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RITMOS BIOLÓGICOS DE PARÂMETROS FISIOLÓGICOS EM PEIXES AMAZÔNICOS

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Manaus-Amazonas

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Resumo

O objetivo desse estudo foi avaliar os ritmos biológicos de parâmetros fisiológicos em juvenis de oscar (*Astronotus ocellatus*) e matrinxã (*Brycon amazonicus*). ***Capítulo 1*** - Alguns parâmetros fisiológicos que são indicadores de gasto energético e, estão relacionados a condições estressantes, apresentam variação circadiana relacionada ao ciclo claro/escuro para ambas as espécies. *B. amazonicus* apresentou maior número de parâmetros fisiológicos que apresentaram ritmos diários (Htc, Hb, MCH, MCHC, glicose, triglicerídeos e colesterol total) em comparação com *A. ocellatus* (Htc, MCV, triglicerídeos e proteínas totais). Estes resultados podem contribuir para uma maior adequação de condições que proporcionem melhor bem-estar e desempenho produtivo para esses animais quando mantidos em sistemas artificiais. ***Capítulo 2*** **–** Apresenta resultados do efeito do ciclo lunar em juvenis de matrinxã, que indicam que as concentrações de melatonina, cortisol, parâmetros metabólicos e indicadores de estresse oxidativo são modulados tanto pelo claro/escuro quanto pelas fases da lua (lua cheia e nova) para o matrinxã. Nossa pesquisa fornece a primeira percepção sobre a influência do ciclo lunar nos parâmetros fisiológicos em matrinxã, e esses resultados destacaram que a luz da lua é relevante para investigar como os peixes lidam com os ciclos ambientais. ***Capítulo 3*** **-** refere-se ao ritmo circadiano da melatonina plasmática e ocular, e que a luminosidade atua como um modulador dos níveis desse hormônio. Os níveis de melatonina plasmática foram maiores no período escuro para *A. ocellatus* e *B. amazonicus*. Em *A. ocellatus*, o padrão rítmico de melatonina na retina é independente do período de escuro. No entanto, para *B. amazonicus*, os níveis de melatonina ocular mostraram mudanças marcantes no ciclo claro/escuro, com valores menores durante o período de luz e maiores no escuro. Observou-se redução dos níveis de melatonina plasmática à exposição ao pulso de luz para *A. ocellatus* e *B. amazonicus*, indicando que melatonina plasmática apresenta alta sensibilidade à luz. Os resultados destacam a importância da avaliação dos ritmos biológicos (circadiano e infradiano) para parâmetros fisiológicos em espécies de interesse comercial e que são susceptíveis a situações estressantes em condições de criação. As informações geradas podem subsidiar o avanço de novas tecnologias que possam ser utilizadas por programas de gestão ambiental e, consequentemente, contribuir para o bem-estar dos peixes, um dos fatores essenciais para uma aquicultura sustentável.

Palavras-chave: ritmo circadiano, ciclo lunar, melatonina, matrinxã, ciclídeo

Abstract

The aim of this study was to evaluate the biological rhythms of physiological parameters in juvenile oscar (*Astronotus ocellatus*) and matrinxã (*Brycon amazonicus*). C***hapter 1*** shows that some physiological parameters that are indicators of energy expenditure and, therefore, related to stressful conditions, present circadian variation related to the light/dark cycle for both species. *B. amazonicus* showed a greater number of physiological parameters that showed daily rhythms (Htc, Hb, MCH, MCHC, glucose, triglycerides and total cholesterol) compared to *A. ocellatus* (Htc, MCV, triglycerides and total proteins). These results may contribute to a better adequacy of conditions that provide better welfare and productive performance for these animals when kept in artificial systems. C***hapter 2*** shows responses on the lunar cycle effect of the juvenile matrinxã, indicating that the concentrations of melatonin, cortisol, metabolic parameters and oxidative stress indicators are modulated by both light/dark and moon phases (full and new moon). Our research provides the first insight into the influence of the lunar cycle on physiological parameters in matrinxã, and these results highlighted that moonlight is relevant for investigating how fish deal with environmental cycles. C***hapter 3*** refers to the circadian rhythm of plasma and ocular melatonin and that luminosity (light/dark cycle and light pulse) acts as a modulator of this hormone levels. Plasma melatonin levels were higher in the dark period for *A. ocellatus* and *B. amazonicus*. In *A. ocellatus*, the rhythmic pattern of melatonin in the retina is independent of the dark period. However, for *B. amazonicus*, ocular melatonin levels showed marked changes in the light/dark cycle, with lower values during the light period and higher values in the dark. A reduction in plasma melatonin levels was observed on exposure to pulse light for *A. ocellatus* and *B. amazonicus*, indicating that plasma melatonin is highly sensitive to light. In summary, the results highlight the importance of evaluating biological rhythms (circadian and infradian) for physiological parameters in species of commercial interest and susceptible to stressful situations in rearing conditions. In addition, the information generated can support the advancement of new technologies that may be used by environmental management programs and, consequently, contribute to the well-being of fish, essential for sustainable aquaculture.

Key-words: circadian rhythm, lunar cycle, melatonin, matrinxã, cichlid.

Sumário

1. Introdução Geral

A maioria dos seres vivos exibe ritmos biológicos como estratégia para sincronizar suas atividades comportamentais e fisiológicas às mudanças cíclicas no ambiente, fundamentais a sua sobrevivência (Ali, 1992; Andreatta e Tessmar-Raible, 2020; Kaiser e Neumann, 2021). Oscilações periódicas em certos processos fisiológicos permitem que um organismo ou uma população coordene a atividade comportamental, fisiológica ou reprodutiva de uma espécie e entre espécies de um ecossistema integrado, garantindo que suas necessidades diárias possam ser realizadas na hora mais apropriada do dia, mês ou ano (Leatherland et al., 1992).

Ritmos biológicos são considerados eventos cíclicos, que tem como principal regulador os ciclos ambientais, nos quais os animais podem se aclimatar (Hastings et al., 2007; Albrecht, 2012; Schibler et al., 2015). Todas as formas de vida exibem alguns tipos de ritmos, que podem ser classificados de acordo com sua periodicidade: ritmos ultradianos, que são ciclos que se repetem em intervalos de até 20 horas; circadianos que são ciclos de 24 horas; e infradianos que são ciclos que se repetem em intervalos maiores do que 28 horas (Schulz e Leuchtenberger, 2006; Marques e Menna-Barreto, 2011). Dentre esses ritmos, o ciclo circadiano é considerado o mais importante fator ambiental sincronizador dos ritmos biológicos (Vera et al., 2007). De certa forma, alguns desses ritmos expressam correlação com os ciclos geofísicos (Raible et al., 2017), um deles é ciclo infradiano, bem representado pelo ciclo lunar.

O ciclo claro-escuro é um tipo de ciclo circadiano que afeta uma variedade de funções fisiológicas e comportamentais em peixes (Chen et al., 2002). O ritmo circadiano é definido pela ocorrência em um período de 24 horas (Marques e Menna-Barreto, 2011), de acordo com o tempo de rotação da Terra em torno do seu próprio eixo (Froy, 2010). Essa ritmicidade está relacionada à atividade dos animais e compreende uma série de comportamentos, como por exemplo, a busca por alimentos, defesa e a reprodução (Montoya et al., 2010).

O ritmo lunar tem demonstrado efeitos modulatórios no comportamento e na fisiologia para várias espécies, incluindo os peixes (Tessmar-Raible et al., 2011; Raible et al., 2017; Andreatta e Tessmar-Raible, 2020), uma vez que a claridade do luar em determinadas fases lunares, reduz a síntese de melatonina pineal e plasmática nessas espécies de estudo, como por exemplo, *Siganus canaliculatus* (Rahman et al., 2004), *Siganus guttatus* (Takemura et al., 2004a) e *Siganus argenteus* (Takemura et al., 2004b).

Nesse sentido, a regulação dos sistemas fisiológicos é realizada através de mecanismos sincronizados com os fatores ambientais (Evans, 1998; Kulczykowska et al., 2010). Esta sincronização é mediada pelo Núcleo Supraquiasmático do hipotálamo (NSQ) do Sistema Nervoso Central (SNC), pela retina e pela glândula pineal, que secreta melatonina, hormônio essencial na sensibilidade às variações dos períodos de luz (Maitra et al., 2013; Veras et al., 2013).

Assim, informações fóticas são convertidas em um sinal endócrino na forma do hormônio melatonina, produzido na glândula pineal e liberado na corrente sanguínea, funcionando como o marcador central que regula os ritmos diários do comportamento e fisiologia dos animais (Ekström e Meissl, 2003; Falcón et al., 2007; Falcón et al., 2010; Li et al., 2012; Wilczynski e Lutterschmidt, 2016). De outra forma, a melatonina produzida na retina pode estar envolvida em proteção parácrina e adaptação de retina e também tem sido relatada na ritmicidade circadiana (Falcón et al., 2003; Migaud et al., 2007).

De acordo com Veras et al. (2013), existe uma grande plasticidade nos ritmos biológicos dos peixes, sendo a variação da preferência do regime de luz espécie específica e dependente do período de desenvolvimento em que se encontram os animais. Nesse sentido, uma ampla variedade de variáveis fisiológicas exibe ritmos biológicos em vertebrados. Entre eles, a existência de ritmos em hormônios e indicadores do metabolismo de carboidratos e lipídeos tem sido relatada (Kriegsfeld e Silver 2006; Haus, 2007; De Pedro et al., 2008; Tonsfeldt e Chappell, 2012; Tsang et al., 2014).

A melatonina, por exemplo, é regulada pelo ciclo diário de luz/escuro (Nikaido et al., 2010), sendo liberada apenas durante o período de escuro e inibida em presença de luz, conforme observado para *Dicentrarchus labrax* (Villamizar et al., 2012). Assim, esta ritmicidade torna-se a base para eventos fisiológicos e comportamentais, incluindo a atividade locomotora, síntese e secreção de hormônios. Além disso, a melatonina está relacionada a redução do estresse oxidativo, atuando como um potente doador de elétrons, detoxificando, dessa forma, todas as espécies reativas de oxigênio, contribuindo para a diminuição do estresse oxidativo (Okatani et al., 2000; Yerer et al., 2003; Pandi-Perumal et al., 2006; Pandi-Perumal et al., 2008 Othman et al., 2008; Fagundes et al., 2010).

Portanto, os animais são frequentemente influenciados por estímulos ambientais, sendo estes, fundamentais para a expressão dos ritmos endógenos, mostrando que o ciclo circadiano e o ciclo infradiano atuam como importantes sincronizadores dos ritmos

biológicos (Wright et al., 2006; Falcón et al., 2010; Assis e Oster, 2021; Poehn et al., 2022; Zurl et al., 2022).

Assim, avaliar o efeito do ritmo circadiano e do ciclo lunar sobre os parâmetros fisiológicos é de grande importância, pois permite o entendimento da modulação endócrina no metabolismo dos peixes, e consequentemente, o desenvolvimento de técnicas que visem o bem-estar dos peixes, sejam eles de ambiente natural ou artificial.

Apesar de vários estudos relatarem a existência de ritmos biológicos para parâmetros fisiológicos e comportamentais em peixes (López-Olmeda et al., 2006, 2012; Oliveira et al., 2013; Vera et al., 2014; Paredes et al., 2015; Fortes -Silva et al., 2018), pouco se sabe sobre o perfil diário e lunar nas variáveis hematológicas em espécies amazônicas.

Astronotus ocellatus é uma espécie de grande interesse comercial, especialmente como peixe ornamental, sendo criada por aquaristas em todo mundo, e sua carne muito valorizada na região amazônica, o que caracteriza a espécie com significativo potencial para a aquicultura (Fabregat et al., 2006; Gonçalves-de-Freitas e Mariguela, 2006, Daaddy, 2012; Carvalho et al., 2017). Inserida na ordem dos perciformes, *A. ocellatus* pertence à família Cichlidae, com exemplares amplamente distribuídos na bacia amazônica, ocorrendo desde a Ilha de Marajó até alguns tributários do Peru e da Venezuela (Marcon, 1996).

Originário da bacia amazônica, *A. ocellatus* é conhecido como acará-açu ou apaiari. É uma espécie bentopelágica, diurna, de hábito alimentar onívoro, mas com tendência à carnivoria (Fontenele, 1982; Santos et al., 1984; 2009). Apresenta comportamento territorialista durante as fases de alevino e juvenil, exibe hábito gregário, mas quando adultos, costumam viver em grupos menores ou isoladamente (Braga, 1962; Beeching, 1992). Vivem preferencialmente em lagos de várzea e igapó, e podem ser encontrados por toda extensão da bacia amazônica (Marcon, 1996).

Embora o oscar seja de relevância econômica tanto para a piscicultura ornamental quanto para produção de proteína animal, exibe elevadas taxas de comportamento agressivo (Gonçalves-de-Freitas e Mariguella, 2006), que pode aumentar o estresse fisiológico, um dos principais problemas de bem-estar na criação e manutenção de peixes em sistemas artificiais (Carvallino et al., 2023).

Outra espécie de grande importância na região amazônica é o matrinxã (*Brycon amazonicus*), também nativo da bacia amazônica (Howes, 1982), é uma espécie onívora (Izel et al., 2004), bentopelágica, habita lagos e rios de águas brancas, claras e pretas

(Goulding et al., 1988; Ferreira, 1993; Saint-Paul et al., 2000; Do Vale, 2003; Siqueira-Souza e Freitas, 2004; Yamamoto, 2004). Apresenta hábito diurno, faz migração reprodutiva no início da enchente, formando cardumes em direção ao encontro dos rios de águas brancas e pretas para desovar (Lima e Araújo-Lima, 2004; Granado-Lorencio et al., 2005; Soares et al., 2008). Devido sua importância na pesca, é também um dos peixes mais utilizados na aquicultura regional, apresenta grande relevância comercial, por isso é a segunda espécie nativa mais produzida na região Norte (Santos et al., 2009; SIDRA-IBGE, 2021).

No entanto, existem problemas fundamentais na produção desta espécie, principalmente devido ao estado de estresse causado pelo manejo que ocorre em sistemas artificiais, como por exemplo, despesca, transporte, biometria e manutenção da qualidade de água, que por sua vez, afeta a reprodução, a larvicultura e o crescimento dos juvenis, comprometendo sua expansão produtiva na região amazônica (Bernardino et al., 1993; Brandão, 2009; Souza et al., 2014).

Assim, os peixes são constantemente submetidos a condições estressoras que são inevitáveis, por meio de agentes antropogênicos, que podem ser crônicas ou recorrentes (Huntingford et al., 2006). Neste cenário, as respostas comportamentais e fisiológicas ao estresse não têm mecanismos adaptativos e é potencialmente prejudicial aos peixes (Huntingford et al., 2006). Portanto, indicadores fisiológicos são importantes marcadores na avaliação dos ritmos biológicos em peixes, pois permite identificar os períodos do dia em que ocorrem as maiores variações dos parâmetros sanguíneos nos peixes. A identificação dessas variações poderá evitar problemas com antecedência para não prejudicar o bem-estar dos peixes.

Estudos anteriores avaliaram as respostas fisiológicas e comportamentais de *A. ocellatus* (Muusze et al., 1998; Gutierre et al., 2016) e *B. amazonicus* (Lopes et al., 2018; Ferreira et al., 2020**)** decorrentes da exposição a diferentes fatores ambientais. Entretanto, apesar de existirem, há poucas informações sobre a existência de ritmos biológicos e o efeito do ciclo claro-escuro e do ciclo lunar em peixes de água doce nos indicadores de gasto energético para essas duas espécies de peixes amazônicos de interesse comercial.

O ciclo claro-escuro e o ciclo lunar apresentam forte influência sobre o ritmo biológico dos animais, e podem interferir no gasto energético, bem como em outros parâmetros fisiológicos. Em algumas espécies de peixes, o ciclo claro-escuro e as fases da lua podem modular o comportamento e a fisiologia desses animais. Sendo assim, pesquisas relacionadas aos ritmos biológicos são de fundamental importância para a

manutenção dos peixes e, consequentemente a otimização dos sistemas de produção. Por esta razão, *A. ocellatus* e *B. amazonicus* foram escolhidas para esse estudo por serem espécies de alta representatividade comercial na Amazônia, e que estão suscetíveis a práticas de manejo em sistemas artificiais, o que pode desencadear situações potencialmente estressoras, comprometendo a homeostase dos peixes.

Logo, o estudo dos ritmos diário e lunar dos parâmetros fisiológicos em juvenis de *A. ocellatus* e *B. amazonicus*, permitirá a compreensão dos possíveis efeitos das variações diárias e das fases da lua no metabolismo dos peixes, e pode indicar o melhor horário para manusear os animais, e consequentemente um menor impacto das práticas de manejo para não comprometer a saúde e o bem-estar dos peixes, considerando que qualquer interferência nesses ritmos pode causar estresse nos animais.

Esta pesquisa fornece dados relevantes que poderão ser utilizadas por programas ambientais de manejo e piscicultores a fim de estabelecer medidas que favoreçam as manipulações durante a criação intensiva, dos ambientes experimentais e durante as atividades de pesca do oscar e do matrinxã, melhorando o manejo dos estoques naturais e, consequentemente, o bem-estar dos peixes, o que é fundamental para a manutenção dos animais, seja em ambiente natural ou artificial.

De acordo com essas predições, este projeto tem por objetivo avaliar os ritmos biológicos de parâmetros fisiológicos em juvenis de *A. ocellatus* e *B. amazonicus*. Assim, espera-se que a fase escura do ritmo circadiano e a fase da lua nova do ritmo infradiano possam atuar na modulação dos indicadores de gasto energético e melhorar o bem-estar dos peixes. Diante disso, foram avaliados os efeitos dos ritmos circadiano e lunar em indicadores hematológicos, metabólicos e hormonais (cortisol e melatonina) em peixes amazônicos. Os resultados obtidos estão apresentados em três capítulos:

Capítulo 1: Ritmo diário de alguns parâmetros sanguíneos em dois peixes amazônicos, *Astronotus ocellatus* e *Brycon amazonicus*.

Capítulo 2: A exposição à luz do luar afeta as mudanças dia/noite na melatonina e nos parâmetros metabólicos em peixes amazônicos?

Capítulo 3: Ritmo diário da melatonina plasmática e ocular em peixes amazônicos.

Serão apresentadas as considerações finais que envolvem as principais conclusões e as implicações deste estudo para futuras pesquisas.

Capítulo 1_

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Daily rhythm of some blood parameters in two Amazonian fish, *Astronotus ocellatus* and *Brycon amazonicus*

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Abstract

We evaluated the effect of the light–dark (LD) cycle on energy expenditure indicators for two species of Amazonian fish of commercial interest, oscar (*Astronotus ocellatus*) and matrinxã (*Brycon amazonicus*). Fish were exposed to a 12:12 h LD cycle, with lights on at 'zeitgeber time' (ZT) = 0 h. Six animals were used for blood collection every 4 h for 24 h (six time points, three in the light period and three in the dark period). Most haematological parameters exhibited daily rhythms with the acrophase at the end of the dark phase or the beginning of the light period for both species, which may be related to the greater energy demand of these species during the day. For *A. ocellatus*, triglycerides and total protein showed acrophases at ZT = 13:32 ± 2:45 h and ZT = 23:42 ± 1:55 h, respectively, while the other plasma parameters showed no significant daily differences. In *B. amazonicus*, significant rhythms were observed for glucose levels (acrophase at ZT

= 04:16 ± 2:47 h), triglycerides (ZT = 6:51 ± 4:06 h) and total cholesterol (ZT = 23:22 ± 3:45 h). However, total protein and cortisol levels did not show rhythmicity in this species. Our results highlight the importance of the evaluation of biological rhythms for plasma physiological parameters related to energy expenditure in species with commercial interest and susceptible to stressful situations in farming conditions.
Keywords: blood count, circadian rhythm, plasma metabolite, stress, teleost

1. Indrodution

Biological rhythms are observed in living organisms as a strategy for adjusting to environmental cycles (temperature and light) and, in teleost fish, these are regulated by endogenous oscillators that are controlled by a genetically encoded internal timing system (Ali, 1992; Toloza-Villalobos et al., 2015; Sánchez-Vázquez et al., 2019). According to Steindal and Whitmore (2019), central clock (i. e. suprachiasmatic nucleus in mammals) does not appear to be necessary for fish. However, this does not mean that there is no potential interaction between tissue clocks within the fish body. Moreover, a whole variety of hormonal signals, including rhythmic melatonin cues, could also be influencing tissue-specific, daily oscillations (Steindal, & Whitmore, 2019). Several natural and anthropogenic factors can act as circadian disrupters in fish and, consequently, affect multiple physiological processes (Zheng et al., 2021). So, we consider it important to know the daily rhythm of fish to understand the effect of potential environmental disruptions, including in an aquaculture system.

These rhythmic variations synchronize the animal with the environment and provide adaptive advantages by anticipating periodic events and programming physiological responses to occur at specific times of the day or year, thus increasing the probability of success and minimizing energy demand (Gerkema, 1992; Aranda et al., 1999). In addition, knowledge of biological rhythms has applications in the context of aquaculture (Parker, 1984; Mattos et al., 2017), fish physiology (Yufera et al., 2014; Cowan et al., 2017), behaviour (Montoya et al., 2010; Mattos et al., 2016), stress responses (Almeida et al., 2018; Tian et al., 2019), immune system (Bowden, 2008) and the productive performance of fish (Sánchez-Vázquez et al., 1995; Pedrosa et al., 2019).

Environmental changes arising from aquaculture practices can act as a synchronizer of biological rhythms, although they must meet several conditions in order to be considered “zeitgebers”, a term that is defined for exogenous synchronizers, which are classified as either photic (luminosity) or non-photic (temperature, feeding and

stressors) (López-Olmeda et al., 2006; López-Olmeda, 2017; BlancoVives et al., 2011; Vera et al., 2014). In fact, the light–dark cycle plays a very relevant role in influencing biological rhythms in fish, with effects on body development (Blanco-Vives et al., 2011), the immune system (Binuramesh & Michael, 2011), locomotion (López-Olmeda et al., 2012), feeding (Mattos et al., 2016), metabolism (Paredes et al., 2015), release of enzymes and hormones (Yufera et al., 2014; Cowan et al., 2017) and oxidative stress indicators (Tian et al., 2019).

Different parameters that indicate energy expenditure, and are also associated with the physiological stress response, vary during the day and, consequently, present well-characterized biological rhythms (Cowan et al., 2017; Fortes-Silva et al., 2018; Ren et al., 2020). Cortisol, for example, has been the focus of studies on rhythms, mainly because it presents daily patterns in a wide variety of teleost (Montoya et al., 2010; Oliveira et al., 2013; Brüning et al., 2015), although rhythm features such as MESOR (average), amplitude (maximum oscillation) and acrophase (peak time) are species specific (Vera et al., 2014). Under intensive aquaculture conditions, cortisol, as well as other indicators of acute and/or chronic stress (e.g. erythrogram and plasma metabolites), present variation in the synthesis and release depending on the duration and intensity of the management procedures to which the fish are subjected (Conte, 2004; Huntingford et al., 2006). In this context, it is reasonable to suggest that stress responses are not the same at all periods of the light–dark cycle, highlighting the importance of considering the most appropriate time of day when stress indicators are evaluated (Oliveira et al., 2013; Guerra-Santos et al., 2017).

For this reason, acquiring knowledge of the daily rhythm of cortisol, haematological parameters and plasma metabolites of different fish species is an important approach for aquaculture, since these variables can be used as physiological indicators of stress and energy expenditures (Wendelaar-Bonga, 1997). Moreover, haematological parameters are important indicators of fish health status in the aquaculture system (Fazio, 2019) and they are affected by endogenous and exogenous factors (Ahmed et al., 2020).

In general, it is argued that knowledge regarding physiology related to stress and the effect of environmental changes under the rhythm of these physiological parameters in animals kept in an artificial system would enable the identification of adverse conditions in the environment and the development of methodologies or procedures that may mitigate the effects on the health and welfare of fish (Conte, 2004; Martins et al.,

2012). Parker (1984) suggests that production efficiency and fish quality can be potentiated if the activities during the production process are carried out considering the biological rhythm of the species. This author also points out that environmental conditions must be considered in production systems and that routine practices during the production chain need to be altered based on information from chronobiology. In this context, chronobiology is an important tool that can assist us to achieve the objectives of aquaculture, especially in the Amazon region, since it has high potential for studies of this nature due to the high diversity of viable fish species both for aquaculture and the fisheries sector.

Although several studies reported the existence of circadian rhythm for physiological and behavioural parameters in fish (López-Olmeda et al., 2006, 2012; Oliveira et al., 2013; Vera et al., 2014; Paredes et al., 2015; Fortes-Silva et al., 2018), little is known about the daily profile of haematological variables in Amazonian species with interest in aquaculture. Among these species is the "oscar" (*Astronotus ocellatus*), which has high potential for tropical aquaculture, with significant trade in world fish keeping, its meat being highly valued in the Amazon region (Santos et al., 2009; Oliveira et al., 2013). The 'matrinxã' (*Brycon amazonicus*) is also native to the Amazon basin, and it is the second most produced fish species in the northern region of Brazil (SIDRA-IBGE, 2018). However, there are fundamental problems in the production of this species, especially due to the stress state caused by the management that occurs in artificial systems, which in turn affects the reproduction, larviculture and growth of juveniles (Bernardino et al., 1993; Souza et al., 2014), compromising its production expansion in the Amazon region. Previous studies have evaluated physiological and behavioural responses of A. ocellatus (Muusze et al., 1998; Gutierre et al., 2016) and *B. amazonicus* (Lopes et al., 2018; Ferreira et al., 2020), resulting from exposure to different environmental factors. However, nothing is known about the existence of rhythms and the effect of the light– dark cycle on energy expenditure indicators for these two species of Amazonian fish of commercial interest. Thus, our study aims to investigate the daily rhythm of haematological parameters in juvenile *A. ocellatus* and *B. amazonicus* in order to propose the ideal periods for management and manipulation in fish-breeding systems and experimental environments, as well as improving the welfare of these fish species.

2. Materials and Methods

2.1. Acquisition and acclimation of animals

This study was performed in the Laboratory of Physiology and Behavior of Aquatic Animals at the Federal University of Amazonas (UFAM), Amazonas, Brazil. Juvenile *B. amazonicus* (mean weight ± SD: 64 ± 4 g; mean length ± SD: 15.75 ± 0.36 cm) were provided by a fish hatchery (Aquaculture Station of the Experimental Farm [FAEXP] at UFAM), and juvenile *A. ocellatus* (mean weight ± SD: 130 ± 29 g; mean length ± SD: 16.12 ± 1.38 cm) were captured in their natural environment under licence No. 60643–3, which was obtained from the Chico Mendes Institute for Biodiversity Conservation (ICMBIO). All fish had no physical injuries and they were acclimated for 15 days in a 500-L polyethylene tank (1 animal/5 L) which contained internal biological filters for the maintenance of the water quality for a period of at least 15 days. The light intensity in the light period was maintained at 150 ± 42 lx and a photoperiod of 12:12 h light:dark (LD) cycle, with lights on at 06:00 h (zeitgeber time 0 h, ZT 0 h) and lights off at 18:00 h (ZT18 h). These factors were monitored with the aid of a lux meter (MLM-1011, Minipa, Brazil) and time data recorder (HL TM24H, Hardline, Brazil). The tanks were filled with artesian well water with 50% renewal every 3 days, and the water presented the following values for the measured parameters: temperature (27.0 ± 0.4°C), pH (6.1 ± 0.8), dissolved oxygen (5.5 ± 1.0 mg/L) and total ammonia (0.02 ± 0.01 mg/L). The fish were fed with a commercial feed to satiety (28% protein, Gabi, Brazil) twice a day. All the described procedures were performed for the two species under study.

The experiment was conducted according to the principles of ethics in animal experimentation (CONCEA) and was approved by Ethics Commission on the Use of Animals (CEUA-UFAM), case number No. 066/2018.

