

# Biological activities of recombinant Manchurian trout FSH and LH: their receptor specificity, steroidogenic and vitellogenic potencies

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## Abstract

Gonadotropins (GTHs), FSH and LH, play central roles in vertebrate reproduction. Here, we report the production of biologically-active recombinant FSH (r-mtFSH) and LH (r-mtLH) of an endangered salmon species, Manchurian trout (*Brachymystax lenok*), by baculovirus in silkworm (*Bombyx mori*) larvae. The biological activities of the recombinant hormones were analyzed using COS-7 cell line transiently expressing either amago salmon FSH or LH receptor. The steroidogenic potency of the r-mtFSH and r-mtLH was examined by a culture system using rainbow trout follicles *in vitro*. *In vivo*, bioactivity was assessed by measuring ovarian weight, oocyte diameter, and plasma steroid hormone levels in female rainbow trout. Moreover, inducing potency of milt production were examined *in vivo* using goldfish. Our results demonstrated that the r-mtFSH and r-mtLH were successfully produced in the baculovirus-silkworm system and recognized by their cognate receptors specifically *in vitro*. The production of estradiol-17 $\beta$  (E2) and testosterone (T) was stimulated by the r-mtFSH and r-mtLH respectively, from the full-grown follicles of rainbow trout, whereas both E2 and T were increased by relatively higher doses of the recombinant hormones from the follicles of the maturing stage. In *in vivo* assay, injection of the r-mtFSH but not r-mtLH increased ovarian weight, oocyte diameter, and plasma E2 levels in immature rainbow trout. Injection of both r-mtFSH and r-mtLH induced milt production in male goldfish. In conclusion, the present study strongly suggests that the r-mtFSH and r-mtLH have distinct biological properties, such as a specific responsiveness for the cognate receptor, steroidogenic, and vitellogenic activities for ovarian follicles in salmonids. These recombinant FSH and LH may be applied for future studies on the gonadal development and maturation in fishes as well as the endangered salmon species.

*Journal of Molecular Endocrinology* (2007) **38**, 99–111

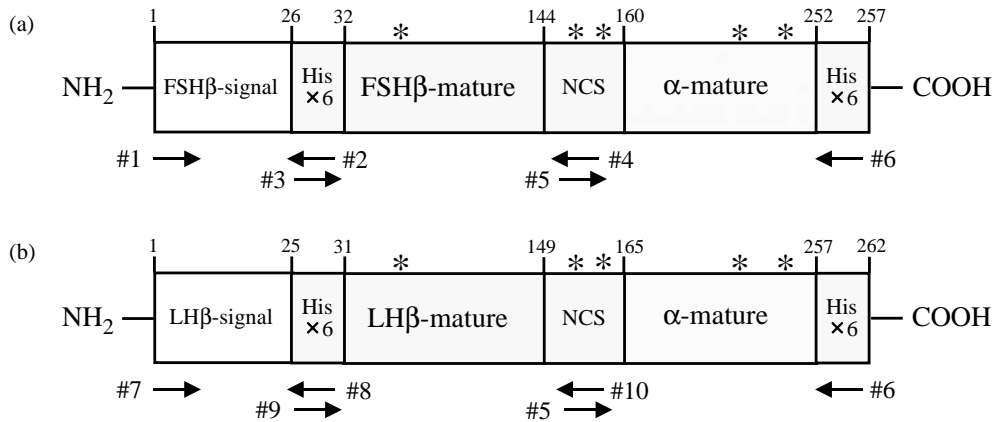
## Introduction

The gonadotropins (GTHs), follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are produced in the pituitary gland of all vertebrates including fish species and they are the key regulators of gonadal development and reproduction. Together, with thyrotropin (TSH) and chorionic gonadotropin (CG), GTHs are members of the glycoprotein hormone family and each member is a heterodimer consisting of two subunits termed  $\alpha$  and  $\beta$  (Pierce & Parsons 1981, Suzuki *et al.* 1988a, Swanson *et al.* 2003). These heterodimeric glycoproteins are reported to carry highly conserved glycosylation sites, and the attached oligosaccharides are related to the hormone's biosynthesis, half-life, and biological activity both in fishes as well as in mammals (Ulloa-Aguirre *et al.* 1999, Zenkevics *et al.* 2000, Klein *et al.* 2003). These hormones react to specific receptors at the reproductive organs which is an essential requirement for their physiological action

(Simoni *et al.* 1997, Dufau 1998, Kumar & Trant 2001, Bogerd *et al.* 2005).

In fishes, FSH is considered to regulate early phases of gametogenesis, such as vitellogenesis and spermatogenesis, whereas LH is responsible for the final maturation processes, such as oocyte maturation, ovulation, spermiation, and milt production (Yaron *et al.* 2003, Kobayashi *et al.* 2006). Despite differences in detailed steroidogenic potency, both FSH and LH stimulated *in vitro* production of estradiol-17 $\beta$  (E2) by vitellogenic ovarian tissues in chum salmon, *Oncorhynchus keta* (Suzuki *et al.* 1988b), coho salmon, *O. kisutch* (Planas *et al.* 2000), goldfish, *Carassius auratus* (Van Der Kraak *et al.* 1992), red seabream, *Pagrus major* (Tanaka *et al.* 1993), and tuna, *Thunnus obesus* (Okada *et al.* 1994), and *in vitro* 11-ketotestosterone (11-KT) production by spermatogenic testicular tissue in coho salmon (Planas & Swanson 1995). In contrast, only LH stimulates *in vitro* production of the maturation-inducing steroid, 17 $\alpha$ ,20 $\beta$ -dihydroxy-4-pregnen-3-one





**Figure 1** Schematic diagrams of recombinant single-chain Manchurian trout FSH (r-mtFSH) (a) and LH (r-mtLH) (b). Primers (#1–#10) used for overlapping PCR strategy are listed in Table 1. The numbers above each box refer to the position of amino acids in each fragment or gonadotropin subunit. Asterisks above each box indicate the putative *N*-linked glycosylation sites (Asn-X-Ser/Thr).

