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Molecular cloning and regulation of mRNA expression of the thyrotropin β and glycoprotein hormone α subunits in red drum, *Sciaenops ocellatus*

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Abstract Full-length cDNAs for thyrotropin β (TSHβ) and glycoprotein hormone α (GSUα) subunits were cloned and sequenced from the red drum (*Sciaenops ocellatus*). The cDNAs for TSHβ (877 bp) and GSUα (661 bp) yielded predicted coding regions of 126 and 94 amino acid proteins, respectively. Both sequences contain all invariant cysteine and putative glycosylated asparagines characteristic of each as deduced by comparison with other GSUα and TSHβ sequences from representative vertebrate species. Multiple protein sequence alignments show that each subunit shares highest identity (79% for the TSHβ and 86% for the GSUα) with perciform fish. Furthermore, in a single joint phylogenetic analysis, each subunit segregates most closely with corresponding GSUα and TSHβ subunit sequences from closely related fish. Tissue-specific expression assays using RT-PCR showed expression of the TSHβ subunit limited to the pituitary. GSUα mRNA was predominantly expressed in the pituitary but was also detected in the testis and ovary of adult animals. Northern hybridization revealed the presence of a single transcript for both TSHβ and GSUα, each close in size to mRNA transcripts from other species. Dot blot assays from total RNA isolated from *S. ocellatus* pituitaries showed that in vivo T_3 administration significantly diminished mRNA expression of both the TSHβ and GSUα subunits and that goitrogen treatment caused a significant induction of TSHβ mRNA only. Both TSHβ and GSUα mRNA expression in the pituitary varied significantly in vivo over a 24-h period. Maximal expression for both TSHβ and GSUα occurred during the early scotophase in relation to a peak in T_4 blood levels previously documented. These results suggest the production of TSH in this species which may serve to drive daily cycles of thyroid activity. Readily quantifiable, variable, and thyroid hormone-responsive pituitary TSH expression, coupled with previously described dynamic daily cycles of circulating T_4 and extensive background on the growth, nutrition, and laboratory culture of red drum, suggests that this species will serve as a useful model for experimental studies of the physiological regulation of TSH production.

Keywords Thyrotropin · Pituitary glycoprotein hormone subunits · Red drum · Thyroid

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Introduction

Thyrotropin (TSH or thyroid-stimulating hormone) is a glycoprotein hormone synthesized and secreted by pituitary thyrotrophs (Szkudlinski et al. 2002). TSH stimulates the thyroid gland to synthesize and secrete the thyroid hormones triiodothyronine (T_3) and tetraiodothyronine (T_4) (Fisher 1996; Ingbar and Braverman 1975). Enzymatic deiodination in peripheral tissues can convert T_4 to the much more biologically active T_3 (Eales and Brown 1993; Fisher 1996; Köhrle 1999) that, in turn, regulates diverse physiological processes including metabolism, growth, reproduction, and in some fish and amphibians, metamorphosis (Cyr and Eales 1996; Evans 1988; Tata 2006; Yamano and Miwa 1998). Thyroid hormones can limit their own production by inhibiting TSH secretion at the level of the hypothalamus and pituitary (Chin et al. 1993; Shupnik 2000). Such negative feedback can be counterbalanced through hypothalamic release of trophic factors that promote TSH secretion.

The significance of TSH in the regulation of fish thyroid function is still poorly understood (MacKenzie et al. 2009). Because TSH directly drives thyroid hormone synthesis and secretion, the regulation of TSH production should be critically important in controlling thyroid hormone provision to tissues (MacKenzie et al. 2009). Indeed, in the central model of control of thyroid function, centrally located controlling elements (e.g., hypothalamus and pituitary) play a primary role in thyroid hormone provision to target tissues through TSH secretion. The importance of the role of TSH in centrally regulating thyroid hormone availability in mammals has been well documented (Eales and Brown 1993). However, deiodinase enzymes expressed in peripheral tissues may also play a critical role in determining T_3 availability to targets (Eales 1985; Eales and Brown 1993; Schweizer et al. 2008). Eales and Brown (1993) proposed that peripheral control predominates in fish, suggesting that it is the more evolutionarily ancient control mechanism. Furthermore, Eales and Brown argue that the relative importance of central control has increased in more recent vertebrate groups. However, because little is known about the synthesis and secretion of TSH in fish, the relative contribution of central mechanisms to overall thyroid function remains unclear.

Previous work on the red drum (*Sciaenops ocellatus*) suggests that this may be an excellent species in which to further examine the contributions of central systems to the control of fish thyroid function. Previous studies in our laboratory have found dynamic diurnal regulation of T_4 secretion (Leiner et al. 2000; Leiner and MacKenzie 2001) apparently driven by a central oscillator that may mediate its effect through the regulation of TSH secretion (Leiner and MacKenzie 2003). If the dynamic daily T_4 cycles reflect a precise hypothalamic and feedback regulation of TSH that is amenable to experimental manipulation, studies of the regulation of red drum TSH production may significantly contribute to our understanding of the central regulation of thyroid function in fish.

The first objective of this study was therefore to clone and characterize both the thyroid-stimulating hormone beta (TSHβ) and the glycoprotein subunit alpha (GSUα) polypeptides that make up the TSH protein in *S. ocellatus*. Although both subunits have been previously characterized for diverse vertebrate species, few studies have examined their simultaneous expression in juvenile fish. The relationship between the α and β TSH subunits' expression in fish is difficult to evaluate in reproductively active adults because the GSUα subunit is shared with the gonadotropins. Using juvenile fish, we hope to minimize the contribution of gonadotropins to pituitary GSUα subunit expression. The second and third objectives of this study were to examine tissue-specific expression of both subunits in the pituitary gland and other tissues and to determine whether expression of both subunits in the pituitary is subject to negative feedback regulation by T_3. Finally, our last objective was to document the coordinated changes in TSHβ and GSUα that occur throughout a 24-h cycle in a time course consistent with the hypothesis that TSH secretion is driving daily T_4 cycles.

Materials and methods

Animals and sampling

Red drum fingerlings were obtained from the Texas Parks and Wildlife Department's hatcheries at Sea Center Texas in Lake Jackson or the CCA/CPL

Marine Development Center in Corpus Christi and were reared in the laboratory on Aquamax (PMI Nutrition, Brentwood, MO) diet. Fish were fed once daily in the morning to apparent satiation unless otherwise noted. Captive fish were held in 8000-L tanks at 25°C, 12L:12D photoperiod in 6ppt recirculating artificial seawater (SuperSalt, Fritz Industries, Mesquite, Texas). Wild adult *S. ocellatus* were caught by hook and line from Gulf of Mexico waters adjacent to the Dow Chemical Co. plant in Freeport, Texas. Gonad samples were collected from wild adult fish that were in the early gonadal recrudescent stage in August (Craig et al. 2000). All other samples were taken from juvenile laboratory-reared fish. Samples from 4 to 6 fish were pooled for RNA extraction. Laboratory fish were held in 80- (MMI experiment) or 400 (all other experiments)-liter experimental tanks for at least 2 weeks of acclimation before treatment. For injection and tissue sampling, fish were anesthetized in tricaine methanesulfonate (Finquel, Argent Laboratories, Redmond, Washington). Blood was collected in heparinized tubes and held on ice for up to 1 h. Following centrifugation, plasma was frozen at −80°C until thyroid hormone analysis using Coat-A-Count Total T_4 and Coat-A-Count Total T_3 kits (Siemens, Los Angeles, CA), both of which quantify hormone concentration in diluted samples utilizing an antibody-coated tube method. For the T_4 assay, sensitivity (defined as the lowest standard on the linear portion of the standard curve) was 0.25 ng/ml and cross-reactivity with T_3 was 2%; inter- and intra-assay variabilities (calculated as coefficient of variation) were 8.2 and 3.2%, respectively (manufacturer's data). For the T_3 assay, sensitivity was 0.2 ng/ml; cross-reactivity with T_4 was 0.5%; and inter- and intra-assay variabilities were 7.6 and 5.5%. In both assays, serial dilutions of red drum plasma diluted parallel to the standard curve. Tissues for RNA extraction were removed within 5 min of terminal anesthesia and transferred directly to TRIzol reagent (Invitrogen, Carlsbad California).

