11-Ketotesterone (Kt-11), Estradiol (E2) Level and Cytochrome P450 (Bgcyp19a) Transcription in the Testis of Male Blue Gourami *(Trichogaster Trichopterus)*

Science

KEYWORDS : Platelet-rich plasma, preparation, principles, standardization

Gad Degani

Faculty of Science and Technology, Tel-Hai Academic College, Upper Galilee 12210, Israel; MIGAL – Galilee Technology Center, P.O. Box 831, Kiryat Shmona 11016, Israel

ABSTRACT

The present study examine 11-ketotesterone (KT-11), estradiol (E2) level and cytochrome P450 (bgCYP19a) in the testis of blue gourami during maturation, sexual behavior and spermatogenesis. A significantly higher level of 11-KT was found in mature fish compared to juveniles. Testis estradiol (E2) level was lower in juveniles compared to adults, but the difference was not statistically significant. The mRNA level of bgCYP19a in the testes of juvenile males did not differ significantly (t-test, $p > 0.05$) from its level in the transcription of mature non-nest- building (non-reproductive) males. However, the bgCYP19a in the testes of reproductive males (nest-builders) increased significantly (t-test; $p < 0.05$) in reproductive males.

Introduction

Hormone control, gametogenesis, oogenesis and spermatogenesis have been studied in fish and are described in detail in several reviews, e.g.[1]. However, compared to fish species grown in aquaculture, knowledge about hormone control reproduction in the Labyrinthici suborder is very limited. The blue gourami (*Trichogaster trichopterus*) belongs to the Anabantidae family, which contains 16 genera and about 50 species distributed throughout most of southern Asia, India and Central Africa. This family belongs to the Labyrinthici suborder, which is characterized by the presence of an air-filled breathing cavity (labyrinth) located above the gills under the operculum. The blue gourami is a model study of fish reproduction and growth [2]. It is multi-spawning and male-dependent, with asynchronic ovary development [3].The sexual behavior of the male affects oocytes maturation of the female before fertilization. Gonad development and reproduction as in all vertebrates are controlled by the brain-pituitary-gonad axis (BPG). In blue gourami, relatively many hormones involved in BPG are studied in females. Gonadotropin-releasing hormone 1(GnRH1)[4] and 3 (GnRH3)[5], and pituitary adenylate cyclase activating polypeptide (PACAP – the long and short form [PRP])[6] control follicle-stimulating hormone (FSH) and luteinizing hormone (LH) [7]. FSH and LH control oogenesis by various steroids [8-11]. The key enzyme synthesis of estrogens from androgens is catalyzed by the heme-binding enzyme cytochrome P450 aromatase (CYP19) in females [12]. Spermatogenesis in blue male gourami has been studied much less. The brain and pituitary hormones in females are similar, but the steroid androgens involved differ in terms of controlling spermatogenesis, and all the different stages have been described [3, 13, 14].

The proposed study will examine 11-ketotesterone (KT-11), estradiol (E2) level and cytochrome P450 (bgCYP19a) in the testis of blue gourami during maturation, sexual behavior and spermatogenesis.

Materials and Methods

Fish

Blue gourami (*T. trichopterus*), maintained and bred in MIGAL laboratories, were used in this study. The fish were grown in containers measuring 3 x 3 x 0.5 m, at a temperature of 27ºC and under a light regime of 12 h light:12 h darkness. The fish were fed an artificial diet (45% protein, 7% fat) supplemented by live food (Artemia salina).

Males were divided into three groups according to gonadal stage and reproductive behavior: (1) juveniles; (2) mature non-reproductive; and (3) mature reproductive (nest-builders) Tissue distribution of the testis were taken for 11-ketotesterone (KT-11), estradiol (E2) level and cytochrome P450 (bgCYP19a), as described previously by [15].

For sampling, each fish was anesthetized in a clove oil bath (0.25 mg/l), and weight and length were recorded. Tissues (testis) were removed and stored in RNA Later buffer (Ambion Inc., Austin, TX). The gonads were removed, weighed and a portion was taken for histology. Total RNA was extracted from RNA Later stored tissues using the RNeasy Total RNA Kit (QIAGEN, Alameda, CA) according to manufacturer's recommendations [12].

