UNIVERSIDADE ESTADUAL DE MARINGÁ
CENTRO DE CIÊNCIAS AGRÁRIAS

EFEITO DA METIONINA E O RISCO DE PREDAÇÃO NA FISIOLOGIA, GENÉTICA E SOBREVIVÊNCIA DO ZEBRAFISH (*Danio rerio*)

Autora: Jaísa Casetta
Orientador: Prof. Dr. Ricardo Pereira Ribeiro
Coorientadora: Prof[a]. Dr[a]. Eliane Gasparino

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Estado do Paraná
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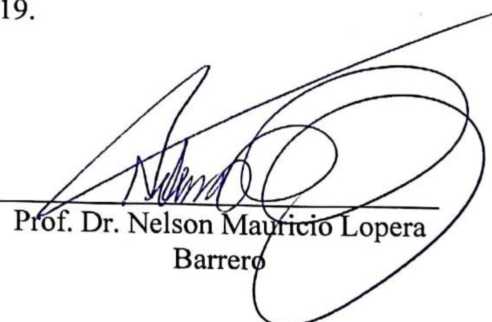

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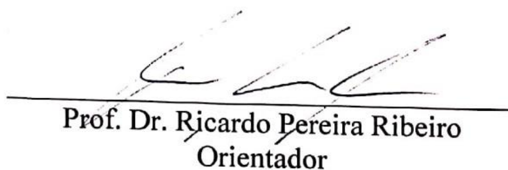

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Orientador

"Não importa o que aconteça, continue a nadar!"

(Walters Graham - Procurando Nemo)

Aos meus pais, Valdenir Casetta e Arminda Maria Casetta,

pelo amor e por me ensinarem a nunca desistir dos meus objetivos.

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BIOGRAFIA

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Em fevereiro de 2019, submeteu-se à banca examinadora para a defesa de dissertação apresentada, como parte das exigências para obtenção do título de mestre em Zootecnia.

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RESUMO

A metionina é um aminoácido limitante em dietas comerciais na maioria dos peixes, e sua insuficiência além de causar limitação no crescimento, pode reduzir a eficiência alimentar, e pode afetar o sistema oxidante do organismo. Nesse sentido, os objetivos deste trabalho foram avaliar o efeito da metionina e o risco de predação no desempenho, histologia intestinal, cortisol, expressão gênica e sobrevivência do zebrafish (*Danio rerio*). No primeiro experimento, 360 animais de um ano de idade foram divididos em três tratamentos relacionados à suplementação de metionina: Sem suplementação de metionina (SM); suplementada com DL-Metionina 99% - aminoácido na sua forma livre (DL) e suplementada com metionina na forma dipeptídeo DL-Metionil-DL-Metionina 95% (MM). Os peixes foram mantidos sem contato com predador no aquário (SO) ou com contato direto com predador no aquário (CO), por 48 horas ou 20 dias, dependendo da fase experimental avaliada. O estresse por risco de predação reduziu o ganho de peso dos animais com 20 dias e os peixes alimentados com suplementação de metionina, independente da fonte utilizada, apresentaram ganho de peso superiores quando comparados aos SM. Não houve diferença estatística nos niveis de cortisol em função do risco de predação e da suplementação de metionina. Não houve efeito significativo da interação entre o risco de predação e a suplementação de metionina, para o gene *PEPT1*. Os peixes com 48 horas de exposição ao estresse, com risco de predação, apresentaram maior expressão do mRNA dos genes *SOD2, CAT* e *GPX1*. Em relação ao consumo prolongado das dietas, observou-se menor expressão desses genes em animais consumindo dietas DL e MM. No segundo experimento, os reprodutores e sua prole, receberam exclusivamente ração formulada com ingredientes convencionais suplementados com metionina: DL, MM e um tratamento controle sem suplementação de metionina (SM). Através do monitoramento continuo dos animais, após eclosão, observou-se que as larvas abriram a boca com 3 dias pós-fertilização (dpf) e a partir desse momento, iniciou-se a alimentação com 3 e 5 dpf para avaliar a sobrevivência das larvas

em função das dietas dos pais até 20 dpf. Para realização do experimento, zebrafish proveniente de programa de melhoramento genético, foram utilizados para a obtenção dos ovos. 1200 larvas com 3 dpf foram divididos em três tratamentos relacionados à suplementação de metionina: SM, DL e MM seguindo a mesma dieta que seus pais. Os animais tiveram 2 períodos de início de alimentação com 3 ou 5 dpf. Não se observou diferença significativa na taxa de sobrevivência entre o início da alimentação com 3 ou 5 dpf. Através da literatura consultada essa é a primeira vez que esse fato é relatado em zebrafish consumindo dietas formuladas exclusivamente com ingredientes convencionais. Observou-se também que independente da fonte de metionina utilizada, a sobrevivência foi maior em larvas dos tratamentos DL e MM. Em conclusão, a suplementação com metionina é fundamental para garantir tolerancia celular, fornecendo poder redutor para os sistemas antioxidantes, que é responsável por neutralizar os radicais livre. Além de auxiliar na geração de larvas precoces para capacidade de ingerir alimentos e garantir maior sobrevivência dessas formas jovens.