Cloning, polymerase chain reaction (PCR) and 5′ and 3′ rapid amplification of cDNA ends (5′ and 3′ RACE) for TSHβ and GSUα

Amplification of an internal red drum TSHβ and GSUα fragment (232 bp) was achieved using standard PCR protocols (for annealing temperatures, see

Table 1 Primer names and sequences used in this study

Oligonucleotide primers				
Primer	Nucleotide sequences	Direction	PCR application	Temp[a] (°C)
TSHβ_1	5′-ACC ATT TGC ATG GGC TTC TGC-3′	Forward	Internal fragment	45
TSHβ_2	5′-TGT GGG CAC ACT CAT CAC TGT-3′	Reverse	Internal fragment	45
TSHβ_3	5′-TGG CTG TGC GGT ATT CCA CCT TGT CG-3′	Forward	3′ RACE	57
TSHβ_4	5′-CCC TGT CTT CAC CTA CCC TGT GGC TC-3′	Reverse	5′ RACE	57
TSHβ_5	5′-AAA ACA TGG AGA CCG CAG TGT TCA CCT GC-3′	Forward	RT-assay	63
TSHβ_6	5′-AGG GAT CAT GTA GTT GCT TTG GCC AG-3′	Reverse	RT-assay	63
GSUα_1	5′-AGC TCC TGT GTA TCA GTG CAT GG-3′	Forward	Internal fragment	60
GSUα_2	5′-AGT GCT GCA ATG GCA GTC TGT G-3′	Reverse	Internal fragment	60
GSUα_3	5′-ACA TCA CCT CGG AGG CGA CGT GCT GTG T-3′	Forward	3′ RACE	68
GSUα_4	5′-CGG CCA CCT CTA TCT CGT AGC TGT GCT T-3′	Reverse	5′ RACE	67
GSUα_5	5′-TCC AAC ACC TCT CAA GGC C-3′	Forward	RT-assay	57
GSUα_6	5′-GAG AAA CAC AAT CTG TTG CCA AGC-3′	Reverse	RT-assay	57
β-actin$_1$	5′-TCT GTT GGC TTT GGG GTT CAG G-3′	Forward	RT-assay	60
β-actin$_2$	5′-TGG GCC AGA AGG ACA GCT ACG-3′	Reverse	RT-assay	60

Primer direction and application is indicated. Both internal cDNA and RACE cDNA fragments were generated for cloning and sequence determination. Sequence information from cloned fragments was used to design primers for the reverse transcription polymerase chain reaction assay (RT-PCR assay) used to evaluate tissue-specific expression presented in Fig. 5

[a] PCR annealing temperature

Table 1) with oligonucleotide primers designed against conserved areas within the coding region of other fish sequences. Sequence information from the internal fragment allowed design of sequence-specific primers that were paired with corresponding forward and reverse primers provided with the GeneRACER kit (Invitrogen) to obtain 5′ and 3′ RACE products. RACE reactions were performed following the manufacturer's protocol. Desalted oligonucleotides were synthesized by Integrated DNA Technologies (Coraville, IA). All primer sequences used in this study, with the exception of RACE kit primers, are listed in Table 1.

Molecular cloning of RACE products and chemical transformation of recombinant plasmids into *E. coli* were conducted using the TOPO TA Cloning kit (Invitrogen) following the manufacturer's protocols. Luria broth (LB) plates containing 75 μg/ml kanamycin or 50 μg/ml ampicillin (Sigma, St. Louis, MO) were streaked with transformation reactions and incubated overnight at 37°C. Three or more independent colonies per transformation were inoculated in 3 ml liquid culture (1× LB plus antibiotic) overnight. Recombinant plasmids from overnight cultures were isolated using the Concert High Purity Plasmid Miniprep kit (GibcoBRL, Life Technologies). Isolated plasmid DNA was subjected to *Eco*RI digestion and electrophoresed on 1% agarose gels at ~100 V using 1× TAE (40 mM Tris–acetate, 1 mM EDTA, pH 8.3) as running buffer and ethidium bromide for UV visualization. Recombinant plasmids in which *Eco*RI digestion revealed an insert were sequenced. Primers that bound within the vector's multiple cloning site were used to prime sequencing reactions at the sequencing facility of Tulane University Medical School, New Orleans, LA. Alternatively, sequencing reactions were performed on a thermocycler using a mixture of primer, plasmid, and BigDye™ reagent using the manufacturer's protocol (Applied Biosystems (ABI), Carlsbad California) followed by electrophoresis at the Gene Technology Laboratory at Texas A&M University.

RT-PCR was performed using the Omniscript reverse transcriptase kit (Qiagen, Valencia, CA) following the manufacturer's protocol. PCR on the reverse-transcribed product were carried out using Go*Taq* Green Master Mix (Promega, Fictburg, WI) following the manufacturer's protocol. Annealing temperatures are listed in Table 1.

Isolation of total RNA, electrophoresis, Northern blotting and dot blotting

Freshly collected tissue samples were suspended in TRIzol reagent (Invitrogen) and homogenized by repetitive aspiration through a 20-gauge syringe needle. Homogenized samples were either immediately processed according to the TRIzol protocol or flash frozen in liquid N_2 and stored at −80°C for subsequent processing. Total RNA purity (A_{260}/A_{280} ratio) and concentration (Abs. at A_{260}) was assessed by UV spectrophotometry. Samples were kept at −80°C in DEPC (Sigma)-treated double-deionized water (ddH_2O) until gel electrophoresis.

Total RNA samples were each diluted in DEPC-treated ddH_2O to a concentration providing uniform loading (~5 μg) of samples. Diluted samples were denatured in formaldehyde loading dye (Ambion, Austin TX) at 60–65°C followed by agarose gel electrophoresis (1% agarose; 1× formaldehyde gel buffer, Ambion Inc, Austin, TX) at ~95 volts in 1× MOPS buffer for up to 1.5 h. Total RNA integrity was assessed by UV visualization of ethidium bromide fluorescence of the ribosomal RNA bands. Northern blotting was performed by capillary blotting using Hibond N^+ nylon membranes (Amersham, Cleveland, OH) with 20× SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0), transfer buffer for at least 4 but no longer than 24 h according to the manufacturer's instructions. Transfer efficiency was evaluated by membrane fluorescence. After blotting, membranes were immediately subjected to covalent linkage of blotted RNA to the membrane using a UV cross-linker (UVP, Upland CA).

