Molecular Cloning of cDNAs Encoding Pituitary Glycoprotein Hormone α, FSH β and LH β Subunits in Ayu, Plecoglossus altivelis

Authors: Yoshida, Daisaku, Nagae, Masaki, Ito, Fuminari, and Soyano, Kiyoshi

Source: Zoological Science, 18(7) : 929-936

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.18.929

ZOOLOGICAL SCIENCE **18**: 929–936 (2001)

Molecular Cloning of cDNAs Encoding Pituitary Glycoprotein Hormone α, FSH β and LH β Subunits in Ayu, *Plecoglossus altivelis*

Daisaku Yoshida[1], Masaki Nagae[2], Fuminari Ito[3] and Kiyoshi Soyano[1]*

[1]*Marine Research Institute, Nagasaki University, Nagasaki 851-2213, Japan;*
[2]*Faculty of Environmental Studies, Nagasaki University, Nagasaki 852-8521, Japan;*
[3]*Freshwater Fisheries and Environment Division, National Research Institute of Fisheries Science, Ueda 386-0031, Japan.*

ABSTRACT—Complementary DNAs (cDNAs) encoding follicle-stimulating hormone (FSH) β, luteinizing hormone (LH) β and glycoprotein hormone (GPH) α subunits were isolated and characterized from ayu pituitary using a PCR technique. The FSH β, LH β and GPH α subunit cDNAs were found to be 556, 588 and 621 base pair (bp) long, encoding 384, 432 and 357bp long open reading frames, respectively. The deduced amino acid sequences of the putative mature forms of ayu GPH α and LH β subunits were on average 63% homologous with those of other teleosts, whereas ayu FSH β subunit was on average 42% homologous. These results show that ayu has two different types of gonadotropins (FSH and LH), as in other teleosts. FSH β subunit mRNA was mainly detected during early vitellogenesis and spermatogenesis. In contrast, LH β subunit mRNA was detected during the late phase of gonadal development, suggesting that the primary function of FSH may be to initiate gametogenesis, while LH may have a role in the development of gametes.

INTRODUCTION

In teleosts, gonadotropins (GtHs) are the most important hormones in the regulation of gonadal development and maturation (Nagahama, 1987). It is generally accepted that two forms of GtH, follicle-stimulating hormone (FSH), or GtH I, and luteinizing hormone (LH), or GtH II, exist in all teleosts (Xiong *et al*., 1994). These hormones are heterodimers consisting of two different noncovalently linked polypeptides, glycoprotein hormone (GPH) α and β subunits. The GPH α subunit is common to both FSH and LH, whereas the β subunit is distinct and provides each hormone with a unique biological specificity (Pierce and Parsons, 1981).

Ayu, *Plecoglossus altivelis*, are widely distributed in Japan. It is an annual and amphidromous fish that spawns in rivers. A distinct environment (water current and fine gravel) is necessary for their spawning, and only after reaching such an environment the fish acquire the physiological conditions necessary for spawning. These physiological conditions include an elevation of milt volume and a decrease in spermatocrit value in males (Ito *et al*., 1992), and development of maturational competence of oocyte in females (Soyano *et al*., 1996). These facts suggest that the spawning environment induces endocrine changes that would lead to spawning in ayu. It is probable that GtH(s) are involved in the process. In fact, Hirose *et al*. (1983) reported that LH-RH treatment increased serum levels of GtH II in ayu, followed by an immediate final maturation of oocytes. However, there is little information about the role of GtH in gonadal development and maturation or relationship between physiological conditions induced by the spawning environment and GtH control of gonadal maturation in ayu. To understand the role of GtH, changes in the synthesis and secretion of GtH(s) during gonadal development, maturation and spawning should be observed. Ayu GtH(s), however, have not been isolated or characterized.

In the present study, as an initial step, the cDNAs encoding pituitary FSH β, LH β and GPH α subunits of ayu were isolated and characterized. In addition, changes in mRNA transcription of these subunits during gonadal development and maturation were examined using Northern blot analysis.

MATERIALS AND METHODS

Fish

Juvenile ayu were purchased from a commercial supplier and reared in an outdoor pond from May to October at the National Research Institute of Fisheries Science, Ueda Station. They were fed a commercial diet and maintained under ambient photoperiod and temperature. Ayu, reared at the Ueda station, usually spawn from early to mid October. Pituitary samples for cDNA cloning of ayu GtH subunits were collected during the maturational period (October). Samples to examine the seasonal changes in the levels of GtH mRNA

* Corresponding author: Tel. +81-95-850-7701;
FAX. +81-95-840-1881.
E-mail:soyano@net.nagasaki-u.ac.jp

Table 1. Gonadal stages*[1] and gonadosomatic index (GSI) of ayu

Stages	Date	Germ cell contents*[2]		GSI(%)	
		Female	Male	Female	Male
I	18, June	Perinuclear	Spermatogonia	<0.7	<0.7
II	2, July	Perinuclear	Spermatogonia	<0.7	<0.7
III	4, August	Primary yolk	Spermatocyte	1.4±0.21	0.7±0.09
IV	7, September	Secondary yolk	Spermatotid	9.8±0.62	8.8±0.27
V	2, October	Tertiary yolk	Spermatozoa	23.9±1.10	9.7±0.33

*[1] These were based on oocyte and testes histological characteristics.
*[2] Germ cell contents shown are those which appeared most in each gonad.

using Northern blot analysis were collected in five different periods as shown in Table 1. All pituitary samples were collected immediately after decapitation and stored at –80°C before use.

Preparation of total cytoplasmic RNA and poly- (A)$^+$ RNA

Total cytoplasmic RNA was extracted from approximately twenty pituitaries by single-step guanidium isothiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987) using an ISOGEN kit (Nippongene, Toyama, Japan) according to the manufacturer's instructions.

