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Immunohistochemical changes in production of pituitary hormones during artificial maturation of female Japanese eel *Anguilla japonica*

YUICHI OZAKI,[a]* KOICHI ISHIDA, KOJI SAITO, KAZUHIRO URA, SHINJI ADACHI AND KOHEI YAMAUCHI

Division of Marine Biosciences, Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan

ABSTRACT: Specific antibodies against follicle-stimulating hormone β subunit (FSHβ), prolactin (PRL), and somatolactin (SL) of the Japanese eel *Anguilla japonica* were produced. These antibodies, as well as antibodies against luteinizing hormone β subunit (LHβ) and growth hormone (GH) produced previously, were used to examine changes in the production of pituitary hormones in female eels during maturation induced by salmon pituitary homogenate (SPH) injection. Immunohistochemical observations showed a decrease in FSH production after SPH injection, suggesting that SPH inhibits FSH production. In contrast, LH production increased markedly with maturation. The number of GH producing cells decreased gradually during maturation, possibly because of inhibition by exogenous GH present in the SPH and/or endogenous insulin-like growth factor-I produced by the stimulation of salmon GH. Although changes in the number of PRL producing cells with maturation were not evident, the number of SL producing cells showed a peak at the late vitellogenic stages, and thereafter decreased to the migratory nucleus stage. These results suggest that GH and SL are involved in sexual maturation in SPH injected eels, as in other fishes.

KEY WORDS: **gonadotropin, growth hormone, immunohistochemistry, Japanese eel, maturation, prolactin, somatolactin.**

INTRODUCTION

The Japanese eel *Anguilla japonica* is a highly valued species in Japan, which is cultured in captivity. However, Japanese eels do not mature under normal culture conditions. Salmon pituitary homogenate (SPH) or extract (SPE) injection is used extensively to induce maturation in female Japanese eels in captivity. Recently, it was reported that preleptocephali, which were obtained from females matured by SPE injection, could be successfully reared on a slurry-type diet made from shark eggs, and that these larvae could grow to the leptocephalus and glass eel developmental stages.[1,2] However, the survival rate of the larvae is low, and transition to the leptocephalus stage is not achieved routinely. This effect may be caused by abnormal gonadal development resulting in poor egg quality. To improve maturation induction, it is essential to further investigate the regulation mechanisms of ovarian development controlled by endogenous and exogenous hormones.

Gonadotropin is an important pituitary hormone regulating gonadal development. In many teleosts, two types of gonadotropins have been identified: (i) tetrapod follicle-stimulating hormone (FSH); and (ii) luteinizing hormone (LH), which consist of a heterodimer composed of a common α subunit and a specific β subunit (FSHβ and LHβ).[3] The complementary DNAs (cDNAs) encoding Japanese eel FSHβ and LHβ have been cloned and changes in gonadotropin transcripts during artificial maturation have been studied extensively.[4–8] However, little is known about the expression profiles of other pituitary hormones during artificial maturation.

Growth hormone (GH), prolactin (PRL), and somatolactin (SL) are the main hormones produced in the pituitary gland. Commonly in teleosts, gonadotropins and GH are produced in the

*Corresponding author: Tel: 81-55-981-5849. Fax: 81-55-981-5849. Email: yozaki@lab.nig.ac.jp

[a]Present address: Genetic Strains Research Center, National Institute of Genetics, Mishima, Shizuoka 411-8540, Japan.

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proximal pars distalis (PPD). PRL and SL producing cells are located in the *rostral pars distalis* (RPD) and *pars intermedia* (PI), respectively. Because of their considerable structural similarity, GH, PRL, and SL are thought to have evolved from a common ancestral molecule by gene duplication, and belong to the GH–PRL family. In several fishes, the GH–PRL family has been implicated in reproduction.[9–17] To better understand the hormonal regulation of ovarian development induced by SPH injection, it is necessary to know the expression profiles of these hormones. Immunohistochemistry enables such expression to be addressed easily. In a previous study, we produced the specific antibody against Japanese eel GH (anti-GH).[18] Specific antibodies against eel gonadotropin α subunit and LHβ (anti-LHβ) were also produced previously.[19]

In the present study, we produced specific antibodies against Japanese eel FSHβ, PRL, and SL, and confirmed changes in the production of gonadotropins in pituitary glands of artificially maturating eels by immunohistochemistry. Associated changes in the production of GH-family hormones were also examined.

MATERIALS AND METHODS

Animals

Cultivated Japanese eels were purchased from a commercial dealer and transferred to recirculating seawater tanks at 20°C. Female eels (300–1000 g, $n = 20$) were selected to receive weekly intramuscular injections of 40 mg/kg body weight of SPH: acetone-dried chum salmon *Oncorhynchus keta* pituitary glands suspended in eel Ringer (150 mM NaCl, 3.0 mM KCl, 3.5 mM $MgCl_2$, 5.0 mM $CaCl_2$, 10 mM Hepes, pH 7.4). Eels were not fed after purchase. Following terminal anesthesia in ethyl aminobenzoate, pituitary glands were collected from more than three eels at each vitellogenic stage. The developmental stage of eels after SPH injections was defined by the diameter of the largest group of ovarian follicles following a previously reported method:[20] follicle diameters at early, mid-vitellogenic stage, late vitellogenic stage, and the migratory nucleus stage were <300, 300–500, 500–700, and >700 μm, respectively.

