UNIVERSIDADE ESTADUAL PAULISTA – UNESP

CENTRO DE AQUICULTURA DA UNESP

Novas investigações sobre o desempenho reprodutivo de pacu (*Piaractus mesopotamicus*) envolvendo variações no uso de prostaglandina $F_{2\alpha}$ exógena e no horário de indução hormonal

Rafael Tomoda Sato

Jaboticabal, São Paulo
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Sumário

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RESUMO GERAL

Ao longo da última década desenvolvemos um protocolo de hipofisação associado a prostaglandina $F_{2\alpha}$ ($PGF_{2\alpha}$) que aumentou a previsibilidade na desova do pacu, mas ainda assim, falhas na ovulação persistem em algumas fêmeas tratadas. Por isso, esta tese teve como objetivo principal aprimorar este protocolo, investigando dois novos aspectos: a) a associação entre ovulação bem-sucedida com o momento de aplicação e a concentração de $PGF_{2\alpha}$ sintética (manuscrito 1); e b) a associação entre ovulação bem-sucedida com o horário de aplicação da segunda dose hormonal, considerando especialmente os ritmos circadianos de melatonina (MTN) na espécie (manuscrito 2). Os manuscritos foram compostos por dois experimentos cada; e testes - piloto não relatados. No experimento 1 (manuscrito 1), 24 fêmeas foram hipofisadas e receberam 2 mL/peixe de $PGF_{2\alpha}$ sintética no momento da segunda dose (2D); ou cinco horas (5H) ou sete horas (7H) após a segunda dose da hipofisação. No experimento 2 (manuscrito 1), as fêmeas foram induzidas com cinco doses de $PGF_{2\alpha}$ variando de 1,0 a 7,0 mL/kg aplicadas no momento da segunda dose da hipofisação. O manuscrito 2, sobre ritmos circadianos de MTN, teve o experimento 1 composto por três grupos: controle (sem indução); controle salino (indução com solução salina); e hipofisação. Neste experimento, os animais tiveram o sangue coletado a cada quatro horas, ao longo de 24 horas antes de ovulação (7h dia um até 7h dia 2), para quantificação dos níveis plasmáticos de MTN. No experimento 2 (manuscrito 2), com base nos níveis circadianos de MTN, o desempenho reprodutivo com indução hormonal iniciada às 19 horas (19H) e 0 horas (0H) foi comparado. Observamos no primeiro manuscrito, sobre variações no momento de aplicação da $PGF_{2\alpha}$ exógena, uma taxa de desova de 66,7% nos grupos 2D e 5H e de 50% no grupo 7H. Além disso, apenas o grupo 2D não apresentou desovas ruins (fecundidade <35.000 ovócitos/kg peixe). No experimento 2, sobre doses de $PGF_{2\alpha}$ exógena, as doses mais elevadas (5,5 e 7,0 mL/kg) foram as únicas que propiciaram 100% de desova, porém a viabilidade embrionária nestes grupos não foi satisfatória. No segundo manuscrito, sobre MTN, seus níveis foram similares ao longo de 24 horas entre os grupos controle e hipofisados. Quando analisados independentemente do tratamento, observamos que os níveis de MTN aumentam no início da noite às 19 horas do dia 1 e diminuem às 15 horas do dia 2. No experimento 2, as taxas de ovulação foram de 75% e 50% para os grupos 19H e 0H, respectivamente. O grupo 19H não apresentou desovas ruins. Além disso, o grupo (19H) obteve uma produção total de larvas (números absolutos) de 737.567, enquanto o grupo (0H) obteve 250.762. Portanto, nossas observações com o primeiro manuscrito não confirmam a especulação teórica que, aplicando $PGF_{2\alpha}$ mais próximo da ovulação e/ou elevando sua dose, mitigariam as falhas de desova ainda recorrentes com o protocolo em uso (estabelecido por Criscuolo-Urbinati et al., (2012)). Porém, em ambos experimentos, as fortes correlações positivas entre níveis de $PGF_{2\alpha}$ no momento da ovulação e fecundidade indicam que investigações neste sentido devem ser continuadas. No segundo manuscrito, apesar do desempenho reprodutivo similar entre os grupos, a produção de larvas superior do grupo 19H indica que fêmeas submetidas a um maior período escuro pós-segunda dose possam apresentar maior sucesso na ovulação e na viabilidade embrionária. Portanto, este estudo consolida o uso da $PGF_{2\alpha}$ exógena como ferramenta indutora da ovulação do pacu e traz fortes indícios iniciais, que precisam ser aprofundados, mostrando que a MTN tem associação importante com a ovulação da espécie e o início do período noturno é o mais adequado para indução hormonal, entre os períodos por nós testados.

Palavras-chave: falhas na ovulação, fotoperíodo, hipofisação, peixes migradores, prostaglandina, ritmos circadianos.

ABSTRACT

Over the last decade we have developed a pituitary protocol associated with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) that increased the predictability of pacu spawning, but even so, failures in ovulation persist in some treated females. Therefore, this thesis had as main objective to improve this protocol, investigating two new aspects: a) the association between successful ovulation with the moment of application and the concentration of synthetic $PGF_{2\alpha}$ (manuscript 1); and b) the association between successful ovulation and the time of application of the second hormonal dose, especially considering the melatonin circadian rhythms (MTN) in the species (manuscript 2). The manuscripts were composed of two experiments each; and tests - pilot not reported. In experiment 1 (manuscript 1), 24 females were hypophysed and received 2 mL/fish of synthetic $PGF_{2\alpha}$ at the time of the second dose (2D); either five hours (5H) or seven hours (7H) after the resolving dose of hypophysation. In experiment 2 (manuscript 1), females were induced with five doses of $PGF_{2\alpha}$ ranging from 1.0 to 7.0 mL/kg applied at the time of the resolving dose of hypophysation. Manuscript 2, about circadian rhythms of MTN, had experiment 1 composed of three groups: control (without induction); saline control (induction with saline solution); and hypophysation. In this experiment, the animals had their blood collected every four hours, over the 24 hours before ovulation (7h day one to 7h day 2), to quantify the plasma levels of MTN. In experiment 2 (manuscript 2), based on circadian levels of MTN, reproductive performance with hormone induction starting at 7 pm (19H) and midnight (0H) was compared. In the first manuscript, on variations in the moment of application of exogenous $PGF_{2\alpha}$, a spawning rate of 66.7% in the 2D and 5H groups and of 50% in the 7H group. Furthermore, only the 2D group did not have poor spawning (fecundity <35,000 oocytes/kg fish). In experiment 2, on exogenous $PGF_{2\alpha}$ doses, the highest doses (5.5 and 7.0 mL/kg) were the only ones that provided 100% spawning, but embryonic viability in these groups was not satisfactory. In the second manuscript, on MTN, its levels were similar over 24 hours between the control and pituitary groups. When analyzed independently of treatment, we observed that MTN levels increase in the early evening at 7 pm on day 1 and decrease at 3 pm on day 2. In experiment 2, ovulation rates were 75% and 50% for groups 19H and 0H, respectively. The 19H group did not present bad spawns. Furthermore, the group (19H) obtained a total production of larvae (absolute numbers) of 737,567, while the group (0H) obtained 250,762. Therefore, our observations with the first manuscript do not confirm the theoretical speculation that, applying $PGF_{2\alpha}$ closer to ovulation and/or increasing its dose, would mitigate the still recurrent spawning failures with the protocol in use (established by Criscuolo-Urbinati et al., (2012)). However, in both experiments, the strong positive correlations between $PGF_{2\alpha}$ levels at the time of ovulation and fertility indicate that investigations in this direction should be continued. In the second manuscript, despite the similar reproductive performance between the groups, the higher larval production of the 19H group indicates that females subjected to a longer post-resolving dose dark period may be more successful in ovulation and embryonic viability. Therefore, this study consolidates the use of exogenous $PGF_{2\alpha}$ as a tool to induce pacu ovulation and provides strong initial evidence, which needs to be deepened, showing that MTN has an important association with the ovulation of the species and the beginning of the nocturnal period is the most appropriate for hormonal induction, between the periods we tested.

Keywords: ovulation failures, photoperiod, hypophysation, migratory fish, prostaglandin, circadian rhythms.

INTRODUÇÃO GERAL

1. Características gerais da espécie-alvo

O pacu (*Piaractus mesopotamicus*) é um caracídeo neotropical onívoro, nativo das bacias dos rios Paraná, Paraguai e Uruguai. Esta espécie possui características zootécnicas favoráveis como resistência a doenças e baixas temperaturas, boa aceitação pelo consumidor e rápido crescimento em várias condições de cultivo (Jomori et al., 2003; Souza et al., 2003; Gelman et al., 2004; Moro et al., 2013; Kuradomi et al., 2017; Mourad et al., 2018; Urbinati e Takahashi, 2020), e por essas razões, possui destacada importância comercial no continente americano, e mais recentemente, no mundo (Gelman et al., 2004; Valladão et al., 2018). No Brasil, existem mais de 14.500 estabelecimentos que cultivam o pacu, distribuídos principalmente nas regiões Sul, Sudeste e Centro-

Oeste, geralmente cultivados em viveiros escavados ou em tanques-rede (Moro et al., 2013; Peixe BR, 2020). Vários aspectos da produção de pacu já foram estabelecidos, como as exigências nutricionais (Abimorad & Carneiro, 2007; Abimorad et al., 2008; Honorato et al., 2016), manejo da larvicultura (Jomori et al., 2003, 2008; Leitão et al., 2011; Portella et al., 2014) e fisiologia (Biller-Takahashi et al., 2015; Takahashi et al., 2017; Marinho de Mello et al., 2019; Zanuzzo et al., 2019). O abate geralmente ocorre com um ano de idade, quando os animais têm cerca de 1 kg (Silva et al., 1997; Souza et al., 2003; Reis Neto et al., 2012; Mourad et al., 2018).

Esta espécie pertence a um grupo de peixes comercialmente conhecido como 'peixes redondos' (pacu; tambaqui (*Colossoma macropomum*); e pirapitinga, (*Piaractus brachypomus*)), bem como seus híbridos interespecíficos (tambacu, tambaqui x pacu; patinga, pacu x pirapitinga; e tambatinga, tambaqui x pirapitinga), que formam o segundo grupo de peixes mais produzidos no Brasil, representando aproximadamente 27% da produção aquícola brasileira em 2021 (IBGE, 2022). Neste mesmo ano, a produção brasileira atingiu 841 mil toneladas (Peixe BR, 2022). Além disso, as exportações deste grupo de peixes subiram expressivamente de 2019 para 2020, crescendo aproximadamente 650% para o tambaqui e 410% para o pacu (Peixe BR, 2021).

Como normalmente ocorre em peixes reofílicos (migração no período de reprodução), o ciclo reprodutivo do pacu é sazonal e a desova é do tipo total (Schorer et al., 2016; Urbinati e Takahashi, 2020). A diferenciação de suas gônadas em ovários se inicia aos 150 dias (Barbosa et al., 2022). A primeira maturação é atingida com aproximadamente 3 anos e 34 cm de comprimento total (Ferraz de Lima et al., 1984), se mantendo apta a reprodução pelo menos até os 10 anos (Schorer et al., 2016). Sua época de reprodução vai de outubro a março na América do Sul (Schorer et al., 2016; Urbinati e Takahashi, 2020).

2. Questões atuais sobre a indução hormonal de peixes migradores tropicais

Na natureza, fatores ambientais como aumento das chuvas, da temperatura e do fotoperíodo (estação das chuvas) estimulam os peixes reofílicos a migrarem em direção à cabeceira dos rios para reprodução, fenômeno conhecido como piracema (Carolsfeld et.al., 2003; Lima et al., 2013). Paralelamente, ocorre a liberação de hormônios gonadotrópicos e produção de esteroides sexuais, responsáveis pela maturação gonadal e desova (Hainfellner et al., 2012a; 2019; Kuradomi et al., 2017). Em cativeiro, mesmo não sendo capazes de realizar a migração, estas espécies apresentam maturidade gonadal (vitelogênese) (Schorer et al., 2016; Kuradomi et al., 2017), porém, necessitam ser induzidas hormonalmente para atingir a ovulação (Mylonas et al., 2010; Borella et al., 2020; Kuradomi & Batlouni, 2018; Sato et al., 2020).

A técnica de indução mais comumente aplicada para peixes reofílicos de importância comercial é a hipofisação, que consiste na aplicação de hormônios gonadotrópicos por meio do extrato bruto de hipófise de peixes doadores maduros, comumente carpas (*Cyprinus carpio*) (EBHC), em peixes receptores, com o objetivo de atingir a maturação final dos ovócitos e ovulação (Zaniboni-Filho e Weingartner, 2007). Os primeiros trabalhos de hipofisação foram desenvolvidos na década de 30, paralelamente com Houssay na Argentina (1930) (Houssay, 1930) e Von Ihering no Brasil (1934) (Von Ihering e de Azevedo, 1934). Para o pacu, os protocolos de hipofisação iniciais foram estabelecidos com extrato de hipófise de salmão (Castagnolli & Donaldson, 1981) e de carpa (Godinho & Godinho, 1986). Protocolos de hipofisação foram usados e aprimorados por décadas e hoje em dia são os mais utilizados e eficientes para espécies migradoras tropicais, inclusive o pacu (Criscuolo-Urbinati et al., 2012; Hainfellner et al., 2012b; Ittzés et al., 2015; Pereira et al., 2017; 2018; Kuradomi e Batlouni, 2018; Borella et al., 2020; de Souza et al., 2020; Sato et al., 2020; Roza de Abreu et al., 2020; 2021).

Em contrapartida, em diversas outras espécies, o uso da hipofisação vem sendo substituído por produtos sintéticos (Mylonas et al., 2010). O uso de hormônios sintéticos em espécies migradoras tropicais, principalmente o hormônio liberador de gonadotropinas (GnRH), apresenta resultados negativos (Paulino et al., 2011; Pereira et al., 2017; 2018; Roza de Abreu et al., 2020; 2021) ou positivos (Viveiros et al., 2015; Souza et al. 2018). O principal problema encontrado ainda é a mortalidade embrionária mesmo quando doses convencionais de hormônios sintéticos são usadas (Pereira et al., 2017; 2018;

de Souza et al., 2020), como também, a ausência de indução a maturação final e ovulação (Roza de Abreu et al., 2020; 2021). Deste modo, devido aos resultados ainda inconsistentes, deve-se considerar, a curto e médio prazo, a necessidade da continuidade e aprimoramento do uso do EBHC para a produção de espécies migradoras tropicais, uma vez que sua produção é praticamente toda obtida por meio desta técnica.

3. Endocrinologia na maturação final dos ovócitos e ovulação em peixes hipofisados

O EBHC utilizado na hipofisação, além dos diversos hormônios hipofisários, contém o hormônio luteinizante (LH) (Aizen et al., 2012), o qual possui receptores nos ovários que, quando ativados, desencadeiam uma cascata de eventos (mediados por esteroides gonadais), culminando na maturação final dos ovócitos (retomada da meiose, condensação cromossômica e migração e quebra da vesícula germinativa (GVBD)) e ovulação (Nagahama e Yamashita, 2008; Lubzens et al., 2010; Tokarz et al., 2015). A maturação final dos ovócitos é regulada por hormônios indutores, conhecidos como “MIS” (*maturation-inducing steroids*), sendo o 17α-20β-dihidroxi-4-pregnen-3-one (DHP), o indutor mais comum encontrado em teleósteos (Nagahama e Yamashita, 2008; Tokarz et al., 2015; Takahashi et al., 2018). Durante este processo destaca-se a respectiva redução e aumento dos níveis plasmáticos de 17β-estradiol (E_2) e DHP, um evento denominado de “*steroidogenic shift*” (Levavi-Zermonsky e Yaron, 1986; Tokarz et al., 2015; Sato et al., 2020). O DHP é sintetizado a partir da conversão do esteroide 17α-hidroxiprogesterona por meio da ação da enzima 20β-hidroxiesteroide desidrogenase (20β-HSD), cuja síntese é controlada pelo LH (Nagahama e Yamashita, 2008; Tokarz et al., 2015) (Figura 1).

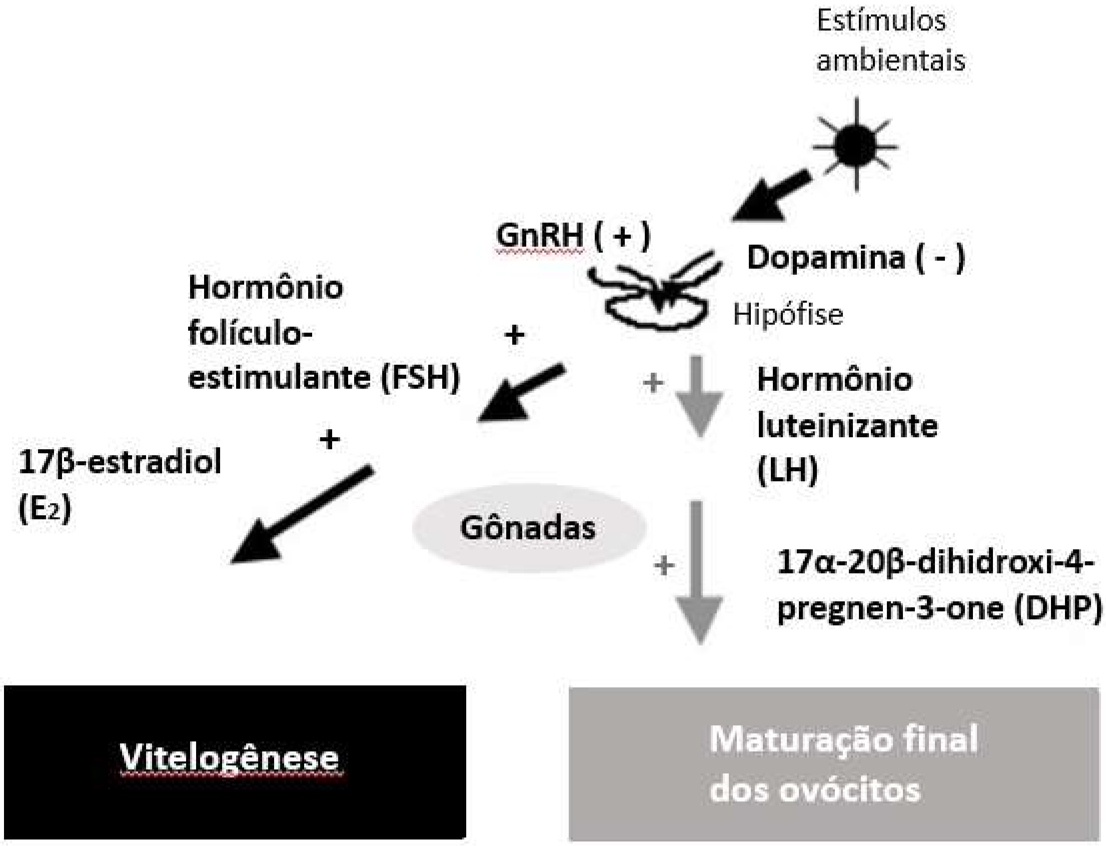

Figura 1. Esquema adaptado de Mylonas et al. (2010) do eixo hipotálamo-hipófise-gônadas em peixes. GnRH, hormônio liberador de gonadotropina. Fonte: Mylonas et al., 2010.

Após a maturação final ocorre o processo de ovulação (ruptura folicular), no qual as conexões entre o ovócito e folículo devem ser rompidas, liberando os ovócitos na cavidade ovariana ou na cavidade abdominal, dependendo da espécie (Lubzens et al., 2010). Neste processo destacam-se as prostaglandinas como eficientes indutores da ovulação em vários teleósteos (Jalabert e Szollosi, 1975; Stacey e Pandey, 1975; Fujimori et al., 2011 e 2012; Takahashi et al., 2013; Knight e Van der Kraak, 2015; Tang et al., 2017; Kuradomi e Batlouni, 2018). As prostaglandinas são eicosanoides sintetizadas a partir do ácido araquidônico (ácidos graxos de 20 carbonos da família ômega-6) por meio de cascata enzimática que se inicia com sua clivagem dos fosfolipídios de membrana pela ação da enzima fosfolipase A_2. Em seguida, as enzimas prostaglandina-endoperóxido-sintase-1 (Ptgs1) e prostaglandina-endoperóxido-sintase-2 (Ptgs2), também conhecidas como ciclooxigenases-1 e -2 (COX-1 e -2), atuam na oxidação do ácido araquidônico, formando a prostaglandina H_2, que posteriormente será convertida em vários subtipos de prostaglandinas (Fujimori et al., 2011 e 2012; Ogiwara e Takahashi, 2016; Tang et al., 2017; Takahashi et al., 2018) (Figura 2). Destes subtipos sintetizados, a prostaglandina $F_{2\alpha}$ ($PGF_{2\alpha}$) (Jalabert e Szollosi, 1975; Kagawa et al., 2003; Lister e Van Der Kraak, 2008; 2009; Joy e Singh, 2013; Knight e Van Der Kraak, 2015; Kuradomi e Batlouni, 2018) e a prostaglandina E_2 (PGE_2) (Fujimori et al., 2011 e 2012; Ogiwara e Takahashi, 2016; Tang et al., 2017) são os principais subtipos relacionadas à ovulação (Takahashi et al., 2018). Desta forma, a ação e eficiência das distintas prostaglandinas parece ser espécie-específica (Takahashi et al., 2018), demandando estudos particulares nas espécies de interesse, como no caso do pacu.

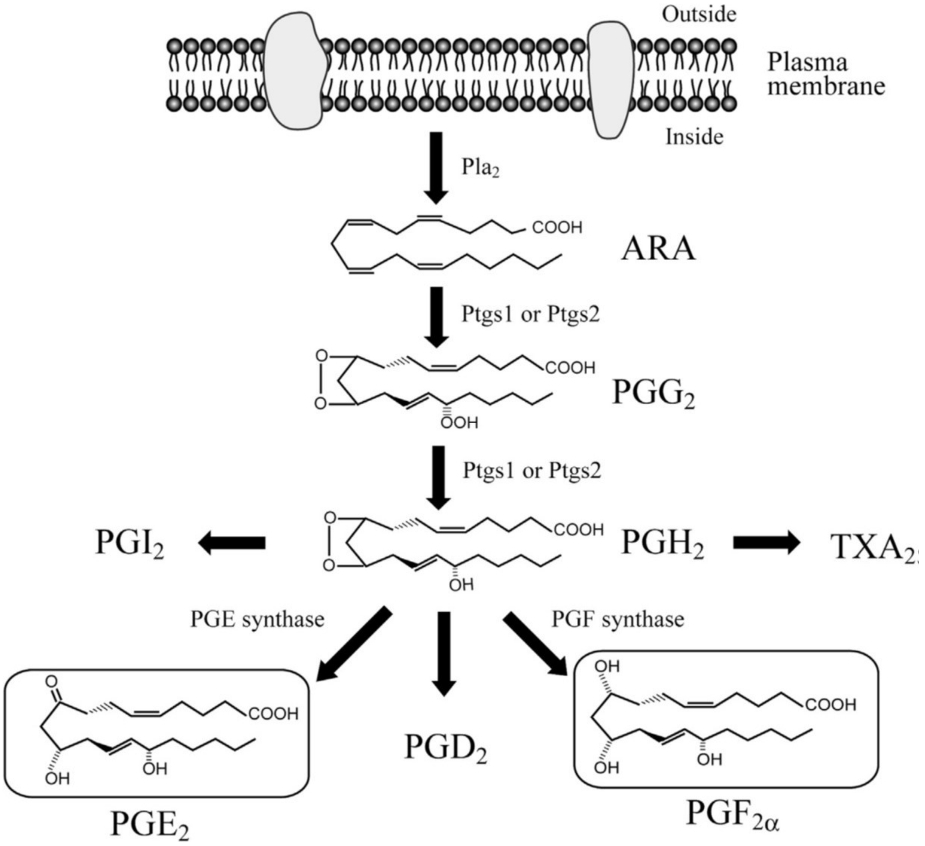

Figura 2. Biossíntese das prostaglandinas. ARA, ácido araquidônico; PGG2, prostaglandina G2; PGH2, prostaglandina H2; PGI2, prostaglandina I2; TXA2, tromboxanos A2; PGD2, prostaglandina D2; PGE_2, prostaglandina E2; PGF2α, prostaglandina $F_{2\alpha}$; Pla_2, fosfolipase A_2; Ptgs 1 ou 2, prostaglandina-endoperóxido-sintase-1 ou 2. Fonte: Takahashi et al., 2018.

