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Connective Tissue is the Main Constituent of the Dorsal Hump, a Secondary Male Sexual Characteristic, in Pink Salmon (*Oncorhynchus gorbuscha*)

Short title: DORSAL HUMP MORPHOLOGY IN PINK SALMON

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ABSTRACT

Mature male Pacific salmon (Genus *Oncorhynchus*) develop a dorsal hump, as a secondary male sexual characteristic, during the spawning period. Previous gross anatomical studies have indicated that the dorsal humps of salmon are mainly composed of cartilaginous tissue (Davidson, 1935). However, the histological and biochemical characteristics of such humps are poorly understood. In this study, the detailed microstructures and components of the dorsal humps of pink salmon (*O. gorbuscha*) were analyzed using histochemical techniques and electrophoresis. In mature males, free interneural spines and neural spines were located in a line near to the median septum of the dorsal hump. No cartilaginous tissue was detected within the dorsal hump. Fibrous and mucous connective tissues were mainly found in three regions of the dorsal hump: i) the median septum, ii) the distal region, and iii) the crescent-shaped region. Both the median septum and distal region consisted of connective tissue with a high water content, which contained elastic fibers and hyaluronic acid. It was also demonstrated that the lipid content of the dorsal hump connective tissue was markedly decreased in the mature males compared with the immature and maturing males. Although, the crescent-shaped region of the hump consisted of connective tissue, it did not contain elastic fibers, hyaluronic acid, or lipids. In an ultrastructural examination, it was found that all of the connective tissues in the dorsal hump were composed of collagen fibers. Gel electrophoresis of collagen extracts from these tissues found that the collagen in the dorsal hump is composed of type I collagen, as is the case in salmon skin. These results indicate that in male pink salmon the dorsal hump is formed as a result of an increase in the amount of connective tissue, rather than cartilage, and the growth of free interneural spines and neural spines.

Keywords: pink salmon; dorsal hump; connective tissue;
secondary sexual characteristic; collagen

INTRODUCTION

Male anadromous Pacific salmon *Oncorhynchus* spp. develop a prominent dorsal hump, as a secondary sexual characteristic, at maturity (Davidson, 1935; Groot and Margolis, 1991). In Pacific salmon, larger dorsal humps are associated with enhanced mating success during intrasexual competition. In addition, Schroder (1981) reported that the dorsal hump plays a role in preventing attacks from other males in chum salmon (*Oncorhynchus keta*). Studies on pink and sockeye salmon have also suggested that developing a larger dorsal hump might increase a male's competitiveness (Keenleyside and Dupuis, 1988; Quinn and Foote, 1994). Among Pacific salmon species, male pink salmon (*O. gorbuscha*) and sockeye salmon (*O. nerka*) develop the most exaggerated dorsal humps (Vladykov, 1962; Burgner, 1991; Heard, 1991). However, there have only been a few anatomical studies on the dorsal humps of salmon, and much remains to be elucidated. Previous gross anatomical studies of mature male pink salmon (Davidson, 1935 in *Journal of Morphology*) and lacustrine sockeye salmon (Nakamura, 1942) reported that the dorsal hump is mainly composed of cartilage. These studies reported that as salmon enter rivers the amount of cartilaginous tissue increases around the dorsal median septum, above the dorsal posterior cones, and around the supracarinalis anterior. Based on these historical gross anatomical studies, many researchers believe that the dorsal hump is composed of cartilage (e.g., Fleming and Reynolds, 2004; Butts et al., 2012). This suggests that the fast growth of cartilage, e.g., via processes similar to those observed during the development of chondrosarcoma, induces salmon dorsal hump formation, but this is pure speculation because the detailed structure and histological properties of the dorsal hump have never been confirmed. Although previous gross anatomical studies of salmon dorsal humps are highly rated by salmon researchers, including us, these studies were based on macroscopic observations. So, it is necessary to reveal more details about the microstructure of salmon dorsal humps based on microscopic observations to gain a better understanding of their ecological function and the process responsible for their formation. In addition, an osteological study of *Oncorhynchus* species demonstrated the presence of both neural spines and free interneural spines in the anterior dorsal region (Hikita, 1962). However, the roles played by free interneural spines and neural spines in the formation of the dorsal hump are unclear. It is assumed that these bone tissues grow during dorsal hump development, but the growth patterns of these structures are largely unknown. Furthermore, it has been reported that the water content of the dorsal hump increases and its lipid content decreases as sexual maturity progresses (Robinson and Mead, 1970; Hendry and Berg, 1999). However, there is still a lot of uncertainty about these changes. Although cartilaginous tissue is probably responsible for the high water content of the dorsal

hump, the biochemical features of dorsal hump are unclear, e.g., it remains to be confirmed whether it contains mucopolysaccharides. In addition, upriver-migrating salmon consume lipids as their primary energy source, so reductions in their lipid stores during maturation would not be surprising, as described in various biochemical studies (e.g., Hendry and Berg, 1999; Crossin et al., 2003; Bower et al., 2011). However, most of these studies focused on the muscle tissue in the dorsal hump as a source of lipids, rather than cartilaginous tissue. Accordingly, there are various questions regarding the histological reason for the decrease in the lipid content of the dorsal hump as well as whether the abovementioned increase in its water content is related to the development of cartilage.

The main goal of the present study is to examine the detailed microstructures of the dorsal humps of salmon and clarify the changes that occur in these structures during sexual maturation. For this purpose, we performed microscopic anatomical analyses of the anterior dorsal tissues of pink salmon at different stages of maturation. In addition, to biochemically characterize the dorsal hump we examined collagen extracts from various regions in the dorsal hump by electrophoresis. Thereby, we attempted to clarify the internal microstructure of the dorsal hump in male pink salmon.

MATERIALS AND METHODS

Animals

We examined the anterior dorsal tissue of pink salmon, *Oncorhynchus gorbuscha*, at different stages of secondary sexual characteristic development; i.e., immature, maturing, and mature salmon. Information about the external morphology of the fish and their collection are shown in Fig. 1 and Table 1. The immature fish were collected from the Northwest Pacific Ocean (43°N, 155°E) by anglers aboard the training ship Oshoro Maru (Hokkaido University) in May 2010. Maturing fish were captured by set nets in the coastal waters off Shibetsu, eastern Hokkaido, in September 2010 and landed at Shibetsu fishing port. Mature spawning fish from the Shibetsu River were provided by the Nemuro Salmon Propagation Association in September 2010 and September 2012. Using these fish, we microscopically investigated the differences in dorsal tissue structure between the sexes and each stage of maturation.

We measured the body weight and length (fork length: FL) of each fish and determined their sex via a visual inspection of the gonads. We isolated anterior dorsal tissue and used it in the anatomical analyses. For the water content and collagen analyses, dorsal tissue was also dissected from the mature males and stored at -30°C until use.

Macroscopic anatomy

Figure 2 includes a photograph and schematic diagram of a cross-section of a dorsal hump. At the macroscopic level, the findings regarding the internal structure of the dorsal hump obtained in this study were consistent with those reported in previous studies (Davidson, 1935; Nakamura, 1942).

To visualize the bony tissues in the dorsal hump, cleared and double stained skeletal specimens were prepared according to the method of Dingerkus and Uhler (1977) with slight modifications: dorsal tissue samples from pink salmon were fixed in 33 g/L formaldehyde in distilled water (DW) for more than 1 week, rinsed in tap water for 1 day, and then immersed in Alcian blue solution [0.1 g/L Alcian blue 8GX (Wako, Osaka, Japan) in 760 mL/L ethanol and 200 mL/L glacial acetic acid] for 2 days. The specimens were then hydrated in a descending ethanol series and DW for 1 day, before being cleared in a solution of 10 g/L trypsin (EC 3.4.21.4, Difco, Franklin Lakes, NJ, USA) in sodium tetraborate solution (7.8 g/L sodium tetraborate in DW) for 1-2 weeks. Next, the specimens were immersed in a solution of 1 g/L alizarin red S in 5 g/L potassium hydroxide for 2 days, dehydrated in a graded 5 g/L potassium hydroxide: glycerol series (3:1, 1:1, 1:3), and then stored in pure glycerol containing 0.05 g/L thymol powder to aid tissue preservation.

Basic histology

Dorsal tissue samples were fixed in Bouin's solution for 72 h, dehydrated using a graded ethanol series, and embedded in paraffin (Histosec; Merck, Darmstadt, Germany) for cross-sectioning. Then, tissue sections (approximate thickness: 8 μm) were prepared on a rotary microtome (Leica RM2125 RTS; Leica, Nussloch, Germany) and mounted on glass slides.

