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cDNA CLONING AND mRNA EXPRESSION OF GROWTH HORMONE IN BELONTIIDAE (ANABANTOIDEI SUBORDER)

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Abstract

Complete cDNA encoding for the growth hormone (GH) of the Belontiidae fish family (Anabantoidei suborder) was cloned by RACE PCR using several sets of degenerate oligonucleotides. GH cDNA of the pearl gourami (*Trichogaster leeri*; pgGH), cloned from the pituitary, included the 5' and 3' noncoding sequences of 44 bp and 181 bp, respectively, and consisted of 840 bp that encoded for a prehormone of 204 amino acid (aa) residues. GH cDNA of the blue gourami (*T. trichopterus*; bgGH) and its deduced aa sequence had the same lengths as those of the pgGH, with nucleotide and aa identities of 97% and 99%, respectively. GH cDNA cloned from the dwarf gourami (*Colisa lalia*; dgGH) differed from both pgGH and bgGH. The identity of the dgGH cDNA nucleotides was 88%, compared to pgGH and bgGH. However, the identity of the deduced dgGH aa sequence was 97% when compared to bgGH and 96% when compared to pgGH. Nucleotides of GH cDNA of the fighting fish (*Betta splendens*; ffGH) had an identity of 82% to those of pgGH and bgGH, and 81% to the dwarf gourami. Higher identity was found among the aa sequences than among the nucleotide sequences. The identity of the cloned aa ffGH compared to bgGH, pgGH, and dgGH was 93%, 92%, and 91%, respectively.

Higher levels of GH mRNA were found in females in immature, vitellogenic, and mature stages than in males in various stages of gonadal development. No significant differences in the GH transcription level were found between immature and mature females and males. However, the mRNA level decreased significantly during sexual behavior in males. GH sequence and expression may be used as systematic markers for Belontiidae fish and possibly other fish.

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Introduction

Information on growth hormone (GH) in species belonging to the Anabantoidei suborder is very limited. The 16 known genera contain about 80 species, distributed throughout most of southern Asia, India, and central Africa (Forselius, 1957; Vierke, 1988). However, the systematic characteristics of the Anabantoidei suborder of fish are not in agreement and many synonymies are used. According to Vierke (1988), taxonomists classify the labyrinth fishes into four families: Anabantidae (genera *Sandelia, Ctenopoma, Anabans*), Belontiidae (genera *Trichopsis, Trichogaster, Sphaerichthys, Pseudosphromeus, Parosphromeus, Malpulutta, Hlostoma, Ctenops, Colisa, Betta, Belontia*), Osphromenide (genus *Osphronemus*), and Helostomatidae (genus *Helostoma*).

The structure and evolution of fish have been examined in terms of DNA composition (for review see Dores et al., 1996; Degani, 2004), microsatellites (DeWoody et al., 2000), and guppy (*Poecilia reticulata*) variation. GH is a pituitary hormone that regulates the development and somatic growth in vertebrates (Perez-Sanchez, 2000). The sequence of GH markers has become the marker of choice in many fish species for genetic studies, since it is an important hormone. GH has been studied extensively, and the GH cDNA nucleotide sequences of many teleosts are available (Company et al., 2000; Anathy et al., 2001; Goldberg et al., 2004).

The GH sequence is only one of the molecular parameters that may support phylogenetic analysis within Anabantoidei and such information, together with other parameters, will improve our knowledge of Anabantoidei.

The study of GH expression during the reproductive cycle could contribute to understanding the interactions between somatotropic and gonadotropic axes at the pituitary level and elucidating the effects of GH on fish reproduction. GH mRNA and protein are expressed soon after hatching (Funkenstein and Cohen, 1996; Yang et al., 1999) and persist during the growth and reproductive stages (Marchant and Peter, 1986; Gomez et al., 1999), although the growth rate slows down in maturing and spawning fish (Le Bail, 1988).

The purposes of this study were to clone and sequence the GH cDNA of four species of the Belontiidae family (*Trichogaster trichopterus*, *T. leeri*, *Colisa lalia*, *Betta splendens*) and to measure mRNA expression during gonadal development and the reproductive cycle in male and female *T. trichopterus*.

