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EXPRESSION OF THE GROWTH HORMONE AND INSULIN-LIKE GROWTH FACTOR 1 GENES IN MALE AND FEMALE BLUE GOURAMI (*TRICHOGASTER TRICHOPTERUS*) AT DIFFERENT TEMPERATURES

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ABSTRACT

*In this study, the GH and IGF-I of blue gourami (*Trichogaster trichopterus*) were examined at different temperatures of 23°C, 27°C and 31°C. GH mRNA expression in the pituitary of males and females at various temperatures was examined using RT-PCR. The mean of mRNA levels was higher in the pituitary glands of females maintained in 23°C and 27°C than in males. However, no significant difference in GH mRNA expression was observed in the pituitary glands of females maintained at 23°C, 27°C and 31°C (ANOVA p>0.05). The GH expression is high in females at 23°C and in males at 31°C. The mean of IGF-I expression in females was high at temperatures of 27°C and 31°C compared to 23°C in brain, liver and ovary, and only 27°C in muscle. In males, a high mean of IGF-I mRNA levels was detected in brain, liver and muscle at 27°C and at all three temperatures in the testis (23°C, 27°C and 31°C); the difference among mRNA levels was not significant. In males, a high mean of IGF-I mRNA levels was detected in brain, liver, ovary and muscle at 27°C but not in the testis.*

Keywords: Blue gourami, Growth hormone, IGF-I, MRNA expression, Temperature.

Contribution/Originality

This study describes, for the first time, the effect of temperature on GH and IGF-I transcription involved in reproduction in the male and female blue gourami model of an Anabantidaes family.

1. INTRODUCTION

In teleosts, as in other vertebrate, growth and reproduction are tightly regulated, mainly via the hypothalamus-pituitary axis. The pituitary adenylate cyclase-activating polypeptide (PACAP) and its related peptide (PRP) are members of the vasoactive intestinal peptide (VIP)/secretin/glucagons family. Both peptides are encoded by a single gene (PRP-PACAP), which is further cleaved to release them to four different peptides. In vertebrates, PACAP is a neuropeptide that is associated

with the release of 63 pituitary GH [1], whereas PRP is active in teleosts and not in mammals [2]. Previously, a model of molecular evolution of these peptides suggested that the mammalian growth hormone-releasing hormone (GHRH) evolved from the non-mammalian GHRH-like peptide. However, more recently, a new GHRH peptide was discovered that is homologous to mammalian GHRH, implying that the GHRH-like peptide is homologous to the mammalian PACAP-related peptide (PRP). In fish, PACAP has a more potent effect on GH 87 secretion than does PRP [3, 4]. In addition, PACAP increased LH secretion and transcription in goldfish and tilapia, probably by a paracrine mechanism [4-6]. In rats, PACAP stimulates a variety of ovarian functions, such as the synthesis of estradiol (E) and progesterone (P) in ovarian granulosa cells through the cAMP signaling pathway [7]. However, PRP did not alter GtH release and transcription in the teleost examined [1].

The blue gourami (*Trichogaster trichopterus*) growth hormone (GH) is a 22 kDa single-chain polypeptide [8-10]. Together with prolactin, placental lactogen and somatolactin, it forms a family of related polypeptide hormones whose sequences seem to have evolved from a common ancestor [11, 12]. GH has been studied extensively, and its cDNA nucleotide sequences are available for many teleosts [13-19].

Growth hormone GH is a pituitary hormone that regulates development and somatic growth in vertebrates [20]. 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 [9, 10].

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 [21, 22], and persist during the growth and reproductive stages [23], although the growth rate slows down in maturing and spawning fish [7, 24-26].

The effect of temperature on reproduction and growth-related factors in blue gourami males under nonreproductive and reproductive conditions was studied by David and Degani [27], and Levy et al. [28].

The relative mRNA levels of brain gonadotropin-releasing hormone 3 (GnRH3), pituitary adenylate cyclase-activating polypeptide (PACAP), insulin-like growth factor-1(IGF-1), pituitary b-luteinizing hormone (β LH) and prolactin were significantly higher when the fish were maintained at 27°C than at 23°C or 31°C. β-Follicle-stimulating hormone (β FSH) mRNA levels were significantly lower when maintained at 31°C than at the other temperatures. Nests were observed only in males under reproductive conditions. In these fish, higher mRNA levels of GnRH3, PACAP, β FSH, β LH and prolactin were detected at 27°C and higher mRNA levels of IGF-1 were detected at 23°C when compared with other temperatures of maintenance or with fish that did not build nests. In conclusion, we propose that temperature has a greater effect on the transcription of genes associated with reproduction than on those pertaining to growth [27].

