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DIEGO LUIZ DOS SANTOS RIBEIRO

BIOLOGIA REPRODUTIVA E EXPRESSÃO GÊNICA GONADAL EM *Genyatremus luteus* (BLOCH, 1790) - (Teleostei, Perciformes: Haemulidae)

São Luís – MA
2022

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Tese apresentada ao Programa de Pós-Graduação em Biodiversidade e Biotecnologia – Rede BIONORTE, como requisito parcial para obtenção do título de Doutor.

Orientador: Prof. Dr. José Ribamar de Souza Torres Junior

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Ouça conselhos e aceite instruções, e acabará sendo sábio. Muitos são os planos no coração do homem, mas o que prevalece é o propósito do Senhor.

(Provérbios 19:20-21)

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RESUMO

Genyatremus luteus, conhecido popularmente como peixe-pedra, é uma espécie estuarino-marinha, de habitat preferencialmente costeiro, vivendo especialmente em estuários e lagunas. Essa espécie tem grande potencial para a exploração pesqueira extrativista no estado do Maranhão. Porém, pouco se sabe sobre a biologia reprodutiva nessa espécie. Desta forma, objetivou-se com o trabalho avaliar a biologia reprodutiva e expressão gênica gonadal em *G. luteus* na Ilha do Maranhão. Foram utilizados 21 exemplares machos e 28 fêmeas. De acordo com as caraterísticas histológicas dos testículos observada na análise histológica, os machos foram agrupados em três estádios reprodutivos: imaturos (n=7), em maturação (n=7) e maduros (n=7) e as fêmeas, por sua vez, com base nas características histológicas dos folículos ovarianos e no desenvolvimento do oócito foram também classificadas: imaturas (n=7), em maturação (n=7), maduro (n=7) e desovado (n=7). Os níveis plasmáticos dos hormônios E2, 11-KT e 17α-OHP foram medidos por meio de ensaio imunoenzimático (ELISA). A expressão gênica dos receptores de estrogênio (*er*) e LH (*lhr)* foi analisada por PCR em tempo real. Nos machos, a concentração plasmática de 11-KT apresentou um pico na fase de maturação final. As maiores concentrações plasmáticas de 17α-OHP foram encontradas no estádio imaturo, as quais diminuiram nos estágios subsequentes. Por outro lado, o 17β-estradiol (E2) apresentou maior concentração no estádio em maturação. A expressão de *er* aumentou significativamente ao longo do desenvolvimento maturacional dos testículos. O mRNA para o receptor de LH diminuiu do estádio imaturo para o estádio em maturação com consequente pico de expressão no estádio maduro. Houve alta correlação entre a expressão gênica dos receptores e os níveis de esteróides plasmáticos, especialmente com E2. Nas fêmeas, a maior concentração plasmática de E2 foi encontrada em espécimes maduros. As concentrações plasmáticas de 17α-OHP aumentaram significativamente desde o estágio imaturo até o estágio final de maturação ovariano. O estágio reprodutivo maturacional não teve nenhum efeito nas concentrações plasmáticas de 11-KT. A expressão de *er* nos ovários aumentou significativamente ao longo da maturação ovariana e permaneceu alta nos estágios de maturação e desova. A expressão de *lhr* apresentou tendência semelhante ao longo do processo de maturação, embora tenha atingido seu pico na fase de desova. Em conclusão, este estudo caracterizou pela primeira vez, sob os aspectos celular, endócrino e molecular, os diferentes estádios de maturação reprodutiva em machos e fêmeas 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 e ovariana, apesar de não se relacionarem necessariamente com o índice gonadossomático.

Palavras-chave: Roncador, Receptor de gonadotrofinas; Estradiol; Gônadas; Reprodução.

RIBEIRO, Diego Luiz dos Santos. **Reproductive biology and gonadal gene expression in *Genyatremus luteus* (BLOCH, 1790) - (Teleostei, Perciformes: Haemulidae).** 2022. 100 f. Tese (doutorado). Graduate Program in Biodiversity and Biotechnology – Rede BIONORTE. Federal University of Maranhão, São Luís, 2022.

ABSTRACT

Genyatremus luteus, popularly known as stonefish, is an estuarine-marine species with a preferentially coastal habitat, living especially in estuaries and lagoons. This species has great potential for extractive fisheries in the state of Maranhão. However, little is known about the reproductive biology of this species. Thus, the objective of this work was to evaluate the reproductive biology and gonadal gene expression in *G. luteus* on the island of Maranhão. 21 male and 28 female specimens were used. According to the histological characteristics of the testes observed in the histological analysis, males were grouped into three reproductive stages: immature (n=7), maturing (n=7) and mature (n=7) and females, in turn, based on the histological characteristics of the ovarian follicles and the development of the oocyte were also classified: immature (n=7), maturing (n=7), mature (n=7) and spawned (n=7). Plasma levels of hormones E2, 11-KT and 17α-OHP were measured by enzyme immunoassay (ELISA). Estrogen (*er*) and LH (*lhr*) receptor gene expression was analyzed by real-time PCR. In males, the plasma concentration of 11-KT showed a peak in the final maturation phase. The highest plasma concentrations of 17α-OHP were found in the immature stage, which decreased in the subsequent stages. On the other hand, 17β-estradiol (E2) showed higher concentration in the maturation stage. The expression of er significantly increased along the maturational development of the testes. The mRNA for the LH receptor decreased from the immature stage to the maturation stage with a consequent peak of expression in the mature stage. There was a high correlation between receptor gene expression and plasma steroid levels, especially with E2. In females, the highest plasma concentration of E2 was found in mature specimens. Plasma concentrations of 17α-OHP increased significantly from the immature stage to the final stage of ovarian maturation. The maturational reproductive stage had no effect on plasma 11-KT concentrations. The expression of er in the ovaries significantly increased throughout ovarian maturation and remained high in the maturation and spawning stages. The expression of lhr showed a similar trend throughout the maturation process, although it reached its peak in the spawning phase. In conclusion, this study characterized for the first time, under the cellular, endocrine and molecular aspects, the different stages of reproductive maturation in males and females of *G. luteus*, demonstrating that the gene expression profile for er and lhr receptors, as well as plasma concentrations of 11-KT and E2 were directly related to testicular and ovarian maturation, although not necessarily related to the gonadosomatic index.

Keywords: Grunts, Gonadotropin Receptor; Estradiol; Gonads; Reproduction.

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1 INTRODUÇÃO

Os peixes teleósteos estuarinos e marinhos representam importantes recursos pesqueiros, sobretudo de subsistência. Entretanto, a sustentabilidade destes recursos está ameaçada, pois a sua exploração predatória produz grandes impactos nas populações naturais. Estudos sobre a biologia reprodutiva das espécies, etnoconhecimento dos pesquisadores, associados ao conhecimento socioambiental, são necessários para que futuras medidas de manejo e conservação sejam adotadas, visando a manutenção dos ecossistemas (COCHRANE, 2002).

Os peixes da família Hamulidae, pertencentes à ordem Perciformes, também estão intimamente inseridos neste cenário. A família congrega cerca de 145 espécies existentes, atualmente classificadas em 17 gêneros, dentre os quais já foram listados no oceano Atlântico, *Anisotremus, Conodon, Orthopristis, Pomadasys, Genyatremus* e *Boridae* (NELSON, 2006; JOHNSON, 1980; TAVERA *et al.*, 2012).

A espécie *Genyatremus luteus*, popularmente conhecida no Maranhão como "peixe-pedra" ou "roncador", foi classificada como uma espécie estuarino-marinha, de habitat preferencialmente costeiro, vivendo especialmente em estuários e lagunas, sobre fundos de lama, areia e pedras (GIARRIZZO e KRUMME, 2007). Apresenta grande potencial para a exploração pesqueira extrativista (ALMEIDA *et al.*, 2005) e, sobretudo, para a perspectiva do avanço da piscicultura marinha no Estado (FAPEMA, 2017), devido à qualidade da carne e ao valor econômico (MARQUES *et al.*, 2016).

As informações acerca da biologia reprodutiva e maturação gonadal do *G. luteus* ainda são escassas. Em um estudo realizado no Golfo de Paria - Venezuela, Gómez *et al.* (2002) observaram que comprimento padrão correspondente à primeira maturação sexual (L50), em ambiente natural, foi estimado entre 29,5 a 38,8 cm. Já em um estudo realizado no Maranhão, com espécimes capturados e mantidos em sistema fechado, Noleto Filho *et al.* (2012) observaram indivíduos sexualmente maturos em um grupo com comprimento final médio de 18,0 ± 2,9 cm e peso de 117,9 ± 46,5 g. Ainda, o mesmo grupo de pesquisa obteve êxito na indução de desova em indivíduos com média de 24,0 cm de comprimento e 315,7 g de peso, e espermiação com média de 19,7 cm e 159,8 g, respectivamente (MARQUES *et al.*, 2016).

Nos peixes teleósteos, assim como em outros vertebrados, os processos de maturação do oócito e ovulação estão intimamente ligados. Estes são desencadeados primariamente por

um sinal endócrino, que é caracterizado por um aumento da gonadotrofina (JALABERT *et al.*, 1991; SCHULZ *et al.*, 2010). O estágio de diferenciação alcançado por todo o folículo (ovócito, granulosa e teca) é determinante e inclui o desenvolvimento de características específicas, tais como complexo enzimático, capacidades de sinalização endócrina e parácrina, além de propriedades mecânicas do folículo (JALABERT *et al.*, 1991; LEVAVI-SIVAN *et al.*, 2010). Enquanto este estágio de diferenciação ou maturação folicular (BOBE *et al.*, 2003) não for atingido, a estimulação precoce do folículo pelas gonadotrofinas pode resultar em falhas na maturação ovocitária, em maturação não seguida de ovulação (DE MONTALEMBERT *et al.*, 1978), ou ovulação de oócitos de má qualidade (JALABERT *et al.*, 1978).

Atingir competências de maturação e ovulatórias em folículos ovarianos de peixes teleósteos envolve também a expressão de genes orquestrada no período pós-vitelogênico, incluindo aumento ou diminuição de enzimas esteroidogênicas durante a transição de estrogênio para progestágeno (NAGAHAMA; YAMASHITA, 2008), bem como alterações em receptores de gonadotropina, reguladores parácrinos, proteases (BOBE *et al.*, 2004), e genes relacionados com a inflamação (BOBE *et al.*, 2006; 2009; MELO *et al.*, 2015; NOBREGA *et al.*, 2015; LI *et al.*, 2021).

Produtos de genes relacionados com competências maturacionais e ovulatórias, no entanto, podem também ser sintetizados no início de desenvolvimento, como folículos vitelogênicos que são sensíveis a estimulação exógena de gonadotropina *in vivo* e podem ser induzidas a sofrer maturação oocítica final e ovulação (ZOHAR *et al.*, 2001; ROTILI *et al.*, 2022).

Apesar de alguns poucos aspectos da biologia do *G. luteus* já terem sido estudados (ALMEIDA *et al.*, 2005; NOLETO-FILHO *et al.*, 2011; MARQUES *et al.*, 2016; FERNANDES *et al.*, 2017), muitas características voltadas à população encontrada no litoral do estado do Maranhão ainda são desconhecidas. Além disso, evidências científicas sobre a sazonalidade reprodutiva, estágios de maturação gonadal e expressão gênica gonadal dessa espécie, no litoral do Maranhão, ainda permanecem não elucidadas.

Por estas razões, é premente a necessidade de estudos reprodutivos mais aprofundados que poderão gerar valiosas ferramentas de suporte ao diagnóstico, avaliação de parâmetros e decisões de manejo e conservação da espécie em nossa realidade local, além de fomentar iniciativas de exploração sustentável, para que os estoques pesqueiros e o equilíbrio da fauna marinha nativa sejam mantidos.

Atualmente, a comunidade científica e as entidades responsáveis pelo manejo e conservação de recursos naturais ainda necessitam de informações relacionadas aos aspectos biológicos e reprodutivos dos peixes da família Hamulidae, objetivando orientar e assegurar a sua exploração sustentável. Esta pesquisa propõe avaliar sob a mesma ótica, a expressão gênica gonadal, o perfil de esteroides sexuais e o *status* maturacional reprodutivo de *Genyatremus luteus* na Ilha do Maranhão. Nesta perspectiva, buscar-se-á, também, contemplar novos subsídios para viabilizar o manejo e conservação da espécie em nosso litoral.

