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Authors: Salam, MdAbdus, Ota, Yuki, Ando, Hironori, Fukuwaka, Masa-aki, Kaeriyama, Masahide, et al.

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High Population Density of Juvenile Chum Salmon Decreased the Number and Sizes of Growth Hormone Cells in the Pituitary

Md. Abdus Salam[1]*, Yuki Ota[1], Hironori Ando[1], Masa-aki Fukuwaka[2], Masahide Kaeriyama[3] and Akihisa Urano[1]

[1]*Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan,*
[2]*Hokkaido National Fisheries Research Institute, Kushiro 085-0802, Japan and*
[3]*Hokkaido Tokai University, Sapporo 005-8601, Japan*

ABSTRACT—Juveniles of chum salmon (*Oncorhynchus keta*) held at high population density were apparently smaller than those held at medium and low population densities. The effects of high population density on pituitary growth hormone (GH) cells in juvenile chum salmon were examined using immunocytochemical and *in situ* hybridization techniques. The ratio of GH-immunoreactive (ir) area to the whole pituitary was almost constant in all of the high, medium and low population density groups, although the number and sizes of GH-ir cells were decreased in the high population density group. Image-analysis of GH-ir cells indicated the presence of a population of heterogenous cells, in which medium or rather strongly stained smaller cells and as extreme weakly stained larger cells. The medium or rather strongly stained smaller cells predominated in the high population density group, while weakly stained larger cells in the low population density group. *In situ* hybridization study showed somewhat different distributions and intensities of hybridization signals for mRNAs encoding GH I and II precursors. The area showing signals for GH II mRNA in the high population density group was significantly smaller than those in the medium and low population density groups. In contrast, the sizes of areas showing signals for GH I mRNA did not differ among the groups, although the intensity was slightly higher in the high population density group. These results indicate that high population density decreased the number of weakly immunoreactive larger GH cells, and also suppressed expression of the gene encoding GH II precursor, which may result in retarded somatic growth.

INTRODUCTION

Juveniles of many animal species including salmonids in high population density are generally smaller than those in low population density. High population density is believed to be stressful to juveniles. The stress response in teleost fish has remarkable effects on somatic growth. Environmental stressors reduced the growth rate in rainbow trout (Peters and Schwarzer, 1985; Pickering, 1990). Moving from low population density to high population density caused significant physiological stress to juvenile coho salmon (Wedemeyer, 1976). However, there are few reports concerning the response to stressor caused by high population density to synthesis and release of growth hormone (GH) in juvenile salmonids.

Growth hormone plays a crucial role in somatic growth (Donaldson *et al*., 1979). However, the response of GH cells to stressful stimuli in teleost fishes is unclear. Controversial reports have been published on the plasma GH response to stressors in teleost fishes. Plasma GH concentrations increased in goldfish (Cook and Peter, 1984) and rainbow trout (Kakizawa *et al*., 1995), decreased in rainbow trout (Pickering *et al*., 1991; Fabridge and Leatherland, 1992), or did not change in salmonids (Wagner and McKeown, 1986) after application of different types of stressors, such as handling and confinement.

The complete nucleotide sequences of salmon growth hormone (sGH) cDNAs have been determined in several salmonid species (e.g., Sekine *et al*., 1985). The cloned sGH cDNAs are classified into two groups, those for sGH I and sGH II precursors. The overall homology of the coding regions between sGH I and sGH II cDNAs is about 94% in amino acid sequence and 95% in nucleotide sequence (Sekine *et al*., 1989). Such information, in combination with a use of GH antiserum, enabled us to examine changes in synthetic activity of GH cells in the pituitaries of juvenile salmon.The aim of present study was thus to evaluate effects of high population

* Corresponding author: Tel. +81-11-706-2995;
FAX. +81-11-706-4448.
E-mail: salam@sci.hokudai.ac.jp

density on synthetic activity of pituitary GH cells using immuno and molecular cytochemical techniques. GH-producing cells were immunostained and also hybridized in the pituitary sections of juvenile chum salmon with specific oligonucleotide probes for sGH I and II mRNAs.

MATERIALS AND METHODS

Animals

Eggs of chum salmon were artificially fertilized in the Chitose Salmon Hatchery (Hokkaido, Japan) in late October and hatched in December,1994. The alevins were maintained in indoor tanks for two months. Then, a cohort of juvenile chum salmon was divided into three population density groups designated as high (group 1, ca 44000 fish/m^3), medium (group 2, ca 35000 fish/m^3) and low population densities (group 3, ca 9000 fish/m^3). Fish of these three groups were maintained separately in three tanks (0.46 m×0.66 m×0.3 m) under natural photoperiod. The water temperature ranged from 7.8 to 8.4°C, and the water inflow was 10 l/min to each tank. Juveniles of all these three groups were fed with dry pellets equivalent to 3% of body weight per day. Body weight was measured every two weeks and feeding rates were corrected accordingly. Fork length was measured by a caliper to the nearest 0.1 mm. Juveniles were reared under these conditions for four months. Mortality of fish was 65.4% in the high population density group, 8.3% in the medium population density group and 1.6% in the low population density group. Condition factors were calculated using the formula, body weight/fork length^3×1000. Fish were killed by decapitation for sampling (five fish from each group) in June, 1995. Sexes of sampled animals could not be determined since the gonads were not distinguishable at this stage.

Tissue preparation

Decapitated intact heads were fixed in phosphate buffered 4% paraformaldehyde (pH 7.5) at 4°C overnight. It was not necessary to decalcify the skull bones since they did not interfere to cut tissue sections. The samples were dehydrated through a series of graded ethanol and were embedded in paraplast. They were then sagittaly sectioned at 4 μm thickness. The same angle was maintained to cut tissue sections for all three groups to avoid medio-lateral variation of pituitary sections. Tissue sections were divided into three parallel sets as a manner that sections from different parts of the pituitary should be mounted on single gelatinized slides. The same procedure was done for each fish from each group. The sections were deparaffinized, rehydrated through graded ethanol, and were washed in phosphate-buffered saline (PBS, 0.01 M, pH 7.5).