For hematoxylin and eosin (HE) staining, deparaffinized sections were stained with Delafield's hematoxylin for 20 min. After being washed in DW, the sections were then stained with 5 g/L aqueous eosin for 10 min, before being dehydrated in a graded alcohol series and xylene and then mounted with Entellan new (Merck, Darmstadt, Germany). All of the light microscopic observations including the histochemical examinations were performed using a microscope (Eclipse 80i, Nikon, Tokyo, Japan) equipped with a digital camera (EOS 5D, Canon, Tokyo, Japan).

For Masson's trichrome (MT) staining, deparaffinized sections were stained with Mayer's hematoxylin for 6 min. Subsequently, the sections were treated with 10 g/L aqueous phosphotungstic acid and 10 g/L aqueous sodium citrate for 30 sec each and then stained with 10 g/L acetic acid containing 10 g/L Biebrich scarlet for 30 sec. Next, the sections were treated with aqueous mordant phosphotungstic acid and phosphomolybdic acid (each 25 g/L) for 2 min each and then stained with aniline blue solution (25 g/L acetic acid containing 25 g/L aniline blue) for 4 min. Finally, the sections were rinsed in 10 mL/L acetic acid, dehydrated with ethanol and xylene, and mounted with Entellan new. This staining procedure resulted in collagen fibers being stained a bright blue color by the aniline blue.

To detect elastic fibers in the dorsal tissue, elastica van Gieson (EVG) staining was performed according to the following standard procedure: deparaffinized sections were stained with resorcin-fuchsin solution (Muto, Tokyo, Japan) for 90 min, differentiated in ethanol, and washed with tap water. The sections were then stained in Weigert's iron hematoxylin for 10 min, washed with DW, and differentiated in acid alcohol. Finally, the sections were stained in van Gieson solution for 3 min, dehydrated with ethanol and xylene, and mounted with Entellan new. The EVG staining resulted in the elastic fibers being stained black by the resorcin-fuchsin.

To identify the main cell types present in each dorsal hump component, we examined the dorsal hump tissue samples with a transmission electron microscope (TEM). Ultrastructural samples were prepared as follows: small dorsal tissue samples were fixed in a mixture of 20 g/L paraformaldehyde and 25 g/L glutaraldehyde in 0.1 M phosphate buffer at 4 °C. They were then postfixed in a solution of 10 g/L osmium

tetroxide in the same buffer for 2 h at 4°C before being dehydrated in a graded series of acetone and finally embedded in epoxy resin. Semi-thin sections of dorsal tissue were also prepared on an ultramicrotome (Leica EM UC7 RT, Leica, Vienna, Austria) and stained with a mixture of 5 g/L methylene blue, 5 g/L azure II, and 5 g/L borax in DW. These sections were examined using light microscopy. Ultrathin sections of the dorsal region were also prepared on the abovementioned ultramicrotome and stained with 25 g/L samarium acetate and 26.6 g/L lead citrate. Then, they were examined with a JEM-1011 TEM (JEOL Ltd., Tokyo, Japan) equipped with a digital camera (iTEM, Olympus, Münster, Germany).

Histochemical examinations of the bony and connective tissue

Pre-embedding osmium tetroxide staining (modified Ciaccio method; Exbrayat, 2013) was performed to detect lipids in the dorsal tissue: tissue samples were rinsed in DW and 0.1M phosphate buffer at 4°C, incubated in 10 g/L osmium tetroxide and 50 g/L potassium dichromate solution for 16 h at 4°C, and embedded in Histosec. Then, the sections were subjected to HE staining, as described above.

To detect acid mucopolysaccharides (including hyaluronic acid; HA) in dorsal tissue, Alcian blue and hematoxylin (AH) staining was performed at pH 2.5: tissue samples were fixed in 33 g/L formaldehyde in DW and embedded in Histosec. The samples were then decalcified with 50 g/L formic acid if necessary, before being sectioned into 8 μm thick slices and mounted on glass slides. Deparaffinized sections were treated with 30 mL/L acetic acid for 3 min, before being stained with Alcian blue solution (30 mL/L acetic acid containing 10 g/L Alcian blue, pH 2.5) for 30 min and rinsed in 30 mL/L acetic acid for 3 min. Next, the sections were stained with Mayer's hematoxylin for 10 min, dehydrated with an absolute ethanol and xylene series, and mounted with Entellan new. AH staining was also performed at pH 1.0, using 0.1 N HCl instead of 30 mL/L acetic acid (Myers et al., 2008). To assess the specificity of this method for detecting HA, 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 was performed at pH 2.5.

Osteoid staining was performed to differentiate between osteoid tissue and mineralized bone matrix tissue, according to the method described by Ralis and Watkins (1992).

Analysis of water content

The water content of each dorsal hump component was determined and compared to clarify the source of the high water content of the dorsal hump. Samples from the three

major tissues that comprise the dorsal hump [muscle, skin, and loose connective tissue (distal region; wet weight: 5-10 g)] were dissected from mature males (n=3). Water content was determined gravimetrically by drying samples at 103°C for 24 h and measuring their water loss (AOAC, 1990).

Determination of the type of collagen in dorsal hump tissue

To determine the levels of each collagen subtype in the dorsal hump, biochemical analyses of collagen extracts from dorsal hump tissue were performed by electrophoresis. All of the crude collagen extraction procedures were performed at 4°C. Three regions of non-skeletal and non-muscular tissue were dissected from the dorsal humps of mature pink salmon (the median septum, the distal region, and the crescent-shaped region). Skin from mature male pink salmon was also dissected as a type I collagen-containing control tissue, as reported in *Oncorhynchus* species previously (Matsui et al., 1991). Each tissue was washed with DW and cut into small pieces (5×5 mm). Fat was removed with ethanol three times. After being washed with DW, the tissues were continuously stirred in HCl (pH 2.0) containing 1 g/L porcine pepsin (EC 3.4.23.1; 1:10,000, Wako Pure Chemical Industries, Osaka, Japan). Then, the mixtures were centrifuged at 2,200 *g* at 4°C for 1 h, and the resultant supernatants were collected. The crude collagen in each supernatant was precipitated by adding NaCl to a final concentration of 1 M and then centrifuged at 2,200 *g* for 90 min. The resultant precipitates were dissolved in HCl (pH 2.0). This process was repeated three times to purify the collagen. Each collagen-containing solution was dialyzed against a 50 times volume of DW for 24 h, and the resultant dialysates were lyophilized and stored at -30°C until use. To determine collagen subtype, 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970).

Statistical analysis

Statistical calculations were performed with IBM SPSS Statistics 20.0 (IBM Japan, Tokyo, Japan). ANOVA and Scheffe's *F*-test were used for inter-group comparisons of the length and diameter of the free interneural spines, as well as comparisons of the water contents of the samples of the three dorsal hump tissue types obtained from the mature males. Body length (FL), the length and diameter of the free interneural spines, the angle between each interneural spine and the body axis, and the water content of each dorsal hump component are expressed as mean and standard deviation values.

RESULTS

Macroscopic anatomy

The dorsa of the cleared and double stained skeletal specimens contained two bone tissues, free interneural spines and neural spines (Fig. 3). It was demonstrated that in males the free interneural spines extended distally and vertically relative to the body axis during maturation. In the mature males, the length and diameter of the longest and thickest free interneural spines were significantly larger than those of the other groups (Scheffe's *F*-test; d.f.=3, 20; $P < 0.001$; Table 2, Fig. 3C). However, the free interneural spines of the female fish did not elongate during sexual maturation (Fig. 3D). Neural spines also seemed to be involved in the development of the dorsal hump, but the elongation pattern of these spines varied greatly among individual males. In the immature period, the neural spines took the form of two symmetrical spine bones (Fig. 3A), and no longitudinal splitting of the free interneural spines was observed.

Microstructure of the salmon dorsal hump

The gross anatomical observations of the internal structure of the dorsal hump obtained in this study were similar to those described in previous reports (Davidson, 1935; Fig. 2). However, cartilaginous tissue was not observed in the dorsal tissue of any fish during light microscopy (Figs. 4 and 5). Instead, connective tissue, including a large amount of adipose-like tissue, was observed in the corresponding regions. In the immature and maturing males, a small amount of collagen fibers were detected in these connective tissues by MT staining (Figs. 6A-B). In the mature males, connective tissue, including adipose-like tissue, was observed throughout the distal region and median septum (Figs. 4C-D and 5C-D). In addition, in the crescent-shaped region connective tissue was observed above the dorsal posterior cones (Figs. 4E and 5E). It was demonstrated that these connective tissues were mainly composed of collagen fibers (Figs. 6C-E). Furthermore, EVG staining detected elastic fibers in the median septum and distal region (Figs. 7B-D and 7F), as well as in blood vessels (Fig. 7A). However, no elastic fibers were present in the crescent-shaped region (Fig. 7E).