Palavras-chave: *Danio rerio*, defesa antioxidante, metionina, sobrevivência, transportador de peptídeos 1

ABSTRACT

Methionine is a limiting amino acid in commercial diets in most of fish, and its insufficiency can cause growth restriction, reduce feed efficiency, and can affect the body's antioxidant system. The objective of this study was to evaluate the effect of methionine and the risk of predation on the performance, intestinal histology, cortisol, gene expression and survival of zebrafish (*Danio rerio*). In the first experiment, 360 one-year-old animals were divided into three treatments related to methionine supplementation: no methionine supplementation (SM); supplemented with DL methionine 99% -amino acid in free form (DL) and supplemented with methionine in the dipeptide form DL-Methionyl-DL-Methionine 95% (MM). Fish were kept without contact with the predator in the aquarium (SO) or with direct contact with predator in the aquarium (CO), for 48 hours or 20 days, depending on the experimental phase evaluated. Stress due to predation risk reduced the weight gain of animals with 20 days and the fish fed with methionine supplementation, regardless of the source used, presented higher weight gain when compared to SM. There was no statistical difference in cortisol levels due to the risk of predation and methionine supplementation. There was no significant effect on the interaction between the risk of predation and methionine supplementation, for *PEPT1* gene. Fish with 48 hours of exposure to stress with risk of predation presented higher mRNA expression of the *SOD2*, *CAT* and *GPX1* genes. In relation to prolonged consumption of the diets, lower expression of these genes in animals consuming diets DL and MM was observed. In the second experiment, breeders and their offspring, received exclusively feed formulated with conventional ingredients supplemented with methionine: DL, MM and a control treatment without methionine supplementation (SM). Through the continuous monitoring of the animals after hatching, it was observed that the larvae opened their mouth 3 days post fertilization (dpf), and from this moment, feeding started with 3 and 5 dpf to evaluate the survival of the larvae, according to the parents' diets, until 20 dpf. For the experiment, zebrafish from a breeding

program were used to obtain the eggs. 1200 larvae at 3 dpf were divided into three treatments related to methionine supplementation: SM, DL and MM, following the same diet as their parents. The animals had two periods for the beginning of feeding, with 3 or 5 dpf. No significant difference in the survival rate between the beginning of feeding with 3 or 5 dpf was observed. Through literature consulted, this is the first time that this fact is reported in zebrafish consuming diets formulated exclusively with conventional ingredients. We also observed that, regardless of the source of methionine used, survival was higher in larvae of DL and MM treatments. In conclusion, methionine supplementation is important to ensure cell tolerance, providing reducing power for antioxidant systems, which is responsible for neutralizing free radicals, besides helping in the generation of early larvae ability to ingest food and ensure greater survival of these young forms.

Key words: antioxidant defense, *Danio rerio*, peptide transporter 1, methionine, survival

I. INTRODUÇÃO

1.0 Zebrafish

1.1 Características

O zebrafish é um peixe tropical de pequeno porte, originário da Ásia, encontrado no nordeste da Índia, Bangladesh e Nepal (Spence et al., 2008). Os adultos normalmente chegam a 40 mm de comprimento total, caracterizado por possuir um corpo fusiforme e comprimido lateralmente, com cinco a sete listras no sentido horizontal, na cor azul escuro, que inicia na parte posterior do opérculo até a nadadeira caudal (Barman, 1991; Spence et al., 2008). A coloração de machos e fêmeas é semelhante, porém os machos podem sem caracterizados por uma coloração mais amarelada e nadadeiras anais maiores (Schilling, 2002).

O Zebrafish (*Danio rerio*) destaca-se como modelo animal utilizado em pesquisas científicas em diferentes áreas, devido a seu genoma estar totalmente sequenciado e a semelhança do seu genoma com o genoma humano (Vilella et al., 2009). Além da genética, atributos como pequeno tamanho, robustez, ciclo de vida curto, prolificidade, transparência dos embriões e larvas, tolerância a grande variação de parâmetros ambientais, facilidade e baixo custo de produção, favorecem a sua utilização em ambientes de pesquisa (Nasiadka e Clark, 2012).

A reprodução do zebrafish é influenciada pelo fotoperíodo (14 horas claro e 10 horas escuro) e pela temperatura da água (28°C), pelo fato de encontrarem essas condições em ambiente natural para o acasalamento, para se ter êxito ao reproduzi-los em laboratório é preciso imitar as condições naturais (Spence et al., 2008). Esses peixes apresentam rápida maturação sexual, que ocorre a cerca de 100 dias de idade (Brundo e Salvaggio, 2018), sendo que, uma fêmea pode depositar a cerca de 200 ovos por dia. Os

ovos possuem fertilização e desenvolvimento embrionário externo, e eclodem rapidamente (aproximadamente três dias pós fertilização). Esse rápido desenvolvimento embrionário, resulta numa rápida formação dos principais órgãos após a fertilização (Brundo and Salvaggio, 2018). Porém o ciclo produtivo do zebrafish não é completamente compreendido, gerando interesse em estudos nessa área, para se estabelecer os melhores padrões de criação tanto para reprodução quanto manutenção desses animais. O zebrafish pode viver em média de três a cinco anos (Kishi et al., 2009), sendo classificado nos estágios de desenvolvimento conforme a Tabela 1.

Tabela 1- Estágio de desenvolvimento do zebrafish (*Danio rerio*).

Estágio	**Dias**	**Comprimento (mm)**	**Descrição**
Larva jovem	3	3.5	Nada livremente, posicionamento vertical
Larva	14	6	Bexiga natatória cheia, procura de alimento, Crescimento
Juvenil	30	10	Nadadeiras e padrão de pigmentação dos Adultos
Adulto jovem	90	20	Reprodução
Adulto	1000	40-50	final da vida

Adaptado de Nusslein-Volhard and Dahm (2002).