Immunocytochemistry

GH cells were immunostained using a Vectastain ABC (Avidin-Biotin-Peroxidase Complex) kit. Rehydrated sections were preincubated in 1% normal goat serum in PBS in a moisture chamber at room temperature (RT) for 30 min. The proper dilution of antiserum was checked by serial dilutions. The primary antiserum, rabbit anti-chum salmon GH was diluted 1: 64000 in PBS containing 0.5% bovine serum albumin (BSA). It was then applied to slides of one set out of three sets and incubated at 4°C for 48 hr. The rabbit anti-chum salmon GH antiserum (Lot No. 8502) was provided by Prof. H. Kawauchi, Kitasato University, Japan. The sections were then incubated in a solution of biotinylated anti-rabbit IgG at RT for 30 min followed by ABC complex (30 min at RT). After wash, sections were immersed in a DAB solution (3, 3' diaminobenzidine tetrahydrochloride, 0.1%; H_2O_2 , 0.02%; tris-buffer, 0.05 M, pH 7.5) at 20°C for 10 min. The specificity of the antiserum was confirmed by an absorption test using chum salmon GH also provided by Prof. H. Kawauchi. Immunostaining was completely prevented by absorption with the corresponding antigen of chum salmon GH (74.25 ng/ml). Pituitary sections from all of the high, medium and low population density groups were immunostained in the same run to maintain the same experimental condition among groups, since immunoreactive differences were accounted for analysis.

In situ hybridization

After rehydration, the sections were preincubated with a hybridization buffer containing 20 mM Tris-HCl, 1 M NaCl, 6 mM ethylenediamine-tetra-acetic acid (EDTA), Denhardt's reagent (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 10 mM dithiothreitol (DTT) and 100 μg/ml denatured calf thymus DNA in the moisture chamber at 42°C for 1 hr. The sequence of oligonucleotide probes for GH I (5'-CAGAGAAGAACATCTTTATTGAAACATGAA-3'; 30-mer) and GH II (5'-TCCATCTGTGGACATACCTAAAGATTGGAG-3'; 30-mer) were selected from the specific regions of GH I and GH II mRNAs. The oligonucleotide probes for GH I and GH II were labeled at the 3' ends with [α-^{35}S] dATP (NEN, USA) by a 3' end labeling system (Amersham, UK). The specific activities of GH I and GH II probes were 4.6×10^9 cpm/μg and 2.9×10^9 cpm/μg, respectively. The radiolabeled probes were diluted with hybridization buffer in which 10% dextran sulphate was added. The probes were then applied to each slide (88 μl/slide). Afterwards, tissue sections with overlying coverslips were incubated in a moisture chamber at 46°C for 16 hr. Coverslips were removed in 2× saline-sodium citrate (SSC) (1× SSC is 150 mM sodium chloride and 15 mM sodium citrate, pH 7.0) at RT. Then the sections were washed in 2× SSC at RT for 20 min, twice in 1× SSC at 45°C for 30 min each, and 2× SSC at RT for 10 min. They were dehydrated through a series of graded ethanol containing 0.3 M ammonium acetate and air-dried, were dipped in a emulsion (Konica NR-M2 diluted 2:1 in distilled water), air-dried again and exposed at 4°C for 4 days. After development in Kodak D-19, the sections were counterstained with hematoxylin-eosin. Finally, the sections were dehydrated through a series of graded ethanol, cleared in xylene and mounted under coverslips with permount. It is hereby noted that the samples from all groups were hybridized in the same batch to ensure the same condition of the experiment.

Measurement of pituitary and GH-ir area

Camera-lucida drawing was made on peripheral, para-sagittal and mid-sagittal sections for both whole pituitary and GH-ir area. Then the pituitary areas were cut off and weighed. Similarly, GH-ir areas were cut off and weighed. These values were then calibrated as μm^2, and the greatest values of individuals were used for later image analysis and statistical analysis.

Image analysis

Five sections from each pituitary gland were selected for image analysis. The selected sections contained the largest GH-immunoreactive (ir) areas as found by camera-lucida drawing in all fish of each group. They were the para-sagittal and mid-sagittal parts of the pituitary that included a combination of cells from the peripheral and central parts of GH-immunoreactive (ir) area. The photomicrographs of these sections were taken with reversible film (Kodak ektachrome) with a multipurpose microscope (Nikon Microphot-FXA, Nikon, Japan) and were scanned with a film scanner (Polaroid Scanner, Polaroid Corp., USA) at 1350 dpi for image analysis. Immunoreactivity and sizes of all recognizable GH-ir cells of the scanned sections were measured by a computer-aided Image Pro-Plus software (Media Cybernetics, USA). Background image of the GH-ir sections was not subtracted, since it was minimal.

For *in situ* hybridization analysis, five sections were also selected from each pituitary for all groups. They contained the largest hybridized areas as measured by a computer-aided Image Pro-Plus software. The photomicrographs of these sections were taken using the dark-field of a microscope (Nikon Microphot-FXA, Nikon, Japan), and were scanned as above for densitometric image analysis. Hybridized areas of GH I and II mRNAs and intensity of hybridization signals

were determined by the Image Pro-Plus software. The background intensity around the specifically hybridized area was subtracted from the hybridization signals for GH I and II mRNAs.

Statistical analysis

Data are expressed as mean±SEM. After one way analysis of variance (ANOVA), the differences among groups were tested by Tukey's significant difference procedure (TSDP). The correlation between immunoreactivity and cell sizes was analyzed using linear correlation method. Gauss Model was used to test the presence of numbers of peaks (populations of cells) in the frequency distribution histograms of immunoreactivity using a computer software, Microcal Origin (Microcal Software, Inc. USA).