These connective tissues did not exhibit the characteristics of cartilage (e.g., a territorial matrix, avascular tissue, and rounded chondrocytes) at the light microscopic level. In the mature females, connective tissue similar to that observed in the mature males was found in the median septum and the distal region, but the females possessed much less of this tissue than the mature males (Figs. 4F, 5F, 6F, and 7F). In light microscopic observations of semi-thin sections, fat-containing adipocytes were indicated by osmium-black formation, and blood vessels were observed in the connective tissues in the dorsal hump (Fig. 8A), but no evidence of cartilage was

detected, which agrees with the other staining results. As for the ultrastructure of the dorsal hump, collagen fibers and typical fibroblasts were observed (Fig. 8B).

Characterization of the connective tissues in the dorsal hump

The lipid content of adipose tissue was assessed in wide visual fields of connective tissue according to pre-embedding osmium-black formation in paraffin-embedded sections (Fig. 9). As a result, it was demonstrated that the connective tissue of the median septum and the distal region was rich in lipids in the immature and maturing periods (Fig. 9A). However, the adipocytes in the corresponding regions of the mature males contained few or no lipids (Figs. 9B-C) although high magnification examinations found that the adipocytes in other regions did contain fat (Fig. 7A). The lipid content of the dorsal connective tissue decreased during maturation. It should also be noted that the crescent-shaped region did not contain any adipose tissue (Fig. 9D). In the mature female fish, the lipid content of connective tissue in the median septum also decreased during maturation; however, the females did not exhibit as marked a reduction as the male fish (Fig. 9E).

Acid mucopolysaccharides were not detected in the dorsal connective tissue of the immature or maturing fish. In contrast, in mature males the connective tissue in the distal region and the median septum of the dorsal hump contained acid mucopolysaccharides (Figs. 10A-B), while that in the crescent-shaped region did not. Hyaluronidase digestion effectively reduced Alcian blue-reactivity in the connective tissue in the distal region and the median septum of the dorsal hump, demonstrating that HA is a major mucopolysaccharide in both regions (Figs. 10C-D). In addition, the mature females also developed connective tissue that contained HA in the median septum and the distal region, although they possessed much less of this tissue than the mature males (data not shown). On the other hand, sulfated mucopolysaccharides, which are a characteristic component of the extracellular matrixes (ECM) of cartilaginous tissues, were not detected by AH staining at pH 1.0 in the connective tissue of the dorsal hump (data not shown).

These histochemical results are summarized in Table 3.

The water contents of the three different tissues that comprise the dorsal hump (muscle, skin, and connective tissue) were determined. The connective tissue exhibited a significantly higher water content (95.5±0.18%) than the other components of the dorsal hump (Scheffe's F-test; d.f.=2, 6; P <0.001).

Using SDS-PAGE, two distinct α chains, α1 and α2, which had molecular masses of approximately 120 kilodaltons (kDa) and 100 kDa, respectively, and are typical components of type I collagen (Kimura et al., 1987), were detected in collagen extracts

from the skin. All of the collagen samples extracted from the three components of the dorsal hump exhibited similar band patterns to those obtained from the skin (Fig. 11). However, type II collagen, which is a typical component of cartilaginous tissue, was not detected in the connective tissue of the dorsal hump.

Bony tissue in the dorsal hump

Osteoid staining successfully distinguished between the well-mineralized bone matrix (red) and less-mineralized matrix tissue (blue) in the dorsal tissues (Fig. 12). Free interneural spines and neural spines were observed as well-mineralized bones in all three developmental stages (Figs. 12A-C). In the mature males, the proximal and distal parts of the free interneural spines were surrounded by osteoblasts and osteoid tissue (less-mineralized bone matrix tissue) (Figs. 12B-C). Although the free interneural spines displayed prominent osteoblasts at both their distal and proximal tips, macroscopic observations suggested that they grew distally. Less-mineralized bone matrix tissue was much more common in the neural spines of the mature males (Fig. 12D).

DISCUSSION

Analysis of the dorsal endoskeleton showed that not only the neural spines reported by Davidson (1935) and Nakamura (1942), but also free interneural spines, are involved in the formation of the dorsal hump. In mature males, free interneural spines were present in the median septum of the dorsal hump, and protruded distally and vertically relative to the body axis. In addition, light microscopic observations also detected evidence of rapid bone growth in the free interneural spines. These results suggest that the vertical elongation pattern and rapid growth of free interneural spines facilitate the development of a larger dorsal hump. However, although there was histological evidence to indicate that the neural spines exhibit rapid growth we were unable to evaluate the elongation of these spines at the macroscopic level. Thus, a more advanced osteological analysis of neural spine development during salmon maturation is necessary. On the other hand, neural spines were found to take the form of two symmetrical spinal bones protruding from a single vertebra in all specimens, and neither the neural spines nor free interneural spines showed signs of the longitudinal splitting described by Davidson (1935) and Nakamura (1942). Previous osteological studies of Salmonidae (e.g., Totland et al., 2011) have also indicated that neural spines originally protrude distally as two symmetrical spines from a single vertebra, although they might be observed as single spinal structures at the macroscopic level in the immature period.

Histochemical and biochemical analyses demonstrated that the dorsal humps of pink salmon are composed of connective tissue, rather than cartilage. It is widely considered that the dorsal humps of Salmonidae are mainly composed of cartilaginous tissue, especially in the median septum, the cartilaginous crescent above the dorsal posterior cones, and the cartilaginous mass around the supracarinalis muscle (Davidson, 1935; Nakamura, 1942). The general histological characteristics of cartilage have been reported to be as follows: it is found in articular tissue, avascular, and composed of rounded chondrocytes (Benjamin, 1990; Benjamin and Ralphs, 2004; Witten et al., 2010). Biochemically, cartilage contains large amounts of sulfated mucopolysaccharides and type II collagen as its major ECM molecules (Witten et al., 2010). However, these molecules were not detected in the connective tissue of the dorsal hump, and it is clear that the connective tissue in the dorsal humps of pink salmon is mainly produced by fibroblasts, rather than chondrocytes. Thus, we concluded that connective tissue, rather than cartilage, participates in the development of the dorsal hump in pink salmon. In particular, the connective tissues in the median septum and the distal region of the dorsal hump were found to contain HA. These HA-rich connective tissues might be similar to gelatinous tissues such as that found in the mammalian umbilical cord (Wharton’s jelly; Hadidian and Pirie, 1948; Catini and

Gheri, 1983). Previous studies (Robinson and Mead, 1970; Bower et al., 2011) reported that the dorsal humps of pink salmon have high water contents although these studies referred to the whole dorsal hump or fillet, rather than to dissected connective tissues. The present study revealed that the high water content of the dorsal hump is mainly derived from the development of connective tissue. It is generally accepted that HA exhibits a strong water retention ability (Nakamura et al., 1993), and the loose and/or less organized-collagen fibers of water-retaining connective tissue are highly suited to the acquisition of a larger dorsal hump. The kype; i.e., a hooked nose, is another secondary sexual characteristic of male salmonids (Tchernavin, 1938). As the kype might be a specialized feature for attacking other males (Gross, 1984; Hutching and Myers, 1987), its structure requires physical strength. Hence, the kype is mainly composed of skeletal tissue, which arises as a spongiosa-like meshwork, rather than a solid bone mass, and this structure suggests that the bony tissue of the kype is composed of the least amount of materials possible and provides the maximum level of mechanical stability (Witten and Hall, 2002, 2003). In contrast, the dorsal hump might serve as a shield against attacks from other males (Schroder, 1981), act as a status indicator (Keenleyside and Dupuis, 1988; Quinn and Foote, 1994), and be useful for preventing other males (i.e., competitors) from gaining access to spawning females (Fleming and Gross, 1994). These studies suggest that developing a larger dorsal hump increases male competitiveness. Our results indicate that the development of the dorsal hump strongly depends on increasing the amount of connective tissue with high water content. Hence, the presence of HA-containing connective tissue in the dorsal humps of pink salmon facilitates the development of a larger dorsal hump due to the strong water retention ability of HA. Such humps might be particularly useful during the spawning period, when salmon stop feeding.