Preparation of specific antibodies against eel pituitary hormones

To produce the antibody against eel FSHβ (anti-FSHβ), a rabbit was immunized with a synthetic oligopeptide (CFTQDSVYKSSLKSYPQQ), which was designed from the deduced amino acid sequence of eel FSHβ[6] conjugated with keyhole limpet hemocyanin (KLH). The antiserum was collected, absorbed with KLH dissolved in 10 mM PBS (pH 7.5), and centrifuged at 10 000 *g* for 1 h. The supernatant was used as anti-FSHβ.

To produce specific antibodies against eel PRL and SL (anti-PRL and anti-SL), recombinant eel PRL and SL were prepared as previously reported[21] and used as antigen. In brief, cDNA fragments of Japanese eel PRL and SL encoding only the mature peptide were subcloned into a pQE-30 expression vector (Qiagen, Hilden, Germany) so as to create a 6 × histidine tag on the expressed protein. After subcloning, recombinant proteins were expressed in *Escherichia coli* and purified by affinity chromatography using a metal chelate absorbent nickel–nitrilotriacetic acid agarose column (Qiagen). Rabbits were immunized with the purified recombinant proteins. The antisera were collected and used as anti-PRL and anti-SL.

Electrophoresis and Western blot analysis

Western blot analysis was performed to confirm the specificity of anti-PRL and anti-SL. Immature male eel pituitary glands were homogenized in

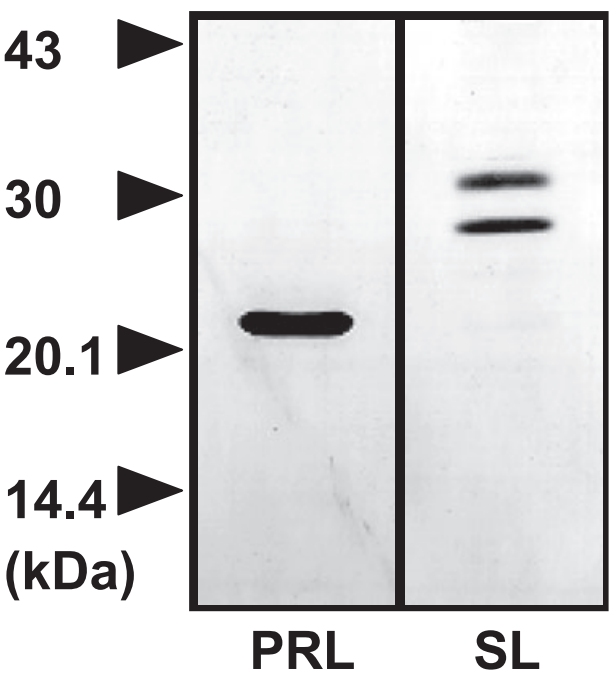

Fig. 1 Western blot analysis of eel pituitary glands using anti-PRL and anti-SL.