RESULTS

Somatic growth

Fork length, body weight and condition factor were significantly greater in the low population density fish than in the high population density animals (Fig. 1).

Immunocytochemistry

The low population density group fish had larger pituitaries including larger GH-ir areas compared with the high population density group (Figs. 2, 3A). In the high population density group, medium or rather strongly stained small GH-ir cells were compactly distributed in the proximal pars distalis (PPD), while weakly stained cells distributed sparsely in the periphery of GH-ir area (Fig. 2A). On the other hand, medium or rather strongly stained small cells and weakly stained cells were intermingled in the PPD of the low population density group (Fig. 2B). The peripheral part of GH-ir area was dominantly occupied by weakly stained cells (Fig. 2B). The ratios of GH-ir area versus the whole pituitary area were not significantly different among groups (Fig. 3B). The numbers of GH-ir cells per pituitary sections were greater in the medium and low population density group than that in the high population density group (Fig. 3C). The number of GH-ir cells versus pituitary area (1 μm^2) were not significantly different among groups (Gr 1, 0.007±0.001; Gr 2, 0.007±0.0004; Gr 3, 0.006±0.0005).

Image analysis showed that the sizes of GH-ir cells in the medium and low population density groups were larger than that in the high population density group (Fig. 4, $p<0.01$). The mean staining intensity in GH cells were, however, similar among the three groups (mean±SEM), although frequency distributions of immunoreactivity were different (Fig. 5).

The symmetrical bell-shaped histograms as found in the high population density group was rather asymmetrically skewed to right in the medium and low population density groups, indicating that medium or rather strongly stained small GH-ir cells were predominant in the high population density group (Fig. 5). Analysis of these skewed histograms using a Gauss Model by curve fitting agreed to show a tendency of two peaks, i. e., the presence of two populations of GH-ir cells in the medium and low population density groups rather than one peak in the high population density group (Fig. 5).

Furthermore, analysis of the cell sizes and immunoreactivity using Scatter plot distributions in the high and low population density groups indicated that GH-ir cells in the pituitaries of the low population density group comprised of a population of heterogenous cells, weakly stained larger cells and medium or somewhat strongly stained smaller cells with many transitional stages (Fig. 6). Weakly stained larger GH cells were noticeable in the low population density group compared with the high population density group (Fig. 6, arrow).

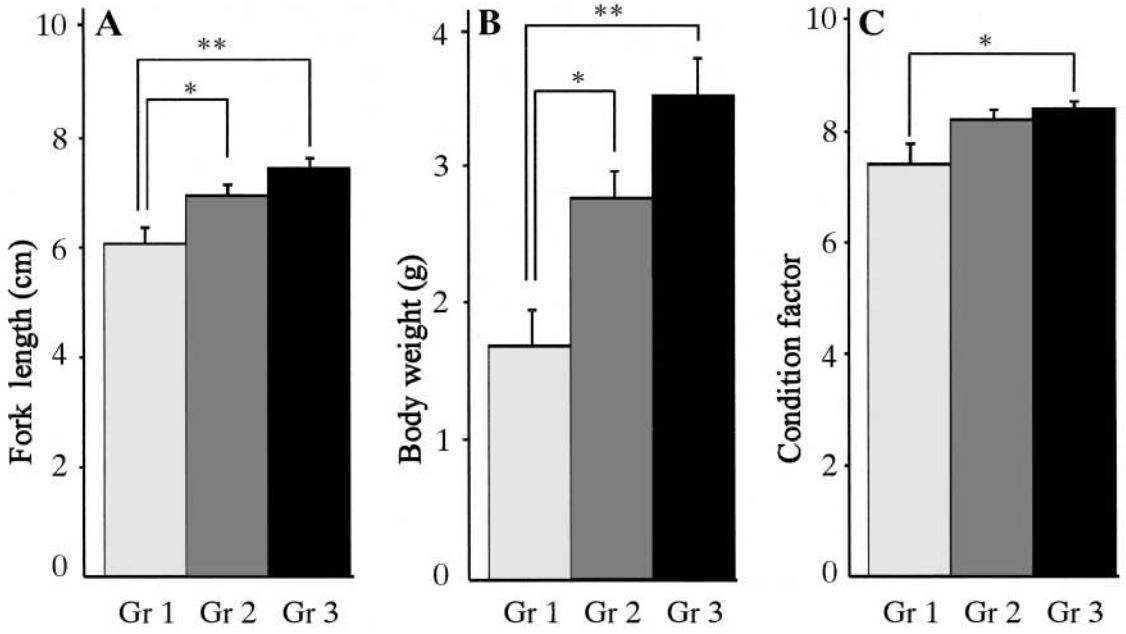

Fig. 1. Fork length (A), body weight (B) and condition factor (C) in the high (Gr 1), medium (Gr 2) and low (Gr 3) population density groups. Values are mean±SEM (n=5). *, $p<0.05$; **, $p<0.01$ compared with Gr 1 by Tukey' significant different procedure (TSDP).

