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Involvement of 11-ketotestosterone in hooknose formation in male pink salmon (*Oncorhynchus gorbuscha*) jaws

Hideaki Kudo [a,*], Tomoaki Kimura [a], Yuya Hasegawa [a], Takashi Abe [a], Masaki Ichimura [b], Shigeho Ijiri [a]

[a] *Faculty of Fisheries Sciences, Hokkaido University, 3-1-1 Minato-cho, Hakodate, Hokkaido 041-8611, Japan*

[b] *Shibetsu Salmon Museum, Kita 1, Nishi 6, Shibetsu, Hokkaido 086-1631, Japan*

[*] Correspondence author.

Hideaki Kudo, Laboratory of Humans and the Ocean, Faculty of Fisheries Sciences, Hokkaido University, Japan

E-mail: hidea-k@fish.hokudai.ac.jp

Tel & Fax: +81 138 40 5602

ABSTRACT

Mature male Pacific salmon (*Oncorhynchus* spp.) develop a hooknose, as a secondary male sexual characteristic, during the spawning period. It is likely that androgens regulate hooknose formation. However, endocrinological and histochemical details about the relationship between androgens and hooknose formation are poorly understood. In this study, we performed assays of serum androgens, detection of androgen receptor (AR) in hooknose tissues, external morphological measurement of hooknose-related lengths, and microscopic observation of hooknose tissues of pink salmon (*O. gorbuscha*) at different stages of sexual maturation. Expression of the *arβ* gene was detected in hooknose tissues of males but not females. The elongation of these tissues was mediated directly via androgens. Serum 11-ketotestosterone (11-KT) concentrations indicated a significant positive correlation with both jaw lengths during sexual maturation of males. In the upper jaw, cartilage tissue developed during hooknose formation, and AR-immunoreactive chondrocytes were located in the rostal-vetral regions of hooknose cartilage in maturing male. The chondrocytes in maturing males before entering into rivers exhibited rich-cytoplasm with high cell activity than at other sexual development stages. On the other hand, in the lower jaw, the development of the spongiosa-like bone meshworks. AR-immunoreactivity was detected in a proportion of the osteocytes and osteoblast-like cells in the spongiosa-like bone meshworks. These results indicate that hooknose formation in pink salmon, which is associated with the buildup of a structure with sufficient strength that it can be used to attack other males on the spawning ground, is regulated by 11-KT.

Keywords:

11-ketotestosterone, androgen receptor, hooknose, secondary sexual characteristic, pink salmon, cartilage, bone

1. Introduction

The hooknose of Salmonidae is widely known as a male secondary sexual characteristic (e.g., Fleming and Reynolds, 2004). Hooknose formation in mature males involves elongation of both the upper and lower jaws and inward bending (Tchernavin, 1938). This formation is synchronized with the development of the breeding teeth (Johnson et al., 2006). The hooknose has been regarded as a specialized weapon for fighting other males at the spawning ground in coho salmon (*Oncorhynchus kisutch*; Gross, 1984). The hooknose in male Arctic charr (*Salvelinus alpinus*) is regarded as an indicator of social status in males because the lower jaw (kype) of the socially dominant male at the spawning ground is more developed compared to other males (Haugland et al., 2011). These studies suggested that males with more developed hooknoses have a greater chance than males with underdeveloped ones of being chosen as a breeding partner. Anatomical studies of hooknoses of male Atlantic salmon (*Salmo salar*) have reported in detail the remarkably developed lower jaw (i.e., kype; Witten and Hall, 2002, 2003). These reports indicated that the elongation of the lower jaw occurs because both the spongiosa-like bone and skeletal needles add to the tips of the dentary bone, which is one of the main parts of the lower jaw in the splanchnocranium. In addition, chondroid bones with intermediate characteristics between bone and cartilage (Beresford, 1981) are also involved in lower jaw development other than the hooknose formation in Atlantic salmon and other teleosts (Witten and Hall, 2002; Gillis et al., 2006; Witten et al., 2010; Huysseune and Verraes, 1986; Taylor et al., 1994). However, the structures and hooknose-related cells involved are unknown in Pacific salmon (*Oncorhynchus* spp.). Moreover, although there is one report on the macroscopic anatomy of the upper jaw of pink salmon (*O. gorbuscha*; Davidson, 1935), the detailed structure of the hooknose of the upper jaw of Salmonids is almost completely unknown. Recently, the bioactivity of proteoglycan in the nasal cartilage that is the origin of the upper jaw of adult chum salmon (*O. keta*) has been studied in detail, and industrial applications (e.g., materials of cosmetics and health supplements) have resulted

from this (Kakizaki et al., 2011). However, the developmental mechanism and regulatory factors of nasal cartilage tissues are still unknown.

Androgens in teleosts as well as in other vertebrates induce the expression of male secondary sexual characteristics (e.g., Borg, 1994; Taborsky, 2008). 11-Ketotestosterone (11-KT), a specific androgen of fish, is inferred to be the main functional androgen whose activity is greater than that of testosterone (T; Miura et al., 1991). Previous reports indicated that intraperitoneal injection of 11-KT induced hooknose formation in male sockeye salmon (*O. nerka*; Idler et al., 1961), and a positive correlation was observed between plasma 11-KT concentration and nose length (distance from the middle of the eye to the tip of the snout) in Chinook salmon (*O. tshawytscha*) jacks (Butts et al., 2012). Androgen receptor (AR), a nuclear receptor, is a protein with a molecular weight of 90–100 kDa that exists in the nucleus and/or cytosol (e.g., Todo et al., 1999; Ikeuchi et al., 1999; Bain et al., 2015). In rainbow trout (*O. mykiss*) testis, two isoforms (*ar*α and *ar*β) of the AR gene were cloned in a molecular biological study (Takeo and Yamashita, 1999). However, the expression patterns of *ar*s are unknown in other tissues and other Pacific salmon species, including pink salmon. Previous reports described that high expression levels of *ar*s were observed in the dorsal and anal fins of mature male of medaka (*Oryzias latipes*; Ngamniyom et al., 2009), and AR-immunoreactive cells were located in the adipose fin, another male secondary sexual characteristics, of male mature brown trout (*S. trutta*) (Hisar et al., 2012). For the reasons given above, it is likely that 11-KT regulates hooknose formation, and that hooknose tissues are one of the target organs for 11-KT in male salmon. However, the relationship between 11-KT and hooknose formation is poorly understood. In particular, the localization of AR has not been investigated in the cartilage tissues of teleost fish.

The main goal of the present study was to clarify the relationship between androgens and hooknose formation of Pacific salmon male. For this purpose, we performed endocrinological analyses of serum androgen concentrations and AR expression levels in hooknose tissues of

pink salmon at different stages of maturation. In addition, to morphologically characterizing hooknose formation we measured the external morphology of hooknose-related lengths and examined the microscopic anatomy of hooknose tissues in male and female pink salmon at different stages.

2. Materials and methods

2.1. Animals

We examined the upper and lower jaw tissues in both sexes of pink salmon, *Oncorhynchus gorbuscha*, at different stages of secondary sexual characteristic development; i.e., immature, maturing, and mature fish. Information about the external morphology of the head and their collection are shown in Figure 1 and Table 1. 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 2014 and May 2015. Maturing fish were captured using set nets in the coastal waters off Shibetsu, eastern Hokkaido, in September 2014 and September 2015 and landed at Shibetsu Fishing Port. Mature spawning fish from the Shibetsu River were provided by the Nemuro Salmon Propagation Association in September 2014 and September 2015. Fish were immobilized in carbonated water, and then blood samples were taken using polyethylene syringes equipped with 19 gauge needles from the caudal vein, allowed to clot overnight at 4°C and centrifuged at 10,000 rpm for 15 min. Serum was collected and stored at -30°C until use.

Fish were photographed on their left body side and we measured fork length (FL; distance from tip of the snout to center of the fork in the caudal fin), body mass, and gonad mass. Then, upper and lower jaw tissues were isolated surgically.

The experiment was carried out in accordance with the guideline of the Hokkaido University Animal Care and Use Committee.

2.2. External morphology

On each animal, after external morphological lengths were measured using digital images of animals and digital image processing software (AreaQ 2.8.32; http://www.vector.co.jp/soft/dl/winnt/art/se492571.html), both upper and lower jaw lengths were compared among groups (Fig. 2). This software was used to confirm that the measured FL

and the processed FL were equivalent (linear regression, $p < 0.0001$, R^2=0.99; Mann–Whitney U test, p=0.91). In this study, length of the mid-eye to hypural plate-length (MEH; a measurement unbiased by the snout elongation) was measured for adjustment, and the ratios (each length/MEH) were used for comparison among groups, to prevent bias solely resulting from body size (Pitcher et al., 2009).