A taxa de mortalidade no início no desenvolvimento do zebrafish é alta (Mizgirev e Revskoy, 2010), e pode ser atribuído à alimentação, tanto pela dificuldade em encontrar alimento, quanto pelo tamanho da boca pequena para aprisioná-lo. Essa alta mortalidade na fase de larva pode dificultar a obtenção de número de animais suficientes para estudos. A criação de animais com desenvolvimento mais rápido, com maior facilidade em ingerir alimento com menor idade de desenvolvimento, pode auxiliar na redução da mortalidade. Uma ferramenta para alcançar essa aptidão seria o melhoramento genético, que permite a seleção e acasalamento de indivíduos com as características desejadas (Turra et al., 2012; Xu et al., 2015). O núcleo de pesquisa PeixeGen vem trabalhando na implantação de um programa de melhoramento genético que visa produzir animais maiores e mais homogêneos fenotipicamente para serem utilizados em pesquisas científicas.

1.2 Trato gastrointestinal

O intestino do zebrafish adulto ocupa grande parte da cavidade abdominal. É dividido em três diferentes segmentos: intestino anterior, médio e posterior. O intestino

anterior conhecido também como bulbo intestinal possui um diâmetro maior que o lúmen do intestino médio e posterior, por esse motivo pode desempenhar o papel de reservatório de alimento, já que o zebrafish não possui estômago verdadeiro (Wallace et al., 2005; Brugman, 2016).

Com cinco dias pós fertilização (dpf) o sistema digestório da larva já está formado e diferenciado em boca, cavidade oral, faringe, esôfago, intestino, reto e ânus, ou seja, já é funcional (Verri et al., 2003). A sequência do trato digestório é apresentada na Figura 1.

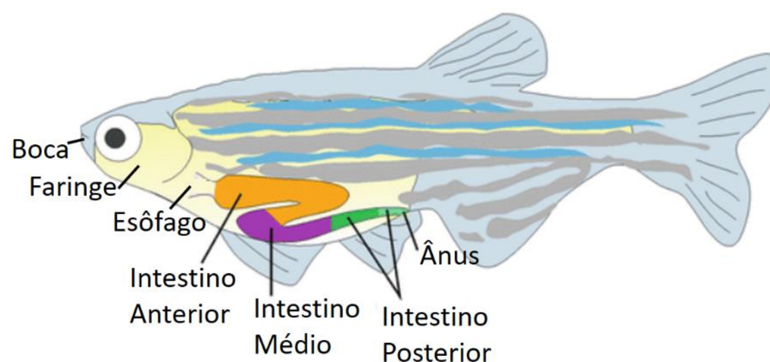

Figura 1- Esquema da divisão do intestino do zebrafish, adaptado de Lobert et al. (2016).

O segmento anterior do intestino possui as vilosidades intestinais mais altas quando comparadas com o segmento posterior e médio, e representa o principal local de digestão de proteínas e lipídeos. Provavelmente essa porção está relacionada com a absorção dos nutrientes, uma vez que as enzimas digestivas e transportadores de solutos estão presentes nessa região. Já o intestino posterior possui as vilosidades mais curtas e não é observado a presença de enterócitos absortivos, possivelmente estão envolvidos na absorção de água (Wallace et al., 2005; Dammski et al., 2011).

Por não possuir estômago e nem a presença de glândulas gástricas, não é necessário um pH ácido para a digestão e absorção. De acordo com Nalbant et al. (1999) em condições normais o pH do intestino de zebrafish adulto é alcalino, maior que 7,5. O zebrafish ainda é desprovido de criptas intestinais, célula de Paneth e glândulas submucosas, características que tornam o intestino desse peixe em um órgão mais simples quando comparado a outros vertebrados (Wang et al., 2010).

Dessa forma, para que ocorra a digestão é necessário que o alimento encontre enzimas digestivas no lúmen do intestino, sendo essas produzidas principalmente pelo pâncreas (NRC, 2011). De modo geral, as proteínas, lipídeos e carboidratos vindos das dietas, ao chegarem no intestino são quebrados pelas proteases alcalinas, lipases e

amilases, respectivamente (Bakke et al., 2010). As enzimas além de fazerem a quebra dos nutrientes, permite que eles sejam absorvidos e posteriormente utilizados pelo animal.

1.3 Transportador de peptídeos - PEPT1

As proteínas são quebradas pelas enzimas digestivas, e podem ser absorvidas pelo enterócitos como dipeptídeos, tripeptídeos ou aminoácidos livres. Nos vertebrados após a digestão das proteínas no trato gastrointestinal, a absorção intestinal dos aminoácidos livres é diferente da absorção dos pequenos peptídeos, enquanto os aminoácidos livres devem ser transportados atendendo alguns requisitos, os pequenos peptídeos podem ser transportados por um único transportador conhecido como transportador de peptídeos 1 (PEPT1) (Daniel e Kottra, 2004).

O PEPT1 é um transportador eletrogênico, presente na membrana da borda em escova do epitélio intestinal, que carrega o substrato do lúmen do intestino junto com o H+ (simporte dependente de H+) para o citosol, em que os peptídeos são quebrados em aminoácidos livres e absorvidos para corrente sanguínea (Wang et al., 2017). Como característica principal, o PEPT1 possui uma alta capacidade e baixa afinidade, ou seja, baixa afinidade em se ligar ao substrato, e alta capacidade, relacionado com a velocidade de transporte e o número de substratos transportados (Wang et al., 2017). Dessa forma, torna-se importante no transporte de di e tripeptídeos, sendo que esses são usados de forma eficiente no metabolismo, desenvolvimento e crescimento dos peixes (Dabrowski et al., 2003; Zhang et al., 2006).

