Effect of Sex Steroids on the mRNA Levels of Gonadotropin I and II Subunits in the Goldfish *Carassius auratus*

Young Chang Sohn, Yasutoshi Yoshiura,* Makito Kobayashi, and Katsumi Aida

Department of Aquatic Bioscience, Graduate School of Agricultural and Life Sciences,
The University of Tokyo, Bunkyo, Tokyo 113-8657, Japan

(Received February 9, 1998)

In order to clarify the role of gonadal sex steroids in the expressions of gonadotropin (GTH) subunit genes in goldfish, we examined the effect of testosterone (T), estradiol-17 β (E₂), and 11-ketotestosterone (11-KT) on the pituitary mRNA levels of GTH I β , GTH II β and α subunits by Northern blot analysis in male and female fish at sexually early recrudescence and mature stages. Fish were implanted with a blank Silastic capsule, or capsules containing T, E₂, or 11-KT (200 μ g), and kept at 20°C for 2 weeks. In early recrudescence fish (GSI: male, 2.6 ± 0.2 ; female, 2.6 ± 0.6), the mRNA of GTH I β in T, E₂, or 11-KT implanted fish showed significantly lower levels than those in the blank implanted fish in both sexes. The mRNA levels of GTH II β in T and E₂ implanted fish showed slightly increased levels whereas decreased levels were observed in 11-KT implanted fish in both sexes. The mRNA levels of the α subunit in 11-KT and E₂ implanted females were lower than those in the blank implanted females, but no differences were observed in males. In sexually mature fish (GSI: male, 3.5 ± 0.3 ; female, 7.5 ± 1.5), no clear effects of T, E₂, and 11-KT were observed on the mRNA levels of the all GTH subunits except for an increased level of GTH II β in E₂ implanted males. Thyrotropin β subunit mRNA levels were also measured in these fish but showed no clear changes by steroid hormone treatment except for a decrease by 11-KT and E₂ treatments in sexually early recrudescence females. These results indicate that the expressions of GTH I β and II β subunit genes are differentially regulated by sex steroids in the goldfish at the early recrudescence stage. The results also suggest that the responsiveness of GTH gene expression to sex steroids differs depending on gonadal maturity.

Key words: gonadotropin, mRNA, sex steroid, goldfish

In teleosts, as in other vertebrates, the gonadal activity is regulated by gonadotropins (GTH I and GTH II in fish and follicle-stimulating hormone and luteinizing hormone in other vertebrates), which are glycoproteins consisting of two subunits, α and β . The α subunit is common to the two GTHs, and its association with a unique β subunit in each case determines the hormone's specific function.¹⁾ In salmonids, it has been reported that GTH I stimulates early gonadal development (e.g., vitellogenesis and spermatogenesis) and GTH II modulates the later stages (e.g., final oocyte maturation, ovulation, and spermiation).^{2–4)} However, the roles of two types of GTH and the regulation of their production and release are not fully understood in other teleost species.

The production of GTHs in the pituitary gland is known to be controlled by various factors. Among the factors, sex steroids produced in the gonad under the stimulation of GTHs are known to play an important regulatory role in GTHs syntheses. Stimulatory effects of sex steroids on GTH II content and mRNA levels in the pituitary have been shown in immature rainbow trout *Oncorhynchus mykiss*^{5–7)} and European eel *Anguilla anguilla*.^{8–10)} The stimulatory effects with an acute treatment of steroids are also known in the goldfish *Carassius auratus*.¹¹⁾ However, in teleosts in which gonadal maturation has been initiated,

stimulation by sex steroids on GTH II production has reported to be less effective in comparison to those in immature fish.^{7,11,12)} In sexually mature goldfish, sex steroids, when given at physiological doses, did not affect the mRNA level of GTH II β subunit¹¹⁾ or GTH II content in the pituitary.¹²⁾ In rainbow trout, treatment of testosterone or estradiol-17 β slightly increased the GTH II β mRNA level in maturing fish, but no effects were observed in spawning fish.⁷⁾ These studies indicate that there is a difference of sensitivity of the pituitary gland to sex steroids depending on the maturity of the fish.