Neste contexto, um padrão sequencial de eventos precedentes à ovulação, que envolve inicialmente um aumento nos níveis de DHP e em seguida, uma ação das prostaglandinas, foi descrito tanto para o pacu (Sato et al., 2020), como para outras espécies de peixes estudadas (Takahashi et al., 2018). Em pacu, Sato et al. (2020) observaram pico plasmático de $PGF_{2\alpha}$ próximo a desova, precedido por um pico plasmático de DHP na retomada da meiose. O estudo com perca amarela (*Perca flavescens*), demonstrou que o DHP estimula a ovulação *in vitro* aumentando concomitantemente os níveis de $PGF_{2\alpha}$ e PGE_2 (Berndtson et al., 1989; Goetz, 1997). Para a enguia japonesa (*Anguilla japonica*), o DHP induz ovulação *in vitro* por meio da síntese de prostaglandinas em células foliculares (Kagawa et al., 2003). No zebrafish (*Danio rerio*), observou-se que, em tecidos ovarianos, o DHP estimula um aumento na expressão gênica da enzima Ptgs2 (Knight & Van Der Kraak, 2015).

Essas funções coordenadas entre o DHP e as prostaglandinas, bem como seus respectivos receptores, foram, mais recentemente, explorados em algumas espécies modelo. A expressão do receptor de PGE_2, EP4b (*ptger4b*), mostrou ser regulada por um mecanismo envolvendo um receptor nuclear de progestina (Pgr) em medaka (*Oryzias latipes*) (Hagiwara et al., 2014; Takahashi et al., 2018) e em zebrafish (Tang et al., 2017). Em medaka, há evidências para a ligação do DHP com o receptor Pgr e subsequente associação com a região promotora de o gene *ptger4b,* o qual será transcrito e expresso (Hagiwara et al., 2014). Ainda neste contexto, nesses mesmos estudos (Hagiwara et al., 2014; Tang et al., 2017), os autores demonstraram que a síntese de Pgr é estimulada pelo LH, via proteína quinase A (PKA), ou seja, o LH proveniente da hipofisação regula tanto a maturação final dos ovócitos (estimula a produção de DHP), como a ovulação (participa da síntese do receptor de prostaglandinas e, em algumas espécies, da própria prostaglandina (Tang et al., 2017)) (Figura 3).

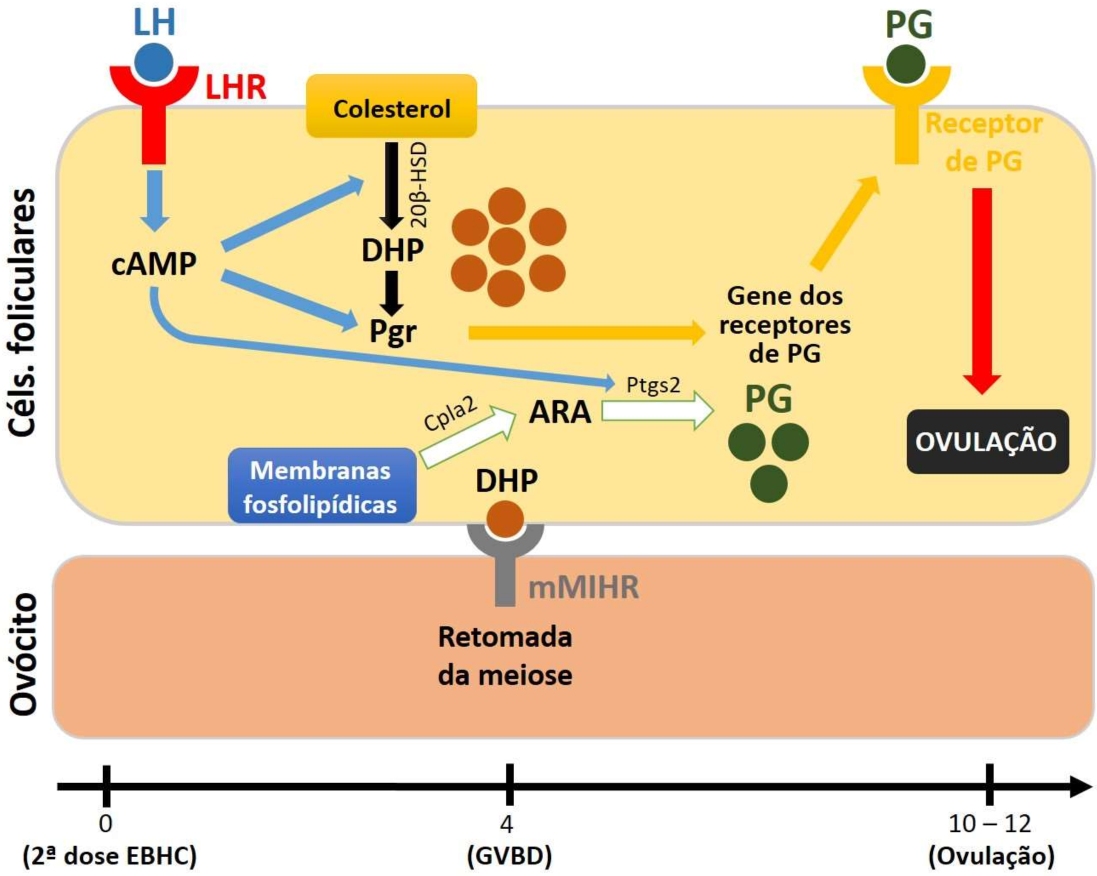

Figura 3. Esquema compilado da maturação final dos ovócitos e ovulação na hipofisação em células foliculares e ovócito (Nagahama e Yamashita, 2008; Hagiwara et al., 2014; Tang et al., 2017; Takahashi et al., 2018; Sato et al., 2020). LH: hormônio luteinizante; cAMP: adenosina monofostato cíclico; DHP: 17α-20β-dihidroxi-4-pregnen-3-one; mMIHR: receptor de membrana de hormônios indutores de maturação; 20β-HSD: 20β-hidroxiesteroide-desidrogenase; Pgr: receptor de progestina; PG: prostaglandina; Ptgs2: prostaglandina-endoperóxido-sintase-2; Cpla2: fosfolipase A2 citosólica. Fonte: próprio autor.

4. Falhas na ovulação em fêmeas hipofisadas

Contudo, apesar de apresentar resultados mais consistentes, a hipofisação também apresenta problemas, geralmente relacionados a imprevisibilidade da ovulação bem-sucedida, principalmente em pacu, tornando uma das principais limitações para a exploração de todo o seu potencial (Criscuolo-Urbinati et al., 2012; Kuradomi e Batlouni, 2018). Essas disfunções reprodutivas causam enormes prejuízos ao setor produtivo e consequentemente a cadeia produtiva dos peixes, pois muitas horas de trabalho são perdidas, hormônios são desperdiçados e geralmente o número de larvas não é suficiente para povoar os tanques previamente preparados para a larvicultura (Zohar & Mylonas, 2001; Lubzens et al., 2010; Mylonas et al., 2010), um passo que requer muitas horas de trabalho e insumos, além de ser fundamental para se obter formas jovens para a fase de crescimento (Jomori et al., 2003). Neste contexto, o domínio reprodutivo do pacu ou de qualquer espécie é necessário e imperativo, uma vez que falhas na ovulação de fêmeas injetadas geralmente levam à morte das matrizes, impossibilitando a manutenção de um plantel apropriado e a realização de cruzamentos dirigidos para obtenção de famílias melhoradas, como no caso do pacu, no qual Mastrochirico-filho et al. (2019) alcançaram um plantel resistente a bactéria *Aeromonas hidrophila.*

Por estas razões, este tema têm sido alvo de pesquisas do Laboratório de reprodução de peixes do CAUNESP (Centro de Aquicultura da UNESP) ao longo das últimas décadas. Neste cenário, estudos desenvolvidos em nosso laboratório mostraram que as falhas observadas são decorrentes do processo de indução hormonal, o qual é capaz de provocar a migração da vesícula germinativa e GVBD (maturação final), porém é ineficaz em induzir a ovulação em algumas fêmeas de diversas espécies migradoras tropicais como o matrinxã (*Brycon amazonicus*) (Hainfellner et al., 2012b), o piau (*Leporinus friderici*) (de Souza et al., 2020), a piapara (*Leporinus elongatus*) (Pereira et al., 2018), piauçu (*Leporinus macrocephalus*) (Pereira et al., 2017), o lambari *(Astyanax altiparanae*) (Roza de Abreu et al., 2020 e 2022) e o pacu (Criscuolo-Urbinati et al., 2012; Kuradomi e Batlouni, 2018). Este tipo de falha também foi demonstrado no estudo de Jalabert et al. (1977), em que fêmeas de carpas induzidas a um mesmo tratamento hormonal que não desovaram, apresentavam, assim como em nossos trabalhos, ovócitos GVBD retidos nos ovários.

5. Indução à hipofisação associado com PGF$_{2\alpha}$ sintética e suas perspectivas

Conhecendo o potencial das prostaglandinas para induzir a ovulação e tentando mitigar suas falhas no pacu, em 2012 publicamos um estudo no qual mostramos que o uso de PGF$_{2\alpha}$ (2 mL de Ciosin® por peixe contendo 0,25 mg/mL de cloprostenol sódico) concomitante com a segunda dose da hipofisação convencional elevou a taxa de ovulação do pacu, com consequente aumento da frequência de folículos pós-ovulatórios (Criscuolo-Urbinati et al., 2012). Porém, posteriormente (resultados não publicados), notamos que mesmo adicionando $PGF_{2α}$ à hipofisação, algumas fêmeas continuavam apresentando ovulação tardia, com liberação de ovócitos com sangue, em forma de “grumos” (falha parcial na ovulação), que comprometem sua fertilização.

Esses achados nos levaram a avaliar os níveis de $PGF_{2α}$ endógena no momento da ovulação em fêmeas desovadas e não desovadas com o objetivo de determinar se a $PGF_{2α}$ estava de fato associada com a ovulação bem-sucedida nesta espécie. Os resultados obtidos mostraram que os níveis plasmáticos de $PGF_{2α}$ de todas as fêmeas desovadas eram expressivamente superiores à maioria dos níveis das fêmeas que não desovavam. No entanto, algumas raras fêmeas que não desovavam, apresentaram níveis de $PGF_{2α}$ similares às fêmeas que desovaram, indicando que seus níveis no momento da ovulação não explicam completamente as razões sobre o sucesso ou fracasso da ovulação nesta espécie (Kuradomi e Batlouni, 2018). A este respeito, já foi demonstrado que, em algumas espécies, um pico de $PGF_{2α}$ pouco antes a ovulação precede a desova (Lister & Van Der Kraak, 2008; Knight & Van Der Kraak, 2015; Tang et al., 2017), mas por outro lado, também é conhecido que há um intervalo de ação das prostaglandinas, normalmente horas antes da ovulação induzida (Jalabert & Szöllösi, 1975; Stacey & Pandey, 1975).

Considerando estes resultados, seria imprescindível conhecer a concentração plasmática de $PGF_{2α}$ e esteroides gonadais entre a segunda dose da hipofisação e a desova. Para isso, fêmeas de pacu foram hipofisadas com ou sem $PGF_{2α}$ exógena (2 mL de Ciosin) ao longo do processo de maturação final e ovulação, a fim de estabelecer relações entre os níveis de $PGF_{2α}$ e esteroides gonadais com os eventos que constituem a desova: maturação final e ovulação durante a desova induzida. Para isso também foram analisadas as frequências dos diferentes tipos de ovócitos quanto à posição da vesícula germinativa (central, excêntrica, GVBD e ausente). Como resultados nós observamos que a sequência clássica de eventos que precede a ovulação (elevação dos níveis de DHP, na maturação final, e aumento nos níveis de prostaglandina, na ovulação (Lister & Van Der Kraak, 2008; Nagahama & Yamashita, 2008; Tokarz et al., 2015; Takahashi et al., 2018)), também ocorre no pacu. Além disso, sugerimos que o incremento na taxa de ovulação obtido com hipofisação convencional associada a $PGF_{2α}$, descrito por Criscuolo-Urbinati et al. (2012), foi possivelmente devido à elevação precoce e mais extensa dos níveis circulantes de $PGF_{2α}$ quando comparado com a hipofisação convencional. Desta forma, conhecendo o perfil hormonal das fêmeas de pacu durante a indução hormonal, no presente trabalho, nós propusemos avaliar duas novas variáveis ainda não testadas: o momento de aplicação da $PGF_{2α}$ exógena e a concentração da dose de $PGF_{2α}$ aplicada.

6. Melatonina e ritmos biológicos

Sabe-se que além da $PGF_{2α}$, diversas outras variáveis e/ou substancias atuam de forma integrada ou não para provocar a ovulação dos peixes. Entre inúmeras variáveis e processos, pouco conhecidos, é conhecido que o fotoperíodo é um dos principais fatores que regulam os processos comportamentais e fisiológicos, inclusive os reprodutivos (Chattoraj et al., 2005; Falcon et al., 2007; 2010; de Alba et al., 2019; Paredes et al., 2019). Esta variável se apresenta de forma circadiana, ou seja, alternância entre período de luz e o período escuro ao longo de aproximadamente 24 horas e tem como um de seus principais agentes um neurohormônio chamado melatonina (MTN) (Falcon et al., 2007; 2010). A MTN está associada a diversos mecanismos reprodutivos, desde a modulação do eixo hipotálamo-hipófise-gônadas (Chattoraj et al., 2005; 2008; Falcon et al., 2010; Takahashi et al., 2018) até a ovulação (Ogiwara e Takahashi, 2016).

A MTN é um neurohormônio pertencente ao grupo das indolaminas, conhecido por ser um "*zeitgeber*", ou seja, possui importante papel na regulação dos relógios circadianos dos vertebrados, que transmitem informações rítmicas (circadianas e circanuais) aos animais (Falcon et al., 2007; 2010; Martinez-Chavez et al., 2008). Este hormônio é sintetizado principalmente na glândula pineal de maneira periódica, resultando em níveis sanguíneos elevados durante a noite e níveis reduzidos durante o dia (Falcon et al., 2010). Nas células pineais, sua síntese é iniciada a partir do triptofano, sendo divididas em duas etapas enzimáticas: 1) formação da serotonina a partir do triptofano por meio das enzimas triptofano hidroxilase e aminoácido aromático descarboxilase; 2) conversão de serotonina em MTN pela ação das enzimas arylalkylamine N-acetyltransferase (AANAT) e hydroxyindole-O-methyltransferase (HIOMT) (Falcon et al., 2010; Ogiwara e Takahashi, 2016).

Na maioria das espécies, a glândula pineal se localiza anexada por um pedúnculo delgado ao teto do diencéfalo, em sua porção posterior, geralmente localizado abaixo de uma abertura no crânio por onde a luminosidade é captada. Em mamíferos, a retina registra sinais periódicos de luminosidade (ritmo circadiano), os quais atingem o núcleo supraquiasmático do hipotálamo, que, por sua vez sinalizam a glândula pineal por meio da via multissináptica, controlando assim secreção de MTN. Já em peixes, em resposta ao fotoperíodo, a retina e a pineal transmitem dois tipos de informações, as neurais (da retina e da glândula pineal que chegam ao diencéfalo ventral) e as hormonais (via MTN). Na retina, a MTN é um fator autócrino e/ou parácrino, já a MTN pineal é liberada no líquido cefalorraquidiano e no sangue, atingindo alvos específicos através dos receptores de MTN, como no hipotálamo, (Falcon et al., 2007; Zohar et al., 2010); na hipófise (Khan e Thomas, 1996; Falcon et al., 2010); e em outros tecidos, como nas gônadas (Chattoraj et al., 2005; 2008; Carnevalli et al., 2011; Ogiwara e Takahashi, 2018) (Figura 4).

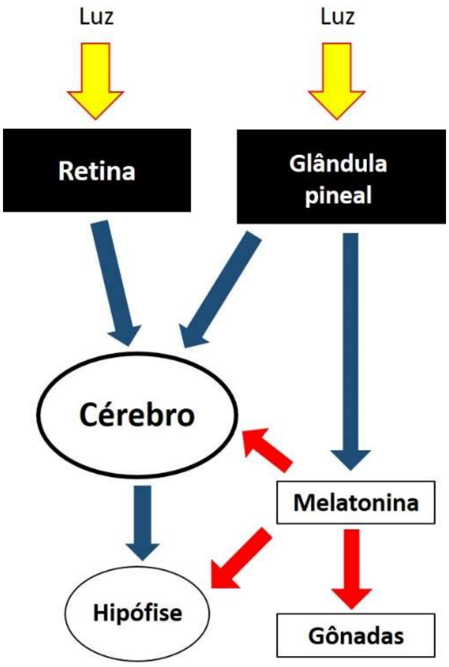

Figura 4. Controle do fotoperíodo nas funções neuroendócrinas adaptado de Falcon et al. (2007). Flechas amarelas indicam emissão de luz. Flechas azuis indicam informações neurais. Flechas vermelhas indicam informações retransmitidas pela melatonina. Fonte: Falcon et al., 2007.

Dessa forma, a MTN atua modulando a sincronização de vários processos rítmicos comportamentais e fisiológicos, como locomoção, termorregulação, migração, crescimento, eixo hipotálamo-hipófise-gônadas, sistema imunológico e metabolismo (Martinez-Chavez et al., 2008; Falcon et al., 2010; Takahashi et al., 2018). Em peixes, essa alternância circadiana entre período de luz e o período escuro influencia comportamentos como atividade locomotora, pigmentação da pele, consumo de oxigênio, termorregulação, ingestão alimentar e formação de cardume. Além disso, os peixes também detectam essas mudanças de fotoperíodo anualmente (circanuais), atuando em funções fisiológicas como no crescimento, resposta imune, migração reprodutiva e reprodução (Falcon et al., 2007; 2010).

7. O papel da melatonina na reprodução de peixes

Os ritmos de MTN possuem importante papel na modulação do eixo hipotálamo-hipófise-gônada em peixes e podem impactar tanto a estação reprodutiva (ritmos circanuais), como o próprio sucesso da desova (ritmos circadianos) (Chattoraj et al., 2005; Falcon et al., 2010; Ogiwara e Takahashi, 2016), mecanismos estes quase que completamente inexplorado na reprodução de espécies migradoras de produção. A manipulação do fotoperíodo causa oscilações nos níveis de MTN no organismo, que por sua vez podem atuar: a) no hipotálamo, modulando a secreção de GnRH (Takahashi et al., 2018) ou GniH (hormônio inibidor de gonadotropinas) (Zohar et al., 2010) via kisspeptina (Zohar et al., 2010; Carnevalli et al., 2011; Maitra et al., 2013); b) na hipófise, regulando a síntese do hormônio folículo estimulante (FSH) e do LH (Khan e Thomas, 1996; Falcon et al., 2010); c) e nas gônadas: controlando a produção de hormônios reprodutivos, como DHP e prostaglandinas (Chattoraj et al., 2005; 2008; Carnevalli et al., 2011; Ogiwara e Takahashi, 2018). Estes estímulos de MTN podem tanto aumentar quanto diminuir a secreção desses hormônios de acordo com a oscilação de seus níveis. Neste contexto, em estudo com machos e fêmeas de zebrafish, Paredes et al. (2019) observaram maiores níveis de GnRH no período escuro (maiores níveis de MTN), enquanto as maiores expressões de *lhβ* e *fshβ* foram registradas no período de luz (menores níveis de MTN). Por outro lado, em machos e fêmeas de tilápia (*Oreochromis niloticus*), níveis maiores de expressões de lhβ e fshβ foram encontradas no período de escuro e maiores níveis de GnRH no período de luz (de Alba et al., 2019). Além disso, fêmeas de corvina do Atlântico (*Micropogonias undulatus*) apresentaram maiores níveis de LH plasmático no período inicial de escuro (Khan e Thomas, 1996). Deste modo, as oscilações hormonais circadianas observadas durante o fotoperíodo parecem ser espécie-específicas, diferenciando-se da estratégia reprodutiva de cada peixe (Blanco-Vives e Sánchez-Vázquez, 2009; Paredes et al., 2019).

Em fêmeas de corvina do Atlântico, a aplicação de MTN elevou os níveis plasmáticos de LH e estimulou, *in vitro*, a secreção de LH pela hipófise (Khan e Thomas, 1996). Chattoraj et al. (2005) observaram que, em ovócitos de carpas (*Catla catla*; *Labeo rohita*; e *C. Carpio*), a MTN acelerou a ação do DHP quando adicionado quatro horas antes do DHP, resultando em uma antecipação da maturação final (GVBD). Resultados semelhantes foram encontrados em folículos de zebrafish *in vitro*, demonstrando aumento na taxa de GVBD com adição de MTN ao tratamento de DHP (Carnevali et al., 2011). Além disso, em

C. catla, foi possível atingir a maturação final somente com a incubação de MTN (Chattoraj et al., 2008). Em estudo com fêmeas de medaka, Ogiwara e Takahashi (2016) observaram que o uso de antagonistas de MTN (luzindole) em folículos ovarianos *in vitro* reduziu os níveis de PGE_2 e foi um potente inibidor da ovulação. No mesmo estudo, a MTN foi associada ao aumento da expressão da fosfolipase citosolica A2 grupo 4a (*pla2g4a*), enzima que libera o ácido araquidônico das membranas fosfolipídicas com subsequente conversão em PGE_2. Além disso, Ogiwara e Takahashi (2016) observaram que a MTN ativa a proteína moesina A (Msna), nos folículos, e quando fosforilada, atua similarmente à PGE_2, na despolimerização do citoesqueleto de actina nas células da granulosa propiciando a ovulação. A Msna, é uma das proteínas do complexo ezrina-radixina-moesina (ERM) e atua como principal ligante entre a membrana plasmática e citoesqueleto de actina, estando associado a mudanças na forma celular (Takahashi et al., 2018).

Como já descrito acima, várias espécies migradoras nativas de produção apresentam problemas relacionados com a ovulação (Hainfellner et al., 2012b; Pereira et al., 2017; 2018; de Souza et al., 2020; Roza de Abreu et al., 2020 e 2022), principalmente o pacu (Criscuolo-Urbinati et al., 2012;; Kuradomi e Batlouni, 2018). As metodologias de hipofisação foram criadas na década de 30 (Houssay, 1930; Von Ihering, 1934), e foram sendo aprimoradas até os dias atuais, entretanto, algumas práticas são aplicadas de forma arbitrária, sem nenhuma investigação científica prévia, como é o caso do horário de indução hormonal. Para fins de produção, conhecer o horário natural de desova de uma espécie pode ajudar muito em ter sucesso na sua reprodução em cativeiro, como é o caso do zebrafish, já estabelecido que desova sempre nas primeiras horas de luz do dia (Blanco-Vives e Sánchez-Vásquez, 2009; Paredes et al., 2019). Infelizmente, desde a invenção da hipofisação, os peixes de produção vêm sendo induzidos em horários aleatórios e arbitrários de acordo com os interesses e comodidade humana. Considerando que existem ritmos circadianos de substancias que governam a desova em ambiente natural, o conhecimento dos mesmos poderia ajudar a ter mais sucesso na reprodução induzida em cativeiro, uma vez que poderíamos induzir os peixes em seus momentos “favoráveis” aproveitando da síntese e liberação natural de substancias indutoras endógenas e concatenando o uso de substancias exógenas indutoras de forma sinérgica, para que a desova fosse impulsionada pelos dois eixos: exógeno e endógeno.

