NAIARA MELO

ESTRATÉGIAS ALIMENTARES EM PEIXES E SUAS RESPOSTAS FISIOLÓGICAS ASSOCIADAS AO DESEMPENHO

LAVRAS-MG
2023

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Zootecnia, área de concentração em Produção e Nutrição de Não Ruminantes, para a obtenção do título de Doutor.

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NAIARA MELO

ESTRATÉGIAS ALIMENTARES EM PEIXES E SUAS RESPOSTAS FISIOLÓGICAS ASSOCIADAS AO DESEMPENHO

FEEDING STRATEGIES AND THEIR PHYSIOLOGICAL RESPONSES ASSOCIATED WITH PERFORMANCE IN FISH

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Zootecnia, área de concentração em Produção e Nutrição de Não Ruminantes, para a obtenção do título de Doutor.

APROVADA em 30 de Janeiro de 2023.

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2023

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RESUMO

A alimentação constitui a maior parte dos custos na piscicultura intensiva, portanto adotar estratégias alimentares que produzam peixes saudáveis com máxima taxa de crescimento e alta eficiência alimentar é importante para o sucesso produtivo. Desta forma, a presente tese trata de questões com alta demanda de conhecimento em três manuscritos. No primeiro estudo investigamos os efeitos da frequência de alimentação nos parâmetros de crescimento, eficiência alimentar, sobrevivência, deformidades, composição corporal próxima e crescimento muscular em *Chirostoma estor* em um experimento com duração de 45 dias. Os peixes (peso médio:180,57 ± 3,02 mg) foram alimentados nas frequências de 4,8 e 12 alimentações ao dia^{-1}. Ao final do período experimental, os peixes alimentados 12 vezes ao dia^{-1} exibiram o maior desempenho de crescimento ($P < 0,05$). A taxa de conversão alimentar foi afetada ($P < 0,05$) pela frequência de alimentação. A sobrevivência dos peixes foi semelhante ($P > 0,05$) entre os tratamentos, enquanto, as deformidades ($P < 0,05$). nos animais foram menos incidentes nos animais alimentados com a maior frequência. A composição corporal proximal dos peixes foi afetada pelos tratamentos, conforme a frequência de alimentação aumentou, o conteúdo de lipídios aumentou significativamente, mas o conteúdo de cinzas diminuiu. Houve diferença ($P < 0,05$) para os diâmetros das fibras musculares entre os animais tratamentos. Portanto, nas condições em que o experimento foi realizado, a frequência alimentar de doze alimentações ao dia promoveu melhor desempenho para juvenis de *C. estor* e menor porcentagem de deformidades. O segundo manuscrito teve como objetivo avaliar o efeito da fase de alimentação restrita e realimentação nas respostas compensatórias do crescimento, sobrevivência, enzimas digestivas, histomorfologia intestinal, composição proximal, metabolitos plasmáticos e atividades antioxidante em *Colossoma. macropomum*. Os peixes (peso médio: 7,12 ± 0,18 g) foram alimentados com cinco taxas de alimentação (FR = 2%, 4%, 6%, 8% e 10% do peso vivo por dia $^{-1}$) durante 30 dias (fase de alimentação restrita) e depois realimentados até a saciedade aparente por mais 30 dias (fase de realimentação). Os parâmetros de crescimento aumentaram ($P < 0,05$) com o aumento da taxa de alimentação, enquanto que a eficiência alimentar diminuiu. A redução da taxa de alimentação afetou negativamente ($P < 0,05$) as variáveis fisiológicas, todavia, após a realimentação estas variáveis foram em grande parte, restauradas ($P > 0,05$). Nossos resultados indicam que a taxa de alimentação de 6% dia^{-1} proporcionou o maior crescimento e eficiência alimentar, enquanto o crescimento completo foi alcançado com 4% dia^{-1} após a realimentação. Considerando as respostas fisiológicas pode-se concluir que a diminuição da taxa de alimentação em 2% dia^{-1} pode desencadear catabolismo de reservas endógenas, fator impeditivo para o crescimento compensatório. O terceiro manuscrito foi investigou os efeitos da fome nas respostas antioxidantes utilizando uma abordagem de diagnóstico celular (em vários tecidos) em *Colossoma macropomum*. Em conclusão, nossos resultados confirmam que as respostas antioxidantes são especificas de cada tecido e que o jejum de 7 dias provou estresse oxidativo no fígado e intestino do tambaqui.

Palavras- chave: Aquicultura. Alimentação. Crescimento. Estresse oxidativo. Peixes nativos.

ABSTRACT

Feed constitutes the majority of costs in intensive fish farming, so adopting feeding strategies that produce healthy fish with maximum growth rate and high feed efficiency is important for productive success. Therefore, this thesis addresses questions with high knowledge demand in three manuscripts. In the first study, we investigated the effects of feeding frequency on growth parameters, feed efficiency, survival, deformities, body composition, and muscle growth in *Chirostoma estor* in a 45-day experiment. Fish (weight: 180.57 ± 3.02 mg) were fed at frequencies of 4.8 and 12 feedings per day. At the end of the experimental period, fish fed 12 times a day exhibited the highest growth performance ($P < 0.05$). Feed conversion rate was affected by feeding frequency ($P < 0.05$). Fish survival was similar between treatments ($P > 0.05$), while deformities were less incident in animals fed more frequently ($P < 0.05$). The proximal body composition of the fish was affected by the treatments, as lipid content increased significantly with increased feeding frequency, but ash content decreased. Muscle fiber diameters differed among the treated animals ($P < 0.05$). Therefore, under the conditions of the experiment, a feeding frequency of twelve feedings a day promoted better performance for juveniles of *C. estor* and a lower percentage of deformities. The second manuscript aimed to evaluate the effect of restricted feeding and refeeding phases on the compensatory responses of growth, survival, digestive enzymes, intestinal histomorphology, proximal composition, plasma metabolites, and antioxidant activities in *Colossoma macropomum*. Fish (average weight: 7.12 ± 0.18 g) were fed with five feeding rates (FR = 2%, 4%, 6%, 8%, and 10% of live weight per day) for 30 days (restricted feeding phase) and then re-fed to apparent satiation for another 30 days (re-feeding phase). Growth parameters increased with increasing feeding rate ($P < 0.05$), while feed efficiency decreased. The reduction in feeding rate negatively affected physiological variables ($P < 0.05$); however, after refeeding, these variables were largely restored ($P > 0.05$). Our results indicate that a feeding rate of 6% per day provided the highest growth and feed efficiency, while full growth was achieved at 4% per day after refeeding. Considering the physiological responses, it can be concluded that a decrease in the feeding rate by 2% per day can trigger catabolism of endogenous reserves, which is an impeding factor for compensatory growth. The third manuscript investigated the effects of starvation on antioxidant responses using a cellular (multi-tissue) diagnostic approach in *Colossoma macropomum*. In conclusion, our results confirm that antioxidant responses are tissue-specific, and a 7-day fasting period induced oxidative stress in tambaqui liver and intestine.

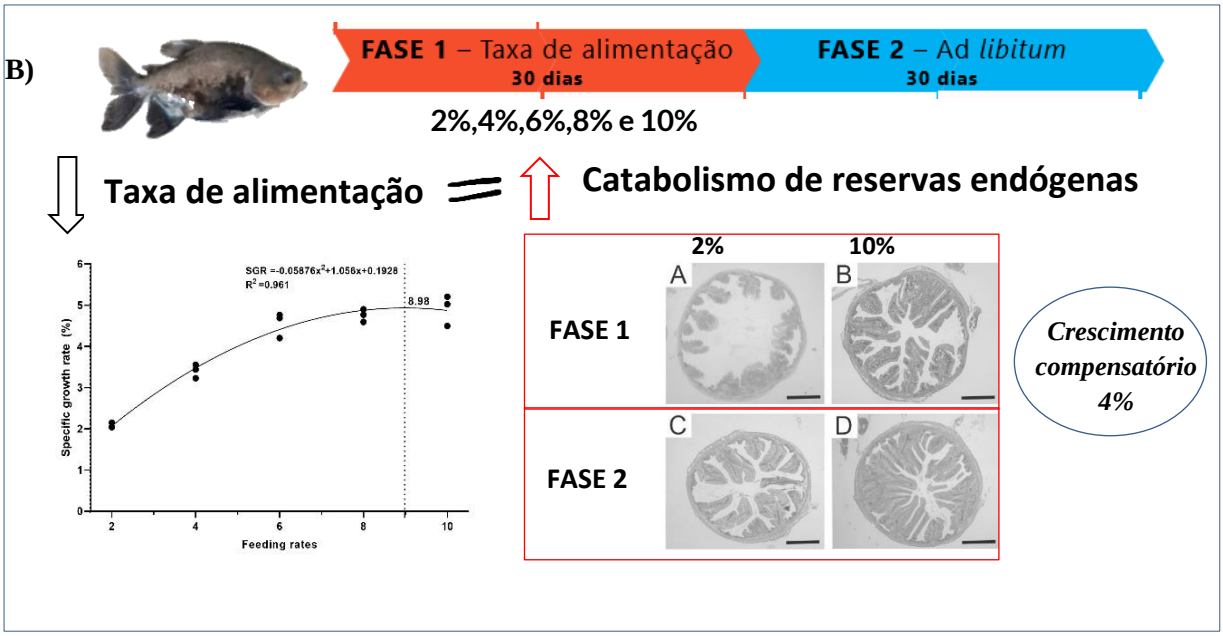
Key words: Aquaculture. Feeding. Growth. Oxidative stress. Native fish.

Resumo interpretativo

ESTRATÉGIAS ALIMENTARES EM PEIXES E SUAS RESPOSTAS FISIOLÓGICAS ASSOCIADAS AO DESEMPENHO

Elaborado por **Naiara Melo** orientada por **Luis David Solis Murgas**

A piscicultura representa o setor de produção animal que mais cresceu nos últimos anos. Esse crescimento é atribuído à geração e transferência de pacotes tecnológicos, com destaque para as estratégias alimentares. O objetivo da alimentação na piscicultura é fornecer as necessidades nutricionais para uma boa saúde, crescimento ótimo e mínimo desperdício, a fim de otimizar a produção. A presente pesquisa buscou contribuir para o desenvolvimento deste setor por meio de estudos sobre a interação da estratégia alimentar nas respostas fisiológicas de duas espécies de peixes de interesse comercial. No primeiro estudo foi observado que as larvas de peixe branco alimentados doze vezes ao dia apresentam melhor crescimento e desenvolvimento, com destaque para a redução das deformidades esqueléticas. No estudo realizado com juvenis de tambaqui, verificou-se que a baixa oferta de alimento provocou uma redução no crescimento. Além disso, a espécie apresentou crescimento compensatório quando as condições de alimentação foram retomadas. No terceiro estudo, foi observado que o jejum causou efeitos fisiológicos no fígado e intestino do tambaqui. Ambos os órgãos apresentaram estresse oxidativo devido à escassez de alimento.

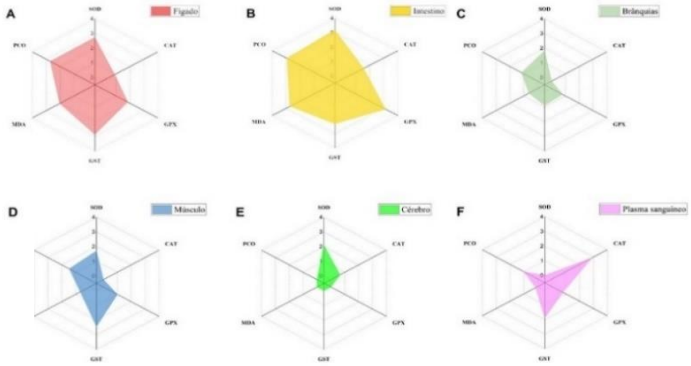


C)

Jejum

Estresse Oxidativo

- *Fígado*
- *Intestino*
- *Brânquias*
- *Musculo*
- *Cérebro*
- *Plasma Sanguíneo*



Danos Oxidativos: Fígado e Intestino

Tese de doutorado em Zootecnia na UFLA, defendida em 30/01/2023

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PARTE I

1. INTRODUÇÃO

Nos últimos 30 anos a produção de organismos aquáticos aumentou exponencialmente, tornando a aquicultura um dos setores da produção animal que mais cresceu neste período (AHMED; THOMPSON; GLASER, 2019). Com crescimento médio de 6,7% ao ano entre 1990 e 2020, a produção aquícola excedeu a pesca de captura e já responde pela metade do consumo mundial de pescados (FAO, 2022). Em volume de produção, o subsetor da aquicultura foi dominado pela piscicultura, que representa o principal produto da aquicultura em muitos países. A contribuição da produção de peixes tem se mantido entre 63-68% nas últimas duas décadas (ELANGOVAN et al., 2017). Estima-se que o crescimento da produção de organismos aquáticos tende aumentar ainda mais em decorrência do aumento da população mundial, que deverá passar dos atuais 8,0 bilhões de indivíduos para 9,0 bilhões de pessoas em 2030 (FAO, 2020). No entanto, o principal desafio para o setor é realizar a produção de forma sustentável e reduzir os custos envolvidos (DIANA et al., 2013; GRAFTON; DAUGBJERG; QURESHI, 2015).

Vários autores atribuem a expansão da piscicultura às melhorias em torno dos alimentos, incluindo a composição dos alimentos para nutrição e digestibilidade, além de relação custo-benefício e técnicas de alimentação adequadas (DIANA et al., 2013; GRAFTON; DAUGBJERG; QURESHI, 2015; TACON, 2020). Além disso, a alimentação dos organismos aquáticos é um dos fatores mais importantes na aquicultura, devido aos altos custos de alimentação (ANRAS et al., 2007).

O crescimento dos peixes depende principalmente de um suprimento adequado de nutrientes, tanto em quantidade quanto em qualidade, independente do sistema de produção em que são cultivados (CYRINO, 2008). Tanto a subalimentação como a superalimentação podem afetar o crescimento e o desenvolvimento dos animais (KÜÇÜK et al., 2014; OLIVEIRA-JÚNIOR et al., 2021). Portanto, o manejo alimentar adequado é fundamental do ponto de vista econômico e zootécnico (RODRIGUES et al., 2019), pois traz inúmeros benefícios, entre eles: melhora o crescimento, sobrevivência, contribui para minimizar o desperdício de alimentos, além de reduzir a heterogeneidade do lote e o comportamento agressivo (CARVALHO et al., 2018; DADA; FAGBENRO; FASAKIN, 2002; MANLEY et al., 2015). Adicionalmente, as práticas de alimentação que exploram a trajetória de crescimento dos peixes são essenciais para

melhorar a produção, os custos de alimentação, otimizar a mão de obra e reduzir os problemas de qualidade de água (ALI; NICIEZA; WOOTTON, 2003; ASHOURI et al., 2020).

Apesar dos avanços nos protocolos de alimentação, diversas pesquisas ainda vêm sendo realizadas todos os anos. Esse fato ocorre, pois os protocolos de alimentação são específicos para cada espécie em seu estágio de desenvolvimento. Portanto, tornam-se necessárias pesquisas que visam melhorar as estratégias alimentares de acordo com as lacunas encontradas de cada espécie. Considerando a importância do tema, a presente tese aborda dois assuntos com alta demanda de conhecimento para produtores e pesquisadores. O primeiro e segundo manuscrito avaliam, respectivamente, os efeitos da frequência alimentar em uma espécie agastrica (*Chirostoma estor*), ameaçada de extinção, e a influência de taxas de alimentação e realimentação sobre o crescimento, sobrevivência e respostas fisiológicas da espécie nativa mais produzida no Brasil, o tambaqui (*Colossoma macropomum*). O terceiro manuscrito faz uma abordagem dos biormarcadores de estresse oxidativo em *C. macropomum.*

2. REFERENCIAL TEÓRICO

2.1 Morfologia e alimentação em peixes

O trato gastrointestinal dos peixes pode ser subdividido em quatro regiões topográficas: porção cefálica, porção anterior, intestino médio e posterior. Subdivisões morfofuncionais adicionais podem ser sobrepostas, em que porção cefálica é comporta pela boca e faringe, cuja função é adquirir alimento e processá-los (GERMAN; HORN, 2006; GONÇALVES et al., 2012). A porção anterior é composta pelo esôfago e estomago, onde começa a digestão química dos alimentos, no entanto, em alguns peixes, a quebra mecânica dos alimentos também pode ocorrer parcialmente ou totalmente no estômago. O intestino médio é o responsável pela maior proporção do comprimento do intestino e é onde a digestão química é continuada e a absorção ocorre principalmente. O intestino posterior é a seção final do intestino, que inclui o reto; embora em alguns casos não haja distinção morfológica entre intestino médio e posterior (AL-HUSSAINI, 1949; DABROWSKI; PORTELLA, 2005; WOOTTON, 1990).

Considerando que os peixes teleósteos representam um grupo altamente diversificado, composto por mais de 20.000 espécies que vivem em todos os ambientes aquáticos, não é de surpreender que a descrição acima da anatomia básica do sistema digestivo não pode ser

aplicada universalmente (WILSON; CASTRO, 2010). Os peixes consomem uma ampla gama de alimentos, sendo que a ingestão se dá de diversas formas (OLSSON, 2011). Enquanto algumas espécies se alimentam de organismos microscópicos vegetais, outras apresentam-se como predadoras vorazes, o que resulta em distintos hábitos alimentares, cada qual conferindo variações na morfologia e fisiologia do sistema digestório (WOOTTON, 1990). As características morfofisiológicas do sistema digestório estão diretamente associadas ao hábito alimentar de cada espécie e é resultado de longos períodos de evolução. Estas características vão desde a estrutura bucal, que é responsável pela captura do alimento, passando pela forma e tamanho do estômago e intestino, até a produção de enzimas digestivas que implicam na digestão, absorção e aproveitamento dos nutrientes (GONÇALVES et al., 2013). Devido a diferenças na morfologia e fisiologia do sistema digestório entre as espécies de peixes, o tipo de alimento e o tamanho da refeição estão geralmente relacionados aos hábitos alimentares distintos. Isto se reflete, por exemplo, no tamanho do estômago, definindo o intervalo de tempo entre as alimentações (KAPOOR; SMIT; VERIGHINA, 1976).

Peixes carnívoros, tendem a apresentar um estômago grande, com alta capacidade de distensão, podendo aumentar seu tamanho em até quatro vezes, além de realizar o processo de digestão. mais lentamente. Enquanto os peixes onívoros e herbívoros tendem a realizar várias alimentações em curto período de tempo, portanto, possuem estômagos menores, porém com uma grande quantidade de músculos, que proporcionam fortes contrações para triturar os alimentos ingeridos, normalmente com maior conteúdo em matéria fibrosa e menor digestibilidade (GONÇALVES et al., 2012).

O comprimento do intestino também pode ser usado como indicador morfológico do nível trófico em ecologia nutricional (GERMAN; HORN, 2006; MICHL, 2017). No entanto, o comprimento intestinal é influenciado por vários outros fatores, além da dieta, que incluem o tamanho do peixe, forma do corpo, histórico recente de alimentação e ontogenia (WOOTTON, 1990). De forma geral, espécies herbívoras tendem a possuir intestinos mais longos, tendo em vista que os itens que compõem a dieta destes animais são pouco digestíveis e de baixo valor nutricional. Por outro lado, os carnívoros geralmente possuem intestinos curtos, já que se alimentam de alimentos mais digestíveis e com alto valor nutricional. Embora alguns autores sugiram que os peixes onívoros tendem a ter intestino de tamanho intermediário, variando conforme a complexidade dos alimentos consumidos pela espécie (RUST, 2003), Kramer e Bryant (1995) alertaram que o comprimento do intestino como reflexo da dieta deve ser aplicado apenas à identificação de categorias amplas. Portanto, dietas ricas em itens de difícil

digestão e baixa digestibilidade, como a de espécies herbívoras e algumas onívoras, seriam compensadas por uma alimentação mais frequente e o comprimento intestinal, para atendimento das exigências nutricionais e aumento do tempo de retenção da digestão, respectivamente (GONÇALVES et al., 2012; SADO et al., 2020).

2.2 Manejo alimentar na piscicultura

O objetivo da alimentação dos peixes é fornecer as necessidades nutricionais para uma boa saúde, ótimo crescimento, ótimo rendimento e mínimo desperdício dentro de custos razoáveis, de modo a otimizar a produção (SCHMITTOU; JIAN; CRAMBER, 1998). Ao contrário dos animais terrestres, onde o excesso de ração pode ser recuperado quando o animal está satisfeito, esta prática é algo impraticável na piscicultura, e o alimento não consumido não pode ser aproveitado (JOBLING et al., 2012).

O crescimento dos peixes depende principalmente de um suprimento adequado de nutrientes, tanto em quantidade quanto em qualidade, independente do sistema de produção em que são cultivados (CYRINO, 2008). A superalimentação pode aumentar o custo de produção do peixe e causar a deterioração da qualidade da água, o que pode eventualmente reduzir o crescimento do peixe (AMADOU et al., 2019), por outro lado, uma alimentação deficiente afeta o crescimento, aumenta a competição por alimento e contribui para a heterogeneidade do lote (MANLEY et al., 2015). Portanto, o manejo alimentar adequado é adequado é fundamental do ponto de vista econômico e zootécnico (RODRIGUES et al., 2019), pois traz inúmeros benefícios entre eles; melhora o crescimento, sobrevivência, contribuem para minimizar o desperdício de alimentos (OKOMODA et al., 2019; RODRIGUES et al., 2019), além de reduzir a heterogeneidade do lote e o comportamento agressivo (MANLEY et al., 2015; RIBEIRO; FORSYTHE; QIN, 2015).