Dot blotting was performed using 1.1 μg of RNA per sample loaded onto Hibond N^+ nylon membranes using vacuum suction through a Minifold filtration manifold dot blotting apparatus (Schleicher and Schuell). Statistical determinations used 3 dots per treatment group for the negative feedback experiments (Fig. 6) and 4 dots per treatment group for the cycle experiment (Fig. 7). Hibond N^+ nylon membranes were briefly presoaked in 20× SSC before samples were loaded. Samples were prepared in a denaturing buffer consisting of 5 parts formamide, 1.62 parts formaldehyde, and 10 parts 10× MOPS. Samples were then brought up to a volume of 320 μl using DEPC-treated water and heated to 65°C for 15 min. Lastly, 80 μl of 20× SSC was added to the

samples directly before application onto the membrane. Dot blot apparatus wells were rinsed with 20× SSC to ensure that all samples were fully loaded onto the membrane. After blotting, membranes were treated and stored as described for Northern blots previously.

Production of inserts, labeling, and hybridization

The 3′ RACE products of both red drum TSHβ and GSUα were used as probes for Northern and dot blottings. Segments of the red drum β-actin and the 18S ribosomal subunit were cloned into plasmids as housekeeping control genes for Northern and dot blot probing. The 239-bp β-actin probe was ligated into a pCR 2.1 plasmid (Invitrogen). The 595-bp TSHβ probe, 358-bp GSUα probe, and 778-bp 18S ribosomal subunit probe were ligated into pCR 4-TOPO-plasmids (Invitrogen). Glycerol stocks of *E. coli* containing plasmids of red drum TSHβ, GSUα, β-actin, and 18S ribosomal subunit were streaked on 1× LB plates plus antibiotic. Single colonies were grown in 1× LB plus antibiotic overnight cultures and the plasmids isolated using the Concert High Purity Plasmid Maxiprep kit (GibcoBRL, Life Technologies). Maxipreped plasmids were sequenced to confirm identity with the original clone. The plasmid insert released using *Eco*RI digestion was electrophoresed and gel purified using the Qiaquick Gel Extraction kit (Qiagen). Between 25 and 100 ng of insert was ^{32}P labeled using the DECAprime II random priming DNA labeling kit (Ambion, Applied Biosystems) following the manufacturer's protocol. Probe consisting of ^{32}P-labeled insert was separated from unincorporated α^{32}P-dCTP by centrifugation through Spin-50 mini-Sephadex columns (BioMax). Salmon sperm DNA (Ambion, Applied Biosystems), as carrier, was added just before denaturation of probe. Probes were denatured by boiling and then added to membrane blots undergoing prehybridization at 65°C in a hybridization oven. Blots were prehybridized and hybridized in the Quikhybe solution (Stratagene, Cedar Creek, TX) following the manufacturer's protocol for both hybridization and washing. After washing, blots were sealed to preserve moisture and exposed to a phosphoimaging plate followed by image development and analysis using the BAS-5000 phosphoimager (Fujifilm, Tokyo, Japan). Bands representing hybridization of TSHβ or GSUα probes were quantified by dividing their measured pixel density by the pixel density of a probed housekeeping gene using the Multigauge 2.3 software (Fugifilm). *S. ocellatus* 18S RNA was used as the housekeeping gene except for the cycle experiment (Fig. 7) in which *S. ocellatus* β-actin was used since 18S RNA appeared to cycle over a 24-h period. Blots were stripped between hybridizations according to the manufacturer's protocol for Hibond N^+ membranes.

Multiple protein sequence alignment and phylogenetic analysis

Multiple protein sequence alignments of selected vertebrate TSHβ and GSUα subunits were performed using the AlignX software (Vector NTI Advance 10.3.0, Invitrogen). A phylogenetic tree of selected vertebrate TSHβ and GSUα subunits was constructed by the Mega 4.0.1 software (www.megasoftware.net) using the neighbor-joining method with 1000 replicates. MacKenzie et al. (2009) lists the GenBank accession numbers and references of selected vertebrate TSHβ and GSUα subunits were used to generate the multiple protein sequence alignments and phylogenetic analysis. The signal peptide was identified using the program SignalP (Center for Biological Sequence Analysis, Technical University of Denmark, www.cbs.dtu.dk).

Methimazole and T_3 administration to juvenile fish

Goitrogen treatment was administered once a day via ip injection of methimazole (100 μg/g body weight) dissolved in saline prepared daily immediately before use. The control group received injection of saline without goitrogen. On the 12th day, blood samples were collected from the caudal vasculature and pituitaries were removed and pooled for the extraction of total RNA from 41 to 38 fish of 135 g (±3.1 sem) average mass in the methimazole and control groups, respectively. The T_3 treatment was given once daily via ip injection of T_3 (15 μg/g body weight) dissolved in saline. The control group received injection of saline without T_3. On the eighth day, blood samples were collected from the caudal vasculature and pituitaries were removed for the extraction of total RNA from 6 to 7 fish of 270 g

(±15.6 sem) average mass in the T_3 and control groups, respectively. The T_3 injections lasted for 7 days with no injection on the fourth day. Insufficient RNA was available from this initial T_3 experiment for statistical analysis so a second T_3 experiment was conducted using the same protocol as above with 3 fish of 245 g (±43.0 sem) average mass in each T_3 and control groups.

Daily rhythm of TSHβ and GSUα mRNA expression in juvenile fish

Animals were left undisturbed with a 12L:12D photoperiod for 1 month. Animals were fed a commercial diet to apparent satiation at random times during photophase as previously described (Leiner and MacKenzie 2001). Individual tanks containing 12 experimental fish were assigned to each time point. On the day of the experiment, blood samples were collected from the caudal vasculature of all 12 fish in each tank at each sampling time. Pituitaries were then removed for the extraction of total RNA. Three pituitaries were pooled for each replicate yielding 4 pooled samples for each time point.

Statistics

Multiple comparisons for statistical significance were performed using Mann–Whitney *U*-tests with a $P \leq 0.05$. Spearman's correlation tests were used to evaluate the correlation of expression between the α and β subunits.

Results

Cloning and sequencing of TSHβ and GSUα

Two contiguous full-length cDNA sequences were assembled from overlapping PCR products and subjected to BLAST analysis (NCBI). BLAST confirmed the sequences to be TSHβ (Fig. 1A) and GSUα (Fig. 1B). The full-length TSHβ cDNA sequence is 877 bp long and is comprised of a 72-bp 5′ untranslated region (5′ UTR), followed by a 438-bp nucleotide coding block and ending in a 356-bp 3′ untranslated region (3′ UTR) containing a consensus polyadenylation site. Translation of the coding block yielded a TSHβ protein sequence 146 amino acids long. The GSUα clone comprised a 661-bp full-length cDNA containing a 29-bp 5′ UTR followed by a nucleotide coding block of 396 bp and ending in a 212-bp 3′ UTR containing a consensus polyadenylation site. Translation gave a 132 amino acid GSUα sequence. Further analysis defined the signal sequence as spanning amino acids 1 (start methionine)-38.

