UNIVERSIDADE ESTADUAL PAULISTA – UNESP

CENTRO DE AQUICULTURA DA UNESP

Indução à maturação final e ovulação de lambari (*Astyanax altiparanae*) com análogos do hormônio liberador de gonadotropinas (GnRH)

Mariana Roza de Abreu

Engenheira de Aquicultura

Jaboticabal, São Paulo

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Título: Indução à maturação final e ovulação de lambari (*Astyanax altiparanae*) com análogos do hormônio liberador de gonadotropinas (GnRHa)

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Sumário

Dedicatória

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Resumo

A presente tese foi dividida em três capítulos, no primeiro é apresentada uma revisão bibliográfica dos temas relacionados à tese, os outros dois capítulos são manuscritos gerados a partir dos experimentos realizados durante o doutorado. No manuscrito I (capítulo II) avaliamos o desempenho reprodutivo de fêmeas de lambari (*Astyanax altiparanae*) usando diferentes protocolos de reprodução em cativeiro, como a administração de análogo do hormônio liberador de gonadotrofina de salmão (sGnRHa) e extrato de pituitária de carpa (CPE), bem como sem o uso de terapia hormonal. Foram realizados dois experimentos consecutivos com machos e fêmeas maduros de lambari (seis meses de idade) usando dose única (Exp. 1) ou fracionada (10% + 90% - intervalo de 12h) (Exp. 2). No Exp. 1, seis doses de sGnRHa (variando de 1 a 160 μg kg^{-1}) foram aplicadas em três repetições (cinco fêmeas e 10 machos cada); no Exp. 2, três doses de sGnRHa (10, 100 e 1000 μg kg^{-1}) foram aplicadas em quatro repetições (cinco fêmeas e 10 machos cada). Em ambos os experimentos, 6 mg de CPE kg^{-1} e solução salina foram usados como controles positivo e negativo, respectivamente. A quantificação de 17α-20β-dihidroxi-4-pregnen-3-ona (17,20β-P) e níveis plasmáticos de prostaglandina F2α e avaliações histológicas ovarianas foram introduzidas no Exp. 2 separando fêmeas desovadas e não desovadas. Usando uma única dose, a taxa de desova foi baixa e semelhante entre todos os grupos (de 0 a 20%). Usando doses fracionadas, a taxa de desova de CPE (60%) foi maior do que todos os outros grupos (de 10 a 25%), com exceção da dose mais alta de sGnRHa (40%). A desova e elevação dos níveis de 17,20β-P ocorreram em algumas fêmeas do controle negativo (Exp. 2). Portanto, a terapia com doses fracionadas de CPE melhorou a ovulação de lambari, mas não foi condicional para a obtenção da ovulação. Apenas as fêmeas não desovadas induzidas com doses fracionadas de CPE, mas não com sGnRHa e nem no controle negativo, atingiram a maturação final. Além disso, a desova nos grupos com sGnRHa ocorreu em uma extensão semelhante à do controle negativo e os grupos tratados com sGnRHa não mostraram qualquer resposta dose-dependente considerando todas variáveis avaliadas. Portanto, é possível que a desova nos grupos sGnRHa se deva apenas ao agrupamento de peixes, semelhante ao controle negativo. Em conclusão, a dose fracionada de CPE foi o único tratamento que provocou maturação final em todas as fêmeas tratadas e um aumento significativo no desempenho reprodutivo em comparação com todos os outros tratamentos. No manuscrito II (capítulo III) avaliamos o efeito do análogo do hormônio liberador do hormônio luteinizante (LHRHa) com e sem domperidona no desempenho reprodutivo de lambari e na liberação de 17,20β-P e $PGF_{2\alpha}$, bem como avaliação histológica das estruturas ovarianas em dois experimentos independentes e consecutivos. No primeiro experimento cinco doses de LHRHa (20, 60, 180, 540 e 1620 μg kg^{-1}) foram testadas com três repetições cada, enquanto no segundo experimento a dose de 180 μg kg^{-1} foi testada em combinação com 20 mg kg^{-1} de domperidona (D) com quatro repetições. Em ambos os experimentos utilizamos solução salina 0,9% como controle negativo e 6 mg de CPE kg^{-1} como controle positivo e as fêmeas de todos os grupos receberam duas injeções (10% + 90% - intervalo de 12h). As doses testadas de LHRHa não apresentram resultados superiores aos do controle negativo. Associando o LHRHa com D não foram obtidos resultados superiores ao LHRHa sem associação, sugerindo que a dopamina parece não apresentar um papel no controle neuroendócrino da reprodução dessa espécie ou que este antidopaminérgico não é o mais indicado para o lambari. O grupo tratado com CPE foi o único com melhor desempenho que o controle negativo, indicando que o CPE foi a melhor terapia hormonal para induzir a desova em lambari (entre os testados por nós). Houve uma diminuição nas taxas de fertilização e eclosão que coincidiram com as duas doses mais altas de LHRH (540 e 1620

μg kg^{-1}), sugerindo que essas doses podem ter causado uma superestimulação da hipófise na produção de LH, a qual pode ter afetado a qualidade dos ovos.

Palavras-chave: tratamento com hormônios exógenos, hormônios esteroides sexuais, reprodução induzida, desenvolvimento ovariano, maturação e ovulação de oócitos, prostaglandina, manejo de reprodutores.

Abstract

The present thesis was divided into three chapters, in the first one a review of the themes related to the thesis is presented, the other two chapters are manuscripts prepared from the experiments carried out during the doctorate. In the manuscript I (chapter II) we evaluated lambari (*Astyanax altiparanae*) reproductive performance using different protocols of reproduction in captivity, such as the administration of analogue of salmon gonadotropin-releasing hormone (sGnRHa) and carp pituitary extract (CPE) as well as without the use of hormonal therapy. We performed two consecutive experiments with mature lambari males and females (six months old) using single (Exp. 1) or fractioned doses (10% + 90% - 12h interval) (Exp. 2). In Exp. 1, six sGnRHa doses (ranging from 1 to 160 μg kg^{-1}) were applied in three replicates (five females and 10 males each); in Exp. 2, three sGnRHa doses (10, 100 and 1000 μg kg^{-1}) were applied in four replicates (five females and 10 males each). In both experiments 6 mg of CPE kg^{-1} and saline solution were used as positive and negative controls, respectively. Quantification of 17α-20β-dihydroxy-4-pregnen-3-one (17,20β-P) and prostaglandin $F_{2\alpha}$ plasma levels and ovarian histological evaluations were introduced in Exp. 2 via the separation of spawned and unspawned females. Using a single dose, the spawning rate was low and similar between all groups (from 0 to 20%). Using fractioned doses, the spawning rate of CPE (60%) was higher than all other groups (from 10 to 25%), with the exception of the highest sGnRHa dose (40%). Spawning and elevation of 17,20β-P levels occurred in some females of the negative control (Exp. 2). Therefore, only CPE fractioned doses therapy improved lambari ovulation but were not conditional for obtaining ovulation. Only unspawned females treated with fractioned CPE doses, but neither sGnRHa nor negative control, attained final oocyte maturation.In addition, spawning in sGnRHa groups occurred in a similar extent as in negative control and sGnRHa-treated groups did not show any dose dependent response considering all variables measured. Therefore, it was possible that spawning in sGnRHa groups were just due to grouping of fish, similar to negative control. In conclusion, the fractioned dose of CPE was the only treatment that provoked final oocyte maturation in all treated females and a significant enhance in reproductive performance comparing to all other treatments. In the manuscript II (chapter III) we evaluated the effect of luteinizing hormone-releasing hormone ethylamide analogue (LHRHa) with and without domperidone on reproductive performance of lambari and the quantification of plasma levels of 17,20β-P and $PGF_{2\alpha}$, as well as histological evaluation of ovarian structures in two independent and consecutive experiments. In the first, five doses of LHRHa (20, 60, 180, 540 and 1620 μg kg^{-1}) were tested with three replicates each, while in the second the dose of 180 μg kg^{-1} was tested in combination with 20 mg kg^{-1} domperidone (D) with four replicates. In both experiments, we used 0.9% saline as a negative control and 6 mg CPE kg^{-1} as a positive control and the females of all groups received two injections (10%; and 90% after 12 h). The doses we tested of LHRHa did not show results superior to those of the negative control. When associating LHRHa with D, we did not obtain results superior to LHRHa without association, suggesting that dopamine does not seem to play a role in the neuroendocrine

control of the reproduction of this species or that this antidopaminergic is not the most suitable for lambari. The group treated with CPE was the only one with better performance than the negative control, indicating that CPE was the best hormonal therapy to induce spawning in lambari (among those tested by us). There was a decrease in fertilization and hatching rates that coincided with the two highest doses of LHRH (540 and 1620 μg kg^{-1}), suggesting that these doses may have caused an overstimulation of the pituitary in the production of LH, which may have affected egg quality.

Keywords: exogenous hormone treatment, sex steroids hormones, controlled reproduction, ovarian development, oocyte maturation and ovulation, prostaglandin, broodstock management.

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Revisão Bibliográfica

Lista de Abreviações

FAO – Food & Agriculture Organisation

MPA – Ministério da Pesca e Aquicultura

GnRH – Hormônio liberador de gonadotropinas

FSH – Hormônio folículo estimulante

LH – Hormônio luteinizante

E_2 - 17β-Estradiol

MIS – Hormônio indutor da maturação (do inglês - *Maturation inducing steroid*)

17,20β-P - 17α-20β-diidroxi-4pregnen-3-ona

DHP - 17α-20β-diidroxi-4pregnen-3-ona

20β-HSD - 20β-hidroxiesteróide desidrogenase

PGs – Prostaglandinas

COX – Enzimas ciclooxigenases

$PGF_{2\alpha}$ – Prostaglandina $F_{2\alpha}$

PGE_2 – Prostaglandina E_2

GnIH – Hormônio inibidor de gonadotropinas

GABA – Ácido gama amino butírico

NPY – Neuropeptídeo Y

T – Testosterona

ARA – Ácido araquidônico

nPR – Receptor nuclear de progesterona

PGr – Receptor de prostaglandinas

CGPs – Células germinais primordiais

GVBD – Quebra da vesícula germinativa (do inglês – *germinal vesicle break-down*)

Mmp15 – Metaloproteinase-15 de matriz

EP4b – Receptor de prostaglandina E_2

CPE – Extrato hipofisário de carpa (do inglês – *carp pituitary extract*)

MAPA – Ministério da Agricultura Pecuária e Abastecimento

GnRHa – Análogo do hormônio liberador de gonadotropinas

mGnRHa – Análogo do hormônio liberador de gonadotropinas de mamíferos

sGnRHa – Análogo do hormônio liberador de gonadotropinas de salmão

LHRHa – Análogo do hormônio liberador do hormônio luteinizante

CPO – Complexos pós-ovulatórios

CONCEA – Conselho Nacional de Controle de Experimentação Animal

CAUNESP – Centro de Aquicultura da UNESP

SD – Desvio padrão

1Gn – 1 µg de sGnRHa kg^{-1}

10Gn – 10 µg de sGnRHa kg^{-1}

20Gn – 20 µg de sGnRHa kg^{-1}

40Gn – 40 µg de sGnRHa kg^{-1}

80Gn – 80 µg de sGnRHa kg^{-1}

160Gn – 160 µg de sGnRHa kg^{-1}

100Gn – 100 µg de sGnRHa kg^{-1}

1000Gn – 1000 µg de sGnRHa kg^{-1}

PV – Oócitos pré-vitelogênicos

CA – Oócitos com alvéolo cortical

EV – Oócitos vitelogênicos iniciais

FV - Oócitos vitelogênicos finais

AT – Oócitos atrésicos

POF – Folículos pós-ovulatórios

IT – Tecidos intersticiais

OMC – Competência maturacional dos oócitos (do inglês – *oocyte maturational competence*)

GC – Células da granulosa

MIH – Hormônio indutor da maturação (do inglês – *maturation-inducing hormone*)

hCG – Gonadotropina coriônica humana

FOM – Maturação final dos oócitos

D – Domperidona

20 LHRH – 20 µg de LHRHa kg^{-1}

60 LHRH – 60 µg de LHRHa kg^{-1}

180 LHRH – 180 µg de LHRHa kg^{-1}

540 LHRH – 540 µg de LHRHa kg^{-1}

1620 LHRH – 1620 µg de LHRHa kg^{-1}

180 LHRH + 20 D – 180 µg de LHRHa kg^{-1} + 20 mg de domperidona kg^{-1}

20 D - 20 mg de domperidona kg^{-1}

ELISA – do inglês – *Enzyme Linked ImmunoSorbent Assay*

cAMP – adenosina 3',5'-monofosfato cíclico

1. CAPÍTULO I

Revisão Bibliográfica

Contextualização da Aquicultura

Em 2018 a aquicultura representou 46% do total de 178,5 milhões de toneladas da produção global de animais aquáticos (FAO, 2020). Nas estatísticas anuais da FAO podemos perceber que a produção pela pesca permanece estática desde a década de 80, enquanto que a aquicultura está em constante crescimento. Além disso, o consumo per capita por pescados aumentou no decorrer dos anos, com uma taxa média de aproximadamente 1,5% ao ano, passando de 9 kg per capita em 1961 para 20,5 kg per capita em 2018 (FAO, 2020). Com o consumo aumentando e a estagnação da pesca, a aquicultura ganha importância para suprir a demanda por estes alimentos.

A produção mundial da aquicultura em 2018, excluindo plantas aquáticas, foi de 82,1 milhões de toneladas (FAO, 2020). Esse valor cai para 30,8 milhões de toneladas quando se exclui a produção da China. Desde o início da década de 90 a China produz mais peixes do que toda a produção do resto do mundo combinada (FAO, 2018). O abastecimento de pescado nesse país pela aquicultura ultrapassa a pesca desde 1993 e em 2018 a aquicultura representou 76,5% na produção total de pescado (FAO, 2020). Diferentemente do que é observada na aquicultura brasileira, a produção aquícola da China é baseada em 95% nas espécies nativas daquele continente, principalmente nas espécies de carpa (MPA, 2014).

A piscicultura brasileira teve um crescimento de 4,9% e produziu 758 mil toneladas em 2019, sendo liderada pela tilápia, a qual representou 57% da produção nacional (Peixe BR, 2020). As espécies nativas, sendo representadas por um grupo de espécies, onde o Tambaqui apresenta maior destaque, representou 38% da produção brasileira: 288.040 toneladas em 2019 (Peixe BR, 2020). Outras espécies, entre as quais se destacam Carpas, Panga e Trutas, representam 5% da produção brasileira de peixes cultivados em 2019, com 37.900 toneladas, principalmente na região Sul (Peixe BR, 2020).

No relatório do MPA de 2014 é relatado que o principal estímulo para a produção das espécies exóticas parece estar mais relacionado à existência de informações básicas para a criação do que às características das espécies. Com isso, as pesquisas para obter informações básicas das espécies nativas contribuem para a formação de pacotes tecnológicos e consequentemente para a ascensão da produção comercial dessas espécies. A cadeia produtiva da piscicultura se inicia com a produção de “sementes” tendo como um dos principais objetivos obter um grande número de ovos viáveis com grande

sobrevivência das larvas. Portanto, estudos em reprodução de teleósteos nativos auxiliam a indústria da aquicultura em suprir a crescente demanda por produto.

Reprodução e controle da maturação final e ovulação em fêmeas de teleósteos

O eixo hipotálamo-hipófise-gônadas

Em geral, a reprodução de fêmeas dos peixes teleósteos é regulada pela interação do sistema nervoso com o sistema endócrino, e esta interação é realizada pelo eixo hipotálamo-hipófise-gônada (Figura 1) (Borella et al., 2014). Os sinais ambientais (i.e. fotoperíodo, temperatura e períodos de chuva) em conjunto com características sociais da espécie (i.e. presença de outros indivíduos, densidade da população, proporção de sexo) são percebidos pelos órgãos sensoriais (i.e. pineal, retina, linha lateral) e transmitidos a várias partes do cérebro culminando no hipotálamo, estimulando a síntese e liberação do hormônio liberador de gonadotropinas (GnRH) (Muñoz-Cueto, 2009; Yaron e Levavi-Sivan, 2011). As terminações dos neurônios do hipotálamo enviam sinais às células gonadotrópicas da adenohipófise, que respondem aumentando ou diminuindo a secreção de duas gonadotropinas distintas: hormônio folículo estimulante (FSH) e hormônio luteinizante (LH) (Yaron e Levavi-Sivan, 2006; Pankhurst e Munday, 2011; Yaron e Levavi-Sivan, 2011).

As gonadotropinas (FSH e LH) são glicoproteínas heterodiméricas formadas por duas subunidades: uma subunidade α em comum e uma subunidade β específica, que determina a especificidade do hormônio (Levavi-Sivan et al., 2010). Nos ovários, as gonadotropinas exercem suas funções através da ligação com seus receptores acoplados à proteína G, regulando a esteroidogênese através de enzimas específicas (Borella et al., 2014; Liu e Lin, 2017). O FSH está envolvido no controle da gametogênese e crescimento vitelogênico dos folículos (vitelogênese) (Mañanós et al., 2008), quando as células da teca secretam testosterona que difunde nas células da granulosa onde o citocromo P450 aromatase está localizado para converter a testosterona em 17β-estradiol (E_2) (Nagahama, 1994; Senthilkumaran et al., 2004). No final da gametogênese ocorre um aumento na síntese de LH pela hipófise, induzindo uma mudança na esteroidogênese (do inglês – *steroidogenic shift*) (Mañanós et al., 2008), com o LH estimulando a produção do hormônio indutor da maturação (MIS) (Nagahama, 1994). Na maioria dos teleósteos o MIS mais eficiente em induzir a maturação final dos oócitos é a 17α-20β-diidroxi-4-pregnen-3-ona (17,20β-P – "DHP") (Nagahama e Yamashita, 2008). Sua produção provém da 17α-

hidroxiprogesterona, que é produzida na camada das células da teca e atravessa a membrana basal para então ser convertida em DHP nas células da granulosa através da enzima 20β-hidroxiesteróide desidrogenase (20β-HSD) (Nagahama, 1997). O MIS induz a maturação final dos oócitos através da sua ligação aos seus receptores de membrana e seguido pela ativação de fatores que promovem a maturação, cdc2-quinase e ciclina-B (Yaron e Levavi-Sivan, 2011). Além disso, o MIS está envolvido nos processos de ovulação, através da sua ligação com seus receptores nucleares e consequentemente agindo como indutor da expressão de receptores de prostaglandinas (PGs) (Takahashi et al., 2013). As PGs são eicosanoides produzidas a partir da via do ácido araquidônico, com a participação das enzimas ciclooxigenases (COX), e agem através de ligação com receptores específicos na superfície celular (Fujimoro et al., 2011, 2012). A ligação entre PGs e seus receptores é requerida para a ovulação de peixes no momento que ocorre a ruptura do folículo (Takahashi et al., 2013). Dentre os subtipos de prostaglandinas, a $PGF_{2\alpha}$ é considera a mais eficiente na indução à ovulação de teleósteos (Kagawa e Nagahama, 1981; Kagawa et al., 2003), no entanto, a PGE_2 é o subtipo envolvido na ovulação de medaka (Takahashi et al., 2013). Os esteroides sexuais secretados nas gônadas, por sua vez, realizam uma comunicação de *feedback* (positivo ou negativo) com o hipotálamo aumentando ou diminuindo a liberação de gonadotropinas (Liu e Lin, 2017).

Enquanto o GnRH é considerado o principal fator que estimula a produção das gonadotropinas na hipófise e a reprodução de peixes, sua ação estimuladora pode sofrer oposição pelo seu antagonista, a dopamina (Muñoz-Cueto, 2009; Dufour et al., 2010). A dopamina é um neurotransmissor do grupo das catecolaminas (Dufour et al., 2005) e apresenta duas classes de receptores (D1 e D2) acoplados à proteína G (Rangel-Barajas et al., 2015). A dopamina inibe a liberação de LH basal e estimulada por análogos do GnRH, através da ligação a receptores do tipo D2 localizados nas células gonadotrópicas da hipófise (Chang et al., 1990; Van der Kraak et al., 1998) e pode também agir inibindo a liberação de GnRH através da sua ligação com seus receptores do tipo D1 no corpo celular dos neurônios que produzem o GnRH (Yu et al., 1991). A dopamina parece ter diferentes funções fisiológicas e adaptativas, dependendo da espécie (Zohar et al., 2010). Em fêmeas de goldfish a dopamina é importante para evitar a ovulação em condições ambientais adversas, enquanto nos machos o papel da dopamina é prevenir a espermiação antes da ovulação (revisado por Zohar et al., 2010). Na enguia (*Anguilla anguilla*) (Dufour et al., 2005) e na tainha (*Mugil cephalus*) (Aizen et al., 2005) a dopamina está envolvida na

regulação do desenvolvimento da puberdade. No entanto, a ação inibitória da dopamina não parece ser geral para todos os teleósteos (Dufour et al., 2010; Zohar et al., 2010), como por exemplo, em espécies marinhas como a corvina (*Micropogonoias undulates*) (Copeland e Thomas, 1989), dourada (*Sparus auratus*) (Zohar et al., 1995) e robalo (*Dicentrarchus labrax*) (Mateos et al., 2002), bem como em uma espécie de Ciprinídeo o *Tinca tinca* (Podhorec et al., 2016).

O hormônio inibidor de gonadotropinas (GnIH) é um neuropeptídeo hipotalâmico, que foi primeiramente isolado em aves como um inibidor da liberação de LH agindo diretamente na hipófise (Tsutsui et al., 2000) ou no hipotálamo, através de inervações nas células de GnRH (Bentley et al., 2008). Em peixes, o papel do GnIH na reprodução ainda não foi totalmente compreendido (Muñoz-Cueto et al., 2017; Di Yorio et al., 2019). É relatado que os estudos do GnIH e seus efeitos sobre a reprodução e outros processos fisiológicos só foi abordado em algumas espécies de peixes, e os resultados obtidos são, em alguns casos, conflitantes (Muñoz-Cueto et al., 2017). Um trabalho *in vitro* com explantes de hipófise e lâminas do encéfalo de machos de lambari foi realizado com o objetivo de estudar o efeito do GnIH adicionado no meio de cultura (0 ou 100nM) com ou sem GnRH2 (0 ou 100nM) (Branco et al., 2018). Estes autores propõe dois papéis regulatórios do GnIH; o GnIH diminuiu o efeito estimulador de GnRH2 nos níveis de *fshβ* e *lhβ* e o GnIH sozinho, ou na presença de GnRH2, estimulou a expressão de *gnrh2*, demonstrando que o GnIH exerce um papel regulador sobre o eixo reprodutivo de machos de lambari modulando a célula gonadotrópica e as funções neuronais do GnRH.

O ácido gama amino butírico (GABA), outro neurotransmissor atuante no eixo, age estimulando a síntese/liberação de LH e de GnRH e inibindo a de dopamina (Kah et al., 1992; Trudeau et al., 2000). O neuropeptídeo Y (NPY) está envolvido na regulação da alimentação em peixes (Silverstein e Plisetskaya, 2000) e na síntese de LH (Cerdá-Reverter et al., 1999). O sistema kisspeptina e seu receptor desempenha papel importante no início da puberdade em mamíferos, ativando o GnRH e indiretamente estimulando a síntese de FSH e LH (Roa et al., 2008). Em peixes, a função deste sistema ainda não é totalmente compreendida, mas parece também estar envolvido no controle da puberdade (Elizur, 2009), estimula a liberação de LH em goldfish (Li et al., 2009), porém não é necessário para a reprodução de zebrafish (linhagens mutantes férteis e com desenvolvimento ovariano normal) (Liu et al., 2017; Liu e Lin, 2017).