Os peixes não se alimentam continuamente durantes as 24 horas do dia, mas concentram sua atividade alimentar em uma fase específica, apresentando padrões de atividade diurnos ou noturnos (STEINBERG, 2018; ZHDANOVA; REEBS, 2005). O conhecimento da fase do dia em que os animais preferem se alimentar tem uma implicação importante no cultivo, pois permite que os peixes sejam alimentados no horário ideal do dia para otimizar a utilização dos nutrientes da dieta e reduzir o estresse produzido pela alimentação fora da fase de atividade (EBRAHIMI et al., 2017; GÓMEZ-BORONAT et al., 2018). Desta forma, além do controle nutricional, é importante que os peixes sejam alimentados em horários fixos e que este

protocolo seja mantido, uma vez que a maioria dos peixes possuem um mecanismo chamado de atividade antecipatória de alimentação, que promove um processo interno, englobando ritmos biológicos e ritmos alimentares (HERRERO et al., 2005; ZEYTIN; SCHULZ; UEBERSCHÄR, 2016).

O uso de estratégias nutricionais para maximizar a produção, minimizando os custos de produção e o lançamento de efluentes e consequentemente o impacto ambiental tem crescido consideravelmente ao longo das últimas décadas (DIANA et al., 2013). Embora o crescimento dos peixes seja afetado por diversos fatores, um regime alimentar otimizado é considerado crucial para o máximo crescimento dos peixes (PILLAY; KUTTY, 2005). Melhorias muito significativas na qualidade das rações e nas práticas de alimentação foram alcançadas através da determinação da taxa de alimentação e frequência alimentar para várias espécies de peixes cultivadas(GUO et al., 2020; MELO et al., 2023; OLIVEIRA et al., 2019).

2.2.1 Taxa de alimentação

O crescimento ideal e a eficiência alimentar só podem ser alcançados se uma quantidade suficiente de ração for fornecida, completamente ingerida e digerida (BAKKE; GLOVER; KROGDAHL, 2010). A taxa de alimentação, também conhecida como taxa de arraçoamento ou nível de alimentação, representa a quantidade de alimento a ser fornecida diariamente em relação ao seu peso vivo, sendo expressa na forma de porcentagem (%) da biomassa. (JOBLING et al., 2012). Determinar a quantidade de ração a ser fornecida para os peixes é fundamental não só para saúde e bem estar dos animais, mas também para minimizar o desperdício de alimento e o acumulo de resíduos que afetam negativamente a qualidade da água (BISWAS et al., 2005; MIZANUR et al., 2014). A taxa de alimentação a ser fornecida a uma dada espécie varia durante o ciclo de cultivo em função da qualidade da água, da, da densidade de estocagem, das interações sociais, fotoperíodo e, principalmente da qualidade da dieta, temperatura da agua e fase de desenvolvimento dos peixes (BOLLIET; AZZAYDI; BOUJARD, 2007; LI et al., 2021; LÓPEZ-OLMEDA; NOBLE; SÁNCHEZ-VÁZQUEZ, 2012) e deve ser ajustada de acordo com as condições individuais de cada sistema de cultivo (ANRAS et al., 2001).

Como os peixes se alimentam para satisfazer suas necessidades energéticas, dietas com níveis excessivos de energia podem resultar em diminuição do consumo de ração e redução do ganho de peso (LIU et al., 2013; SUN; HASSAN; LI, 2016). Da mesma forma, uma dieta com conteúdo energético inadequado pode resultar em ganho de peso reduzido porque o peixe não

pode consumir ração suficiente para satisfazer suas necessidades de energia para crescimento (NOBRE et al., 2019; PIROZZI; BOOTH; ALLAN, 2010).

A temperatura da água é uma das variáveis ambientais mais importantes que afeta diretamente o metabolismo, o crescimento e outros processos fisiológicos dos organismos aquáticos (COWAN; AZPELETA; LÓPEZ-OLMEDA, 2017; WU; LUO; WANG, 2015). Em geral, a temperatura ideal aumenta a taxa metabólica e a demanda por alimento, enquanto que em baixas ou altas temperaturas as taxas metabólicas são suprimidas resultando em baixas taxas de alimentação e crescimento (PINTO et al., 2020). Dias-Koberstein et al (2004) verificaram o comportamento alimentar de juvenis de *Piaractus mesopotamicus* em duas temperaturas de cultivo e concluíram que o consumo diário de ração foi influenciado pela temperatura, proporcionando taxa de alimentação de 2,29% e 2,97% do peso vivo dos peixes ao dia para as temperaturas 23 e 27 ºC, respectivamente.

Nas fases iniciais de desenvolvimento, os peixes exigem maior quantidade de energia e nutrientes para o metabolismo por unidade de peso vivo (GISBERT; MORAIS; MOYANO, 2013; RØNNESTAD et al., 2013). Nestas fases, os peixes apresentam uma maior taxa de crescimento, consumindo uma quantidade percentual maior de alimento em relação ao peso vivo de peixes com peso maior. Esse percentual de ração consumida em relação ao peso corporal diminui à medida que os peixes crescem e suas taxas metabólicas diminuem (LEEUWEN; ROSENFELD; RICHARDS, 2012; OKOMODA et al., 2019). As taxas de alimentação para larvas e juvenis variam de 4 a 20% do peso vivo, diminuindo em adultos para valores inferior a 4% do peso vivo (NRC, 2011; LALL; DUMAS, 2022).

Vários métodos são usados para estimar a taxa de alimentação diária, isso inclui cálculos baseados no uso de gráficos de alimentação, equações de alimentação e previsões de crescimento que são estimados a partir do ganho em peso, sobrevivência e conversão alimentar (SUN; HASSAN; LI, 2016). No entanto, todas essas estimativas não levam em consideração o sistema de cultivo e nem as flutuações de curto ou longo prazo no apetite em resposta a fatores fisiológicos e ambientais(CARTER, 2015; HOULIHAN; BOUJARD; JOBLING, 2008). Claramente, a quantidade de alimentação deve ser determinada através do monitoramento minucioso do consumo de ração, crescimento e eficiência alimentar ao longo de vários ciclos de crescimento (JOBLING, 1995; NOBRE et al., 2019). Além disso, fatores como composição centesimal, metabolitos plasmáticos, enzimas digestivas e parâmetros de estresse devem ser levados em consideração ao estimar o nível ideal de alimentação, visto que todas estas variáveis

exercem influência no crescimento dos peixes (FURNÉ et al., 2009; GILANNEJAD et al., 2021; LI et al., 2016; SAKYI et al., 2021).

2.2.2 Frequência alimentar

A frequência alimentar representa o número de vezes que a ração é fornecida ao dia para os peixes (LEE; PHAM, 2010) e caracteriza-se como um manejo importante para atender as necessidades fisiológicas dos peixes e garantir o melhor crescimento no menor tempo de cultivo influenciando diretamente o retorno econômico dos sistemas de produção (EL-ARABY; AMER; KHALIL, 2020; GUO et al., 2018; OH; MARAN; PARK, 2018). Adequar a frequência alimentar pode estimular a procura pelo alimento em momentos pré-determinados e contribuir com o desempenho dos peixes (GILANNEJAD et al., 2020; LÓPEZ-OLMEDA; NOBLE; SÁNCHEZ-VÁZQUEZ, 2012).

Frequência alimentar baixa pode não garantir alimento suficiente para o crescimento e sobrevivência, além de induzir o comportamento agressivo pela competição do alimento, enquanto que um aumento de frequência geralmente melhora a eficiência da produção, melhorando o crescimento e minimizando o desperdício de ração (KESTEMONT; BARAS, 2001; MANLEY et al., 2015). No entanto, a alimentação em excesso leva não só à redução da eficiência alimentar e ao aumento dos custos de produção, mas também à acumulação de resíduos que afetam negativamente a qualidade da água (BISWAS et al., 2005; VERAS et al., 2016). Muitos estudos confirmam que existe uma frequência alimentar ideal para cada espécie cultivada e está varia de acordo com vários fatores como; habito alimentar, fase de desenvolvimento e alimentação anterior (LEE; CHO; KIM, 2000; RODRIGUES et al., 2019; WU et al., 2015).

Quanto ao hábito alimentar da espécie, peixes com estômagos grandes são morfologicamente capazes de ingerir presas grandes porque distendem seus estômagos para aumentar a capacidade de armazenamento (GONZÁLEZ-BERGONZONI et al., 2020), permitindo que sejam saciados após uma única e grande refeição. Portanto espécies com estômago maiores, como a de espécies carnívoros requerem alimentação menos frequente para atingir o crescimento máximo. Este comportamento foi demonstrado em um estudo com juvenis de *Rachycentron canadum* (carnívoro - 110 g) alimentados uma, duas, três, quatro e seis vezes ao dia. Costa-Bomfim et al. (2014) não observaram diferenças nas variáveis de desempenho zootécnico, sugerindo que a frequência alimentar ideal para espécie seja de apenas uma vez ao

dia nesta fase de desenvolvimento. Peixes herbívoros com estômagos pequenos e intestino longo necessitem de altas frequências de alimentação para atingir o melhor crescimento alguns estudos. Em *Mugil liza* (herbívoro -13,68 g) quatro frequências de alimentação foram testadas (1 vez ,3,5 e 7 vezes ao dia) e os resultados demonstraram que as altas frequências de alimentação melhoraram as variáveis de crescimento (5 e 7 vezes ao dia), enquanto que os peixes perderam peso quando alimentados na menor frequência diária (SILVA et al., 2020).

Em relação à fase de desenvolvimento, o tamanho da refeição irá aumentar com a idade, mas a frequência de vezes que este alimento será ofertado será inversamente proporcional ao seu tamanho (BAKKE; GLOVER; KROGDAHL, 2010; COUNCIL, 2011). Portanto, maior frequência alimentar proporciona melhor crescimento nos peixes mais jovens em relação aos adultos, pois os peixes nas fases iniciais de desenvolvimento apresentam elevadas exigências nutricionais e baixa capacidade digestiva. Este comportamento foi demonstrado em alguns estudos com tilápia, *Oreochromis* sp (BHUJEL, 2014) que durante o seu ciclo de vida a frequência alimentar variou de 8 a 2 alimentações diárias. Resultados semelhantes também foram obtidos por Pullin e Lowe - McConnell (1982) que recomendam alimentar a tilápia 12 vezes ao dia quando seu peso é de 20 g, mas apenas 2 vezes ao dia quando pesam 200 g. Com a truta arco-íris, *Oncorhynchus mykiss* pesando 0,3 g o crescimento foi melhor quando alimentada oito vezes ao dia, mas quando pesavam 15 g a alimentação recomendada foi de 3 vezes ao dia (Piper 1982).

O efeito da frequência de alimentação no desempenho dos peixes tem sido estudado para várias espécies cultivadas, onde as frequências de alimentação ótimas foram determinadas, avaliando vários parâmetros de desempenho, como crescimento, eficiência alimentar, composição proximal, metabolitos plasmáticos, crescimento muscular e atividade das enzimas digestivas (GUO et al., 2018; LI et al., 2014; LIU; LIAO, 1999; WU et al., 2021; ZHOU et al., 2003).

2.2.3 Restrição alimentar e realimentação

A restrição alimentar, definida como redução ou falta de alimento disponível para consumo é uma prática amplamente utilizada na piscicultura (ABOLFATHI et al., 2012; PY; ELIZONDO-GONZÁLEZ; PEÑA-RODRÍGUEZ, 2022). Manipulação na taxa de alimentação, frequência alimentar diária e no número de dias de alimentação na semana são estratégias de alimentação que envolve a restrição alimentar parcial e realimentação que visa não somente a

economia de ração, mas sim, maximizar o desempenho através do crescimento compensatório (ALI et al., 2016; KÄNKÄNEN; PIRHONEN, 2009; WON; BORSKI, 2013).

O crescimento compensatório (CG) é um fenômeno em que um animal com crescimento previamente reduzido expressa taxas de crescimento mais altas do que seus coespecíficos que não experimentaram depressão no crescimento (ALI; NICIEZA; WOOTTON, 2003). Na restrição alimentar, o CG é expresso como um crescimento significativamente mais rápido em relação aos indivíduos que não experimentaram nenhum tipo de privação alimentar (HASANPOUR et al., 2021; SALGADO-ISMODES; TAIPALE; PIRHONEN, 2020; URBINATI; SARMIENTO; TAKAHASHI, 2014).

A compensação do crescimento pode ocorrer de forma parcial onde os animais privados não atingem no mesmo tempo o tamanho, mas mostram alta taxa de crescimento e melhora na eficiência alimentar; compensação total em que os animais atingem o mesmo tamanho dos animais não privados, sobre compensação os animais que tinham experimentado uma alimentação restrita atinge um tamanho maior do que os animais não restritos e não compensação não ocorrendo respostas compensatórias (ALI; NICIEZA; WOOTTON, 2003). Os mecanismos que contribuem para o ocorrência do CG ainda não é totalmente compreendido, mas alguns mecanismos, como hiperfagia e melhor conversão alimentar, foram identificados como impulsionadores do crescimento acelerado (PY; ELIZONDO-GONZÁLEZ; PEÑA-RODRÍGUEZ, 2022; WON; BORSKI, 2013).

Resultados de estudos sobre CG em peixes mostram resultados inconsistentes. A compensação foi observada na maioria dos estudos (FAVERO et al., 2020; GALLARDO-COLLÍ et al., 2020), mas uma capacidade limitada de crescimento compensatório foi relatada em outros (ARGÜELLO-GUEVARA et al., 2020; STUMPF et al., 2020). Para as espécies que apresentam CG, o uso desta estratégia alimentar é uma alternativa para otimizar a produção, reduzir os custos com alimentação sem comprometer o crescimento (TORFI MOZANZADEH et al., 2021).

2.3 Espécies estudadas

2.3.1 *Chirostoma estor*

Chirostoma estor, popularmente conhecido como pescado blanco, é um peixe de água doce da família Atherinopsidae (Dyer and Chernoff, 1996). Esta família é constituída por um grupo de espécies de distribuição cosmopolita que na sua maioria habita ambientes marinhos

ou salobres, como exemplo o peixe rei *Odonthestes bonariensis* (BAIGÚN; COLAUTTI; GROSMAN, 2009; MARTÍNEZ-PALACIOS et al., 2019; URIBE-ALCOCER; OLVERA-GARCÍA; DÍAZ-JAIMES, 2002). O pescado blanco é uma espécie endêmica da região do lago de Pátzcuaro (Michoacan, México) e constituiu por muito tempo a base da pesca deste local, sendo recurso econômico de subsistência da região e de muitas famílias (FLORESCANO; SÁNCHEZ DÍAZ, 2018). No entanto, devido a fatores como a sobrepesca, a introdução de espécies exóticas e contaminação dos corpos de água em que habita, resultou na redução de sua população, tornando-o uma espécie ameaçada de extinção (ARELLANES-CANCINO et al., 2021).

C. estor é um filtrador zooplanctófago que apresenta pequena boca superior protrátil, com dentes faríngeos, numerosos espinhos nos arcos branquiais e na mandíbula inferior possui quatro fileiras de dentes discretos, simples e cônicos (MARTÍNEZ-PALACIOS et al., 2019). Embora seja um peixe zooplanctófago, quando adultos podem se alimentar de pequenos crustáceos (MARTÍNEZ-PALACIOS et al., 2006). É uma espécie agástrica e com intestino curto, cujo coeficiente intestinal de juvenis e adultos é 0,3 (MARTÍNEZ-PALACIOS et al., 2007; ROSS et al., 2006). Este peixe é ovíparo, sendo que sua reprodução se intensifica nos meses quentes, embora possa desovar ao longo do ano (CORONA-HERRERA et al., 2016; MOTTA et al., 2021).

Figura 1. Pescado blanco, *Chirostoma estor*

Fonte: Google imagens

Em comparação com outras espécies de peixes de água doce, *C. estor* se destaca por apresentar alto valor nutricional, o que ocorre devido a capacidade de produzir altas proporções de ácidos graxos polinsaturados de cadeia longa, especificamente o ácido docosaexaenoico (DHA), característica esta que o torna uma espécie emergente da aquicultura (FONSECA-MADRIGAL et al., 2012; MARTÍNEZ-PALACIOS et al., 2020).

Nos últimos 20 anos, o cultivo controlado desta espécie vem sendo realizado em escala experimental pelos pesquisadores da Universidad Michoacana de San Nicolás de Hidalgo - México. Desde então, grandes avanços foram alcançados, tendo já sido realizado o ciclo completo da espécie em laboratório. O ciclo produtivo inclui desde a obtenção da desova, incubação e eclosão dos ovos, a passagem de larvas a juvenis, o crescimento dos juvenis a adultos, até a geração de novos reprodutores (CORONA-HERRERA et al., 2018; FLORESCANO; SÁNCHEZ DÍAZ, 2018). Em relação a nutrição e alimentação desta espécie, vários estudos foram direcionados para estabelecer os requisitos básicos e otimizar as estratégias de alimentação durante os estágios iniciais (JUÁREZ-GUTIÉRREZ et al., 2021; MARTÍNEZ-ANGELES et al., 2022; MARTÍNEZ-PALACIOS et al., 2007; OSPINA-SALAZAR et al., 2016; RÍOS-DURÁN et al., 2016; TOLEDO-CUEVAS et al., 2011). No entanto, não foi possível dar o salto para o cultivo comercial, devido à falta de informação nas diferentes etapas que ainda devem ser solucionadas. Um destes pontos não resolvidos ocorre na fase juvenil, em que ainda são desconhecidas as práticas alimentares corretas, sendo um ponto chave para o desenvolvimento da cadeia produtiva da espécie.

2.3.2 *Colossoma macropomum*

O tambaqui (*Colossoma macropomum*) (Cuvier, 1818) é um caracídeo de água doce, pertence à classe Actinopterygii, ordem Characiformes, família Serrasalminae (BUCKUP; MENEZES, 2007) é um peixe nativo das bacias do rio Amazonas , rio Orinoco e seus afluentes, sendo comumente, encontrado da foz do rio Xingu, no Estado do Para até o médio rio Ucaiali, no Peru (VAL; DE OLIVEIRA, 2021; WOYNÁROVICH; VAN ANROOY, 2019).

Com habito alimentar onívoro, consome sementes e frutos nos períodos de cheia e zooplâncton durante a seca (GOULDING; CARVALHO, 1982). Por conta de sua alimentação, a dentição do tambaqui é própria para consumo de alimentos duros,), formada por duas fileiras de dentes molariformes fortes, separadas por um espaço triangular (ROUBACH; SAINT-PAUL, 1994; VAL; DE OLIVEIRA, 2021). Possui um estômago elástico e bem definido, coeficiente intestinal de XX, bem como cecos pilóricos desenvolvidos e provavelmente voltados para a digestão de alimentos de origem vegetal (VALLADÃO; GALLANI; PILARSKI, 2018). É uma espécie reofilica e, portanto é necessária a indução hormonal para que ocorra a reprodução em cativeiro(CASTRO et al., 2020).

Figura 2. Tambaqui; *Colossoma macropomum*

Fonte: Arquivo pessoal

O tambaqui apresenta algumas características importantes para produção em cativeiro, como rusticidade, rápido crescimento, alta produtividade, resistência à hipóxia e fácil adaptação a dietas artificiais (PAULINO et al., 2018; RODRIGUES, 2018). Apesar da espécie ser a mais produzida comercialmente no Brasil, informações limitadas estão disponíveis sobre o manejo alimentar dessa espécie (GUIMARÃES; MARTINS, 2015; RODRIGUES, 2018).

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PARTE II – Artigo I

Feeding frequency has a determinant role in growth performance, skeletal deformities, and body composition in the Mexican pike silverside (*Chirostoma estor*), an agastric short-intestine fish (Teleostei: Atheriniformes)

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Abstract

Several fish species with potential for aquaculture diversification possess agastric short-intestine anatomical configuration. The absence of a stomach or long intestine in fish may imply specific feeding frequency strategies for optimal growth. Because adequate feeding frequencies are paramount for thriving fish culture, the presente study aimed to evaluate the effects of feeding frequency on growth performance, feeding efficiency, survival, deformities, proximate body composition, and muscle growth in Mexican pike silverside (Chirostoma estor: Atherinopsidae) a short-intestine agastric fish. Fish with an average weight of 180.57 ± 3.02 mg were randomly distributed in twelve tanks and fed four (M4), eight (M8), and twelve (M12) times a day until apparent satiety for 45 days. Significantly higher growth (70%) was found in M12 treatment compared to M4. There was no difference in survival among treatments. However, significantly fewer deformities were found in the M12 treatment compared to M8 and M4 treatments. Increased feeding frequency produced fish with higher lipids, with a concomitant reduction in ash content. Muscle fiber diameters were significantly different in all treatments: M12 (32.97 ± 3.13 μm), M8 (30.28 ± 4.59 μm), and M4 (26.74 ± 4. 42 μm). These results reflect the importance of feeding strategies in accordance to fish habits and their digestive configurations and may be relevant for Other emerging species with similar digestive morphology and lower in the trophic chain, which are essential for aquaculture diversification and sustainability.

