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Comparative Anatomy of the Dorsal Hump in Mature Pacific Salmon

Short title: DORSAL HUMP VARIATION IN PACIFIC SALMON

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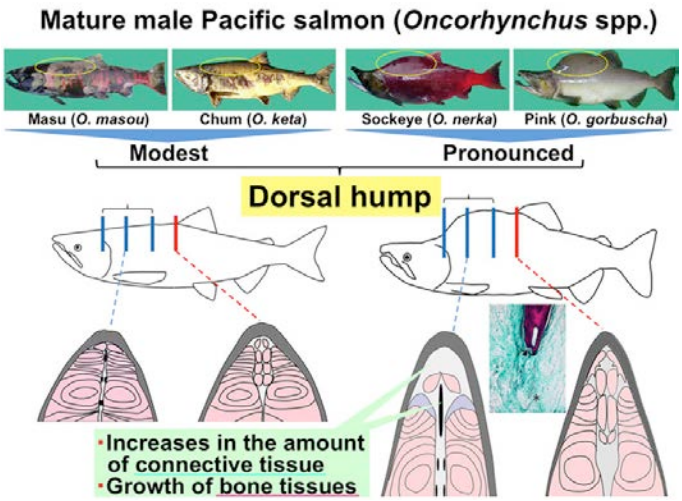

ABSTRACT

Mature male Pacific salmon (Genus *Oncorhynchus*) demonstrate prominent morphological changes, such as the development of a dorsal hump. The degree of dorsal hump exaggeration depends on the species in Pacific salmon. It is generally accepted that sockeye (*O. nerka*) and pink (*O. gorbuscha*) salmon develop most pronounced dorsal humps in mature male Pacific salmon. The internal structure of the dorsal hump in pink salmon has been confirmed in detail. However, the detailed structure in other species remains to be elucidated. In this study, the dorsal hump morphologies were analyzed in four Pacific salmon species inhabiting Japan, masu (*O. masou*), sockeye, chum (*O. keta*), and pink salmon. The internal structure of the dorsal humps also depended on the species; sockeye and pink salmon showed conspicuous development of connective tissue and growth of bone tissues in the dorsal tissues. On the other hand, masu and chum salmon exhibited less-pronounced increases in connective tissues and bone growth. Hyaluronic acid was clearly detected in dorsal hump connective tissue by histochemistry, except for in masu salmon. The lipid content in dorsal hump connective tissue was richer in masu and chum salmon than in sockeye and pink salmon. These results revealed that the patterns of dorsal hump formation differed among species, and especially sockeye and pink salmon develop pronounced dorsal humps through both increases in the amount of connective tissue and the growth of bone tissues. In contrast, masu and chum salmon develop their dorsal humps by the growth of bone tissues, rather than the development of connective tissue.

Keywords: Pacific salmon; secondary sexual characteristic; connective tissue; water content; lipid consumption.

INTRODUCTION

The dorsal hump of Pacific salmon, *Oncorhynchus* spp., is widely known as a male secondary sexual characteristic (e.g., Fleming and Reynolds, 2004). In particular, male pink salmon (*O. gorbuscha*) and sockeye salmon (*O. nerka*) develop particularly pronounced humps on their back before the dorsal fin (Burgner, 1991; Heard, 1991) among anadromous Pacific salmon species. The dorsal hump of male salmon is regarded as an indicator of social status in males, because male salmon with larger dorsal humps have more chances to be chosen as a breeding partner compared with males with smaller humps (Keenleyside and Dupuis, 1988; Quinn and Foote, 1994). In addition, Schroder (1981) suggested that a dorsal hump serves as a shield against attacks from other males in chum salmon (*O. keta*) around the spawning redd. 11-ketotestosterone, a specific androgen of fish, is inferred to be an inducer for expression of the secondary sexual characteristic including the dorsal hump in Chinook salmon (*O. tshawytscha*; Butts et al., 2012). Hence, it is believed that a larger dorsal hump in Pacific salmon provides the male with enhanced mating success during intrasexual competition at the spawning ground, but it also increases the risks of predation by bears (*Ursus* spp.) and of stranding in shallower habitats (Quinn et al., 2001).

Recently, we reported the internal structure of the pronounced dorsal hump in pink salmon (Susuki et al., 2014). It was found that dorsal humps of pink salmon are mainly occupied by fibrous and mucous connective tissue with high water content at the dorsal median septum and distal tip of the dorsal tissue, instead of the cartilage described previously by Davidson (1935). Therefore, it is now apparent that dorsal humps are not formed from cartilage, but it is still not clear why males of Pacific salmon species differ so greatly in their dorsal hump morphology (see Fig. 1). In spite of these obvious morphological differences among closely related species, researchers sometimes have regarded salmon dorsal humps as the same characteristic (e.g., Fleming and Reynolds, 2004) without distinction. It is easily assumed that male sockeye salmon, that form similarly pronounced dorsal humps as pink salmon, also exhibit such phenomena in the dorsal tissues. At the same time, it has also been speculated that masu (*O. masou*) and chum salmon, which generally form more modest dorsal humps than sockeye and pink salmon, exhibit less-pronounced development of connective tissue and bone growth; otherwise their dorsal humps could be formed in different ways. However, nothing has been determined about the internal structures of these dorsal humps.

Mature male pink salmon gain their larger dorsal humps through its high water content of connective tissue, which that may derived from hyaluronic acid that develops after sexual maturation (Susuki et al., 2014). It was also suggested that subcutaneous

adipose tissues in the dorsal tissue are replaced by fibrous and mucous connective tissue during dorsal hump formation. In general, lipids are regarded as the primary energy source for upstream migration in salmon (Hendry and Berg, 1999; Kinnison et al., 2003), so lipid consumption in the dorsal tissue is not surprising. However, the lipid consumption pattern might differ among species, and its difference might lead to the variation in developmental pattern of dorsal hump connective tissue, and finally, dorsal hump size and shape. Therefore, we examined the characteristics of dorsal hump connective tissues to gain a better understanding of dorsal hump development and variation in anadromous Pacific salmon.

In this study, we compared the internal morphologies of dorsal hump in mature males between the modest (masu and chum salmon) and the pronounced (sockeye and pink salmon) dorsal hump species in Pacific salmon inhabiting Northern Japan to address the differences. We examined the detailed internal structure and histochemical characteristics of the dorsal humps among these four species, as well as the water and lipid content in the dorsal hump connective tissues to confirm the relationship between them. Thereby, we attempted to understand the variation in dorsal hump morphologies in some Pacific salmon species.