2.3. Histochemistry

Upper and lower jaw tissues were fixed in 3.7% formaldehyde in distilled water (DW) or Bouin's solution, and then decalcified with 5% formic acid in DW if necessary. Next, samples were dehydrated using a graded ethanol series and embedded in paraffin (Histosec; Merck, Darmstadt, Germany). Sagittal sections approximately 8 μm in thickness were prepared on a rotary microtome (Leica RM2125 RTS; Leica, Nussloch, Germany) and mounted on glass slides. After air-dry, they were then stored at 4°C until use and stained with Delafield's hematoxylin and eosin (HE) or other histochemical stains, as described below. All of the microscopic observations, including immunohistochemical examinations, were performed using a microscope (Eclipse 80i, Nikon, Tokyo, Japan) equipped with a digital camera (EOS 5D mark II, Canon, Tokyo, Japan).

For the detection of collagen fibers, Masson’s trichrome (MT) staining was performed in accordance with the method of Flint and Lyons (1975). Ralis’s 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 with blue, while mineralized bone matrix was indicated by red color.

2.4. Serum androgen assay

For androgen measurements, steroids were extracted from 200 μl serum according to the method by Kagawa et al. (1981). After extraction, testosterone (T) level was measured by a

time-resolved fluoroimmunoassay (TR-FIA) following the method of Yamada et al. (1997). TR-FIA for 11-KT was re-evaluated according to the method by Yamada et al. (1997, 2002). In brief, 11-KT-BSA conjugate was generated by the method of Hosoda et al. (1979) and Asahina et al. (1995). The concentration of 0.05 μg/ml for 11-KT-BSA was immobilized by physical adsorption to the wells of microtiter plate (Greiner bio-one, Kremsmuenster, Austria). After washes and blocking with 0.1 % BSA, 50 μl of standards 11-KT or extracted serum samples and 150 μl of anti-11-KT rabbit serum, FKA118 (Cosmo-Bio, Tokyo, Japan) at x48,000 dilution factor were dispensed. After the immunoreaction and washes, europium (Eu)-labeled goat anti-rabbit IgG (PerkinElmer, Waltham, Massachusetts) was treated. After washing, the intensity of dissociated Eu was measured by ARVO Sx 1420 maltilabel counter (Wallac, Oy, Finland). Cross-reaction of T in the 11-KT TR-FIA, which was expressed as a percentage at a level of 50 % replacement for 11-KT, was less than 4 % (Supplemental data 1). Intraassay coefficient of variations (CVs) was 4.9 % (n=8) for the low concentration (0.59 ng/ml) and 4.3 % (n=8) for the high concentration (11.35 ng/ml). Interassay CVs were 6.6 % (n=5) for the low concentration (0.44 ng/ml) and 8.9 % (n=5) for the high concentration (10.23 ng/ml) (Supplemental data 2).

2.5. Gene expression of *ar*

The upper jaw and the testis tissues of male pink salmon were immediately immersed in RNAlater (Thermo Fisher Scientific, Waltham, MA) and stored at -30°C until RNA extraction. Total RNA was prepared from the above tissues using ISOGEN (Nippongene, Tokyo, Japan). Poly $(A)^+$ RNA was isolated using an Oligotex-dT30<Super>mRNA Purification Kit (Takara, Otsu, Japan). Oligonucleotides used as PCR primers are shown in Table 2. The primer pairs (ARα_F, ARα_R and ARβ_F, ARβ_R) were designed against both AR cDNAs from rainbow trout (*O. mykiss*) testis (Takeo and Yamashita 1999; GenBank accession no. AB012095 and AB012096, respectively). In brief, poly $(A)^+$ RNA from the upper jaw and the testis were

reverse transcribed using a Random Primer pd$(N)_6$ and PrimeScript Reverse Transcriptase (Takara) in accordance with the manufacturer's instructions. PCR was performed using the following conditions, first cycle, denaturation at 94°C for 3 min, 35 cycles of incubation: 30 s at 94°C, 30 s at 63°C and 1 min at 72°C using Ex Taq polymerase (Takara, Tokyo, Japan) and a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). To normalize the relative expression of *ar*, salmon elongation factor 1α (*ef1α*) was amplified (annealing at 60°C) as a housekeeping gene (Jorgensen et al., 2006; Shimomura et al., 2012). Amplicons were separated by 1.5% agarose gel electrophoresis with ethidium bromide dye and visualized using a Benchtop UV Transilluminator M-20E (UVP) equipped with BioPyramid MBP-01 (MeCan Imaging, Fujimino, Japan). Then, each single band-specific amplicon was quantified using ImageJ ver. 4.4 (NIH) with the digital images from the electrophoresis gels. The amplicon of partial *arβ* (approximately 450 bp) run on a 1.5% agarose gel was excised and purified using a QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA, USA), and then direct sequencing (Hokkaido System Science, Sapporo, Japan) was performed using the above primer pair for *arβ*.

2.6. Immunohistochemistry

Upper and lower jaw tissues were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) at 4°C for 72 h, and embedded in Histosec). Then, sections of approximately 5 μm in thickness were prepared. Serial sagittal sections of both jaw tissues were prepared in accordance with the procedure described above. After deparaffinization and rehydration, the sections were blocked with 0.3% H_2O_2 in absolute methanol for 15 min to deactivate endogenous peroxidases. After rinsing with phosphate-buffered saline (PBS), the sections were incubated with antigen-retrieval solution (L.A.B. Solution; Polysciences, Warrington, PA) for 10 min at room temperature (RT), and then rinsed with PBS. Antigen-retrieved sections were incubated with 10% bovine serum albumin in PBS for 60 min at RT followed

by incubation in a humid chamber with one of the following: 2 μg/ml rabbit anti-human AR polyclonal antibody (AR-C19; Santa Cruz Biotechnology, Santa Cruz, CA), or 4 μg/ml mouse anti-rat proliferating cell nuclear antigen (PCNA) monoclonal antibody (PC10; Santa Cruz Biotechnology) for 16 h at 4°C. After rinsing in PBS, sections were reacted using the indirect immunoperoxidase method (Histofine Simple Stain MAX-PO Multi, Nichirei, Tokyo, Japan). The peroxidase complex was visualized by treatment with a freshly prepared diaminobenzidine tetrahydrochloride (0.1 mg/mL) solution with 0.01% H_2O_2 for 5 min at RT. The AR polyclonal antibody by this study was generated against C-terminus region (a part of ligand-binding domain) of human AR (position: 870-919). This antigenic 50 amino acid sequence has 62% of homology to rainbow trout AR. In addition, the specificity of the AR-immunoreactivities was confirmed by replacing the primary antibody with antibody preabsorbed with antigenic peptide (AR C-19 blocking peptide; Santa Cruz).

2.7. Statistical analysis

Statistical calculations were performed using IBM SPSS Statistics 20.0 (IBM Japan, Tokyo, Japan). Dunn's test following Kruskal–Wallis test compared the both upper and lower jaw lengths, serum androgens, and the expression levels of *ar* among each stage for secondary sexual characteristic development. All values are expressed as mean and standard deviation (SD). Differences were considered significant at $p < 0.05$.

3. Results

3.1. External morphology

Both upper and lower jaw lengths increased during maturation in both sexes (Fig. 3). The mean upper jaw lengths of mature males and females were approximately 1.9- and 1.4-fold more elongated than those of immature males and females, respectively (Fig. 3A). The mean lower jaw lengthsof mature males and females were approximately 1.6- and 1.4-fold more elongated than those of immature males and females, respectively (Fig. 3B). Sex differences within the same developmental stage were confirmed only for upper jaw lengths in mature fish ($p = 0.009$).

3.2. Histochemistry

Adipose tissue was observed only in the rostral inner regions of the upper jaw in immature fish (Fig. 4A). Masson trichrome staining demonstrated that these regions of all upper jaws were mainly composed of light green-stained cartilage tissues, including collagen fibril-enriched extracellular matrix and chondrocytes with cartilage cavities (Figs. 4D-I). Although maturing males had rich cytoplasm chondrocytes with high cell activity (Fig. 4H), Chondrocytes of mature males showed shrinkage nuclei and achromatophil cytosol with the degeneration (Fig. 4I) of cartilage tissues.