2.2. Experimental design

After the acclimation period, 36 juvenile *A. ocellatus* (six animals per tank, six tanks in total) and 18 juvenile *B. amazonicus* (three animals per tank, six tanks in total) were grouped in 500L polyethylene tanks for a 24 h period (12:12 h LD cycle). During this period, fish of both the species were fasted to avoid the influence of this procedure on the energy expenditure indicators to be measured.

In studies of circadian rhythms, when the lights are turned on, this period is called zeitgeber (synchronizer) time (ZT) and this corresponds to ZT0 (in this study = 06:00 h); and when the lights are turned off, this period corresponds to ZT12 (in this study = 18:00 h) (López-Olmeda et al., 2016). Every 4-h interval, all animals in the same tank (n = 6 for *A. ocellatus* and n = 3 for *B. amazonicus*) were used for blood collection, totalling

six samples: three in the light period (8:00 h/ZT2, 12:00 h/ZT6 and 16:00 h/ZT10) and three in the dark period (20:00 h/ZT14, 00:00 h/ZT18 and 04:00 h/ZT22).

Most of the studies regarding the daily rhythm of physiological parameters evaluate the effect of environmental factors, such as feeding period and photoperiod, which act as modulators of the circadian cycle in fish (Spieler & Noeske, 1984; Bayarri et al., 2009; Montoya et al., 2010; Tian et al., 2019). However, this study analysed the daily pattern of these physiological indicators of energy expenditure without the influence of external factors that could act as synchronizers of the biological rhythm, and for this, the animals were kept fasted during the 24 h of the experimental period. In addition, the photoperiod was controlled for 12 h light:12 h dark, which avoided the effect of manipulation by these factors on the cyclic variation in the physiological variables analysed.

2.3. Blood collection

At the end of the experimental period, fish were anaesthetized with eugenol (64 µl/L), as indicated by Ferreira et al. (2020), and immediately punctured in the caudal vein region to obtain blood samples with the aid of 0.8 ml disposable syringes containing heparin (5000 units/ml) as an anticoagulant. During the dark period, animal manipulation and blood collection were performed under low intensity red light (<0.3 lux) to avoid the effect of luminosity on physiological parameters, in accordance with Oliveira et al. (2007).

2.4. Blood parameters

Haematological analyses were performed immediately after blood collection at each moment of the cycle. The concentration of blood haemoglobin (Hb, g/dl) was analysed using the cyanmethaemoglobin method (Kampen & Zijlstra, 1964). The erythrocyte count (RBC, 10^6/µl blood) was performed via optical reading in a Neubauer chamber of blood samples fixed in formalin–citrate (1:200), according to the usual method used for fish. Haematocrit (Htc, %) was determined using the microhaematocrit method (Goldenfarb et al., 1971), in which the capillary tubes were centrifuged at 13,000 rpm for 6 min. The erythrocyte indices, mean corpuscular volume (MCV, fL), mean corpuscular haemoglobin (MCH, pg/cell) and the concentration of mean corpuscular haemoglobin (MCHC, g/dl) were calculated from the haematological parameters (Wintrobe, 1934).

The remaining blood collection material was centrifuged for plasma separation (3000 rpm/15 min) and kept at -20°C for biochemical analysis. Plasma levels of glucose (mg/dl), triglycerides (mg/dl), total cholesterol (mg/dl) and total proteins (g/dl) were obtained using commercial enzymatic–colorimetric kits (In Vitro Diagnostica Ltda; Itabira/ MG, Brazil), which were specific for each constituent. The plasma concentration of cortisol was analysed using the immunoenzyme method (ELISA) and a commercial kit (DRG International, Germany), as performed by Barry et al. (1993). The reference values were approximate to those described by Muusze et al. (1998) and Ferreira et al. (2020) for *A. ocellatus* and *B. amazonicus*, respectively, with the intra-trial variation of 4.05%.

2.5. Data analysis

The Cosinor analysis was used to detect the existence of significant rhythmicity of the haematological parameters, metabolites and plasma cortisol, performed with the software "EL TEMPS" (v. 1.179, Prof. Diez-Noguera, University of Barcelona, Spain). The Cosinor analyses is based on the least-square approach of time-series data with a cosine function of a known period of the type $Y = M + A*[Cos (\Omega t + \Phi)]$, where M is the MESOR, A is the amplitude, Ω is the angular frequency (3600/24 h for daily rhythms) and Φ is the acrophase, according to López-Olmeda et al. (2012) and Ren et al. (2020). The Cosinor analyses also provide the statistical significance of the rhythm by and F-test of the variance accounted for by the waveform versus a straight line of zero amplitude (null hypothesis).

In order to detect the existence of statistical differences between time points, the SPSS Software (IBM, version 22.0, Armonk, NY, USA) was used. Data normality was previously evaluated using the Shapiro–Wilk test and the homogeneity of variance was verified using the Levene test. All data are presented as mean ± SEM. As distribution was normal, data on energy expenditure indicators (haematological parameters and indices, and plasma metabolites) were compared between periods by the use of one-way ANOVA, followed by Duncan's post hoc test. We also compared the mean cortisol concentration of the three collection moments (grouped data) between the light and dark periods, using an independent Student's t-test. Statistical analyses were performed with the level of significance set at $p < 0.05$.

3. Results

In regards to *A. ocellatus*, significant daily rhythms were observed for Htc (Cosinor, p = 0.029) and MCV (p = 0.004), with acrophases at 03:17 ± 4:18 h and 4:47 ± 2:59 h respectively (Table 1; Figures 1 and 3). Htc decreased at the beginning of the dark period (ZT14) compared with the end of the same period (p = 0.020) (ZT22; Figure 1). MCV increased at the beginning of the light period (ZT2) and decreased at the beginning of the dark period (p = 0.006) (ZT14; Figure 1). No daily rhythm was observed for Hb, RBC, MCH and MCHC (p > 0.05; Table 1), although the concentrations of Hb and RBC were significantly (ANOVA, p = 0.001) higher at ZT22 (Figure 1).

For *B. amazonicus*, rhythmicity was observed for most haematological parameters, such as Hb (acrophase: 21:56 ± 2:43 h; p = 0.001), Htc (acrophase: 4:38 ± 4:04 h; p = 0.024), MCH (acrophase: 7:28 ± 2:43 h; p = 0.001) and MCHC (acrophase: 19:40 ± 3:03 h; p = 0.006) (Table 1; Figures 1 and 3). No significant differences were observed for RBC and MCV (p > 0.05; Figure 1). Hb (p = 0.005) and MCHC (p = 0.013) were higher in the dark period (ZT22; Figure 1), while Htc (p = 0.016) and MCH (p = 0.002) were higher in the early light period (ZT2; Figure 1).

The total triglyceride and protein levels showed acrophase at 13:32 ± 2:45 h (p = 0.001) and 23:42 ± 1:55 h, respectively (p = 0.001; Table 1; Figures 2 and 3), and the other plasma parameters did not show significant difference between the periods of the light–dark cycle for *A. ocellatus* (p > 0.05). Triglycerides had higher plasma levels at the beginning of the dark period (p = 0.004), while total proteins decreased during the same period (p = 0.001) (ZT14; Figure 2). Despite not presenting a circadian rhythm, the plasma cortisol level increased at the beginning of the dark period (ZT14) compared with the light period (ZT2; p = 0.007; Figure 2).

In *B. amazonicus*, a circadian variation was observed through Cosinor analysis for glucose (acrophase: 04:16 ± 2:47 h; p = 0.001), triglycerides (acrophase: 6:51 ± 4:06 h; p = 0.025) and total cholesterol (acrophase: 23:22 ± 3:45 h; p = 0.011) (Table 1; Figures 2 and 3). The level of total proteins did not show rhythmicity (p > 0.05; Table 1); however, there was a significant increase in this parameter at the end of the light period (p = 0.036; ZT10) compared with the beginning of the same period (ZT2; Figure 2). Glucose levels were higher in the middle of the light period (ZT6) and lower in the middle of the dark period (ZT18) (p = 0.005; Figure 2). Triglycerides increased at the beginning of the light period (ZT2) compared with the dark period (ZT18) (p = 0.002; Figure 2). No daily rhythm was observed for cortisol concentration in *B. amazonicus* (p = 0.184; Table 1; Figure 2). However, when the mean concentration of this hormone of the three collection

moments between periods of light was compared with that of the dark period, higher plasma levels were observed during the light phase (237.84 ± 15.74 ng/ml) when compared with the dark phase (186.06 ± 13.80 ng/ml) (Independent t-test, p = 0.025).

Table 1. Mesor, amplitude, acrophase, and statistical significance values of the plasma physiological parameters subjected to Cosinor analysis for *Astronotus ocelattus* and *Brycon amazonicus*.

Parameters	**Species**	**Mesor**	**Amplitude**	**Acrophase** (ZT hours)	**Significance** (p)
Hb (g/dL)	*A. ocellatus*	-	-	-	NS
	B. amazonicus	7.29 ± 0.75	1.15 ± 1.35	21:56 ± 2:43	0.001
Htc (%)	*A. ocellatus*	22.96 ± 2.70	2.69 ± 4.90	3:17 ± 4:18	0.029
	B. amazonicus	34.84 ± 4.36	4.49 ± 7.85	4:38 ± 4:04	0.024
RBC (106/µL)	A. ocellatus	-	-	-	NS
	B. amazonicus	-	-	-	NS
MCV (fL)	*A. ocellatus*	132.10 ± 13.60	17.16 ± 24.29	4:47 ± 2:59	0.004
	B. amazonicus	-	-	-	NS
MHC (pg/cell)	*A. ocellatus*	-	-	-	NS
	B. amazonicus	8.79 ± 6.81	9.94 ± 12.26	7:28 ± 2:43	0.001
MCHC (g/dL)	*A. ocellatus*	-	-	-	NS
	B. amazonicus	21.50 ± 4.24	5.31 ± 7.63	19:40 ± 3:03	0.006
Glucose (mg/dL)	*A. ocellatus*	-	-	-	NS
	B. amazonicus	67.39 ± 14.60	21.89 ± 26.27	04:16 ± 2:47	0.001
Triglycerides (mg/dL)	*A. ocellatus*	135.63 ± 26.00	37.26 ± 45.65	13:32 ± 2:45	0.001
	B. amazonicus	274.04 ± 48.87	50.03 ± 87.99	6:51 ± 4:06	0.025
Cholesterol total (mg/dL)	*A. ocellatus*	-	-	-	NS
	B. amazonicus	21.81 ± 20.22	159,14 ± 29.42	23:22 ± 3:45	0.011
Total Protein (mg/dL)	*A. ocellatus*	2.56 ± 0.42	0.80 ± 0.76	23:42 ± 1:55	0.001
	B. amazonicus	-	-	-	NS
Cortisol (ng/mL)	*A. ocellatus*	-	-	-	NS
	B. amazonicus	-	-	-	NS

NS: non-significant; Errors are mean ± SEM, fiducial limits set at 95%. Abbreviations: Hb, haemoglobin; Htc, haematocrit; RBC, red blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration.

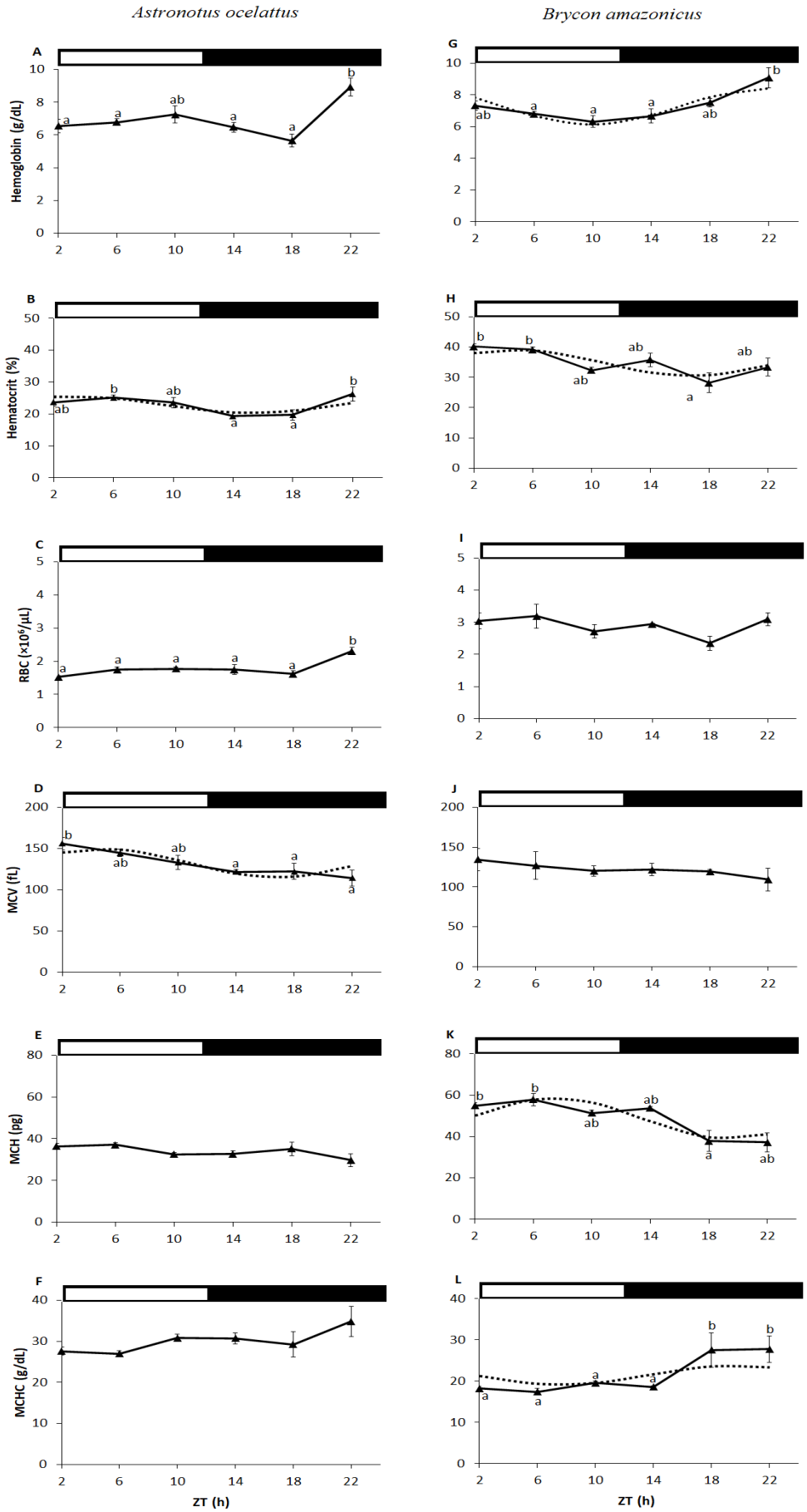

Figure 1. Daily changes in physiological parameters of *Astronotus ocelattus* (A–F) and *Brycon amazonicus* (G–L). A/G- Hb; B/H- Htc; C/I- RBC; D/J- MCV; E/K- MCH and F/L- MCHC. Values represent the mean ± S.E.M. ($n = 6$ / time point, *A. ocellatus*; $n = 3$/time point, *B. amazonicus*). White and black bars represent light and darkness respectively. Different letters indicate significant differences between time points (Duncan, $p < 0.05$). The sinusoidal dashed line represents the adjustment to a rhythm calculated by the Cosinor analysis whenever this analysis was statistically significant ($p < 0.05$). RBC, red blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration.

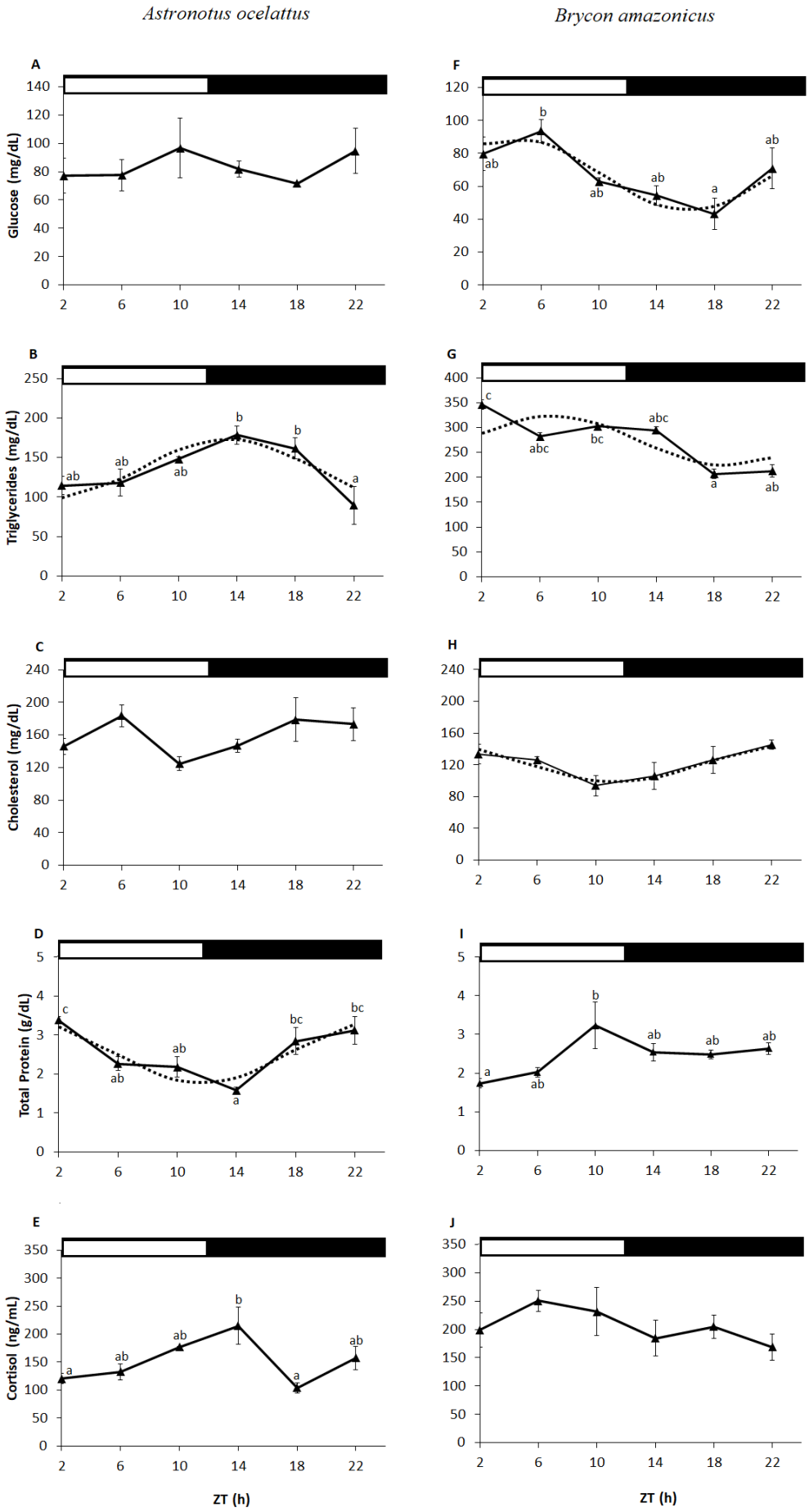

Figure 2. Daily changes in physiological parameters of *Astronotus ocelattus* (A–E) and *Brycon amazonicus* (F–J). A/F – Glucose; B/G – Triglycerides; C/H – Cholesterol; D/I – Total protein and E/J – Cortisol. Values represent the mean ± S.E.M. (*n* = 6/time point, *A. ocellatus*; *n* = 3/time point, *B. amazonicus*). White and black bars represent light and darkness respectively. Different letters indicate significant differences between time points (Duncan, $p < 0.05$). The sinusoidal dashed line represents the adjustment to a rhythm calculated by the Cosinor analysis whenever this analysis was

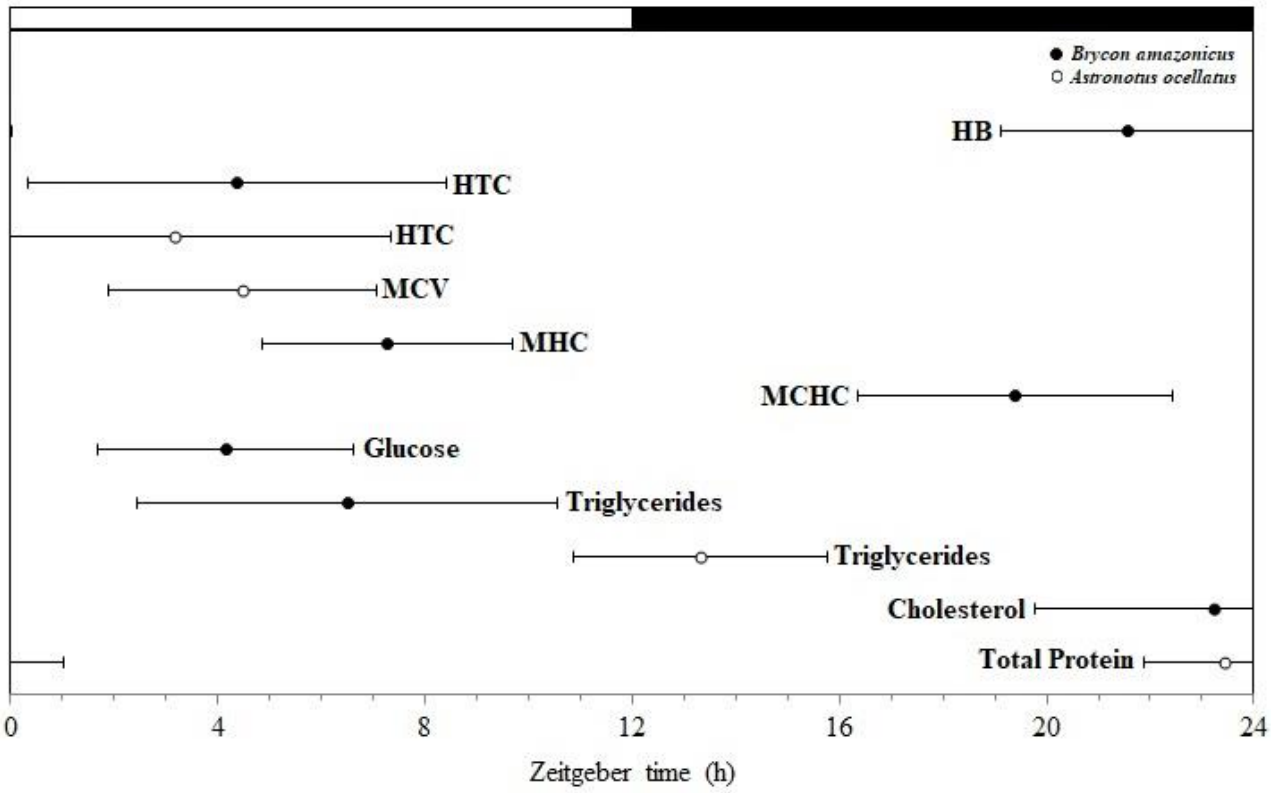

Figure 3. Acrophases map for the physiological parameters (Cosinor, $p < 0.05$). The acrophase is indicated by a circle, black and white for *Brycon amazonicus* and *Astronotus ocelattus* respectively. The confidence intervals (set at 95%) are indicated by the lateral bars. White and black bars above the graph represent light and darkness respectively.

4. Discussion

The haematological indices presented mean values similar to those described in other studies for *A. ocellatus* (Baptista et al., 2016) and *B. amazonicus* (Tavares-Dias et al., 2008; Ferreira et al., 2020), indicating the reference values and that the methodology used was suitable.

The concentration of HB, Htc, MCH and MCHC exhibited their acrophase at the end of the dark phase or beginning of the light period for *B. amazonicus*. Considering that the haematological parameters are related to the blood's oxygen transport capacity (Witeska, 2013), these results are consistent with the high level of swimming activity of *A. ocellatus* that has been reported during daytime (Soares et al., 2008), which may reflect a higher respiratory demand during this period. In fact, the release of erythrocytes by the spleen, and subsequent increase in Htc and RBC values, are directly related to the increased activity of animals (Fánge, 1992; Glomski et al., 1992). In addition, the higher values of MCH and MCHC may have been due to the increase in Hb, since these erythrocyte índices are directly proportional to the concentration of haemoglobin present in the blood (Wintrobe, 1934).

For *A. ocellatus*, the higher values of Hb and RBC at the end of the dark period and the acrophase for Htc and MCV at the beginning of the light period also suggest the triggering of physiological adjustments related to a greater energy demand during the day since *A. ocelattus* has a diurnal habit (Santos et al., 2009). In fact, erythrocyte indices may vary among species and within the same species, presenting a direct relationship with fish activity. For example, more active fish, such as tuna and other pelagic fish, tend to have higher MCV values when compared with benthic and sedentary species due to the higher demand of 02 for animals with higher activity (Clauss et al., 2008). In addition, the same pattern of rhythmic response observed for Htc and MCV corroborates the direct relationship between these two haematological indicators (Wintrobe, 1934).

The daily rhythm pattern for plasma glucose concentration varies among fish species. In the present study, the absence of daily rhythm for glucose levels in *A. ocellatus* coincides with the results obtained for *Oreochromis niloticus*, another species of cichlid (Almeida et al., 2018) and *Centropristis striata* (Ren et al., 2020), as well as for *Tinca tinca* and *Oncorhynchus mykiss* when subjected to food deprivation (De Pedro et al., 2005; Polakof et al., 2007). For *B. amazonicus*, the observation of a daily glycaemic rhythm with acrophase at ZT = 04:16 ± 2:47 h may indicate the preparation for the greatest energy demand during the day, considering its high metabolic and behavioural activity that is predominant during daytime (Ferreira et al., 2020; Soares et al., 2008). These results are consistent with the findings of Guerra-Santos et al. (2017), which show higher glycaemic values in the period of greater locomotor activity in *Oreochromis niloticus*.

In teleost, the circadian profile of plasma cortisol is considered to be species specific and related to the activity pattern (diurnal or nocturnal) of the animal (Ellis et al., 2012; López-Olmeda et al., 2013). However, Cowan et al. (2017) suggested the need for further studies to demonstrate the relationship between the higher plasma cortisol concentration and the activity pattern of the fish species. The peak activity of this hormone may also be associated with the period of fish feeding, suggesting its synchronization with the feeding cycle (Spieler & Noeske, 1984; López-Olmeda et al., 2009; Montoya et al., 2010; Guerra-Santos et al., 2017). Thus, the 24-h fasting period to which the animals were submitted in this study may have influenced the absence of significant rhythms for cortisol in both *A. ocelattus* and *B. amazonicus*. Although no daily rhythm was observed for A. ocellatus, plasma cortisol concentrations remained stable until the end of the light phase, with values ranging from 120.50 ± 4.02 ng/ml (ZT2) to

136.24 ± 0.58 ng/ml (ZT10). However, a significant peak (214 ± 0.61 ng/ml) was observed at the beginning of the dark phase (ZT14), which is similar to that found for other fish species (Vera et al., 2014; Almeida et al., 2018; Tian et al., 2019). This fact can be explained since the increase in cortisol occurred shortly after the transition from the light phase to the dark phase, which is characterized as an abrupt environmental change in laboratory conditions and may have reflected in a physiological preparation as a result of a potentially stressful condition. In fact, Saito et al. (2004) showed that the plasma cortisol level did not present daily rhythm for *Oncorhynchus keta*, but an increase in this hormone was observed over the 24-h period, which may be associated with a stressful response due to capture and transport procedures. Pickering et al. (1982) also suggested that cortisol concentrations in different fish species typically rise a few minutes after exposure to a moderate acute stressor, peak and return to baseline values within approximately 6 h. Thus, this response may have been observed in the present study, since the baseline values returned within this time range, after the peak of cortisol which may be due to an acute response to the change in ambient luminosity. On the other hand, this hormone increase may not be a response to the change from dark to light period, but it may have endogenous control.

