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Somatolactin Transcription during Oogenesis in Female Blue Gourami (*Trichogaster trichopterus*)

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Abstract

Somatolactin (SL), a specific pituitary hormone belonging to the prolactin (PRL) super family, is involved in background adaptation, osmoregulation, reproduction and fatty acid metabolism. The goal of this study was to examine the gene transcription of SL changes in the ovary of blue gourami females (*Trichogaster trichopterus*) during oogenesis by quantitative real-time polymerase chain reaction (Real-Time PCR). Somatolactin in the pituitary was higher in females at low vitellogenesis compared to females with oocytes in maturation, and the difference was significant ($p < 0.05$). No significant differences were found in mRNA levels between low and high vitellogenesis, and high vitellogenesis and maturation. The findings of this and previous studies demonstrate that SL, growth hormone (GH) and PRL are involved in oogenesis in blue gourami; however, considerably more studies are required in order to separate the functions of these hormones.

Keywords

Female Blue Gourami, Oogenesis, RNA, Somatolactin

1. Introduction

Blue gourami (*T. trichopterus*) is a tropical fish. It serves as a useful model for studying the role of endocrine regulation in reproduction [1]. Blue gourami (*T. 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 breath-

ing cavity (labyrinth) located above the gills under the operculum [1]. Blue gourami is multi-spawning and male-dependent, with asynchronic ovary development [2]. The gonadotropin-releasing hormone (GnRH) plays a central role in the control of blue gourami reproduction by affecting various hormones, e.g., gonadotropins (GtHs), which in turn regulate gametogenesis and steroidogenesis. In addition, final oocyte maturation occurs in females characterized by a large percentage of high vitellogenic oocytes in the ovary, only in the presence of a mature male [3]. Sexual glucosteroids 17α-hydroxyprogesterone(17-P), 17α, 20β-Dihydroxy-4-pregnen-3-one (17, 20P) and 17β-estradiol (E_2) are secreted into the water; they might serve as chemical pheromones during reproductive behavior and be involved in preparing females to spawn via the promotion of oogenesis E_2 and 17, 20P plasma levels [4] [5]. Thus, gonadal stages in females can be controlled and examined separately in the laboratory [2] [6]. SL, GH and PRL are pituitary hormones closely related to hormones belonging to the growth hormone family [7]. All these hormones are involved in many biological functions, including growth and reproduction [8].

Blue gourami GH is a 22 kDa single-chain polypeptide [9]-[11]. The GnRH and pituitary adenylate cyclase-activating polypeptide (PACAP) hormones family controls the GH axis and the GTH axis, enabling them to stimulate differential biological functions in oogenesis regulation [12]-[14]. In some species, including salmonids, there are two forms, SLα and SLβ [15]. The SL receptor (SLR) has recently been cloned, and phylogenetic analysis shows that it is similar to previously cloned GH receptors (GHRs) of non-salmonids. The ligand-specificity of GHR/SLR is unclear. Little is known about the role of SLs in sexual maturation of fish [15]. The aim of this study is to increase our knowledge about the regulatory role of SLs during oogenesis in fish, focusing on the female blue gourami model of the Labyrinthici suborder.

2. Methods

2.1. Fish and Sampling

T. trichopterus (blue gourami) females and males purchased from local pet shops were separated into different containers and grown for about a month. The fish were grown in aquaria at 27˚C under a light regime of 12 h light: 12 h darkness, fed twice a day with commercial tropical fish food (Tetra Bits 47.5% protein, 6.5% fat) and supplemented once a day with frozen live food (*Artemia salina*) [16]. Males were divided into two groups: 1) mature males (isolated from females); and 2) individual mature males under reproductive conditions that include plants adapted for nest-building (**Figure 1**). Females were divided into three groups: 1) non-mature females (BW = 1.13 ± 0.38 g) (number = 12)—the ovary of these fish contains mostly oocytes at their pre-vitellogenic stage; 2) mature females that were kept in a group without males (6.85 ± 1.17 g) (number = 12); and 3) mature females that were kept with nest-building males for 24 hours (7.20 ± 1.20 g) (number = 8) (**Figure 1**). The latter