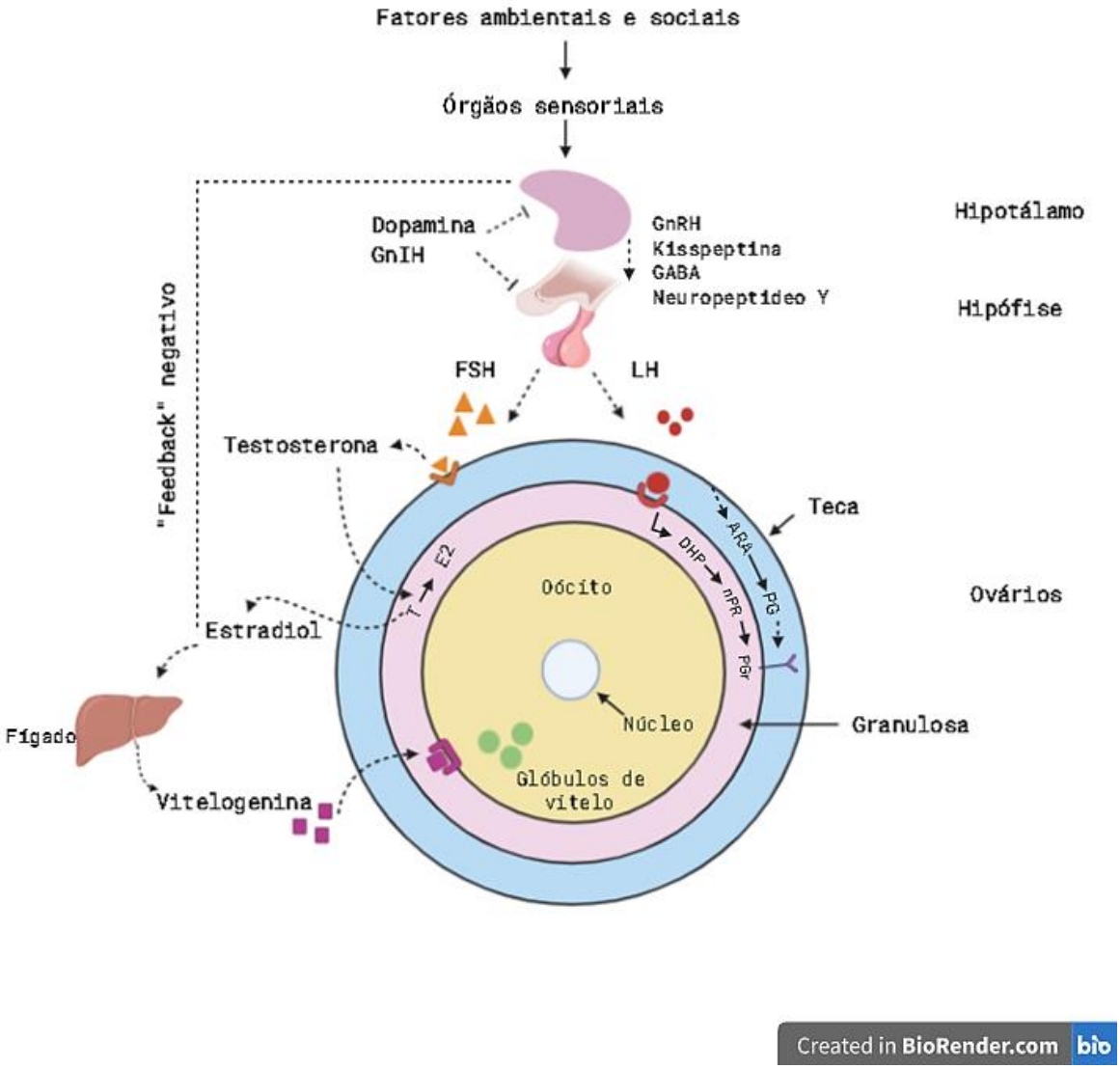

Figura 1. Esquema representativo do eixo hipotálamo-hipófise-gônada em peixes. GnIH, hormônio inibidor de gonadotropinas; GnRH, hormônio liberador de gonadotropinas; GABA, ácido gama amino butírico ; FSH, hormônio folículo estimulante; LH, hormônio luteinizante; T, testosterona; E2, 17β-estradiol; ARA, ácido araquidônico; PG, prostaglandinas; DHP, 17α,20β-diidroxi-4-pregnen-3-ona; nPR, receptor nuclear de progesterona; PGr, receptores de prostaglandinas. ---| (inibitório); ---> (ação positiva). (Adaptado de: Muñoz-Cueto, 2009; Clelland e Peng, 2009; Takahashi et al., 2013). Criado com BioRender.com

Desenvolvimento ovariano em fêmeas: oogênese, maturação e ovulação

Oogênese é o processo de crescimento celular e diferenciação de células germinais primordiais (CGPs) a oócitos maduros (Mañanos *et al*., 2008). A incorporação, síntese e processamento dos componentes oocitários, que ocorre durante a oogênese, desempenham um importante papel no desenvolvimento de oócitos de qualidade, que poderão ser fertilizados e gerar embriões normais (Bobe e Labbé, 2010).

A oogênese pode ser descrita em seis principais etapas: 1) formação das CGPs (células da linhagem germinativa); 2) transformação das CGPs em oogônias (diferenciação sexual) e a proliferação das oogônias mitoticamente; 3) transformação das oogônias em oócitos primários (início da meiose I); 4) crescimento celular, formação das camadas foliculares da granulosa e da teca, formação dos alvéolos corticais e incorporação de vitelo enquanto a meiose I fica estacionada na fase de diplóteno na profáse I; 5) retomada da meiose (maturação final dos oócitos); e 6) expulsão do oócito do seu folículo (ovulação) (Patiño e Sullivan, 2002; Lubzens et al., 2010; Yaron e Levavi-Sivan, 2011; Kagawa, 2013) (Figura 2).

As etapas da oogênese são controladas por meio de um mecanismo hormonal, por exemplo, a transformação das CGPs em oogônias e a proliferação das oogônias mitoticamente ocorrem sob a estimulação do E_2 (Yaron e Levavi-Sivan, 2011). O DHP além de estimular as oôgonias a entrarem em meiose I, também estimula a retomada da meiose nos oócitos pós-vitelogênicos (Lubzens et al., 2010) (Figura 2).

Os oócitos em desenvolvimento ficam circundados por uma camada folicular (camada das células da granulosa) e uma camada mais externa do envelope folicular (camada das células da teca) (Kagawa, 2013). A camada das células da granulosa é composta por células de um único tipo morfológico, entretanto, essas células podem passar por alterações morfológicas durante o desenvolvimento oocitário (Dos Santos-Silva et al. 2015), já a camada das células da teca é composta por fibroblastos, fibras de colágenos, vasos sanguíneos e células que produzem esteroides (Kagawa, 2013).

Durante a fase de crescimento celular os oócitos ficam “estacionados” na prófase da meiose I, e é nessa fase que ocorre a formação da zona radiata, dos alvéolos corticais e a vitelogênese (Figura 2). Na vitelogênese, a maioria dos nutrientes lipídicos e proteicos requeridos para o desenvolvimento embrionário e larval é estocada no oócito (Reading et al., 2018). O FSH é a gonadotropina chave dessa fase, pois ela estimulará a produção de testosterona nas células da teca, a qual é convertida em E_2 nas células da granulosa, e então via corrente sanguínea o E_2 age no fígado induzindo a produção de vitelogenina, que por sua vez, se liga aos seus receptores na membrana dos oócitos que então é processada em glóbulos de vitelo (Reading e Sulivan, 2011; Reading et al., 2018). Os oócitos maduros (que completaram a vitelogênese) podem permanecer em repouso nesta fase por vários meses (“long arrest”), até que a meiose seja retomada e os processos de maturação final iniciem por gatilhos sociais e ambientais (Yaron e Levavi-Sivan, 2011).

A maturação final dos oócitos (FOM) ocorre antes da ovulação e é um pré-requisito para o sucesso da fertilização; este processo, na maioria dos teleósteos, inicia com a produção do DHP, mediada pelo LH (Yaron e Levavi-Sivan, 2011). O DHP se liga, então, aos seus receptores na membrana plasmática do oócito e ativa os fatores promotores da maturação (cdc2-quinase e cliclina-B) (Nagahama e Yamashita, 2008). Morfologicamente o processo de maturação final resulta na migração do núcleo em direção ao polo animal e a posterior desintegração da sua membrana (GVBD), condensação cromossômica, montagem do fuso meiótico e formação do primeiro corpúsculo polar (Nagahama e Yamashita, 2008). A quebra da vesícula germinativa (GVBD) corresponde à primeira divisão meiótica imediatamente seguida pela preparação da segunda divisão que vai manter os oócitos "estacionados" na metáfase II até a fertilização ("short arrest") (Bobe et al., 2008) (Figura 2).

A ovulação é a liberação de um oócito maduro do seu folículo na cavidade ovariana (na maioria dos teleósteos) ou na cavidade abdominal (em salmonídeos e no pirarucu) (Jalabert, 2005). Este processo requer a separação do oócito da camada das células da granulosa, a ruptura da camada folicular e a expulsão do oócito (Bobe et al., 2008). A ovulação é um processo que depende de mecanismos comportamentais e fisiológicos (Knight e Van Der Kraak, 2015), como por exemplo, a expressão de receptores nucleares de progesterona (nPR) e de receptores de prostaglandinas, a liberação de prostaglandinas (Takahashi et al., 2013), e ativação da região promotora de metaloproteinases de matriz (e.g. mmp15), uma protease envolvida na ruptura do folículo (Hagiwara et al., 2020). Assim como a maturação final a ovulação também é mediada pelo LH, estimulando a expressão de nPR e dos receptores de prostaglandinas. As prostaglandinas tem um papel fundamental no mecanismo de ovulação (Hagiwara et al., 2020) e sua síntese em vertebrados não-mamíferos ocorre durante a ovulação natural ou induzida (Takahashi et al., 2013). Em medaka, um modelo biológico, PGE_2 induz a ovulação através da sua ligação com o seu receptor (EP4b) imediatamente antes do momento da ovulação (Takahashi et al., 2013). Em zebrafish, outro modelo biológico, os níveis ovarianos de $PGF_{2\alpha}$ foram maiores próximos à ovulação (Knight & Van der Kraak, 2015; Lister & Van der Kraak, 2008).

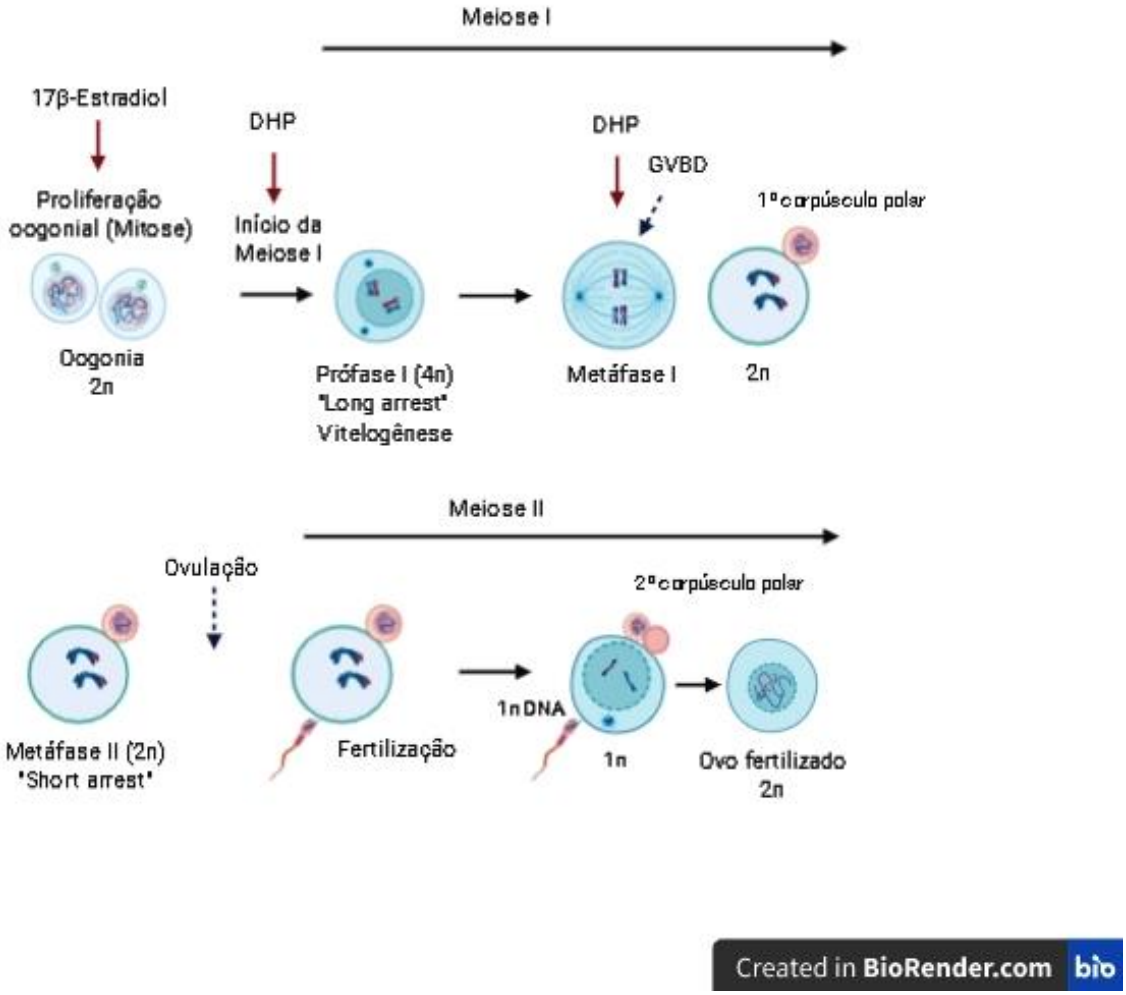

Figura 2. Estágio da oogênese em peixes e sua regulação endócrina. Adaptado de Yaron e Levavi-Sivan, 2011. Criado com BioRender.com

Indução da maturação final e ovulação

No habitat natural, o ciclo reprodutivo e as mudanças endócrinas nos peixes acontecem naturalmente e a desova ocorre no momento em que as condições ambientais e sociais são as mais apropriadas para a sobrevivência da prole e para a sua própria sobrevivência (Mañanós et al., 2008). No entanto, quando as condições ambientais estão restritas como em cativeiro, acontece um bloqueio nos processos da reprodução na grande maioria das espécies. Esse bloqueio é devido a disfunções reprodutivas que podem ser classificadas em três tipos: 1) falha em completar a vitelogênese (oócitos imaturos para serem induzidos à ovulação com hormônios exógenos); 2) ausência da maturação ovocitária final, e; 3) ausência da ovulação (Zohar e Mylonas, 2001). A mais simples

disfunção reprodutiva é a ausência de desova que ocorre em salmonídeos cultivados (Mylonas et al 2010). Nessas espécies, vitelogênese, maturação final dos oócitos e ovulação ocorrem naturalmente, mas a desova não acontece, provavelmente, devido à perda do comportamento de desova e/ou falta de substrato apropriado para postura dos ovos.

A grande maioria das espécies nativas brasileiras, que apresentam potencial para aquicultura, realiza migração em direção à nascente dos rios durante a época reprodutiva (espécies reofílicas) (Paulino et al., 2011). Nos sistemas de cultivo, o comportamento migratório é impedido, resultando na falha da resposta endócrina apropriada para realizar maturação final dos oócitos e ovulação (Pereira et al., 2009). Dessa forma, os ovários se desenvolvem até estágio de vitelogênese completa e entram no "período de dormência" (Andrade e Yasui, 2003). O "período de dormência" pode durar desde poucas semanas até alguns meses, nas espécies brasileiras (Zaniboni-Filho e Nuñer, 2004) ou até que as condições ambientais estejam adequadas. Na falta dos estímulos ambientais, ocorrerá o processo de atresia folicular que leva a reabsorção dos oócitos vitelogênicos, devido a fatores ambientais críticos (i.e. oxigênio, temperatura, etc.) e por manejo inadequado durante a maturação final (Harvey e Carolsfeld, 1993; Murgas et al., 2009).

Como as condições ambientais em cativeiro não se tornarão adequadas para estas espécies, é necessário selecionar os indivíduos que completaram a vitelogênese e induzir a maturação final e ovulação por meio de terapias hormonais.

Hipofisação

No Brasil o método mais utilizado para induzir a maturação final e desova de peixes é a aplicação de extrato hipofisário de carpa (CPE), conhecido como hipofisação (Zaniboni-Filho e Weingartner, 2007). Os primeiros trabalhos utilizando a hipofisação em peixes reofílicos foram desenvolvidos paralelamente na Argentina (Houssay, 1930) e no Brasil (Ihering, 1935) (Zaniboni-Filho e Nuñer, 2004). O local de ação do CPE é diretamente sobre as gônadas e nele estão contidos todos os hormônios produzidos pela hipófise, sobretudo as gonadotrofinas FSH e LH, que estimulam os ovários a secretarem os hormônios esteroides sexuais que desencadeiam os eventos da maturação final dos ovócitos culminando com a desova (Viveiros et al., 2011). O CPE pode ser injetado na cavidade celomática ou via intramuscular nos reprodutores, em duas aplicações, sendo que o tempo entre as aplicações e a dosagem hormonal distribuída entre as doses varia de

acordo com a exigência de cada espécie (Crepaldi et al., 2006), com o grau de maturação dos reprodutores e com o método escolhido para fazer a aplicação (Zaniboni-Filho e Weingartner, 2007). De modo geral a dosagem mais utilizada nas fêmeas é de 0,5 mg/kg na 1ª dose e 5,0 mg/kg na 2ª dose (Andrade e Yasui, 2003), podendo ser também de 0,6 mg/kg na 1ª dose e 5,4 mg/kg na 2ª dose (Kuradomi e Batlouni, 2018) ou com uma aplicação de 0,25 mg/kg 12h antes do tratamento convencional (duas doses) (Zaniboni-Filho e Barbosa, 1991). O intervalo entre as aplicações pode variar entre 6 e 24h (Zaniboni-Filho e Barbosa, 1996).

A hipofisação é uma técnica simples e amplamente utilizada que apresenta diversas vantagens, como por exemplo, a praticidade dos procedimentos, a utilização de equipamentos simples, e o fato de que outros hormônios contidos na hipófise podem apresentar efeito sinérgico (Donaldson e Hunter, 1983). No entanto, apesar de ser uma metodologia altamente difundida ainda apresenta algumas problemáticas e incertezas, como, a variável porcentagem de fêmeas que responderão positivamente ao tratamento hormonal, a ovulação parcial dos ovários e a retenção dos oócitos maduros na papila urogenital das fêmeas (Hainfellner et al., 2012; Pereira et al., 2016; Pereira et al., 2018 ; Souza et al., 2020). Além dos problemas associados às fêmeas, a hipofisação demonstra outros entraves, como, a presença de outros tipos de hormônios e osmorreguladores na hipófise do peixe doador; a variabilidade do conteúdo de gonadotropina das hipófises; e o potencial na transmissão de doenças entre o peixe doador e os reprodutores (Zohar e Mylonas, 2001; Lokman et al., 2015). Essas problemáticas além de acarretarem em perdas econômicas, comprometimento do estoque de reprodutores e perda de material com grande potencial genético, fizeram com que o Ministério da Agricultura Pecuária e Abastecimento (MAPA) proibisse a utilização da hipófise de carpa em muitos estados brasileiros (Viveiros et al., 2013).

Análogos ao hormônio liberador de gonadotropinas (GnRHa) e antagonistas do receptor da dopamina

Os análogos ao GnRH são nonapeptídeos devido à retirada da glicina presente originalmente na décima posição (Zaniboni-Filho e Nuñer, 2004). Além disso, há uma substituição do aminoácido na posição 6 por Alanina ou Arginina, na posição dextro (D), para o GnRHa de mamífero e o GnRHa de salmão, respectivamente (revisado em Zohar e Mylonas, 2001; Zaniboni-Filho e Nuñer, 2004). Sua síntese tem como objetivo tornar esses análogos mais resistentes à degradação enzimática e com uma maior afinidade aos seus

receptores do que o GnRH natural (revisado em Zohar e Mylonas, 2001). Ao contrário do CPE, no qual o LH é exógeno e age diretamente sobre as gônadas, os GnRHa agem na hipófise, induzindo a liberação do LH endógeno que, por sua vez, atua nas gônadas para induzir a esteroidogênese e o processo de maturação final e ovulação (Mylonas et al., 2010). Mesmo nas espécies que apresentam disfunções reprodutivas em cativeiro a hipófise pode sintetizar e liberar as gonadotropinas endógenas, quando dado tratamento e doses apropriadas de GnRHa (Zohar e Mylonas, 2001).

O uso de GnRHa para indução de desova de peixes apresenta diversas vantagens, como: não desencadeia uma resposta imune e pode ser usado novamente em épocas de desova subsequente com nenhuma redução na sua eficácia; atua em um nível superior do eixo hipotálamo-hipófise-gônadas, consequentemente, pode fornecer uma estimulação mais equilibrada dos eventos reprodutivos e uma melhor integração destes eventos com outras funções fisiológicas; e pode ser sintetizado e obtido em forma pura, e, portanto, não carrega o risco de transmissão de doenças (Zohar e Mylonas, 2001).

Alguns produtos sintéticos estão disponíveis no mercado, como por exemplo, a busserelina (produto comercial: Conceptal®), a qual apresenta a serina inserida na posição dextro em substituição ao sexto peptídeo ([D-Ser(tBu)^{6},Pro^{9}-NEt]-GnRHa) (Zaniboni-Filho e Nuñer, 2004, e a gonadorrelina, a qual é um decapeptídeo com a mesma sequência do GnRHa natural (Zapletal e Pavlik, 2008). No entanto, os mais utilizados para indução à maturação final e ovulação de peixes são os análogos dos hormônios liberadores de gonadotropina de mamíferos ([D-Ala^{6}, Pro^{9},NEt]-mGnRHa = [des-Gly^{10}, D-Ala^{6}]-LHRHa) e de salmão ([D-Arg^{6}, Pro^{9}, NEt]-sGnRHa) (Zaniboni-Filho e Nuñer, 2004).

Mesmo com o desenvolvimento de diversos análogos de GnRH, foi observado que algumas espécies não respondiam positivamente a esses produtos, o que levou à descoberta da ação inibitória da dopamina. Essa descoberta apresenta importantes implicações na aquicultura, pois as condições ambientais em cativeiro geralmente levam a um bloqueio na maturação e ovulação de oócitos (Dufour et al., 2005), impossibilitando a reprodução dessas espécies. Nesse contexto, um método de indução hormonal que combina um agonista de GnRH e um antagonista do receptor de dopamina do tipo D2 (e.g. pimozida, domperidona, metoclopramida) foi proposto por dois pesquisadores Lin e Peter, chamado de método “Linpe” (Peter et al., 1988). Há no mercado produtos que fazem essa associação, como: o Ovaprim-C®, desenvolvido pelo Laboratório Syndel do Canadá, associa sGnRHa com domperidona, (Zaniboni-Filho e Nuñer, 2004); o Ovopel®,

desenvolvido na Hungria, contem na sua formulação mGnRHa e metoclopramida (DAS, 2004); o Ovatide, formulado pela M/S Hemmo Pharma, na Índia, possuindo seus ingredientes biológicos ativos similares ao do Ovaprim, porém com menor viscosidade e custo (Dhawan e Kaur, 2004).