From the total cytoplasmic RNA, poly- (A)$^+$ RNA was isolated using Oligotex dT30<super> (TaKaRa Shuzo, Ootu, Japan). Total cytoplasmic RNA (50 μl) was added to 150 μl of elution buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1.0% SDS) and 200 μl Oligotex dT30 latex. After incubation at 65°C for 5 min, it was immediately placed on ice for 3 min. The above mixture was added to 40 μl 5 M NaCl and incubated at 37°C for 10 min to bind poly- (A)$^+$ RNA to the latex. The latex was centrifuged and the aqueous portion removed. The latex was then suspended in 200 μl TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA) and incubated at 65°C for 5 min to elute the poly- (A)$^+$ RNA. After centrifugation, the poly- (A)$^+$ RNA contained in the aqueous phase was collected by ethanol precipitation, and resuspended in 10 μl diethylepyrocarbonate (DEPC) H_2O. Total cytoplasmic and poly- (A)$^+$ RNA concentrations were assessed by spectrophotometric absorbance at 260 nm.

Double-stranded cDNA synthesis

Double-stranded cDNAs were obtained using the ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. After the addition of phosphorylated *Eco*RI linkers and *Xho*I digestion, cDNAs were inserted into the EcoRI-XhoI site of the Uni-ZAP XR vector (Stratagene, La Jolla, CA) in a sense orientation.

Cloning of ayu GtH subunit cDNAs

The locations of the oligonucleotide primers used in PCR for cDNA cloning of ayu GtH subunits are shown in Fig. 1. The primer sets, A and B, were based on the conserved sequences of each GtH subunit cDNA as follows: chum salmon, *Oncorhynchus keta* (Sekine *et al.*, 1989), striped bass, *Morone saxatilis* (Hassin *et al.*, 1995) and mummichog, *Fundulus heteroclitus* (Lin *et al.*, 1992) for FSH β; chum salmon (Sekine *et al.*, 1989) and common carp, *Cyprinus carpio* (Chang *et al.*, 1988) for LH β; chum salmon (Kitahara *et al.*, 1988) and common carp (Huang *et al.*, 1991) for GPH α. Primer sequences were:

FSH β subunit A: 5'TGCAGYTGGTYSTCATGG 3'
FSH β subunit B: 5'CWYCTCRTAGGACCASTC 3'
LH β subunit A: 5'ATCTGCAGYGGYCACTGC 3'
LH β subunit B: 5'ACAGTCRGAMGTGTCCAT 3'
GPH α subunit A: 5'GGMTGTGAGGARTGYAMACTSAA 3'
GPH α subunit B: 5'GCWACGCAGCATGTRGCTTCAGA 3'
(R: A/G, Y: C/T, S: C/G, W: A/T, M: A/C)

The primer sets, C and D, for each ayu GtH subunit were designed after obtaining the sequence information of the cDNA fragments amplified by the above primer A and B sets: Uni-ZAP XR vector sequences upstream (ZAP-F) and downstream (ZAP-R) of the cDNA inserts were used for external primers. Primers were synthesized on an Applied Biosystems DNA synthesizer (model 392, Foster City, CA).

The reaction mixture for PCR contained 2.5 U *Taq* and 1 x the supplied buffer (Ex *Taq*, TaKaRa Shuzo), together with 50 ng Uni-ZAP XR vector inserted pituitary cDNA, 0.2 mM of each dNTP, and 0.5 μM of the upstream and downstream primers, in a total volume of 20 μl. The following cycle was repeated 35 times: denaturation at

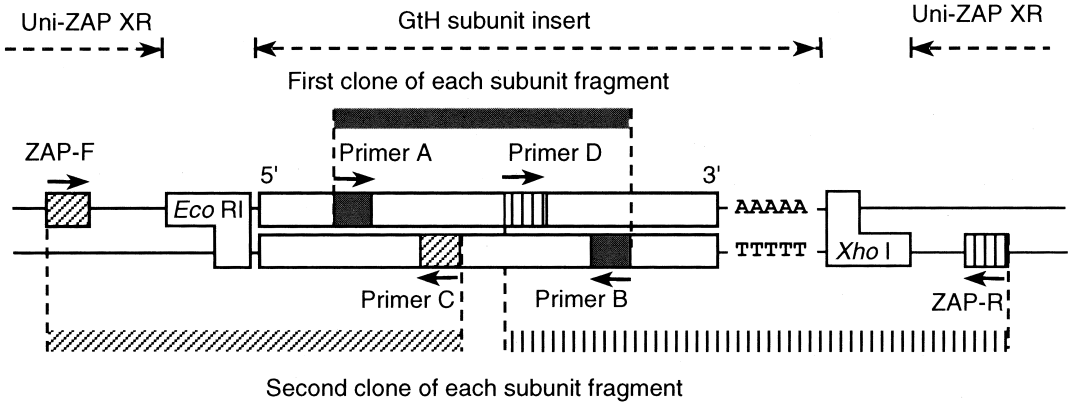

Fig. 1. Schematic representation of FSH β, LH β and GPH α subunit cDNA inserts (with asymmetric, *Eco* RI and *Xho* I arms) in Uni-ZAP XR vector (not drawn to scale). Gray boxes (primers A and B) depict the relative locations of the target sequences for the oligonucleotide primers used in the PCR reaction. Primers C and D were specific sequences of each subunit. Directions of the primers are indicated by arrows. Clones of the PCR products are indicated by solid lines above the diagram. ZAP-F and ZAP-R represent primers corresponding to the Uni-ZAP XR vector sequence located upstream and downstream of the GtH cDNA insert, respectively.

94°C for 20 sec, annealing at 50–55°C for 30 sec and extension at 72°C for 1 min.