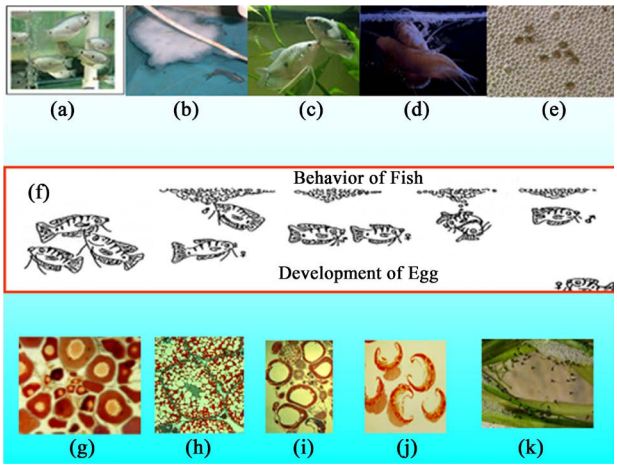

Figure 1. (a) Mature fish found in the group with no sexual behavior; (b) Nest-building male found in a separate aquarium; (c) Female and male in one aquarium, sexual behavior under the nest; (d) Mating, the female lays the eggs and the male fertilizes them, the fertilized eggs in the nest; (f) Different stages of sexual behavior; (g) Oocytes in a juvenile female (no vitellogenesis); (h) Oocytes in advanced vitellogenes is in a female found in groups; (i) Oocytes in maturation in females during sexual behavior of the male; (j) Oocytes in ovulation; (k) Larvae on the day of hatching in the nest.

showed oocytes at their final maturation stage. Fish were sampled and the gonads (small pieces of the ovary) were dissected [17]. The pituitaries were collected from females at various stages of gonadal development from non-mature females, from mature females that were kept in a group without males, and from mature females that were kept in a group without males [18] [19].

2.2. Histological Analysis

Gonad samples were fixed in Bouin and subsequently processed for light microscopy. Paraffin sections of 6 µm were stained with hematoxylin and eosin, as previously described [17] (**Figure 1**).

2.3. SL mRNA Extraction

SL mRNA levels were determined in the groups of female blue gourami: 1) non-mature females, the ovary of these fish contains mostly oocytes at their pre-vitellogenic stage (low vitellogenesis); 2) mature females that were kept in a group without males; and 3) mature females that were kept with nest-building males for 24 hours. For sampling, each fish was anesthetized in a clove oil bath (0.25 mg/l), and weight and length were recorded. Tissues (pituitary for mRNA SL expression studies) were removed and stored in RNA Later buffer (Ambion Inc., Austin, TX). The gonads were removed and weighed, and a portion was taken for histology. Total RNA was extracted from RNA Later stored tissues by means of the RNeasy® Total RNA Kit (QIAGEN, Alameda, CA) according to manufacturer's recommendations [8]. First-strand cDNA was synthesized from 2 µg of total RNA by the Superscript System (Invitrogen, Carlsbad, CA). The single-strand cDNA was used to amplify a cDNA internal fragment using gene-specific primers [15].