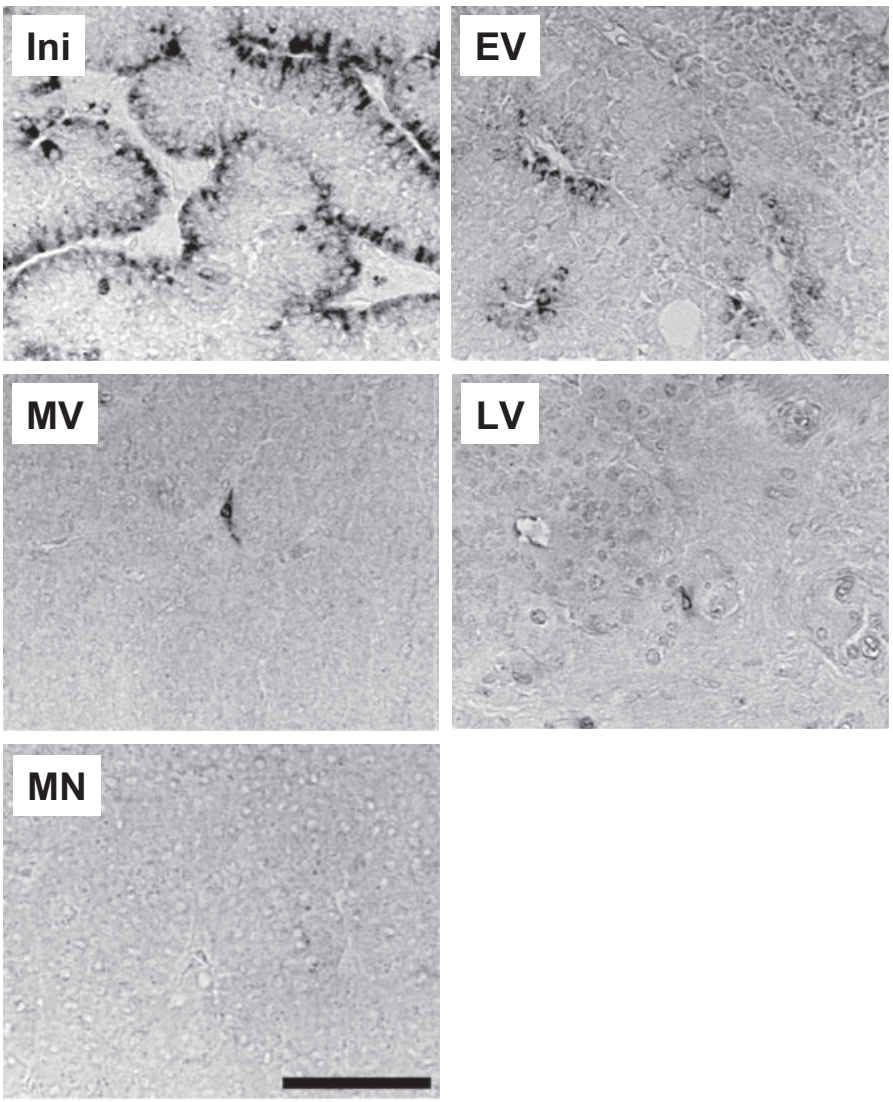

Fig. 2 *Proximal pars distalis* mid-sagittal sections immunostained with anti-FSHβ, of eels at each vitellogenic stage. Ini, before SPH injection; EV, early vitellogenic stage; MV, mid-vitellogenic stage; LV, late vitellogenic stage; MN, migratory nucleus stage. Bar, 50 μm.

20 mM Tris-HCl (pH 8.0) and the homogenate was centrifuged at 10 000 *g* for 1 h at 4°C. The supernatant was then mixed with an equal volume of sample buffer (125 mM Tris-HCl, 4% (w/v) sodium dodecyl sulfate (SDS), 20% (v/v) glycerol, 0.05% (w/v) bromophenol blue) with reduction by 10% 2-β-mercaptoethanol (2ME) and boiled for 10 min.

SDS–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a Tris-glycine buffer system.[22] For Western blot analysis, the proteins separated on the acrylamide gel were transferred to a polyvinylidine difluoride membrane (Millipore, Bedford, MA, USA). The membrane was incubated and shaken for 1 h in Tris-buffered saline (TBS, 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl) containing 10% skimmed milk to block non-specific binding, and then immersed overnight at 4°C in a solution containing antibodies against each pituitary hormone at a dilution of 1:1000 in TBS containing 10% skimmed milk. After washing with TBS containing 0.025% Tween 20 and then with TBS, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat antirabbit IgG (Bio-Rad, Hercules, CA, USA) diluted to 1:1000 in TBS for 3 h. After washing, HRP activity was visualized using a freshly prepared solution of 0.06% 4-chloro-1-napthol in TBS containing 0.06% H_2O_2.

Immunohistochemistry

Immunohistochemistry was performed as in a previous study.[23] In brief, pituitary glands were fixed in Bouin–Hollande sublimate, dehydrated through a series of ethanols, and embedded in paraffin. The embedded pituitary glands were cut sagittally at 4-μm thickness and sections were stained immu-

nohistochemically with antibodies against pituitary hormones described above. The specificities of anti-LHβ and anti-GH have been confirmed in previous studies.[18,19]

Measurement of immunoreactive cells

Measurements of anti-FSHβ, anti-LHβ, and anti-GH immunoreactive cells were performed as reported previously.[23] The number of immunoreacted cells was counted in a 1.4-mm^2 area of the PPD in randomly selected sections. The mean of the three sections was expressed as cells/mm^2 in a pituitary gland.

For measurements of immunoreacted cells with anti-PRL and anti-SL, all immunoreacted cells per section were counted in randomly selected sections. The mean of the three sections was expressed as cells/section in a pituitary gland.

Results were presented as means ± standard error of the mean (SEM) for more than three eels at each developmental stage. Data analysis was carried out using a non-parametric statistical test (Kruskal–Wallis test), followed by *post hoc* Bonferroni adjustment. Significance was accepted at $P < 0.05$.

RESULTS

Specificities of anti-PRL and anti-SL

Western blot analysis showed only one band of approximately 22 kDa that strongly immunoreacted with anti-PRL. In Western blot analysis using anti-SL, two bands were observed at approximately 31 and 27 kDa (Fig. 1).

Changes in production of pituitary hormones during artificial maturation

Anti-FSHβ immunoreactive cells were observed only in the PPD. The number of immnoreactive cells prior to SPH injection was markedly greater than in post-treatment eels and very few anti-FSHβ positive cells were observed during sexual maturation (Figs 2 and 3). In pretreatment eels, there were few anti-LHβ positive cells and these, like the anti-FSHβ cells, were only observed in the PPD. However, in contrast to anti-FSHβ, the number of anti-LHβ positive cells increased greatly along with ovarian development. Moreover, the LHβ producing cells became larger with ovarian development (Figs 3 and 4).