The effect of temperature on oogenesis and hormone gene expression related to reproduction and growth in the blue gourami female maintained under non-reproductive and reproductive conditions [28] was examined. In females under nonreproductive conditions, vitellogenic oocytes, gonadotropin-releasing hormone 3 (GnRH3), β luteinizing hormone (βLH) and growth hormone (GH) mRNA levels were affected by temperature changes. In females maintained under reproductive conditions with non-reproductively active males, a percentage of females in the final oocyte maturation (FOM) stage, pituitary adenylyl cyclase activating polypeptide (PACAP and PRP-PACAP), gonadotropins and GH mRNA levels were affected due to temperature changes [28]. In females maintained under reproductive conditions with reproductively active males, also GnRH3 and insulin-like growth factor 1 (IGF-1) were affected by temperature changes. In conclusion, in blue gourami females, changes in environmental temperature affect oogenesis through changes in brain and pituitary hormone mRNA levels [28].

The purpose of this study was to measure mRNA expression of GH and IGF-I found at three temperatures, 23ºC, 27ºC and 31C ºC, in male and female *T. trichopterus*.

2. MATERIALS AND METHODS

2.1. Fish

Mature females and blue gourami males (*T. trichopterus*), maintained and bred at MIGAL Laboratories in northern Israel, were used in this study. The fish were maintained in containers measuring 2x2x0.5 m at temperatures of 23°C, 27°C and 31°C for three weeks in groups of 10 fish, 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). The pituitaries were collected from females and males (3.5 ± 0.6 g) at the end of three weeks [27].

For sampling, each fish was anesthetized in a clove oil bath (0.25 mg/l), and weight and length were recorded. Tissues (brains, gonads, livers, pituitaries, muscle for tissue) 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.

2.1.1. 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 [29].

2.1.2. IGF-I and GH mRNA Extraction

IGF-I and GH mRNA levels were determined in the groups of male and female blue gourami reared under different temperature conditions (23°C, 27°C and 31°C). 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 GH expression studies and liver, muscles, gonads and brain for IGF-I mRNA 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 [12].

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 (Table 1).

2.1.3. Real-Time PCR

In order to compare the levels of the bgGH and bgIGF-I mRNAs, their relative abundance was normalized with an endogenous reference, the rRNA of the 18S subunit by the comparative threshold cycle (C_T) method.

This method was validated using serial dilutions (0.5, 0.1, 0.02, 0.01 and 0.005) of cDNA preparations from the pituitary (for GH) and the liver (for IGF-1). The amplification efficiencies of each target mRNA and 18S rRNA were compared by plotting ΔC_T versus log (template) according to the method of [30].

Gene-specific primers for the measurement of mRNA levels by RT-PCR were designed by means of the Primer3 Software and are listed in Table 1.

Primers P1 and P2 amplified a 154 bp fragment of 18S rRNA reference. Primers P3 and P4 amplified a 188 bp fragment of bgGH cDNA. Primers P5 and P6 amplified a 136 bp fragment of bgIGF-I cDNA. Amplification of the bgGH, IGF-I and 18S rRNA cDNAs was performed simultaneously in separate tubes and in duplicate, and the results were analyzed using Q-Gene software.

Dissociation-curve analysis was run after each real-time experiment to ensure that there was only one product. To control for false positives, a reverse-transcriptase negative control was run for each template and primer pair.

2.1.4. Statistical Analysis

Data are presented as the mean ± SEM. The significance of the differences between group means of mRNA levels was determined by one-way analysis of variance (ANOVA) followed by the Student Newman-Keuls (SNK) test using the Graph-Pad Prism software, with the level of significance in different groups set at $p < 0.05$.

Table-1. Primers used in this study.

Primer	Position	Primer Sequence
P1	329-348	TTCTCGATTCTGTGGGTGGT
P2	482-463	GAACGCCACTTGTCCCTCTA
P3	67-86	TTCACAACCGCTATGGACAA
P4	254-235	TGACGCTGCTCTTCAATCTG
P5	52-71	TCCTGTAGCCACACCCTCTC

3. RESULTS

The GH mRNA expression pituitary of females and males at various temperatures was examined by RT-PCR represented in Figures 1 and 2, respectively. The mean of mRNA levels was

higher in the pituitary glands of females maintained at 23°C and 27°C than those in males. However, no significant difference of GH mRNA expression in the pituitary glands of females maintained at 23°C, 27°C and 31°C (ANOVA $p>0.05$) was observed. A high expression of GH was found in females at 23°C and in males at 31°C (Figs. 1 and 2).

Figure-1. Relative mRNA levels (mean ± SE) of GH in pituitary glands from blue gourami females at different temperatures. There were 662 stages of reproduction: juvenile, mature non-reproductive and mature reproductive.