the mature protein without stop codon) or the mtLH $\beta$  (23 amino acids of signal sequence and 124 amino acids of the mature protein without stop codon), and mature mtGTH $\alpha$  were generated by overlapping PCR method with designated primers (Fig. 1 and Table 1: #1–4 for mtFSH $\beta$ ; #5–6 for mtGTH $\alpha$ ; #7–10 for mtLH $\beta$ ). Some substitutions were introduced in the primers to maximize the translation from predicted initiation site (#1 and #7) and to generate hexahistidyl peptide (His-Tag; #2–3 and #8–9). In each cDNA construct, a synthetic DNA encoding Ser-Gly-Ser-Asn-Ala-Thr-Gly-Ser-Gly-Ser-Asn-Ala-Thr-Ser-Gly-Ser (*N*-linked glycosylation sequence (NCS); #4–5 and #10) was inserted between the  $\beta$  and  $\alpha$  chain by overlapping PCR strategy, since a recombinant human FSH containing the NCS was defined as a longer-acting agonist than native FSH (Klein *et al.* 2003). In addition, an Eco RI site was placed at the 5'-end of the DNA constructs and an Xho I site immediately following the terminator codon of the common GTH $\alpha$  subunit. PCR was performed in 50  $\mu$ l final volume containing the subunit cDNA templates (Choi *et al.* 2005), 5  $\mu$ l 10 $\times$  reaction buffer, 2 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTP, 2  $\mu$ M each primer, and 2.5 U LA Taq DNA polymerase (Takara Bio, Otsu, Shiga, Japan). After an initial 5 min denaturing step at 94  $^{\circ}$ C, 30 cycles of amplification were performed using a cycle profile of 94  $^{\circ}$ C for 30 s, 55  $^{\circ}$ C for 30 s, and 72  $^{\circ}$ C for 1.0 min. After the last cycle, elongation was extended to 10 min at 72  $^{\circ}$ C. Next, the PCR products were cut with Eco RI and Xho I, purified by gel-extraction and inserted into Eco RI–Xho I sites of a transfer vector (pYNG: Katakura Industries, Sayama, Saitama, Japan). Finally, the plasmid DNA was sequenced in both strands by chain-termination method using a Big Dye Terminator Ready Reaction Mix (Applied Biosystems, Boston, MA, USA) and an Applied Biosystems' Prism 377 DNA Sequencer.

### Production of recombinant mtFSH and mtLH

Recombinant Manchurian trout FSH (r-mtFSH) and LH (r-mtLH) were produced by the Superworm System (Katakura Industries), as previously described (Kobayashi *et al.* 2006). Briefly, the pYNG transfer vector harboring mtFSH or mtLH cDNA and baculovirus (*Bombyx mori* nucleopolyhedrovirus; CPd strain) genomic DNA were cotransfected into *B. mori* culture cells (BmN; Maeda 1989). Recombinant viruses, into which mtFSH and mtLH cDNAs were introduced by homologous recombination, were screened by the end-point dilution method on 96-well microplates (Maeda 1989). After propagation of the recombinant baculoviruses in *B. mori* culture cells, the recombinant baculovirus carrying cDNA of the mtFSH or mtLH was infected into silkworm larvae of the fifth instar early stage. The infected silkworm larvae were reared by artificial diet at 25  $^{\circ}$ C. Six or seven days after inoculation, hemolymph of the larvae containing r-mtFSH or r-mtLH was collected and centrifuged at 100 000 *g* for 1 h. The supernatants (approximately 30 ml per each recombinant protein) were stored at –80  $^{\circ}$ C until analysis.

### Protein purification and de-*N*-glycosylation

Histidine-tagged hormones were purified by Ni-NTA agarose beads (Qiagen) following the manufacturer's instructions. Briefly, a 50% suspension of the beads (500  $\mu$ l) was added to a sample tube containing 5 ml hemolymph supernatant and incubated on a shaker at 4  $^{\circ}$ C for 2–3 h. The beads were centrifuged briefly and supernatant was removed at 4  $^{\circ}$ C. The remaining beads were packaged in a plastic column and washed with 10 ml washing buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 20 mM imidazole and 0.05% Tween 20, pH 8.0) twice. The Ni-affinity proteins were then eluted with 900  $\mu$ l

elution buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 500 mM imidazole and 0.05% Tween 20, pH 8.0). To remove excessive salt and imidazole, the eluted samples (300 µl) were centrifuged at 4 °C for 30 min with a centrifugal filter device (Amicon 10 000 MWCO, Millipore Corp. Bedford, MA, USA). The filter device was washed with 300 µl PBS (PBS, pH 7.6) twice, and each recombinant hormone was finally eluted with the same volume of PBS and kept at 4 °C. Protein concentration was measured by a spectrophotometer (Ultrospec3100pro, Amersham Biosciences) at 595 nm using a Bradford reagent (Sigma-Aldrich).

N-linked glycosylation state of the recombinant proteins was examined with N-glycosidase F (PNGase F; Sigma-Aldrich) according to the instructions of the manufacturer and our recent report (Shin *et al.* 2006). Briefly, an aliquot (10 µl) of the eluted samples was added to 10 µl denaturing buffer consisting of 0.45% SDS, 1 M Tris-HCl (pH 8.0), and 0.1 M 2-mercaptoethanol. The resulting solution was heated at 100 °C for 5 min and cooled on ice. Next, 1, 5, or 25 mU PNGase F were added to this solution, followed by incubation at 37 °C for 16 h. Finally, the reaction mixture was applied to western blotting.

### Electrophoresis and western blot analysis

SDS-PAGE was performed using 12.5% polyacrylamide gels and proteins were transferred to a nitrocellulose membrane (Pall Corp., Ann Arbor, MI, USA). After blocking, the membrane was sequentially incubated in a ratio of 1:2000 dilution of polyclonal rabbit antibodies (anti-goldfish GTH $\alpha$ , Kobayashi *et al.* 2006; anti-His-Tag, Santa Cruz Biotech., Santa Cruz, CA, USA) overnight at 4 °C and a peroxidase-conjugated polyclonal antibody to rabbit IgG (1:2000; Santa Cruz Biotech.) for 2 h at room temperature. After washing, the membrane was incubated in a chemiluminescent detection reagent (Amersham Biosciences) according to the manufacturer's directions and bands were visualized by exposure to X-ray film (Amersham Biosciences).

### In vitro cAMP assay

Ligand-stimulated intracellular cAMP production was examined by cAMP responsive reporter and enzyme-immunoassay (EIA) system using COS-7 cells transiently transfected with amago salmon FSH-R or LH-R expression plasmid (Oba *et al.* 1999a,b; kind gifts from Dr Yoshitaka Nagahama, National Institute for Basic Biology, Okazaki, Aichi, Japan). COS-7 cells were maintained at 37 °C with 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic-antimycotic reagent (GIBCO, Invitrogen).