Real-time PCR

In order to compare the levels of CYP19a mRNAs in the testis, their relative abundance was normalized with an endogenous reference, the rRNA of the 18S subunit, according to the comparative threshold cycle (CT) method [16].This method was validated using serial dilutions (0.5, 0.1,0.02, 0.01 and 0.005) of cDNA preparations from the testis (for CYP19a). The amplification efficiencies of each target mRNA and 18S rRNA were compared by plotting DCT versus log (template), according to the method of [17]. Linear regressions of the plots showed R2 values and slopes for CYP19a of 0.999 and 3.808, respectively. Gene-specific primers for the measurement of mRNA levels of CYP19a mRNA and 18S rRNA by RT-PCR were designed using Primer3 Software (Perkin-Elmer, Foster City, CA) and are listed in Table 1. Primers P1 and P2 amplified a 136 bp fragment of CYP19a cDNA; primers P3 and P4 amplified a 153 bp product of 18S rRNA.

Total RNA was prepared from individual brains or ovaries using Trizol (Invitrogen, Carlsbad, CA), and each sample was reverse-transcribed at 57ºC using the Reverse-iT1st Strand Synthesis Kit (ABgene, Surrey, UK) and random hexamers, according to manufacturer's protocols.

Amplification of the CYP19a and 18S rRNA cDNAs was performed simultaneously in separate tubes and in duplicate, and the results were analyzed using Q-Gene software (BioTechniques 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 reverse-transcriptase negative control was run for each template and primer pair.

Table 1: Primers used for the cloning and expression of blue gourami CYP19a.

Primer	Position	Primer sequence
P1	175-195	5'-ATCGGGAGCCTGGTAGAACT-30
2P	1 311-291	50-AACACGCACTGCTTGACATC-30
P3	329-348	50-TTCTCGATTCTGTGGGTGGT-30
P4	482-463	50-GAACGCCACTTGTCCCTCTA-30

Histology

Testis samples were fixed in Bouin and subsequently processed for light microscopy. Paraffin sections of 6 lm were stained with hematoxylin and eosin (Fig. 1).

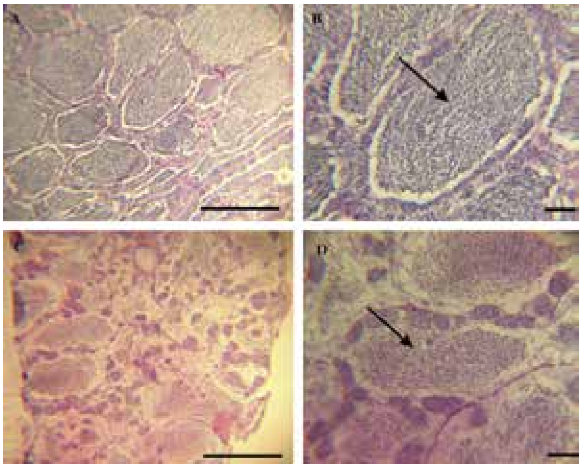

Fig. 1 Histological section showing different stages of testis development in blue gourami. (A) Testis of mature non-reproductive fish, bar = 170 lm. (B) Testis of mature non-reproductive fish (note the concentration of spermatozoa in the middle of the lobule; see arrow), bar = 35 lm. (C) Testis of mature reproductive fish during sexual behavior, bar = 170 lm. (D) Testis of mature reproductive fish during sexual behavior (note the decrease in the quantity of spermatozoa in the center of the lobule of reproductive fish compared to non-reproductive fish; see arrow), bar = 35 lm.

Statistical analysis

The relative mRNA expression levels were calculated from each set of quantified data. All results are presented as means ± SE. When appropriate, data were subjected to logarithmic transformation before being analyzed by one-way ANOVA, followed by Tukey's multiple range test. A probability level of $p < 0.05$ was used as the criterion for the significance of differences between groups. The significance of difference between sex ratios of control and treated groups was tested using the Fisher exact test.