MATERIALS AND METHODS

Animals

Anterior dorsal tissue of mature male Pacific salmon (*Oncorhynchus*) species inhabiting Hokkaido, Japan (masu, sockeye, chum and pink salmon; Fig. 1) were collected during their spawning seasons (Table 1); masu salmon were collected from the Yurappu River System, and live fish were provided by Yurappu hatchery (Oshima Salmon Propagation Association) in September 2013; sockeye salmon from the Bibi River population were provided by Chitose Field Station (Hokkaido National Fisheries Research Institute) in October 2013; chum salmon were collected from the Yurappu River in November 2013; and pink salmon from the Shibetsu River population were provided by Shibetsu Salmon Museum (Nemuro Salmon Propagation Association) in September 2013. Fish were selected as fully mature males through examining their spermiation. Most of these fish were obtained after being stripped of sperm in an artificial propagation program, so gonad weights and precise body weights of these fish were not obtained. Because these fish were sampled immediately after death, we are sure that there were no problems in the use of these fish in our analyses. For this reason, data on the body weight and gonad weight were omitted in this study. Artificial spawning handlings did not have any effects on dorsal hump morphology. Additionally, immature pink salmon were used and collected from the Northwest Pacific Ocean (43°N, 155°E) as part of a resource survey program by the training ship "Oshoro Maru IV" (Hokkaido University) in May 2013, to examine the change in dorsal subcutaneous adipose tissue. Fish were photographed on the left body side and body lengths were measured (fork length, FL). Then anterior dorsal tissues were isolated surgically.

Internal gross morphology

Dorsal hump sizes of the four species were compared from the photographs (Fig. 1). Internal gross morphology of the dorsal humps was also observed macroscopically. To understand their internal structure, dorsal humps of these animals were appropriately divided into four sections (described as 1/4, 2/4, 3/4, and 4/4 sections). After observing the internal structure, we regarded the 4/4 section as the posterior part of the dorsal hump and others as anterior parts according to their structural similarities (described in the results). Then the dorsal tissue was subjected to the histochemical and compositional analyses described next.

Histochemistry

Dorsal tissue samples were fixed in 3.7% formaldehyde in 0.1 M phosphate buffer (PB) for 48 h (except for the samples used in ALP staining below). Samples were then

decalcified with 5% formic acid, if necessary [except for the samples used in alkaline phosphatase (ALP) and tartarate resistant acid phosphatase (TRAP) staining]. Next, samples were dehydrated using a graded ethanol series, and embedded in paraffin (Histosec; Merck, Darmstadt, Germany) for cross-sectioning. Finally, tissue sections were prepared on a rotary microtome (approximate thickness: 8 μm: Leica RM2125 RTS; Leica, Nussloch, Germany) and mounted on glass slides.

Masson's trichrome staining was performed in accordance with the method of Witten and Hall (2003); deparaffinized sections were stained with Mayer's hematoxylin for 10 min, then washed in running tap water for 10 min and with distilled water. Second, sections were stained with xylidine ponceau for 2 min (mordant 0.5% xylidine ponceau 2R in 1% acetic acid and 0.5% acid fuchsin in 1% acetic acid). Next, the sections were treated with 1% phosphomolybdic acid for 4 min, rinsed, and stained with light green solution (2% light green in 2% citric acid, and we diluted this solution to 10 times with distilled water prior to use) for 90 sec. Finally, the sections were dehydrated with ethanol and xylene, and mounted with Entellan new. This staining procedure resulted in collagen fibers being stained a bright green in color by the light green.

To detect elastic fibers in the dorsal tissue, elastica van Gieson (EVG) staining was performed using the method of Susuki et al. (2014). Elastic fibers were indicated by dark-purple coloration with this staining.

For the detection of reticular fibers, staining (a modified method of Gordon and Sweet's silver staining; Gordon and Sweet, 1936) was conducted as follows; deparaffinized sections were treated with 0.5% potassium permanganate solution for 5 min, then washed in running tap water and distilled water. Subsequently sections were treated with 2% oxalic acid solution for 1 min, and washed in tap water and rinsed in distilled water for 5 min. Sections were then treated with alum iron solution for 50 sec. After washing in tap water for 5 min, and rinsing in distilled water for 2 min (three times), sections were stained with Tollens' reagent (ammoniacal silver nitrate solution) for 10 min. Next, sections were treated with ethanol for 1 sec and reducing reagent (a mixture composed of formalin stock solution: 2% alum iron solution: distilled water = 1: 2: 97), washed in tap water and distilled water. To replace silver particles with gold ones, sections were then treated with 0.1% gold chloride (III) solution for 3 h and washed in tap water. Then, sections were treated with fixing solution (5% sodium thiosulfate solution) for 5 min, washed in tap water for 5 min, and dehydrated and finally mounted with Entellan new. Reticular fiber was visualized by the deposition of gold particles (blackish staining).

Pre-embedding osmium tetroxide staining was performed to detect lipids in the dorsal hump connective tissue, by the modified Ciaccio method (Exbrayat, 2013; Susuki et al.,

2014). Lipid droplets in adipose tissue were indicated by osmium-black formation.

To detect acid mucopolysaccharides (including hyaluronic acid) in dorsal tissue, alcian blue and hematoxylin staining at pH 2.5 and pH 1.0 was performed in accordance with a standard procedure (Myers et al., 2008). In this study, nuclei were stained with Mayer's hematoxylin for 10 min. To assess the specificity of this method for detecting hyaluronic acid, adjacent sections were digested with 250 U/mL *Streptomyces* hyaluronidase (EC 3.2.1.35; Merck Millipore, Massachusetts, USA) in 100 mM acetate buffer for 2 h at 60°C before AH staining at pH 2.5. Acid mucopolysaccharides were stained light blue.

Osteoid staining was performed to differentiate between osteoid tissue and mineralized bone matrix tissue, in accordance with the method described by Ralis and Watkins (1992). Osteoid was stained blue, whereas mineralized bone matrix was indicated in red.

To detect activated osteoblasts, ALP staining was performed using a commercial staining kit (TRAP/ALP stain kit; Wako, Osaka, Japan). In this staining method, samples were fixed in 3.7% formaldehyde in 0.1 M PB for 16 h and decalcified with a solution that was composed of 100 ml 0.2 M ethylenediaminetetraacetic acid in 0.1 M phosphate buffer (pH 7.2) and 0.4 ml 1% zinc sulfate. Staining was conducted as described in the protocol; deparaffinized sections were washed in distilled water, rinsed in 0.1 M Tris-HCl buffer (pH 9.4) for 10 min, stained with ALP staining premix substrate (5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium chloride) solution for 1 h, then washed in distilled water for 1 min (three times). After nuclear staining by methyl green for 5 sec, sections were washed in distilled water, dehydrated with ethanol and xylene, and finally mounted with Entellan new. ALP-activity was indicated by a purple color. In parallel, TRAP staining was conducted with a same staining kit. Deparaffinized sections were washed in distilled water, stained with TRAP staining solution (kit content) for 60 min at 37°C. Then sections were washed in distilled water for 1 min (three times), stained with methyl green for 5 sec, dehydrated and finally mounted with Entellan new.