A maior atividade do PEPT1 em zebrafish é em pH alcalino, sugerindo que essa é uma característica única do zebrafish, pela ausência de estomago (Verri et al., 2003). A expressão desse gene (*PEPT1*) foi identificado em larvas de zebrafish com apenas 2 dpf, porém com atividade baixa, e o máximo de expressão de 4 a 7 dpf (Verri et al., 2003). Isso pode ser um bom indicativo de capacidade de transporte de di e tripeptídeos já na primeira semana de desenvolvimento larval.

Conhecer o transportador e suas características é de extrema importância do ponto de vista econômico e biológico, pois permite o melhor entendimento de como os aminoácidos são absorvidos, quais são os fatores que contribuem para influenciar positiva ou negativamente neste processo. O processo de absorção impacta diretamente na quantidade de aminoácidos disponíveis para as células dos diferentes tecidos corporais, responsáveis por maximizar o ganho de peso e a conversão alimentar dos animais, além

de minimizar o impacto ambiental. Dessa forma, é possível otimizar as dietas com as fontes de proteínas, permitindo melhor crescimento dos peixes (Wang et al., 2017), e consequentemente melhorar a relação custo/benefício das rações. O crescimento saudável dos teleósteos, depende da composição nutritiva do alimento, digestão, absorção, transporte e metabolismo de nutrientes (Cahu e Infante, 2001).

2.0 Desempenho dos peixes: Estresse e estresse oxidativo

2.1 Estresse

O estresse é considerado um estado fisiológico privado da homeostase causado por uma alteração ambiental (Squires, 2010), ocasionado por estímulos intrínsecos ou extrínsecos designados estressores (Bonga, 1997). Nos vertebrados situações estressantes desencadeiam sinalizações através de hormônios, peptídeos e neurotransmissores (Joëls e Baram, 2009; Tejpal et al., 2009; Creel et al., 2013), que prepara o indivíduo para uma situação perigosa (Roozendaal e McGaugh, 2011), desencadeando um estímulo que permite ao animal fugir da situação ameaçadora (Wingfield, 2003). A resposta do animal em relação ao estressor é, por consequência, um resposta adaptativa, que possibilite ao animal responder imediatamente a uma circunstância perigosa (Hill et al., 2016). Níveis moderados de estresse estão ligados a algumas respostas positivas (Bonga, 1997), como aprendizagem e consolidação da memória (Sapolsky et al., 1986). Já o estresse crônico afeta negativamente o comportamento e a fisiologia do animal (Wingfield, 2013).

A resposta fisiológica causada pelo estresse pode ser dividida em três fases subsequentes: alarme, resistência e exaustão (Selye, 1950). Na fase de alarme, ocorre a liberação do cortisol, sendo este o principal hormônio corticosteroide liberado em resposta ao estresse em zebrafish (Mommsen et al., 1999). Com o aumento do nível de cortisol, transcorre a tentativa do corpo de compensar o distúrbio e restabelecer o equilíbrio, passando então para a segunda fase, a de resistência, com alterações do sistema respiratório e cardiovascular (Rodnick e Planas, 2016). Dependendo do tempo que o animal ficar exposto ao estressor, esse é levado à exaustão (terceira fase) (Selye, 1950). No zebrafish a concentração de cortisol do corpo inteiro, é utilizado como indicador de estresse primário no peixe, sendo que os níveis de cortisol em animais não estressados variam de 2,1 a 4,7 ng g^{-1} (Barton, 2002; Ramsay et al., 2006).

A resposta fisiológica causada pelo estresse em zebrafish é semelhante a resposta ao estresse em humanos (Gerlai, 2010). O eixo hipotálamo-hipófise-inter-renal (HHI) é

responsável por controlar as respostas ao estresse (Bonga, 1997). O animal ao se encontrar em uma situação de estresse estimula o sistema nervoso central que ativa diversos mecanismos em seu organismo como resposta fisiológica (Barton, 2002). Dessa forma, ocorre a produção de cortisol pelo eixo HHI que é estimulado pelo fator de liberação de corticotrofina (CRF), especialmente pelo hipotálamo, que estimula a hipófise a liberar o hormônio adenocorticotrófico (ACTH), que chega até o tecido inter-renal para estimular a liberação de cortisol (Barton, 2002). O cortisol promove no peixes estressados a ação recuperadora da homeostase inicial, promovendo a melhora na energia disponível no organismo (Bonga, 1997).

O estresse no meio aquático tornou-se um assunto muito importante (Navarro e Navarro, 2012). Há diversas fontes de estresse que podem afetar os animais, como: confinamento, alta densidade, baixa concentração de oxigênio, predador, entre outros, que acarreta em alterações no metabolismo, e como consequência queda no crescimento (Oba et al., 2009). O estresse causa alteração no sistema fisiologico, sendo capaz de aumentar a produção das espécies reativas de oxigênio (ROS), podendo levar ao estress oxidativo, sendo este o aumento significativo de ROS e diminuição da capacidade redutora de pares redox celulares (Schafer e Buettner, 2001), como consequência altera a atividade das enzimas antioxidantes que atuam no estresse oxidativo (Abdalla, 2015). De acordo com McIntosh e Sapolsky (1996) os glicocorticoides podem ocasionar um aumento de 10% nos ROS.

