Brazilian Journal of Biology®

ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

Original Article

Differential gene expression pattern and plasma sex steroids during testicular development in *Genyatremus luteus* (Perciforme: Haemulidae) (Bloch, 1790)

Padrão diferencial de expressão gênica e esteróides sexuais plasmáticos durante o desenvolvimento testicular em *Genyatremus luteus* (Pisces: Haemulidae) (Bloch, 1790)

D. L. S. Ribeiro[a], L. S. S. Ribeiro[b], N. P. C. Bezerra[c], J. M. Silva[c], K. S. Noleto[a], F. A. Souza[d], A. V. Carvalho-Neta[e], Z. S. Almeida[e†], D. P. Chaves[b], and J. R. S. Torres-Junior[f*]

[a]Universidade Federal do Maranhão – UFMA, Rede de Biodiversidade e Biotecnologia da Amazônia Legal – BIONORTE, São Luís, MA, Brasil
[b]Universidade Estadual do Maranhão – UEMA, Departamento de Patologia, São Luís, MA, Brasil
[c]Universidade Estadual do Maranhão – UEMA, Departamento de Engenharia de Pesca, São Luís, MA, Brasil
[d]Fundação Oswaldo Cruz – FIOCRUZ, Instituto Oswaldo Cruz, Laboratório de Imunomodulação e Protozoologia, Rio de Janeiro, RJ, Brasil
[e]Universidade Estadual do Maranhão – UEMA, Departamento de Química e Biologia, São Luís, MA, Brasil
[f]Universidade Federal do Maranhão – UFMA, Departamento de Oceanografia e Limnologia, São Luís, MA, Brasil
[†]*in Memorian*

Abstract

The aim of the current study is to evaluate gene expression patterns of LH (*lhr*) and estrogen (*er*) receptors and plasma steroid levels during testicular development in *Genyatremus luteus*. Males were histologically classified as immature (n=7), maturing (n=7) and mature (n=7), based on the cellular structure of their testes. Plasma 11-KT concentration recorded peak at the final maturation stage. The highest plasma 17α-OHP concentrations were observed at the immature stage; they decreased at the maturation and mature stages. On the other hand, 17β-estradiol (E2) recorded higher concentrations at the maturation stage. *Er* expression has significantly increased along the maturational development of animals' testes. The mRNA observed for the LH receptor has decreased from immature to maturing stage; it presented expression peak at the mature stage. There was high association between receptor gene expression and plasma steroid levels, mainly E2. The current study was the first to feature different reproductive maturation stages in male *G. luteus* specimens, based on cellular, endocrine and molecular aspects. In addition, it has shown that the gene expression profile for *er* and *lhr* receptors, as well as plasma 11-KT and E2 concentrations, are directly linked to testicular maturation, although they are not necessarily associated with the gonadosomatic index.

Keywords: receptors, estradiol, gonadal maturation, reproduction, grunt.

Resumo

O objetivo deste estudo foi avaliar os padrões de expressão gênica dos receptores de LH (*lhr*) e de estrogênio (*er*) e dos níveis de esteróides plasmáticos durante o desenvolvimento testicular de *Genyatremus luteus*. Os machos foram classificados histologicamente em imaturos, em maturação e maduros, de acordo com a estrutura celular dos testículos. A concentração plasmática de 11-KT apresentou um pico na fase de maturação final (P<0.05). As maiores concentrações plasmáticas de 17α-OHP foram encontradas no estádio imaturo (P<0.05), com consequente diminuição nos estádios em maturação e maturo. O 17β-estradiol (E2) apresentou maiores níveis de concentração no estádio em maturação (P<0.05). A expressão de *er* aumentou significativamente ao longo do desenvolvimento maturacional dos testículos (P<0.05). O mRNA para o receptor de LH diminuiu do estádio imaturo para o estádio em maturação (P<0.05) com consequente pico de expressão no estádio maduro. Houve alta relação entre a expressão gênica dos receptores e os níveis de esteróides plasmáticos, especialmente com E2. Em conclusão, este estudo caracterizou pela primeira vez, sob os aspectos celular, endócrino e molecular, os diferentes estádios de maturação reprodutiva em machos de *G. luteus*, demonstrando que o perfil da expressão gênica para os receptores *er* e *lhr*, bem como as concentrações plasmáticas de 11-KT e E2 foram diretamente relacionados à maturação testicular, apesar de não se relacionarem necessariamente com o índice gonadossomático.

Palavras-chave: receptores, estradiol, maturação gonadal, reprodução, roncador.

*e-mail: jose.torres@ufma.br
Received: March 13, 2022 – Accepted: July 20, 2022

This is an Open Access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

The hypothalamic-pituitary gonadal (HPG) axis is the most important endocrine system controlling the reproductive process in teleosts, as well as in other vertebrate species; it is activated at puberty onset (Okuzawa, 2002). Spermatogenesis is mainly regulated by the pituitary gland, which secretes hormones capable of stimulating gonadal steroid hormones.

In teleosts, are produced in distinct cells forming their own functional networks (Levavi-Sivan et al., 2010; Golan et al., 2016; Santiago-Andres et al., 2021). Steroid hormones such as testosterone and estradiol are significantly involved in the transmission of gonadotropic signals in vertebrates. However, in many male teleosts, the testosterone derivative, 11-ketotestosterone (11-KT) plays a central role in controlling gonad development and spermatogenesis (Devlin and Nagahama, 2002; Schulz et al., 2010).

FSH and LH act by activating their cognate receptors expressed in gonadal cells (Levavi-Sivan et al., 2010). Thus, Leydig cells are stimulated by FSH to release 11-ketotestosterone (11-KT), which activates Sertoli cells in order to produce activin B. Then, activin B induces spermatogonial mitosis to enable spermatocyte formation and spermatogenesis (Miura and Miura, 2003; Levavi-Sivan et al., 2010).

Furthermore, during the early stages of spermatogenesis, FSH regulates the expression of a variety of genes in the teleost fish test (Sambroni et al., 2013; Nóbrega et al., 2015; Rajakumar and Senthilkumaran, 2020), including anti-Müllerian hormone (AMH) (Skaar et al., 2011; Oliveira et al., 2021) and insulin growth factor-3 (IGF3) (Sambroni et al., 2013; Melo et al., 2015; Nobrega et al., 2015; Li et al., 2021). AMH acts by inhibiting the management of spermatogonia and IGF3 regulates the expression of genes related to spermatogonia (Skaar et al., 2011; Nóbrega et al., 2015). Thus, changes in plasma 11-KT levels, FSH and LH gene expression and AMH and IGF3 expression are indicators that indicate the onset of puberty in teleost fish.

Sperm maturation is regulated by LH, which induces maturation-inducing steroid production (MIS; 17α,20β-dihydroxy-4-pregnen-3-one in most fish species, and 17α,20β-21-trihydroxy- 4-pregnen-3-one in different marine perciformes) (Nagahama, et al., 1994; Cavaco et al., 2001; Zohar et al., 2010). MIS production in sperm is mediated by 20β-hydroxysteroid dehydrogenase (20βHSD), which converts the 17α-hydroxyprogesterone synthesized in Leydig cells. Specific enzymes are activated in response to MIS production and this process increases seminal plasma pH and the number of cAMPs in sperm, which, in their turn, induce sperm capacitation and spermiation (Miura and Miura, 2003; Hatef and Unniappan, 2019).

Knowledge about spermatogenesis in fish is limited to some species used in basic research and/or in aquaculture biotechnology, such as Atlantic salmon (*Salmo salar*) (Maugars and Schmitz, 2006; Trombley and Schmitz, 2013; Schulz et al., 2019), zebrafish (*Danio rerio*) (Kwok et al., 2005; Assis et al., 2018; Fallah et al., 2021), European seabass (*Dicentrarchus labrax*) (Rocha et al., 2007; Molés et al., 2011; Pinto et al., 2018), Japanese eel (*Anguilla japonica*) (Jeng et al., 2007; Kazeto et al., 2008; Suzuki et al., 2020), rainbow trout (*Oncorhynchus mykiss*) (Sambroni et al., 2007; Middleton et al., 2019) and Nile tilapia (*Oreochromis niloticus*) (Aizen et al., 2012; Thönnes et al., 2020).

Species *Genyatremus luteus* belongs to order Perciformes and to family Haemulidae, which is popularly known as the grunt fish family. It is classified as estuarine-marine species, with preferentially coastal habitat (Giarrizzo and Krumme, 2007). This species is widely distributed in South America, from Eastern Colombia to Brazil, where it inhabits coastal water with sand, rocks and muddy bottoms, mostly estuaries and lagoons (Cervigón, 1966; Artigas et al., 2003).

The investigated species has shown great potential to be used for extractive fishery (Almeida et al., 2005) and, above all, to enhance marine fish farming due to its meat quality and economic value (Marques et al., 2016). Studies about *G. luteus* available in the literature often address seasonal variations in its commercial capture (Fernandes et al., 2015), hormone-induced spawning (Marques et al., 2016) and species' diet in Northeastern Brazil (Almeida et al., 2005; Fernandes et al., 2017). According to Noleto-Filho et al. (2012), *Genyatremus luteus* has aquaculture-related potential, since it is capable of adapting to captive environments and shows trophic plasticity.