No entanto, existem raros relatos sobre horário de desova de peixes migradores nativos em ambiente natural, o que nos força a propor testes em cativeiro sem este conhecimento prévio. Em um cenário mais aplicado, considerando os ritmos circadianos naturais das espécies, mesmo que desconhecidos, mas que possivelmente variam com fotoperíodo, Muniz et al. (2008) induziram fêmeas de tambaqui em dois momentos distintos, aplicando a segunda dose hormonal no início do período escuro (desova prevista ao amanhecer), ou no início do período de luz (desova prevista ao anoitecer). Como resultado, os autores observaram que somente fêmeas induzidas no período escuro apresentaram desovas. Considerando que a segunda dose é a dose com maior concentração de hormônio (dose definitiva), responsável pela GVBD nos ovócitos e ovulação, esses resultados nos mostram uma possível interferência do fotoperíodo nos hormônios modulados pelo ritmo circadiano.

Portanto, conhecendo a importância e os efeitos que a MTN possui sobre os hormônios reprodutivos, é possível sugerir que, a partir do conhecimento das oscilações circadianas de MTN do pacu, o ajuste no período de indução hormonal com maiores níveis circadianos deste hormônio, poderá resultar em uma otimização no desempenho reprodutivo, principalmente das taxas de ovulação. Não obstante, dada a sabidamente comprovada ação sinérgica de MTN e prostaglandinas (Ogiwara e Takahashi, 2016; Takahashi et al., 2018) este trabalho se propôs a avaliar de forma inicial possíveis associações entre níveis endógenos da primeira e variações nas concentrações e tempo de aplicação exógena da segunda no sucesso de ovulação do pacu.

OBJETIVOS GERAIS

O objetivo deste trabalho foi avaliar o efeito de diferentes momentos de aplicação e doses de $PGF_{2\alpha}$ sintética sobre o desempenho reprodutivo de fêmeas de pacu. Além disso, tivemos como segundo objetivo analisar se existem e como ocorrem as oscilações circadianas plasmáticas de MTN nesta espécie, em associação com o processo de hipofisação ou não. Por fim, de posse do conhecimento das oscilações circadianas de MTN, propusemos dois distintos horários de indução hormonal e avaliamos possíveis diferenças no desempenho reprodutivo e se estas diferenças estavam associadas aos níveis de MTN durante o período de indução hormonal.

OBJETIVOS ESPECÍFICOS

Manuscrito 1:

- Avaliar se as fêmeas induzidas com $PGF_{2\alpha}$ sintética mais próxima à ovulação apresentam melhores parâmetros reprodutivos;
- Observar se fêmeas induzidas com $PGF_{2\alpha}$ sintética mais próxima à ovulação apresentam maiores níveis plasmáticos de $PGF_{2\alpha}$ e DHP no momento da ovulação;
- Avaliar se o aumento da dose de $PGF_{2\alpha}$ sintética proporciona melhor desempenho reprodutivo;
- Analisar se há correlação entre os níveis plasmáticos de $PGF_{2\alpha}$ e DHP no momento da ovulação e parâmetros reprodutivos.

Manuscrito 2:

- Avaliar as oscilações plasmáticas de MTN em 24 horas
- Analisar se a indução hormonal altera as oscilações circadianas plasmáticas de MTN;
- Avaliar se fêmeas induzidas no início da noite (19 horas) apresentam melhores parâmetros reprodutivos que fêmeas induzidas à meia-noite (0 horas);
- Analisar se fêmeas induzidas no início da noite (19 horas) apresentam maiores níveis plasmáticos de MTN, DHP e $PGF_{2\alpha}$ que fêmeas induzidas à meia-noite (0 horas).

MANUSCRITO 1

Reproductive performance of pacu (*Piaractus mesopotamicus*) induced by hypophysation with prostaglandin $F_{2\alpha}$ applied at different doses and periods

Manuscrito nas normas da revista *Aquaculture*

Reproductive performance of pacu (*Piaractus mesopotamicus*) induced by hypophysation with prostaglandin $F_{2\alpha}$ applied at different doses and periods

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Abstract

Over the last decade, we have developed a hypophysation protocol associated with exogenous prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) that increased predictability in pacu (*Piaractus mesopotamicus*) spawning, but still, ovulation failures persist in some females. Thus, in order to improve spawning rate, the objective of this study was to evaluate two new variables: the moment of application of exogenous $PGF_{2\alpha}$ and the $PGF_{2\alpha}$ dose. To that, in experiment 1, 24 hypophised females received 2 mL/fish of $PGF_{2\alpha}$ either at the time of resolving dose (2D) or five (5H) or seven hours after resolving dose (7H) (n=6 per group). In experiment 2, hypophysed females were induced with $PGF_{2\alpha}$ at the time of resolving dose with five doses ranging from 1.0 to 7.0 mL/kg (n=2 per group). In both, reproductive parameters were recorded. For better comparison of ovulation rates, relative fecundities lower than 35,000 oocyte/kg fish were considered as poor-quality ovulation. At the time of ovulation, females had their blood collected to determine the plasma levels of 17α-20β-dihydroxy-4-pregnen-3-one (DHP) and $PGF_{2\alpha}$. In experiment 1, groups 2D and 5H had an ovulation rate of 66.7% while 7H had 50%. Only the 2D group did not show poor-quality ovulation. In experiment 2, groups that received the highest doses of exogenous $PGF_{2\alpha}$ (i.e., 5.5 and 7.0 mL/kg) were the only ones that did not show ovulation failure (i.e., poor-quality ovulation or non-spawned female), but the fertility and hatching rates in these groups were not satisfactory. There was no effective gain in reproductive performance when postponing the application of $PGF_{2\alpha}$ closer to ovulation nor increasing the conventional 2mL/fish doses. $PGF_{2\alpha}$ and DHP plasma levels were similar among treatments, but higher than controls and basal levels in both experiments. A strong positive correlation between $PGF_{2\alpha}$ levels and fecundity were observed together with a strong negative correlation between fecundity and accumulated thermal units. Taken together, spawning failures, and low embryo viability observed here, especially related to late spawning females, could not be mitigated by changing the time of application and/or increasing the 2mL $PGF_{2\alpha}$/fish used in the conventional protocol. Ovulation failures, as well as the occurrence of low-quality late ovulations, seems to be associated with other factors that need to be explored, among them we highlight circadian rhythms of substances that control reproduction such as melatonin, DHP and $PGF_{2\alpha}$, as well as the pattern of expression and localization of their receptors.

Keywords: prostaglandin synthetic, ovulation failures, migratory fish, hormonal induction

1. Introduction

Pacu (*Piaractus mesopotamicus*) is a highly prized Neotropical characid for human feed and fishery that presents favorable zootechnical features, such as hardness, resistance to low temperatures, and fast growth in many growing conditions (Jomori et al., 2003; Souza et al., 2003; Gelman et al., 2004; Moro et al., 2013; Mourad et al., 2018; Urbinati and Takahashi, 2020).

This species together with tambaqui (*Colossoma macropomum*) and pirapitinga (*Piaractus brachypomus*) and its interspecific hybrids belong to a fish group commercially known as “round fish”. This group is the second one more produced in Brazil, representing about 27% of production of farmed fish in 2021 (IBGE, 2022). In this same year, Brazilian production reached 841 thousand tons (Peixe BR, 2022). Moreover, the exportation of this fish group increased by 650% for tambaqui and 410% for pacu from 2019 to 2020 (Peixe BR, 2021).

Pacu is a rheophilic species and therefore needs hormonal induction to reproduce in captivity. The carp pituitary extract is the primary protocol for inducing native migratory fishes in Brazil, including for pacu (Criscuolo-Urbinati et al., 2012; Kuradomi and Batlouni, 2018; Borella et al., 2020). This methodology, however, still presents failures related to female ovulation response and consequently causes spawning success unpredictability (Criscuolo-Urbinati et al., 2012; Kuradomi and Batlouni, 2018).

In this scenario, knowing the prostaglandins potential as efficient ovulation inducers in several teleosts (Jalabert and Szollosi, 1975; Stacey and Pandey, 1975; Kagawa et al., 2003; Fujimori et al., 2011; 2012; Joy and Singh, 2013; Takahashi et al., 2013; 2018; Knight and Van der Kraak, 2015; Tang et al., 2017; Kuradomi and Batlouni, 2018), we published in 2012 a study showing that the use of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (2 ml of

Ciosin® per fish, containing 0.25 mg/ml of cloprostenol sodium) together with the resolving dose of classic hypophysation increased ovulation rate in pacu (Criscuolo-Urbinati et al., 2012). Nevertheless, we noticed later (unpublished results) that even after adding $PGF_{2\alpha}$ to the hypophysation, some females continued to show late ovulation with the release of oocytes in the form of "clumps" with blood which compromised their fertilization (i.e., partial ovulation failure).

These findings led us to assess endogenous $PGF_{2\alpha}$ levels at the time of ovulation in ovulated and non-ovulated pacu females induced with classic hypophysation in order to determine whether $PGF_{2\alpha}$ was indeed associated with successful ovulation in this species (Kuradomi and Batlouni, 2018). In that study, we showed that the $PGF_{2\alpha}$ plasma levels of all ovulated females were significantly higher than the levels of most non-ovulated females (Kuradomi and Batlouni, 2018). However, some rare non-ovulated females showed $PGF_{2\alpha}$ plasma levels similar to females that ovulated, suggesting that $PGF_{2\alpha}$ plasma levels at the time of ovulation did not fully explain the success or failure of ovulation in this species. In this regard, it has already been shown that, in some species, a peak of $PGF_{2\alpha}$ occurs shortly before the time of ovulation (Lister & Van Der Kraak, 2008; Knight & Van Der Kraak, 2015; Tang et al., 2017), but on the other hand, it is also known that there is an interval of action of prostaglandins, normally hours before the induced ovulation (Jalabert and Szöllösi, 1975; Stacey and Pandey, 1975).

As the aforementioned information was not available for pacu, we developed a study to evaluate the pharmacokinetics of this substance in plasma and observed that the application of $PGF_{2\alpha}$ associated with classic hypophysation in pacu altered circulating $PGF_{2\alpha}$ levels when compared to the females that received only classic hypophysation (Sato et al., 2020). We showed that the application of exogenous $PGF_{2\alpha}$ triggered two peaks of circulation $PGF_{2\alpha}$, between one hour after the resolving dose and 8-12 hours after the resolving dose (time of ovulation); while in females with only hypophysation occurred only one peak between 8-12 hours after the resolving dose (Sato et al., 2020). Hence, the present study aims to add and evaluate two new variables to the protocol of hypophysation with exogenous $PGF_{2\alpha}$: the moment of application of exogenous $PGF_{2\alpha}$ after the resolving dose of hypophysation; and the concentration of the $PGF_{2\alpha}$, and thus associate them with successful ovulation.

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 003837/17.

2.1. Origin, characteristics and management of fish

The experiments were carried out during the pacu reproductive season, in December 2017 (experiment 1) and December 2018 (experiment 2) at the UNESP Aquaculture Center (CAUNESP), located in Jaboticabal, São Paulo, Brazil (21º15'17"S and 48º19'20"W). The broodstock was maintained throughout the year in 200 m² earthen ponds (at a density of 0.80 kg/m^2), supplied with a constant flow of approximately 20 L/min. The fish were fed apparent satiety twice a day (approximately 1-4% of the biomass) with commercial feed Omnivores Growth - Fri-Acqua, containing 28% crude protein, 3.5% ether extract, 12% ash and 9% of fibrous matter, according to the manufacturer's information. The fish used were produced at CAUNESP from crossings carried out with the broodstock already existing at the Institution.

Water parameters were determined every two weeks (7:00 a.m.). Temperature and conductivity were measured using a HANNA probe, HI-98311; pH was measured using a KASVI probe, K39-0014P and dissolved oxygen with a BERNAUER-424 AQUACULTURE probe, F-1550A. The mean ± standard deviation of pH, dissolved oxygen concentration, conductivity and water temperature, were, at 5.71 ± 0.88, 6.43 ± 0.13 mg/L, 34.45 ± 10.60 µS/cm and 24.50 ± 2.70 °C, and, respectively, in experiment 1 and, 5.82 ± 0.65, 5.98 ± 0.33 mg/L, 34.26 ± 12.05 µS/cm and 25.35 ± 2.86 °C respectively, in experiment 2.

2.2. Broodstock selection

Females were initially selected based on external characteristics, such as a swollen abdomen (Schorer et al., 2016) and hemorrhagic urogenital papilla (Kuradomi et al., 2017) and later, by the degree of development of the intra-ovarian oocytes sampled by ovarian biopsies, choosing females with a frequency greater than or equal to 30% of oocytes with germinal vesicle shifted towards the periphery (Kuradomi and Batlouni, 2018; Schorer et al., 2016). Males were selected by releasing semen under slight abdominal pressure (Kuradomi et al., 2016). Then, selected animals were transported to the laboratory in transport boxes and maintained in 750 L tanks coupled to a water recirculation system at constant temperature (experiment 1: 26.08 ± 0.27ºC; experiment 2: 26.82 ± 0.26ºC) under natural photoperiod (in both: approximately 13 hours and 20 minutes of photoperiod).

2.3. Hormonal induction protocol

Hormonal induction was divided into two intramuscular injections with an interval of 24 hours between them. Previously the hormonal induction females were anesthetized with 100 mg/L of benzocaine solution (Sigma-Aldrich, Saint Louis, USA). Females received two doses of crude carp pituitary extract (CPE) (0.6 and 5.4 mg/kg) suspended in 0.25 mL/kg of 0.9% saline solution. The control group only received 0.9% saline solution in both injections and the same volume (i.e., 0.25 mL/kg). The induction with exogenous $PGF_{2\alpha}$ was performed via intramuscular injection with 2 mL of Ciosin®/fish (containing 0.25 mg/mL of cloprostenol, MSD Animal Health) for experiment 1. The males received 3 mg/kg of CPE at the time of the resolving dose for females.

2.4. Experiment 1

2.4.1. Experimental design

Twenty-four adult females with five years old and 3,097.50 ± 180.71 g of body mass were distributed in a completely randomized design with four groups (n = 6 females per group): control group (C), classic hypophysation associated with exogenous $PGF_{2\alpha}$ applied at the resolving dose (2D); classic hypophysation associated with exogenous $PGF_{2\alpha}$ applied five hours after the resolving dose (5H); classic hypophysation associated with exogenous $PGF_{2\alpha}$ applied seven hours after the resolving dose (7H) (Figure 1). We determined the application time of exogenous $PGF_{2\alpha}$ based on our previous study (Sato et al., 2020), where we observed a peak of endogenous $PGF_{2\alpha}$ between eight and 12 hours after the resolving dose. To assess the baseline conditions of females, we added an initial group (IN), in which we randomly selected six females from the same lot that did not receive hormonal induction for blood sampling.

Pacu spawns within a range of 276-323 accumulated thermal units (ATU), that is, between 10 and 12 hours after the resolving dose at 27 °C (Criscuolo-Urbinati et al., 2012). ATU was calculated as the sum of the water temperature (°C) over time (h) after the resolving dose (Ceccarelli et al., 2000). In order to minimize injuries related to sampling handling, we carried out only one blood sampling, the spawned females with up to 340 ATU; and unspawned females, which had not spawned before 340 ATU (Kuradomi and Batlouni, 2018).

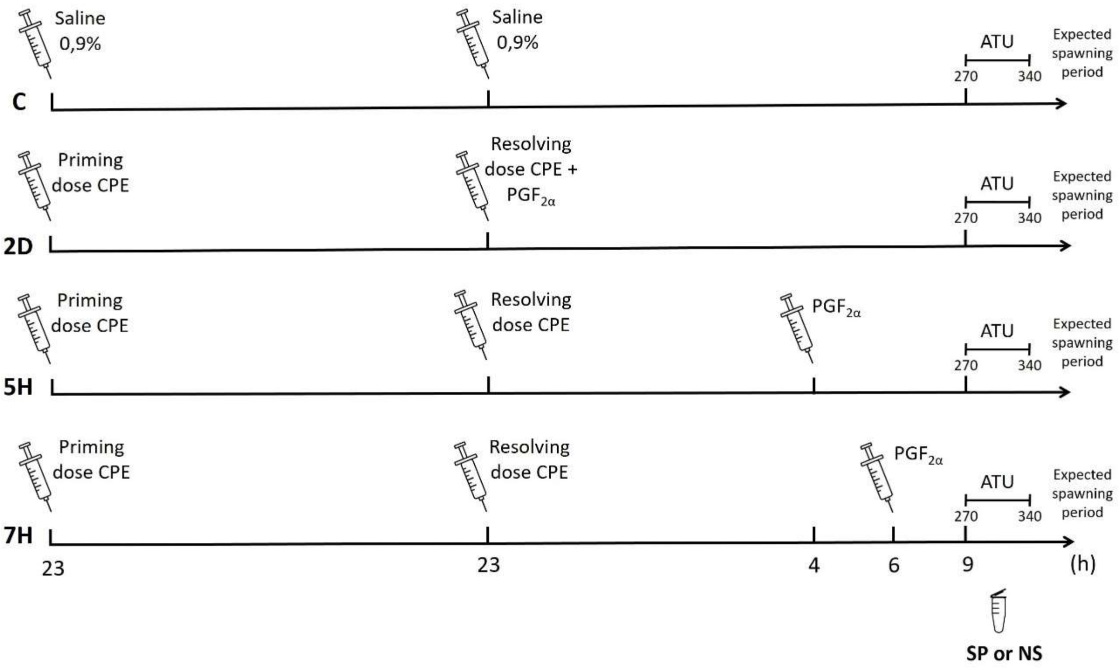

Figure 1. Experiment 1. Experimental design during hormonal induction in *P. mesopotamicus* females distributed into 4 groups: saline control (C); classic hypophysation with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in the resolving dose (Criscuolo-Urbinati et al., 2012) (2D); classic hypophisation with $PGF_{2\alpha}$ 5 hours after the resolving dose (5H); and classic hypophysation with $PGF_{2\alpha}$ 7 hours after the resolving dose (7H). Samples were collected at the time of ovulation for females spawned up to 340 ATU (SP) or non-spawned females after 340 ATU (NS) (microtube). In addition, six random females collected before hormonal induction. CPE: carp pituitary extract. ATU: Accumulated thermal units.

2.4.2. Reproductive performance

After observing the onset of reproductive behavior of the females in the tanks (i.e., restlessness and muscle spasms in the abdomen) and/or observing the first oocytes released at the bottom of the tanks, the process of extruding the oocytes was carried out through abdominal massage (between 270 and 340 ATU). The oocytes were extruded into a dry container and the oocyte mass and spawning time were recorded.

Fertilization was obtained by adding a pool of semen from three males (in the proportion of 0.5 mL of pooled semen for each 50 g of oocytes). The sperm concentration in pacu ranges from approximately 4.7 to 6.9 x 10^{10} cells/mL (Kuradomi et al., 2016) and the average number of oocytes present in one gram of spawn is around 1,200 (Cecarelli et al., 2000). The total spawning mass, in grams, was multiplied by 1200 to obtain the estimated number of oocytes released. Females with fecundity lower than 35,000 oocytes/kg of fish were considered females with poor-quality ovulation. The oocytes were fertilized and hydrated according to conventional methodology, adding water after mixing the gametes. Then, 10 mL of eggs from each female were transferred to 8 L acrylic incubators in triplicate at an average temperature of 26.8 ± 0.3 ºC. Data were collected for ovulation rate, ATU and relative fecundity. The estimated rates of fertility (blastopore closure) and hatching (tail moving and fully unfolded) were performed about 12 and 18 hours after eggs fertilization, respectively (Schorer et al., 2016; Kuradomi and Batlouni, 2018). To obtain fertility and hatching rates roughly 100 eggs from each incubator (triplicate) were classified as viable or non-viable embryos.

The following parameters were recorded:

Ovulation rate (%) = number of ovulated females / total number of females x 100

ATU = mean of water temperature (ºC) x latency period (h)

Relative fecundity = number of oocytes released / female body mass (g)

Fertility rate (%) = number of viable embryos / total number of eggs x 100

Hatching rate (%) = number of viable embryos / total number of eggs x 100

2.4.3. Blood sampling

For sample collection, fish were anesthetized with 100 mg/L of benzocaine; blood samples were collected from the caudal vein using heparin-treated syringes. The plasma was separated by centrifugation at 1000×g for 15 min at 4 °C and then stored at -80 °C until measurement of the steroid hormone concentrations. For $PGF_{2\alpha}$ dosage, 10 µM indomethacin was added to the blood immediately after collection to inhibit prostaglandin synthesis. The plasma levels of 17α-20β-dihydroxy-4-pregnen-3-one (DHP) and $PGF_{2\alpha}$ 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 DHP and $PGF_{2\alpha}$ plates were performed at an absorbance of 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 analysis, intra- and inter-assay variability were assessed and we obtained the respective following variations: 0.55 to 17.27% and 1.71 to 18.53% for DHP; 2.88 to 19.31% and 4.71 to 20.53% for $PGF_{2\alpha}$.

2.4.4. Statistical analysis

All statistical tests were performed using the STATISTICA software (StatSoft, Inc., Tulsa, OK, USA) and Excel (Microsoft, Redmond, USA). Normality and homogeneity of the variances were tested using the Shapiro-Wilk's test and Levene's test, respectively. Parametric data (i.e., reproductive performance) were analyzed using the variance test (one-way ANOVA) followed by Tukey's test. Kruskal-Wallis's test was used for non-parametric data (DHP and $PGF_{2\alpha}$ plasma levels) followed by Dunn's test for multiple comparisons. The plasma levels of DHP and $PGF_{2\alpha}$ were correlated with the reproductive performance parameters (regardless of group) using the non-parametric Spearman's rank correlation test. In the correlation event, negligible correlation is considered with ρ (rô) values from 0.0 to 0.10, weak from 0.10 to 0.39, moderate from 0.40 to 0.69, strong from 0.70 to 0.89 and very strong from 0.90 to 1.0, according to a conventional method of interpreting correlation coefficients (Schober et al., 2018). Significant differences were accepted with a P value <0.05. Data were expressed as mean ± standard error or median (first quartile; third quartile) and correlation in ρ value.

2.5. Experiment 2

2.5.1. Experimental design

Ten adult females with six years old and 2,870.50 ± 112.89 g of body mass were distributed in a completely randomized design distributed into five groups (n = 2 females per group) with increasing and equidistant doses: T1, classic hypophysation associated with 1.0 mL/kg of exogenous $PGF_{2\alpha}$; T2, classic hypophysation associated with 2.5 mL/kg of exogenous $PGF_{2\alpha}$; T3, classic hypophysation associated with 4.0 mL/kg of exogenous $PGF_{2\alpha}$; T4, classic hypophysation associated with 5.5 mL/kg of exogenous

$PGF_{2\alpha}$; and T5, classic hypophysation associated with 7.0 mL/kg of exogenous $PGF_{2\alpha}$. The dose of 1.0 mL/kg was base the protocol initially proposed by Criscuolo-Urbinati et al., (2012). Samplings were carried out in a single period, at the time of ovulation the spawned females with up to 340 ATU; and unspawned females, which had not spawned before 340 ATU (Kuradomi and Batlouni, 2018). Each fish was considered an experimental unit.