Materials and Methods

Fish. Belontiidae species were imported to Israel from southern Asia and India. The fish were maintained in MIGAL's laboratory in northern Israel (Degani and Veit, 1990). The species belonged to the Trichogasternae subfamily and included blue gourami (*Trichogaster trichopterus* Pallas) and pearl gourami (*T. leeri* Bleeker), native to Sumatra, Thailand, Borneo, and Malaysia, dwarf gourami (*Colisa lalia* Hamilton), native to India, and fighting fish (*Betta splendens* Regan), native to Singapore and Thailand.

Five pituitaries from each species were excised to clone GH. For mRNA expression, the pituitaries and gonads of 15 males (five from each stage - juvenile, mature, and nest building) and 15 females (five from each stage of ovary development - juvenile, vitellogenic, and mature) were sampled. The level of mRNA in each pituitary was measured by means of real-time polymerase chain reaction (PCR).

cDNA cloning. The cDNA of blue gourami GH (bgGH) was cloned according to rapid amplification of cDNA ends (RACE)-PCR protocol for the amplification of cDNA ends (Frohman, 1990; Degani et al., 2003a; Goldberg et al. 2004). The degenerate primer for the 3' RACE (P1, Table 1) was designed according to the sequence of relative genes of *T. trichopterus* (accession no. Af157633 in GenBank; Goldberg et al., 2004), and was used for 3' RACE along with the adapter primer provided in the kit. The combinations of primers used in this study are shown in Table 1.

Sequence assembly and analysis. The cDNA sequences were assembled using the

Table 1. Primers used in this study.

Primer	*cDNA target*	*Used*	*Nucleotide sequence*	*Direction in relation to mRNA*
P1*	GH	3' RACE-PCR	5' GC(ACGT)TG(CT)TT(CT)AA(AG)AA(AG)G	5' > 3'
P2	GH	5' RACE-cDNA synthesis	5' AAGAACAGGACTACAGAACAC	3' > 5'
P3	GH	5' RACE- PCR	5' GCATTTAGCCACAGTCAG	3' > 5'
P4	GH	Complete cDNA cloning	5' AGAAGTGAACCTGAACCTGTATC	5' > 3'
P5	GH	Complete cDNA cloning	5' CATTGTGCTGGAACTGG	3' > 5'
P6	GH	GH RealTime PCR	5' TTCACAACCGCTATGGACAA 3'	5' > 3'
P7	GH	GH RealTime PCR	5' TGACGCTGCTCTTCAATCTG 3'	3' > 5'
P8	GH	18S RealTime PCR	5' TTCTCGATTCTGTGGGTGGT 3'	5' > 3'
P9	GH	18S RealTime PCR	5' GAACGCCACTTGTCCCTCTA 3'	3' > 5'
dT_{17}-adapter	Universal	cDNA synthesis	5' GACTCGAGTCGACATCGA$(T)_{17}$	
Adapter	Universal	RACE-PCR	5' GACTCGAGTCGACATCG	

* Degeneracy fold, × 64; corresponding conserved amino acid sequence, ACFKKD.

GAP4 software package (Bonfield et al., 1995). Sequence analysis was carried out using the GCG software package (Ver. 9.0, Madison, WI). Multiple sequence alignments were performed using the Clustal W program. A dendrogram, based on multiple alignments, was created by the Clustal W program using the cluster analysis neighbor-joining method.

Real-time PCR. To compare bgGH mRNA levels, the relative abundance of mRNA was normalized with an endogenous reference, the 18S subunit of mRNA, by the comparative threshold cycle (C_T) method, according to Muller et al. (2002) and modified by Levavi-Sivan et al. (2004).

Total RNA was prepared from individual pituitaries by Trizol (Invitrogen, Carlsbad, CA) and each sample was reverse-transcribed at 57°C by Reverse-iTTM 1st Strand Synthesis Kit (ABgene, Surrey, UK) and random hexamers, according to the manufacturers' protocols. Gene-specific primers employed in the real-time PCR were designed with Primer 3 Software. The primers used for bgGH amplified a 188-bp product and corresponded to nucleotides (nt) 67-86 and 254-235 (P6 and P7, respectively, accession no. AF157633). Primers for 18S mRNA (P8 and P9) amplified a 154-bp product. Amplifications of bgGH and 18S mRNA cDNAs were performed simultaneously in duplicate in separate tubes and results were analyzed with Q-Gene software (BioTechniques Software Library; www.BioTechniques.com). Dissociation-curve analysis was run after each real-time experiment to ensure that there was only one product. A reverse-transcriptase negative control was run for each template and primer pair as a control for false positives.