Immunohistochemistry

To detect the proliferating cells in dorsal humps, dorsal tissues were fixed in 4% paraformaldehyde in 0.1 M PB at 4°C for 24 h, and embedded in Histosec (Merck, Darmstadt, Germany). Then samples were sectioned with approximately 5 μm thickness. To inhibit endogenous peroxidase, deparaffinized sections were treated with 0.3% hydrogen peroxide in methanol for 30 min followed by a wash in running tap water for 5 min. Then sections were subjected to microwave antigen retrieval by modified method

of Cuevas et al. (1994). Sections were placed in a TPX staining jar (AS ONE, Osaka, Japan) filled with citrate buffer (pH 6.0), and irradiated with microwave oven for 5 min. After antigen retrieval, sections were cooled at room temperature for 30 min. Then sections were incubated with normal goat serum for 10 min, and subjected to immunohistochemical staining with 2 μg/ml monoclonal antibody against Proliferating Cell Nuclear Antigen (PCNA, PC10: Santa Cruz Biotechnology, dilution rate: 1:100) in accordance with the procedure described by Kudo et al. (2000). Then, Histofine Simple Stain MAX PO Multi (secondary antibody: Nichirei Biosciences, Tokyo, Japan) and 3,3'-diaminobenzidine were used for visualizing immunohistochemical reactive cells. Sections were washed in tap water, dehydrated with ethanol and xylene, and finally mounted with Entellan new.

Water and lipid contents

Dorsal hump connective tissue from each species was subjected to the quantification of water and lipid content. Water content was determined gravimetrically by drying samples at 103°C for 24 h (AOAC, 1990), and lipid content was determined by Folch's method (Folch et al., 1957). In brief, dorsal hump connective tissue was cut into small pieces and homogenized with Folch's reagent (chloroform/methanol = 2:1 v/v), then the lipid extract was collected and the solvent was evaporated using a rotary evaporator (AS ONE, Osaka, Japan).

Statistical analysis

For the comparisons of lipid and water content of each dorsal hump connective tissue, ANOVA and Scheffe's *F*-test were conducted. The SPSS version 20 (SPSS Japan Inc., Tokyo, Japan) was used in these statistical procedures. Body length (fork length: FL), the lipid and water content of each dorsal hump connective tissue is expressed as the mean and standard deviation.

RESULTS

Internal gross morphology

At the macroscopic level, the dorsal humps of four Pacific salmon species are shown in Figure 2. Connective tissue was observed at the midline of the dorsal hump in all species. The amount of connective tissue in the dorsal hump depended on the species; it was extremely small in modest dorsal hump species (masu and chum salmon: Fig. 2A), whereas pronounced hump species (sockeye and pink salmon) showed larger amounts (Fig. 2B). Anterior dorsal sections (1/4–3/4 parts) showed similar structures in each species, and the amount of connective tissue was clearly large in 3/4 parts in pronounced hump species. More detail, consistent with previous reports (Davidson, 1935; Nakamura, 1942; Susuki et al., 2014), pink and sockeye salmon exhibited crescent-shaped connective tissue above the dorsal posterior cones, whereas others did not. However, the crescent-shaped connective tissue of sockeye was slightly smaller than the pink salmon. Posterior dorsal sections (4/4 sections) were clearly distinguishable from other sections, and their internal structures were mostly consistent among the four species [Figs. 2A (ii) and 2B (ii)], and were occupied by large amounts of dorsal fin muscle, and a small amount of connective tissue at their midline regions. In pink salmon, crescent-shaped connective tissue was absent only in this section.

Histochemistry

Masson's trichrome staining demonstrated that all dorsal hump connective tissues were mainly composed of collagen fibers (Fig. 3), and its amount was quite large in pronounced hump species. In these connective tissues, elastic fibers were also richer in pronounced hump species, while in modest hump species they were very scarcely and observed only in the vicinity of blood vessels (Fig. 4). The amount of elastic fibers was slighter than that of collagen fibers, even in sockeye and pink salmon. Reticular fibers, which are included in typical adipose tissue, were very scarce in the dorsal hump connective tissues (Figs. 5A-D). However, reticular fibers were clearly observed in the dorsal tissue of immature male pink salmon (Fig. 5E).

Lipids in the dorsal connective tissues were visualized by the formation of osmium black (Fig. 6). In this staining, masu salmon showed the more abundant presence of lipid droplets in the dorsal hump connective tissues than sockeye and pink salmon. Some chum salmon showed abundant presence lipids, but fewer than that of masu salmon. Alcian blue and hematoxylin staining (pH 2.5) with hyaluronidase digestion was performed to detect hyaluronic acid in the dorsal connective tissues. It was shown that dorsal hump connective tissue in sockeye, chum, and pink salmon contained hyaluronic acid (Figs. 7B-D). Sockeye and pink salmon had a large amount of the

hyaluronic acid than chum salmon in the dorsal connective tissue. In contrast, only masu salmon did not show the presence of hyaluronic acid in this tissue (Fig. 7A). Alcian blue and hematoxylin staining at pH 1.0 demonstrated that sulfated mucopolysaccharides, which are regarded as a representative component of cartilage (see Witten et al., 2010), were not involved in development of these connective tissues (data not shown). These characteristics of connective tissues were consistent with ones for the posterior sections.

Osteoid staining showed numerous osteoblasts around the dorsal bone tissues in sockeye and pink salmon (Figs. 8B and D), on the other hand there were fewer osteoblasts in masu and chum salmon (Figs. 8A and C). In all species in this study, the bone tissues of the dorsal humps were well-mineralized (indicated by red staining; Fig. 8). Osteoblasts were larger in pronounced hump species than in modest hump species. Furthermore, in pronounced hump species osteoblasts were surrounded by mineralized and non-mineralized bone matrix, which arose from rapid bone growth. ALP staining detected numerous activated osteoblasts in the dorsal humps of pronounced hump species (Figs. 9B and D) but not in modest hump species (Fig. 9C). However, no osteoclasts were observed in the vicinity of bone tissue in any of the dorsal humps in this study. PCNA immunostaining also demonstrated that pronounced hump species had many proliferative fibroblasts and osteoblasts in their dorsal humps (Fig. 10). In contrast, modest hump species showed no PCNA-immunoreactive cells in their dorsal humps. Information obtained from the histochemical analyses is summarized in Table 2.

Water and lipid contents

Results of these analyses are summarized in Table 3. Pronounced hump species showed higher water content in their dorsal hump connective tissues than modest hump species (ANOVA, $P < 0.01$), and there were no significant differences between sockeye and pink salmon or between masu and chum salmon. Unlike water content, the lipid content in their connective tissues was higher in modest hump species, than one in pronounced hump species ($P < 0.05$). There were larger individual differences in water and lipid contents in chum and pink salmon than in masu and sockeye salmon (see Table 3). As expected, there was a strong negative correlation between water and lipid contents in these connective tissues (Pearson's coefficient $r = -0.832$, $P = 1.84 \times 10^{-5}$).