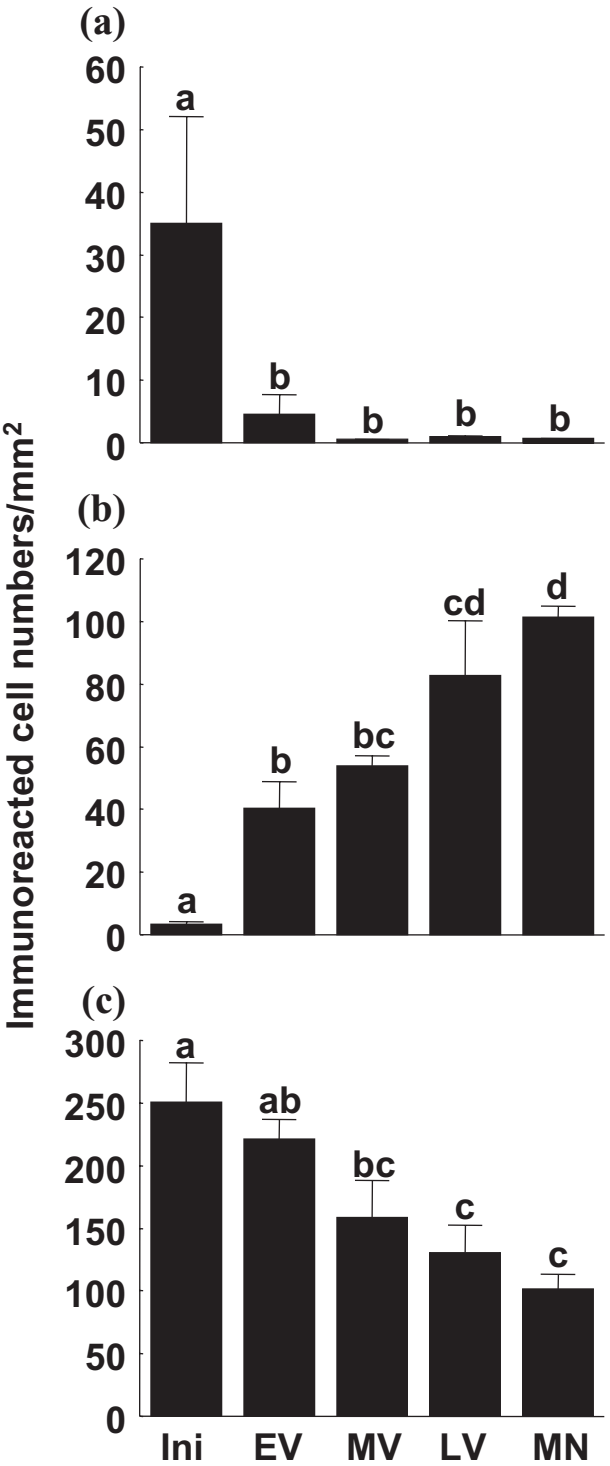

Fig. 3 Changes in numbers of cells immunoreacted with (a) anti-FSHβ, (b) anti-LHβ, and (c) anti-GH during artificial maturation. Ini, before SPH injection; EV, early vitellogenic stage; MV, mid-vitellogenic stage; LV, late vitellogenic stage; MN, migratory nucleus stage. Means with different letters are significantly different between stages, $P < 0.05$; error bars, standard error of the mean (>3 eels) at each developmental stage.

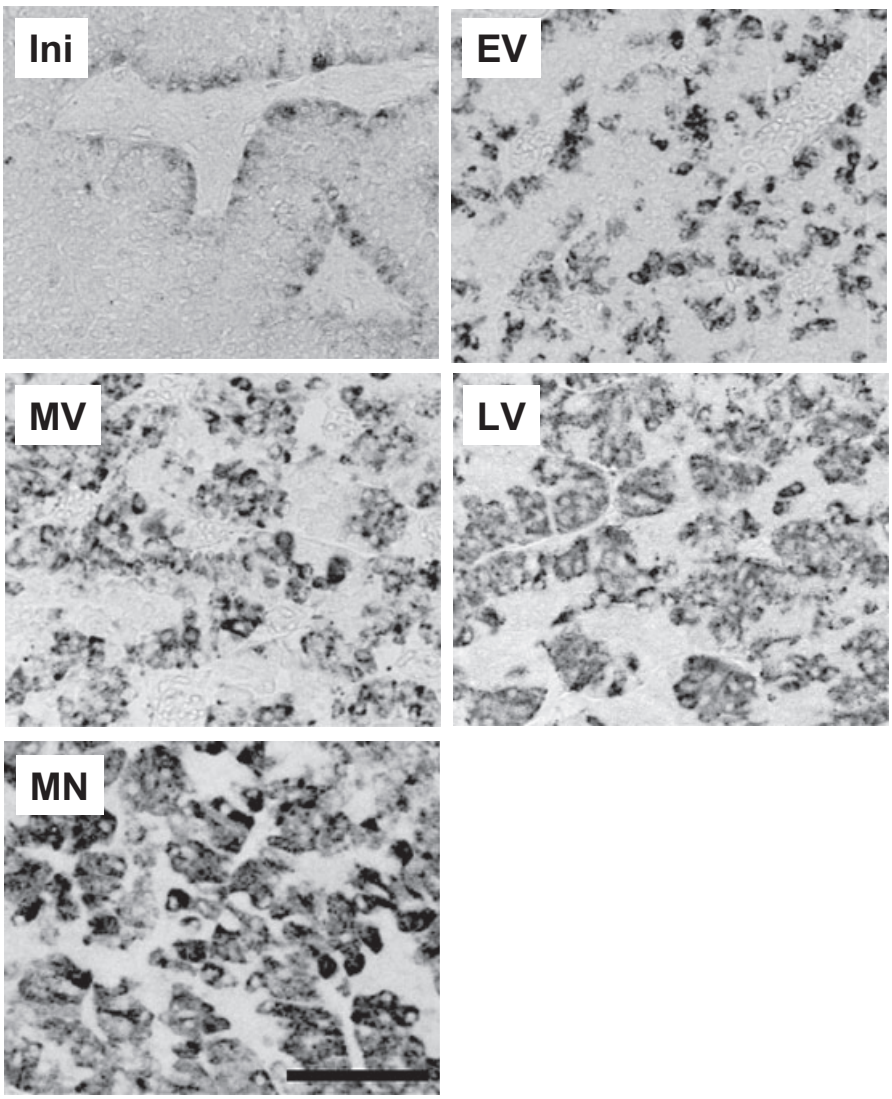

Fig. 4 *Proximal pars distalis* mid-sagittal sections immunostained with anti-LHβ, of eels at each vitellogenic stage. Ini, before SPH injection; EV, early vitellogenic stage; MV, mid-vitellogenic stage; LV, late vitellogenic stage; MN, migratory nucleus stage. Bar, 50 μm.