The rapid resume to the initial level of cortisol (ZT18) agrees with the lack of variation in blood glucose observed for *A. ocellatus*, since increases in plasma levels of cortisol and glucose are directly related (Carneiro et al., 2002). According to Mommsen et al. (1999), the increase in cortisol acts as a gluconeogenic signal by increasing the level of glucose in the blood in fish. In fact, this relationship can be evidenced because, although *B. amazonicus* did not present daily rhythm for cortisol, there was a higher concentration of this hormone in the light period, which may be associated with the glucose acrophase observed in the early morning (04:16 ± 2:47 h).

Cortisol can also promote increased levels of free fatty acids in plasma (Butler, 1973), thus increasing plasma cholesterol in some fish species (Poursaeid et al., 2015). In addition, cholesterol is the precursor of steroid hormones such as glucocorticoids, evidencing a direct relationship between cholesterol availability and cortisol synthesis (Miller, 1988; Sanderson, 2006). Although no cortisol rhythm has been observed, the rhythmicity of cholesterol with acrophase at 23:22 ± 3:45 h may indicate the higher cortisol synthesis evidenced by the higher concentration of this hormone in the light period for *B. amazonicus*.

A daily plasma triglycerides profile was observed for *A. ocellatus* and *B. amazonicus*, suggesting the body's attempt to seek greater input to maximize the use of energy substrate. According to Tocher (2003), lipids can function as an energy substrate for fish, and are important for providing support arising from increased metabolic activity. Higher triglyceride levels were observed in *B. amazonicus* in the early hours of the day (ZT2), with a significant decrease halfway through the dark phase (ZT18) and the acrophase at ZT = 6:51 ± 4:06 h. This response may be associated with greater mobilization of energy reserves due to greater activity of the species in the daytime (Soares et al., 2008; Rodrigues et al., 2017), which is a relationship also suggested for other parameters analysed in this study (e.g. Hb, Htc and blood glucose).

According to Fortes-Silva et al. (2018), the circadian variation in plasma proteins is complex as a result of the daily pattern of specific proteins; however, these data are important in assessing the health status of fish. In addition, Mommsen et al. (1999) report that changes in total protein concentrations are related to increased cortisol, which results in increased gluconeogenesis and protein catabolism activity. Thus, the results of the daily rhythm of this physiological parameter for *A. ocellatus* (acrophase: 23:42 ± 1:55 h) indicate that this circadian variation may be due to the increase in cortisol at the beginning of the dark period observed for the species. For *B. amazonicus*, no daily rhythmicity was observed, as also reported by De Pedro et al. (2005) and Fortes-Silva et al. (2018) for *Tinca tinca* and *Lophiosilurus alexandri* respectively.

Brycon amazonicus presented a greater number of physiological parameters that showed daily rhythms (Htc, Hb, MCH, MCHC, glucose, triglycerides and total cholesterol) compared with *A. ocellatus* (Htc, MCV, triglycerides and total proteins). This difference suggests that *B. amazonicus* presents greater biochemical modulation evidenced by the rhythmicity of energy expenditure indicators and relative to the increase in metabolic demand during the light period. In fact, *B. amazonicus* is a species that has a high rate of locomotion, aggressiveness and territoriality (Ferraz & Gomes, 2009; Ferreira et al., 2020; Souza et al., 2014), which is reflected by a high demand for O 2 transport and availability of energy substrates (Alvarenga & Volpato, 1995; Wendelaar-Bonga, 1997). *A. ocellatus*, presents also diurnal behaviour and it is a territorial fish (Beeching, 1992; Santos et al., 2009), although less activity can be observed when compared with *B. amazonicus*, and this is evidenced by the lower frequency of aggressive items exhibited by *A. ocellatus* (Gonçalves-de-Freitas & Mariguela, 2006) as compared

with *B. amazonicus* (Ferreira et al., 2020) in conditions of social challenge (mirror test) in an equivalent time period.

In summary, it can be observed that some physiological parameters that are indicators of energy expenditure and, therefore, related to stressful conditions, such as Hb, Htc, MCV, MCH, MCHC, glucose, triglyceride, cholesterol and total protein, present circadian variation for *A. ocellatus* and *B. amazonicus*. Therefore, care should be taken to assess the stress response and/or well-being in fish, especially when these parameters are measured at different times of the light–dark cycle, or when comparisons are made of the results of different studies or between treatments in the same study (López-Olmeda et al., 2012; Ren et al., 2020). Both species studied are of commercial interest and are susceptible to management practices in an artificial system that triggers potentially stressful situations. In this context, knowledge of the rhythms of physiological parameters in fish is essential in order to evaluate the time-dependent effects of treatments that induce stressful responses (e.g. manipulation) and also to maximize the efficiency of the administration of exogenous substances, as in the case of *B. amazonicus* whose reproduction depends on hormonal induction (Bernardino et al., 1993; Romagosa et al., 2001). In addition, this work may contribute to a greater adequacy of conditions that provide better welfare and productive performance for these animals when kept in artificial systems.

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Conflict of Interest

The authors declare no potential conflict of interest.

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Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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Capítulo 2_

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Does exposure to moonlight affect day/night changes in melatonin and metabolic parameters in Amazonian fish?

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Abstract

Lunar cycle modulates the rhythmic activity patterns of many animals, including fish. The effect of the moonlight cycle on daily melatonin and metabolic parameters was evaluated in matrinxã (Brycon amazonicus) subjected to external natural lighting. Eighty juvenile were distributed in 4 tanks of 1m^3 (20 fish/tank) and divided into two groups. One group was exposed to the full moon and the other group to the new moon for 30 days, which corresponds to the duration of the lunar period. At the end of the lunar phase, 6 fish from each group were anesthetized to collect blood, tissue and eye samples at midday and midnight. The comparison between the light and dark periods revealed a significant increase in plasma and ocular melatonin in the last period. However, there was

no significant difference for plasma melatonin between moons. Ocular melatonin presented higher concentrations during the new moon. Glucose, total proteins, cortisol, liver glutathione and gill lipid peroxidation were higher in the full moon compared to in the new moon. Plasma triglyceride was higher during the night for the full moon, and the opposite was found for the new moon. Total cholesterol values were higher at night regardless the moon phase. Glutathione in the gills and lipid peroxidation in the liver showed no significant differences. These results highlight the importance of considering both the day and lunar cycles for melatonin and metabolic parameters in species of commercial interest and susceptible to stressful situations in rearing conditions.

Keywords: biological rhythm, melatonin, metabolism, lunar cycle, fish

1. Introduction

Depending on its relative position, the illumination reflected from the sun by the moon changes during the lunar month. Moonlight modulates the rhythmic activity patterns of many living organisms and this may be transduced by changes in neural and hormonal levels such as melatonin and other hormones, as well as specific metabolites (Chakraborty, 2020). According to Andreatta and Tessmar-Raible (2020), connections between metabolic/endocrine pathways and moon-controlled rhythms are evidenced for a variety of species. However, in teleost fish, these mechanisms are still elusive, particularly in freshwater species.

Changes in moonlight during the phases of the moon synchronize lunar rhythms in organisms in various ways, such as, providing time cues to synchronize important biological aspects at individual and populational levels (Kronfeld-Schor et al., 2013; Gaston et al., 2017). Takemura et al. (2010) reported that the intensity of the light from the moon influences the physiological and behavioral activities of fish inhabiting tropical and subtropical waters. Indeed, the lunar cycle plays a relevant role in influencing the biological rhythms of fish, and has an effect on reproduction (Oliveira et al., 2009; Ikegami et al., 2014; Golmoradizadeh et al., 2021), molecular mechanisms (Steindal and Whitmore, 2019; Andreatta and Tessmar-Raible, 2020), predation behavior (Palmer et al., 2017) and the release of hormones such as melatonin and sexual steroids (Oliveira et al., 2010).

Biological rhythms, irrespectively from being a direct response to the environment or driven by endogenous oscillators, are also mediated by changes in

hormonal levels and metabolism (Pittendrigh, 1960; Ali, 1992). The evolution of biological clocks, for example, has probably been favored by the regularity of geophysical cycles and the advantages that organisms have in reacting to regular changes in the environment and anticipating these changes in order to prepare properly for them, thus maximizing their ecological fitness (Sellix, 2016; Raible et al., 2017). In addition, an essential factor in the regulation of biological rhythms is the input of melatonin rhythms, a key hormone involved in the regulation of many rhythmic processes (Reiter et al., 1997; Migaud et al., 2010; Falcón et al., 2010, 2011; ViviD and Bentley, 2018). The amplitude/duration of the nocturnal melatonin rhythm is finely tuned by environmental light intensity, photoperiod and temperature, thus providing the animal with daily, seasonal and lunar information (Reiter, 1993; Porter et al., 2001; Takemura et al., 2006; Brüning et al., 2015). In vertebrates, melatonin synthesis occurs mainly in the pineal gland, but in teleost fish it also occurs in the retina (Dodt, 1963; Omura and Oguri, 1969; Cahill, 1996; Vuilleumier et al., 2007; Vera et al., 2014). Pineal melatonin, in addition to controlling circadian activities, regulates seasonal reproduction, the rhythmicity of locomotor activity, the immune response and also acts as an antioxidant agent (Falcón et al., 2011; Wiechmann and Sherry, 2013). In contrast, the retinal melatonin pattern, despite exhibiting a daily rhythmicity, may presents peaks at different times of the day, depending on the species (Cahill et al., 1991; Falcón et al., 2003).

According to Oliveira et al. (2007), the nocturnal production of melatonin is very sensitive to light, i.e., a light intensity as low as 0.3 lx (full moon) is able to reduce plasma levels of melatonin. Also, in some marine fish species, the low illumination presents at a full moon can significantly reduce melatonin levels (e.g., Takemura et al., 2006; Oliveira et al., 2010; Park et al., 2014; Fukunaga et al., 2019). In short, melatonin translates environmental information about light or darkness into a hormonal signal to cells and organs, thereby synchronising behavioral and physiological rhythms (Falcón et al., 2020).

In addition to melatonin, parameters that indicate energy expenditure in fish also show rhythmic variations (Oliveira et al., 2013; Brüning et al., 2015; Cowan et al., 2017; Fortes-Silva et al., 2018; Ren et al., 2020). Oxidative stress indicators can also vary due to environmental changes and present rhythmicity associated with the light-dark cycle and the phases of the moon (Tal et al., 2011; Vera et al., 2014). However, little is known about the effect of moonlight on the rhythm of these physiological parameters. In this sense, further research is needed to understand the influence of the lunar cycle on fish in

order to fully understand the effect of potential environmental disturbances in aquatic systems such as nocturnal light contamination.

Knowing how moonlight modulates physiological and behavioral mechanisms in fish is crucial for understanding how chronobiological changes caused by anthropogenic impacts (e.g., light pollution and temperature changes, among others) and natural factors in aquatic ecosystems (e.g., Fogarty and Marhaver, 2019; Shlesinger and Loya, 2019). In fact, exposure to natural and artificial factors can generate temporal disturbances that lead to misalignment of physiology and metabolism (i.e. chronodisruption), thus obscuring the natural order of alternating periods of light and darkness at all levels of organization (Gaston et al., 2017; Falcón et al., 2020; Zheng et al., 2021). Studies of the effects of the moon on fish have also shown that spawning, migration, activity, feeding, physiology, and vulnerability to commercial or recreational fishing may be synchronized to lunar cycles (Ali, 1992; Ikegami et al., 2014; Vinson and Angradi, 2014). Besides its ecological relevance, little attention has been paid to this issue in freshwater tropical fish, such as the matrinxã (*Brycon amazonicus*), which is a native species of the Amazon Basin and of great importance in this region (Brazil, Peru and Colombia), since it is the second most-produced local fish species (FAO, 2022). In this context, this study aimed to investigate the influence of the moonlight cycle on physiological parameters in matrinxã (*Brycon amazonicus*) exposed to external natural lighting.

According to Sánchez-Vázquez et al. (2019), there is a need to properly take into account the role of biological rhythms when discussing fish welfare issues. As such, we intend to provide information that will contribute to the advance of new technologies that can be used by environmental management programs and, consequently, safeguard the welfare of fish, which is essential for sustainable and responsable aquaculture.

2. Materials and Methods

2.1. Area of study and ethical approval

This study was conducted in a dam (60 m wide x 3 m deep) with continuous water circulation located at the Experimental Farm of the Federal University of Amazonas (FAEXP/UFAM), Manaus, Brazil, (2° 38′ 39” S, 60° 03′ 11” W).

The experiment was carried out in accordance with the ethical principles of the Brazilian National Council for Animal Experimentation Control (CONCEA) and was

approved by the Ethics Commission in the Use of Animals (CEUA-UFAM) under process No. 066/2018.

2.2. Experimental design

Eighty juvenile *Brycon amazonicus* (271.0 ± 5.9 g; 23.3 ± 0.2 cm; mean ± SEM) obtained from the Aquaculture Station at FAEXP/UFAM were stored in four 1 m^3 net cages (20 animals per tank) and divided into two groups. After acclimation, one group was exposed to the full moon (0.1 lx [midnight] and 697.5 ± 80.5 lx [midday]) and the other group to the new moon (0.0lx [midnight]; 656.5 ± 50.5 lx [midday]), with 2 tanks per group and for 30 days. Both groups remained in their tanks exposed to the moon for the full lunar cycle (i.e., new moon from 7/2/ 2019 to 7/31/2019 and full moon from 6/17/2019 to 7/16/2019). The animals were stocked on the first day of each moon phase and blood samples were performed on the last day of each moon period.

After 30 days of each treatment (full moon and new moon), 6 animals were collected at midnight and 6 specimens were sampled at midday for the analysis of plasma and ocular melatonin concentration, plasma metabolites and oxidative stress indicators. The fish were anesthetized with 64 µl/L of eugenol (Biodinâmica, Ibiporã, Brazil; Ferreira et al., 2020), and immediately punctured in the caudal vein region in order to obtain blood samples with the aid of 1 ml disposable syringes containing heparin (Cristália, Itapira, Brazil). Subsequently, fish biometrics were performed: body weight of 319.0 ± 15.3 g and standard length of 25.6 ± 0.3 cm (full moon); body weight of 291.5 ± 4.8 g and standard length of 24.3 ± 0.5 cm (new moon).

Then, all the fish were killed by medullary section for eye, liver and gills collection. These samples were immediately frozen in liquid nitrogen and subsequently stored in a freezer - 80 °C for further analysis. Blood samples were centrifuged for plasma separation (3000 rpm*15 min^{-1}) and kept at - 80 °C for analysis of hormones (melatonin and cortisol) and plasma metabolites.

During the whole experimental period, the fish were fed with commercial feed (32% crude protein, Guabi, Indaiatuba, Brazil) until apparent satiety twice a day. The fish were fasted for a period of 24 h prior to collection to avoid the influence of feeding on the indicators of energy expenditure to be measured. During the dark period, for both phases of the lunar cycle, animal manipulation and tissue collection were performed under only moonlight and low intensity red light (0.2 ± 0.1 lx) to avoid the effect of luminosity

on physiological parameters. In fact, Oliveira et al. (2007) recommend <0.3 lx for nighttime collection.

The water quality parameters (temperature 29.7 ± 0.3 °C, dissolved oxygen 5.8 ± 0.1 mg/L and pH 7.5 ± 0.2) were monitored daily using a multiparameter probe (Askso, AK88, São Leopoldo, Brazil). The values of these parameter are considered appropriate for this species (Brasil, 2005).

2.3. Melatonin analysis

The melatonin analyses were carried out at the University of Murcia (Murcia, Spain). Eye samples were thawed, individually weighed and homogenized, discarding the cornea and crystalline lens, and maintained at 4 ºC in 1 ml NaCl 0.9%. Plasma and homogenized eyecup samples were extracted and purified using C18 phase extraction columns (IBL, Hamburg, Germany), after which an enzyme-linked immunosorbent assay (ELISA) (Melatonin ELISA kit, IBL, Hamburg, Germany) was performed, as previously described by Bayarri et al. (2002). Total eye protein was quantified using the Lowry method (Lowry et al., 1951), with melatonin values expressed in pg*mg of protein^{-1}. Plasma melatonin concentration was expressed in pg*ml^{-1}.

2.4. Metabolites and plasma cortisol

The levels of glucose (mg*dl^{-1}), triglycerides (mg*dl^{-1}), total cholesterol (mg*dl^{-1}) and total proteins (g*dl^{-1}) were measured using commercial enzymatic-colorimetric kits (In Vitro Diagnostica Ltda; Itabira, Brazil) that were specific for each constituent. Plasma cortisol concentration (ng*ml^{-1}) was analyzed using a commercial ELISA kit (Cortisol ELISA kit, IBL, Marburg, Germany), as described by Montoya et al. (2010).

2.5. Oxidative stress

Liver and gill samples were homogenized in 0.1 M sodium phosphate buffer (pH 7.0). Samples were weighed and homogenized 1:20 in 0.1 M sodium phosphate buffer (w/w). After homogenization, the material was used to quantify the total protein for glutathione concentration (GSH) and for lipid peroxidation analyses. Total protein was quantified using Bradford's method (Bradford, 1976). The concentration of GSH (µmol*g of tissue^{-1}) was measured spectrophotometrically at 412 nm, according to Beutler (1984). The method used was based on the reaction between GSH and 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), which results in the formation of trinitrobenzene

(TNB), a yellow product. Lipid peroxidation was estimated via malondialdehyde production (MDA; nmol*mg of protein^{-1}) using a thiobarbituric acid reactive substance (TBARS), a method described by Draper and Hadley (1990).

2.6. Data analysis

All data are presented as mean ± SEM. Data were tested for normality using the Levene test. Plasma and ocular melatonin levels, metabolic parameters, and oxidative stress indicators were compared between the moon phases (full moon and new moon) and the light and dark periods (midday and midnight) via two-way ANOVA, followed by the multiple-comparisons test. Statistical analyses were performed using SPSS Software, version 22.0. Level of significance was set at $p<0.05$.

3. Results

Plasma melatonin was lower during the light phase (midday) for the full and new moons, and increased significantly at night during both moon phases ($F = 9.320$; $p = 0.009$; Fig. 1A; Table 1). However, no interaction was observed between the phases of the moon and the day/night changes for plasma melatonin ($F = 0.005$; $p = 0.943$; Fig. 1A). For ocular melatonin, interaction was observed between the variables tested ($F = 4.933$; $p = 0.045$), with lower melatonin concentrations during midday for the new moon. Ocular melatonin was higher during the night of the new moon compared to the night of the full moon (Fig. 1B; Table 1).

Plasma glucose showed interaction between the phases of the moon and the periods of light and dark ($F = 23.940$; $p = 0.001$; Fig. 2A; Table 1), being higher at midnight during full moon. There was also interaction between moon phases and time of the day for plasma triglyceride levels ($F = 36.768$; $p = 0.001$; Fig. 2B; Table 1). Thus, opposite profiles were observed depending on the lunar phase: during full moon higher values were obtained at midnight whereas during new moon higher concentrations were observed at midday.

There was no interaction between the phases of the moon and the light/dark periods for plasma concentrations of total cholesterol ($F = 0.674$; $p = 0.422$; Fig. 3A; Table 1), total proteins ($F = 0.001$; $p = 23.940$; Fig. 3B; Table 1) and cortisol ($F = 0.198$; $p = 0.661$; Fig. 4; Table 1). When light and dark periods were compared, cholesterol values were high at midnight, both during the full moon and during the new moon ($F = 29.392$; $p = 0.001$; Fig. 3A). Total proteins ($F = 4.872$; $p = 0.040$; Fig. 3B) and cortisol

(F = 4.310; p = 0.050; Fig. 4) showed significant difference only between the two phases of the moon, with higher values observed during the full moon.

Liver GSH values were higher during the full moon when compared to during the new moon. The comparison between the light and dark periods showed significant difference during the full moon, with high GSH values during the dark period (F = 7.595; p = 0.013; Fig. 5A; Table 1). There was no significant difference in GSH values between light and dark periods for the new moon (p > 0.05). GSH in the gills showed no significant differences between time of the day (midnight/ midday), nor between the two phases of the lunar cycle (F = 3.836; p = 0.065; Fig. 5B; Table 1).

Interaction between the phases of the moon and the periods of light and dark was observed for the MDA in the gills (F = 6.291; p = 0.021; Fig. 6B; Table 1). Thus, this parameter was higher during the full moon in relation to the new moon when considering the light period. However, no significant differences were observed for the MDA in the liver between the two phases of the moon, nor between the light and dark periods of both lunar phases (F = 3.043; p = 0.097; Fig. 6A; Table 1).

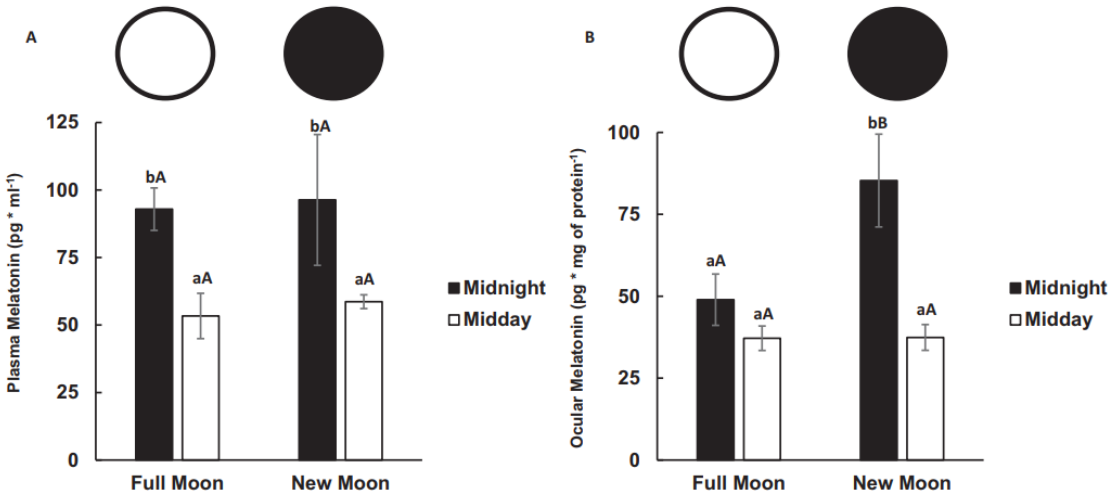


Figure 1. Plasma (pg*ml^{-1}; A) and ocular (pg*mg of protein^{-1}; B) melatonin concentrations in matrinxã during full and new moons. White columns correspond to midday samplings, black columns to midnight samplings. All values represent mean ± S.E.M., n = 6 fish per treatment. Means with different lowercase letters (a, b) are significantly different between periods, and uppercase letters (A, B) are different between moons (two-way ANOVA, p < 0.05).

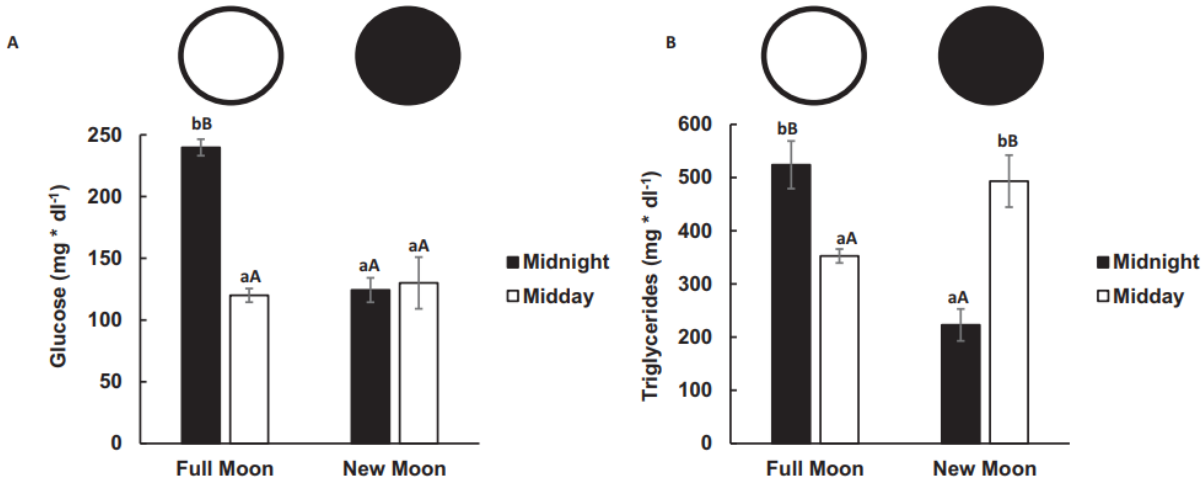


Figure 2. Glucose (mg*dl^{-1}; A) and triglycerides (mg*dl^{-1}; B) concentrations in matrinxã during full and new moons. White columns correspond to midday samplings, black columns to midnight samplings. All values represent mean ± S.E.M., n= 6 fish per treatment. Means with different lowercase letters (a, b) are significantly different between periods, and uppercase letters (A, B) are different between moons (two-way ANOVA, $p<0.05$).

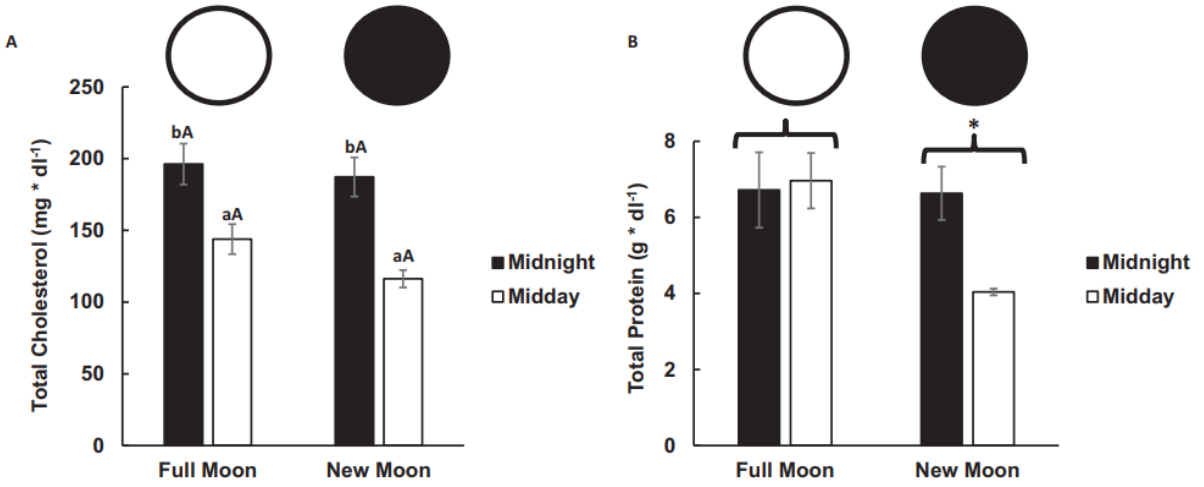


Figure 3. Total cholesterol (mg*dl^{-1}; A) and total protein (g*dl^{-1}; B) concentrations in matrinxã during full and new moons. White columns correspond to midday samplings, black columns to midnight samplings. All values represent mean ± S.E.M., n= 6 fish per treatment. Means with different lowercase letters (a, b) are significantly different between periods. Asterisk correspond to significant difference between moons (two-way ANOVA, $p<0.05$).