2.2 Estresse oxidativo

O oxigênio é obrigatório na oxidação de compostos orgânicos e produção de energia para o metabolismo celular (Comhair e Erzurum, 2002). A oxidação é uma etapa primordial da via aeróbica do organismo, e por algumas alterações biológicas os radicais livres são produzidos espontaneamente pelo organismo animal, e passam a ser designado como ROS (Betteridge, 2000). ROS são moléculas não estáveis e seus elétrons não encontram-se emparelhados, passando a ser bastante reativos (Baynes e Dominiczak, 2015). No organismo, quando em baixas concentrações atua na produção de energia, fagocitose, sinalização intercelular, regulação do crescimento celular e na síntese de substâncias biológicas importantes (Barreiros et al., 2006). No entanto, o excesso de ROS apresenta efeitos prejudiciais, tais como danos nas biomoléculas de lipídeos, proteínas e DNA (Willemsen et al., 2011). A injúria do ROS nas membranas e lipoproteínas, está relacionada com o desenvolvimento de muitas doenças, que podem ser degenerativas e

inflamatórias (Gasparovic et al., 2013). Já o dano proteico é proporcionado pela formação de carbonila (grupos proteicos), que podem induzir a proteólise nas bases do DNA, quebrando as fitas do material genético, além de poder causar lesão celular, necrose e apoptose como consequência de sua toxicidade em tecidos ou órgãos (Halliwell, 1994).

As formas de ROS mais ocorrentes podem ser radicalares, como o radical hidroxila ($^{\cdot}OH$) e radical superóxido ($O_2^{-\cdot}$) e não radicalares como o peróxido de hidrogênio (H_2O_2) (Echtay, 2007; Ray et al., 2012). Nos processos biológicos dos animais aeróbicos, a maior parte do ROS é produzida na cadeia respiratória mitocondrial, resultado do oxigênio que não foi reduzido à água. Para a formação de água é necessário que o oxigênio sofra redução e incorpore quatro elétrons ao final da cadeia de transporte de elétrons no interior da mitocôndria, caso ocorra alguma redução do oxigênio com menor número de elétrons, serão formados intermediários metabólicos reativos com grande instabilidade, ou seja, haverá a produção de ROS (Rains e Jain, 2011). ROS são os produtos intermediários da redução do oxigênio em água, como mostra a Figura 2.

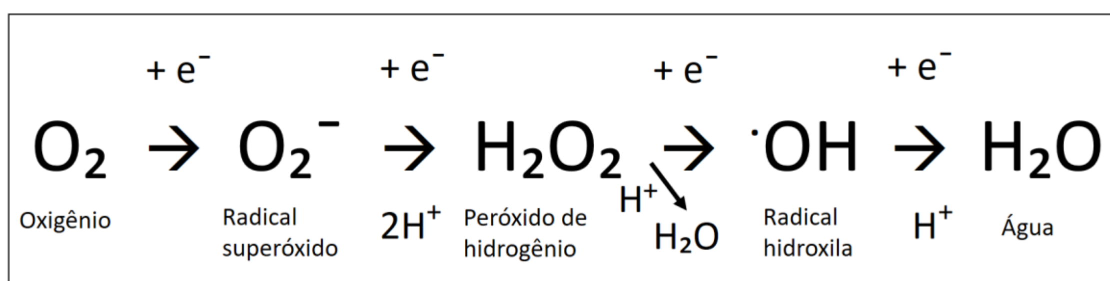

Figura 2- Esquema de redução do O_2 em água e o conjunto de produtos intermediários formado pelas espécies reativas: radical superóxido, peróxido de hidrogênio e o radical hidroxila. (Fonte: Adaptado de Imlay, 2003).

O superóxido ($O_2^{-\cdot}$) é formado na redução do O_2 com um elétron, e a partir dessa espécie reativa, as demais são geradas. A redução de dois elétrons de oxigênio ou a dismutação do O_2 pela enzima superóxido dismutase (SOD) (Gutteridge e Mitchell, 1999), leva a formação do peróxido de hidrogênio (H_2O_2). Pela ausência de elétrons desemparelhados na última camada o H_2O_2 não é um radical livre, porém, é uma ROS que pode participar da síntese de $^{\cdot}OH$ (Gutteridge e Mitchell, 1999; Comhair e Erzurum, 2002), ou pode ser convertido em água, pelas enzimas catalase (CAT) e glutationa peroxidase (GPX) (Dasuri et al., 2013).

Tanto o peróxido de hidrogênio (H_2O_2) quanto o radical superóxido ($O_2^{-\cdot}$) na presença de metais reativos como o ferro e cobre, podem participar na síntese de radical

hidroxil ($OH^{\cdot}$) (Reação de Fenton ou Haber-Weiss) (Machado, 2014). O radical hidroxil possui alta reatividade e é lesivo para as células (Piacenza et al., 2009) por não possuir sistema enzimático de defesa (Yu, 1994).

O organismo desenvolveu formas de defesa quanto aos danos causados pelo ROS, essa defesa é conhecida como antioxidante e é preciso estar sempre ativa em quantidades suficientes para garantir a proteção do organismo, sendo que a produção de energia é constante e é o principal gatilho de formação de ROS (Barreiros et al., 2006). O estresse oxidativo é causado por um desequilíbrio entre a formação de espécies reativas de oxigênio (ROS) e as enzimas de defesas antioxidantes. Esse desequilíbrio pode ser causado pela alta produção de ROS e também pela deficiência das defesas antioxidantes (Halliwell e Gutteridge, 2015).