DISCUSSION

Spawning adult anadromous Pacific salmon from four species (i.e., masu, sockeye, chum and pink salmon) are found in rivers of Japan (Augerot, 2005). Our present study revealed the differences in internal morphologies of dorsal hump among these four species of Pacific salmon. Dorsal humps of these four species were classifiable into the modest-type (masu and chum salmon) and the pronounced-type (sockeye and pink salmon). Masu and chum salmon showed their highest points of the dorsal humps at the point of insertion of the dorsal fin, and they exhibited less connective tissue in the dorsal humps. On the other hand, the highest points of dorsal humps in some sockeye and pink salmon were located slightly anterior to the dorsal fin, where the largest amount of connective tissue was observed in their dorsal humps. It was suggested that the amount of connective tissue in dorsal tissues represents the difference in dorsal hump morphology among these species. To fully understand the variations in salmon dorsal hump morphology, it is necessary to conduct determinate research that includes non-Japanese Pacific salmon species, such as steelhead trout (*O. mykiss*), coho, and Chinook salmon in the future. However, it is expected that the structures of dorsal hump in these three species are similar with modest dorsal hump species (masu and chum salmon).

All dorsal hump connective tissues in this study were mainly composed of collagen fibers. Elastic fibers participated to a lesser degree but were richer in dorsal hump connective tissue of pronounced hump species than in the modest hump species. We performed a detection of reticular fibers in dorsal humps to demonstrate the changes occurred in salmon dorsal tissues. Reticular fibers were present in the adipose tissue of immature pink salmon, but they were scarce in the dorsal hump connective tissues of mature animals. Typically, collagen and elastic fibers gives appropriate strength and flexibility to loose connective tissue, whereas reticular fibers provide a supporting network to the structures and are usually found in typical adipose cells (Ushiki, 2002). Our previous study suggested that (fibrous and mucous) dorsal hump connective tissue is derived from subcutaneous adipose tissue in pink salmon (Susuki et al., 2014). Accordingly, it is strongly indicated by the present study, that it might be no longer adipose tissues, but was another connective tissue. As mature female pink salmon, male masu and chum salmon in this study contained more lipids in their dorsal hump connective tissues than male sockeye and pink salmon. Hence, we consider that the patterns of lipid mobilization in dorsal subcutaneous adipose tissues differed between sexes or among these species, and were more enhanced in male sockeye and pink salmon, whose dorsal humps were relatively larger than male masu and chum salmon. It could be important in male sockeye and pink salmon to deposit large quantities of lipids

in their dorsal subcutaneous adipose tissue for developing a larger dorsal hump at maturity, before they cease feeding, because it would be replaced by fibrous and mucous connective tissue at maturity. It was also confirmed that the water content of dorsal hump connective tissues affected dorsal hump exaggeration in these species. However, in this study, we could not clarify how dorsal subcutaneous adipose tissue changed into fibrous (and mucous except for masu salmon) connective tissue. It could be implied that adipose cells in dorsal subcutaneous adipose tissue might include dedifferentiated fat cells. It was shown that dedifferentiated fat cells occurred under mechanical stress in mammalian model (Matsumoto et al., 2008; Liao et al., 2015). Dedifferentiated mammalian fat cells showed a fibroblast-like appearance, and were further shown to transdifferentiate into osteoblasts and other cell types (Ullah et al., 2013; Liao et al., 2015). Dorsal hump formation involves numerous fibroblasts and osteoblasts, and our results may support the possibility that dorsal subcutaneous adipose tissue could include such cells and become a source of dorsal hump connective tissue in sockeye and pink salmon. In contrast, masu and chum salmon might lack, or show low, potentials of this dedifferentiation mechanism. If not, dorsal hump connective tissue could be newly formed by potentially presented fibroblasts, osteoblasts, chondrocytes and other cells, or transitions of them. More investigations, such as in vivo bioluminescence imaging techniques, detection of marker proteins and quantification of marker gene expression levels in salmon dorsal hump will be required to confirm this process.

It is generally accepted that the lipid content of an animal tissue will have a negative correlation with water content, which was also shown in the muscle tissue of some Pacific salmon species (e.g., Robinson and Mead, 1970; Kinnison et al., 2001; 2003; Bower et al., 2011). In this study, we also found a similar pattern in the dorsal connective tissues. Hence, we concluded that the decrease of lipid content in mature male salmon associated with the increase in water content in dorsal hump connective tissues, and this might serve as an important force for developing a larger dorsal hump. As described above, there was clear difference in lipid mobilization pattern in dorsal subcutaneous adipose tissues among species; 1) sockeye and pink salmon: lipid content in the dorsal hump was almost exhausted, and 2) masu and chum salmon: lipid content of adipose tissue was relatively conserved. Hyaluronic acid synthesis may also support larger dorsal hump formation through its strong water retention ability (Nakamura et al., 1993). On this point, masu salmon was the only exception among four species in this study. In molecular phylogenetic study, masu salmon was the most primitive species, and chum and pink salmon diverged most recently among Pacific salmon (Murata et al., 1996). Masu salmon lacks hyaluronic acid synthesis ability in the dorsal hump

connective tissue. So there may be a correlation between the acquisition of synthetic ability of hyaluronic acid and phylogenetic relationship in these species. However, chum salmon showed the slight presence of hyaluronic acid in dorsal hump connective tissue, but apparently developed smaller dorsal humps than sockeye and pink salmon. So it may be concluded that hyaluronic acid does facilitate larger dorsal hump formation through its water retention property, but itself alone does not always provide a crucial key to induce the expression of an pronounced dorsal hump. We considered it more important to induce an increase in connective tissue itself and pronounced bone growth, rather than hyaluronic acid synthesis, for gaining a larger dorsal hump for mature male salmon. Furthermore, chum salmon is considered to be a more derived (or evolutionary) species than sockeye salmon (Murata et al., 1996). Hence we could imply that there is little correlation between the expression of larger dorsal hump and phylogenetic relationship. Hyaluronic acid is biosynthesized from glucose which is one of the energy sources *in vivo* (e.g., O'Regan et al., 1994). Males in species that development a pronounced hump (i.e., sockeye and pink salmon) can store many hyaluronic acids in the hump, so they may have a surplus of energy for the upstream migration. Generally, pink salmon spawns at the lower reach of the river near the coast (Heard, 1991), and sockeye salmon spawns at the influent river of the large lake (Burgner, 1991). Both species were shorter than the modest-hump species (masu and chum salmon) in the distance between the spawning site and the open large space (i.e., coast and/or lake). This distance may be one of the important factors about the difference in hump development.

Osteoid staining showed prominent bone growth in pink and sockeye salmon. Furthermore, ALP staining detected activated osteoblasts, and PCNA immunostaining also showed the presence of proliferative fibroblasts and osteoblasts in the dorsal humps of pink and sockeye salmon. Therefore, it appears evident that the intensity of bone growth and cell proliferation in dorsal hump connective tissues play pivotal roles in dorsal hump formation. Sockeye and pink salmon undoubtedly express the most pronounced dorsal humps among Pacific salmon species, and our anatomical results provided a reasonable background to this knowledge. On the contrary, we did not detect any osteoclasts around the bone tissues in the dorsal humps (not only from TRAP staining but also from other stainings), although they were detected in kype skeleton of Atlantic salmon (*Salmo salar*), during not only its resorption but also its formation (Witten and Hall, 2002; 2003). From this result, we assumed that osteoclasts had little or nothing to do with dorsal hump formation in Pacific salmon species. It has been regarded that semelparous *Oncorhynchus* species have the most elaborate secondary sexual characters (Davidson, 1935; Tchernavin, 1938; Vladykov, 1954; 1962), and

invest a total energy into a single reproductive chance before a inevitable death (Fleming and Reynolds, 2004). It may be reasonable to assume that the dorsal hump formation in semelparous *Oncorhynchus* species has little or nothing to do with osteoclastic bone resorption, whereas kype development of iteroparous Atlantic salmon utilize the osteoclastic ability. This difference in post-spawning death and energy requirement for survival may result in the difference in bone resorption ability in salmonids. We assumed that the kype of Atlantic salmon could require the participation of osteoclasts for its regression and, therefore, been morphologically reversible to some extent (Roule, 1933; Witten and Hall, 2003), whereas dorsal hump formation in male semelparous Pacific salmon species does not necessarily involved osteoclasts, and has not been regarded as reversible because of their death after spawning (except for some resident-type male). Regulatory factors of dorsal hump formation remain to be addressed in future studies.