In the first experiment we found some difficulties to guarantee that the fish would be induced at the same time; and for this reason, in this experiment the number of fish per treatment was reduced to ensure that all fish were injected at the same time (priming and resolving doses) avoiding the same problems. The suspicion that the time of hormonal induction interferes with spawning due to circadian rhythms of spawning inducing substances is being evaluated in other experiments and has shown to be an important variable. Since injecting fish weighing ~3kg, at the same time, requires large teams, in order to have accurate data, we opted for more treatments with fewer repetitions and carried out an individual analysis without considering the data statistically. We also emphasize here that due to the high fecundity of the species, >50,000 oocytes/kg of fish, normally a maximum of two or three females are injected at a time by commercial farmers, mainly because larviculture ponds (1000 m^2) are usually populated with approximately 100-200 larvae/m^2 (Ceccarelli et al., 2000).

2.5.2. Reproductive performance

The methodology and reproductive performance parameters were the same as described in experiment 1.

2.5.3. Blood sampling

Blood collection and methodology, as well as quantification of plasma levels of $PGF_{2\alpha}$ and DHP were the same as described for experiment 1. To validate the analysis, intra- and inter-assay variability were assessed and we obtained the respective following variations: 0.30 to 12.32% and 2.11 to 17.14% for DHP; 4.66 to 19.26% and 4.79 to 18.71% for $PGF_{2\alpha}$.

2.5.4. Results Analysis

Descriptive analysis (individual) was performed for reproductive performance and hormone levels. For the correlation analysis (spearman correlation) the same software used in experiment 1 was used, as well as assumptions such as normality and homoscedasticity. Significant differences were accepted with a P value <0.05.

3. Results

3.1. Experiment 1

3.1.1. Reproductive performance

Groups 2D and 5H had an ovulation rate of 66.7% while group 7H had 50%. The 2D group did not show poor-quality ovulation (i.e., fecundity <35,000 oocytes/kg fish), whereas the 5H and 7H groups showed 25% and 66.7% of poor-quality ovulation, respectively. The average values of ATU and fecundity, fertility and hatching rates were similar among all groups that spawned (2D, 5H and 7H) (p>0.05) (Table 1).

Table 1. Experiment 1. Reproductive performance of *Piaractus mesopotamicus* females submitted to classic hypophysation associated with exogenous $PGF_{2\alpha}$ applied in different periods.

Groups	Ovulation rate (%)	Poor-quality ovulation (%)*	ATU	Relative fecundity	Fertility rate (%)	Hatching rate (%)
C	0/6 (0%)	-	-	-	-	-
2D	4/6 (66.7%)	0/4 (0%)	288.6 ± 6.2	70,065 ± 10,369.8	52.5 ± 11.8	47.4 ± 7.7
5H	4/6 (66.7%)	1/4 (25%)	289.3 ± 11.0	73,050 ± 21,634.7	63.3 ± 15.4	55.8 ± 14.0
7H	3/6 (50.0%)	2/3 (66.7%)	309.2 ± 9.8	53,092 ± 26,428.9	44.9 ± 19.0	29.9 ± 14.1

C: 0.9% saline control; 2D: classic hypophysation + 2 mL/fish of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in the resolving dose; 5H: classic hypophysation + 2 mL/fish of $PGF_{2\alpha}$ five hours after the resolving dose; 7H: classic hypophysation + 2 mL/fish of $PGF_{2\alpha}$ seven hours after the resolving dose. ATU: accumulated thermal units. Relative fecundity: number of oocytes released/kg fish. The variables ATU, relative fecundity, fertility rate and hatching rate were similar among groups (p>0.05). Data expressed as mean ± SE.

* Female considered as poor-quality ovulation presented fecundity < 35,000 oocyte/kg fish

3.1.2. Plasma levels of $PGF_{2\alpha}$ and DHP

All groups showed wide internal individual variation in $PGF_{2\alpha}$ plasma levels at the time of ovulation (2D, values between 1.81 and 150.45 ng/mL; 5H, values between 3.61 and 555, 52 ng/ml; 7H, values between 3.16 and 261.43 ng/ml). $PGF_{2\alpha}$ levels in groups 2D with a median of 43.36 (first quartile: 2.30; third quartile: 53.49 ng/mL), 5H with a median of 87.65 (first quartile: 14.78; third quartile: 167.18 ng/mL) and 7H with a median of 34.02 (first quartile: 5.25; third quartile: 97.18 ng/mL) were more than 200 times higher than the levels of group C with a median of 0.16 (first quartile: 0.15; third quartile: 0.17 ng/mL) and the IN group with a median of 0.10 (first quartile: 0.06; third quartile: 0.13 ng/mL) ($p<0.05$) (Figure 2A).

Similarly, plasma levels of DHP were about ten times higher in 2D with median of 0.21 ng/mL (first quartile: 0.13; third quartile: 0.33 ng/mL), 5H with median of 0.25 ng/mL (first quartile: 0.17; third quartile: 0.45 ng/mL) and 7H with median of 0.26 ng/mL (first quartile: 0.16; third quartile: 0.44 ng/mL) compared to group C with median of 0.02 ng/mL (first quartile: 0.01; third quartile: 0.02 ng/mL) and group IN with median of 0.02 ng/mL (first quartile: 0.01; third quartile: 0.02 ng/mL) ($p<0.05$) (Figure 2B).

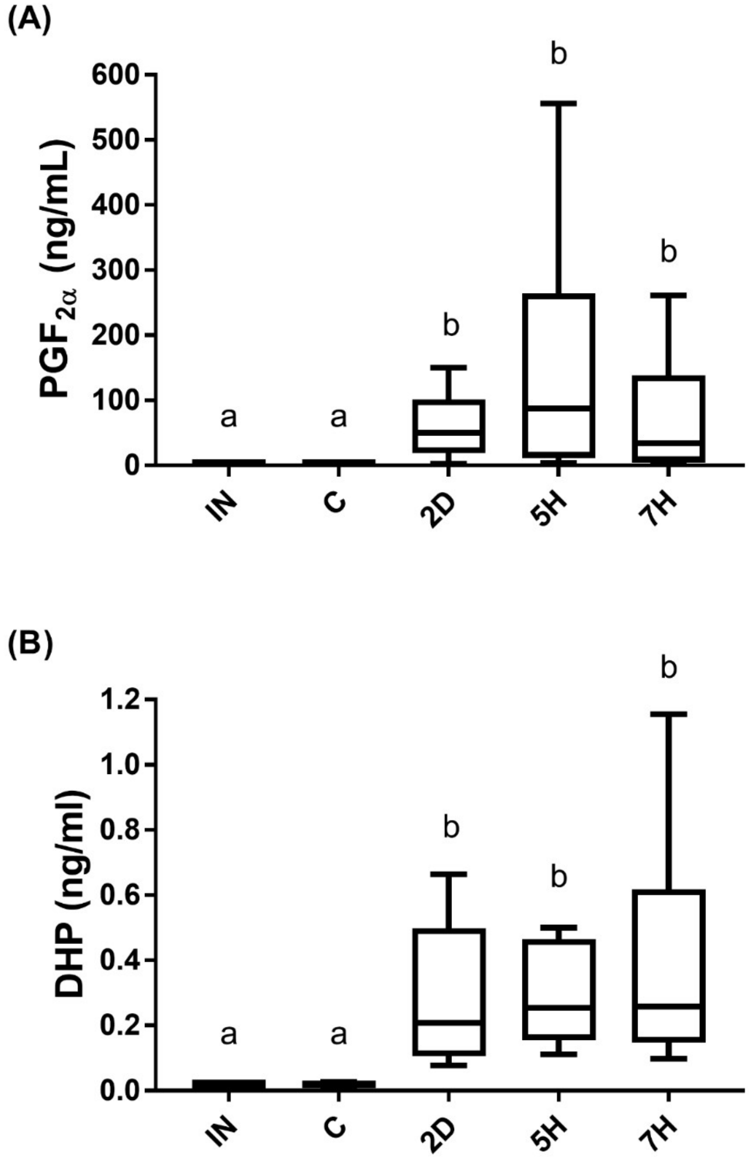

Figure 2. Experiment 1. Plasma levels of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (A) and 17α-20β-dihydroxy-4-pregnen-3-one (DHP) (B) in *Piaractus mesopotamicus* females at the time of ovulation of spawned females with up to 340 ATU; and unspawned females, which had not spawned before 340 ATU (n=6). IN: 6 random females collected before hormone induction; C: saline control; 2D: classic hypophysation + $PGF_{2\alpha}$ at resolving dose; 5H: classic hypophysation + $PGF_{2\alpha}$ five hours after resolving dose; 7H: classic hypophysation + $PGF_{2\alpha}$ seven hours after resolving dose. Data are expressed as median ± Q1 and Q3. Different letters indicate significant differences between groups ($p<0.05$).

3.1.3. Correlation

Correlations between variables were analyzed regardless of group. Following the method by Schober et al. (2018), strong positive and negative correlations were observed ($p<0.05$). (Table 2).

Table 2. Experiment 1. Spearman's correlations

Correlation	**ρ**	**Rate**
$PGF_{2\alpha}$ x Fecundity	0,83	Strong
Fecundity x Fertility rate	0,81	Strong
Fecundity x Hatching rate	0,88	Strong
Fecundity x ATU	-0,78	Strong
ATU x Fertility rate	-0,79	Strong
ATU x Hatching rate	-0,84	Strong

$PGF_{2\alpha}$: prostaglandin $F_{2\alpha}$; Fecundity: number of oocytes released/kg fish; Fertility and Hatching rate: number of viable embryos/total number of eggs×100; ATU: accumulated thermic units;
Rate: following (Schober et al., 2018)

3.2. Experiment 2

3.2.1. Reproductive performance

Groups T4 and T5 were the only ones in which, females did not show partial or complete ovulation failures (i.e., 100% of spawned females). In group T4, we observed the number “8” female with high fertility (91.9%) and hatching (88.0%) rates in contrast to female number “7” with low rates (21.5% of fertility and 19.0% of hatching). Females from the T5 group had low fertility (mean ± DP 31.5 ± 8.9%) and hatching (mean ± DP 30.7% ± 7.9%) rates (Table 3).

Females number “2” (T1) and “5” (T3) did not spawn until 340 ATU. Female number “4” (T2) was considered as having poor-quality ovulation, in addition, it had the highest ATU value (338) among all females and low fertility and hatching rates of 15.9% and 5.1%, respectively (Table 3).

Table 3. Experiment 2. Individual reproductive performance of *Piaractus mesopotamicus* females submitted to classic hypophysation associated with exogenous $PGF_{2\alpha}$ applied in different concentrations.

Groups	Relative fecundity	ATU	Fertility rate (%)	Hatching rate (%)	$PGF_{2\alpha}$ (ng/mL)	DHP (ng/mL)
1 - T1	142,080	299.0	71.0	60.1	66.67	0.21
2 - T1	-	-	-	-	449.04	0.08
3 - T2	184,920	322.4	68.6	67.8	161.37	0.30
4 - T2	33,720*	338.0	15.9	5.1	48.92	0.08
5 - T3	-	-	-	-	3.61	0.03
6 - T3	50,160	299.0	73.2	71.6	38.20	0.21
7 - T4	195,480	325.0	21.5	19.0	516.39	0.22
8 - T4	77,400	312.0	91.9	88.0	296.14	0.20
9 - T5	132,360	306.8	25.2	25.1	99.54	0.26
10 - T5	119,760	301.6	37.9	36.4	63.10	0.17

T1: classic hypophysation + 1.0 mL/kg of $PGF_{2\alpha}$ in the resolving dose; T2: classic hypophysation + 2.5 mL/kg of $PGF_{2\alpha}$ in the resolving dose; T3: classic hypophysation + 4.0 mL/kg of $PGF_{2\alpha}$ in the resolving dose; T4: classic hypophysation + 5.5 mL/kg of $PGF_{2\alpha}$ in the resolving dose; T5: classic hypophysation + 7.0 mL/kg of $PGF_{2\alpha}$ in the resolving dose. Dash (-) represents the non-spawning female. Relative fecundity: number of oocytes released/kg fish. ATU: accumulated thermal units. $PGF_{2\alpha}$: Prostaglandin $F_{2\alpha}$. DHP: 17α-20β-dihydroxy-4-pregnen-3-one.
* Female considered as poor-quality ovulation presented fecundity < 35,000 oocyte/kg fish

3.2.2. Plasma levels of $PGF_{2\alpha}$ and DHP

As in experiment 1, in this experiment, females showed wide individual variation in plasmatic levels of $PGF_{2\alpha}$ regardless of the group (3.61 to 516.39 ng/mL) (Table 3). The highest $PGF_{2\alpha}$ levels in spawned females were observed in the T4 group (female number "7" = 516.39 ng/mL and "8" = 296.14 ng/mL). Even though female number "2" (T1) presented 449.04 ng/mL $PGF_{2\alpha}$ plasma levels, it did not spawn (Table 3).

Reduced plasma levels of DHP were observed for females that did not ovulate (female number "2" = 0.08 ng/mL and "5" = 0.03 ng/mL) and for female number "4"

(0.08 ng/mL), which had poor-quality ovulation. Spawned females (females number "1", "3", "6", "7", "8", "9", "10") showed DHP plasma levels among 0.17 and 0.30 ng/mL (Table 3).

3.2.3. Correlation

Table 4. Experiment 2. Spearman's correlation.

Correlation	ρ	Rate
$PGF_{2\alpha}$ x Fecundity	0,71	strong
DHP x Fecundity	0,78	strong

$PGF_{2\alpha}$: prostaglandin $F_{2\alpha}$; DHP: 17α-20β-dihydroxy-4-pregnen-3-one; Fecundity: number of oocytes released/kg fish
Rate: following (Schober et al., 2018)

4. Discussion

In this study, we did not observe gains in reproductive performance that would justify the change in the hormone induction protocol previously proposed by Criscuolo-Urbinati et al., 2012 (i.e., hypophysation associated with 2 mL of exogenous $PGF_{2\alpha}$ at the time of the resolving dose).

In experiment 1, 2D and 5H groups had an ovulation rate of 66.7% while group 7H had only 50%. Furthermore, we observed that the third intervention (application of $PGF_{2\alpha}$ 5 and 7 hours after the resolving dose) caused the presence of poor-quality ovulation (2D, 0%; 5H, 25.0%; 7H, 66.7 %), that is, low fecundity, less than 35,000 oocyte/kg of fish. Poor-quality ovulation usually appear as "clumps" and with blood, making fertilization unfeasible, which corroborates the results of this experiment, in which relative fecundity has a strong positive correlation with fertility and hatching rates, demonstrating that low fecundities are associated with low fertility and hatching rates. Another point is that the females that received the third intervention (5H and 7H) did not spawn earlier than the females in the 2D group. The anticipation of spawning (lower UTA) would possibly result in a higher embryo viability, since we observed that the UTA obtained a strong negative correlation with fertility and hatching rates. Therefore, postponing $PGF_{2\alpha}$ application closer to ovulation did not anticipate the spawning moment and resulted in poor quality ovulation with low embryonic viability.

The unsatisfactory reproductive parameters observed in the 5H and 7H groups may be due to the third intervention, caused by the application of $PGF_{2\alpha}$ between the resolving dose and ovulation, which maybe generates additional stress to the broodstock, impairing the quality of gametes and their reproductive performance, as described in study on trouts (Campbell et al., 1992; 1994; Contreras-Sánchez et al., 1998). Cortisol, secreted in response to stress, acts by inhibiting reproductive hormones, which can delay ovulation, decrease egg production (fecundity) and fertility and hatching rates, in addition to increasing the number of abnormal larvae (Morgan et al., 1999; Murugananthkumar and Sudhakumari, 2022).

We did not observe an association between the application of $PGF_{2\alpha}$ closer to ovulation (5H and 7H) with higher $PGF_{2\alpha}$ plasma levels at the time of ovulation. We demonstrated, in a previous study (Sato et al., 2020), that classic hypophysation together with exogenous $PGF_{2\alpha}$ application (at the time of hypophysation resolving doses), provoked a plasma peak of $PGF_{2\alpha}$ one hour after resolving doses (due to exogenous $PGF_{2\alpha}$) with a subsequent rapid decrease; and a second and lower $PGF_{2\alpha}$ plasma peak occurs 8-12 hours after resolving doses (provoked by endogenous $PGF_{2\alpha}$ production) (Sato et al., 2020). Thus, considering this study, we assume here that the exogenous $PGF_{2\alpha}$ plasma peak, caused by the administration of this substance in the 5H and 7H groups, occurred, respectively, 6 and 8 hours after the resolving dose, i.e., before the time of ovulation (plasma sampling time at ovulation was approximately 12 hours after resolving doses). So we consider that that the levels detected here were the endogenous peak, triggered by hypophysation 8-12 hours post-resolving dose, according to Sato et al., (2020).

The considerable variation in endogenous $PGF_{2\alpha}$ levels in pacu females undergoing hypophysation has already been reported by us (Kuradomi and Batlouni, 2018), and we observed the same pattern here in this study. Even so, the absence or inconstancy of a pattern of response to the use of prostaglandins has been reported since the first studies evaluating the role of this substance in inducing ovulation in several species of fish (Jalabert and Szöllösi, 1975; Stacey and Pandey, 1975; Goetz and Theofan, 1979; Berndtson et al., 1989). Therefore, obtaining a response pattern concerning the dose-response (amount injected x plasma concentration) is still a challenge to be overcome. Individual variations in endogenous production strongly interfere with this ratio.

Regarding DHP, we observed higher plasma levels in $PGF_{2α}$ treated females (2D, 5H and 7H) at the time of ovulation compared to initial and control females. This fact together with the similar levels among 2D, 5H and 7H at the time of ovulation, suggests that the third intervention (application of $PGF_{2α}$ in 5H and 7H groups) did not interfere with DHP levels at the time of ovulation. DHP levels are directly associated with prostaglandins and their receptors (Berndtson et al., 1989; Goetz, 1997; Kagawa et al., 2003; Hagiwara et al. 2014; Knight & Van Der Kraak, 2015). Hagiwara et al. (2014) showed that expression of the PGE_2 receptor, EP4b (ptger4b), is regulated by a mechanism involving a nuclear progestin receptor (Pgr) in medaka (*Oryzias latipes*). In this species, there is evidence for DHP binding with the Pgr and subsequent association with the promoter region of the *ptger4b* gene (PGE receptor), which will be transcribed and expressed (Hagiwara et al., 2014). However, in this context, the DHP peak found in the 2D, 5H and 7H groups at the time of ovulation seems to be related to hypophysation itself with no obvious relation with exogenous $PGF_{2α}$ treatment, in accordance with Sato et al. (2020) and Batlouni and Kuradomi (2018).

In this scenario, the maintenance of $PGF_{2α}$ application at the time of the resolving dose is still shown as the best option. Thus, we corroborate the previous study (Sato et al., 2020), where the greater predictability in spawning and ovulation success with this protocol possibly occurs due to the earlier and longer increase in circulating levels of $PGF_{2α}$ compared to hypophysation alone, i.e., a prolonged action (longer action interval) of this substance, as in goldfish (Stacey and Pandey, 1975) and medaka (Fujimori et al., 2011). Therefore, we carried out experiment 2, testing different doses of $PGF_{2α}$ in order to provide higher levels of $PGF_{2α}$ after its application and possibly increase its action interval.

In experiment 2, we observed wide inter-individual variations in $PGF_{2α}$ plasma levels at the time of ovulation and no dose-dependent relationship between higher doses of $PGF_{2α}$ and its higher levels at the time of ovulation. As previously mentioned, this aspect was expected, since exogenous $PGF_{2α}$ levels increase shortly after its application, decreasing rapidly in a few hours (Sato et al., 2020). The strong positive correlation between levels of $PGF_{2α}$ and relative fecundity was also observed in experiment 1. In this context, considering that $PGF_{2α}$ acts in the disruption of bonds between follicle and oocyte (Lubzens et al., 2010), it is reasonable to suggest that high levels of this hormone, at the time of ovulation, may be more associated with increased fecundity than ovulation rate.

In the second experiment, the highest doses of Ciosin® (5.5 and 7.0 mL/kg) were the only ones that showed 100% ovulation and absence of poor-quality ovulation, however, low fertility and hatching rates were found. Considering that there is an action interval of $PGF_{2\alpha}$ in this species proposed by Sato et al. (2020), these good results in ovulation may be due to the increase in its dose possibly provided by higher and longer circulating levels of $PGF_{2\alpha}$ after its application. Although, we noticed that the females were prostrate, mainly at the time of ovulation, probably due to the large volume injected into the females of these groups, which may have hindered embryo survival. This is the first study testing such a broad spectrum of *in vivo* doses of $PGF_{2\alpha}$ in induced spawning and, despite poor embryonic survival, it seems that increasing the dose may improve ovulation rate. Thus, there must be an optimum curve for $PGF_{2\alpha}$ action that needs to be determined in further studies, including *in vitro* tests together with that basic information about the pattern of prostaglandins and DHP receptors in this species.

Still concerning experiment 2, we observed some problems related to the handling of the fish and its consequences presented negative effects that masked the possible positive effects of increasing the concentration of exogenous $PGF_{2\alpha}$. Firstly, we found difficulty in injecting large volumes of Ciosin®, especially in higher concentrations. Moreover, at the moment of injection, the females presented agitation greater than usual of the animal due to the large volume injected (some females received about 20 mL). Finally, after the application, the females that received larger doses remained prostrate and apathetic at the bottom of the tank throughout the hormone induction and after spawning, we observed swollen and reddish areas in the injection region.

Thus, considering that the analyses performed in this manuscript we concluded that the modifications proposed here cannot mitigate the spawning failure still observed in the $PGF_{2\alpha}$ protocol nor solve the problem with low embryo viability of late spawning females. Given this scenario, we are evaluating on ongoing research the second possibility proposed here, which is the adequacy of the timing of resolving dose according to the circadian rhythms of substances with a direct function in spawning, such as melatonin (Chattoraj et al., 2005; Ogiwara and Takahashi, 2016; Takahashi et al., 2018).

Declaration of Competing Interest

We declare that we have no conflict of interest.

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5. References

Berndtson, A. K., Goetz, F. W., & Duman, P., 1989. In vitro ovulation, prostaglandin synthesis, and proteolysis in isolated ovarian components of yellow perch (Perca flavescens): Effects of 17α,20β-dihydroxy-4-pregnen-3-one and phorbol ester. Gen. Comp. Endocrinol., 75(3), 454–465. https://doi.org/10.1016/0016-6480(89)90181-0

Borella, M. I., Chehade, C., Costa, F. G., Jesus, L. W. O., Cassel, M., Batlouni, S. R., 2020. The brain-pituitary-gonad axis and the gametogenesis. In B. Baldisserotto, E. C. Urbinati, J. E. P. Cyrino (Eds). Biology and Physiology of Freshwater Neotropical Fish (pp. 315–341). Academic Press (Elsevier).

Campbell, P. M., Pottinger, T. G., & Sumpter, J. P., 1992. Stress reduces the quality of gametes produced by rainbow trout. Biol. Reprod., 47(6), 1140–1150. https://doi.org/10.1095/biolreprod47.6.1140

Campbell, P.M., Pottinger, T.G., Sumpter, J.P., 1994. Preliminary evidence that chronic confinement stress reduces the quality of gametes produced by brown and rainbow trout. Aquacult., 120, 151-169. https://doi.org/10.1016/0044-8486(94)90230-5

Ceccarelli P. S., Senhorini J. A., Volpato G., 2000. Dicas em piscicultura - perguntas e respostas. Botucatu: Ed. Santana, 247p, 2000.