Spongiosa-like meshworks, which consist of ponceau xylidine-stained bone matrix, were observed on the rostral outside of dentary bone in all lower jaw (Figs. 5A-C). Dense spongiosa-like structure elongated distally into the connective tissue in mature male (Fig. 5E), but this structure did not elongate in immature males (Fig. 5D) and females (Fig. 5F). Connective tissues, including dense collagen fibers and fibroblasts (Fig. 5G), had developed into the region at the tip from the spongiosa-like meshworks in all developmental stages. Sharpey's fibers, which consist of a bundle of collagen fibers connecting periosteum to bone were observed on the ventral side of these structures (Fig. 5H). Chondroid bones, for which

bone matrix exists around cartilage-like cells, were observed in rostral regions of these structures (Fig. 5I).

In osteoid staining at the tip of these structures, developing ossificating sites were not observed between the well-mineralized ossification zone and the osteoid zone in the lower jaw of immature males (Fig. 6A), but in maturing and mature males, developing ossificating sites were observed (Fig. 6B). Osteoblasts surrounded by osteoid matrix, which was self-secreted in these regions of maturing and mature males (Fig. 6B).

3.3. Serum androgens

Serum concentrations of 11-KT and T at different stages of secondary sexual characteristic development are shown in Figure 7. Serum 11-KT concentrations in males were significantly higher compared with those in females at maturing ($p < 0.001$) and mature ($p < 0.0001$) stages (Fig. 7A). These concentrations increased markedly during sexual maturation in male ($p < 0.0001$-0.001, Fig. 7A). Serum T concentrations showed no significant changes during sexual maturation in both sexes (Fig. 7B).

Relationships between both serum androgens and both jaw lengths are shown in Figure 8. A significant positive correlation between 11-KT and upper jaw length ($R^2 = 0.5119$, $r = 0.639$, $p < 0.05$), 11-KT and lower jaw length ($R^2 = 0.5071$, $r = 0.712$, $p < 0.05$) was observed during sexual maturation in males. However, the significant relationship was detected in neither combination with mature males only ($p > 0.05$) as well as previous report of Chinook salmon anadromous males (Butt et al. 2012).

3.4. Expression of *ars* in upper jaw cartilage

Amplified cDNA fragments of both *arβ* and *ef-1α* at the expected sizes (459 and 141 bp, respectively) were detected in male upper jaw cartilage and testis using RT-PCR, with each specific primer pairs (Fig. 9A). The nucleotide sequence of the partial *arβ* amplicon

(LC318624) indicate high homology (99%) with the corresponding region of *arβ* (AB012096) previously examined in rainbow trout testis (Takeo and Yamashita, 1999). For this reason, the deduced amino acid sequence was identical to rainbow trout *arβ*. This amplicon included the sequence of both DNA- and ligand-binding domains (Mangelsdorf et al., 1995). Relative expression levels of the *arβ* mRNA in the male upper jaw cartilage tissue were not significantly different among the different stages of secondary sexual characteristic development, although the expression level in maturing males was highest (Fig. 9B). Expression of *arα* was not detected in the upper jaw cartilage using the primer pair and method in the present study.

3.5. AR-immunohistochemistry

AR-immunohistochemistry of cartilage tissue in the upper jaw demonstrated immunoreactivity for AR in the nuclei and/or perinuclear regions of chondrocytes at the rostal-vetral regions near the connective tissues in all developmental stages of males (Figs. 10A-C). Immunoreactivities for AR in maturing fish were markedly more intense compared with other developmental stages (Fig. 10B). In particularly, the strong immunoreactivities were remarkable in males compared with females among maturing fish (Fig. 10D). In the lower jaw, immunoreactivity for AR was observed in some osteocytes and osteoblast-like cells near the tip of the dentary bone (Figs. 10E-G). These AR-immunoreactive cells in the dense spongiosa-like structure in maturing males (Fig. 10F) also were more intense compared with other developmental stage males (Figs. 10E, G). In addition, immunohistochemistry using a pre-absorbed AR antibody showed no detectable positive-immunoreactivity in adjacent sections of rostral regions in the upper jaw of maturing males (Fig. 10I).

Although there was not the detection on the same section, immunoreactivity for proliferating cell nuclear antigen (PCNA) was observed in equivalent cell-population of AR-immunoreactive cells in semi-adjacent sections of the rostral regions of maturing male

upper jaw (Fig. 11).

Discussion

The present external morphological analysis indicated that both the upper and lower jaws of male pink salmon elongated during sexual maturation. Previous reports demonstrated that the kype (hook) on the tip of the lower jaw developed prominently in mature males of Atlantic salmon (Tchernavin, 1937, 1938; 1944; Witten and Hall, 2002, 2003). On the contrary, in the hooknose of pink salmon, upper jaw length elongated significantly during sexual maturation, and lower jaw length was similar in both sexes. The difference between the genus *Oncorhynchus* (including pink salmon), in which male secondary sexual characteristics are remarkable in the upper jaw, and genus *Salmo* (including Atlantic salmon), in which those are remarkable in the lower jaw (Tchernavin, 1938), may reflect their evolution or a difference in life history (i.e., semelparity or iteroparity).

Previous studies reported that changes occurred in blood androgen concentrations of other Pacific salmon species in the spawning period (e.g., chum salmon, Ueda et al., 1991, masu salmon *O. masou*, Munakata et al., 2001). Both species males indicated the peak of 11-KT concentrations before final maturation. The present study revealed that serum 11-KT increased significantly in male pink salmon during sexual maturation, and the change in serum T was not similar to the increase in 11-KT. In addition, although a significantly positive correlation between 11-KT and both jaw lengths was observed, no correlation existed for T. These results suggest that 11-KT profoundly affects hooknose formation in male pink salmon. In analysis of Chinook salmon of only mature male, anadromous-type showed no positive correlation between the upper jaw and both 11-KT and T (Butts et al., 2012). However, a significantly similar relationship was observed in jacks-type (Butts et al., 2012). Jacks-type are presumably resident in their natal rivers their entire lives, reach sexual maturity precociously, and employ a sneaking tactic, by darting from nearby refuges, to steal fertilizations from anadromous males (Healey, 1991). Because pink salmon does not show life-history polymorphisms, river-resident males do not exist (Heard, 1991). Therefore, all

present samples were equivalent to anadromous males. The present study demonstrated that significant relationships between androgens and jaw lengths were not observed only in the mature stage, although a significant positive correlation between 11-KT and both jaw lengths was observed during sexual maturation in males. These results suggested that hooknose formation and the functions of androgens in both jaws had already finished at the mature stage in anadromous males. Moreover, the actions of androgens in the lower jaw and in pre-mature stage males were clarified for the first time by this study.

Gene expression levels of *arβ* and AR-immunoreactivities were confirmed for the first time in the present study in the hooknose tissues of salmonid jaws. These results indicated that the hooknose tissue is one of the target tissues for androgens during hooknose formation. The present study was not able to detect the expression of *arα* in the jaw tissues of pink salmon. In a previous 11-KT injection experiment, the transcriptional activity of *arβ* increased compared with *arα* in male three-spined sticklebacks (*Gasterosteus aculeatus*) kidney (Olsson et al., 2005). However, *arα* was more functional in the actions of AR than *arβ* in rainbow trout testis (Takeo and Yamashita, 1999). We have only limited information on the involvement of *arα* in hooknose formation.