Approximately,  $5 \times 10^4$  cells were cultured in 24-well plates and transfection was performed using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. In the reporter gene assay, 200 ng of each receptor expression plasmid and pCRE-luciferase reporter plasmid containing four copies of the cAMP response element (Stratagene, La Jolla, CA, USA) along with a *LacZ* expression vector, pRSV- $\beta$ -galactosidase (internal control plasmid) were cotransfected into the cells. About 24 h after transfection, the cells were changed into fresh medium supplemented with 10% FBS and treated with r-mtFSH or r-mtLH. After 6 h hormone treatment, the cells were subsequently washed with ice-cold PBS and lysed with 100 µl  $1 \times$  lysis buffer (Promega). Luciferase activity was measured with an assay buffer (1 mM luciferin, 2.16 mM ATP, 10.8 mM MgCl<sub>2</sub>, 90 mM KH<sub>2</sub>PO<sub>4</sub>) using a microplate-luminometer (EG&G Berthold, Bad Wildbad, Germany) and normalized by the  $\beta$ -galactosidase values. Transfection experiments were performed in triplicate and repeated at least twice. In measuring the intracellular cAMP concentration, the cells transiently transfected with 200 ng FSH-R or LH-R plasmid as mentioned above were lysed and applied to BIOTRAK cAMP EIA system (Amersham Biosciences) according to the manufacturer's instructions.

### In vitro sex steroid production by rainbow trout follicles

The experimental fish were kindly provided by Salmon Research Center, East Sea Fisheries Research Institute, Yang-Yang, Gangwon-do, Korea. In December 2004, ovarian follicles from one sexually mature female rainbow trout (gonadosomatic index (GSI; gonad weight  $\times$  100/body weight), 17.9%) and one maturing female (GSI, 12.6%) were used in the present experiment. Following decapitation, ovaries were immediately dissected and kept in ice-cold HEPES-NaOH-buffered trout balanced-salt solution (TBSS; pH 7.5) according to the methods of Kagawa *et al.* (1982). The ovarian follicles were incubated in 24-well culture plates containing 1 ml of TBSS (5 or 10 follicles/well) with various doses of r-mtGTHs in a humidified incubator at 15 °C for 18 h. The incubated media were collected and kept at -20 °C until RIA for steroid hormones.

### In vivo effects of r-mtGTHs on female rainbow trout

In February 2005, 1-year-old immature rainbow trout (average body weight (BW),  $201 \pm 10$  g; average GSI,  $0.05 \pm 0.01$ ), which were reared in fresh water of a race-way tank (Salmon Research Center, Yang-yang, Korea), were anesthetized with 2-phenoxyethanol (0.5 ml/l)

and randomly assigned to one of three treatment groups ( $n=7$  per group): hemolymph-derived Ni-affinity proteins (negative control), r-mtFSH, or r-mtLH. All proteins were diluted to a concentration of 80  $\mu\text{g}/\text{ml}$  PBS containing BSA (1 mg/ml). Hormone was administered as a single i.p. injection at a dose of 10  $\mu\text{g}/100$  g BW. Blood samples were collected from the fish 18, 24, and 72 h after the injection, taken from the caudal vasculature with a heparinized syringe and needle after anesthetization with 2-phenoxyethanol. Blood samples were centrifuged at 4000 g and plasma was stored at  $-80^\circ\text{C}$  until RIA. The fish were killed 72-h post-injection by decapitation and body as well as gonad weights were recorded. Parts of the gonads were fixed in 50 ml Bouin's solution for 24 h over night and then processed in ascending concentrations of alcohol, embedded in paraffin, and sectioned at a thickness of 7  $\mu\text{m}$  on a rotary microtome. Every section at intervals of 100  $\mu\text{m}$  was mounted on a microscope slide and stained with hematoxylin–eosin solution. To examine the effects of recombinant hormones on ovarian follicles *in vivo*, all the ovarian section were viewed with a light microscope (Olympus CX31, Shinjuku, Tokyo, Japan) and follicle diameters were measured in the order of larger size ( $n=10$ ; each fish) without repeated measurements.

## RIA

Concentrations of T and E2 in the medium or blood plasma were measured by RIAs as previously described (Aida *et al.* 1984, Kobayashi *et al.* 1988). Rabbit anti-E2-6-CMO-BSA and anti-T-6-CMO-BSA sera were purchased from Cosmo-Bio Co. Ltd (Koto, Tokyo, Japan). Non-radioactive steroids to be used as standards were purchased from Steraloids Inc. (Wilton, NH, USA). Radio-labeled T and E2 ( $[2,4,6,7\text{-}^3\text{H}]\text{-T}$  and  $[2,4,6,7\text{-}^3\text{H}]\text{-E2}$ ) were purchased from Amersham Biosciences.

The sensitivities of the assays were 12.5 pg/ml and 10 pg/ml for E2 and T respectively. The intra- and inter-assay coefficients of variations at the 50% binding were 3.4% ( $n=3$ ) and 11.5% ( $n=6$ ) for E2, 2.3% ( $n=3$ ) and 12.5% ( $n=6$ ) for T respectively. The cross-reactions of E2 antibody to estrone and estriol were 0.5 and 0.9% respectively. Cross-reactivities with T and DHP in the E2 measurement were  $<0.01\%$ . The T antibody cross-reacted with dihydrotestosterone (2.7%), androsten-3,17-dione (0.5%), 11-KT (0.5%) and androstenedione (0.35%), and all other steroids tested showed  $<0.001\%$ .

## Bioassays for milt production

The milt production in male goldfish by the r-mtFSH or r-mtLH was examined according to a previous report (Kobayashi *et al.* 2006). Briefly, in the milt

production assay, sexually mature spermated male goldfish (average BW, 14.6 g) were starved for 7–10 days to suppress milt production and then injected with hemolymph containing recombinant hormones. Milt production of the experimental fish was examined 24 h after injection by gentle stroking of the abdomen.

## Statistical analysis

All data were expressed as the mean  $\pm$  S.E.M. Statistical significance was determined by one-way ANOVA followed by Duncan's multiple-range test ( $P<0.05$ ).