Histology. Histological sections (6 µm) of ovaries were obtained by means of a Reichert (Austria) microtome and stained with Mallory trichrome. Identification of development stages of female gonads followed Jackson et al. (1994) and of male gonads Degani et al. (2003a).

Statistical analysis. Data are presented as means±SEM. The significance of differences between group means of hormone or mRNA levels was determined by one-way analysis of

variance (ANOVA) followed by Student Newman-Keuls (SNK) test using Graph-Pad Prism software (GraphPad, San Diego, CA) with a significance level of $p<0.05$.

Results

Rresults showed the similarity in GH sequence among the various species of Belontiidae, leading us to hypothesize that GH can be a molecular marker for the systematic study of reproduction in Belontiidae.

GH cDNA, encoding for a prehormone of 204 aa residues, was cloned from the *T. leeri* pituitary. It consisted of 840 bp that included the 5' and 3' noncoding sequences of 42 bp and 183 bp, respectively (Fig. 1). When the cDNA and deduced peptide of *T. trichopterus* were compared to those of *T. leeri,* they were found to be of the same length. The identity between them was 97% on the nucleotide level and 99% on the amino acid level (Fig. 2; accession no. for *T. leeri* GH mRNA in GenBank is AY873789).

In contrast, GH cDNA cloned from *C. lalia* differed from both *T. leeri* and *T. trichopterus*. *Colisa lalia* GH cDNA, including the 5' and 3' noncoding sequences of 42 bp and 183 bp, respectively (accession no. for *C. lalia* GH mRNA in GenBank is AY873788), consisted of 840 bp and encoded a prehormone of 204 aa residues. The nucleotide identity of GH cDNA of *C. lalia* was 88% compared to the cDNA of *T. leeri* and *T. trichopterus* (*T. trichopterus* GH mRNA, complete cds AF157633). On the protein level, the identity of *C. lalia* was 97% and 96% compared to *T. trichopterus* and *T. leeri*, respectively.

The GH cDNA with the greatest variation was cloned from the pituitary of *B. splendens* (accession no. *B. splendens* GH mRNA in GenBank is AY873790). It consisted of 814 bp that encoded for a prehormone of 204 aa residues and included the 5' and 3' noncoding sequences of 28 bp and 171 bp, respectively. The nucleotide identity between GH cDNA cloned from *B. splendens* and GH cDNA of *T. leeri* and *T. trichopterus* was 82%. When compared to GH cDNA of *C. lalia,* the identity was 81%. The identity on the amino acid level was higher than on the nucleotide level, with levels of 93%, 92%, and 91%, respectively, for *T. trichopterus*, *T. leeri,* and *C. lalia*.

The identities of the Belontiidae were more similar to other fish in the Perciformes order than to fish of other orders (Salmoniformes, Siluriformes, Cypriniformes, Anguilliformes) with the lowest similarity found between Belontiidae and Anguilliformes (Table 2).

Fig. 3a shows the gonadal morphology of the males. The gonads were used only to determine the reproduction stage. In all three groups (juveniles, mature, and nest building), the same cellular stages (spermatogonia, spermatocyte I, spermatocyte II, spermatid, and spermatozoa) were found, but in different proportions. No morphological differences were observed between testes from the two latter groups but there were fewer spermatozoa in the center of the lobule of mature reproductive males processed after spawning. The testes of mature reproductive and mature non-reproductive fish were similar while testes of juvenile fish contained relatively few spermatozoa. Ovarian stages of vitellogenesis and maturation are presented in Figs. 3b and 3c.

The expression of GH mRNA was greater in female *T. trichopterus*, a model for the Belontiidae family, than in males at all stages of gonadal development (Fig. 4). No significant differences were found in GH transcription level between immature and mature females but the mRNA level dropped significantly during sexual activity in males. GH mRNA in the testis was significantly lower than in the pituitary gland. It was $3 \times 10^{-6} \pm 4 \times 10^{-6}$ GH mRNA/18S (mean±SE) in mature males and slightly increased during nest-building to $4 \times 10^{-6} \pm 1.5 \times 10^{-5}$ GH mRNA/18S (mean±SE) but the difference was not significant. GH transcription in the ovary was very low and not detected by real-time PCR.