Keywords: Feeding strategies, Fish performance, Stomachless fish, Atherinopsidae

1. INTRODUCTION

In addition to the nutritional requirements, developing an optimal diet and feeding strategy for any fish species requires knowledge of the digestive physiology and its associated feeding habits (Solovyev and Gisbert, 2016). Agastric fish, rigorously defined as lacking acid-peptic digestion as adults, comprise approximately 20–27% of known species (Wilson and Castro, 2010). Several species have developed accessory anatomical apparatus or gut modifications such as specialized pharyngeal teeth, gizzards, pyloric caeca, long guts, or short-but-wide diameter (Horn, 1989; Stevens and Hume, 2004; Manjakasy et al., 2009; Egerton et al., 2018) to compensate the lack of acid-peptic digestion in a stomach. Notably, despite several short-gut agastric species from the Belonidae family having been described from a nutritional ecology standpoint (Manjakasy et al., 2009), to our knowledge, no representatives of fish with this digestive configuration exist in commercial aquaculture, and their feeding requirements for optimal growth performance are unknown. This information is relevant because feeding frequency can contribute to production costs (De Silva et al., 2007; Tian et al., 2015; Abdel-Aziz et al., 2021).

The Mexican pike silverside (*Chirostoma estor)* is a zooplanktivorous freshwater fish species, endemic to Lake Patzcuaro, Michoacan, Mexico (Martínez-Palacios et al., 2006; Martínez-Chavez et al., 2014). This species has high regional socioeconomic importance, but due to overfishing and habitat degradation, it is under threat of extinction (Martínez-Chavez et al., 2018; Davila-Camacho et al., 2019). Compared to other freshwater fish species, *C. estor* stands out for its high nutraceutical and economic value (Martínez-Chavez et al., 2018; Martínez-Palacios et al., 2020). The latter is mainly due to its cultural importance, organoleptic properties, and ability to synthesize high proportions of long-chain Omega 3 polyunsaturated fatty acids, specifically docosahexaenoic acid (DHA) (Fonseca- Madrigal et al., 2012; Martínez-Palacios et al., 2020). Thus, it is na emerging species with excellent potential for aquaculture diversification needs (Little et al., 2016; Martínez-Palacios et al., 2020). Like Other carnivore fishes from the Atherinomorpha clade, *C. estor* lacks a stomach, has a short intestine (relative gut length: 0.7), is prey selective (ram suction feeding), and feed processing occurs in well-developed pharyngeal teeth (Ross et al., 2006; Manjakasy et al., 2009; Martínez-Palacios et al., 2019). However, although significant advances in husbandry for the conservation and commercial production of *C. estor* have been successfully developed (Martínez-Chavez et al., 2014; Corona-Herrera et al., 2016; Ospina-Salazar et al., 2016; Ríos-Dur´an et al., 2016;

Martínez-Chavez et al., 2018; Juarez-Gutierrez et al., 2021; Motta et al., 2021), further knowledge on the feeding habits (feeding frequency) associated to the anatomical alimentary canal is required to maximize growth performance and reduce skeletal deformities due to inadequate feeding strategies of this zooplanktophagous fish under culture. Previous research on *C. estor* has shown higher growth rates when frequently fed under continuous light (Corona-Herrera et al., 2016, 2018). Furthermore, a preliminary study suggested that an increase in feeding frequency (eight times a day) was associated with higher growth and a significant reduction in skeletal deformities (unpublished data). Considering this information and the species digestive biology and feeding habits of *C. estor*, it is hypothesized that a higher feeding frequency may be essential to reach optimal growth levels and feeding efficiency under culture.

Thus, the present study aimed to evaluate the effects of feeding Frequency on growth performance, feeding efficiency, survival, deformities, proximate body composition, and muscle growth in *C. estor*.

2. MATERIALS AND METHODS

2.1 Experimental system and fish

The experimental setup consisted of twelve 3 m diameter circular tanks (9 m3) in a continuous water flow system (5–8 L min^{1}). Each tank had a PVC central drain pipe and a 2 mm fish screen to prevent fish from escaping. A blower provided continuous aeration through two ceramic air stones in all the tanks. In each tank, 70 juvenile *C. estor* (mean weight: 180.57 ± 3.02 mg), obtained from breeding stock maintained at the IIAF-UMSNH facilities, were randomly distributed. Water temperature was measured twice a day with a mercury thermometer; dissolved oxygen and pH with an oximeter (YSI 55/25, Yellow Springs, Ohio, USA) and potentiometer (Fisher-Scientific Model Accumet®, Waltham, Massachusetts, USA); ammonium and nitrite were determined by spectrophotometry with a YSI Photometer (model 9500, Yellow Springs, Ohio, USA). During the experiment, the water temperature, pH and dissolved oxygen were 22.05 ± 0.84 ◦C, 7.31 ± 0.10, and 6.56 ± 0.33 mg L^{1}, respectively. Nitrite and nitrate were not detected. The trial was performed in a greenhouse under natural photoperiod conditions (Lat 19.68939489239912◦, Long 101.24929071179976◦).

2.2 Experimental design

A 45-day feeding trial was carried out in a completely randomized design, in which three feeding frequencies were tested, four (M4), eight (M8), and twelve (M12) feedings per day. Each treatment was evaluated in quadruplicate groups. All feedings took place from 7 am to 6 pm, and the interval between them was 3.7 h for M4, 1.6 h for M8, and 1 h for M12.

Fish were manually fed until apparent satiety (fullness) at each feeding frequency schedule, with a formulated diet containing 431 g Kg-1 crude protein, 55.4 g Kg-1 crude lipid between 1 and 2 mm according to the size of fish. Feed consumption was measured for each tank daily before and after each feeding time according to the methodology from Dwyer et al. (2002). Fish mortality was checked daily before the first feeding of the day.

At the end of the experiment, all fish were fasted for 12 h and anesthetized with ice to being weighed individually to assess growth performance. Survival and deformities were also recorded. Skeletal deformities were evaluated based on direct observation of all individuals during the weighing process and taking into account the mortality (Perrott et al., 2018). The final weight (FW), weight gain (WG), daily weight gain (DWG), specific growth rate (SGR), feed intake (FI), feed conversion ratio (FCR), protein consumption (PC), protein efficiency ratio (PER), survival and deformity rate were calculated using the following equations :

WG (%)=((Individual Final wet weight-Individual Initial wet weight)/(Initial wet weight)*100

DWG (mg day^{-1}) = (Individual Final wet weight-Individual Initial wet weight)/days

SGR (%)=((Ln Individual Final wet weight-Ln Individual Initial wet weight)/days)*100

FI (mg day^{-1}) = ((Feed consumption per tank (g)/number of fish)/days))*1000

FCR = FI/DWG

PC (mg day^{-1}) = FI*(Dietary protein level (%)/100)

PER = DWG/PC

Survival rate (%) = (final fish number)/ (initial fish number)*100

Deformity rate (%) = (fish with deformity)/(final fish number)*100

2.3 Proximal analysis

The proximate composition of whole fish (8 fish per replicate) was analyzed using the Association of Official Analytical Chemists standards (AOAC, 2000). Moisture (oven drying at 105 °C to constant weight); crude protein (Leco FP-528, Dumas method; Ebeling, 1968), crude lipid (Soxtec Avanti 2050); and crude ash (incineration at 550 °C, Fisher Scientific®) were determined. All chemical analyzes were performed in triplicate.

2.4 Muscle tissue histology

At the end of the experiment, eight juveniles from each treatment were randomly collected for histological analysis of muscle fibers. Sampled fish were fixed in Davidson's solution for 48 h. An incision was made in the abdomen of each specimen for better fixation. The whole fish (except the tail) was dehydrated in increasing ethanol concentrations, diaphanized in xylol, embedded, and included in paraffin. Paraffin blocks were transversely sectioned at a thickness of 5 µm using a microtome (Leica Jung-Histocit 820). The histological sections near the dorsal fin were distributed in glass slides and stained with hematoxylin and eosin (Feldman and Wolfe, 2014). Digital images were obtained at 100X (Carl Zeiss Model Axioskop 40) and analyzed (Image-Pro Plus version 6.0) to measure the smallest diameter of 200 muscle fibers per animal in a fragment of the muscle region. Subsequently, the muscle fibers were distributed into three classes according to their diameter: D1 = fibers smaller than 20 µm in diameter (hyperplastic fibers), D2 = fibers with a diameter between 20 and 50 µm (increasing intermediate fibers), and D3 = fibers with more than 50 µm in diameter (increasing hypertrophic fibers) (Melo et al., 2020).

2.5 Statistical analysis

Data were tested for homoscedasticity and normality using the Levene and Shapiro-Wilk tests. Growth, proximate composition, and muscle histology data were analyzed using One-way ANOVA followed by Tukey's *post hoc* test. All analyzes were conducted using Minitab 18.1. Significance was assigned at 0.05. All data are expressed as mean ± standard deviation.

3. RESULTS

3.1 Growth and feeding behavior

‘ The results of growth and feeding efficiency are shown in Table 1, and weight distribution can be observed in Figure 1. The highest growth in terms of FW (p= 0.003), WG (p= 0.001), DWG (p= 0.004) and SGR (p= 0.001) occurred in group M12, differing from groups M4 and M8. No significant differences in feed intake were observed among treatments; however M12 presented better feeding efficiency in terms of FCR and PER (Table 1). There was no significant difference in the final survival among treatments, but there was a significant difference in deformities in fish (p 0.009), being lowest in M12 (3.59 ± 2.03%) compared to treatments M8 (16.99 ± 8.66%) and M4 (21.78 ± 6.93%) (Fig. 2).

3.2 Proximate analysis

The initial and final proximal composition data of the different treatments are presented in Table 2. The moisture content and crude protein were not affected by the feeding frequency. However, crude lipids were higher (p= 0.002), in groups M12 and M8 than in treatment M4. In contrast, crude ash content, which was higher (p= 0.001) in treatment M4 compared to M8 and M12 treatments

3.3 Muscle tissue histology

Histological evidence of muscle fibers and quantitative results of the muscle fibers are presented in Figure 3. Regarding mean diameters (μm) of muscle fibers, there was only a significant difference (p= 0.021) between the animals in M12 treatment (32.97±3.13 μm) compared to M4 (26.74 ± 4.42 μm) (Fig. 3B).

Regarding the frequency distribution of muscle fibers according to diameter classes, treatments M4 and M8 had a similar distribution pattern, with a more significant number of fibers in class D2 (20-50 μm), followed by fibers in class D1 (< 20 μm) with few larger fibers of class D3 (> 50 μm). Treatment M12, on the other hand, had a more significant number of intermediate fibers, but no statistical difference was observed between D1 and D3 class fibers. In addition, M12 had more D3 class fibers than treatments M8 and M4 (Fig. 3C).

Table 1 Growth and feed utilization parameters of juveniles of *Chirostoma estor* fed to satiety with different feeding frequencies.

Parameters	Treatments			
	M4	**M8**	**M12**	**p-value**
IW[1] (mg fish^{-1})	184.82 ± 13.62	178.9 ± 29.1	178.0 ± 27.3	0.912
FW[2] (mg fish^{-1})	936.3 ± 154.7^{b}	1108 ± 230^{b}	1573.4 ± 195.4^{a}	0.003
FI[3] (mg day^{-1} fish^{-1})	40.23 ± 9.32	47.58 ± 11.79	47.28 ± 2.47	0.451
PC[4] (mg day^{-1})	17.34 ±4.02	20.42 ± 5.07	20.62 ±1.08	0.451
WG[5] (%)	404.4 ± 56.6^{b}	515.5 ± 70.7^{b}	790.6 ± 89.0^{a}	0.001
DWG[6] (mg day^{-1})	16.7 ± 3.17^{b}	21,6 ± 4.56^{b}	31.01 ± 3.89^{a}	0.004
SGR[7] (%)	3.59 ± 0.26^{b}	4.03 ± 0.26^{b}	4.85 ± 0.23^{a}	0.001
FCR[8]	2.40 ± 0.25^{b}	2.21 ± 0.28^{b}	1.53 ± 0.13^{a}	0.001
PER[9]	0.97 ± 0.09^{b}	1.02±0.10^{b}	1.52±0.13^{a}	0.001

Data is expressed as mean ± standard deviation.
Means with different lowercase letters in the same row are significantly different ($p < 0.05$).
M4 - 4 meals/day; M8 – 8 meals/day; M12 – 12 meals/day.
[1] Initial wet weight. [2] Final wet weight. [3] Feed intake expressed on a dry-weight basis. [4] Protein consumption expressed on a dry-weight basis. [5] Weight gain. [6] Daily weight gain. [7] Specific growth rate. [8] Food conversion ratio. [9] Protein efficiency ratio

Table 2 Average body proximate composition (% wet weight) of juveniles of *Chirostoma estor* fed with different feeding frequencies.

Parameters	*Treatments*			
	Initial fish	**M4**	**M8**	**M12**	**P-value**
Protein	14.70 ±0.3	17.55 ±0.12	17.10 ±0.49	17.25 ±0.09	0.151
Lipids	1.01 ±0.04	2.42 ± 0.21^{c}	3.42 ± 0.42^{b}	4.02 ± 0.11^{a}	0.002
Ash	3.12 ±0.17	2.76 ± 0.09^{a}	2.50 ± 0.08^{b}	2.46 ± 0.04^{b}	0.001
Moisture	81.51 ±0.52	77.25 ±0.08	77.08 ±1.15	76.52 ±0.08	0.315

Data are expressed as mean ± standard deviation, n= 3
Means with different lowercase letters in the same row are significantly different ($p < 0.05$).
M4 - 4 meals day^{-1}; M8 – 8 meals day^{-1}; M12 – 12 meals day^{-}

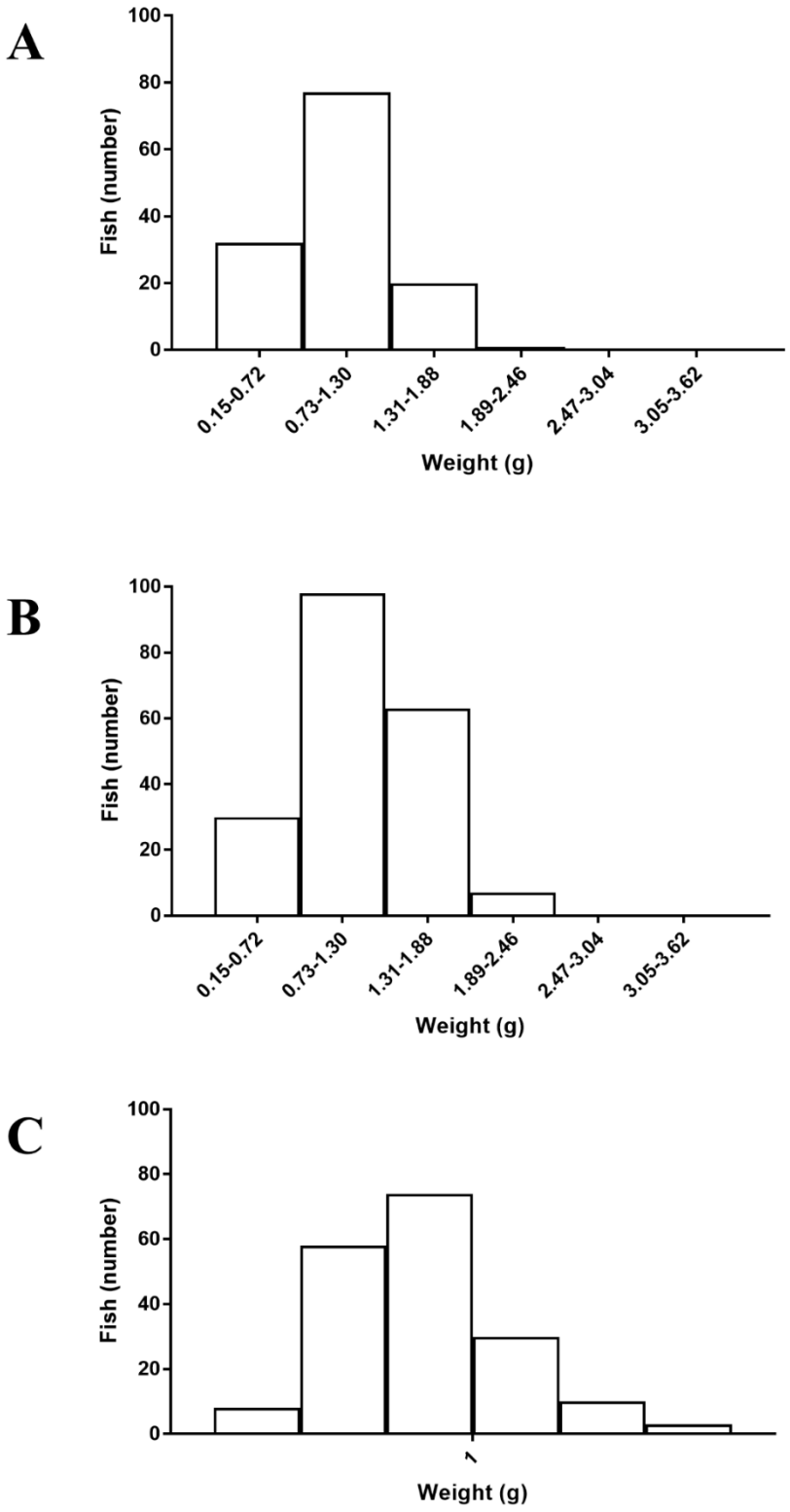

Figure 1. Weight distribution (mg) of juveniles of *Chirostoma estor* fed with different feeding frequencies.

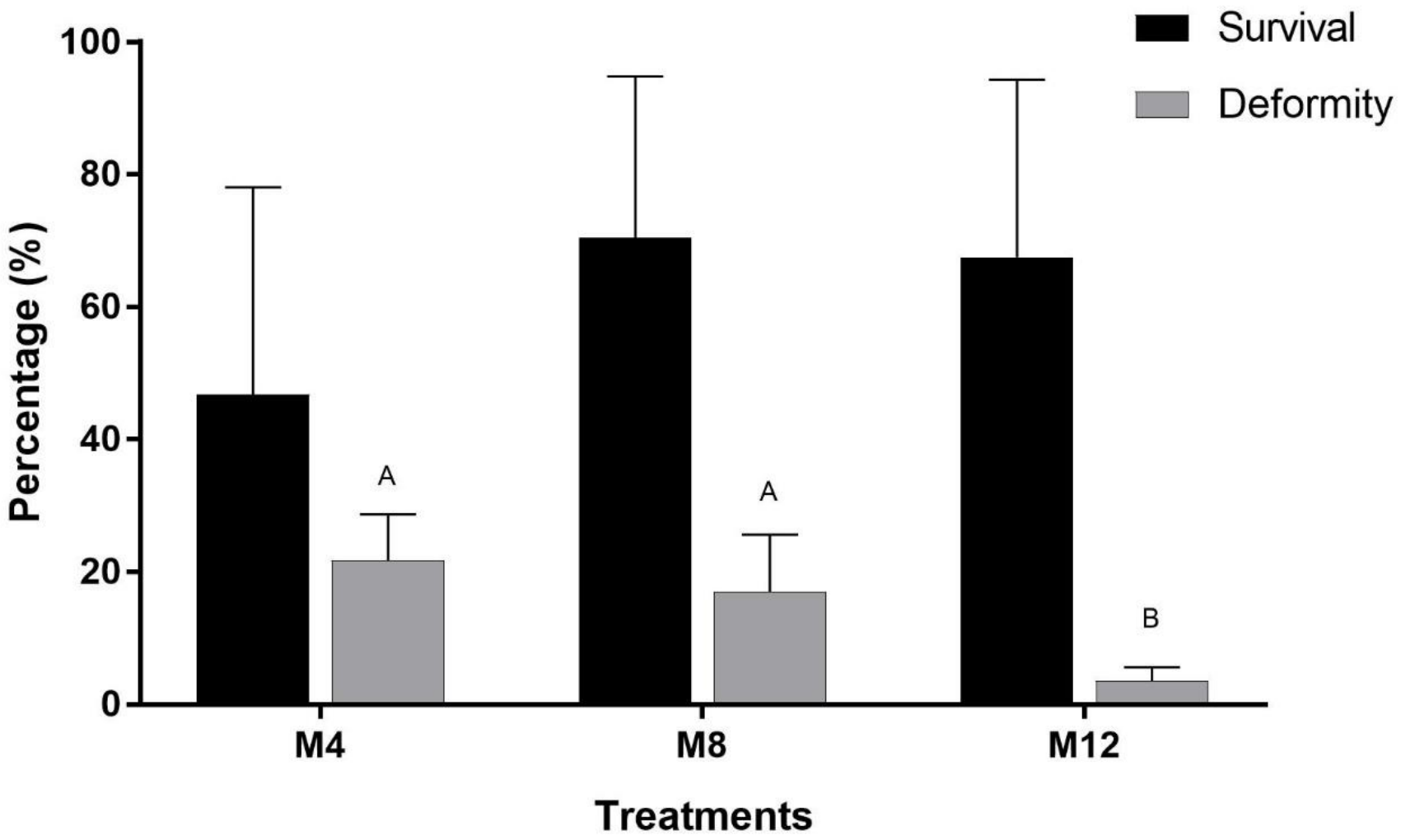

Figure 2. Survival and deformity of juveniles of *Chirostoma estor* fed with different feeding frequencies. Different uppercase letters indicate significant differences ($p < 0.05$)