## Results

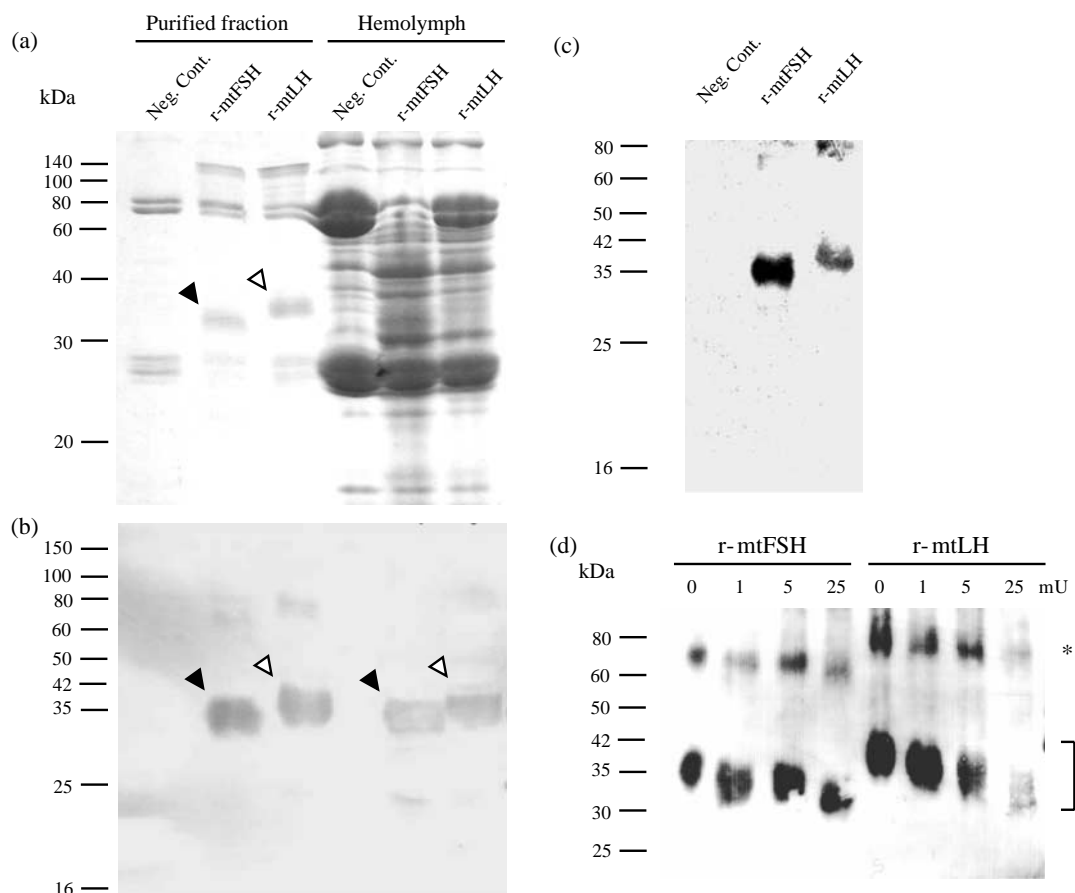
### Production of recombinant Manchurian trout GTHs

To study the biological activities and physiological significances of the two GTHs in the Manchurian trout, we produced recombinant Manchurian trout GTHs using baculovirus-silkworm larvae system as a bioreactor. In a SDS-PAGE analysis, a specific band corresponding to a molecular size of approximately 35 kDa for r-mtFSH or 38 kDa for r-mtLH was shown in the Ni-affinity fraction from hemolymph of silkworm larvae that were infected with the recombinant baculovirus carrying mtFSH or mtLH cDNA (Fig. 2a). Based on spectrophotometric absorbance and protein moiety in SDS-PAGE, the purified recombinant hormones were calculated to 60 and 180  $\mu\text{g}$  per larva for the r-mtFSH and r-mt-LH respectively. In contrast, there were no equivalent bands at 35 and 38 kDa in the fraction from non-infected silkworm hemolymph. Furthermore, western blot analysis using a goldfish-GTH $\alpha$  antibody confirmed that the 35 and 38 kDa molecules are the r-mtFSH and the r-mtLH (Fig. 2b). In addition, anti-His-Tag probe detected the same bands in a western blot (Fig. 2c).

To examine N-linked glycosylation states of the r-mtFSH and the r-mtLH, the purified hormones were incubated with PNGase F at  $37^\circ\text{C}$  for 16 h and the resulting reactants were applied to western blot by the goldfish GTH $\alpha$  antibody. After digestion with the enzyme, the recombinant proteins decreased in molecular mass to 31–33 kDa as shown in Fig. 2d.

### *In vitro* cAMP production assays

The functional characteristics of the r-mtFSH and r-mtLH were investigated using the COS-7 cells transiently transfected with salmon GTH-R expression plasmid, cAMP responsive reporter (CRE-luc), and EIA systems. In the CRE-luc examination, luciferase expression was significantly and selectively activated in



**Figure 2** SDS-PAGE, western blot, and de-*N*-glycosylation analyses of recombinant Manchurian trout FSH (r-mtFSH) and LH (r-mtLH). (a) SDS-PAGE (12.5%) was conducted under reducing conditions with hemolymph of silkworm or Ni-affinity purified fraction as indicated. (b) r-mtFSH and r-mtLH were detected by SDS-PAGE (12.5%) under reducing conditions followed by western blot using an anti-goldfish GTH $\alpha$  antibody. Black and white arrow heads in (a) and (b) indicate r-mtFSH and r-mtLH bands respectively. (c) Western blot using an antibody for His-Tag. (d) Purified r-mtFSH and r-mtLH were incubated at 37 °C for 16 h without or with increasing amounts of PNGase F treatments (1–25 mU), followed by western blot using the goldfish GTH $\alpha$  antibody. Non-specified bands are indicated by an asterisk.

the FSH-R-expressing cells by the r-mtFSH and in the LH-R-expressing cells by the r-mtLH respectively, in dose-dependent manners (Fig. 3a), but the response of the latter was much more potent. In the EIA system, cAMP production levels in response to the r-mtFSH or r-mtLH also increased in the cognate receptor-transfected cells, and hCG (5  $\mu$ g/ml) stimulated the cAMP production in LH-R-cells only (Fig. 3b).