Sexual maturation and spermatogenesis are complex processes that require the highly coordinated regulation of several genes. Although the endocrine system plays crucial role in these processes, the exact mechanisms involved in the sexual maturation of fish remain poorly understood. Studies focused on featuring gonadotropin receptors (*fshr* and *lhr*) have been recently carried out to help better understanding the role played by them in regulating sex steroid hormones and testicular development in several fish species (Maugars and Schmitz, 2008; Burow et al., 2020; Kitano et al., 2022).

Thus, it is essential investigating the hormonal profile and gene expression of cell receptors during the gonadal development of male *G. luteus* individuals to help better understanding the mechanisms involved in their sexual maturation process. The aim of the present study was to evaluate the sex steroid profile and differential expression of reproductive hormone receptors in male *G. luteus* individuals at different sexual maturation stages.

2. Materials and Methods

2.1. Fish and sample collection

The present research was approved by the Ethics Committee on Animal Use (CEUA) of Federal University of Maranhão, under protocol n. 23115.004707 / 2017-50, as well as authorized by the Biodiversity Authorization and Information System (ICMBio - SISBIO), under protocol number 66551.

Male *Genyatremus luteus* specimens were captured through artisanal fishing with the aid of hand line made of two hooks, based on validated methodologies (Almeida et al., 2005; Marques et al., 2016). Sampling took place in São José Bay (02º 43 '03 "S, 44 12' 03" W),

Maranhão State Coast, Coastal Equatorial Amazon, Brazil, from June to September 2019.

Immediately after capture, the animals were anesthetized by immersion in a benzocaine solution at a concentration of 0.1 g/L, and a blood sample was collected by puncturing the fish gills with a heparinized syringe (Liquemine, Roche®). The samples were kept on ice until centrifugation (1,500g for 10 min); the plasma was aliquoted and frozen at -80°C until the time of hormonal analysis.

Subsequently, specimens were euthanized by exposure to freezing cold water and subjected to laparotomy right away in order to enable collecting gonad fragments (5 x 5 mm) that were individually stored in RNAlater (Ambion®) and kept frozen at -20ºC, for RNA extraction purposes.

Fish biometric data were measured with ichthyometer, at 1 mm measurement scale. The following biometric parameters were measured: total length (TL), which corresponded to the distance (in cm) from the tip of the cranial end to the end of the caudal fin; and partial length (PL), which corresponded to the distance from the tip of the cranial end to the last vertebra. Subsequently, total weight (TW) was recorded in precision scale (accuracy = 0.01 grams).

Gonads were analyzed based on macroscopic aspects such as size, color and vascularity; they were also excised and weighed to calculate the gonadosomatic index (GSI), by taking into consideration variables such as body weight rate and total weight of testis, based on the following formula [GSI = (gonad weight / body weight) x 100] (Vazzoler, 1996). In addition, fragments of the median region of fish's testis were collected for histological analysis, after fixation in Bouin's solution and processing based on routine histological methods (Yoshida, 1964).

Males were categorized based on the macroscopic aspects of their gonads, as well as on the histological configuration of testicular lobes and spermatogenic cell lineages. It was done by following the gonadal maturation scale developed for Hamulids, according to Shinozaki-Mendes et al. (2013), and adapted to three maturation stages, namely: immature (n=7), maturing (n=7), mature (n=7) - 21 specimens, in total.

2.2. Steroid analyses

Plasma 17β-estradiol (E2), 17-α-hydroxy-progesterone (17α-OHP) and 11-ketotestosterone (11KT) levels were determined through Enzyme-linked immunosorbent assays (ELISA) (IBL International, Hamburg, Germany, for E2; and 17α-OHP, and Cayman Chemicals Company, Michigan, USA, for 11KT). Tests were carried out based on manufacturers' recommendations. Pilot trials were initially conducted by using five dilutions (1: 1, 1: 2, 1: 4, 1: 8 and 1:16) in two samples at different maturation stages (immature and mature). It was done to establish appropriate dilutions to assays' detection limits - 1:2 dilution was established for assays comprising all three hormones. In addition, plasma samples were analyzed in duplicate, whereas test kits were validated by calculating the intra and inter-assay coefficients of variation (% CV). The detection limit of the assay was 10.6 pg / mL, for E2; 30 pg / mL, for 17α-OHP; and 1.3 pg / mL, for 11-KT. Absorbance measurements were performed in microplate reader (Spectra MAX 250). Plasma E2 and 17-OHP concentrations were determined at wavelength of 450 nm, whereas 11KT concentrations were determined at wavelength of 405 nm. E2, T and 11-KT (pg / mL) sample concentrations were calculated based on a standard curve by applying a logit-log curve fit, based on the following equation: LogitDO: log (DO / (100- DO)), as recommended by the manufacturer.

2.3. Total RNA isolation and reverse transcription

Total RNA was extracted from individual testis samples in 1 mL of Trizol (Invitrogen; Carlsbad, CA, USA) for *er* and *lhr* expression analysis, based on manufacturer's instructions. RNA pellet was dissolved in DEPC-treated water and quantified based on its absorbance at OD260 / OD280. RNA integrity was assessed in 1.5% agarose gel. All RNA samples were treated with DNase (DNase I, Applied Biosystems, Washington, UK); cDNA was synthesized with 1.5 µg of RNA by using the commercial kit SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA), based on manufacturer's instructions, with final volume of 20 µL per reaction. Oligo-dT primers were used in this rapid reverse transcriptase kit for reverse transcription processes; cDNA was diluted 3 times in Nuclease-Free Water and stored at -20°C.

2.4. Quantitative real-time PCR (qPCR)

Primers used for *lhr* and β-actin were described by Rhody et al. (2015) and Wang et al. (2009), respectively. Primers used for estrogen receptor were designed in Primer 3 software, based on the AB007453.1 sequence, which is available in the NCBI database, as described in Table 1. To evaluate the specificity of the primers, cDNAs from *G. luteus* were submitted to conventional PCR. The specific fragments were purified using the Wizard SV Gel and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) and an ABI PRISM 3100 Genetic Analyzer. The *lhr* and *er* mRNA sequences obtained for *G. luteus* were confirmed by alignment with previously deposited sequences at GenBank http://www.ncbi.nlm.nih.gov/) using the BLASTN interface.

Duplicates of each cDNA sample were amplified through SYBR quantitative real-time PCR (qPCR). This procedure was followed by melting curve analysis to check whether each PCR product only has a unimodal dissociation curve. The qPCR was performed based on using 2.5 µL of cDNA, 10 µM of the specific pair primer for each gene and 12.5 µL of SYBR Green qPCR SuperMix (Thermofisher, Carlsbad, CA), at final volume of 25 µL per reaction.

PCR efficiency of each primer pair was evaluated based on standard curves from a graded series of diluted cDNA (3-fold; 9-fold; 27-fold; 81-fold; 243-fold); it was done to make sure that the PCR efficiency of the selected primer pair would range from 90% to 100% (R2 > 0.99).

The following qPCR parameters were herein adopted by using the ABI 7500 Real-time PCR System (Applied Biosystems, USA): 50ºC for 2 min, 95ºC for 10 min, 40 cycles of 95ºC for 15 s and 60ºC for 1 min. Data were analyzed based on the comparative Ct method (Livak and

Table 1. List of genes and primers used in quantitative PCR.

Gene	Primers	Genbank Accession No.	Annealing Temperature (°C)
lhr-F	TCCTCCTGGTGTGGACCCAGTT	KF314819	62
lhr-R	TCGGGTTGCAGGCTCTCAAAGG		
er-F	ACATGTACCCCGAAGACAGC	AB007453.1	60
er-R	CAGGGGTGGAGTGGCTATAA		
β-actin-F	AATCGCCGCACTGGTTGTTG	AY190686	57
β-actin-R	ACGATACCAGTGGTACGACC		

Schmittgen, 2001). Ct values were normalized based on β-actin expression (Wang et al., 2009).

2.5. Statistical analysis

Frequencies of each gonadal maturation stage were analyzed through Fisher's exact test, based on Sampaio (2002), in GraphPadInstat® software (GraphPad Software, San Diego, CA). The remaining data analyses were performed in the Analysis System for Windows SAS® software (version 9.4. SAS Inst. Inc., Cary, NY).

Shapiro-Wilk test (SAS PROC UNIVARIATE application) was used to investigate residue normality for each variable. Data transformation was carried out, whenever necessary (logarithm to basis 10 - Log10 X), in order to meet the assumptions in the analysis of variance.

Continuous dependent variables of normal distribution (ichthyological data) were expressed as mean and standard error of the mean (mean ± SEM). They were subjected to ANOVA; means recorded for each gonadal maturation stage were compared to each other through Duncan's test (PROC GLM of SAS). E2, 17-OHP and 11KT concentrations, as well as differential cDNA expression for estrogen (*er*) and LH (*lhr*) receptors, presented non-normal distribution. Thus, the model was adjusted for Poisson distribution and its associations with animals' maturational reproduction status were subjected to Poisson regression analysis (PROC GLIMMIX of SAS), based on Wang et al. (2014) and Svensson et al. (2019).