In comparison to the studies on the regulation of GTH II production, information on the regulation of the GTH I production is very limited. In salmonids, it is reported that treatment of steroids in immature fish had no effects on GTH I synthesis *in vitro*⁷⁾ and *in vivo*.¹³⁾ However, steroidal regulation of GTH I in other teleosts has not been examined. In the present study, we investigated the *in vivo* effects of long-term treatment of testosterone (T), estradiol-17 β (E₂), and 11-ketotestosterone (11-KT) on the production of GTH I and GTH II in goldfish, measuring the steady-state mRNA levels of GTH I β and II β , and α subunits in the pituitary. We also examined the responsiveness of the GTH mRNA levels to sex steroids in fish at different stages of gonadal maturity. In addition, pituitary

* Present address: Laboratory of Reproductive Biology, Department of Developmental Biology, National Institute for Basic Biology, Okazaki 444-8585, Japan.

mRNA levels of β subunit of thyrotropin (TSH), another member of pituitary glycoprotein hormones, were also investigated.

Materials and Methods

Experiment 1 (Early Recrudescent Fish)

Goldfish in sexually early recrudescence were obtained from a local dealer in September, 1995 and kept in an outdoor tank under natural conditions. The fish were transferred to a 1000 l indoor recirculating tank at 20°C under 14 h light-10 h dark beginning on October 4, 1995. On October 11, the experimental fish were randomly selected and the fish received Silastic capsule implant containing a sex steroid (Male: T, $N=15$; E_2 , $N=16$; 11-KT, $N=20$. Female: T, $N=15$; E_2 , $N=14$; 11-KT, $N=10$) or a blank capsule (Male, $N=21$; Female, $N=9$). Some fish were sacrificed before the experiment to evaluate gonadal maturity by calculating gonadosomatic index (GSI, gonad weight $\times 100$ /body weight) and by histological examination. Average body weight (BW) and GSI of the sacrificed fish were as follows (mean $\pm$ SEM): male; BW 30 ± 3 g, GSI $2.6 \pm 0.2\%$ ($N=8$); female; BW 24 ± 2.6 g, GSI $2.6 \pm 0.6\%$ ($N=8$). The males had testes at early spermatogenesis stage, and the females had ovaries containing the oocytes at the perinucleolous stage and yolk vesicle stage (data not shown). The experimental fish were fed with commercial trout pellets at 2% BW every day, and they were sampled after a duration of two weeks.

Experiment 2 (Mature Fish)

Sexually mature goldfish were obtained from a local dealer in April, 1996 and kept in an outdoor tank under natural conditions. On May 28, 1995, the fish were transferred to a 1000 l indoor recirculating tank, and kept under the same environmental conditions as described in Experiment 1. On June 3, experimental fish received Silastic capsule implant containing a sex steroid (Male: T, $N=16$; E_2 , $N=16$; 11-KT, $N=18$. Female: T, $N=14$; E_2 , $N=14$; 11-KT, $N=12$) or a blank capsule (Male, $N=15$; Female, $N=15$), and were reared as described in Experiment 1. Some fish were sacrificed before the experiment to examine GSI and gonad. Average BW and GSI of the sacrificed fish were as follows: male; BW 33 ± 2 g, GSI $3.5 \pm 0.3\%$ ($N=14$); female; BW 46 ± 6 g, GSI $7.5 \pm 1.5\%$ ($N=12$). The male had testes with an abundance of spermatocytes and spermatids in the seminal lobule, and the females had ovaries containing the oocytes at the secondary and tertiary yolk stages (data not shown). The experimental fish were fed with commercial trout pellets at 2% BW every day, and they were sampled after a duration of two weeks.