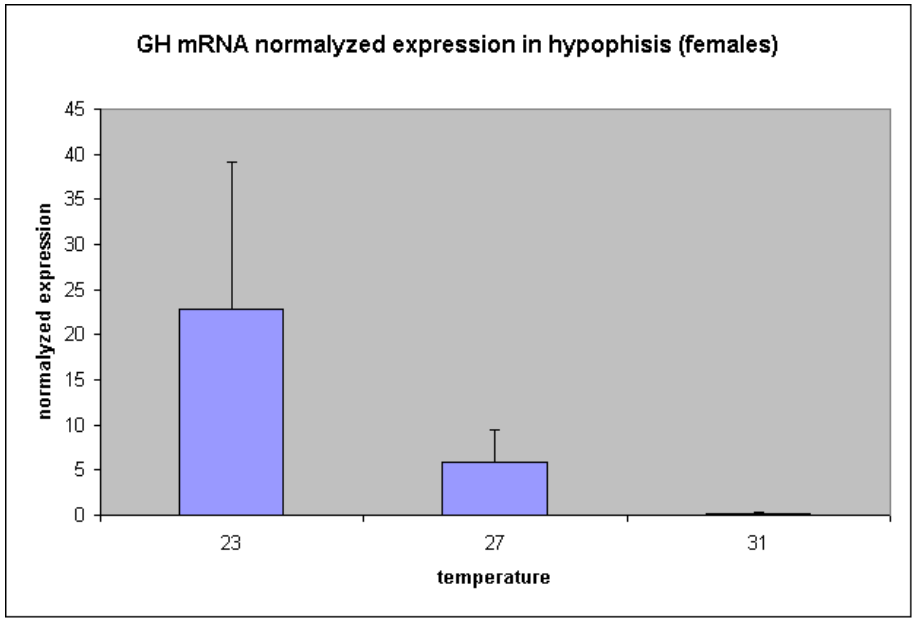
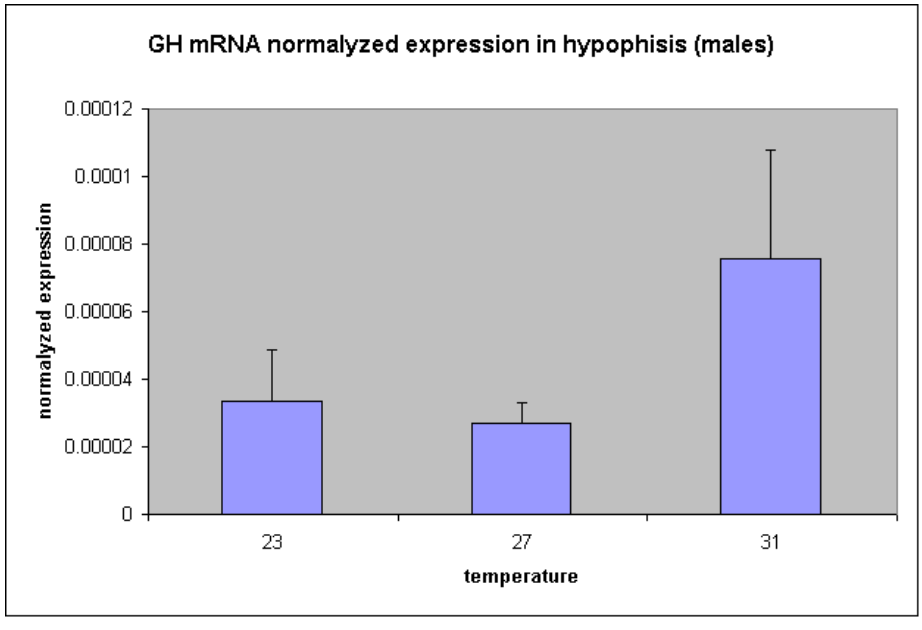


Figure-2. Relative mRNA levels (mean ± SE) of GH in pituitary glands from blue gourami males at different temperatures.



The mean of IGF-I expression found in females was high at temperatures of 27°C and 31°C compared to 23°C in brain, liver and ovary, and in muscle only at 27°C (ANOVA, $p < 0.05$) (Figs. 3, 4 and 5).

In males, a high mean of IGF-I mRNA levels was detected in brain, liver and muscle at 27°C, but at all three temperatures in the testis (23°C, 27°C and 31°C); the difference among mRNA levels is not significant (ANOVA, $p > 0.05$) (Figs. 6-10).

Figure-3. Relative mRNA levels (mean ± SE) of IGF-I in brain from blue gourami females at different temperatures.