The association between variables was investigated based on the Principal Component Analysis (PCA) method, in Statistica 7.1 software (Statsoft, Inc., 2007), according to which, two graphically produced axes represented the strongest data pattern. In order words, it explains the important role played by the two main components in total data variation. Significance level of 5% was adopted to reject H0 (null hypothesis), i.e., significance level lower than 0.05 has evidenced the effect of classificatory variables and of interactions among them.

3. Results

3.1. Reproductive maturational stages and GSI analyses

The testes of *G. luteus* individuals were featured as paired, elongated, and fusiform organs located inside the coelomic cavity, dorsolaterally to the gas bladder and dorsally to the digestive tract. Macroscopic analysis has evidenced different coloration, consistency, and vascularization patterns in the gonads of male *G. luteus* individuals, depending on their maturational development stage. Immature testes have shown translucent color, whereas the mature ones presented milky-white color.

Specimens were microscopically classified into three maturation stage: a) Immature, b) Maturing, c) Mature (Figure 1). The spent stage was not observed in the herein investigated animals. Male germ cell types were determined by taking into consideration the histological appearance of the cytoplasm, nucleus, and cell size. Based on these observations, spermatogenic cells were identified as follows: primary (sg1) and secondary (sg2) spermatogonia; primary (sc1) and secondary (s2) spermatocytes; spermatids (sd) and sperm.

The analyzed specimens presented total body length ranging from 13 cm to 22.5 cm (mean body length was 17.18 ± 0.73 cm); minimum and maximum standard length of 11 cm and of 20 cm, respectively, (mean standard length was 14.29 ± 0.63); and furcal length ranging from 12.5 cm to 23 cm (mean furcal length was 16.33 ± 0.70). Total weight ranged from 38 g to 220g (mean weight was 96.95 ± 12.96), and the combined weight of the two stimulators ranged from 0.10g to 1.3g (mean combined weight was 0.33 ± 0.07). The comparison based on maturational stage did not show any statistical difference in ichthyometric and GSI data between the analyzed groups (Table 2).

3.2. Plasma steroids

Plasma 11-KT concentration has significantly increased at the final maturation stage ($P<0.05$), although it did not show significant difference in the transitional process of reproductive maturation in comparison to that of the immature status ($P>0.05$) (Figure 2). The highest plasma 17α-OHP concentrations were observed at the immature stage ($P<0.05$). These concentrations have shown progressive decrease at the maturation and mature stages. There was increase in plasma 17β-estradiol concentrations from the immature stage to the maturing one ($P<0.05$), as well as decreased concentrations of it at the mature stage ($P<0.05$).

3.3. Gene Expression Profiles for er and lhr

Estrogen receptor mRNA expression has significantly increased throughout the maturational development of

Table 2. Ichthyometric and GSI data analyzed during the reproductive cycle of male *Genyatremus luteus* specimens. Data are presented as mean ± SEM.

Variable	Reproductive Maturational Stages		
	Immature	Maturing	Mature
Total Length (cm)	15.78± 0.79	16.64± 1.60	19.10 ± 1.04
Furcal Length (cm)	14.95 ± 0.79	15.75 ± 1.51	18.28 ± 0.98
Standard Length (cm)	13.07 ± 0.64	13.92 ± 1.45	15.85 ± 0.88
Total Weight (g)	68.00 ± 10.60	100.71 ± 28.34	122.14 ± 58,78
Eviscerated Weight (g)	61.78 ± 9.55	91.28 ± 26.93	111.71 ± 20.68
Gonadal weight (g)	0.27 ± 0.10	0.27 ± 0.10	0.45 ± 0.07
GSI (%)	1.54 ± 0.31	1.72 ± 0.58	2.14 ± 0.77

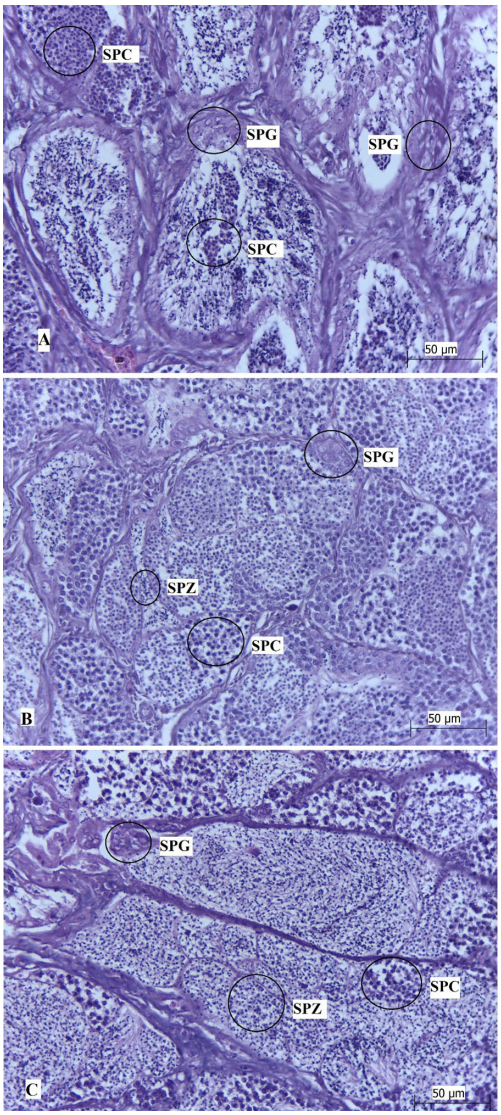

Figure 1. Photomicrographs of germ cell and testes development stages of *Genyatremus luteus*. Stages were determined as (A) Immature, (B) Maturing, (C) Mature. Abbreviations are as follows: SPG, spermatogonia; SPC, spermatocyte; SPZ, spermatozoa. All panels were at 60x magnification.

the testes (P<0.05), as shown in Figure 3A. LH receptor expression has decreased from the immature stage to the maturing one (P<0.05), but it peaked at the mature stage (Figure 3B).

3.4. Association among ichthyometric variables, GSI, hormonal profile and gene expression

The Principal Component Analysis (PCA) presented in Figure 4 has shown association among ichthyological parameters, gonadosomatic index, hormonal profile, and testis gene expression of *lhr* and *er* in male *G. luteus* individuals. Results have shown that the two main components (together) explained 59.78% of total data variation, with emphasis on Component 2, since its ichthyometric variables were more representative due to the fact of having longer vectors that were closer to Component 2 axis.

Plasma E2 concentration, and *er* and *lhr* gene expression, were highly related to each other, since they formed sharp angles between the irrespective vectors determined by greater mature stage representation for these parameters. Likewise, plasma 11-KT concentration and total weight (TW) were also highly related to each other. Based on Figure 3, variables mostly contributing to Component 1 were *lhr* and *er* expression, as well as the ichthyological parameters, which were inversely related to each other.

4. Discussion

The current research provided new information about physiological and transcriptional mechanisms capable of regulating gonadal development in male *G. luteus* individuals. Morphological and histological criteria were used to classify male individuals according to the following reproductive stages: immature, when spermatogonia and spermatocytes were observed in specimens; maturing, when animals presented spermatocytes undergoing active spermatogenesis and lumen formation in the lobule devoid of sperm; and mature, when animals presented peripheral region filled with germ cells, in addition to more central acini and lumen densely filled with spermatozoa. Such findings agree with what has been described for most

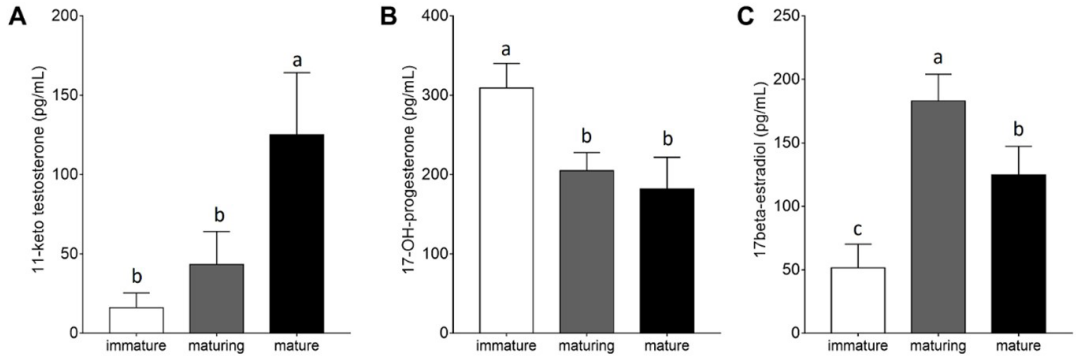

Figure 2. Steroid concentrations in the blood plasma of male *Genyatremus luteus* individuals during their reproductive cycle. (A) 11-ketotestosterone. (B) 17 α-hidroxy progesterone. (C) 17β-estradiol. Data are represented as mean ± SEM. abc: indicates statistically significant difference ($p<0.05$).