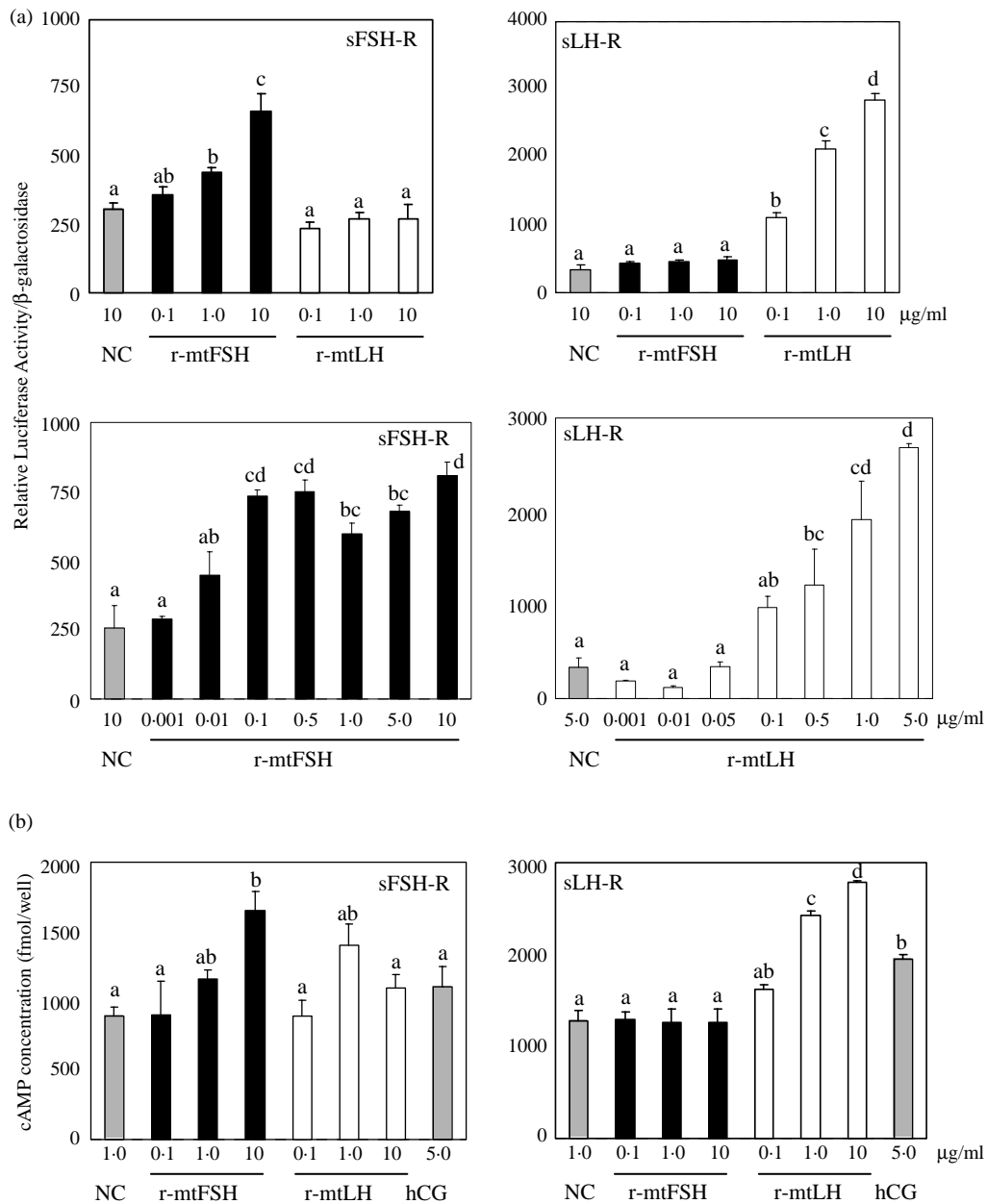
#### ***In vitro* effects of r-mtGTHs on sex steroid production**

To investigate steroidogenic actions of the r-mtFSH and the r-mtLH in salmonid ovary, we examined the steroidogenic response of rainbow trout intact follicles at two different stages of oocyte maturation. In full-grown follicles from a mature female (GSI=17.9), the production of E2 and T was significantly stimulated by

the r-mtFSH and r-mtLH respectively, at a dose of 100 ng/ml (Fig. 4a). In contrast, E2 and T increased by growing follicles from a maturing female (GSI=12.6) in response to both recombinant hormones at higher doses (1  $\mu$ g/ml; Fig. 4b).

#### ***In vivo* effects of r-mtGTHs on ovarian development in female rainbow trout**

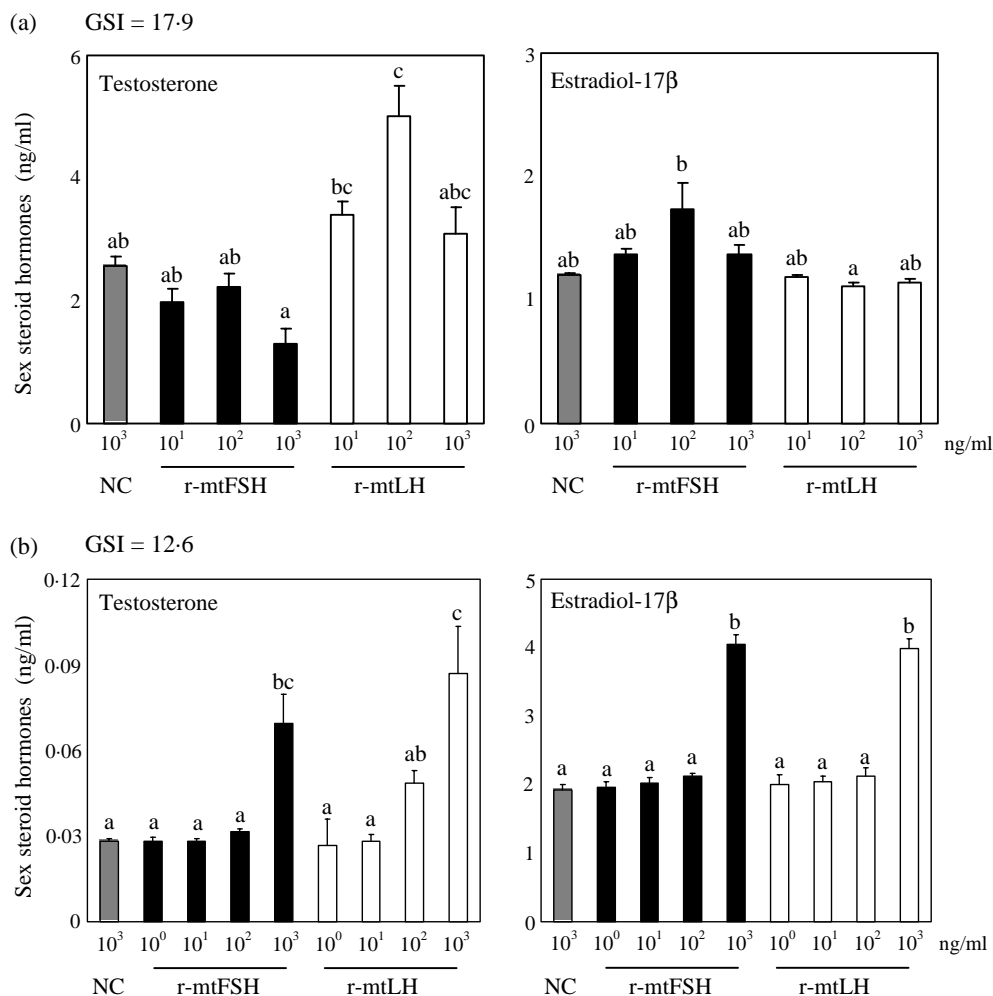
Mean GSI and follicle diameter following a single injection of control protein, r-mtFSH or r-mtLH in immature rainbow trout are shown in Fig. 5a. The injection of r-mtFSH, but not r-mtLH, increased mean GSI and follicle diameters significantly as compared with those of control fish, although the r-mtLH slightly increased GSI value. In histological examination, vitellogenic follicles at early stages (cortical alveoli