Multiple protein sequence alignment and sequence identity table for TSHβ and GSUα

The sequence of *S. ocellatus* TSHβ was compared to fourteen other TSHβ proteins from representative vertebrate groups (Fig. 2a). The *S. ocellatus* TSHβ protein exhibited regions of identity common to other TSHβ proteins, including the 12 invariant cysteine residues and one invariant putative asparagine glycosylation site. Regions of similarity were higher between the *S. ocellatus* sequence and other fish and highest between *S. ocellatus* and one other perciform, *Pseudolabrus sieboldi* (Otha et al. unpublished, GenBank Accession No. BAF81902). Among fish, the identity table (Fig. 3a) showed *S. ocellatus* TSHβ protein ranged from 79% identity (*P. sieboldi*) to 59% (*D. rerio*). Identities between *S. ocellatus* and tetrapod vertebrates ranged from 37 to 40%.

Multiple sequence alignment of the *S. ocellatus* GSUα with 14 other GSUα proteins identified from representative vertebrate species is shown in Fig. 2b. The *S. ocellatus* GSUα shared the same regions of identity present in other GSUα proteins, including the ten invariant cysteines and two putative asparagine glycosylation sites. *S. ocellatus* GSUα shared higher overall similarity with other fish species and highest similarity with the two other perciform species, *Sebastes schlegelii* and *P. sieboldi*. The first AUG codon of the *S. ocellatus* GSUα was chosen as the start codon because it was preceded with A at position-3 (mammalian mutagenesis studies have shown that almost all ribosomes will initiate at a start codon preceded by an A at position-3; (Kozak 2002). The long signal sequence shared between *S. ocellatus* and *S. schlegelii* has also been observed in other fish GSUα sequences. The identity table generated from the alignment (Fig. 3b) showed that the *S. ocellatus* GSUα protein had highest identity to the perciform fish *S. schlegelii* and *P. sieboldi*, with values of 86% and 85%, respectively. Identity with other fish ranged from 58 to 60% and from 51 to 54% with tetrapod vertebrates.

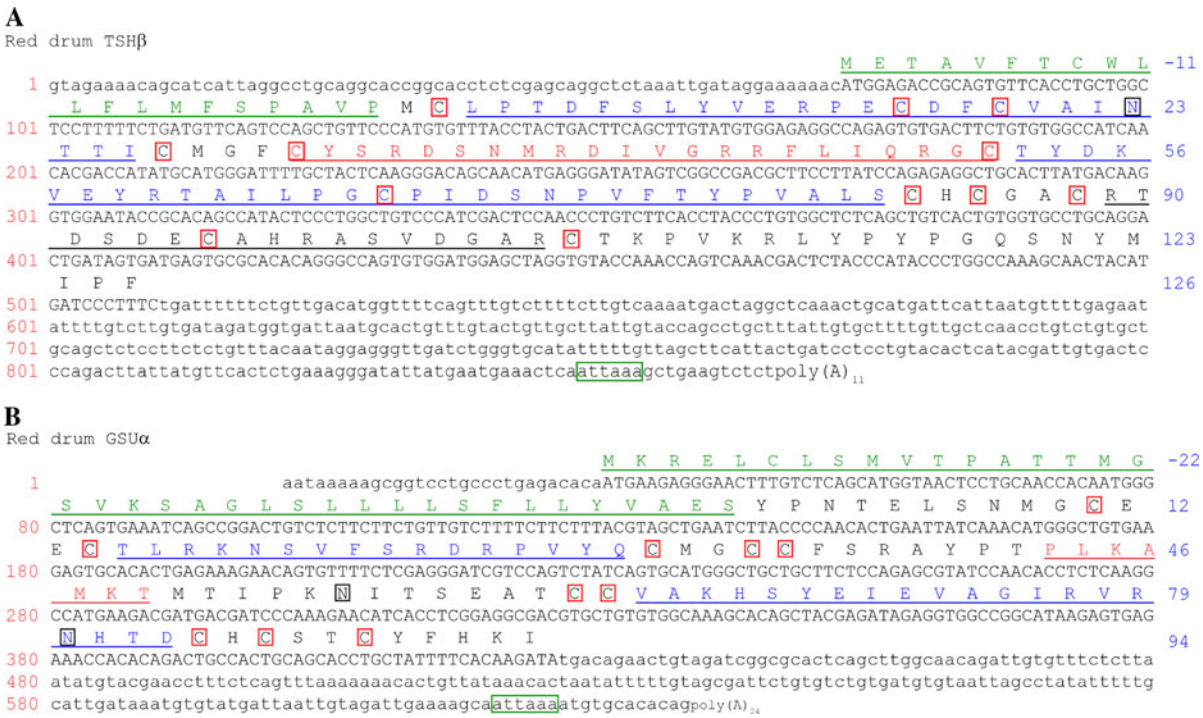

Fig. 1 Nucleotide and translated amino acid sequences for **A** TSHβ (GenBank Accession GU144513) and **B** GSUα (Genbank GU144512) cDNAs cloned from *S. ocellatus*. Nucleotides shown in *lower case* at the beginning and end of each sequence designate 5′ and 3′ UTR, respectively. Nucleotides designating coding sequence are indicated in *upper case*. Nucleotides are specified by *red numbers on the left*. *Single letter code* for each amino acid is designated above each corresponding codon and is specified by *blue numbers on the right*. All designated sites and sequences specified are presumptive based on sequence analysis and comparison (see Discussion). For both (**A**) and (**B**), signal sequences are in *green letters underlined in green*, *hair pin loops* are shown in *blue letters underlined in blue*, and polyadenylation sites are *boxed in green* at the end of the 3′ UTR. Invariant cysteines are *boxed in red*. A boxed "N" signifies a glycosylated asparagine as deduced from its alignment with glycosylated asparagines found in other TSHβ or GSUα proteins shown in Fig. 2. For (**A**), the long loop is shown in *red letters underlined in red* and the seatbelt is emboldened and *underlined in black*. For (**B**) *red letters underlined in red* indicate an α-helix

Joint phylogenetic tree for TSHβ and GSUα

A phylogenetic tree (Fig. 4) was generated that includes the GSUα and TSHβ from *S. ocellatus* along with the other proteins from the multiple protein sequence alignments. Both the TSHβ and GSUα of *S. ocellatus* clustered with other members of TSHβ and GSUα proteins, respectively. *S. ocellatus* TSHβ segregated to the same clade as other fish TSHβ proteins and formed a branching pair with *P. sieboldi,* the other perciform species. For the GSUα tree, *S. ocellatus* clusters within fish forming a clade with the two other perciform fish (*P. sieboldi* and *S. schlegelii)*.