2.4. Real-Time PCR

The mRNA levels of SL in the gourami pituitary were determined [20]. The SL in the pituitary and the relative abundance of mRNA were normalized with the mRNA of the endogenous reference gene, 18 S subunit of rRNA (18 S rRNA), using the comparative threshold cycle (CT) method according to [21]. The relative amount of each gene was calculated by the formula 2_DCT, where DCT corresponds to the difference between the CT measured for each target gene and that determined for 18S rRNA. To validate this method, serial dilutions were prepared from a brain cDNA sample, and the efficiencies of gene amplifications were compared by plotting _DCT versus log (template) according to the method of [22]. Linear regressions of the plots showed R 2 values of 0.99 and efficiency values of >95%. Gene-specific primers for real-time PCR were designed using Primer 3 Software [12]. Syber Green Master Mix (ABgene) was added to each of the above PCR mixtures, and amplification was carried out in a Rotor Gene 3000 Sequence Detection System (Corbett Research, Sydney, Australia) under the following conditions: for GnRH1 and GnRH2 (Accession numbers KF113107 and KF113108, respectively), initial denaturation at 95˚C for 15 min, followed by 40 cycles of 95˚C for 10 sec, 52˚C for 20 sec, 72˚C for 20 sec, and 82˚C for 15 sec; for detecting SL and r18S CT values, the reactions were carried out as previously reported [23]. Amplifications of each target gene and reference gene (18 S rRNA) cDNAs were performed in separate tubes in duplicate, and the results were analyzed using Q-Gene software (Bio Techniques Software Library at: www.BioTechniques.com). Dissociation-curve analysis was run after each real-time experiment to ensure that there was only one product. To control for false positives, a non-template negative control was run for each primer pair.

2.5. Analysis

The phylogenetic tree showing the relationship between SL amino acid sequences was generated by maximum Clustal W using DNA Star WI Megalign software. All sequences were obtained from the NCBI Gene bank according to the accession numbers. Data of the transcription of each gene are presented as mean ± SEM. The significance of the differences between group means of hormone mRNA levels was determined by one-way analysis of variance (ANOVA). In order to compare between non-mature females and the ovary of these fish containing mostly oocytes at their pre-vitellogenic stage, mature females that were kept in a group without males, and mature females, student's t-test data analysis was performed using the Statistical Package for the Social Sciences 17.0 software (Chicago: SPSS Inc.). The comparison differences were considered to be statistically significant at $p < 0.05$.

3. Results

In order to determine whether SL is expressed in brains and pituitaries, total RNA from these organs of gourami fish was reverse-transcribed. Reactions using cDNA derived from brain RNA and a set of primers specific to a segment of the gourami SL gene produced a fragment (**Figure 2**) of *T. trichopterus*. The partial length of cDNA sequence of somatolactin compared to other fish species is shown in **Figure 2**. A comparison between the amino acid sequences of SL sequences was made using the Clustal W method. The phylogenetic tree showing the relationship between somatolactin amino acid sequences compared to differences inteloses is presented in **Figure 3**.