The connective tissues in the median septum and distal region of the dorsal hump contained HA, lipids, and elastic fibers, while that in the crescent-shaped region did not. Accordingly, we assume that the connective tissue in the crescent-shaped region differs from those in the distal region and median septum. Moreover, both mature males and mature females possessed HA-containing connective tissue in the distal region and median septum of their dorsal tissue, although the females only possessed an extremely small amount of this tissue. This suggests that the development of such connective tissue during sexual maturation is not limited to males, but only males are able to induce prominent dorsal development. The present study also obtained histological evidence that suggested that the adipose tissue in the dorsal hump is replaced by fibrous and mucous connective tissue in male fish during maturation. This process might be due to lipid consumption; i.e., the consumption of an energy store during upriver migration in

the spawning period (Hendry and Berg, 1999; Kinnison et al., 2003). In mature females, the median septum was also found to contain lipids; however, the females did not develop dorsal humps even though they developed similar connective tissue (containing HA and collagen fibers). Therefore, energy mobilization in dorsal tissue might differ between the sexes, and lipid consumption in dorsal tissue might be associated with dorsal hump formation. Further studies are required to elucidate the mechanism regulating the formation of the dorsal hump in salmon, as well as other salmonid species.

In conclusion, this study provided evidence that the dorsal humps of male pink salmon develop via the elongation of bony tissues in the median septum and increases in the amount of connective tissue with a high water content; i.e., connective tissue composed of type I collagen and HA. It was also clearly demonstrated that in pink salmon the dorsal hump is not composed of cartilaginous tissue.

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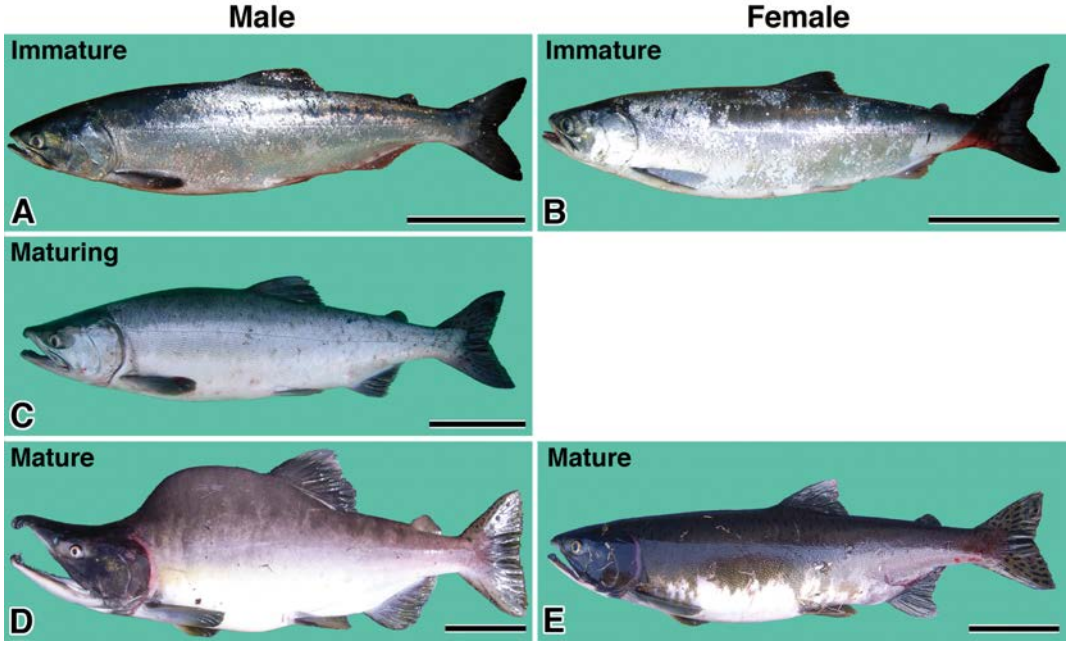

Fig. 1. *Oncorhynchus gorbusha*, differences in the external morphologies at different stages of secondary sexual characteristic development. (A) immature male, (B) immature female, (C) maturing male, (D) mature male, (E) mature female. Scale bars: 100 mm.

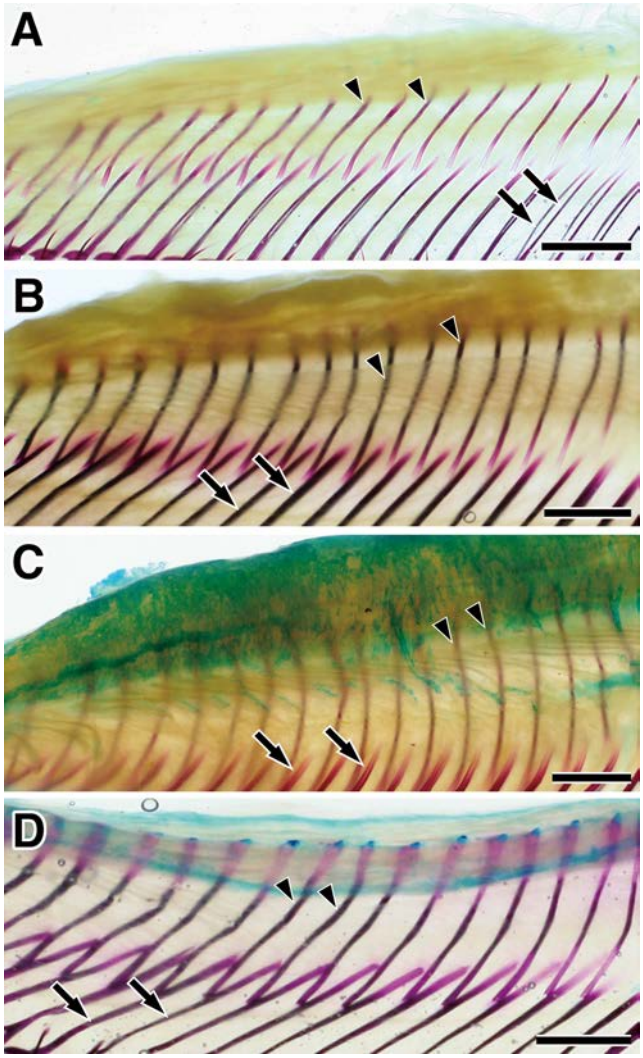

Fig. 2. *Oncorhynchus gorbusha*, cleared and double stained skeletal specimens of the dorsal regions at different stages of secondary sexual characteristic development. Arrows and arrowheads indicate neural spines and free interneural spines, respectively. (A) Immature male. In the immature males, the neural spines took the form of a pair of spines in the dorsal region [arrows in (A)]. (B) Maturing male. (C) Mature male. The blue region represents Alcian blue-positive connective tissue. (D) Mature female. Scale bars: 10 mm.

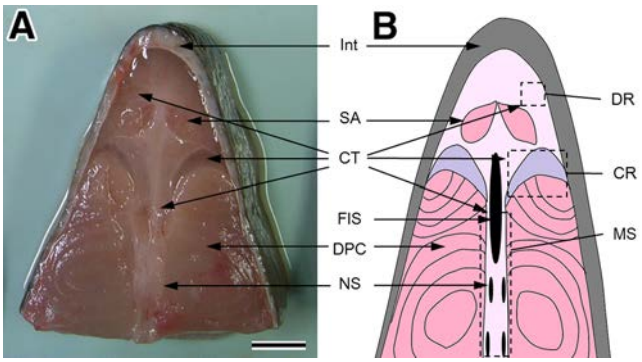

Fig. 3. *Oncorhynchus gorbusha*, photograph (A) and schematic illustration (B) of a cross-section of the dorsal hump of a mature male. In the cross-sections observed in this study, the internal structure of the dorsal hump was found to be as shown in (B). CR, crescent-shaped region; CT, connective tissue; DR, distal region; DPC, dorsal posterior cone; FIS, free interneural spine; Int, integument; MS, median septum; NS, neural spine; SA, supracarinalis anterior. Scale bar: 10 mm.