Sex Steroid Implantation

The preparation of the Silastic capsules containing steroids was conducted according to the method described by Kobayashi *et al.*^{14,15} with minor modifications. Ten milligram of T, E_2 , or 11-KT was initially dissolved in 300 μ l of ethanol. The ethanol solutions including sex steroids were then mixed with 2.5 ml of sesame oil. The ethanol was evaporated overnight at room temperature. Silastic tubing (0.78 mm i.d., 1.25 mm o.d., Dow Corning) were cut at 2.0 cm length and filled with 50 μ l of the prepared T,

E_2 , or 11-KT solution. The final sex steroid concentration of the capsule was 4 μ g/ μ l sesame oil. Capsules filled with 50 μ l sesame oil only served as the blank controls. The ends of the tubing were then sealed with Medical Adhesive (Silicone Type A, Dow Corning).

After the fish were anesthetized with 2-phenoxy-ethanol (0.5 ml/l), an incision of approximately 0.3 cm in length was made on the left side of the body near the anus just a few millimeters apart from the medial line of the abdomen for the implantation of the capsules. The capsules were inserted into the body cavity through this incision. No suture was made after the implantation process. In Experiment 1, each fish was implanted with either one blank capsule, or one sex steroid capsule. In Experiment 2, each fish received either one blank capsule, one T or 11-KT capsule, or two E_2 capsules. Since the release of E_2 from the Silastic capsule was less than those of T and 11-KT in Experiment 1, two capsules were implanted in Experiment 2. After the implantation, the fish were kept in 0.02% Elbarg (10% sodium nifrostyrenate, Ueno Pharmac. Co. Ltd.) overnight to prevent bacterial infection and then transferred to a 1000 l recirculating tank at a water temperature of 20°C.

Sampling of Pituitaries and Blood

In each experiment, the pituitaries and blood were sampled from each fish, two weeks after implantation. Before sampling, the fish were anesthetized with 2-phenoxy-ethanol (0.5 ml/l). BW was measured and blood sample was taken from the caudal vasculature with a heparinized syringe and needle. After decapitation, the pituitary glands were collected, immediately frozen in liquid nitrogen, and stored at -80°C until Northern blot analysis were conducted. Total RNA preparations from two to three pooled samples (4–7 fish per sample) were applied to the analysis. The gonads were removed and weighed for calculation of GSI, and blood samples were centrifuged at $2,000 \times g$ for 5 minutes. The resulting plasma was stored at -30°C until assayed.

Quantification of mRNA by Northern Blot Analysis

RNA extraction and Northern blot analysis were performed as described in a previous report.¹⁶ Briefly, total RNA was extracted from the pooled pituitaries with RNA extraction solution, Isogen (Nippon Gene) according to the manufacturer's protocol. The concentration of RNA was determined spectrophotometrically by reading absorbance at 260 nm for each sample before gel electrophoresis. Total RNA (1 μ g for α and GTH II β , or 10 μ g for GTH I β and TSH β) was denatured in 50% formamide for 15 min at 65°C , subjected to electrophoresis on a 0.9% agarose/formamide gel, and transferred to Hybond N⁺ nylon membrane (Amersham Corp.). The integrity of total RNA was verified by ethidium bromide staining of 28S and 18S rRNA on the gel by an imaging analyzer system (Molecular Analyst, V2.1 and Bio-Rad Gel Doc 1000 Gel Documentation system, Richmond, CA, USA) before hybridization. The membranes were hybridized with randomly labeled [α -³²P]dCTP cDNA probes^{17–19} encoding goldfish pituitary glycoprotein $\alpha 2$, GTH I β and II β , and TSH β subunits. The membranes were washed in $2 \times \text{SSC}$ for 15 min at room temperature and in $1 \times \text{SSC}$ containing 0.5% SDS for 15 min at 65°C . The specificity of probes

