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βFSH, βLH and Growth Hormone Gene Expression in Blue Gourami (*Trichogaster trichopterus*, Pallas 1770) During Spermatogenesis and Male Sexual Behavior

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ABSTRACT—The relationship between gonadal development (histological evidence for spermiogenesis and/or spermatogenesis), sexual behavior (nest-building) and mRNA levels of gonadotropins (βFSH and βLH) and growth hormone (GH) in the male pituitary was investigated. Amplification of βFSH cDNA showed a significantly higher mRNA level in mature males (whether sexually active or not) than in juveniles. However, following PCR amplification of βLH cDNA, a significantly higher mRNA level was found in the sexually active group compared to the sexually inactive group. These results suggest that FSH may participate in spermatogenesis, whereas LH is more involved in spermiogenesis. The GH mRNA level increased slightly during the maturation process but no significant differences were found between the groups studied.

Key words: teleost, RACE-PCR, cloning, gonadotropin, pituitary

INTRODUCTION

Spermatogenesis in teleosts, as in other vertebrates, involves a complex interaction of various hormones along the brain-pituitary-gonad axis (Degani *et al*., 1998; Lee *et al*., 1998). One of these hormones is gonadotropin (GtH), a glycoprotein consisting of two subunits, α and β, which are non-covalently bound. There are two distinct GtHs in the pituitary gland of teleosts: FSH (or GtH-I) and LH (or GtH-II). These proteins share a common α subunit but differ in their β subunits, which confer the immunological and biological specificity to each hormone.

The participation of FSH and LH in the female teleost reproductive cycle has been described thoroughly (Nagahama *et al*., 1994). However, less information has been published on the effects of FSH and LH on gametogenesis in the male. Lee *et al*. (1998) described the relationship between steroids and GtH during spermatogenesis. Apparently, hormonal control is similar to that found in females, with FSH being responsible for the first stages of spermatogenesis and LH for the final stages. In the salmonid species, spermatogenesis is regulated by pituitary FSH and LH. FSH levels rise in the plasma during the early stage of spermatogenesis (spermiogenesis) and decline during spermiation, whereas the FSH level is very low during spermiogenesis and increases during spermiation (Swanson, 1991; Prat *et al*., 1996). The salmonids exhibit a difference in sensitivity to FSH and LH in the testis; the sensitivity to LH increases towards the final stages of spermatogenesis (Planas and Swanson, 1995; Swanson *et al*., 1999).

The synthesis and secretion of gonadotropins are regulated by positive and negative factors that act at the brain, pituitary and gonad levels. Most studies describing changes in the levels of gonadotropins during the teleost reproductive cycle are related to the secretion of gonadotropin, but not to its synthesis. Molecular biology techniques have made it possible to examine changes in the mRNA levels of the gonadotropins during the gonadal cycle. Changes in the expression of FSH and LH during the gonadal cycle have been described recently (Jackson *et al*., 1999; Gomez *et al*., 1999; Weil *et al*., 1995; Meiri *et al*., 1995; Sohn *et al*., 1999).

Pituitary growth hormone (GH), a single-chain polypeptide, plays an essential role in the regulation of growth and development by promoting the division, differentiation and enlargement of cells (Corin *et al*., 1990). The GH gene has been cloned from several teleost species (Venkatesh and Brenner, 1997). Only one type of GH gene has been cloned

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in most teleosts, but two GH genes have been found in salmonids and tilapia (see reviews in Venkatesh and Brenner, 1997; Melamed *et al*., 1998).

The biological activities of GH have been examined in many fish species (Sakata *et al*., 1993). The metabolic effects of GH include increased protein synthesis, increased use of fat for energy production, and decreased glucose utilization throughout the body (Copeland and Nair, 1994; Salmon *et al*., 1991). GH in fish also influences osmoregulation (Sakamoto and Hirano, 1993) and reproduction (Kraak *et al*., 1990; Le Gac *et al*., 1992).

Little information has been published on the GH level in the plasma of teleosts during growth and maturation, but accumulating data strongly point to the fact that GH is involved in the reproductive cycle of fish. Bjornsson *et al*. (1995) found that the GH level is higher in sexually mature Atlantic salmon (*Salmo salar*) than in immature fish. Le Gac *et al*. (1992) observed an increase in the GH level in male rainbow trout (*Salmo gairdneri*) at the beginning of sperm production, concomitant with an increase in the level of 17α, 20β-dihydroxy-progesterone (17, 20-P). Degani *et al*. (1996, 1998) found that the increase of GH in the plasma of both male and female carp (*Cyprinus carpio*) correlates to the gonadosomatic index.