2.3 Sistema de defesa antioxidante enzimáticos e não enzimáticos

Para restaurar o equilíbrio redox e neutralizar os efeitos dos oxidantes, as defesas antioxidantes do organismo podem ser enzimáticas ou não enzimáticas. Pode-se então avaliar o estresse oxidativo através da mensuração dos agentes antioxidantes enzimáticos, sendo os de maior importância: superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPX) (Lucio et al., 2018). Já os principais antioxidantes de defesa não enzimática são vitaminas hidrossolúveis e lipossolúveis, nutrientes de origem mineral (zinco, cobre, manganês, selênio e ferro) e derivados de plantas (bioflavonoides) (Halliwell e Gutteridge, 2015).

2.4 Defesa antioxidante enzimáticos: Superóxido dismutase (SOD), Catalase (CAT) e Glutationa peroxidase (GPX)

A Superóxido dismutase é considerada a primeira enzima que ataca o radical superóxido ($O_2^{-\cdot}$), quando este é formado no organismo pelos processos de oxidação do oxigênio (Halliwell e Gutteridge, 2015). Através da dismutação a SOD é capaz de catalisar a conversão do radical superóxido em peróxido de hidrogênio (H_2O_2) (Yu, 1994). Após algumas horas de exposição do composto ou mecanismo que induzem a produção de ROS, a SOD já é ativada (Van der Oost et al., 2003). A mensuração de sua atividade é muito utilizada para estimar os efeitos que conduzem a produção de ROS (Simonato et al., 2016).

A SOD pode ser encontrada em todos animais aeróbicos, além de ser a enzima em maior quantidade no organismo, podendo ser identificada de duas formas, contendo Cobre-Zinco (CuZnSOD) encontrada no citoplasma e contendo Manganês (MnSOD), localizada na mitocôndria (Halliwell e Gutteridge, 2015).

A catalase atua sobre o peroxido de hidrogênio (H_2O_2), que por sua vez é convertido em água e oxigênio molecular (Van der Oost et al., 2003). A ação da catalase depende de doadores de H e da concentração de H_2O_2 no organismo, pois quando o H_2O_2 está em baixa concentração, a função de transformá-lo em água é atribuída a glutationa peroxidasse (Halliwell e Gutteridge, 1985, 2015). Outra função antioxidandante dessa enzima é minimizar os riscos de formação do radical OH via reação de Haber-Weiss e Fenton, catalizada principalmente por ions metálicos de cobre e ferro (Birben et al., 2012). Devido ao tempo de vida curta, esse radical dificultosamente será neutralizado pelo organismo, promovendo assim, grande poder reativo e lesivo (Halliwell e Gutteridge, 2015).

Tanto a CAT quanto a GPX evitam o acúmulo de radical superóxido e de peróxido de hidrogênio para que não aconteça produção excessiva de radical hidroxil, pois para esse composto o sistema enzimático de defesa é inexistente (Yu, 1994).

O sistema de defesa da glutationa (GSH) depende da ação das enzimas glutationa oxidase (GO), glutationa peroxidase (GPX) e glutationa redutase (GR). Para reduzir o H_2O_2 e transformá-lo em água, glutationa (GSH) é utilizada como agente redutor, para isso, sofre ação da glutationa oxidase (GO) e glutationa peroxidase (GPX), que é transforma em glutationa dissulfeto (GSSG), para assim comprir o seu papel (Rover Júnior et al., 2001). A glutationa dissulfeto (GSSG) é reduzida pela glutationa redutase (GR) que regenera a glutationa (GSH), dessa forma, GSH consegue reiniciar novas reações para reduzir especies oxidantes (Huber et al., 2008).

A avaliação e mensuração dos genes das enzimas antioxidantes relacionados ao estresse oxidativo tem sido realizada na tentativa de decifrar os eventos fisiológicos ligados a este processo, uma vez que a produção de espécies reativas de oxigênio (ROS) e sua eliminação, são mecanismos regulados pelo sistema antioxidante do organismo (Abdalla, 2015).

3.0 Estresse e metionina sobre absorção intestinal e estresse oxidativo

3.1 Estresse e absorção

Os peixes têm a capacidade de alterarem sua homeostasia para se adaptar as novas condições quando submetidos a estresse, mas como consequência diminui seu desempenho por desviarem sua energia a fim de restabelecer o equilíbrio do organismo (Squires, 2010). Além de afetar o desempenho, o estresse pode afetar negativamente o comportamento, crescimento, reprodução, sistema imunológico e fisiologia (Pickering e Pottinger, 1995; Da Silveira et al., 2009). Com isso, as funções do trato gastrointestinal sofrem alterações a curto e a longo prazo em resposta ao estresse, englobando efeitos negativos na microflora, capacidade regenerativa, fluxo sanguíneo da mucosa intestinal, além de alteração da motilidade e secreção gastrointestinal (Konturek et al., 2011).

De acordo com Davis et al. (2016) a microbiota intestinal realiza uma importante função no comportamento relacionado ao estresse, pois os micróbios de alguma forma comunicam-se bidireccionalmente com o sistema nervoso central e modula respostas de estresse e comportamento. Para diminuir as respostas do estresse, como comportamento ansioso e depressivo, estudos foram realizados com cepas de Lactobacillus e / ou Bifidobacterium e mostraram aliviar esses sintomas (Bravo et al., 2011; Savignac et al., 2014; Savignac et al., 2015). Outro estudo avaliando a suplementação de metionina, demonstrou que a mesma teve efeitos no equilíbrio da microflora intestinal, aumentando a proliferação de bactérias benéficas e deprimindo o crescimento de bactérias nocivas, promovendo dessa forma, imunidade humoral (Tang et al., 2009).