Chattoraj, A., Bhattacharya, S., Basu, D., Bhattacharya, S., Bhattacharya, S., Maitra, S.K., 2005. Melatonin accelerates maturation inducing hormone (MIH): induced oocyte maturation in carps. Gen. Comp. Endocrinol. 140, 145–155. https://doi.org/10.1016/j.ygcen.2004.10.013

Contreras-Sánchez, W. M., Schrek, C. B., Fitzpatrick, M. S., Pereira C. B., 1998. Effects of stress on the reproductive performance of rainbow trout (Oncorhynchus mykiss). Biol. Reprod. 58, 439-447. https://doi.org/10.1095/biolreprod58.2.439

Criscuolo-Urbinati, E., Kuradomi, R.Y., Urbinati, E.C., Batlouni, S.R., 2012. The administration of exogenous prostaglandin may improve ovulation in pacu (Piaractus mesopotamicus). Theriogenology 78, 2087–2094. https://doi.org/10.1016/j.theriogenology.2012.08.001

Fujimori C., Ogiwara K., Hagiwara A., Rajapakse S., Kimura A., Takahashi T., 2011. Expression of cyclooxygenase-2 and prostaglandin receptor EP4b mRNA in the ovary of the medaka fish, Oryzias latipes: Possible involvement in ovulation. Mol. Cell. Endocrinol., 332: 67–77. https://doi.org/10.1016/j.mce.2010.09.015

Fujimori, C., Ogiwara, K., Hagiwara, A., Takahashi, T., 2012. New evidence for the involvement of prostaglandin receptor EP4b in ovulation of the medaka, Oryzias latipes. Mol. Cell. Endocrinol., 362(1–2), 76–84. https://doi.org/10.1016/j.mce.2012.05.013

Gelman, A., Drabkin, V., Sachs, O., Chechic, K., Gabay, I., Glatman, L., 2004. Pacu (Piaractus mesopotamicus) a new fish species in Israeli aquaculture: Possibility of utilization. Developments in Food Science, 42(C), 75–83. https://doi.org/10.1016/S0167-4501(04)80010-4

Goetz, F. W., Theofan G., 1979. In vitro stimulation of germinal vesicle breakdown and ovulation of yellow perch (Perca flavescens) oocytes. Effects of 17α-hydroxy-20β-dihydroprogesterone and prostaglandins. Gen. Comp. Endocrinol., 37:273–285. https://doi.org/10.1016/0016-6480(79)90001-7

Goetz, F. W., 1997. Follicle and Extrafollicular Tissue Interaction in17a,20b-Dihydroxy-4-pregnen-3-one-Stimulated Ovulation and Prostaglandin Synthesis in the Yellow Perch (Perca flavescens) Ovary. Gen. Comp. Endocrinol., 105, 121–126. https://doi.org/10.1006/gcen.1996.6807

Hagiwara, A., Ogiwara, K., Katsu, Y., Takahashi, T., 2014. Luteinizing Hormone-Induced Expression of Ptger4b, a Prostaglandin E2 Receptor Indispensable for Ovulation of the Medaka Oryzias latipes, Is Regulated by a Genomic Mechanism Involving Nuclear Progestin Receptor1. Biol. Reprod., 90(6), 1–14. https://doi.org/10.1095/biolreprod.113.115485

IBGE “Instituto Brasileiro de Geografia e Estatística”, 2022. Produção da Pecuária Municipal 2021, 11

Jalabert, B., Szöllösi, D., 1975. In vitro ovulation of trout oocytes : Effect of prostaglandins on smooth muscle-like cells of the theca. Prostaglandins, 9(5), 765–778. https://doi.org/10.1016/0090-6980(75)90113-6

Jomori, R. K., Carneiro, D. J., Malheiros, E. B., Portella, M. C., 2003. Growth and survival of pacu Piaractus mesopotamicus (Holmberg, 1887) juveniles reared in ponds or at different initial larviculture periods indoors. Aquacult., 221(1–4), 277–287. https://doi.org/10.1016/S0044-8486(03)00069-3

Joy, K.P., Singh, V., 2013. Functional interactions between vasotocin and prostaglandins during final oocyte maturation and ovulation in the catfish Heteropneustes fossilis. Gen. Comp. Endocrinol. 186, 126–135. https://doi.org/10.1016/j.ygcen.2013.02.043.

Knight, O.M., Van Der Kraak, G., 2015. The role of eicosanoids in 17α,20β-dihydroxy-4-pregnen-3-one-induced ovulation and spawning in Danio rerio. Gen Comp. Endocrinol. 213, 50–58. https://doi.org/10.1016/j.ygcen.2014.12.014.

Kuradomi, R. Y., De Souza, T. G., Foresti, F., Schulz, R. W., Bogerd, J., Moreira, R. G., Furlan, L. R., Almeida, E. A., Maschio, L. R., Batlouni, S. R., 2016. Effects of re-stripping on the seminal characteristics of pacu (Piaractus mesopotamicus) during the breeding season. Gen. Comp. Endocrinol., 225, 162–173. https://doi.org/10.1016/j.ygcen.2015.06.007

Kuradomi, R. Y., Foresti, F., Batlouni, S. R., 2017. The effects of sGnRHa implants on Piaractus mesopotamicus female breeders. An approach addressed to aquaculture. Aquacult. Int., 25(6), 2259–2273. https://doi.org/10.1007/s10499-017-0186-2

Kuradomi, R.Y., Batlouni, S.R., 2018. PGF2α and gonadal steroid plasma levels of successful and unsuccessful spawning Piaractus mesopotamicus (Teleostei, Characiformes) females. Aquacult. Int. 26, 1083–1094. https://doi.org/10.1007/s10499-018-0269-8

Kagawa, H., Gen, K., Okuzawa, K., Tanaka, H., 2003. Effects of luteinizing hormone and follicle-stimulating hormone and insulin-like growth factor-I on aromatase activity and P450 aromatase gene expression in the ovarian follicles of red seabream, Pagrus major. Biol. Reprod. 68, 1562–1568. https://doi.org/10.1095/biolreprod.102.008219

Lister, A.L., Van Der Kraak, G., 2008. An investigation into the role of prostaglandins in zebrafish oocyte maturation and ovulation. Gen. Comp. Endocrinol. 159 (1), 46–57. https://doi.org/10.1016/j.ygcen.2008.07.017

Lubzens, E., Young, G., Bobe, J., Cerdà, J., 2010. Oogenesis in teleosts: how fish eggs are formed. Gen. Comp. Endocrinol. 165 (3), 367–389. https://doi.org/10.1016/j.ygcen.2009.05.022

Morgan, M. J., Wilson, C. E., Crim, L. W., 1999. The effect of stress on reproduction in Atlantic cod. J. Fish Biol., 54(3), 477–488. https://doi.org/10.1111/j.1095-8649.1999.tb00629.x

Moro, G. V., Rezende, F. P., Alves, A. L., Hashimoto, D. T., Varela, E. S., Torati, L. S., 2013. Espécies de peixe para piscicultura. In Embrapa. Piscicultura de água doce: multiplicando conhecimentos (pp. 36-37). Embrapa pesca e aquicultura.

Mourad, N. M. N., Costa, A. C., Freitas, R. T. F., Serafini, M. A., Neto, R. V. R., Felizardo, V. O., 2018. Weight and morphometric growth of Pacu (Piaractus mesopotamicus), Tambaqui (Colossoma macropomum) and their hybrids from spring to winter. Pesquisa Veterinaria Brasileira, 38(3), 544–550. https://doi.org/10.1590/1678-5150-PVB-4808

Murugananthkumar, R., Sudhakumari, C., 2022. Understanding the impact of stress on teleostean reproduction. Aquac. Fisheries, 7 (2022), 553-561. https://doi.org/10.1016/j.aaf.2022.05.001

Ogiwara, K., Takahashi, T., 2016. A Dual Role for Melatonin in Medaka Ovulation: Ensuring Prostaglandin Synthesis and Actin Cytoskeleton Rearrangement in Follicular Cells1. Biol. Reprod., 94(3), 1–15. https://doi.org/10.1095/ biolreprod.115.133827

Peixe BR, 2021. Anuário 2021 Peixe BR da piscicultura, 34.

Peixe BR, 2022. Anuário 2022 Peixe BR da piscicultura, 12–14.

Sato, R. T., Kuradomi, R. Y., Calil, M. C., Silva, L. M. J., Roza de Abreu, M., Figueiredo-Ariki, D. G., Batlouni, S. R., 2020. Resumption and progression of meiosis and circulating levels of steroids and prostaglandin F2α of Piaractus mesopotamicus induced by hypophysation with prostaglandin F2α. Aquac. Res. 52 (3), 1026–1037. https://doi.org/10.1111/are.14957

Schober, P.; Boer, C.; Schwarte, L.A. 2018. Correlation Coefficients. Anesthesia & Analgesia, 126: 1763-1768. https://doi.org/10.1213/ANE.0000000000002864

Schorer, M., Moreira, R. G., Batlouni, S. R., 2016. Selection of pacu females to hormonal induction: Effect of age and of evaluation methods. Boletim do Instituto De Pesca, 42(4), 901–913. https://doi.org/10.20950/1678-2305.2016v42n4p901

Souza, V. L., Urbinati, E. C., Martins, M. I. E. G., Silva, P. C., 2003. Avaliação do Crescimento e do Custo da Alimentação do Pacu (Piaractus mesopotamicus Holmberg, 1887) Submetido a Ciclos Alternados de Restrição Alimentar e Realimentação. Revista Brasileira De Zootecnia, 32, 19–28. https://doi.org/10.1590/S1516-35982003000100003

Stacey, N. E., Pandey, S., 1975. Effects of indomethacin and prostaglandins on ovulation of goldfish. Prostaglandins, 9(4), 597–607. https://doi.org/10.1016/0090-6980(75)90065-9

Takahashi, T., Fujimori, C., Hagiwara, A., Ogiwara, K., 2013. Recent Advances in the Understanding of Teleost Medaka Ovulation: The Roles of Proteases and Prostaglandins. Zoological Science, 30(4), 239–247. https://doi.org/10.2108/zsj.30.239

Takahashi, T., Hagiwara, A., Ogiwara, K., 2018. Prostaglandins in teleost ovulation: A review of the roles with a view to comparison with prostaglandins in mammalian ovulation. Mol. Cell. Endocrinol., 461, 236–247. https://doi.org/10.1016/j.mce.2017.09.019

Tang, H., Liu, Y., Li, J., Li, G., Chen, Y., Yin, Y., Lin, H., 2017. LH signaling induced ptgs2a expression is required for ovulation in zebrafish. Mol. Cell. Endocrinol. 447, 125–133. https://doi.org/10.1016/j.mce.2017.02.042.

Urbinati, E. C., Takahashi, L. S., 2020. Pacu (Piaractus mesopotamicus). In B. Baldisseroto (org). Espécies nativas para piscicultura no Brasil (pp 169-170). Editora UFSM.

MANUSCRITO 2

Circadian melatonin rhythms and its associations on reproductive performance of pacu (*Piaractus mesopotamicus*)

Manuscrito nas normas da revista *Aquaculture*

Circadian melatonin rhythms and its associations on reproductive performance of pacu (*Piaractus mesopotamicus*)

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Abstract

This study aimed to describe the circadian rhythm of melatonin (MTN) in pacu (*Piaractus mesopotamicus*) females and based on these results, evaluate the reproductive performance at different times of application of the resolving dose of hypophysation. For this, in experiment 1, 18 females were divided into three groups (n=6 per group): control (no hormone induction); saline control (0.9% NaCl); and hypophysation (0.6 and 5.4 mg/kg carp pituitary extract). Animals in all three groups had their blood collected every four hours between 7 am on the first day and 7 am on the second day for quantification of MTN plasma levels over 24 hours (i.e., seven collections). In experiment 2, based on the results of experiment 1, we selected two times to apply the resolving dose of hypophysation associated with exogenous prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), at 7 pm (19H) (increased MTN levels) and at midnight (0H), and thereby compare the reproductive performance of the groups and the plasma levels of $PGF_{2\alpha}$, 17α-20β-dihydroxy-4-pregnen-3-one (DHP) and MTN. The results showed that MTN plasma levels were elevated at the beginning of the night at 7 pm of day 1 and decreased at 3 pm of day 2, regardless of whether they were hypophysized or not. Ovulation rates were 75% and 50% for the 19H and 0H groups, respectively. The 19H group did not show poor-quality spawning (i.e., fecundity <35,000 oocytes/kg fish). In addition, in absolute numbers, the 19H group produced almost three times as many larvae (737,567) as the 0H group (250,762). The ATU values were similar between the groups, indicating that the timing of induction does not interfere with the latency period. $PGF_{2\alpha}$ and DHP levels were similar between groups at each collection but higher at the time of ovulation. MTN levels were also similar between groups. We observed higher MTN levels at the time of ovulation only in the 19H group, which spawned near sunrise. Furthermore, MTN levels at ovulation were positively correlated with fecundity, fertility rate, hatching rate and the number of hatched larvae/kg fish. The highest embryo viability of the 19H group suggests that there may be an association between spawning success and the timing of the resolving hormone dose for pacu. These results indicate a possible optimization in reproductive performance when females are subjected to a more extended period of darkness after the resolving dose, with its application near the beginning of the night (19H group).

Keywords: circadian rhythm, migratory fish, ovulation rate, spawning

1. Introduction

The photoperiod is one of the main factors that regulate behavioral and physiological processes, including those reproductive (Chattoraj et al., 2005; Falcon et al., 2007; 2010; de Alba et al., 2019; Paredes et al., 2019). This variable presents itself in a circadian manner, that is, alternating between a light period and dark period over approximately 24 hours and has as one of its main agents a neurohormone called melatonin (MTN) (Falcon et al., 2007; 2010).

MTN is one of the main hormones modulating circadian clocks in vertebrates, sending rhythmic information (i.e., circadian and circannual rhythms) to animals. This hormone is synthesized in the pineal gland following a circadian rhythm, resulting in high blood levels at night and low levels during the day (Falcon et al., 2010; Ogiwara and Takahashi, 2016), and MTN levels during the day are species-specific. Falcon et al., (2010) have defined three different types of MTN profiles throughout the day (a) discrete peak in late dark phase – cod *Gadus morhua* (b) discrete peak in mid dark phase – Nile tilapia (*Oreochromis niloticus*) (c) Prolonged peak through the majority of the dark phase – Atlantic salmon and rainbow trout. Circadian plasma oscillations of MTN can vary according to the annual reproductive phase of the species (Maitra et al., 2013). In pineal cells, MTN is synthesized from tryptophan via a metabolic pathway involving serotonin and several enzymes, among them arylalkylamine N-acetyltransferase (AANAT) and hydroxyindole-O-methyltransferase (HIOMT) (Falcon et al., 2010). In fish, in response to photoperiod, the retina and pineal organ transduce two types of information, neural (reaching the ventral diencephalon) and hormonal (via MTN). In the retina, the MTN is an autocrine and/or paracrine factor, whereas the pineal MTN is released in the cerebrospinal fluid and in the blood, reaching specific targets through the MTN receptors, such as the hypothalamus, the pituitary itself and other tissues (gonads, liver, among others).

Switching between light and dark periods in approximately 24 hours (circadian rhythm) influences behaviors such as locomotor activity, skin pigmentation, oxygen consumption, thermoregulation, food intake, and schooling. In addition, animals also detect these photoperiod changes annually (circannual rhythms), acting on physiological functions such as growth, immune response, migration, and reproduction (Falcon et al., 2007; 2010). The manipulation of photoperiod causes oscillations in MTN levels in the body, which in turn may alter: in the hypothalamus, the secretion of gonadotropin-releasing hormone (GnRH) (Takahashi et al, 2018) or gonadotropin-inhibitory hormone (GnIH) (Zohar et al., 2010; Maitra et al., 2013) via kisspeptin (Zohar et al., 2010; Maitra et al., 2013); in the pituitary gland, the synthesis of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Falcon et al., 2010); and in the gonads, the production of reproductive hormones, such as 17α-20β-dihydroxy-4-pregnen-3-one (DHP) and prostaglandins (Chattoraj et al., 2005; 2008; Carnevalli et al., 2011; Ogiwara and Takahashi, 2016). These MTN stimuli can both increase and decrease the secretion of these hormones according to the oscillation of their levels. In this context, in a study with male and female zebrafish (*Danio rerio*), Paredes et al. (2019) observed higher GnRH levels in the dark period (higher MTN levels), while the highest *lhβ* and *fshβ* expressions were recorded in the light period (lower MTN levels). On the other hand, in males and females of tilapia (*Oreochromis niloticus*), higher levels of *lhβ* and *fshβ* expressions were found in the dark period and higher levels of GnRH in the light period (de Alba et al., 2019). Furthermore, female Atlantic croaker (*Micropogonias undulatus*) had higher plasma LH levels in the early dark period (Khan and Thomas, 1996). Thus, the circadian hormonal oscillations observed during the photoperiod seem to be species-specific, differing from the reproductive strategy of each fish (Blanco-Vives and Sánchez-Vázquez, 2009; Paredes et al., 2019).

In Atlantic croaker females, application of MTN raised LH plasma levels and stimulated, in vitro, pituitary LH secretion (Khan and Thomas, 1996). Chattoraj et al. (2005) observed that, in carp oocytes (Catla catla; Labeo rohita; and C. Carpio), MTN accelerated the action of DHP when added four hours before DHP, resulting in an anticipation of final maturation (GVBD). Similar results were found in zebrafish follicles in vitro, demonstrating increased GVBD rate with the addition of MTN to DHP treatment (Carnevali et al., 2011). Furthermore, in *C. catla*, final maturation could be reached with MTN incubation alone (Chattoraj et al., 2008). In a study with medaka (*Oryzias latipes*) females, Ogiwara and Takahashi (2016) observed that the use of MTN antagonists (luzindole) in ovarian follicles in vitro reduced prostaglandin E_2 (PGE_2) levels and was a potent ovulation inhibitor. In the same study, MTN was associated with increased expression of cytosolic phospholipase A2 group 4a (*pla2g4a*), an enzyme that releases arachidonic acid from phospholipid membranes with subsequent conversion to PGE_2. In addition, Ogiwara and Takahashi (2016) observed that MTN activates moesin A (associated with cell shape changes) in follicles, a protein that, when phosphorylated acts similarly to PGE_2 (i.e., acts in depolymerizing the actin cytoskeleton in granulosa cells propitiating ovulation).

Despite several studies linking circadian rhythms and reproduction in fish, little information is found in South American aquaculture species. Muniz et al. (2008), seeking to employ the concept of circadian rhythm (photoperiod) with the period of hormonal induction of tambaqui (*Colossoma macropomum*) applied the resolving dose at the beginning of the dark period (spawning expected at dawn), or at the beginning of the light period (spawning expected at dusk). The authors observed that only females induced in the dark period spawned. In this scenario, it is possible to suggest that, from the knowledge of the circadian oscillations of MTN, the adjustment in the hormone induction period with higher circadian levels of this hormone, may result in an optimization in ovulation rates, a major problem found in South American migratory species reared in captivity (Hainfellner et al, 2012; Pereira et al., 2017; 2018; De Souza et al., 2020; Roza de Abreu et al., 2020 and 2022), especially of pacu (*Piaractus mesopotamicus*) (Criscuolo-Urbinati et al., 2012; Kuradomi and Batlouni, 2018).

Pacu, the target species of this study, is rheophilic and needs to be hormonally stimulated for induction to spawn in captivity. Protocols using carp pituitary extract (i.e., hypophysation) are the main and most common methods for induced reproduction of South American migratory species (Borella et al., 2020; Criscuolo-Urbinati et al., 2012; Kuradomi & Batlouni, 2018). However, this methodology still presents shortcomings related to ovulation, and consequently, the unpredictability of spawning success (Criscuolo-Urbinati et al., 2012; Kuradomi and Batlouni, 2018).

Some species as the zebrafish have a constant spawning schedule, in this case in the morning (Blanco-Vives e Sánchez-Vásquez, 2009; Paredes et al., 2019). Knowing that the endogenous rhythm of substances such as MTN regulate the reproduction and ovulation of the species, the knowledge of the spawning time in a natural environment could provide a synergy between endogenous and exogenous agents (hormonal induction) favoring the reproductive performance making the species ovulate at their usual time in nature. However, this information does not exist for pacu and most migratory native species.

Therefore, considering this lack of knowledge, the present study had as an initial objective to determine in captivity and in an unprecedented way the circadian plasma oscillation pattern of MTN in pacu over 24 hours and to evaluate if there are differences between females submitted or not to hypohysation. As a second objective, we evaluated the plasma profiles of DHP, $PGF_{2\alpha}$ and MTN by subjecting pacu females to hormone induction in two distinct periods routinely used in fish farms. We evaluated if females exposed to a longer period of darkness between resolving dose and ovulation showed any gain in reproductive performance.

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 003837/19.

2.1. Origin, characteristics and management of fish

The experiments were carried out during the pacu reproductive season, in November 2020 (experiment 1) and December 2021 (experiment 2) at the UNESP Aquaculture Center (CAUNESP), located in Jaboticabal, São Paulo, Brazil (21º15'17"S and 48º19'20"W). The broodstock was maintained throughout the year in 200 m^2 earthen ponds (at a density of 0.80 kg/m^2), supplied with a constant flow of approximately 20 L/min. The fish were fed apparent satiety twice a day (approximately 1-4% of the biomass) with commercial feed Omnivores Growth - Fri-Acqua, containing 28% crude protein, 3.5% ether extract, 12% ash and 9 % of fibrous matter, according to the manufacturer's information. The fish used were produced at CAUNESP from crossings carried out with the broodstock already existing at the Institution.

Water parameters were determined every two weeks at 7 am. Temperature and conductivity were measured using a HANNA probe, HI-98311; pH was measured using a KASVI probe, K39-0014P and dissolved oxygen with a BERNAUER-424 AQUACULTURE probe, F-1550A. The mean ± standard deviation of pH, dissolved oxygen concentration, conductivity and water temperature, were, at 6.05 ± 0.72, 6.21 ± 0.42 mg/L, 32.10 ± 15.65 µS/cm and 25.21 ± 3.71 °C, respectively, in experiment 1 and, 6.12 ± 0.31, 5.80 ± 0.55 mg/L, 33.89 ± 11.24 µS/cm and 24.24 ± 3.56 °C respectively, in experiment 2.

2.2. Broodstock selection

Females were selected based on external characteristics, such as a swollen abdomen (Schorer et al., 2016) and hemorrhagic urogenital papilla (Kuradomi et al., 2017). Males were selected by releasing semen under slight abdominal pressure (Kuradomi et al., 2016). Then, selected animals were transported to the laboratory in transport boxes and maintained in 750 L tanks coupled to a water recirculation system at constant temperature (experiment 1: 26.78 ± 0.22ºC; experiment 2: 27,04 ± 0.25ºC). Each fish was considered as an experimental unity.

2.3. Hormonal induction protocol

Hormonal induction was divided into two intramuscular injections with an interval of 24 hours between them. Females were anesthetized with 100 mg/L of benzocaine solution (Sigma-Aldrich, Saint Louis, USA) and received two doses of crude carp pituitary extract (CPE) (0.6 and 5.4 mg/kg) suspended in 0.25 mL/kg of 0.9% saline solution. The saline group only received 0.9% saline solution in both injections and the same volume (i.e., 0.25 mL/kg), whereas the control group did not receive injection during the experimental period. The induction with exogenous $PGF_{2\alpha}$ was performed via intramuscular injection with 2 mL of Ciosin®/fish (containing 0.25 mg/mL of cloprostenol, MSD Animal Health) for experiment 2. The males received 3 mg/kg of CPE at the time of the resolving dose for females.