**Figure 3** *In vitro* biological activities, as assessed by cAMP production, of r-mtFSH and r-mtLH. (a) Effects of r-mtFSH and r-mtLH on COS-7 cells transiently cotransfected with a cAMP-responsive reporter gene construct (pCRE-luciferase) and amago salmon FSH-R or LH-R. (b) Intracellular cAMP concentration in COS-7 cells transiently transfected with amago salmon FSH-R or LH-R (EIA method). NC, negative control. Values sharing the same letter do not significantly differ ( $P < 0.05$ ).

and/or oil droplet stages) were frequently observed in the ovaries of r-mtFSH-injected fish (Fig. 5a), whereas ovaries in control and r-mtLH-injected fish did not contain these stages of follicles. Plasma levels of E2 fish receiving the r-mtFSH were also elevated from 18-h post-injection and attained maximum levels at 24 h (Fig. 5b). At 72-h post-injection, E2 levels still showed

higher concentration in the r-mtFSH-injected fish when compared with those of control and r-mtLH-injected fish, but significant differences were not observed among the three groups. In r-mtLH-treated fish, plasma T levels and mean GSI values were not significantly different from those of control fish, although these parameters showed a tendency to increase.



**Figure 4** Effects of r-mtFSH and r-mtLH on testosterone (T) and estradiol-17β (E2) production *in vitro* by intact ovarian follicles from sexually mature (a) and maturing female rainbow trout (b). The ovarian follicles were incubated in 24-well culture plates containing 1 ml of HEPES-NaOH-buffered trout balanced-salt solution (pH 7.5) with indicated doses of recombinant GTHs at 15 °C for 18 h. NC, negative control. Values sharing the same letter do not differ significantly ( $P < 0.05$ ).

### Milt production in goldfish

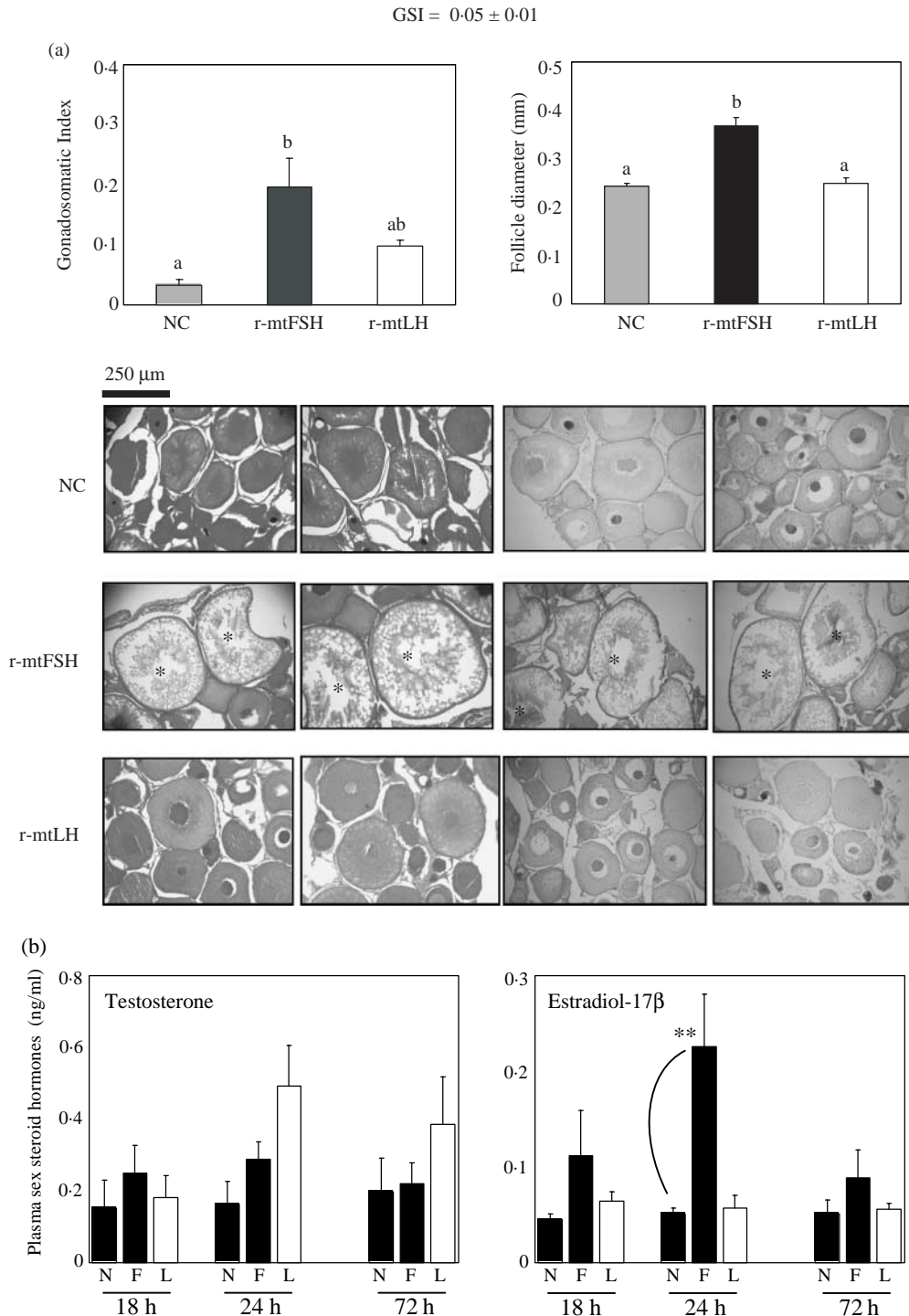
Injection of hemolymph containing r-mtFSH or r-mtLH (5 or 20 μl/g BW) induced milt production in male goldfish as did hCG (10 U/g BW; Table 2).