In conclusion, in this study we clarified the morphological differences of dorsal hump between modest-type and the pronounced-type in Japanese Pacific salmon species; (1) all dorsal humps in this study were mainly composed of collagen fibers, (2) hyaluronic acid participated in dorsal hump formation except for in masu salmon, (3) dorsal subcutaneous adipose tissue was clearly reduced and might be a source of dorsal hump connective tissue, (4) there was a strong relationship between lipid consumption and water retention in dorsal hump connective tissue, and (5) fibroblasts and osteoblasts play important roles in promoting pronounced dorsal hump formation. As expected, development of connective tissue and bone growth greatly contributed to dorsal hump formation, although the extent depended on the species. The findings in this study clearly explained how the dorsal hump morphology in Japanese Pacific salmon differed so much among species.

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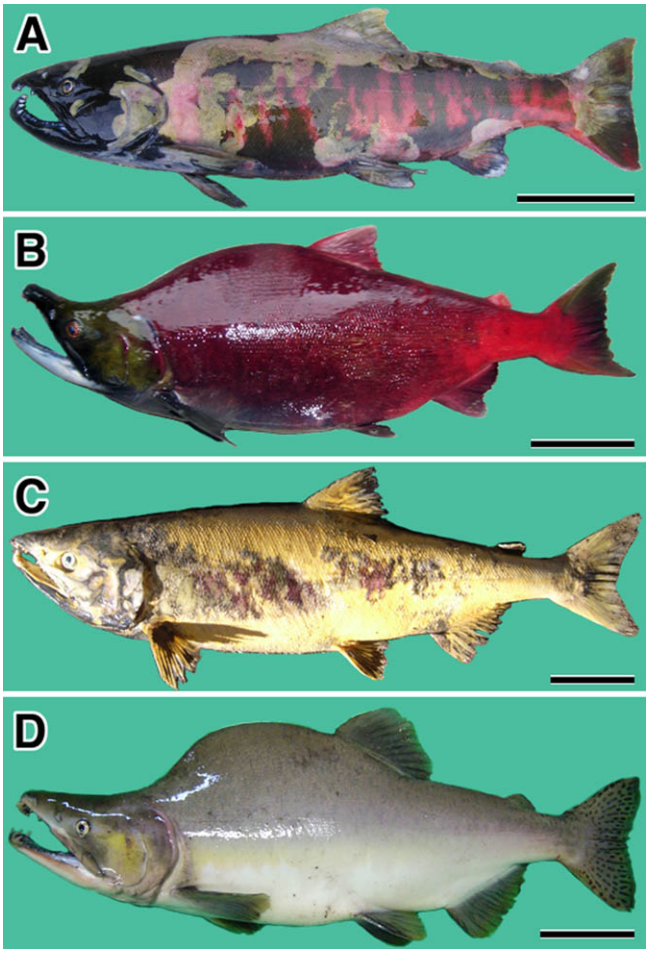

Fig. 1. External gross anatomy of mature male Pacific salmon species in Japan. (A) masu salmon, (B) sockeye salmon, (C) chum salmon, and (D) pink salmon. Scale bars: 100 mm.

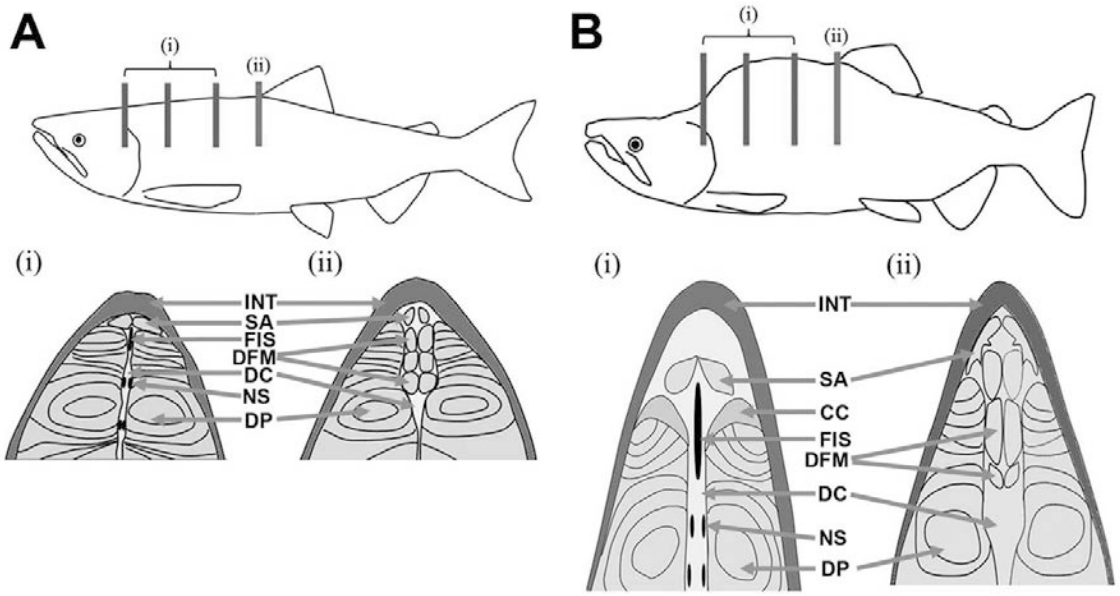

Fig. 2. Approximate external and internal gross anatomy of (A) masu and chum salmon, and (B) sockeye and pink salmon. Each dorsal hump was approximately divided into four sections, and the sections were examined at the order (1)–(4). In the cross-sections observed in this study, the internal structure of each dorsal hump is also shown. Abbreviations: CC, crescent-shaped connective tissue; DC, dorsal hump connective tissue; DFM, dorsal fin muscle; DP, dorsal posterior cone; FIS, free interneural spine; Int, integument; NS, neural spine; SA, supracarinalis anterior. As described in the results, obvious CR was observed only in pink salmon.