Blue gourami (*Trichogaster trichopterus*) belongs to the Anabantidae family, which contains 16 genera and about 50 species distributed throughout most of Southern Asia, India and Central Africa. This family belongs to the Labyrinthici suborder, which is characterized by the presence of an air-filled breathing cavity (the labyrinth). The blue gourami shows a very complex social and spawning behavior, and has therefore become a common subject for ethological studies (Hodges and Behre, 1953; Cheal and Davis, 1974; McKinnon and Liley, 1986). The onset of the reproductive period in the male is marked by the development of nuptial coloration, initiation of nest-building behavior and increasing aggressiveness. *T. trichopterus* utilizes a floating bubble nest for egg deposition. This nest is composed of air bubbles enveloped in oral mucus that are released at the surface where they tend to stick together. Cheal and Davis (1974) found that the presence of a nest is not a prerequisite for spawning; in many cases, the female was able to spawn without a nest. However, in many cases when spawning took place without a nest, the eggs were found dispersed over the water surface and many did not develop (unpublished data).

Recently, cDNA sequences of blue gourami βFSH and βLH have been cloned and the mRNA of these hormones quantified in the female during the gonadal cycle (Jackson *et al*., 1999). Mañanos *et al*. (1997) measured the LH levels in the plasma and the pituitary, as well as the steroidal testosterone (T) and progesterone (P) in the plasma and testis in male blue gouramis during sexual behavior.

The purpose of the present study was to examine βFSH, βLH and GH gene expression during the sexual behavior (nest-building) of male blue gourami.

MATERIALS AND METHODS

Fish

Male blue gouramis, bred and maintained at MIGAL Laboratories in northern Israel, were used in this study. The fish were grown in containers measuring 2×2×0.5 m, at a temperature of 27°C, and under a light regime of 12L 12D. The fish were fed an artificial diet (45% protein, 7% fat) supplemented by live food (*Artemia salina*). Males and females were maintained separately in groups of about 20 each in containers of 1 m^3. After two weeks, fish were paired by transferring a male into the presence of a mature female in a small aquarium (40×20×20 cm) containing *Elodea*. In most instances, the male started to build the nest on the plant within a few hr.

After weighing and measuring each specimen, testes were taken for histological examination in order to determine the stage of gonadal development, and freshly excised pituitaries were processed immediately for RNA extraction. Donors of pituitary and testes were: 1) mature males that had started to build a nest after coupling (considered as reproductive or sexually active); 2) mature males that were kept in a group without females, hence did not exhibit nest-building behavior (considered as non-reproductive or non-sexually active); and 3) immature males (juveniles).

Histological examination

The pieces of testes were fixed in Bouin and processed until they were embedded in paraffin. Histological sections of 2–5 μm were obtained by using a Reichert-Jung (Austria) microtome. The trichrome of Mallory was used to stain the sections (Humason, 1979).

Measurement of βFSH-I, βLH-II and GH mRNA levels by RT-PCR

The βFSH, βLH and GH mRNA levels were measured by reverse transcriptase (RT) PCR using 18S rRNA as an internal standard (Jackson *et al*., 1999). This method was employed because the very small size of the blue gourami pituitary gland precluded the use of Northern blotting, which requires relatively large amounts of RNA (Kawasaki, 1990). 18S rRNA was used as an internal standard for the measurements since it is an abundant RNA and its expression is considered stable (Amoureux *et al*., 1997). The 18S rRNA levels were observed to have a greater uniformity than those of other commonly used internal standards, such as β actin (Ambion Technical Bulletin No. 151, 1997). The 18S level also proved to be stable for the blue gourami and did not vary during different reproductive stages (Jackson *et al*., 1999).

Total RNA was extracted individually from each pituitary by means of the Rneasy® total RNA kit (QIAGEN, Hilden, GR). In most cases, 40–70 ng/μl of total RNA were obtained from each pituitary.