### Discussion

In the present study, we demonstrated that (1) biologically-active recombinant Manchurian trout GTHs can be produced by a baculovirus-silkworm larvae system; (2) the recombinant hormones selectively activate their cognate receptors and produce sex steroids in ovarian follicles *in vitro*; and (3) the hormones have the potency to stimulate steroidogenesis and ovarian development of rainbow trout *in vivo*,

and these are effective for milt production in goldfish. This is the first report on production of biologically active recombinant GTHs in salmonids, although pioneering researches on the fish GTHs and GTH receptors were carried out using salmon species (Suzuki *et al.* 1988a,b, Yan *et al.* 1992, Miwa *et al.* 1994, Oba *et al.* 1999a,b).

In order to produce heterodimeric and biologically active recombinant GTHs, single-chain mtFSH and mtLH were constructed in which the carboxyl terminus of the mtFSHβ or mtLHβ subunit was fused to the amino terminus of the mature mtGTHα subunit with an intermediate polypeptide sequence (NCS). In mammals, a recombinant human FSH containing the NCS extended half-life in the blood by 7.3 h, which is twofold longer than native recombinant FSH (Klein *et al.* 2003).



**Figure 5** *In vivo* effects of r-mtFSH and r-mtLH on ovarian development (a) and sex steroid hormone production (b) in sexually immature female rainbow trout. r-mtFSH or r-mtLH was administered as a single i.p. injection at a dose of 10 µg/100 g BW. Blood samples were collected at 18, 24, and 72-h post-injection and plasma sex steroid levels were measured by RIA. The ovaries were weighed for GSI calculation and a part of the ovary was individually fixed in Bouin's solution for histological examination (H&E staining). Single asterisk and double asterisks indicate vitellogenic follicles at early stages and significant differences at  $P < 0.01$  respectively. Values sharing the same letter do not differ significantly ( $P < 0.05$ ). NC (N), hemolymph of silkworm larvae as a negative control; F, r-mtFSH; L, r-mtLH.

**Table 2** Bioassays for r-mtFSH and r-mtLH inducing milt production in male goldfish

	No response	Milt produced	n	Dosage
Silkworm hemolymph	7	0	7	b
r-mtFSH	1	6	7	a
r-mtFSH	0	7	7	b
r-mtLH	1	6	7	a
r-mtLH	1	6	7	b
hCG	1	6	7	c

a, 5 µl/g Body weight (BW); b, 20 µl/g BW; c, 10 U/20 µl/g BW; n, fish number.

Binding potency of the NCS-FSH to the human FSH receptor and signal transduction was also comparable with those of native FSH. In the present investigation, SDS-PAGE followed by western blot analyses using antibodies for both goldfish GTH $\alpha$  subunit and His-Tag revealed that the tethered mtFSH $\beta/\alpha$  and mtLH $\beta/\alpha$  proteins are successfully produced in the silkworm larvae. Furthermore, molecular masses of the r-mtFSH and r-mtLH corresponded to 35 and 38 kDa respectively, indicating that the proteins were N-glycosylated; if not both proteins should be detected as 29 kDa molecules. In fact, an N-glycosidase F treatment decreased the molecular masses of r-mtFSH and r-mtLH to 31 and 33 kDa respectively, confirming their properties as glycoproteins. An extra and non-specified band at high molecular mass ranges (70–80 kDa) was shown by the western blots. Although we have no clear explanation for these bands, these results may imply that unexpected dimerization occurred in the recombinant hormones, judged by their molecular sizes and glycosylated forms. The different molecular sizes between r-mtFSH and r-mtLH are most likely due to the different signal sequences of the proteins rather than N-linked moieties, since excessive treatments of N-glycosidase F (~100 mU) did not change the molecular masses and evidently, the amino terminus of r-mtFSH was started from mature mtFSH $\beta$ , whereas the r-mtLH had intact signal sequence of mtLH $\beta$  (data not shown). In a previous report, we noted that the different secretion pattern between recombinant mtFSH- and mtLH-expressing CHO cells may be due to the higher hydrophobicity of signal peptides in mtLH $\beta$  than that of mtFSH $\beta$  (Choi *et al.* 2005).

To date, several recombinant GTHs of fishes have been produced in various bioreactors, such as yeast (Kamei *et al.* 2003, Kasuto & Levavi-Sivan 2005), transgenic fish (Morita *et al.* 2004), soil amoeba (Vischer *et al.* 2003), S2 Drosophila cell line (Zmora *et al.* 2003), CHO cells (Choi *et al.* 2005, So *et al.* 2005), and silkworm larvae (Kobayashi *et al.* 2006). Although goldfish recombinant GTHs produced in silkworm larvae (Kobayashi *et al.* 2006) were characterized as

biologically active hormones, information of the direct effects of recombinant GTHs to their cognate receptors is essential to examine the function of r-mtFSH and r-mtLH. Evaluation of the binding properties of r-mtFSH and r-mtLH to amago salmon GTH receptors and signal transduction *in vitro* was conducted by intracellular cAMP productivities using a cAMP responsible reporter and a direct EIA assay. In both systems, r-mtFSH and r-mtLH were highly specific for their cognate receptors, but the r-mtLH:LH-R interaction was potent in terms of signal transduction when compared with that of the r-mtFSH:FSH-R. It is noteworthy that magnitude of the LH interaction with LH-R was greater than that of FSH to FSH-R in both the Manchurian trout and chum salmon (Oba *et al.* 1999a,b). Opposite to the situation in the salmon species, however, the African catfish FSH responded to the cognate FSH-R with higher potency (EC<sub>50</sub>: 0.31 ng/ml) than that of African catfish LH to the LH-R (EC<sub>50</sub>: 26.76 ng/ml; Vischer *et al.* 2003). In contrast to the high specificity of r-mtFSH and r-mtLH for their receptors, purified chum salmon FSH activated both types of GTH receptors with a slight preference for FSH-R, while LH did only LH-R (Oba *et al.* 1999a,b). In *in vitro* ligand autoradiography, coho salmon FSH showed a specific interaction with FSH-R, whereas LH interacted with both FSH-R and LH-R (Miwa *et al.* 1994), which is a similar situation in African catfish (Vischer *et al.* 2003), channel catfish (Zmora *et al.* 2003), and zebrafish (So *et al.* 2005). Although we have no clear explanation for these different observations, these results may be attributed to high plasticity of gonadotropin-receptor interaction in teleosts and the use of heterologous GTHs from a different species.