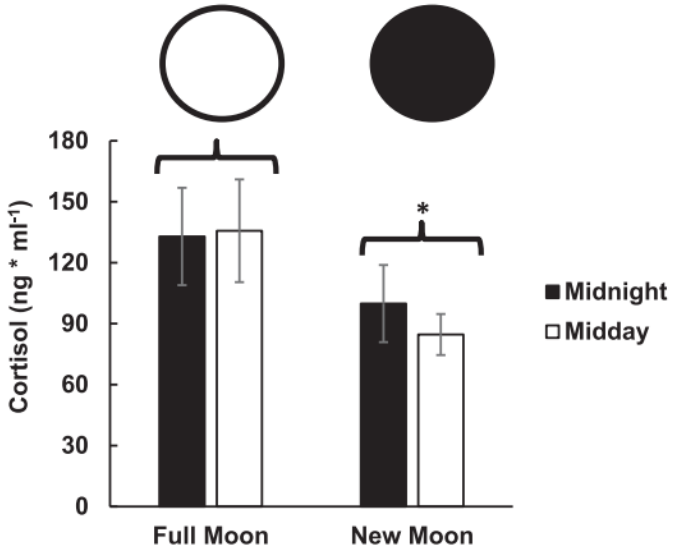


Figure 4. Plasma cortisol concentration (ng*ml^{-1}) in matrinxã during full and new moons. White columns correspond to midday samplings, black columns to midnight samplings. All values represent mean ± S.E.M., n= 6 fish per treatment. Asterisk correspond to significant difference between moons (two-way ANOVA, $p<0.05$).

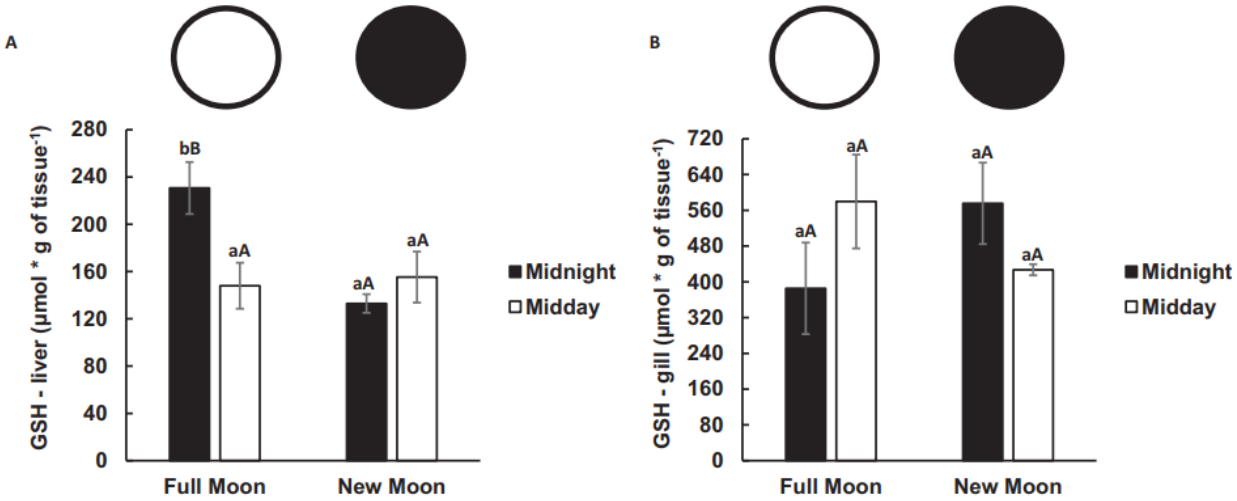


Figure 5. Glutathione (GSH) concentration in the liver (µmol*g of tissue^{-1}; A) and gill (µmol*g of tissue^{-1}; B) in matrinxã during full and new moons. White columns correspond to midday samplings, black columns to midnight samplings. All values represent mean ± S.E.M., n= 6 fish per treatment. Means with different lowercase letters (a, b) are significantly different between periods, and uppercase letters (A, B) are different between moons (two-way ANOVA, $p<0.05$).

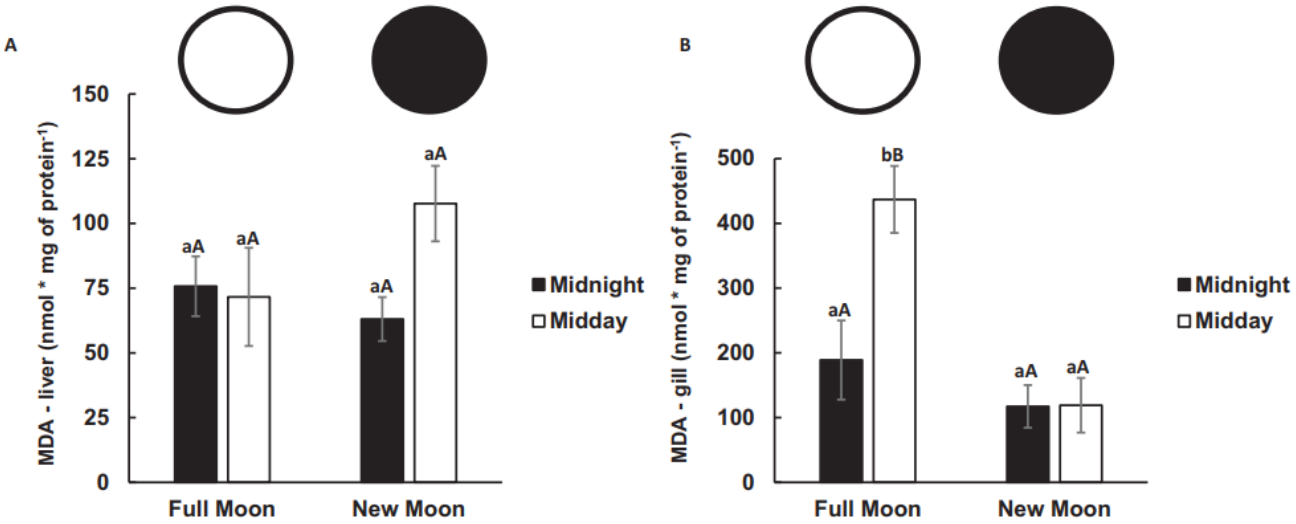
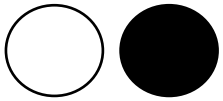
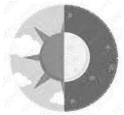
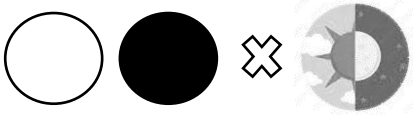


Figure 6. Malondialdehyde (MDA) concentration in the liver (nmol*g of protein^{-1}; A) and gill (µmol*g of protein^{-1}; B) in matrinxã during full and new moons. White columns correspond to midday samplings, black columns to midnight samplings. All values represent mean ± S.E.M., n= 6 fish per treatment. Means with different lowercase letters (a, b) are significantly different between periods, and uppercase letters (A, B) are different between moons (two-way ANOVA, $p<0.05$).

Table 1. Statistical analysis of melatonin and metabolic parameters considering the effect of lunar phase (full/new moon), periods (midday/midnight) and the interaction of the variables.

Parameter	Lunar phase 	Periods 	Interaction 
Plasma melatonin (pg*ml^{-1})	NS	*	NS
Ocular melatonin (pg*mg of protein^{-1})	**	*	**
Plasma glucose (mg*dl^{-1})	**	**	*
Triglycerides (mg*dl^{-1})	*	NS	*
Total cholesterol (mg*dl^{-1})	NS	**	NS
Total proteins (g*dl^{-1})	*	NS	NS
Plasma cortisol (ng*ml^{-1})	*	NS	NS
GSH - liver (µmol*g of tissue^{-1})	*	NS	*
GSH - gill (µmol*g of tissue^{-1})	NS	NS	NS
MDA - liver (µmol*g of protein^{-1})	NS	NS	NS
MDA - gill (µmol*g of protein^{-1})	**	*	**

NS = non-significant; **p < 0.01; *p < 0.05.

4. Discussion

Effects of moon phases on behavioral or other aspects are not known for the studied species (*Brycon amazonicus*). In fact, little information about the biological rhythm in matrinxã has been described (e.g., Lopes et al., 2022). We have thought that these aspects need to be investigated and we also consider that moonlight can interfere with hormone synthesis, metabolism and reproduction, despite the scarce information for freshwater fish.

We hypothesize that the full moon phase (i.e., higher light intensity) stimulates general fish activity and hence an increase in energy expenditure indicators in *B. amazonicus*, and that the new moon (i.e., lower light intensity) can modulate these indicators with low concentrations, associated with greater production of melatonin in the dark period. In fact, we found clear effects of the day/night and moon cycles on melatonin concentrations, metabolic parameters and oxidative stress indicators for the matrinxã. Plasma and ocular melatonin showed a characteristic profile, with low values at midday and high values at midnight. In fact, the reduction in light intensity at night is perceived by fish and may reflect in the greater production of melatonin by the pineal gland and also by the eyes, as reported by Kashiwagi et al. (2013).

On the other hand, moonlight did not modulate the plasma melatonin concentration for *B. amazonicus*. This response differs from the pattern exhibited for some species of fish exposed in both phases of the moon, considering that the synthesis of melatonin is extremely sensitive and inhibited by exposure to light and shows greater synthesis in dark periods (Rahman et al., 2004a; Rahman et al., 2004b; Takemura et al., 2004) and, therefore, it is affected by the light of the moon (Andreatta and Tessmar-Raible, 2020). Our results indicate that *B. amazonicus* may have a plasma melatonin rhythm that is not very sensitive to moonlight or that the intensity threshold was not able to inhibit melatonin secretion at night during the full moon phase. In addition, the matrinxã may also be a bimodal species, with variable levels of plasma melatonin throughout the night, as described by Oliveira et al. (2010).

Andreatta and Tessmar-Raible (2020) suggested that the intensity and length of moonlight exposure and tissue-specific melatonin levels may contribute to increasing the specificity of melatonin signaling in the characterization of different lunar phases. In fact, in *Siganus canaliculatus*, ocular melatonin under natural conditions was higher during the new and waning moons compared to the full and crescent moons (Rahman et al., 2004b). A similar response was found in our study under natural conditions, with higher

concentrations of ocular melatonin during the new moon. Thus, the light of the moon is perceived by the fish's eye and has an impact on the fluctuation of melatonin in the retina, and it can be metabolized in situ, which prevents the release of this substance into the blood (Grace et al., 1991; Kashiwagi et al., 2013). According to Fukunaga et al. (2020), the ability to perceive differences in moonlight is essential for lunar periodicity, since the luminosity of moonlight is markedly lower than that of sunlight. Thus, the retina and the pineal organ are the main candidates for the perception and transduction of moonlight. This response, in turn, triggers a reduction in the synthesis and release of melatonin at lower light intensity, which directly affects physiological and behavioral responses in fish. In addition, higher concentrations of melatonin reduce locomotor activity and aggressiveness in fish (Falcón et al., 2011; Amaral et al., 2020), which may reflect higher metabolic demand and the need for energy substrate availability.

We expected an increase in plasma metabolic parameters in the matrinxã during full moon, since the lower concentration of melatonin at lower light intensity may increase the total number of aggressive interactions for the species (Amaral et al., 2020) and, consequently, it may trigger higher energy expenditure (Alvarenga and Volpato, 1995). The higher concentration of plasma glucose at midnight during full moon may suggest a higher energy demand in these animals with a high metabolic rate. Indeed, plasma cortisol concentrations exhibited their highest levels during the full-moon phase. In this sense, moonlight seems to play a key role in raising cortisol levels. This relationship has been evidenced by Lopes et al. (2022), who reported *B. amazonicus* exhibiting a higher concentration of this hormone in the light period, and this may be associated with the glycemic acrophase observed in the early morning (04:16 ± 2:47 h). Increases in plasma glucocorticoids have a wide range of other metabolic effects, including increased lipolysis and the synthesis and degradation of proteins (Mommsen et al., 1999; Thau et al., 2022).

The highest plasma cholesterol levels were observed at midnight in both phases of the moon, with a decrease at midday in both phases as well. Thus, the highest concentration of plasma cholesterol at midnight corroborates with Lopes et al. (2022) who identified a rhythmicity of cholesterol with acrophase at 23:22 ± 3:45 h, which indicates higher cortisol synthesis that was evidenced by the higher concentration of this hormone in the light period for *B. amazonicus*. In our study, the highest concentration of cortisol also occurred at the full moon, which was probably modulated by the high incidence of moonlight.

Elevated plasma triglyceride levels were observed for both phases of the moon, which reveals the body's attempt to seek greater input to maximize the use of the energy substrate due to greater metabolic activity (Tocher, 2003). However, the response to periods of light and dark varied depending on the phase of the moon. For example, the full moon phase caused high triglyceride levels at midnight, which may indicate the fish need to seek a greater energy supply at night during full moon, due to the higher environmental luminosity, lower concentration of melatonin and, possibly, greater activity of the matrinxã (Amaral et al., 2020). Notwithstanding, triglyceride levels exhibited the opposite response when compared between periods of the new moon, with a decrease at midnight, and increased levels at midday. This response may be associated with a greater mobilization of energy reserves due to the greater activity of the species during the day (Soares et al., 2008; Rodrigues et al., 2017). The total proteins values were higher during the full moon phase when compared to new moon, regardless of the time of the day. According to Mommsen et al. (1999), changes in total protein concentrations were related to increased cortisol, which results in increased gluconeogenesis and protein catabolism activity. Thus, the results for this physiological parameter indicated that the elevated levels in the full moon may have been due to the increase in cortisol that was also observed in the full moon phase for both periods.

Although indicators of oxidative stress may also show variation in accordance with the phases of the moon, little information about this relationship has been described for vertebrates. Basically, studies were only found relating the reproductive period, the phase of the moon and the production of free radicals in invertebrate species (e.g., coral and shrimp; Murphy et al., 2019; Bautista-Covarrubias et al., 2020). As such, our results provide new data on the relationship between moonlight and the production of components of the antioxidant system (melatonin and GSH) and lipid peroxidation (MDA) in fish. Greater activation of the antioxidant system (GSH in the liver) was observed during the full moon and during the dark period. This result may be due to the lower concentration of melatonin, especially at midday, which can increase the activity (i.e., aggressiveness) and energy demand of fish (Amaral et al., 2020) and which, in turn, can increase respiratory rate resulting in exacerbated production of reactive oxygen species (ROS), including free radicals (Wendelaar-Bonga, 1997). This higher production of ROS can stimulate the production of components of the antioxidant system, such as GSH, which minimizes the harmful effect of oxidative stress (Poljsak et al., 2013; Chowdhury and Saikia, 2020). In fact, our results suggest that the higher GSH

concentration in the liver at midnight and during the full moon may have reduced lipid peroxidation, which was evidenced by the absence of significant differences for MDA in the liver between the two phases of the moon and the light and dark periods.

The higher concentration of MDA in the gills at the midday during the full moon indicates that there was a greater degradation of cell membranes as a result of the production of free radicals due to the high intensity of light under these conditions (expressed in 100%; Ikegami et al., 2014). In addition, GSH in the gills did not show significant differences between the sampling times, nor between the moon phases, which may indicate the absence of activation of this antioxidant system in the gills. Fish exposed to the new moon phase at midday exhibited lower rates of lipid peroxidation in the gills. In this sense, it is likely that the low intensity or absence of light has contributed to these low concentrations, possibly through the action of melatonin. In fact, melatonin has antioxidant properties, and acts directly on the elimination of free radicals, assisting antioxidant defense enzymes and stimulating the synthesis of mRNA from the glutamylcysteine synthetase enzyme, which is responsible for the biosynthesis of GSH (Urata et al., 1999; Ramis et al., 2015; Reiter et al., 2018). Melatonin can also decrease the formation of MDA, probably due to its ability to interact with bilayer lipids, thus preventing changes in membrane fluidity and contributing to the reduction of lipid peroxidation (García et al., 1997; Ramis et al., 2015), as has been found for *Oncorhynchus mykiss* (Gülçin et al., 2009).

In summary, it can be concluded that, in *B. amazonicus*, hormonal, metabolic parameters and oxidative stress indicators are modulated by both the day/night and by moon phases. In fish, the pineal organ and its hormone melatonin are likely to be the mediators between environmental cycles and biological rhythms (Amano et al., 2000; Bromage et al., 2001; Bayarri et al., 2004). Thus, we suggest that melatonin may at least partially mediate the described rhythmic changes in physiological processes for matrinxã, as also reported by Zimecki (2006). Although the exact mechanism of the influence of the moon on living organisms needs further study, knowledge of this type of biorhythm may be relevant in order to investigate how fish cope with environment cycles.

Our research also provides the first insight into the influence of the lunar cycle on hormonal and metabolic parameters in *Brycon amazonicus*. Although the lunar periodicity acts as an external regulator, it is necessary to evaluate how this environmental factor synchronizes physiological and behavioral parameters that directly affect the

reproduction and capture in the natural environment of the matrinxã, a valuable species for the diversification of aquaculture in South American countries.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Capítulo 3_

Manuscrito será elaborado nas normas do periódico Chronobiology International.

Ritmo diário da melatonina plasmática e ocular em peixes amazônicos

Resumo

Foram avaliados os ritmos diários da melatonina plasmática e ocular e o efeito do pulso de luz no período escuro sobre a síntese desse hormônio em peixes amazônicos de interesse comercial, oscar (*Astronotus ocellatus*) e matrinxã (*Brycon amazonicus*). Foram realizados dois experimentos: Experimento 1- Trinta e seis juvenis de oscar (130 ± 29g; 16,12 ± 1,38cm) e dezoito juvenis de matrinxã (64 ± 4g; 15,75 ± 0,36cm) foram expostos a um ciclo de 12 h de luz: 12 h de escuro, com as luzes acesas às 06:00 h (zeitgeber time, ZT = 0). Seis animais foram utilizados para coleta de sangue a cada 4 h por 24 h (seis momentos, três no período claro e três no escuro). Experimento 2- um pulso de luz foi fornecido no período de escuro (00:00 h) durante uma hora. Para isso, 18 juvenis de oscar (169,40 ± 1,24g; 17,31 ± 0,29cm) e 09 juvenis de matrinxã (239,64 ± 13,70g; 21,89 ± 0,30cm) foram utilizados em 3 coletas de sangue: ZT6 (12:00 h), ZT18 (00:00 h) e ZT18 + pulso (n= 6 para *A. ocelattus* e n= 3 para *B. amazonicus* em cada coleta). A melatonina plasmática exibiu acrofase no meio do período escuro para *A. ocellatus* (ZT= 18:42 ± 03:01 h) e *B. amazonicus* (ZT= 16:00 ± 2:57 h). Em *A. ocellatus* foi demonstrado ritmo diário para a melatonina ocular (ZT= 06:34 ± 5:41 h), com maiores níveis durante o dia, indicando um perfil inverso da liberação plasmática. Em *B. amazonicus*, a melatonina ocular exibiu um perfil noturno (ZT= 15:43 ± 2:10 h). Em ambas as espécies, o pulso de luz à meia-noite (ZT18 + pulso) diminuiu a concentração plasmática de melatonina para valores semelhantes aos obtidos no período claro. Não houve diferença significativa para os níveis de melatonina ocular em resposta à um pulso de luz. De modo geral, foi observado ritmo diário para a melatonina plasmática e ocular, e que o pulso de luz no meio do período escuro diminuiu a concentração plasmática de melatonina para ambas as espécies. Assim, ajustes de luz são relevantes para favorecer a produção de melatonina e podem ser utilizados como mitigadores fisiológicos de estresse e gasto energético, favorecendo o bem-estar e o desempenho produtivo de espécies de peixes de interesse comercial na região amazônica.

Palavras-chave: ritmicidade diária, plasma, retina, metabolismo, aquicultura

1. Introdução

Entre as várias ações da melatonina já comprovadas, a variação rítmica ao longo do tempo é uma das que foram mais descritas (Reiter, 1993; Zhdanova, 2005; Reiter et al., 2013; Cipolla-Neto e Amaral, 2018). A melatonina é o "tradutor neuroendócrino" do ciclo claro/escuro (Falcón, 1999) e, em peixes, o perfil rítmico da melatonina está sob o controle dos relógios circadianos, sendo considerada um importante modulador dos ritmos comportamentais e fisiológicos diários (Sánchez-Vázquez et al., 2019a). Entre os fatores ambientais que sincronizam e desenvolvem ritmos circadianos em animais, a luz é um dos mais importantes, particularmente, na mudança diária entre luz e escuridão (Aschoff, 1981; Carr et al., 2006; Ziv e Gothilf, 2006).

A melatonina é produzida principalmente pela glândula pineal e liberada na circulação sanguínea com altas concentrações à noite e baixas concentrações durante o dia. Em peixes, o ritmo da melatonina plasmática, proveniente da transdução dos ciclos de luz e escuridão na pineal, influencia a coordenação temporal de muitos processos biológicos (Ekström e Meissl, 1997). A melatonina também é sintetizada na retina de peixes teleósteos, apresentando marcada ritmicidade diária, usualmente, mas não exclusivamente, associada a células fotorreceptoras (Cahill, 1996; Vuilleumier et al., 2007; Vera e Migaud, 2014).

Existem dados bem fundamentados sobre a distribuição dos locais de ligação da melatonina em uma ampla variedade de tecidos corporais (Kulczykowska et al., 2006; Sauzet et al., 2008). De fato, esse hormônio pode estar envolvido em vários processos fisiológicos, a maioria deles exibindo ritmos diários e/ou sazonais, como no desenvolvimento corporal (Blanco-Vives et al., 2011), no sistema imune (Binuramesh e Michael, 2011), na locomoção (López-Olmeda et al., 2012), na alimentação (Mattos et al., 2016), no metabolismo (Paredes et al., 2015), na liberação de enzimas e hormônios (Yufera et al., 2014; Cowan et al., 2017) e em indicadores de estresse oxidativo (Tian et al., 2019).

A caracterização do ritmo diário da melatonina em diferentes espécies de peixes é uma abordagem importante para a aquicultura, uma vez que essa variável pode ser utilizada como mitigador fisiológico de estresse e gasto energético, especialmente por seu papel antioxidante (Sánchez-Vázquez et al., 2019a; Vera et al., 2014). Dessa forma, os processos homeostáticos e circadianos estão estreitamente interligados, de modo que as perturbações circadianas levam ao comprometimento do bem-estar dos animais (Gnocchi e Bruscalupi, 2017; Sánchez-Vázquez et al., 2019b).

A intensidade da luz é um fator fundamental em sistemas de produção de peixes (Espinosa et al., 2020), e alguns trabalhos mostraram que o pulso de luz no meio da fase escura também pode alterar o padrão rítmico dos níveis de melatonina em peixes (Bayarri et al., 2002; 2003; Oliveira et al., 2007). De acordo com Falcón et al. (1987), Bolliet et al. (1995) e Cahill (1996) a luz suprimiu o ritmo endógeno da melatonina, enquanto um pulso de luz durante a noite inibe a produção de melatonina. De fato, para as espécies como o robalo (*Centropristis striata*) e a truta (*Salvelinus fontinalis*), quanto maior é a intensidade do pulso de luz no meio da fase escura, menor o nível de melatonina plasmática para essas espécies (Bayarri et al., 2002; Zachmann et al., 1992).

De acordo com Sanchez-Vázques et al. (2019a), a poluição luminosa à noite deve ser reprimida, proporcionando aos peixes um ambiente favorável à produção de melatonina, uma vez que esse hormônio é liberado à noite e interage nas respostas ao estresse, atenuando os efeitos adversos nos peixes (Sanchez-Vázquez et al., 2019b). Além disso, foi relatado por Bayarri et al. (2002) que o limiar de luz de 6mW/cm2 é suficiente para modificar os conteúdos de melatonina em *Centropristis striata*. Portanto, o fornecimento de luz adequado é fundamental em sistemas de aquicultura, a fim de preservar a síntese de melatonina em espécies de peixes de interesse comercial.

Apesar de vários estudos relatarem a existência de ritmo diário da melatonina em peixes, assim como os efeitos de um pulso de luz no meio da fase escura, como por exemplo, *Dicentrarchus labrax* (Bayarri et al., 2004a), *Solea senegalensis* (Bayarri et al., 2004b; Oliveira et al., 2007; 2013), *Argyrosomus regius* (Oliveira et al., 2018), nenhum estudo foi relatado sobre o perfil diário e um pulso de luz nos níveis de melatonina em espécies amazônicas com interesse para a aquicultura.

O oscar (*Astronotus ocellatus*), por exemplo, espécie de ornamental que possui alto potencial para a aquicultura tropical, com comércio significativo na criação mundial de peixes, sendo sua carne muito valorizada na região amazônica (Gonçalves-de-Freitas e Mariguela, 2006; Santos et al., 2009). O matrinxã (*Brycon amazonicus*) é outra espécie bastante promissora na aquicultura, especialmente por seu alto potencial zootécnico e econômico (Gomes e Urbinati, 2010), é nativa da bacia amazônica, sendo a segunda espécie nativa mais produzida na região Norte (SIDRA-IBGE, 2021). No entanto, existem problemas fundamentais na produção desta espécie, principalmente devido ao estado de estresse causado pelo manejo que ocorre em sistemas artificiais que, por sua vez, afeta a reprodução, a larvicultura e o crescimento dos juvenis (Gomes et al., 2000; Gomes e Urbinati, 2010), comprometendo sua expansão produtiva.

Este estudo foi, portanto, desenvolvido para descrever a existência de ritmos diários e resposta a um pulso de luz nos níveis de melatonina plasmática e ocular em juvenis de oscar (*Astronotus ocellatus)* e matrinxã (*Brycon amazonicus*). A abordagem dos ritmos de melatonina, bem como a resposta a um pulso de luz no período escuro é de fundamental importância para compreender a influência do ciclo claro/escuro nos níveis de melatonina no metabolismo dos peixes. Diante disso, essas respostas permitirão a elaboração de estratégias inovadoras a respeito de ajustes de luz que poderão impulsionar a manutenção e o manejo dos peixes em ambiente controlado, fornecendo um ambiente favorável a produção de melatonina, e consequentemente, a saúde e o bem-estar dos peixes.

2. Material e Métodos

2.1 Aquisição e aclimatação dos animais

Os juvenis de matrinxã (*Brycon amazonicus*) foram oriundos da Estação de Aquicultura da Fazenda Experimental (FAEXP) da Universidade Federal do Amazonas (UFAM). Os espécimes de oscar (*Astronotus ocellatus*) foram adquiridos em um exportador de peixes ornamentais localizado no município de Manaus, Amazonas, Brasil.

Área de estudo, nota ética e licença para coleta dos animais

Este estudo foi realizado no Laboratório Experimental de Fisiologia e Comportamento de Animais Aquáticos da Universidade Federal do Amazonas (UFAM), Amazonas, Brasil. Os peixes foram aclimatados em caixa de polietileno de 500 L (01 animal/5 L) contendo filtros biológicos internos para a manutenção da boa qualidade da água por um período de, no mínimo, 15 dias. A intensidade luminosa foi mantida em 150 ± 42 lx com o uso de 06 lâmpadas led 9w bulbo branca fria, e o fotoperíodo em 12 horas claro: 12 horas escuro (06:00 às 18:00h), com o auxílio de um luxímetro (MLM-1011, Minipa, Brasil) e de um temporizador digital (HL TM24H, Hardline, Brasil), respectivamente. Os tanques foram supridos com água proveniente de poço artesiano, que foi renovada parcialmente (20%) a cada três dias e apresentou os seguintes valores para os parâmetros medidos: temperatura = 27 ± 0,4 ºC, pH = 6,1 ± 0,8, oxigênio dissolvido = 5,5 ± 1,0 mg/L e amônia total = 0,02 ± 0,01 mg/L. Os peixes foram alimentados com ração comercial (28% de proteína; Multifós; Rondônia, Brasil) oferecida duas vezes ao dia (início da manhã e final da tarde) até a saciedade aparente. Todos os procedimentos descritos foram realizados para as duas espécies em estudo.

A captura dos espécimes de oscar em ambiente natural foi regulamentada por meio da licença n^0 60643-3 obtida junto ao Instituto Chico Mendes de Conservação da Biodiversidade (ICMBIO). Este estudo está de acordo com os Princípios Éticos na Experimentação Animal (CONCEA) e foi aprovado pela Comissão de Ética no Uso de Animais (CEUA) da UFAM, Manaus, AM, certificado n^0 066/2018.