The present histochemical analyses of upper jaws indicated that the major portion of hooknose tissues was occupied by cartilage tissues, including collagen fibril-enriched extracellular matrix and chondrocytes with cartilage cavities. In addition, the activities of chondrocytes in maturing males were higher than in other stages, and chondrocytes in mature males showed cell shrinkage and degeneration. These results suggested that cartilage tissues enlarged because activated chondrocytes produced abundant extracellular matrix, and as a result, the upper jaw elongates. These chondrocytes in the rostal-ventral regions of hooknose cartilage had strong AR expression in maturing males. In humans, ARs are expressed in chondrocytes at sites of bone formation in growth plates from developing bone (Abu et al., 1997; Noble et al., 1999) and in thyroid cartilage (Claassen et al., 2006). It seems reasonable

to suppose that chondrocytes in these regions were regulated by 11-KT, and were involved in upper jaw elongation and in the formation of the hook-shape. AR-immunoreactivity was observed in a proportion of cells, with PCNA immunoreactivity used as the marker of cell proliferation. AR may be expressed in a limited set of proliferating cells, or for a very short period in proliferating cells in the hooknose cartilage in the upper jaw. Elongation of the lower jaw originated in the development of spongiosa-like bone meshworks in the rostral outside of the dentary bone in the lower jaws as well as the kypes of Atlantic salmon (Witten and Hall, 2002; 2003). In mammals, previous studies reported that spongiosa-like bone had high strength with a small bone mass (Parfitt, 1988). In addition, these structures were made stronger by the joining of Sharpey's fibers (e.g., Beresford, 1981; McKee et al., 1996) in the lower jaws of pink salmon. Because anadromous salmon fast during the spawning migration (Hendry and Berg, 1999), mature anadromous salmon suffer from mineral deficiency (van Someren, 1937). In the lower jaw of mature male salmon, it was supposed that this structure was adapted to build a bone structure of a high strength in calcium deficiency conditions. In fact, a low-mineralized ossification region was abundantly present in these structures in maturing and mature males. This low-mineralized ossification region may have slow or imperfect progress of ossification, reflecting the calcium deficiency. AR-immunoreactivity was localized in a proportion of the osteocytes and osteoblast-like cells in the rostral regions of spongiosa-like meshworks in the lower jaws. It was estimated that androgens are involved in the development of bone meshworks in the lower jaws by both osteocytes and osteoblast-like cells. In fact, osteoblasts in human trabecular (spongy) bones and growth plates from developing bones expressed *ar* genes (Colvard et al., 1989) and AR proteins (Abu et al., 1997), respectively. The actions of androgens may be essential to the development of sponge-form bone tissues. On the other hand, chondroid bone has been described previously in growth sites around bone tissues in the kype of mature males (Witten and Hall, 2003) and the dentary bone of juveniles (Gills et al., 2006) in Atlantic salmon and other teleosts

(Huysseune and Verraes, 1986; Taylor et al., 1994). In the present study, although remarkable AR-immunoreactivity in the component cells of chondroid bones was not observed, chondroid bones were present in the rostral regions of spongiosa-like meshworks in the lower jaws of maturing and mature males. As mentioned above, hooknose formation in pink salmon differed in development pattern between the upper and lower jaws. Cartilage tissues developed in the upper jaw and both spongiosa-like bone meshworks and connective tissues developed in the lower jaw, respectively. In terms of osteology, the upper jaw unites with the neurocranium and has a high strength, and the free lower jaw had a low strength (Vladykov, 1962). Shock protection when biting requires strong structures such as bone tissues in the lower jaw.

In conclusion, in this study we performed endocrinological and anatomical analyses of hooknose formation in male pink salmon. The main findings were as follow: (1) expression of *arβ* gene and AR-immunoreactivity were detected in hooknose tissues of males, and elongation of these tissues was mediated via androgens (Fig. 12); (2) serum 11-KT concentrations showed a significant positive correlation between both upper jaw length and lower jaw length during sexual maturation in males; (3) in the upper jaws, cartilage tissue developed during hooknose formation, and AR-immunoreactive chondrocytes were located in the rostal-vetral regions of hooknose cartilage in maturing males; (4) the chondrocytes in maturing males before entering the river showed higher cell activities than in other sexual development stages; and (5) in the lower jaws, the development of spongiosa-like bone meshworks and the connective tissues were confirmed similar to the previous report on the kype of Atlantic salmon. These findings suggested that hooknose formation of pink salmon built up structures with sufficient strength that can be used for attacking other males in the spawning ground by regulation of 11-KT in calcium deficiency conditions.

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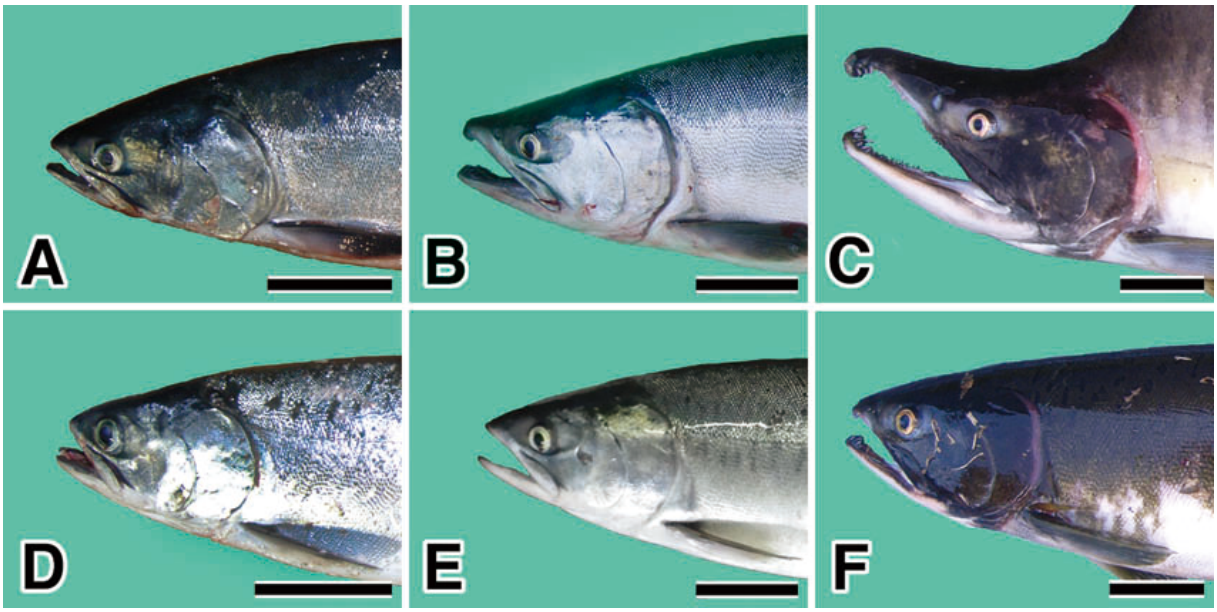

Fig. 1. External morphologies of the heads of pink salmon at different stages of secondary sexual characteristic development. (A) Immature male, (B) maturing male, (C) mature male, (D) immature female, (E) maturing female, and (F) mature female. Scale bars: 50 mm.

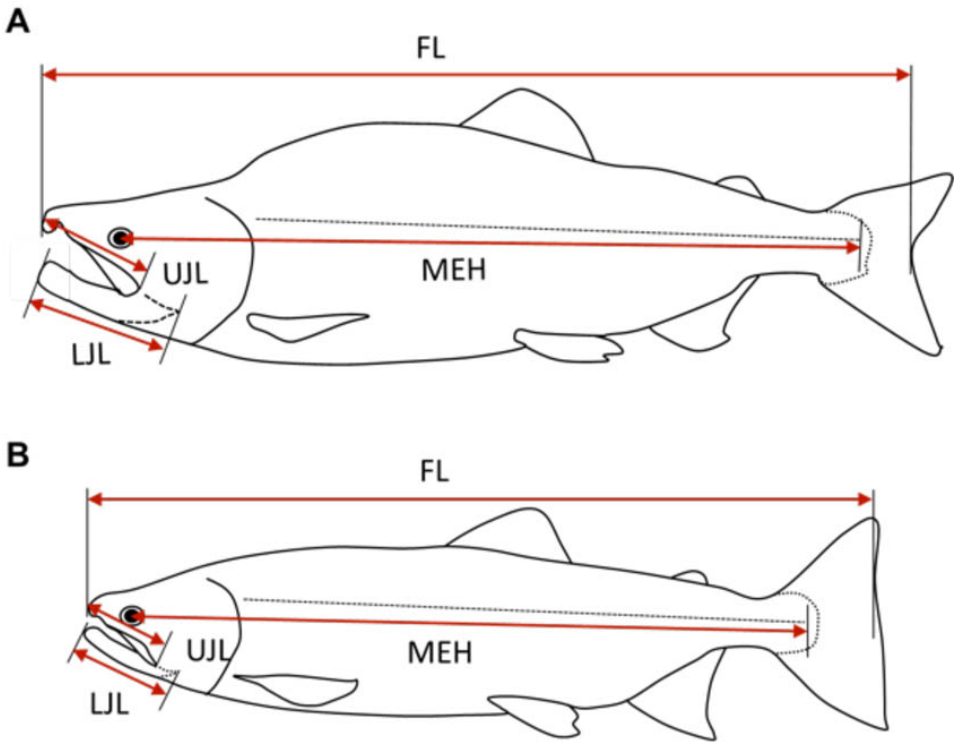

Fig. 2. Body measurements associated with the hooked nose of (A) male and (B) female pink salmon. (FL) fork length, (LJL) lower jaw length, (MEH) mid eye to hypural flexure length, (UJL) upper jaw length.