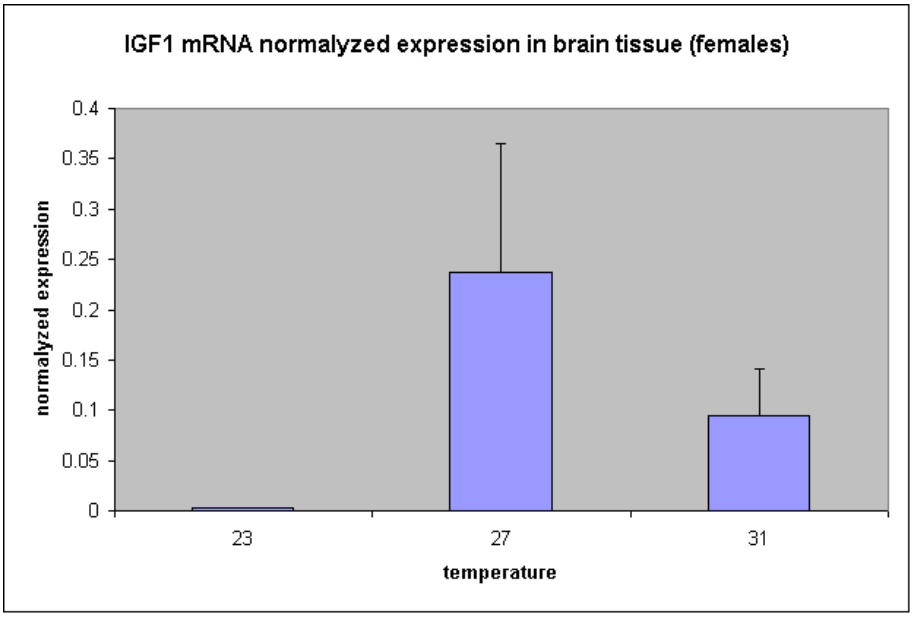


Figure-4. Relative mRNA levels (mean ± SE) of IGF-I in liver from blue gourami females at different temperatures.

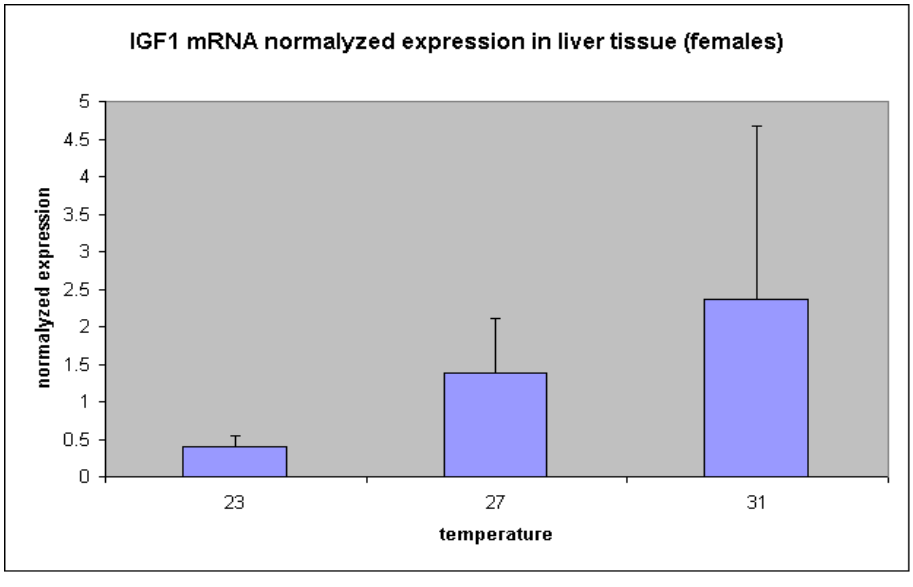


Figure-5. Relative mRNA levels (mean ± SE) of IGF-I in ovary from blue gourami females at different temperatures.

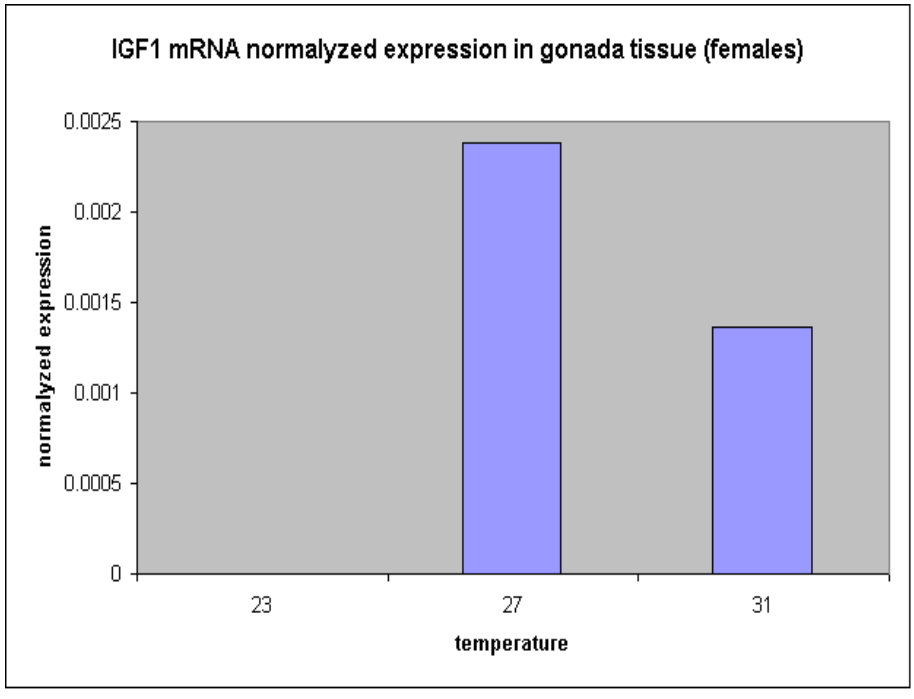


Figure-6. Relative mRNA levels (mean ± SE) of IGF-I in muscle from blue gourami females at different temperatures.