Os transportadores de dipeptídeos parece ser menos afetados por problemas de agressões a mucosa intestinal, quando comparado com os transportadores para aminoácidos individuais, por exemplo, em casos de desnutrição proteica-energética verifica-se a redução na absorção de leucina, mas a absorção do dipeptideo glicina-leucina não é afetada (Matthews e Adibi, 1976). Em casos de doenças ou desafios que possam levar a diminuição absortiva pelos enterócitos, a administração de dipeptídeos e tripeptideos podem atuar como fator de prevenção de desnutrição proteica. A busca por alimentos ou microrganismos que melhorem a microflora e a absorção intestinal é uma necessidade, visto as inúmeras consequências que o estresse pode causar no sistema gastrointestinal.

3.2 Dipeptídeo de metionina

A metionina está entre os aminoácidos essenciais mais limitantes na alimentação de peixes, e sua deficiência além de causar limitação no crescimento reduz a eficiência alimentar (Niu et al., 2018). O dipeptideo de metionina pode potencialmente ser utilizado

nas rações dos organismos aquáticos, como a principal finalidade de maximizar a eficiência de absorção e disponibilização de metionina para o organismo, favorecendo assim o desenvolvimento corporal. Diante desses benefícios, nos últimos anos o dipeptídeo de metionina tem sido utilizado como suplementação da dieta de animais aquáticos (Mamauag et al., 2012; Façanha et al., 2018; Niu et al., 2018). Isso porque, estudos relacionados ao sistema de transporte intestinal de aminoácidos na sua forma livre, bem como na forma de dipeptídeos e tripeptídeos têm revelado diferença significativa no mecanismo pelo qual estas substâncias são absorvidas (Chen et al., 2002; Dabrowski et al., 2003; Daniel, 2004), em que foram verificados pequenos peptídeos intactos (2-3 peptídeos) podem ser transportados de forma muito mais eficiente, em relação aos aminoácidos livres. Uma vez que, o gasto energético para transportar os aminoácidos livres é o mesmo que o observado para transportar dipeptídeos e tripeptídeos, mostrando maior eficiência energética corporal quando se absorve dipeptídeos e tripeptídeos. Ainda, o transporte de aminoácidos livres necessita de alta especificidade entre substrato e transportador (Daniel, 2004), sendo alguns aminoácidos livres absorvidos pelo mesmo sistema de transporte, podendo haver maior absorção de um aminoácido em detrimento ao outro, causando desequilíbrios entre quantidades de aminoácidos no organismo, podendo ocasionar menor eficiência alimentar.

Como os peptídeos podem ser transportados por um único transportador conhecido como já visto, muitos estudos têm sido realizados para avaliar quais são os possíveis fatores envolvidos com a regulação da expressão do gene *PEPT1* (Boll et al., 1994; Pan et al., 1997), além de verificar sua distribuição nos tecidos corporais (Chen et al., 1999; Chen et al., 2002). Já que a expressão do *PEPT1* pode ser regulada pela quantidade e qualidade da dieta disponível no lúmen intestinal, além de que a restrição alimentar também parece interferir na expressão do *PEPT1* (Gilbert et al., 2008). De acordo com Burini e Campana (1993), a atividade dos transportadores de aminoácidos livres e pequenos peptídeos na borda em escova do intestino parece não estar diretamente relacionada a mecanismos neurais ou endócrinos, dependendo apenas da presença dos substratos a serem absorvidos em contato com as células intestinais.

A partir da compreensão de como a absorção de di e tripeptideos ocorre, e em virtude da sua maior eficiência, a administração de dipeptideos nas rações tem sido proposta como uma das possíveis soluções para os problemas relacionados com a otimização qualitativa do componente proteico da dieta (Dolomatov et al., 2014). Essa indicação se dá em função da eficiência na absorção dos dipeptídeos e tripeptídeos em

relação aos aminoácidos livres, uma vez que, em quantidades equivalentes dessas fontes de aminoácidos, os di e tripeptídeos são absorvidos mais rapidamente, além de não competirem pelo mesmo transportador, como ocorre com os aminoácidos livres (Frenhani e Burini, 1999). Estudo utilizando o dipeptídeo de metionina na alimentação de crustáceos e peixes, tem demonstrado que essa fonte de metionina é duas vezes mais eficiente no crescimento animal, quando comparado a outras fontes de metionina, isso porque, o uso do dipeptideo além de melhorar a absorção de metionina pode ter efeito positivo sobre o crescimento do animal (dados do grupo de P & D aqua da Evonik). Diversos estudos que evidenciam que os aminoácidos sintéticos são bem aproveitados pelo animais aquáticos (Li et al., 2009; Espe et al., 2014; Niu et al., 2016) e rações suplementadas com dipeptídeo são mais eficientes quando comparadas aos aminoácidos livres (Niu et al. (2018)).

3.3 Metionina no estresse oxidativo.

A metionina desempenha diversas funções no organismo animal, na Figura 3 é apresentado de forma simplificada o metabolismo da metionina.

Além de diversas funções a metionina pode minimizar os efeitos causados pelo aumento na produção de espécies reativas de oxigênio (ROS), gerado pelo estresse elevado. Uma vez que metionina pode agir, no sistema antioxidante, como precursor da glutationa através da cisteína, que pode ser sintetizada no organismo por meio da via metabólica da metionina reduzindo os níveis de ROS gerado (Luo e Levine, 2009; Del Vesco et al., 2015). A cisteína por sua vez pode participar da biossíntese da glutationa, que pode ser produzido tanto pela cisteína como pelos aminoácidos glicina e ácido glutâmico. Na biossíntese da cisteína, via transulfuração, a homocisteína além de participar da biossíntese da cisteína (Stipanuk, 1986) estudos sugerem que cerca de 50% da glutationa gerada é através da homocisteína, sendo que, ao sofrer estresse oxidativo a via de transulfuração é estimulada e inibe as enzimas responsáveis pela síntese de cisteína e metionina (Mosharov et al., 2000; Persa et al., 2004).