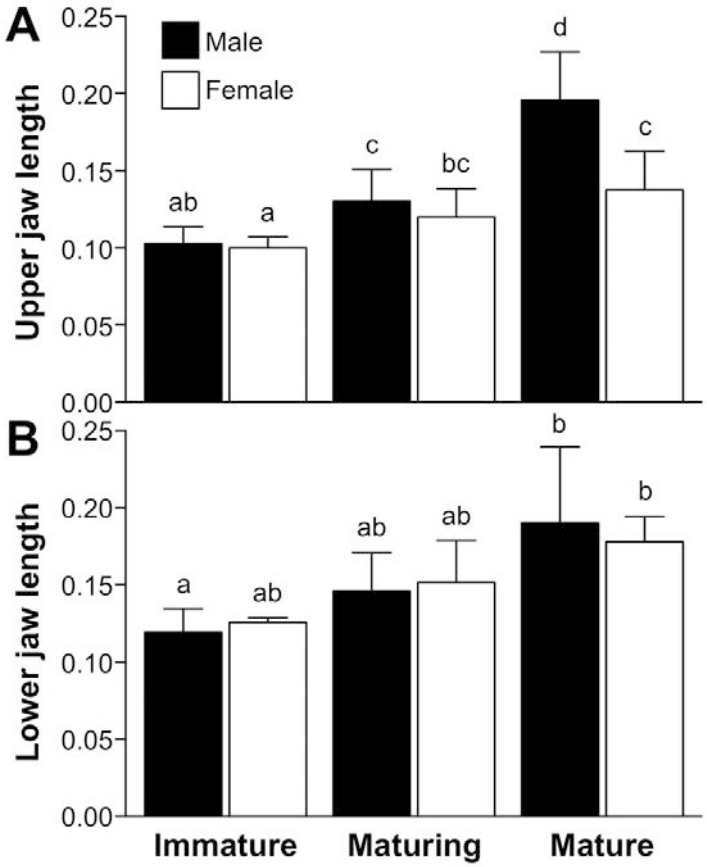

Fig. 3. Comparisons of the upper jaw length (A) and lower jaw length (B) at different stages of secondary sexual characteristic development in male and female pink salmon. Variables are obtained by dividing the upper and lower jaw lengths by the mid eye to hypural flexure length. Values represent the mean ± standard deviation. Bars sharing the same symbol are not significantly different from each other ($p < 0.05$).

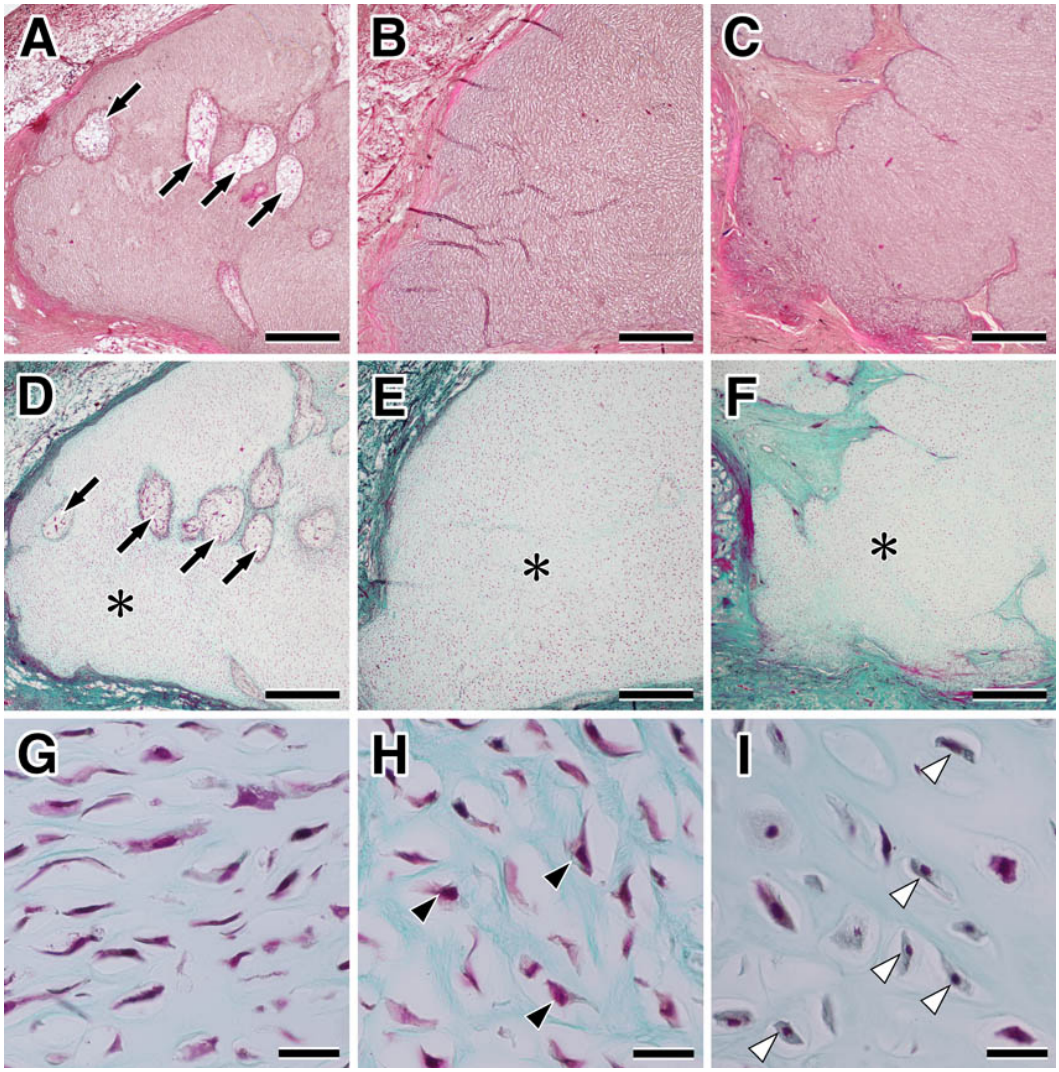

Fig. 4. Photomicrographs of sagittal sections of rostral regions of upper jaws in males at different stages of secondary sexual characteristic development. (A, D, G) immature, (B, E, H) maturing, and (C, F, I) mature. (A-C) hematoxylin and eosin staining, (D-I) Masson trichrome staining. Arrows indicate adipose-like tissue. Asterisks indicates the presence of collagen fibril-enriched extracellular matrix. Black arrowheads indicate cytoplasm-rich cells with high cell activity. White arrows indicate shrinkage nuclei and achromatophil cytosol with degeneration in chondrocytes. Scale bars in Figs. 4A-F and 4G-I: 500 μm and 50 μm, respectively.