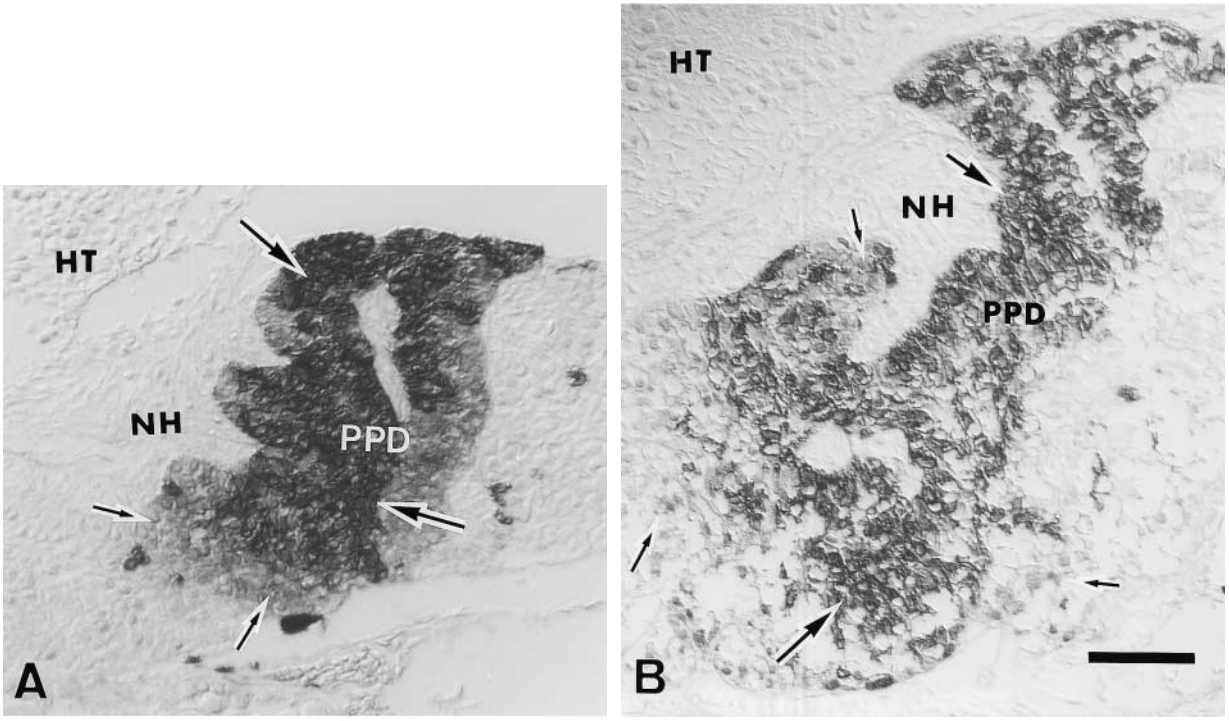

Fig. 2. Photomicrographs of GH-ir areas in the sagittal view of the pituitaries of juvenile chum salmon kept in different population densities. A, high population density fish; B, low population density fish. Note the medium or rather strongly stained cells (larger arrows) and faintly or weakly stained cells (smaller arrows) in the high and low population density fish. HT, hypothalamus; NH, neurohypophysis; PPD, proximal pars distalis. Scale bar: 50 μm.

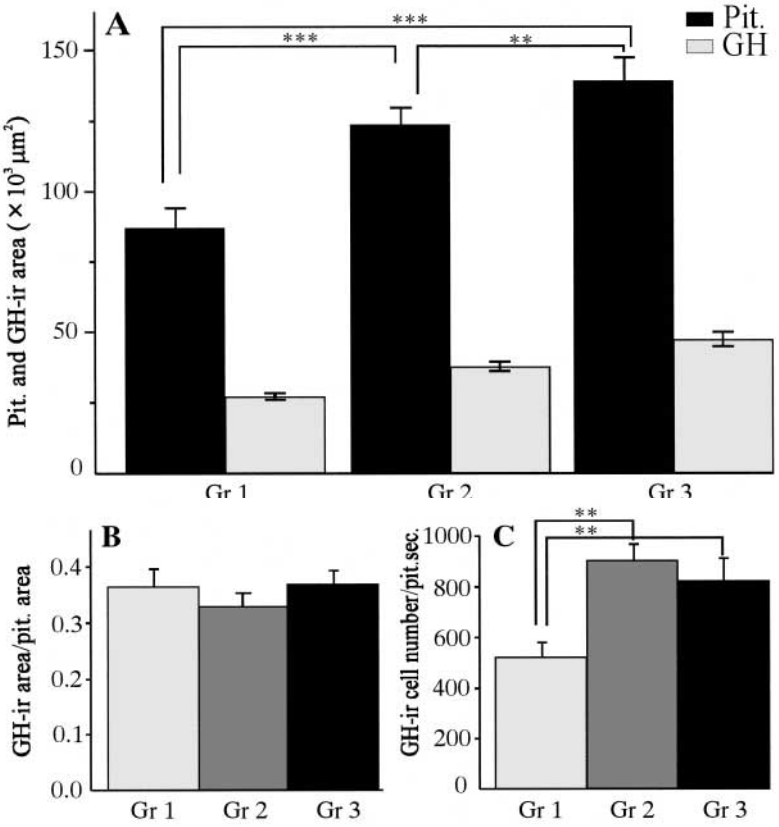

Fig. 3. Sizes of pituitaries and GH-ir areas (A), their ratios (B), and the numbers of GH-ir cells per individual pituitary sections (C) which included the largest GH-ir area. Gr 1, high population density group; Gr 2, medium population density group; Gr 3, low population density group. Values are mean±SEM (n=5). **, $p<0.01$; ***, $p<0.001$ compared with Gr 1 by TSDP.