Most cells in the PPD of preinjection eels immunoreacted with anti-GH. However, the number of GH cells gradually decreased along with ovarian development to levels similar to cells that immunoreacted with anti-LHβ in the migratory nucleus stage (Figs 3 and 5). Cells immunoreacted with anti-PRL were observed only in the RPD. The number of anti-PRL positive cells in preinjection eels was significantly greater than in SPH treated eels. Cells reacted strongly with anti-PRL in eels that had not yet received an SPH injection, and it was clear that almost all cells formed follicles. No changes were observed in the number of PRL immunoreactive cells with maturation, and it was not clear whether the PRL producing cells formed follicles after the mid-vitellogenic stage (Figs 6 and 7). Most cells around the neural lobe in the PI were immunoreacted with anti-SL. The number increased until it peaked at the late vitellogenic stage, and then decreased to the migratory nucleus stage (Figs 7 and 8).

DISCUSSION

Immunohistochemical observation showed that changes in the production of gonadotropin hormones agreed well with previously observed changes in gonadotropin transcripts. Expression profiles of gonadotropins have been well studied in female Japanese eels during artificial maturation. First, LHβ cDNA was cloned and it was shown that levels of the transcripts increased during artificial maturation.[4,5] Subsequently, FSHβ cDNA was identified,[6] and changes in gonadotropin transcripts were examined. FSHβ transcripts decreased sig-

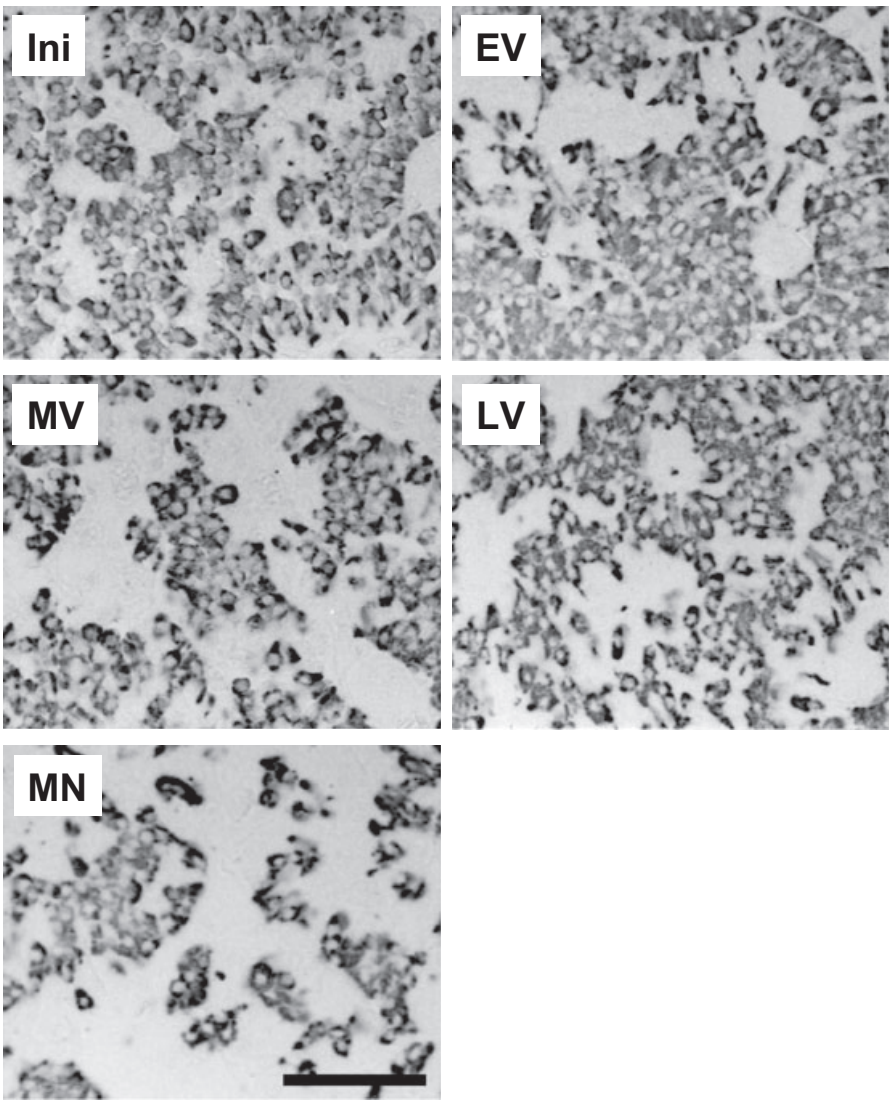

Fig. 5 *Proximal pars distalis* mid-sagittal sections immunostained with anti-GH, of eels at each vitellogenic stage. Ini, before SPH injection; EV, early vitellogenic stage; MV, mid-vitellogenic stage; LV, late vitellogenic stage; MN, migratory nucleus stage. Bar, 50 μm.

nificantly after SPH injection and LHβ transcripts increased along with ovarian development.[8]

Ikeuchi *et al.*[19] produced specific antibodies against gonadotropin α subunit, thyroid-stimulating hormone (TSH)β, and LHβ, and estimated the number of FSH producing cells in the PPD by subtraction of anti-LHβ immunoreactive cells from cells that immunoreacted with the antibody against gonadotropin α subunit. In the present study, to examine changes in the production of gonadotropins, we produced anti-FSHβ.