In order to measure βFSH, βLH and GH mRNA levels, the cDNA pool for the 3' end was synthesized using an AMV RT (Promega), as described by Frohman (1990). The primer for the synthesis of the 3' cDNA end consisted of an oligo dT (17 bases) linked to a unique 17 mer adapter and a mixture of random decamers. At least five specimens from each of the following stages of the reproductive cycle were used in this study: spermatogenesis (immature males), males maintained together to inhibit nest-building (non-reproductive males) and mature nest-building males (mature reproductive males).

Amplification of cDNA of βFSH, βLH and 18S rRNA

Each amplification of βFSH, βLH and GH cDNA was coupled to an amplification of the 18S rRNA internal standard. All amplifications were conducted in separate tubes since many attempts to use multiplex PCR resulted in considerable interference between the different reactions. The concentration of each primer pair and the number of cycles were calibrated carefully to allow for amplifications that were linearly dependent on the initial concentration of the target

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cDNA. βFSH, βLH and GH cDNAs were amplified with gene-specific primers (see Table 1) at a concentration of 6.25 pmol/reaction. 18S rRNA cDNA was amplified with specific primers obtained from Ambion as part of its Quantum RNA kit at a concentration of 5 pmol/reaction. The reagents, except for the cDNA and the primers, were prepared as a batch for each set of reactions to minimize possible variations that might be independent of the initial cDNA concentrations. The PCR amplifications were carried out in 50 μl reactions using 2.5 units of Taq polymerase (Promega), a suitable reaction

Table 1. Sequence of primers for cDNA synthesis and PCR

Primer Name	cDNA Target	Use	Nucleotide Sequence
P1	βFSH	Complete cDNA cloning	5'GTCTGTACAGATGTTTAGAGAG
P2	βFSH	Complete cDNA cloning	5'AACGTGGGATGAATGATGAGTG
P3	βLH	Complete cDNA cloning	5'CTGGCTAACCTGCCGCTGACAC
P4	βLH	Complete cDNA cloning	5'TTGCTTTTGGTTTGCTGTGCAG
P5	GH	Complete cDNA cloning	5'AGAAGTGAACCTGAACCTGTATC
P6	GH	Complete cDNA cloning	5'CATTGTGCTGGAACTGG
dT_{17}-adapter	Universal	cDNA synthesis	5' GACTCGAGTCGACATCGA$(T)_{17}$
Adapter	Universal	RACE PCR	5'GACTCGAGTCGACATCG