2.2. Experimento 1: *Ritmos diários de melatonina plasmática e ocular*

Após o período de aclimatação, 36 juvenis de oscar (6 animais por caixa, totalizando 6 caixas) e 18 juvenis de matrinxã (3 animais por caixa, totalizando 6 caixas) foram agrupados em caixas de polietileno de 500 L por um período de 24 horas. O fotoperíodo foi controlado para 12 horas claro:12 horas escuro (06:00 h às 18:00 h), sem a influência de fatores externos que pudessem atuar como arrastadores ou sincronizadores do ritmo biológico, o que evitou o efeito da manipulação destes fatores na variação cíclica dos níveis de melatonina plasmática e ocular e, para isso, os animais foram mantidos em jejum durante as 24 horas do período experimental.

Em estudos de ritmos circadianos, quando as luzes são acesas, esse período é denominado tempo *zeitgeber* (sincronizador) (ZT), correspondendo a ZT0 (=06:00 h); e quando as luzes são apagadas, esse período corresponde a ZT12 (=18:00 h) (López-Olmeda et al., 2016). A cada intervalo de quatro horas, todos os animais de uma mesma caixa (n= 6 para *A. ocelattus* e n= 3 para *B. amazonicus*) foram utilizados para a coleta de sangue, totalizando seis amostragens: três no período claro (ZT2/8:00 h, ZT6/12:00 h e ZT10/16:00 h) e três no período escuro (ZT14/20:00 h, ZT18/00:00 h e ZT22/04:00 h).

A cada intervalo de 4 horas, os peixes foram anestesiados com eugenol (64 µL/L), conforme realizado por Ferreira et al. (2020), e imediatamente puncionados na região da veia caudal para a obtenção das amostras de sangue com o auxílio de seringas descartáveis de 1 mL, contendo heparina (5.000 unidades/mL) como anticoagulante. Em seguida, foi realizada a biometria (peso corporal e comprimento padrão), sendo utilizados animais de peso corporal (*A. ocelattus*: 130 ± 29g e *B. amazonicus*: 64 ± 4g; média ± DP) e comprimento padrão (*A. ocelattus*: 16,12 ± 1,38cm e *B. amazonicus*: 15,75 ± 0,36cm; média ± DP). Durante o período escuro, a manipulação dos animais e as coletas de sangue foram realizadas sob luz vermelha de baixa intensidade (0,21 ± 0,10 lx), pois, de acordo com Oliveira et al. (2007), a luz vermelha não inibe a produção noturna de melatonina e, portanto, pode ser usada com segurança durante a amostragem noturna.

2.3. Experimento 2*:* Efeito do pulso de luz no período de escuro sobre os níveis de melatonina plasmática e ocular

Neste estudo, foram realizadas 3 coletas de sangue: ZT6 (12:00 h), ZT18 (00:00 h) e ZT18 + um pulso de luz fornecido durante uma hora (293,40 ± 2,40 lx) no meio do período escuro (Bayarri et al., 2002). Para isso, foram realizadas coletas de 18 juvenis de oscar e 09 juvenis de matrinxã (n= 6 para *A. ocelattus* e n= 3 para *B. amazonicus*, em cada coleta), conforme os procedimentos do experimento 1 para o tempo de agrupamento, anestesia e coleta de sangue. Em seguida, foi realizada a biometria (peso corporal e comprimento padrão), sendo utilizados animais de peso corporal (*A. ocelattus*: 169,40 ± 1,24g e *B. amazonicus*: 239,64 ± 13,70g; média ± DP) e comprimento padrão (*A. ocelattus*: 17,31 ± 0,29cm e *B. amazonicus*: 21,89 ± 0,30cm; média ± DP). Durante o período escuro, a manipulação dos animais e as coletas de sangue foram realizadas sob luz vermelha de baixa intensidade (0,21 ± 0,15 lx) para evitar o efeito da luminosidade nos parâmetros fisiológicos, conforme evidenciado por Oliveira et al. (2007).

2.4. Análise de melatonina

As análises de melatonina foram realizadas na Universidade de Murcia (Murcia, Espanha). As amostras de olhos, descartando-se a córnea e o cristalino, foram descongeladas, pesadas individualmente e homogeneizadas, a 4°C em 1 ml de solução salina tamponada com fosfato. Amostras oculares e de plasma foram homogeneizadas, extraídas e purificadas usando colunas de extração de fase (Waters, Massachusetts, EUA) em centrífuga, e após isso, foi utilizado um kit comercial ELISA (Melatonin ELISA kit, IBL, Hamburg, Germany), conforme descrito por Bayarri et al. (2002)**.** A proteína total do olho foi quantificada pelo método de Lowry (Lowry et al., 1951), sendo os valores de melatonina expressos em $pg*mg^{-1}$ de proteína. A concentração da melatonina plasmática foi expressa em $pg*ml^{-1}$.

2.5. Análise de dados

Os dados foram analisados quanto à existência de valores discrepantes e, em seguida, testados quanto à normalidade pelo teste de Shapiro-Wilk. Todos os resultados foram expressos como a média ± erro padrão (EP), sendo $p< 0,05$ o nível de significância estabelecido em todas as análises estatísticas.

Experimento 1: A análise Cosinor foi utilizada para avaliar a atividade rítmica das concentrações de melatonina plasmática e ocular, sendo ajustada para a função Cosinor

(Y= M+A*[Cos (Ωt + Φ)]), onde M é o mesor, A é a amplitude, Ω é a frequência angular (360^0/24h para ritmos diários) e Φ é a acrofase, conforme realizado por López-Olmeda et al. (2012) e Ren et al. (2020). Para a realização dessas análises foi utilizado o software El Temps (v.1, 179 Dr. Díez Noguera, Barcelona). Os níveis plasmáticos e oculares de melatonina foram comparados entre os períodos por meio da ANOVA de uma via, seguido pelo teste de Tukey para comparações múltiplas.

Experimento 2: Os níveis plasmáticos e oculares de melatonina foram comparados entre os três períodos (ZT6, ZT18 e ZT18 +P) por meio da ANOVA de uma via, seguido pelo teste de Tukey.

3. Resultados

Em relação a *A. ocellatus*, foram observados ritmos diários significativos para a melatonina plasmática (Cosinor, p= 0,006) e ocular (Cosinor, p= 0,049) com acrofases às 18:42 ± 03:01 h e 06:34 ± 5:41 h, respectivamente (Figura 1). A melatonina plasmática apresentou níveis baixos no início do período claro e foi maior no final do período escuro (ANOVA, F= 3,55; p= 0,02). A melatonina ocular apresentou ritmo circadiano, mas não houve diferença significativa dos níveis de melatonina entre os períodos (ANOVA, F= 1,35; p= 0,29; Figura 1).

Foi observada variação diária para a melatonina plasmática (acrofase: 16:00 ± 2:57 h; p= 0,005) e ocular (acrofase: 15:43 ± 2:10 h; p= 0,001) em *B. amazonicus* (Figura 1). A melatonina plasmática apresentou níveis baixos durante o dia e níveis mais elevados período escuro, principalmente no ZT14 (ANOVA, F= 4,24; p= 0,02; Figura 1). A melatonina no olho apresentou níveis baixos durante o dia e foi significativamente maior no início (ZT14) e no meio do período escuro (ZT18) (ANOVA, F= 18,03; p= 0,001; Figura 1).

Os níveis plasmáticos de melatonina apresentaram valores baixos no período claro (ZT6) e altos no período escuro (ZT18) para *A. ocellatus* (ANOVA, F= 8,65; p= 0,01) e *B. amazonicus* (ANOVA, F= 16,97; p= 0,002). O pulso de luz ofertado no ZT18 (i.e., ZT 18 + P) diminuiu significativamente a concentração plasmática de melatonina para valores semelhantes aos obtidos no período claro (p<0,05; Figura 2). Não houve diferença significativa para os níveis de melatonina ocular para ambas as espécies (*A. ocellatus*: ANOVA, F= 0,66; p= 0,53 e *B. amazonicus*: ANOVA, F= 1,13; p= 0,38).

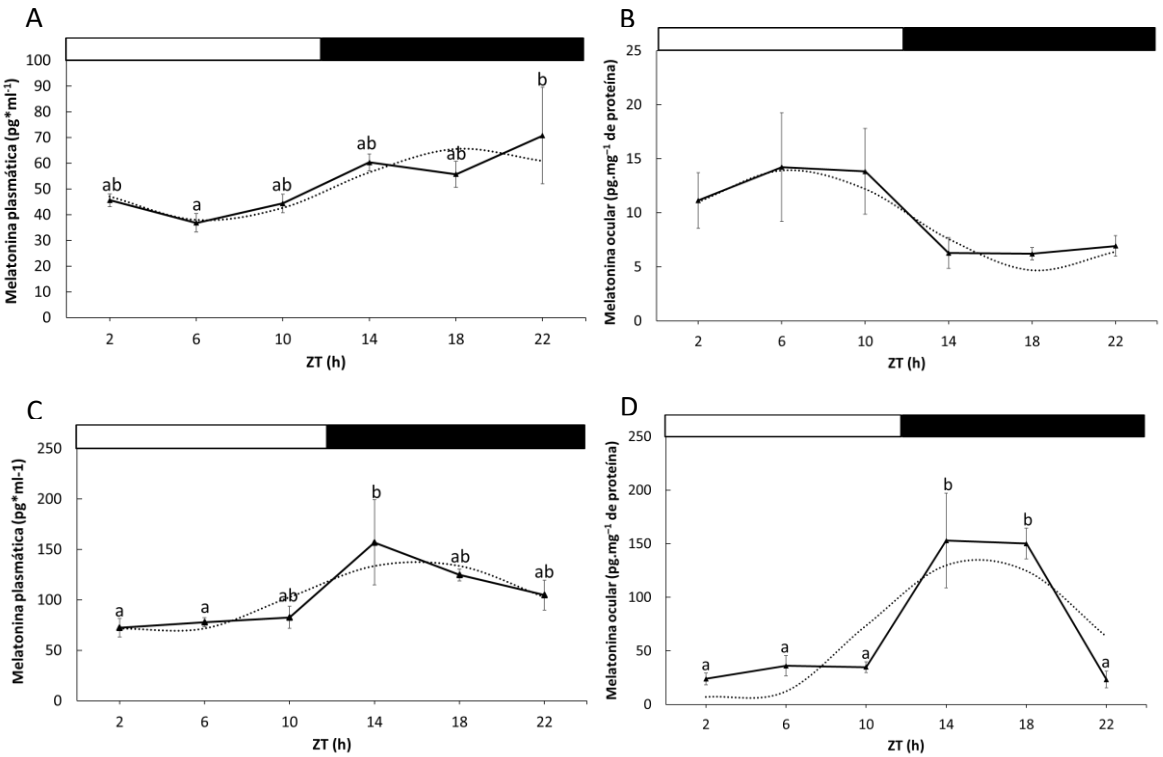

Figura 1. Ritmo diário da melatonina plasmática e ocular para *Astronotus ocelattus* (A–B) e *Brycon amazonicus* (C–D). Valores estão representados como média ± E.P. (*n* = 6/tempo de coleta, *A. ocellatus*; *n* = 3/tempo de coleta, *B. amazonicus*). Barras brancas e pretas representam o período de claro e escuro, respectivamente. Letras diferentes indicam diferença significativa entre aos pontos de coleta (ZT) (Tukey, $p < 0.05$). A linha tracejada sinusoidal representa o ajuste a um ritmo calculado pela análise Cosinor sempre que esta análise foi estatisticamente significativa (Cosinor, $p < 0.05$).

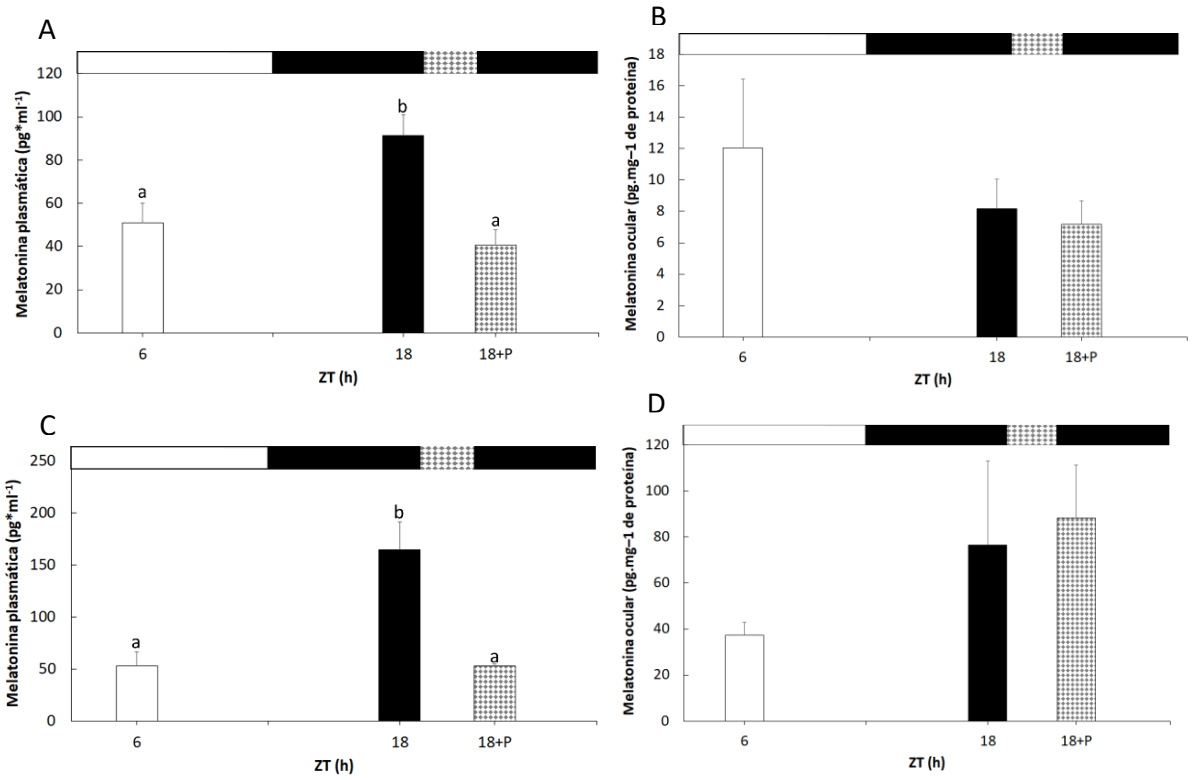

Figura 2. Níveis de melatonina plasmática e ocular para *Astronotus ocelattus* (A–B) e *Brycon amazonicus* (C–D) em relação ao ZT6, ZT18 e ZT18 e pulso de luz (ZT18+P). Valores estão representados como média ± E.P. (*n* = 6/tempo de coleta, *A. ocellatus*; *n* = 3/tempo de coleta, *B. amazonicus*). Letras diferentes indicam diferença significativa entre aos pontos de coleta (ZT) (Tukey, $p < 0.05$).

4. Discussão

Os níveis de melatonina plasmática foram maiores no período escuro para *Astronotus ocellatus* e *Brycon amazonicus*. Esse perfil ritmico é semelhante ao padrão de variação circadiana encontrado para outras espécies de peixes teleósteos, tais como, *Dicentrarchus labrax* e *Tinca tinca* (Bayarri et al., 2003; Vera et al., 2005). Assim, é provável que essa variação diária do ciclo claro/escuro possa afetar a amplitude do sinal de melatonina por meio de um controle direto na quantidade da proteína arilalquilamina *N*-acetiltransferase ($AANAT_2$), enzima sintetizadora da melatonina-via glândula pineal, com maior atividade de $AANAT_2$ durante a noite (Fálcon et al., 2010).

De acordo com Falcón et al. (2010) e Migaud et al. (2010), a ritmicidade noturna da melatonina mostra três tipos de perfis (A, B e C) que são observados em diferentes vertebrados, incluindo peixes. O perfil A é caracterizado pela presença de um pico discreto de melatonina no final da fase escura; O perfil B indica um pico discreto no meio

da fase escura e o perfil C caracteriza-se por um pico prolongado durante a maior parte da fase escura. Para ambas as espécies estudadas, foi observado um rápido aumento da melatonina após o início da fase escura (ZT14), que foi prolongado até o final da mesma (ZT22), inserindo-se no perfil do tipo C, que é descrito para várias espécies de teleósteos (Bayarri et al., 2002; Bayarri et al., 2004b; Falcón et al., 2010; Migaud et al., 2010; Oliveira et al., 2018).

Apesar do perfil de melatonina exibir um padrão semelhante ao encontrado para outras espécies de peixes, as espécies deste estudo apresentaram acrofases distintas para a produção de melatonina durante a noite. Em *A. ocellatus*, a acrofase foi exibida em ZT = 18:42 ± 3:01h, no final do período escuro. Por outro lado, em *B. amazonicus* a acrofase ocorreu em ZT = 16:00 ± 2:57h, início do período escuro.

De acordo com Cipolla-Neto et al. (2014), a melatonina é uma molécula essencial na associação entre as variações ambientais e o ritmo circadiano dos processos fisiológicos e comportamentais necessários para a otimização do balanço energético e manutenção do metabolismo, o que pode ser comprovado por meio dos ritmos comportamentais específicos de cada espécie (Porter, 1998; Mayer, 2000; Zhdanova et al., 2001).

Portanto, os resultados para ambas as espécies corroboram a produção diária de melatonina e regulação fisiológica nos organismos durante a noite através de seus efeitos imediatos, além de auxiliar na preparação dessas reações para o período claro mediante seus efeitos prospectivos que só aparecerão ao cessar a produção de melatonina no início da manhã, e consequentemente pelos seus efeitos prolongados, que serão expressos ao longo do dia seguinte (Amaral e Cipolla-Neto, 2018; Cipolla-Neto e Amaral, 2018).

Nesse contexto, Lopes et al. (2022), mostraram acrofase para alguns indicadores de gasto energético no final do período escuro em *A. ocellatus*, que apresenta maior atividade durante o dia, o que aumenta sua demanda metabólica neste período. Essa resposta sugere uma preparação para o dispêndio de energia no início do período de luz para essa espécie, uma vez que nesse período, a produção de melatonina cessa, funcionando como um marcador central na regulação dos ritmos diários do comportamento e fisiologia dos animais (Falcón et al., 2010). Do mesmo modo, o histórico anual da produção diária de melatonina prepara o sistema endócrino, sinalizando a aproximação das estações do ano (Cipolla-Neto e Amaral, 2018).

De modo similar e considerando que a ausência ou os baixos níveis de melatonina durante o dia podem refletir no aumento dos índices de gasto energético dos organismos,

Lopes et al. (2022) identificaram que *B. amazonicus* apresentou ritmo diário para a maioria dos parâmetros fisiológicos com acrofase durante o período claro para Htc, Hb, HCM, CHCM, glicose, triglicerídeos e colesterol total o que corrobora com os baixos níveis de melatonina plasmática durante o dia indicado neste estudo.

Brycon amazonicus também apresenta alta atividade metabólica e comportamental durante o dia, o que favorece maior atividade, ingestão de alimentos, armazenamento de energia e aumento do cortisol (Soares et al., 2008; Wehr et al., 2001). Também pode-se destacar que os níveis de melatonina plasmática para matrinxã registrados neste estudo foram semelhantes ao encontrado por Lopes et al. (2023), com maiores níveis de melatonina exibidos no período escuro (meia noite) para a mesma espécie.

Diferente do observado para a melatonina plasmática, *A. ocellatus* apresentou maiores níveis de melatonina ocular no período claro, com acrofase em ZT = 6:34 ± 5:41 h. Isso pode ser sugerido, uma vez que, para algumas espécies de peixes, esse padrão entre melatonina plasmática e ocular pode ser invertido (Gern et al. 1978; Iigo et al. 1997a; Bayarri et al., 2003; Besseau et al., 2006), sendo esses resultados decorrentes da plasticidade que organismos têm frente às mudanças ambientais (Iigo et al., 2007; Falcón et al., 2010) ou de funções específicas que a melatonina apresenta, sendo sua síntese dissociada do ciclo claro/escuro (Schwartz et al., 2009).

Embora a produção de melatonina exiba um padrão rítmico diário, sua exibição na retina é bem diferente da glândula pineal, atingindo seu pico a noite ou em diferentes momentos do dia ou modificando a fase do ritmo durante as estações, dependendo da espécie (Cahill et al., 1991; Falcón et al., 2003). Por exemplo, o perfil inverso de melatonina pode ser explicado pela existência de duas isoformas diferentes de AANAT (arilalquilamina N-acetiltransferase; $AANAT_1$- enzima da retina; $AANAT_2$- enzima da pineal), enzimas sintetizadoras de melatonina e que podem suprimir a produção do hormônio durante a noite (Klein, et al., 1997; Vuilleumier et al., 2007; Falcón et al., 2001; Falcón et al., 2007; Falcón et al., 2010; Iuvone et al., 2005).

Os resultados deste estudo sugerem uma função local para a melatonina ocular (Vera et al., 2014), uma vez que a melatonina retiniana atua de maneira autócrina/parácrina e alguns dados indicam que esse hormônio não é liberado na circulação (Falcón et al., 2010). Além disso, a $AANAT_1$ pode estar relacionada às propriedades antioxidantes, atuando na eliminação de radicais livres e também como um composto antiapoptótico na retina, que pode estar ligada ao efeito indutor da melatonina

sobre as enzimas antioxidantes, bem como seu efeito supressor sobre os compostos pró-oxidantes (Hardeland e Pandi-Perumal, 2005; Del Valle Bessone et al., 2019).

Nesse contexto, o oscar estaria classificado como uma regulação da produção e liberação de melatonina como não especializado, ou seja, a glândula pineal é exclusivamente responsável pela percepção e produção de melatonina nesta espécie, conforme o proposto por Migaud et al. (2007). Além disso, sugere-se que a melatonina produzida pelos olhos nessa espécie não contribuiria para os níveis plasmáticos desse hormônio. A distinção entre a produção de melatonina da pineal e da retina podem ser explicadas pelas diferentes funções funcionais, com a melatonina da glândula pineal fornecendo um indicador endócrino confiável do ciclo dia-noite (Falcón et al., 2007), enquanto a melatonina ocular pode estar envolvida na proteção parácrina e adaptação da retina (Falcón et al., 2003; Besseau et al., 2006).

De modo geral, o padrão noturno da melatonina ocular em peixes está sincronizado com o ambiente claro/escuro (Takeuchi et al., 2014). No entanto, algumas espécies exibem um padrão diurno de melatonina nos olhos, com níveis mais altos durante o período de luz, tais como, *Oncorhynchus mykiss*, *Salvelinus fontinalis*, *Dicentrarchus labrax*, *Fundulus heteroclitus*, *Oreochromis niloticus* e *Takifugu rubripes* (Gern et al., 1978; Iigo et al., 1997; Iigo et al., 2007; Sánchez-Vázquez et al., 1997; García-Allegue et al., 2001). Dessa forma, *A. ocellatus* segue o mesmo padrão descrito para as espécies acima, sendo a síntese de melatonina na retina independente do período de escuro.

Por outro lado, os níveis de melatonina ocular mostraram mudanças marcantes no ciclo claro/escuro, com valores menores durante o período de luz e maiores no escuro para *B. amazonicus*, assim como para os níveis de melatonina plasmática. Nesse sentido, a regulação da produção e liberação da melatonina para o matrinxã estaria caracterizada como um sistema intermediário, o que sugere que tanto os olhos como a glândula pineal são fundamentais para suprir os ritmos de melatonina, como por exemplo, no robalo (*Dicentrarchus labrax*; Oshima et al., 1989; Falcón et al., 2007, pássaros (Jimenez et al., 1995; Brandstatter, 2003) e anfíbios (Wright et al., 2006).

Conforme descrito por Bayarri et al. (2003) e Vera et al. (2005) para *Dicentrarchus labrax* e *Tinca tinca*, respectivamente, observou-se redução dos níveis de melatonina plasmática à exposição ao pulso de luz para *A. ocellatus* e *B. amazonicus*. Essa resposta pode ser explicada pelo fato de melatonina plasmática apresentar alta sensibilidade à luz, com limiares próximos a 10-20 lux (Zachmann et al., 1992; Bayarri et al., 2002; Oliveira et al., 2007). Para a *Tinca tinca*, a melatonina mostrou maior

sensibilidade à luminosidade, de modo que uma intensidade de pulso de luz de 0,3 lux diminuiu a melatonina plasmática para níveis semelhantes aos obtidos no período claro (Vera et al., 2005). Neste estudo, o pulso de luz apresentou intensidade de 293,40 ± 2,40 lx, indicando que o oscar e o matrinxã respondem prontamente a oferta de luz no período noturno, com menor concentração de melatonina plasmática, mas sem efeito significativo na melatonina ocular.

A menor concentração de melatonina decorrente do pulso de luz no período de escuro (ZT18 + pulso) pode desencadear maior gasto energético para as espécies, devido ao aumento das interações agressivas em decorrência da menor disponibilidade de melatonina circulante, conforme descrito por Amaral et al. (2020) para *B. amazonicus*. De fato, a exposição ao pulso de luz à meia-noite pode induzir uma maior mobilização energética nos peixes, uma vez que os níveis de melatonina foram suprimidos durante o período escuro. Maiores taxas metabólicas durante a fase de luz também foram observadas para *Oreochromis niloticus*, devido as interações comportamentais exibidas nesta fase, o que aumentou seus níveis de atividade e, portanto, sua taxa metabólica (Biswas et al., 2002).

De acordo com Ferreira et al. (2020), *B. amazonicus* é uma espécie com alta atividade metabólica e comportamental e pode reagir rapidamente à um estimulo estressor. Um ambiente de luz desconfortável estimula o sistema visual dos peixes e resulta em uma resposta ao estresse. Isso pode subsequentemente interromper o sistema imunológico e resultar em resultados adversos (Song e Choi, 2019).

Assim, a luminosidade pode atuar como um estressor e afetar o metabolismo dessa espécie, conforme descrito por Lopes et al. (2023) que relataram maiores níveis de cortisol na luz do luar e durante a fase de lua cheia. Portanto, a exposição à luminosidade durante a noite deve ser evitada, proporcionando aos peixes um ambiente favorável à produção de melatonina (Sánchez-Vázquez et al., 2019a). Sob outra perspectiva, a melatonina ocular não exibiu resposta significativa ao pulso de luz para ambas as espécies, o que pode indicar que a intensidade de luz oferecida não afetou a síntese de melatonina na retina. Dessa forma, é importante considerar as características da luz para controlar adequadamente a produção de melatonina e seus processos fisiológicos relacionados (Bayarri et al., 2002).

Este estudo fornece as primeiras informações sobre os ritmos diários da melatonina e sua resposta à um pulso de luz no período de escuro em peixes amazônicos com alta representatividade para a aquicultura. De maneira geral, foi constatado ritmo

diário para a melatonina plasmática e ocular em *A. ocellatus* e *B. amazonicus*. Além disso, o pulso de luz ofertado no meio da fase escura (ZT18 + P) diminuiu significativamente a concentração plasmática de melatonina para ambas as espécies.

Estudos do ritmo circadiano da melatonina, bem como o pulso de luz no meio do período escuro são relevantes para a produção de espécies de peixes de interesse comercial, uma vez que a produção dessa indolamina é influenciada pelo ciclo claro-escuro e afeta as principais funções fisiológicas em peixes, devido sua função antioxidante, imunoestimulante e antiestresse. Assim, os resultados indicam que os ajustes de luz são relevantes para favorecer a produção de melatonina e podem ser utilizados como mitigadores fisiológicos de estresse e gasto energético, favorecendo o bem-estar e o desempenho produtivo de espécies de peixes de interesse comercial na região amazônica.

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Considerações Finais

Nos capítulos da presente tese foram apresentados e discutidos os resultados que investigaram os ritmos circadiano (claro/escuro) e infradiano (ciclo lunar) e seus efeitos na fisiologia de duas espécies de peixes amazônicos.