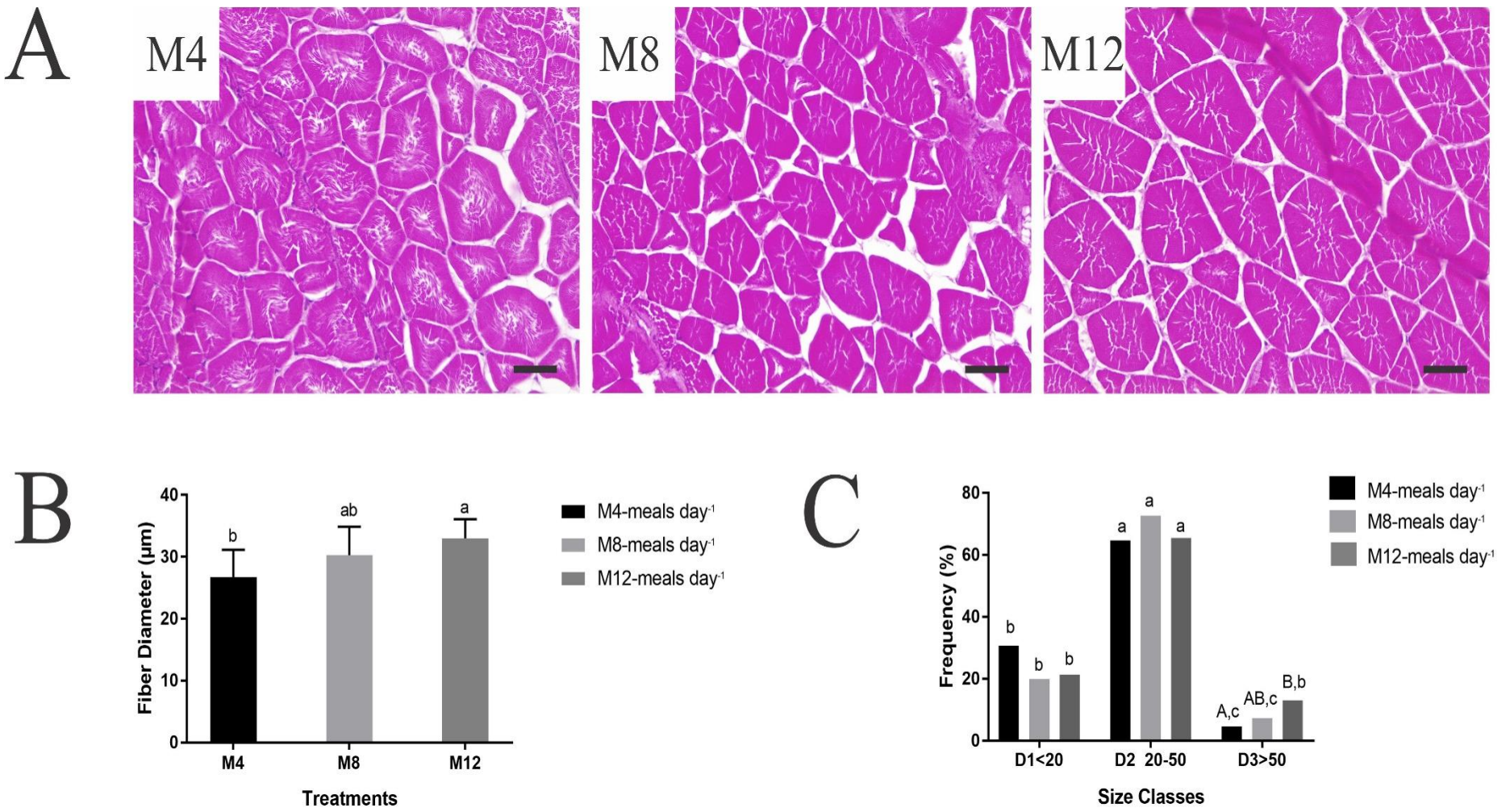

Figure 3. A) Histological sections of muscle of juveniles of *Chirostoma estor* stained with Hematoxylin and Eosin. **B)** Mean ± standard deviation of muscle fiber diameter in juveniles of *Chirostoma estor* fed different feeding frequencies. Treatments presenting different letters indicate a significant difference (p<0 .05). **C)** Mean ± standard deviation of the frequency distribution in the different muscle fiber classes of juveniles of *Chirostoma estor* fed different feeding frequencies. Different lower case letters indicate significant differences between treatments and different upper case letters within the same treatment (p<0 .05).

4. DISCUSSION

Early fish larvae generally lack a functional stomach and have an immature digestive system, conditioning larvae to feed frequently on live prey (Cahu et al., 1998; Yúfera et al., 1999; Zambonino Infante and Cahu, 2001). On the other hand, juveniles and adults of commercial fish species, generally gastric, are fed only a few times a day (i.e., 4 times a day) according to their digestive system (Xie et al., 2011). However, in species with foraging, aggressive feeding behaviors, or small stomachs, multiple daily meals are likely to improve performance indicators (Davis and Hardy, 2022). To our knowledge, there are no short-intestine-agastric fish species in commercial aquaculture; therefore, there is a lack of information regarding optimal feeding frequency in these digestive fish models.

In this study, despite the different meal frequencies offered to apparent satiety, total daily food consumption was similar between all treatments. Interestingly this resulted in 18% and 70% more growth when fed 8 and 12 times a day, respectively, compared to 4 times a day during 45 days. This suggests differential digestibility rates across treatments, which could be associated with evacuation times and food volume (Jobling, 1981). In fact (although not significant), an inverse relationship between feeding frequency and the amount of feed intake was observed (Fig. Supp. 1). It has been shown that feeding frequency, meal size, and timing can affect food transit, apparent digestibility, and thus nutrient utilization in gastric species with different feeding habits and gut morphology (Jobling, 1981; NRC, 2011; Gilannejad et al., 2019; Gilannejad et al., 2021). For example, allocation of full daily meal offered in a single ration provoked fast gut filling and apparent reduced digestibility in both Solea senegalensis and *Sparus aurata,* while the opposite occurred when the daily meal amount was offered several times a day (Gilannejad et al., 2019).

The results in this short-intestine-agastric model also suggest that fast gut filling and reduced apparent digestibility could also be occurring in *C. estor*. In S. senegalensis, lower gene expression of key digestive enzymes occurs when the feeding protocol is limited to a single meal a day (Gilannejad et al., 2021). Omic approaches (transcriptomics, microbiomics, and metabolomics) in C. estor are currently ongoing to shed light on the mechanisms and effects of different feeding frequencies in the digestion and nutrient uptake in this short-intestine-agastric species.

In agreement with the previously discussed, feeding efficiency was improved in the most frequent feeding treatment (M12). This is similar to what was found in *Carassius auratus*, where better growth and feed efficiency were observed when fed 24 times a day compared to 12 (Zhou et al., 2003). However, the effects of feeding frequency in several other fish species have different outcomes (Kiaalvandi et al., 2011; Costa- Bomfim et al., 2014; Tian et al., 2015; Okomoda et al., 2019; Wu et al., 2021). The latter can be explained by variables such as the age of fish in each case but are more likely due to the different digestive modes (gastric or agastric) and by the accessory anatomical apparatus or gut modifications that differ among species that are associated with their feeding habits and rhythms (Stevens and Hume, 2004; Lopez-Olmeda and Sanchez Vazquez, 2010; Egerton et al., 2018). Therefore, this suggests that when comparing feeding frequency across different studies, each species alimentary canal configuration and nutritional ecology, and their opportunistic plasticity in different environments (i.e., wild vs. captivity) should be considered, as previously suggested in atherinopsids (Martínez-Palacios et al., 2019). For example, according to its digestive configuration, *C. estor* benefits from increased feeding frequency in captivity, which may resemble the continuous foraging feeding habits in the wild.

In this study, decreased feeding frequency also contributed to a higher incidence of externally visible skeletal deformities, significantly higher in treatments M4 and M8 despite total daily food consumption being similar (Fig. 2). The appearance of vertebral deformities in fish such as lordosis, kyphosis, and scoliosis are mainly due to high incubation temperatures of eggs, genetic factors, and nutritional deficiencies (Smedley et al., 2016; Clarkson et al., 2021; Eissa et al., 2021). Therefore, sub-optimal feeding frequencies in on-growing fish can cause skeletal deformities as in the present study, suggesting that a 12 times a day feeding frequency is adequate to reduce the deformity incidence under the experimental conditions tested.

The feeding regime often affects body composition and can be used as a quality indicator of fish growth (Ali et al., 2016; Cadorin et al., 2021). In our study, no significant differences were found in crude protein and moisture levels among different treatments (Table 2); this can be explained because no differences were found in the amount of feed consumed. This suggests that low feeding frequencies were sufficient to provide dietary protein above the maintenance level of fish, as indicated by Cadorin et al. (2021), despite lower growth performance.

There was, however, an increment in whole body crude lipids as feeding frequency increased. This is in agreement with previous observations in this and other species (Martínez-Chávez et al., 2014; Wu et al., 2015; Okomoda et al., 2019). This is a desired aspect in *C. estor* as high percentages of body fat represent high levels of long-chain Omega 3 fatty acids (Fonseca-Madrigal et al., 2014; Martínez-Palacios et al., 2020). A hypothesis by Xie et al. (2011) proposes that less energy expenditure caused by lower food competition in frequent-feeding treatments may cause higher body lipid deposition.

Fish growth is determined by muscle growth and occurs through changes in signaling pathways regulating the proliferation and differentiation of myogenic cells (Johnston, 1999; Rowlerson and Veggetti, 2001; Koganti et al., 2021). The appearance of small new muscle fibers is caused by hyperplasia, which increases in size through hypertrophic growth (Johnston et al., 2011; Leitao et al., 2011). According to Rowlerson and Veggetti (2001), fibers with diameters smaller than 20 μm indicate the occurrence of hyperplasia, and diameters >50 μm are associated with hypertrophy. In our study, group M12 presented a significant number of larger diameter fibers than M4, where the number of smaller diameter fibers was higher (Fig. 3B). This suggests that the decrease in feeding frequency reduced the available nutrients for protein synthesis and muscle growth, which is supported by the protein efficiency ratio, which showed significant differences among treatments in this study. Corroborating our results, Xu et al. (2019) found a gradual decrease in muscle fibers with a reduction in growth performance in *Ctenopharyngodon idella.*

The increased feeding frequency in M12 resulted in a more significant number of muscle fibers with a diameter higher than 50 μm. In this sense, the decrease in total ash observed in the M12 group is likely due to muscle and bone in fast-growing fish, which is a desired characteristic for market value (de Oliveira-Júnior et al., 2021). Thus, the different distributions of muscle fibers found in the present study demonstrate that the feeding strategies tested affected the dynamics of skeletal muscle growth in *C. estor*. Further studies are required in *C. estor* to assess whether feeding frequencies can be further optimized, especially under continuous illumination photoperiod where enhanced growth has been achieved (Martínez-Chávez et al., 2014; Corona-Herrera et al., 2018).

Furthermore, logistic (automatic feeders) and bioengineering implications such as dissolved oxygen uptake requirements of fish fed more frequently than common gastric species should be considered for commercial applications in this and other species with similar feeding protocols.

5. CONCLUSION

The present study provides evidence that an increased frequent feeding (at least twelve feedings a day) improved feed efficiency, promoted higher growth, and reduced skeletal deformities in *C. estor* juveniles, a short intestine, agastric, zooplanktophagous fish. These results here could be relevant for other species, low in the trophic chain, with similar digestive configurations, such as anchovies and sardines, which are milestone species for aquaculture diversification and sustainability.

Data availability

No data was used for the research described in the article.

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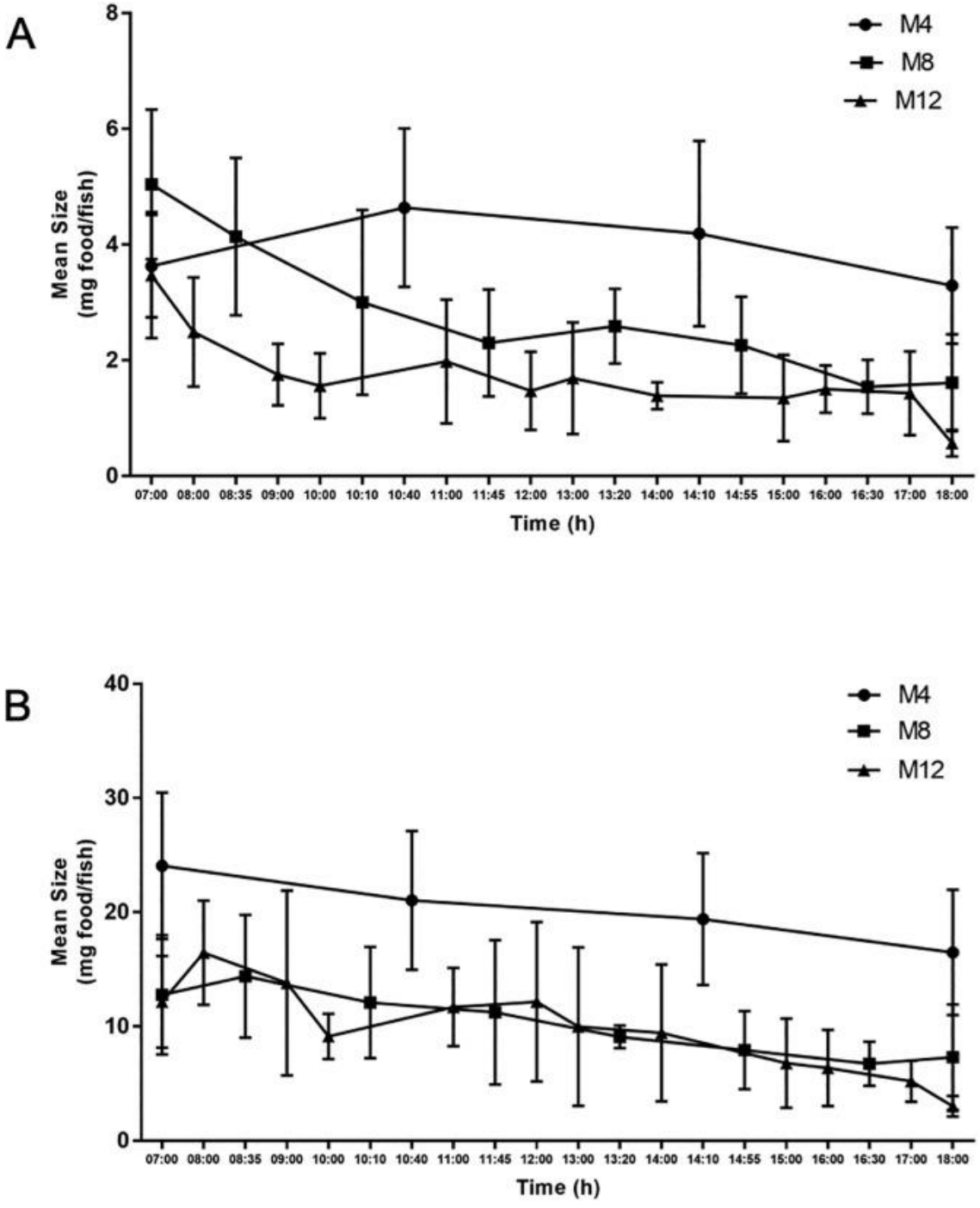
The following is the supplementary data related to this article. The following is the supplementary data related to this article.



Supplementary Figure 1. Weight distribution (mg) of juveniles of *Chirostoma estor* fed with different feeding requencies.

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PARTE III - Artigo II

Physiological responses associated with compensatory growth of *Colossoma macropomum* submitted to different feeding rates

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Abstract

Colossoma macropomum, popularly known as tambaqui, is the most commercially cultivated native species in South America. Our study aimed to evaluate a series of endpoints related to growth response and physiological parameters in *C. macropomum* juveniles submitted to different feeding rates (FR) and subsequent refeeding. Fish were fed at five different FR (2%, 4%, 6%, 8%, and 10% of body weight per day) for 30 days (restricted feeding phase) and then refed until apparent satiation for another 30 days (refeeding phase). Growth parameters increased ($P < 0.05$) with increasing FR. The FR for maximum growth was estimated to be 8.98% day $^{-1}$. The reduction in FR negatively affected ($P < 0.05$) the activity of digestive enzymes, intestinal histomorphometry, proximal composition, plasma metabolites, and antioxidant capacity. However, after refeeding, these physiological variables were mostly restored ($P > 0.05$). Our results indicate that a FR of 6% day^{-1} provided the best growth and feed conversion, while full growth was achieved at 4% day^{-1} after refeeding. Collectively, our data suggest that a FR of 2% day^{-1} can trigger the catabolism of endogenous reserves, an impeding factor for compensatory growth.

Keywords: digestive enzymes; feeding strategies; oxidative stress; tambaqui; zootechnical performance.

1. INTRODUCTION

Tambaqui (*Colossoma macropomum*) is a freshwater fish species native to the Amazon and Orinoco river basins (Val and de Oliveira, 2021; Woynárovich and Van Anrooy, 2019). With an omnivorous feeding habit, it has productive potential due to its robustness, reproduction in captivity, adaptability to artificial diets, fast growth, and the possibility of production in different cultivation systems (Boaventura et al., 2021; Paulino et al., 2018; Santos et al., 2021; Santos et al., 2018; Souza et al., 2018). *C. macropomum* is the second most produced fish species in Brazil, and the most commercially cultivated native species in South America, although its production has also spread throughout the world, mainly in Asian countries such as China, Indonesia, Malaysia, Myanmar, and Vietnam (Amanajás and Val, 2022; Valladão et al., 2016). In recent years, interest in this species has been growing and includes the search for more efficient farming strategies to increase productivity and fish health (Guilherme et al., 2022; Luz et al., 2021; Nakayama et al., 2022; Vieira et al., 2022).

Given that feeding costs represent the highest operating expenses in fish farming, optimal fish growth, and feed efficiency require not only a balanced diet, but also appropriate feeding strategies (Cadorin et al., 2021; Melo et al., 2023; Veras et al., 2014). Thus, feeding control based on the determination of the ideal feeding rate (FR) is essential to maximize growth rates and improve feed efficiency, in addition to minimizing size heterogeneity, feed waste, and water quality deterioration in fish farming, among others (Oliveira et al., 2021; Ul Hassan et al., 2021). In addition to being species-specific, the optimal FR will vary according to fish size, age, stocking density, water quality, feed quality, and feeding strategy (Bolliet et al., 2001; Okomoda et al., 2019; Rodde et al., 2020).

Although restricted feeding in terms of FR results in underfeeding, the induction of compensatory growth (CG) through the application of feed restriction and refeeding has received great attention as a feeding strategy in fish farming (Ali et al., 2003; Dar et al., 2020; Xu et al., 2019). After periods of restricted feeding, several fish species can compensate for reduced growth when Feeding rates are retake (reviewed by Py et al., 2022). As an example, Mozanzadeh et al. (2021) reported that juveniles of *Acanthopopagrus latus* showed complete CG of final weight when fish were subjected to restricted FR (6% and 8%) compared to fish fed at 10% FR for 30 days followed by

refeeding for the same period. On the other hand, in addition to affecting growth, feeding restriction and refeeding can cause physiological changes in fish, requiring investigation of these responses (Hasanpour et al., 2021; Tamadoni et al., 2020).

A few studies have already evaluated different FR in tambaqui (Nakayama et al., 2022; Porto et al., 2018) as well as some dietary strategies for inducing CG (Assis et al., 2020; Roa et al., 2019; Santos et al., 2018). However, these studies were mainly focused on growth responses. In this sense, there is a lack of information regarding physiological responses in *C. macropomum* associated with FR and CG. Thus, our study was designed to determine the optimal FR and subsequent refeeding on growth performance and physiological effects on *C. macropomum*. As such, we measured a series of endpoints including growth parameters, the activity of digestive enzymes, intestinal histomorphometry, proximal composition, plasma metabolites, and antioxidant capacity.

2. MATERIALS AND METHODS

The experiment was carried out at the fish farming station of the Animal Science Department of the Federal University of Lavras (UFLA), Lavras, Minas Gerais, Brazil. The experimental procedures were approved by the Ethics Committee on Experimental Animal Use (CEUA) of UFLA, under protocol number 29/2017.

2.1 Fish maintenance

The fish were obtained from the Aquaculture Laboratory (LAQUA) of the Federal University of Minas Gerais (UFMG, Brazil) and acclimated to laboratory conditions for 30 days. After acclimatization, *C. macropomum* juveniles (average weight: 7.12 ± 0.18 g) were randomly distributed in a thermoregulated water recirculation system equipped with 15 circular fiberglass tanks (capacity of 100 liters each) with aerated, filtered water (sand filter and biofilters) and ultraviolet sterilized lamp.

The animals were kept under a photoperiod of 12:12 h (light/dark), with the lights on at 7:00 am and off at 7:00 pm, using an automated system (Ref. 8769, Brasfort, São Paulo, Brazil). Water temperature (analog mercury thermometer) and dissolved oxygen (digital oximeter AT 170, Alfakit, Florianópolis, Brazil) were measured daily. The pH,

ammonia, and nitrite were measured weekly using colorimetric kits (Alfakit, Florianópolis, Brazil). During the experiment, water temperature, dissolved oxygen, pH, ammonia, and nitrite were 28.38 ± 0.34 ˚C, 5.20 ± 0.40 mg L^{-1}, 6.91 ± 0.22, 0.23 ± 0.11 mg L^{-1}, and 0.07 ± 0.04 mg L^{-1}, respectively.