A tese foi dividida em capítulos, sendo que é apresentada a introdução e revisão de literatura seguida do Capítulo 1, onde é apresentado o artigo intitulado: “Differential gene expression pattern and plasma sex steroids during testicular development in *Genyatremus luteus* (Perciforme: Haemulidae) (Bloch, 1790)” e no Capítulo 2 consta o artigo intitulado “Steroid profile and ovarian gene expression in *Genyatremus luteus* (Teleostei, Perciformes: Haemulidae) during sexual maturation”.

2 OBJETIVOS

2.1 Objetivo geral

Avaliar a biologia reprodutiva e expressão gênica gonadal em *Genyatremus luteus* em diferentes estágios de maturação gonadal na Ilha do Maranhão.

2.2 Objetivos específicos

- Avaliar os parâmetros ictiométricos de *Genyatremus luteus;*
- Caracterizar os estágios de maturação gonadal pela análise macroscópica e histológica;
- Verificar os níveis de esteroides sexuais em *Genyatremus luteus* de acordo com os estágios reprodutivos;
- Identificar o padrão de expressão gênica do receptor do estrógeno (ER) e receptor do hormônio luteinizante (LHR) na maturação gonadal;
- Correlacionar os parâmetros ictiométricos aos níveis de esteroides sexuais e ao padrão de expressão gênica gonadal.

3 REVISÃO DE LITERATURA

3.1 Reprodução em peixes teleósteos

Em peixes, como em outros vertebrados, é sabido que a reprodução é regulada por uma extensa rede de sinais reguladores endócrinos, parácrinos e autócrinos ao longo do eixo cérebro-hipófise-gônada (CHG) (ZOHAR *et al.*, 2010). Entre esses, o hormônio liberador de gonadotrofina (GnRH) é considerado o ponto de partida do eixo CHG, embora outros neuropeptídeos (por exemplo, kisspeptina, Neurokinina B e F, hormônio inibidor da gonadotrofina) estejam emergindo como importantes reguladores (BIRAN *et al.*, 2012; MOUSSAVI *et al.*, 2012; TENA-SEMPERE *et al.*, 2012).

Esses neuro-hormônios regulam a síntese e secreção de duas gonadotrofinas hipofisárias (hormônio folículo-estimulante (FSH) e hormônio luteinizante (LH), que por sua vez regulam a esteroidogênese gonadal e a gametogênese (PETER E YU, 1997; SWANSON *et al.*, 2003; ZOHAR *et al.*, 2010) O modo de ação e regulação do FSH e LH foi investigado em várias espécies de peixes (YARON *et al.*, 2003; KAH E DUFOUR, 2011; LEVAVI-SIVAN *et al.*, 2010). Por exemplo, na truta arco-íris *Oncorhynchus mykiss*, a regulação gonadotrópica nos estágios iniciais da gametogênese (vitelogênese ou espermatogênese) é tipicamente caracterizada por uma elevação no mRNA do FSH, enquanto o LH atinge o pico no momento da ovulação e espermatogênese (BOBE *et al.*, 2004; SAMBRONI *et al.*, 2007a; MIDDLETON *et al.*, 2019).

Como alternativa, em algumas espécies de peixes com desova parcelada, a expressão do mRNA de FSH e LH (e seus receptores) aumenta simultaneamente enquanto os oócitos estão se desenvolvendo em taxas diferentes para atingir um pico na desova, como demonstrado em *Dicentrarchus labrax* (MIGAUD *et al.*, 2012; PINTO *et al.*, 2018), bacalhau do Atlântico *Gadus morhua* (COWAN *et al.*, 2012; DE ALMEIDA *et al.*, 2011; MITTELHOLZER *et al.*, 2009) e *Hippoglossus hippoglossus* (WELTZIEN *et al.*, 2003).

O conhecimento das particularidades reprodutivas de uma espécie é importante para se compreender o comportamento das populações de peixes, favorecendo a correta administração desses recursos (SENTHILKUMARAN; KAR, 2021). As características reprodutivas e ictiométricas geram dados que indicam o estágio de desenvolvimento do ciclo gonadal, época e local de desova, comprimento e idade média em que os indivíduos iniciam o processo reprodutivo. Estas informações são subsídios necessários e importantes na

regulamentação da pesca, dentro de um programa de manejo, permitindo a tomada de medidas para a preservação dos estoques (MARSHALL *et al.*, 2021).

Tanto em ambientes naturais como em ambientes aquaculturais, a reprodução tem papel central nos processos produtivos, garantindo não apenas a propagação das espécies, mas também, a combinação de informações genéticas entre dois ou mais indivíduos (STRÜSSMANN & NAKAMURA, 2002). A reprodução de peixes envolve diversos fatores relacionados às estratégias reprodutivas, tipos de desova, comportamento parental, fatores ambientais e suas relações com o comportamento reprodutivo e fatores relacionados à gametogênese (JALABERT, 2005; DUFOUR *et al.*, 2010; ALIX *et al.*, 2020).

Quanto às estratégias reprodutivas, a maioria dos peixes teleósteos reproduzem-se sexualmente e, na maioria dos casos, caracteriza-se pela existência de sexos separados, sendo que cada indivíduo produz apenas espermatozoides ou oócitos (PIFERRER, 2009). A determinação sexual em peixes pode ser classificada em: (a) genotípica, onde o sexo se determina no momento da fecundação e ocorre diferenças genéticas entre os sexos e (b) fenotípica, que é diretamente dependente de fatores ambientais, onde não existem diferenças genéticas consistentes entre os sexos e o mesmo se define algum tempo após a fertilização, podendo este tempo ser em dias, meses ou anos (VALENZUELA *et al.*, 2003).

O desenvolvimento sexual dos peixes teleósteos pode ser afetado por vários fatores, incluindo idade, gênero, tamanho e, em muitos casos, fatores ambientais (JALABERT, 2005). Dependendo da espécie, os peixes se tornarão sexualmente maturos em vários estágios de suas vidas, com alguns sendo maturos e prontos para se reproduzir no nascimento, enquanto outros vivem por vários anos antes de se tornarem maturos. Tipicamente, os peixes teleósteos atingem a maturidade entre 1 e 5 anos, com os exemplos mais extremos levando mais de 10 anos para atingir a maturidade sexual (WOOTTON, 1990).

Em muitas espécies de peixes teleósteos, a maturação é controlada pela taxa de crescimento, com indivíduos de crescimento mais rápido alcançando a maturação primeiro (PIFERRER, 2001). Comumente, as fêmeas exibirão taxas de crescimento mais rápidas e, portanto, atingirão a maturação antes dos machos da mesma espécie (PIFERRER, 2001). Tradicionalmente, não importa o tamanho na maturidade, machos pequenos ou grandes são igualmente aptos em termos de reprodução (JONSONN E JONSONN, 1993). Os machos grandes podem competir pelas fêmeas, enquanto os machos menores podem aproveitar os locais abertos de desova, mas ambos podem se reproduzir com sucesso geralmente equivalente.

Quanto às fêmeas, no entanto, quanto maior seu tamanho, mais óvulos poderá produzir (JONSONN E JONSONN, 1993; PIFERRER, 2001).

Independentemente do modelo utilizado para a reprodução, todos os eventos reprodutivos de teleósteos ocorrem de forma cíclica. Primeiro, os gametas se desenvolvem, amadurecem e então ocorre a desova, onde o óvulo é fertilizado pelo espermatozoide (COWARD *et al.* 2003). Muitos teleósteos sobrevivem vários anos e participam de vários eventos de acasalamento ao longo de suas vidas. A desova em regiões temperadas geralmente ocorre na primavera e durará um período distinto de tempo (WEITZMAN, 2014).

A duração dos ciclos de reprodução pode variar desde quatro semanas até alguns anos, com algumas espécies reproduzindo várias vezes por ciclo e outras apenas reproduzindo uma vez em toda a sua vida. Existem muitas espécies de peixes teleósteos, especialmente espécies marinhas, em que os tempos reprodutivos são completamente desconhecidos (WEITZMAN, 2014).

Aproximadamente 12 espécies de teleósteos produzem jovens vivos, e são considerados ovovivíparos, caso em que os ovos são retidos no ovário onde os descendentes se desenvolvem completamente até o nascimento, ou vivíparos, caso em que os ovos abrigam os filhotes por um tempo muito curto e elas são incubadas internamente e nutridas por uma estrutura semelhante à placenta do ovário ou do útero (WEITZMAN, 2014). Em ambos os casos, esta fertilização interna e o subsequente nascimento de juvenis vivos é muito raro e ocorre apenas em cerca de 2-3% das espécies de teleósteos (COWARD *et al.*, 2003).

Mais comumente, no entanto, são as espécies ovíparas, nas quais as fêmeas liberam ovos no ambiente. As espécies ovíparas podem ser subdivididas em ovulíparas, nas quais os oócitos maturos são liberados da fêmea e fertilizados no ambiente externo, ou espécies zigoparíferas, nas quais o óvulo é fertilizado internamente e permanece dentro da fêmea por um curto período de tempo antes de ser liberado o ambiente (COWARD *et al.*, 2003). Em casos de espécies ovíparas, todo o desenvolvimento embrionário ocorre fora do corpo materno, toda a oferta de nutrientes é através da gema, e juvenis eclodem uma vez desenvolvidos por quebrando o envelope do ovo (COWARD *et al.*, 2003).

Quanto à produção de ovos, os teleósteos de água doce são tipicamente caracterizados como produtores de ovos demersais. Estes ovos são tipicamente ricos em vitelo e enterrados, colocados ou ligados a um substrato de cascalho ou planta. Os ovos demersais geralmente

eclodem em jovens que exibem rapidamente comportamentos e formas corporais semelhantes às dos peixes adultos (POUGH *et al.*, 2005). Em contraste, teleósteos marinhos produzem principalmente ovos pelágicos, que possuem pouca reserva de gema e podem flutuar livremente na água. Os jovens produzidos a partir de ovos pelágicos são geralmente pequenos e tendem a ser muito diferente em aparência dos adultos, embora ainda reconhecível para espécies (POUGH *et al.* 2005).

Os peixes teleósteos exibem comportamento parental. Sargent e Gross (1986) definem o cuidado parental em animais como uma associação entre pai (s) e filhos que aumentará a chance de sobrevivência da prole. Como muitos outros aspectos associados à reprodução em teleósteos, o cuidado parental também é altamente variável entre as espécies. São descritas quatro classes gerais de cuidados parentais em teleósteos: ausência de cuidados, cuidados masculinos, cuidados biparentais e cuidados femininos, sendo que a ausência de cuidados é a forma mais comum e cuidado feminino, a menos comum (SARGENT E GROSS, 1986). O cuidado centrado no macho é mais comum nos peixes que estabelecem ninhos e os protegem durante o desenvolvimento da prole. A duração do cuidado parental geralmente termina na incubação ou logo após, mas existem situações extremas em que os pais protegem seus filhotes por um período muito mais longo de tempo (OLIEVERA *et al.*, 2005).

Com o decorrer do ciclo reprodutivo dos peixes, uma série de modificações marcantes ocorrem em suas gônadas (NIKOLSKY, 1963), a principal modificação ocorre no tamanho e peso das gônadas, que em grande parte é devido ao acúmulo de material de reserva nos oócitos em maturação (VAZZOLER, 1996).

O índice gonadossomático (IGS) constantemente é utilizado como indicativo do estado funcional dos ovários, uma vez que este expressa a porcentagem que as gônadas representam do peso total ou do peso corporal dos indivíduos (VAZZOLER, 1996).

O IGS é um indicador bastante eficiente para determinar o grau de desenvolvimento e o período reprodutivo (ISAAC-NAHUM; VAZZOLER, 1983). Vazzoler (1996) verificou um aumento no volume do ovário nas fases finais do desenvolvimento ovocitário e consequentemente no peso destes que se reflete em um aumento do valor do IGS nesta fase, podendo este aumento ser utilizado como indicativo do período reprodutivo.