2.4. Experiment 1

2.4.1. Experimental design

Eighteen adult females with 3,412.50 ± 192.89 g of body mass (mean ± SEM) were distributed in a completely randomized design into three groups (n = six females per group): control group (C), fish were not handled; saline control (CS), fish received 0.9%

saline solution in both injections; and hypophysation (H), classic hypophysation with carp pituitary extract (Figure 1).

On day 1 of the experiment, sunrise (i.e., beginning of the day) occurred at 5:14 am and sunset (i.e., beginning of the night) at 6:29 pm, totaling a photoperiod of 13 hours and 11 minutes. On day 2, sunrise also occurred at 5:14 am. Due to the constant management of the females, throughout the hypophysation, we did not carry out reproductive performance analyzes.

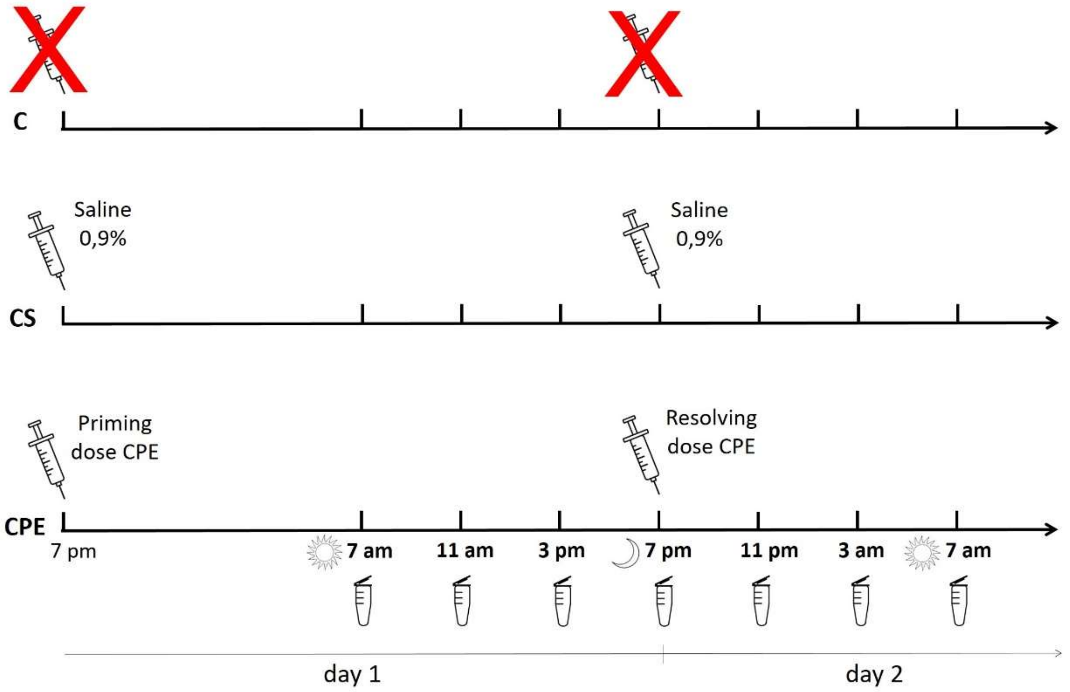

Figure 1. Experiment 1. Experimental design of induction over time in *Piaractus mesopotamicus* females distributed in three groups: C (control – fish were not handled); CS (saline control – fish received 0.9% saline solution in both injections); and CPE (classic hypophysation with carp pituitary extract) (n=6 females per group). Seven collections were performed every four hours between 7 am on day one and 7 am on day two (represented by microtubes). The moon represents the beginning of the night, and the sun represents the beginning of the day. CPE: carp pituitary extract. ATU: accumulated thermal units.

2.4.2. Melatonin plasma levels quantification

Blood collections were performed every four hours between 7 am on day one and 7 am on day two, totaling 7 collections per animal (Figure 1). For blood collection, fish were anesthetized with 100 mg/L of benzocaine (Sigma-Aldrich, Saint Louis, USA); blood samples were collected from the caudal vein using heparin-treated syringes. The plasma was separated by centrifugation at 1000×g for 15 min at 4 °C and then stored at -80 °C until measurement of the MTN hormone concentrations. The plasma levels of MTN were quantified by Enzyme-Linked ImmunoSorbent Assay (ELISA) using commercial kits (IBL International, Hamburg, Germany) following the manufacturer's instructions. The readings plates were performed at an absorbance of 405 nm, using an Epoch2 plate reader (BioteK Instruments, Inc., Highland Park, Winooski, USA), and all samples were read in duplicate. To validate the MTN levels analysis, intra- and inter-assay variability were assessed and we obtained the respective following variations: 2.31 to 16.28% and 3.47 to 17.35%.

2.4.3. Statistical analysis

All statistical tests were performed using the STATISTICA software (StatSoft, Inc., Tulsa, OK, USA) and Excel (Microsoft, Redmond, USA). Normality and homogeneity of the variances were tested using Shapiro-Wilk and Levene's test, respectively.

MTN plasma levels showed normal distribution and homoscedasticity being compared by repeated measures ANOVA test followed by a multiple comparisons test (Fisher LSD). Significant differences were accepted at a p-value <0.05. Data were expressed as mean ± standard error of the mean (SEM).

2.5. Experiment 2

2.5.1. Experimental design

Eight adult females with 3,176.25 ± 292.17 g of body mass (mean ± SEM) were distributed in a completely randomized design into two groups (n = four females per group): 7 pm group (19H), classic hypophysation associated with exogenous $PGF_{2\alpha}$ applied at 7 pm; midnight group (0H), classic hypophysation associated with exogenous $PGF_{2\alpha}$ applied at midnight (Figure 2). On the day of the experiment (resolving dose), sunset occurred at 6:55 pm, and the next morning (spawning day) sunrise occurred at 5:19 am, totaling a photoperiod of 13 hours and 36 minutes. Knowing that ovulation in this species normally occurs between 276 and 323 ATU (Criscuolo-Urbinati et al., 2012), that is, between 10 and 12 hours after the resolving dose at 27ºC, we stipulate an expected spawning period between 270 and 340 ATU for this study.

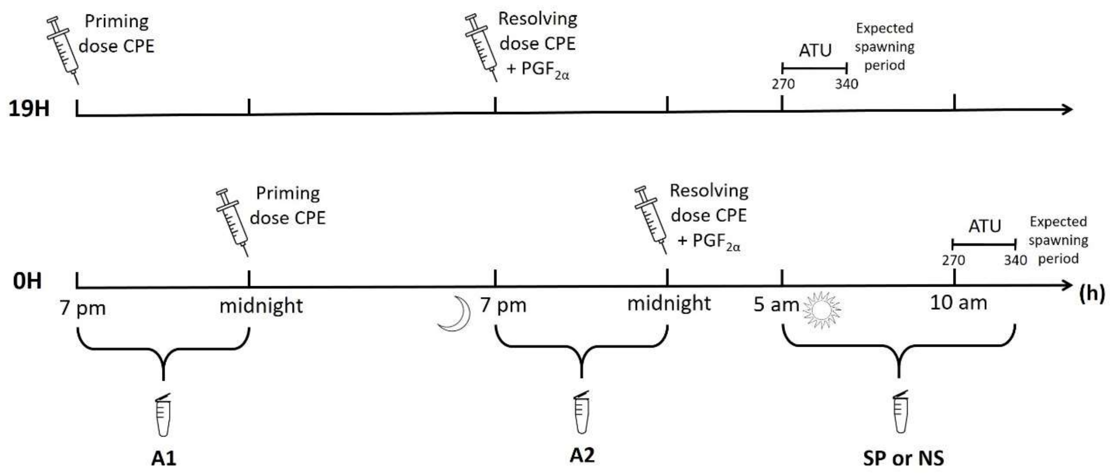

Figure 2. Experiment 2. Experimental design of different times of hypophysation associated with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in *Piaractus mesopotamicus* females distributed in two groups: induction at 7 pm (19H) and induction at midnight (0H), in spawned females (SP) (up to 340 ATU) or non-spawned females (UN) (considered for not spawning before 340 ATU) (n=4 females per group). The moon represents the beginning of the night, and the sun represents the beginning of the day. CPE: Carp pituitary extract. ATU: accumulated thermal units.

2.5.2. Reproductive performance

After observing the reproductive behavior of the females in the tanks (i.e., restlessness and muscle spasms in the abdomen) and/or observing the first oocytes released at the bottom of the tanks (Kuradomi and Batlouni, 2018), the process of extruding the eggs was carried out through abdominal massage (between 260 and 340 ATU). The oocytes were extruded into a dry container and the oocyte mass and spawning time were recorded. Females with fecundity lower than 35,000 oocytes/kg of fish were considered females with poor-quality spawning. Females that presented poor-quality spawning (i.e., partial ovulation failure) released oocytes in the form of “clumps” with blood which compromised their fertility and hatching rates.

Fertilization was obtained by adding a pool of semen from three males (in the proportion of 0.5 mL of pooled semen for each 50 g of oocytes). The sperm concentration in pacu ranges from approximately 4.7 to 6.9 x 10^{10} cells/mL (Kuradomi et al., 2016) and the average number of oocytes present in one gram of spawn is around 1,200 (Cecarelli et al., 2000). The oocytes were fertilized and hydrated according to conventional methodology, adding water after mixing the gametes. Then, 10 mL of eggs from each female were transferred to 8 L acrylic incubators in triplicate at an average temperature of 26.8 ± 0.3 ºC. Data were collected for ovulation rate, ATU, relative fecundity, number of hatched larvae/kg of fish and total number of hatched larvae. The estimated rates of fertility (blastopore closure) and hatching (tail moving and fully unfolded) were performed about 12 and 18 hours after eggs fertilization, respectively (Schorer et al., 2016; Kuradomi and Batlouni, 2018). To obtain fertility and hatching rates roughly 100 eggs from each incubator (triplicate) were classified as viable or non-viable embryos.

The following parameters were recorded:

Ovulation rate (%) = number of ovulated females / total number of females x 100

ATU = mean of water temperature (ºC) x latency period (h)

Relative fecundity = number of oocytes released / female body mass (kg)

Fertility rate (%) = number of viable embryos / total number of eggs x 100

Hatching rate (%) = number of viable embryos / total number of eggs x 100

Number of hatched larvae/kg of fish = number of released oocytes × hatching rate (%) / female body mass (kg)

Total number of hatched larvae = sum of all females in each group

2.5.3. *Plasma levels quantification of DHP, $PGF_{2\alpha}$ and MTN*

Blood collections were performed at three time points: at the time of the first dose (A1); at the time of the resolving dose (A2); and at the time of ovulation (SP or NS) when females were classified as spawned up to 340 ATU or non-spawned females, which did not spawn before 340 ATU (Kuradomi and Batlouni, 2018) (Figure 2). The blood collection procedure and its storage were performed as described in experiment 1. For $PGF_{2\alpha}$ dosage, 10 µM indomethacin was added to the blood immediately after collection to inhibit prostaglandin synthesis. The plasma levels quantification was carried out by ELISA using commercial kits for 17α-20β-dihydroxy-4-pregnen-3-one (DHP) and $PGF_{2\alpha}$ (Cayman Chemical Company, Ann Arbor, MI, USA) and MTN (IBL International, Hamburg, Germany) following the manufacturer's instructions. The readings of DHP, $PGF_{2\alpha}$ and MTN plates were performed at an absorbance of 412, 405 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 analysis, intra- and inter-assay variability were assessed and we obtained the respective following variations: 0.45 to 18.12% and 0.71 to 17.89% for DHP; 1.54 to 18.37% and 8.71 to 19.41% for $PGF_{2\alpha}$; 0.62 to 13.25% and 0.78 to 16.66% for MTN.

2.5.4. Statistical analysis

The statistical tests were performed with the same software used in experiment 1, as well as assumptions such as normality and homoscedasticity. The mean reproductive performance of spawned females (including females with poor-quality spawning) showed normal distribution and homoscedasticity and were compared by the t-test. Plasma levels were correlated with reproductive performance parameters (independent of group) using Spearman's nonparametric rank correlation test. In the correlation event, negligible correlation is considered with ρ (rô) values from 0.0 to 0.10, weak from 0.10 to 0.39, moderate from 0.40 to 0.69, strong from 0.70 to 0.89 and very strong from 0.90 to 1.0, according to a conventional method of interpreting correlation coefficients (Schober et al., 2018). Significant differences were accepted with a P value <0.05. Data were expressed as mean ± SEM and correlation in ρ value.

3. Results

3.1. Experiment 1

3.1.1. Melatonin plasma levels

We first observed that the circadian rhythm of MTN in plasma was similar between groups (p>0.05) (Figure 3).

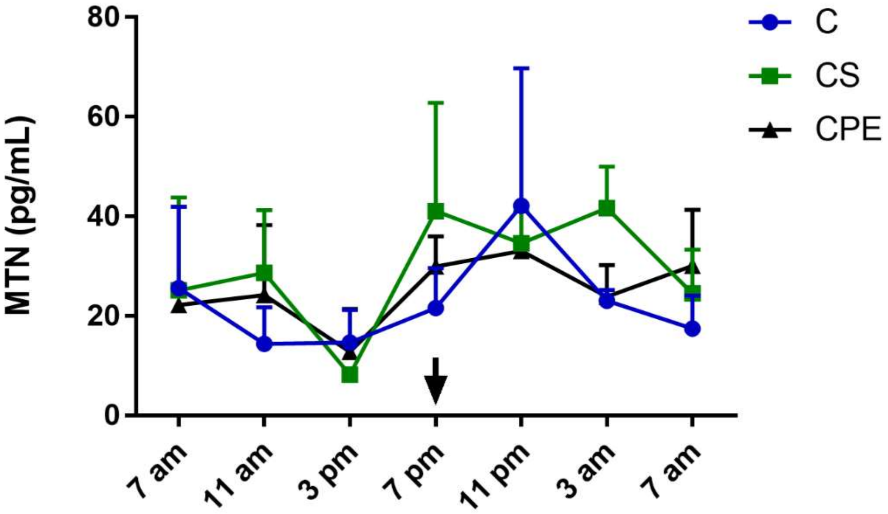

Figure 3. Experiment 1. Circadian plasma profile of Melatonin (MTN) in *Piaractus mesopotamicus* females distributed into three groups: C (control – fish were not handled); CS (saline control – fish received 0.9% saline solution in both injections); and CPE (classic hypophysation with carp pituitary extract) (n=6

females per group) at 7 am on day one until 7 am on day two (seven collections). Data are expressed as mean ± SEM. There were no significant differences among groups ($p>0.05$). Arrow indicates the moment of the resolving dose.

We, therefore, used all females, regardless of group, in order to analyze the circadian rhythm of MTN throughout the day. Thus, the plasma profile of MTN starts at 7 am (26.04 ± 6.91 pg/mL), decrease in the afternoon (3 pm) (10,44 ± 2,89 pg/mL) ($p<0.05$), increase at the beginning of the night (7 pm) (31.91 ± 6,05 pg/mL) ($p<0.05$) and remain similar until the beginning of the morning (7 am) (27.38 ± 4.42 pg/mL) ($p>0.05$) of the following day (Figure 4).

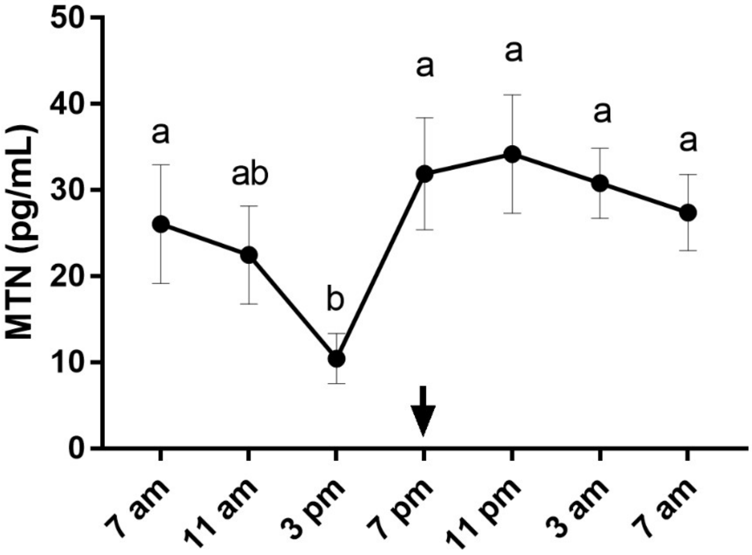

Figure 4. Experiment 1. Circadian plasma profile of Melatonin (MTN) in *Piaractus mesopotamicus* females (n=18) from 7 am on day one to 7 am on day two (seven collections). Data are expressed as mean ± SEM. Different letters indicate significant differences over time ($p<0.05$). Arrow indicates the moment of the resolving dose.

3.2. Experiment 2

3.2.1. *Plasma levels of PGF$_{2α}$, DHP and MTN*

There were no significant differences in the plasma levels of PGF$_{2α}$, DHP and MTN between the groups (19H and 0H) regardless of the time of collection ($p > 0.05$) (Figure 5).

At ovulation, PGF$_{2α}$ levels were notably higher than the levels of these substances determined at collections A1 (≈1,300-fold) and A2 (≈1,200-fold) ($p<0.05$) (Figure 5A).

Similar to $PGF_{2\alpha}$, DHP levels were higher at ovulation compared to the levels of these substances determined in collections A1 (≈17-fold) and A2 (≈ 14-fold) ($p<0.05$) (Figure 5B).

Regarding MTN, females in the 0H group showed similar levels among collections, while females in the 19H group obtained higher levels at the time of ovulation when compared to collections A1 (≈4-fold) and A2 (≈6-fold) ($p<0.05$) (Figure 5C).

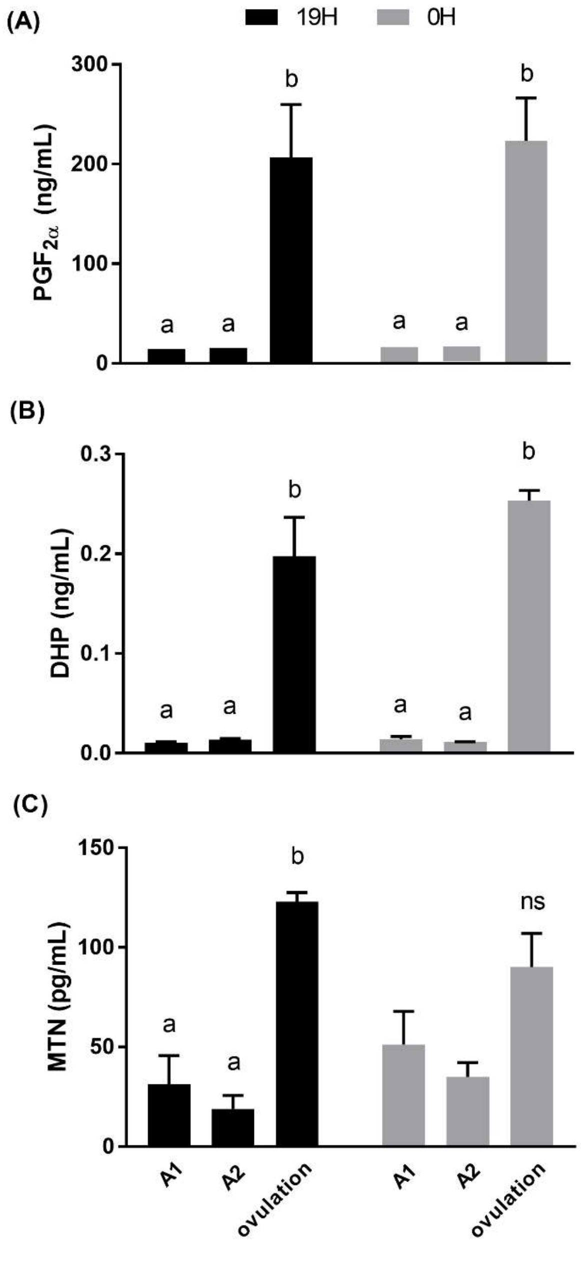

Figure 5. Experiment 2. Plasma levels of (A) prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), (B) 17α-20β-dihydroxy-4-pregnen-3-one (DHP) and (C) melatonin (MTN) in *Piaractus mesopotamicus* females at the time of the priming dose (A1); the resolving dose (A2); and ovulation, in spawned females (up to 340 ATU) or non-spawned females (considered for not spawning before 340 AUT) (n=4). 19H: hypophysation at 7 pm; 0H: hypophysation at midnight. Data expressed as mean ± SEM. Different letters indicate significant differences among collections ($p<0.05$). There were no significant differences among groups regardless of the time of collection ($p>0.05$). Plasma levels of MTN in the 0H group were similar among collections (ns) ($p>0.05$).

3.2.2. Reproductive performance

The 19H group, which stayed longer between resolving dose and ovulation in the dark (from 7 pm to 5:19 am - about 10 hours), showed 75% of ovulation rate, while the

0H group, which stayed approximately 5 hours in the dark after resolving dose (from midnight to 5:19 am), showed 50% of ovulation rate. In addition, females in the 19H group did not show poor-quality spawning (i.e., <35,000 oocyte/kg). Relative fecundity, ATU, fertility and hatching rates, and the number of hatched larvae/kg fish were similar between groups (p>0.05) (Table 1).

Table 1. Experiment 2. Reproductive performance of *Piaractus mesopotamicus* submitted to hormonal induction at two different times.

Groups	**Ovulation rate**	**Poor-quality spawning***	**ATU**	**Relative fecundity**	**Fertility rate (%)**	**Hatching rate (%)**	**Number of hatched larvae/kg fish**	**Total number hatched larvae**
19H	3/4 (75%)	0/3 (0%)	309.7 ± 12.8	114,120 ± 17,498.7	80.0 ± 15.3	71.3 ± 18.4	86,236 ± 28,414	737,567
0H	2/4 (50%)	1/2 (50%)	305.0 ± 5.8	77,820 ± 44,820	58.5 ± 33.4	49.0 ± 36.2	54,346 ± 50,126	250,762

19H: application of resolving dose of hypophysation + prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) at 7 pm; 0H: application of resolving dose of hypophysation + $PGF_{2\alpha}$ at midnight. The females submitted to hypophysation with two doses (0,6mg/kg e 5,4mg/kg) with an interval of 24 hours between doses associated with $PGF_{2\alpha}$ in the resolving dose. ATU: Accumulated thermal units; Relative fecundity: number of released oocytes/kg fish. Data expressed by mean ± SEM.
*Female considered as poor-quality spawning presented fecundity < 35,000 oocyte/kg fish

In the individual analysis, we highlight that female 1 (19H group) and 6 (0H group) presented low hatching rates (35.5 and 12.8%), the former with relatively high ATU values (330 ATU) and the latter associated with low fecundity (33,000 oocytes/kg fish) (Table 2). Females 2 and 4 (19H group) and female 8 (0H group) showed satisfactory reproductive performance, i.e., high fecundities, fertility and hatching rates and number of hatched larvae/kg fish.

Table 2. Experiment 2. Individual analysis of reproductive performance and plasma hormone levels of *Piaractus mesopotamicus* females at the time of ovulation and submitted to hormonal induction at two different times.