2.2 Experimental design

This experiment was composed of five FR and lasted for 60 days. The experiment was divided into two phases, including a 30-day restricted feeding phase (phase 1) and a 30-day refeeding phase (phase 2). Each experimental condition was tested in triplicate and each tank was composed of 20 fish. During phase 1, fish were fed at five FR (2%, 4%, 6%, 8%, and 10% of live body weight per day). The amount of feed for each FR was adjusted halfway through phase 1 (day 15). The fish were fed commercial feed (AQUOS TROPICAL; particle size 2 to 4 mm, 320 g kg^{-1} crude protein, 7.5 g kg^{-1} of crude fat, 120 g kg^{-1} of ash, and 4.5 g kg^{-1} of fiber) divided equally into four meals a day according to the FR for 30 days, making sure no feed was left uneaten.

In phase 2, the fish were fed with the same commercial feed provided during phase 1, but the feed was provided for all treatments until visual satiation four times a day for 30 days. Feed consumption was measured for each tank daily before and after each feeding according to Dwyer et al. (2002).

2.3 Sample collection

During the feeding trial, sampling was performed at the end of each phase after 16h of fasting. The fish were anesthetized with eugenol (25 μL L^{-1}) and individually weighed with a precision electronic scale (Bel®, model S2202H, Piracicaba, Brazil) for growth analysis. Blood was collected from the caudal vein (n=6 fish per tank, totaling 18 fish per treatment) with a syringe containing EDTA (4%), immediately centrifuged at 1000g (Beckman Coulter Microfuge® 20R) for 10 min, and the plasma collected and stored at -80°C for further analysis. Then, all fish were euthanized by medullary section and the internal organs were dissected. Samples were placed in cryogenic tubes, frozen in liquid nitrogen, and stored at -80 °C for subsequent determination of antioxidant status (liver) and digestive enzyme activity (intestine) and fixed for histological analysis

(intestine) according to the methods described in 2.6 section. Then, the whole fish was stored for determination of proximate composition.

2.4 Growth parameters

The growth performances of fish were calculated according to the equations established by Jobling (1995).

Weight gain (WG, %) = [(*Individual final wet weight - Individual initial wet weight*)/(*Initial wet weight*)]*100

Specific growth rate (SGR, %) = [(*Ln individual final wet weight - Ln individual initial wet weight*)/*days*]*100

Feed intake (FI, g day $^{-1}$) = [(*Feed consumption per tank* (g)/*number of fish*)/*days*)]

Feed conversion ratio (FCR) = *Feed intake*/*weight gain*

Survival (%) = (*final fish number*)/ (*initial fish number*) *100

Where Ln = *natural logarithm and days = the number of experimental days*.

2.5 Digestive enzymes

Intestinal tissues were weighed on an analytical scale (Shimadzu AUW220D) and homogenized (Ultra Turrax T18) in an ice-cold buffer solution (tris-HCl buffer, pH=7.5) in a 1:5 ratio (weight: volume). Then, the samples were centrifuged (Beckman Coulter Microfuge® 20R) at 3300g for 15 min at 4 ºC and the supernatant was separated, divided into aliquots (5 aliquots; each containing 500 µL), and kept at -80°C for subsequent determination of enzymatic activities.

Amylase (E.C.3.2.1.1), lipase (E.C. 3.1.1.3), and alkaline phosphatase (E.C. 3.1.3.1) activities were evaluated using commercial kits (Gold Analisa Cat. 311, 304 and 440, respectively) adapted for fish intestine samples. Chymotrypsin activity (E.C 3.4.21.1) was determined by using N-benzoyl-L-tyrosine ethyl ether (BTEE) as substrate and absorbance read at 256 nm (Worthington, 1991). Trypsin activity (E.C 3.4.21.4) was

determined using Nα-benzoyl-L-arginine-4-nitroanilide (BApNA) as substrate and absorbance read at 410 nm (Erlanger et al., 1961).

The protein content of the crude extracts was determined by the Bradford method (1976). All samples were analyzed in triplicate using a microplate reader (Multiskan GO, Thermo Scientific, Waltham, Massachusetts, USA). Digestive enzyme results are expressed as specific activity (U mg protein^{-1}).

2.6 Intestinal histomorphometry

Fragments (~1 cm) of the midgut portion were washed in saline (0.9%) and fixed in 10% buffered formalin for 48 h. After dehydration in graded concentrations of ethanol (70-100%), the samples were clarified in xylol and embedded in paraffin. Six sections (4 μm) per fragment were obtained using a microtome (Lupetec MRP09) and placed on histological slides. The slides were stained with hematoxylin and eosin (Feldman and Wolfe, 2014) and each section was photo-documented with a digital camera (AxioCam ICc3; Zeiss) attached to a microscope (Axio Scope; Zeiss) with a 20x magnification. The images were used to determine the mucosal height as well as the length and width of the villi (average value was calculated for 15 villi/sample) using the Image Pro-Plus software (Media Cybernetics, Rockville, USA).

2.7 Whole-body proximate composition

The proximate composition of whole fish was analyzed according to the Association of Official Analytical Chemists (AOAC, 2000) standards. Moisture was evaluated by drying the samples in an oven at 105 ˚C until constant weight; crude protein by the Kjeldahl method after acid digestion, lipid by the Soxhlet method, and ash by incineration at 550 ˚C in a muffle furnace (Fisher Scientific®). All analyzes were performed in triplicate.

2.8 Plasma metabolites

Commercial kits were used to quantify glucose (Glucose HK Liquiform, Cat. 137), cholesterol (Cholesterol Liquiform, Cat. 76), triglycerides (Triglycerides Liquiform, Cat. 87), aspartate aminotransferase (Kovalent, Cat. 47) and alanine aminotransferase (Kovalent Cat. 51) in plasma. Analyzes were performed in triplicate in 96-well plates and absorbance was read in a spectrophotometer (Multiskan GO, Thermo Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions.

2.9 Oxidative stress

For the preparation of the crude extract, liver samples were weighed (Shimadzu AUW220D) and homogenized (Ultra Turrax T18) in ice-cold saline solution (NaCl 0.138 M; KCl 0.0027 M; pH 7.2) in the proportion of 1:10 (weight: volume). After centrifugation (Beckman Coulter Microfuge® 20R) at 3300g for 15 min at 4°C, the supernatant was separated, divided into aliquots (6 aliquots; each containing 500 µL), and kept at -80°C for further analysis.

Catalase activity (CAT; EC 1.11.1.6) was measured by assessing H_2O_2 reduction at 240 nm according to Aebi (1984). The activity of superoxide dismutase (SOD; EC 1.15.1.1) was determined based on the auto-oxidation of pyrogallol by the method of Madesh and Balasubramanian (1997) and absorbance read at 570 nm. Glutathione peroxidase (GPX; EC 1.11.1.9) activity was determined by measuring the oxidation rate of nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of H_2O_2 using reduced glutathione (GSH) and glutathione reductase (GR) as substrates at 340 nm (Günzler et al., 1984) Glutathione reductase (GR; EC 1.8.1.7.) activity was determined by measuring NADPH oxidation at 340 nm, as described by Carlberg and Mannervik (1975). Glutathione-S-transferase (GST; EC 2.5.1.18) activity was quantified at 340 nm using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate according to Habig et al. (1974).

Lipid peroxidation was assessed by measuring the production of thiobarbituric acid reactive substances (TBARS) at 530 nm (Buege and Aust, 1978). Protein carbonyl (PC) content was determined after the derivation of 2,4-Dinitrophenylhydrazine (DNPH) (Reznick and Packer, 1994).

The Bradford method was used to determine the protein levels of the extracts. All samples were analyzed in triplicate using a microplate reader (Multiskan GO, Thermo

Scientific, Waltham, Massachusetts, USA). Enzyme activities were expressed as specific activities.

2.10 Statistical analysis

Data were analyzed using the IBM SPSS software version 25 (IBM Corp. USA) and are expressed as mean ± standard deviation. All data were analyzed for normality (Shapiro Wilk test) and homogeneity (Levene test) of their variances and transformed using the Box-Cox model when not normal. ANOVA followed by Tukey's post hoc test was used to assess differences among treatments in their respective phases. Except for zootechnical performance variables, differences in the same treatment at different stages were compared using the T-test. The probability level of 0.05 was used to reject the null hypothesis. The maximum feeding rate was determined based on the specific growth rate using second-order polynomial regression.

3. RESULTS

3.1 Growth parameters

The results obtained from the feeding trial are shown in Table 1. These results indicate that the growth performance of *C. macropomum* juveniles was affected ($P < 0.05$) by feeding and refeeding rates. However, survival was not ($P > 0.05$) affected by the treatments.

After the restricted feeding phase, the final weight, weight gain, and specific growth rate were higher ($P < 0.05$) in the FR 6%, FR 8%, and FR 10% groups, while the feed conversion ratio was higher ($P < 0.05$) in the FR 8% and FR 10% groups. The second-degree polynomial relationship between specific growth rate and feeding rate estimated that the FR for maximum growth was 8.98% (Fig 1).

At the beginning of the refeeding, the final weight was significantly different ($P < 0.05$) as the fish were fed with different FR during phase 1. At the end of phase 2, the final weight was lower ($P < 0.05$) in the FR 2% group, while weight gain and specific

growth rate were lower ($P < 0.05$) in the FR 10% group compared to the FR 4% group. The FR 2% group exhibited the highest ($P < 0.05$) feed conversion ratio in phase 2.

3.2 Digestive enzymes

The specific activity of different digestive enzymes during the restricted feeding and refeeding phase are shown in Fig 2. The specific activity of digestive enzymes including chymotrypsin, amylase, lipase, and alkaline phosphatase showed significant changes ($P < 0.05$) among different FRs in phase 1. Briefly, chymotrypsin activity (Fig. 2B) in the FR 8% group was greater ($P < 0.05$) than in the FR 2% group. Amylase (Fig. 2C) and alkaline phosphatase (Fig. 2E) activities were lower ($P < 0.05$) in the FR 2% group compared to the FR 4%, FR 6%, and FR 8% groups. Lipase (Fig. 2D) was lower ($P < 0.05$) in FR 2% than FR 8% and FR 10% groups. Trypsin activity (Fig. 2A) did not differ ($P > 0.05$) among different groups in phase 1.

After the refeeding phase, specific trypsin activity (Fig. 2A) in the FR 2% and FR 4% groups was higher ($P < 0.05$) than in the other groups. There were no differences in chymotrypsin, amylase, lipase, and alkaline phosphatase activities in the refeeding phase. Also, when comparing different phases (phase 1 *versus* phase 2), there was compensation in the specific activity of trypsin, chymotrypsin, lipase, and alkaline phosphatase ($P < 0.05$) at the end of the refeeding phase for the FR 2% group. Besides, for the FR 4% group, the specific activities of trypsin and chymotrypsin were higher ($P < 0.05$) in the refeeding phase.

3.3 Intestinal histomorphometry

The results of midgut histomorphometry during the restricted feeding and refeeding phase are shown in Fig. 3. Feeding rates affected ($P < 0.05$) villi height (Fig. 3A) in the restricted feeding phase and refeeding phase, the same was observed for villi length (Fig. 3B) in the restricted feeding phase. There was no effect ($P > 0.05$) of treatments on mucosal height (Fig. 3C).

3.4 Whole-body proximate composition

Results of the proximate composition of *C. macropomum* juveniles during the restricted feeding and refeeding phases are shown in Table 2. In the restricted feeding phase, lipid and moisture contents showed significant changes ($P < 0.05$) among different treatments. The FR 2% group had the highest moisture but lowest lipid content, which was significantly ($P < 0.05$) lower than the lipid content observed in fish from the FR 10% group. The protein and ash contents in phase 1 and all variables of the centesimal composition of phase 2 were not affected by the different treatments ($P > 0.05$).

3.5 Plasma metabolites

The results of plasma metabolite levels of *C. macropomum* juveniles during the restricted feeding and refeeding phase are presented in Table 3. In the restricted feeding phase, the FR 2% group had a lower value ($P < 0.05$) of total cholesterol and triglycerides and higher ($P < 0.05$) AST compared to the FR 8% and FR 10% groups. Glucose in phase 1 and ALT and phase 2 were unaffected by the different treatments ($P > 0.05$). After refeeding, there was an increase in glucose and triglycerides in the FR 2%, FR 4%, and FR 6% groups, cholesterol in FR 2%, and a 39.9% decline in AST in this group.

3.6 Oxidative stress

The results of the oxidative stress indices of *C. macropomum* juveniles during the restricted feeding and refeeding phase are shown in Table 4. In the restricted feeding phase, SOD and CAT activities were higher in the FR 2% compared to the other groups, while GPX activity was higher in the FR 2% compared to the FR 4% and FR 6% groups. GR activity was higher in the FR 8% and FR 10% groups and different from the FR 2% group. GST activity was not affected by different treatments. Regarding oxidative damage, the levels of lipid peroxidation and carbonyl protein (MDA and PCO)were higher in FR 2% compared to the other groups. After refeeding, GPX and GR activities were higher in FR 2% compared to the other groups. Also, when comparing different phases (phase 1 *versus* phase 2), we found that the FR 2% group showed a reduction in SOD ($p=0.0063$), CAT ($p=0.0156$), lipid peroxidation ($p=0.0472$), and carbonyl protein activity ($p=0.0077$) and an increase in GR activity ($p=0.0052$).

Table 1. Effects of feeding rate on growth performance of *Colossoma macropomum* during restricted feeding (30 days) and refeeding (30 days) phases.

Growth parameters	Feeding rates (% body weight day^{-1})					*P*-value
	2	4	6	8	10	
Restricted feeding phase						
Initial weight (g fish^{1})	7.17 ± 0.08	7.08 ± 0.15	7.13 ± 0.12	7.02 ± 0.07	7.04 ± 0.22	0.634
Final weight (g fish^{1})	13.39 ± 0.14^{c}	19.69 ± 0.58^{b}	27.99 ± 2.21^{a}	29.23 ± 1.13^{a}	30.91 ± 4.24^{a}	0.000
Weight gain (%)	86.63 ± 3.34^{c}	178.35 ± 13.73^{b}	297.80 ± 35.04^{a}	316.68 ± 19.26^{a}	338.03 ± 47.26^{a}	0.000
Specific growth rate (% day fish^{1})	2.07 ± 0.06^{c}	3.40 ± 0.17^{b}	4.55 ± 0.31^{a}	4.75 ± 0.14^{a}	4.91 ± 0.37^{a}	0.000
Feed intake (g day fish^{1})	0.14 ± 0.00^{e}	0.29 ± 0.00^{d}	0.50 ± 0.00^{c}	0.75 ± 0.02^{b}	0.93 ± 0.03^{a}	0.000
Feed conversion ratio	0.78 ± 0.02^{a}	0.85 ± 0.04^{a}	0.88 ± 0.11^{a}	1.22 ± 0.05^{b}	1.43 ± 0.23^{b}	0.001
Survival (%)	100	100	100	100	100	-
Refeeding phase						
Initial weight (g fish^{1})	13.39 ± 0.14^{c}	19.69 ± 0.58^{b}	27.99 ± 2.21^{a}	29.23 ± 1.13^{a}	30.91 ± 4.24^{a}	0.000
Final weight (g fish^{1})	28.49 ± 3.55^{b}	47.28 ± 3.45^{a}	54.41 ± 1.48^{a}	56.56 ± 4.92^{a}	57.40 ± 7.49^{a}	0.002
Weight gain (%)	113.02 ± 28.81^{ab}	140.39 ± 20.09^{a}	95.48 ± 20.99^{ab}	93.43 ± 13.59^{ab}	85.99 ± 7.71^{b}	0.042
Specific growth rate (% day fish^{1})	2.50 ± 0.44^{ab}	2.91 ± 0.29 ^{a}	2.22 ± 0.35^{ab}	2.20 ± 0.24^{ab}	2.06 ± 0.14 ^{b}	0.044
Feed intake (g day fish^{1})	1.07 ± 0.12	1.22 ± 0.12	1.10 ± 0.23	1.35 ± 0.21	1.13 ± 0.18	0.353
Feed conversion ratio	2.60 ± 0.43^{b}	1.62 ± 0.38^{a}	1.49 ± 0.15^{a}	1.78 ± 0.08^{ab}	1.55 ± 0.33^{a}	0.007
Survival (%)	100	100	100	100	100	-

Values are expressed as mean ± standard deviation. Different letters in the lines indicate significant differences among feeding rates (one-way ANOVA followed by Tukey's post hoc test, $P < 0.05$). N = 20 fish per biological replicate (tank); 60 fish per treatment in total.

Table 2. Mean body composition (% wet weight) of *Colossoma macropomum* juveniles during the restricted feeding phase (30 days) and refeeding phase (30 days).

Proximate composition	Feeding rates (% body weight day^{-1})					
	2	4	6	8	10	*P*-value
Restricted feeding phase						
Protein	15.62 ± 0.76	16.20 ± 0.58	16.75 ± 0.61	17.43 ± 0.77	17.20 ± 0.92	0.073
Lipids	6.33 ± 1.00^{b}	7.03 ± 0.31^{b}	7.69 ± 0.52^{ab}	8.00 ± 0.61^{ab}	8.71 ± 0.44^{a}	0.008
Ash	5.01 ± 0.18	5.29 ± 0.30	5.36 ± 0.19	5.24 ± 0.43	5.28 ± 0.46	0.734
Moisture	70.92 ± 1.32^{a}	68.94 ± 0.81^{ab}	67.67 ± 0.97^{ab}	66.86 ± 2.21^{b}	66.72 ± 1.40^{b}	0.025
Refeeding phase						
Protein	16.17 ± 0.64	16.98 ± 0.82	17.75 ± 1.15	17.96 ± 0.47	17.85 ± 0.50	0.071
Lipids	6.93 ± 0.25	7.52 ± 0.56	7.19 ± 0.65	7.70 ± 0.72	8.34 ± 0.60	0.103
Ash	4.87 ± 0.27	4.96 ± 0.06	5.13 ± 0.09	5.05 ± 0.08	4.85 ± 0.16	0.187
Moisture	67.98 ± 1.74	67.53 ± 0.61	67.19 ± 1.27	67.23 ± 0.83	66.30 ± 0.98	0.957

Values are expressed as mean ± standard deviation. Different letters in the lines indicate significant differences among feeding rates (one-way ANOVA followed by Tukey's post hoc test, $P < 0.05$). N = 6 fish per biological replicate (tank); 18 fish per treatment in total.

Table 3. Plasma metabolites of *Colossoma macropomum* juveniles during the restricted feeding phase (30 days) and refeeding phase (30 days).

Plasma metabolites	Feeding rates (% body weight day^{-1})					*P*-value
	2	4	6	8	10	
Restricted feeding phase						
Glucose (mg dL^{-1})	76.43 ± 8.00	83.09 ± 5.49	84,80 ± 3.34	88,22 ± 2.86	88.79 ± 3.44	0.074
Cholesterol (mg dL^{-1})	86.12 ± 14.61^{c}	115.51 ± 15.78^{abc}	108.26 ± 7.85^{bc}	126.81 ± 19.8^{ab}	152.21 ± 12.15^{a}	0.003
Triglycerides (mg dL^{-1})	131.21 ± 14.29^{b}	161.71 ± 15.90^{ab}	163.65 ± 16.28^{ab}	190.34 ± 18.70^{a}	194.10 ± 28.50^{a}	0.016
Aspartate aminotransferase (U L^{-1})	61.56 ± 11.00 ^{a*}	42.15 ± 5.02^{ab}	48.67 ± 9.29^{ab}	40.43 ± 6.27 ^{b}	38.31 ± 4.04^{b}	0.023
Alanine aminotransferase (U L^{-1})	17.20 ± 2.14**	14.54 ± 0.94	13.37 ± 3.70	15.88 ± 1.53	14.27 ± 1.40	0.293
Refeeding phase						
Glucose (mg dL^{-1})	98.04 ± 5.37*	99.06 ± 7.74*	100.13 ± 5.24*	95.11 ± 6.89	96.98 ± 6.77	0.892
Cholesterol (mg dL^{-1})	116.90 ± 11.97*	137.00 ± 7.63	120.07 ± 11.50	117.52 ± 9.25	134.10 ± 7.74	0.077
Triglycerides (mg dL^{-1})	203.40 ± 24.70**	191.99 ± 9.10*	192.96 ± 8.14*	197.18 ± 6.23	214.48 ± 11.33	0.305
Aspartate aminotransferase (U L^{-1})	41.28 ± 5.48	43.28 ± 10.15	38.25 ± 5.19	33.72 ± 6.14	37.13 ± 9.02	0.586
Alanine aminotransferase (U L^{-1})	9.78 ± 1.75	11.80 ± 2.22	12.15 ± 2.59	13.27 ± 2.49	14.52 ± 2.47	0.219

Values are expressed as mean ± standard deviation. N = 6 fish per biological replicate (tank); 18 fish per treatment in total. Different letters in the lines indicate significant differences among feeding rates (one-way ANOVA followed by Tukey's post hoc test, $P < 0.05$). T-test was used to compare the same feeding rate in different treatment phases: restricted feeding and refeeding (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Table 4. Antioxidant enzyme activity (U mg of protein), protein carbonylation index (PC, U mg of protein), and lipid peroxidation index (TBARs, U mg of protein) of *Colossoma macropomum* juveniles during the restricted feeding (30 days) and refeeding phases (30 days).