was previously assessed by dot blot hybridization with cDNAs of $\alpha 1$, $\alpha 2$, GTH I β , GTH II β , and TSH β under the same conditions as the Northern blot analysis.¹⁶⁾ Hybridization signals were observed only in homologous combinations of cDNA and cDNA probes, except for the α subunit. It has been shown that goldfish have two types of α subunit, $\alpha 1$ and $\alpha 2$, which share 95% nucleotide sequence identity.¹⁸⁾ In the present study, cDNA encoding $\alpha 2$ subunit was used as a labeled probe which hybridized with $\alpha 1$ and $\alpha 2$ cDNA equally.¹⁶⁾ Therefore, the data obtained in Northern blot analysis used in this study represents the total of $\alpha 1$ and $\alpha 2$ subunits. The hybridized signals which were expressed in units of PSL (photostimulated luminescence) were quantified by a Fujix BAS 1000 Bio-Imaging Analyzer (Fuji Film). To evaluate the mRNA levels of the pituitary glycoprotein subunits, the membranes were hybridized with labeled [α -³²P]dCTP cDNA probe encoding goldfish β -actin¹⁷⁾ under the same condition as previously described for Northern blot analysis. The relative mRNA levels of pituitary $\alpha 2$, GTH I β and II β , and TSH β subunits were obtained as hybridized signals of Northern blot hybridization quantified by the imaging analyzer standardized with 1) those of β -actin and 2) total RNA. Since both methods gave no difference in the results, the data standardized with total RNA were indicated in the present study.

Radioimmunoassays (RIA)

Plasma GTH II levels were measured for comparison with the mRNA levels of pituitary GTH II subunit, using a silver carp GTH II RIA.^{20,21)} Since RIA system for cyprinid GTH I is not available, plasma GTH I level is not measured. Plasma levels of sex steroids were measured by RIA according to the method of Aida *et al.*²²⁾ Details on the RIA for each steroid have been described previously.^{23,24)}

Statistics

ANOVA followed by Duncan's multiple range test was applied for statistical analyses.

Results

Experiment 1

Sexually early recrudescence goldfish of both sexes were reared for 2 weeks after implantations of capsules containing sex steroids, or blank capsules. Figure 1 summarizes the effects of sex steroids on the mRNA levels of the pituitary glycoprotein hormone α , GTH I β , GTH II β , and TSH β subunits. The mRNA levels of the α subunit in 11-KT and E₂ implanted females were significantly ($P < 0.05$) lower than those of T or blank capsule-implanted females, but no marked differences were observed in males. The mRNA levels of GTH I β of all steroid-implanted fish in both sexes showed significantly ($P < 0.01$) lower levels compared to those of the blank capsule-implanted fish. The mRNA levels of GTH II β in the E₂- and T-implanted females and E₂-implanted males were significantly ($P < 0.05$) higher than those of blank control fish, whereas 11-KT implanted fish showed decreased GTH II β mRNA levels in both sexes ($P < 0.05$). The mRNA levels of TSH β of 11-KT- and E₂-implanted females showed significantly lower levels ($P < 0.05$) compared to those of blank capsule-implanted

Fig. 1. Effects of sex steroids on pituitary mRNA levels of glycoprotein hormone subunits in early recrudescence goldfish in Experiment 1.

Each fish was implanted with one blank capsule (B), or one capsule containing 200 μ g of 11-ketotestosterone (K), estradiol-17 β (E), or testosterone (T), and was reared at 20°C for 2 weeks. The data represents the ratio to the values of blank implanted control fish, given 100. Data represent mean $\pm$ SEM (all males, and E and T in females, $N = 3$; B and K in females, $N = 2$). Columns with different letters are significantly ($P < 0.05$) different.

fish. No significant differences were observed in males.

Plasma GTH II showed no significant differences between sex steroid treated and blank control female fish. However, E₂ implanted males were found to have significantly higher levels (Fig. 2). In the steroid implanted fish, plasma levels of implanted steroids were significantly ($P < 0.05$) higher than the respective steroid levels of the control fish (Fig. 3).

Experiment 2

In sexually mature fish, most of the mRNA levels of all the GTH subunits were not affected by steroid implantation (Fig. 4). Only in E₂ implanted males, the GTH II β mRNA levels were significantly ($P < 0.05$) higher than those of blank control fish. The mRNA levels of TSH β subunit also showed no significant differences between sex steroids and blank capsule-implanted fish in both sexes.

Plasma GTH II levels in males showed no significant differences among the experimental groups, although the

Fig. 2. Plasma GTH II levels in early recrudescence goldfish in Experiment 1.