The main product in each PCR was subcloned into pCR 2.1 vectors using a TA cloning system (Original TA Cloning Kit, Invitrogen, Carlsbad, CA). The resulting plasmid DNAs were purified by an alkaline lysis method (Sambrook *et al.*, 1989). And both strands sequenced using a *Taq* dye primer cycle sequencing kit (Applied Biosystems). Sequence analysis and comparisons were carried out using DNASIS software (Hitachi Co. Ltd., Tokyo, Japan).

Northern blot analysis

Total cytoplasmic RNA (2.5 μg) from five pituitaries of ayu were separated by electrophoresis on 1% agarose gels containing formaldehyde, and transferred onto nylon membrane (Hybond-N^+, Amersham, Bucks, U.K.). Images of ethidium bromide stained gels were captured by a gel documentation system (Printgraph, ATTO, Tokyo, Japan). Probes were labeled with digoxigenin (DIG)-dUTP using a PCR DIG Probe Synthesis Kit (Roche, Barsel, Switzerland) according to the manufacturer's instructions. Membranes were prehybridized at 42°C for 2 h in 5 × SSPE (0.75 M NaCl, 43.25 mM NaH_2PO_4, 6.25 mM EDTA) containing 50% formamide, 2% Denhardt's solution, 0.02% sodium dodecyl sulfate (SDS) and denatured calf thymus DNA (100 μg/ml). Hybridization was carried out at 42°C for 12 h in the same solution as described above containing denatured DIG-dUTP labeled cDNA probe. Membranes were washed at 65°C for 2 × 10 min in 2 × SSPE containing 0.1% SDS, for 2 × 20 min in 1 × SSPE containing 0.1% SDS and for 2 × 20 min in 0.1 × SSPE containing 0.1% SDS. After washing, labeled mRNA on membranes was detected using a DIG Luminescent Detection Kit (Roche Diagnostics K.K., Tokyo, Japan), according to the instructions of the manufacturer, and copied using x-ray film. Developed photo images and images of ethidium bromide stained gels were analyzed using image-analysis software (NIH image 1.61, National Institute of Health, USA) to measure the intensity of each hybridization signal and 18S rRNA. Data was standardized by dividing the levels of each subunit by that of 18S rRNA.

RESULTS AND DISCUSSION

Double-stranded cDNA synthesis and PCR amplification

Two hundred micrograms of total cytoplasmic RNA was obtained from approximately 20 pituitaries. From this total cytoplasmic RNA, approximately 2.5 μg poly- $(A)^+$ RNA was isolated. This poly- $(A)^+$ RNA was reverse-transcribed to cDNA and ligated into Uni-ZAP XR vector for PCR amplification.

PCR amplification of partial FSH β, LH β and GPH α subunit cDNAs produced major bands of approximately 240, 180 and 170 bp using Primer A and B sets, respectively. After sub-cloning these three fragments, several clones were sequenced. The sequencing analysis revealed that these clones encoded part of the open-reading frames of FSH β, LH β and GPH α subunits.

The PCR amplification of the 5'-end portions of FSH β, LH β and GPH α subunits was performed using Primer ZAP-F and C sets. The main products of PCR for FSH β, LH β and GPH α were approximately 140, 260 and 190 bp, respectively. Sequencing analysis revealed that these products contained 5'-end portions of each subunit cDNA.

The fragments containing 3'-end portions of FSH β, LH β and GPH α subunits were amplified using Primer D and ZAP-R sets. The main products of PCR for FSH β, LH β and GPH α were approximately 380, 330 and 450 bp, respectively. Sequencing analysis indicated that these products contained 3'-end cDNAs of each subunit, encoding the open reading frames of the C-terminal portions.

The second clones, 5' and 3' PCR products, were identical to the portion where they overlapped the first clone (first PCR fragment). The full length sequence of each subunit cDNA was compiled from these three fragments. In addition, the full length of each subunit was amplified using specific primers and sequenced for reconfirmation. These results indicated that ayu has two different types of GtH, FSH and LH, as in other teleosts.

Sequence determination and analysis

The ayu FSH β and LH β subunit cDNAs were 556 and 588 bp long and contained full 384 and 432 bp open reading frames, respectively. From a comparison with FSH β and LH β subunits of other teleosts, the deduced amino acid sequences indicated that the signal peptide comprised of 15 and 23 amino acids and the mature subunit comprised of 113 and 121 amino acids (Fig. 2-A and 2-B), respectively. Alignment of the deduced amino acid sequence of mature FSH β and LH β subunits from ayu with those of other telesots is shown in Fig. 3-A and 3-B. The positions of 12 cysteines and 1 putative N-linked glycosylation site in LH β were completely conserved among these teleosts. However, the position and number of cysteines in FSH β varied among teleosts. Many teleost FSH β subunits contain 12 cysteines (ayu, masu salmon, striped bass, etc.) at specific positions. Mummichog and Japanese eel FSH β subunits also contain 12 cysteines, but the position of the third cysteine in these fish is clearly different from that of the teleosts mentioned above. Furthermore, goldfish FSH β contains 13 cysteines. In addition to the variation in the position and number of cysteine residues in FSH β, the percentage homology of ayu FSH β with those of other teleosts (36–46%) was lower than that of LH β (48–69%). Thus, our results show that the primary structure of LH β has been conserved better than that of FSH β during teleost evolution, as described by Kato *et al.* (1993).

The ayu GPH α subunit cDNA was 621 bp long, containing full 357 bp open reading frame. From a comparison with GPH α subunits of other teleosts, the deduced amino acid sequence indicated that the signal peptide comprised of 23 amino acids and the mature subunit comprised of 96 amino acids (Fig. 2-C). Alignment of the deduced amino acid sequence of mature GPH α subunit from ayu with those of other teleosts is shown in Fig. 3-C. The positions of 10 cysteines and 2 putative N-linked glycosylation sites were completely conserved. The percentage of homology was 73% with masu salmon α1, 67% with masu salmon α2 and Japanese eel, and in the range of 54–64% with other teleosts.