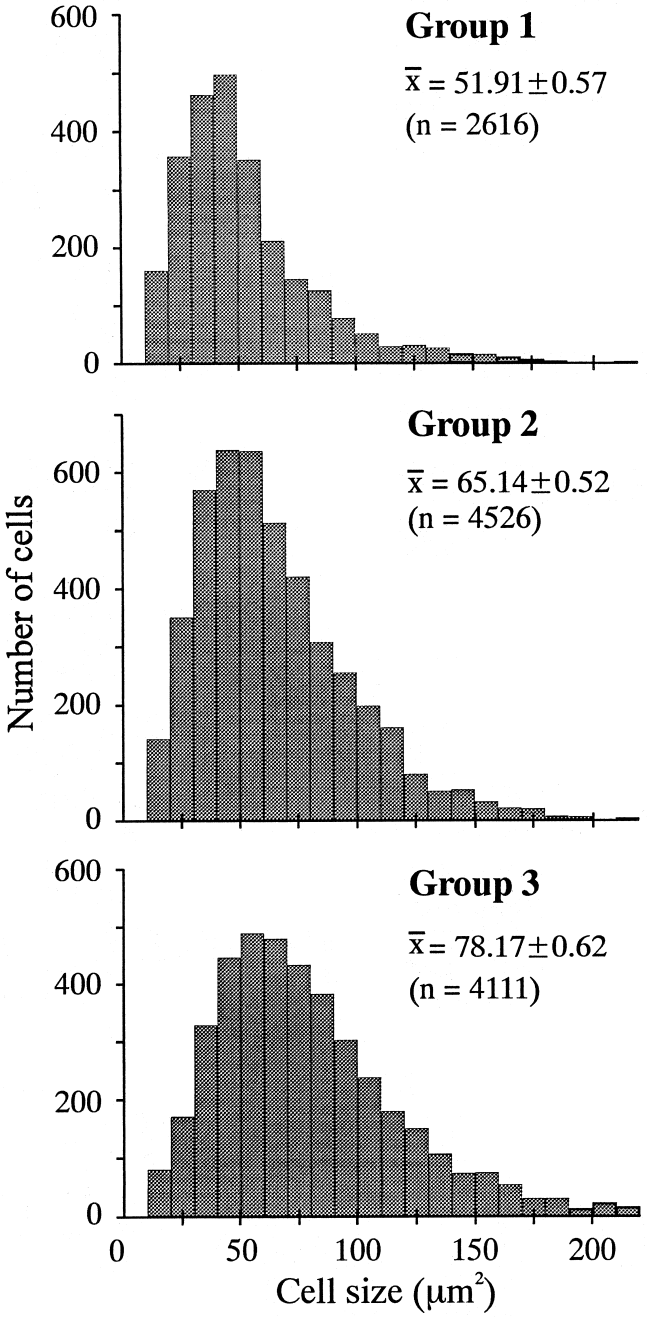

Fig. 4. Histograms showing the frequency distributions of pituitary GH-ir cell sizes in the high (group 1), medium (group 2) and low (group 3) population density groups. Note that the greater numbers of larger cells in the groups 2 and 3. The numbers of cells analyzed: group 1, 2616; group 2, 4526; group 3, 4111.

In situ hybridization

The areas which contained hybridization signals for GH I and II mRNAs were localized almost in the same region of the pituitary where GH-immunoreactive (ir) cells were distributed (Figs. 2, 7). The size of hybridized areas and signal intensity of GH I did not differ among groups (Figs. 8A and 8B). The hybridized areas showing signals for GH II mRNA were significantly larger in the medium and low population density group (Fig. 8C) than those in the high population density group. In the low population density group, the area showing GH II mRNA signals included the periphery of GH-ir area in which weakly stained GH-ir cells were located (see Figs. 2B and 7D), while the areas for GH I mRNA signals occupied in the central part of GH-ir area (Fig. 7B). The intensity of hybridization signals for GH II mRNA was slightly but significantly stronger in the low population density group (Fig. 8D).

DISCUSSION

In the present study, we first found that, in the pituitary of juvenile chum salmon, GH-ir cells consisted of heterogenous cells, at least weakly stained larger cells and medium or somewhat strongly stained smaller cells. The pituitary GH-ir areas in the low population density group included a considerable number of weakly stained larger cells, whereas those in the high population density group dominantly contained medium or somewhat strongly stained smaller cells. Thus, the average sizes of GH-ir cells in the medium and low population density group were larger than those in the high population density group. Although not clearly described by the authors, rainbow trout GH-ir cells in serum-free culture consisted of faintly stained cells and medium to strongly stained cells (Yada *et al.*, 1991). This result supports the present findings that GH-ir cells in the salmonid pituitary are composed of a population of heterogenous cells, which may differ in secretory activity.

The difference in the features of GH-ir cells mentioned above indicates that high population density decreased or suppressed secretory activity of a portion of pituitary GH-ir cells in juvenile chum salmon. Interpretation of changes in immunoreactivity and cell sizes in terms of secretory activity is not always so straightforward. In the case of salmonid pituitary, active GH cells in serum-free culture showed a drastic decrease in immunoreactivity and hypertrophy accompanied by a decreased number of secretory granules and dilated rough endoplasmic reticulum (Yada *et al.*, 1991). The dilation of rough endoplasmic reticulum indicates enlargement of cell sizes, and the decrease in the number of secretory granules indicate high secretory activity of pituitary cells. In addition, the amount of accumulated hormone in the pituitary cells determines the degree of immunological reaction. Thus weakly stained GH cells indicated less amount of hormone stored in these cells, as reported in striped bass, *Morone saxatilis* (Huang and Specker, 1994). Therefore, weakly stained larger cells in low population density fish may have higher secretory activity.