Each fish was implanted with one blank capsule (B), or one capsule containing 200 μ g of 11-ketotestosterone (K), estradiol-17 β (E), or testosterone (T), and was reared at 20°C for 2 weeks. Data represent mean $\pm$ SEM ($N=8$). Columns with different letters are significantly ($P<0.05$) different.

levels in steroid treated fish tended to be lower than those in blank control fish (Fig. 5). In females, plasma GTH II levels in T implanted fish were significantly ($P<0.05$) higher than those of blank control fish. In the steroid implanted fish plasma levels of implanted steroids were significantly ($P<0.05$) higher than the respective steroid levels of blank control fish, except for T implanted males and E₂ implanted females (Fig. 6).

Discussion

The goal of the present study was to investigate whether steroids observed during gonadal maturation were also involved in GTH production in the pituitary. It is well known that plasma steroid levels in goldfish exhibit a gradual rise and remain elevated during gonadal maturation and there are no acute variations with the exception at the time of spawning.²⁴⁻²⁶ Therefore, long-term effects of sex steroids on GTH mRNA levels were investigated. The first task of the investigation was to demonstrate that the steroid levels observed during gonadal maturation could be chronically influenced using Silastic steroid implants. From the results observed in the current investigation, it is clear that the various steroid capsule implants were successful in elevating the respective steroid levels over control fish with the exception of T in mature males and E₂ in mature females. One possible explanation for these observations is that steroid levels of mature fish were already elevated and the dosages used in the respective implants

Fig. 3. Plasma sex steroid levels in early recrudescence goldfish in Experiment 1.

Each fish was implanted with one blank capsule (B), or one capsule containing 200 μ g of 11-ketotestosterone (K), estradiol-17 β (E), or testosterone (T), and was reared at 20°C for 2 weeks. Data represent mean $\pm$ SEM ($N=8$). Columns with different letters are significantly ($P<0.05$) different.

did not result in a further increase in serum levels. However, most of the steroid implantation in sexually early recrudescence and mature fish were found to be effectively increased and sustained over the course of two weeks.

The results of Experiment 1 show that the treatment with T, E₂, or 11-KT exerts an inhibitory effect on the steady-state mRNA level of the GTH I β subunit in sexually recrudescence goldfish. This is consistent with the elevation of GTH I β mRNA levels by ovariectomy and a decrease in the levels by steroid replacement in sexually recrudescence and mature goldfish (Kobayashi *et al.*, in preparation). These results indicate that the expression of the GTH I β gene is mediated through an inhibitory control mechanism by sex steroids in early recrudescence goldfish. It remains to be elucidated whether this inhibitory effect of a steroid is at the levels of the pituitary or indirect through hypothalamic factors and whether all three steroids function in the same manner.

It has been reported that in immature salmonids no significant effects of sex steroids were observed on GTH I β mRNA levels.^{7,13} The different results obtained for salmonid and goldfish investigations may be due to differences in the maturity of the fishes used. In the present study, we observed marked inhibitory effects of sex steroids on GTH I β in early recrudescence goldfish but not in mature fish. It is of interest to examine whether sex

Fig. 4. Effects of sex steroids on pituitary mRNA levels of glycoprotein hormone subunits in sexually mature goldfish in Experiment 2.

Each fish was implanted with one blank capsule (B), or one capsule containing 200 μ g of 11-ketotestosterone (K) or testosterone (T), or two capsules containing 200 μ g of estradiol-17 β (E), and was reared at 20°C for 2 weeks. The data represents the ratio to the values of blank implanted control fish, given 100. Data represent mean $\pm$ SEM ($N=3$). Columns with different letters are significantly ($P<0.05$) different.

steroids also exert an inhibitory effect on GTH I β mRNA levels in sexually immature or juvenile goldfish. In addition to the difference in the maturity of fish in the studies of salmonids and the present study, there may be different regulatory mechanisms for GTH I β gene expression between salmonid and goldfish species. Since goldfish are characterized as a multiple-spawning teleosts²⁶⁾ and salmonids are annual spawners which spawn only once during their spawning season,²⁷⁾ it is possible that the regulatory system of GTH gene expression may be different for the different patterns of gonadal development.