Northern hybridization analysis

Northern blot analysis using cloned FSH β, LH β and GPH α cDNAs as probes showed specific hybridization of each with a single band of approximately 700 bp (Fig. 4). Since the expression of FSH β (GtH I β) and LH β (GtH II β) subunit

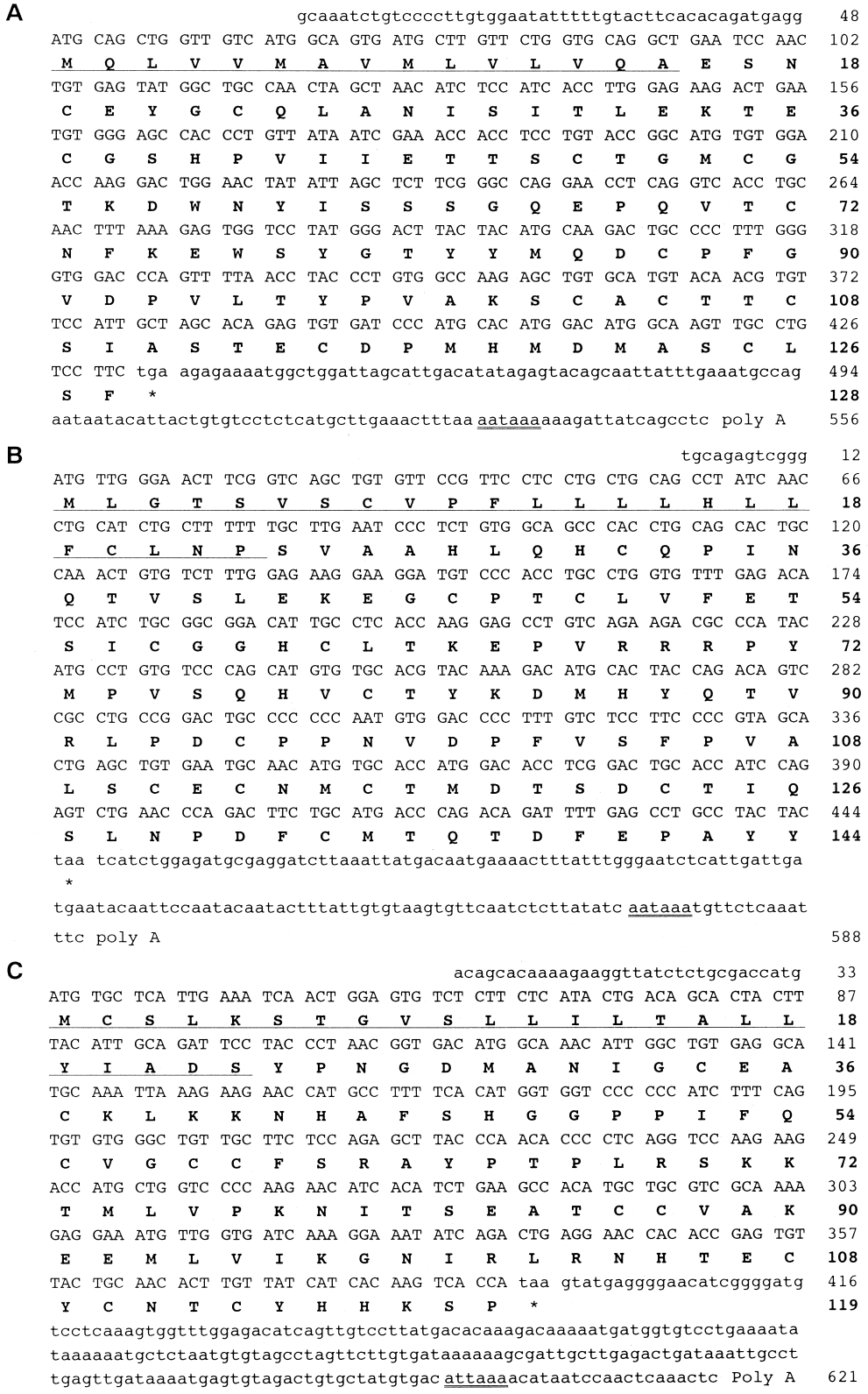

Fig. 2. Nucleotide sequence of the cDNAs encoding the FSH β (A), LH β (B) and GPH α (C) subunits of ayu (upper line) and the deduced amino acid sequences (lower line). The nucleotide and deduced amino acid residue number are shown on the right. The underlined region indicates the putative signal peptide. The doubly underlined region indicates the putative poly-A signal sequence.