Discussion

GH is a very important hormone for regulating developmental and somatic growth in vertebrates (Perez-Sanchez, 2000). Numerous studies have therefore been carried out on cloned GH and mRNA expression in fish (Ayson et al., 2000; Chen et al., 2003; Degani et al., 2003a; Inoue et al., 2003). In this study,

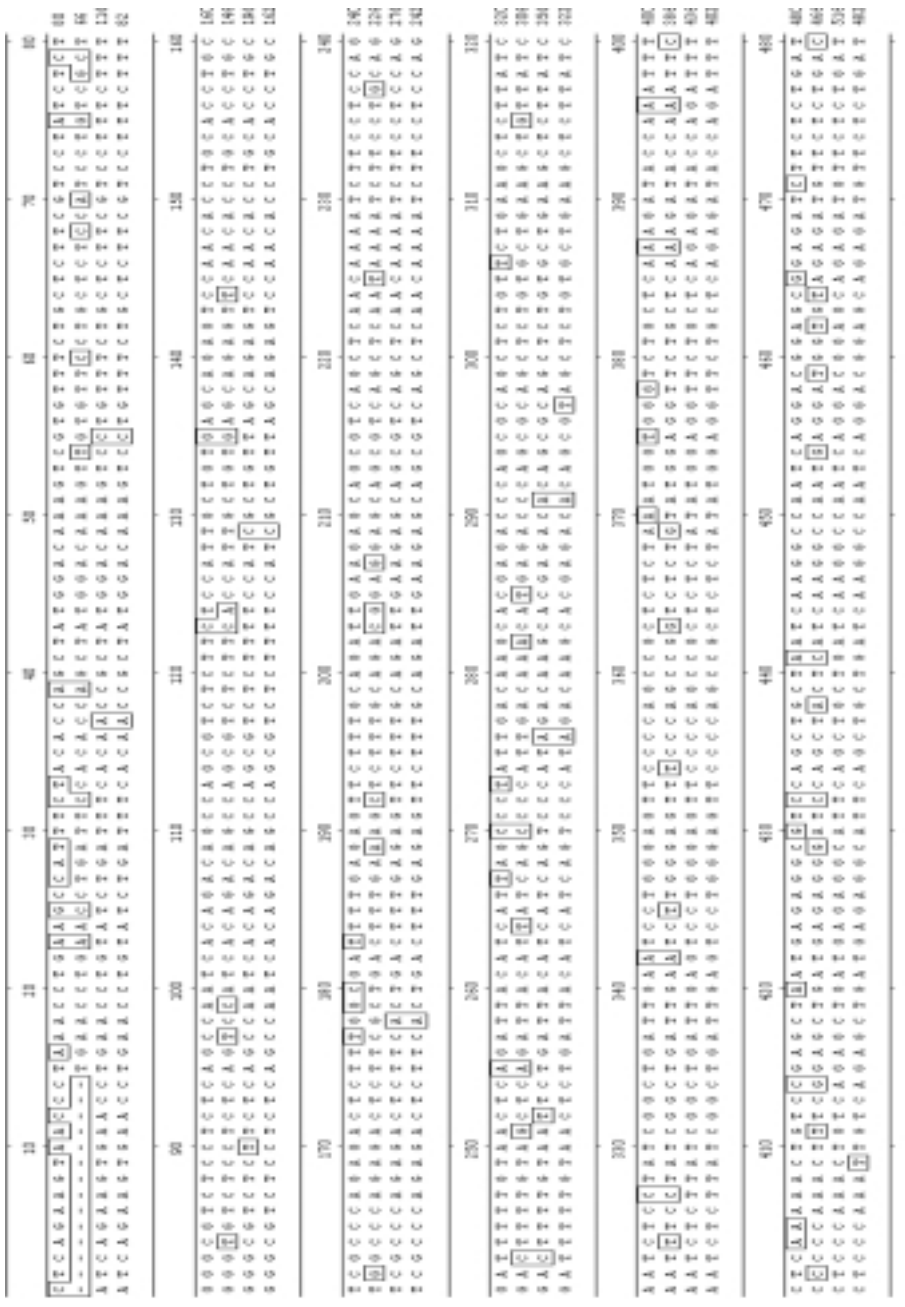

Fig. 1. Comparison of nucleotide sequences of growth hormones from four species of the Belontiidae family (*Trichogaster trichopterus*, *T. leeri*, *Colisa lalia*, *Betta splendens*). Differences are marked.