Os primeiros estudos testando os análogos nas espécies reofílicas brasileiras foram realizados na década de 80 com o tambaqui (*Colossoma macropomum*) (Bernardino e Ferrari, 1987) e pacu (*Piaractus mesopotamicus*) (Carolsfeld et al., 1988). Passando para os anos 90 podemos citar dois trabalhos nesse mesmo contexto. Zaniboni-Filho (1995) confrontou os custos na indução do pacu com o uso do LHRHa e da técnica de hipofisação, além disso, buscou estabelecer a dosagem de LHRHa necessária para induzir a maturação final e ovulação para esta espécie. Com a maior dose de 20 µg LHRHa kg^{-1} as fêmeas não desovaram, já as tratadas com 10 ou 15 µg de LHRHa kg^{-1} desovaram e apresentaram melhores resultados em relação à taxa de fertilização e quantidade de oócitos por peso de fêmea para àquelas induzidas com 15 µg kg^{-1}. Quanto aos resultados para os custos, para induzir um quilo de peixe foram necessários U$ 4,90 para o CPE e U$ 1,08 para o LHRHa. Em 1996 Zaniboni-Filho e Resende testaram um protocolo onde uma dose "priming" de 0,25 mg de CPE kg^{-1} foi administrada dois ou três dias antes da indução com LHRHa D-Ala^6 (dose única de 10 µg kg^{-1} ou dose fracionada 1,5 µg + 15 µg kg^{-1} com 12h de intervalo) em curimba, pacu e tambaqui. Esse protocolo resultou em taxa de desova de 92,8 a 100% e fertilização de 51,2 a 80,1%, demonstrando que a aplicação da dose "priming" é efetiva e permite uma maior qualidade e quantidade de gametas.

Se analisarmos o cenário das pesquisas nos últimos dez anos (2010 - 2020), podemos perceber que as informações sobre a utilização dessas substâncias para as nossas espécies são extremamente escassas e pouco conclusivas e que a utilização dos análogos ainda está longe de virar uma realidade no setor produtivo nacional (Tabela 1). Com isso, a hipofisação continua sendo o método mais utilizado nas pisciculturas e nos centros de pesquisa, mesmo com todas as problemáticas (citadas anteriormente) e poucos avanços dessa técnica.

Tabela 1. Literatura dos últimos dez anos (2010 - 2020) sobre o uso de hormônios sintéticos na indução à maturação e ovulação de espécies nativas brasileiras. ND – não determinado.

Espécie	Produto	Dosagem	Taxa de desova (%)	Fertilização (%)	Eclosão (%)	Referência
Curimba (*Prochilodus lineatus*)	LHRHa (decapeptídeo)	20 µg/peixe + 50 µg/peixe (14h intervalo)	16,67	ND	ND	Itani et al., 2010
	LHRHa (nonapeptídeo)	0,8 µg/peixe + 2 µg/peixe (14h intervalo)	40			
Pacu (*P. mespotamicus*) Piracanjuba (*Brycon orbygnianus*) Curimba (*P. lineatus*)	Buserelina	0,125 ml/kg + 0,25 ml/kg + 0,75 ml/kg (12 horas de intervalo)	ND	40 0 0	ND	Paulino et al., 2011
Lambari (*Astyanax altiparanae*)	Gonadorrelina	80 µg/kg	100%	ND	ND	Felizardo et al., 2012
Curimba (*P. lineatus*)	Ovaprim (sGnRHa + DOM)	0,05 + 0,45 ml/kg	100	48	39	Viveiros et al., 2013
		0,125 + 0,375 ml/kg (14h de intervalo)	100	52	48	
Curimba (*P. lineatus*)	Gonadorrelina	60 + 60 µg/kg (12h intervalo)	20	0,0	ND	Andrade et al., 2014
	Buserelina	0,25 + 0,5 mL/kg (12h de intervalo)	40	81,8		
Jundiá (*Rhandia quelen*)	Ovaprim	0,5 ml/kg	87,5	82,1	ND	Ittzés et al., 2014
	sGnRHa	10 µg/kg	0	-		
	sGnRHa + MET	10 µg/kg + 20 mg/kg	37,5	74,0		
Piauçu (*Leporinus macrocephalus*)	Conceptal (mGnRHa) + MET	7 µg/kg + 10 mg/kg	100	6,25	0	Pereira et al., 2016
Tambaqui (*Colossoma macropomum*)	Ovopel (mGnRHa + MET)	0,2 pellet/kg	100	71,4	56,8	Souza et al., 2018
		0,4 pellet/kg	62,5	50,8	33,1	
Piapara (*Leporinus elongatus*)	Conceptal (mGnRHa) + MET	3,5 µg/kg + 10 mg/kg	100	1,4	0,4	Pereira et al., 2018
		5,0 µg/kg + 10 mg/kg	100	0,0	0,0	
Piau três pintas (*Leporinus friderici*)	mGnRHa + MET	40 µg + 20 mg (dose única)	70	0,08	0	Souza et al., 2020
		4 µg + 2 mg (6h de intervalo) 8 µg + 4 mg	50	0,09	0	

Espécie Modelo: *Astyanax altiparanae*

Astyanax altiparanae, popularmente conhecido como lambari-do-rabo-amarelo, é um characiforme de pequeno porte com produção em ascensão no Brasil. Sua produção aumentou 136,07% entre os anos de 2016 (237 T) e 2017 (554 T) (IBGE, 2017). O aumento da produção se reflete, também, no fato de ser a quinta em número de estabelecimentos de produção, com 23.028 unidades no Brasil em 2017 (IBGE, 2017). Estes estabelecimentos se concentram nas regiões sul e sudeste do país, com climas mais amenos, porém há produção de lambari em todas as regiões do país (incluindo norte e nordeste com climas mais quentes), demonstrando ser uma espécie que pode se adaptar a diversos tipos de clima (IBGE, 2017). O principal destino de sua produção é o mercado de iscas-vivas (Silva e Fernandes, 2011; Sussel, 2015), principalmente para pescadores de espécies carnívoras e há, inclusive, discussões sobre a elaboração de políticas públicas no Brasil aventando a possibilidade de ser usado como substituto da sardinha (*Sardinela* spp.) na pesca do atum, a fim de reduzir a pressão pesqueira sobre os estoques naturais de sardinha (Sussel, 2015; Valladão et al., 2016), a qual encontra-se em sobrepesca (FAO, 2018). Se estas políticas públicas se tornarem viáveis, estima-se que a demanda anual será de 600 milhões de lambaris destinados para a pesca industrial do atum (Sussel, 2015), aumentando a demanda pela produção nacional. Outra destinação crescente para os alevinos desta espécie são os diversos programas de peixamento do Brasil, setor que absorve a produção de milhões de alevinos todos os anos. Além disso, como peixe de baixo nível trófico, *A. altiparanae* tem grande potencial para ser produzido de forma sustentável e, assim, promover o desenvolvimento socioeconômico, melhorar a segurança alimentar e conservar os recursos naturais no Brasil (Fonseca et al. 2017). Nos centros de pesquisa, o lambari vem sendo utilizado como modelo biológico em estudos *in vitro* (Branco et al., 2018), em reprodução (Jesus et al., 2017; Lira et al., 2018; Brambila-Souza et al., 2019), nutrição (Bertolini et al., 2017) e em manipulação cromossômica e de gametas (de Bem et al., 2012; Yasui et al., 2015; Adamov et al., 2016; Nascimento et al., 2017; Santos et al., 2018).

As informações sobre a biologia reprodutiva dos lambaris em geral são bastante controversas, pois a espécie é ora classificada como de desova total (Rodrigues et al., 1992), ora como de desova parcelada (Lira et al., 2018) ou até como tendo suas desovas ocorrendo em função de condições ambientais favoráveis (Garutti, 1989). O mesmo ocorre com relação ao fato de ser ora classificada como uma espécie migradora (Harvey e

Carolsfeld, 2003) ou não, realizando apenas migrações laterais de curta distância (Suzuki et al., 2005). No entanto, mesmo com todas estas controvérsias sua reprodução em cativeiro é facilmente obtida, a despeito de ausência de conhecimento sobre a eficiência do processo e do potencial reprodutivo da espécie.

O lambari é normalmente submetido ao sistema semi-natural de desova (Lira et al., 2018; Brambila-Souza et al., 2019), pois por ser de pequeno porte a extrusão manual dos gametas pode acarretar em altas taxas de mortalidade dos reprodutores (Porto-Foresti et al., 2018). Os casais são induzidos com hormônios exógenos e alocados em unidades experimentais que podem ser caixas d'água ou até mesmo nos próprios viveiros de piscicultura para que o pareamento e a desova ocorram (Porto-Foresti et al., 2018). A desova ocorre, geralmente, com 160 horas-grau após a indução hormonal com extrato bruto de hipófise de carpa (CPE) (Porto-Foresti et al., 2018) ou entre 240,3 e 250,3 horas-grau com LHRH-gonadorrelina (Felizardo et al., 2012), os ovos são levemente adesivos e bastante resistentes (Sato et al., 2006). A fecundidade absoluta de uma fêmea de lambari varia na literatura e é estimada entre 4.119 e 7.443 oócitos por fêmea (Felizardo et al., 2012) e entre 11.086 e 31.720 oócitos por fêmea (Sato et al., 2006). Esta inconsistência nos dados apresentados na literatura sobre o lambari dificulta a avaliação do potencial reprodutivo e a definição de protocolos mais eficientes de reprodução da espécie.

Com relação à sua reprodução em cativeiro para fins comerciais, protocolos e métodos de avaliação de desempenho apresentam o mesmo padrão de controvérsias anteriormente apresentado com relação à sua biologia reprodutiva, o que leva ao desconhecimento da eficiência do sistema de reprodução empregado. A desova pode ser obtida apenas agrupando os casais, com redução do nível da água, sem indução hormonal (Chehade et al., 2015). Além disso, pode ser utilizado CPE em dose única de 3mg kg^{-1} (de Bem et al., 2012; Nascimento et al., 2017) ou 6mg kg^{-1} (Sato et al., 2006; Lira et al., 2018) ou duas doses, fracionando 10% na primeira dose e 90% na segunda dose hormonal (Porto-Foresti et al., 2018). Sabe-se também que a espécie responde a tratamentos hormonais com dose única de 80 µg de LHRH-gonadorrelina kg^{-1} (Felizardo et al., 2012) e com dose única de 5.000 IU de gonadotropina coriônica humana (hCG) kg^{-1} (Brambila-Souza et al., 2019). No entanto, analisando as referências acima citadas, podemos perceber que muitos destes trabalhos não avaliam o desempenho reprodutivo, mas sim utilizam os protocolos como forma de obter formas jovens de lambari para outros tipos de estudo, como, reversão sexual (de Bem et al., 2012) e ferramentas para triploidia (Nascimento et al., 2017). Em

outro caso as informações sobre as taxas de fertilização e eclosão e o potencial para obtenção de embriões viáveis não foram apresentadas (Felizardo et al., 2012). Na desova sem indução hormonal foi analisado apenas a porcentagem de complexos pós-ovulatórios (CPO) nos ovários após 24h de manejo, com aproximadamente 10% de CPO e sem informação do número de fêmeas que apresentavam CPO (Chehade et al., 2015).

Apesar de todos estes métodos levarem a obtenção de alevinos, a ausência de informações precisas sobre a eficiência dos protocolos impede que o máximo de formas jovens seja obtido por desova. Certamente o potencial reprodutivo da espécie pode ser muito maior do que o atingido atualmente e esta otimização poderia ajudar a atender a demanda por formas jovens. Além disso, para o desenvolvimento de programas de manutenção da variabilidade genética (usados em peixamentos) e de melhoramento genético (usados para aumento da produtividade da espécie) cruzamentos dirigidos sem falhas na ovulação são necessários. Nossas observações foram suportadas pela literatura mostrando uma ampla variação na fecundidade da espécie, porém não existem relatos sobre as taxas de ovulação ou se apresentaram a eficácia em se obter embriões viáveis.

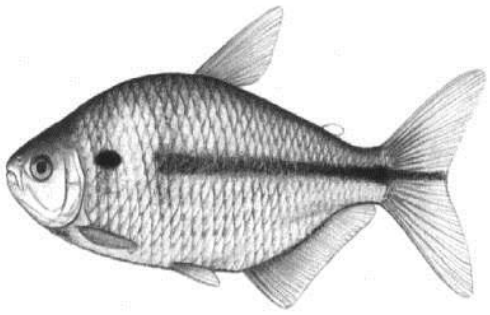

Figura 3. ***Astyanax altiparanae.*** Ilutração por Oscar Akio Shibatta.

Instrumentos da Pesquisa

Perguntas

- O uso de análogos do GnRH pode induzir maturação final e ovulação em fêmeas de *A. altiparanae*? Se sim, os parâmetros reprodutivos podem ser melhores do que com outros protocolos de indução à desova?
- Os níveis de 17,20β-P estão relacionados com a maturação final e ovulação em fêmeas de *A. altiparanae*?

- Os níveis de $PGF_{2\alpha}$ estão relacionados com a ovulação em fêmeas de *A. altiparanae*?
- A inibição do sistema dopaminérgico através do uso de domperidona (método Linpe) pode melhorar o desempenho reprodutivo de fêmeas de *A. altiparanae*?

Hipóteses

- É possível induzir a desova de fêmeas de *A. altiparanae* utilizando hormônios sintéticos.
- A indução à reprodução artificial produz aumento nos níveis plasmáticos 17,20β-P promovendo a maturação final e ovulação nas fêmeas de *A. altiparanae*.
- Os níveis plasmáticos de $PGF_{2\alpha}$ são superiores em fêmeas que desovam do que em fêmeas que não desovam.
- A associação entre análogos do GnRH e do antagonista dos receptores da dopamina (domperidona) pode apresentar resultados diferentes, intensificando ou prejudicando o desempenho reprodutivo de fêmeas de *A. altiparanae*.

Objetivos

Geral

Avaliar o uso de hormônios sintéticos (sGnRHa e LHRHa) no desempenho reprodutivo e na produção de substâncias envolvidas com a maturação final e ovulação (17,20β-P e $PGF_{2\alpha}$) em fêmeas de *A. altiparanae*.

Específicos

- Avaliar o desempenho reprodutivo de fêmeas de lambari induzidas com sGnRHa e LHRHa;
- Avaliar a necessidade da aplicação de um antagonista dos receptores da dopamina em conjunto com sGnRHa e LHRHa;
- Associar os níveis plasmáticos de 17,20β-P e PGF2α das fêmeas que ovularam e não ovularam, nos diferentes tratamentos, com maturação final e ovulação;
- Avaliar os efeitos dos tratamentos nas porcentagens de oócitos em GVBD e folículos pós-ovulatórios nos ovários após o tempo para a ovulação.

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2. CAPÍTULO II

Reproductive performance of lambari (*Astyanax altiparanae*) in a seminatural system using different protocols

Manuscrito nas normas do periódico “Aquaculture Research”

Reproductive performance of lambari (*Astyanax altiparanae*) in a seminatural system using different protocols

Abstract

We evaluated lambari (Astyanax altiparanae) reproductive performance by administration of gradually increasing doses of analogue of salmon gonadotropin-releasing hormone (sGnRHa). We performed two consecutive experiments applying six sGnRHa single doses in three replicates (ranging from 1 to 160 µg kg^{-1}) (Exp. 1) and three sGnRHa fractioned doses in four replicates (10% + 90% - 12h interval) (10, 100 and 1000 µg kg^{-1}) (Exp. 2) in six months old breeders. In both experiments 6 mg of Carp pituitary extract (CPE) kg^{-1} and saline solution (without hormonal therapy) were used as positive and negative controls, respectively. Reproductive performance was recorded for both experiments, and quantification of 17α-20β-dihydroxy-4-pregnen-3-one (17,20β-P) and prostaglandin $F_{2\alpha}$ plasma levels and ovarian histological evaluations were introduced in Exp. 2. Using a single dose, the spawning rate was low and similar between all groups (from 0 to 20%). Using fractioned doses, the spawning rate of CPE (60%) was higher than other groups (from 10 to 25%), but similar to 1000 µg of sGnRHa kg^{-1} (40%). 17,20β-P increased at the final sampling regardless of treatment (including controls) or successful spawning, suggesting that the simple grouping of fish caused this increase. 17,20β-P and $PGF_{2\alpha}$ plasma levels did not explain ovulation failures in this species. CPE fractioned doses improved lambari ovulation and provided final oocyte maturation in all evaluated females, however, hormonal induction were not conditional for obtaining ovulation since spawning was registered in negative control. Concluding, CPE fractioned doses protocol can be applied to increase reproductive performance of this species.

Keywords: brood-stock management, controlled reproduction, egg maturation and ovulation exogenous hormone treatment, ovarian development, prostaglandin, sex steroids hormones.

1. Introduction

Lambari (*Astyanax altiparanae*) is a small-sized characid fish (Silveira & Vaz-dos-Santos, 2015), formerly known as *Astyanax bimaculatus* (Garutti & Britsky, 2000), with emerging interest for aquaculture in South America for food consumption, but primarily as live bait (Silva & Fernandes, 2011, Sussel, 2015). Its production increased 136.07% in Brazil from 2016 (237 tons) to 2017 (554 tons) (IBGE, 2018) and the species is currently produced in 23,028 fish farms (Peixe BR, 2020). Lambari is a low-trophic level fish with a large potential to be produced in a sustainable way, such as in integrated multi-trophic systems (Fonseca, Costa-Pierce & Valenti, 2017), and it is also one of the main species used for restocking fish programs in South American rivers, where they are reintroduced in areas with reservoirs to mitigate the environmental impact of hydroelectric dams (Duke

Energy, 2013). Its captive production serves dozens of fish programs across the American continent via the production of millions of larvae every year (Duke Energy, 2013). Lambari is being considered as a live bait substitute for sardines (*Sardinella* spp.) in tuna fishing, in order to reduce fishing pressure on natural sardine stocks (Sussel, 2015, Valladão, Gallani & Pilarski, 2016), which are overfished (FAO , 2018). Due to these diverse forms of use and versatility for several purposes, the actual demand for larvae is increasing (IBGE, 2018) and has attracted the attention of many farmers to provide accurate information about the effectiveness of existing protocols.

Information about its reproductive biology is quite controversial, and spawning is considered single-batch (Rodrigues, Campos, Santos, Junior & Camara, 1992) or multiple-batch (Lira, Kuradomi, Souza, Hainfellner & Batlouni, 2018) or spawning changes according to environmental conditions (Garutti, 1989). The same changes occur in its natural reproductive behavior, and it is sometimes classified as a migratory species (Harvey & Carolsfeld, 2003) or a species that just performs short-distance lateral migrations (Suzuki, Bulla, Agostinho & Gomes, 2005). The reproduction of this species in captivity is obtained with (Sato, Sampaio, Fenerich-Verani & Verani, 2006, Lira et al., 2018, de Bem, Fontanetti, Senhorini & Parise-Maltempi, 2012, Felizardo et al., 2012, Nascimento et al., 2017, Porto-Foresti, Castilho-Almeida, Senhorini & Foresti, 2018) or without hormonal induction (Chehade, Cassel & Borella, 2015). The hypophysation is the most widely used method and one of the main controversial points concerns the number of doses, since lambari is reported to be induced by single (Sato et al., 2006, de Bem et al., 2012, Felizardo et al., 2012, Nascimento et al., 2017, Lira et al., 2018) or fractioned carp pituitary extract (CPE) doses (Porto-Foresti et al., 2018).

Exogenous hormones such as CPE and analogue of salmon gonadotropin-releasing hormone (sGnRHa) have been shown to induce maturation, ovulation and spawning performance in both wild and cultured fish species (Zohar & Mylonas, 2001, Vazirzadeh et al., 2011, Zadmajid, 2016, Zadmajid, Bashiri, Sharafi & Butts, 2018, Souza, Kuradomi, Rodrigues & Batlouni, 2020). However, the efficiency of exogenous hormones for induction of spawning exhibits considerable variation between species, and may be species-specific, for either dose of hormonal treatment, administration method, or degree of sexual maturity at time of treatment (Zohar & Mylonas, 2001, DiMaggio, Broach & Ohs, 2014, Zadmajid & Butts, 2018). For Neotropical fishes, the physiological effects of synthetic hormones used to induce spawning remain poorly understood, and they can vary according to the nature of the molecule and the species of fish (Carneiro & Mikos, 2008,

Acuña & Rangel, 2009, Paulino, Miliorini, Murgas, Lima & Felizardo, 2011, Felizardo et al., 2012, Viveiros et al., 2013, Ittzés, Szabó, Kronbauer & Urbányi, 2014, Pereira, Boscolo, Moreira & Batlouni, 2016, Souza et al., 2018). To the best of our knowledge, the use of GnRHa to enhance lambari's reproduction has been reported in only one study. In that study, a single dose of 80 μg of luteinizing hormone-releasing hormone (LHRH-gonadorelin) kg^{-1} was tested, showing similar results to CPE treatment on reproductive parameters of the females (number of spawned females and absolute fecundity) (Felizardo et al., 2012). However, there is no information available regarding the effects of synthetic hormone on fertilization, hatching rates and larval quality in this species.

Because lambari is considered a good biological model (small size and attainment of sexual maturity at approximately four months in captivity), some studies used traditional spawning induction protocols to obtain fries for sexual reversion (de Bem et al., 2012) and triploid assays (Nascimento et al., 2017), but without reproductive performance evaluation. No studies established an improved induced-spawning protocol to supply the productive sector demand. Reproductive performance remains unknown, primarily the spawning rate (i.e., the number of females that spawn). Moreover, preliminary experiments conducted in our laboratory has shown that since the species has relatively small eggs and many females are placed in the spawning units at the same time, it is possible that the apparent large number of eggs obtained is masking a low spawning rate. Notably, there is no scientific evidence confirming whether the species must be hormonally induced because spontaneous spawns are observed without hormonal induction (Chehade et al., 2015, Brambila-Souza et al., 2019). Whether and at what intensity the hormonal induction protocol may increase the spawning rate and reproductive performance are not known. The lack of published information allowed the productive sector to define the "best" and the "worst" protocols by itself, even in the absence of an appropriate scientific background, and lambari farmers themselves are looking for more accurate information to meet the demand.

Therefore, the present study analyzed for the first time the reproductive performance of lambari females using hypophysation in single or fractioned doses, which are the most widely used and described protocol, and protocols using the analogue of salmon gonadotropin-releasing hormone (sGnRHa) and spawning without the use of hormones. To evaluate the efficiency of each treatment, we compared the plasma levels of substances involved in final oocyte maturation and ovulation (17α-20β-dihydroxy-4-pregnen-3-one (17,20β-P) (Brambila-Souza et al., 2019) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Figueiredo-Ariki,

2019) and the association of these levels with treatments, doses and the biological processes that promote spawning (i.e., final oocyte maturation and ovulation).

2. Material and Methods

This study was performed in agreement with the precepts of the National Council for the Control of Animal Experimentation (CONCEA), and it was approved by the Animal Ethics and Welfare Committee from UNESP, Jaboticabal, SP, Brazil, under permission number 003838/17.