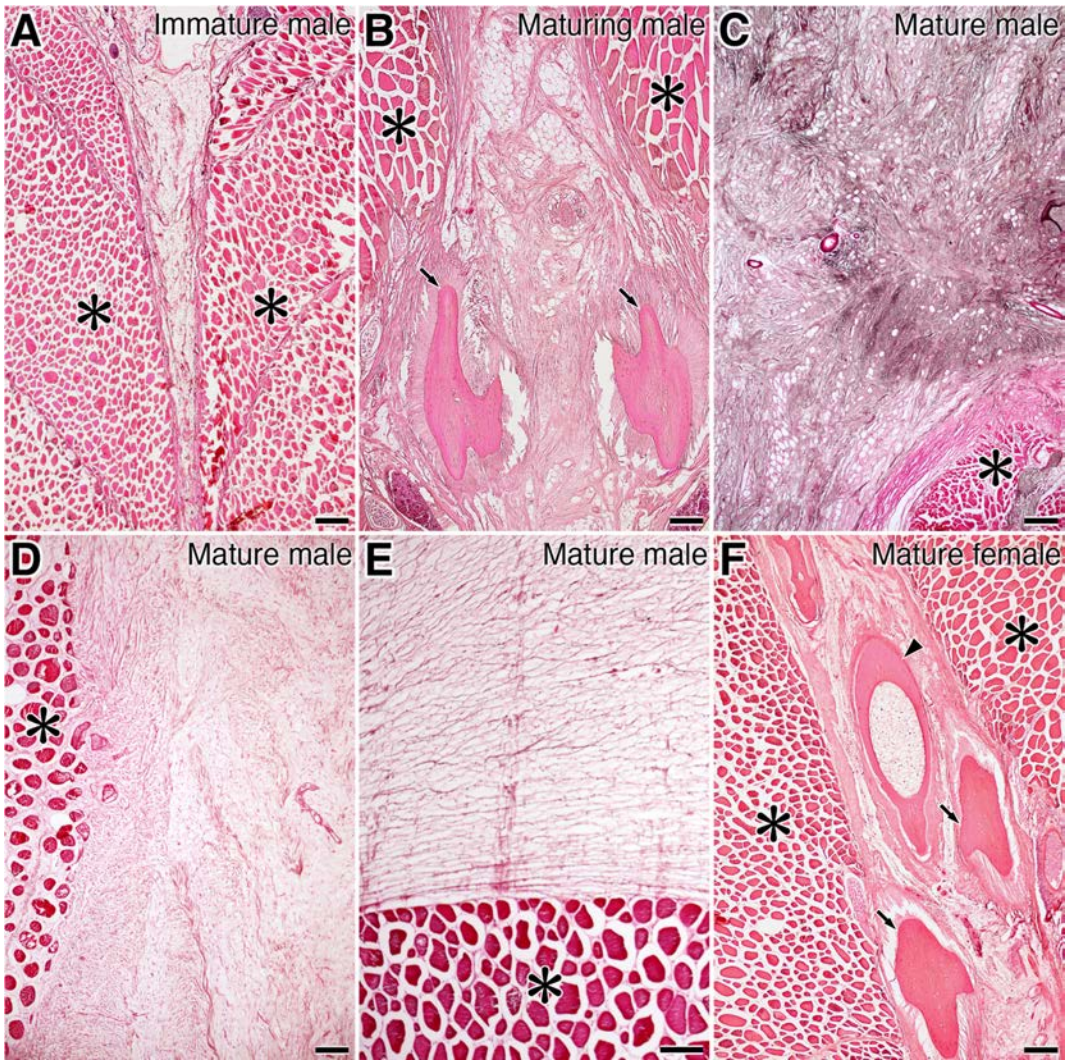

Fig. 4. *Oncorhynchus gorbusha*, low magnification photomicrographs of cross-sections of dorsal tissue stained with hematoxylin and eosin. Immature male (A), maturing male (B), mature male [(C), (D), and (E)], and mature female (F). Arrows indicate neural spines. The arrowhead indicates a free interneural spine. Asterisks indicate dorsal posterior cones (muscle tissue), except for the asterisk in (C), which indicates the supracarinalis anterior (another muscle tissue). (A) Dorsal median septum of an immature male fish. A small amount of connective tissue is present. (B) Median septum of a maturing male. This figure shows a slight increase in the amount of connective tissue around the neural spines. (C) Connective tissue in the distal region around the supracarinalis anterior of a mature male. Adipose-like tissue was observed in some of the connective tissue. (D) Connective tissue in the median septum of a mature male. (E) Crescent-shaped region located above the posterior cones in a mature male. (F) Median septum of a mature female. A slightly increased amount of connective tissue was observed compared with the immature period, although the females still possessed much less connective tissue than the mature males. Scale bars: 200 μm.

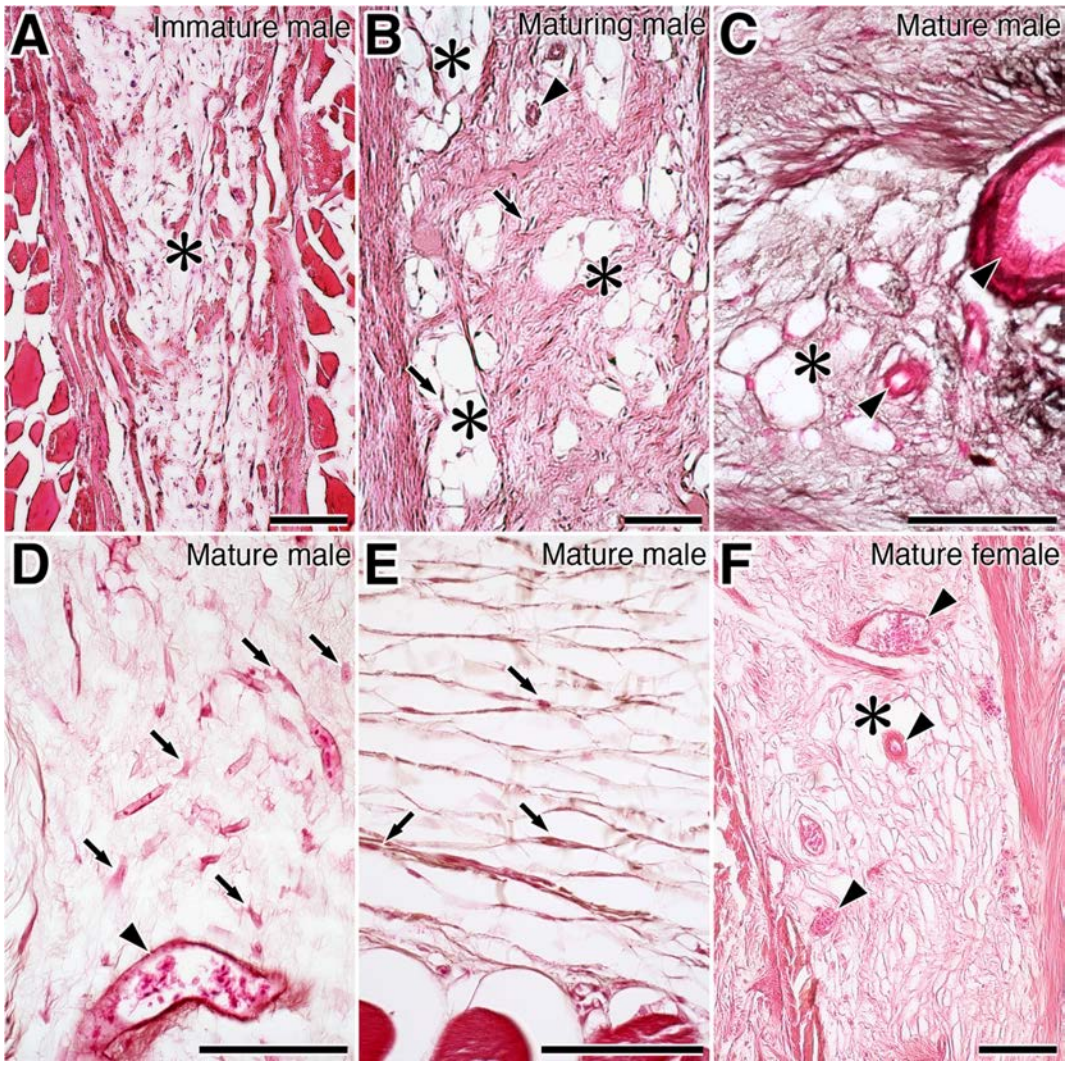

Fig. 5. *Oncorhynchus gorbusha*, high magnification photomicrographs of cross-sections of dorsal tissue stained with hematoxylin and eosin. (A) Dorsal median septum of an immature male fish. (B) Median septum of a maturing male. (C) Connective tissue in the distal region of a mature male. (D) Connective tissue in the median septum of a mature male. (E) Crescent-shaped region above the posterior cones of a mature male. It was demonstrated that the fiber orientation of this connective tissue differed from those of the other connective tissues. (F) Median septum of a mature female. No cells in these tissues displayed the typical features of chondrocytes, such as a rounded shape and the presence of a territorial matrix. Typical fibroblasts (arrows) and blood vessels (arrowheads) were observed in these tissues, suggesting that they were not cartilaginous. Asterisks indicate adipose-like tissue. Scale bars: 100 μm.