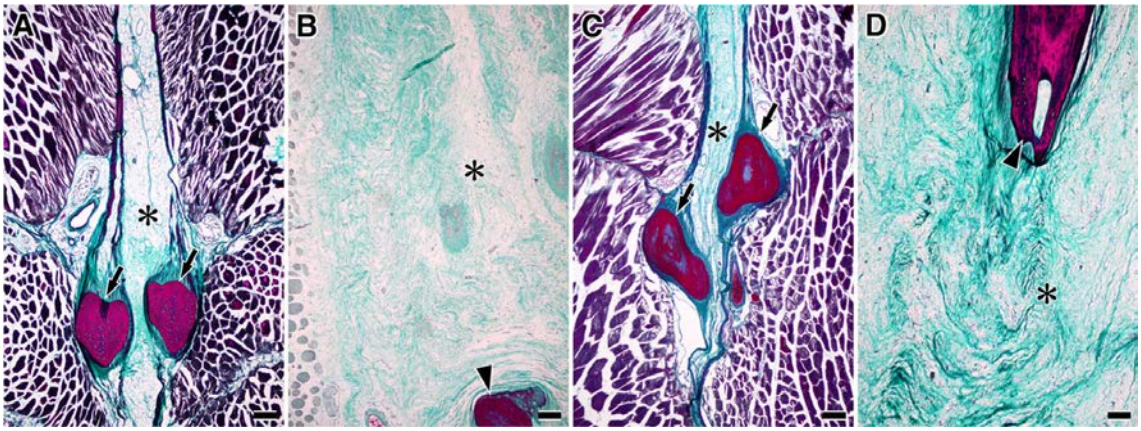

Fig. 3. Photomicrographs of cross-sections of dorsal tissues stained with Masson's trichrome stain. Each figure shows the dorsal midline region of (A) masu salmon, (B) sockeye salmon, (C) chum salmon, and (D) pink salmon. Arrows indicate neural spines. The arrowhead indicates a free interneural spine. Asterisks indicate each dorsal hump connective tissue. In this staining, collagen fibers are indicated by light green (pale green color). (A) Dorsal median septum of a mature male masu salmon. The amount of collagenous connective tissue is much smaller than sockeye and pink salmon. (B) Median septum of a mature male sockeye salmon. This figure shows the abundant presence of collagenous connective tissue around the free interneural spines. (C) Median septum of a mature male chum salmon. As in masu salmon, a small amount of collagenous connective tissue was observed. (D) Connective tissue in the median septum of a mature male pink salmon. As in sockeye salmon, pink salmon also showed abundant connective tissue in the dorsal hump. Scale bars: 200 μm.

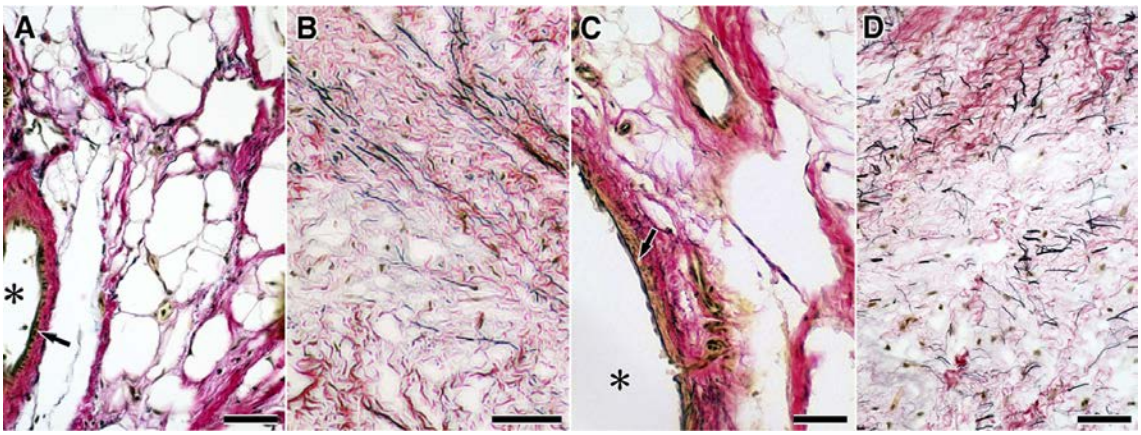

Fig. 4. Photomicrographs of cross-sections of dorsal tissues stained with EVG stain. Each figure shows the dorsal hump connective tissue in (A) masu salmon, (B) sockeye salmon, (C) chum salmon, and (D) pink salmon. Resorcin-fuchsin in this staining colored the elastic fibers purple-black. Asterisks and arrows indicate blood vessels and the tunica intima and media, respectively. (A) Dorsal median septum of a masu salmon. Elastic fibers were clearly observed in blood vessels but were scarce in the dorsal hump connective tissue. (B) Dorsal median septum of a sockeye salmon. Sockeye salmon showed a richer presence of elastic fibers than masu and chum salmon in the dorsal hump connective tissue. (C) Dorsal hump connective tissue of a chum salmon. As in masu salmon, most elastic fibers were detected only in blood vessels and not in connective tissue. (D) Connective tissue in the median septum of a pink salmon. This connective tissue showed a relatively richer presence of elastic fibers than masu and chum salmon, and as much as that of sockeye salmon. Scale bars: 50 μm.

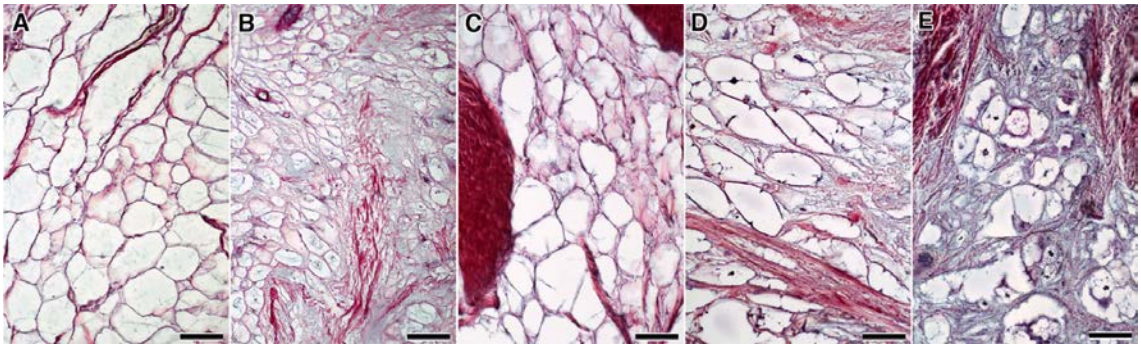

Fig. 5. Photomicrographs of cross-sections of dorsal tissues stained with reticular fiber stain. Each figure shows the dorsal median septum of a mature male (A) masu salmon, (B) sockeye salmon, (C) chum salmon, (D) pink salmon, and of an (E) immature male pink salmon. (A)-(D) Mature animals did not exhibit obvious reticular fibers (blackish staining) in their dorsal hump connective tissue, whereas (E) immature male fish clearly showed the presence of reticular fibers in their dorsal subcutaneous adipose tissue. Scale bars: 50 μm.