Because the deduced amino acid sequences of GTH–TSH family share high homology, synthetic oligopeptide designed from an amino acid sequence specific for eel FSHβ was used as antigen in the present study. In Western blot analysis of eel pituitary gland using anti-FSHβ, an immunoreactive band was not observed (data not shown). In immunohistochemical observations, however, anti-FSHβ positive cells were detected only in the PPD, and changes in the number of positive cells were well correlated with changes in the amount of transcripts.[8] TSHβ producing cells were present in RPD in eel,[19] and in the present study, anti-FSHβ positive cells were different from anti-LHβ positive cells. It is not rare that an antibody can be used only for either Western blot analysis or immunohistochemistry. These results suggest that anti-FSHβ produced in the present study could be used to specifically detect FSHβ producing cells by immunohistochemistry.

Reported changes in gonadotropin expression profiles with maturation have varied between fish species. In a previous study, however, comparisons with the New Zealand longfinned eel *Anguilla dieffenbachia*, which is very closely related to the Japanese eel, suggested that ovaries did not develop normally in SPH-injected Japanese eels; the FSHβ

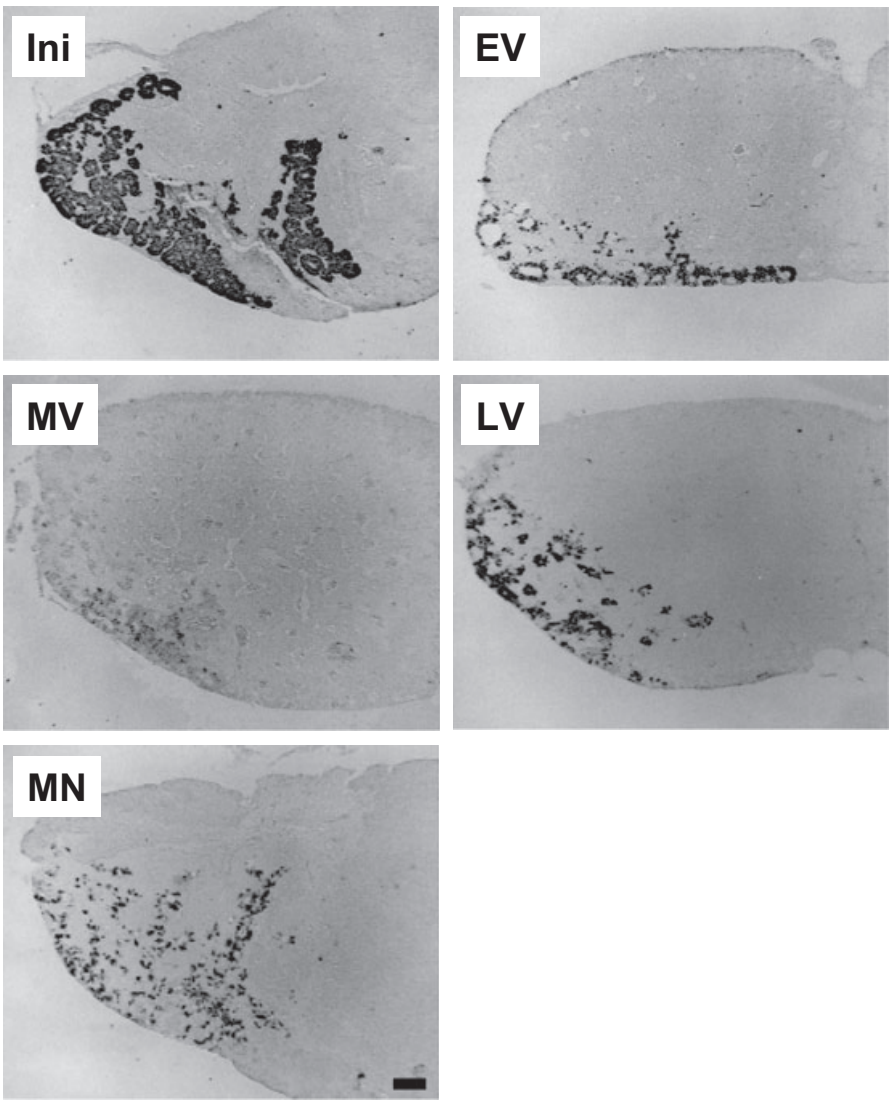

Fig. 6 *Rostral pars distalis* mid-sagittal sections immunostained with anti-PRL, of eels at each vitellogenic stage. Ini, before SPH injection; EV, early vitellogenic stage; MV, mid-vitellogenic stage; LV, late vitellogenic stage; MN, migratory nucleus stage. Bar, 50 μm.

transcripts in wild maturing longfinned eels were low through the previtellogenic stage, and then increased to the mid-vitellogenic stage.[8] Taken together, these results suggest that FSH plays an important role in the early stages of ovarian development in naturally maturing eels and that SPH injections inhibit FSH expression.