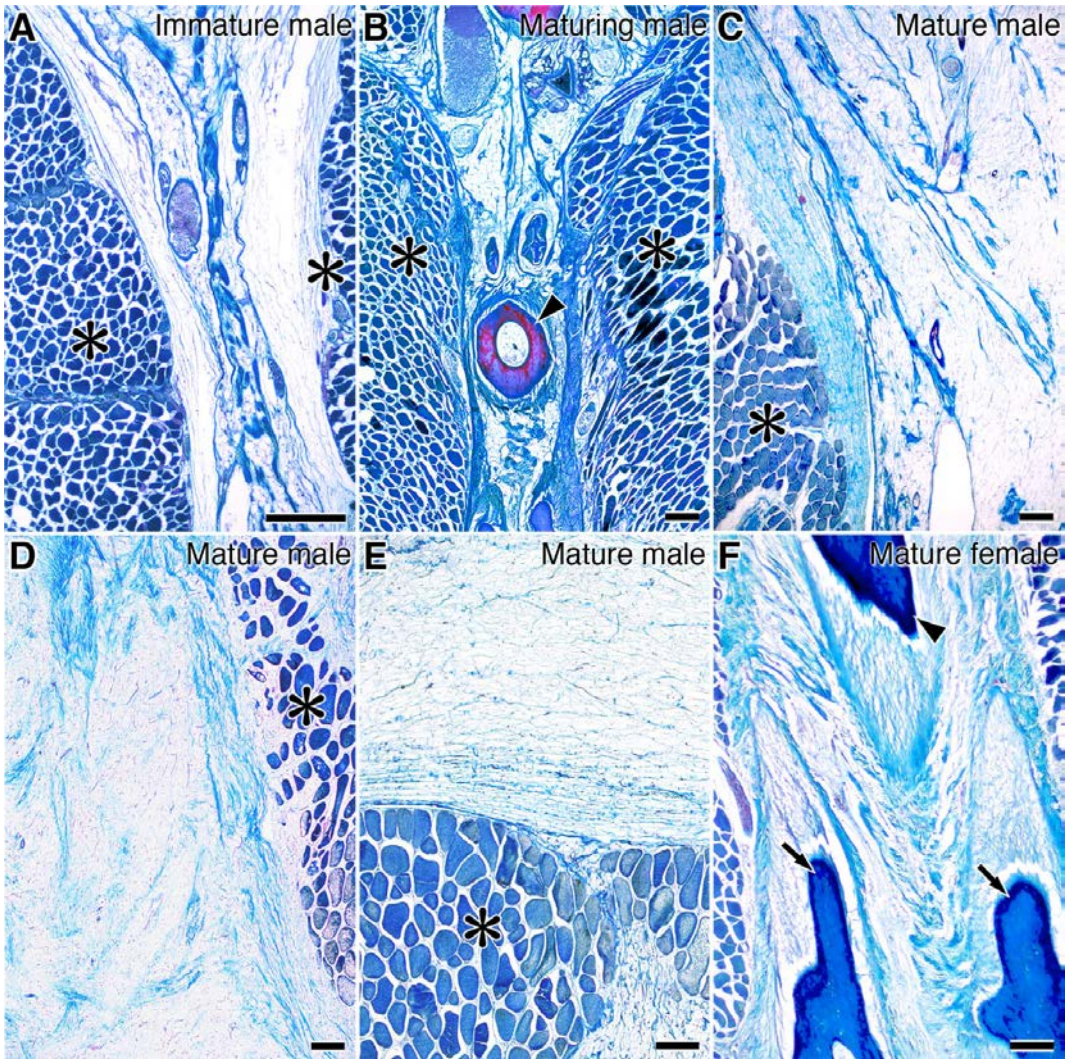

Fig. 6. *Oncorhynchus gorbusha*, photomicrographs of cross-sections of dorsal tissue stained with Masson's trichrome stain. The aniline blue in Masson's trichrome stain colored collagen fibers bright blue. (A) Median septum of an immature male fish. (B) Median septum of a maturing male. (C) The distal region of a mature male. (D) The median septum of a mature male. (E) Crescent-shaped region above the posterior cones of a mature male. (F) Median septum of a mature female. Arrowheads indicate free interneural spines. In mature males, the connective tissues in the dorsal hump were mainly composed of collagen fibers. Arrows indicate neural spines. Asterisks indicate muscle tissue, as the supracarinalis anterior in (C) and the dorsal posterior cones in other panels. Scale bars: 200 μm.

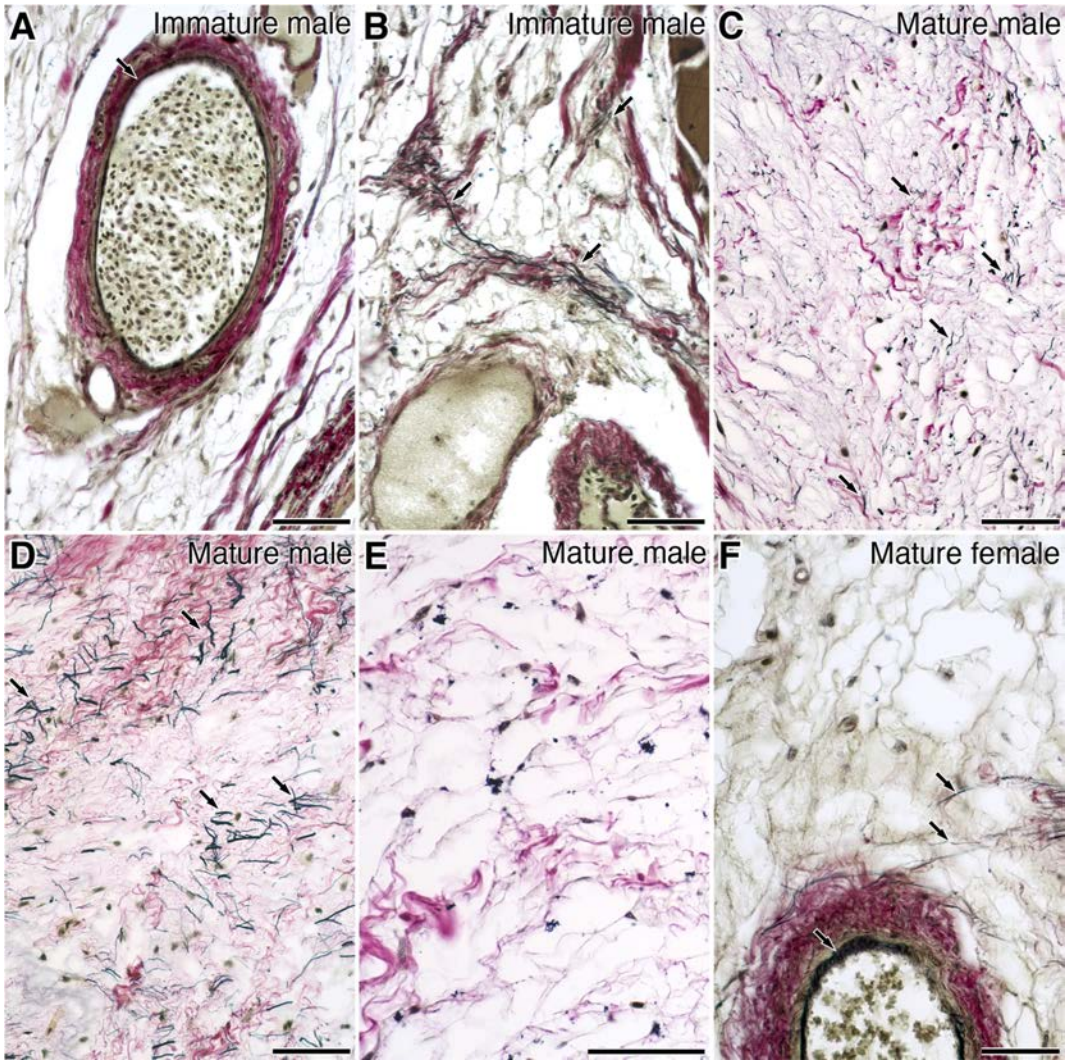

Fig. 7. *Oncorhynchus gorbusha*, photomicrographs of cross-sections of dorsal tissue stained with elastica-van Gieson stain. The resorcin-fuchsin in elastica-van Gieson stain colored the elastic fibers black (arrows). (A) Median septum of an immature male. Elastic fibers were clearly detected in the tunica intima and media of blood vessels. (B) Median septum of an immature male. (C) Connective tissue in the distal part of a mature male. A small number of elastic fibers were detected in this connective tissue. (D) Connective tissue in the median septum of a mature male. As in the other regions, elastic fibers were also observed in this connective tissue. (E) Crescent-shaped region above the posterior cones of a mature male. Elastic fibers were not detected in this region of connective tissue. (F) Median septum of a mature female. Scale bars: 50 μm.