Oxidative stress	Feeding rates (% body weight day^{-1})					*P*-value
	2	4	6	8	10	
Restricted feeding phase						
SOD[1]	102.20 ± 19.72^{a**}	32.19 ± 7.04^{b}	33.03 ± 7.45^{b}	44.95 ± 8.47^{b}	36.70 ± 8.53^{b}	0.000
CAT[2]	239.85 ± 37.61^{a}	146.09 ± 30.78^{b}	141.04 ± 25.17^{b}	152.32 ± 28.43^{b}	110.73 ± 23.60^{b}	0.004
GPX[3]	5.63 ± 1.23^{a}	3.03 ± 0.93^{b}	3.31 ± 0.67^{b}	4.26 ± 0.43^{ab}	3.93 ± 0.47^{ab}	0.018
GR[4]	28.56 ± 5.86^{b}	51.00 ± 10.08^{ab}	49.78 ± 13.22^{ab}	58.23 ± 8.84^{a}	57.96 ± 5.02^{a}	0.017
GST[5]	30.74 ± 5.97	32.70 ± 6.35	26.10 ± 5.01	28.73 ± 8.18	25.33 ± 6.56	0.622
PC[6]	96.01 ± 18.94^{a**}	47.24 ± 15.70^{b}	51.79 ± 12.83^{b}	54.70 ± 12.41^{b}	34.62 ± 7.92^{b}	0.003
TBARS[7]	2.81 ± 0.59^{a}	1.17 ± 0.15^{b}	0.98 ± 0.28^{b}	0.77 ± 0.18^{b}	0.67 ± 0.18^{b}	0.000
Refeeding phase						
SOD[1]	39.36 ± 6.53	43.34 ± 10.80	51.51 ± 16.45	48.01 ± 17.27	52.06 ± 10.84	0.719
CAT[2]	132.53 ± 26.41*	137.92 ± 31.49	146.29 ± 41.46	124.07 ± 14.89	148.89 ± 24.27	0.828
GPX[3]	5.79 ± 0.97^{a}	3.11 ± 0.69^{b}	3.59 ± 0.76^{b}	3.65 ± 0.77^{b}	3.29 ± 0.46^{b}	0.008
GR[4]	79.46 ± 14.84^{a**}	42.92 ± 6.97^{b}	45.41 ± 12.34^{b}	46.84 ± 7.08^{b}	47.03 ± 9.58^{b}	0.000
GST[5]	25.33 ± 4.90	22.12 ± 3.67	29.65 ± 1.33	29.48 ± 4.16	21.94 ± 4.27	0.106
PC[6]	35.38 ± 9.48	43.90 ± 8.90	50.31 ± 6.56	49.05 ± 13.16	47.98 ± 7.80	0.357
TBARS[7]	1.79 ± 0.18^{a*}	1.63 ± 0.39^{ab}	1.00 ± 0.21^{c}	1.09 ± 0.15^{bc}	0.81 ± 0.17^{c}	0.002

Values are expressed as mean ± standard deviation. N = 3 fish per biological replicate (tank); 9 fish per treatment in total. Different letters in the lines indicate significant differences among feeding rates (one-way ANOVA followed by Tukey's post hoc test, $P < 0.05$). T-test was used to compare the same feeding rate in different treatment phases: restricted feeding and refeeding (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

[1]Superoxide dismutase. [2]Catalase. [3]Glutathione peroxidase. [4] Glutathione reductase. [5]Glutathione-S-transferase. [6]Protein carbonyl. [7]Thiobarbituric Acid Reactive Substances - Lipid peroxidation indicator.

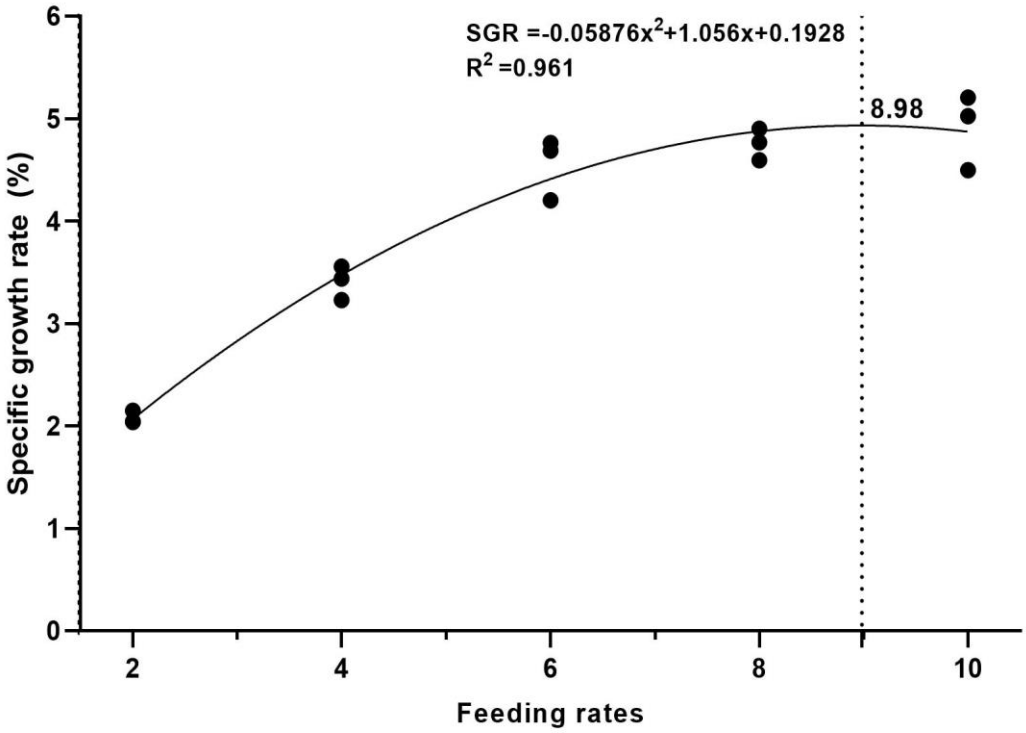

Figure 1. The maximum feeding rate of *Colossoma macropomum* juveniles based on specific growth rate determined by second-order polynomial regression.

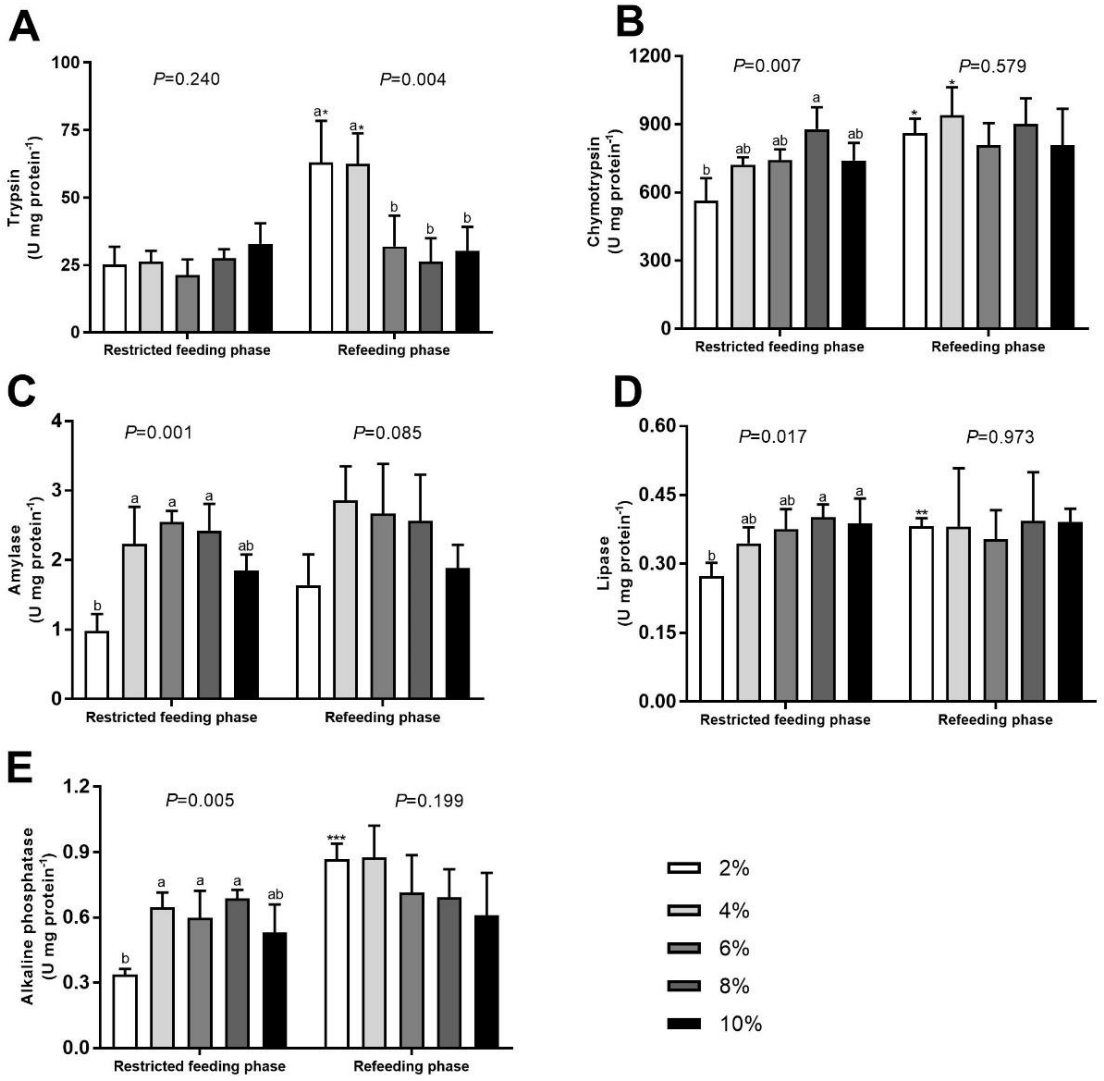

Figure 2. Specific activities of trypsin (A), chymotrypsin (B), amylase (C), lipase (D), and alkaline phosphatase (E) of *Colossoma macropomum* juveniles during the restricted feeding phase (30 days) and refeeding phase (30 days). Values are expressed as mean ± standard deviation. N = 3 fish per biological replicate (tank); 9 fish per treatment in total. Different letters in the lines indicate significant differences among feeding rates (one-way ANOVA followed by Tukey's post hoc test, $P < 0.05$). T-test was used to compare the same feeding rate in different treatment phases: restricted feeding and refeeding (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

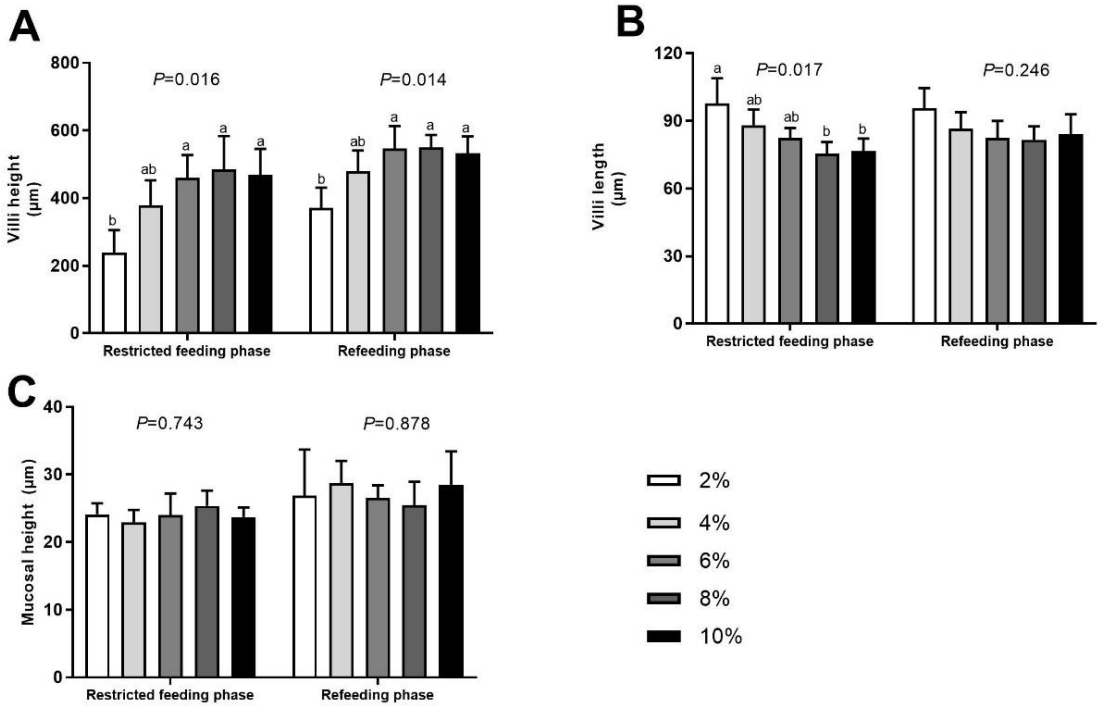

Figure 3. Intestinal histomorphometry of villi height (A), villi width (B) and Mucosal height of *Colossoma macropomum* juveniles during the restricted feeding phase (30 days) and refeeding phase (30 days). Values are expressed as mean ± standard deviation; n= 3 fish per biological replicate; 9 fish per treatment in total. Different letters in the lines indicate significant differences among feeding rates (one-way ANOVA followed by Tukey's post hoc test, $P < 0.05$). T-test was used to compare the same feeding rate in different treatment phases: restricted feeding and refeeding (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

4. DISCUSSION

Feed is a crucial parameter for fish growth and feed efficiency. In this sense, understanding the relationship between FR and growth response is essential to optimize feeding strategies (Alanärä et al., 2001; Carter, 2015). The present study sought to understand the general growth performance pattern of *C. macropomum* juveniles in response to different feeding levels. The results of our study indicate that the optimal FR for the growth of *C. macropomum* juveniles grown in a water recirculation system that results in maximum growth is 8.89% day $^{-1}$. However, Silva et al. (2007) suggested a FR of 10 % day $^{-1}$ for *C. macropomum* juveniles (2 g) kept in net cages. On the other hand, Porto et al. (2018) observed that the daily FR for *C. macropomum* juveniles (30 g) grown in a net tank is 6% day $^{-1}$. Discrepancies between our recent findings and those of other studies for this species may be due to differences in the developmental stage of the fish, as well as in experimental conditions, stocking density, feeding strategy, and diet quality (Kestemont et al., 2003; Lee et al., 2019).

Feed utilization followed a different pattern than weight gain in this study. The values of feed conversion increased with the increase of the FR, being the best ones obtained with levels lower than those that allowed the maximum growth. Similar results were also reported in other fish species, such as *Sparus aurata* (Mihelakakis et al., 2002), *Megalobrama amblycephala* (Tian et al., 2015), *Myxocyprinus asiaticus* (Yuan et al., 2010), *Heteropneustes fossilis* (Khan and Abidi, 2010) and *Pseudobagrus ussuriensis* (Bu et al., 2017). According to Eroldoğan et al. (2004), the best feeding levels would be those in which fish are fed at a submaximal level. Increasing the feeding rate above the optimal level results in overfeeding and reduced feed efficiency, as fish are not able to utilize excess nutrients for growth (Baloi et al., 2014; Guo et al., 2020). Indeed, in commercial fish farming, the ideal feeding rate should consider both growth and feed conversion to optimize economic profitability (Jauralde et al., 2011).

Among the advantages of adopting restriction and refeeding strategies in fish farming is the compensatory growth of the animals, which use some physiological mechanisms, such as hyperphagia and improved feed conversion for growth recovery (Py et al., 2022; Won and Borski, 2013; Xiao et al., 2012). In our study, the total compensatory growth in fish from the FR4% group can be, at least in part, attributed to an improvement in feed conversion ratio and not to an increase in feed intake. Improved feed efficiency after fasting may also be associated

with increased digestive enzyme activities or nutrient digestibility (Furné et al., 2008; Lingam et al., 2019).

Changes in growth performance are commonly associated with differences in digestive enzyme activities, which reflect the degree of digestion and absorption of nutrients (Caruso et al., 2014; Krogdahl and Bakke-McKellep, 2005). The FR 2% group showed depressed activity of chymotrypsin, amylase, lipase, and alkaline phosphatase enzymes. These results are consistent with changes in growth performance obtained in this study. The decrease in pancreatic enzymes may be related to the absence of digestive products that act directly on pancreatic acinar cells to stimulate enzyme production and secretion, while the decrease in intestinal enzymes may be related to the reduction of the surface area for absorption and intestinal mucosa (Gisbert et al., 2011; Rønnestad et al., 2013). We observed a decrease in intestinal digestive activities in the FR 10% group. We believe that these enzymes were influenced by overeating, overloading the digestive system, and increasing peristalsis, thus preventing food from being efficiently digested and absorbed (Oyarzún et al., 2019). At the end of refeeding, trypsin activity was overcompensated by 149% and 137% in the FR 2% and FR 4% groups, respectively. The increase in trypsin secretion in these groups may be the result of increased protein intake previously reduced by the FR (Bakke et al., 2010). In addition to trypsin, there was compensation in the activity of chymotrypsin, lipase, and alkaline phosphatase activities in FR 2% and chymotrypsin in the FR 4% group. The maintenance of enzymatic activity at FR 4% during the restricted feeding phase may be one of the factors that ensured compensatory growth after refeeding (Ali et al., 2003).

Alkaline phosphatase is mainly distributed in the intestinal brush border membrane, and its role is associated with the hydrolysis of inorganic phosphates for energy production and nutrient absorption in the intestine (Castro-Ruiz et al., 2021; Gisbert et al., 2018). According to Lallès (2019), feed intake is one of the main drivers of alkaline phosphatase enzymatic activity in fish. The results of this study indicated that alkaline phosphatase activity was lower for fish fed with FR 2%, but its activity was compensated for refeeding phase.

The digestive capacity of fish is also related to the absorption surface area of the intestinal mucosa (Bakke et al., 2010). In our study, the morphometric parameters analyzed in the midgut, except for the thickness of the intestinal mucosa, were affected by the feeding rate. Compromised intestinal mucosa, evidenced by the shortening of the villi and the wide space between them, leads to a decrease in the ability to assimilate and absorb nutrients, which may be responsible for the decline in the activities of digestive enzymes found in the FR 2% group.

Changes in the structure of the intestinal mucosa were also found in *Rutilus rutilus caspicus* (Abolfathi et al., 2012) and *Pterygoplichthys disjunctivus* (German et al., 2010) deprived of food. The intestinal mucosa is a dynamic structure and is highly flexible to the availability of nutrients (Abolfathi et al., 2012; Hall and Bellwood, 1995). Our results suggest that the increase in food availability in the refeeding phase provided sufficient nutrients to the intestinal lumen for recovery of the intestinal epithelium (Wilson and Castro, 2010).

Changes in body composition are related to endogenous and exogenous availability of food, elucidating changes in the centesimal composition of the whole body in response to the feeding strategy as an indicator of the nutritional status of fish (Gallardo-Collí et al., 2020; Liu et al., 2022). Body composition can be affected by FR, but the changes that occur in protein, lipid, ash, and moisture contents differ between species (Guo et al., 2020; Khan and Abidi, 2010; Kim et al., 2021). We verified a reduction in the body lipid content of tambaqui in the FR 2 % group compared to FR 8% and FR 10% treatments. The inverse trend occurred in the moisture content which decreased with increasing FR. Inverse relationships between the percentages of lipids and moisture were also observed for other fish species, such as *Sardinella brasiliensis* (Baloi et al., 2017), *Dicentrarchus labrax* (Eroldoğan et al., 2004) and *Argyrosomus regius* (Bu et al., 2017). A possible explanation was proposed by McCue (2010) who stated that the excess food consumed by fish at higher FR is converted into energy reserves and stored in adipose tissues, mainly in the form of lipids, replacing water. In contrast to lipids, body protein showed a not statistical significance decline in fish fed the lowest FR. These results suggest that the mobilization of fat as the main energy source continues until a critical threshold is reached, after which tissue protein becomes the main energy source (Krogdahl and Bakke-McKellep, 2005; Navarro and Gutiérrez, 1995; Pérez-Jiménez et al., 2011). After refeeding, the proximal composition was restored in the FR 2% and FR 4% groups compared to the other treatments, indicating that the fish were able to restore the previously reduced lipid levels.

Plasma nutrient depletion is inevitable during starvation and feed restriction (Pérez-Jiménez et al., 2011). Plasma metabolites, except for glucose and alanine aminotransferase, were affected by FR. Changes in serum levels were also found in *Ictalurus punctatus*, *Sardinella brasiliensis* (Baloi et al., 2017), and *Cyprinus carpio* (Shimeno et al., 1997) under these conditions. We found that the lower plasma concentrations of triglycerides and cholesterol in fish that received a daily diet of 2%, compared to levels above 8%, probably occurred due to nutrient insufficiency. Glucose is an important energy substrate responsible for providing energy to tissues and cells (Polakof et al., 2012). However, during starvation, a decrease in

plasma glucose levels is commonly observed in fish (Ashouri et al., 2019; Jafari et al., 2018; Tamadoni et al., 2020). We verified that despite the differences in the nutritional status of the animals, the glycemic levels remained unaltered for all groups. We believe that the glucose requirement to meet the energy demand was met by the hydrolysis of hepatic glycogen or by gluconeogenesis (Li et al., 2018; Navarro and Gutiérrez, 1995).