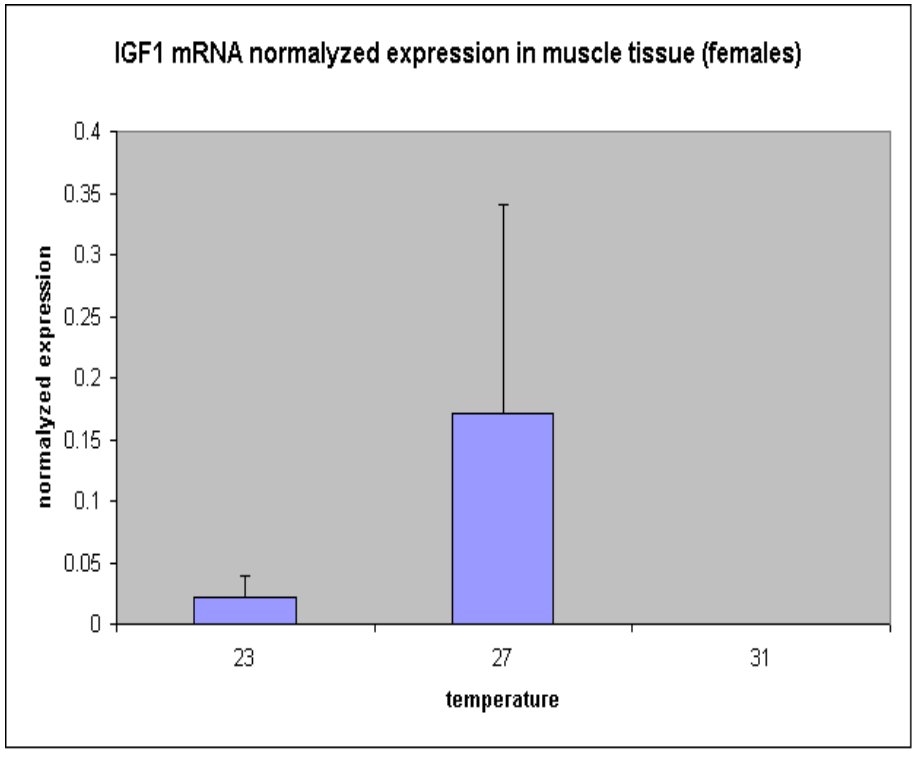


Figure-7. Relative mRNA levels (mean ± SE) of IGF-I in brain from blue gourami males at different temperatures.

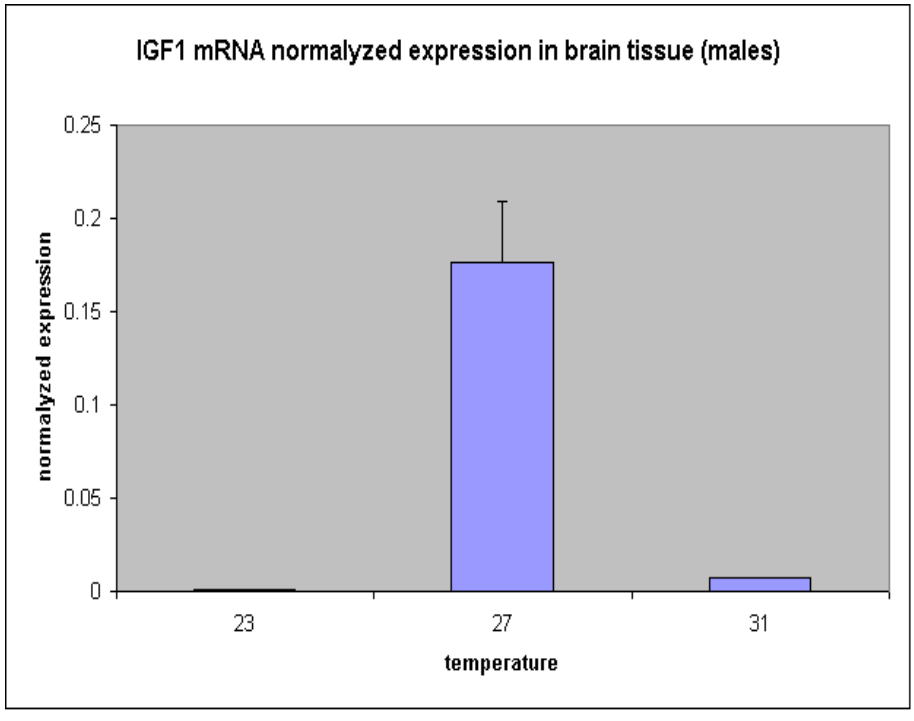


Figure-8. Relative mRNA levels (mean ± SE) of IGF-I in liver from blue gourami males at different temperatures.