3.2 Fatores ambientais na determinação e diferenciação sexual

Pesquisas mostram que, em espécies de peixes temperados, o eixo cérebro-hipófise-gônada (CHG) é influenciado por fatores externos, como variações sazonais nas condições ambientais, incluindo fotoperíodo e temperatura (MIGAUD *et al.*, 2010). No entanto, embora a maioria das pesquisas tenha focado nos requisitos fototérmicos de espécies de peixes temperados, outros sinais ambientais, como o ciclo das marés lunar ou inundações durante a estação chuvosa, estão envolvidos na regulação da reprodução de espécies de peixes tropicais e subtropicais, isso se deve às mudanças sazonais relativamente pequenas de temperatura e fotoperíodo que ocorrem nos trópicos. (JOHANNES, 1978; TAKEMURA *et al.*, 2004).

Os efeitos da temperatura sobre os processos bioquímicos e fisiológicos são fundamentais para definir o desempenho de diversos processos nos animais, especialmente nos ectotérmicos como os peixes (FANGUE *et al.*, 2006). A variação da temperatura corpórea em peixes influencia várias funções fisiológicas como consumo de oxigênio, alimentação e digestibilidade (EVANS, 2006), afetando também aspectos funcionais como o crescimento e a aptidão reprodutiva (HAM *et al.*, 2003; OSTROWSKI *et al.*, 2011; IMHOLT *et al.*, 2011).

A elevação da temperatura da água tem um efeito negativo sobre o crescimento em decorrência do aumento da exigência energética, mas em contrapartida observa-se um efeito positivo na eficiência da transformação da energia dos alimentos em energia metabolizável (ÁRNASON *et al.*, 2009). Os peixes nas fases jovens demonstram um ligeiro aumento na taxa de crescimento com o aumento da temperatura, passando por um pico e caindo rapidamente quando a temperatura se torna adversa. Desta forma, a temperatura ótima para o peixe, representada pelo pico de crescimento, é espécie-específica e varia ao longo da vida (IMSLAND *et al.*, 1996). A temperatura ideal para muitas espécies para a conversão alimentar e crescimento diminui à medida que os peixes crescem (IMSLAND *et al.*, 1996; ÁRNASON *et al.*, 2009).

Vários registros de disfunções gonadais decorrente de temperaturas adversas em peixes foram descritos por Glasser *et al.* (2004). As técnicas histológicas auxiliam na identificação e reconhecimento destes distúrbios, pois permitem a pesquisa tecidual e celular no interior das gônadas. Além disto, a histologia permite inferir sobre a fecundidade dos peixes e é uma técnica amplamente utilizada em ambos os casos (PARENTI; GRIER, 2004; BAPARY; TAKEMURA, 2010).

O comprimento médio de primeira maturação gonadal, a época de maturação e o tipo de desova são traços reprodutivos moldados pelo ambiente que determinam a sobrevivência das populações (WINEMILLER; DONALD, 1989).

A maioria das espécies de peixes inicia seu desenvolvimento gonadal em uma época anterior àquela de reprodução completando sua maturação no momento em que as condições ambientais forem adequadas à fecundação e ao desenvolvimento da prole (VAZZOLER, 1996).

Os estímulos ambientais, tais como, fotoperíodo, temperatura, qualidade da água, pluviosidade e alterações nos níveis da água, são responsáveis pela modulação da reprodução dos teleósteos, variações estas que são percebidas através de órgãos sensoriais e transduzidas pelo sistema nervoso, que por sua vez controla o sistema endócrino (MIGAUD *et al.*, 2010). Os peixes teleósteos são sazonais, ou seja, se reproduzem sempre na mesma época do ano, que pode ser diferente para cada espécie.

A sincronização entre as variáveis ambientais adequadas para cada espécie, o desenvolvimento gonadal e a desova garantem uma maior de sobrevivência da prole (BALDISSEROTTO, 2002; ZANIBONI-FILHO E WEINGARTNER, 2007). Dessa forma, em espécies de clima temperado e latitudes mais elevadas, a reprodução se dá com o aumento das temperaturas e comprimento dos dias, ou seja, na primavera, enquanto que nos peixes tropicais e subtropicais o período reprodutivo parece estar relacionado com o aumento da pluviosidade ou ainda movimentos sazonais nas correntes oceânicas (BROMAGE *et al.*, 2001).

3.3 Esteroidogênese gonadal

Além da modulação por estímulos externos, como chuva, fotoperíodo e temperatura, a reprodução em teleósteos é controlada endogenamente pelo sistema neuroendócrino, através do eixo hipotálamo-hipófise-gônadas. A partir dos estímulos ambientais captados por receptores sensoriais, há transdução pelo sistema nervoso que estimulam o hipotálamo a sintetizar e liberar o hormônio liberador de gonadotrofinas (GnRH), que irão estimular as células gonadotróficas localizadas na adeno-hipófise a sintetizar e liberar o hormônio folículo estimulante (FSH) e hormônio luteinizante (LH). Esses hormônios são transportados pela corrente sanguínea e atingem as gônadas, onde estimulam a produção dos hormônios esteroides gonadais, responsáveis pelo desenvolvimento e maturação dos gametas (KUMAKURA *et al.*, 2003; WELTZIEN *et al.*, 2004; BOMBARDELLI *et al.*, 2006; LEVAVI-SIVAN *et al.*, 2010).

O controle da reprodução em teleósteos é possível devido ao Hormônio liberador de gonadotrofina (GnRH) que desempenha diferentes tipos de reações intracelulares para a liberação do hormônio folículo estimulante (FSH) e hormônio luteinizante (LH), que executam um importante papel no desenvolvimento gonadal e através desse estímulo podem atingir o fator de crescimento dos peixes através da ligação entre as células de Leyding, que é receptora do LH, e células de Sertoli, correspondente ao FSH, ocorrendo então a proliferação espermatogonial. As gonadotrofinas (FSH e LH) são cruciais devido a sua participação no processo gonadal. O FSH age nos primeiros estágios de maturação, enquanto o LH influencia nos processos finais da espermatogênese (BALDISSEROTTO, 2013).

O FSH atua nas células da teca nas fêmeas, ocasionando a conversão do colesterol em testosterona. Em seguida, a testosterona é carreada para as células foliculares e, sob a ação do FSH, é aromatizada em 17β-estradiol (17β-E) através da ação da enzima P450-aromatase (NAGAHAMA *et al.*, 1995). O 17β-E é responsável pela produção de vitelogenina e coreogenina pelos hepatócitos (FAUSTO *et al.*, 2004; SENTHILKUMARAN *et al.* 2004). A vitelogenina é uma fosfolipoglicoproteína, sintetizada nas células do fígado e carreada por via sanguínea ao ovário onde é endocitada e reduzida em proteínas vitelínicas: lipovitelina e fosvitina (MATSUBARA; SAWANO 1995). A coreogenina é precursora da proteína do invólucro do ovo de peixes, responsável pela proteção do ovo e embrião em desenvolvimento (FAUSTO *et al.* 2004).

A produção de 17α-hidroxiprogesterona (17α-OHP) pelas células da teca é induzida pela queda do FSH, seguida pelo aumento do LH, e posteriormente convertida em hormônio indutor da maturação final, 17α-20β-dihidroxi-4-pregnen-3-one (DHP) ou 17α-20β-trihidroxi-4-pregnen-3-one pela enzima 20β-hidroxiesteroide-desidrogenase (NAGAHAMA & YAMASHITA, 2008; LUBZENS *et al.*, 2010). O DHP promove a maturação ovocitária final, evento essencial para ovulação e desova (GARCIA-LOPEZ *et al.* 2007; NAGAHAMA & YAMASHITA 2008).

Nos machos, o FSH estimula a transformação do colesterol em pregnenolona nas células de Leydig. Posteriormente, a pregnenolona é convertida em 11-ketotestosterona e testosterona (T) por reações enzimáticas no retículo endoplasmático liso. Os dois andrógenos atuam no desenvolvimento do testículo e de caracteres sexuais secundários, e influenciam o comportamento reprodutivo (HAIDER, 2007; OHTA *et al.* 2007). A testosterona atua na maturação testicular, iniciando a divisão das espermatogônias, e início da liberação de

espermatozoides no lume dos túbulos seminíferos (WELTZIEN *et al.*, 2004; SCHULZ *et al.*, 2010).

Outra ação do FSH é estimular a conversão de testosterona em estrógeno pela enzima P450-aromatase. A proliferação, auto-renovação de espermatogônias e a diferenciação das células germinativas, é resultante da ação do17β-E (SCHULZ; MIURA 2002; PIFERRER; BLAZQUEZ 2005; MIURA *et al.* 2006), tendo comprovada ação na proliferação de células de Sertoli em testículos espermiados (CHAVES-POZO *et al.* 2007). Durante a maturação das células germinativas, altos níveis de T e 17β-E inibem a liberação do FSH pela hipófise. O LH, mais ativo nas fases mais tardias da espermatogênese, estimula as células de Leydig a produzir 17α-OHP, a qual é convertida em DHP, responsável pela diferenciação e maturação dos espermatozoides (YAMADA *et al.* 2002).

Em criação de peixes em pisciculturas, os perfis de esteroides sexuais são variáveis importantes e alguns trabalhos foram realizados com espécies neotropicais sob condições de cultivo (GAZOLA; BORELLA 1997; BARCELLOS *et al.* 2001; BARCELLOS *et al.* 2002; ARANTES *et al.*, 2010; ARANTES *et al.* 2011). Apesar de pouco exploradas, tais informações são igualmente importantes para a reprodução na natureza, visto que, alterações ambientais podem desencadear desequilíbrios endócrinos que limitam a propagação e conservação de peixes nativos (ARANTES *et al.* 2010; DOMINGOS *et al.* 2012b).

3.4 Genes associados à reprodução

3.4.1 Receptores de gonadotrofinas

As gonadotrofinas (GtH), hormônio folículo-estimulante (FSH) e hormônio luteinizante (LH), são proteínas heterodiméricas que consistem em uma subunidade α comum (glicoproteína α), que é ligada de forma não covalente a uma subunidade β específica a hormônios (FSHβ e LHβ) (Pierce e Parsons, 1981). Na gônada, as GtHs exercem suas ações através dos receptores (GtHR; FSHR e LHR), que são membros dos receptores acoplados à proteína G (COSTAGLIOLA *et al.*, 2005; CALTABIANO *et al.*, 2008). Estes, por sua vez, atuarão diretamente regulando as diferentes fases do processo de gametogênese e a liberação dos gametas, ou, indiretamente, por meio da produção e atividade de fatores de crescimento e outras moléculas sinalizadoras (LEVAVI-SIVAN *et al.*, 2010; ZOHAR *et al.*, 2010).

Nos peixes, os genes GtHR são predominantemente expressos nas gônadas. Essa distribuição limitada de tecidos destaca a importância de ambos os hormônios glicoproteicos na regulação da fisiologia gonadal, como demonstrado em mamíferos (PAKARAINEN *et al.*, 2007). Em salmão, foi proposto que o FSHR se localize nas células tecais e granulosa da fêmea e nas células de Sertoli durante todo o desenvolvimento dos machos, enquanto o LHR se localiza nas células da granulosa do folículo pré-ovulatório e nas células de Leydig (MIWA *et al.*, 1994; YAN *et al.*, 1992). Trabalhos em enguia japonesa (OHTA *et al.*, 2007) e peixe-gato africano (GARCIA-LOPEZ *et al.*, 2009), pertencentes às ordens anguilliformes e siluriformes, demonstraram a presença da proteína FSHR ou expressão do gene FSHR nas células Leydig.

Além de seu papel na regulação da esteroidogênese, a análise dos perfis de expressão gonadal dos genes GtHR durante o ciclo reprodutivo sugere seu envolvimento em períodos críticos da gametogênese de peixes. Nas fêmeas, a expressão do gene FSHR aumenta nos ovários à medida que os oócitos previtelogênicos entram na fase inicial de crescimento vitelogênico, atingem um pico na vitelogênese média e diminuem quando os oócitos atingem o estágio de crescimento total (KWOK *et al.*, 2005). Em *Dicentrarchus labrax*, os transcritos de FSHR são detectados nas células foliculares ao redor dos oócitos pré-vitelogênicos e precoces, mas não nos oócitos totalmente crescidos (ROCHA *et al.*, 2007a). Na enguia, os tratamentos E2 ou T não induziram um aumento dos níveis de transcrição de FSHR no ovário, sugerindo que o padrão de expressão do gene FSHR observado durante a vitelogênese precoce não se deve a uma ação direta dos esteroides sexuais (JENG *et al.*, 2007). Tomadas em conjunto, essas observações apoiam o envolvimento do FSHR no recrutamento de novos oócitos e no início da vitelogênese.