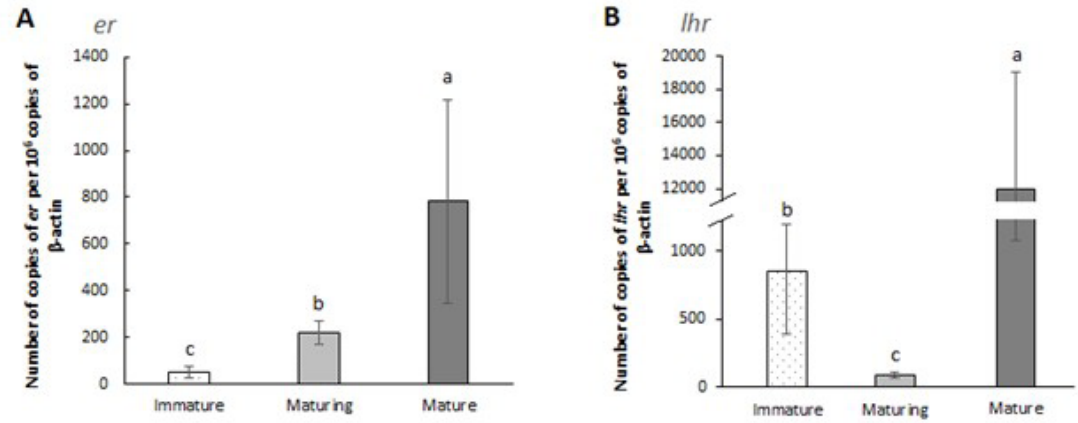

Figure 3. Gene expression of *er* (A) and *lhr* (B) in the male gonad of *Genyatremus luteus* individuals at different maturation stages. Columns represent arithmetic mean and standard error of the mean. [abc] Different letters indicate statistically significant difference ($p<0.05$).

teleost species (Brown-Peterson et al., 2011; Nishimura and Tanaka, 2014; Siqueira-Silva et al., 2019; Felicio et al., 2021).

The herein analyzed ichthyological parameters and GSI data did not show variation during testicular development in *G. luteus*. Male individuals are often smaller than the female ones, likely due to males' selection for early maturation and to the need of less reproductive effort, which reduces male growth and nutritional demand (Parker, 1982; Endler, 1983; Andersson, 1994; Barbieri et al., 2001).

Low plasma 11-KT levels were herein detected in immature males, although they have increased during gonadal development until reaching peak concentration at mature stage. Studies have shown that 11-KT stimulates all spermatogenesis stages, including the early mitotic phase, through the stimulus produced by FSH in the pituitary gland (Miura et al., 1991; Planas and Swanson, 1995; Middleton et al., 2019). Gonadotropin signal transduction stimulates 11-ketotestosterone production (11-KT), which is one of the main androgens found in fish (Miura et al., 1991); 11-KT affects target cell function by activating androgens' nuclear receptors.

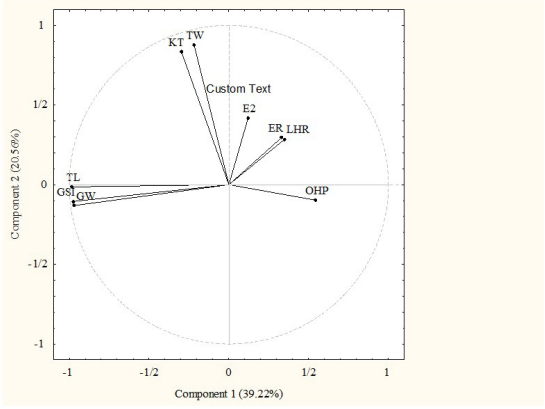

Figure 4. Principal component analysis (PCA) used to classify the influence of *lhr* and *er* gene expression, plasma steroids (11-KT, 17-OHP and E2), ichthyological parameters and GSI on male *G. luteus* individuals. Legend: LHR = LH receptor; ER = estrogen receptor; KT = 11-ketotestosterone; E2 = 17β-estradiol; OHP = 17-α-hydroxy-progesterone; TW = total weight; TL = total length; GW = gonad weight; GSI = gonadosomatic index.

Study conducted with African catfish (*Clarias gariepinus*) has shown that 11-KT stimulates spermatogenesis whereas other androgens (T, DHT, and androstenedione) have no effects on it (Cavaco et al., 1998; 2001). Plasma FSH and 11KT levels in Chinook Salmon and Rainbow Trout increase during spermatogenesis in spring, whereas plasma LH levels remain low or undetectable until spermiogenesis and spermiation (Prat et al., 1996; Gomez et al., 1999; Campbell et al., 2003).

Forsgren and Young (2012) reported that treatment with Androgen receptor (*Ar*) antagonist has inhibited 11-KT effects on spermatogenesis, a fact that suggests that 11-KT acts via *Ar* in *Oncorhynchus kisutch*. Taken together, seasonal changes in 11-KT, as well as its stimulating effects on spermatogenesis in male individuals and on follicular development in female individuals, have evidenced physiological roles played by 11-KT in regulating reproduction processes in fish.

Plasma E2 concentrations in male *G. luteus* individuals recorded low levels at the immature stage, although these levels increased and peaked at the mature and mature stages, respectively. According to Miura et al. (1999), E2 plays important role in the spermatogonial turnover of Japanese eel (*Anguilla japonica*). These very same authors have shown that low E2 concentrations acted at the early spermatogonial development stages through estradiol receptors in Sertoli cells, which stimulated and maintained spermatogonia proliferation before the progression to later spermatogenesis stages. Plasma E2 levels in trout have shown transient elevation early in the reproductive cycle (Gomez et al., 1999).

The role played by E2 during spermatogenesis is not fully understood. It is suggested that this hormone participates in different biological processes, such as spermatogonial stem cell renewal, lipid metabolism, protein metabolism and folding, as well as in intercellular chemical communication (Amer et al., 2001; Pinto et al., 2006). Furthermore, estrogens are also known to influence both male and female behavior (Filby et al., 2012).

Plasma 17-OHP levels in *G. luteus* remained high during all maturation stages; the highest levels of it were observed at the immature stage. During spermatogenesis, 17α-OHP is converted into 17α,20 β-dihydroxy-4-pregnen-3-one (DHP), which is the hormone accounting for inducing spermiation and for stimulating sperm motility (Baynes and Scott, 1985; Miura et al., 1992). Furthermore, 17α-OHP is also precursor of other sex steroids such as T and E2 (Yaron and Levavi-Sivan, 2011). This finding suggests that the decrease 17α-OHP concentration observed during testicular maturation in *G. luteus* may be associated with increasing conversion of this hormone into T, E2 or DHP.

Er expression has gradually increased during testicular development in *G. luteus*. Study conducted with male *Anoplopoma fimbria* individuals recorded increased transcription levels for three estrogen receptors (*er1, er2a, er2b*) during testicular development, mainly for the *er1* gene, which was highly correlated to *lhb* mRNA levels (Guzmán et al., 2018). This outcome suggests that estrogens can act in gonadotropic cells by activating estrogen response elements and the *lhb* promoter gene. On the other hand, Morini et al. (2017) conducted a study with *Anguilla anguilla* and showed higher *er* expression at its early development stage (spermatogonia A). All three *er* expression types have significantly decreased at the spermatogonia and spermatocyte stages, as well as remained low until the end of spermatogenesis (Morini et al., 2017). This outcome suggests that E2 acts as spermatogonial renewal factor mediated by estrogen receptors.

With respect to the gene expression of LH receptor, mRNA levels remained low during the immature and maturation stages, although they have considerably increased at the mature stage; this finding suggests the participation of this receptor at the end of testicular development. Such results were also observed in male *Oncorhynchus mykiss, Salmo salar L, Seriola quinqueradiata* and *Oryzias latipes* individuals and showed that *lhr* expression has steadily increased as testicular maturation progressed (Rahman et al., 2003; Kusakabe et al., 2006; Maugars and Schmitz, 2008; Burow et al., 2020). There are few studies in the literature about the regulation of gonadotropin receptors in male fish. *Lhr* mRNA levels in mammals are differentially regulated by its cognate hormone, depending on the gonadal development stage (Ascoli et al., 2002, Walker and Cheng, 2005). Experimental studies conducted with mice have shown that *lhr* mRNA levels in Leydig cells have significantly increased after LH and testosterone presence (Shan et al., 1995). On the other hand, immature swine Leydig cells exposed to recombinant LH presented *lhr* downregulation (Lejeune et al., 1998).

Suzuki et al. (2020) conducted a study with *Anguilla japonica* and established a cell line capable of producing recombinant LH, in order to evaluate the binding specificity to its receptor, and its effects on steroidogenesis *in vitro*. They identified *lhr* mRNA in Sertoli and Leydig cells; in addition, they reported low LH receptor expression levels in immature testes, like the ones observed in the current study. Chauvigné et al. (2014) have shown that LH directly induces spermiogenesis through the *lhr* located in spermatid cell membranes.