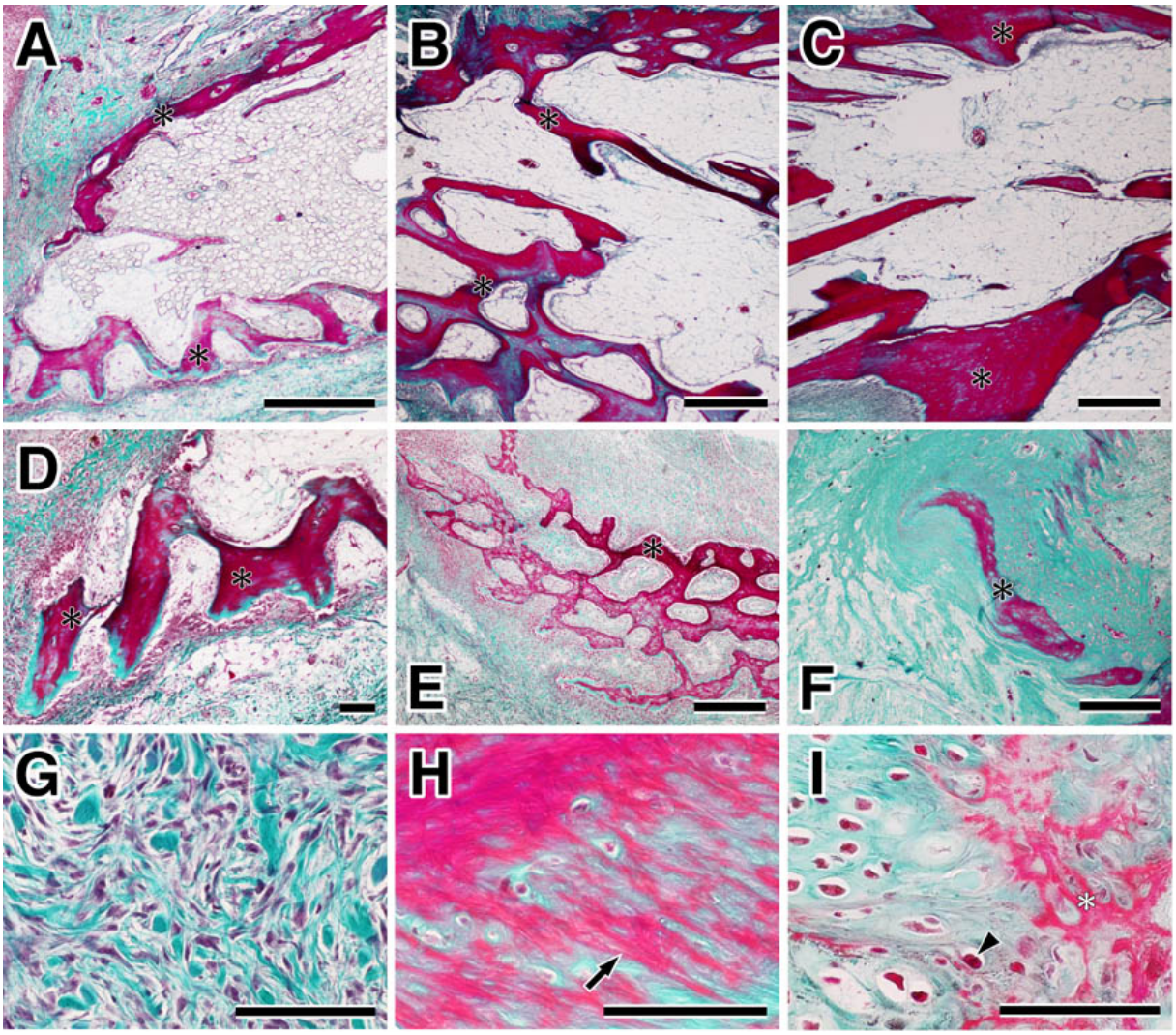

Fig. 5. Photomicrographs of sagittal section of rostral regions in male (A-E, G-I) and female (F) lower jaws at different stages of secondary sexual characteristic development stained with Masson trichrome. (A, D) immature, (B, G, H) maturing, and (C, E, F, I) mature. Dense spongiosa-like structures elongated distally into the connective tissue in mature males (E), but this structure did not elongate in immature males (D) or females (F). Many fibroblasts were observed in distal connective tissue in maturing males (G). Black asterisks indicate the spongiosa-like meshwork. Arrow indicates Sharpey's fiber bone (H). Arrowhead indicates cartilage-like cells, and a white asterisk indicates bone matrix (I). Scale bars in Figs. 5A-C, E, F and Figs 5D, G-I: 500 μm and 100 μm, respectively.

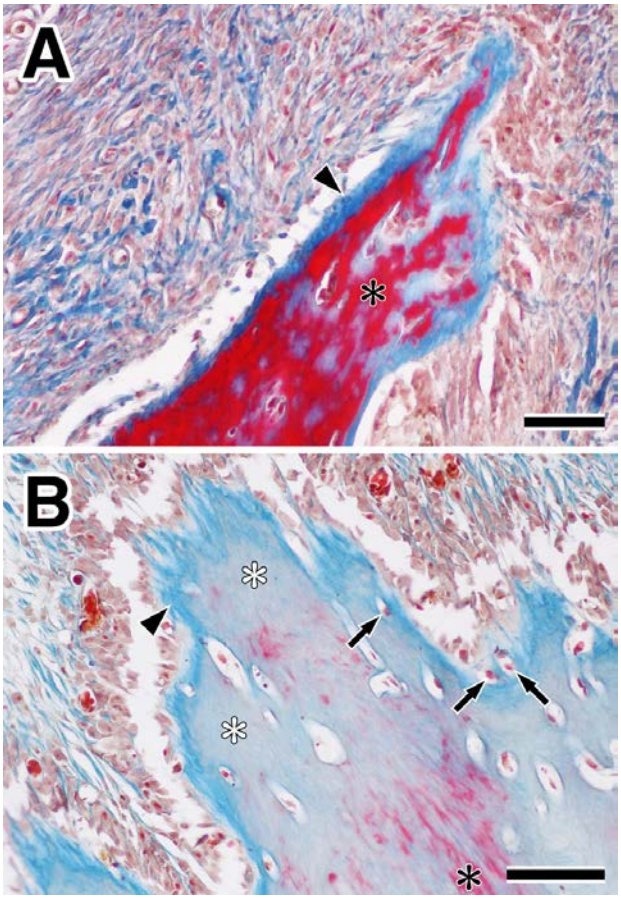

Fig. 6. Photomicrographs of sagittal section at the tip of the dense spongiosa-like structure in immature (A) and maturing (B) male lower jaws stained with Ralis's osteoid staining. Developing ossificating sites were not observed between the ossification zone (red staining, black asterisks) and osteoid zone (blue staining, arrowheads) in immature males, but in maturing males, developing ossificating sites (light-blue staining, white asterisk) were observed. Arrows indicate osteoblasts surrounded by substrate with its own secretions. Scale bars: 100 μm.

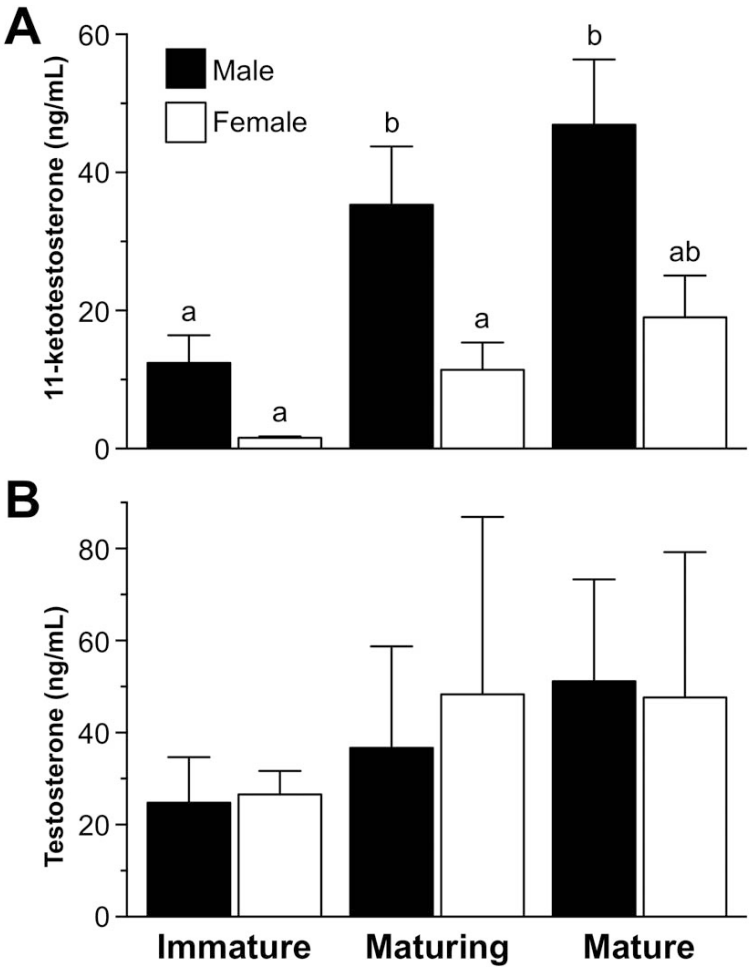

Fig. 7. Comparisons of the serum 11-ketotestosterone (A) and testosterone (B) levels at different stages of secondary sexual characteristic development in male (solid columns) and female (open columns) pink salmon. Data represent the mean ± standard deviation. Symbols sharing the same letters are not significantly different from each other ($p < 0.001$).