Results

The histology of the testes in juvenile non-reproductive and reproductive males of blue gourami is presented in Figure1, classified into the following categories: immature testes without any germ cells (stage A); testes containing a small number of spermatogonia (stage B); and mature testes exhibiting active spermatogenesis with spermatocytes and spermatids (stage C).

The level of 11-ketotestosterone (11-KT) in the testis of juveniles and adults blue gourami is shown in Figure 2.

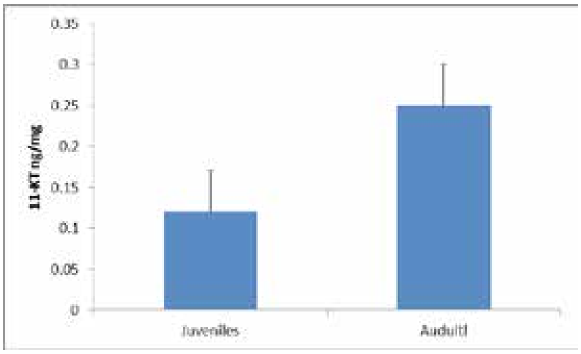

Fig. 2: 11-ketotesterone (KT-11) level in testis. Each histogram represents the average of five independent measurements (means + SE).

A significantly higher level of 11-KT was found in mature fish compared to juveniles. Testis estradiol (E2) level was lower in juveniles compared to adults, but the difference was not statistically significant (Fig. 3). The level of 11-KT in the testes varied between 0.1 and 0.25 ng/mg (Fig. 2) and was significant higher than the E2 level variation of between 0.023 and 0.02 (Fig. 3).

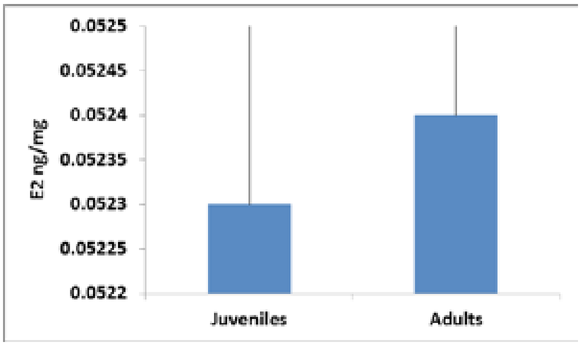

Fig. 3: Estradiol (E2) level in testis. Each histogram represents the average of five independent measurements (means + SE).

The mRNA level of bgCYP19a in the testes of juvenile males did not differ significantly (t-test, $p > 0.05$) from its level in the transcription of mature non-nest- building (non-reproductive) males. However, the bgCYP19a in the testes of reproductive males (nest-builders) increased significantly (t-test; $p < 0.05$) in reproductive males (Fig. 4).

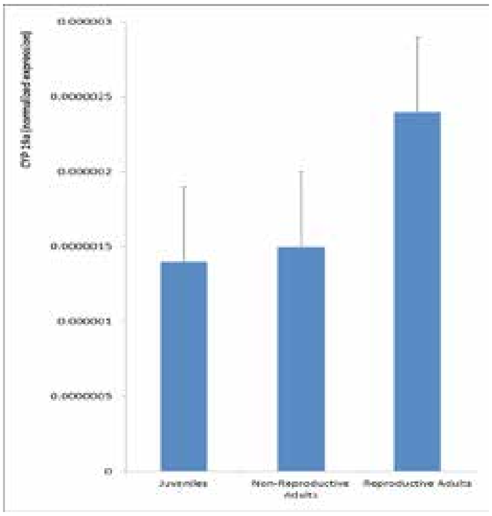

Fig. 4. CYP19a mRNA level in testis representing the average of five independent measurements (means + SE). A significant difference (t-test) exists between the level of mRNA in adult reproductive males compared to juveniles and adult non-reproductive males.