Serum AST and ALT markers are commonly used to assess liver function in fish (Coz-Rakovac et al., 2008). The increase in AST as in our study was also observed in *Sebastes schlegeli* fed at the lowest FR, which was possibly due to underfeeding stress (Mizanur et al., 2014). Another possible explanation for the increase in plasma AST levels would be the mobilization of protein as a substrate for gluconeogenesis to satisfy energy demands (Ashouri et al., 2019) since factors such as excessive protein intake, hunger, or stress cause increased catabolism of amino acids for energy production (Dabrowski and Guderley, 2003; Li et al., 2009; Pérez-Jiménez et al., 2011).

Plasma metabolites were restored after refeeding, indicating that these parameters are easily restored to normal once the restriction ceases (Navarro and Gutiérrez, 1995; Pérez-Jiménez et al., 2011; Py et al., 2022). The nutrients contained in the diet are quickly digested and made available in the blood, normalizing cholesterol and triglyceride levels (Gisbert et al., 2004; Rønnestad et al., 2017). Furthermore, the return to normal AST levels indicates that protein degradation has possibly ceased (Mizanur et al., 2014).

Among all antioxidant enzymes, SOD, CAT, GPX, and GR constitute the main enzymatic defense mechanisms against oxidative stress (Furné et al., 2009; Pérez-Jiménez et al., 2011). The increase in SOD activity in FR 2% indicates a high production of superoxide anion (O_2^-) and greater generation of H_2O_2, which may be the reason for the high hepatic activities of CAT and GPX. Although there is no general pattern regarding the behavior of the main primary antioxidant defenses in aquatic organisms, our results are consistent with other species in which short or long-term starvation increased the activities of hepatic antioxidant enzymes (Furné et al., 2009; Morales et al., 2004). We hypothesize that due to restricted feeding, the animals in the FR 2% group were consuming their endogenous reserves to meet their energy needs. In this sense, oxidative phosphorylation would also be more active because of the need for ATP. It is known that the increase in ATP production leads to an increase in the production of reactive oxygen species (ROS). Therefore, we believe that this is the reason for the increased activity of detoxifying enzymes in the FR 2% group. Although antioxidant defense mechanisms were enhanced, some ROS managed to escape the detoxifying enzyme

systems and induced lipid peroxidation and protein oxidation in the liver of *C. macropomum* in the FR 2% group. The decrease in GR activity may be responsible for the failure of antioxidant defenses in FR 2% since the reduction in the activity of this enzyme can cause a decline in the regeneration of GSSG into GSH, as observed in *Dicentrarchus labrax* (Sinha et al., 2015). Furthermore, the parallel increase of GPX with GR at FR 8% and FR 10% indicates an efficient turnover of GSH, suggesting a highly effective GSH-based antioxidant system in mitigating oxidative damage mediated by increased FR. Proving this efficiency, TBARS and PC levels were low compared to FR 2%, indicating that the processes of lipid peroxidation and protein oxidation were not activated by the excess of ROS production, and consequently, lipids and proteins were protected against oxidation.

Refeeding promoted a reduction in SOD and CAT activity without a decrease in GPX activity and an increase of 178.2% in GR activity in FR 2%. In this group, the PC level was similar to the other treatments, indicating that protein carbonylation was neutralized after refeeding. The TBARS level decreased by 36.2%, but its value remained high compared to those observed in the other treatments, except for FR 4% which increased by 15.8%. Possibly, ATP generation was increased by refeeding to compensate for the previous reduction in food, therefore, a greater amount of ROS was induced during this phase, leading to lipid peroxidation (Birben et al., 2012; Schieber and Chandel, 2014). Furthermore, the refeeding period (30 days) may not have been sufficient to allow lipid peroxidation levels to return to the values of the other groups.

5. CONCLUSIONS

In conclusion, our results showed that feeding *C. macropomum* juveniles with a FR of 6 % day $^{-1}$ resulted in better growth and feed efficiency. In addition to growth, FR influenced physiological responses, indicating that feeding fish with 2% day^{-1} resulted in reduced enzymatic activity, compromised intestinal health and intermediary metabolism, and caused lipid and protein damage. With refeeding, the altered physiological variables tended to normalize, but growth was not restored in animals fed with 2% day^{-1}. However, total compensatory growth was observed in tambaqui juveniles fed at a FR of 4% day^{-1}.

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PARTE IV – Artigo III

Sensibilidade de diferentes órgãos e tecidos como biomarcadores de estresse oxidativo em *Colossoma macropomum* submetidos ao jejum

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Artigo que será submetido para publicação em revista científica

Resumo

O presente estudo foi conduzido para avaliar os efeitos da fome nas respostas de biomarcadores oxidativos e de defesas antioxidantes utilizando uma abordagem de diagnóstico celular (em vários tecidos) em *Colossoma macropomum*. Os peixes foram separados em dois grupos: controle (alimentados) e jejum (7 dias). Ao final do teste de alimentação os peixes foram amostrados para avaliação dos biomarcadores de estresse oxidativo (MDA-peroxidação lipídica e PCO- proteína carbonil) e defesas antioxidantes (SOD-superóxido dismutase; CAT-catalase; GPX-glutationa peroxidase; e GST-glutationa-S-transferase) no fígado, intestino, brânquias, músculo, cérebro e plasma sanguíneo. Os resultados mostraram aumento nas concentrações de MDA e PCO no fígado e intestino dos peixes em jejum. A atividade das enzimas SOD e GPX aumentaram no tecido hepático e intestinal em relação aos seus respectivos controles, enquanto no tecido branquial ocorreu redução da atividade das enzimas SOD e CAT. Houve redução também da atividade da CAT no músculo, enquanto no cérebro não houve alterações nos biomarcadores de estresse oxidativo. O plasma sanguíneo apresentou baixa resposta oxidativa. Em conclusão, nossos resultados confirmam que as respostas antioxidantes são especificas de cada tecido e que o jejum de 7 dias provou estresse oxidativo em *C. macropomum* no fígado e intestino do *C. macropomum*.

Palavras chaves: tambaqui, fome, peroxidação lipídica, oxidação proteica, defesas antioxidantes

1. INTRODUÇÃO

As espécies reativas de oxigênio (ROS) são subprodutos naturais do metabolismo aeróbico e desempenham um papel essencial no controle fisiológico a nível celular do sistema biológico (Barim-Öz, 2018; Brookes, 2005; Mailloux, 2020). Os principais elementos ROS incluem radicais livres como, superóxido ($O_2^{\bullet-}$) e hidroxila ($OH^{\bullet}$) e não radicais, como peroxido de hidrogênio (H_2O_2) (Birben et al., 2012; Halliwell and Gutteridge, 2015). Níveis estacionários de ROS são intrínsecos ao funcionamento normal das células, cumprindo as funções de sinalização celular e homeostase (Dröge, 2002). Todavia, quando produzidos em excesso ou quando as defesas celulares não são capazes de metabolizá-lo, ocorre o estresse oxidativo, danificando os componentes celulares (Juan et al., 2021; Schieber and Chandel, 2014). Sistemas enzimáticos e não enzimáticos como a superóxido dismutase (SOD), catalases (CAT), glutationa peroxidases (GPx) e GSH-S- transferase são os principais mecanismos de defesa antioxidante responsáveis pelo equilíbrio redox (Dröge, 2002; Schieber and Chandel, 2014).

As defesas antioxidantes dos peixes podem ser afetadas por uma infinidades de condições como, flutuações da temperatura, alterações na disponibilidade de oxigênio, poluição por diversos tipos de contaminantes, captura, transporte e manuseio dos animais, desnutrição e restrição alimentar (Corona-Herrera et al., 2018; Lushchak, 2016; Pérez-Jiménez et al., 2011; Py et al., 2022). Estudos demonstraram que o estresse oxidativo alimentar induzido pela fome provoca alterações nas defesas antioxidantes em diferentes magnitudes, e ocasiona unanimemente, aumento da peroxidação lipídica (Bayir et al., 2011; Hasanpour et al., 2021; Yang et al., 2019; Zheng et al., 2016) e, oxidação de proteínas (Bassam AL-Salahy and Ibrahim, 2018; Florescu (Gune) et al., 2021) embora ainda há poucos trabalhos que mensurem este dano induzido pela inanição.

A extensão do dano oxidativo e das respostas nos sistemas de defesa antioxidante em peixes pode variar consideravelmente entre os diferentes órgãos e tecidos , como demonstrado em *Clarias gariepinus* (Bassam AL-Salahy and Ibrahim, 2018),*Salmo trutta* (Bayir et al., 2011) e *Acipenser naccarii e Oncorhynchus mykiss* (Furné et al., 2009). Mesmo com as diferenças nos resultados acima, multi-tecidos foram mensurados em poucos estudos, enquanto, o fígado, brânquias e/ou sangue são os principais órgãos avaliados (Dar et al., 2019; Hasanpour et al., 2021; Huang et al., 2016; Yang et al., 2019; Zheng et al., 2016), mas há também estudos que analisem tais níveis exclusivamente no sistema digestivo (Antonopoulou et al., 2013; Florescu (Gune) et al., 2021; Pan et al., 2022) e músculo (Hidalgo et al., 2017; Wu et al., 2020).

Embora o interesse pelo estresse oxidativo alimentar tenha aumentado nos últimos anos, existe uma forte necessidade de expandir os estudos para outros órgãos e tecidos e avaliar a sensibilidade a resposta antioxidante induzido pelo manejo alimentar. Portanto, neste estudo comparativo, objetivamos investigar as respostas de biomarcadores oxidativos e de defesas antioxidantes em diferentes órgãos e tecidos (fígado, intestino, brânquias, musculo, cérebro, e plasma sanguíneo) em juvenis de *Colossoma macropomum*.

2. MATERIAL E MÉTODOS

O experimento foi conduzido no departamento de zootecnia da Universidade Federal de Lavras (UFLA), Lavras, Minas Gerais, Brasil. Os procedimentos experimentais e coletas de amostras foram aprovados pelo Comitê de ética no Uso de Animais (CEUA) da UFLA, sob protocolo Nº 29/2017.

2.1 Peixes e design experimental

Trinta juvenis de *C. macropomum* provenientes da criação no Laboratório de Aquacultura (LAQUA) da Universidade Federal de Minas Gerais (UFMG, Brasil) foram aclimatados nas unidades experimentais por 2 semanas. As unidades experimentais foram compostas por seis tanques de 100 L, com cinco animais cada, em um sistema de recirculação de água termorregulado com aeração, filtragem e esterilização por ultravioleta. Os animais foram mantidos sob fotoperíodo de 12:12 (claro/escuro), sendo as luzes acesas às 07:00h e apagadas às 19:00h, mediante um sistema automatizado (timer Brasfort 8769). A temperatura da água (termômetro analógico de mercúrio) e o oxigênio dissolvido (oxímetro digital AT 170, Alfakit®) foram medidos diariamente. O potenciômetro (pH) e amônia a cada sete dias por meio de kits colorimétricos (Alfakit®). Durante o período experimental, a temperatura da água, oxigênio dissolvido, pH, e amônia foram 28.26 ± 0.07◦C, 6.38 ± 0.45 mg L^{-1}, 6.98 ± 0.38 mg L^{-1} e 0.09 ± 0.05 mg L^{-1}, respectivamente.

Os peixes foram alimentados três vezes ao dia (8:00, 12:00 e 16:00 h) com ração comercial extrusada (AQUOS TROPICAL; tamanho de partícula 2 a 4 mm, 320 g/kg de proteína bruta, 7,5 g/kg de gordura bruta, 120 g/kg de cinzas e 4,5 g/kg de fibra). Ao iniciar o teste de alimentação, peixes de tamanho uniforme (peso 73.23 ± 4.45 g) foram submetidos a

dois grupos experimentais: peixes controle (alimentados) e peixes em jejum por 7 dias (jejum). Cada condição experimental foi testada em triplicata.

2.2 Amostragem

Ao final do teste de alimentação, os peixes foram anestesiados com eugenol (25 μL^{-1}) e o sangue coletado da veia caudal (n = 3 peixes por tanque, totalizando 9 peixes por tratamento) com seringa contendo EDTA (4%) e imediatamente centrifugado a 1000*g* (MCT-16000) por 10 min, e o plasma coletado e armazenado a -80°C até a análise. Após a coleta de sangue, todos os peixes foram sacrificados por secção medular e o fígado, intestino, brânquias, cérebro e uma porção do músculo branco da região dorsal foram coletados e armazenados em tubos criogênico marcados individualmente e imediatamente congelados em nitrogênio líquido e armazenadas a -80 °C.

2.3 Tratamento das amostras e métodos analíticos

Os tecidos foram pesados (Shimadzu AUW220D) e homogeneizados (Ultra Turrax T 18 Ika) em solução salina gelada (NaCl 0,138 M; KCl—0,0027 M; pH 7,2) na proporção de 1:10 (peso:volume). Após a centrifugação a 7000*g* por 15 min a 4 ºC, o sobrenadante foi separado, dividido em alíquotas (4 alíquotas e cada uma continha 500 µl) e mantidos a -80°C até analise.

Todas as amostras foram analisas em triplicata usando um leitor de microplacas (Multiskan GO, Thermo Scientific, Waltham, Massachusetts, EUA). O intervalo entre a coleta e finalização das análises foi de uma semana.

A peroxidação lipídica foi avaliada através da quantificação do malondialdeído (MDA) medindo a produção de substâncias reativas ao ácido tiobarbitúrico (TBARS) a 530 nm (Gatta et al., 2000). O teor de proteína carbonilada (PCO) foi determinado após derivação de 2,4-Dinitrophenylhydrazine (DNPH) (Reznick and Packer 1994).

A atividade da catalase (CAT; EC 1.11.1.6) foi medida após a redução do H_20_2 a 240 nm de acordo com Aebi (1984). A atividade da superóxido dismutase (SOD; EC 1.15.1.1) foi determinada com base na auto oxidação do pirogalol pelo método de Madesh e Balasubramanian (1997) e absorbância lida a 570 nm. A atividade da glutationa peroxidase

(GPX; EC 1.11.1.9) foi determinada medindo a taxa de oxidação do fosfato de dinucleótido de nicotinamida e adenina (NADPH) na presença de H_20_2, Glutationa reduzida (GSH) e Glutationa redutase (GR) a 240 nm de acordo com Flohé e Günzler (1984). A atividade da glutationa glutationa-S-transferase (GST; EC 2.5.1.18) foi quantificada a 340 nm utilizando o 1-cloro-2,4-dinitrobenzeno (CDNB) como substrato (Habig et al. (1974).

O método de Bradford foi utilizado para determinação dos níveis de proteína dos extratos. As atividades enzimáticas foram expressas como atividade específica em unidades por miligrama de proteína (U mg proteína^{-1}) de tecido.

2.4 Análise estatística

Todas as análises estatísticas foram realizadas com o software GraphPad version 9 (La Jolla, CA, USA). As diferenças entre o grupo controle e o grupo em jejum fígado foram analisadas pelo teste *T* de Student para amostras independentes ($*P < 0.05$, $**P < 0.01$ e $**P < 0.001$). Os dados são expressos como média ± erro padrão da média. O mapa de calor foi construído para cada grupo em cada variável através da porcentagem relativa entre os tecidos atribuindo o valor de 100 ao tecido que apresenta maior atividade.

O cálculo do Índice Integrado de Respostas de Biomarcadores (IBR) foi realizado conforme metodologia proposta por Beliaeff and Burgeot (2002) e posteriormente modificada por Sanchez et al., (2013). Os valores do IBR foram plotados em gráficos de radar para cada tecido, onde os valores mais altor de IBR indicam pior estado de saúde (organismos estressados).

3. RESULTADOS

As concentrações dos biomarcadores de danos oxidativos no fígado, intestino, brânquias, músculo, cérebro e plasma sanguíneo de *C. macropomum* estão apresentados na Figura 1. O jejum resultou em concentrações significativamente maiores de MDA (Figura 1A) e PCO (Figura 1B) no fígado ($P = 0.0361$ e $P = 0.0323$) e intestino ($P= 0.0012$ e $P = 0.0032$) em relação aos seus respectivos controles. Os demais tecidos não apresentaram diferenças nas concentrações de MDA e PCO.

As respostas das enzimas antioxidantes no fígado, intestino, brânquias, músculo, cérebro e plasma sanguíneo de *C. macropomum* estão apresentados na Figura 2. O jejum resultou em diferenças significativas na atividade da SOD (Figura 2A) no fígado ($P = 0.0428$), intestino ($P = 0.0190$), brânquias ($P = 0.0003$) e plasma sanguíneo ($P = 0.0021$). Nas brânquias, músculo e plasma sanguíneo, a atividade da CAT (Figura 2B) resultou em diferenças ($P < 0.05$) em relação aos seus respectivos controles. A atividade da GPX (Figura 2C) no fígado ($P = 0.0095$) e intestino ($P = 0.0003$) foram maiores durante o período de jejum. A atividade da GST (Figura 2D) não apresentou mudanças ($P > 0.05$) entre os tratamentos em nenhum dos tecidos avaliados.

Para resumir os principais resultados obtidos nos diferentes parâmetros estresse oxidativo em *C. macropomum* o mapa de calor (Figura 3) evidencia as respostas do sistema antioxidante nos diferentes tecidos em cada grupo. Os resultados obtidos no mapa de calor do grupo controle (Figura 3A) indicaram que a maior porcentagem relativa da SOD, GPX, GST e PCO foi encontrada nas brânquias. O fígado apresentou maior porcentagem relativa da CAT, enquanto a maior porcentagem relativa de MDA foi ocorreu no cérebro. Nas grupo em jejum (Figura 3B) a maior porcentagem relativa da SOD, CAT e MDA foi encontrada no fígado. As brânquias apresentaram maior porcentagem relativa de GST e PCO. A maior porcentagem relativa de GPX ocorreu no intestino. No plasma sanguíneo foi encontrado a menor porcentagem relativa de todas as variáveis analisadas nos dois grupos experimentais.

Os gráficos de radar usando o índice IBR para as respostas dos biomarcadores de estresse oxidativo e defesas antioxidantes de cada tecido são representados na Figura 4. Os valores de IBR foram diferente entre os tecidos com maior valor obtido no intestino (Figura 4B; IBR 15.60) seguido pelo fígado (Figura 4A; IBR 13.26). No tecido muscular (Figura 4D) e no plasma sanguíneo (Figura 4F) os valores de IBR foram 7.67 e 7.13, respectivamente. No tecido branquial (Figura 4B) foi encontrado IBR 5.36. O tecido cerebral (Figura 4E) exibiu o menor valor deste índice (IBR 2.26).