According to previous reports, *lhr* in coho salmon (*Oncorhynchus kisutch*) and African catfish (*C. gariepinus*) is selective for its cognate gonadotropin (Yan et al., 1992; Vischer et al., 2003). In addition to LH, FHS can also interact with and activate the LH receptor in other species such as amago salmon (*O. rhodurus*) and rainbow trout (*O. mykiss*) (Oba et al., 1999a; 1999b; Sambroni et al., 2007). We believe that this very same process may take place in *G. luteus*, and it would explain the higher *lhr* expression (although low) observed at the immature stage that, in its turn, could be activated by FSH. However, it is necessary conducting further studies to confirm this hypothesis.

Data resulting from the principal component analysis (PCA) have shown high correlation between the gene expression of LH and ER receptors, and the serum levels of E2 and 11-KT. This outcome suggests that LH triggers spermatogenic events through steroid production in *G. luteus* (Levavi-Sivan et al., 2010).

The same results observed in the current study had already been reported for Japanese eel (*A. japonica*) by Suzuki et al. (2020), who conducted an experiment using a cell line with recombinant Fsh and Lh, and observed that reFsh has stimulated its cognate receptor, whereas reLh

has activated both receptors. Furthermore, reFsh and reLh induced testicular 11-KT production. Effective reLh doses were apparently lower and their effects emerged faster than those of reFsh.

In conclusion, the current study was the first to feature different reproductive maturation stages in male *G. luteus* individuals, based on cellular, endocrine, and molecular aspects. In addition, it has evidenced that the gene expression profile observed for estrogen and LH receptors, as well as plasma 11-KT and E2 concentrations, are directly linked to testicular maturation in *G. luteus*, although they are not necessarily associated with the gonadosomatic index.

Acknowledgements

The authors are grateful to CAPES, for the financial support and for granting the scholarship to the first author. They would also like to thank Maranhão State Research, Scientific and Technological Development Support Foundation (FAPEMA - Fundação de Amparo à Pesquisa e ao Desenvolvimento Científico e Tecnológico do Maranhão) for the financial support provided to this research (UNIVERSAL Process n. 00640/17).

References

AIZEN, J., KOWALSMAN, N., KOBAYASHI, M., HOLLANDER, L., SOHN, Y.C., YOSHIZAKI, G., NIV, M.Y. and LEVAVI-SIVAN, B., 2012. Experimental and computational study of inter- and intra-species specificity of gonadotropins for various gonadotropin receptors. *Molecular and Cellular Endocrinology*, vol. 364, no. 1-2, pp. 89-100. http://dx.doi.org/10.1016/j.mce.2012.08.013. PMid:22954681.

ALMEIDA, Z.S., NUNES, J.L.S. and ALVES, M.G.F.S., 2005. Dieta alimentar de *Genyatremus luteus* (Bloch, 1790) - (Teleostei, Perciformes: Haemulidae) na Baía de São José, Maranhão, Brasil. *Atlântica*, vol. 27, no. 10, pp. 39-47. http://dx.doi.org/10.5088/atlântica.v27i1.2207.

AMER, M.A., MIURA, T., MIURA, C. and YAMAUCHI, K., 2001. Involvement of Sex Steroid Hormones in the Early Stages of Spermatogenesis in Japanese Huchen (*Hucho perryi*). *Biology of Reproduction*, vol. 65, no. 4, pp. 1057-1066. http://dx.doi.org/10.1095/biolreprod65.4.1057. PMid:11566726.

ANDERSSON, M., 1994. *Sexual selection. Monographs in behavior and ecology*. Princeton, New Jersey: Princeton University Press, 624 p.

ARTIGAS, L.F., VENDEVILLE, P., LEOPOLD, M., GUIRAL, D. and TERNON, J.F., 2003. Marine biodiversity in french guiana: estuarine, coastal, and shelf ecosystems under the influence of amazonian waters la biodiversidad marina en guyana francesa: los ecosistemas de estuarios, las costas y plataformas bajo la influencia de las aguas amazonicas. *Gayana*, vol. 67, no. 2, pp. 302-326. http://dx.doi.org/10.4067/S0717-65382003000200013.

ASCOLI, M., FANELLI, F. and SEGALOFF, D.L., 2002. The Lutropin/Choriogonado-tropin receptor, a 2002 perspective. *Endocrine Reviews*, vol. 23, no. 2, pp. 141-174. http://dx.doi.org/10.1210/edrv.23.2.0462. PMid:11943741.

BARBIERI, G., SALLES, F.A. and CESTAROLLI, M.A., 2001. Reproductive and nutritional dynamics of *Salminus maxillosus* Valenciennes, 1849 (Pisces, Characidae) at Mogi Guaçu river, state of São Paulo, Brasil. *Acta Scientiarum*, vol. 23, pp. 441-444. http://dx.doi.org/10.4025/actascibiolsci.v23i0.2698.

BAYNES, S.M. and SCOTT, A.P., 1985. Seasonal variations in parameters of milt production and in plasma concentration of sex steroids of male rainbow trout (*Salmo gairdneri*). *General and Comparative Endocrinology*, vol. 57, no. 1, pp. 150-160. http://dx.doi.org/10.1016/0016-6480(85)90211-4. PMid:3972242.

BROWN-PETERSON, N.J., WYANSKI, D.M., SABORIDO-REY, F., MACEWICZ, B.J. and LOWERRE-BARBIERI, S.K., 2011. A standardized terminology for describing reproductive development in fishes. *Marine and Coastal Fisheries*, vol. 3, no. 1, pp. 52-70. http://dx.doi.org/10.1080/19425120.2011.555724.

BUROW, S., MIZRAHI, N., MAUGARS, G., VON KROGH, K., NOURIZADEH-LILLABADI, R., HOLLANDER-COHEN, L., SHPILMAN, M., ATRE, I., WELTZIEN, F.A. and LEVAVI-SIVAN, B., 2020. Characterization of gonadotropin receptors Fshr and Lhr in Japanese medaka, *Oryzias latipes. General and Comparative Endocrinology*, vol. 285, pp. 113276. http://dx.doi.org/10.1016/j.ygcen.2019.113276. PMid:31536722.

CAMPBELL, B., DICKEY, J.T. and SWANSON, P., 2003. Endocrine changes during puberty onset in male spring Chinook salmon, *Oncorhynchus tshawytscha. Biology of Reproduction*, vol. 69, no. 6, pp. 2109-2117. http://dx.doi.org/10.1095/biolreprod.103.020560. PMid:12930714.

ASSIS, L.H.C., NÓBREGA, R.H., GÓMEZ-GONZÁLEZ, N.E., BOGERD, J. and SCHULZ, R.W., 2018. Estrogen-induced inhibition of spermatogenesis in zebrafish is largely reversed by androgen. *Journal of Molecular Endocrinology*, vol. 60, no. 4, pp. 273-284. http://dx.doi.org/10.1530/JME-17-0177. PMid:29476039.

CAVACO, J.E.B., BOGERD, J., GOOS, H.J.T. and SCHULZ, R.W., 2001. Testosterone inhibits 11–ketotestosterone-induced spermatogenesis in African catfish, *Clarias gariepinus. Biology of Reproduction*, vol. 65, no. 6, pp. 1807-1812. http://dx.doi.org/10.1095/biolreprod65.6.1807. PMid:11717145.

CAVACO, J.E.B., VILROKX, C., TRUDEAU, V.L., SCHULZ, R.W. and GOOS, H.J.T., 1998. Sex steroids and the initiation of puberty in male African catfish (*Clarias gariepinus*). *The American Journal of Physiology*, vol. 275, no. 6, pp. R1793-R1802. http://dx.doi.org/10.1152/ajpregu.1998.275.6.R1793. PMid:9843868.

CERVIGÓN, F., 1966. *Los peces marinos de Venezuela*. 2nd ed. Caracas: Fundación Científica Los Roques, vol. 4, 254 p.

CHAUVIGNÉ, F., ZAPATER, C., GASOL, J.M. and CERDÀ, J., 2014. Germ-line activation of the luteinizing hormone receptor directly drives spermiogenesis in a nonmammalian vertebrate. *Proceedings of the National Academy of Sciences of the United States of America*, vol. 111, no. 4, pp. 1427-1432. http://dx.doi.org/10.1073/pnas.1317838111. PMid:24474769.

DEVLIN, R.H. and NAGAHAMA, Y., 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture*, vol. 208, no. 3-4, pp. 191-364. http://dx.doi.org/10.1016/S0044-8486(02)00057-1.

ENDLER, J.A., 1983. Natural and sexual selection on color patterns in poeciliid fishes. *Environmental Biology of Fishes*, vol. 9, no. 2, pp. 173-190. http://dx.doi.org/10.1007/BF00690861.

FALLAH, H.P., RODRIGUES, M.S., ZANARDINI, M., NÓBREGA, R.H. and HABIBI, H.R., 2021. Effects of gonadotropin-inhibitory hormone on early and late stages of spermatogenesis in ex-vivo culture of zebrafish testis. *Molecular and Cellular Endocrinology*, vol. 520, pp. 111087. http://dx.doi.org/10.1016/j.mce.2020.111087. PMid:33249103.