Discussion

Sexual behavior, environment, pheromones and hormones affect the spermatogenesis of blue gourami, as discussed in this paper. The specific contribution of the present study is not only the results that describe the involvement of 11-KT,E_2 and bgCYP19a during testis development and spermatogenesis, but also tries to summarize all the knowledge and propose a quality model whose parameters affect spermatogenesis in model fish belonging to the Labyrinthici suborder that have not been described in detail [1, 2]. In the present study, together with other studies on sexual behavior and steroid control, spermatogenesis improves our knowledge of reproduction control of male blue gourami. Degani 1989 [18] studied the environmental effect on sexual behavior and nest-building of blue gourami, and showed that temperature affects the male's sexual behavior. Nest-building occurs and eggs are laid in a temperature range of 23° C to 29°C. Under light conditions, most nests were built during the first two days. In general, more nests were built in darkness. The effect of temperature on hypothalamus and pituitary hormone-controlling reproduction and spermatogenesis was studied by David and Degani (2011) [14]. They found that in male blue gourami, the relative mRNA levels of brain GnRH3, PACAP, insulin-like growth factor-1(IGF-1), βLH and prolactin were significantly higher when the fish were maintained at 27°C than at 23°C or 31°C. βFSH mRNA levels were significantly lower when the fish were maintained maintained at 31°C than at the other temperatures. In reproductive males, nests were observed to have higher mRNA levels of GnRH3, PACAP, bFSH, βLH and prolactin at 27°C. In the present study, the hormones controlled by hypothalamus and gonadotropins hormones, 11-KT and E2 level in the testes of mature fish, and bgCYP19a in the testes of reproductive males (nest-builders) increased significantly compared to non-reproductive males. Moreover, it supports the hypothesis that not only the environment factor affected the BPG by hormones but also the pheromones. Davies and Pilotte (1975) [19] and Ingersoll and Lee (1980) found no response by the male to any possible chemical signal by the female [20]. Additional studies are required to prove if it affects all or only some components of BPG, however our hypothesis is that the pheromones affect sexual behavior and the hormones control reproduction, e.g., gonadotropins, 11-KT and E2 level [13].

Based on the results of the present study and our knowledge of sexual behavior, and pheromone and hormone control of spermatogenesis in blue gourami, we proposed the model described in Figure 5.

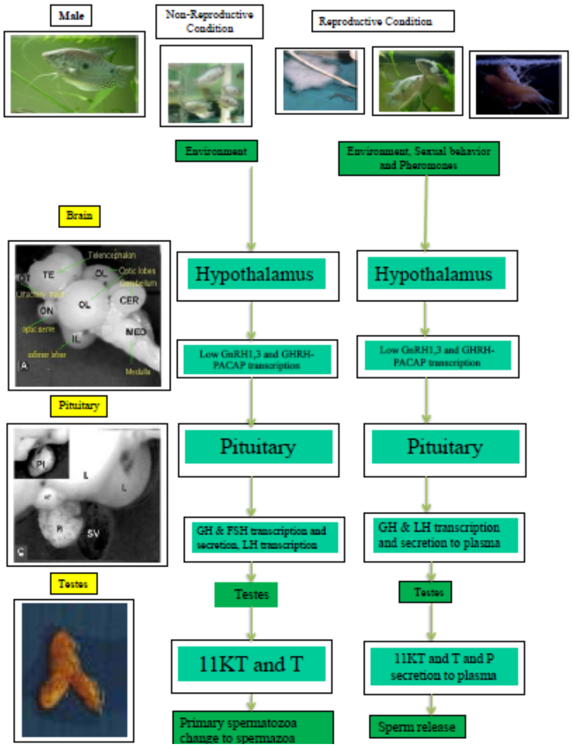

Fig. 5. Based on previous studies in our laboratory and the present study, we subjected quality models of the relationship between sexual behavior, pheromones and hormones involved in spermatogenesis in blue gourami.