Yada *et al.* (1991) also showed that the salmonid pituitary in serum-free culture continuously released a large amount of accumulated and newly synthesized GH. The presence of weakly immunoreactive larger GH-ir cells in the low and medium population density group indicate that some parts of GH-ir areas in the pituitaries actively synthesized and released the hormone. The higher secretory activity of GH cells in these parts may be responsible for the elevation of plasma GH levels found in juvenile salmonids during parr-

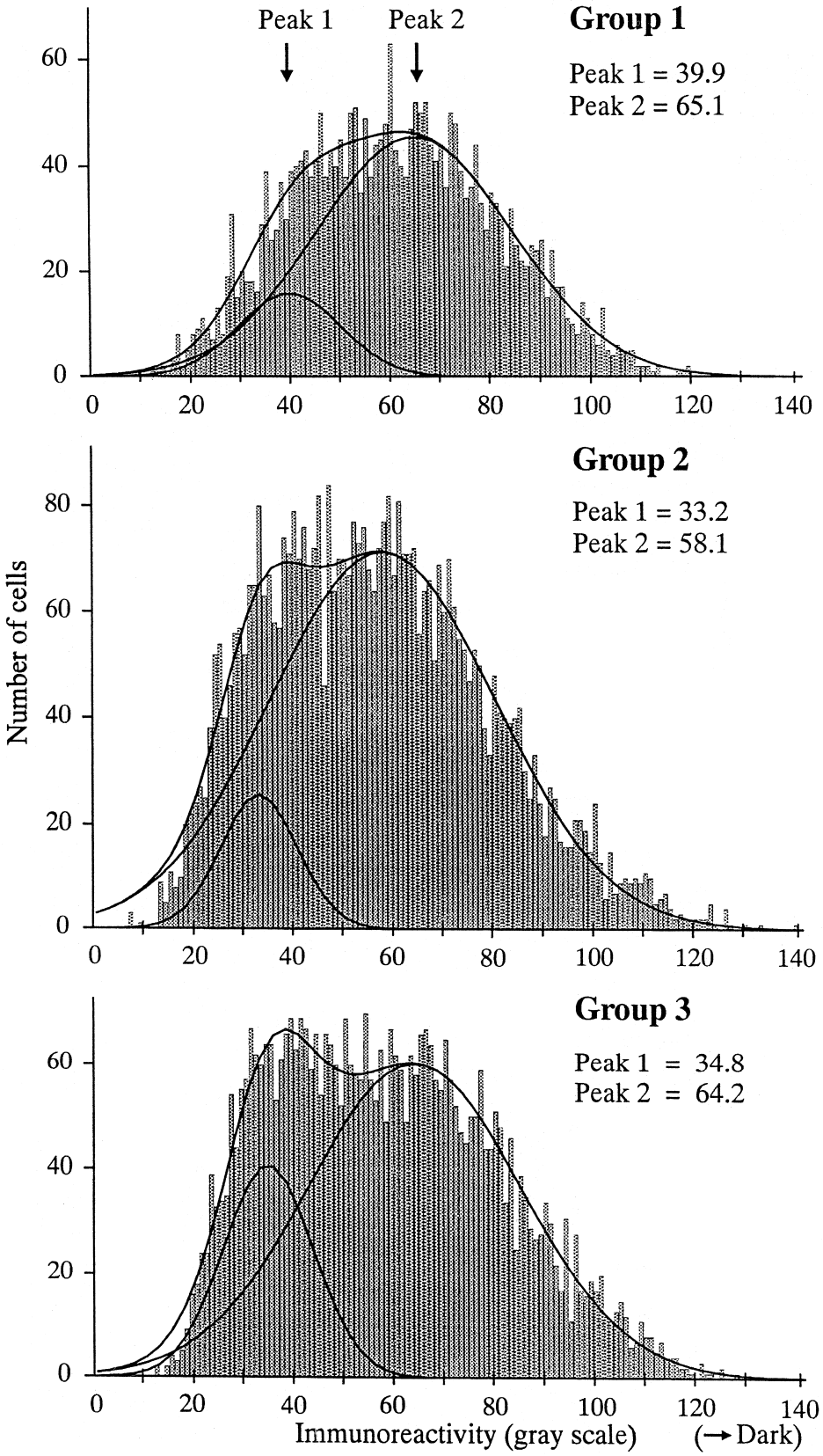

Fig. 5. Histograms showing the frequency distributions of GH immunoreactivity expressed as arbitrary unit from the gray scale values in the high (group 1), medium (group 2) and low (group 3) population density groups. Note the asymmetrical distribution histograms of immunoreactive cells, skewed to right by the presence of two peaks (one sharp peak and another blunt peak) in the groups 2 and 3. The numbers of cells analyzed: group 1, 2616; group 2, 4526 and group 3, 4111.

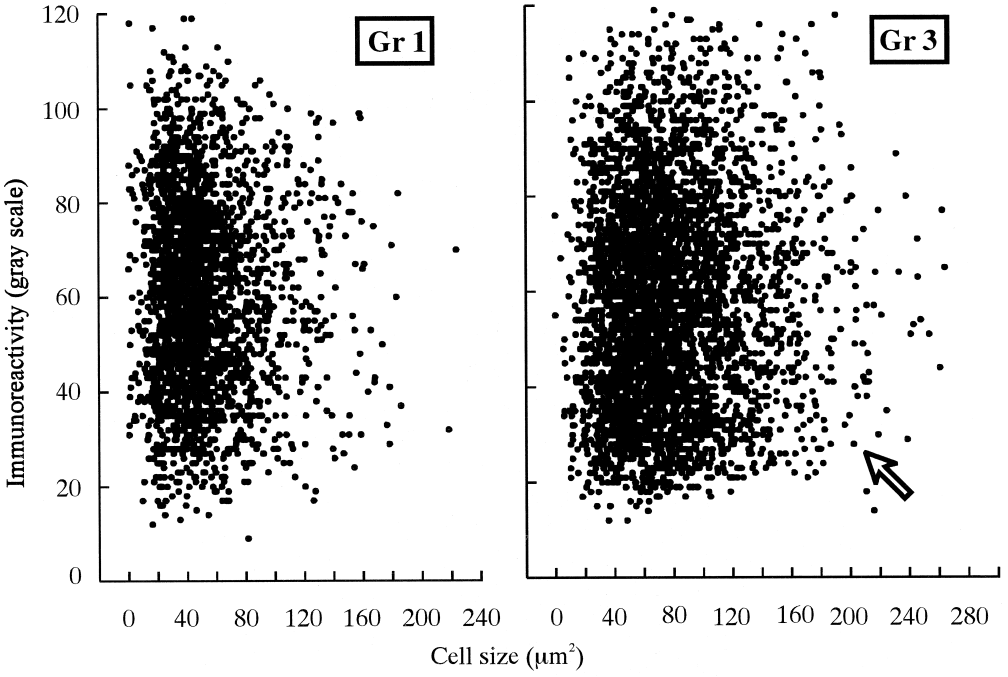

Fig. 6. Scatter plot distributions between GH-ir cell sizes and their immunoreactivity in the high (Gr 1) and low (Gr 3) population density groups. Note the distributions of weakly stained larger cells (arrow) in the group 3. The numbers of cells analyzed: 2616 in group 1, and 4111 in group 3.