The biological activity of the recombinant r-mtFSH and r-mtLH was further evaluated in steroidogenic effects on intact ovarian follicles of rainbow trout *in vitro*. As expected, both r-mtFSH and r-mtLH were capable of inducing T and E2 production in maturing ovarian follicles which are generally in agreement with the potency of purified FSH and LH in coho salmon (Planas *et al.* 2000), although the magnitude of steroidogenic effect of coho salmon FSH and LH was greater than that of r-mtFSH and r-mtLH effects on the maturing follicles. In fully grown follicles from mature rainbow trout, on the other hand, r-mtFSH but not r-mtLH had a weak stimulatory effect on E2 production, whereas only r-mtLH stimulated the production of T at a dose of 100 ng/ml. Considering that in red seabream gradually increased levels of aromatase mRNA during oocyte growth dramatically decreased in follicles at the migratory nucleus and the mature stages (Gen *et al.* 2001), no effect of r-mtLH on the E2 production by mature follicles of rainbow trout is at least in part, due to decreased aromatase expression level in the follicles. Further research is clearly needed to determine the

precise activities of r-mtFSH and r-mtLH during final oocyte maturation in salmonids.

In the present study, biological activities of recombinant Manchurian trout FSH and LH were further examined with respect to their *in vivo* effects, i.e. ovarian development and production of sex steroids. In immature rainbow trout, a single injection with r-mtFSH but not r-mtLH significantly increased mean GSI and follicle diameters as compared with those of control fish, at 3 days post-injection. Furthermore, vitellogenic oocytes were frequently observed and plasma E2 levels at 24-h post-injection were significantly higher in the r-mtFSH-injected fish than in control fish. These data are consistent with a previous report that FSH but not LH function in stimulating *in vitro* and *in vivo* VTG uptake into vitellogenic follicles of rainbow trout within 24 h (Tyler *et al.* 1991). Moreover, E2 is also able to induce VTG mRNA expression, protein synthesis and release in juvenile rainbow trout within 48 h (Arukwe *et al.* 2001). Thus, it is strongly suggested that r-mtFSH elevated plasma E2 levels and in turn E2 stimulated VTG synthesis and to some extent its uptake into immature ovarian follicles of rainbow trout. Plasma T levels showed an increasing tendency by r-mtLH at 24- and 72-h-post-injection, while E2 was not changed by the hormone throughout the examinations. These results suggest that the r-mtLH did not augment ovarian aromatase activity in immature rainbow trout. Recently, Kagawa's research group defined clearly the expression and activities of ovarian P450 aromatase, which are mainly controlled by LH but not FSH during oocyte development of red seabream (Gen *et al.* 2001, Kagawa *et al.* 2003). In the rainbow trout, however, FSH-containing fraction but not LH-fraction was able to increase E2 production by early vitellogenic follicles *in vitro*, although highly purified GTHs were not used (Nagler & Idler 1991). These results may in part be explained by differences of gonadal development patterns between rainbow trout, which undergoes synchronous ovarian development and red seabream having an asynchronous type of ovary.

In the bioassays for milt production in a heterogeneous species, goldfish, both r-mtFSH and r-mtLH were as effective as hCG for milt production in male goldfish. Recently, Kobayashi *et al.* (2006) reported that recombinant goldfish FSH and LH produced by silkworm larvae induced milt production in male goldfish with a similar magnitude. In coho salmon (Planas & Swanson 1995) and red seabream (Kagawa *et al.* 1998), both FSH and LH stimulated testicular 11-KT production *in vitro*. Furthermore, 11-KT was slightly effective in inducing spermiation in goldfish *in vivo* (Ueda *et al.* 1985). These results suggest that both r-mtFSH and r-mtLH activate a final phase of testicular development via, at least partly, 11-KT production in male fish.

In summary, biologically active r-mtFSH and r-mtLH have been produced successfully in the baculovirus-silkworm system. The r-mtFSH and r-mtLH were highly specific for their cognate receptors, although the efficacy of signal transduction by r-mtLH was stronger than that of r-mtFSH. The production of E2 and T were stimulated by the r-mtFSH and r-mtLH respectively, from fully grown follicles of rainbow trout *in vitro*, whereas both E2 and T increased by the follicles of maturing stage reacting with both r-mtFSH and r-mtLH. In immature rainbow trout, a single injection with r-mtFSH increased ovarian weights and follicular diameters at 3 days post-injection. Furthermore, in the r-mtFSH-injected fish, vitellogenic oocytes were frequently observed and plasma E2 levels were higher than those of control fish. In r-mtLH-treated fish, plasma T levels and GSI values were not significantly different from those of control fish, although these parameters showed a tendency to increase. Both r-mtFSH and r-mtLH were highly effective for milt production in male goldfish.

## Acknowledgements

We thank Dr Yoshitaka Nagahama, National Institute for Basic Biology, Okazaki, Aichi, Japan, for supplying the amago salmon GTH receptor plasmids. We also thank Mi-Ae Kim of Kangnung National University for technical support and the personnel of Salmon Research Center, East Sea Fisheries Research Institute, Yang-Yang, Gangwon-do, Korea, especially Drs Chae Sung Lee and Cheul Ho Lee for providing and maintenance of experimental fish. This work was supported by Grants-in-Aid from the JSPS, Japan, for Japan–Korea Basic Scientific Cooperation (M K), and grants from KOSEF for Joint Research Project under Korea–Japan Basic Scientific Cooperation Program and from the Ministry of Maritime Affairs and Fisheries, Korea, for KSGP and MarineBio21 program (Y C S). H Ko and W Park are recipients of a graduate fellowship provided by the Brain Korea (BK21) program sponsored by the Ministry of Education and Human Resources Development, Korea. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 9 October 2006

Accepted 8 November 2006

Made available online as an Accepted Preprint on

17 November 2006