sea temperatures.

This review has identified a significant gap in our knowledge of the salmon musculoskeletal system. That being the function, formation, onset, and pattern of mineralisation of IBs. Further research on characterising and understanding IB development and identifying the genetic and epigenetic risk factors affecting bone development and mineralisation, may identify mitigations which could improve overall skeletal health and the welfare of salmonids in aquaculture.

AUTHOR CONTRIBUTIONS

Brianne A. Lyall: Writing – original draft; writing – review and editing. **P. Eckhard Witten:** Writing – review and editing. **Chris G. Carter:** Writing – review and editing. **Matthew R. Perrott:** Writing – review and editing. **Jane E. Symonds:** Writing – review and editing. **Seumas P. Walker:** Writing – review and editing. **Zac Waddington:** Writing – review and editing. **Gianluca Amoroso:** Writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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