Esta abordagem tem o objetivo de propor períodos adequados para o manejo e a manipulação em sistemas de criação de peixes e ambientes experimentais, bem como contribuir para o bem-estar dessas espécies. Os resultados mostraram que os ritmos biológicos dos peixes são influenciados pelos ciclos claro/escuro e pelo ciclo lunar (lua cheia e lua nova), favorecendo maior modulação metabólica nessas espécies.

A descrição de ritmos e o efeito do ciclo claro/escuro sugerem que as condições ambientais devem ser consideradas nos sistemas de produção e que as práticas rotineiras durante a cadeia produtiva precisam ser alteradas com base nas informações da cronobiologia.

Neste sentido, os resultados indicam que os parâmetros hormonais, metabólicos e indicadores de estresse oxidativo são modulados tanto pelo período de claro e escuro quanto pelas fases da lua. Os resultados compilam informações relevantes para melhor compreender o padrão diário de luz e escuridão, e as fases da lua nos indicadores de gasto energético e hormonais dos peixes em condições naturais e artificiais.

A presente tese acrescenta informações detalhadas e inovadoras sobre a manutenção de peixes em sistemas artificiais. Os peixes são expostos ao ciclo claro/escuro em um período de 24 horas, os parâmetros fisiológicos que são indicadores de gasto energético e, portanto, relacionados a condições estressantes, como Hb, Htc, MCV, MCH, MCHC, glicose, triglicerídeos, colesterol e proteína total, apresentam variação circadiana para *A. ocellatus* e *B. amazonicus*. Já o ciclo lunar demonstrou que os parâmetros hormonais (melatonina e cortisol), metabólicos e indicadores de estresse oxidativo são modulados tanto pelo claro/escuro quanto pelas fases da lua. E quando os peixes foram expostos à um pulso de luz durante o período de escuro, houve uma redução dos níveis plasmáticos melatonina em ambas as espécies, indicando que a luz pode atuar como um potencial estressor para os peixes, devido a maior mobilização de energia em função do aumento das interações agressivas, decorrentes dos baixos níveis de melatonina circulante.

Este estudo constatou que a melatonina pode promover efeitos na atenuação das variáveis associadas ao metabolismo energético dos peixes, alterando o perfil hematológico e bioquímico, reduzindo o estresse oxidativo associado à peroxidação

lipídica, estimulando a atividade antioxidante através da enzima GSH e inibindo o aumento dos níveis de cortisol plasmático.

Assim, pretende-se fornecer informações que contribuam para o avanço de novas tecnologias que possam ser utilizadas por programas de gestão ambiental e contribuir para o bem-estar dos peixes, essencial para uma aquicultura sustentável e responsável. A condução de trabalhos envolvendo abordagens mais integrativas a respeito dos ritmos biológicos em peixes, incluindo os componentes abióticos e bióticos, permitirá encontrar respostas mais consistentes sobre a relação dos peixes com as variações temporais do ambiente.

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ANEXOS

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ORIGINAL ARTICLE

Daily rhythm of some blood parameters in two Amazonian fish, *Astronotus ocellatus* and *Brycon amazonicus*

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Abstract

We evaluated the effect of the light–dark (LD) cycle on energy expenditure indicators for two species of Amazonian fish of commercial interest, oscar (*Astronotus ocellatus*) and matrinxã (*Brycon amazonicus*). Fish were exposed to a 12:12 h LD cycle, with lights on at 'zeitgeber time' (ZT) = 0 h. Six animals were used for blood collection every 4 h for 24 h (six time points, three in the light period and three in the dark period). Most haematological parameters exhibited daily rhythms with the acrophase at the end of the dark phase or the beginning of the light period for both species, which may be related to the greater energy demand of these species during the day. For *A. ocellatus*, triglycerides and total protein showed acrophases at ZT = 13:32 ± 2:45 h and ZT = 23:42 ± 1:55 h, respectively, while the other plasma parameters showed no significant daily differences. In *B. amazonicus*, significant rhythms were observed for glucose levels (acrophase at ZT = 04:16 ± 2:47 h), triglycerides (ZT = 6:51 ± 4:06 h) and total cholesterol (ZT = 23:22 ± 3:45 h). However, total protein and cortisol levels did not show rhythmicity in this species. Our results highlight the importance of the evaluation of biological rhythms for plasma physiological parameters related to energy expenditure in species with commercial interest and susceptible to stressful situations in farming conditions.

KEYWORDS
blood count, circadian rhythm, plasma metabolite, stress, teleost

1 | INTRODUCTION

Biological rhythms are observed in living organisms as a strategy for adjusting to environmental cycles (temperature and light) and, in teleost fish, these are regulated by endogenous oscillators that are controlled by a genetically encoded internal timing system (Ali, 1992; Toloza-Villalobos et al., 2015; Sánchez-Vázquez et al., 2019). According to Steindal and Whitmore (2019), central clock (i.e. suprachiasmatic nucleus in mammals) does not appear to be necessary for fish. However, this does not mean that there is no potential interaction between tissue clocks within the fish body. Moreover, a whole variety of hormonal signals, including rhythmic melatonin cues, could also be influencing tissue-specific, daily oscillations (Steindal, & Whitmore, 2019). Several natural and anthropogenic factors can act as circadian disrupters in fish and, consequently, affect multiple physiological processes (Zheng et al., 2021). So, we consider it important to know the daily rhythm of fish to understand the effect of potential environmental disruptions, including in an aquaculture system.

These rhythmic variations synchronize the animal with the environment and provide adaptive advantages by anticipating periodic events and programming physiological responses to occur at specific times of the day or year, thus increasing the probability of success and minimizing energy demand (Gerkema, 1992; Aranda et al., 1999).

In addition, knowledge of biological rhythms has applications in the context of aquaculture (Parker, 1984; Mattos et al., 2017), fish physiology (Yufera et al., 2014; Cowan et al., 2017), behaviour (Montoya et al., 2010; Mattos et al., 2016), stress responses (Almeida et al., 2018; Tian et al., 2019), immune system (Bowden, 2008) and the productive performance of fish (Sánchez-Vázquez et al., 1995; Pedrosa et al., 2019).

Environmental changes arising from aquaculture practices can act as a synchronizer of biological rhythms, although they must meet several conditions in order to be considered "zeitgebers", a term that is defined for exogenous synchronizers, which are classified as either photic (luminosity) or non-photic (temperature, feeding and stressors) (López-Olmeda et al., 2006; López-Olmeda, 2017; Blanco-Vives et al., 2011; Vera et al., 2014). In fact, the light–dark cycle plays a very relevant role in influencing biological rhythms in fish, with effects on body development (Blanco-Vives et al., 2011), the immune system (Binuramesh & Michael, 2011), locomotion (López-Olmeda et al., 2012), feeding (Mattos et al., 2016), metabolism (Paredes et al., 2015), release of enzymes and hormones (Yufera et al., 2014; Cowan et al., 2017) and oxidative stress indicators (Tian et al., 2019).

Different parameters that indicate energy expenditure, and are also associated with the physiological stress response, vary during the day and, consequently, present well-characterized biological rhythms (Cowan et al., 2017; Fortes-Silva et al., 2018; Ren et al., 2020). Cortisol, for example, has been the focus of studies on rhythms, mainly because it presents daily patterns in a wide variety of teleost (Montoya et al., 2010; Oliveira et al., 2013; Brüning et al., 2015), although rhythm features such as MESOR (average), amplitude (maximum oscillation) and acrophase (peak time) are species specific (Vera et al., 2014). Under intensive aquaculture conditions, cortisol, as well as other indicators of acute and/or chronic stress (e.g. erythrogram and plasma metabolites), present variation in the synthesis and release depending on the duration and intensity of the management procedures to which the fish are subjected (Conte, 2004; Huntingford et al., 2006). In this context, it is reasonable to suggest that stress responses are not the same at all periods of the light–dark cycle, highlighting the importance of considering the most appropriate time of day when stress indicators are evaluated (Oliveira et al., 2013; Guerra-Santos et al., 2017).

For this reason, acquiring knowledge of the daily rhythm of cortisol, haematological parameters and plasma metabolites of different fish species is an important approach for aquaculture, since these variables can be used as physiological indicators of stress and energy expenditures (Wendelaar-Bonga, 1997). Moreover, haematological parameters are important indicators of fish health status in the aquaculture system (Fazio, 2019) and they are affected by endogenous and exogenous factors (Ahmed et al., 2020).

In general, it is argued that knowledge regarding physiology related to stress and the effect of environmental changes under the rhythm of these physiological parameters in animals kept in an artificial system would enable the identification of adverse conditions in the environment and the development of methodologies or procedures that may mitigate the effects on the health and welfare of fish (Conte, 2004; Martins et al., 2012). Parker (1984) suggests that production efficiency and fish quality can be potentiated if the activities during the production process are carried out considering the biological rhythm of the species. This author also points out that environmental conditions must be considered in production systems and that routine practices during the production chain need to be altered based on information from chronobiology. In this context, chronobiology is an important tool that can assist us to achieve the objectives of aquaculture, especially in the Amazon region, since it has high potential for studies of this nature due to the high diversity of viable fish species both for aquaculture and the fisheries sector.

Although several studies reported the existence of circadian rhythm for physiological and behavioural parameters in fish (López-Olmeda et al., 2006, 2012; Oliveira et al., 2013; Vera et al., 2014; Paredes et al., 2015; Fortes-Silva et al., 2018), little is known about the daily profile of haematological variables in Amazonian species with interest in aquaculture. Among these species is the "oscar" (*Astronotus ocellatus*), which has high potential for tropical aquaculture, with significant trade in world fish keeping, its meat being highly valued in the Amazon region (Santos et al., 2009; Oliveira et al., 2013). The 'matrinxã' (*Brycon amazonicus*) is also native to the Amazon basin, and it is the second most produced fish species in the northern region of Brazil (SIDRA-IBGE, 2018). However, there are fundamental problems in the production of this species, especially due to the stress state caused by the management that occurs in artificial systems, which in turn affects the reproduction, larviculture and growth of juveniles (Bernardino et al., 1993; Souza et al., 2014), compromising its production expansion in the Amazon region. Previous studies have evaluated physiological and behavioural responses of *A. ocellatus* (Muusze et al., 1998; Gutierre et al., 2016) and *B. amazonicus* (Lopes et al., 2018; Ferreira et al., 2020), resulting from exposure to different environmental factors. However, nothing is known about the existence of rhythms and the effect of the light–dark cycle on energy expenditure indicators for these two species of Amazonian fish of commercial interest. Thus, our study aims to investigate the daily rhythm of haematological parameters in juvenile *A. ocellatus* and *B. amazonicus* in order to propose the ideal periods for management and manipulation in fish-breeding systems and experimental environments, as well as improving the welfare of these fish species.

2 | MATERIALS AND METHODS

2.1 | Acquisition and acclimation of animals

This study was performed in the Laboratory of Physiology and Behavior of Aquatic Animals at the Federal University of Amazonas (UFAM), Amazonas, Brazil. Juvenile *B. amazonicus* (mean weight ± SD: 64 ± 4 g; mean length ± SD: 15.75 ± 0.36 cm) were provided by a fish hatchery (Aquaculture Station of the Experimental Farm [FAEXP] at UFAM), and juvenile *A. ocellatus* (mean weight ± SD: 130 ± 29 g; mean length ± SD: 16.12 ± 1.38 cm) were captured in their natural

environment under licence No. 60643–3, which was obtained from the Chico Mendes Institute for Biodiversity Conservation (ICMBIO). All fish had no physical injuries and they were acclimated for 15 days in a 500-L polyethylene tank (1 animal/5 L) which contained internal biological filters for the maintenance of the water quality for a period of at least 15 days. The light intensity in the light period was maintained at 150 ± 42 lx and a photoperiod of 12:12 h light:dark (LD) cycle, with lights on at 06:00 h (zeitgeber time 0 h, ZT 0 h) and lights off at 18:00 h (ZT18 h). These factors were monitored with the aid of a lux meter (MLM-1011, Minipa, Brazil) and time data recorder (HL TM24H, Hardline, Brazil). The tanks were filled with artesian well water with 50% renewal every 3 days, and the water presented the following values for the measured parameters: temperature (27.0 ± 0.4°C), pH (6.1 ± 0.8), dissolved oxygen (5.5 ± 1.0 mg/L) and total ammonia (0.02 ± 0.01 mg/L). The fish were fed with a commercial feed to satiety (28% protein, Gabi, Brazil) twice a day. All the described procedures were performed for the two species under study.

The experiment was conducted according to the principles of ethics in animal experimentation (CONCEA) and was approved by Ethics Commission on the Use of Animals (CEUA-UFAM), case number No. 066/2018.

2.2 | Experimental design

After the acclimation period, 36 juvenile *A. ocellatus* (six animals per tank, six tanks in total) and 18 juvenile *B. amazonicus* (three animals per tank, six tanks in total) were grouped in 500-L polyethylene tanks for a 24 h period (12:12 h LD cycle). During this period, fish of both the species were fasted to avoid the influence of this procedure on the energy expenditure indicators to be measured.

In studies of circadian rhythms, when the lights are turned on, this period is called zeitgeber (synchronizer) time (ZT) and this corresponds to ZT0 (in this study = 06:00 h); and when the lights are turned off, this period corresponds to ZT12 (in this study = 18:00 h) (López-Olmeda et al., 2016). Every 4-h interval, all animals in the same tank ($n = 6$ for *A. ocellatus* and $n = 3$ for *B. amazonicus*) were used for blood collection, totalling six samples: three in the light period (8:00 h/ZT2, 12:00 h/ZT6 and 16:00 h/ZT10) and three in the dark period (20:00 h/ZT14, 00:00 h/ZT18 and 04:00 h/ZT22).

Most of the studies regarding the daily rhythm of physiological parameters evaluate the effect of environmental factors, such as feeding period and photoperiod, which act as modulators of the circadian cycle in fish (Spieler & Noeske, 1984; Bayarri et al., 2009; Montoya et al., 2010; Tian et al., 2019). However, this study analysed the daily pattern of these physiological indicators of energy expenditure without the influence of external factors that could act as synchronizers of the biological rhythm, and for this, the animals were kept fasted during the 24 h of the experimental period. In addition, the photoperiod was controlled for 12 h light:12 h dark, which avoided the effect of manipulation by these factors on the cyclic variation in the physiological variables analysed.

2.3 | Blood collection

At the end of the experimental period, fish were anaesthetized with eugenol (64 μl/L), as indicated by Ferreira et al. (2020), and immediately punctured in the caudal vein region to obtain blood samples with the aid of 0.8 ml disposable syringes containing heparin (5000 units/ml) as an anticoagulant. During the dark period, animal manipulation and blood collection were performed under low-intensity red light (<0.3 lux) to avoid the effect of luminosity on physiological parameters, in accordance with Oliveira et al. (2007).

2.4 | Blood parameters

Haematological analyses were performed immediately after blood collection at each moment of the cycle. The concentration of blood haemoglobin (Hb, g/dl) was analysed using the cyanmethaemoglobin method (Kampen & Zijlstra, 1964). The erythrocyte count (RBC, 10^6/μl blood) was performed via optical reading in a Neubauer chamber of blood samples fixed in formalin–citrate (1:200), according to the usual method used for fish. Haematocrit (Htc, %) was determined using the microhaematocrit method (Goldenfarb et al., 1971), in which the capillary tubes were centrifuged at 13,000 rpm for 6 min. The erythrocyte indices, mean corpuscular volume (MCV, fL), mean corpuscular haemoglobin (MCH, pg/cell) and the concentration of mean corpuscular haemoglobin (MCHC, g/dl) were calculated from the haematological parameters (Wintrobe, 1934).

The remaining blood collection material was centrifuged for plasma separation (3000 rpm/15 min) and kept at −20°C for biochemical analysis. Plasma levels of glucose (mg/dl), triglycerides (mg/dl), total cholesterol (mg/dl) and total proteins (g/dl) were obtained using commercial enzymatic–colorimetric kits (In Vitro Diagnostica Ltda; Itabira/MG, Brazil), which were specific for each constituent. The plasma concentration of cortisol was analysed using the immunoenzyme method (ELISA) and a commercial kit (DRG International, Germany), as performed by Barry et al. (1993). The reference values were approximate to those described by Muusze et al. (1998) and Ferreira et al. (2020) for *A. ocellatus* and *B. amazonicus*, respectively, with the intra-trial variation of 4.05%.

2.5 | Data analysis

The Cosinor analysis was used to detect the existence of significant rhythmicity of the haematological parameters, metabolites and plasma cortisol, performed with the software "EL TEMPS" (v. 1.179, Prof. Diez-Noguera, University of Barcelona, Spain). The Cosinor analyses is based on the least-square approach of time-series data with a cosine function of a known period of the type $Y = M + A^*[\cos(\Omega t + \Phi)]$, where M is the MESOR, A is the amplitude, Ω is the angular frequency (3600/24 h for daily rhythms) and Φ is the acrophase, according to López-Olmeda et al. (2012) and Ren et al. (2020). The

Cosinor analyses also provide the statistical significance of the rhythm by and F-test of the variance accounted for by the waveform versus a straight line of zero amplitude (null hypothesis).

In order to detect the existence of statistical differences between time points, the SPSS Software (IBM, version 22.0, Armonk, NY, USA) was used. Data normality was previously evaluated using the Shapiro–Wilk test and the homogeneity of variance was verified using the Levene test. All data are presented as mean ± SEM. As distribution was normal, data on energy expenditure indicators (haematological parameters and indices, and plasma metabolites) were compared between periods by the use of one-way ANOVA, followed by Duncan's post hoc test. We also compared the mean cortisol concentration of the three collection moments (grouped data) between the light and dark periods, using an independent Student's *t*-test. Statistical analyses were performed with the level of significance set at $p < 0.05$.

3 | RESULTS

In regards to *A. ocellatus*, significant daily rhythms were observed for Htc (Cosinor, $p = 0.029$) and MCV ($p = 0.004$), with acrophases at 03:17 ± 4:18 h and 4:47 ± 2:59 h respectively (Table 1; Figures 1 and 3). Htc decreased at the beginning of the dark period (ZT14) compared with the end of the same period ($p = 0.020$) (ZT22; Figure 1). MCV increased at the beginning of the light period (ZT2) and decreased at the beginning of the dark period ($p = 0.006$) (ZT14; Figure 1). No daily rhythm was observed for Hb, RBC, MCH and MCHC ($p > 0.05$; Table 1), although the concentrations of Hb and RBC were significantly (ANOVA, $p = 0.001$) higher at ZT22 (Figure 1).

For *B. amazonicus*, rhythmicity was observed for most haematological parameters, such as Hb (acrophase: 21:56 ± 2:43 h; $p = 0.001$), Htc (acrophase: 4:38 ± 4:04 h; $p = 0.024$), MCH (acrophase: 7:28 ± 2:43 h; $p = 0.001$) and MCHC (acrophase: 19:40 ± 3:03 h; $p = 0.006$) (Table 1; Figures 1 and 3). No significant differences were observed for RBC and MCV ($p > 0.05$; Figure 1). Hb ($p = 0.005$) and MCHC ($p = 0.013$) were higher in the dark period (ZT22; Figure 1), while Htc ($p = 0.016$) and MCH ($p = 0.002$) were higher in the early light period (ZT2; Figure 1).

The total triglyceride and protein levels showed acrophase at 13:32 ± 2:45 h ($p = 0.001$) and 23:42 ± 1:55 h, respectively ($p = 0.001$; Table 1; Figures 2 and 3), and the other plasma parameters did not show significant difference between the periods of the light–dark

TABLE 1 Mesor, amplitude, acrophase and statistical significance values of the plasma physiological parameters subjected to Cosinor analysis for *Astronotus ocelattus* and *Brycon amazonicus*

Parameters	Species	Mesor	Amplitude	Acrophase (ZT hours)	Significance (*p*)
Hb (g/dl)	*A. ocellatus*	–	–	–	NS
	B. amazonicus	7.29 ± 0.75	1.15 ± 1.35	21:56 ± 2:43	0.001
Htc (%)	*A. ocellatus*	22.96 ± 2.70	2.69 ± 4.90	3:17 ± 4:18	0.029
	B. amazonicus	34.84 ± 4.36	4.49 ± 7.85	4:38 ± 4:04	0.024
RBC (10^6/μl)	*A. ocellatus*	–	–	–	NS
	B. amazonicus	–	–	–	NS
MCV (fl)	*A. ocellatus*	132.10 ± 13.60	17.16 ± 24.29	4:47 ± 2:59	0.004
	B. amazonicus	–	–	–	NS
MHC (pg/cell)	*A. ocellatus*	–	–	–	NS
	B. amazonicus	48.79 ± 6.81	9.94 ± 12.26	7:28 ± 2:43	0.001
MCHC (g/dl)	*A. ocellatus*	–	–	–	NS
	B. amazonicus	21.50 ± 4.24	5.31 ± 7.63	19:40 ± 3:03	0.006
Glucose (mg/dl)	*A. ocellatus*	–	–	–	NS
	B. amazonicus	67.39 ± 14.60	21.89 ± 26.27	04:16 ± 2:47	0.001
Triglycerides (mg/dl)	*A. ocellatus*	135.63 ± 26.00	37.26 ± 45.65	13:32 ± 2:45	0.001
	B. amazonicus	274.04 ± 48.87	50.03 ± 87.99	6:51 ± 4:06	0.025
Cholesterol total (mg/dl)	*A. ocellatus*	–	–	–	NS
	B. amazonicus	121.81 ± 20.22	159,14 ± 29.42	23:22 ± 3:45	0.011
Total Protein (mg/dl)	*A. ocellatus*	2.56 ± 0.42	0.80 ± 0.76	23:42 ± 1:55	0.001
	B. amazonicus	–	–	–	NS
Cortisol (ng/ml)	*A. ocellatus*	–	–	–	NS
	B. amazonicus	–	–	–	NS

Note: Errors are mean ± SEM, fiducial limits set at 95%.

Abbreviations: Hb, haemoglobin; Htc, haematocrit; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; NS, non-significant; RBC, red blood cells.

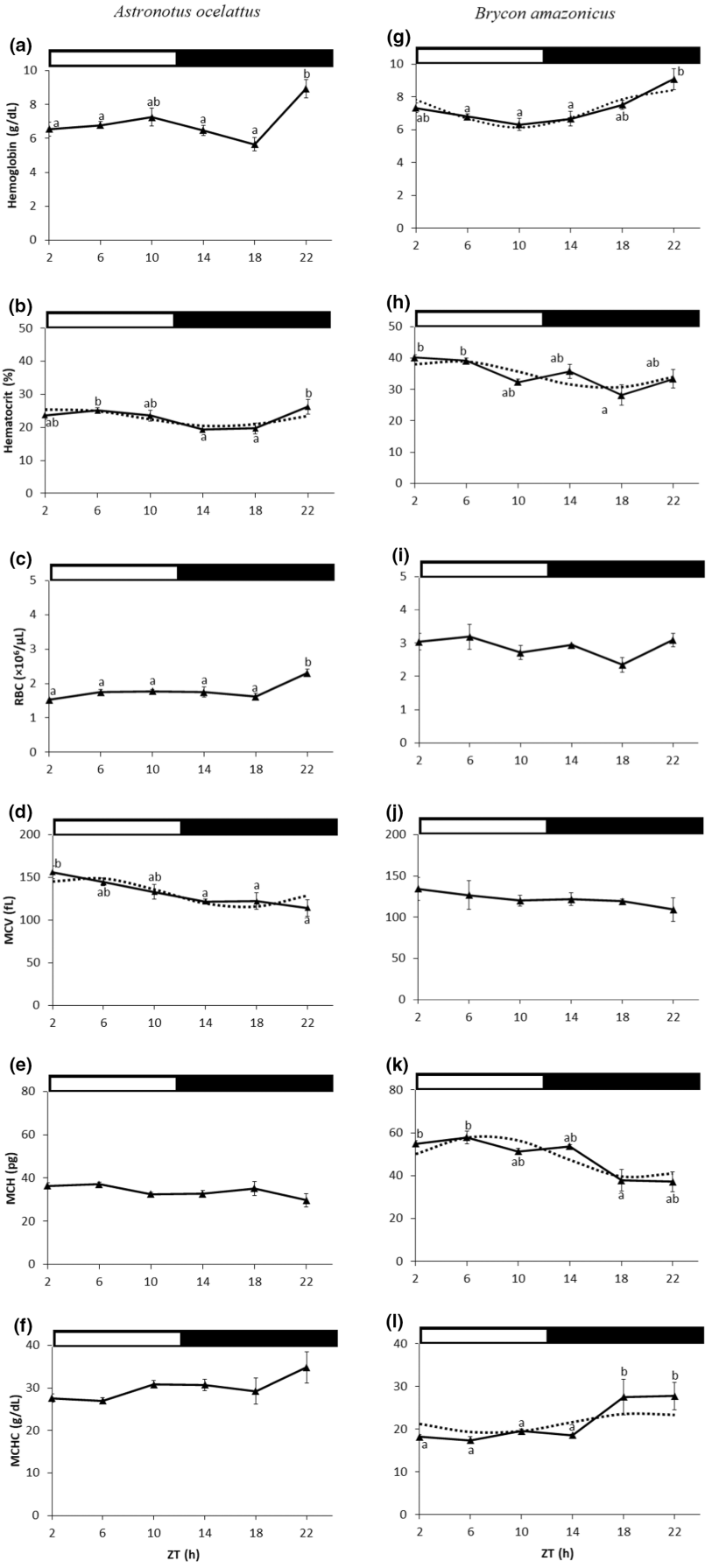


FIGURE 1 Daily changes in physiological parameters of *Astronotus ocelattus* (a–f) and *Brycon amazonicus* (g–l). a/g- Hb; b/h- Htc; c/i- RBC; d/j- MCV; e/k- MCH and f/l- MCHC. Values represent the mean ± S.E.M. ($n = 6$ / time point, *A. ocellatus*; $n = 3$/time point, *B. amazonicus*). White and black bars represent light and darkness respectively. Different letters indicate significant differences between time points (Duncan, $p < 0.05$). The sinusoidal dashed line represents the adjustment to a rhythm calculated by the Cosinor analysis whenever this analysis was statistically significant ($p < 0.05$). RBC, red blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration.

cycle for *A. ocellatus* ($p > 0.05$). Triglycerides had higher plasma levels at the beginning of the dark period ($p = 0.004$), while total proteins decreased during the same period ($p = 0.001$) (ZT14; Figure 2). Despite not presenting a circadian rhythm, the plasma cortisol level increased at the beginning of the dark period (ZT14) compared with the light period (ZT2; $p = 0.007$; Figure 2).

In *B. amazonicus*, a circadian variation was observed through Cosinor analysis for glucose (acrophase: 04:16 ± 2:47 h; $p = 0.001$), triglycerides (acrophase: 6:51 ± 4:06 h; $p = 0.025$) and total cholesterol (acrophase: 23:22 ± 3:45 h; $p = 0.011$) (Table 1; Figures 2 and 3). The level of total proteins did not show rhythmicity ($p > 0.05$; Table 1); however, there was a significant increase in this parameter at the end of the light period ($p = 0.036$; ZT10) compared with the beginning of the same period (ZT2; Figure 2). Glucose levels were higher in the middle of the light period (ZT6) and lower in the middle of the dark period (ZT18) ($p = 0.005$; Figure 2). Triglycerides increased at the beginning of the light period (ZT2) compared with the dark period (ZT18) ($p = 0.002$; Figure 2). No daily rhythm was observed for cortisol concentration in *B. amazonicus* ($p = 0.184$; Table 1; Figure 2). However, when the mean concentration of this hormone of the three collection moments between periods of light was compared with that of the dark period, higher plasma levels were observed during the light phase (237.84 ± 15.74 ng/ml) when compared with the dark phase (186.06 ± 13.80 ng/ml) (Independent *t*-test, $p = 0.025$).

4 | DISCUSSION

The haematological indices presented mean values similar to those described in other studies for *A. ocellatus* (Baptista et al., 2016) and *B. amazonicus* (Tavares-Dias et al., 2008; Ferreira et al., 2020), indicating the reference values and that the methodology used was suitable.