2.1.Animals and Maintenance

Mature lambari males and females (six months old) that were used in Experiment 1 (Exp. 1) and Experiment 2 (Exp. 2) were reared in 200 m^2 earthen ponds at a density of approximately 50 fish m^{-2} at Centro de Aquicultura da Unesp (CAUNESP) in Jaboticabal, São Paulo, Brazil (21°15'17''S, 48°19'20''W). These earthen ponds are used for breeders maintenance and were supplied with a constant water flow between 15 and 20 L min^{-1}, and the water temperature ranged from 24.0 to 26.5 °C (mean ± SD = 25.28 ± 0.81 °C) during Exp. 1 (November 2016) and from 22.8 to 25.4 °C (mean ± SD = 24.1 ± 0.68 °C) during Exp. 2 (November 2017). The fish were fed a commercial diet (36% crude protein) *ad libitum* twice daily.

The animals included in both experiments were selected for their external characteristics of mature individuals: bulging belly and hemorrhagic urogenital papilla (for females) and the presence of spikes on the anal fin (for males) (Lira et al., 2019). The selected fish were transported to the laboratory, acclimatized and kept in 750 L boxes in a closed recirculation system before the onset of the experiments. The water temperature, dissolved oxygen concentration and photoperiod were, at 26.6 ± 0.3 °C, 8.73 ± 0.03 mg L^{-1} and 12 h light:12 h dark, respectively, in Exp. 1 and 27.9 ± 0.5 °C, 7.4 ± 0.28 mg L^{-1} and 12 h light:12 h dark, respectively, in Exp. 2.

2.2.Experimental Design (Figure 1 and Table 1)

2.2.1. Exp.1- Reproductive performance of A. altiparanae females induced with a single intraperitoneal injection of sGnRHa or CPE

In this experiment, a single dose (positive control and sGnRHa treatments) or a single injection with saline solution (Control) was applied. We defined 6 mg of CPE kg^{-1} in a single dose as the positive control because it is the most widely used hormonal induction protocol for this species (Sato et al., 2006, de Bem et al., 2012, Felizardo et al., 2012, Nascimento et al., 2017, Lira et al., 2018). Fish in the negative control were handled as in the other treatments, and the vehicle (0.9% saline solution) was applied. Six different concentrations of sGnRHa [D-Arg^{6}, Pro^{9}, NEt]-sGnRHa (Syndel Laboratories Inc., Vancouver, Canada) 1, 10, 20, 40, 80 and 160 μg kg^{-1} (denoted 1Gn, 10Gn, 20Gn, 40Gn, 80Gn and 160Gn), a positive and a negative control were applied using three replicates (eight groups x three replicates) (Podhorec et al., 2011, Felizardo et al., 2012). Each replicate was considered an experimental unit. The experimental units were 10 L boxes with constant water flow and contained an egg collecting net at the 0.5 mm water outlet. Five females (18.41 ± 4.77 g) and ten males (9.38 ± 2.32 g) were placed inside each experimental unit. Therefore, each experimental unit was composed of 15 animals in the sexual proportion of 1 female: 2 males (Brambila-Souza et al., 2019). We used a completely randomized experimental design with eight groups x three replicates x 15 animals per replicate for a total of 360 animals (Table 1).

Table 1. Experimental groups used in this study.

	Treatments	Denotation	Number of replicates*	Number of females per replicate	Total number of injected females
Experiment 1 – Single dose	0.9% NaCl solution	Control	3	5	15
	Carp pituitary extract (6 mg kg^{-1})	CPE	3	5	15
	sGnRHa (1 µg kg^{-1})	1Gn	3	5	15
	sGnRHa (10 µg kg^{-1})	10Gn	3	5	15
	sGnRHa (20 µg kg^{-1})	20Gn	3	5	15
	sGnRHa (40 µg kg^{-1})	40Gn	3	5	15
	sGnRHa (80 µg kg^{-1})	80Gn	3	5	15
	sGnRHa (160 µg kg^{-1})	160Gn	3	5	15
Experiment 2 – Fractionated dose (10% + 90% - 12h interval)	0.9% NaCl solution	Control	4	5	20
	Carp pituitary extract (6 mg kg^{-1})	CPE	4	5	20
	sGnRHa (10 µg kg^{-1})	10Gn	4	5	20
	sGnRHa (100 µg kg^{-1})	100Gn	4	5	20
	sGnRHa (1000 µg kg^{-1})	1000Gn	4	5	20

* Each replicate was considered an experimental unit. The experimental units were 10 L boxes with constant water flow and contained an egg collecting net at the 0.5 mm water outlet.

The experiment started when females and males were induced with the application of a single dose and were allocated to the experimental units at 11 *p.m.* (day zero, hour zero) (Figure 1). Males were induced with a single dose of 3 mg CPE kg^{-1} (all treatments and controls) (Brambila-Souza et al., 2019). The injected volume (40 µL for each 10 g of fish) was the same for all groups. The experiment lasted approximately 12 h after hormonal injection, following the theoretical spawning period for the species (Felizardo et al., 2012, Porto-Foresti et al., 2018). The total egg production of an experimental unit was calculated by collecting the total amount of eggs produced until the end of the experiment (12 hours after the resolving or single dose). After this period, small amounts of eggs were rarely found, but were not considered for analysis.

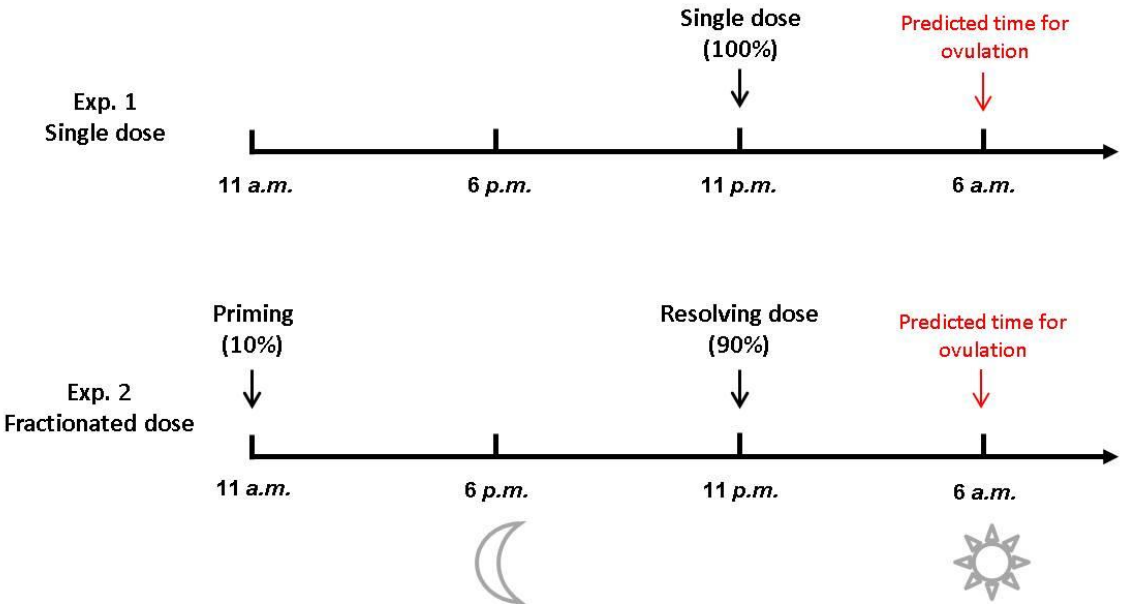

Figure 1. Experimental design. Exp. 1: Single dose of sGnRHa in six concentrations (1, 10, 20, 40, 80 and 160 µg kg^{-1}), one positive control (6 mg of CPE kg^{-1}) and one negative control (saline solution 0.9%). Three replicates with five females and ten males each. **Exp. 2:** Fractionated dose of sGnRHa (10% + 90%) in three concentrations (10, 100 and 1000 µg kg^{-1}), one positive control (6 mg of CPE kg^{-1}) and one negative control (saline solution 0.9%). Four replicates with five females and ten males each.

All females were evaluated after post-spawning period and classified as spawned (releasing oocytes by gentle abdominal compression) and unspawned (remained with bulging belly and did not eliminate residual oocytes) (Lira et al., 2018) and their length (cm) and body mass (g) were recorded.

2.2.1.1.Reproductive Performance

The experimental units were checked for ovulation every half hour as from 3:30 *a.m.* until 11:00 *a.m.* and when eggs were observed in the collecting net another 30 min interval was waited to collect eggs and measure the spawning volume in a graduated cylinder. After that, a 10 mL aliquot of eggs from the first spawning of each experimental unit was allocated to individual 6.5 L incubators to determine fertility and hatching rates (one incubator per experimental unit). The average temperatures conditions for egg incubation were 26.6 ± 0.3°C (Exp. 1) and 27.9 ± 0.5°C (Exp. 2). Fertility rate (percentage of fertilized oocytes) was measured approximately 3 h post-fertilization at the gastrula stage and hatching rate (percentage of larvae that hatched) was measured approximately 9 h post-fertilization when caudal fin detachment was observed in each embryo prior to hatching. To obtain both parameters, 100 eggs per incubator were collected randomly, and egg counts were performed under a LEICA M50 stereomicroscope (LEICA Microsystems, Wetzlar, Germany).

The following parameters were recorded:

a) Latency period = hours between the resolving dose and first spawn observed in each replicate,
b) Replicates with spawned females = number of replicates with spawn / total number of replicates,
c) Spawned females per replicate = number of spawned females per replicate/total number of female per replicate,
d) Spawning rate (%) = average or median percentage of spawned females per treatment,
e) Relative fecundity (mL g^{-1}) = total eggs volume / total sum of female body weight per replicate,
f) Fertility rate (%) = number of viable eggs x 100 / 100 (total number of collected eggs), and
g) Hatching rate (%) = number of embryo caudal fin detachment x 100 / 100 (total number of collected eggs).

2.2.2. Exp. 2 – Effects of sGnRHa and CPE administration in fractioned doses in A. altiparanae females

All treatments and controls were applied in fractionated doses. The experiment started at the time the females received their first hormonal doses and were allocated to the experimental units at 11 *a.m.* (day zero, hour zero) (Figure 1). Four females were randomly selected before treatment to represent the baseline conditions (Initial) to assess the effects after treatments. The experiment lasted approximately 12 h after resolving dose, following the theoretical spawning period for the species (Felizardo et al., 2012, Porto-Foresti et al., 2018).

Females in all groups received two intraperitoneal injections (one comprised of 10% of the total dose administered at 11 *a.m.* and a second dose 12 h later (at 11 *p.m.*) comprising the resolving dose). Due to the extreme heterogeneity and low spawning rate obtained in most treatments in Exp. 1, the highest dose of sGnRHa and number of replicates were increased. Males were grouped with females after the second dose of females and received a single dose of 3 mg CPE kg^{-1}. The volume injected (40 µL for each 10 g of fish) was the same for all groups. The following experimental groups were used: three doses of sGnRHa (10, 100 and 1000 µg of sGnRHa kg^{-1}, denoted: 10Gn, 100Gn and 1000Gn), one positive control (6 mg of CPE kg^{-1}), and a negative control (0.9% saline solution). The doses tested in this experiment were based on the results of Exp. 1. Each treatment was performed in four replicates (five groups x four replicates x 15 animals per replicate = 300 animals.). Each replicate was considered an experimental unit. The experimental units were the same as Exp. 1 with five females (16.36 ± 2.74 g) and ten males (10.3 ± 1.08 g) were placed inside each experimental unit. Within each experimental unit, the number of animals and the sex ratio of 1 female: 2 males from Exp. 1 were repeated (Table 1).

One spawned and one unspawned female from each replicate (confirmed by ovarian histology) were randomly chosen approximately 12 h post resolving dose (at the end of the trial) for ovarian stereological evaluation and the measurement of plasma levels of 17,20β-P (Jesus et al., 2017, Brambila-Souza et al., 2019) and $PGF_{2\alpha}$ (Figueiredo-Ariki, 2019).

2.2.2.1.Reproductive Performance

The same parameters of reproductive performance used in Exp. 1 (section 2.2.1.1) were applied in Exp. 2.

2.2.2.2.Sampling procedure

For sample collection, fish were anesthetized with 100 mg L^{-1} of benzocaine (Sigma-Aldrich, Saint Louis, USA) approximately 12 h post resolving dose, and blood samples were collected from the caudal vein using heparinized syringes and needles. The collected blood was separated into aliquots (approximately 60 µL each), including one aliquot with 10 µM indomethacin to inhibit prostaglandin synthesis (Kuradomi & Batlouni, 2018). The plasma was separated via centrifugation at 3,000 rpm for 10 min at 4°C and frozen at -80°C for the subsequent 17,20β-P and $PGF_{2\alpha}$ assays. After blood collection, length (cm) and body mass (g) were recorded.

After this procedure, females were euthanized via a lethal dose of benzocaine (500 mg L^{-1}) and a section of the spinal cord at the level of the operculum. To remove the ovaries, a ventral incision was made in the caudal-cranial direction from the urogenital pore. The ovaries were removed, and a portion was sectioned and fixed in modified Karnovsky's solution (4% paraformaldehyde and 2% glutaraldehyde in Sorensen phospate buffer pH 7.2) for histological characterization. The fixed ovary samples were treated using routine histology techniques: 70% alcohol washes, serial dehydration, inclusion in historesin (Technovit 7100, Kulzer Histo-Technik), sectioned at 3 µm thickness, fixed on slides and stained with hematoxylin-eosin.

2.2.2.3.Plasma levels of 17,20β-P and $PGF_{2\alpha}$

The plasma levels of $PGF_{2\alpha}$ and 17,20β-P were quantified using enzyme linked immunosorbent assays (ELISA) and commercial kits (Cayman Chemical Company, Ann Arbor, MI, USA) following the manufacturer's instructions. The readings of $PGF_{2\alpha}$ and 17,20β-P plates were performed at absorbances of 405 and 412 nm, respectively, using an Epoch2 plate reader (BioteK Instruments, Inc., Highland Park, Winooski, USA), and all samples were read in duplicate. To validate the kits, intra- and interassays were performed, and we obtained the following variations: 0.125 to 20.127% and 5.342 to 16.932% for $PGF_{2\alpha}$, 0.042 to 19.317% and 1.784 to 14.891% for 17,20β-P.

2.2.2.4.Volume density

The histological slides of the ovaries were analyzed under a light microscope to identify the different types of structures observed following Cassel et al. (2017). After this identification, an analysis of the volume density of the ovaries of a spawned female and a unspawned female per replicate from all treatments and four females before hormonal

induction using a light microscope and a grid of 352 points of intersection (Criscuolo-Urbinati, Kuradomi, Urbinati & Batlouni, 2012, Pereira et al., 2016, Souza et al., 2020). The grid was applied and counted in three fields in the middle region of the ovary for a total of 1056 points for each female using a 5x objective. The method of Criscuolo-Urbinati et al. (2012) was used with some modification. Points were classified according to the histological descriptions of *A. altiparanae* by Cassel et al. (2017): previtellogenic (PV), cortical alveoli (CA), early vitellogenic with incomplete vitellogenesis and cytoplasm not filled with yolk (EV), final vitellogenic with cytoplasm filled with yolk (FV), mature vitellogenic oocyte with germinal vesicle breakdown (GVBD), atretic (AT), postovulatory follicles (POF), and interstitial tissue (IT). Artifacts were rarely observed, and points with artifacts were excluded from the total number of points used to obtain the percentages.

2.3.Statistical analyses

The assumptions of normality and homogeneity of the variances were tested using the Cramer-von Mises and Levene tests, respectively. Parametric variables were analyzed using the variance test (one-way ANOVA) followed by Tukey's test (for more than two groups) or the t test (for two groups). The Kruskal-Wallis test was used for nonparametric variables followed by Dunn's test for multiple comparisons (for more than two groups) or Mann-Whitney test (for two groups). Log-transformation was performed on data of 17,20β-P plasma levels for spawned females to achieve the assumptions of normality.

The variables:

- Latency period: Mann-Whitney test (Exp. 1) and Kruskal-Wallis test (Exp. 2).
- Spawning rate: Kruskal-Wallis test (Exp. 1) and one-way ANOVA followed by Tukey's test (Exp. 2).
- Relative fecundity: Kruskal-Wallis test (Exp. 1) and one-way ANOVA followed by Tukey's test (Exp. 2).
- Fertility and hatching rates: T test (Exp. 1) and one-way ANOVA followed by Tukey's test (Exp. 2).
- 17,20β-P plasma levels: one-way ANOVA followed by Tukey's test (Exp. 2).
- $PGF_{2\alpha}$ plasma levels: Kruskal-Wallis test (Exp. 2).
- Volume density: Kruskal-Wallis test followed by Dunn's test or one-way ANOVA followed by Tukey's test, depending on normality and homogeneity of the variances (Exp. 2).

3. Results

3.1.Experiment 1

3.1.1. Reproductive performance

Ovulation occurred between five hours and twenty minutes and nine hours after the single dose (at 26.6 ± 0.3°C), and no significant difference was found between treatments (CPE and 10Gn) ($p>0.05$) (Table 2). The other groups could not compose the statistical analysis due to the small number of females that spawned (Table 2). Spawning was observed in all replicates only in the CPE group, but spawning was absent in at least one replicate in all other groups (Table 2).Spawning was not recorded in any replicates in the negative control and 1Gn groups (Table 2). Spawning was recorded in 2/3 of 10Gn and 1/3 of 20Gn to 160Gn replicates (Table 2). The number of spawned females per replicate was extremely heterogeneous and low. Spawning was rarely observed in more than half of injected females in the same replicate. Regardless of the treatment, 0/5 females spawned from 15/24 replicates (62.5% of the replicates), only 1/5 of the females spawned in 4/24 replicates (16.6% of the replicates), and only 2/5 of the females spawned in 2/24 replicates (8.8% of the replicates). Only 3/24 replicates presented more than 50% spawned females: (3/5) for the CPE and 80Gn group and 4/5 for one replicate from the 160Gn treatment.

The median values of relative fecundity were statistically similar between groups ($p>0.05$). The average fertility and hatching rates were over 60% in all groups, and there was no significant difference between CPE and 10Gn (groups with more than one replicate spawned) ($p>0.05$) (Table 2).

Table 2. Experiment 1. Effects of 0.9% NaCl, 6 mg of CPE kg^{-1} and different doses of sGnRHa in a single intraperitoneal injection on the reproductive performance of *A. altiparanae* females.

Groups	Latency period (h)	Replicates with spawned females[1]	Spawned females per replicate[2]			Spawning rate (%)	Relative fecundity (mL g^{-1})	Fertility rate (%)	Hatching rate (%)
			R1	R2	R3				
Control	-	0/3	0/5	0/5	0/5	0 (0, 0)	0.00 (0.00, 0.00)	-	-
CPE	5.3 (5.3, 6.17)	3/3	1/5	3/5	1/5	20 (20, 60)	0.44 (0.29, 0.59)	64.42 ± 8.49	61.24 ± 9.82
1Gn	-	0/3	0/5	0/5	0/5	0 (0, 0)	0.00 (0.00, 0.00)	-	-
10Gn	7.66 (6.3, 9.0)	2/3	1/5	2/5	0/5	20 (0, 40)	0.24 (0.00, 0.46)	71.68 ± 6.10	62.79 ± 6.34
20Gn	8.5	1/3	1/5	0/5	0/5	0 (0, 20)	0.00 (0.00, 0.25)	96.41	84.00
40Gn	5.3	1/3	2/5	0/5	0/5	0 (0, 40)	0.00 (0.00, 0.48)	92.71	87.61
80Gn	5.3	1/3	3/5	0/5	0/5	0 (0, 60)	0.00 (0.00, 0.38)	80.17	75.25
160Gn	8.5	1/3	4/5	0/5	0/5	0 (0, 80)	0.00 (0.00, 0.53)	80.00	72.32

Data are expressed as mean ± SD or median (first quartile, third quartile). There were no significant differences among groups for the variables evaluated.
Latency period was analyzed by Mann-Whitney test between CPE and 10Gn group. Spawning rate and relative fecundity were analyzed by Kruskal-Wallis test. Fertility and Hatching rate were analyzed by T test between CPE and 10Gn group.
[1] Replicates with spawned females: number of spawning replicates /total number of replicates.
[2] Spawned females per replicate: Number of females spawned/total number of females. R1) Replicate 1, R2) Replicate 2, R3) Replicate 3.

3.2.Experiment 2

3.2.1. Reproductive performance

Ovulation occurred between four hours and ten minutes and eight and a half hours after the resolving dose (at 27.9 ± 0.5°C). The latency period of 1000Gn was longer than CPE group ($p<0.05$) (Table 3). Spawning was observed in all groups in this experiment, including the negative control (Control) (Table 3). Spawning was recorded in 4/4 replicates in CPE and 1000Gn groups. For the control group and other sGnRHa groups (10Gn and 100Gn) spawning was recorded in 3/4 and 2/4 of replicates, respectively (Table 3). In this experiment (fractioning the dose), spawning was observed in 15/20 replicates (75%) regardless of the treatment. The spawning rate (spawned females per replicate) and relative fecundity were higher for CPE (60 ± 16.33% and 1.15 ± 0.55 mL g^{-1}, respectively) compared to the other groups, except for its similarity to 1000Gn (40 ± 16.33% and 0.51 ± 0.29 mL g^{-1}, respectively) (Table 3). However, the spawning rate and relative fecundity of 1000Gn was similar to the other groups ($p>0.05$) (Table 3). Fertility and hatching rates were all over 70% and similar between groups ($p>0.05$) (Table 3).

Table 3. Experiment 2. Effects of 0.9% NaCl, 6 mg of CPE kg^{-1} and different doses of sGnRHa in two intraperitoneal injections on the reproductive performance of *A. altiparanae* females.

Groups	Latency period (h)	Replicates with spawned females[1]	Spawned females per replicate[2]				Spawning rate (%)	Relative fecundity (mL g^{-1})	Fertility rate (%)	Hatching rate (%)
			R1	R2	R3	R4				
Control	5 (5, 5) [ab]	3/4	2/5	2/5	1/5	0/5	25 ± 19.15 [b]	0.30 ± 0.21 [b]	93.15 ± 8.59 [a]	88.42 ± 12.01 [a]
CPE	4.17 (4.17, 4.17) [a]	4/4	3/5	4/5	3/5	2/5	60 ± 16.33 [a]	1.15 ± 0.55 [a]	77.05 ± 9.29 [a]	71.97 ± 14,07 [a]
10Gn	5 (5, 5) [ab]	2/4	0/5	1/5	0/5	1/5	10 ± 11.55 [b]	0.21 ± 0.24 [b]	97.31 ± 1.27 [a]	97.16 ± 1.35 [a]
100Gn	4.92 (4.17, 5.6) [ab]	2/4	1/5	0/5	0/5	1/5	10 ± 11.55 [b]	0.19 ± 0.22 [b]	93.68 ± 2.23 [a]	88.17 ± 7.40 [a]
1000Gn	7 (5.5, 8.5) [b]	4/4	3/5	2/5	2/5	1/5	40 ± 16.33 [ab]	0.51 ± 0.29 [ab]	93.08 ± 4.77 [a]	91.55 ± 7.88 [a]

Data are expressed as mean ± SD or median (first quartile, third quartile). Groups with the same letters were not significantly different.
Latency period was analyzed by Kruskal-Wallis test. Spawning rate, relative fecundity, fertility and hatching rate were analyzed by one-way ANOVA followed by Tukey test.
[1] Replicates with spawned females: number of spawning replicates /total number of replicates.
[2] Spawned females per replicate: Number of females spawned/total number of females. R1) Replicate 1, R2) Replicate 2, R3) Replicate 3, R4) Replicate 4.