REFERENCE

[1] R.W.S.a.T. Miura, Spermatogenesis and its endocrine regulation, Fish Physiology and Biochemistry, 26 (2002) 43–56. | [2] G. Degani, Blue gourami (Trichogaster trichopterus) model for Labyrinth fish, Laser Pages Publishing, Israel, 2001. | [3] G. Degani, K. Jackson, D. Goldberg, R. Sarfati, R.R. Avtalion, betaFSH, betaLH and growth hormone gene expression in blue gourami (Trichogaster trichopterus, Pallas 1770) during spermatogenesis and male sexual behavior, Zoolog Sci, 20 (2003) 737-743. | [4] G. Levy, G. Degani, Involvement of GnRH, PACAP and PRP in the reproduction of blue gourami females (Trichogaster trichopterus), J Mol Neurosci, 48 (2012) 603-616. | [5] G. Levy, Y. Gothilf, G. Degani, Brain gonadotropin releasing hormone3 expression variation during oogenesis and sexual behavior and its effect on pituitary hormonal expression in the blue gourami, Comp Biochem Physiol A Mol Integr Physiol, 154 (2009) 241-248. | [6] G. Levy, G. Degani, Evidence of a reproduction-related function for pituitary adenylate cyclase-activating polypeptide-related peptide in an Anabantidae fish, J Mol Endocrinol, 46 (2011) 101-110. | [7] G. Degani, TRANSCRIPTION OF FSH-β, LH-β AND GROWTH HORMONE IN | FEMALE TRICHOGASTER TRICHOPTERUS (PALLAS 1770) | TREATED WITH GnRH ANALOG, The Molecular Basis | Of Environmental Adaptation, 1-5 (2004) 45-57. | [8] G. Degani, The effects of human chorionic gonadotropin on steroid changes in Trichogaster trichopterus, Comp Biochem Physiol A 96 (1990) 525-528. | [9] G. Degani, R. Boker, Sensitivity to maturation-inducing steroids and gonadotropin in the oocytes of blue gourami Trichogaster trichopterus (Anabantidae, Pallas, 1770), Gen Comp Endocrinol, 85 (1992) 430-439. | [10] G. Degani, R. Boker, Vitellogenesis level and the induction of maturation in the ovary of the blue gourami Trichogaster trichopterus (Anabantidae, Pallas 1770). J. Exp. Zool., 263 (1992b) 330 - 337. | [11] G. Degani, Gal, E. & Vaya, J. , In vitro biosynthesis of steroids in ovary of female Trichogaster trichopterus (Pallas, 1770), Comp. Biochem. Physiol., 109 (1994) 715-723. | [12] M. Ezagouri, S. Yom-Din, D. Goldberg, K. Jackson, B. Levavi-Sivan, G. Degani, Expression of the two cytochrome P450 aromatase genes in the male and female blue gourami (Trichogaster trichopterus) during the reproductive cycle, Gen Comp Endocrinol, 159 (2008) 208-213. | [13] E. Mananos, Y. Zohar, G. Degani, The relationship between gonadotropin and sexual behavior of male Trichogaster trichopterus (Pallas), Indian J. Fish, 44 (1997) 239-246. | [14] D. David, G. Degani, Temperature affects brain and pituitary gene expression related to reproduction and growth in the male blue gouramis, Trichogaster trichopterus, J Exp Zool A Ecol Genet Physiol, 315 (2011) 203-214. | [15] G. Degani, K. Jackson, D. Goldberg, R. Sarfati, R.R. Avtalion, betaFSH, betaLH and growth hormone gene expression in blue gourami (Trichogaster trichopterus, Pallas 1770) during spermatogenesis and male sexual behavior, Zoolog Sci., 20 (2003) 737-743. | [16] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods, 25 (2001) 402-408. | [17] P.Y. Muller, H. Janovjak, A.R. Miserez, Z. Dobbie, Processing of gene expression data generated by quantitative real-time RT-PCR, Biotechniques., 32 (2002) 1372-1374, 1376, 1378-1379. | [18] G. Degani, The effect of temperature, light, fish size and container size on breeding of Trichogaster trichopterus., Israeli Journal of Aquaculture Bamidgeh, 41 (1989) 67 73. | [19] D.R.E.a.P.N. J., f, ttraction to conspecific | and non-specific chemical stimuli in male and | female Macropodus opercularis (Teleosti, Anabantoidai), Biol. Bihav., 13 (1975) 191-196. | [20] I.D.W.a.L.C. T., Belontiidae chemosignal | activity: interspecific attraction and perceived | chemosignal similarity, Behav. Neurol. Biol., 29 (1980) 463-472.