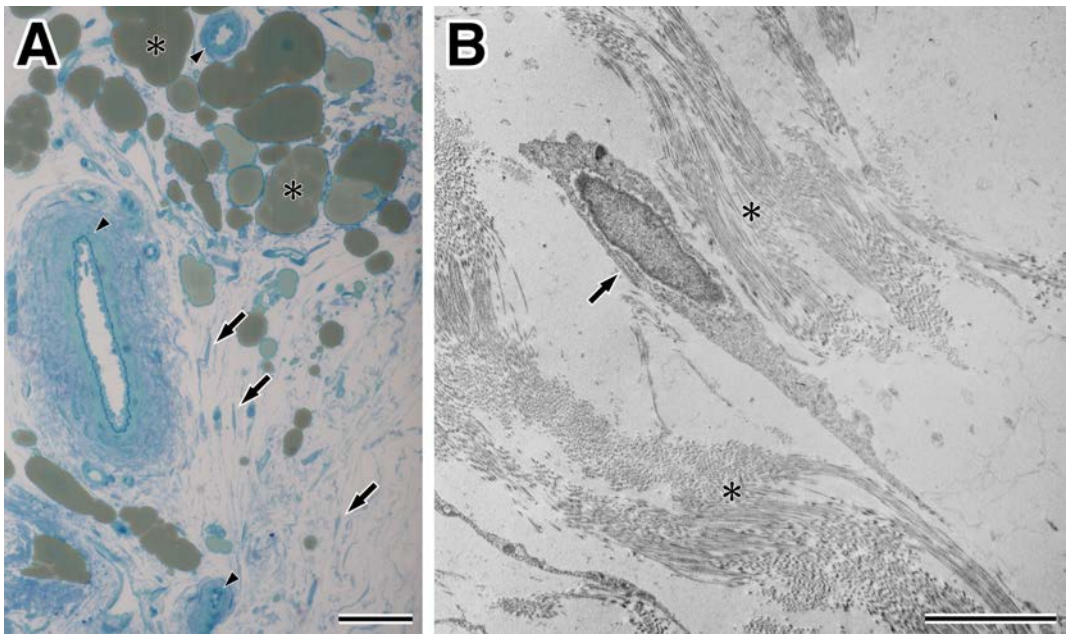

Fig. 8. *Oncorhynchus gorbusha*, photomicrograph of a semi-thin section (A) and an electron micrograph of an ultrathin section (B) of the distal region of a mature male. (A) Scattered fibroblasts [arrows in (A)] and vascular vessels [arrowheads in (A)] were observed in this region. The lipids in adipocytes were stained by osmium-black [asterisks in (A)]. Scale bar in (A): 50 μm. (B) Ultrastructure of the dorsal region. Typical fibroblasts [arrow in (B)] and collagen fibers [asterisks in (B)] were observed in the connective tissue, but no chondrocytes were present. Scale bar in (B): 5 μm.

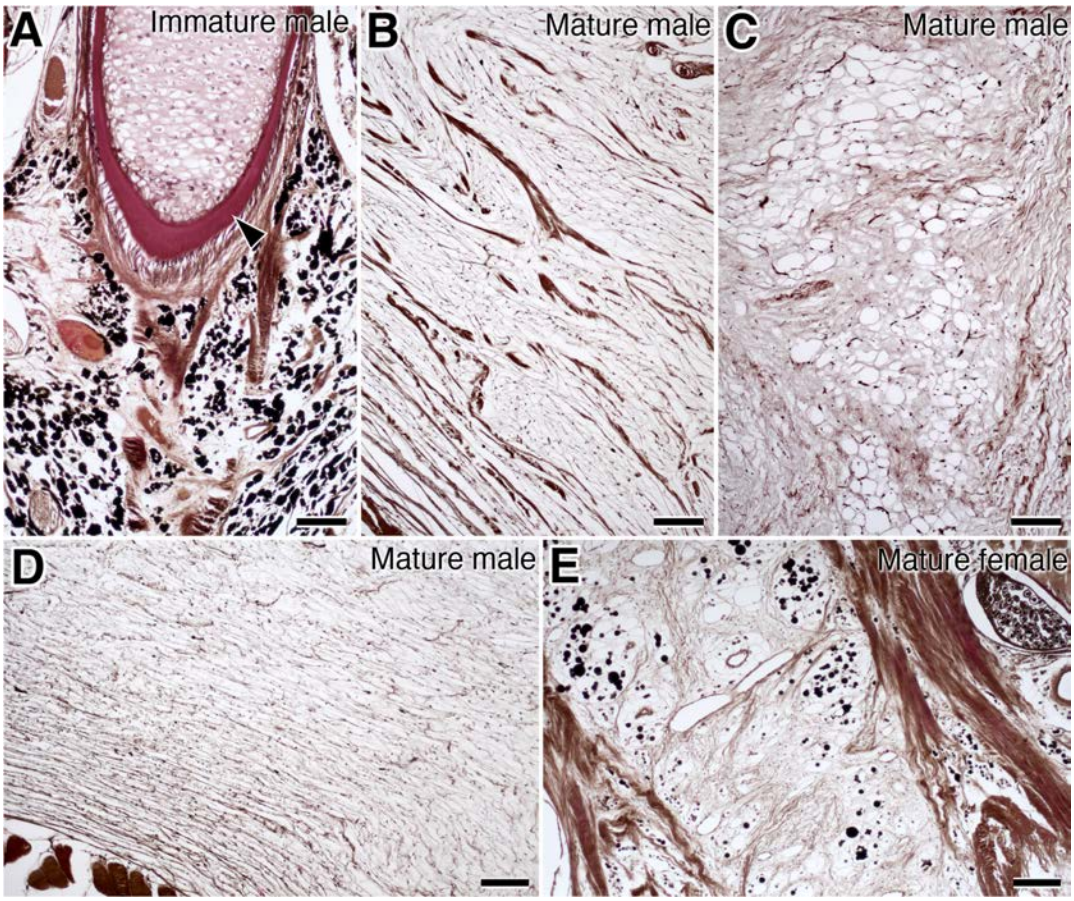

Fig. 9. *Oncorhynchus gorbusha*, photomicrographs of cross-sections of dorsal tissue stained with pre-embedding osmium staining. This staining procedure detected lipids via osmium-black formation. (A) Median septum of an immature male fish. The arrowhead indicates a free interneural spine. (B) Connective tissue in the distal region of a mature male. (C) Connective tissue in the median septum of a mature male. (D) Crescent-shaped region above the posterior cones of a mature male. (E) Median septum of a mature female. Scale bars: 100 μm.