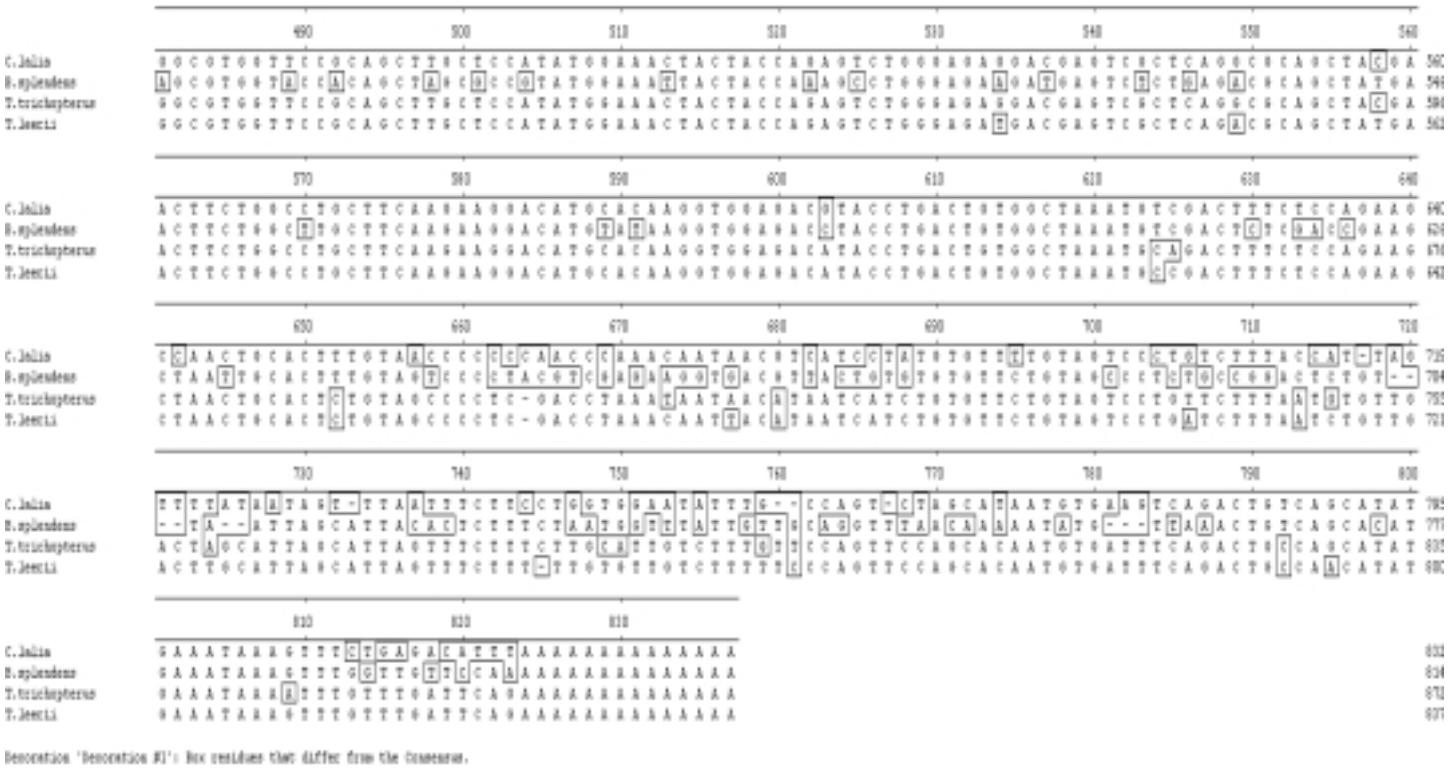

Fig. 1. Cont'd

	Trichogaster leeri	*Colisa lalia*	*Betta splendens*
Nucleotide identity			
Trichogaster trichopterus	97	88	82
Trichogaster leeri		88	82
Colisa lalia			81
Amino acid identity			
Trichogaster trichopterus	99	97	93
Trichogaster leeri		96	92
Colisa lalia			91