Tissue-specific expression

RT-PCR demonstrated that TSHβ expression was localized strongly to the pituitary (Fig. 5a). In addition, the relative size of the TSHβ subunit mRNA was significantly smaller than that of the genomic sequence indicating the presence of one or more introns within the TSHβ gene. Absence of a genomic product in all other samples in Fig. 5 indicates the absence of contaminating genomic DNA. RT-PCR of the same tissues (Fig. 5b) showed GSUα expression to be strongest in the pituitary but also detectable in the ovaries and testes. β-actin

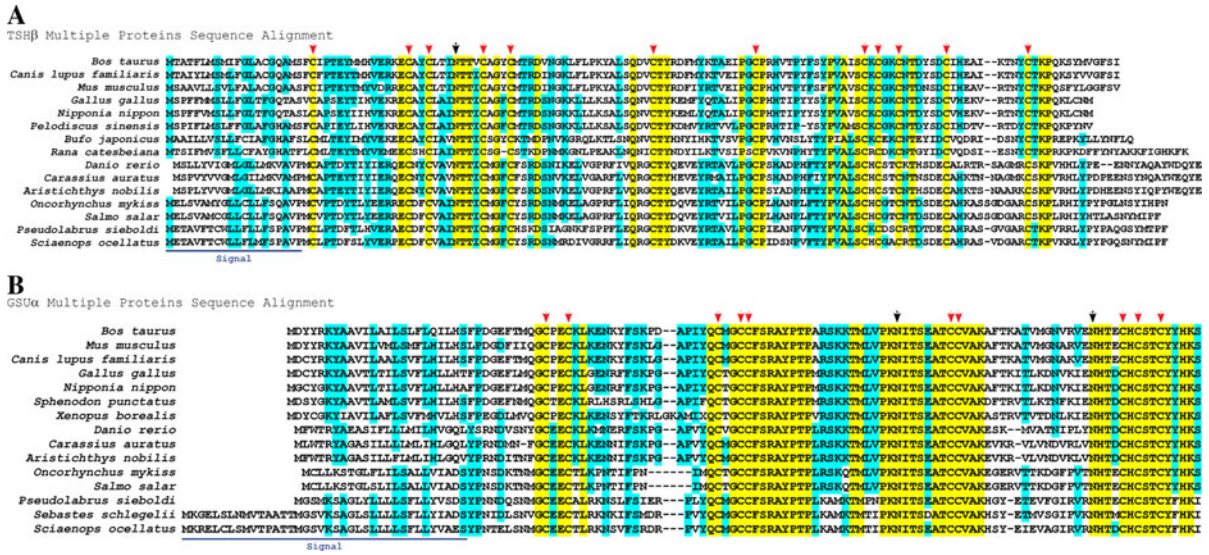

Fig. 2 Multiple protein sequence alignments for fourteen predicted **a** TSHβ and **b** GSUα proteins, with *S. ocellatus* located at the *bottom*. Mammals are *Bos Taurus*; *Mus musculus*; *Canus lupus familiaris*. Birds are: *Gallus gallus*; *Nipponia Nippon*. Reptiles are: *Pelodiscus sinensis*; *Sphenedon punctatus*. Amphibians are *Bufo japonicus*; *Rana catesbeiana*; *Xenopus borealis*. Fish are *Danio rerio*; *Carassius auratus*; *Aristichthys nobilis*; *Oncorhynchus mykiss*; *Salmo salar*; *Pseudolabris sieboldi*; *Sebastes schlegelii*. The common names associated with these sequences are listed in MacKenzie et al. (2009). For both alignments, the presumptive N-terminal signal sequence is *underlined, red arrows* indicate the conserved cysteine residues, and *black arrows* indicate glycosylated asparagine residues. *Yellow shading and green shading* indicate regions of identity and similarity, respectively

(Fig. 5c) served as a positive control to confirm an amplifiable cDNA pool in all samples.

Effect of goitrogen and thyroid hormone treatment

Northern analysis revealed that T_3 treatment of *S. ocellatus* produced a dramatic reduction in steady-state TSHβ mRNA levels in the pituitary compared to saline-injected controls (Fig. 6a). A similar but less dramatic pattern was seen for GSUα. Injection of the goitrogen methimazole resulted in moderate elevation of steady-state TSHβ mRNA expression in the pituitary compared to saline-injected controls (Fig. 6a). A similar but less dramatic effect was seen for GSUα. All Northerns yielded only one band corresponding to the size of the mRNA product as predicted from the full-length cDNA for both TSHβ and GSUα. T_3 treatment significantly increased both circulating T_3 (from 5.4 ± 0.9 in controls to 2886 ± 237.5 ng/ml in treated) and T_4 (from 0.7 ± 0.3 to 130.5 ± 12.1 ng/ml), whereas MMI treatment significantly reduced both T_3 (from 5.5 ± 0.7 to 0.7 ± 0.07) and T_4 (from 2.21 ± 0.9 to below the 0.1 ng/ml sensitivity of the assay). Elevated T_4 measured from T_3-injected fish was likely due to cross-reactivity of T_3 in the T_4 RIA.

Replicated feedback experiments were performed using a semiquantitative dot blot procedure normalized to 18S ribosomal RNA expression. Results showed a statistically significant 1.6 fold induction of TSHβ after MMI (Fig. 6b) and a statistically significant 55% reduction after T_3 treatment compared to saline-injected controls (Fig. 6b). In the same samples assayed for GSUα mRNA, fish injected with T_3 showed a significant 71% reduction in GSUα expression, whereas methimazole did not produce a statistically significant change in GSUα mRNA over saline-injected controls. A significant correlation was found between TSHβ and GSUα expression in dot blot experiments ($r^2 = 0.687$, $P = 0.042$ for MMI-treated animals, $r^2 = 0.617$, $P = 0.064$ for T_3-treated animals). T_3 treatment significantly increased both circulating T_3 (from 5.2 ± 0.6 in controls to 2601.3 ± 218.8 ng/ml in treated fish) and T_4 (from 1.0 ± 0.3 to 107.6 ± 14.3 ng/ml), whereas

A
TSHβ Identity

	Bos taurus	*Canis lupus familiaris*	*Mus musculus*	*Gallus gallus*	*Nipponia nippon*	*Pelodiscus sinensis*	*Bufo japonicus*	*Rana catesbeiana*	*Danio rerio*	*Carassius auratus*	*Aristichthys nobilis*	*Oncorhynchus mykiss*	*Salmo salar*	*Pseudolabrus sieboldi*	***Sciaenops ocellatus***
Bos taurus		93	80	67	66	71	48	48	37	39	39	41	41	38	**40**
Canis lupus familiaris			83	67	67	72	51	51	38	39	39	44	44	40	**40**
Mus musculus				66	66	68	48	47	37	39	39	45	45	40	**41**
Gallus gallus					96	80	50	48	41	43	44	46	46	41	**42**
Nipponia nippon						80	51	47	41	42	44	45	45	40	**42**
Pelodiscus sinensis							53	49	43	45	45	47	47	42	**43**
Bufo japonicus								66	36	37	37	38	38	34	**35**
Rana catesbeiana									34	34	36	38	38	35	**37**
Danio rerio										83	84	64	63	56	**59**
Carassius auratus											89	65	62	53	**56**
Aristichthys nobilis												67	64	55	**57**
Oncorhynchus mykiss													93	68	**73**
Salmo salar														66	**70**
Pseudolabrus sieboldi															**79**
Sciaenops ocellatus															