3.2.2. Plasma levels of 17,20β-P

In Exp. 2, all spawned females, regardless of group, showed an increase in 17,20β-P plasma levels at the final sampling compared to baseline levels (Initial) (Figure 2). At the final sampling, 17,20β-P plasma levels in unspawned females were higher in CPE-treated females compared to the other groups ($p<0.05$) (Figure 2). Values in the 100Gn and 1000Gn groups were higher, and values of the 10Gn and negative control groups were similar to baseline levels (Initial) ($p<0.05$) (Figure 2). We did not observe a dose-dependent effect in the sGnRHa-treated groups for spawned and unspawned females. The profiles of the spawned and unspawned females in the same group were similar for 17,20β-P, except for the increased values of unspawned CPE females (Figure 2).

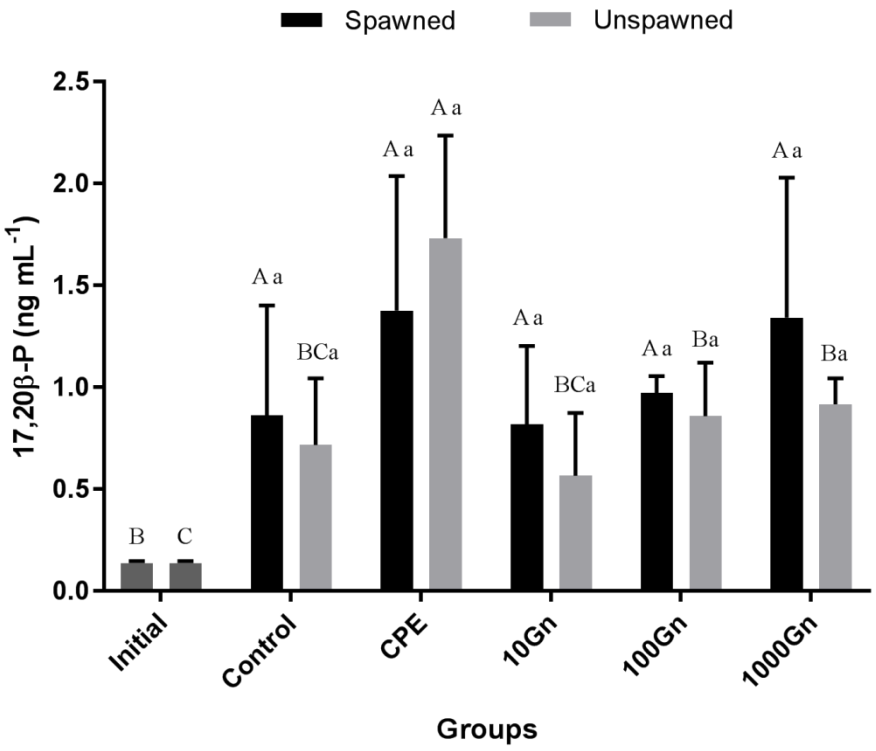

Figure 2. Experiment 2. 17,20β-P plasma levels of *A. altiparanae* females sampled 12 hours after the resolving dose injection. **Initial** bars refer to four females randomly selected before hormonal induction, which represent the baseline collection. Each Initial bar represents the statistical comparison between spawned and unspawned females. One spawned and one unspawned female for each replicate were randomly chosen for this analysis. Data are expressed as the means ± SD. **Spawned females:** The significant differences between Initial and spawned females from different treatments are expressed by different upper cases according to the Tukey's test. **Unspawned females**: The significant differences between Initial and unspawned females from different treatments are expressed by different upper case letters according to the Tukey's test. The significant differences between spawned females and unspawned females from the same treatment are expressed by different lower case letters according to the T test.

3.2.3. Plasma levels of $PGF_{2\alpha}$

The levels of $PGF_{2\alpha}$ in spawned and unspawned females were similar between groups and compared to the initial group ($p>0.05$) (Figure 3). $PGF_{2\alpha}$ levels were only different between spawned and unspawned females in the 10Gn group, with higher levels in spawned females ($p<0.05$) (Figure 3).

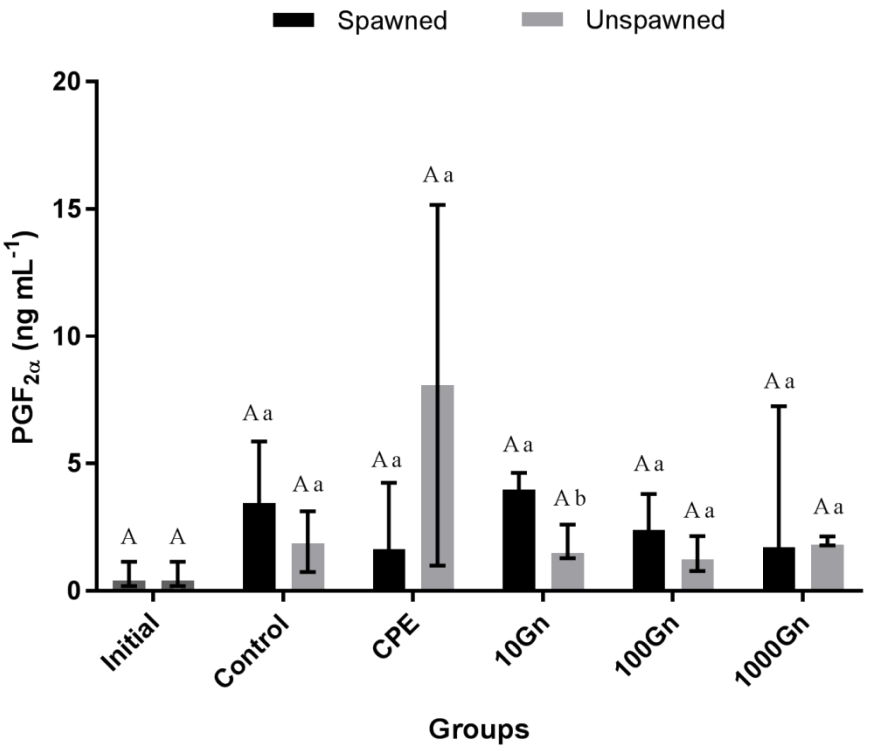

Figure 3. Experiment 2. $PGF_{2\alpha}$ plasma levels of *A. altiparanae* females sampled 12 hours after the resolving dose injection. **Initial bars** refer to four females randomly selected before hormonal induction, which represent the baseline collection. Each Initial bar represents the statistical comparison between spawned and unspawned females, respectively. One spawned and one unspawned female for each replicate were randomly chosen for this analysis. Data are expressed as the medians and 5-95 percentiles. **Spawned females:** The significant differences between Initial and spawned females from different treatments are expressed by different upper case letters according to the Kruskal-Wallis test. **Unspawned females**: The significant differences between Initial and unspawned females from different treatments are expressed by different upper case letters according to the Kruskal-Wallis test. The significant differences between spawned females and unspawned females from the same treatment are expressed by different lower case letters according to the T test or Mann–Whitney U test.

3.2.4. Volume density

Stereological evaluation revealed that ovaries from initial females were 87.13 ± 2.12% composed of final vitellogenic stage oocytes (FV) (data not shown). Neither POF nor GVBD oocytes were observed in these females, which shows that these females were ready to spawn but had not ovulated at the beginning of the experiment onset. Among spawned females, volume density of different oocyte types was similar in all groups

(Figure 4A). For unspawned females, GVBD (53.45 ± 22.8%) and FV (3.77 ± 2.61%) were higher and lower, respectively, in CPE-treated females compared to the other groups ($p<0.05$). GVBD oocytes retained in the ovaries of unspawned females were only observed (3 of 3 females analyzed) in the CPE-treated group (Figure 4B).

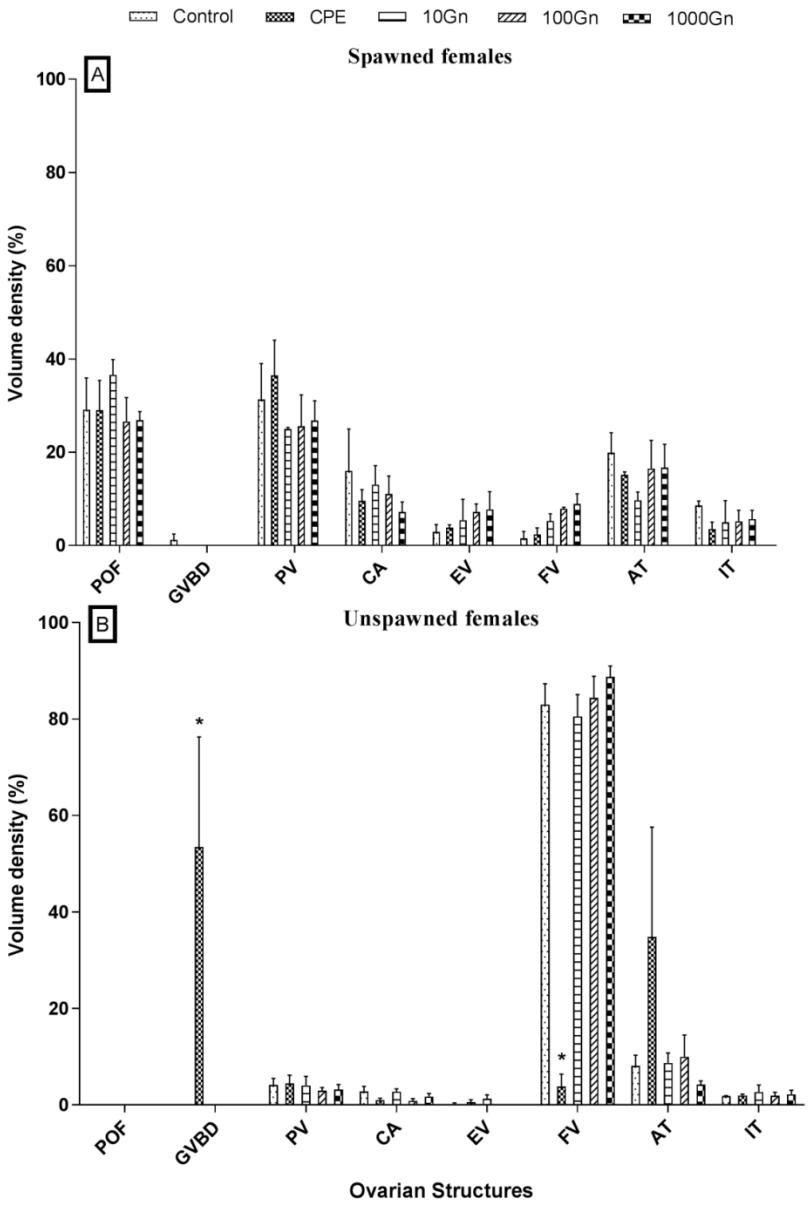

Figure 4. Experiment 2. Volume densities in percentage values (±SEM) of different types of ovarian structures sampled 12 hours after the resolving dose injection. Asterisk indicates significant differences between treatments for each type of ovarian structure. **(A) Spawned females**: GVBD and EV were analyzed using the Kruskal-Wallis test. POF, PV, CA, FV, AT and IT were analyzed using one-way ANOVA. **(B) Unspawned females**: GVBD, EV, AT and IT were analyzed using the Kruskal-Wallis test. PV, CA and FV were analyzed using one-way ANOVA. **Ovarian structures: PV** previtellogenic, **CA** cortical alveoli, **EV** early vitellogenic, **FV** final vitellogenic, **GVBD** mature vitellogenic oocyte with germinal vesicle breakdown, **POF** postovulatory follicle, **AT** atretic and **IT** interstitial tissue.

4. Discussion

The present study obtained similar reproductive performances, including all GnRH treatments, negative and positive controls, when lambari were treated with a single dose (Exp 1). However, the use of 6 mg of CPE kg^{-1} in fractioned doses (10% + 90%) significantly increased the spawning rates compared to all of the other fractioned groups, with the exception of 1000Gn (Exp. 2) that was similar to all groups. Moreover, fractionated CPE was the only treatment that increased the mean percentage of spawned females 2.4-fold based on absolute numbers and increased the egg volume obtained per gram of injected fish 3.83-fold, compared to the negative control. These results allow us to clearly and undoubtedly conclude that CPE fractioned doses intensified and increased lambari reproduction in captivity and must be applied instead of a single CPE dose protocol (the most used nowadays) or any sGnRHa protocols tested here.

Two important physiological processes are known to be related to the effective results obtained here using CPE fractioned doses instead of a single dose: the acquisition of "oocyte maturational competence" (Patiño, Thomas & Yashizaki, 2003) and the "stereoidogenic shift" prior to ovulation (Levavi-Zermonsky & Yaron, 1986). The so-called "oocyte maturational competence" was described *in vivo*, when a priming dose (0.6 mg of CPE kg^{1}) induced germinal vesicle migration concomitantly with the acquisition of oocyte responsiveness to 17,20β-P, in carps (*Cyprinus carpio*) (Jalabert, Breton, Brzuska, Fostier & Wieniawski, 1977). Also in carps, the onset of germinal vesicle migration preceded the detection of 17,20β-P in the plasma (Levavi-Zermonsky & Yaron, 1986), after two injections (priming + resolving dose) of pituitary homogenate (Patiño et al., 2003). Concerning the "steroidogenic shift", many studies demonstrated that this shift in steroidogenesis during spawning induction is necessary for final oocyte maturation and ovulation to occur (Rahman et al., 2001, Senthilkumaran, Yoshikuni & Nagahama, 2004, Chaube, Singh & Joy, 2014). In addition, a distinct shift in steroidogenesis is well documented in teleosts by exogenous hormonal therapy (Podhorec et al., 2016, Zadmajid, Mirzaee, Hoseinpour, Vahedi & Butts, 2017). "Steroidogenic shift' is characterized by a 17,20β-P level peak during ovulation with a simultaneously decrease in the estradiol (E_2) levels (Levavi-Zermonsky & Yaron, 1986). The occurrence of both process (oocyte maturation competence and stereoidogenic shift) are related to the administration of fractioned doses of the pituitary homogenate (Levavi-Zermonsky & Yaron, 1986).

In this study we focused on the reproductive performance using different protocols, therefore the process of steroidogenic shift could not be evaluated since a single sampling was performed to avoid interference with breeding behavior. But we intend to explore this process in the future and find the reasons for the low performance found, since even using fractioned CPE doses (the best protocol), 40% of induced females did not spawn, showing that the relative fecundity of 1.15 ml of eggs per gram of fish obtained could be intensively increased. The successfully spawned females of CPE-fractioned doses group (60%) yielded 150,693 larvae (absolute number calculated using the spawned volume and hatching rate), and the percentage of females with spawning failure (40%) could theoretically produce approximately 100,462 more larvae. In addition to the losses in the production of larvae with a percentage of treated female failures in ovulation, there is a waste of hormone, labor and professional costs, corroborating that the reproductive performance of this species can be can be greatly improved.

Concerning possible reasons for ovulation failures, we observed that only the unspawned females of the fractioned CPE group (and all individuals) retained GVBD oocytes (meiosis resumption) in the ovaries, while all unspawned females from other groups retained only oocytes with intact germinal vesicles. This specific type of failure, which causes GVBD but not ovulation, was in deep studied using fractioned CPE doses in common carp (Jalabert et al., 1977) and in some tropical reophilic species, such as matrinxã (*Brycon amazonicus*) (Hainfellner, Souza, Moreira, Nakaghi & Batlouni, 2012), piau (*Leporinus friderici*) (Souza et al., 2020), piapara (*Leporinus elongatus*) (Pereira, Boscolo, Moreira & Batlouni, 2018) and piaçu, (*Leporinus macrocephalus*) (Pereira et al., 2016). All of these descriptions mentioned unovulated GVBD oocytes retained in the ovaries, but it is still unknown the reasons for such partial failure. Considering that in the present study unspawned and spawned fractionated CPE-treated females had similar 17,20β-P plasma levels, it seems that other substances, that act specifically on ovulation such as vasotocin (Chaube et al., 2014) and prostaglandins (Joy & Singh, 2013) (among many other) should play an important role in this process and need to be studied, as well as their respective receptors. Among these substances, in the present study we evaluated $PGF_{2\alpha}$ plasma levels and they were similar among all treatments, at the final sampling (12h post resolving dose), and did not explain the ovulation failures. Moreover, unlike 17,20β-P plasma levels, the grouping of couples per se (negative controls) did not significantly increase the plasma levels of $PGF_{2\alpha}$ between spawned females compared to baseline levels (initial).

In this concern it must be considered that the interval of action of 17,20β-P and prostaglandins is species-specific and it generally occurs hours before ovulation (Knight & Van der Kraak, 2015, Tang et al., 2017). Ovulation is a complex process that includes a sequential pattern of events (Bobe, Montfort, Nguyen & Fostier, 2006, Knight & Van der Kraak, 2015). The first increase in 17,20β-P levels must be followed by the subsequent action of other substances, primarily prostaglandins, for ovulation, as described for other fish species studied (Takahashi, Hagiwara & Ogiwara, 2018), and seems to precede ovulation in many species. In yellow perch, 17,20β-P stimulated ovulation *in vitro* via an increase in $PGF_{2\alpha}$ and prostaglandin E_2 (PGE_2) levels (Berndtson, Goetz & Duman, 1989, Goetz, 1997). For Japanese eel, 17,20β-P induced ovulation *in vitro* via the synthesis of prostaglandins in follicular cells (Kagawa, Gen, Okuzawa & Tanaka, 2003). In the ovarian tissues of zebrafish, 17,20β-P stimulated an increase in the gene expression of *ptgs2* (Knight & Van der Kraak, 2015). The present study prioritized the evaluation of reproductive performance (avoiding manipulating animals) and we only performed blood samplings at before injection and 12 h post resolving dose. Therefore, it is not possible to conclude the associations of 17,20β-P and $PGF_{2\alpha}$ with final oocyte maturation and ovulation due to limited samplings, but the specific intervals of action of these substances are being evaluated on ongoing issues, including *in vitro* assays.

Still concerning possible explanations for failures on ovulation, but now evaluating groups, which failures included absence of final oocyte maturation and ovulation (negative controls and treatments with sGnRHa), we emphasize that even the 1000Gn group (the highest dose) did not cause meiosis resumption, since 100% of unspawned females showed only intact germinal vesicle oocytes, but not GVBD oocytes. Plasma 17,20β-P levels of unspawned and spawned sGnRHa-treated females were similar for any groups, which highlights that the species likely has a dopaminergic system that must be inhibited by dopamine antagonists, as for *Carrassius auratus* (Peter, Crim, Goos & Crim, 1978), *Onchorhynchus mykiss* (Vacher, Ferrière, Marmignon, Pellegrini & Saligaut, 2002), *Mugil cephalus* (Aizen, Meiri, Tzchori, Levavi-Sivan & Rosenfeld, 2005), *Oreochromis mossambicus* (Jiang, Lian & He, 2016) and *Astatotilapia burtoni* (Bryant, Greenwood, Juntti, Byrne & Fernald, 2016) and many other species. Moreover, the intermediate reproductive performance obtained using 1000 μg sGnRHa kg^{-1} highlights that future approaches testing dopamine inhibitors together with sGnRHa may change the current scenario. Even unusual and much higher than that the doses used for hormonal induction for neotropical (Acuña & Rangel, 2009, Viveiros et al., 2013, Pereira et al., 2016, Pereira

et al., 2018, Souza et al., 2018, Souza et al., 2020), cyprinids (Podhorec et al., 2012a, Podhorec et al., 2012b, Podhorec et al., 2016) and salmonids fishes (Arabaci, Diler & Sari, 2004), the 1000 µg sGnRHa kg^{-1} treatment resulted in fertility and hatching rates above 90%, indicating that the use of this synthetic treatment is not a problem for this species in this concern.

In that regard, some studies showed a negative effect of synthetic hormones on the production of viable embryos in neotropical species at doses much lower than the doses used in the present study. A dose of 8 µg of mGnRHa kg^{-1} in *Leporinus macrocephalus* (Pereira et al., 2016) and doses of 40 µg of mGnRHa kg^{-1} and 12 µg of mGnRHa kg^{-1} for *Leporinus friderici* (Souza et al., 2020) resulted in embryos death, and a dose of 10 µg of sGnRHa kg^{-1} in *Colossoma macropomum* resulted in low fertility and hatching rates (Acuña & Rangel, 2009). Moreover, for South-American reophilic tropical species, the action of dopamine inhibitors remains misunderstood due to the controversial results found in the literature. The use of Ovopel (mGnRHa + metoclopramide) for *Colossoma macropomum* provoked 100% of spawning rate and satisfactory fertilization (71.4%) and hatching rates (56.8%) (Souza et al., 2018). In contrast, using the same association for *Leporinus macrocephalus*, 100% spawning was obtained but without viable embryos (Pereira et al., 2016). For *Rhamdia quelen*, no females ovulated after treatment with the same product (Carneiro & Mikos, 2008). The spawning rate for *Colossoma macropomum* using Ovaprim (sGnRHa + domperidone) was 100%, but with low fertility and hatching rates (11.1% and 19.7%, respectively) (Acuña & Rangel, 2009). These results support the fact that an inhibition of the dopaminergic system stimulates ovulation in these species, but its use is still associated with embryonic death, requiring many tests and evaluations.

In conclusion, we demonstrated that the use of conventional hypophysation (CPE fractioned doses) was the most adequate hormonal therapy for lambari captivity spawning for providing better reproductive performance (among the protocols we tested). In addition, CPE fractioned doses promoted meiosis resumption (oocytes in GVBD in post-spawning ovary) in all injected females unlike the sGnRHa-treated groups and negative control. The simple grouping of male and female breeders, regardless treatment, was associated with increased levels of 17,20β-P, but not $PGF_{2\alpha}$ plasma level, at the final sampling (12 h post resolving dose) in comparison to basal levels. A clear association between 17,20β-P and $PGF_{2\alpha}$ plasma levels with final oocyte maturation and ovulation has not been observed, which must be explored in future approaches including more sampling points and *in vitro* studies.

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Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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3. CAPÍTULO III

The effects of LHRHa with and without dopamine antagonist on reproductive performance in lambari *Astyanax altiparanae*

Manuscrito nas normas do periódico “Aquaculture”

The effects of LHRHa with and without dopamine antagonist on reproductive performance in lambari *Astyanax altiparanae*

Abstract

For reophilic tropical species the involvement of dopamine in the inhibition of luteinizing hormone (LH) release remains unknown, justifying the studies in this concerning. The present study evaluated the effect of luteinizing hormone-releasing hormone ethylamide analogue (LHRHa) with and without domperidone on reproductive performance of lambari and the quantification of plasma levels of substances involved with final oocyte maturation and ovulation (17α-20β-dihydroxy-4-pregnen-3-one (17,20β-P) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)), as well as histological evaluation of ovarian structures in two independent and consecutive experiments. In the first (Exp. 1), five doses of LHRHa (20, 60, 180, 540 and 1620 μg kg^{-1}) were tested with three replicates each, while in the second (Exp. 2) the dose of 180 μg kg^{-1} was tested in combination with 20 mg kg^{-1} domperidone (D) with four replicates. In both experiments, two control groups were formed, 0.9% saline (control) and 6 mg CPE kg^{-1} and females from all groups received two doses (10%, and 90% after 12 h). The use of LHRHa associated with D did not improve reproductive performance of lambari and in both experiments CPE-treated group was the only one performed better than control, indicating that CPE is the best hormonal therapy to induce final oocyte maturation and ovulation in lambari. There was a decrease in fertilization and hatching rates that coincided with the two highest doses of LHRH (540 and 1620 μg kg^{-1}), suggesting that these doses may have caused an overstimulation of pituitary in producing LH that affected egg quality.