Expression of the GTH II β gene is known to be stimulated by T treatment in immature teleosts.^{6,7,9,10)} In immature goldfish, it is reported that an acute treatment with T showed a stimulatory effect on the expression of GTH II β gene.¹¹⁾ In the present study, the levels of GTH II β mRNA by a chronic treatment with T or E₂ only resulted in a slight increase in early recrudescence goldfish and almost no increase in mature fish. The magnitude of the stimulatory effects between the previous investigations and the present study may be partly due to the maturity of fish. In rainbow

Fig. 5. Plasma GTH II levels in sexually mature goldfish in Experiment 2.

Each fish was implanted with one blank capsule (B), or one capsule containing 200 μ g of 11-ketotestosterone (K) or testosterone (T), or two capsules containing 200 μ g of estradiol-17 β (E), and was reared at 20°C for 2 weeks. Data represent mean $\pm$ SEM ($N=6$).

trout, the stimulatory effects of T on GTH II β mRNA were significant in immature fish, slight in maturing fish, and not observed in spawning fish.^{7,13)} Whether GTH II β expression of the earlier stages of sexual maturation in goldfish is stimulated by sex steroids wants further investigation.

It is notable that 11-KT exerts an inhibitory effect on both GTH I β and GTH II β mRNA levels in sexually early recrudescence fish of both sexes. This nonaromatizable androgen may act in a different way than other steroids on GTH β subunit genes, especially for the GTH II β subunit gene.

In sexually mature fish (Experiment 2), no significant changes were observed in the mRNA levels of all GTH subunits by sex steroids except for E₂ on GTH II β in males. These results suggest that GTH synthesis in mature fish, which were already exposed to elevated endogenous steroids, do not respond to sex steroids as observed in mature salmonid species.^{7,13)} However, the mechanism of how the responsiveness to sex steroids decrease in mature fish is not clear.

TSH β mRNA levels did not show any remarkable changes by steroid implantation. Only in 11-KT- and E₂-implanted recrudescence females, the TSH β mRNA levels decreased slightly. It seems that the expression of TSH β gene is regulated by thyroid hormones rather than sex steroids in goldfish, since it has been demonstrated that thyroid hormone administration depressed TSH β mRNA levels (Yoshiura *et al.*, in preparation), whereas ovariectomy did not show any marked changes in the levels

Fig. 6. Plasma sex steroid levels in sexually mature goldfish in Experiment 2.

Each fish was implanted with one blank capsule (B), or one capsule containing 200 µg of 11-ketotestosterone (K) or testosterone (T), or two capsules containing 200 µg of estradiol-17β (E), and was reared at 20°C for 2 weeks. Data represent mean ± SEM ($N=6$). Columns with different letters are significantly ($P<0.05$) different.

(Kobayashi *et al.*, in preparation).

Plasma GTH II levels did not show a clear correlation to the pituitary mRNA levels of GTH IIβ and α after steroid treatments in both Experiments. Our previous study on environmental manipulation also showed no clear correlations between GTH II mRNA levels and plasma levels.¹⁶ These results suggest that the synthesis and release of GTH II are differentially regulated in goldfish.

Although E₂ and 11-KT are not major gonadal steroids in male and female goldfish, respectively, these steroids showed the same effects in both sexes. The responsiveness to these steroid suggests that male and female goldfish have a common regulatory brain-pituitary system for GTH gene expression. This notion is supported by the fact that 11-KT, a typical male androgen could induce male type sexual behavior and GTH II secretion in female goldfish.^{21,28}

In summary, treatment with T and E₂ exerts an inhibitory effect on GTH Iβ gene expression and only a slight stimulatory effect on GTH IIβ gene expression in early recrudescence goldfish. In the treatment with 11-KT, an inhibitory effect was observed on both GTH Iβ and IIβ gene expressions. These data indicate that in early recrudescence female-goldfish under natural conditions gradual increases of plasma T and E₂ levels suppress the expression of GTH Iβ gene whereas they activate the expression of the GTH IIβ gene. The results in early recrudescence male-goldfish

also suggest that an increase of plasma T level exerts an inhibitory effect on GTH Iβ and a stimulatory effect on GTH IIβ whereas an increase in 11-KT suppresses both GTH Iβ and GTH IIβ. In mature fish, there was no notable effects by the steroids examined on both GTH Iβ and IIβ gene expressions, implying that the responsiveness of GTH gene expression to sex steroids differs depending on gonadal maturity.