Female	Groups	ATU	Relative fecundity	Fertility rate (%)	Hatching rate (%)	Number of hatched larvae/kg fish	$PGF_{2\alpha}$ (ng/mL)	DHP (ng/mL)	MTN (pg/mL)
1	19H	330.0	83,400	49.6	35.5	29.591	167.24	0.16	119.29
2	19H	312.2	144,000	92.2	82.3	118.512	119.16	0.16	123.74
3	19H	-	-	-	-	-	177.37	0.31	113.57
4	19H	286.0	114,960	98.2	96.2	110.605	362.26	0.16	135.17
5	0H	-	-	-	-	-	351.66	0.26	91.47
6	0H	310.8	33,000*	25.1	12.8	4.219	170.23	0.25	42.51
7	0H	-	-	-	-	-	182.34	0.28	116.22
8	0H	299.2	122,640	91.9	85.2	104.472	188.96	0.22	110.76

19H: application of resolving dose of hypophysation + prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) at 7 pm; 0H: application of resolving dose of hypophysation + $PGF_{2\alpha}$ at midnight. The females submitted to hypophysation with two doses (0,6mg/kg e 5,4mg/kg) with an interval of 24 hours between doses associated with $PGF_{2\alpha}$ in the resolving dose. ATU: Accumulated thermal units; Relative fecundity: number of released oocytes/kg fish. Data expressed by mean ± SEM. *Female considered as poor-quality spawning presented fecundity < 35,000 oocyte/kg fish

3.2.3. *Correlation*

Correlations between variables were analyzed regardless of group. Positive correlations from strong to moderate were observed between plasma levels of MTN and either fertility rate or hatching rate or number of larvae per kg of fish and relative fecundity (Table 3). Furthermore, negative and strong correlations were found between $PGF_{2\alpha}$ x ATU, ATU x hatching rate (Table 3).

Table 3. Experiment 2. Spearman's correlation.

Correlation	ρ	Rate
MTN x fertility rate	0,90	Strong
MTN x hatching rate	0,70	Strong
MTN x nº larvae/kg fish	0,80	Strong

MTN x fecundity	0,60	Moderate
$PGF_{2α}$ x ATU	-0,90	Strong
ATU x hatching rate	-0,70	Strong

MTN: melatonin; Fertility and hatching rate: number of viable embryos/total numbers of eggs; Fecundity: number of oocytes released/kg fish; $PGF_{2α}$: Prostaglandin $_{2α}$; ATU: Accumulated thermal units.
Rate: following (Schober et al., 2018)

4. Discussion

In this study, we defined in an unprecedented way the circadian variation of MTN levels in a native tropical species produced throughout South America, the pacu. Our results confirmed like several other species studied, that pacu females present an increase in MTN plasma levels during the dark period. Hence, we observed higher MTN levels at the beginning of the night (7 pm), levels that remain similar until 11 am, reducing at 3 pm. Thus, analyzing it descriptively, we observed a profile similar to that of type b described by Falcon et al. (2010), with an increase in the early evening, a slight MTN peak in the middle of the dark phase, with a slight decrease in the morning. The type b has also been observed in medaka (Ogiwara and Takahashi, 2016), African catfish (*Clarias gariepinus*) (Martinez-Chavez et al., 2008), Nile tilapia (Martinez-Chavez et al., 2008) and common dentex (*Dentex dentex*) (Pavlidis et al., 1999). The basal (mean 11.3 pg/mL) and during the dark period (from 29.9 to 34.2 pg/mL) circulating MTN values were similar to those found in tropical species such as Nile tilapia and African catfish (Martinez-Chavez et al., 2008). It was also observed that there was no difference among the groups, suggesting that MTN levels are not LH-dependent, as found in the study by Ogiwara and Takahashi (2016).

Regarding experiment 2, we observed that the application of the resolving dose earlier (group 19H), caused an ovulation rate of 75% and an absence of poor-quality spawning (fecundity lower than 35,000 oocytes/kg fish), while in group 0H the ovulation rate was 50%, besides presenting 50% of poor-quality spawning. No statistical differences in ATU were observed between groups, suggesting that the timing of induction does not interfere with the latency period (i.e., the interval between resolving dose and ovulation). Despite the low number of replicates, we observed differences in ovulation and poor-quality spawning rates when we changed the period of application of the resolving dose, corroborating the data obtained in a study by Muniz et al. (2008), in which they observed spawning only in the group that the resolving dose was applied between 6 pm and 8 pm (night period), a protocol similar to the one used in the present study. *In vivo* studies like ours and with fish over 2 kg are difficult to carry out, especially in the sampling and timing of hormone induction, because the fish need to be handled all at the same time. In this way, more studies need to be conducted with a greater level of depth in order to begin the baseline knowledge on this subject. However, as already mentioned, the present study and the previous study by Muniz et al. (2008), in tambaqui, indicate that the beginning of the night period may be adequate for the application of the resolving dose in these species.

Even though the two groups presented similar results in fecundity, fertility and hatching rate, the 19H group provided a greater absolute number of hatched larvae than the 0H group (737,567 and 250,762 respectively) with the same number of induced females, in addition to not having any poor-quality spawning (fecundity < 35,000 oocyte/kg fish).

Another point is that female 6 of the 0H group (with poor-quality spawning), had the lowest individual MTN level recorded in the experiment. Furthermore, plasma levels of MTN at the time of ovulation showed a moderate positive correlation with fecundity and a strong positive correlation with fertility and hatching rates and larvae/kg fish. These data corroborate the results of studies that associate high levels of MTN with increased fertility in zebrafish (Carnevali et al., 2011) and in fecundity and number of hatched larvae in killlifish (*Fundulus heteroclitus*) (Lombardo et al., 2014).

Regarding MTN plasma levels in experiment 2, we observed that only females from the 19H group showed higher levels at the time of ovulation compared to the A1 and A2 collections. This result is due to the different collection times in the two groups (19H and 0H), performed at the time of hormone inductions (19 and 0 hours, respectively) and spawning (≈6 and 11 hours, respectively). Corroborating with experiment 1, in experiment 2 we observed similar MTN plasma levels in the 0H group collections (at midnight and 11 am). However, the superiority of MTN levels at the time of ovulation in the 19H group (at approximately 6 am) compared to the A1 and A2 collections found in experiment 2, may be due to the time of ovulation of this group being closer to sunrise (≈40 minutes after sunrise) than the 7 am collection in experiment 1 (≈1 hour and 45 minutes after sunrise), since the rise in circulating levels of MTN occurs at night and decreases during the light period (data from the present study and previous studies: Pavlidis et al, 1999; Martinez-Chavez et al., 2008; Maitra et al., 2013; Ogiwara and Takahashi, 2016).

Plasma levels of $PGF_{2\alpha}$ and DHP at the time of ovulation were much higher compared to their levels in collections A1 and A2, corroborating with studies conducted in our laboratory, in which plasma levels of endogenous $PGF_{2\alpha}$ and DHP were higher near the time of ovulation in pacu (Kuradomi and Batlouni, 2018; Sato et al., 2020). Levels of $PGF_{2\alpha}$ and DHP were similar between groups at each collection, suggesting that elevations of these hormones, in hypophysed females, are more associated with hormone induction than the photoperiod between the resolving dose and ovulation.

Analyzing individually female 6 (considered to have poor-quality spawning) at the time of extrusion presented a bloody and "clumpy" spawning, which justifies its low hatching rate. Female 1 (group 19H) recorded the highest ATU and the lowest fertility and hatching rates. Females 2, 4 (group 19H) and 8 (group 0H), on the other hand, had high fertility and hatching rates and close ATU values. Furthermore, ATU values had a strong negative correlation with hatching rate and with $PGF_{2\alpha}$ levels at the time of ovulation. Therefore, corroborating with our previous data (unpublished data), we observed that, regardless of the group, higher $PGF_{2\alpha}$ levels at the time of ovulation are associated with early spawning, the latter being related to high embryonic viability.

In this scenario, based on our results, it is reasonable to suggest that the induction at 7 pm was more efficient than the induction at midnight, being, at least in this experiment, the best time to apply the resolving dose. Even though in experiment 2 female 1 had a hatching rate considered low (35.5%), it still produced 29,591 larvae/kg fish. As a comparison, female 6, which also had a low hatching rate (12.8%), produced 4,219 larvae/kg fish.

Finally, we conclude that pacu females show similar circadian plasmatic oscillations of MTN as other fish species and we also have an indication that females submitted to a longer period of darkness after the resolving dose may present a higher success in ovulation and, consequently, a higher production of larvae.

Declaration of Competing Interest

We declare that we have no conflict of interest.

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References

Blanco-vives, B., Sanchez-Vasquez, F. J., 2009. Synchronisation to light and feeding time of circadian rhythms of spawning and locomotor activity in zebrafish. Physiology & Behavior, 98 (3), 268-275. https://doi.org/10.1016/j.physbeh.2009.05.015

Borella, M. I., Chehade, C., Costa, F. G., Jesus, L. W. O., Cassel, M., Batlouni, S. R., 2020. The brain-pituitary-gonad axis and the gametogenesis. In B. Baldisserotto, E. C. Urbinati, J. E. P. Cyrino (Eds). Biology and Physiology of Freshwater Neotropical Fish (pp. 315–341). Academic Press (Elsevier).

Carnevali, O., Gioacchini, G., Maradonna, F., Olivotto, I., Migliarini, B., 2011, Melatonin Induces Follicle Maturation in Danio rerio. Plos one, 6 (5), 1-9. https://doi.org/10.1371/journal.pone.0019978

Ceccarelli PS, Senhorini JA, Volpato G. 2000. Dicas em piscicultura - perguntas e respostas. Botucatu: Ed. Santana, 247p, 2000.

Chattoraj, A., Bhattacharya, S., Basu, D., Bhattacharya, S., Bhattacharya, S., Maitra, S.K., 2005. Melatonin accelerates maturation inducing hormone (MIH): induced oocyte maturation in carps. Gen. Comp. Endocrinol. 140, 145–155. https://doi.org/10.1016/j.ygcen.2004.10.013

Chattoraj, A., Seth, M., Maitra, S.K., 2008. Influence of serotonin on the action of melatonin in MIH-induced meiotic resumption in the oocytes of carp Catla catla. Comp. Biochem. Physiol. A 150, 301–306. https://doi.org/10.1016/j.cbpa.2008.03.014

Criscuolo-Urbinati, E., Kuradomi, R.Y., Urbinati, E.C., Batlouni, S.R., 2012. The administration of exogenous prostaglandin may improve ovulation in pacu (Piaractus mesopotamicus). Theriogenology 78, 2087–2094. https://doi.org/10.1016/j.theriogenology.2012.08.001

De Alba, G., Mourad, N. M. N., Paredes, J. F., Sánchez-Vázquez, F. J., López-Olmeda, J. F., 2019. Daily rhythms in the reproductive axis of Nile tilapia (Oreochromis niloticus): Plasma steroids and gene expression in brain, pituitary, gonad and egg. Aquaculture 507: 313–321. https://doi.org/10.1016/j.aquaculture.2019.04.047

De Souza, T.G., Kuradomi, R.Y., Rodrigues, S.M., Batlouni, S.R., 2020. Wild Leporinus friderici induced spawning with different dose of mGnRHa and metoclopramide or carp pituitary extract. Anim. Reprod. 17, 1–15. https://doi.org/10.21451/1984-3143-AR2019-0078.

Falcon J., Besseau L., Sauzet S., Boeuf G., 2007. Melatonin effects on the hypothalamo-pituitary axis in fish. Trends in Endocrinology and Metabolism, 18, 81-88 https://doi:10.1016/j.tem.2007.01.002

Falcon J., Migaud H., Muñoz-Cueto J. A., Carrilo M., 2010. Current knowledge on the melatonin system in teleost fish. Gen Comp Endocrinol 165: 469-482. http://dx.doi.org/10.1016/j.ygcen.2009.04.026

Hainfellner, P., De Souza, T. G., Muñoz, M. E., Freitas, G. A., Batlouni, S. R., 2012. Spawning failure in Brycon amazonicus may be associated with ovulation and not with final oocyte maturation. Arquivo Brasileiro De Medicina Veterinaria E Zootecnia, 64(2), 515–517. https://doi.org/10.1590/S0102-09352012000200038

Khan, I., Thomas, P., 1996. Melatonin Influences Gonadotropin II Secretion in the Atlantic Croaker (Micropogonias undulatus). General and Comparative Endocrinology 104, 231–242. https://doi.org/10.1006/gcen.1996.0166

Kuradomi, R. Y., De Souza, T. G., Foresti, F., Schulz, R. W., Bogerd, J., Moreira, R. G., Furlan, L. R., Almeida, E. A., Maschio, L. R., Batlouni, S. R., 2016. Effects of re-stripping on the seminal characteristics of pacu (Piaractus mesopotamicus) during the breeding season. Gen. Comp. Endocrinol., 225, 162–173. https://doi.org/10.1016/j.ygcen.2015.06.007

Kuradomi, R. Y., Foresti, F., Batlouni, S. R., 2017. The effects of sGnRHa implants on Piaractus mesopotamicus female breeders. An approach addressed to aquaculture. Aquac International. 25, 2259-2273. https://doi.org/10.1007/s10499-017-0186-2

Kuradomi, R.Y., Batlouni, S.R., 2018. PGF2α and gonadal steroid plasma levels of successful and unsuccessful spawning Piaractus mesopotamicus (Teleostei, Characiformes) females. Aquacult. Int. 26, 1083–1094. https://doi.org/10.1007/s10499-018-0269-8

Lombardo, F., Gioacchini, G., Fabbrocini, A., Candelma, M., D'Adamo, R., Giorgini, E., Carnevali, O., 2014. Melatonin-mediated effects on killifish reproductive axis. Comparative Biochemistry and Physiology, Part A 172, 31–38. http://dx.doi.org/10.1016/j.cbpa.2014.02.008

Maitra, S. K., Chatorraj, A., Mukherjee, S., Moniruzzaman., M., 2013. Melatonin: A potent candidate in the regulation of fish oocyte growth and maturation. General and Comparative Endocrinology. 181, 215–222. https://doi.org/10.1016/j.ygcen.2012.09.015

Martinez-Chavez, C. C., Al-Khamees, S., Campos-Mendoza, A., Penman, D. J., Migaud, H., 2008. Clock-controlled endogenous melatonin rhythms in nile tilapia (Oreochromis niloticus niloticus) and african catfish (Clarias gariepinus). Chronobiology International 25(1): 31–49. https://10.1080/07420520801917547

Muniz, J. A., Catanho, M. T., Santos, A. J. G., 2008. Influência do fotoperíodo natural na reprodução induzida do tambaqui, Colossoma macropomum (CUVIER, 1818). B. Inst. Pesca, 34(2): 205 – 211.

Ogiwara, K., Takahashi, T., 2016. A Dual Role for Melatonin in Medaka Ovulation: Ensuring Prostaglandin Synthesis and Actin Cytoskeleton Rearrangement in Follicular Cells1. Biology of Reproduction, 94(3), 1–15. https://doi.org/10.1095/biolreprod.115.133827

Paredes J. F., Cowan M., López-Olmeda J. F., Muñoz-Cueto J. A., Sánchez-Vázquez F. J., 2019. Daily rhythms of expression in reproductive genes along the brain-pituitary-gonad axis and liver of zebrafish. Comp Biochem Physiol A 231: 158–169. https://doi.org/10.1016/j.aquaculture.2019.04.047

Pavlidis, M., Greewood, L., Paalavuo, M., Molsa, H., Laitinen, J. T., 1999. The Effect of Photoperiod on Diel Rhythms in Serum Melatonin, Cortisol, Glucose, and Electrolytes in the Common Dentex, Dentex dentex. General and Comparative Endocrinology 113, 240–250. https://doi.org/10.1006/gcen.1998.7190

Pereira, T. S. B., Boscolo, C. N. P., Moreira, R. G., Batlouni, S. R., 2017. The use of mGnRHa provokes ovulation but not viable embryos in Leporinus macrocephalus. Aquaculture International, 25(2), 515–529. https://doi.org/10.1007/s10499-016-0049-2

Pereira, T. S. B., Boscolo, C. N. P., Moreira, R. G., Batlouni, S. R., 2018. Leporinus elongatus induced spawning using carp pituitary extract or mammalian GnRH analogue combined with dopamine receptor antagonists. Animal Reproduction, 15(1), 64–70. https://doi.org/10.21451/1984-3143-2017-AR983

Roza de Abreu, M., Silva, L.M.J., Figueiredo-Ariki, D.G., Sato, R.T., Kuradomi, R.Y., Batlouni, S.R., 2020. Reproductive performance of Lambari (Astyanax altiparanae) in a seminatural system using different protocols. Aquac. Res. 52 (2), 471–483. https://doi.org/10.1111/are.14905.

Roza de Abreu, M., Silva, L.M.J., Figueiredo-Ariki, D.G., Sato, R.T., Kuradomi, R.Y., Batlouni, S.R., 2022. The effect of LHRHa with and without dopamine antagonist on reproductive performance in lambari Astyanax altiparanae. Aquaculture. Vias de publicação. https://doi.org/10.1016/j.aquaculture.2021.737883

Sato, R.T., Kuradomi, R.Y., Calil, M.C., Silva, L.M.J., Roza de Abreu, M., Figueiredo-Ariki, D.G., Batlouni, S.R., 2020. Resumption and progression of meiosis and circulating levels of steroids and prostaglandin F2α of Piaractus mesopotamicus induced by hypophysation with prostaglandin F2α. Aquac. Res. 52 (3), 1026–1037. https://doi.org/10.1111/are.14957.

Schober, P.; Boer, C.; Schwarte, L.A. 2018. Correlation Coefficients. Anesthesia & Analgesia, 126: 1763-1768. https://doi.org/10.1213/ANE.0000000000002864

Schorer, M., Moreira, R. G., Batlouni, S. R., 2016. Selection of pacu females to hormonal induction: Effect of age and of evaluation methods. Boletim do Instituto De Pesca, 42(4), 901–913. https://doi.org/10.20950/1678-2305.2016v42n4p901

Takahashi, T., Hagiwara, A., Ogiwara, K., 2018. Prostaglandins in teleost ovulation: A review of the roles with a view to comparison with prostaglandins in mammalian ovulation. Molecular and Cellular Endocrinology, 461, 236–247. https://doi.org/10.1016/j.mce.2017.09.019

Zohar, Y., Muñoz-Cueto, J. A., Elizur, A., Kah, O., 2010. Neuroendocrinology of reproduction in teleost fish. General and Comparative Endocrinology, 165, 438–455. http://doi.org/10.1016/j.ygcen.2009.04.017

CONCLUSÕES GERAIS

As modificações propostas no protocolo de hipofisação associado a $PGF_{2α}$ não propiciaram um melhor desempenho reprodutivo se comparado ao protocolo já estabelecido por Criscuolo-Urbinati et al. (2012). A aplicação de $PGF_{2α}$ exógena para além da segunda dose de hipofisação e mais próximo do período de ovulação não trouxe ganhos no desempenho, mas pelo contrário esteve associada a ocorrência de “desovas ruins”, caracterizadas por baixa fecundidade. A outra variável modificada, o aumento da dose de $PGF_{2α}$ exógena, propiciou sim estabilidade na ovulação com taxas de 100% de ovulação e ausência de desovas ruins, contudo, taxas de fertilidade e eclosão não foram satisfatórias. Não observamos associação entre a aplicação de $PGF_{2α}$ mais próximo da ovulação e o aumento de sua dose com níveis plasmáticos superiores de $PGF_{2α}$ no momento da ovulação. Entretanto, as correlações positivas fortes encontradas entre níveis de $PGF_{2α}$ no momento da ovulação e fecundidade, indicam que, apesar de não ser necessariamente relacionados ao sucesso da ovulação, elevados níveis $PGF_{2α}$ no momento da ovulação estão associados ao maior número de ovos liberados pelas fêmeas.

As concentrações plasmáticas de MTN não apresentaram variações entre animais controle e hipofisados. Quando analisados conjuntamente, observamos que os níveis de MTN se elevam no início da noite (19 horas) do dia 1, se mantêm semelhantes até às 11 horas e reduzem às 15 horas do dia 2. Durante a indução hormonal, níveis plasmáticos de MTN, PGF e DHP foram similares entre as fêmeas hipofisadas as 19 horas e 0 horas. Da mesma forma, o desempenho reprodutivo foi similar entre os dois grupos. Porém, o grupo 19H com o mesmo número de fêmeas, apresentou uma produção total (números absolutos) de 737.567 larvas, contra 250.762 do grupo 0H. Além disso, os níveis plasmáticos de MTN no momento da ovulação apresentaram correlação positiva com fecundidade, taxa de fertilidade, taxa de eclosão e larvas/kg peixe. Estes resultados indicam que, apesar de não apresentarem diferenças significativas entre os grupos (níveis hormonais e desempenho reprodutivo), fêmeas submetidas a um maior período escuro pós-segunda dose podem apresentar maior sucesso na ovulação e maior produção de larvas. Este aspecto necessita de explorações mais aprofundadas que envolvem a expressão de receptores das substancias envolvidas, entre outras variáveis e conhecimentos, tais como estudos sobre o horário de desova natural da espécie.

Por fim, este estudo, que testou doses e momentos aplicação de $PGF_{2\alpha}$, não confirmou as vantagens teóricas das alterações propostas em relação ao protocolo vigente, porém consolida a $PGF_{2\alpha}$ exógena como excelente ferramenta para indução a ovulação por suas fortes correlações positivas com fecundidade. Com relação a MTN, apresentamos de forma pioneira o ritmo circadiano desta substancia no pacu e mostramos que, apesar de serem necessários mais investigações, a indução no início do período de escuro pode auxiliar no sucesso reprodutivo da espécie em cativeiro.

REFERÊNCIAS BIBLIOGRÁFICAS

Abimorad, E. G., & Carneiro, D. J. (2007). Digestibility and performance of pacu (Piaractus mesopotamicus) juveniles - fed diets containing different protein, lipid and carbohydrate levels. Aquaculture Nutrition, 13, 1–9. https://doi.org/10.1111/j.1365-2095.2007.00438.x

Abimorad, E. G., Squassoni, G. H., & Carneiro, D. J. (2008). Apparent digestibility of protein, energy, and amino acids in some selected feed ingredients for pacu Piaractus mesopotamicus. Aquaculture Nutrition, 14(4), 374–380. https://doi.org/10.1111/j.1365-2095.2007.00544.x

Aizen, J., Kobayashi, M., Selicharova, I., Sohn, Y. C., Yoshizaki, G., Levavi-Sivan, B., 2012. Steroidogenic response of carp ovaries to piscine FSH and LH depends on the reproductive phase. Gen Comp Endocrinol 178: 28-36.

Barbosa, R. P., Kuradomi, R. Y., Sato, R. T., Batlouni, S. R., 2022. Piaractus mesopotamicus gonad differentiation. Aquaculture reseach, 00:1–10. https://doi.org/10.1111/are.15912

Berndtson, A. K., Goetz, F. W., & Duman, P. (1989). In vitro ovulation, prostaglandin synthesis, and proteolysis in isolated ovarian components of yellow perch (Perca flavescens): Effects of 17α,20β-dihydroxy-4-pregnen-3-one and phorbol ester. General and Comparative Endocrinology, 75(3), 454–465. https://doi.org/10.1016/0016-6480(89)90181-0

Biller-Takahashi, J. D., Takahashi, L. S., Mingatto, F. E., Urbinati, E. C., 2015. The immune system is limited by oxidative stress: Dietary selenium promotes optimal antioxidative status and greatest immune defense in pacu Piaractus mesopotamicus. Fish and Shellfish Immunology, 47(1), 360–367. https://doi.org/10.1016/j.fsi.2015.09.022

Blanco-vives, B., Sanchez-Vasquez, F. J., 2009. Synchronisation to light and feeding time of circadian rhythms of spawning and locomotor activity in zebrafish. Physiology & Behavior, 98 (3), 268-275. https://doi.org/10.1016/j.physbeh.2009.05.015

Borella, M. I., Chehade, C., Costa, F. G., Jesus, L. W. O., Cassel, M., Batlouni, S. R., 2020. The brain-pituitary-gonad axis and the gametogenesis. In B. Baldisserotto, E. C. Urbinati, J. E. P. Cyrino (Eds). Biology and Physiology of Freshwater Neotropical Fish (pp. 315–341). Academic Press (Elsevier).