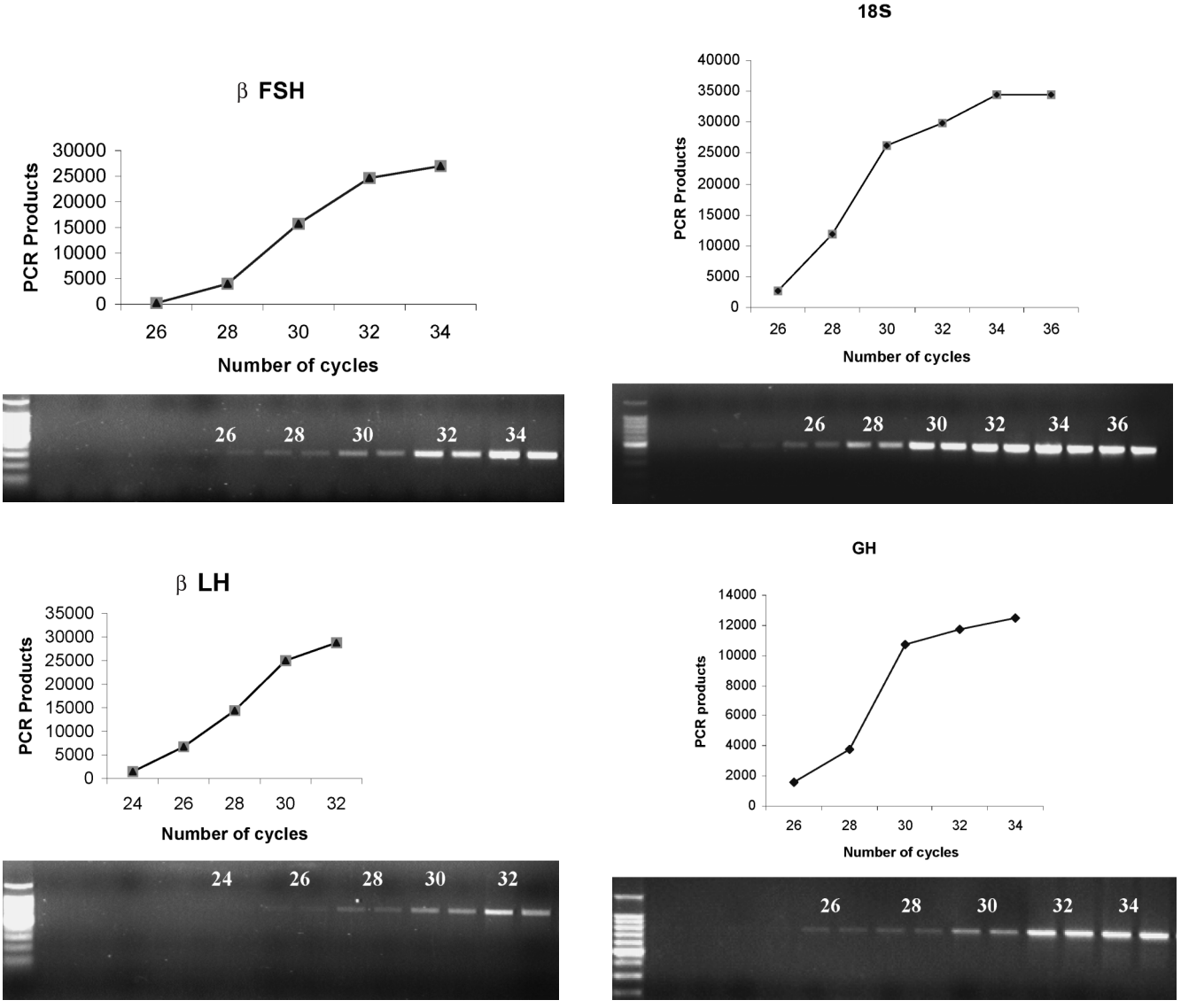


Fig. 1. Determination of the exponential range of amplification for βLH, βFSH, GH and 18S. The amount of PCR products is expressed in arbitrary units of intensity as defined by the densitometry software (PCBAS-2).

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buffer (Promega), 1.5 mM $MgC1_2$, 0.2 mM of each nucleotide, specific primers, 5 µl of cDNA and 20 µl mineral oil.

Determination of cycling parameters

It is important to select the appropriate number of cycles so that the amplification products are clearly visible on an agarose gel, but also that the amplification is in the exponential range and has yet not reached a plateau. The optimal number of cycles was checked for each primer pair using the most concentrated cDNA. Ten reaction tubes for each primer pair were prepared as described above. Following 26 complete cycles, two reaction tubes of each primer pair were removed from the termocycler (every two cycles, from 26 to 34). The PCR products were electrophoresed in agarose gel and quantified as described below (Fig. 1). For βFSH, βLH, GH and 18S, only 30 cycles produced enough PCR products for the semi-quantitative analysis while maintaining linear dependence on the cDNA concentration. Thus, cycling parameters were: 3 min denaturation at 94°C followed by 30 cycles, each consisting of 1 min denaturation at 94°C, 1 min annealing at 54°C and 1 min extension at 72°C.

Quantification of PCR products

The PCR products (half of the PCR reaction) were electrophoresed on 2% agarose gel at 120 mA for 45 min. The DNA was then stained by ethidium bromide (0.03 mg in 100ml distilled water), and imaged with the Eagle Eye II Image analysis system (STRATAGENE). The gels were imaged at three different exposure times to prevent a possible saturation of the images. The resulting images were analyzed with the aid of the PCBAS-2 densitometry software (Raytest, GR).

The variations in the expression of the βFSH, βLH and GH genes at the mRNA level were evaluated by first measuring the amount of specific PCR products and then dividing it by the amount of the amplified internal standard. This normalization step was intended to eliminate the effect of random changes in the experimental setup.

Statistical analysis

The differences between the mRNA levels of the various hormones were analyzed by ANOVA, and the difference of means between mRNA levels by the Student's t-test, with the level of significance in different groups set at $p<0.05$ (Parker, 1986).