Acknowledgments We express our thanks to Dr. C. S. Tamaru, Sea Grant Extension Service, Aquaculture Development Program, School of Ocean Earth Science Technology, Department of Land and Natural Resources, University of Hawaii, for his editorial comments. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan.

References

- 1) J. G. Pierce and T. F. Parsons: Glycoprotein hormones: structure and function. *Ann. Rev. Biochem.*, **50**, 465–495 (1981).
- 2) K. Suzuki, Y. Nagahama, and H. Kawauchi: Steroidogenic activities of two distinct salmon gonadotropins. *Gen. Comp. Endocrinol.*, **71**, 452–458 (1988).
- 3) C. R. Tyler, J. P. Sumpter, H. Kawauchi, and P. Swanson: Involvement of gonadotropin in the uptake of vitellogenin into vitellogenic oocytes of the rainbow trout, *Oncorhynchus mykiss*. *Gen. Comp. Endocrinol.*, **84**, 291–299 (1991).
- 4) F. Prat, J. P. Sumpter, and C. R. Tyler: Validation of radioimmunoassays for two salmon gonadotropins (GTH I and GTH II) and their plasma concentrations throughout the reproductive cycle in male and female rainbow trout (*Oncorhynchus mykiss*). *Biol. Reprod.*, **54**, 1375–1382 (1996).
- 5) L. W. Crim, R. E. Peter, and R. Billard: Onset of gonadotropic hormone accumulation in the immature trout pituitary gland in response to estrogen or aromatizable androgen steroid hormones. *Gen. Comp. Endocrinol.*, **44**, 374–381 (1981).
- 6) K.-Y. Trinh, N. C. Wang, C. L. Hew, and L. W. Crim: Molecular cloning and sequencing of salmon gonadotropin β subunit. *Eur. J. Biochem.*, **159**, 619–624 (1986).
- 7) F. Xiong, D. Liu, Y. L. Dreon, H. P. Elsholtz, and C. L. Hew: Differential recruitment of steroid hormone response elements may dictate the expression of the pituitary gonadotropin IIβ subunit gene during salmon maturation. *Mol. Endocrinol.*, **8**, 782–793 (1994).
- 8) R. Counis, S. Dufour, G. Ribot, B. Querat, Y.-A. Fontaine, and M. Jutisz: Estradiol has inverse effects on pituitary glycoprotein hormone α-subunit messenger ribonucleic acid in the immature European eel and the gonadectomized rat. *Endocrinol.*, **121**, 1178–1184 (1987).
- 9) B. Querat, A. Hardy, and Y. A. Fontaine: Regulation of the type-II gonadotropin α and β subunit mRNAs by estradiol and testosterone in the European eel. *J. Mol. Endocrinol.*, **7**, 81–86 (1991).
- 10) Y. S. Huang, M. Schmitz, N. Le Belle, C. F. Chang, B. Querat, and S. Dufour: Androgens stimulate gonadotropin-II β-subunit in eel pituitary cells *in vitro*. *Mol. Cell. Endocrinol.*, **131**, 157–166 (1997).
- 11) D. Huggard, Z. Khakoo, G. Kassam, S. S. Mahmoud, and H. R. Habibi: Effect of testosterone on maturational gonadotropin subunit messenger ribonucleic acid levels in the goldfish pituitary. *Biol. Reprod.*, **54**, 1184–1191 (1996).
- 12) M. Kobayashi and N. E. Stacey: Effects of ovariectomy and steroid hormone implantation on serum gonadotropin levels in female goldfish. *Zool. Sci.*, **7**, 715–721 (1990).
- 13) J. T. Dickey and P. Swanson: Development of RNase protection assays for quantification of gonadotropin (GTH I and GTH II) subunit transcript levels in coho salmon (*Oncorhynchus kisutch*), in "Reproductive Physiology of Fish" (ed. by F. W. Goetz and P. Thomas), FishSymp 95, Austin, 1995, p. 28.
- 14) M. Kobayashi, K. Aida, and I. Hanyu: Induction of gonadotropin surge by steroid hormone implantation in ovariectomized and sex-