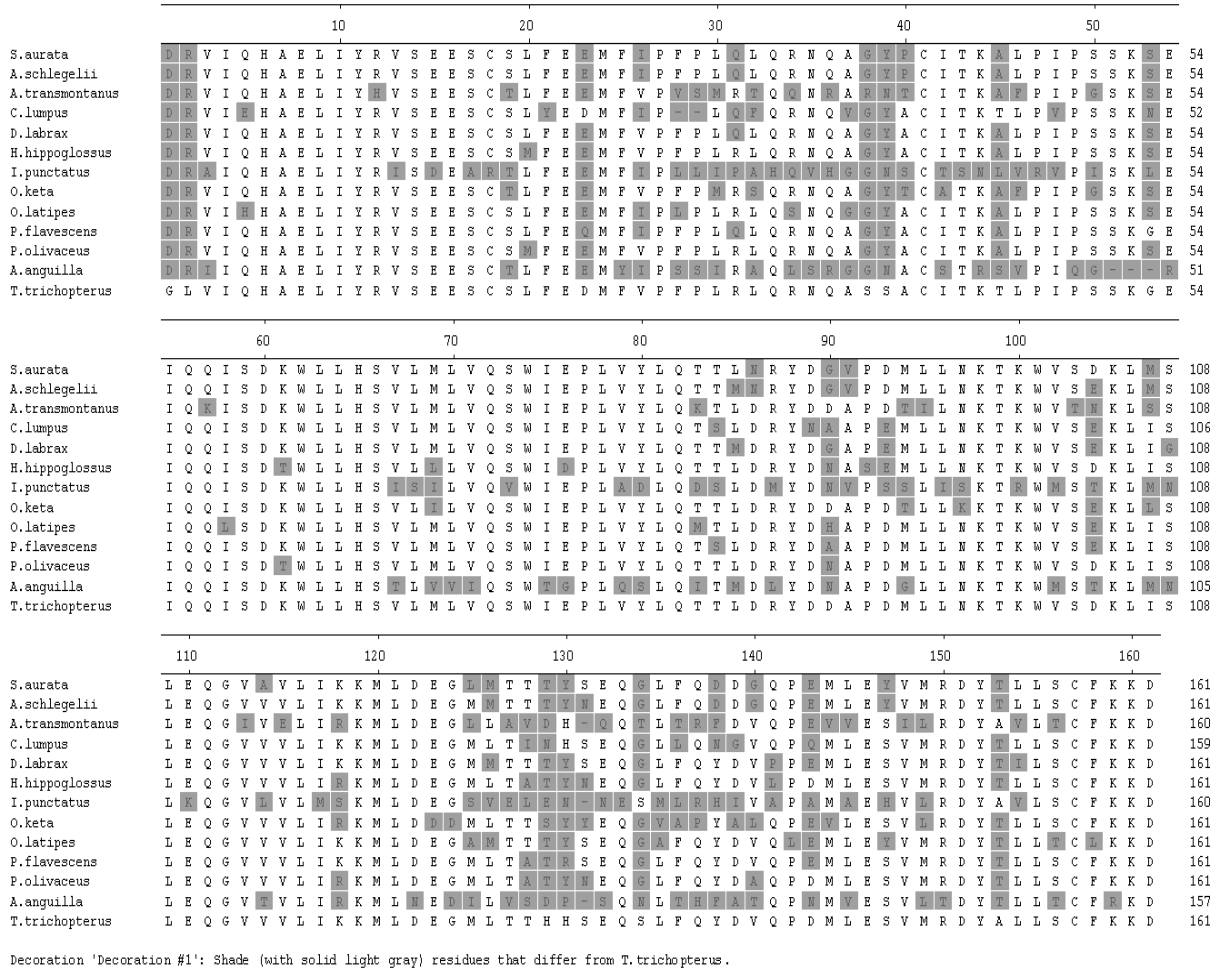

Figure 2. Comparison of cDNA sequences of *Trichogaster trichopterus* somatolactin to various fish species.

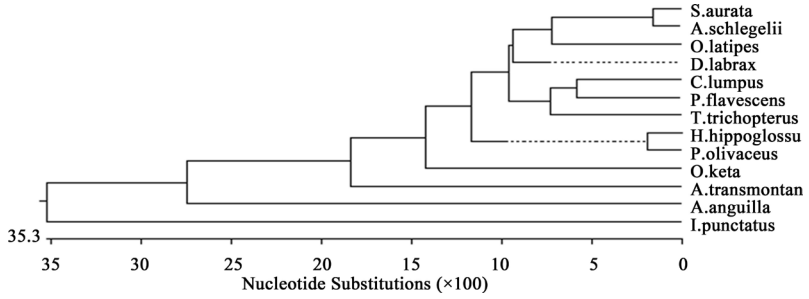

Figure 3. Phylogenetic tree showing the relationship between SL amino acid sequences. The tree was generated by maximum Clustal W using DNA Star WI Megalign software. All sequences were obtained from NCBI Gene bank according to the accession numbers provided in **Figure 2**.