B
GSUα Identity

	Bos taurus	*Mus musculus*	*Canis lupus familiaris*	*Gallus gallus*	*Nipponia nippon*	*Sphenodon punctatus*	*Xenopus borealis*	*Danio rerio*	*Carassius auratus*	*Aristichthys nobilis*	*Oncorhynchus mykiss*	*Salmo salar*	*Pseudolabrus sieboldi*	*Sebastes schlegelii*	***Sciaenops ocellatus***
Bos taurus		91	93	82	81	75	75	60	62	60	60	60	56	54	**54**
Mus musculus			92	79	78	75	75	62	63	61	59	59	56	55	**53**
Canis lupus familiaris				84	82	79	77	61	61	61	59	59	55	54	**53**
Gallus gallus					98	82	75	61	63	63	57	57	51	50	**52**
Nipponia nippon						82	75	61	63	63	57	57	51	50	**52**
Sphenodon punctatus							74	58	58	57	58	58	51	50	**52**
Xenopus borealis								59	58	58	60	60	50	49	**51**
Danio rerio									80	79	58	58	57	58	**59**
Carassius auratus										95	60	60	59	59	**60**
Aristichthys nobilis											60	60	58	59	**59**
Oncorhynchus mykiss												99	60	61	**58**
Salmo salar													60	61	**58**
Pseudolabrus sieboldi														81	**85**
Sebastes schlegelii															**86**
Sciaenops ocellatus															

Fig. 3 Identity tables for **a** TSHβ and **b** GSUα generated by multiple protein sequence alignment (Fig. 2). *Numbers* designate amino acid identity between each listed protein *on the left* and all other listed proteins. Identities between the *S. ocellatus* protein and a corresponding version from another species are shown by the emboldened numbers in the column labeled ***Sciaenops ocellatus***

MMI treatment significantly reduced both T_3 (from 5.5 ± 0.7 to 0.7 ± 0.1) and T_4 (from 2.2 ± 0.9 ng/ml to <0.1 ng/ml).

Daily changes in TSHβ mRNA, GSUα mRNA, and T_4 levels

In fish held on a standard laboratory photoperiod of 12L:12D, steady-state levels of TSHβ mRNA were lowest mid-morning and increased to significantly higher levels three hours after the beginning of scotophase (Fig. 7a). A similar but less pronounced pattern in diurnal expression of GSUα was seen (Fig. 7b). Circulating T_4 was lowest 3 h after the beginning of the photophase and peaked at significantly higher levels around the beginning of scotophase, as has been previously observed for this species. A significant correlation was found between TSHβ and GSUα expression in the daily cycle experiment ($r^2 = 0.432$, $P < 0.001$).

Discussion

Both TSHβ and GSUα full-length cDNAs generated from cloning were suitable for the development of hybridization assays to characterize the in vivo expression of red drum TSH during daily cycles and under conditions of thyroid hormone and goitrogen manipulation of negative feedback. Our identification of the two TSH subunits from *S. ocellatus*, TSHβ and GSUα, is based on analysis of the translated amino acid sequences of two full-length cDNA sequences, tissue-specific expression profiles, and physiological regulation of transcripts expressed in the pituitary.

The TSHβ protein and GSUα protein sequences deduced from their respective cDNAs were compared in separate multiple protein sequence alignments with those of other representative vertebrates and showed identities as high as 79% for TSHβ and as high as 85% for GSUα compared with TSHβ and GSUα

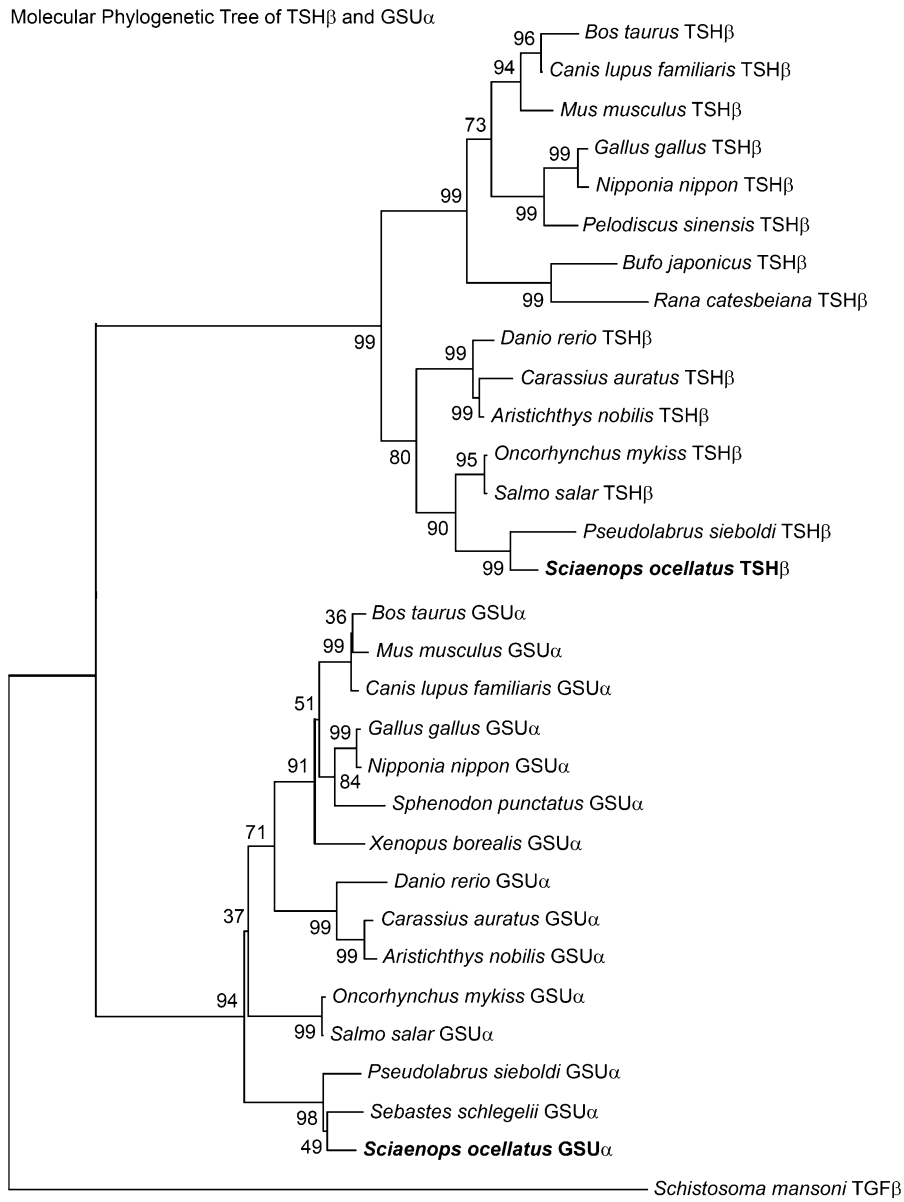

Fig. 4 Joined phylogenetic tree for TSHβ and GSUα proteins. *Schistosoma mansoni* transforming growth factor β (TGFβ) was chosen as a representative cystine-knot growth factor protein for the outgroup (GenBank Accession No. AB164156). *Numbers at branch points* are bootstrap values generated from one-thousand iterations using the neighbor-joining method (see Material and Methods). *S. ocellatus* branches are labeled *in bold*

counterparts reported from other fish species. The TSHβ and GSUα we report for *S. ocellatus* also contain the same invariant stretches of amino acid sequences in the same relative positions of other TSHβ and GSUα found across all vertebrate classes. These invariant regions are necessary for the formation of the "seat-belt" region of TSHβ, hairpin loops (for both TSHβ and GSUα), and alpha helices (in GSUα) that together contribute to proper subunit dimerization and receptor binding (Szkudlinski et al. 2002).