Além disso, aumentos na expressão do gene FSHR foram correlacionados com eventos posteriores do ciclo reprodutivo, incluindo a maturação oocitária e ovulação (BOBE *et al.*, 2003; KWOK *et al.*, 2005; SAMBRONI *et al.*, 2007). Da mesma forma, a expressão do gene FSHR nas gônadas masculinas mostra flutuação moderada durante o início do ciclo reprodutivo, mas aumenta significativa e consistentemente na espermiação em todas as espécies de peixes estudadas (KUSAKABE *et al.*, 2006; MAUGARS e SCHMITZ, 2006, 2008; RAHMAN *et al.*, 2003; ROCHA *et al.*, 2009; SAMBRONI *et al.*, 2007). Os transcritos de FSHR foram localizados nas células que circundam os espermatócitos (presumivelmente células de Sertoli), mas também nas células de Leydig (GARCIA-LOPEZ *et al.*, 2009). Isto

apoia fortemente que o gene FSHR também está envolvido na produção de esteroides nos machos.

Em relação ao LHR, sua expressão gênica está consistentemente correlacionada com os processos de desova e espermiação em todas as espécies de peixes estudadas até o momento (KUMAR *et al.*, 2001a; KWOK *et al.*, 2005; SAMBRONI *et al.*, 2007). Altos níveis de LHR foram encontrados em folículos ovarianos com oócitos vitelogênicos crescidos (GARCÍA-LÓPEZ *et al.*, 2011; KITANO *et al.*, 2011; NYUJI *et al.*, 2013). Os altos níveis de LHR em *Seriola dumerili* encontrados durante o período de desova podem estar associados à presença de oócitos vitelogênicos totalmente crescidos, uma vez que, os níveis de LH plasmático aumentaram gradualmente durante o desenvolvimento ovariano e permaneceram em altos níveis durante o período de desova (NYUJI *et al.*, 2016). Isto sugere que a síntese e secreção de LH e a expressão de LHR são reguladas positivamente após a conclusão da vitelogênese.

3.4.2 Receptor de estrógeno

O estradiol (E2) é um importante hormônio esteróide que atua na regulação da diferenciação sexual, desenvolvimento ovariano e produção hepática de vitelogenina em fêmeas (LASSITER *et al.*, 2002; NAGLER *et al.*, 2012; NI *et al.*, 2013). O mecanismo de ativação de sinalização do E2 envolve a ligação aos seus receptores de estrogênio cognato (ERs) na célula ou na membrana plasmática da célula (EDWARDS 2005; THOMAS *et al.*, 2006; LEVIN *et al.*, 2009; NAGLER *et al.*, 2010).

Em peixes, diferentes funções para os subtipos de ERs durante oogênese, vitelogênese e desenvolvimento testicular foram sugeridos com base em suas diferenças na afinidade de ligação (MENUET *et al.*, 2002), na expressão gênica entre tecidos masculinos e femininos (HALM *et al.*, 2004; LAFONT *et al.*, 2016) e em padrões de expressão sazonais em tecidos reprodutivos como gônadas, fígado e cérebro (LAFONT *et al.*, 2016).

Em fêmeas de robalo, a expressão gênica do receptor de estrógeno Er1 e Er2α têm um padrão sazonal específico nos ovários, no qual os níveis de expressão aumentaram fortemente ao final da vitelogênese e atingiram um pico durante a maturação (ZAPATER *et al.*, 2018). Por outro lado, a expressão de Er2β, cuja expressão aumentou com o início da vitelogênese, semelhante ao observado em peixe-martelo coreano e enguia europeia (LAFONT *et al.*, 2016). Em outros teleósteos, os níveis de Er1 foram mais semelhantes aos de Er2β, aumentando

durante a vitelogênese, enquanto os níveis de Er2α não mudam em nenhum momento durante o ciclo reprodutivo (CHAKRABORTY *et al.*, 2011; NAGASAWA *et al.*, 2014). Além disso, altos níveis de E2 foram encontrados nos ovários vitelogênicos do robalo, atingindo a produção máxima na pós-vitelogênese e diminuindo durante a maturação para atingir os níveis mais baixos de atresia (ZAPATER *et al.*, 2018). O E2 plasmático nesses mesmos animais mostra um perfil diferente, com níveis mais altos atingidos durante a pós-vitelogênese, mas são mantidos durante a maturação e atresia (ROCHA *et al.*, 2009).

Estudos de *knockdown* em hepatócitos de peixes dourados demonstraram que Esr1 é o principal subtipo envolvido na indução de vitelogênese, enquanto os subtipos Esr2 regulam a expressão de esr1 no fígado (NELSON *et al.*, 2010). Além disso, a perda de Esr2α e Esr2β em fêmeas de peixe-zebra *knockouts* levou a uma interrupção da foliculogênese no estágio pré-vitelogênico, seguida pela reversão de sexo entre fêmeas (LU *et al.*, 2017).

A presença de pelo menos um tipo de receptor de estrógeno foi demonstrada no testículo de muitas espécies de teleósteos, como o *Ictalurus punctatus* e a *Sparus aurata* (WU *et al.*, 2001; CHAUVIGNÉ *et al.*, 2017). No testículo de peixes dourados, apenas os transcritos de ERβ foram detectados (TCHOUDAKOVA *et al.*, 1999), enquanto que na corvina do Atlântico, o mRNA de ERα e o ERβ são co-expressos (XIA *et al.*, 2000; SOCORRO *et al.*, 2000; HAWKINS *et al.*, 2000). Por outro lado, nos testículos juvenis da enguia japonesa, a expressão de ERβ foi restrita apenas às células de Sertoli. No entanto, o mesmo estudo demonstrou que o estrogênio *in vitro* foi capaz de induzir a divisão espermatogonial, mostrando claramente o papel do "hormônio sexual masculino" no ciclo espermatogênico (MIURA et a., 1999).

A regulação do desenvolvimento e fertilidade de gametas masculinos por meio da interação entre estrógenos e ERs é evidente também em testículos maturos de *Ictalurus punctatus*. Nessa espécie os genes ERα e ERβ são co-transcritos nos epitélios germinativo e não-germinativo dos testículos (WU *et al.*, 2001). Estudos evolutivos moleculares realizados em *Spinibarbus denticulatus* demonstraram a expressão em espécimes masculinos de ERα e ERβ, cada um presente com duas isoformas após duplicação de genes. No testículo, foram detectadas isoformas ERα1 e ERβ1. A expressão de ERα1 aumentou gradualmente durante o período de recrudescência, enquanto a expressão de ERβ1 foi alta no estágio inicial de recrutamento, diminuiu no recrutamento tardio e, finalmente, aumentou novamente no período de recrutamento completo (ZHU *et al.*, 2008).

No bacalhau do Atlântico (*Gadus morhua*), três sequências de cDNA parciais de ER (denominadas esr1, esr2a e esr2b) foram clonadas. Todas as três formas estavam presentes em células de Leydig imaturas e maturas. A análise da expressão gênica revelou um padrão de expressão sexualmente dimórfica dos três paralelos de ER no testículo e ovário, com maiores conteúdos de esr1 e esr2a no testículo antes da desova (NAGASAWA *et al.*, 2014).

Recentemente, quatro receptores de estrogênio (denominados esr1a, esr1b, esr2a e esr2b) foram identificados nos testículos da truta arco-íris (*Oncorhynchus mykiss)*. A RT-PCR quantitativa revelou que, nesse peixe, os estrogênios têm funções fisiológicas discretas durante os estágios inicial e final da espermatogênese: no primeiro estágio, apenas o esr1b é bastante expresso; neste último, a expressão esr1a e esr2a aumenta consideravelmente; além disso, os andrógenos são capazes de regular positivamente esr1a, esr2a e esr2b testicular (DELALANDE *et al.*, 2001).

3.5 Espécie em estudo

Genyatremus luteus, conhecido popularmente como peixe-pedra ou roncador, é uma espécie de peixe teleósteo, e está classificado na família Haemulidae, ordem Perciformes. No total, 17 gêneros e cerca de 145 espécies formam essa família (NELSON, 2006), que habitam regiões tropicais e subtropicais, principalmente águas marinhas e estuarinas (LINDEMAN e TOXEY, 2002). O nome da família (*grunts*) deriva do som produzido pelo ranger dos dentes faríngeos que é amplificado pela bexiga natatória, atuando como caixa de ressonância (BAUCHOT, 1992; LINDEMAN; TOXEY, 2002).

A espécie possui ampla distribuição na América do Sul, podendo ser encontrado desde a porção leste da Colômbia até o Brasil, habitando águas costeiras com areia, pedras e fundos lamacentos, especialmente estuários e lagunas (CERVIGÓN, 1966; ARTIGAS *et al.*, 2003). É classificada como uma espécie estuarino-marinha (GIARRIZZO; KRUMME, 2007) e oportunista (CARVALHO-NETA *et al.*, 2011), pois utiliza o estuário para completar apenas uma etapa do ciclo de vida, utilizando a área para reprodução e/ou alimentação.

G. luteus é uma espécie demersal de médio porte (ESPÍRITO SANTO *et al.*, 2005), cujos tamanhos variam entre 4,5 a 21,4 cm na costa amazônica brasileira (FERNANDES *et al.*, 2017) e entre 9,5 a 32,0 cm na baía de São José, estado do Maranhão (ALMEIDA *et al.*, 2005). Em estudo no Golfo de Paria na Venezuela, Gomez *et al.* (2002) verificaram que a primeira

maturação nesta espécie, em ambiente natural, ocorre com 2 a 3 anos de vida, apresentando um comprimento total entre 14,7 a 34,5 cm.

Há dimorfismo sexual nesta espécie em relação ao comprimento e peso, que são superiores nas fêmeas, sugerindo que o maior crescimento destas tende a maximizar o sucesso reprodutivo (FONTELES FILHO, 2011), com relação direta entre o tamanho das fêmeas e a quantidade e tamanho dos ovos (FERNANDES *et al.*, 2017). Noleto-Filho *et al.* (2012) afirmam que esta espécie possui desova parcelada e sincrônica, que se caracteriza por apresentar dois grupos distintos e bem definidos, o que permite que larvas sejam encontradas durante o ano todo (BARLETTA e SAINT-PAUL, 2010).

O peixe-pedra é considerado uma das espécies mais capturadas pela pesca artesanal, principalmente nos estados do Maranhão e Pará, devido a sua grande abundância e apreciação pela população, servindo como fonte de alimento e renda (ISAAC *et al.*, 2011; ALMEIDA *et al.*, 2011; BENTES *et al.*, 2012; FERNANDES *et al.*, 2015). Além disso, Noleto-Filho *et al.* (2011) afirmam que *Genyatremus luteus* apresenta potencial aquícola, pois, em estudo realizado com a espécie em cativeiro, observou-se uma boa adaptação ao ambiente confinado, demonstrando plasticidade trófica.

*CAPÍTULO 1**

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

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Short running title: Testicular regulation in *Genyatremus luteus* (Perciforme: Haemulidae)

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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.

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

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.

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 *et al.*, 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.*, 2013a,b; Nobrega *et al.*, 2015; Rajakumar *et al.*, 2020), including anti-Müllerian hormone (AMH) (Skaar *et al.*, 2011; Oliveira *et al.*, 2021) and insulin growth factor-3 (IGF3) (Sambroni *et al.*, 2013a,b; Melo *et al.*, 2015; Nobrega *et al.* 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; Nobre *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; Castro 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*. (2011), *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.

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		

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 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, 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.