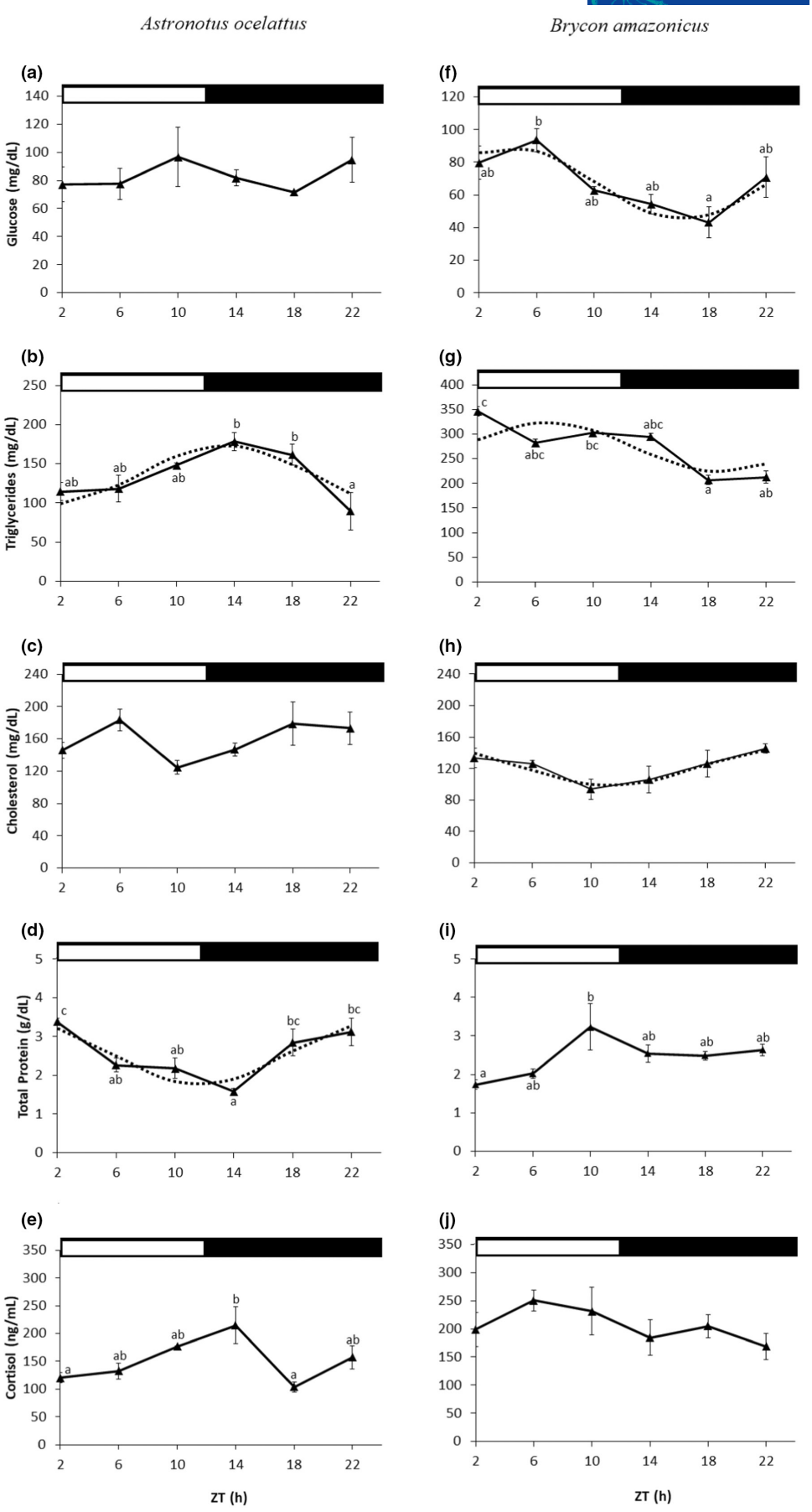
The concentration of HB, Htc, MCH and MCHC exhibited their acrophase at the end of the dark phase or beginning of the light period for *B. amazonicus*. Considering that the haematological parameters are related to the blood's oxygen transport capacity (Witeska, 2013), these results are consistent with the high level of swimming activity of *A. ocellatus* that has been reported during daytime (Soares et al., 2008), which may reflect a higher respiratory demand during this period. In fact, the release of erythrocytes by the spleen, and subsequent increase in Htc and RBC values, are directly related to the increased activity of animals (Fánge, 1992; Glomski et al., 1992). In addition, the higher values of MCH and MCHC may have been due to the increase in Hb, since these erythrocyte indices are directly proportional to the concentration of haemoglobin present in the blood (Wintrobe, 1934).

For *A. ocellatus*, the higher values of Hb and RBC at the end of the dark period and the acrophase for Htc and MCV at the beginning of the light period also suggest the triggering of physiological adjustments related to a greater energy demand during the day since *A. ocelattus* has a diurnal habit (Santos et al., 2009). In fact, erythrocyte indices may vary among species and within the same species, presenting a direct relationship with fish activity. For example, more active fish, such as tuna and other pelagic fish, tend to have higher MCV values when compared with benthic and sedentary species due to the higher demand of 0_2 for animals with higher activity (Clauss et al., 2008). In addition, the same pattern of rhythmic response observed for Htc and MCV corroborates the direct relationship between these two haematological indicators (Wintrobe, 1934).

The daily rhythm pattern for plasma glucose concentration varies among fish species. In the present study, the absence of daily rhythm for glucose levels in *A. ocellatus* coincides with the results obtained for *Oreochromis niloticus*, another species of cichlid (Almeida et al., 2018) and *Centropristis striata* (Ren et al., 2020), as well as for *Tinca tinca* and *Oncorhynchus mykiss* when subjected to food deprivation (De Pedro et al., 2005; Polakof et al., 2007). For *B. amazonicus*, the observation of a daily glycaemic rhythm with acrophase at ZT = 04:16 ± 2:47 h may indicate the preparation for the greatest energy demand during the day, considering its high metabolic and behavioural activity that is predominant during daytime (Ferreira et al., 2020; Soares et al., 2008). These results are consistent with the findings of Guerra-Santos et al. (2017), which show higher glycaemic values in the period of greater locomotor activity in *Oreochromis niloticus*.

In teleost, the circadian profile of plasma cortisol is considered to be species specific and related to the activity pattern (diurnal or nocturnal) of the animal (Ellis et al., 2012; López-Olmeda et al., 2013). However, Cowan et al. (2017) suggested the need for further studies to demonstrate the relationship between the higher plasma cortisol concentration and the activity pattern of the fish species. The peak activity of this hormone may also be associated with the period of fish feeding, suggesting its synchronization with the feeding cycle (Spieler & Noeske, 1984; López-Olmeda et al., 2009; Montoya et al., 2010; Guerra-Santos et al., 2017). Thus, the 24-h fasting period to which the animals were submitted in this study may have influenced the absence of significant rhythms for cortisol in both *A. ocelattus* and *B. amazonicus*. Although no daily rhythm was observed for *A. ocellatus*, plasma cortisol concentrations remained stable until the end of the light phase, with values ranging from 120.50 ± 4.02 ng/ml

FIGURE 2 Daily changes in physiological parameters of *Astronotus ocelattus* (a–e) and *Brycon amazonicus* (f–j). a/f – Glucose; b/g – Triglycerides; c/h – Cholesterol; d/i – Total protein and e/j – Cortisol. Values represent the mean ± S.E.M. ($n = 6$/time point, *A. ocellatus*; $n = 3$/time point, *B. amazonicus*). White and black bars represent light and darkness respectively. Different letters indicate significant differences between time points (Duncan, $p < 0.05$). The sinusoidal dashed line represents the adjustment to a rhythm calculated by the Cosinor analysis whenever this analysis was statistically significant ($p < 0.05$).


Astronotus ocelattus
Brycon amazonicus
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
Glucose (mg/dL)
Triglycerides (mg/dL)
Cholesterol (mg/dL)
Total Protein (g/dL)
Cortisol (ng/mL)
ZT (h)
ZT (h)

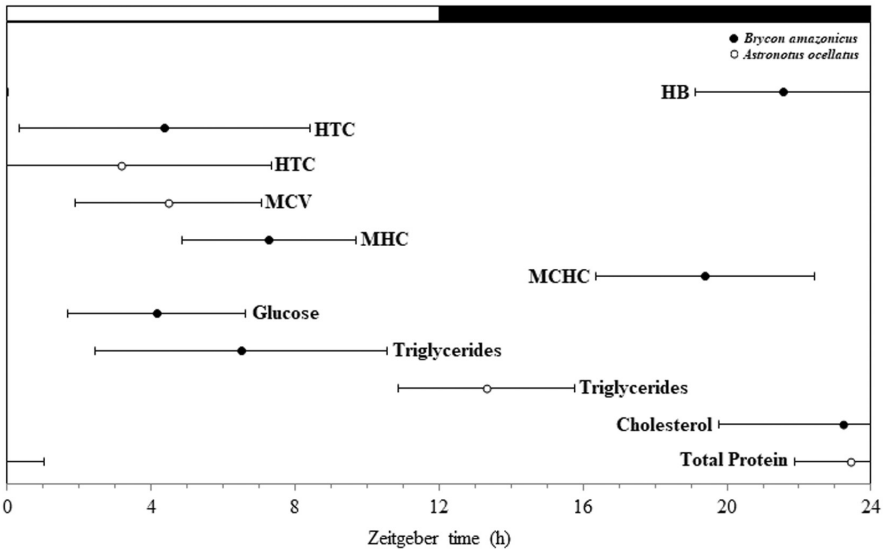


FIGURE 3 Acrophases map for the physiological parameters (Cosinor, $p < 0.05$). The acrophase is indicated by a circle, black and white for *Brycon amazonicus* and *Astronotus ocelattus* respectively. The confidence intervals (set at 95%) are indicated by the lateral bars. White and black bars above the graph represent light and darkness respectively.

(ZT2) to 136.24 ± 0.58 ng/ml (ZT10). However, a significant peak (214 ± 0.61 ng/ml) was observed at the beginning of the dark phase (ZT14), which is similar to that found for other fish species (Vera et al., 2014; Almeida et al., 2018; Tian et al., 2019). This fact can be explained since the increase in cortisol occurred shortly after the transition from the light phase to the dark phase, which is characterized as an abrupt environmental change in laboratory conditions and may have reflected in a physiological preparation as a result of a potentially stressful condition. In fact, Saito et al. (2004) showed that the plasma cortisol level did not present daily rhythm for *Oncorhynchus keta*, but an increase in this hormone was observed over the 24-h period, which may be associated with a stressful response due to capture and transport procedures. Pickering et al. (1982) also suggested that cortisol concentrations in different fish species typically rise a few minutes after exposure to a moderate acute stressor, peak and return to baseline values within approximately 6 h. Thus, this response may have been observed in the present study, since the baseline values returned within this time range, after the peak of cortisol which may be due to an acute response to the change in ambient luminosity. On the other hand, this hormone increase may not be a response to the change from dark to light period, but it may have endogenous control.

The rapid resume to the initial level of cortisol (ZT18) agrees with the lack of variation in blood glucose observed for *A. ocellatus*, since increases in plasma levels of cortisol and glucose are directly related (Carneiro et al., 2002). According to Mommsen et al. (1999), the increase in cortisol acts as a gluconeogenic signal by increasing the level of glucose in the blood in fish. In fact, this relationship can be evidenced because, although *B. amazonicus* did not present daily rhythm for cortisol, there was a higher concentration of this hormone in the light period, which may be associated with the glucose acrophase observed in the early morning (04:16 ± 2:47 h).

Cortisol can also promote increased levels of free fatty acids in plasma (Butler, 1973), thus increasing plasma cholesterol in some fish species (Poursaeid et al., 2015). In addition, cholesterol is the precursor of steroid hormones such as glucocorticoids, evidencing a direct relationship between cholesterol availability and cortisol synthesis (Miller, 1988; Sanderson, 2006). Although no cortisol rhythm has been observed, the rhythmicity of cholesterol with acrophase at 23:22 ± 3:45 h may indicate the higher cortisol synthesis evidenced by the higher concentration of this hormone in the light period for *B. amazonicus*.

A daily plasma triglycerides profile was observed for *A. ocellatus* and *B. amazonicus*, suggesting the body's attempt to seek greater input to maximize the use of energy substrate. According to Tocher (2003), lipids can function as an energy substrate for fish, and are important for providing support arising from increased metabolic activity. Higher triglyceride levels were observed in *B. amazonicus* in the early hours of the day (ZT2), with a significant decrease halfway through the dark phase (ZT18) and the acrophase at ZT = 6:51 ± 4:06 h. This response may be associated with greater mobilization of energy reserves due to greater activity of the species in the daytime (Soares et al., 2008; Rodrigues et al., 2017), which is a relationship also suggested for other parameters analysed in this study (e.g. Hb, Htc and blood glucose).

According to Fortes-Silva et al. (2018), the circadian variation in plasma proteins is complex as a result of the daily pattern of specific proteins; however, these data are important in assessing the health status of fish. In addition, Mommsen et al. (1999) report that changes in total protein concentrations are related to increased cortisol, which results in increased gluconeogenesis and protein catabolism activity. Thus, the results of the daily rhythm of this physiological parameter for *A. ocellatus* (acrophase: 23:42 ± 1:55 h) indicate that this circadian variation may be due to the increase in cortisol at the beginning of the dark period observed for the species. For *B. amazonicus*, no daily rhythmicity was observed, as also reported by De Pedro et al. (2005) and Fortes-Silva et al. (2018) for *Tinca tinca* and *Lophiosilurus alexandri* respectively.

Brycon amazonicus presented a greater number of physiological parameters that showed daily rhythms (Htc, Hb, MCH, MCHC, glucose, triglycerides and total cholesterol) compared with *A. ocellatus* (Htc, MCV, triglycerides and total proteins). This difference suggests

that *B. amazonicus* presents greater biochemical modulation evidenced by the rhythmicity of energy expenditure indicators and relative to the increase in metabolic demand during the light period. In fact, *B. amazonicus* is a species that has a high rate of locomotion, aggressiveness and territoriality (Ferraz & Gomes, 2009; Ferreira et al., 2020; Souza et al., 2014), which is reflected by a high demand for O_2 transport and availability of energy substrates (Alvarenga & Volpato, 1995; Wendelaar-Bonga, 1997). *A. ocellatus*, presents also diurnal behaviour and it is a territorial fish (Beeching, 1992; Santos et al., 2009), although less activity can be observed when compared with *B. amazonicus*, and this is evidenced by the lower frequency of aggressive items exhibited by *A. ocellatus* (Gonçalves-de-Freitas & Mariguela, 2006) as compared with *B. amazonicus* (Ferreira et al., 2020) in conditions of social challenge (mirror test) in an equivalent time period.

In summary, it can be observed that some physiological parameters that are indicators of energy expenditure and, therefore, related to stressful conditions, such as Hb, Htc, MCV, MCH, MCHC, glucose, triglyceride, cholesterol and total protein, present circadian variation for *A. ocellatus* and *B. amazonicus*. Therefore, care should be taken to assess the stress response and/or well-being in fish, especially when these parameters are measured at different times of the light–dark cycle, or when comparisons are made of the results of different studies or between treatments in the same study (López-Olmeda et al., 2012; Ren et al., 2020). Both species studied are of commercial interest and are susceptible to management practices in an artificial system that triggers potentially stressful situations. In this context, knowledge of the rhythms of physiological parameters in fish is essential in order to evaluate the time-dependent effects of treatments that induce stressful responses (e.g. manipulation) and also to maximize the efficiency of the administration of exogenous substances, as in the case of *B. amazonicus* whose reproduction depends on hormonal induction (Bernardino et al., 1993; Romagosa et al., 2001). In addition, this work may contribute to a greater adequacy of conditions that provide better welfare and productive performance for these animals when kept in artificial systems.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization, Bruno Olivetti de Mattos, Ana Caroliny Cerdeira Lopes, Francisco Javier Sánchez-Vázquez and Thaís Billalba Carvalho; Methodology, Bruno Olivetti de Mattos, Ana Caroliny Cerdeira Lopes, Francisco Javier Sánchez-Vázquez and Thaís Billalba Carvalho; Formal analysis, Bruno Olivetti de Mattos, Gonzalo de Alba and Thaís Billalba Carvalho; Investigation, Ana Caroliny Cerdeira Lopes, Jhomaxon de Souza Gonçalves, Bruno Olivetti de Mattos, Jaydione Luiz Marcon, Francisco Javier Sánchez-Vázquez, Gonzalo de Alba and Thaís Billalba Carvalho; Resources, Bruno Olivetti de Mattos, Jaydione Luiz Marcon, Francisco Javier Sánchez-Vázquez and Thaís Billalba Carvalho; Data curation, Ana Caroliny Cerdeira Lopes, Jhomaxon de Souza Gonçalves and Thaís Billalba Carvalho; Writing—original draft preparation, Ana Caroliny Cerdeira Lopes, Jhomaxon de Souza Gonçalves, Bruno Olivetti de Mattos, Jaydione Luiz Marcon, Francisco Javier Sánchez-Vázquez, Gonzalo de Alba and Thaís Billalba Carvalho; Writing—review and editing, Ana Caroliny Cerdeira Lopes, Jhomaxon de Souza Gonçalves, Bruno Olivetti de Mattos, Jaydione Luiz Marcon, Francisco Javier Sánchez-Vázquez, Gonzalo de Alba and Thaís Billalba Carvalho; Supervision, Bruno Olivetti de Mattos, Francisco Javier Sánchez-Vázquez and Thaís Billalba Carvalho. All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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Does exposure to moonlight affect day/night changes in melatonin and metabolic parameters in Amazonian fish?

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ABSTRACT

Lunar cycle modulates the rhythmic activity patterns of many animals, including fish. The effect of the moonlight cycle on daily melatonin and metabolic parameters was evaluated in matrinxã (*Brycon amazonicus*) subjected to external natural lighting. Eighty juvenile were distributed in 4 tanks of 1m^3 (20 fish/tank) and divided into two groups. One group was exposed to the full moon and the other group to the new moon for 30 days, which corresponds to the duration of the lunar period. At the end of the lunar phase, 6 fish from each group were anesthetized to collect blood, tissue and eye samples at midday and midnight. The comparison between the light and dark periods revealed a significant increase in plasma and ocular melatonin in the last period. However, there was no significant difference for plasma melatonin between moons. Ocular melatonin presented higher concentrations during the new moon. Glucose, total proteins, cortisol, liver glutathione and gill lipid peroxidation were higher in the full moon compared to in the new moon. Plasma triglyceride was higher during the night for the full moon, and the opposite was found for the new moon. Total cholesterol values were higher at night regardless the moon phase. Glutathione in the gills and lipid peroxidation in the liver showed no significant differences. These results highlight the importance of considering both the day and lunar cycles for melatonin and metabolic parameters in species of commercial interest and susceptible to stressful situations in rearing conditions.

1. Introduction

Depending on its relative position, the illumination reflected from the sun by the moon changes during the lunar month. Moonlight modulates the rhythmic activity patterns of many living organisms and this may be transduced by changes in neural and hormonal levels such as melatonin and other hormones, as well as specific metabolites (Chakraborty, 2020). According to Andreatta and Tessmar-Raible (2020), connections between metabolic/endocrine pathways and moon-controlled rhythms are evidenced for a variety of species. However, in teleost fish, these mechanisms are still elusive, particularly in freshwater species.

Changes in moonlight during the phases of the moon synchronize lunar rhythms in organisms in various ways, such as, providing time cues to synchronize important biological aspects at individual and populational levels (Kronfeld-Schor et al., 2013; Gaston et al., 2017). Takemura et al. (2010) reported that the intensity of the light from the moon influences the physiological and behavioral activities of fish inhabiting tropical and subtropical waters. Indeed, the lunar cycle plays a relevant role in influencing the biological rhythms of fish, and has an effect on reproduction (Oliveira et al., 2009; Ikegami et al., 2014; Golmoradizadeh et al., 2021), molecular mechanisms (Steindal and Whitmore, 2019; Andreatta and Tessmar-Raible, 2020), predation behavior (Palmer et al., 2017) and the release of hormones such as melatonin and sexual steroids (Oliveira et al., 2010).

Biological rhythms, irrespectively from being a direct response to the

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environment or driven by endogenous oscillators, are also mediated by changes in hormonal levels and metabolism (Pittendrigh, 1960; Ali, 1992). The evolution of biological clocks, for example, has probably been favored by the regularity of geophysical cycles and the advantages that organisms have in reacting to regular changes in the environment and anticipating these changes in order to prepare properly for them, thus maximizing their ecological fitness (Sellix, 2016; Raible et al., 2017). In addition, an essential factor in the regulation of biological rhythms is the input of melatonin rhythms, a key hormone involved in the regulation of many rhythmic processes (Reiter et al., 1997; Migaud et al., 2010; Falcón et al., 2010, 2011; ViviD and Bentley, 2018). The amplitude/duration of the nocturnal melatonin rhythm is finely tuned by environmental light intensity, photoperiod and temperature, thus providing the animal with daily, seasonal and lunar information (Reiter, 1993; Porter et al., 2001; Takemura et al., 2006; Brüning et al., 2015). In vertebrates, melatonin synthesis occurs mainly in the pineal gland, but in teleost fish it also occurs in the retina (Dodt, 1963; Omura and Oguri, 1969; Cahill, 1996; Vuilleumier et al., 2007; Vera et al., 2014). Pineal melatonin, in addition to controlling circadian activities, regulates seasonal reproduction, the rhythmicity of locomotor activity, the immune response and also acts as an antioxidant agent (Falcón et al., 2011; Wiechmann and Sherry, 2013). In contrast, the retinal melatonin pattern, despite exhibiting a daily rhythmicity, may presents peaks at different times of the day, depending on the species (Cahill et al., 1991; Falcón et al., 2003).

According to Oliveira et al. (2007), the nocturnal production of melatonin is very sensitive to light, i.e., a light intensity as low as 0.3 lx (full moon) is able to reduce plasma levels of melatonin. Also, in some marine fish species, the low illumination presents at a full moon can significantly reduce melatonin levels (e.g., Takemura et al., 2006; Oliveira et al., 2010; Park et al., 2014; Fukunaga et al., 2019). In short, melatonin translates environmental information about light or darkness into a hormonal signal to cells and organs, thereby synchronising behavioral and physiological rhythms (Falcón et al., 2020).

In addition to melatonin, parameters that indicate energy expenditure in fish also show rhythmic variations (Oliveira et al., 2013; Brüning et al., 2015; Cowan et al., 2017; Fortes-Silva et al., 2018; Ren et al., 2020). Oxidative stress indicators can also vary due to environmental changes and present rhythmicity associated with the light-dark cycle and the phases of the moon (Tal et al., 2011; Vera et al., 2014). However, little is known about the effect of moonlight on the rhythm of these physiological parameters. In this sense, further research is needed to understand the influence of the lunar cycle on fish in order to fully understand the effect of potential environmental disturbances in aquatic systems such as nocturnal light contamination.

Knowing how moonlight modulates physiological and behavioral mechanisms in fish is crucial for understanding how chronobiological changes caused by anthropogenic impacts (e.g., light pollution and temperature changes, among others) and natural factors in aquatic ecosystems (e.g., Fogarty and Marhaver, 2019; Shlesinger and Loya, 2019). In fact, exposure to natural and artificial factors can generate temporal disturbances that lead to misalignment of physiology and metabolism (i.e. chronodisruption), thus obscuring the natural order of alternating periods of light and darkness at all levels of organization (Gaston et al., 2017; Falcón et al., 2020; Zheng et al., 2021). Studies of the effects of the moon on fish have also shown that spawning, migration, activity, feeding, physiology, and vulnerability to commercial or recreational fishing may be synchronized to lunar cycles (Ali, 1992; Ikegami et al., 2014; Vinson and Angradi, 2014). Besides its ecological relevance, little attention has been paid to this issue in freshwater tropical fish, such as the matrinxã (*Brycon amazonicus*), which is a native species of the Amazon Basin and of great importance in this region (Brazil, Peru and Colombia), since it is the second most-produced local fish species (FAO, 2022). In this context, this study aimed to investigate the influence of the moonlight cycle on physiological parameters in matrinxã (*Brycon amazonicus*) exposed to external natural lighting. According to Sánchez-Vázquez et al. (2019), there is a need to properly take into account the role of biological rhythms when discussing fish welfare issues. As such, we intend to provide information that will contribute to the advance of new technologies that can be used by environmental management programs and, consequently, safeguard the welfare of fish, which is essential for sustainable and responsable aquaculture.

2. Materials and methods

2.1. Area of study and ethical approval

This study was conducted in a dam (60 m wide x 3 m deep) with continuous water circulation located at the Experimental Farm of the Federal University of Amazonas (FAEXP/UFAM), Manaus, Brazil, (2° 38′ 39” S, 60° 03′ 11” W).

The experiment was carried out in accordance with the ethical principles of the Brazilian National Council for Animal Experimentation Control (CONCEA) and was approved by the Ethics Commission in the Use of Animals (CEUA-UFAM) under process No. 066/2018.

2.2. Experimental design

Eighty juvenile *Brycon amazonicus* (271.0 ± 5.9 g; 23.3 ± 0.2 cm; mean ± SEM) obtained from the Aquaculture Station at FAEXP/UFAM were stored in four 1 m^3 net cages (20 animals per tank) and divided into two groups. After acclimation, one group was exposed to the full moon (0.1 lx [midnight] and 697.5 ± 80.5 lx [midday]) and the other group to the new moon (0.0 lx [midnight]; 656.5 ± 50.5 lx [midday]), with 2 tanks per group and for 30 days. Both groups remained in their tanks exposed to the moon for the full lunar cycle (i.e., new moon from 7/2/2019 to 7/31/2019 and full moon from 6/17/2019 to 7/16/2019). The animals were stocked on the first day of each moon phase and blood samples were performed on the last day of each moon period.

After 30 days of each treatment (full moon and new moon), 6 animals were collected at midnight and 6 specimens were sampled at midday for the analysis of plasma and ocular melatonin concentration, plasma metabolites and oxidative stress indicators. The fish were anesthetized with 64 μl/L of eugenol (Biodinâmica, Ibiporã, Brazil; Ferreira et al., 2020), and immediately punctured in the caudal vein region in order to obtain blood samples with the aid of 1 ml disposable syringes containing heparin (Cristália, Itapira, Brazil). Subsequently, fish biometrics were performed: body weight of 319.0 ± 15.3 g and standard length of 25.6 ± 0.3 cm (full moon); body weight of 291.5 ± 4.8 g and standard length of 24.3 ± 0.5 cm (new moon).

Then, all the fish were killed by medullary section for eye, liver and gills collection. These samples were immediately frozen in liquid nitrogen and subsequently stored in a freezer −80 °C for further analysis. Blood samples were centrifuged for plasma separation (3000 rpm*15 min^{-1}) and kept at −80 °C for analysis of hormones (melatonin and cortisol) and plasma metabolites.

During the whole experimental period, the fish were fed with commercial feed (32% crude protein, Guabi, Indaiatuba, Brazil) until apparent satiety twice a day. The fish were fasted for a period of 24 h prior to collection to avoid the influence of feeding on the indicators of energy expenditure to be measured. During the dark period, for both phases of the lunar cycle, animal manipulation and tissue collection were performed under only moonlight and low intensity red light (0.2 ± 0.1 lx) to avoid the effect of luminosity on physiological parameters. In fact, Oliveira et al. (2007) recommend <0.3 lx for nighttime collection.

The water quality parameters (temperature 29.7 ± 0.3 °C, dissolved oxygen 5.8 ± 0.1 mg/L and pH 7.5 ± 0.2) were monitored daily using a multiparameter probe (Askso, AK88, São Leopoldo, Brazil). The values of these parameter are considered appropriate for this species (Brasil, 2005).