Multiple protein sequence alignments of the *S. ocellatus* TSHβ and GSUα also illustrate the invariant cysteine positions essential for the formation of the cystine-knot structure that is characteristic of each subunit (Szkudlinski et al. 2002). Other signature aspects of the protein structures are the positional locations of the putative N-linked glycosylation site for the *S. ocellatus* TSHβ and the two putative N-linked glycosylation sites for *S. ocellatus* GSUα that are positionally identical to the respective TSHβs and GSUαs of other vertebrates. Finally, the TSHβ of *S. ocellatus* contains the same positional location for the teleost-specific CMGFC amino acid sequence known to facilitate dimerization between

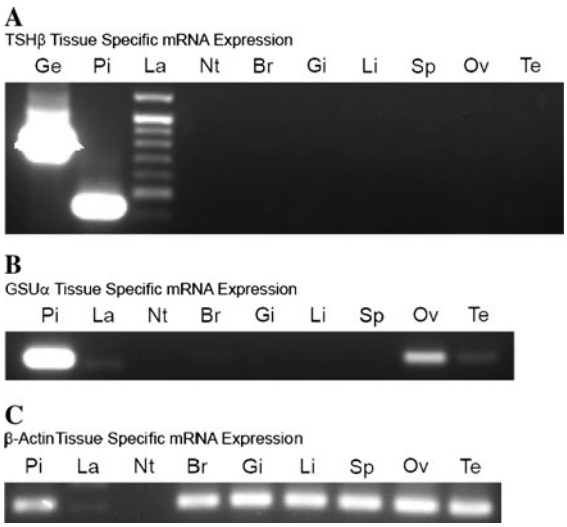

Fig. 5 RT-PCR showing tissue-specific expression of **a** TSHβ, **b** GSUα, and **c** β-actin in indicated tissues in *red drum* (see Table 1 for oligonucleotide sequences used). All tissues were collected from laboratory animals except wild-collected gonads. Legend for tissue identification is as follows: Ge, genomic DNA; Pi, Pituitary; La, Ladder; Nt, No template control; Br, Brain; Gi, Gill; Li, Liver; Sp, Spleen; Ov, Ovary; Te, Testis. The genomic DNA PCR yielded a much larger amplicon indicating the presence of 1 or more introns

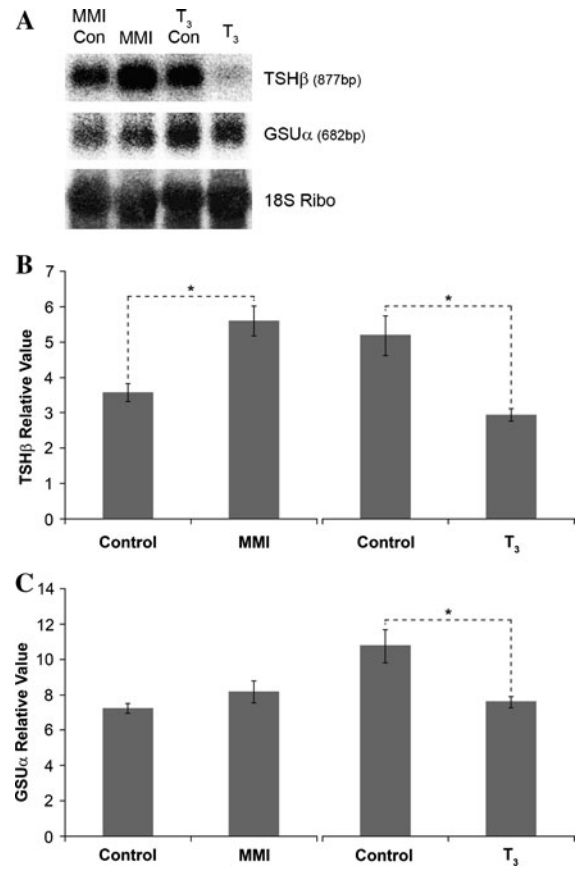

Fig. 6 Effects of negative feedback and its goitrogen treatment on steady-state levels of TSHβ mRNA and GSUα mRNA expression in *S. ocellatus* as measured by **a** Northern blot and **b** dot blot. **a** Treatment groups were as follows: MMI Con, methimazole vehicle control; MMI, methimazole treated; T_3 Con, T_3 vehicle control; T_3, T_3 treated. Results are from 3 consecutive hybridizations with 3 different radiolabeled cDNA inserts (18S ribosomal, TSHβ, GSUα) performed on the same blot. **b** The same experimental regime as (**a**) using semi-quantitative dot blot hybridization. Saline-injected control is shown paired with its respective treatment group. Each statistically significant difference between a treatment group and its matched control is indicated by *an asterisk over dashed lines* connecting the two groups

the TSHβ and GSUα subunits in all teleost fish examined to date (Chatterjee et al. 2001).

The GSUα and TSHβ protein sequences were used to generate a phylogenetic tree including numerous other GSUαs and TSHβs. The tree shows that the *S. ocellatus* GSUα sequence clusters within the vertebrate GSUα branch. Moreover, it is grouped within the fish GSUα cluster and is most closely positioned with other perciform GSUα sequences. Correspondingly, the TSHβ of *S. ocellatus* clusters within the vertebrate TSHβ branch and further resolves within the fish TSHβ cluster and in which it is most closely joined to another perciform TSHβ sequence. These results underscore and corroborate our conclusions from multiple protein sequence alignments regarding the identity of our reported sequences.

Whereas previous studies have suggested a low content of TSH in teleost fish pituitaries (MacKenzie et al. 2009), pituitary expression of the mRNA's representing TSHβ and GSUα was readily detectable using a PCR or hybridization-based assay in the pituitary of *S. ocellatus*. Using cDNA probes to detect steady-state levels of TSHβ and GSUα mRNA, we detected a single 877-bp product for TSHβ and a single 661-bp product for GSUα in Northern blots of pituitary total RNA. The size measured on Northern blots of each mRNA representing each of the two TSH subunits corresponded to the size of its respective full-length cDNA. Likewise, both subunits could be detected using a cDNA-specific RT-PCR assay on total RNA isolated from the pituitary. Interestingly, multiple transcripts of TSHβ and GSUα mRNA have been detected and characterized in other fish species