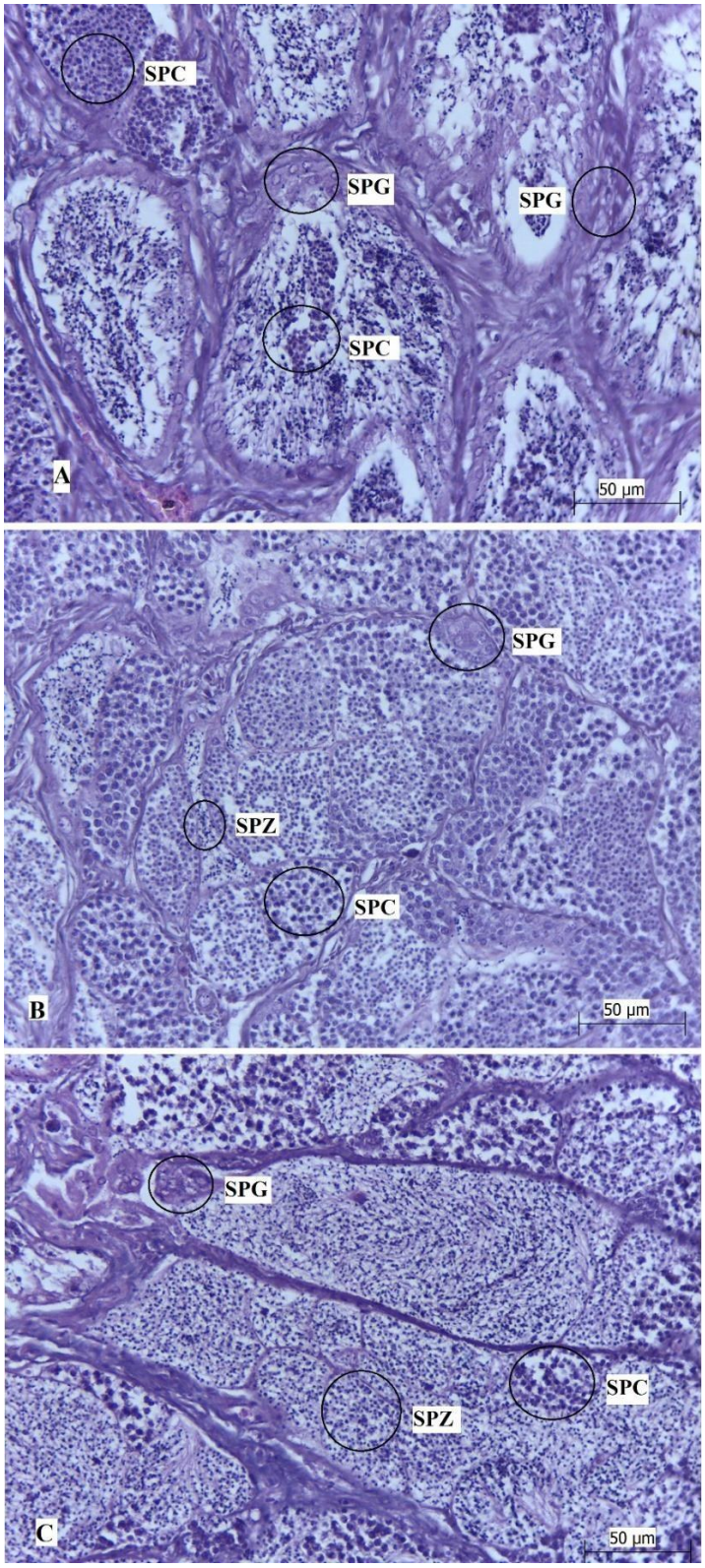
3 Results

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.

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 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).

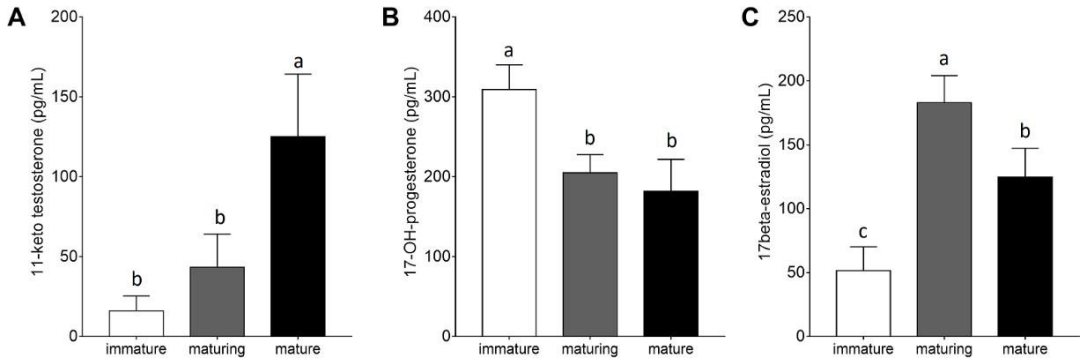
Table 2. Ichthyometric and GSI data analyzed during the reproductive cycle of male *Genyatremus luteus* specimens. Data are presented as mean ± SEM.

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

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 ($P<0.05$).

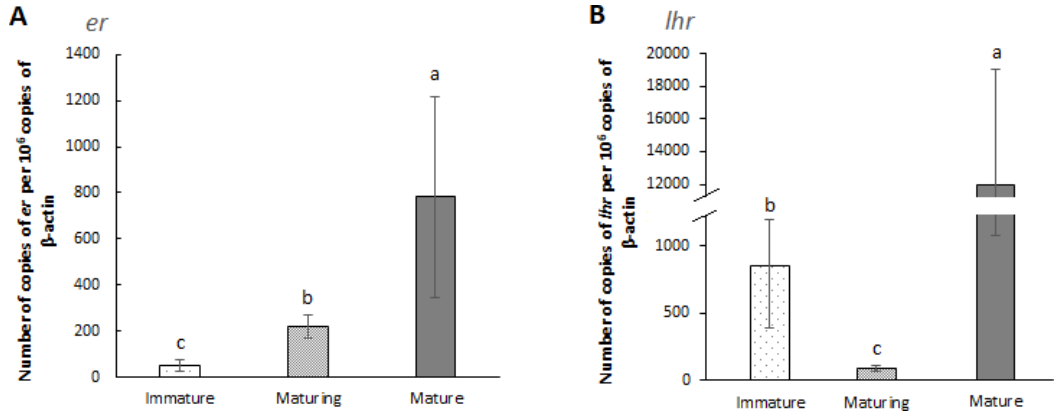
Figure 2. Steroid concentrations in the blood plasma of male *Genyatremus luteus* individuals during their reproductive cycle. (A) 11-ketotestosterone. (B) 17 α-hidroxy progesterone. (C) 17β-estradiol. Data are represented as mean ± SEM. *indicates statistically significant difference ($p<0.05$).



3.3 Gene Expression Profiles for *er* and *lhr*

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

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 ($p<0.05$).

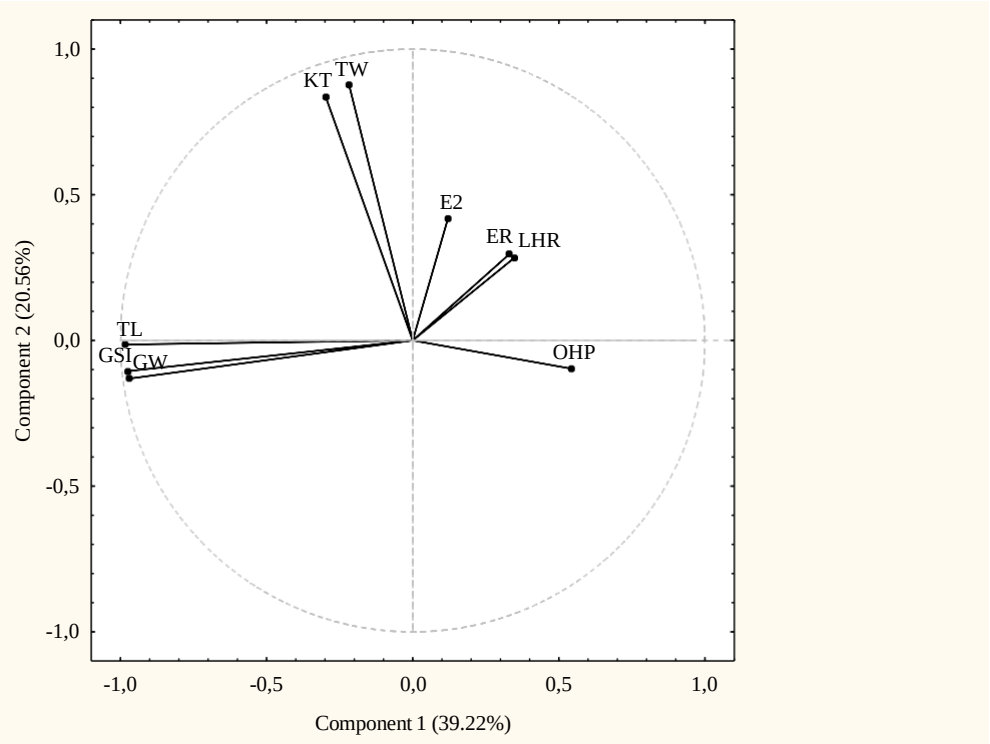


3.4 Association among ichthyometric variables, GSI, hormonal profile and gene expression

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 4, variables mostly contributing to Component 1 were *lhr* and *er* expression, as well as the ichthyological parameters, which were inversely related to each other.

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.



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 teleost species (Brown-Peterson *et al.*, 2011; Nishimura and Tanaka, 2014; Siqueira-Silva *et al.*, 2018; 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.

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.

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*CAPÍTULO 2**

***Artigo submetido à revista Boletim do Instituto de Pesca.**

Steroid profile and ovarian gene expression in *Genyatremus luteus* (*Teleostei*, Perciformes: Haemulidae) during sexual maturation*

Perfil de esteróides e expressão gênica ovariana em *Genyatremus luteus* (Teleostei, Perciformes: Haemulidae) durante a maturação sexual

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ABSTRACT

The aim of this study was to evaluate the histological features, sex steroid profile and ovarian gene expression throughout the ovarian maturation development of *Genyatremus luteus* in Brazilian Coastal Equatorial Amazon. Specimens were categorized into different gonadal maturation stages (Immature (n=7), Maturing (n=7), Mature (n=7) and Spawned (n=7)) based on histological features of ovarian follicles and on oocyte development. Plasma E2, 11-KT and 17α-OHP levels were measured through ELISA assay. Gene expression of estrogen (*er*) and LH (*lhr*) receptors in the ovary was analyzed by real-time PCR. The highest plasma E2 concentration was found in mature specimens (P <0.05). Plasma 17α-OHP concentrations have significantly increased from the immature to the final maturation stage (P <0.05). Maturational reproductive stage did not have any effect on plasma 11-KT concentrations. *Er* expression in the ovaries has significantly increased throughout ovarian maturation (P <0.05) and remained high at mature and spawned stages. *Lhr* expression has shown similar trend throughout the maturation process, although it reached its peak at the spawned stage (P <0.05). In conclusion, the gene expression profile of *er* and *lhr*, and plasma E2 and 17α-OHP concentrations are proportional to oocyte development and play important role in regulating ovarian development and ovulation in *G. luteus*.

Keywords: Gonadotropin receptor; Estradiol; Gonads; Reproduction; Grunts.

RESUMO

O objetivo deste estudo foi avaliar as características histológicas, o perfil de esteroides sexuais e a expressão gênica ovariana ao longo do desenvolvimento da maturação ovariana de *Genyatremus luteus* na Amazônia equatorial costeira brasileira. Os espécimes foram categorizados em diferentes estágios de maturação gonadal (imaturo (n=7), em maturação (n=7), maduro (n=7) e desovado (n=7)) com base nas características histológicas dos folículos ovarianos e no desenvolvimento do oócito. Os níveis plasmáticos de E2, 11-KT e 17α-OHP foram medidos por meio de ensaio ELISA. A expressão gênica dos receptores de estrogênio (*er*) e LH (*lhr)* no ovário foi analisada por PCR em tempo real. A maior concentração plasmática de E2 foi encontrada em espécimes maduros (P <0,05). As concentrações plasmáticas de 17α-OHP aumentaram significativamente desde o estágio imaturo até o estágio final de maturação (P <0,05). O estágio reprodutivo maturacional não teve nenhum efeito nas concentrações plasmáticas de 11-KT. A expressão de *er* nos ovários aumentou significativamente ao longo da maturação ovariana (P <0,05) e permaneceu alta nos estágios de maturação e desova. A expressão de *lhr* apresentou tendência semelhante ao longo do processo de maturação, embora

tenha atingido seu pico na fase de desova (P <0,05). Em conclusão, o perfil de expressão gênica de *er* e *lhr*, e as concentrações plasmáticas de E2 e 17α-OHP são proporcionais ao desenvolvimento oocitário e desempenham papel importante na regulação do desenvolvimento ovariano e ovulação em *G. luteus*.