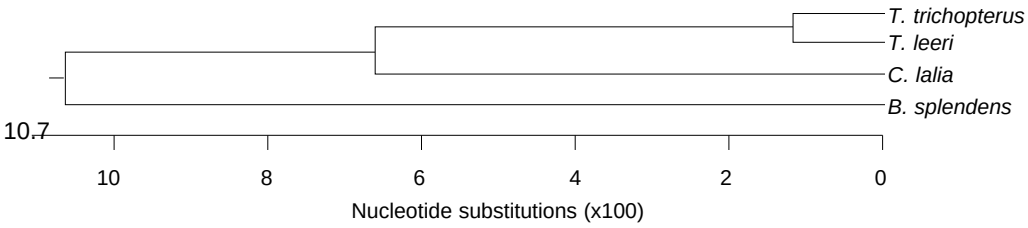

Fig. 2. Nucleotide and amino acid identity (%) among Belontiidae growth hormones and a phylogenetic tree constructed with the MegAlign program (DNASTAR) by the Clustral W method. Branch length represents evolutionary distance.

the complete cDNA encoding of the GH of four species of the Belontiidae family (Anabantoidei suborder) was examined. Two of the species, *T. trichopterus* and *T. leeri*, are morphologically and geographically distributed. *C. lalia* does not have an overlapping geographic distribution and is more similar to *B. splendens*, which is distributed in a small area covered by the *Trichogaster* genus. The gene loci of species belonging to the *Colisa* genus had a higher degree of similarity to that of the *Trichogaster* species than to that of the *Betta* (Degani and Veit, 1990).

The GH sequence analysis is described in many fish but few orders and information on the Anabantoidei suborder is very limited. GH cDNA sequence analysis is a useful tool for studying the systematic relationships between species belonging to a family and it seems to be a good support for parameters used in systematics. The results of the present study are in agreement with results in other fish species (Dores et al., 1996). Two species belonging to the same genus, *T. leeri* and *T. trichopterus*, were very similar to each other when compared to two species of other genera, *C. lalia* and *B. splendens*. The results of this study concur with a previous starch gel electrophoresis study that examined 22 isozyme systems found in the muscle and liver of ten species from the Anabantidae family (Degani and Veit, 1990). In that study, the gene loci of species belonging to the *Colisa* genus revealed a high degree of similarity to that of the *Trichogaster* genus. In the present study, there was less difference between GH of *Trichopterus* and GH of *Colisa* than between these two and GH of *Betta*. From a geographic point of view, *Trichogaster* and *Colisa* are sympatric species while *Betta* is not

Table 2. Amino acid and nucleotide identity (%) between GH of the Belontiidae *Trichogaster trichopterus* (blue gourami; B), *T. leeri* (pearl gourami; P), *Colisa lalia* (dwarf gourami; D), and *Betta splendens* (fighting fish; F) and other Perciforms or fish of other orders.

			Identity (%)							
			Nucleotide				*Amino acid*			
Class/Order	*Species*	*GH accession no.*	*B*	*P*	*D*	*F*	*B*	*P*	*D*	*F*
Perciformes	*Sparus aurata*	U01301	81	80	80	79	86	86	86	85
	Acanthopagrus butcheri	X59377	82	82	81	80	86	86	85	84
	Oreochromis niloticus	M26916	79	78	78	77	82	82	81	79
Salmoniformes	*Oncorhynchus kisutch*	M19999	67	66	66	65	64	64	66	61
	Oncorhynchus tshawytscha	S50867	68	68	67	65	64	64	65	62
	Oncorhynchus mykiss	M24683	69	68	68	66	65	65	65	63
	Salmo salar	X14305	68	68	68	67	65	65	65	63
Siluriformes	*Pangasianodon gigas*	L27835	63	63	63	59	56	56	55	55
	Clarias gariepinus	AF416487	63	63	62	60	56	56	55	54
Cypriniformes	*Cyprinus carpio*	M27000	64	64	64	62	56	55	55	54
	Ctenopharyngodon idella	M27094	65	64	64	64	54	53	53	53
	Hypophthalmichthys nobilis	X60473	64	63	64	64	54	54	53	53
	Hypophthalmichtys molitrix	X60475	63	63	63	63	54	54	53	53
Anguilliformes	*Anguilla anguilla*	AY148493	59	58	58	57	44	44	44	44
	Anguilla japonica	M24066	59	58	58	57	44	44	44	44