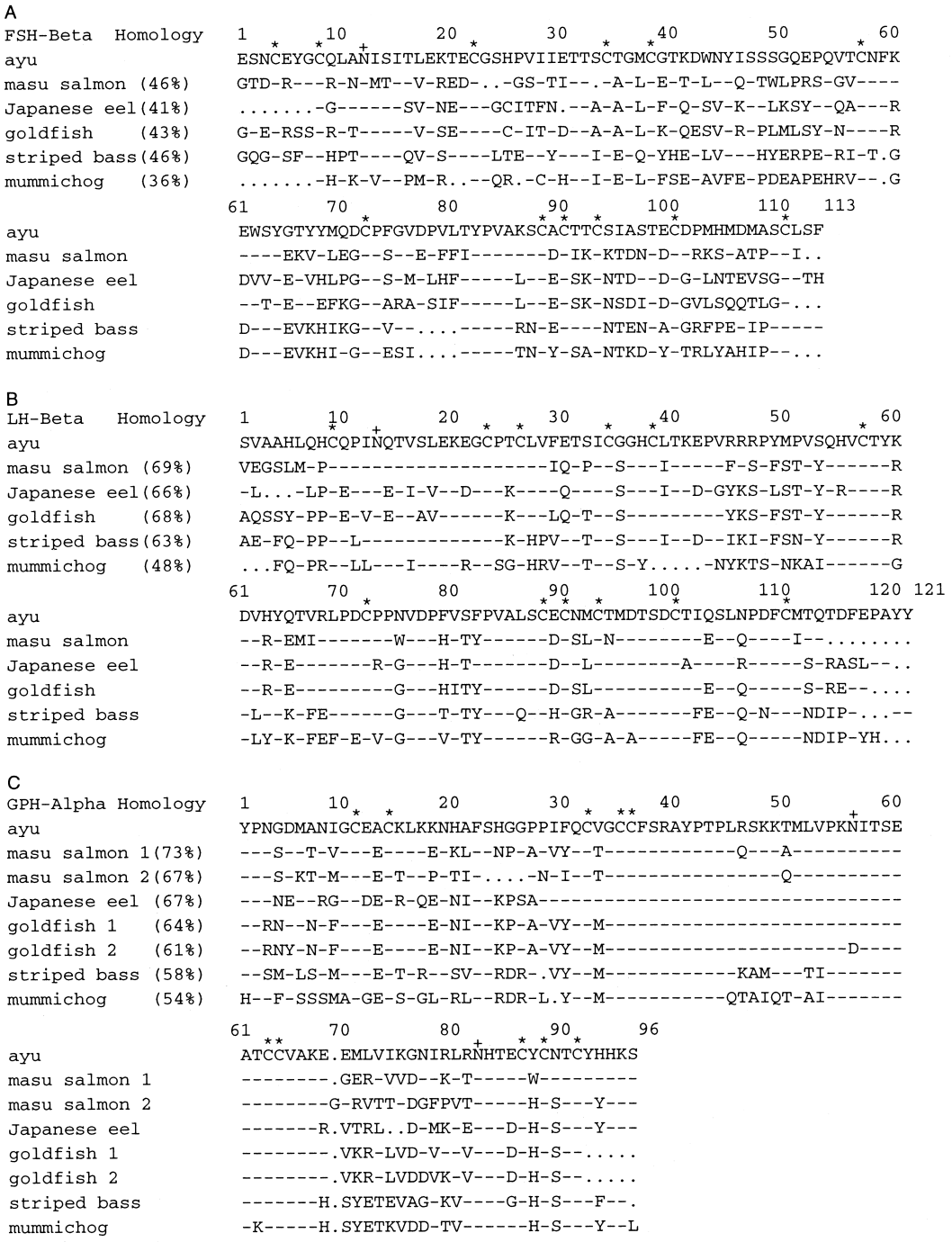

Fig. 3. Alignment of the amino acid sequence of the putative mature FSH β (A), LH β (B) and GPH α (C) subunits of ayu, *Plecoglossus altivelis*, with those of the masu salmon, *Oncorhynchus masou*, (Gen *et al.*, 1993; Kato *et al.*, 1993), Japanese eel, *Anguilla japonica*, (Nagae *et al.*, 1996; Yoshiura *et al.*, 1999), goldfish, *Carassius auratus*, (Kobayashi *et al.*, 1997; Yoshiura *et al.*, 1997), striped bass, *Morone saxatilis*, (Hassin *et al.*, 1995), mummichog, *Fundulus heteroclitus* (GenBank accession No. U12923; Lin *et al.*, 1992). The percentage shows homology with deduced amino acid sequence of ayu GtH mature subunits. Cysteine residues are indicated by an asterisk (*). Putative N-linked glycosylation sites are indicated by plus (+) signs. Dashes indicate amino acid residues that are identical to those of the ayu amino acid sequence. Gaps are marked by periods.

genes changed during gonadal development, the obtained images were standardized by the intensity of the ethidium bromide stained gel images of 18S rRNA using image-analysis software (NIH-image 1.61) (Fig. 5).

In the present study, the expression of FSH β mRNA in the pituitaries of females was not detected at an early immature stage (stage I: the early perinucleous oocyte) (Fig. 5-B), but then became detectable at a late immature stage (stage II). The intensity of the detected FSH β mRNA bands was highest during early vitellogenesis (stage III), and decreased