Carolsfeld, J. Harvey, B. Raer, A. Ross, C., 2003. Migratory fishes of South America: Biology, Fisheries, and Conservation Status. Ottawa: World Fisheries Trust, 373.

Castagnolli, N., Donaldson, E. M., 1981. Induced ovulation and rearing of the pacu (Colossoma mitrei). Aquaculture, 25, 275–279. https://doi.org/10.1016/0044-8486(81)90189-7

Carnevali, O., Gioacchini, G., Maradonna, F., Olivotto, I., Migliarini, B., 2011, Melatonin Induces Follicle Maturation in Danio rerio. Plos one, 6 (5), 1-9.

Chattoraj, A., Bhattacharya, S., Basu, D., Bhattacharya, S., Bhattacharya, S., Maitra, S.K., 2005. Melatonin accelerates maturation inducing hormone (MIH): induced oocyte maturation in carps. Gen. Comp. Endocrinol. 140, 145–155.

Chattoraj, A., Seth, M., Maitra, S.K., 2008. Influence of serotonin on the action of melatonin in MIH-induced meiotic resumption in the oocytes of carp Catla catla. Comp. Biochem. Physiol. A 150, 301–306.

Criscuolo-Urbinati, E., Kuradomi, R.Y., Urbinati, E.C., Batlouni, S.R., 2012. The administration of exogenous prostaglandin may improve ovulation in pacu (Piaractus mesopotamicus). Theriogenology 78, 2087–2094. https://doi.org/10.1016/j.theriogenology.2012.08.001

de Alba, G., Mourad, N. M. N., Paredes, J. F., Sánchez-Vázquez, F. J., López-Olmeda, J. F., 2019. Daily rhythms in the reproductive axis of Nile tilapia (Oreochromis niloticus): Plasma steroids and gene expression in brain, pituitary, gonad and egg. Aquaculture 507: 313–321.

de Souza, T.G., Kuradomi, R.Y., Rodrigues, S.M., Batlouni, S.R., 2020. Wild Leporinus friderici induced spawning with different dose of mGnRHa and metoclopramide or carp pituitary extract. Anim. Reprod. 17, 1–15. https://doi.org/10.21451/1984-3143-AR2019-0078.

Falcon, J., Besseau, L., Sauzet, S., Boeuf, G., 2007. Melatonin effects on the hypothalamo-pituitary axis in fish. Trends in Endocrinology and Metabolism, 18, 81-88 https://doi.org/10.1016/j.tem.2007.01.002.

Falcon, J., Migaud, H., Muñoz-Cueto, J. A., Carrilo, M., 2010. Current knowledge on the melatonin system in teleost fish. Gen Comp Endocrinol 165: 469-482.

Ferraz de Lima, J.A., Barbieri, G., Verani, J.R., 1984. Período de reprodução e idade a primeira maturação gonadal de pacu (Colossoma mitrei) em ambiente natural, (Rio

Cuiabá, - Pantanal de Mato Grosso). Anais do III Simpósio Brasileiro de Aqüicultura, São Carlos, SP

Fujimori, C, Ogiwara, K, Hagiwara, A, Rajapakse, S, Kimura, A, Takahashi, T., 2011. Expression of cyclooxygenase-2 and prostaglandin receptor EP4b mRNA in the ovary of the medaka fish, Oryzias latipes: Possible involvement in ovulation. Mol Cell Endocrinol 332: 67–77

Fujimori, C., Ogiwara, K., Hagiwara, A., Takahashi, T., 2012. New evidence for the involvement of prostaglandin receptor EP4b in ovulation of the medaka, Oryzias latipes. Molecular and Cellular Endocrinology, 362(1–2), 76–84. https://doi.org/10.1016/j.mce.2012.05.013

Gelman, A., Drabkin, V., Sachs, O., Chechic, K., Gabay, I., Glatman, L., 2004. Pacu (Piaractus mesopotamicus) a new fish species in Israeli aquaculture: Possibility of utilization. Developments in Food Science, 42(C), 75–83. https://doi.org/10.1016/S0167-4501(04)80010-4

Godinho, H. P., Godinho, A. L., 1986. Induced Spawning of the pacu, Colossoma mitrei (Berg 1895), by hypophysation with crude pituitary extract. Aquaculture, 55, 69–73. https://doi.org/10.1016/0044-8486(86)90057-8

Goetz, F. W., 1997. Follicle and extrafollicular tissue interaction in 17α,20β-dihydroxy-4-pregnen-3-one-stimulated ovulation and prostaglandin synthesis in the yellow perch (Perca flavescens) ovary. General and Comparative Endocrinology, 105(1), 121–126. https://doi.org/10.1006/gcen.1996.6807

Hagiwara, A., Ogiwara, K., Katsu, Y., Takahashi, T., 2014. Luteinizing Hormone-Induced Expression of Ptger4b, a Prostaglandin E2 Receptor Indispensable for Ovulation of the Medaka Oryzias latipes, Is Regulated by a Genomic Mechanism Involving Nuclear Progestin Receptor1. Biology of Reproduction, 90(6), 1–14. https://doi.org/10.1095/biolreprod.113.115485

Hainfellner, P., de Souza, T. G., Moreira, R. G., Nakaghi, L. S. O., Batlouni, S. R., 2012a. Gonadal steroids levels and vitellogenesis in the formation of oocytes in Prochilodus lineatus (Valenciennes) (Teleostei: Characiformes). Neotropical Ichthyology, 10(3):601-612. https://doi.org/10.1590/S1679-62252012005000021

Hainfellner, P., de Souza, T. G., Muñoz, M. E., Freitas, G. A., Batlouni, S. R., 2012b. Spawning failure in Brycon amazonicus may be associated with ovulation and not with final oocyte maturation. Arquivo Brasileiro De Medicina Veterinaria E Zootecnia, 64(2), 515–517. https://doi.org/10.1590/S0102-09352012000200038

Hainfellner, P., Kuradomi, R. Y., de Souza, T. G., Sato, R. T., Figueiredo-Ariki, D. G., De Freitas, G. A., Queiroz, L., Valenti, W. C., Valenti, P. M., Ge, W., Batlouni, S. R., 2019. Reproductive cycle of the Amazonian planktivorous catfish Hypophthalmus marginatus (Siluriformes, Pimelodidae). Aquaculture Research, 50, 3382–3391. https://doi.org/10.1111/are.14296

Honorato, C. A., de Almeida, L. C., Camilo, R. Y., Moraes, G., Nunes, C. D. S., Carneiro, D. J., 2016. Dietary carbohydrate and food processing affect the digestive physiology of Piaractus mesopotamicus. Aquaculture Nutrition, 22(4), 857–864. https://doi.org/10.1111/anu.12308

Houssay, B. A., 1930. Accion sexual de la hipofisis em los peces y reptiles. Rev Soc Arg Biol, v.6, p.686-688, 1930.

IBGE “Instituto Brasileiro de Geografia e Estatística”, 2022. Produção da Pecuária Municipal 2021, 11.

Ittzés, I., Szabó, T., Kronbauer, E. K., Urbanyi, B., 2015. Ovulation induction in jundia (Rhamdia quelen, Heptapteridae) using carp pituitary extract or salmon GnRH analogue combined with dopamine receptor antagonists. Aquac Res 46: 2924–2928.

Jalabert, B., Szöllösi, D., 1975. In vitro ovulation of trout oocytes : Effect of prostaglandins on smooth muscle-like cells of the theca. Prostaglandins, 9(5), 765–778. https://doi.org/10.1016/0090-6980(75)90113-6

Jalabert, B., Breton, B., Brzuska, E., Fostier, A., Wieniawski, J., 1977. A new tool for induced spawning: The use of 17α-hydroxy-20β-dihydroprogesterone to spawn carp at low temperature. Aquaculture, 10(4), 353–364. https://doi.org/10.1016/0044-8486(77)90126-0

Jomori, R. K., Carneiro, D. J., Malheiros, E. B., Portella, M. C., 2003. Growth and survival of pacu Piaractus mesopotamicus (Holmberg, 1887) juveniles reared in ponds or at different initial larviculture periods indoors. Aquaculture, 221(1–4), 277–287. https://doi.org/10.1016/S0044-8486(03)00069-3

Jomori, R. K., Ducatti, C., Carneiro, D. J., Portella, M. C., 2008. Stable carbon (δ13C) and nitrogen (δ15N) isotopes as natural indicators of live and dry food in Piaractus mesopotamicus (Holmberg, 1887) larval tissue. Aquaculture Research, 39(4), 370–381. https://doi.org/10.1111/j.1365-2109.2007.01760.x

Joy, K.P., Singh, V., 2013. Functional interactions between vasotocin and prostaglandins during final oocyte maturation and ovulation in the catfish Heteropneustes fossilis. Gen. Comp. Endocr. 186, 126–135. https://doi.org/10.1016/j.ygcen.2013.02.043.

Kagawa, H., Gen, K., Okuzawa, K., Tanaka, H., 2003. Effects of luteinizing hormone and follicle-stimulating hormone and insulin-like growth factor-I on aromatase activity and P450 aromatase gene expression in the ovarian follicles of red seabream, Pagrus major. Biol. Reprod. 68, 1562–1568. https://doi.org/10.1095/biolreprod.102.008219.

Khan, I., Thomas, P., 1996. Melatonin Influences Gonadotropin II Secretion in the Atlantic Croaker (Micropogonias undulatus). General and Comparative Endocrinology 104, 231–242. https://doi.org/10.1006/gcen.1996.0166

Knight, O.M., Van Der Kraak, G., 2015. The role of eicosanoids in 17α,20β-dihydroxy-4-pregnen-3-one-induced ovulation and spawning in Danio rerio. Gen. Comp. Endocrinol. 213, 50–58. https://doi.org/10.1016/j.ygcen.2014.12.014.

Kuradomi, R. Y., Foresti, F., Batlouni, S. R., 2017. The effects of sGnRHa implants on Piaractus mesopotamicus female breeders. An approach addressed to aquaculture.

Aquaculture International, 25(6), 2259–2273. https://doi.org/10.1007/s10499-017-0186-2

Kuradomi, R.Y., Batlouni, S.R., 2018. PGF2α and gonadal steroid plasma levels of successful and unsuccessful spawning Piaractus mesopotamicus (Teleostei, Characiformes) females. Aquacult. Int. 26, 1083–1094. https://doi.org/10.1007/s10499-018-0269-8

Leitão, N. J., Pai-Silva, M. D., de Almeida, F. L. A., Portella, M. C., 2011. The influence of initial feeding on muscle development and growth in pacu Piaractus mesopotamicus larvae. Aquaculture, 315(1–2), 78–85. https://doi.org/10.1016/j.aquaculture.2011.01.006

Levavi-Zermonsky, B., Yaron, Z., 1986. Changes in gonadotropin and ovarian steroids associated with oocytes maturation during spawning induction in the carp. General and Comparative Endocrinology, 62(1), 89–98. https://doi.org/10.1016/0016-6480(86)90097-3

Lima, R.V.A., Bernardino, G., Val-Sella, M.V., Fava-de-Moraes, F., Schemy, R.A., Borella, M.I., 1991. Tecido germinativo ovariano e ciclo reprodutivo de pacus (Piaractus mesopotamicus Holmberg, 1887) mantidos e cativeiro. Bol Téc CEPTA. 4, 1-46.

Lima, A. F., Moro, G. V., Kirschnik, L. N. G, Barroso, R. M., 2013. Reprodução, larvicultura e alevinagem de peixes. In Embrapa. Piscicultura de água doce: multiplicando conhecimentos (pp 306-307). Embrapa pesca e aquicultura.

Lister, A. L., Van Der Kraak, G. J., 2008. An investigation into the role of prostaglandins in zebrafish oocyte maturation and ovulation. Gen. Comp. Endocrinol. 159 (1), 46–57. https://doi.org/10.1016/j.ygcen.2008.07.017

Lister, A. L., Van Der Kraak, G. J., 2009. Regulation of prostagladin synthesis in ovaries of sexually mature zebrafish (Danio rerio). Mol Reprod Dev 76: 106

Lubzens, E., Young, G., Bobe, J., Cerdà, J., 2010. Oogenesis in teleosts: how fish eggs are formed. Gen. Comp. Endocrinol. 165 (3), 367–389. https://doi.org/10.1016/j.ygcen.2009.05.022

Marinho de Mello, M. M., de Fátima Pereira de Faria, C., Zanuzzo, F. S., Urbinati, E. C., 2019. β-glucan modulates cortisol levels in stressed pacu (Piaractus mesopotamicus) inoculated with heat-killed Aeromonas hydrophila. Fish and Shellfish Immunology, 93(2019), 1076–1083. https://doi.org/10.1016/j.fsi.2019.07.068

Martinez-Chavez, C. C., Al-Khamees, S., Campos-Mendoza, A., Penman, D. J., Migaud, H., 2008. Clock controlled endogenous melatonin rhythms in Nile tilapia (Oreochromis niloticus niloticus) and African catfish (Clarias gariepinus). Chronobiol. Int. 25, 31–49

Mastrochirico-filho, V. A., Ariede, R. B., Freitas, M. V., Lira, L. V. G., Agudelo, J. F. G., Pilarski, F., Reis Neto, R. V., Yáñez, J. M., Hashimoto, D. T., 2019. Genetic parameters for resistance to Aeromonas hydrophila in the Neotropical fish pacu (

Piaractus mesopotamicus). Aquaculture, 513(2019), 734442. https://doi.org/10.1016/j.aquaculture.2019.734442

Moro, G. V., Rezende, F. P., Alves, A. L., Hashimoto, D. T., Varela, E. S., Torati, L. S., 2013. Espécies de peixe para piscicultura. In Embrapa. Piscicultura de água doce: multiplicando conhecimentos (pp. 36-37). Embrapa pesca e aquicultura.

Mourad, N. M. N., Costa, A. C., Freitas, R. T. F., Serafini, M. A., Neto, R. V. R., Felizardo, V. O., 2018. Weight and morphometric growth of Pacu (Piaractus mesopotamicus), Tambaqui (Colossoma macropomum) and their hybrids from spring to winter. Pesquisa Veterinaria Brasileira, 38(3), 544–550. https://doi.org/10.1590/1678-5150-PVB-4808

Muniz, J. A., Catanho, M. T., Santos, A. J. G., 2008. Influência do fotoperíodo natural na reprodução induzida do tambaqui, Colossoma macropomum (CUVIER, 1818). B. Inst. Pesca, 34(2): 205 – 211.

Mylonas, C. C., Fostier, A., Zanuy, S., 2010. Broodstock management and hormonal manipulations of fish reproduction. General and Comparative Endocrinology, 165(3), 516–534. https://doi.org/10.1016/j.ygcen.2009.03.007

Nagahama, Y., Yamashita, M., 2008. Regulation of oocyte maturation in fish. Development, Growth & Differentiation, 50, 195–219. https://doi.org/10.1016/S0070-2153(08)60565-7

Ogiwara, K., Takahashi, T., 2016. A Dual Role for Melatonin in Medaka Ovulation: Ensuring Prostaglandin Synthesis and Actin Cytoskeleton Rearrangement in Follicular Cells1. Biology of Reproduction, 94(3), 1–15. https://doi.org/10.1095/biolreprod.115.133827

Paredes J. F., Cowan M., López-Olmeda J. F., Muñoz-Cueto J. A., Sánchez-Vázquez F. J., 2019. Daily rhythms of expression in reproductive genes along the brain-pituitary-gonad axis and liver of zebrafish. Comp Biochem Physiol A 231: 158–169.

Paulino M. S., Sampaio M., Miliorini A. B., Murgas L. D. S., Lima F. S. M., Felizardo V. O., 2011. Desempenho reprodutivo do pacu, piracanjuba e curimba induzidos com extrato de buserelina. Bol Inst Pesca 37: 39–45.

Peixe BR, 2020. Anuário 2020 Peixe BR da piscicultura, 60–61.

Peixe BR, 2021. Anuário 2021 Peixe BR da piscicultura, 34.

Peixe BR, 2022. Anuário 2022 Peixe BR da piscicultura, 12–14.

Pereira, T. S. B., Boscolo, C. N. P., Moreira, R. G., Batlouni, S. R., 2017. The use of mGnRHa provokes ovulation but not viable embryos in Leporinus macrocephalus. Aquaculture International, 25(2), 515–529. https://doi.org/10.1007/s10499-016-0049-2

Pereira, T. S. B., Boscolo, C. N. P., Moreira, R. G., Batlouni, S. R., 2018. Leporinus elongatus induced spawning using carp pituitary extract or mammalian GnRH analogue combined with dopamine receptor antagonists. Animal Reproduction, 15(1), 64–70. https://doi.org/10.21451/1984-3143-2017-AR983

Portella, M. C., Jomori, R. K., Leitão, N. J., Menossi, O. C. C., Freitas, T. M., Kojima, J. T., Lopes, T. S., Clavijo-Ayala, J. A., Carneiro, D. J., 2014. Larval development of indigenous South American freshwater fish species, with particular reference to pacu (Piaractus mesopotamicus): A review. Aquaculture, 432, 402–417. https://doi.org/10.1016/j.aquaculture.2014.04.032

Reis Neto, R. V., Serafini, M. A., Tadeu, R., De Freitas, F., Allaman, I. B., Mourad, N. M. N., Lago, A. A., 2012. Performance and carcass traits in the diallel crossing of pacu and tambaqui. Revista Brasileira De Zootecnia, 41(12), 2390–2395. https://doi.org/10.1590/S1516-35982012001200002

Roza de Abreu, M., Silva, L.M.J., Figueiredo-Ariki, D.G., Sato, R.T., Kuradomi, R.Y., Batlouni, S.R., 2020. Reproductive performance of Lambari (Astyanax altiparanae) in a seminatural system using different protocols. Aquac. Res. 52 (2), 471–483. https://doi.org/10.1111/are.14905.

Roza de Abreu, M., Silva, L.M.J., Figueiredo-Ariki, D.G., Sato, R.T., Kuradomi, R.Y., Batlouni, S.R., 2022. f. Aquaculture. https://doi.org/10.1016/j.aquaculture.2021.737883

Sato, R.T., Kuradomi, R.Y., Calil, M.C., Silva, L.M.J., Roza de Abreu, M., Figueiredo-Ariki, D.G., Batlouni, S.R., 2020. Resumption and progression of meiosis and circulating levels of steroids and prostaglandin F2α of Piaractus mesopotamicus induced by hypophysation with prostaglandin F2α. Aquac. Res. 52 (3), 1026–1037. https://doi.org/10.1111/are.14957.

Schorer, M., Moreira, R. G., Batlouni, S. R., 2016. Selection of pacu females to hormonal induction: Effect of age and of evaluation methods. Boletim do Instituto De Pesca, 42(4), 901–913. https://doi.org/10.20950/1678-2305.2016v42n4p901

Silva, J. W. B. E., Bernardino, G., Silva Nobre, M. I., Ferrari, V. A., Mendonça, J. O. J., 1997. Cultivo do pacu Piaractus mesopotamicus (Holberg, 1887) em duas densidades de estocagem no nordeste do Brasil. Boletim Técnico do CEPTA, 10, 61–70.

Souza, V. L., Urbinati, E. C., Martins, M. I. E. G., Silva, P. C., 2003. Avaliação do Crescimento e do Custo da Alimentação do Pacu (Piaractus mesopotamicus Holmberg, 1887) Submetido a Ciclos Alternados de Restrição Alimentar e Realimentação. Revista Brasileira De Zootecnia, 32, 19–28. https://doi.org/10.1590/S1516-35982003000100003

Souza F. N., Martins E. F. F., Corrêa R. A. C., de Abreu J. A., Pires L. B., Streit Jr. D. P., Lopera-Barrero, N. M., Povh J. A., 2018. Ovopel® and carp pituitary extract for induction of reproduction in Colossoma macropomum females. Anim Reprod Sci 195: 53-57.

Stacey, N. E., Pandey, S., 1975. Effects of indomethacin and prostaglandins on ovulation of goldfish. Prostaglandins, 9(4), 597–607. https://doi.org/10.1016/0090-6980(75)90065-9

Takahashi, L. S., Biller-Takahashi, J. D., Mansano, C. F. M., Urbinati, E. C., Gimbo, R. Y., Saita, M. V., 2017. Long-term organic selenium supplementation overcomes the trade-off between immune and antioxidant systems in pacu (Piaractus mesopotamicus). Fish and Shellfish Immunology, 60, 311–317. https://doi.org/10.1016/j.fsi.2016.11.060

Takahashi, T., Fujimori, C., Hagiwara, A., Ogiwara, K., 2013. Recent Advances in the Understanding of Teleost Medaka Ovulation: The Roles of Proteases and Prostaglandins. Zoological Science, 30(4), 239–247. https://doi.org/10.2108/zsj.30.239

Takahashi, T., Hagiwara, A., Ogiwara, K., 2018. Prostaglandins in teleost ovulation: A review of the roles with a view to comparison with prostaglandins in mammalian ovulation. Molecular and Cellular Endocrinology, 461, 236–247. https://doi.org/10.1016/j.mce.2017.09.019

Tang, H., Liu, Y., Li, J., Li, G., Chen, Y., Yin, Y., Lin, H., 2017. LH signaling induced ptgs2a expression is required for ovulation in zebrafish. Mol. Cell. Endocrinol. 447, 125–133. https://doi.org/10.1016/j.mce.2017.02.042.

Tokarz, J., Möller, G., Hrabě De Angelis, M., Adamski, J., 2015. Steroids in teleost fishes: A functional point of view. Steroids, 103, 123–144. ttps://doi.org/10.1016/j.steroids.2015.06.011

Urbinati, E. C., Takahashi, L. S., 2020. Pacu (Piaractus mesopotamicus). In B. Baldisseroto (org). Espécies nativas para piscicultura no Brasil (pp 169-170). Editora UFSM.

Valladão, G. M. R., Gallani, S. U., Pilarski, F., 2018. South American fish for continental aquaculture. Reviews in Aquaculture, 10, 351–369. https://doi.org/10.1111/raq.12164

Viveiros, A. T. M., Goncalves, A. C., Chiacchio, I. M., Nascimento, A. F., Romagosa, E., Leal, M. M., 2015. Gamete quality of streaked prochilod Prochilodus lineatus (Characiformes) after GnRHa and dopamine antagonist treatment. Zygote 23: 212–221

Von Ihering, R., de Azevedo, P., 1934. A curimatã dos açudes nordestinos (Prochilodus argenteus). Archivos do Intituto Biologico, 5, 143-178.

Zaniboni-Filho, E., Weingartner, M., 2007. Técnicas de indução da reprodução de peixes migradores (Induced breeding in migratory fishes). Revista Brasileira de Reprodução Animal. Belo Horizonte, 31, (n.3): 367-373.

Zanuzzo, F. S., Sabioni, R. E., Marzocchi-Machado, C. M., Urbinati, E. C., 2019. Modulation of stress and innate immune response by corticosteroids in pacu (Piaractus mesopotamicus). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 231, 39–48. https://doi.org/10.1016/j.cbpa.2019.01.019

Zohar, Y., Mylonas, C. C., 2001. Endocrine manipulations of spawning in cultured fish: From hormones to genes. Aquaculture, 197(1–4), 99–136. https://doi.org/10.1016/S0044-8486(01)00584-1