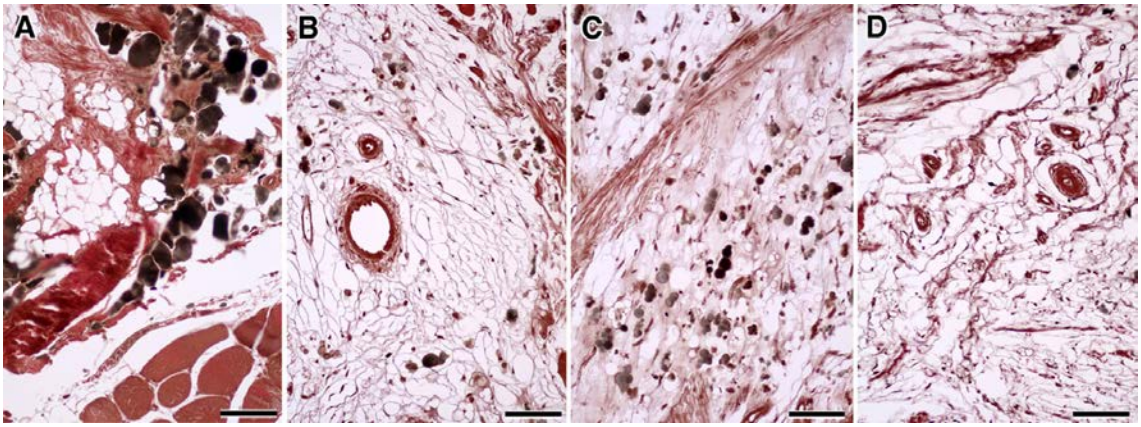

Fig. 6. Photomicrographs of cross-sections of dorsal tissue stained with pre-embedding osmium staining. This staining procedure detected lipids via osmium-black formation. Each figure shows the dorsal median septum of a mature male (A) masu salmon, (B) sockeye salmon, (C) chum salmon, and (D) pink salmon. (A) Median septum of a masu salmon. Lipids content (osmium-black) was observed abundantly in the dorsal hump connective tissue. (B) Median septum of a sockeye salmon. Lipid content in this dorsal hump connective tissue was evidently less abundant than that of masu salmon. (C) Median septum of a chum salmon. Some chum salmon showed abundant lipid content similar to masu salmon, while others showed less (as this figure shows). However, it was apparent that chum salmon contained more lipids in their dorsal hump connective tissue than sockeye and pink salmon. (D) Median septum of a pink salmon. As shown previously, there were negligible lipids droplets in dorsal hump connective tissue of pink salmon. Scale bars: 50 μm.

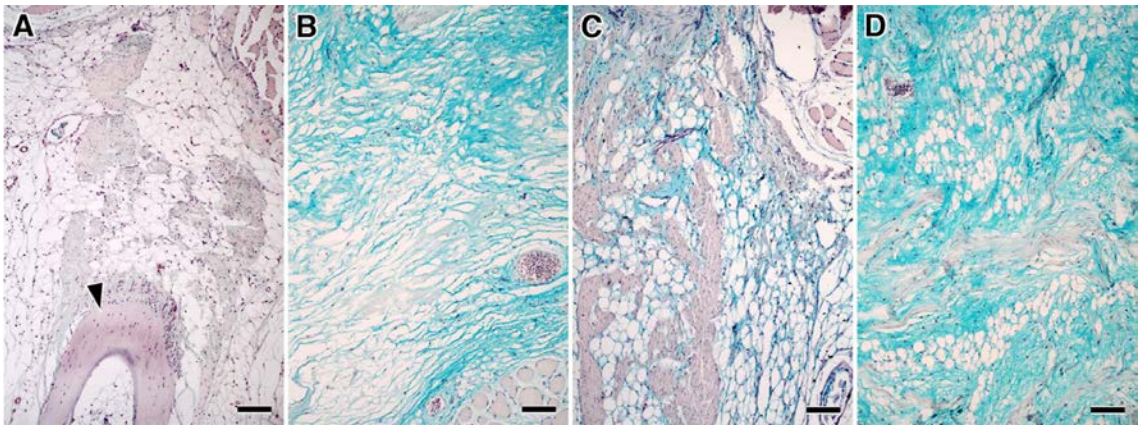

Fig. 7. Photomicrographs of cross-sections of dorsal tissues stained with Alcian blue and hematoxylin stain (pH 2.5). Each figure shows the dorsal hump connective tissue in (A) masu salmon, (B) sockeye salmon, (C) chum salmon, and (D) pink salmon. (A) Median septum of a masu salmon. The arrowhead indicates a free interneural spine. Unlike other species, masu salmon did not exhibit abundant presence of hyaluronic acid in the dorsal hump connective tissue (black asterisks). (B)-(D) Abundant hyaluronic acid was consistently detected in the dorsal hump connective tissues of Pacific salmon species in this study, except for masu salmon. White asterisks indicate the adipose tissue. Scale bars: 100 μm.

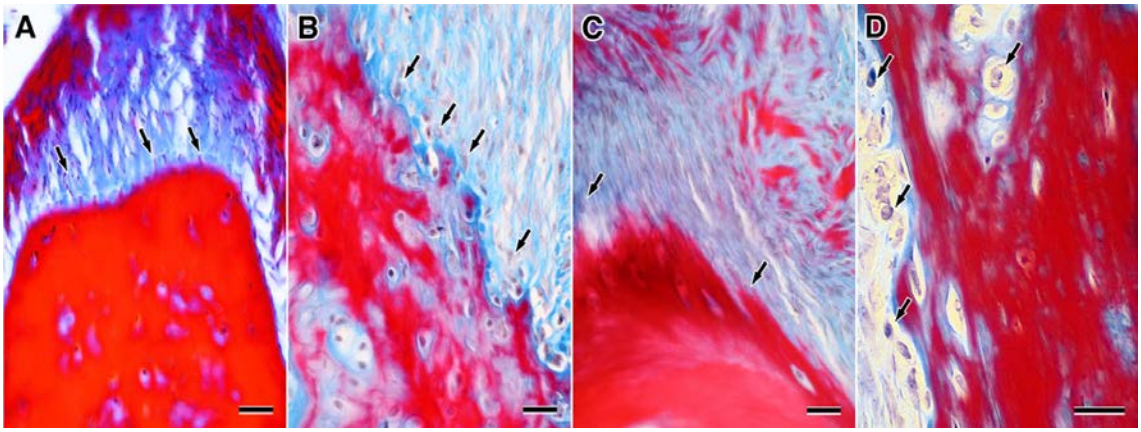

Fig. 8. Photomicrographs of cross-sections of dorsal tissue stained with osteoid staining (Ralis & Watkins, 1992). Well- and less-mineralized bone matrixes were stained red and blue, respectively. The arrows indicate osteoblasts around this bone tissue. (A) A free interneural spine of a male masu salmon. Osteoblasts in this dorsal tissue were smaller and less abundant than those of pronounced hump species (sockeye and pink salmon). (B) A free interneural spine of a male sockeye salmon. Osteoblasts were numerous and larger than those of modest hump species. These cells were also surrounded by mineralized and non-mineralized bone matrix, which indicates rapid growth of bone tissue. (C) A free interneural spine of a male chum salmon. As in masu salmon, small osteoblasts were observed, which were less obvious than those of pronounced hump species. (D) A free interneural spine of a male pink salmon. Pink salmon exhibited numerous, large osteoblasts, similar to sockeye salmon. Further, enclosure of osteoblasts into mineralized and non-mineralized bone matrix was also observed. Scale bars: 50 μm.