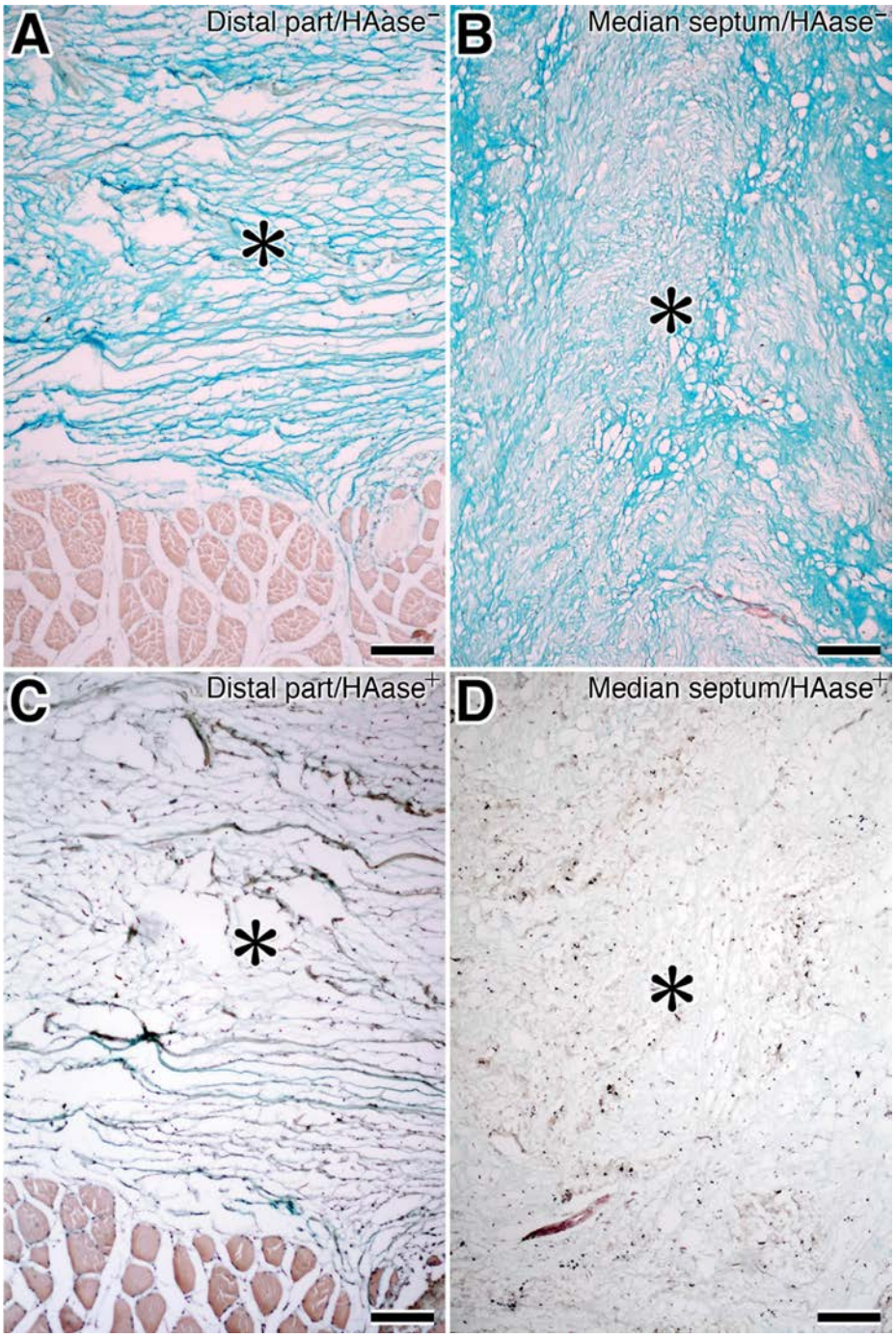

Fig. 10. *Oncorhynchus gorbusha*, photomicrographs of cross-sections of dorsal tissue stained with Alcian blue and hematoxylin at pH 2.5. (A) Connective tissue (asterisks) in the distal region of a mature male [(A), (C)] and the median septum of a mature male [(B), (D)]. The bright blue signals representing acid mucopolysaccharides that were seen in [(A) and (B)] were absent from adjacent sections that had been pre-digested with hyaluronidase [HAase; (C) and (D), respectively]. Scale bars: 100 μm.

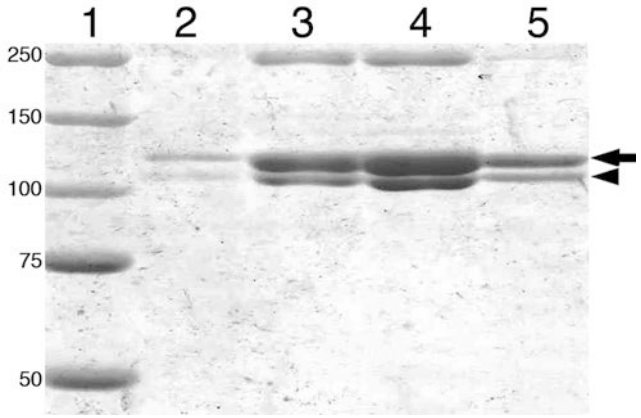

Fig. 11. SDS-PAGE of crude collagen extracts obtained from the connective tissue in the median septum (lane 2), crescent-shaped region (lane 3), or distal region (lane 4) or the skin (lane 5) of the dorsal hump of a mature male pink salmon. The arrow and arrowhead indicate α1(I) and α2(I) chains, respectively. The positions of the molecular mass markers (lane 1), which are expressed in kilodaltons, are indicated on the left side of the figure.

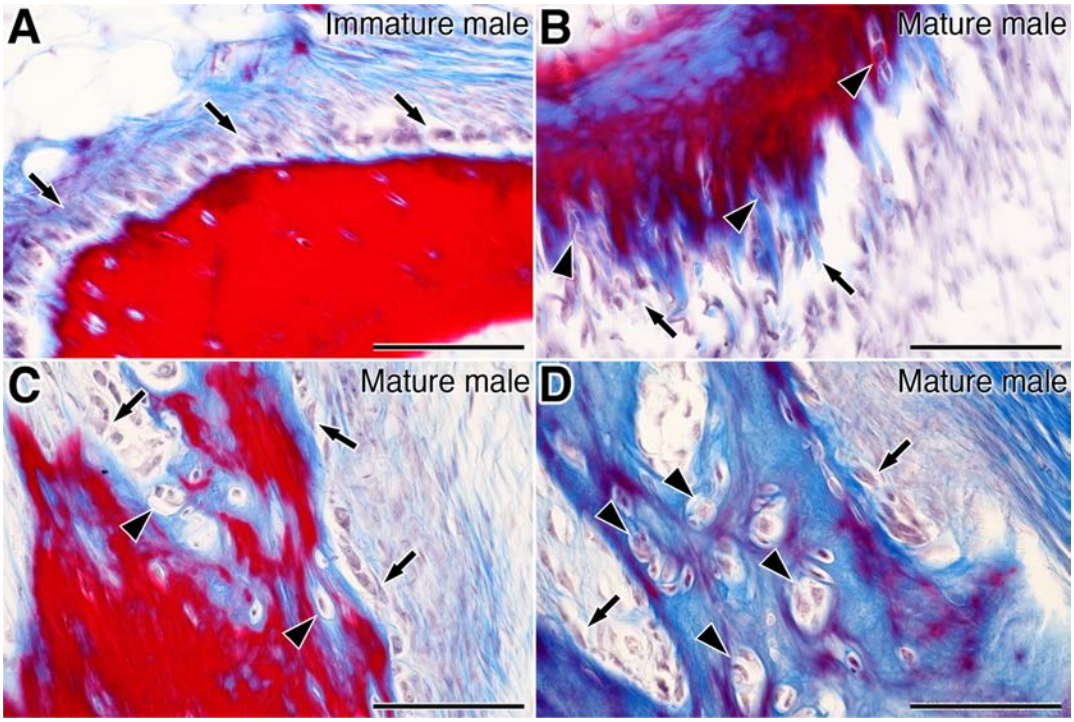

Fig. 12. *Oncorhynchus gorbusha*, photomicrographs of cross-sections of dorsal tissue stained with osteoid staining (Ralis and Watkins, 1992). Well- and less-mineralized bone matrixes were stained red and blue, respectively, by this process. (A) Free interneural spine of an immature male. (B) Proximal region of a free interneural spine in the dorsal hump of a mature male. (C) Distal region of a free interneural spine in the dorsal hump of a mature male. (D) Neural spine in the dorsal hump of a mature male. Arrows indicate osteoblasts located around bony tissues. Arrowheads indicate osteoblasts enclosed in osteoid tissue. Scale bars: 100 μm.

TABLE 1. Body size and sampling sites of pink salmon collected during each maturation stage in this study

	Body length (mm)	Body weight (g)	Number of samples	Sampling site
Immature males	392±15.5	614±71.1	n=12	Northwest Pacific Ocean (43°N, 155°E)
Immature females	394±21.6	631±112	n=8	Northwest Pacific Ocean (43°N, 155°E)
Maturing males	532±38.2	1763±473.2	n=5	Coastal waters off Shibetsu
Mature males	597±33.9	2598±395.7	n=11	Shibetsu River
Mature females	518±25.4	1668±276.1	n=5	Shibetsu River

Body length (mm) and weight (g) are expressed as mean ± S. D. values.

TABLE 2. Inter-group comparison of the length, diameter, and angle to the body axis of free interneural spines in cleared pink salmon skeletal specimens

	Immature males	Maturing males	Mature males	Mature females
Length (mm)	16.8±1.17	24.6±3.13	41.0±5.57 ***	21.9±0.61
Diameter (mm)	0.70±0.04	0.87±0.10	1.40±0.22 ***	0.73±0.05
Angle (°) to body axis	66.0±1.79	79.4±4.22	91.0±2.29	65.0±2.00
Number of samples	n=5	n=5	n=8	n=5

***, $P < 0.005$ (Scheffe's test)

TABLE 3. Results of histochemical observations of dorsal tissue from pink salmon at different stages of maturation

	Immature males	Maturing males	Mature males	Mature females
Distal region:				
Connective tissue	+	+	++	+
Elastic fibers	+	+	+	+
Lipids	++	++	+/-	+/-
Hyaluronic acid	-	-	+	+
Median septum:				
Connective tissue	+	+	++	+
Elastic fibers	+	+	+	+
Lipids	++	++	+/-	+/-
Hyaluronic acid	-	-	+	+
Crescent-shaped region:				
Connective tissue	-	-	++	-
Elastic fibers	-	-	-	-
Lipids	-	-	-	-
Hyaluronic acid	-	-	-	-
Collagen fibers	+	+	++	+
Blood vessels	+	+	++	+

-: not present, +: slightly present, ++: abundant