Keywords: lambari, steroids, prostaglandin, tropical fish, fish reproduction

1. Introduction

Lambari (*Astyanax altiparanae*) is a small-sized Neotropical characid with a significant increase in its aquaculture production in Brazil (136.07% between the years 2016 and 2017) (IBGE, 2018). The species has diverse forms of use, as a human feed and live bait (Silva and Fernandes, 2011, Sussel, 2015) and being useful for several purpose, as in the integrated multitrophic aquaculture (Fonseca et al. 2017), for restocking fish programmes in South American rivers (Hashimoto et al. 2015), and as a biological model in *in vitro* study (Branco et al., 2018), in reproduction (Jesus et al., 2017, Lira et al., 2018, Brambila-Souza et al., 2019), nutrition (Bertolini et al., 2017) and for gamete and chromosome manipulation (de Bem et al., 2012, Yasui et al., 2015, Adamov et al., 2016, Nascimento et al., 2017, Santos et al., 2018).

A previous study showed through histological and hormonal analyses that lambari females kept in captivity were not able to naturally reach final oocyte maturation and

ovulation as well as the luteinizing hormone (LH) level did not change during the spawning season (Jesus et al., 2017), suggesting a reproductive dysfunction in this species. However, natural spawn occurred when females and male were grouped with (Chehade et al., 2015) or without (Brambila-Souza et al., 2019) environmental manipulation. Thus, the lambari intensive culture is dependent on supply of fry from natural reproduction grouping males and females in spawning ponds or, preferably, through artificial reproduction. Hypophysation, using carp pituitary extract (CPE), has been the most widely applied protocol of artificial reproduction for this species (Sato et al., 2006, de Bem et al., 2012, Nascimento et al., 2017 , Lira et al., 2018, Porto-Foresti et al., 2018). Moreover, the species seems to be also responsive to luteinizing hormone-releasing hormone (LHRH-gonadorelin) (Felizardo et al., 2012) and human chorionic gonadotropin (hCG) (Brambila-Souza et al., 2019). Recently we have tested salmon gonadotropin-releasing hormone analogue (sGnRHa) as a hormonal therapy in lambari females in different trials, and our results showed that even with a high dose of sGnRHa (1000 μg kg^{-1}) we could not achieve an improvement in reproductive performance compared to the negative control, suggesting this species may has a dopaminergic neuroendocrine control, that could be blocked through a dopamine receptor antagonist (unpublished data).

It is known that the gonadotropin-releasing hormone analogues (GnRHa) acts stimulating the synthesis and release of LH (Podhorec et al., 2011, 2012a, 2012b, 2016). LH, in turn, promotes a surge of 17α, 20β-dihydroxy-4-pregnen-3-one (17α,20β-P), which provokes final oocyte maturation (FOM) by membrane receptors and the ovulation by nuclear receptors in teleosts (Nagahma & Yamashita 2008, Podhorec et al., 2016). Additionally, the cyclooxygenases and prostaglandins (PGs) act as an important downstream factors stimulated by LH signaling and play an important role in mediating the action of LH on ovulation (Tang et al., 2016, 2017) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is known as the most potent ovulation inductor among the subtypes of PGs (Kagawa & Nagahama, 1981, Kagawa et al., 2003, Criscuolo-Urbinati et al., 2012, Joy & Singh, 2013). In the opposite dopamine has been shown to inhibit the release of basal and GnRHa-stimulated LH by binding to type D2 receptors located on the pituitary gonadotropic cells (Chang et al., 1990, Van der Kraak et al., 1998) in various species such as, *Carrassius auratus* (Peter et al., 1978), *Onchorhynchus mykiss* (Vacher et al., 2002), *Mugil cephalus* (Aizen et al., 2005), *Oreochromis mossambicus* (Jiang et al., 2016), *Astatotilapia burtoni* (Bryant et al., 2016). The discovery of the dopamine inhibitory action has important

implications for aquaculture, since environmental conditions in captivity generally lead to a block in the maturation and ovulation of oocytes (Dufour et al., 2005). Thus, a method of hormonal induction that combines a GnRH agonist and a D2-type dopamine receptor antagonist (e.g. pimozide, domperidone, metoclopramide) was proposed by two researchers Lin and Peter, known as Linpe method (Peter et al., 1988).

For reophilic tropical species the use of Linpe method resulted in controversial results and remaining unclear whether dopamine is involved in the neuroendocrine control of the reproduction of these species, since these studies did not compare the Linpe method with GnRHa alone. Besides that, some results showed a negative effect of this method in egg quality, so the assessment of whether the negative impact on egg results from the Linpe method per se and or from a direct negative effect of D2-type dopamine receptor antagonist is difficult. The use of Ovopel (mammalian GnRHa + metoclopramide) for tambaqui (*Colossoma macropomum*) had good results in reproductive performance with 100% of spawned females and good rates of fertilization (71.4%) and hatching (56.8%) (Souza et al., 2018), in contrast, using the same association for piauçu (*Leporinus macrocephalus*) 100% spawning was obtained, but without viable embryos (Pereira et al., 2016) and for jundiá (*Rhamdia quelen*), no female ovulated after treatment with the same product (Carneiro & Mikos, 2009). The spawning rate for tambaqui using Ovaprim (sGnRHa + domperidone) was 100%, but with low fertility and hatching rates (11.1% and 19.7%, respectively) (Acuña & Rangel, 2009).

Thus, the objectives of the present study were to assess the impact of [des-Gly^{10}, D-Ala^{6}]-luteinizing hormone-releasing hormone ethylamide analogue (LHRHa) treatment, with or without dopamine inhibitor (domperidone), on reproductive performance and on plasma levels of substances involved with final oocyte maturation and ovulation 17,20β-P and $PGF_{2\alpha}$, as well as histological evaluation of ovarian structures in two consecutives experiments. Firstly, we tested a broad range of LHRHa to find the dose with better responses, and then we carried out a second experiment associating this dose with domperidone aiming study whether the Linpe method could improve the reproductive parameters of lambari females.

2. Material and Methods

This study was conducted in agreement with the precepts of the National Council for the Control of Animal Experimentation (CONCEA) and was approved by the Animal Ethics and Welfare Committee from UNESP, Jaboticabal, SP, Brazil, under permission number 003838/17.

2.1.Animals and Maintenance

Mature *A. altiparanae* males and females (six months old) that were used in the Experiment 1 (Exp. 1) and in the Experiment 2 (Exp. 2) were reared in reared in 200 m^2 earthen ponds at a density of approximately 50 fish m^{-2} at Centro de Aquicultura da Unesp (CAUNESP) in Jaboticabal, São Paulo, Brazil (21°15'17''S, 48°19'20''W). The earthen ponds were supplied with a constant water flow between 15 and 20 L min^{-1} and the water temperature ranged from 24.1 to 25.9 ° C (mean ± SD = 24.95 ± 0.63 ° C) in the months of the both experiments. The fish were fed a commercial diet (36% crude protein) *ad libitum* twice a day.

The animals that composed both experiments were selected based on external aspects such as strong irrigation by blood vessels in the ventral region and soft abdomen, in females, as well as the presence of spinelets in the anal fin, in males, characteristics of individuals suitable for reproduction (Porto-Foresti et al., 2018). Then, the selected fish were transported to the laboratory, acclimatized and kept in 750 L boxes in a closed recirculation system before hormonal manipulation. During the experimental period in a closed system laboratory the water temperature, dissolved oxygen concentration and photoperiod were, respectively, at 26.7 ± 0.2 °C, 7.43 ± 0.21 mg L^{-1} and 12h : 12h light : dark, in both experiments.

2.2.Experimental Design

Two independent and consecutive experiments were carried out in the same year (2018), as reported below:

Experiment 1: We defined 6 mg of CPE kg^{-1} in fractionated dose as positive control (CPE), since it has been shown to be the most efficient hormonal induction protocol for this species (unpublished data). In the negative control (Control), fish were handled as in other treatments and the vehicle (0.9% saline solution) was applied. Five different concentrations of LHRHa [des-Gly^{10}, D-Ala^{6}]-LHRH Ethylamide (Syndel Laboratories Inc., Vancouver,

Canada) ranging from 20 to 1,620 μg kg^{-1} (denoted 20 LHRH, 60 LHRH, 180 LHRH, 540 LHRH and 1620 LHRH) a positive and a negative control were applied using three replicates (seven groups x three replicates). Each replicate was considered as an experimental unit. The experimental units were 10 L boxes with constant water flow and contained a 0.5 mm egg collecting net at the water outlet. Five females (16.36 ± 3.21 mg) and ten males (10.3 ± 1.08 mg) were placed inside each experimental unit. Therefore, each experimental unit was composed of 15 animals in the sexual proportion of 2 males: 1 female. Thus, we used a completely randomized experimental design with 7 groups x 3 replicates x 15 animals per replicate, totaling 315 animals (Table 1).

Experiment 2: In this experiment we also defined 6 mg of CPE kg^{-1} in fractionated dose as positive control (CPE) and the negative control (Control), which fish were handled as in other treatments and the vehicle (0.9% saline solution) was applied. The experimental groups were the dose of 180 μg de LHRH kg^{-1}, applied in the experiment 1, in association with 20 mg of domperidone kg^{-1} (180 LRHR + 20D) (Drori et al., 1994, Podhorec et al., 2012a, Ittzés et al., 2014, Cejko & Kucharczyk, 2015) or without association (180 LHRH). Therefore we defined one group that received just 20 mg of domperidone kg^{-1} (20 D). To resuspend domperidone propylene glycol was used (Chaube et al., 2014) and the final volume was adjusted with 0.9% saline solution. LHRHa and domperidone were applied at the same time in one injection. As we had less experimental groups in this experiment we could increase the number of replicates, thus the experimental groups were applied using four replicates (five groups x four replicates). Each replicate was considered as an experimental unit. The experimental units were 10 L boxes with constant water flow and contained a 0.5 mm egg collecting net at the water outlet. Five females (25.40 ± 4.53 mg) and ten males (17.25 ± 2.09 mg) were placed inside each experimental unit. Therefore, each experimental unit was composed of 15 animals in the sexual proportion of 2 males: 1 female. Thus, we used a completely randomized experimental design with 5 groups x 4 replicates x 15 animals per replicate, totaling 300 animals (Table 1).

2.3.General Methods

Females of all groups in both experiments received two intraperitoneal injections (one comprising 10% of the total dose administered at 11 *a.m.* and a second one 12 h later at 11 *p.m.* comprising the resolving dose) (Figure 1). Both experiments started at the time

the females received their first respective hormonal doses and were allocated to the experimental units at 11 *a.m.* (day zero, hour zero). The experiments lasted approximately 12 h after resolving dose, following the theoretical spawning period for the species (Felizardo et al., 2012, Porto-Foresti et al., 2018) (Figure 1). Three females (Exp. 1) and four females (Exp. 2) (the same number of replicates in each experiment) were randomly selected from the same lot before treatment to represent the baseline conditions (Initial) to assess the effects after treatments.

One spawned and one unspawned female from each replicate (confirmed by ovarian histology) were randomly chosen at the time of collection and submitted to ovarian stereological evaluation and had their plasma collected to determine levels of 17,20β-P (Jesus et al., 2017, Brambila-Souza et al., 2019) and $PGF_{2\alpha}$ (Figueiredo-Ariki, 2019). Males were grouped with females at the second dose of females and received a single dose of 3 mg CPE kg^{-1}. The volume injected (40 µL for each 10 g of fish) was the same for all groups.

Table 1. Experimental groups used in both experiments.

	Treatments	Denotation	Number of replicates*	Male : Female ratio/replicate	Total (n°)	
					Male	Female
Exp. 1	0.9% NaCl solution	Control	3	2:1	30	15
	Pituitary carp extract (6 mg kg^{-1})	CPE	3	2:1	30	15
	LHRHa (20 µg kg^{-1})	20 LHRH	3	2:1	30	15
	LHRHa (60 µg kg^{-1})	60 LHRH	3	2:1	30	15
	LHRHa (180 µg kg^{-1})	180 LHRH	3	2:1	30	15
	LHRHa (540 µg kg^{-1})	540 LHRH	3	2:1	30	15
	LHRHa (1620 µg kg^{-1})	1620 LHRH	3	2:1	30	15
Exp. 2	0.9% NaCl solution	Control	4	2:1	40	20
	Pituitary carp extract (6 mg kg^{-1})	CPE	4	2:1	40	20
	LHRHa (180 µg kg^{-1})	180 LHRH	4	2:1	40	20
	180 µg LHRHa kg^{-1} + 20 mg of Domperidone kg^{-1}	180 LHRH + 20 D	4	2:1	40	20
	Domperidone (20 mg kg^{-1})	20 D	4	2:1	40	20

***** Each replicate was considered an experimental unit. The experimental units were 10 L boxes with constant water flow and contained a 0.5 mm egg collecting net at the water outlet.

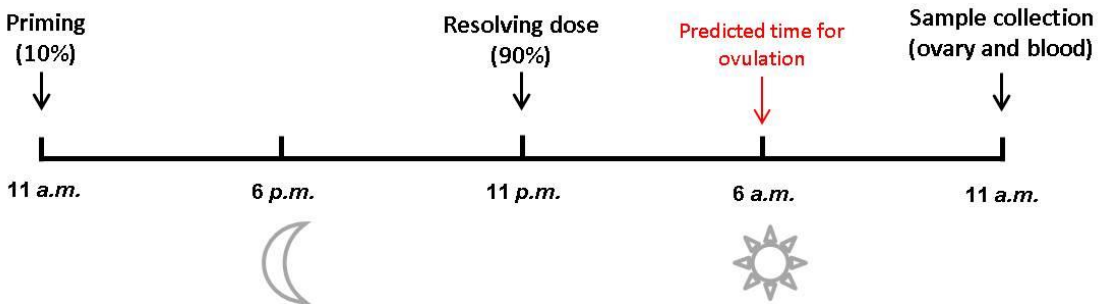

Figure 1. Timeline and experimental design for both experiments.

2.3.1. Reproductive Parameters

The experimental units were checked for ovulation every half hour as from 3 h post resolving dose until 12 h post resolving dose, and when eggs were observed in the collecting net another 30 min interval was waited to collect eggs and measure the spawning volume in a graduated cylinder. After that, a 10 mL aliquot of eggs from each experimental unit was allocated to individual 6.5 L incubators to determine fertility and hatching rates. The average temperatures conditions for egg incubation were 26.7 ± 0.2 °C for both experiments. Fertility rate (percentage of fertilized oocytes) was measured approximately 3 h (80 degree-hours) post-fertilization (PF) at the gastrula stage and hatching rate (percentage of larvae that hatched) was measured approximately 9 h PF (230 degree-hours) when caudal fin detachment was observed in each embryo prior to hatching. To obtain both parameters, 100 eggs per incubator were collected randomly, and egg counts were performed under a LEICA M50 stereomicroscope (LEICA Microsystems, Wetzlar, Germany).

The following parameters were recorded:

a) Latency period = hours between the resolving dose and first spawn observed in each replicate (at 26.7°C),
b) Replicates with spawned females = number of replicates with spawn/total number of replicates,
c) Spawning rate (%) = average or median percentage of spawned females per replicate,
d) Relative fecundity (mL g^{-1}) = total eggs volume/total sum of female body weight per replicate,

e) Fertility rate (%) = number of viable eggs x 100/100 (total number of collected eggs), and
f) Hatching rate (%) = number of embryo caudal fin detachment x 100/100 (total number of collected eggs).

2.3.2. Sampling procedure

For sample collection, fish were anesthetized with 100 mg L^{-1} of benzocaine (Sigma-Aldrich, Saint Louis, USA) approximately 12 h post resolving dose, and blood samples were collected from the caudal vein using heparinized syringes and needles. The collected blood was separated into aliquots (approximately 60 µL each), including one aliquot with 10 µM indomethacin to inhibit prostaglandin synthesis (Kuradomi & Batlouni, 2018). The plasma was separated via centrifugation at 3,000 rpm for 10 min at 4°C and frozen at -80°C for the subsequent 17,20β-P and $PGF_{2\alpha}$ assays. After blood collection, length (cm) and body mass (g) were recorded.

After this procedure, females were euthanized via a lethal dose of benzocaine (500 mg L^{-1}) and a section of the spinal cord at the level of the operculum. To remove the ovaries, a ventral incision was made in the caudal-cranial direction from the urogenital pore. The ovaries were removed, and a portion was sectioned and fixed in modified Karnovsky's solution (4% paraformaldehyde and 2% glutaraldehyde in Sorensen phospate buffer pH 7.2) for histological characterization. The fixed ovary samples were treated using routine histology techniques: 70% alcohol washes, serial dehydration, inclusion in historesin (Technovit 7100, Kulzer Histo-Technik), sectioned at 3 µm thickness, fixed on slides and stained with hematoxylin-eosin.

2.3.3. Plasma levels of 17,20β-P and $PGF_{2\alpha}$

The plasma levels of 17,20β-P and PGF2α were quantified by Enzyme Linked ImmunoSorbent Assay (ELISA) using commercial kits (Cayman Chemical Company, Ann Arbor, MI, USA) following the manufacturer's instructions. The readings of 17,20β-P and $PGF_{2\alpha}$ plates were performed at an absorbance at 412 and 405 nm, respectively, using an Epoch2 plate reader (BioteK Instruments, Inc., Highland Park, Winooski, USA), and all samples were read in duplicate. To validate the kits, intra- and interassays were performed, and we obtained the following variations: 0.164 to 9.650% and 4.580 to 18.262% for 17,20β-P, 1.000 to 11.229% and 2.124 to 16.062% for $PGF_{2\alpha}$.

2.3.4. Volume density

The histological slides of the ovaries were analyzed under a light microscope to identify the different types of structures observed, following Cassel et al. (2017). After this identification, an analysis of the volume density of the ovaries of a spawned female and a unspawned female per replicate from all treatments and four females before hormonal induction (total: Exp. 1 = 33 females and Exp. 2 = 29 females), using a light microscope and a grid of 352 points of intersection (Criscuolo-Urbinati et al., 2012, Pereira et al., 2016, Souza et al., 2020). The grid was applied and counted in three fields in the middle region of the ovary, totaling 1056 points for each female, with a 5x objective. The method of Criscuolo-Urbinati et al. (2012) was used with some modification. Points were classified according to histological descriptions of A. altiparanae by Cassel et al. (2017): previtellogenic (PV), cortical alveoli (CA), early vitellogenic with incomplete vitellogenesis and cytoplasm not filled with yolk (EV), final vitellogenic with cytoplasm filled with yolk (FV), mature vitellogenic oocyte with germinal vesicle breakdown (GVBD), atretic (AT), post-ovulatory follicles (POF), and interstitial tissue (IT). Artifacts were rarely observed, and points with artifacts were excluded from the total number of points used to obtain the percentages.

2.3.5. Statistical analysis

First, the assumptions of normality and homogeneity of the variances were tested using the Cramer-von Mises test and Levene's test, respectively. Parametric variables were analyzed using the variance test (one-way ANOVA) followed by the Tukey's test (for more than two groups) or by T test (for two groups). Kruskal-Wallis test was used for non-parametric variables followed by Dunn's test for multiple comparisons (for more than two groups) or by Mann-Whitney test (for two groups).

As females for each treatment were divided into spawned and unspawned groups to analyze the plasma levels of $PGF_{2\alpha}$ and 17,20β-P, as well as the volume density of ovaries, the statistical analysis were carried out only among treatments where ovulation was seen in more than one replicate.

3. Results

3.1.Reproductive parameters

3.1.1. Experiment 1

The first ovulation within replicates occurred between 4.5 and 8h after resolving dose (at 26.7°C), and no significant difference was found among groups ($p>0.05$) (data no shown). No ovulation was seen in 20 LHRH, while ovulation was seen only in one replicate in control and 60 LHRH and in the other groups ovulation was seen in all replicates (Table 2). Differences were detected among groups in relative fecundity, with the CPE, 180 LHRH, 540 LHRH and 1620 LHRH groups performing significantly better ($p<0.05$), though the LHRH-treated groups were similar to Control (Table 2). No significant differences were found among groups in spawning rate, fertility and hatching rates ($p>0.05$). However the highest doses of LHRH (540 LHRH and 1620 LHRH) resulted in a low mean of fertility (28.32 ± 47.05% and 50.61 ± 42.19%, respectively) and hatching (24.48 ± 40.68% and 41.82 ± 41.23%, respectively) rates with high deviation among replicates (Table 2), thus the selected dose for association with domperidone was 180 LHRH (Exp. 2).

3.1.2. Experiment 2

The first ovulation within replicates occurred between 4 and 9h after resolving dose (at 26.7°C), and no significant difference was found among groups ($p>0.05$) (data no shown). Nevertheless, collected females from 180 LHRH group were still releasing some eggs at the sampling time (12 hours after resolving dose). Ovulation was seen in all groups, with the CPE group showing higher spawning rate and relative fecundity than control and 20 D ($p<0.05$) (Table 2). The 180 LHRH and 180 LHRH + 20 D showed similar reproductive performance for all parameters analyzed (Table 2). No significant differences were found among groups in fertility and hatching rates ($p>0.05$) (Table 2).

Table 2. Reproductive performance of female *A. altiparanae* undergoing hormonal induction in two independents experiments.

Groups	Replicates with spawned females	Spawning rate (%)	Relative fecundity (mL g^{-1})	Fertility rate (%)	Hatching rate (%)
Experiment 1					
Control	1/3	0 (0, 60) [a]	0 (0, 0.39) [ab]	97.00	94.00
CPE	3/3	40 (40, 60) [a]	0.58 (0.53, 0.67) [a]	65.73 ± 16.27 [a]	64.34 ± 17.30 [a]
20 LHRH	0/3	0 (0, 0) [a]	0 (0, 0) [b]	-	-
60 LHRH	1/3	0 (0, 20) [a]	0 (0, 0.35) [ab]	93.97	84.44
180 LHRH	3/3	40 (40, 40) [a]	0.55 (0.39, 0.61) [a]	90.92 ± 5.19 [a]	87.32 ± 4.85 [a]
540 LHRH	3/3	40 (20, 60) [a]	0.16 (0.15, 0.89) [a]	28.32 ± 47.05 [a]	24.48 ± 40.68 [a]
1620 LHRH	3/3	20 (20, 60) [a]	0.62 (0.29, 0.87) [a]	50.61 ± 42.19 [a]	41.82 ± 41.23 [a]
Experiment 2					
Control	1/4	5 ± 10 [b]	0 (0, 0.34) [b]	87.37	85.84
CPE	4/4	60 ± 16.33 [a]	0.97 (0.53, 1.32) [a]	61.14 ± 14.72 [a]	57.09 ± 15.75 [a]
180 LHRH	3/4	35 ± 25.17 [ab]	0.62 (0.15, 0.79) [ab]	72.90 ± 26.28 [a]	58.96 ± 32.47 [a]
180 LHRH+20D	2/4	30 ± 38.3 [ab]	0.16 (0, 1.09) [ab]	80.32 ± 18.03 [a]	76.00 ± 12.73 [a]
20D	1/4	5 ± 10 [b]	0 (0, 0.36) [b]	64.00	59.80

Control saline solution, CPE carp pituitary extract, LHRH [des-Gly10, D-Ala6]-luteinizing hormone-releasing hormone ethylamide analogue. Data are expressed as mean ± SD or median (first quartile, third quartile). Groups from the same experiment, which shows the same letters, were not significantly different.