2.3. Melatonin analysis

The melatonin analyses were carried out at the University of Murcia (Murcia, Spain). Eye samples were thawed, individually weighed and homogenized, discarding the cornea and crystalline lens, and maintained at 4 °C in 1 ml NaCl 0.9%. Plasma and homogenized eyecup samples were extracted and purified using C18 phase extraction columns (IBL, Hamburg, Germany), after which an enzyme-linked immunosorbent assay (ELISA) (Melatonin ELISA kit, IBL, Hamburg, Germany) was performed, as previously described by Bayarri et al. (2002). Total eye protein was quantified using the Lowry method (Lowry et al., 1951; 67,65 ± 2,41 mg*ml^{-1}), with melatonin values expressed in pg*mg of protein^{-1}. Plasma melatonin concentration was expressed in pg*ml^{-1}.

2.4. Metabolites and plasma cortisol

The levels of glucose (mg*dl^{-1}), triglycerides (mg*dl^{-1}), total cholesterol (mg*dl^{-1}) and total proteins (g*dl^{-1}) were measured using commercial enzymatic-colorimetric kits (In Vitro Diagnostica Ltda; Itabira, Brazil) that were specific for each constituent. Plasma cortisol concentration (ng*ml^{-1}) was analyzed using a commercial ELISA kit (Cortisol ELISA kit, IBL, Marburg, Germany), as described by Montoya et al. (2010).

2.5. Oxidative stress

Liver and gill samples were homogenized in 0.1 M sodium phosphate buffer (pH 7.0). Samples were weighed and homogenized 1:20 in 0.1 M sodium phosphate buffer (*w*/w). After homogenization, the material was used to quantify the total protein for glutathione concentration (GSH) and for lipid peroxidation analyses. Total protein was quantified using Bradford's method (Bradford, 1976), with mean values of 3,64 ± 1,22 mg*ml^{-1} for liver and 0,45 ± 0,19 mg*ml^{-1} for gills. The concentration of GSH (μmol*g of tissue^{-1}) was measured spectrophotometrically at 412 nm, according to Beutler (1984). The method used was based on the reaction between GSH and 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), which results in the formation of trinitrobenzene (TNB), a yellow product. Lipid peroxidation was estimated via malondialdehyde production (MDA; nmol*mg of protein^{-1}) using a thiobarbituric acid reactive substance (TBARS), a method described by Draper and Hadley (1990).

2.6. Data analysis

All data are presented as mean ± SEM. Data were tested for normality using the Levene test. Plasma and ocular melatonin levels, metabolic parameters, and oxidative stress indicators were compared between the moon phases (full moon and new moon) and the light and dark periods (midday and midnight) via two-way ANOVA, followed by the multiple-comparisons test. Statistical analyses were performed using SPSS Software, version 22.0. Level of significance was set at $p < 0.05$.

3. Results

Plasma melatonin was lower during the light phase (midday) for the full and new moons, and increased significantly at night during both moon phases (F = 9.320; $p = 0.009$; Fig. 1A; Table 1). However, no interaction was observed between the phases of the moon and the day/night changes for plasma melatonin (F = 0.005; $p = 0.943$; Fig. 1A). For ocular melatonin, interaction was observed between the variables tested (F = 4.933; $p = 0.045$), with lower melatonin concentrations during midday for the new moon. Ocular melatonin was higher during the night of the new moon compared to the night of the full moon (Fig. 1B; Table 1).

Plasma glucose showed interaction between the phases of the moon and the periods of light and dark (F = 23.940; $p = 0.001$; Fig. 2A; Table 1), being higher at midnight during full moon. There was also interaction between moon phases and time of the day for plasma triglyceride levels (F = 36.768; p = 0.001; Fig. 2B; Table 1). Thus, opposite profiles were observed depending on the lunar phase: during full moon higher values were obtained at midnight whereas during new moon higher concentrations were observed at midday.

There was no interaction between the phases of the moon and the light/dark periods for plasma concentrations of total cholesterol (F = 0.674; $p = 0.422$; Fig. 3A; Table 1), total proteins (F = 0.001; $p = 23.940$; Fig. 3B; Table 1) and cortisol (F = 0.198; $p = 0.661$; Fig. 4; Table 1). When light and dark periods were compared, cholesterol values were high at midnight, both during the full moon and during the new moon (F = 29.392; p = 0.001; Fig. 3A). Total proteins (F = 4.872; $p = 0.040$; Fig. 3B) and cortisol (F = 4.310; $p = 0.050$; Fig. 4) showed significant difference only between the two phases of the moon, with higher values observed during the full moon.

Liver GSH values were higher during the full moon when compared to during the new moon. The comparison between the light and dark

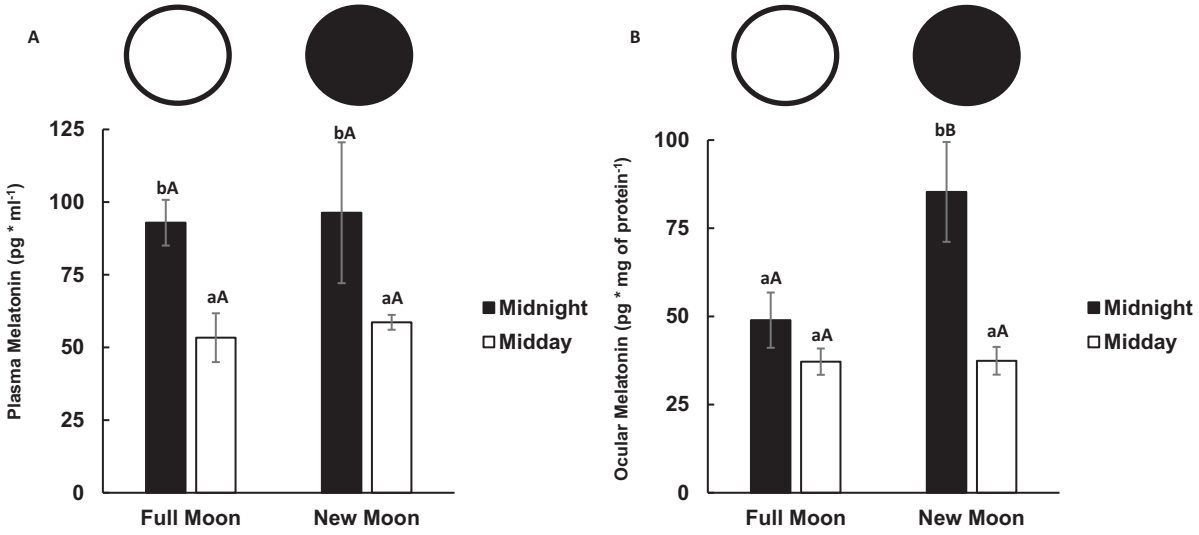

Fig. 1. Plasma (pg*ml^{-1}; A) and ocular (pg*mg of protein^{-1}; B) melatonin concentrations in matrinxã during full and new moons. White columns correspond to midday samplings, black columns to midnight samplings. All values represent mean ± S.E.M., $n = 6$ fish per treatment. Means with different lowercase letters (a, b) are significantly different between periods, and uppercase letters (A, B) are different between moons (two-way ANOVA, $p < 0.05$).

Table 1
Statistical analysis of melatonin and metabolic parameters considering the effect of lunar phase (full/new moon), periods (midday/midnight) and the interaction of the variables.

Parameter	Lunar phase	Periods	Interaction
Plasma melatonin (pg*ml^{-1})	NS	*	NS
Ocular melatonin (pg*mg of protein^{-1})	**	*	**
Plasma glucose (mg*dl^{-1})	**	**	*
Triglycerides (mg*dl^{-1})	*	NS	*
Total cholesterol (mg*dl^{-1})	NS	**	NS
Total proteins (g*dl^{-1})	*	NS	NS
Plasma cortisol (ng*ml^{-1})	*	NS	NS
GSH - liver (μmol*g of tissue^{-1})	*	NS	*
GSH - gill (μmol*g of tissue^{-1})	NS	NS	NS
MDA - liver (μmol*g of protein^{-1})	NS	NS	NS
MDA - gill (μmol*g of protein^{-1})	**	*	**

NS = non-significant; **$p < 0.01$; *$p < 0.05$.

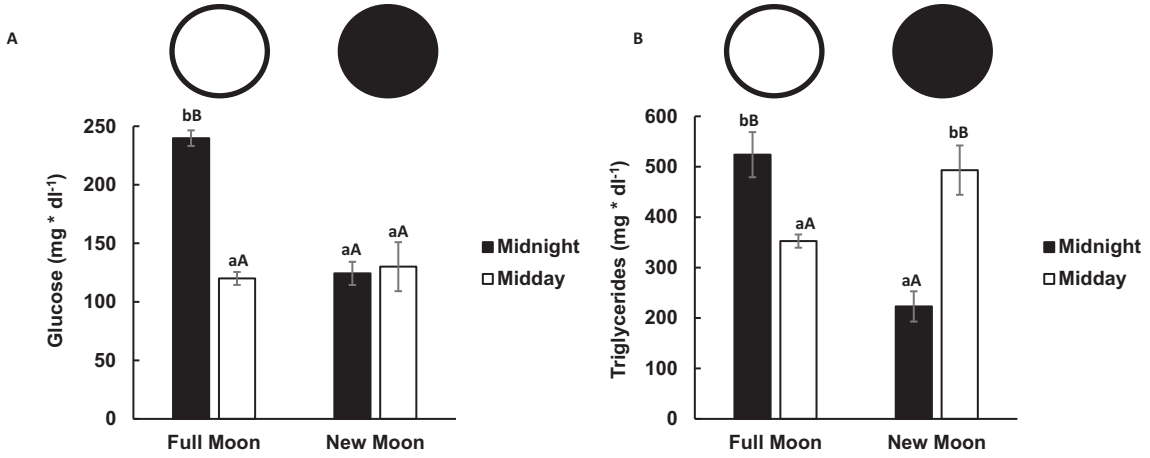

Fig. 2. Glucose (mg*dl^{-1}; A) and triglycerides (mg*dl^{-1}; B) concentrations in matrinxã during full and new moons. White columns correspond to midday samplings, black columns to midnight samplings. All values represent mean ± S.E.M., n = 6 fish per treatment. Means with different lowercase letters (a, b) are significantly different between periods, and uppercase letters (A, B) are different between moons (two-way ANOVA, $p < 0.05$).

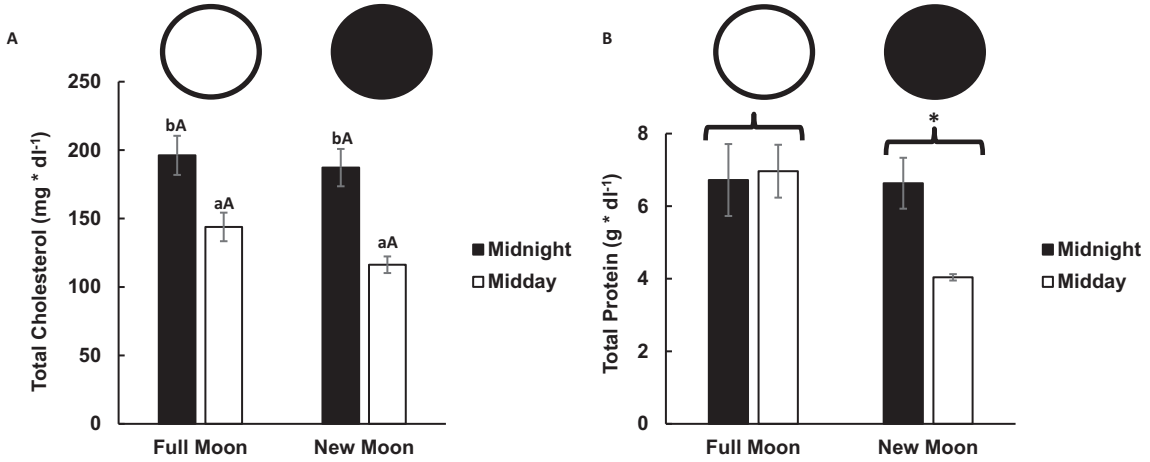

Fig. 3. Total cholesterol (mg*dl^{-1}; A) and total protein (g*dl^{-1}; B) concentrations in matrinxã during full and new moons. White columns correspond to midday samplings, black columns to midnight samplings. All values represent mean ± S.E.M., n = 6 fish per treatment. Means with different lowercase letters (a, b) are significantly different between periods. Asterisk correspond to significant difference between moons (two-way ANOVA, $p < 0.05$).

periods showed significant difference during the full moon, with high GSH values during the dark period (F = 7.595; $p = 0.013$; Fig. 5A; Table 1). There was no significant difference in GSH values between light and dark periods for the new moon ($p > 0.05$). GSH in the gills showed no significant differences between time of the day (midnight/midday), nor between the two phases of the lunar cycle (F = 3.836; $p =$

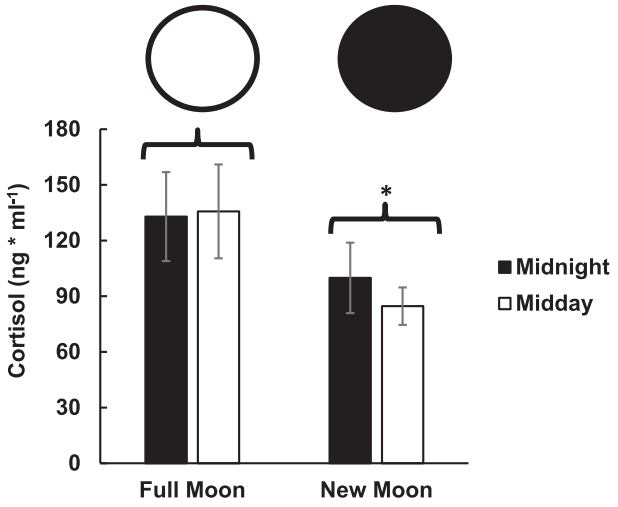

Fig. 4. Plasma cortisol concentration (ng*ml^{-1}) in matrinxã during full and new moons. White columns correspond to midday samplings, black columns to midnight samplings. All values represent mean ± S.E.M., n = 6 fish per treatment. Asterisk correspond to significant difference between moons (two-way ANOVA, $p < 0.05$).

0.065; Fig. 5B; Table 1).

Interaction between the phases of the moon and the periods of light and dark was observed for the MDA in the gills ($F = 6.291$; $p = 0.021$; Fig. 6B; Table 1). Thus, this parameter was higher during the full moon in relation to the new moon when considering the light period. However, no significant differences were observed for the MDA in the liver between the two phases of the moon, nor between the light and dark periods of both lunar phases ($F = 3.043$; $p = 0.097$; Fig. 6A; Table 1).

4. Discussion

Effects of moon phases on behavioral or other aspects are not known for the studied species (*Brycon amazonicus*). In fact, little information about the biological rhythm in matrinxã has been described (e.g., Lopes et al., 2022). We have thought that these aspects need to be investigated and we also consider that moonlight can interfere with hormone synthesis, metabolism and reproduction, despite the scarce information for freshwater fish.

We hypothesize that the full moon phase (i.e., higher light intensity) stimulates general fish activity and hence an increase in energy expenditure indicators in *B. amazonicus*, and that the new moon (i.e., lower light intensity) can modulate these indicators with low concentrations, associated with greater production of melatonin in the dark period. In fact, we found clear effects of the day/night and moon cycles on melatonin concentrations, metabolic parameters and oxidative stress indicators for the matrinxã. Plasma and ocular melatonin showed a characteristic profile, with low values at midday and high values at midnight. In fact, the reduction in light intensity at night is perceived by fish and may reflect in the greater production of melatonin by the pineal gland and also by the eyes, as reported by Kashiwagi et al. (2013).

On the other hand, moonlight did not modulate the plasma melatonin concentration for *B. amazonicus*. This response differs from the pattern exhibited for some species of fish exposed in both phases of the moon, considering that the synthesis of melatonin is extremely sensitive and inhibited by exposure to light and shows greater synthesis in dark periods (Rahman et al., 2004a; Rahman et al., 2004b; Takemura et al., 2004) and, therefore, it is affected by the light of the moon (Andreatta and Tessmar-Raible, 2020). Our results indicate that *B. amazonicus* may have a plasma melatonin rhythm that is not very sensitive to moonlight or that the intensity threshold was not able to inhibit melatonin secretion at night during the full moon phase. In addition, the matrinxã may also be a bimodal species, with variable levels of plasma melatonin throughout the night, as described by Oliveira et al. (2010).

Andreatta and Tessmar-Raible (2020) suggested that the intensity and length of moonlight exposure and tissue-specific melatonin levels may contribute to increasing the specificity of melatonin signaling in the characterization of different lunar phases. In fact, in *Siganus canaliculatus*, ocular melatonin under natural conditions was higher during the new and waning moons compared to the full and crescent moons (Rahman et al., 2004b). A similar response was found in our study under natural conditions, with higher concentrations of ocular melatonin during the new moon. Thus, the light of the moon is perceived by the fish's eye and has an impact on the fluctuation of melatonin in the retina, and it can be metabolized in situ, which prevents the release of this substance into the blood (Grace et al., 1991; Kashiwagi et al., 2013). According to Fukunaga et al. (2020), the ability to perceive differences in moonlight is essential for lunar periodicity, since the luminosity of moonlight is markedly lower than that of sunlight. Thus, the retina and the pineal organ are the main candidates for the perception and transduction of moonlight. This response, in turn, triggers a reduction in the synthesis and release of melatonin at lower light intensity, which directly affects physiological and behavioral responses in fish. In addition, higher concentrations of melatonin reduce locomotor activity and aggressiveness in fish (Falcón et al., 2011; Amaral et al., 2020), which may reflect higher metabolic demand and the need for energy substrate

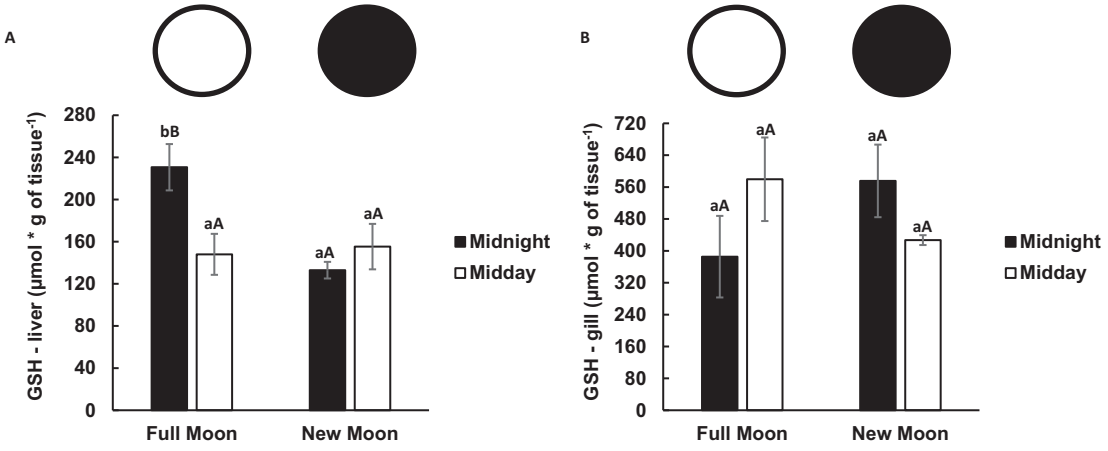

Fig. 5. Glutathione (GSH) concentration in the liver (µmol*g of tissue^{-1}; A) and gill (µmol*g of tissue^{-1}; B) in matrinxã during full and new moons. White columns correspond to midday samplings, black columns to midnight samplings. All values represent mean ± S.E.M., n = 6 fish per treatment. Means with different lowercase letters (a, b) are significantly different between periods, and uppercase letters (A, B) are different between moons (two-way ANOVA, $p < 0.05$).

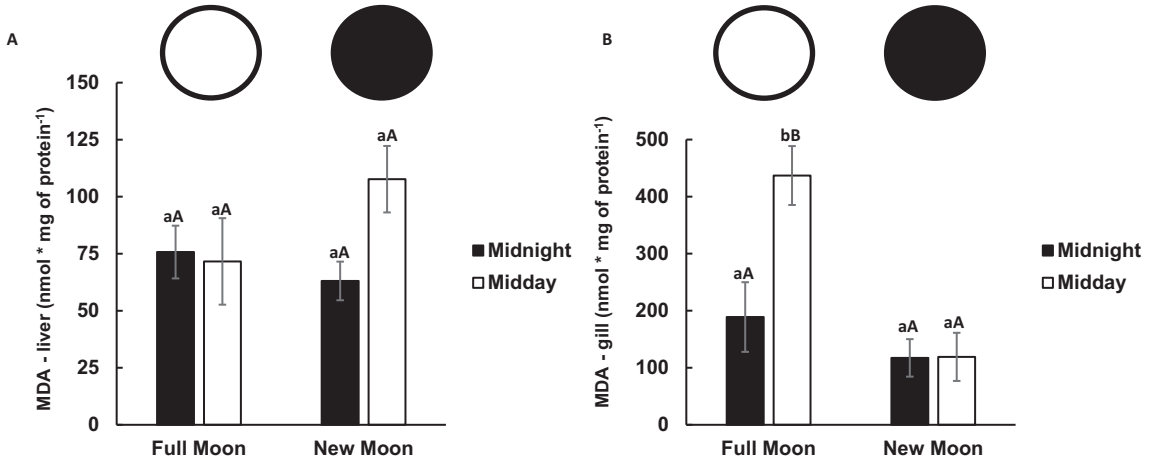

Fig. 6. Malondialdehyde (MDA) concentration in the liver (nmol*g of protein^{-1}; A) and gill (μmol*g of protein^{-1}; B) in matrinxã during full and new moons. White columns correspond to midday samplings, black columns to midnight samplings. All values represent mean ± S.E.M., n = 6 fish per treatment. Means with different lowercase letters (a, b) are significantly different between periods, and uppercase letters (A, B) are different between moons (two-way ANOVA, $p < 0.05$).

availability.

We expected an increase in plasma metabolic parameters in the matrinxã during full moon, since the lower concentration of melatonin at lower light intensity may increase the total number of aggressive interactions for the species (Amaral et al., 2020) and, consequently, it may trigger higher energy expenditure (Alvarenga and Volpato, 1995). The higher concentration of plasma glucose at midnight during full moon may suggest a higher energy demand in these animals with a high metabolic rate. Indeed, plasma cortisol concentrations exhibited their highest levels during the full-moon phase. In this sense, moonlight seems to play a key role in raising cortisol levels. This relationship has been evidenced by Lopes et al. (2022), who reported *B. amazonicus* exhibiting a higher concentration of this hormone in the light period, and this may be associated with the glycemic acrophase observed in the early morning (04:16 ± 2:47 h). Increases in plasma glucocorticoids have a wide range of other metabolic effects, including increased lipolysis and the synthesis and degradation of proteins (Mommsen et al., 1999; Thau et al., 2022).

The highest plasma cholesterol levels were observed at midnight in both phases of the moon, with a decrease at midday in both phases as well. Thus, the highest concentration of plasma cholesterol at midnight corroborates with Lopes et al. (2022) who identified a rhythmicity of cholesterol with acrophase at 23:22 ± 3:45 h, which indicates higher cortisol synthesis that was evidenced by the higher concentration of this hormone in the light period for *B. amazonicus*. In our study, the highest concentration of cortisol also occurred at the full moon, which was probably modulated by the high incidence of moonlight.

Elevated plasma triglyceride levels were observed for both phases of the moon, which reveals the body's attempt to seek greater input to maximize the use of the energy substrate due to greater metabolic activity (Tocher, 2003). However, the response to periods of light and dark varied depending on the phase of the moon. For example, the full moon phase caused high triglyceride levels at midnight, which may indicate the fish need to seek a greater energy supply at night during full moon, due to the higher environmental luminosity, lower concentration of melatonin and, possibly, greater activity of the matrinxã (Amaral et al., 2020). Notwithstanding, triglyceride levels exhibited the opposite response when compared between periods of the new moon, with a decrease at midnight, and increased levels at midday. This response may be associated with a greater mobilization of energy reserves due to the greater activity of the species during the day (Soares et al., 2008; Rodrigues et al., 2017). The total proteins values were higher during the full moon phase when compared to new moon, regardless of the time of the day. According to Mommsen et al. (1999), changes in total protein concentrations were related to increased cortisol, which results in increased gluconeogenesis and protein catabolism activity. Thus, the results for this physiological parameter indicated that the elevated levels in the full moon may have been due to the increase in cortisol that was also observed in the full moon phase for both periods.

Although indicators of oxidative stress may also show variation in accordance with the phases of the moon, little information about this relationship has been described for vertebrates. Basically, studies were only found relating the reproductive period, the phase of the moon and the production of free radicals in invertebrate species (e.g., coral and shrimp; Murphy et al., 2019; Bautista-Covarrubias et al., 2020). As such, our results provide new data on the relationship between moonlight and the production of components of the antioxidant system (melatonin and GSH) and lipid peroxidation (MDA) in fish. Greater activation of the antioxidant system (GSH in the liver) was observed during the full moon and during the dark period. This result may be due to the lower concentration of melatonin, especially at midday, which can increase the activity (i.e., aggressiveness) and energy demand of fish (Amaral et al., 2020) and which, in turn, can increase respiratory rate resulting in exacerbated production of reactive oxygen species (ROS), including free radicals (Wendelaar-Bonga, 1997). This higher production of ROS can stimulate the production of components of the antioxidant system, such as GSH, which minimizes the harmful effect of oxidative stress (Poljsak et al., 2013; Chowdhury and Saikia, 2020). In fact, our results suggest that the higher GSH concentration in the liver at midnight and during the full moon may have reduced lipid peroxidation, which was evidenced by the absence of significant differences for MDA in the liver between the two phases of the moon and the light and dark periods.

The higher concentration of MDA in the gills at the midday during the full moon indicates that there was a greater degradation of cell membranes as a result of the production of free radicals due to the high intensity of light under these conditions (expressed in 100%; Ikegami et al., 2014). In addition, GSH in the gills did not show significant differences between the sampling times, nor between the moon phases, which may indicate the absence of activation of this antioxidant system in the gills. Fish exposed to the new moon phase at midday exhibited lower rates of lipid peroxidation in the gills. In this sense, it is likely that the low intensity or absence of light has contributed to these low concentrations, possibly through the action of melatonin. In fact, melatonin has antioxidant properties, and acts directly on the elimination of free radicals, assisting antioxidant defense enzymes and stimulating the synthesis of mRNA from the glutamylcysteine synthetase enzyme, which is responsible for the biosynthesis of GSH (Urata et al., 1999; Ramis et al., 2015; Reiter et al., 2018). Melatonin can also decrease the

formation of MDA, probably due to its ability to interact with bilayer lipids, thus preventing changes in membrane fluidity and contributing to the reduction of lipid peroxidation (García et al., 1997; Ramis et al., 2015), as has been found for *Oncorhynchus mykiss* (Gülçin et al., 2009).

In summary, it can be concluded that, in *B. amazonicus,* hormonal, metabolic parameters and oxidative stress indicators are modulated by both the day/night and by moon phases. In fish, the pineal organ and its hormone melatonin are likely to be the mediators between environmental cycles and biological rhythms (Amano et al., 2000; Bromage et al., 2001; Bayarri et al., 2004). Thus, we suggest that melatonin may at least partially mediate the described rhythmic changes in physiological processes for matrinxã, as also reported by Zimecki (2006). Although the exact mechanism of the influence of the moon on living organisms needs further study, knowledge of this type of biorhythm may be relevant in order to investigate how fish cope with environment cycles.

Our research also provides the first insight into the influence of the lunar cycle on hormonal and metabolic parameters in *Brycon amazonicus*. Although the lunar periodicity acts as an external regulator, it is necessary to evaluate how this environmental factor synchronizes physiological and behavioral parameters that directly affect the reproduction and capture in the natural environment of the matrinxã, a valuable species for the diversification of aquaculture in South American countries.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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