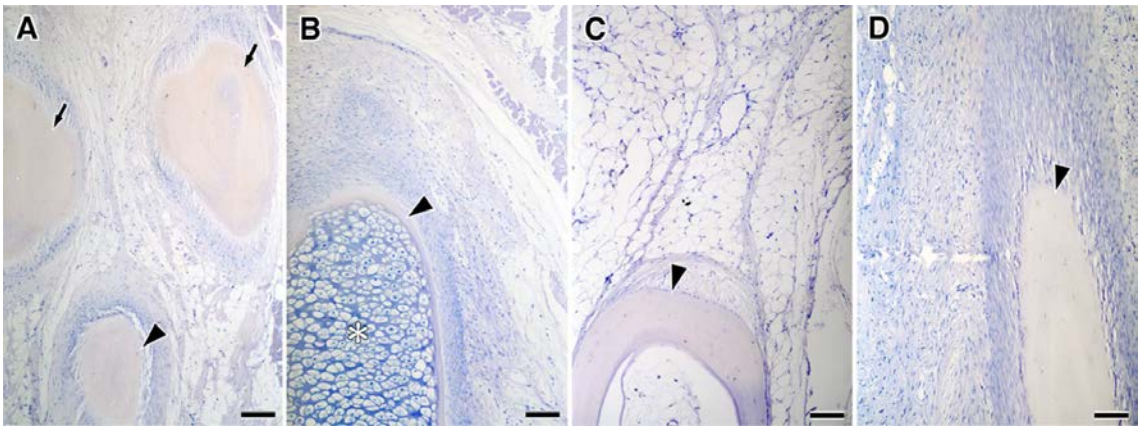

Fig. 9. Photomicrographs of cross-sections of dorsal tissue stained with a ALP staining. Arrows and arrowheads indicate neural spines and free interneural spines, respectively. White asterisk in (B) indicates epiphyseal cartilage of a free interneural spine. In this staining, activated osteoblasts (osteoblasts with high ALP activity) were stained pale purple. (A) A pair of neural spines and a free interneural spine of a male masu salmon. Activated osteoblasts were detected occasionally around these bone tissues. (B) A free interneural spine of a male sockeye salmon. Around this bone tissue, strong ALP activities of osteoblasts were demonstrated. (C) A free interneural spine of a male chum salmon. Chum salmon did not exhibit strong ALP activity of osteoblasts around the free interneural spine. (D) A free interneural spine of a male pink salmon. Pink salmon also displayed activated osteoblasts around the bone tissues, as well as sockeye salmon. Scale bars: 100 μm.

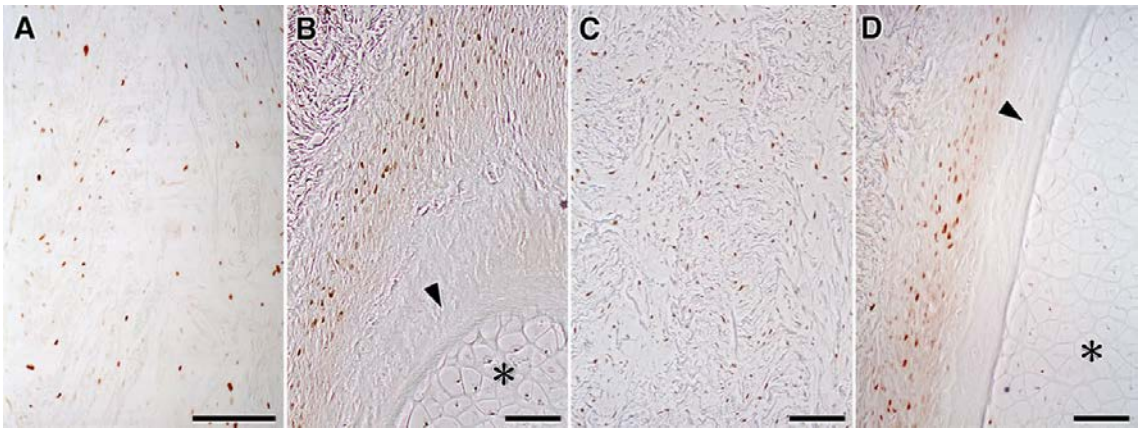

Fig. 10. Photomicrographs of cross-sections of dorsal tissue stained with PCNA immunostaining. Arrowheads indicate free interneural spines, and white asterisks indicate epiphyseal cartilage of free interneural spines. (A) Median septum and (B) a free interneural spine of a sockeye salmon. (C) Median septum and (D) a free interneural spine of a pink salmon. In pronounced hump species, PCNA-immunostaining (indicated by brown staining) was detected in scattered fibroblasts (A, C) and osteoblasts around bone tissues (B, D) in these dorsal humps. On the other hand, modest hump species showed no PCNA-immunoreactive cells in their dorsal hump. Scale bars: 100 μm.

Table 1. Data sets of the fish in this study. Fork length (mm) is expressed as mean ± S. D.

	Fork length (mm)	Number of samples	Population
Masu salmon	549±25.9	n=8	Yurappu River population
Sockeye salmon	533±54.6	n=8	Bibi River population
Chum salmon	803±18.1	n=8	Yurappu River population
Pink salmon	577±52.8	n=8	Shibetsu River population
Immature pink salmon	388±16.3	n=5	Unknown

Table 2. Results of histochemical observations of the dorsal hump of masu, sockeye, chum, and pink salmon.

	Masu	Sockeye	Chum	Pink
Dorsal hump connective tissue:				
Collagen fiber	+	++	+	++
Elastic fiber	+/-	+	+/-	+
Reticular fiber	+/-	+/-	+/-	+/-
Lipid droplets	+	+/-	+	+/-
Hyaluronic acid	-	++	+	++
Sulfated mucopolysaccharides	-	-	-	-
Crescent-shaped connective tissue	-	+/-	-	++
Bone tissue:				
Proliferative osteoblasts	+/-	++	+/-	++
Activated osteoblasts	+	++	+	++
Osteoclasts	-	-	-	-

-: not present, +/-: scarcely present, +: present, ++: abundant

Table 3. Water and lipid compositions of dorsal humps from masu, sockeye, chum, and pink salmon. Each value is expressed as mean ± S. D. Values with different letters show significant differences by Scheffe's *F*-test (a-bc and ab-c: $P < 0.05$, a-c: $P < 0.01$).

	Masu	Sockeye	Chum	Pink
Water content (%)	81.2±1.06 [a]	89.9±1.95 [c]	82.4±2.53 [a]	91.8±3.51 [c]
Lipid content (%)	5.26±0.19 [a]	2.44±0.88 [bc]	4.27±1.00 [ab]	1.66±1.24 [c]