FELICIO, G.R., CORDEIRO, J.G., DUTRA-COSTA, B.P., MAXIMINO, C., BRANCO, G.S., QUIRINO, P.P. and SIQUEIRA-SILVA, D.H., 2021. Gonadal characterization of the Amazonian fish *Serrapinnus*

kriegi (Characidae: cheirodontinae). *Brazilian Journal of Biology = Revista Brasileira de Biologia*, vol. 83, pp. e249158. http://dx.doi.org/10.1590/1519-6984.249158. PMid:34550290.

FERNANDES, S.C.P., BENTES, A.B., PEREIRA, L.J.G., NASCIMENTO, M.S. and BENTES, B.S., 2015. Variação temporal da captura comercial do peixe pedra, *Genyatremus luteus*, desembarcado em um polo pesqueiro da costa norte do Brasil-Península de Ajuruteua- Bragança-PA. *Boletim do Instituto de Pesca*, vol. 41, no. 1, pp. 173-182.

FERNANDES, S.C.P., PEREIRA, L.J.G., NASCIMENTO, M.S., ANDRADE, C.E.R. and BENTES, B., 2017. Biologia populacional do peixe pedra, *Genyatremus luteus* (Bloch, 1790), na costa amazônica brasileira. *Boletim do Instituto de Pesca*, vol. 43, no. 4, pp. 527-541. http://dx.doi.org/10.20950/1678-2305.2017v43n4p527.

FILBY, A.L., PAULL, G.C., SEARLE, F., ORTIZ-ZARRAGOITIA, M. and TYLER, C.R., 2012. Environmental estrogen-induced alterations of male aggression and dominance hierarchies in fish: a mechanistic analysis. *Environmental Science & Technology*, vol. 46, no. 6, pp. 3472-3479. http://dx.doi.org/10.1021/es204023d. PMid:22360147.

FORSGREN, K.L. and YOUNG, G., 2012. Stage-specific effects of androgens and estradiol-17beta on the development of late primary and early secondary ovarian follicles of coho salmon (Oncorhynchus kisutch) in vitro. *Biology of Reproduction*, vol. 87, no. 3, pp. 64. http://dx.doi.org/10.1095/biolreprod.111.098772. PMid:22674392.

GIARRIZZO, T. and KRUMME, U., 2007. Spatial differences and seasonal cyclicity in the intertidal fish fauna from four mangrove creeks in a salinity zone of the Curuçá Estuary, North Brazil. *Bulletin of Marine Science*, vol. 80, no. 3, pp. 739-754.

GOLAN, M., MARTIN, A.O., MOLLARD, P. and LEVAVI-SIVAN, B., 2016. Anatomical and functional gonadotrope networks in the teleost pituitary. *Scientific Reports*, vol. 6, no. 1, pp. 23777. http://dx.doi.org/10.1038/srep23777. PMid:27029812.

GOMEZ, J.M., WEIL, C., OLLITRAULT, M., LE BAIL, P.-Y., BRETON, B. and LE GAC, F., 1999. Growth hormone (GH) and gonadotropin subunit gene expression and pituitary and plasma changes during spermatogenesis and oogenesis in Rainbow Trout (*Oncorhynchus mykiss*). *General and Comparative Endocrinology*, vol. 113, no. 3, pp. 413-428. http://dx.doi.org/10.1006/gcen.1998.7222. PMid:10068502.

GUZMÁN, J.M., LUCKENBACH, J.A., DA SILVA, D.A.M., HAYMAN, E.S., YLITALO, G.M., GOETZ, F.W. and SWANSON, P., 2018. Seasonal variation of pituitary gonadotropin subunit, brain-type aromatase and sex steroid receptor mRNAs, and plasma steroids during gametogenesis in wild sablefish. *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology*, vol. 219-220, pp. 48-57. http://dx.doi.org/10.1016/j.cbpa.2018.02.010. PMid:29496550.

HATEF, A. and UNNIAPPAN, S., 2019. Metabolic hormones and the regulation of spermatogenesis in fishes. *Theriogenology*, vol. 134, pp. 121-128. http://dx.doi.org/10.1016/j.theriogenology.2019.05.021. PMid:31167155.

JENG, S.R., YUEH, W.S., CHEN, G.R., LEE, Y.H., DUFOUR, S. and CHANG, C.F., 2007. Differential expression and regulation of gonadotropins and their receptors in the Japanese eel, *Anguilla japonica*. *General and Comparative Endocrinology*, vol. 154, no. 1-3, pp. 161-173. http://dx.doi.org/10.1016/j.ygcen.2007.05.026. PMid:17597622.

KAZETO, Y., KOHARA, M., MIURA, T., MIURA, C., YAMAGUCHI, S., TRANT, J.M., ADACHI, S. and YAMAUCHI, K., 2008. Japanese eel follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh): production of biologically active recombinant Fsh and Lh by Drosophila S2 cells and their differential actions on the reproductive biology. *Biology of Reproduction*, vol. 79, no. 5, pp. 938-946. http://dx.doi.org/10.1095/biolreprod.108.070052. PMid:18685126.

KITANO, T., TAKENAKA, T., TAKAGI, H., YOSHIURA, Y., KAZETO, Y., HIRAI, T., MUKAI, K. and NOZU, R., 2022. Roles of Gonadotropin Receptors in Sexual Development of Medaka. *Cells*, vol. 11, no. 3, pp. 387. http://dx.doi.org/10.3390/cells11030387. PMid:35159197.

KUSAKABE, M., NAKAMURA, I., EVANS, J., SWANSON, P. and YOUNG, G., 2006. Changes in mRNAs encoding steroidogenic acute regulatory protein, steroidogenic enzymes and receptors for gonadotropins during spermatogenesis in rainbow trout testes. *The Journal of Endocrinology*, vol. 189, no. 3, pp. 541-554. http://dx.doi.org/10.1677/joe.1.06684. PMid:16731785.

KWOK, H.F., SO, W.K., WANG, Y. and GE, W., 2005. Zebrafish gonadotropins and their receptors: cloning and characterization of zebrafish follicle stimulating hormone and luteinizing hormone receptors-evidence for their distinct functions in follicle development. *Biology of Reproduction*, vol. 72, no. 6, pp. 1370-1381. http://dx.doi.org/10.1095/biolreprod.104.038190. PMid:15728795.

LEJEUNE, H., HABERT, R. and SAEZ, J.M., 1998. Origin, proliferation and differentiation of Leydig cells. *Journal of Molecular Endocrinology*, vol. 20, no. 1, pp. 1-25. http://dx.doi.org/10.1677/jme.0.0200001. PMid:9513078.

LEVAVI-SIVAN, B., BOGERD, J., MAÑANÓS, E.L., GÓMEZ, A. and LAREYRE, J.J., 2010. Perspectives on fish gonadotropins and their receptors. *General and Comparative Endocrinology*, vol. 165, no. 3, pp. 412-437. http://dx.doi.org/10.1016/j.ygcen.2009.07.019. PMid:19686749.

LI, J., LIU, Z., KANG, T., LI, M., WANG, D. and CHENG, C.H., 2021. Igf3: a novel player in fish reproduction. *Biology of Reproduction*, vol. 104, no. 6, pp. 1194-1204. http://dx.doi.org/10.1093/biolre/ioab042. PMid:33693502.

LIVAK, K.J. and SCHMITTGEN, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *Methods*, vol. 25, no. 4, pp. 402-408. http://dx.doi.org/10.1006/meth.2001.1262. PMid:11846609.

MARQUES, N.E.F., SAMPAIO, J.A.A., BEZERRA, S.F.B., YAURI, W.M., and SANTOS, R.L., 2016. Indução hormonal à desova do peixe pedra *Genyatremus luteus* (BLOCH, 1790) (TELEOSTEI: HAEMULIDAE). *Boletim do Laboratório de Hidrobiologia*, vol. 26, pp. 32-35.

MAUGARS, G. and SCHMITZ, M., 2006. Molecular cloning and characterization of FSH and LH receptors in Atlantic salmon (*Salmo salar* L.). *General and Comparative Endocrinology*, vol. 149, no. 1, pp. 108-117. http://dx.doi.org/10.1016/j.ygcen.2006.04.011. PMid:16764877.

MAUGARS, G. and SCHMITZ, M., 2008. Expression of gonadotropin and gonadotropin receptor genes during early sexual maturation in male Atlantic salmon parr. *Molecular Reproduction and Development*, vol. 75, no. 2, pp. 403-413. http://dx.doi.org/10.1002/mrd.20767. PMid:17874455.

MELO, M.C., VAN DIJK, P., ANDERSSON, E., NILSEN, T.O., FJELLDAL, P.G., MALE, R., NIJENHUIS, W., BOGERD, J., FRANÇA, L.R., TARANGER, G.L. and SCHULZ, R.W., 2015. Androgens directly stimulate spermatogonial differentiation in juvenile Atlantic salmon (*Salmo salar*). *General and Comparative Endocrinology*, vol. 211, pp. 52-61. http://dx.doi.org/10.1016/j.ygcen.2014.11.015. PMid:25435279.