3.2.17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P)

3.2.1. Experiment 1

With the exception of 1620 LHRH group, all groups showed significantly increased 17,20β-P concentration after the ovulation time in spawned females compared to pre-treatment basal levels (Initial) ($p<0.05$) (Figure 2 A). Concerning to unspawned females only the CPE and 540 LHRH groups showed an increased 17,20β-P plasma levels compared to the Initial group ($p<0.05$) (Figure 2 B). However, the CPE-treated group showed similar levels compared to the other groups ($p>0.05$) (Figure 2 B).

Comparing spawned and unspawned females from the same treatment, with the exception of 1620 LHRH group, in all groups the spawned females showed a higher 17,20β-P concentration than unspawned females ($p<0.05$) (Figure 3).

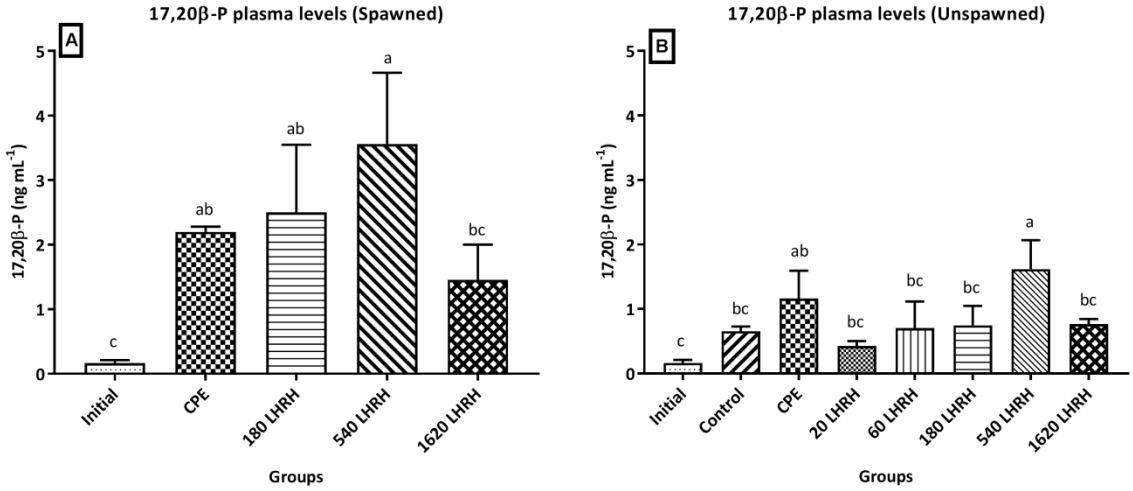

Figure 2. Experiment 1. 17,20β-P plasma levels of *A. altiparanae* females sampled 12 hours after the resolving dose injection. **Initial** refer to three females randomly selected before hormonal induction, which represent the baseline collection. One spawned and one unspawned female for each replicate were randomly chosen for this analysis. **(A)** Spawned females: Data are expressed as the means ± SD. Groups with the same letters were not significantly different according to the Tukey's test. **(B)** Unspawned females: Data are expressed as the means ± SD. Groups with the same letters were not significantly different according to the Tukey's test.

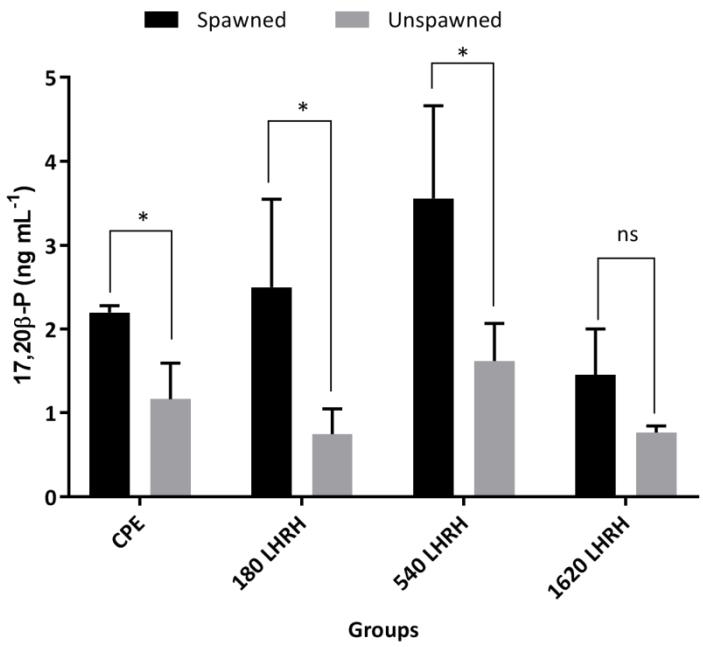

Figure 3. Experiment 1. 17,20β-P plasma levels of *A. altiparanae* females, comparing spawned and unspawned females from the same treatment by T test. Data are expressed as the means ± SD. Asterisk means significantly difference between spawned and unspawned females from the same treatment. * $p<0.05$, ns: non-significant.

3.2.2. Experiment 2

For spawned females the 180 LHRH was the only group that have showed a significantly increased 17,20β-P concentration compared to pre-treatment basal levels ($p<0.05$) (Figure 4 A). Concerning to unspawned females all groups showed an increased 17,20β-P plasma levels compared to the Initial group, with the highest levels for CPE and 180 LHRH groups ($p<0.05$) (Figure 4 B).

Regarding the differences observed between spawned and unspawned females within the same treatment, females from 180 LHRH and 180 LHRH + 20D groups showed a higher 17,20β-P concentration than unspawned females ($p<0.01$ and $p<0.05$) (Figure 5). In the CPE group spawned and unspawned females showed similar 17,20β-P levels ($p>0.05$) (Figure 5).

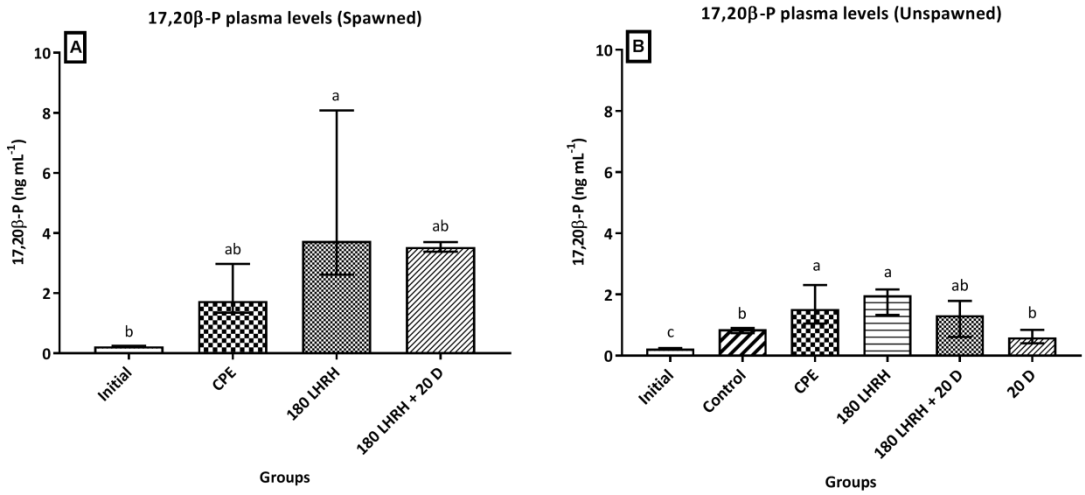

Figure 4. Experiment 2. 17,20β-P plasma levels of *A. altiparanae* females sampled 12 hours after the resolving dose injection. **Initial** refer to four females randomly selected before hormonal induction, which represent the baseline collection. One spawned and one unspawned female for each replicate were randomly chosen for this analysis. **(A) Spawned females:** Data are expressed as the medians and 5-95 percentiles. Groups with the same letters were not significantly different according to the Kruskal-Wallis test. **(B) Unspawned females:** Data are expressed as the medians and 5-95 percentiles. Groups with the same letters were not significantly different according to the Kruskal-Wallis test.

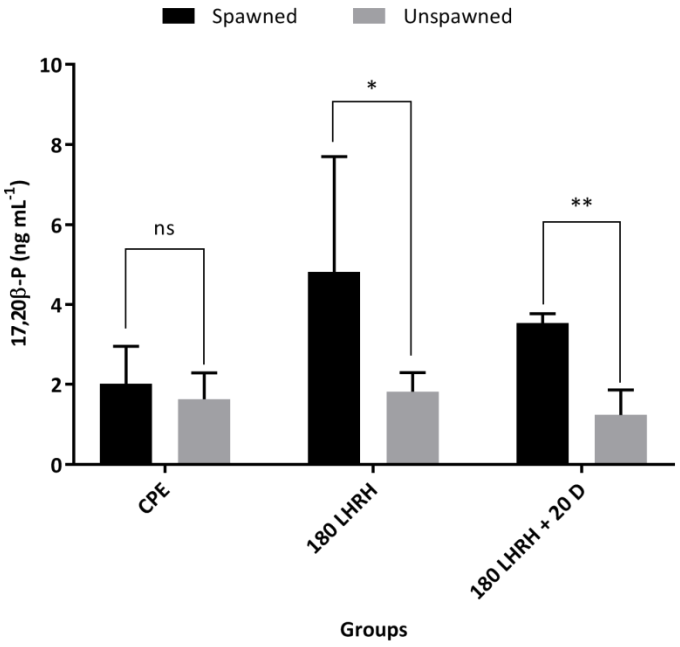

Figure 5. Experiment 2. 17,20β-P plasma levels of *A. altiparanae* females, comparing spawned and unspawned females from the same treatment by T test for CPE and 180 LHRH + 20 D groups and Mann-Whitney's test for 180 LHRH group. Data are expressed as the means ± SD. Asterisk means significantly difference between spawned and unspawned females from the same treatment. * $p<0.05$, ** $p<0.01$, ns: non-significant.

3.3.Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)

3.3.1. Experiment 1

There was no significantly difference in $PGF_{2\alpha}$ plasma level for spawned females ($p>0.05$) (Figure 6 A), while for unspawned females the increase in $PGF_{2\alpha}$ plasma level was associated with the CPE treatment, showing significantly increased $PGF_{2\alpha}$ concentration compared to pre-treatment basal levels and other groups, with exception for 180 LHRH ($p<0.05$) (Figure 6 B).

Regarding the differences observed between spawned and unspawned females within the same group, the unspawned females from 180 LHRH group showed higher $PGF_{2\alpha}$ levels than the spawned females ($p<0.05$) and the opposite was observed in 540 LHRH group with higher $PGF_{2\alpha}$ levels in spawned females ($p<0.05$) (Figure 7).

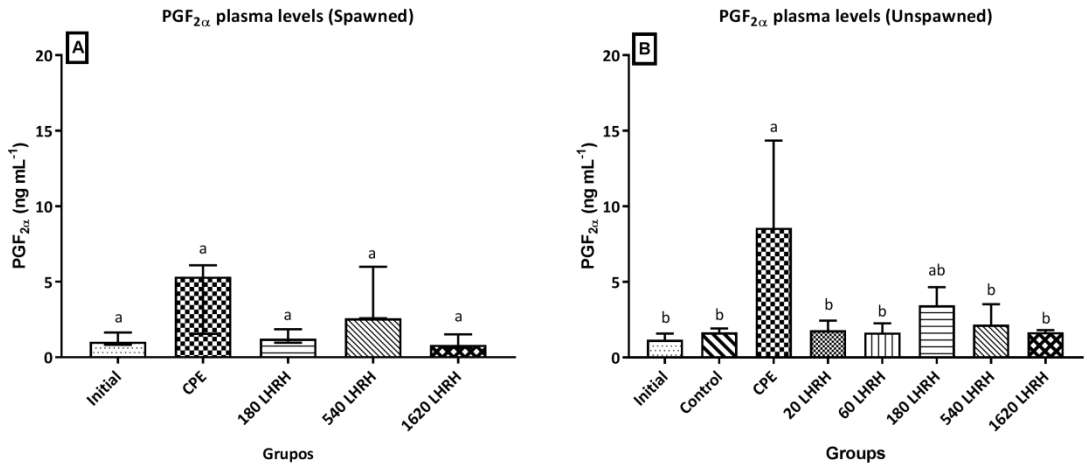

Figure 6. Experiment 1. $PGF_{2\alpha}$ plasma levels of *A. altiparanae* females sampled 12 hours after the resolving dose injection. **Initial** refer to three females randomly selected before hormonal induction, which represent the baseline collection. One spawned and one unspawned female for each replicate were randomly chosen for this analysis. **(A) Spawned females:** Data are expressed as the medians and 5-95 percentiles. Groups with the same letters were not significantly different according to the Kruskal-Wallis test **(B) Unspawned females:** Data are expressed as the means ± SD. Groups with the same letters were not significantly different according to the Tukey's test.

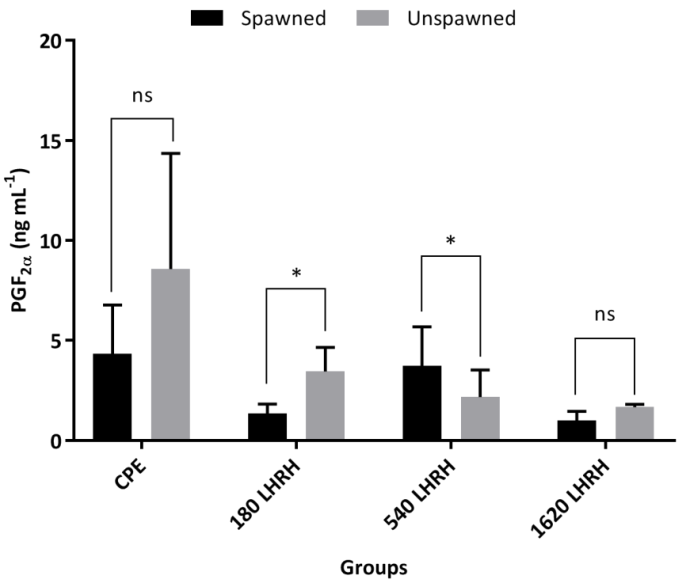

Figure 7. Experiment 1. $PGF_{2\alpha}$ plasma levels of *A. altiparanae* females, comparing spawned and unspawned females from the same treatment by T test for CPE, 180 LHRH and 1620 LHRH groups and Mann-Whitney's test for 540 LHRH group. Data are expressed as the means ± SD. Asterisk means significantly difference between spawned and unspawned females from the same treatment. * $p<0.05$, ns: non-significant.

3.3.2. Experiment 2

For spawned females the 180 LHRH was the only group that have showed a significantly increased $PGF_{2\alpha}$ plasma level compared to pre-treatment basal levels ($p<0.05$) (Figure 8 A). Concerning to unspawned females all groups showed a similar $PGF_{2\alpha}$ concentration compared to the Initial group ($p>0.05$) (Figure 8 B).

Regarding the differences observed between spawned and unspawned females within the same group, the spawned females from 180 LHRH group showed higher $PGF_{2\alpha}$ levels than the unspawned females ($p<0.05$) and the similar levels between spawned and unspawned females were observed in CPE and 180 LHRH + 20 D groups ($p>0.05$) (Figure 9).

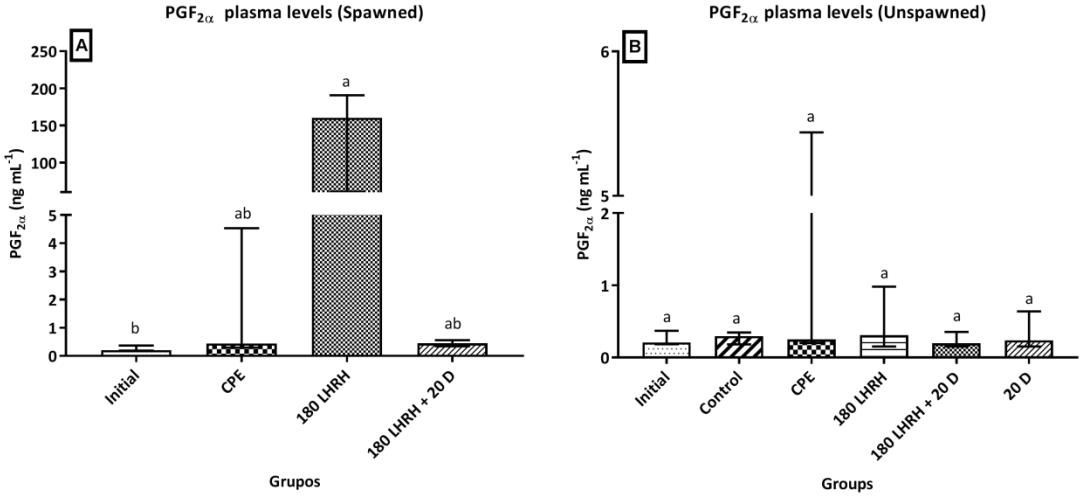

Figure 8. Experiment 2. $PGF_{2\alpha}$ plasma levels of *A. altiparanae* females sampled 12 hours after the resolving dose injection. **Initial** refer to four females randomly selected before hormonal induction, which represent the baseline collection. One spawned and one unspawned female for each replicate were randomly chosen for this analysis. **(A) Spawned females:** Data are expressed as the medians and 5-95 percentiles. Groups with the same letters were not significantly different according to the Kruskal-Wallis test **(B) Unspawned females:** Data are expressed as the medians and 5-95 percentiles. Groups with the same letters were not significantly different according to the Tukey's test.

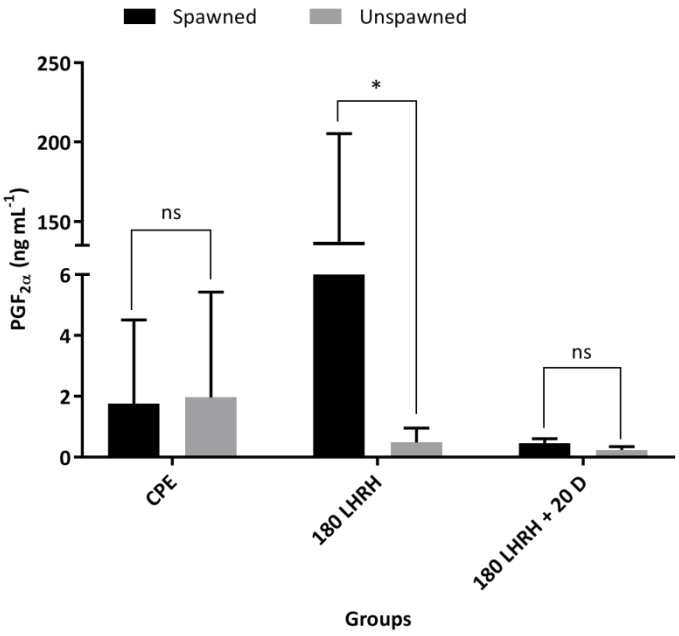

Figure 9. Experiment 2. $PGF_{2\alpha}$ plasma levels of *A. altiparanae* females, comparing spawned and unspawned females from the same treatment by T test for 180 LHRH + 20 D and by Mann-Whitney's test for CPE and 180 LHRH. Data are expressed as the means ± SD. Asterisk means significantly difference between spawned and unspawned females from the same treatment. * $p<0.05$, ns: non-significant.

3.4.Volume density

3.4.1. Experiment 1

Stereological evaluation revealed that ovaries from initial females were 88.26 ± 3.88% (mean ± SEM) composed by final vitellogenic stage oocytes (FV) (data not shown). Neither POF nor GVBD oocytes were observed in these females, which shows that these females were ready to spawn but had not ovulated at the beginning of the experiment onset. No differences among groups were found in volume density for both spawned and unspawned females ($p>0.05$) (Figure 10). GVBD oocytes retained in ovaries of unspawned females were only observed for CPE-treated group (1 of 3 females analyzed) (Figure 10 B).

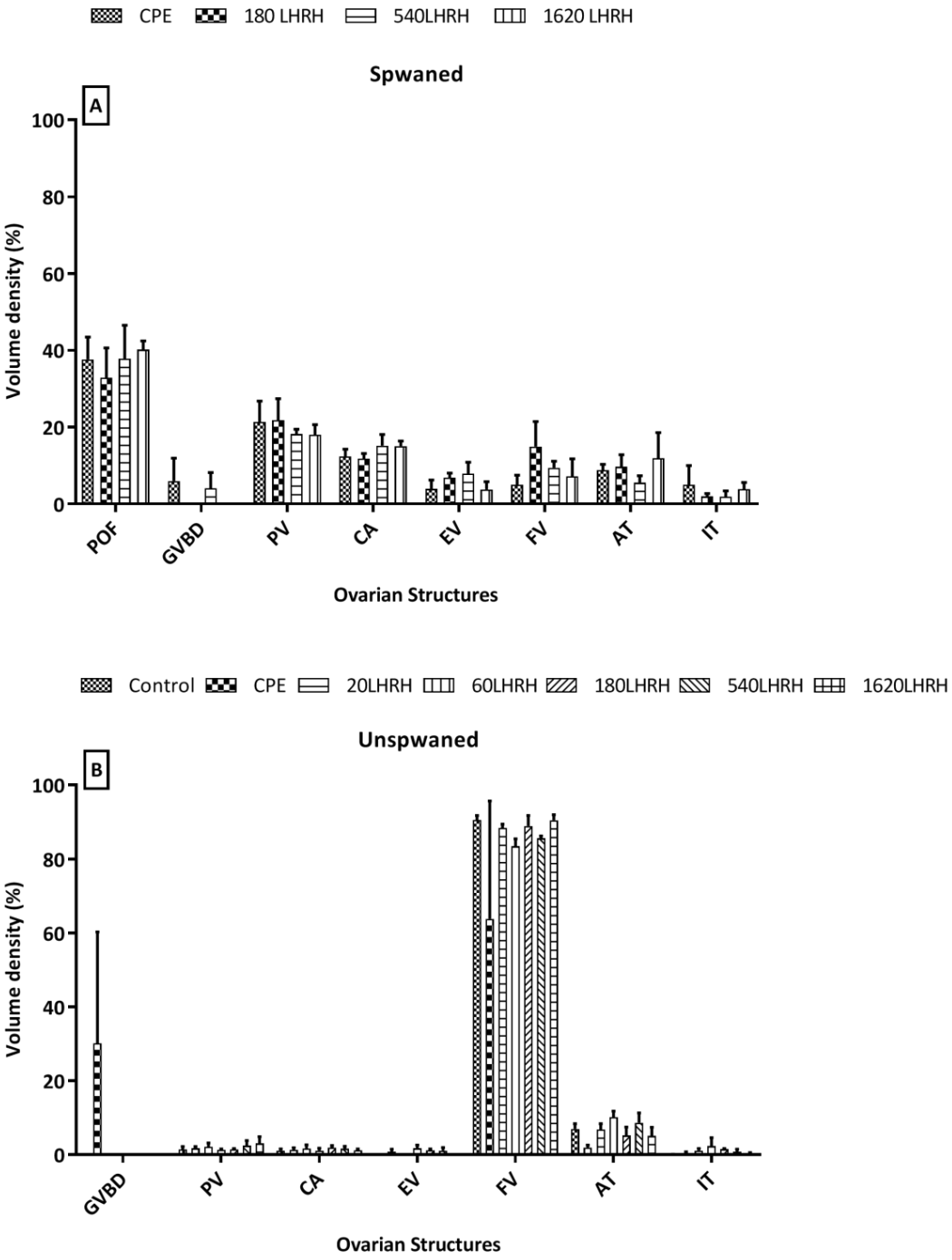

Figure 10. Experiment 1. Volume densities in percentage average values (±SEM) of different types of ovarian structures sampled 12 hours after the resolving dose injection. Asterisk indicates significant differences between treatments for each type of ovarian structure. **(A) Spawned females**: POF, PV, CA, EV, FV and AT were analyzed using the one-way ANOVA test. GVBD and IT were analyzed using Kruskal-Wallis. **(B) Unspawned females**: GVBD, PV, EV, FV and IT were analyzed using the Kruskal-Wallis test. CA and AT were analyzed using one-way ANOVA. **Ovarian structures: PV** previtellogenic, **CA** cortical alveoli, **EV** early vitellogenic, **FV** final vitellogenic, **GVBD** mature vitellogenic oocyte with germinal vesicle breakdown, **POF** postovulatory follicle, **AT** atretic and **IT** interstitial tissue.