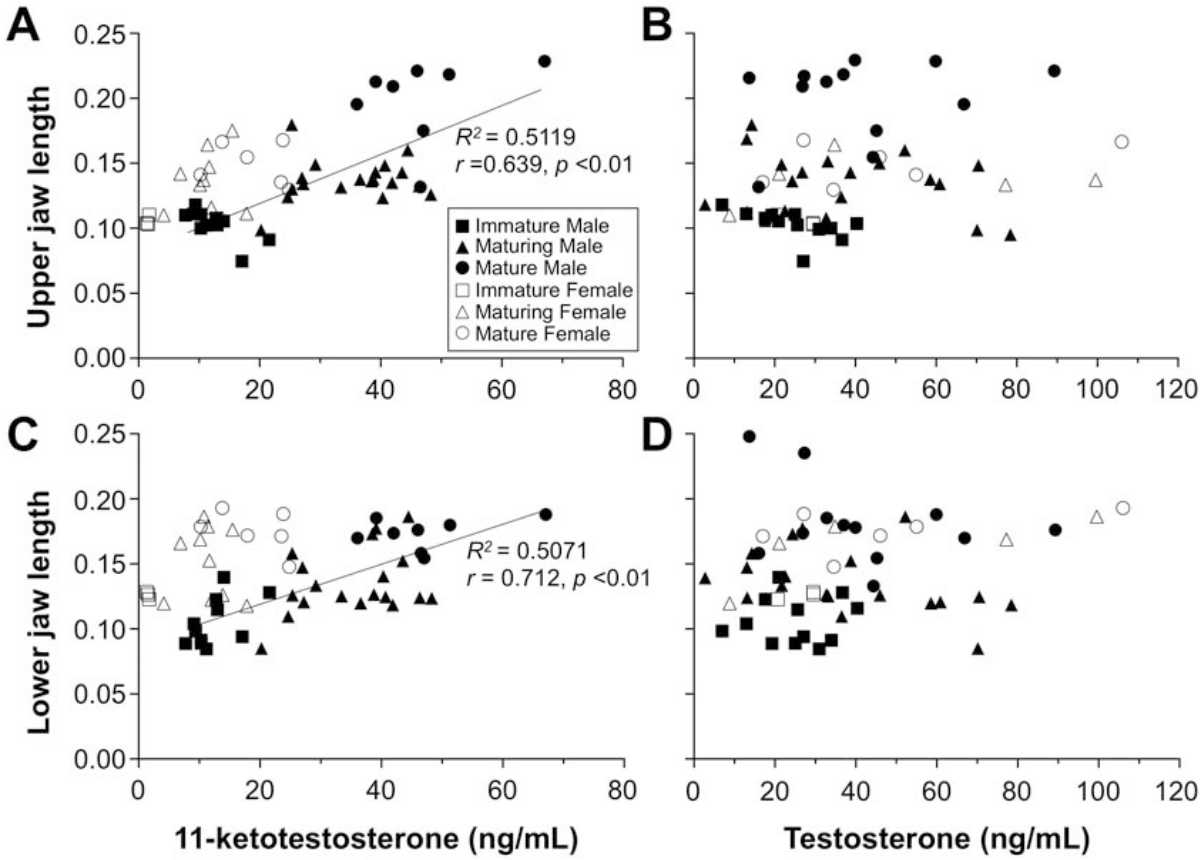

Fig. 8. Relationship between serum androgen, 11-ketotestosterone (11-KT) or testosterone, and jaw length, upper jaw length or lower jaw length in male and female pink salmon. Values are obtained by dividing the both jaw lengths by the mid eye to hypural flexure length. Significant positive correlation between 11-KT and upper jaw length ($R^2 = 0.5119$, $r = 0.639$, $p < 0.01$), 11-KT and lower jaw length ($R^2 = 0.5071$, $r = 0.712$, $p < 0.01$) were observed in males.

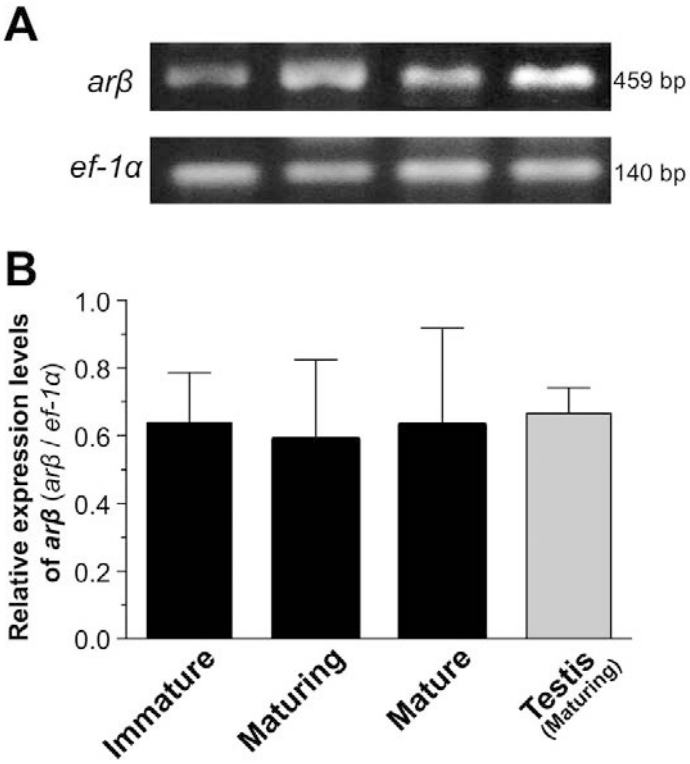

Fig. 9. Agarose gel showing *arβ* and *ef-1α* mRNA expression levels in cartilage tissue in upper jaw of males at each developmental each stage, and in maturing male testis (A). PCR-amplified *arβ* cDNA of approximately 450 bp and *ef-1α* cDNA of approximately 140 bp predicted size. A partial *ef-1α* gene was amplified as a housekeeping gene and used to normalize the relative expression of *arβ* genes. Comparison of the relative expression levels of *arβ* mRNA in male upper jaw cartilage tissue at different stages of secondary sexual characteristic development (B). Values are expressed as means ± standard deviation (n=3). There were no significant differences among the three groups.

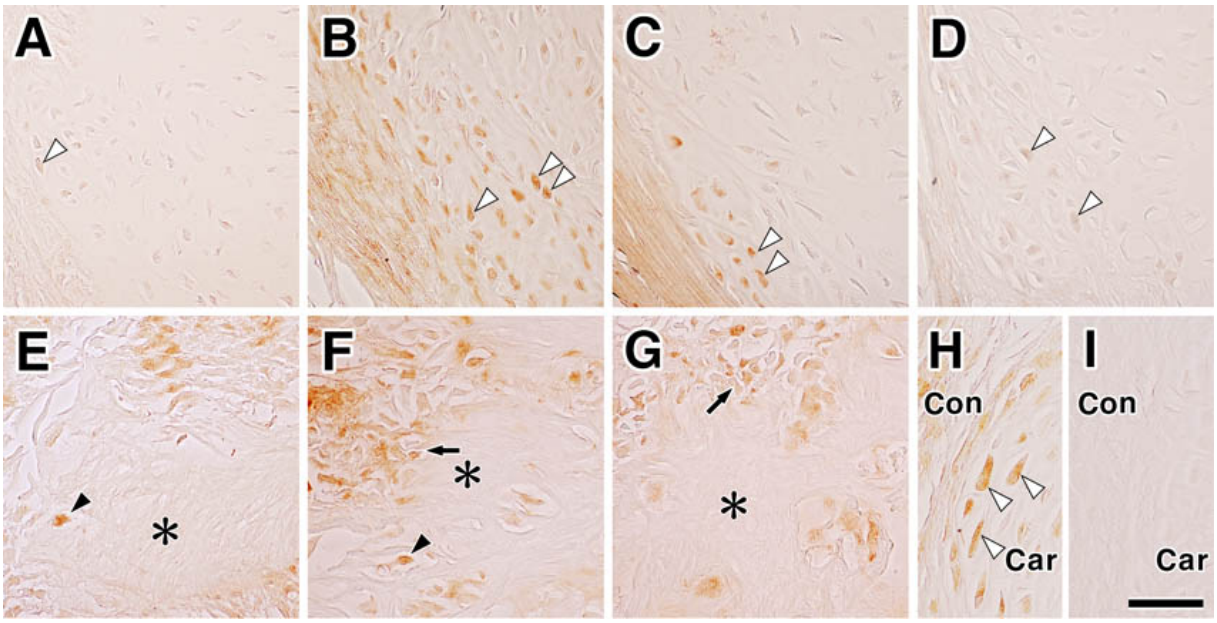

Fig. 10. Immunoreactivity for AR in rostral regions of upper (A-D, H, I) and lower (E-G) jaws at different stages of secondary sexual characteristic development. (A, E) immature male, (B, F, H, I) maturing male, (C, F) mature male, and maturing female (D). Immunoreactivity for AR in rostral regions of maturing male upper jaws using a normal AR antibody (H) and level of immunoreactivity using a pre-absorbed AR antibody with AR antigen C-terminal peptide in a section adjacent to that shown in H (I). White arrowheads indicate AR-immunoreactive chondrocytes. Black arrowheads and arrows indicate AR-immunoreactive osteocytes and osteoblast-like cells, respectively. Asterisks indicate bone matrix. (Car) cartilage tissue, (Con) connective tissue (H, I). Scale bar: 50 μm.