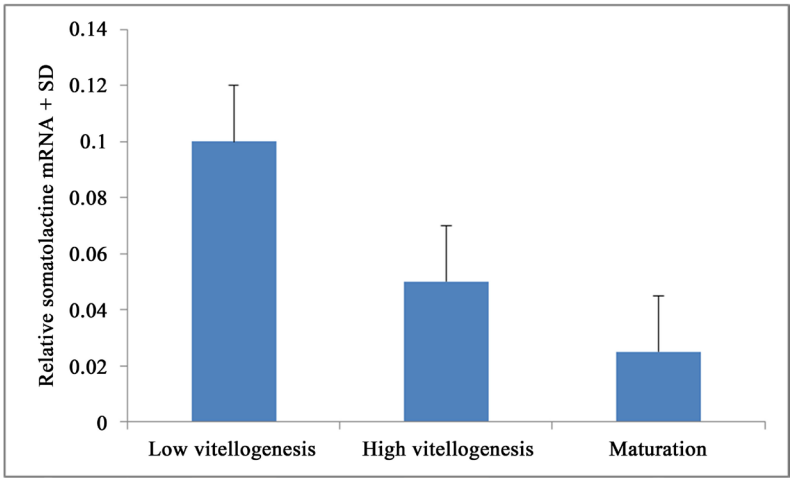

Figure 4. Relative mRNA levels of somatolactin in the pituitary of blue gourami females at different stages of oogenesis: low vitellogenesis, high vitellogenesis and maturation.

The *T. trichopterus* somatolactin sequences are very similar to *Cyclopterus lumpus* and *P. flavescens* (**Figure 3**). *T. trichopterus* somatolactin is evolutionally very distant from *Ictalurus punctatus* and *Anguilla andguilla*. In all the other fish species, the somatolactin sequence is relatively similar to *T. trichopterus*.

The mRNA of somatolactin during oogenesis is shown in **Figure 4**.

The transcription of somatolactin in the pituitary was higher in females at low vitellogenesis compared to females where oocytes were in maturation, and the difference was significant ($p < 0.05$) (**Figure 4**). However, no significant differences were found in mRNA levels between low and high vitellogenesis, and high vitellogenesis and maturation (**Figure 4**).

4. Discussion

SL has been found in all fish species studied and its structure is conserved. In the present study on partial SL of blue gourami (*T. trichopterus*) belonging to the Anabantidae family, it was compared to other fish species for the first time and the mRNA level was measured during oogenesis. The two SL forms are produced by different cells in the pituitary gland of fish [24]. Like GH and PRL, SL is thought to have multiple functions [15]. In the present study, it was found that SL might be involved in the vitellogenesis of female blue gourami and not in maturation.

There is apparently a small difference in mRNA level of PRL in blue gourami. The average level of PRL mRNA in juvenile and low vitellogenetic females was lower than in mature females (at high vitellogenesis and maturation), but the differences were not significant [7]. The GH in blue gourami seem to be involved in a similar way as are the SL and PRL hormones. The expression of GH and mRNA was measured during various stages of oogenesis: immature, low vitellogenesis, high vitellogenesis and final oocyte maturation. The highest levels of GH mRNA were found in immature females during high vitellogenesis and maturation. These results suggest that GH may play a role in the gonadal cycle of female blue gourami. High levels of GH mRNA found during the late stages of the gonadal cycle validate the concept that GH participates in reproduction [9]-[11].

Medaka (*Oryzias latipes*), a small freshwater fish, is a powerful model organism. This mutant exhibits a remarkably pale body color due to constitutively increased white pigment cells. Transcription of medaka SL in the brain (pituitary) is dramatically enhanced or suppressed when the fish is kept in a black or white tank. These results strongly suggest a conserved and major role of SLs in chromatophore regulation [25]. In blue gourami, it is well known that during sexual behavior, the oocytes reach maturation (**Figure 1**) and their color changes consider ably in males but less in females [1]. In this study, it was found that the SL mRNA level is relatively low in females during oocyte maturation.

5. Conclusion

In conclusion, the findings of this and previous studies demonstrate that SL, GH and PRL are involved in ooge-

nesis in blue gourami; however, considerably more studies are required in order to separate the functions of these hormones.

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