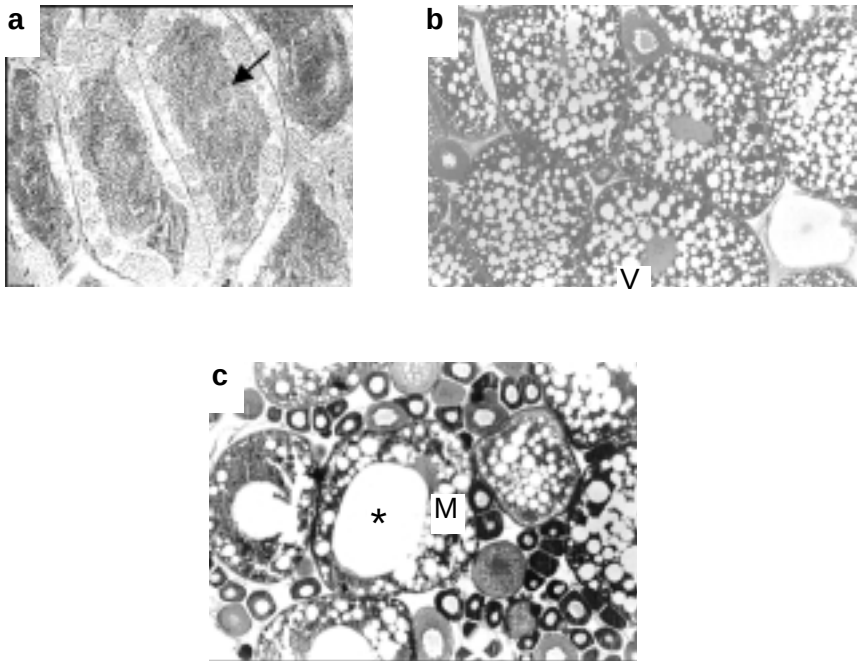

Fig. 3. Histological sections of the blue gourami *Trichogaster trichopterus* showing (a) germinal vesicle breakdown (arrow) in the testes, (b) vitellogenic oocytes (V) in the ovaries, and (c) oocytes at final maturation (M) with formation of the central droplet (asterisk).

(Hoedeman, 1975). The results of Degani and Veit (1990) lead us to conclude that the high polymorphism in some enzymes of some species can be utilized as a genetic marker for these species and as an aid in aquaculture when dealing with these systematic groups.

When the GH sequences of the Belontiidae fish, sequenced for the first time in this study, were compared to those of other fish, it was revealed that the GH of Belontiidae is very similar to that of other fish belonging to the Perciformes order. The identity between the nucleotide sequences of the Belontiidae and Perciformes fishes was 77-82% while the identity between the sequences of the Belontiidae fish and those of Salmoniformes, Siluriformes, Cypriniformes, and Anguilliformes was 57-69%. The percent identity among amino acids was even lower, 44-64%.

As in most teleost GH, four cysteine residues were found in bgGH. In comparison, five cysteine residues were described in goldfish (Law et al., 1996) and other cyprinids (Chang et al., 1992). These cysteine residues are involved in the formation of two disulfide bonds in a pattern analogous to the “big loop little loop” pattern found in the human growth hormone (Vestling et al., 1991) that plays an important role in determining its biological activity (Lewis et al., 1980). A putative glycosylation site was found in bgGH. The existence of this site is a common feature of many teleost GH. N-linked glycosylation might serve as a signal for protein transport to the cell surface (Guan et al., 1985).

GH mRNA expression was relatively higher in female blue gourami than in males. Similar results were found in European eels