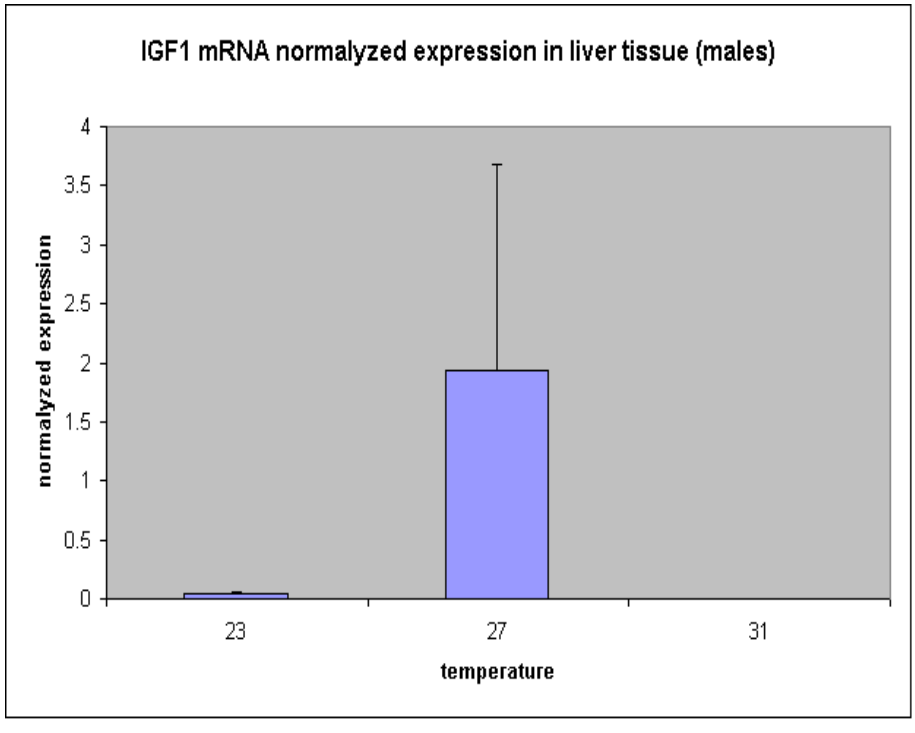


Figure-9. Relative mRNA levels (mean ± SE) of IGF-I in testis from blue gourami males at different temperatures.

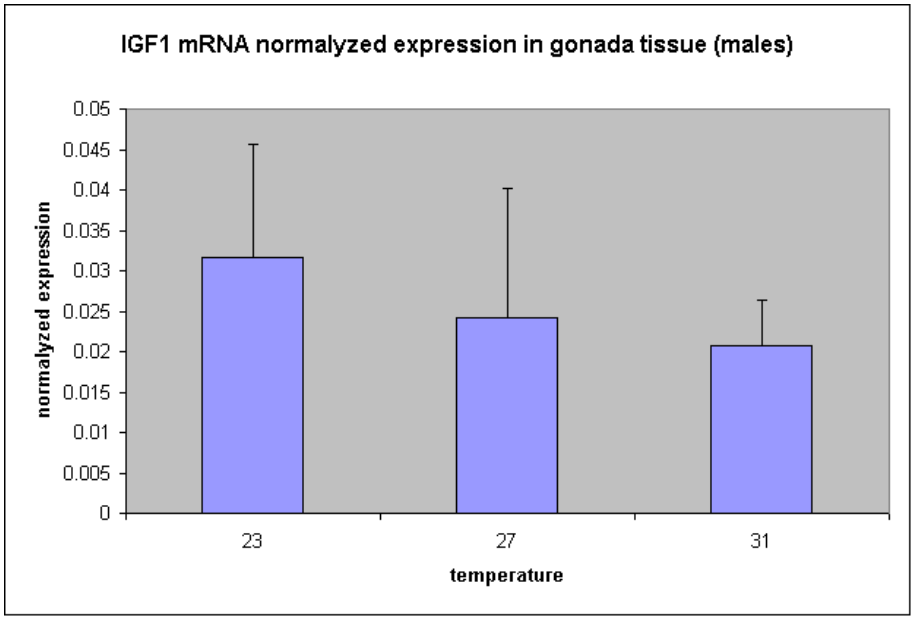
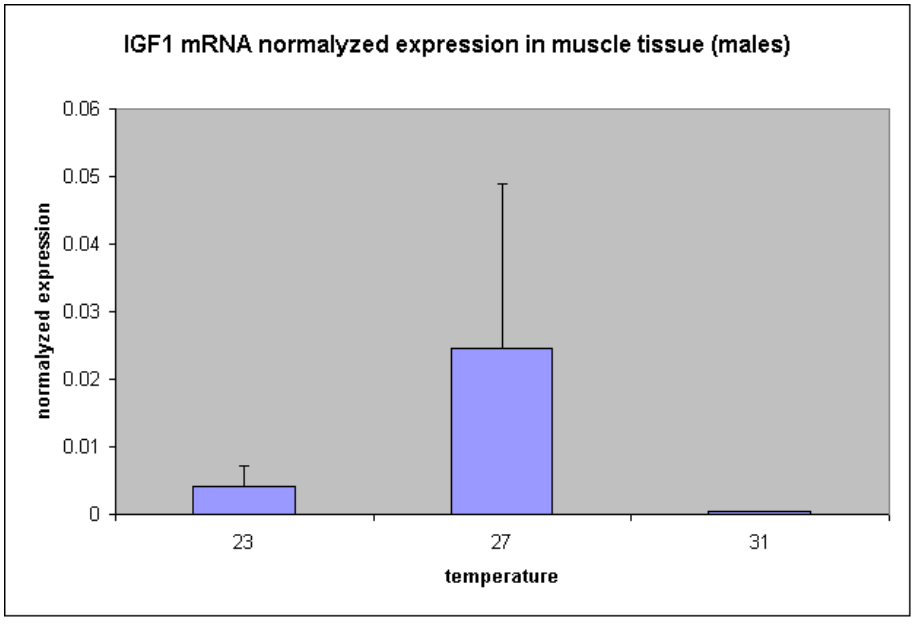