MIDDLETON, M.A., LARSEN, D.A., DICKEY, J.T. and SWANSON, P., 2019. Evaluation of endocrine and transcriptomic markers of male maturation in winter-run steelhead trout (*Oncorhynchus mykiss*). *General and Comparative Endocrinology*, vol. 281,

pp. 30-40. http://dx.doi.org/10.1016/j.ygcen.2019.05.010. PMid:31102580.

MIURA, T. and MIURA, C.I., 2003. Molecular control mechanisms of fish spermatogenesis. *Fish Physiology and Biochemistry*, vol. 28, no. 1, pp. 181-186. http://dx.doi.org/10.1023/B:FISH.0000030522.71779.47.

MIURA, T., MIURA, C., OHTA, T., NADER, M.R., TODO, T. and YAMAUCHI, K., 1999. Estradiol-17β stimulates the renewal of spermatogonial stem cells in males. *Biochemical and Biophysical Research Communications*, vol. 264, no. 1, pp. 230-234. http://dx.doi.org/10.1006/bbrc.1999.1494. PMid:10527870.

MIURA, T., YAMAUCHI, K., TAKAHASHI, H. and NAGAHAMA, Y., 1991. Hormonal induction of all stages of spermatogenesis in vitro in the male Japanese eel (*Anguilla japonica*). *Proceedings of the National Academy of Sciences of the United States of America*, vol. 88, no. 13, pp. 5774-5778. http://dx.doi.org/10.1073/pnas.88.13.5774. PMid:2062857.

MIURA, T., YAMAUCHI, K., TAKAHASHI, H. and NAGAHAMA, Y., 1992. The role of hormones in the acquisition of sperm motility in salmonid fish. *The Journal of Experimental Zoology*, vol. 261, no. 3, pp. 359-363. http://dx.doi.org/10.1002/jez.1402610316. PMid:1321204.

MOLÉS, G., GÓMEZ, A., CARRILLO, M., ROCHA, A., MYLONAS, C.C. and ZANUY, S., 2011. Determination of Fsh quantity and bioactivity during sex differentiation and oogenesis in European sea bass. *Biology of Reproduction*, vol. 85, no. 4, pp. 848-857. http://dx.doi.org/10.1095/biolreprod.111.091868. PMid:21715715.

MORINI, M., PEÑARANDA, D.S., VÍLCHEZ, M.C., TVEITEN, H., LAFONT, A.G., DUFOUR, S., PÉREZ, L. and ASTURIANO, J.F., 2017. The expression of nuclear and membrane estrogen receptors in the European eel throughout spermatogenesis. *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology*, vol. 203, pp. 91-99. http://dx.doi.org/10.1016/j.cbpa.2016.08.020. PMid:27590411.

NAGAHAMA, Y., MIURA, T. and KOBAYASHI, T., 1994. The onset of spermatogenesis in fish. *Ciba Foundation Symposium*, vol. 182, pp. 255-267. http://dx.doi.org/10.1002/9780470514573.ch14. PMID: 7835154.

NISHIMURA, T. and TANAKA, M., 2014. Gonadal development in fish. *Sexual Development: Genetics, Molecular Biology, Evolution, Endocrinology, Embryology, and Pathology of Sex Determination and Differentiation*, vol. 8, no. 5, pp. 252-261. http://dx.doi.org/10.1159/000364924. PMid:25034975.

NÓBREGA, R.H., MORAIS, R.D.V.S., CRESPO, D., WAAL, P.P., FRANÇA, L.R., SCHULZ, R.W. and BOGERD, J., 2015. Fsh stimulates spermatogonial proliferation and differentiation in zebrafish via Igf3. *Endocrinology*, vol. 156, no. 10, pp. 3804-3817. http://dx.doi.org/10.1210/en.2015-1157. PMid:26207345.

NOLETO-FILHO, E.M., YAURI, W.L.M. and LUVIZOTTO-SANTOS, R., 2012. Captura de reprodutores de peixe-pedra *Genyatremus luteus* (Block, 1797) (Pisces: Haemulidae) e manutenção em sistema fechado. *Boletim do Laboratório de Hidrobiologia*, vol. 25, no. 1, pp. 55-60.

OBA, Y., HIRAI, T., YOSHIURA, Y., YOSHIKUNI, M., KAWAUCHI, H. and NAGAHAMA, Y., 1999a. Cloning, functional characterization, and expression of a gonadotropin receptor cDNA in the ovary and testis of amago salmon (*Oncorhynchus rhodurus*). *Biochemical and Biophysical Research Communications*, vol. 263, no. 2, pp. 584-590. http://dx.doi.org/10.1006/bbrc.1999.1346. PMid:10491336.

OBA, Y., HIRAI, T., YOSHIURA, Y., YOSHIKUNI, M., KAWAUCHI, H. and NAGAHAMA, Y., 1999b. The duality of fish gonadotropin receptors: cloning and functional characterization of a second gonadotropin receptor cDNA expressed in the ovary and testis of amago salmon (*Oncorhynchus rhodurus*). *Biochemical and Biophysical Research Communications*, vol. 265, no. 2, pp. 366-371. http://dx.doi.org/10.1006/bbrc.1999.1700. PMid:10558873.

OKUZAWA, K., 2002. Puberty in teleosts. *Fish Physiology and Biochemistry*, vol. 26, no. 1, pp. 31-41. http://dx.doi.org/10.1023/A:1023395025374.

OLIVEIRA, M.A., MARTINEZ, E.R.M., BUTZGE, A.J., DORETTO, L.B., RICCI, J.M.B., RODRIGUES, M.S., VIGOYA, A.A.A., GÓMEZ-GONZÁLEZ, N.E., STEWART, A.B. and NÓBREGA, R.H., 2021. Molecular characterization and expression analysis of anti-Müllerian hormone in common carp (*Cyprinus carpio*) adult testes. *Gene Expression Patterns*, vol. 40, p. 119169. http://dx.doi.org/10.1016/j.gep.2021.119169. PMid:33667682.

PARKER, G.A., 1982. Why are there so many tiny sperm? Sperm competition and the maintenance of two sexes. *Journal of Theoretical Biology*, vol. 96, no. 2, pp. 281-294. http://dx.doi.org/10.1016/0022-5193(82)90225-9. PMid:7121030.

PINTO, P.I., ANDRADE, A.R., ESTÊVÃO, M.D., ALVARADO, M.V., FELIP, A. and POWER, D.M., 2018. Duplicated membrane estrogen receptors in the European sea bass (*Dicentrarchus labrax*): Phylogeny, expression and regulation throughout the reproductive cycle. *The Journal of Steroid Biochemistry and Molecular Biology*, vol. 178, pp. 234-242. http://dx.doi.org/10.1016/j.jsbmb.2017.12.019. PMid:29288793.

PINTO, P.I.S., TEODÓSIO, H.R., GALAY-BURGOS, M., POWER, D.M., SWEENEY, G.E. and CANÁRIO, A.V.M., 2006. Identification of estrogen-responsive genes in the testis of sea bream *(Sparus auratus)* using suppression subtractive hybridization. *Molecular Reproduction and Development*, vol. 73, no. 3, pp. 318-329. http://dx.doi.org/10.1002/mrd.20402. PMid:16267841.

PLANAS, J.V. and SWANSON, P., 1995. Maturation-associated changes in the response of the salmon testis to the steroidogenic actions of gonadotropins (GTH I and GTH II) *in vitro*. *Biology of Reproduction*, vol. 52, no. 3, pp. 697-704. http://dx.doi.org/10.1095/biolreprod52.3.697. PMid:7756464.

PRAT, F., SUMPTER, J.P. and TYLER, C.R., 1996. Validation of radioimmunoassays for two salmon gonadotropins (GTH I and GTH II) and their plasma concentrations throughout the reproductive cycle in male and female Rainbow Trout (*Oncorhynchus mykiss*). *Biology of Reproduction*, vol. 54, no. 6, pp. 1375-1382. http://dx.doi.org/10.1095/biolreprod54.6.1375. PMid:8724367.

RAHMAN, M.A., OHTA, K., YAMAGUCHI, A., CHUDA, H., HIRAI, T. and MATSUYAMA, M., 2003. Gonadotropins, gonadotropin receptors and their expressions during sexual maturation in yellowtail, a carangid fish. *Fish Physiology and Biochemistry*, vol. 28, no. 1, pp. 81-83. http://dx.doi.org/10.1023/B:FISH.0000030481.94719.a0.

RAJAKUMAR, A. and SENTHILKUMARAN, B., 2020. Steroidogenesis and its regulation in teleost-a review. *Fish Physiology and Biochemistry*, vol. 46, no. 3, pp. 803-818. http://dx.doi.org/10.1007/s10695-019-00752-0. PMid:31940121.