Palavras-Chave: Receptor de gonadotrofina; Estradiol; Gônadas; Reprodução; roncador.

INTRODUCTION

Estuarine and marine teleost fish are important fishing resources, mainly for the subsistence of riverside populations. However, the sustainability of these resources is threatened, since their exploitation has had major impact on natural populations living in Coastal Equatorial Amazon (Santos, Santos, 2005). Thus, it is necessary to carry out studies focused on investigating the reproductive biology of the aforementioned fish species, in association with socio-environmental studies, in order to better substantiate future management and conservation measures to be adopted to help maintaining aquatic ecosystems (Cochrane, 2002).

Fish belonging to family Haemulidae, as well as to order Perciformes, are also inserted in this scenario. The aforementioned family comprises approximately 145 species distributed in 17 genera, among which, one finds *Anisotremus, Conodon, Orthopristis, Pomadasys, Genyatremus* and *Boridae,* that were already listed in the Atlantic Ocean (Johnson, 1980; Nelson, 2006; Tavera *et al*., 2012).

Oocyte maturation and ovulation processes in teleost fish are intrincately linked to each other, similar to those of other vertebrates. These processes are primarily triggered by an endocrine signal featured by increased follicle-stimulating hormone (FSH) level (Lubzens *et al*., 2010). FSH acts in theca cells in order to convert cholesterol into testosterone. Then, due to the action of FSH, testosterone is carried to follicular cells and aromatized in 17β-estradiol (E2) through the action of the aromatase cytochrome P450 enzyme (Levavi-Sivan *et al*., 2010). E2 accounts for inducing vitellogenin and choreogenin production by hepatocytes (Senthilkumaran *et al*., 2004). Vitellogenin is carried through the bloodstream to the ovary, where it is incorporated to oocytes during vitellogenesis (Matsubara, Sawano, 1995).

The steroidogenic pathway changes E2 into 17α-hydroxyprogesterone at the end of the maturation stage (17α-OHP). 17a-OHP production by theca cells is induced by FSH decrease. It is followed by LH increase and converted into maturation-inducing hormone, 17α-20β-dihydroxy-4-pregnen-3-one (DHP) or 17α-20β-trihydroxy-4-pregnen-3-one by 20β-hydroxysteroid-dehydrogenase enzyme (Nagahama, Yamashita, 2008; Lubzens *et al*., 2010).

DHP enables the final oocyte maturation, which plays essential role in ovulation and spawning (García-López *et al*., 2007; Nagahama, Yamashita, 2008; Honji, Moreira, 2017).

Achieving maturation and ovulatory competencies in ovarian follicles of teleost fish requires orchestrated gene expression in the post-vitellogenic period, increase or decrease in the number of steroidogenic enzymes during estrogen transition to progestin (Nagahama, Yamashita, 2008), as well as changes in gonadotropin receptors, paracrine regulators, proteases (Bobe *et al*., 2004) and inflammation-related genes (Bobe *et al*., 2006; 2009). However, gene products associated with maturation and ovulatory competencies can also be synthesized at early developmental stage as vitellogenic follicles sensitive to exogenous gonadotropin stimulation *in vivo*, as well as induced to final oocyte maturation and ovulation (Zohar *et al*., 2001).

Although a few *Genyatremus luteus* (Bloch, 1790) biology aspects have already been investigated, studies about hormonal and molecular features associated with ovarian maturation development in this species remain scarce. Gene expression of estradiol (*er*) and LH (*lhr*) receptors in the ovary were analyzed through SYBR real-time PCR assays. Plasma Estradiol (E2), 11-ketotestosterone (11-KT) and 17α-hydroxyprogesterone (17α-OHP) levels were measured in enzyme-linked immunosorbent assay (ELISA) in order to feature endocrine changes taking place during gonadal development in female *G. luteus* individuals. The analysis of ovarian development in female *G. luteus* specimens was based on macroscopic and histological features.

MATERIALS AND METHODS

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.

Female *Genyatremus luteus* individuals were captured through artisanal fishing with hand line made up 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, Amazon Equatorial Coast, Brazil, from June to September 2019.

Blood samples were collected by puncturing the fish gills immediately after capture with heparinized syringe (Liquemine, Roche®); which were centrifuged at 1500g for 10 min; plasma was aliquoted and immediately frozen at -80°C, until hormonal analysis time.

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

Fish biometrics 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 consderation variables such as body weight rate and total weight of ovaries, based on the following formula [GSI = (gonad weight / body weight) x 100] (Vazzoler, 1996). In addition, median region fragments of fish ovaries were collected for histological analysis, after fixation in Bouin's solution and processing based on routine histological methods (Yoshida, 1964). Females were categorized based on macroscopic aspects of their gonads and on the histological configuration of ovarian follicles and oocytes; it was done by following the gonadal maturation scale developed for Hamulids, according to Shinozaki-Mendes *et al.* (2013), and adapted to four maturation stages, namely: immature (n=7), maturing (n=7), mature (n=7) and spawned (n=7).

Steroid analyses

Plasma 17β-estradiol (E2), 17-α-hydroxy-progesterone (17α-OHP) and 11-ketotestosterone (11-KT) levels were determined through Enzyme-linked immunoasorbent assays (ELISA) (IBL International, Hamburg, Germany, for E2 and 17α-OHP, and Cayman Chemicals Company, Michigan, USA, for 11-KT). 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 the detection limits of the assays - 1: 2 dilution was established for assays comprising all three hormones. In addition, plasma samples were analyzed in duplicate, whereas the 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 on microplate reader (Spectra MAX 250). Plasma E2 and 17-

OHP concentrations were determined at wavelength of 450 nm and 11-KT 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 by using the following equation: LogitDO: log (DO / (100- DO)), as recommended by the manufacturer.

Total RNA isolation and reverse transcription

Total RNA was extracted from individual ovary samples in 1 mL of Trizol (Invitrogen; Carlsbad, CA, USA) for er and lhr expression analysis, according to 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) and 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), according to the manufacturer's instructions, with a final volume of 20 µL per reaction. Primers in this rapid reverse transcriptase kit for reverse transcription were Oligo-dT primers. cDNA was diluted 3-fold and stored at -20°C.

Quantitative real-time PCR (qPCR)

The 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 available in the NCBI database, as described in Table 1.

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		

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

qPCR efficiency of each primer pair was evaluated by standard curves from a graded series of diluted cDNA (3-fold; 9-fold; 27-fold; 81-fold; 243-fold) to ensure that PCR efficiency of selected primer pair ranged from 90% to 100% (R2 > 0.99).

qPCR parameters were: 50ºC for 2 min, 95ºC for 10 min, 40 cycles of 95ºC for 15 s and 60ºC for 1 min, using an ABI 7500 Real-timePCR System (Applied Biosystems, USA). Data were analyzed based on the comparative Ct method (Livak, Schmittgen, 2001). Ct values were normalized based on β-actin expression (Wang *et al.*, 2009).

Statistical analysis

Frequencies of each gonadal maturation stage were analyzed through Fisher's exact test, according to Sampaio (2002), in the GraphPad Instat® 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 and means recorded for each gonadal maturation stage were compared to each other through Duncan test (PROC GLM of SAS). E2, 17-OHP and 11-KT 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, 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 the interactions among them.

RESULTS

Reproductive maturational stages and GSI analyses

Macroscopic analysis has shown a pair of tubular gonads covered by fibrous connective tissue capsule (tunica albuginea), which defined the intraovarian limit typical of cystovarian species, whose oocyte visualization in separate was only possible at the most advanced maturity stages. Different size, symmetry, color, consistency, and vascularity patterns in the ovaries of *G. luteus*, were observed, *depending* on their maturation status.

Microscopically the investigated species was classified as gonochoric due to evidence of unisexual gonads in all specimens, regardless of their reproductive maturation stage. Based on the morphology of ovigerous lamellae and oocytes, *G. luteus* specimens were classified into four maturation stages, namely: a) Immature, b) Maturing, c) Mature and d) Spawned (Figure 1a - d). They were also featured as multiple-batch group synchronous species, since they simultaneously presented gonads with follicles and oocytes at different development stages (Figure 2).

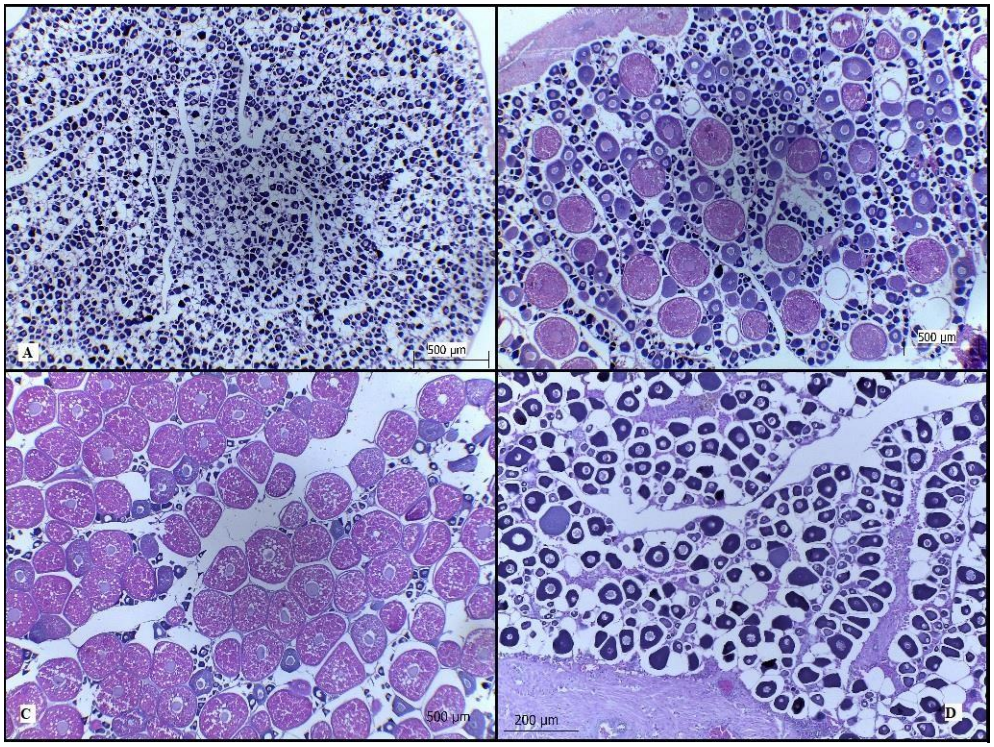

Figure 1. Histological sections of ovarian maturation stages in *Genyatremus luteus*. a. Immature; b. Maturing; c. Mature; d. Spawned. HE 4x; 10x.

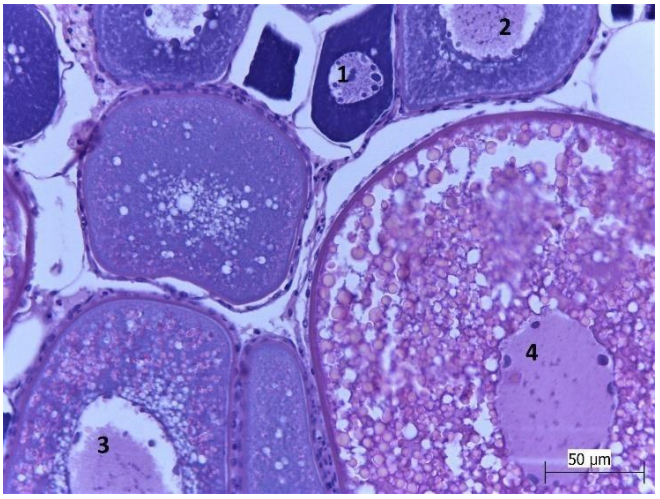

Figure 2. Histological sections of different oocyte developmental stages in *Genyatremus luteus*. Four stages: previtellogenic, perinucleolar (1); previtellogenic, cortical alveoli formation onset (2); early vitellogenic (3); and mid-vitellogenic, found in a single ovary (4).