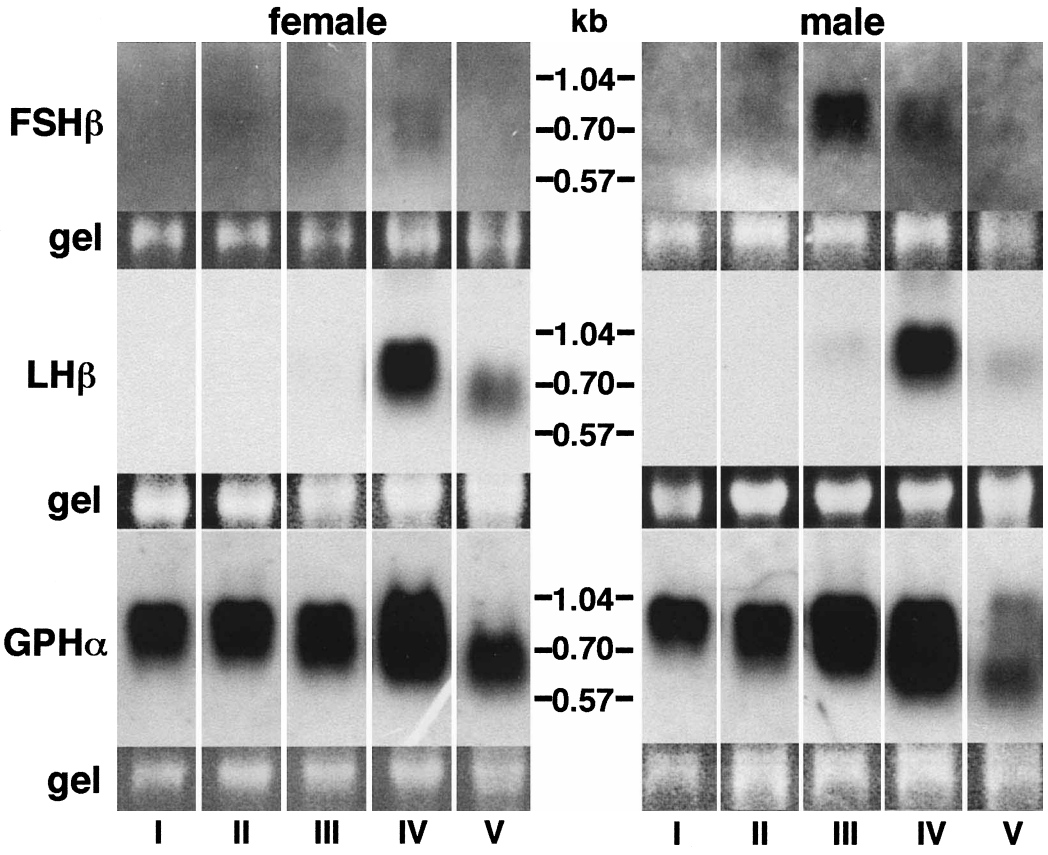

Fig. 4. Northern blot analysis of FSH β, LH β and GPH α mRNAs with ethidium bromide stained gel images of 18S rRNA from the pituitaries of ayu at the five different stages (I to V) of gonadal development shown in Table 1. Each lane contains approximately 5 μg of total RNA. Ayu total RNA was hybridized with DIG-labeled probes of each subunit. The size of each subunit mRNA is indicated between the two images.

during subsequent ovarian developmental stages (stages IV and V). Gomez *et al.* (1999) observed that FSH β mRNA levels as well as the circulating levels of FSH (GtH I) rose during early vitellogenesis and then declined with oocyte development in rainbow trout. Moreover, Prat *et al.* (1996) reported that FSH was associated with the early growth of gonads. These results agree with our finding of increased expression of FSH β subunit during early vitellogenesis. However, it is difficult to understand the function of FSH during early vitellogenesis of ayu from this FSH β gene expression data. Nevertheless, early rise of FSH β strongly suggest that the primary function of FSH in ayu is to initiate vitellogenesis and early growth of vitellogenic oocytes.

In male ayu, FSH β mRNA could be detected at the late immature stage (stage II: spermatogonia) just prior to meiosis. The relative levels of FSH β mRNA peaked at stage III (including spermatocyte) at which the gonadosomatic index (GSI) started to increase. Thereafter, the levels of FSH β mRNA decreased and remained low throughout late spermatogenesis (stages V). This pattern of gene expression of FSH β mRNA in male ayu is similar to that of the female. In contrast, the relative content of FSH β mRNA increased from the spermatogonia stage to the spermiation stage in rainbow trout (Gomez *et al.*, 1999). Moreover, FSH β mRNA levels in male red sea bream increased in association with an increase in GSI levels and gonadal development during sexual maturation (Gen *et al.*, 2000). These results suggest that FSH has an important role in spermatogenesis in fishes with different styles of testicular development. However, assessing the function of FSH in males is more difficult, because the relative levels of FSH β mRNA and circulating levels of FSH protein were much lower than in females. Our results strongly indicate that FSH has a role in the initiation of testicular growth.

Changes in the relative levels of LH β mRNA is shown in Fig. 5-C. A similar pattern of gene expression was observed regardless of sex. Expression of LH β mRNA was not detectable at an immature stage (stages I and II). The intensity of the detected mRNA rose dramatically at the vitellogeneic and spermatogenic stage (stage IV) and then decreased rapidly during late vitellogenesis and spermatogenesis just prior to final maturation, ovulation and spermiation (stage V). This pattern of changes in the expression of LH β was different from that found for other species. The increase of LH β subunit mRNA during gonadal development could be linked to development of the gametes (rainbow trout: Naito *et al.*, 1991; Gomez *et al.*, 1999; Japanese eel: Nagae *et al.*, 1996). In these fishes, the relative content of LH β mRNA always increased until late vitellogenesis and reached maximum lev-