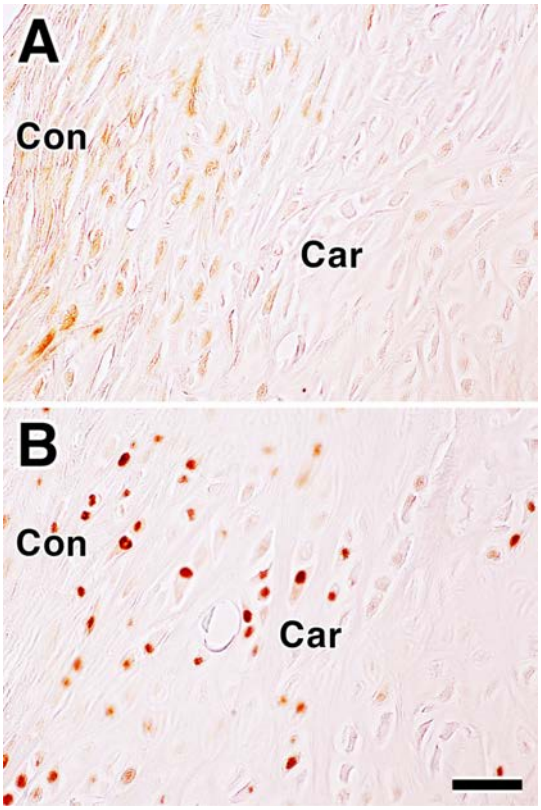

Fig. 11. Immunoreactivity for AR (A) and proliferating cell nuclear antigen (PCNA: B) in rostral regions in maturing male upper jaws. (Car) cartilage tissue, (Con) connective tissue. Scale bar: 50 μm.

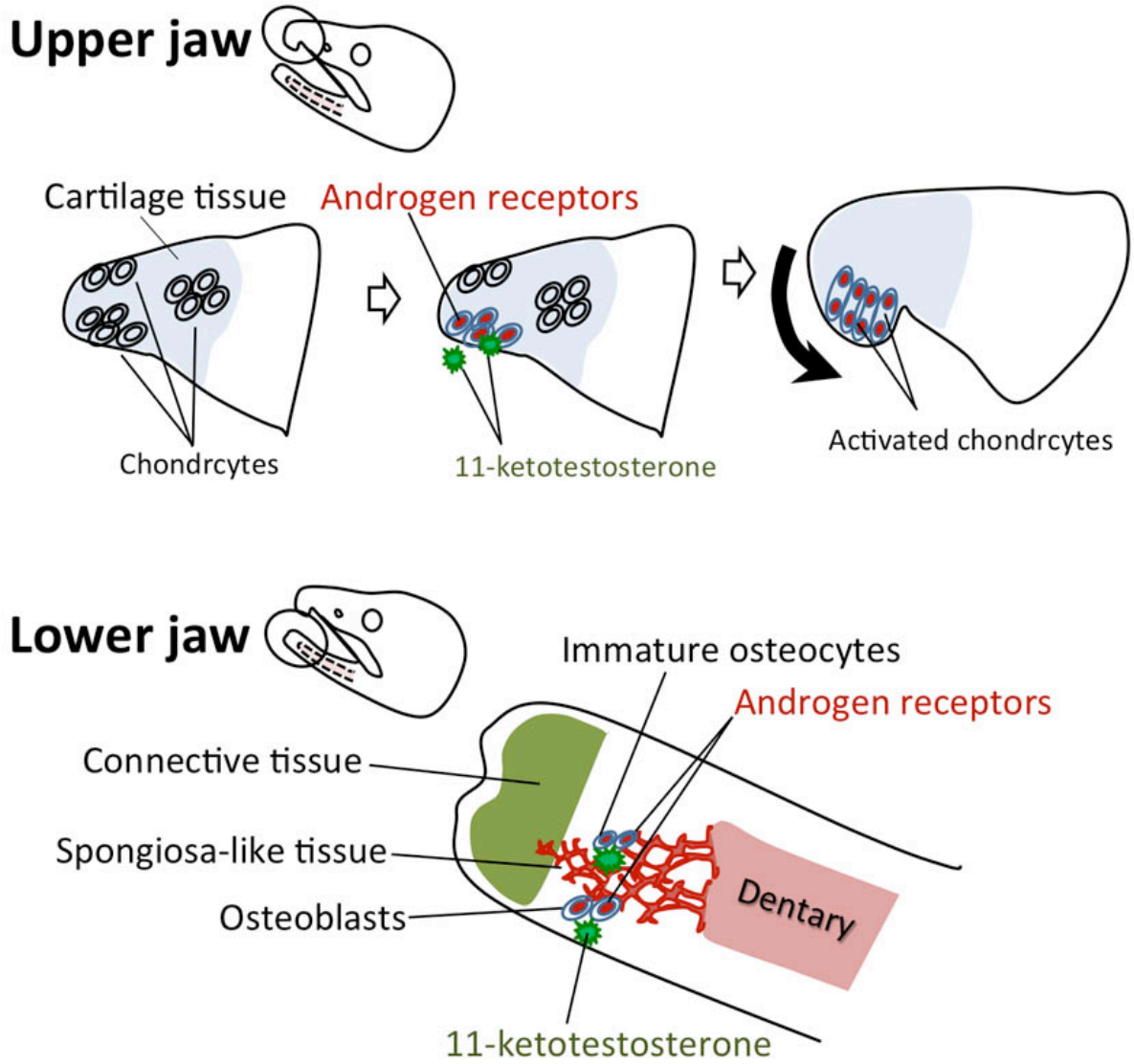

Fig. 12. Schematic illustration of the mechanism of hooked nose formation in the rostral regions of pink salmon jaws.

Table 1. Fish used in this study. Values of fork length (mm), body weight (g), gonad mass (g), and gonad somatic index (GSI: %) are expressed as mean ± standard deviation. N. A. Not available.

Reproductive stage	n	Fork length (mm)	Body mass (g)	Gonad mass (g)	GSI (%)
Immature					
Males	23	419 ± 15.2	804 ± 86.4	2.37 ± 1.05	0.29 ± 0.12
Females	15	404 ± 17.1	728 ± 116	10.4 ± 4.59	1.50 ± 0.52
Maturing					
Males	42	543 ± 32.9	2049 ± 438.4		152 ± 45.6 7.2 ± 1.2
Females	30	508 ± 21.6	1619 ± 199.5		178 ± 31.9 11 ± 1.6
Mature					
Males	26	580 ± 39.6	2255 ± 528.4	N. A.	N. A.
Females	24	525 ± 23.9	1683 ± 351.4	N. A.	N. A.

Table 2. Primer sets used in the RT-PCR analyses for sequence and expression analysis of pink salmon androgen receptor α (*arα*), androgen receptor β (*arβ*), and elongation factor-1α (*ef-1α*).

Name	Primer sequence	GenBank accession No.
ARα_F	5'- GGACATCCATGCACAGATA -3'	AB012095
ARα_R	5'- CATGTGCTGGGGGTT -3'	AB012095
ARβ_F	5'- CACAATATGGACCGAGGCAGGGTAT -3'	AB012096
ARβ_R	5'- GACATGATGGGCAGTTCTTCCKTCT -3'	AB012096
EF-1α_F	5'- GAATCGGCCATGCCCGGTGAC -3'	FJ890356
EF-1α_R	5'- GGATGATGACCTGAGCGGTG -3'	BG933853