Total (TL), furcal (FL) and partial (PL) lengths of immature females were significantly shorter ($P < 0.05$) than those of maturing, mature and spawned females ($P > 0.05$) (Table 2). Spawned females have shown longer lengths, as well as higher total and eviscerated weight ($P < 0.05$).

Table 2. Ichthiometric and gonadosomatic index data recorded throughout the reproductive cycle of female *Genyatremus luteus* individuals. Data were expressed as mean ± SEM.

Variable	Reproductive Maturation Stages			
	Imature	Maturing	Mature	Spawned
Total Length (cm)	18.35 ± 1.22^{c}	23.14 ± 1.73^{b}	24.07 ± 1.83^{b}	29.19 ± 0.24 ^{a}
Furcal Length (cm)	17.21 ± 1.06^{c}	21.57 ± 1.50^{b}	22.57 ± 1.67^{b}	27.44 ± 0.23^{a}
Partial Length (cm)	15.21 ± 1.06^{c}	19.28 ± 1.45^{b}	20.14 ± 1.54^{b}	24.11 ± 0.36^{a}
Total Weight (g)	130.57 ± 36.63^{c}	238.57 ± 40.90^{bc}	267.14 ± 58.78^{b}	414 ± 14.11^{a}
Eviscerated Weight (g)	118.42 ± 35.78^{c}	216.64 ± 37.00^{bc}	240.00 ± 50.28^{b}	379 ± 10.92^{a}
Gonadal weight (g)	1.07 ± 0.75^{b}	3.98 ± 1.60^{ab}	6.41± 2.75^{a}	3.44 ± 0.32^{ab}
Gonadosomatic index (%)	0.48 ± 0.19 ^{b}	1.54 ± 0.55 ^{a}	1.92 ± 0.46 ^{a}	0.84 ± 0.09 ^{ab}

^{abc} Different letters on the same line indicate significant difference between reproductive stages (P <0.05)

Mature females presented gonadal weight higher than that of immature females (P <0.05); these variables did not show statistically significant difference between animals at mature and spawned stages. Immature females recorded GSI value 3.2 and 4 times lower than those of maturing and mature adult females, respectively (P <0.05). Mature and maturing females did not differ from each other in any of the investigated ichthyometric variables (P>0.05) (Table 2).

Plasma steroids

The highest plasma E2 concentrations were found in mature specimens, whereas the lowest concentrations of it were found in spawned specimens (P <0.05). On the other hand, the reproductive maturation transitional process did not mean increased E2 concentrations in comparison to immature status (P> 0.05) (Figure 3).

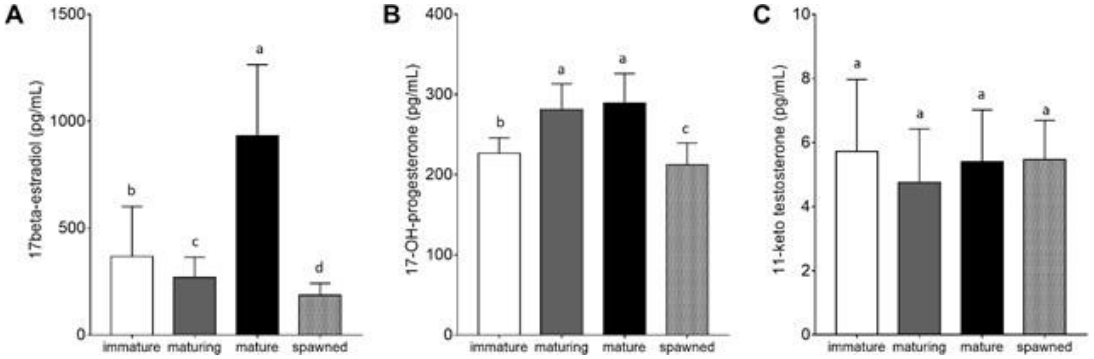

Figure 3. Plasma steroid concentrations throughout the reproductive cycle of female *Genyatremus luteus* individuals. (A) Plasma 17β-estradiol concentration. (B) Plasma 11-ketotestosterone concentration. (C) Plasma 17 α-hidroxyprogesterone concentration. Data were expressed as mean ± SEM. abc Different letters indicate statistically significant difference ($P < 0.05$).

Plasma 17α-OHP concentrations have significantly increased from the immature to the final maturation stage, although they declined again to the lowest concentrations in spawned females ($P < 0.05$). There was no effect of reproductive maturation stage on plasma 11-KT concentrations in female *G. luteus* individuals.

Gene Expression Profiles recorded for *er* and *lhr*

The *er* mRNA expression has significantly increased during ovarian maturation development ($P < 0.05$) and remained high and stable in animals at mature and spawned stages, as shown in Figure 4a. The LH receptor expression has shown similar trend throughout the maturation process. However, there was also significant increase in it since the mature stage, and it reached its peak at spawned stage ($P < 0.05$) (Figure 4b).

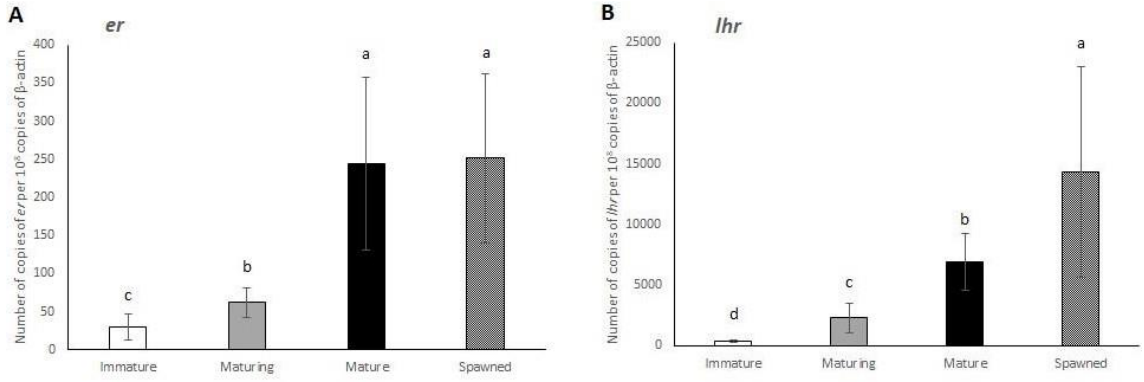

Figure 4. Gene in the gonad of Genyatremus luteus females at different maturation stages. a. *er* expression. b. *lhr* expression. Columns represent arithmetic mean (n = 7) and standard error. abc Different letters indicate statistically significant difference (P < 0.05).

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

The association among ichthyological parameters, gonadosomatic index, hormonal profile and ovarian gene expression of *lhr* and *er* in female *G. luteus* individuals was analyzed through Principal Component Analysis (PCA) (Figure 5). Results have shown that the two main components altogether explained 65.7% of total data variation, with emphasis on Component 1, according to which, ichthyometric variables were more representative due to longer vectors closer to Component 1 axis.

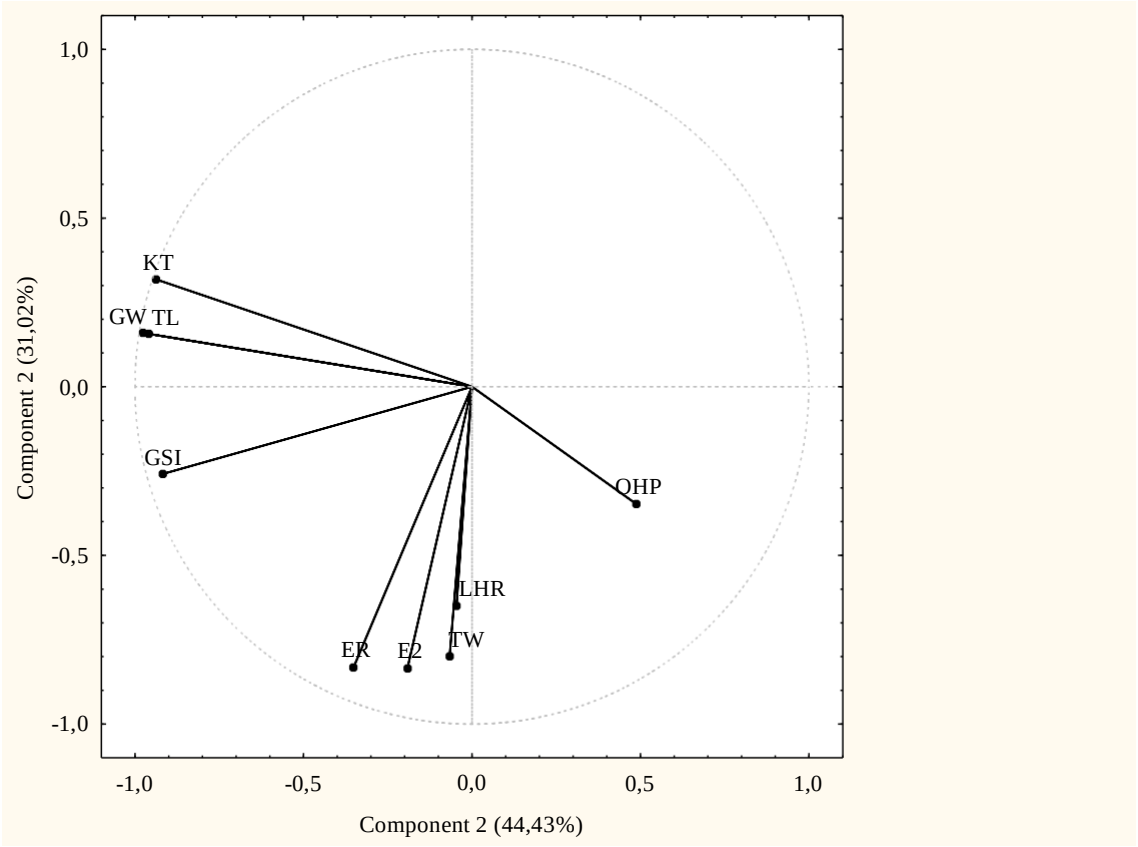

Figure 5. Principal component analysis applied to classify the influence of lhr and er gene expression, ichthyological parameters, GSI and maturation stage on the evaluated variables in *Genyatremus luteus* females.

Plasma E2, GW and GSI concentrations were highly associated with each other, since they formed acute angles between their respective vectors, which were determined by the high representativeness of the mature stage for these parameters. Similarly, *er*, TW and TL expressions were also closely associated with each other, as well as positively correlated to *lhr*

expression - the spawning stage was the most representative of these vectors. Based on Figure 5, 17OHP and 11-KT concentrations were the variables that mostly contributed to Component 2; they were inversely correlated to each other and did not present significant association with other variables.

DISCUSSION

Macroscopic evaluation of the *G. luteus'* ovaries has shown that this organ has the same basic anatomical pattern as that of most teleosts. This finding met data about this species available in the literature. (Noleto-Filho *et al.*, 2018). Its featuring as gonochoric, cystovarian species with group-synchronous oocyte development and multiple-spawning - as evidenced for the first time in this manuscript, according to findings detailed by Wallace and Selman, (1981), Tyler and Sumpter (1996), Blazer (2002) - is similar to another "grunt" species belonging to family Haemulidae, namely: *Haemulon plumieri* (Shinozaki-Mendes *et al.*, 2013).

Sex differentiation based on macroscopic features of developed gonads was evident in *G. luteus*; however, this visual classification in immature individuals was not possible due to their similarity (small, translucent gonad with no apparent vascularization). Thus, the histological analysis enabled performing precise assessment to differentiate animals' sexes and maturation stages to avoid accurate identification issues (West, 1990; Dias *et al.*, 1998; Honji *et al.*, 2009). *G. luteus* oocytes' development did not differ from that of most multiple-spawning teleost species (Wallace, Selman, 1981; Godinho *et al.*, 2005; Amaral *et al.*, 2019).