RESULTS

Fig. 2 illustrates the gonadal morphology of juvenile males, mature non-reproductive males and mature reproductive males (after spawning). In all three groups, the same cellular stages were found but in different proportions (Table 2). No morphological differences could be observed between the testis of males from the two latter groups. However, there was a decrease in the number of spermatozoa in the center of the lobule of mature reproductive males pro-

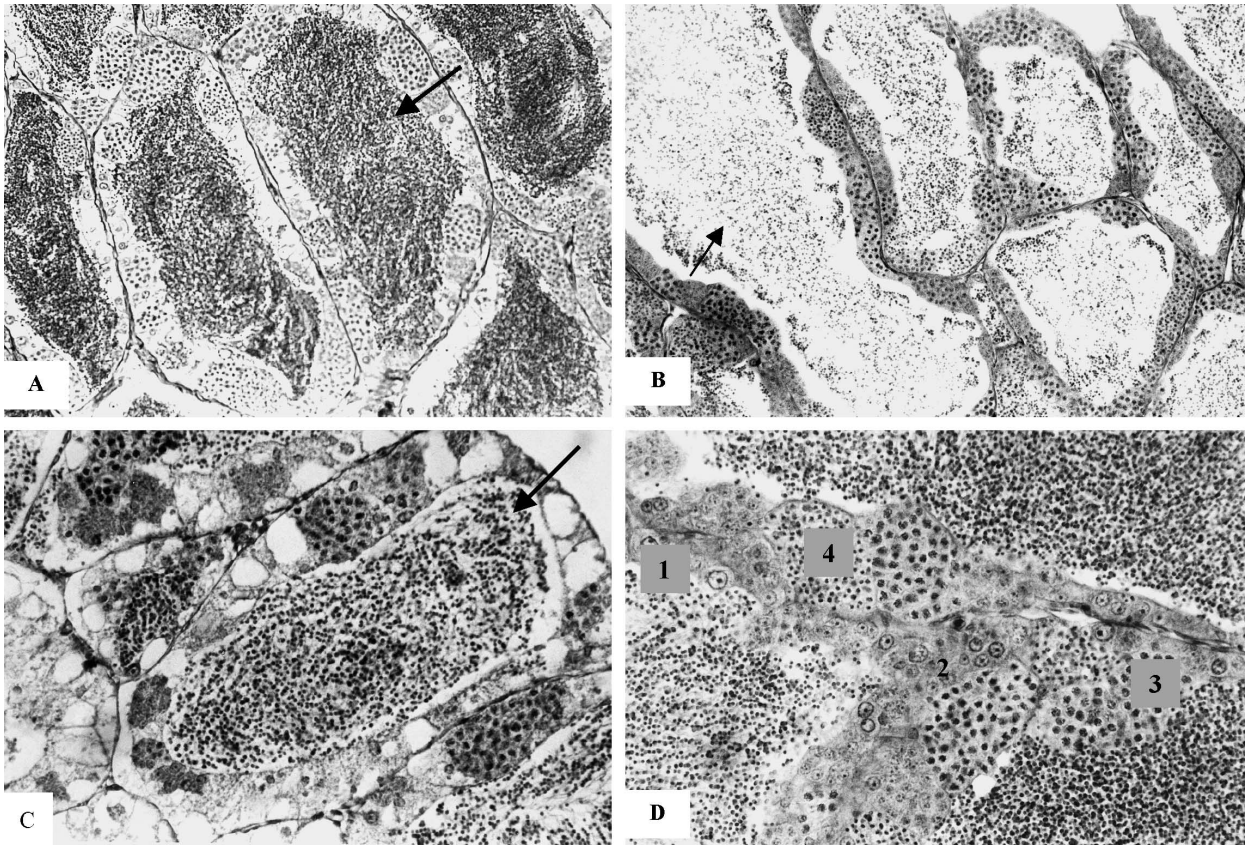


Fig. 2. Histological section showing different stages of gonadal development in the blue gourami male *T. trichopterus*: (A) Mature non-reproductive fish (note the concentration of spermatozoa in the middle of the lobule; see arrow); (B). Mature reproductive fish during sexual behavior (note the decrease in the quantity of the spermatozoa in the center of the lobule of reproductive fish when compared to non-reproductive fish; see arrow); (C). Young males at the beginning of spermiogenesis (note the lower concentration of spermatozoa when compared to fully developed fish); and (D) Early cell stages in *T. Trichopterus*: 1 = spermatogonia, 2 = primary spermatocyte, 3 = secondary spermatocyte, 4 = spermatid H.E.