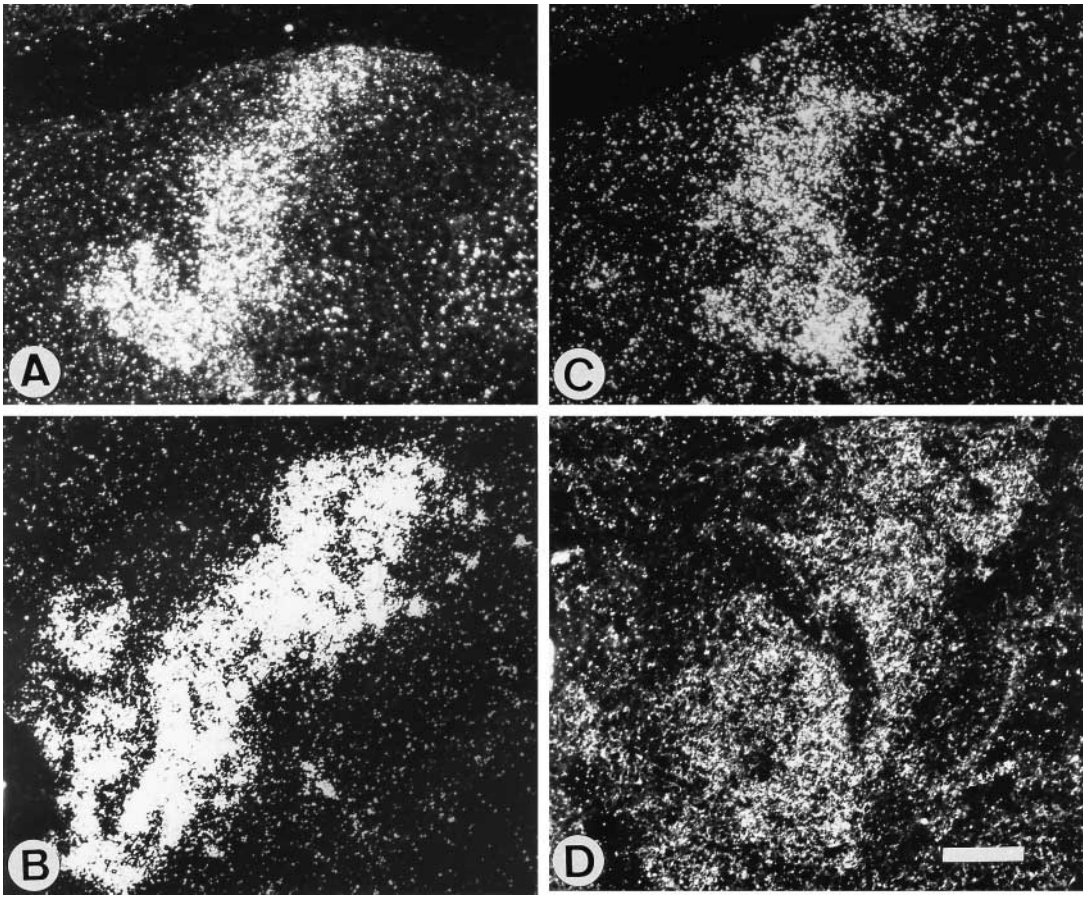

Fig. 7. Dark-field photomicrographs of hybridized areas showing GH I (A and B) and GH II (C and D) mRNA signals in the sagittal view of pituitaries of juvenile chum salmon kept in different population densities. A and C, high population density fish; B and D, low population density fish. Scale bar, 50 μm.