- ually regressed female goldfish. *Gen. Comp. Endocrinol.*, **73**, 469–476 (1989).
- 15) M. Kobayashi, K. Aida, and N. E. Stacey: Induction of testis development by implantation of 11-ketotestosterone in female goldfish. *Zool. Sci.*, **8**, 389–393 (1991).
 - 16) Y. C. Sohn, Y. Yoshiura, M. Kobayashi, and K. Aida: Effects of water temperature and food limitation on pituitary gonadotropin and thyrotropin subunit mRNA levels in the female goldfish, *Carassius auratus*. *Fisheries Sci.*, **64**, 700–706 (1998).
 - 17) Y. Yoshiura, M. Kobayashi, Y. Kato, and K. Aida: Molecular cloning of the cDNAs encoding two gonadotropin β subunits (GTH I β and II β) from the goldfish. *Gen. Comp. Endocrinol.*, **105**, 379–389 (1997).
 - 18) M. Kobayashi, Y. Kato, Y. Yoshiura, and K. Aida: Molecular cloning of cDNA encoding two types of pituitary gonadotropin α subunit from the goldfish. *Gen. Comp. Endocrinol.*, **105**, 372–378 (1997).
 - 19) Y. Yoshiura, M. Kobayashi, K. Kikuchi, K. Aida, and Y. Kato: Molecular cloning of cDNA that encodes the β subunit of teleost thyrotropin from the goldfish. *Proc. Jpn. Soc. Comp. Endocrinol.*, **10**, 47 (1995).
 - 20) M. Kobayashi, K. Aida, and I. Hanyu: Radioimmunoassay for silver carp gonadotropin. *Nippon Suisan Gakkaishi*, **51**, 1085–1091 (1985).
 - 21) M. Kobayashi, K. Furukawa, M.-H. Kim, and K. Aida: Induction of male-type gonadotropin secretion by implantation of 11-ketotestosterone in female goldfish. *Gen. Comp. Endocrinol.*, **108**, 434–445 (1997).
 - 22) K. Aida, T. Kato, and M. Awaji: Effects of castration on the smoltification of precocious male masu salmon *Oncorhynchus masou*. *Nippon Suisan Gakkaishi*, **50**, 565–571 (1984).
 - 23) M. Kobayashi, K. Aida, and I. Hanyu: Gonadotropin surge during spawning in male goldfish. *Gen. Comp. Endocrinol.*, **62**, 70–79 (1986).
 - 24) M. Kobayashi, K. Aida, and I. Hanyu: Hormone changes during ovulation and effects of steroid hormones on plasma gonadotropin levels and ovulation in goldfish. *Gen. Comp. Endocrinol.*, **67**, 24–32 (1987).
 - 25) M. Kobayashi, K. Aida, and I. Hanyu: Annual changes in plasma levels of gonadotropin and steroid hormones in goldfish. *Nippon Suisan Gakkaishi*, **52**, 1153–1158 (1986).
 - 26) M. Kobayashi, K. Aida, and I. Hanyu: Hormone changes during ovulatory cycle in goldfish. *Gen. Comp. Endocrinol.*, **69**, 301–307 (1988).
 - 27) M. Kobayashi, K. Aida, H. Sakai, T. Kaneko, K. Asahina, I. Hanyu, and S. Ishii: Radioimmunoassay for salmon gonadotropin. *Nippon Suisan Gakkaishi*, **53**, 995–1003 (1987).
 - 28) N. E. Stacey and M. Kobayashi: Androgen induction of male sexual behaviors in female goldfish. *Horm. Behav.*, **30**, 434–445 (1996).