It was possible identifying oogonies and oocytes at all gonad maturation stages, including at the spawned stage, and it suggested that the investigated species is continuously available for vitellogenesis and reproductive maturation resumption (Wallace, Selman, 1981; Vazzoler, 1996; Shinozaki-Mendes *et al.*, 2013). Cortical alveolar oocytes were identified at the subsequent oocyte development stage due to the incidence of spherical vacuoles that looked like empty structures - this stage is commonly called endogenous vitellogenesis (Wallace, Selman 1981; Tyler, Sumpter, 1996; Grier *et al.*, 2009; Lubzens *et al.*, 2010).

Vitellogenic oocytes are featured by significant increase in size due to the incorporation of highly acidophilic granules, which accumulate inside the cytoplasm vesicles, in a process known as exogenous vitellogenesis (Peter, Yu 1997; Yaron, Silvan, 2006). This oocyte development stage was essential to classify *G. luteus* gonadal stage as mature. The incidence of post-ovulatory follicles (POF) in the ovaries of *G. luteus* individuals was observed in spawned females; they are featured as structures formed after ovulation and comprise follicular cell layers that, after the expulsion of mature oocytes, keep on presenting broken and

deflated appearance inside the ovaries after spawning (Nagahama, Kagawa, 1982; Selman, Wallace, 1989).

As expected, ichthyometric and GSI values increased as ovarian development progressed. The lowest values were recorded for animals at immature stage, whereas the highest values were recorded for animals at mature stage, who showed normal gonadal development. According to Vazzoler (1996), ichthyometric values associated with GSI are a good reference for the reproductive activity of fish; they can be used to determine gonadal maturation stages, since the maturation of germ cells happens at the same time female gonad weight increases (Le Cren, 1951).

High plasma E2 levels were found in female *G. luteus* individuals during oocyte development; they reached their peak in animals at mature stage and decreased in animals at spawned stage ($p<0.05$). Studies have shown that E2 acts directly on germ cell progression throughout early oogenesis stage; plasma E2 levels of approximately 0.4 ng / mL were recorded during mitotic egg proliferation in Japanese huchen (*Hucho perryi*) and common carp (*Cyprinus carpio*) (Miura *et al.*, 2007; Kazeto *et al.*, 2011), and it evidenced the fundamental role played by E2 in germ cell proliferation since the immature stage, which was also observed in the present study. In addition, E2 plays essential role in vitellogenesis, since it stimulates hepatic vitellogenin synthesis, which promotes oocyte growth and yolk incorporation to it (Honji e Moreira, 2017; Reading *et al.*, 2017). Increased E2 levels associated with high GSI levels in mature *G. luteus* individuals suggests the role played by this steroid in inducing hepatic vitellogenin synthesis. Variations in plasma E2 levels at different maturation stages were also reported in other multiple spawning teleost species, before and during spawning (Rinchard *et al.*, 1997; Rahman *et al.*, 2000; Amaral *et al.*, 2019).

The present study has found increased plasma 17α-OHP levels in *G. luteus* from the immature to the mature stage; these levels have decreased in spawned females. Studies conducted *in vitro* have shown that 17α-OHP was converted into 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) by 20β-hydroxysteroid dehydrogenase (20β- HSD) enzymatic action during the final oocyte maturation (FOM) stage (Matsuyama *et al.*, 1998; Ohta *et al.*, 2002; Matsuyama *et al.*, 2005) – this outcome explains decreased plasma 17α-OHP levels observed in animals at spawned stage. 17α, 20β-DP is the main steroid acting in the maturation and final ovulation of oocytes in most teleosts; it is commonly known as maturation-inducing steroid (MIS) (Lubzens *et al.*, 2010). In addition, 17α-OHP alternatively participates as precursor for its conversion into 17α, 20β, 21-trihydroxy-4-pregnen-3-one (20β-S) in teleost species, such as Atlantic croaker (*Micropogonias undulatus*), spotted seatrout (*Cynoscion nebulosus*) and bamboo

leaf wrasse (*Pseudolabrus sieboldi*) (Trant *et al.*, 1986; Thomas, Trant, 1989; Matsuyama *et al.*, 1998; Tokarz *et al.* 2015; Ogino *et al.*, 2016).

Plasma 11-KT levels in the current study were low at all maturation stages. The highest levels (5.7 pg / mL) were found in immature specimens, and this finding suggests that pre-vitellogenic and vitellogenic oocyte development in *G. luteus* may not require high 11-KT concentrations. Thus, further studies focused on addressing some of these issues associated with the role played by androgens in the sexual development of females belongin to this species should be conducted.

Although 11-KT is considered androgen, some studies have shown that it is involved in controlling the growth of pre-vitellogenic oocytes in some teleost species (Lokman *et al.*, 2007; Endo *et al.* 2008; Tosaka *et al.*, 2010; Hermelink *et al.*, 2011; Wang *et al.*, 2020). Amaral *et al.* (2019) reported lower 11-KT levels in female *A. gigas* individuals than in other species, although these levels increased and reached their peak in animals at mature stage (71.54 pg/mL). The aforementioned authors have suggested that this hormone is not limited to vitellogenesis, it also acts in the final maturation / ovulation stages in this species.

Treatments conducted *in vitro* and *in vivo* with 11-KT in Atlantic cod (*Gadus morhua*) have accelerated the growth and development of pre-vitellogenic oocytes. This outcome has suggested the role played by this androgen in early oocyte growth in controlled environments (Kortner *et al.*, 2009a, b). On the other hand, Kohn *et al.* (2013) did not observe the effects of treatment with 11-KT on ovarian development and total lipid concentrations in the liver of female *Polyprion oxygeneios* individuals. These controversial results were reported in different studies that have suggested that the role played by 11-KT in ovarian development is likely species-dependent.

The current results have shown gradual increase in *er* mRNA expression during gonadal development. Similar results were reported by Zapater *et al.* (2018) in studies conducted with *Dicentrarchus labrax*, which evidenced low ovarian *er* transcription during pre-vitellogenesis, although it increased during vitellogenesis and reached peak expression at mature stage. The regulation of *er* transcript levels may be associated with increased circulatory estradiol-17β (E2) levels during oocyte maturation stages (Silva *et al.*, 2016). It happens because the activation of estrogen receptors depends on cell ligands (E2) (Edwards 2005; Thomas *et al.*, 2006; Levin *et al.*, 2009; Nagler *et al.*, 2010). Thus, if one takes into consideration that 17β-estradiol (E2) is one of the main ligands for estrogen receptor, its greater expression indicates higher levels of this steroid in gonads. Kang *et al.* (2015) reported increased plasma E2 levels in female *Hapalogenys nitens* individuals, based on oocyte

development and on changes in GSI. This factor induced increasing vitellogenin production at mature stage, maintained high levels of it during spawning and, consequently, reduced the levels of it at post-spawning stage, as also observed in the current study. ERs can transduce E2 signals in the ovary to initiate or maintain ovarian development. Study conducted by Hou *et al.* (2017) with female *Oncorhynchus mykiss* individuals has shown positive correlation between increased serum E2 levels and *er* expression during ovarian maturation. The *er* expression level significantly affects the action of E2, since its high expression sensitizes and increases cell response to this hormone (Nelson, Habibi, 2010).

However, estrogens in teleosts and mammals have pleiotropic functions, which are not just limited to the reproductive axis, since they are found in several cell types and tissues, such as those in the immune system (Burgos-Aceves *et al.*, 2016; Amenyogbe *et al.*, 2020). Assumingly, most estrogenic actions are mediated by estrogen receptors (er) that belong to the family of steroid hormone receptors and have different isoforms (ERα and ERβ) that are differentially regulated based on cell type (Nelson, Habibi, 2013). Casanova-Nakayama *et al.* (2018) have evaluated the gene expression of four *er* isoforms in immune system organs, liver and ovaries during the reproductive cycle of female rainbow trout; they reported differential transcription profile for each isoform, as well as changes in the number of transcripts in immune cells and organs, although they differed from those found in animals' liver and gonads. In addition, they found moderate-to-low correlation between the number of *er* gene transcripts and serum E2 concentrations. This outcome suggested that low responsiveness to estrogens may encompass other mechanisms, such as non-genomic pathways or indirect effects. Similarly, the current study has only observed this low correlation in animals at spawned stage, who presented decreased serum E2 levels, despite the high values recorded for *er* transcripts.

A previous study aimed at clarifying *er* function in teleost model (*Oryzias latipes*), based on knockout technology. It has shown that *er2a* -/- females had atresic oviduct, in contrast to *er2a* +/− females that spawned every day and presented oviduct with full opening. This outcome confirmed *er* participation in the development of female genital morphology, mainly of the oviduct (Kayo *et al.*, 2019).

The *lhr* expression profile started increasing at immature stage, kept on increasing through maturational development and reached its peak during spawning. Previous studies with female salmonid individuals have suggested that LH plays important role in regulating the final maturation and ovulation of semelparous individuals (Bobe *et al.*, 2004; Yaron, Sivan, 2006; Sambroni *et al.*, 2007). On the other hand, *lhr* synthesis in fish multiple-batch group

synchronous reproduction presenting varying ovarian development levels (Wallace, Selman, 1981; Brown-Peterson *et al.*, 2011) is likely required for different vitellogenesis stages, as well as for final oocyte maturation, which happen simultaneously. These previous studies have contributed to justify the result obtained in the current study, according to which there was increased *lhr* expression in female *G. luteus* individuals at final maturation stage.

Lhr expression in fish species presenting multiple spawning, such as *Dicentrarchus labrax* (García-López *et al.*, 2011) and *Seriola dumerili* (Nyuji *et al.*, 2016), increases at the same time oocytes develop to reach peak spawning. This fact was also observed in the current study, since *G. luteus* has synchronous spawning in multiple batches and presents residual oocytes at the final maturation stage, during the spawning period. *Oncorhynchus mykiss* recorded remarkable increase in *lhr* expression at the final vitellogenesis stage, and it persisted during oocyte maturation and ovulation. This outcome corroborates the interpretation that both LH and its receptor act in the final maturation of ovarian follicles and oocytes (Gomez *et al.*, 1999). Studies have reported high plasma LH levels during the post-spawning period in rainbow trouts and *Seriola dumerili*, although their role remains unclear (Breton *et al.*, 1998; Gomez *et al.*, 1999, Nyuji *et al.*, 2016), which may be associated with findings in the current study, according to which *lhr* expression in spawned females was also significantly high.

Significant association among ichthyometric parameters, plasma E2 and *er* / *lhr* expression has indicated regulation common to cellular, endocrine and molecular features and led to sexual maturation process in *G. luteus.* Liu *et al.* (2017) have shown that E2 stimulates *lhr* expression through *er* in *Danio rerio*'s ovary. Accordingly, other studies have also shown association between *lhr* and *er*, through positive feedback on LH, in female *Anoplopoma fimbria* individuals treated with E2 injection, mainly in the juvenile phase (Guzmán *et al.*, 2015). Estradiol injections induced increased pituitary LH (Aroua *et al.*, 2007) and ovarian lhβ mRNA levels in *Anguilla anguilla* (Schmitz *et al.*, 2005). Thus, based on results in the current study, estrogen and LH receptors act together in the gonadal development of female *G. luteus* individuals, mainly at spawning stage, whose success depends on specific stimuli aimed at oocyte vitellogenesis and final maturation.

In conclusion, the current study was pioneer in featuring different reproductive maturation stages in female *G. luteus* individuals, based on cellular, endocrine and molecular aspects. It has shown that the gene expression profile of estrogen and LH receptors, as well as plasma E2 and 17α-OHP concentrations, are proportional to oocyte development and play important role in regulating ovarian development and ovulation in *G. luteus.*

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CONCLUSÕES

A partir dos resultados obtidos, pode-se concluir que:

- A variação do IGS, juntamente com os parâmetros ictiométricos, está relacionada com os estádios de maturação gonadal em *G. luteus;*
- A magnitude da expressão gênica para os receptores de estrógeno e do hormônio luteinizante é proporcional ao desenvolvimento maturacional ovariano e testicular, com pico no estádio maturo.
- *er* e *lhr* estão altamente correlacionados entre si e com o estágio maturacional, IGS e peso das gônadas, reforçando a hipótese de sua participação na regulação do desenvolvimento gonadal em *G. luteus*.

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