Figure-10. Relative mRNA levels (mean ± SE) of IGF-I in muscle from blue gourami males at different temperatures.



The different stages of ovary and testis development are represented in Figure 11 and Figure 12, respectively. The ovaries of females maintained at temperatures of 23°C and 31°C were found at the low oocytes vitellogenesis stage compared to the ovaries of females maintained at 27°C (Fig. 11). A high sperm count was found in the testis of males maintained at 23°C and 27°C compared to that of males maintained at 31°C . However, this difference is not very significant (Fig. 12).

Figure-11. Histological section showing different stages of gonadal development in the blue gourami female *T. trichopterus*. A) Perinucleolus stage oocyte. Bar = 25 µm. **B)** Early vitellogenic oocyte. Bar = 23 µm. **C)** Vitellogenic oocyte. Bar = 60µm. **D)** Oocyte final maturation. Bar = 60 µm. Nucleus (N), Cortical alveoli (CA), Lipid vesicle (V), Vitelline granules (Yv).

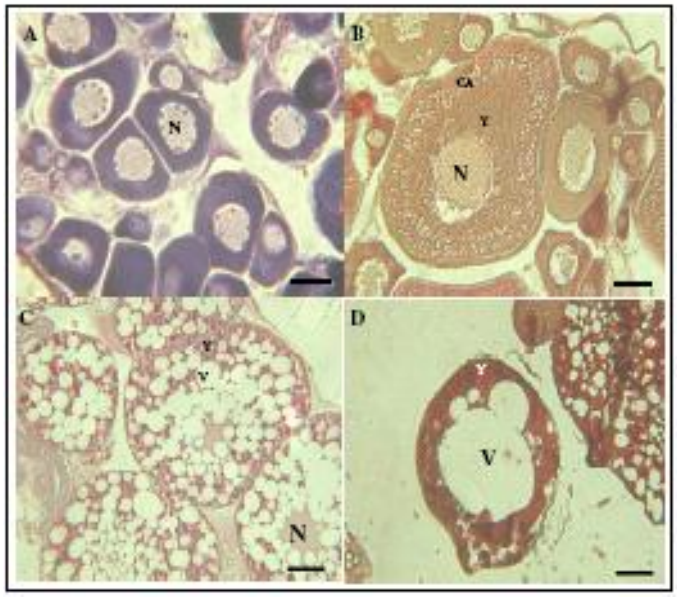
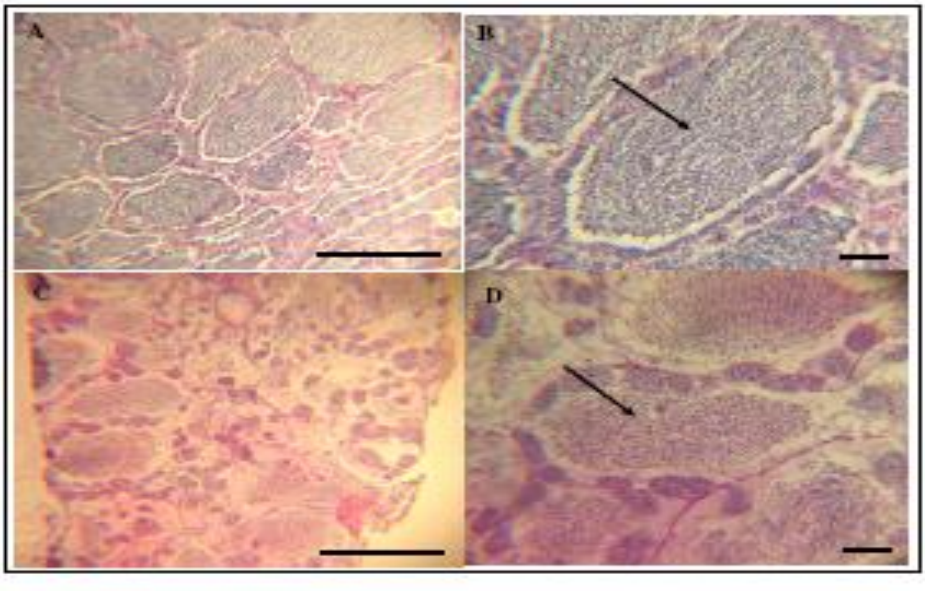