In contrast to the gonadotropins, changes in the expression of GH-family members have rarely been demonstrated in SPH injected eels. It has been established that the GH family is involved in fish reproduction as in other vertebrates.[9–17] In salmonids, plasma GH levels are elevated in association with gonadal maturation, and it is also evident that GH modulates the production of sex steroids.[10] The involvement of GH in reproduction has also been suggested in eels; it was reported that GH had a potentiating effect on estrogen-induced vitellogenin synthesis.[11] In a previous study, we identified a cDNA encoding a Japanese eel GH receptor and showed that the GH receptor was expressed in both female and male gonads.[21] In the present study, however, GH cells decreased with ovarian development. In salmonids, negative feedback control by GH and insulin-like growth factor (IGF)-I has been confirmed.[24] It was also reported that IGF-I inhibited GH production in European eel *Anguilla anguilla*.[25,26] Chum salmon pituitary glands used in the present study to induce eel maturation were collected from migrating salmon that spawn in the river, and it is plausible that the pituitaries collected from these salmons included an appreciable amount of GH, as well as LH. Accordingly, it is possible that the production of GH in maturating eels may be inhibited by exogenous salmon GH and/or endogenous IGF-I produced by the stimulation of salmon GH.

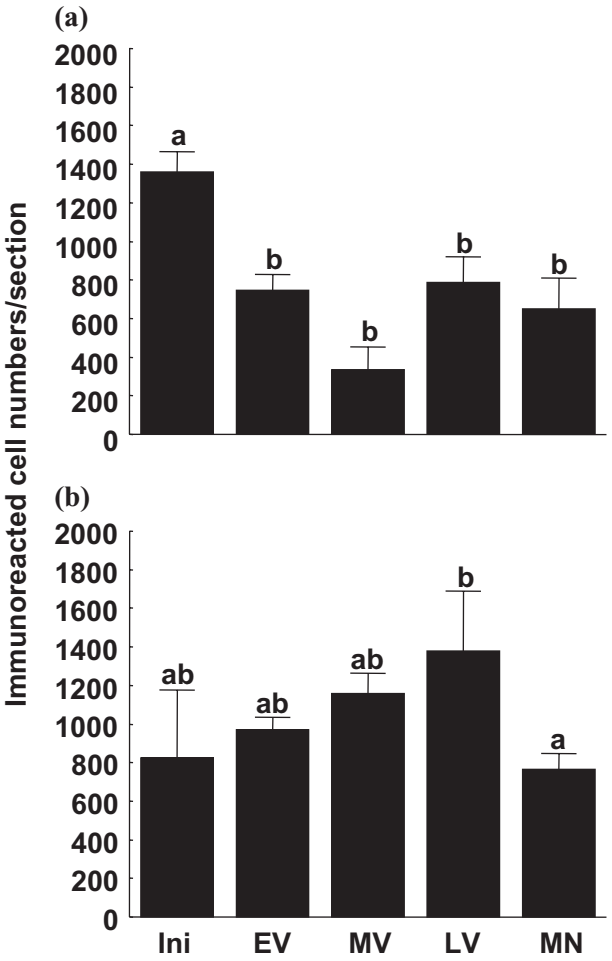

Fig. 7 Changes in numbers of cells immunoreacted with (a) anti-PRL, and (b) anti-SL during artificial maturation. Ini, before SPH injection; EV, early vitellogenic stage; MV, mid-vitellogenic stage; LV, late vitellogenic stage; MN, migratory nucleus stage. Means with different letters are significantly different between stages, $P < 0.05$; error bars, standard error of the mean (>3 eels) at each developmental stage.

It is also possible that the observed decrease in GH cells might be an artifact related to the observed increment of LH producing cells within the counted area. Moreover, it was reported that GH production was significantly increased with long-term starvation in eels as in other fishes,[27] and eels were not fed for 7–8 weeks to develop to the migratory nucleus stage in the present study. Thus, there should be a predicted increase in GH production. Different approaches may be needed for quantification of GH expression during sexual maturation. In teleosts as in other vertebrates, the expression of GH is regulated in a complex way by several factors because of its many physiological roles acting on various tissues, either directly or indirectly by stimulating IGF-I production. Moreover, it has been suggested that there is a complex relationship between the gonadotropin releasing hormone–gonadotropin–sex steroid axis and the GH–IGF axis.[24] Although there is no doubt that GH is also involved in reproduction in artificially maturating eels, further research is needed to elucidate the roles of GH.

In a previous study, Japanese eel PRL was purified from pituitary glands and an antibody against the purified PRL was produced.[28] Results of Western blot analysis in the present study showed that only one band that immunoreacted with anti-PRL was observed at the same size as in the previous study. Moreover, immunohistochemical observation showed that PRL positive cells were found only in the RPD and formed follicles. These results suggested that the anti-PRL antibody is specific.

In the present study, we have shown that the number of PRL producing cells in eels before SPH injection is markedly greater than after SPH injection. It is evident that the observed decrease was caused by the transfer from fresh to sea water. Previously, it was confirmed that PRL levels in plasma and pituitary glands of eels in sea water were much lower than those of eels in fresh water.[29] In teleosts, PRL is well known as a hormone involved in freshwater adaptation.[30] Although the role of PRL in reproduction is rarely clarified, several reports have suggested that it has a role in reproduction, as PRL stimulated steroidogenesis in gonads and is associated with the onset of gonadal development.[12] Localization of the PRL receptor in spermatogonia and oocytes has also been reported.[13] In the present study, however, changes in the production of PRL during sexual maturation were not observed, so it remains unclear whether PRL is involved in eel reproduction.

In Western blot analysis, two bands that immunoreacted with anti-SL were observed. In several fish, similar results have been reported and two forms of SL have been seen in pituitary glands, with the larger band identified as a glycosylated form.[31,32] Immunohistochemical observations revealed that immunoreacted cells were detected only in PI. These results suggested that anti-SL produced in the present study immunoreacted with SL in a specific manner.