3.4.2. Experiment 2

Stereological evaluation revealed that ovaries from initial females were 87.85 ± 1.88% (mean ± SEM) composed by final vitellogenic stage oocytes (FV) (data not shown). Neither POF nor GVBD oocytes were observed in these females, which shows that these females were ready to spawn but had not ovulated at the beginning of the experiment onset.

No significant differences were observed among spawned females volume density of different oocyte types ($p>0.05$) (Figure 11 A). For unspawned females, GVBD (64.32 ± 19.93%) and FV (25.55 ± 20.54%) were higher and lower, respectively, in females for CPE-treated females comparing to other groups ($p<0.01$ and $p<0.05$) (Figure 11 B). GVBD oocytes retained in ovaries of unspawned females were only observed for CPE-treated group (4 of 4 females analyzed) (Figure 11 B).

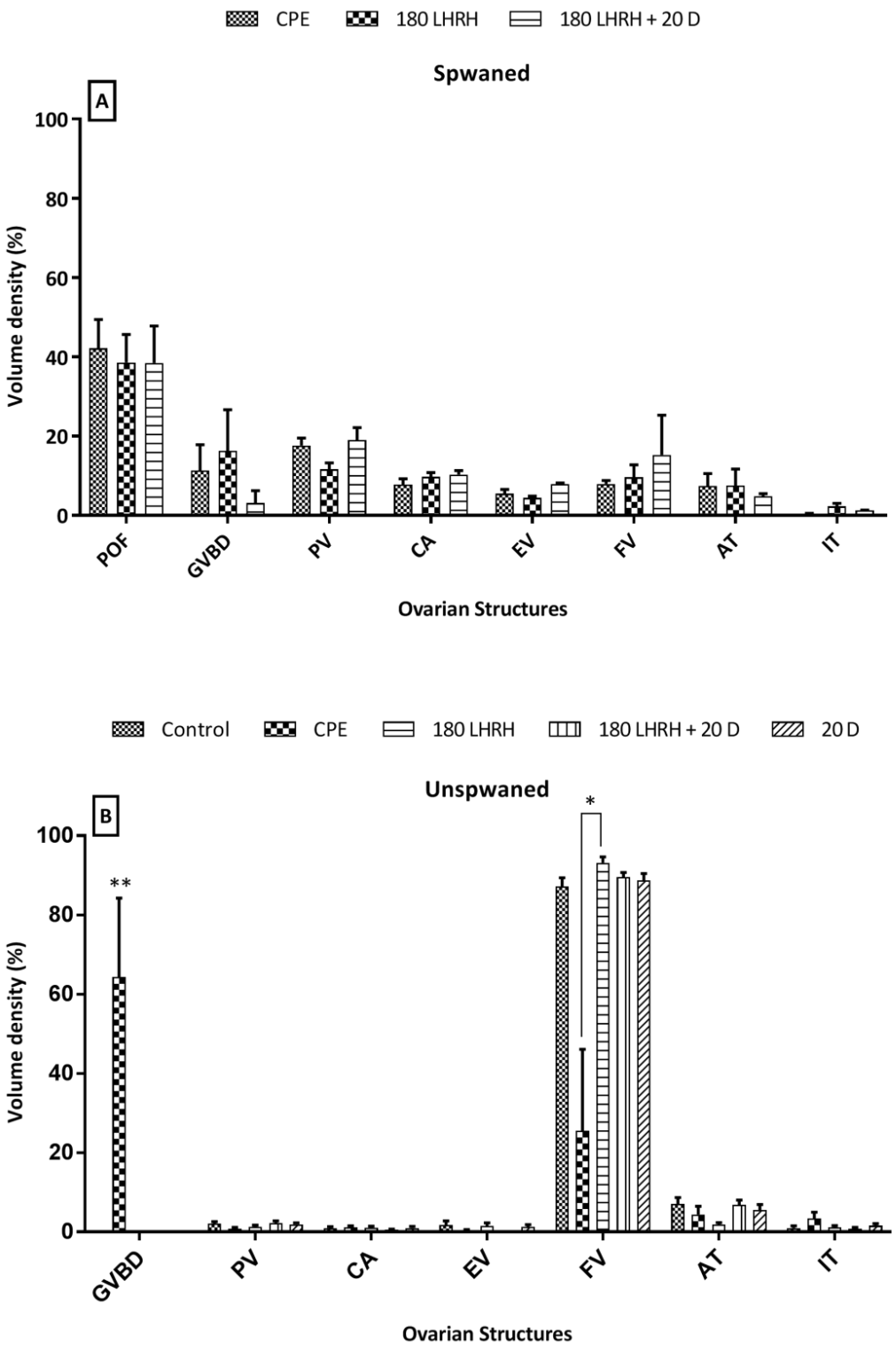

Figure 11. Experiment 2. Volume densities in percentage average values (±SEM) of different types of ovarian structures sampled 12 hours after the resolving dose injection. Asterisk indicates significant differences between treatments for each type of ovarian structure (* $p<0.05$, ** $p<0.01$). **(A) Spawned females**: POF, AT and IT were analyzed using the one-way ANOVA test. GVBD, PV, CA, EV and FV were analyzed using Kruskal-Wallis. **(B) Unspawned females**: GVBD, EV, FV, AT and IT were analyzed using the Kruskal-Wallis test. PV and CA were analyzed using one-way ANOVA. **Ovarian structures: PV** previtellogenic, **CA** cortical alveoli, **EV** early vitellogenic, **FV** final vitellogenic, **GVBD** mature vitellogenic oocyte with germinal vesicle breakdown, **POF** postovulatory follicle, **AT** atretic and **IT** interstitial tissue.

4. Discussion

In this study, we showed for the first time that the application of Linpe method did not improve the reproductive parameters of lambari females. This affirmation is supported by the similar reproductive performance between 180 LHRH and 180 LHRH + 20 D in the Exp.2. A possible explanation for that is the similar stimulation of 17,20β-P and $PGF_{2\alpha}$ release observed in both treatments. The ability of LHRHa alone to induce ovulation in lambari is similar to many species that lack dopamine inhibition of LH release, as in the Atlantic croaker (*Micropogonoias undulates*) (Copeland & Thomas, 1989), sea bream (*Sparus auratus*) (Zohar et al., 1995), sea bass (*Dicentrarchus labrax*) (Mateos et al., 2002) and tench (*Tinca tinca*) (Podhorec et al., 2016). However, the inhibitory role of dopamine was also confirmed in other fish species such as in trout (*Onchorhynchus mykiss*) (Vacher et al., 2002), European ell (*Anguilla anguilla*) (Dufour et al., 2005), grey mullet (*Mugil cephalus*) (Aizen et al., 2005), a catfish (*Heteropneustes fossilis*) (Chaube et al., 2014), Mozambique tilapia (*Oreochromis mossambicus*) (Jiang et al., 2016), and a cichlid fish (*Astatotilapia burtoni*) (Bryant et al., 2016). These results showed that the effect of dopamine receptor antagonists is species-specific and must be investigated in different species.

Although there were no significant differences in reproductive parameters in both experiments, CPE-treated group showed slightly better reproductive performance, because all LHRHa-treated groups performed similarly to the control in both experiments. Therefore, the hypophysation remains the most adequate hormonal therapy to induce final oocyte maturation and ovulation in lambari of the protocols we tested. Females from the group that received only domperidone injections (20 D) have spawned, which could suggest that lambari has a dopaminergic neuroendocrine control, however females from control also spawned and there was no difference between these groups in any of the variables analyzed. The injections handlings and the grouping of males and females at the spawning boxes provoked ovulation in both experiments. This fact corroborates previous studies that show spontaneous spawning in lambari (Chehade et al., 2015, Brambila-Souza et al., 2019). Thus, it was possible that spawning in 20 D was just due to grouping of couples in the experimental units, similar to negative control.

Many studies reported that the role of dopamine in the neuroendocrine control of reproduction is observed, mainly in Cyprinidae (Drori et al., 1994, Cejko and Kucharczyk,

2015). However, for tench (*Tinca tinca*), a species from this family, a low dose of GnRHa without a dopamine receptor antagonist (1 µg kg^{-1}) was efficient in inducing a gradual increase in LH with an ovulation rate of 63% (Podhorec et al., 2011). Other studies with this species indicated that both treatments with and without a dopamine inhibitor association cause an increase in LH plasma levels and high ovulation rates, however GnRHa alone induces gradual LH release, whereas with the inhibitor is demonstrated an immediate LH release peak right after induction (Pohdorec et al., 2012a, Pohdorec et al., 2012b, Pohdorec et al., 2016). This immediate surge may be associated with the deleterious effects on eggs, interfering in fertilization and hatching rates (Pohdorec et al., 2016). In our study, we did not evaluate LH levels, but considering the gonadal steroid levels evaluated (17,20β-P), we have an indirect evidence of excessive levels of LH. In our results, the dose of 540 LHRH stimulated a higher production of 17,20β-P in both spawned and unspawned females, however it did not improve reproductive performance. In addition, it appeared to impair the viability of the eggs, suggesting this LHRHa dose (540 µg kg^{-1}) triggered an overstimulation of pituitary in producing LH, which can potentially affect the quality of eggs (Mateos et al., 2002). In the same context, the highest dose of LHRHa (1620 µg kg^{-1}) also impaired the egg viability, however as we only performed blood samplings at before injection and 12 h post resolving dose, we cannot reject the possibility that a surge in 17,20β-P plasma levels has occurred at another time post-injection. It could be supported by the greater reduction of 17,20β-P plasma levels in 1620 LHRH than other groups measured 12 h post resolving dose. Another hypothesis is 17,20β-P levels in 1620 LHRH did not increase as much as in 540 LHRH due to a negative feedback caused by an overstimulation by hormone treatment, since the impairment in eggs viability was not as evident as in 540 LHRH. Thus, these hypotheses will be considered in the future, but as in the present study the priority was to observe differences on reproductive parameters, we did not want to stress the fish with multiple samplings over time.

Still in that concerning, unspawned females from 540 LHRH and CPE (Exp. 1) and 180 LHRH and CPE (Exp. 2) showed similar 17,20β-P plasma levels. Females from CPE-treated group had unovulated oocytes in GVBD stage, while in 540 LHRH and 180 LHRH all the oocytes had an intact germinal vesicle after spawn period. The association between GVBD and surge in 17,20β-P plasma levels is expected, since it is known that 17,20β-P is the main maturation-inducing hormone (MIH) in lambari (Jesus et al., 2017, Brambila-

Souza et al., 2019) and during the process of final oocyte maturation the 17,20β-P binds to oocyte membrane-specific receptors to active the maturation promoting factor (cdc2-kinase and clicline-B) in the ooplasm that triggers the disintegration of the germinal vesicle and the resumption of meiosis (Nagahama & Yamashita, 2008, Lubzens et al., 2010, Yaron & Levavi-Sivan, 2011). It explains at least the CPE-treated group observations, which a surge of 17,20β-P was associated with reinitiates meiosis (GVBD), however the LHRHa did not induce completion of final oocyte maturation in unspawned females even after an increase of 17,20β-P levels. Earlier studies suggested that, in order to acquire oocyte maturational competence (OMC), in addition to inducing follicular MIH production, LH also induces the sensitivity of the oocyte to MIH (Patiño et al., 2001). This sensitivity is the induction and activation of MIH membrane receptors, through the transfer of cAMP from granulosa cells (GC) to the oocyte through the heterologous gap junctions (communication between GC and oocyte) (Patiño et al., 2003). These observations bring us the reflection about the sensitivity of oocytes to MIH in LHRHa treatments, because we cannot assess whether the LH released was enough to induce all the process of OMC in unspawned females. Therefore, future studies should be carried out in order to quantify the release of LH and the activity of MIH membrane receptors to understand more deeply the processes of final oocyte maturation in lambari. Further, MIH production begins only after an advanced stage of germinal vesicle migration (peripheral position) (Levavi-Zermonky & Yaron, 1986). In our study, the maturation process was evaluated based on nuclear integrity and we did not analyze its position and migration, which prevents us from concluding whether the increase in 17,20β-P levels in females that did not have GVBD occurred due to the migration of the nucleus. In addition, this makes us question whether the latency period determined by the literature (Felizardo et al., 2012, Porto-Foresti et al., 2018) was sufficient for the females of the groups with LHRH to complete the OMC process. Future work with this species should take into consideration the analysis of the nucleus migration over time.

We can conclude here that the spawning failure with any dose of LHRHa was associated with failure to induce final oocyte maturation (FOM), unlike what was observed in the CPE group, in which it was associated with failure in ovulation (at least in 5 of 7 females analyzed). The specific type of failure, that there is FOM but not ovulation, is quite common and well described in CPE, such as in matrinxã (*Brycon amazonicus*) (Hainfellner et al., 2012), piau (*Leporinus friderici*) (Souza et al., 2020), piapara

(*Leporinus elongatus*) (Pereira et al., 2018) and piaçu, (*Leporinus macrocephalus*) (Pereira et al., 2016). It suggests that the spawning success depends of additional physiological and behavioral mechanisms (Knight & Van Der Kraak, 2015), such as expression of nuclear progesterone receptor (nPR), prostaglandins receptors and prostaglandin release (Takahashi et al., 2013) as well as activation of promoter region of matrix metalloproteinase-15 (mmp15), a protease involved in follicle rupture (Hagiwara et al., 2020). On the other hand, we did not find in the literature previous specific reports in any tropical reophilic species describing the failure observed in LHRHa-treated groups, but in our previous study using sGnRHa as a hormonal therapy also in lambari, we could observe the same pattern of reproductive failure (unpublished data).

The increase in 17,20β-P levels may explain much of the ovulation of females, with the exception of 1620 LHRH (Exp. 1) and CPE (Exp. 2) (groups that presented similar levels between spawned and unspawned females). The reason that similar levels of 17,20β-P induce ovulation in only a few females' remains unknown and needs to be further studied in future work. In addition, the high plasma level of $PGF_{2\alpha}$ in 180 LHRH found in the Exp. 2 comparing with in Exp. 1 was probably due the occurrence of ovulating females at the moment of blood collection in Exp. 2. It is known that PGs have a fundamental role in the mechanism of ovulation (Hagiwara et al., 2020) and their synthesis in non-mammalian vertebrates occurs during spontaneous or artificially-induced ovulation (Takahashi et al., 2013). In medaka, an important biological model, PGE_2 induces the ovulation by binding to the its receptor (EP4b) just before the time of ovulation and the PGE2/EP4b signaling is required for fish ovulation at the time that follicle rupture occurs (Takahashi et al., 2013). Moreover, in zebrafish, ovarian $PGF_{2\alpha}$ levels were higher close to ovulation (Knight & Van der Kraak, 2015, Lister & Van der Kraak, 2008). Thus, it is expected that the PGs plasma levels be greater at the moment of ovulation than in females that spawned previously, and that in order to study the role of PGF2α in lambari ovulation, we need to carry out future work with collections over time to take samples at the time of ovulation of all experimental groups.

The increase in 17,20β-P and $PGF_{2\alpha}$ plasma levels was slightly more significant in the CPE-treated group in both experiments, showing an association between the release of both substances and CPE. A model for ovulation process in medaka was proposed, in which the LH surge simultaneously stimulates both the production of 17,20β-P and the expression of nPR, via the activation of LH receptors on the granulosa cells (GC)

(Takahashi et al., 2013). nPR binds to 17,20β-P and acts as a critical transcription factor for EP4b (*ptger4b* - a PGE_2 receptor) gene expression. The interaction EP4b/PGE_2 evokes an intracellular signal transduction reaction in GC that eventually leads to ovulation (Takahashi et al., 2013). In zebrafish both nPR and *ptger4b* are downstream factor of LH signaling required for ovulation (Tang et al., 2016). According to these studies we can clearly see the relationship between these three substances (LH – 17,20β-P – PGs) in the process of final oocyte maturation and or ovulation, and that the substance that initiates this cascade of events is LH. As in hypophysation, using CPE, LH is exogenous and acts directly at the level of the gonad and in LHRHa the action is at the level of pituitary to induce the release of endogenous LH stores, which, in turn act at the level of the gonad to induce steroidogenesis and the process of final oocyte maturation and ovulation (Mylonas et al., 2010), so in treatments with LHRHa the action of hormone therapy it is at higher levels of the axis and the fish itself needs to be physiologically prepared to be able to produce and release LH. Thereby, our results suggest that the LH available in CPE was able to activate this axis in the vast majority of the injected females, unlike the LHRHa-treated groups, where not all the injected females were physiologically capable of producing LH and consequently inducing the 17,20β-P and $PGF_{2\alpha}$ release. Despite that, a large percentage of females in the CPE group did not ovulate (60% - Exp. 1, 40% - Exp. 2), even with similar levels of $PGF_{2\alpha}$ to the females that spawned. This same observation was reported for pacu (*Piaractus mesopotamicus*), which for unknown reasons, some females with similar levels of $PGF_{2\alpha}$ to the females who successfully ovulated, demonstrated failures in ovulation (Kuradomi & Batlouni, 2018). Suggesting that, as for pacu, in lambari the $PGF_{2\alpha}$ levels do not fully explain the success or failure in spawning.

In conclusion, the association between LHRHa and domperidone did not improve the reproductive parameters in lambari females, at least in the dose and inhibitor tested here. Moreover, the use of very high concentrations of LHRHa (540 and 1620 μg kg^{-1}) did not result in better reproductive performance and it seems to impair egg quality, reducing fertility and hatching rates. This observation suggests that these doses may have triggered an overstimulation of pituitary in producing LH, which can potentially affect the quality of eggs. Among all treatments tested here in two experiments, we suggest the use of conventional hypophysation to induce final oocyte maturation and ovulation in lambari. Our suggestion is based on the fact that the CPE group was the only one that showed superior reproductive performance than the negative control group (Control), and in

addition, it was the only one that stimulated final oocyte maturation in practically all of the injected females.

5. References

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4. Considerações finais

Apesar do uso de análogos de GnRH como indutores na reprodução de peixes nativos brasileiros ser estudado desde a década de 80, se analisarmos o cenário atual brasileiro percebemos que as informações sobre a utilização dessas substâncias para as nossas espécies são extremamente escassas e pouco conclusivas. O método mais utilizado no setor produtivo e nos centros de pesquisa continua sendo a hipofisação, mesmo com todas as problemáticas e poucos avanços dessa técnica. Essas problemáticas incluem a imprevisibilidade de uma reprodução bem sucedida, a variabilidade do conteúdo de gonadotropina das hipófises, o potencial de transmissão de doenças para as matrizes, o alto valor de mercado e a falta de autorização da ANVISA para compra e comercialização das hipófises. O desenvolvimento de novos protocolos, que apresentem consistência e reprodutibilidade, é necessário para assegurar o crescimento do setor produtivo. No entanto, estes estudos demandam uma série de experimentos, de tempo e de análises para entender de que forma as novas substâncias e doses testadas atuam nas espécies. No desenvolvimento deste projeto, percebemos a imensidão de pesquisas que precisam ser realizadas para entender as particularidades do lambari e desenvolver o uso de hormônios sintéticos para as espécies nativas.

O que pudemos perceber durante o desenrolar do projeto é que a literatura sobre a reprodução de lambari é escassa e que o paradigma de que a espécie reproduz facilmente deveria ser repensada e melhor analisada por nós. Nos primeiros experimentos testamos uma ampla faixa de doses de [D-Arg^{6}, Pro^{9}, NEt]–sGnRHa e o efeito da associação deste hormônio com domperidona (inibidor da dopamina) (dado não apresentado na tese). Inicialmente optamos pela administração em dose única, devido aos trabalhos já publicados com o lambari, que estimulam a utilização deste protocolo. No entanto, quando analisamos individualmente as fêmeas pós-desova nos surpreendemos com a baixa taxa de desova que encontrávamos, quando aplicado tanto o sGnRHa quanto o protocolo comumente utilizado pelos piscicultores e nos centros de pesquisa (dose única de CPE). Nas pisciculturas essa taxa de desova provavelmente não é percebida, pois é mascarada pela alta fecundidade das fêmeas e pelo aglomerado de fêmeas nos tanques de desova. Porém, compreendemos que essa taxa poderia ser melhorada e deveríamos testar outros protocolos. Com estes resultados, decidimos aumentar a dose de sGnRHa e utilizar doses fracionadas. Com o fracionamento da dose de CPE a taxa de desova teve um incremento de trêz vezes (de 20% para 60%), em contrapartida, utilizando uma dose consideravelmente

alta de sGnRHa (1000 µg kg^{-1}) apenas obtivemos um ganho sutil nos parâmetros reprodutivos, porém não o suficiente para que este tratamento fosse superior estatisticamente ao controle negativo (solução salina). Com isso, decidimos associar a domperidona com a dose de 1000 µg sGnRHa kg^{-1} (experimento não demonstrado na tese), a qual não resultou em melhoras no desempenho reprodutivo e nos fez perceber que, provavelmente, o lambari não seja tão responsivo ao sGnRHa e que deveríamos testar outro hormônio sintético.

Nesse contexto, os experimentos seguintes foram realizados com o LHRH ([des-Gly^{10}, D-Ala^{6}]-LHRH), o qual demonstrou ser mais potente para a espécie, pois requer dose menor do hormônio (180 µg kg^{-1}) para ter desempenho reprodutivo similar ao CPE. Mesmo assim, as doses que testamos de LHRH não apresentram resultados superiores aos do controle negativo. Associando o LHRH com domperidona, também, não obtivemos resultados superiores, sugerindo que a dopamina parece não apresentar um papel no controle neuroendócrino da reprodução dessa espécie. No entanto, outros inibidores dos receptores da dopamina devem ser testados para responder esta pergunta.

Com relação aos efeitos dos hormônios sintéticos testados e do CPE na morfologia dos ovários pós-desova, percebemos que o CPE induz maturação final (GVBD) em praticamente todas as fêmeas injetadas e os sintéticos não induzem maturação final nas fêmeas que não ovulam. A partir disso, acreditamos que futuros trabalhos devem ser feitos levando em conta os processos de maturação final, bem como os de ovulação, como, por exemplo, ativação de receptores de membrana e nuclear de DHP, quantificação de LH no plasma e expressão gênica de *lhβ* nas hipófises de lambari, entre outros.

Com relação às desovas naturais que acontecem com lambari, podemos sugerir que elas ocorrem por um aumento nos níveis de DHP, o qual pode ser por meio do cortisol. No entanto, essa hipótese também deveria ser testada, através da quantificação de cortisol no plasma.

Acreditamos que os resultados aqui mostrados são de importância tanto para a comunidade científica quanto para o setor produtivo e abrem novas perguntas que poderiam ser inseridas em futuros trabalhos.