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Table 2. Germ cell constitution of gourami testis at different developmental stages

Stage	Juveniles	Mature Reproductive (Nest-building)	Mature Non-reproductive (Non-nest-building)
Spermatogonia	+++	+	+
Spaermatocyte I	+	+	+
Spermatocyte II	++	++	++
Spermatid	+	++	++
Spaermatozoa	+ /++	+++	+++

cessed after spawning. The testes of mature reproductive and mature non-reproductive fish were similar; those of juvenile fish contained relatively few spermatozoa (Fig. 2).

Variations in βFSH and βLH and GH mRNA levels during the reproductive cycle

Amplification of βFSH cDNA produced a 306 bp product, that of βLH cDNA a 284 bp product, and that of the internal standard (the cDNA of 18S rRNA) a 430 bp product. The identity of each PCR product was confirmed by DNA sequencing.

The variations of mRNA levels of βFSH are shown in Fig. 3. There was no significant difference (t-test; $p<0.05$) in the βFSH between mature fish, whether non-nest-builders (mature non-reproductive) or nest-builders (mature reproductive). Both levels were significantly higher than in juveniles.

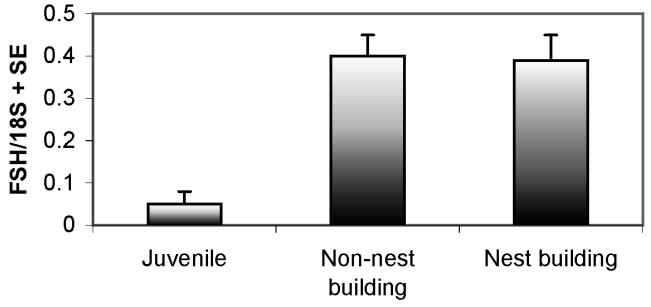


Fig. 3. Variations in βFSH transcript levels during the gonadal cycle. Each histogram represents an average of five independent measurements (mean±SE). The amount of PCR products is expressed in arbitrary units of intensity as defined using the densitometry software (PCBAS-2).

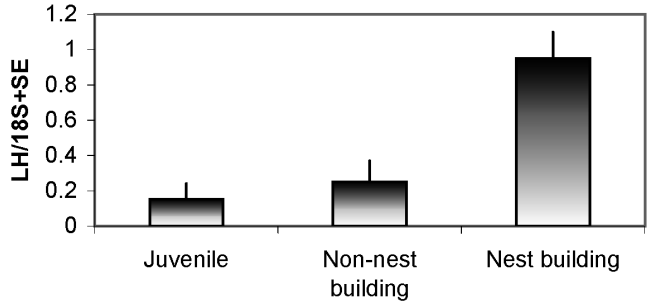


Fig. 4. Variations in βLH transcript levels during the gonadal cycle. Each histogram represents an average of five independent measurements (mean±SE).

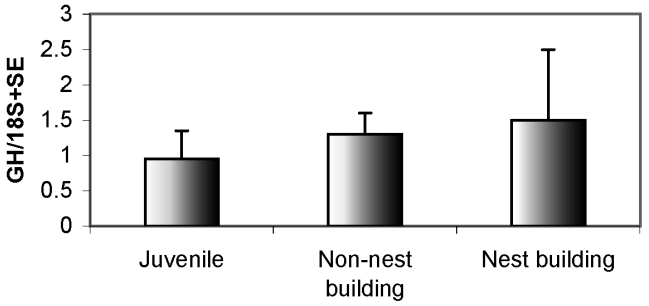


Fig. 5. Variations in GH transcript levels during the gonadal cycle. Each histogram represents an average of five independent measurements (mean±SE).

The variations of mRNA levels of βLH are shown in Fig. 4. Amplifications of cDNA from juveniles and non-reproductive mature fish were low, showing no significant difference (t-test; $p>0.05$) between them.βLH expression rose significantly (ANOVA; $p<0.05$) at the nest-building stage , as manifested by the increased amount of PCR products.

The product generated by the amplification of GH was 733 bp long. The identity of the PCR product was confirmed by DNA sequencing. There were no significant differences in the GH mRNA level (ANOVA or t-test; $p>0.05$) between juveniles, mature non-reproductive and mature reproductive specimens (Fig. 5).