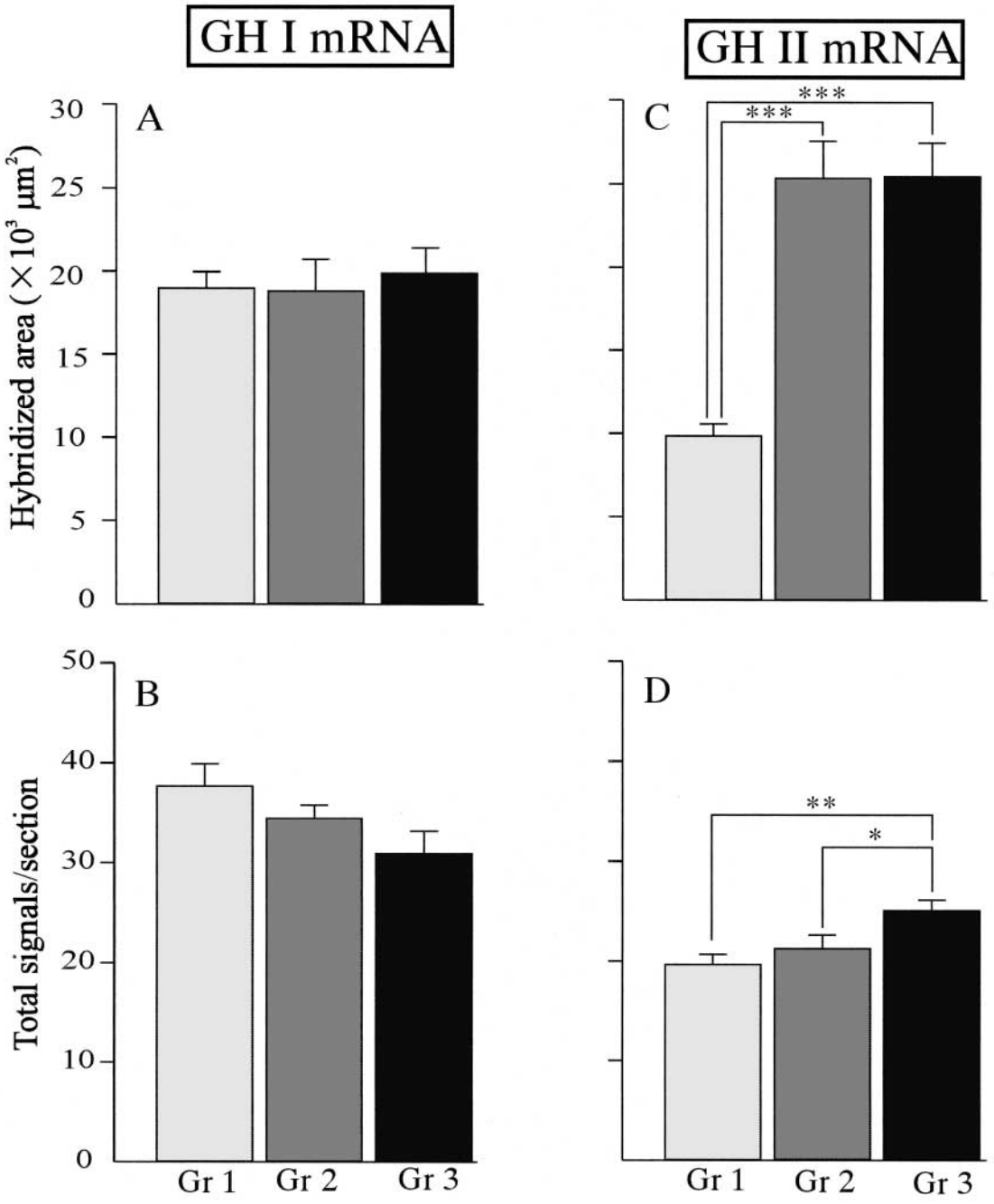

Fig. 8. Sizes of hybridized areas (A, C) and intensity of signals (B, D) for GH I (A, B) and GH II (C, D) mRNAs. Intensity of hybridization signals was expressed in terms of gray scale after background subtraction. Values are the mean±SEM (n=5). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ compared with Gr 1 by TSDP.

smolt transformation in spring when the growth rate is high (Dickhoff *et al.*, 1997).

The greater numbers of GH-ir cells in the medium and low population density groups coincide with the findings from *in situ* hybridization that the sizes of areas showing hybridization signals for GH II mRNA in the medium and low population density groups were larger than that in the high population density group. In contrast, the sizes of areas showing signals for GH-I mRNA did not differ among the groups. It is therefore possible that the expression of GH-II gene was specifically affected by the high population density in growing chum salmon juveniles. This may be true, since the use of radio-labeled oligonucleotide as a molecular probe for *in situ* hybridization can yield highly specific signals with semi-quantitative information on the amount of particular species of mRNA (Hyodo *et al.*, 1988; Urano and Hyodo, 1990).

Changes in the amount of mRNA for a particular hormone are considered to reflect changes in synthetic activity at least

at the transcriptional level. The present study showed small though significant differences in the intensities of hybridization signals for GH II mRNAs among groups.

To date, no functional differences have been found between salmonid GHs from different precursor genes, but a possibility of different physiological function of two forms of GHs can not be ignored. The GH II gene was found to be sex-linked in coho salmon (Forbes *et al*., 1994). In the present study, the somatic growth was suppressed in the high population density group, although hybridized areas and signals for GH I mRNA were unaffected, compared with the low population density group. In contrast, hybridized areas considerably increased in parallel with the growth in the low population density group, which have the highest growth rate. Thus, the differential expression of GH I and GH II mRNAs suggest a different role of GH I and GH II genes as seen in tilapia prolactin mRNAs (Nishioka *et al*., 1993, Shepherd *et al*., 1997). Regulation of two genes may differ as was suggested in two genes encoding precursors of salmon gonadotropin-releasing hormone (Higa *et al*., 1997). The levels of GH mRNA in individual pituitary cells were maintained at around the same level in serum-free culture, in which the salmon pituitary continuously released a large amount of GH (Yada *et al*., 1991). At present, there is no clear line of evidence to explain the above inconsistencies between the levels of mRNAs and the rate of hormone secretion. Possibly, the half-life of GH mRNAs is very short with high turn-over rate. At any rate, the experimental animals used in the present study were sampled in June, when the plasma levels of GH were presumably elevated to promote parr-smolt transformation (Dickhoff *et al*., 1997).

In teleosts, GH cells were very active in secretory function during rapid somatic growth (Nagahama, 1973; Power, 1992), whereas acute stress, such as confinement, handling and crowding, reduced somatic growth (Pickering, 1990; Goede and Barton, 1990; Wendelaar Bonga, 1997), possibly by suppressing secretory activity of GH cells. The present study showed that, compared with the medium and low population density groups, weakly stained larger GH cells were sparse in the pituitary of the high population density group. If high population density was a stressful stimulus, it may act to suppress the number of GH II gene-expressing cells. This suppression presumably resulted in a decrease in the total expression of GH II gene in the pituitary to reduce plasma levels of GH, and thereby retarded somatic growth in these juvenile salmonids.

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