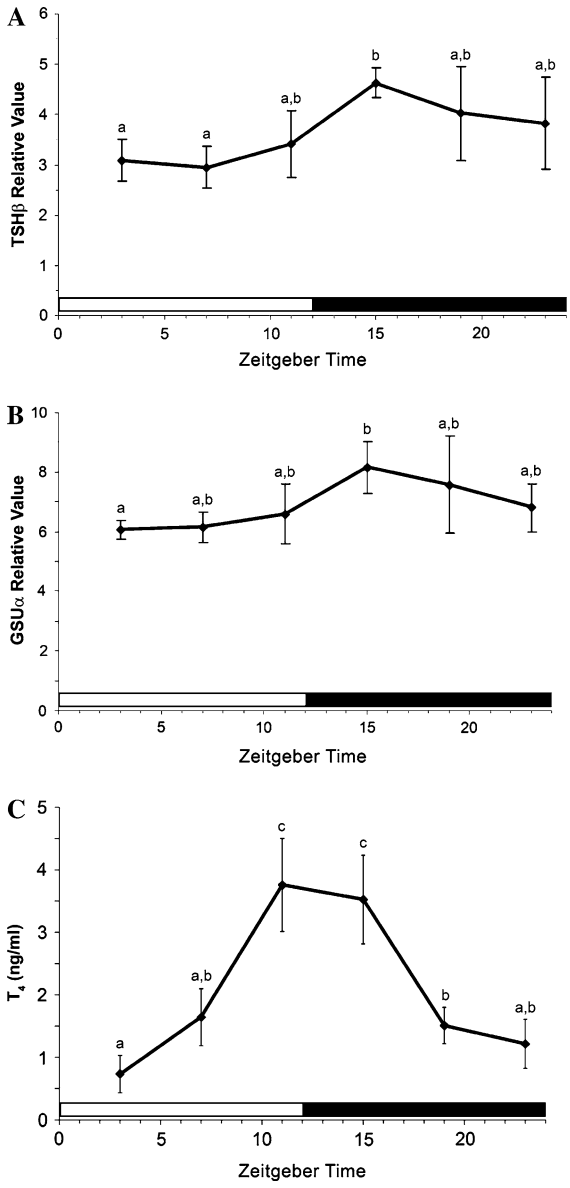

Fig. 7 Daily changes of **a** TSHβ mRNA expression, **b** GSUα mRNA expression, and **c** circulating T_4 over a single 24-h period. Pituitary gland steady-state TSHβ and GSUα mRNA levels are shown after normalization to β-actin expression. Hormone levels are from the same fish from which total RNA was obtained. *Open rectangular bars* on the *x*-axis indicate photophase, and *blackened rectangular bars* on the *x*-axis indicate scotophase. Significant differences are indicated by *unshared letters* between points

(Kobayashi et al. 1997; Lema et al. 2008; Pradet-Balade et al. 1998; Yoshiura et al. 1999). We did not specifically focus on the possibility of multiple transcript expression in this study, but we have uncovered no evidence through cloning, Northern blot analysis, or RT-PCR assay that alternative transcripts or multiple isoforms of either TSHβ or GSUα are expressed in the pituitary of *S. ocellatus.*

Results from the TSHβ and GSUα tissue specificity analysis revealed the expected subunit expression in the pituitary but also the unexpected expression of GSUα in the testis and ovary of adult red drum. Given that the GSUα expressed in the adult gonads was not linked with TSHβ expression, it is plausible that the GSUα expression was instead correlated with gonadotropin expression. Expression of fish gonadotropins and GSUα has been previously described in the ovary of the gilthead seabream (Wong and Zohar 2004). Discovery of expression of both TSHβ and the TSH receptor in a variety of teleost tissues (reviewed in MacKenzie et al. 2009) has suggested that endocrine or paracrine functions exist for extrapituitary TSH, particularly in the gonad. Although we were unable to detect the expression of TSHβ in gonads, we sampled only reproductively mature animals. Further sampling is required to determine whether expression may be more active at other stages of reproductive development.

Our experiments investigating the in vivo effects of alteration of negative feedback showed the expected significant induction of the TSHβ transcript with the goitrogen methimazole and decreased TSHβ expression following T_3 injection. Thus, thyroid hormone feedback on *S. ocellatus* TSHβ is consistent with that found in other fish and throughout mammals, birds, reptiles, and amphibians (Chien et al. 2006; Gregory and Porter 1997; MacKenzie et al. 2009; Manzon and Denver 2004; Shupnik et al. 1989). However, this same T_3 treatment in red drum showed a smaller but still significant diminishment in GSUα mRNA below control, while the methimazole treatment did not have a significant effect upon GSUα mRNA expression. Nevertheless, a significant correlation existed between GSUα expression and TSHβ expression. Together these results suggest that thyroid hormone exerts its strongest effect on TSH expression by regulating the TSHβ subunit.

Unequally matched expression between TSHβ and GSUα can be explained from both a transcriptional and a physiological perspective. Specifically, in mammals, TSHβ and GSUα gene expression are driven by different promoters and are therefore likely to recruit different sets of transcription factors that in turn would

lead to different rates of expression (Shupnik 2000). From a physiological perspective, GSUα is shared by LH and FSH as well as TSH, which raises the possibility that dot blot hybridization represents a summation of GSUα expression from multiple glycoprotein hormone-producing cell types. Since our studies were conducted with juvenile animals, we suggest that the GSUα expression we observed was primarily due to expression in thyrotrophs. This is in contrast to the majority of other studies in adult fish in which GSUα expression has been correlated with the expression of LHβ and FSHβ. However, studies on adult animals would be much less useful for addressing questions of GSUα expression as it relates to total TSH expression because of the confounding variation in gonadotropin expression associated with reproduction. We propose that our ability to measure both GSUα and TSHβ in juvenile animals can help refine our understanding of the regulation of TSH synthesis in fish by minimizing interference from regulation of the gonadotropin subunits.

In addition to measuring both TSH subunits in juvenile animals, we also present data demonstrating our ability to measure both TSH subunits as outputs for the proposed circadian oscillator that drives T_4 secretion in red drum (Leiner and MacKenzie 2003). Dynamic diurnal regulation of T_4 in *S. ocellatus* has been demonstrated over a 12L:12D photoperiod in which circulating T_4 remains low during the scotophase but undergoes a two-step increase (once in the morning and once in the afternoon) during the photophase (Leiner et al. 2000). Since the defining physiological feature of TSH is to drive T_4 secretion from thyroid follicular cells, it is possible that an increase in serum T_4 should be preceded by an increase in serum TSH. Assuming TSH secretion is accurately reflected and preceded by steady-state mRNA levels of the two TSH subunits, we employed a dot blot technique that supports diel regulation of steady-state mRNA levels of both TSH subunits. Our results showed relatively low mRNA levels of both TSH subunits during the photophase, followed by a significant increase in early scotophase and a subsequent scotophase diminishment approaching early photophase levels. Circulating T_4 revealed the same basic pattern reported previously by Leiner et al. (2000). Our results show that mRNA expression of both TSH subunits cycle in phase but that that the peak expression occurs following the peak of circulating T_4 rather than preceding it as might be expected if subunit expression reflected TSH protein secretion form thyrotrophs. However, development of a TSH immunoassay is required to establish the linkage between TSH subunit expression and protein secretion.

In conclusion, we have obtained full-length cDNA clones for both TSH subunits in the warm water, perciform species, *S. ocellatus*. TSH mRNA expression of both subunits is subject to negative feedback by T_3, and expression of the TSH subunits is most prevalent in the pituitary, but extrapituitary GSUα expression has been detected in the gonads of reproductively mature red drum. Finally, we show evidence that TSH mRNA expression is dynamically regulated over a 24-h period in a pattern commensurate with the hypothesis that TSH secretion drives an increase in T_4 secretion. Taken together, this evidence supports an important role for central regulation of thyroid function in this fish.

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