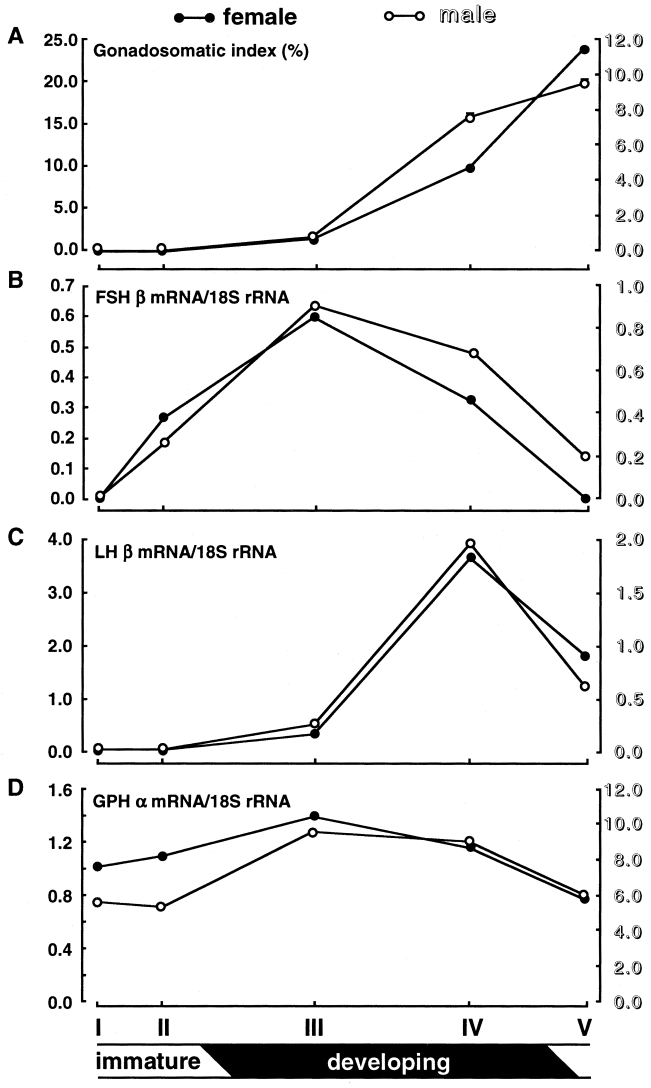

Fig. 5. Changes in gonadosomatic indexes (A), relative levels of FSH β (B), LH β (C) and GPH α (D) mRNA expression during gonadal development in both sexes of ayu. The sampling day and gonadal condition are shown in Table 1.

els in mature fish. In addition, Sohn *et al.*, (1999) reported that LH β mRNA levels in goldfish was highest in spawning season, corresponding to ovarian development. In contrast, LH β mRNA relative content decreased during late vitellogenesis in the ayu. In salmonids, serum concentrations and pituitary content of LH (GtH II) proteins were elevated at the time of ovulation (Suzuki *et al.*, 1988; Prat *et al.*, 1996; Gomez *et al.*, 1999). LH is highly potent in stimulating the production of 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DHP) which is known to be the maturation-inducing hormone (Suzuki *et al.*, 1988). 17α, 20β-DHP is the main maturation-inducing hormone also in ayu (Hirose *et al.*, 1985). Therefore, it is conceivable that LH β mRNA is expressed at the time of final maturation and ovulation in ayu. Unfortunately, samples from this stage are lacking in our present study.

The relative levels of LH β mRNA reached a peak at the optimum time for vitellogenesis and spermatogenesis, while the GSI rapidly increased in both sexes of ayu. Suzuki *et al.* (1988) investigated the effects of FSH and LH on estradiol-17 β production using mid-vitellogenic follicles of amago salmon. They demonstrated the high potential of LH for inducing estradiol-17 β production. Moreover, LH is more potent than FSH in stimulating estradiol-17 β production by vitellogenic oocytes in red sea bream (Tanaka *et al.*, 1995). These data suggest that LH has various effects on steroid synthesis in vitellogenesis. Thus, LH may play an important role in oocyte growth and vitellogenesis in ayu. LH may also be related to androgen synthesis and spermatogenesis, although the biological function of LH in males is not known. It is therefore necessary to investigate the biological function of LH in gametogenesis.

Changes in mRNA levels of GPH α in both sexes are shown in Fig. 5-D. A similar pattern of gene expression was observed regardless of sex. The relative content of mRNA tended to increase at stage III in both sexes, when FSH β mRNA increased sharply. In rainbow trout pituitary glands, GPH α mRNA was expressed in both GtH cells (Naito *et al.*, 1991). The expression pattern of GPH α on FSH cells is different from that on LH cells in the pituitary. Messenger RNA levels of GPH α may be correlated with the synthesis of FSH or LH in GtH cells. The relationship between the expression of GPH α and FSH β or LH β, however, does not explain the pattern of both mRNAs, because FSH, LH and TSH consist of a common GPH α-subunit and a hormone specific β-subunit.

In the present study, the cDNAs of FSH β, LH β and GPH α subunits were cloned from ayu pituitary. We examined the changes in the gene expression of these subunits during gonadal development and maturation in both sexes has been clarified by Northern blot analysis. However, the GtHs mRNA expression in the development of maturational competence and the initiation of final maturation induced by the transfer to a suitable environment for spawning is not clear. To obtain more detailed data on the initiation of final maturation, it is necessary to investigate the gene expression of FSH β, LH β and GPH α subunits during the transfer to a suitable spawning environment by Northern blot analysis and *in situ* hybridization of ayu pituitaries. We are now investigating the gene expression of these GtH subunits during the final maturational stages using these cDNAs as tools.

ACKNOWLEDGEMENTS

We thank the staff of the National Research Institute of Fisheries Science, Ueda station for supplying fish.

REFERENCES

Chang YS, Huang CJ, Huang FL, Lo TB (1988) Primary structures of carp gonadotropin subunits deduced from cDNA nucleotide sequences. Int J Peptide Protein Res 32: 556–564

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156–159

Gen K, Maruyama O, Kato T, Tomizawa K, Wakabayashi K, Kato Y

(1993) Molecular cloning of cDNAs encoding two types of gonadotrophin α subunit from the masu salmon, *Oncorhynchus masou*: construction of specific oligonucleotides for the α1 and α2 subunits. J Mol Endocrinol 11: 265–273

Gen K, Okuzawa K, Senthilkumaran B, Tanaka H, Moriyama S, Kagawa H (2000) Unique expression of gonadotropin-I and -II subunit genes in male and female red seabream (*Pagrus major*) during sexual maturation. Biol Reprod 63: 308–319

Gomez JM, Weil C, Ollitrault M, Le Bail PY, Breton B, Le Gac F (1999) Growth hormone (GH) and gonadotropin subunit gene expression and pituitary and plasma changes during spermatogenesis and oogenesis in rainbow trout (*Oncorhynchus mykiss*). Gen Comp Endocrinol 113: 413–428

Hassin S, Elizur A, Zohar Y (1995) Molecular cloning and sequence analysis of striped bass (*Morone saxatilis*) gonadotrophin-I and -II subunits. J Mol Endocrinol 15: 23–35