RHODY, N.R., DAVIE, A., ZMORA, N., ZOHAR, Y., MAIN, K.L. and MIGAUD, H., 2015. Influence of tidal cycles on the endocrine control of reproductive activity in common snook (*Centropomus undecimalis*). *General and Comparative Endocrinology*, vol. 224, pp. 247-259. http://dx.doi.org/10.1016/j.ygcen.2015.08.007. PMid:26261080.

ROCHA, A., GÓMEZ, A., ZANUY, S., CERDÁ-REVERTER, J.M. and CARRILLO, M., 2007. Molecular characterization of two sea bass gonadotropin receptors: cDNA cloning, expression analysis, and functional activity. *Molecular and Cellular Endocrinology*, vol. 272, no. 1-2, pp. 63-76. http://dx.doi.org/10.1016/j.mce.2007.04.007. PMid:17543442.

SAMBRONI, E., LE GAC, F., BRETON, B. and LAREYRE, J.J., 2007. Functional specificity of the rainbow trout (*Oncorhynchus mykiss*) gonadotropin receptors as assayed in a mammalian cell line. *The Journal of Endocrinology*, vol. 195, no. 2, pp. 213-228. http://dx.doi.org/10.1677/JOE-06-0122. PMid:17951533.

SAMBRONI, E., ROLLAND, A.D., LAREYRE, J.-J. and GAC, F., 2013. Fsh and Lh have common and distinct effects on gene expression in rainbow trout testis. *Journal of Molecular Endocrinology*, vol. 50, no. 1, pp. 1-18. http://dx.doi.org/10.1530/JME-12-0197. PMid:23045716.

SAMPAIO, I.B.M., 2002. *Estatística aplicada à experimentação animal*. 3rd ed. Belo Horizonte: FEPMVZ, 264 p.

SANTIAGO-ANDRES, Y., GOLAN, M. and FIORDELISIO, T., 2021. Functional pituitary networks in vertebrates. *Frontiers in Endocrinology*, vol. 11, pp. 619352. http://dx.doi.org/10.3389/fendo.2020.619352. PMid:33584547.

SCHULZ, R.W., DE FRANÇA, L.R., LAREYRE, J.J., LEGAC, F., CHIARINI-GARCIA, H., NOBREGA, R.H. and MIURA, T., 2010. Spermatogenesis in fish. *General and Comparative Endocrinology*, vol. 165, no. 3, pp. 390-411. http://dx.doi.org/10.1016/j.ygcen.2009.02.013. PMid:19348807.

SCHULZ, R.W., TARANGER, G.L., BOGERD, J., NIJENHUIS, W., NORBERG, B., MALE, R. and ANDERSSON, E., 2019. Entry into puberty is reflected in changes in hormone production but not in testicular receptor expression in Atlantic salmon (*Salmo salar*). *Reproductive Biology and Endocrinology*, vol. 17, pp. 48. http://dx.doi.org/10.1186/s12958-019-0493-8.

SHAN, L., HARDY, D.O., CATTERALL, J.F. and HARDY, M.P., 1995. Efects of luteinizing hormone (LH) and androgen on steady state levels of messenger ribonucleic acid for LH receptors, androgen receptors, and steroidogenic enzymes in rat Leydig cell progenitors in vivo. *Endocrinology*, vol. 136, no. 4, pp. 1686-1693. http://dx.doi.org/10.1210/endo.136.4.7895679. PMid:7895679.

SHINOZAKI-MENDES, R.A., SANTANDER-NETO, J., SILVA, J.R.F. and HAZIN, F.H.V., 2013. Gonad maturation of *Haemulon plumieri* (Teleostei: Haemulidae) in Ceará state, northeastern Brazil. *Brazilian Journal of Biology = Revista Brasileira de Biologia*, vol. 73, no. 2, pp. 383-390. http://dx.doi.org/10.1590/S1519-69842013000200019. PMid:23917566.

SIQUEIRA-SILVA, D.H., DA SILVA RODRIGUES, M. and NÓBREGA, R.H., 2019. Testis structure, spermatogonial niche and Sertoli cell efficiency in Neotropical fish. *General and Comparative Endocrinology*, vol. 273, pp. 218-226. http://dx.doi.org/10.1016/j.ygcen.2018.09.004. PMid:30195025.

SKAAR, K.S., NÓBREGA, R.H., MAGARAKI, A., OLSEN, L.C., SCHULZ, R.W. and MALE, R., 2011. Proteolytically activated, recombinant antimullerian hormone inhibits androgen secretion, proliferation, and differentiation of spermatogonia in adult zebrafish testis organ cultures. *Endocrinology*, vol. 152, no. 9, pp. 3527-3540. http://dx.doi.org/10.1210/en.2010-1469. PMid:21750047.

STATSOFT, INC., 2007 [viewed 13 March 2022]. *Statistica (data analysis software system), version 7* [online]. Available from: www.statsoft.com

SUZUKI, H., KAZETO, Y., GEN, K. and OZAKI, Y., 2020. Functional analysis of recombinant single-chain Japanese eel Fsh and Lh produced in FreeStyle 293-F cell lines: binding specificities to their receptors and differential efficacy on testicular steroidogenesis. *General and Comparative Endocrinology*, vol. 285, pp. 113241. http://dx.doi.org/10.1016/j.ygcen.2019.113241. PMid:31400434.

SVENSSON, K., JUST, A.C., FLEISCH, A.F., SANDERS, A.P., TAMAYO-ORTIZ, M., BACCARELLI, A.A., WRIGHT, R.J., TÉLLEZ-ROJO, M.M., WRIGHT, R.O. and BURRIS, H.H., 2019. Prenatal salivary sex hormone levels and birth-weight-for-gestational age. *Journal of Perinatology*, vol. 39, no. 7, pp. 941-948. http://dx.doi.org/10.1038/s41372-019-0385-y. PMid:31110244.

THÖNNES, M., VOGT, M., STEINBORN, K., HAUSKEN, K.N., LEVAVI-SIVAN, B., FROSCHAUER, A. and PFENNIG, F., 2020. An ex vivo Approach to Study Hormonal Control of Spermatogenesis in the Teleost *Oreochromis niloticus*. *Frontiers in Endocrinology*, vol. 11, pp. 443. http://dx.doi.org/10.3389/fendo.2020.00443. PMid:32793114.

TROMBLEY, S. and SCHMITZ, M., 2013. Leptin in fish: possible role in sexual maturation in male Atlantic salmon. *Fish Physiology and Biochemistry*, vol. 39, no. 1, pp. 103-106. http://dx.doi.org/10.1007/s10695-012-9731-0. PMid:23053613.

VAZZOLER, A.E.A.M., 1996. *Biologia da reprodução de peixes teleósteos: teoria e prática*. Maringá: Eduem, 196 p.

VISCHER, H.F., GRANNEMAN, J.C., LINSKENS, M.H., SCHULZ, R.W. and BOGERD, J., 2003. Both recombinant African catfish LH and FSH are able to activate the African catfish FSH receptor. *Journal of Molecular Endocrinology*, vol. 31, no. 1, pp. 133-140. http://dx.doi.org/10.1677/jme.0.0310133. PMid:12914531.

WALKER, W.H. and CHENG, J., 2005. FSH and testosterone signaling in Sertoli cells. *Reproduction*, vol. 130, no. 1, pp. 15-28. http://dx.doi.org/10.1530/rep.1.00358. PMid:15985628.

WANG, L., LIANG, X.F., ZHANG, W.B., MAI, K.S., HUANG, Y. and SHEN, D., 2009. Amnesic shellfish poisoning toxin stimulates the transcription of CYP1A possibly through AHR and ARNT in the liver of red sea bream *Pagrus major*. *Marine Pollution Bulletin*, vol. 58, no. 11, pp. 1643-1648. http://dx.doi.org/10.1016/j.marpolbul.2009.07.004. PMid:19665739.

WANG, N., WANG, Y., HAO, H., WANG, L., WANG, Z., WANG, J. and WU, R., 2014. A bi-Poisson model for clustering gene expression profiles by RNA-seq. *Briefings in Bioinformatics*, vol. 15, no. 4, pp. 534-541. http://dx.doi.org/10.1093/bib/bbt029. PMid:23665510.

YAN, L., SWANSON, P. and DICKHOFF, W.W., 1992. A two-receptor model for salmon gonadotropins (GTH I and GTH II). *Biology of Reproduction*, vol. 47, no. 3, pp. 418-427. http://dx.doi.org/10.1095/biolreprod47.3.418. PMid:1511095.

YARON, Z. and LEVAVI-SIVAN, B., 2011. Endocrine regulation of fish reproduction. *Encyclopedia of Fish Physiology: from Genome to Environment*, vol. 2, pp. 1500-1508.

YOSHIDA, H.O., 1964. Skipjack tuna spawning in the Marquesas Islands and Tuamotu Archipelago. *Fish Bulletin*, vol. 65, pp. 479-488.

ZOHAR, Y., MUÑOZ-CUETO, J.A., ELIZUR, A. and KAH, O., 2010. Neuroendocrinology of reproduction in teleost fish. *General and Comparative Endocrinology*, vol. 165, no. 3, pp. 438-455. http://dx.doi.org/10.1016/j.ygcen.2009.04.017. PMid:19393655.