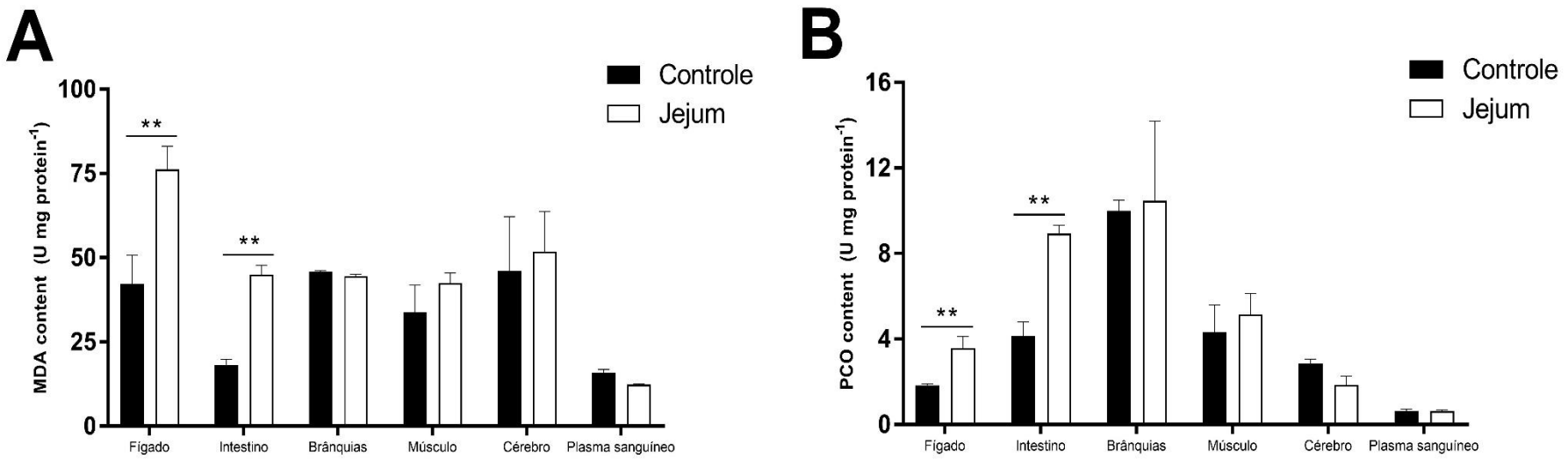

Figura 1. Concentrações dos biomarcadores de dano oxidativo: peroxidação lipídica, MDA (A) e proteína carbonilada, PCO (B) no fígado, intestino, brânquias, musculo, cérebro e plasma sanguíneo de *Colossoma macropomum* submetidos a jejum por 7 dias. Os valores são expressos média ± erro padrão da média (n=3). As diferenças (controle vs jejum) de cada tecido foram comparadas pelo teste *T* (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

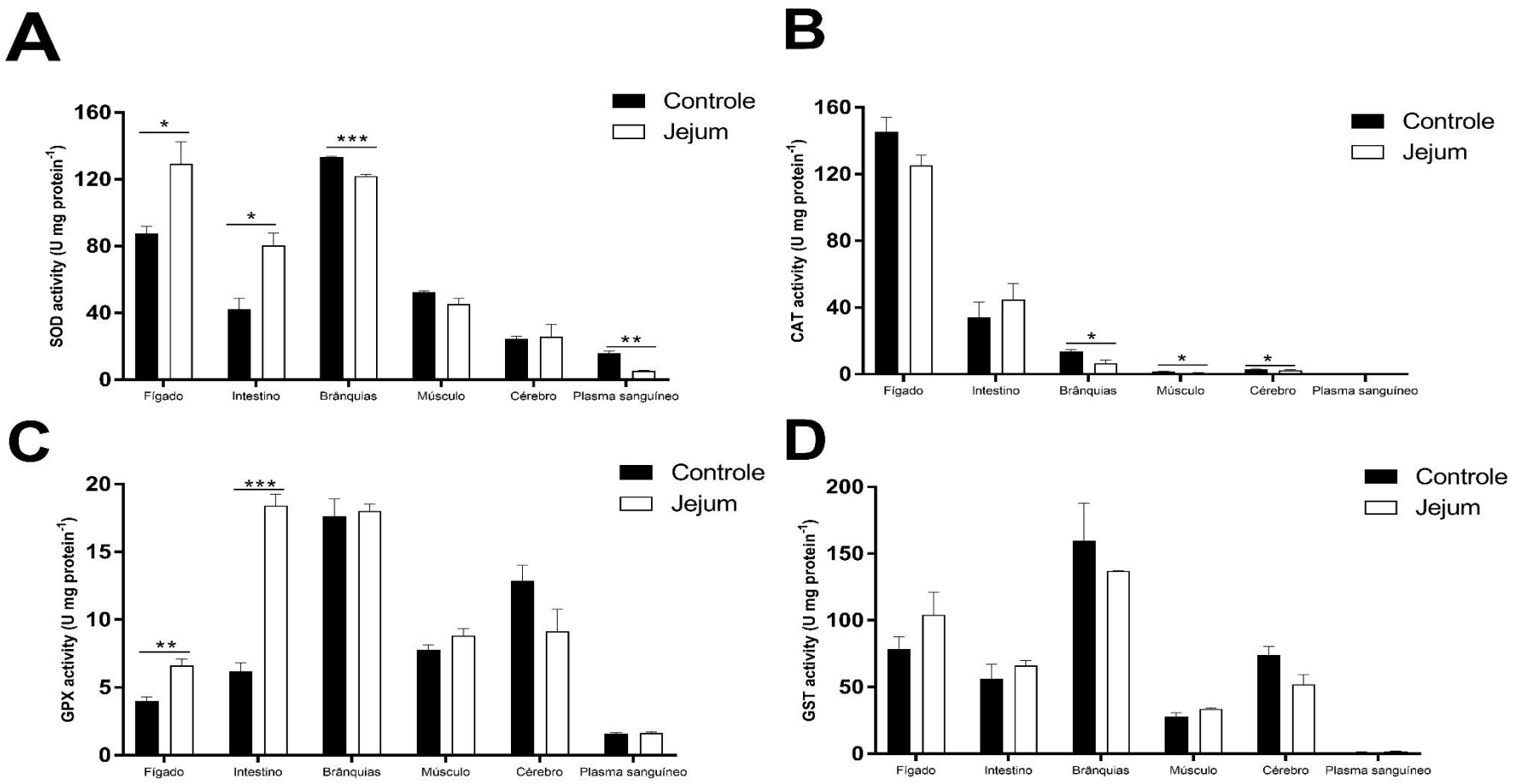

Figura 2. Atividade das enzimas antioxidantes superóxido dismutase, SOD (A); catalase, CAT (B); glutationa peroxidase, GPX (C); e glutationa S transferase GST (D) no fígado, intestino, brânquias, musculo, cérebro e plasma sanguíneo (P. sanguíneo) de *Colossoma macropomum* submetidos a jejum por 7 dias. Os valores são expressos média ± erro padrão da média (n=3). As diferenças (controle vs jejum) de cada tecido foram comparadas pelo teste *T* (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

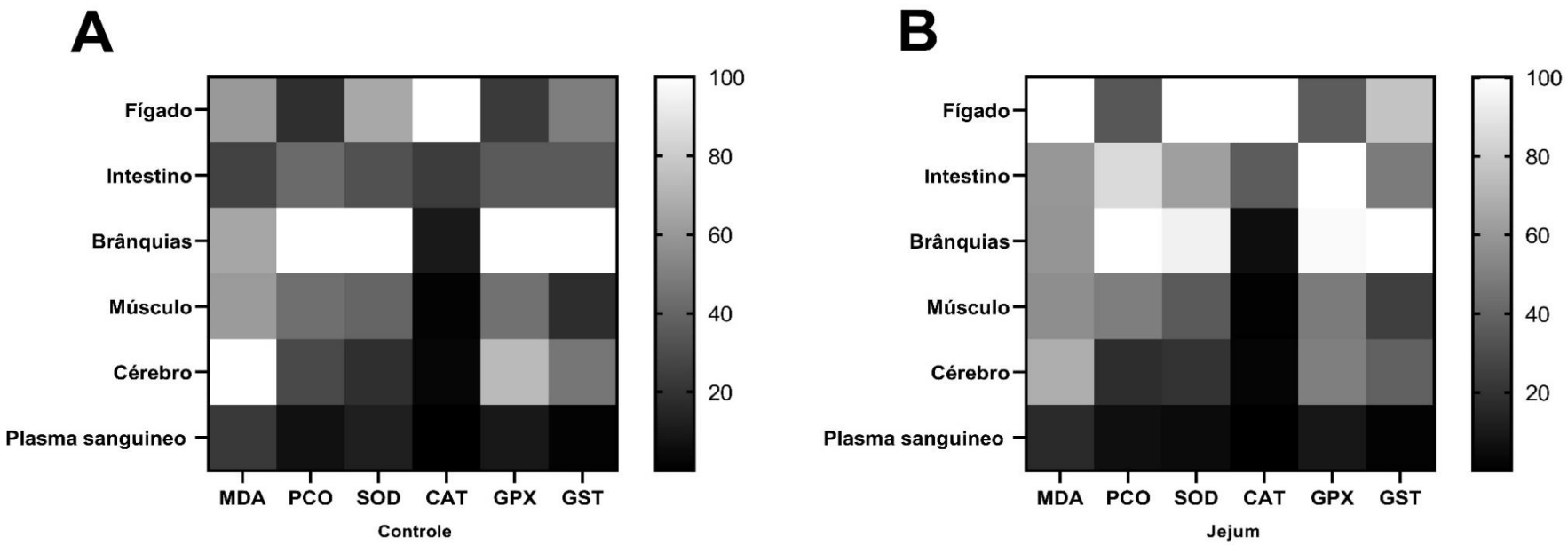

Figura 3. Mapas de calor do controle de de *Colossoma macropomum* no grupo controle (A) e grupo submetidos a jejum por 7 dias (B) dos biomarcadores de dano oxidativo: peroxidação lipídica, MDA; proteína carbonilada, PCO e das enzimas antioxidantes: superóxido dismutase, SOD; catalase, CAT; glutationa peroxidase, GPX e glutationa S transferase, GST. Para cada variável , o valor médio com maior concentração foi considerado como 100%.

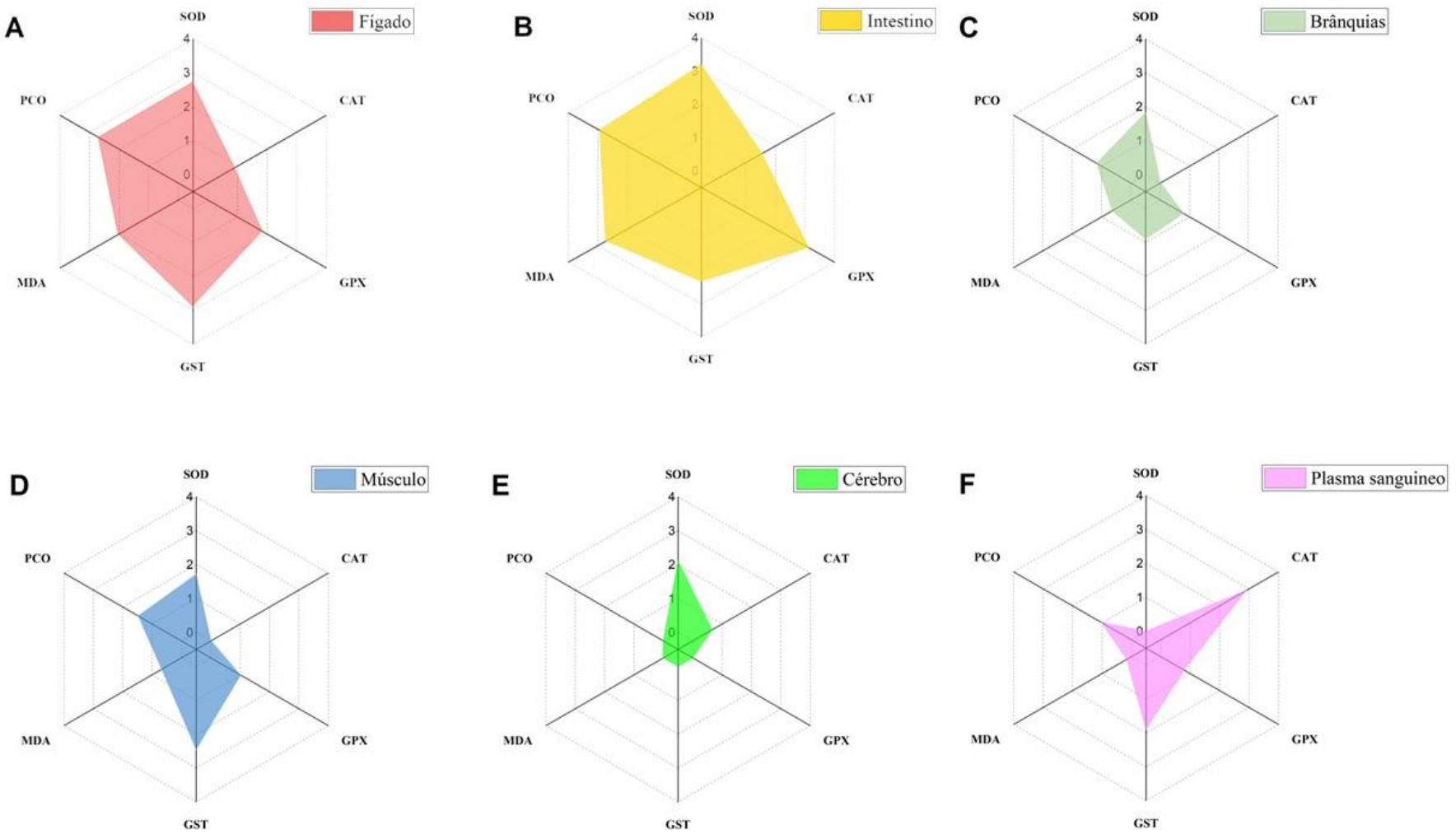

Figura 4. Gráficos de radar usados para determinar as respostas de múltiplos biomarcadores em diferentes tecidos (A) fígado (B) intestino e (C) brânquias (D) músculo (E) cérebro e (F) plasma sanguíneo de *Colossoma macropomum* submetidos a jejum por 7 dias.

4. DISCUSSÃO

Os peixes exibem estratégias adaptativas de sobrevivência para lidar com a fome, dentre as quais incluem converter as reservas endógenas dos tecidos e mobilizar os componentes do corpo para obter energia (McCue, 2010; Navarro and Gutiérrez, 1995; Zaldúa and Naya, 2014). No entanto, tais estratégias podem induzir e/ou agravar o estresse oxidativo devido ao aumento excessivo de ROS gerados pelo metabolismo aeróbico associado as defesas antioxidantes deprimidas, devido principalmente ao desiquilíbrio nutricional (Morales et al., 2011; Pérez-Jiménez et al., 2011). Neste trabalho, um conjunto de parâmetros de estresse oxidativo foram mensurados em diferentes tecidos do *C. macropomum* após jejum de 7 dias. Embora esteja claro que a fome possa levar ao estresse oxidativo alimentar, nossos resultados demonstram que a magnitude do dano e das respostas nos sistemas de defesa antioxidante são específica de cada tecido.

Tendo em vista os tecidos analisados e seus respetivos controles, observamos que o jejum resultou em aumento nas concentrações de MDA e PCO no fígado e intestino, enquanto nas brânquias, sangue, musculo e cérebro as concentrações não foram alteradas. O estresse oxidativo é evidenciado por aumento nos níveis de marcadores de danos oxidativos que podem ser produtos da oxidação de lipídios e proteínas (Birben et al., 2012; Lushchak, 2011; Stadtman and Levine, 2000). O MDA é um dos produtos finais da peroxidação lipídica e as PCO são os produtos finais da oxidação de proteínas, ambos ocorrem tipicamente como resultado do ataque das ROS (Estévez et al., 2019; Hematyar et al., 2019). Portanto, as concentrações de MDA e PCO encontradas em nosso estudo sugerem que a fome resultou em estresse oxidativo no tecido hepático e intestinal. Descobertas comparáveis foram observadas em *Hoplosternum littorale* e *Salmo trutta* após períodos de fome (Bayir et al., 2011; Rossi et al., 2015). No geral, esses relatórios publicados, juntamente com nossas descobertas atuais, destacam a resposta tecido-específica dos marcadores de danos oxidativos induzidos pela fome.

Os níveis de ROS nas células são controlados pela ação concertada de mecanismos de defesa antioxidantes interdependentes (Birben et al., 2012; Schieber and Chandel, 2014). Assim, SOD e CAT fornecem uma primeira linha de defesa contra as espécies reativas de oxigênio. A SOD metaboliza o ânion superóxido ($O_2^{\cdot -}$) para produzir peróxido de hidrogênio (H_2O_2), que é

posteriormente transformado em H_2O e O_2 pela ação da CAT, GPX e várias peroxidases (Juan et al., 2021; Mailloux, 2020). *O* fígado e o intestino estão envolvidos em diferentes funções e os desafios que eles enfrentam também são diferentes (Evans et al., 2013; Rønnestad et al., 2013). O fígado está envolvimento em uma série de atividades metabólicas e processos de desintoxicação, enquanto o intestino é o principal local de digestão e absorção de nutrientes.(Bakke et al., 2010; Wilson and Castro, 2010) Em comum, ambos são constantemente desafiado por muitos radicais livres e, conforme observado em nosso estudo, apresentam semelhanças nas respostas antioxidantes. O aumento na atividade da SOD e GPX no fígado e intestino em relação aos seus respectivos controles não foi efetivamente suficiente para evitar os danos celulares a estes tecidos. Como a consequência do aumento da atividade da SOD resultaria em maior produção de H_2O_2, a atividade elevada da GPX, mas não da CAT, pode indicar uma falha na defesa antioxidante para eliminar o excesso de ROS. (Bacou et al., 2021; Birnie-Gauvin et al., 2017; Chainy et al., 2016).

Considerado como órgão dinâmico, as brânquias desempenham múltiplas funções, incluindo respiração, excreção de amônia e osmorregulação (Evans, 1987; Evans et al., 2005; Huang et al., 2022; Wegner, 2015), portanto, é provável que este órgão possua uma alta capacidade de produzir ROS. Observamos uma redução na atividade da SOD e CAT no tecido branquial, mas que não acarretou em danos a lipídios e proteína. Até o momento, informações muito limitadas estão disponíveis sobre se a fome poderia induzir estresse oxidativo no tecido branquial. Assim, nossos resultados indicam que, ao contrário do fígado e intestino, as brânquias contam com mecanismo de defesa eficiente, baseado na SOD e GPX para extinguir as ROS geradas durante o jejum (Birnie-Gauvin et al., 2017; Morales et al., 2011). Todavia, nossos resultados são contrários ao estudo realizado por Rossi et al, (2015) que demonstraram que a extensão do jejum (7 e 28 dias) ativou as defesas antioxidantes do tecido branquial, através do aumento das enzimas CAT, GPX e GST evitando assim a peroxidação lipídica (Rossi et al., 2015).

Em resposta à fome, o musculo branco do tambaqui apresentou redução na atividade da CAT, mas sem danos oxidativos. Os poucos estudos sobre o impacto da fome nas defesas antioxidantes do musculo de peixes mostraram majoritariamente que as enzimas antioxidantes em sua maioria, são mantidas durante a inanição, evitando aumento nos níveis de peroxidação lipídica (Furné et al., 2009; Guderley et al., 2003). No entanto, em um estudo recente realizado por Hidalgo et al, (2017) foi demonstrado a existência de uma assimetria regional nas defesas antioxidantes no musculo de *Umbrina cirrosa* durante a inanição, relacionada com a contribuição da região para o

metabolismo. Com base na descoberta do estudo acima, podemos sugerir que a região do musculo analisada em nosso trabalho pode ter contribuído nas respostas observadas.

As alterações fisiológicas em resposta ao estresse são iniciadas no sistema nervoso central , sendo o cérebro um dos órgãos com maior atividade metabólica nos peixes (Soengas and Aldegunde, 2002; Urbinati et al., 2020). A presença de altos níveis de ácidos graxos insaturados e defesas antioxidantes relativamente modestas deixam o cérebro vulnerável à ação das ROS e propenso a danos oxidativos (Güller et al., 2020; Ratko et al., 2022). No entanto, as enzimas antioxidantes bem como os marcadores de dano no cérebro não mostraram respostas a fome no período avaliado. Nós acreditamos que as adaptações metabólicas no organismo para suprir as demandas de glicose através da gliconeogênese podem não ter afetado significativamente este órgão no período de 7 dias. Interessantemente, Rossi et al, (2015) não achou respostas com 7 dias em *Hoplosternum littorale* submetidas ao jejum, mas achou com 28 dias (Rossi et al., 2015)

No plasma sanguíneo, encontramos baixa resposta oxidativa, com destaque para diminuição na atividade da SOD e aumento da CAT nos peixes mantidos em jejum. O plasma sanguíneo é um biomarcador do estado fisiológico comumente empregado em peixes e os resultados são arbitrariamente extrapolados para os tecidos (Birben et al., 2012; Monteiro et al., 2006). Curiosamente, o mapa de calor indicou que entre os tecidos analisados, o plasma apresentou a menor porcentagem relativa de todos os parâmetros mensurados independente do estado de alimentação (controle e jejum), corroborando com os resultados de Marcon and Wilhelm Filho, (1999). Isso possivelmente se deve ao número proporcionalmente reduzido de células no sangue em relação a outros tecidos, implicando em uma menor produção dessas enzimas (Mc Donald and Milligan, 1992; Seibel et al., 2021).

Diferente do plasma sanguíneo, observamos que as brânquias apresentaram atividade elevada dos biomarcadores antioxidantes independente do estado de alimentação, já em condições de privação de alimento, estes parâmetros também foram evidentes no tecido hepático e intestinal. Todavia, conclusões a certa das diferenças entre os tecidos devem ser realizada com cautela. O mapa de calor evidenciam o comportamento de cada variável entre os tecidos com base na porcentagem relativa ao tecido que apresentou o maior valor absoluto. Tais diferenças dão uma visão de intensidade sem considerar a contribuição de cada órgão para o metabolismo. Assim, estas diferenças junto com as diferenças encontradas em cada tecido não nos permite tirar conclusões gerais do marcador oxidativo prioritário.

Para obter estas respostas, calculamos o índice integrado de respostas de biomarcadores (IBR), técnica que integra em um único valor as respostas de múltiplos biomarcadores (Beliaeff and Burgeot, 2002; Sanchez et al., 2013). Embora a abordagem IBR seja amplamente utilizada para determinar o estado de saúde dos peixes durante o estresse ambiental (Jiang et al., 2023; Leão-Buchir et al., 2023) é uma ferramenta nova em estudos de nutrição de peixes (Chen et al., 2022; Gokulakrishnan et al., 2022; Meena et al., 2023). No presente estudo, o índice IBR visualizou de forma abrangente o status antioxidante dos diferentes tecidos em resposta a fome, com maior índice encontrado no tecido intestinal seguido pelo tecido hepático, validando assim os achados da comparação de medias e da porcentagem relativa. Curiosamente, no tecido branquial a alta resposta oxidativa não refletiu no IBR, que exibiu o segundo menor índice entre os tecidos. Nesse sentido, nós acreditamos que o intestino e fígado podem ser os marcadores oxidativos prioritários quando se pretende avaliar poucos tecidos em um ensaio de nutrição.

5. CONCLUSÃO

Nossos resultados demonstraram que as respostas dos biomarcadores antioxidantes são especificas de cada tecido e que a fome de 7 dias ocasionou estresse oxidativo no fígado e intestino do Tambaqui. Através da abordagem IBR consideramos que o tecido hepático e intestinal são marcadores oxidativos prioritário em experimentos de nutrição de peixes.

No futuro, pode ser interessante a extensão do tempo de jejum em conjunto com múltiplos endpoints. Isso poderia ajudar na compreensão das estratégias de proteção especifica empregada por esses órgãos para manter o metabolismo quando ameaçados por danos oxidativos induzido pela fome.

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