DISCUSSION

In the present study, differences in the biosynthesis of βFSH, βLH and GH during gonadal development and the male sexual behavior of blue gourami were determined by quantifying their respective mRNAs. The results support the hypothesis that FSH participates in spermatogenesis. FSH transcripts were significantly lower in immature fish in comparison to mature fish. However, spermatozoa could be found in the center of the lobules of immature fish.

In males sampled immediately after spawning, there was a remarkable decrease in the quantity of spermatozoa in the center of the lobule, as observed in histological preparations. It is noteworthy that the blue gourami is a multi-spawning species, and after successful spawning, the same male is able to fertilize another female 24 hr later. Consequently, spermatogenesis occurs continuously in mature fish during sexual behavior.

In females blue gourami, the gonadal development follows an asynchronic pattern, with oocytes found at different developmental stages (Jackson *et al*., 1994). In concerning βFSH expression similar results were observed in females. βFSH transcripts were first detected in immature females during low vitellogenesis (i.e., when the percentage of oocytes found at vitellogenesis was low) and an accentuated increase occurred in mature non-reproductive females

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found at high vitellogenesis (i.e., when the majority of the oocytes were at vitellogenesis). The βFSH mRNA level also remained high in post-vitellogenic females during the final stage of oocyte maturation and spawning. These mature reproductive females able to spawn again after a 24–48 hr break (Jackson *et al*., 1999).

The βLH mRNA level was low in young and mature non-reproductive males, increasing rapidly only in reproductive males (nest-builders) (Fig. 4). The βLH level was also significantly higher in the pituitaries and plasma of reproductive males than in non-reproductive males, as was found by Mañanos *et al*. (1997). Thus, a temporal correlation exists between transcription (βLH mRNA in the pituitary), translation (LH levels in the pituitary) and release (LH levels in the plasma) with respect to this hormone. LH is probably related to the final stages of spermatogenesis/spermiation and triggers the final maturation (acquiring of motility) and release of sperm, probably via steroids (Nagahama *et al*., 1994).

The expression of βLH in the male gourami differs from that found in females (Jackson *et al*., 1999). The expression of βLH gene in females begins during low vitellogenesis (immature or maturating females), with a significant increase in non-reproductive mature females (high vitellogenetic females). It also remains high in mature reproductive females (females found at the final oocyte maturation stage, FOM). Thus, a significant increase of βLH in females is already detected in non-reproductive females (contrary to males, where the increase in βLH level occurs only in reproductive specimens). In females, LH is stored in the pituitary and is released to the plasma only in the presence of the male (Degani *et al*., 1997). The situation in males is different. The delay between LH production and release is not required; LH is probably produced and released by mature reproductive males during nest-building.

The results of this study show that the GH mRNA level remains high, not only during the male's growth (juvenile fish) but also during spermatogenesis and sexual behavior (Fig. 5). Therefore, the GH mRNA level in blue gourami males (as found in the present study) and females (Degani *et al*., 2002) remains high during final stages of the reproductive cycle and during sexual behavior. An increase in GH levels during the final stages of gonadal development was also detected in male and female rainbow trout (Gomez *et al*., 1999). Histological changes in somatotrophs during ovulation and spawning indicate that these cells become markedly more active (Melamed, 1998).

Nest-building males exhibit significantly higher levels of plasma steroids (testosterone, progesterone) than do non-nest-builders (Mañanos *et al*., 1997). This increase in steroid levels could have a positive effect on GH production. The interaction between GH and steroids has been studied recently in teleosts. The injection of recombinant salmon GH in killifish stimulated the growth of the gonads and raised the level of sex steroids circulating in the blood (Singh *et al*., 1988). Injected testosterone can stimulate pituitary mRNA (Huggard *et al*., 1996) or cause an increase in serum concentration of GH in rainbow trout (Holloway and Leatherland, 1997). Le Gac *et al*. (1992) studied the action of GH in the testis of trout, and suggested that at the end of reproductive cycle high GH levels are associated with 17α-hydroxy, 20β-dihydroprogesterone, a progestin necessary for efficient spawning in this fish. Together, these results demonstrate the participation of GH in the reproductive cycle, but more information is needed since there is a difference of action according to species. The specific action of steroids and GnRH on GH gene expression is currently being studied in this laboratory.

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