Hirose K, Nagahama Y, Adachi S, Wakabayashi K (1983) Changes in serum concentrations of gonadotropin, 17α-hydroxy-progesterone and 17α, 20β-dihydroxy-4-pregnen-3-one during synthetic LH-RH-induced final oocyte maturation and ovulation in the ayu *Plecoglossus altivelis*. Bull Japan Soc Sci Fish 49: 1165–1169

Hirose K, Adachi S, Nagahama Y (1985) Changes in plasma steroid hormone levels during sexual maturation in the ayu *Plecoglossus altivelis*. Bull Japan Soc Sci Fish 51: 399–403

Huang CJ, Huang FL, Chang GD, Chang YS, Lo CF, Fraser MJ, Lo TB (1991) Expression of two forms of carp gonadotropin α subunit in insect cells by recombinant baculovirus. Proc Natl Acad Sci USA 88: 7486–7490

Ito F, Yamaguchi M, Soyano K, Yamauchi K (1992) Induction of ovulation by male sex pheromone in the ayu, *Plecoglossus altivelis*. In "Abstracts of 2nd International Symposium on Fish Endocrinology" Ed by B Berton, A Fostier, O Kah, PY Le Bail, M Loir, P Prunet, C Weil, Saint-Malo, France, p 83

Kato Y, Gen K, Maruyama O, Tomizawa K, Kato T (1993) Molecular cloning of cDNAs encoding two gonadotrophin β subunits (GTH-Iβ and -IIβ) from the masu salmon, *Oncorhynchus masou*: rapid divergence of the GTH-Iβ gene. J Mol Endocrinol 11: 275–282

Kitahara N, Nishizawa T, Gatanaga T, Okazaki H, Andoh T, Soma G (1988) Primary structure of two mRNAs encoding putative salmon α-subunits of pituitary glycoprotein hormone. Comp Biochem Physiol 91B: 551–556

Kobayashi M, Kato Y, Yoshiura Y, Aida K. (1997) Molecular cloning of cDNA encoding two types of pituitary gonadotropin α subunit from the goldfish, *Carassius auratus*. Gen Comp Endocrinol 105: 372–378

Lin YWP, Rupnow BA, Price DA, Greenberg RM, Wallace RA (1992) *Fundulus heteroclitus* gonadotropins. 3. Cloning and sequencing of gonadotropic hormone (GTH) I and II β-subunits using the polymerase chain reaction. Mol Cell Endocrinol 85: 127–139

Nagae M, Todo T, Gen K, Kato Y, Young G, Adachi S, Yamauchi K (1996) Molecular cloning of the cDNAs encoding pituitary glycoprotein hormone α- and gonadotropin IIβ-subunits of the Japanese eel, *Anguilla japonica*, and increase in their mRNAs during ovarian development induced by injection of chum salmon pituitary homogenate. J Mol Endocrinol 16: 171–181

Nagahama Y (1987) Gonadotropin action on gametogenesis and steroidogenesis in teleost gonads. Zool Sci 4: 209-222

Naito N, Hyodo S, Okumoto N, Urano A, Nakai Y (1991) Differential production and regulation of gonadotropins (GTH I and GTH II) in the pituitary gland of rainbow trout, *Oncorhynchus mykiss*, during ovarian development. Cell Tissue Res 266: 457–467

Pierce JG, Parsons TF (1981) Glycoprotein hormones: structure and function. Ann Rev Biochem 50: 465–495

Prat F, Sumpter JP, Tyler CR (1996) 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

Sambrook J, Fritsch EF, Manitatis T (1989) "Molecular Cloning: A Laboratory Manual." 2nd ed. Cold spring Harbor Laboratory Press, New York

Sekine S, Saito A, Itoh H, Kawauchi H, Itoh S (1989) Molecular cloning and sequence analysis of chum salmon gonadotropin cDNAs. Proc Natl Acad Sci USA 86: 8645–8649

Sohn YC, Yoshiura Y, Kobayashi M, Aida K (1999) Seasonal changes in mRNA levels of gonadotropin and thyrotropin subunits in the goldfish, *Carassius auratus*. Gen Comp Endocrinol 113: 436–444

Soyano K, Ito F, Yamaguchi M, Yoshida D, Yoshikuni M, Nagahama Y, Adachi S, Yamauchi K (1996) Changes in the sensitivity of the oocyte to maturation-inducing hormone after the transfer to the suitable environment for spawning of the ayu, *Plecoglossus altivelis*. In "Proceedings of the Third Congress of the Asia & Oceania Society for Comparative Endocrinology" Ed by J Joss, Sydney, Australia, pp 190–191

Suzuki K, Nagahama Y, Kawauchi H (1988) Steroidogenic activities of two distinct salmon gonadotropins. Gen Comp Endocrinol 71: 452–458

Tanaka H, Kagawa H, Hirose K (1995) Steroidogenic activities of two distinct gonadotropins in red seabream, *Pagrus major*. In "Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish" Ed by FW Goetz, P Thomas, Austin, Texas, pp 10–12

Xiong F, Suzuki K, Hew CL (1994) Control of teleost gonadotropin gene expression. In "Fish Physiology Vol. XIII" Ed by NM Sherwood, CL Hew, Academic Press, San Diego, pp 135–158

Yoshiura Y, Kobayashi M, Kato Y, Aida K (1997) Molecular cloning of the cDNAs encoding two gonadotropin β subunits (GTH-Iβ and -IIβ) from the goldfish, *Carassius auratus*. Gen Comp Endocrinol 105: 379–389

Yoshiura Y, Suetake H, Aida K (1999) Duality of gonadotropin in a primitive teleost, Japanese eel (*Anguilla japonica*). Gen Comp Endocrinol 114: 121–131

(Received March 2, 2001 / Accepted June 21, 2001)