Figure-12. Histological section showing the different stages of gonadal development in the blue gourami male *T. trichopterus*: A) Mature non-reproductive fish**.** Bar = 170 µm. **B)** Mature non-reproductive fish (note the concentration of spermatozoa in the middle of the lobule; see arrow) Bar = 35 µm. **C)** Mature reproductive after spawning. Bar = 170 µm. **D)** Mature reproductive after spawning (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) Bar = 35 µm.



4. DISCUSSION

Blue gourami belongs to the Perciformes order. A comparison of the deduced amino acid sequence of bgGH clearly shows sequence and the expression in male and female gonadal development [9, 10]. In the present study, we examined the GH and IGF-I of mRNA levels in blue gourami (*Trichogaster trichopterus*) males and females at different temperatures of 23^{o}C, 27^{o}C and 31^{o}C.

Goldberg et al. [9] described the changes in GH expression during the different ovarian stages, and Degani et al. [10] studied the levels of GH mRNA in immature, vitellogenic and mature males during growth and reproduction. In the present study, the temperatures are not affected by spermatogenesis and oogensis. The various temperatures are not affected dramatically by oogenesis or spermatogenesis, or by the expression of GH and IGF-I of mRNA levels. These results are in agreement with previous studies. GH expression was high in juveniles and in mature fish found at the stages of late vitellogenesis and final oocyte maturation (FOM). Only maturing females found in early vitellogenesis (endogenous vitellogenesis) showed a significantly lower GH mRNA level. Recent studies on GH functions have confirmed other physiological effects, in addition to the well-established growth-promoting effects. Among these effects, the participation of GH in reproduction has been studied in several teleosts using different approaches, including gene expression [9] and Degani et al. [31] [10, 32]. GH mRNA was detected shortly after hatching in Sparus aurata [21] and Oncorhynchus mykiss [22] before the organogenesis of the pituitary. In adult animals, GH mRNA was detected outside the pituitary gland in several sites, including the ovary [22]. In our study, high GH mRNA levels were detected in the pituitaries of juveniles (immature fish that are still growing) and full-grown mature fish. These results contrast those described during rainbow trout oogenesis, where significantly higher levels of GH mRNA were found at the stages of exogenous vitellogenesis and post-ovulation, and not in juveniles and fish found at final oocyte maturation [23]. The expression of GH family genes is enhanced in homing chum salmon [33]. The higher levels of GH mRNA in salmonids 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 showed an increase in secretory activity during vitellogenesis [34]. In blue gourami, the occurrence of high GH mRNA levels during the different stages of the gonadal cycle is probably related to a direct influence of this hormone on reproduction, since metabolic and osmoregulatory changes do not occur during this period.

Several studies have suggested that GH may play a role in female gonadal development. A positive correlation between GH concentration and female gonadal development was described by several investigators [34-36]. GH can influence gonadal development by promoting the production of steroids [37]. In the present study, no great variation was observed in gonad stages and nor a high variation in mRNA affected by temperature. It seems that in tropical fish living in a relatively narrow temperature variation, the effect of temperature on mature fish and GH and IGF-I, which are involved in oogensis and spermatogenesis, is low as was found in the present study.

To date, absolute mRNA levels are available only for coho salmon IGF-I in liver and in some extra-hepatic tissues [38], and for IGF-I and IGF-II in rainbow trout liver [39]. Thus, limited

conclusions can be drawn regarding species diversities (or similarities) that would simplify comparisons. The IGF-I mRNA level was discovered to differ among the various tissues of Russian sturgeon. In this investigation, the highest level of IGF-I mRNA expression was detected in the kidney [40]. The IGF-I mRNA expression levels of the kidney, pituitary, and intestine differed from those of other tissues. In the present study, different mRNA levels were found in various tissues but they were not significantly affected by temperature. It is well known that GH has an effect on IGF-I in fish [41], but it seems that its mRNA is expressed at different levels, as has been described in other fish.

In conclusion, the findings demonstrate that the GH and IGF-I mRNA levels are not significantly affected by temperature, but the mean level differed. I hypothesize that if further study will be carried out with more replication, the difference in the high expression of IGF-I at a temperature 27^{o}C might be significant. The expression of mRNA GH differed for males and females, and the difference is not significantly affected by high variations. IGF-I mRNA expression varied from tissue to tissue.

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