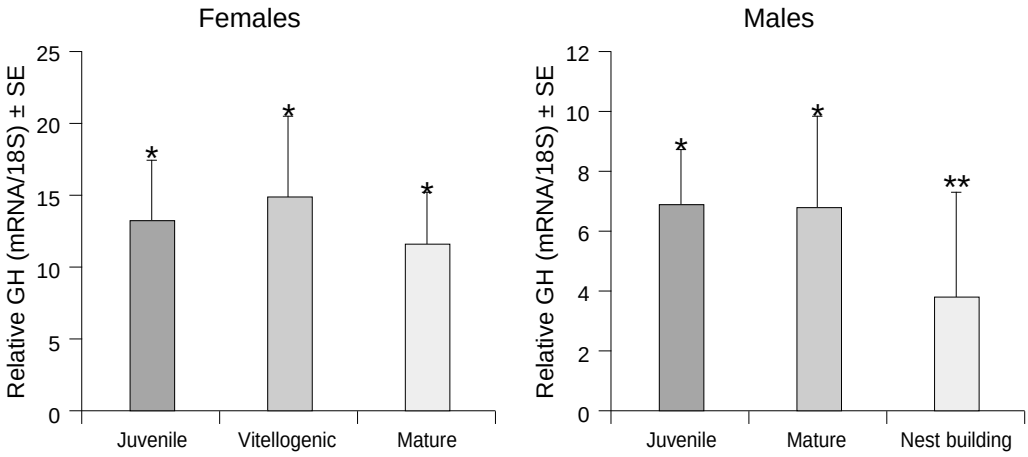

Fig. 4. GH transcript levels in the pituitary during the gonadal cycles of (a) female and (b) male *Trichogaster trichopterus*, measured by real-time PCR. Each histogram represents the average of five independent measurements (mean±SE). Significant differences (*t* test $p<0.05$) are indicated by a different number of asterisks.

(*Anguilla anguilla*), with higher GH transcription levels in females than in males, suggesting that different levels of GH transcription might explain the difference in growth rate between male and female European eels (Degani et al., 2003b). The GH transcription in the pituitary in the present study was dramatically higher than the GH in the gonads.

The present study describes the variation in GH mRNA expression in the pituitary with respect to ovarian stage, as measured by real-time PCR (Levavi-Sivan et al., 2004), a very sensitive method. Goldberg et al. (2004) measured GH expression by reverse transcriptase (RT) PCR which is less sensitive and less accurate than the real-time PCR used in the present study. Measuring by reverse transcriptase (RT) PCR, they discovered that GH mRNA is high in juveniles and mature fish in the stages of late vitellogenesis and final oocyte maturation. The results of the present study are in accordance with these results. The level of mRNA did not change during growth and oogenesis. However, Goldberg et al. (2004) found that only maturing females in early vitellogenesis (endogenous vitellogenesis) had a significantly lower GH mRNA level.

Recent studies on GH functions confirmed other physiological effects in addition to the well-established growth-promoting effects. Among these effects is the participation of GH in reproduction, as has been studied in several teleosts by different approaches including gene expression. GH mRNA was detected shortly after hatching in *Sparus aurata* (Funkenstein and Cohen, 1996) and before organogenesis of the pituitary in *Oncorhynchus mykiss* (Yang et al., 1999). In adult animals, GH mRNA was detected outside the pituitary gland in several sites including the ovary (Yang et al., 1999). In our study, high GH mRNA levels were identified in the pituitaries of juveniles and fully grown mature fish. These results contrast with those described during rainbow trout oogenesis, where significantly higher levels of GH mRNA were discovered at the stages of exogenous vitellogenesis and post ovulation, and not in juveniles or fish in the final oocyte maturation stage (Gomez et al., 1999). The expression of GH family genes in chum salmon is enhanced during swimming to fresh water (Taniyama et

al., 1999). In salmonids the higher levels of GH mRNA during the last stages of oogenesis can be related to metabolic (decrease in eating) and osmoregulatory changes, rather than to direct effects on gonadal development. At the pituitary level, GH-producing cells demonstrated an increase in secretory activity during vitellogenesis (Young and Ball, 1983).

Several studies suggested that GH may play a role in female gonad development. A positive correlation between GH concentration and female gonadal development was described by Young and Ball (1983), Stacey et al. (1984), and Holloway et al. (1999). GH can influence gonad development by promoting production of steroids (Van der Kraak et al., 1990). The physiological concentrations of androgens and estrogens secreted from the gonads stimulate GH expression (Huggard et al., 1996). GH injection of recombinant rainbow trout raised the E_2 level in immature trout and killifish (Singh and Thomas, 1993). Bovine GH enhanced the effect of E_2 *in vitro* on vitellogenin synthesis in the European eel (*A. anguilla*; Kwon and Mugiya, 1994; Peyon et al., 1998). The present study supports the above results that pituitary GH might affect gonads.

In conclusion, GH sequence and expression may serve as a systematic marker for Belontiidae fish and possibly other fish. GH is expressed in the studied fish not only during the growth period, but also during reproduction.

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