Immunohistochemical observations showed that SL production increased to the late vitellogenic stage, suggesting the involvement of SL in sexual maturation. Several studies suggested that SL has many biological functions, as in the case for GH and PRL, and that it may play a role in reproduction.[14] In salmonids, the levels of plasma SL increase during sexual maturation peak at the time

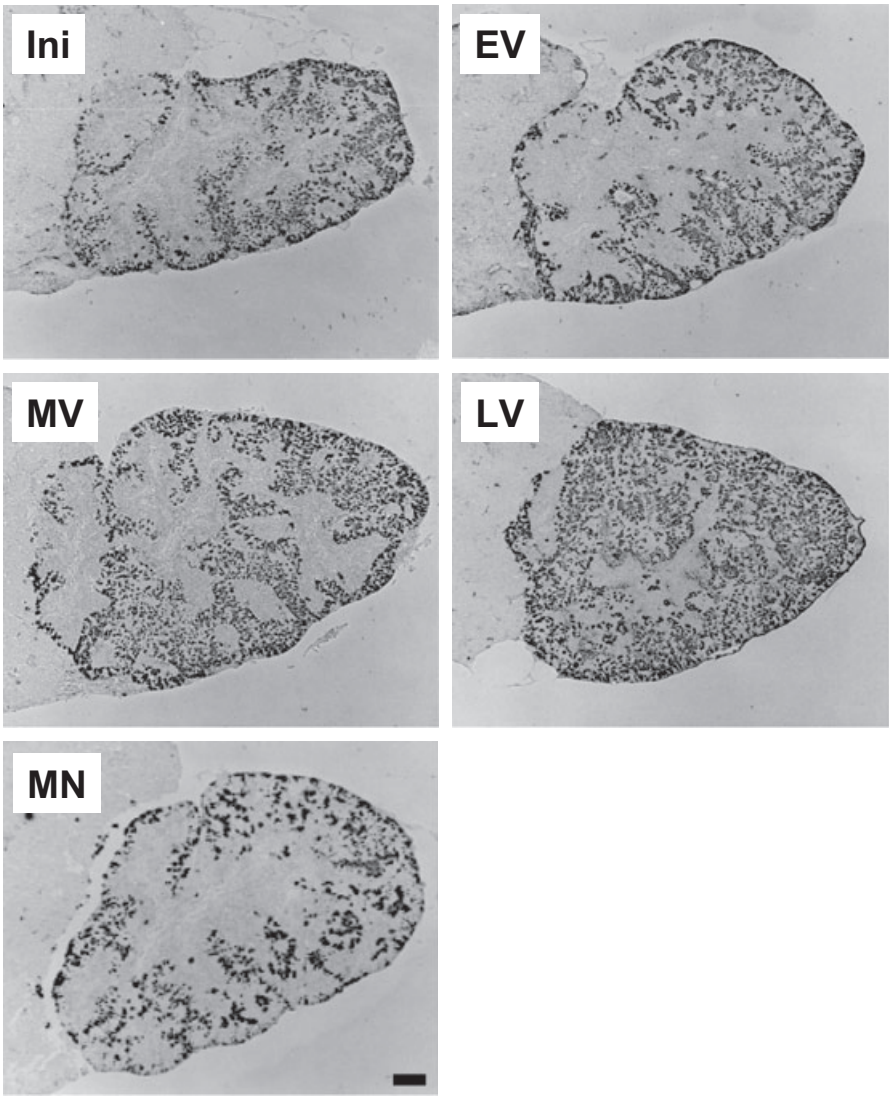

Fig. 8 *Pars intermedia* mid-sagittal sections immunostained with anti-SL, of eels at each vitellogenic stage. Ini, before SPH injection; EV, early vitellogenic stage; MV, mid-vitellogenic stage; LV, late vitellogenic stage; MN, migratory nucleus stage. Bar, 50 μm.

of final maturation in both sexes,[15,16] and SL stimulates production of 11-ketotestosterone and testosterone in males, and estradiol in females.[17] Recent reports suggest that the dominant role of SL is regulation of lipid metabolism when fish are not in the growth stage, and possibly during the reproductive process.[33,34] Moreover, Fukada *et al.*[34] identified a cDNA for the SL receptor for the first time from masu salmon *Oncorhynchus masou* and showed that the SL receptor was expressed in gonads. These results suggest that SL plays a role in sexual maturation in eels as in other fishes, although further research is needed to better characterize SL functions in fish reproduction. In our study, however, SL cells decreased to the migratory nucleus stage, possibly indicating an aberrant expression profile. Although the reason for this observation is still unclear, it is possibly a result of excess circulating estradiol-17β and/or 11-ketotestosterone. It has been reported that the plasma levels of these steroid hormones are significantly increased after the mid-vitellogenic stage.[35]

CONCLUSION

In this study, specific antibodies against Japanese eel FSHβ, PRL, and SL were produced. It was shown that changes in gonadotropin production in pituitary glands of artificially maturating eels were consistent with changes in transcript levels: FSH production was inhibited by SPH injection, and LH production was increased during artificial maturation. The number of GH producing cells decreased gradually during maturation, possibly as a result of inhibition by salmon GH present in the SPH. Additionally, it is suggested that SL may be involved in

sexual maturation of SPH injected eels. Further research is needed to understand the roles of GH-family members in eel reproduction and the antibodies produced in the present study will be useful reagents for those studies.

ACKNOWLEDGMENTS

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