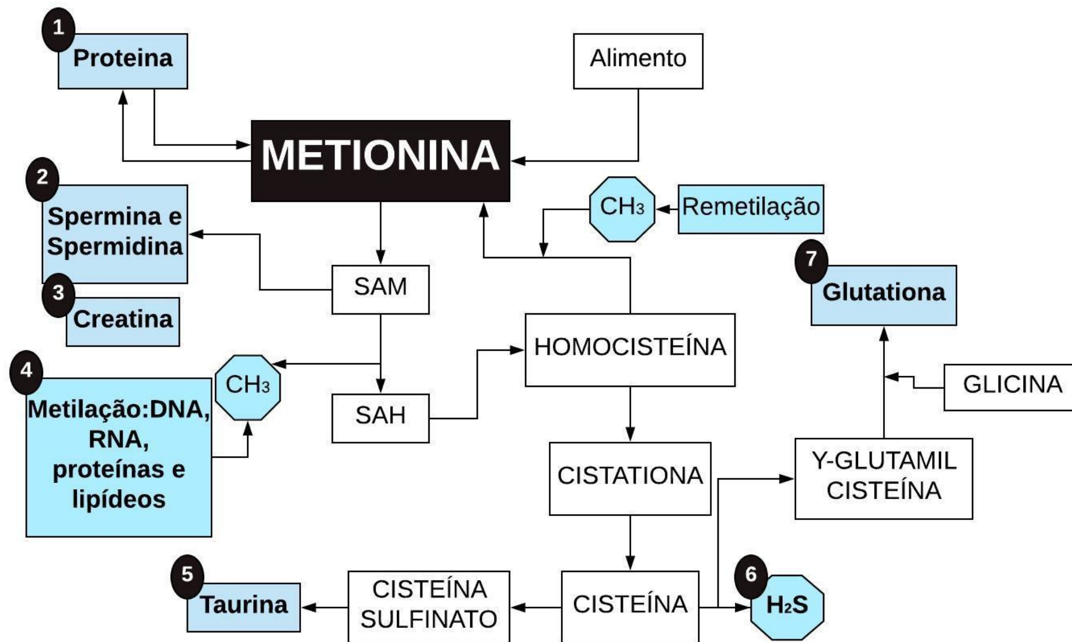

Figura 3- Metabolismo da metionina. 1) Geração de novas proteínas corporais; 2) Síntese de espermina e espermidina, envolvidas no metabolismo celular; 3) Síntese de creatina, utilizada pelo organismo para fornecer energia durante o exercício; 4) Ligação ou substituição de um grupo metila no RNA, DNA, proteína e lipídios; 5) Síntese de taurina, importante para digestão, além de ação antioxidante; 6) síntese de sulfeto de hidrogênio (ação anti-inflamatória); 7) Síntese da glutationa, importante sistema antioxidante. Adaptado de: Stipanuk (2004), Mato et al. (2008), Nelson and Cox (2008), Salway (2009) e Sakomura et al. (2014).

Dessa forma a síntese da glutationa através da homocisteína pode ser potencializada. A metionina além de participar da síntese da glutationa pode fornecer defesa antioxidante diretamente para a molécula em que estão localizadas, reagindo com ROS e protegendo essas moléculas (Luo e Levine, 2009). Dessa forma, níveis adequados de metionina devem ser fornecidos e absorvidos pelo animal, para que este tenha maior eficiência no sistema antioxidante.

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II. OBJETIVOS GERAIS

Avaliar o efeito da suplementação do dipeptídeo de metionina sobre as enzimas antioxidantes intestinais, expressão do gene *PEPT1,* resposta histológica no intestino e nível de cortisol no zebrafish (*Danio rerio*) durante o desafio provocado por risco de predação.

Avaliar a influência da nutrição materna sobre a abertura de boca e sobrevivência no desenvolvimento inicial de larvas de zebrafish alimentadas com ração suplementada com metionina.

1 # III. Intestinal antioxidant enzymes and PEPT1 gene expression,
2 histology and cortisol in zebrafish in response to methionine dipeptide
3 and risk of predation

4

5 (Chemosphere)

6

7 **ABSTRACT**

8 The aim of this study was to verify the effect of methionine and risk of predation on gut
9 histology, cortisol whole body level, and gene expression in zebrafish. A total of 360 one
10 year-old animals were divided into three treatments related to methionine
11 supplementation: no methionine supplementation (SM); supplemented with DL
12 methionine 99% amino acid in its free form (DL) and supplemented with methionine in
13 the dipeptide form DL-Methionyl-DL-Methionine 95% (MM). The fish were maintained
14 without contact with predator *Astronotus ocellatus* in the aquarium (SO) or with direct
15 contact with predator in the aquarium (CO) (for 48 hours or 20 days, depending on the
16 experimental phase evaluated). Stress due to predation risk reduced weight gain by 20
17 days (SO = 0.142 and CO = 0.085g). Methionine supplementation, regardless of the
18 source, presented higher weight gain when compared to SM (SM = 0.077g, DL = 0.130g
19 and MM = 0.133g). No statistical significant differences were found in cortisol levels due
20 to the risk of predation and methionine supplementation. The dipeptide transporter
21 (PEPT1) had no significant effect on the interaction between the risk of predation and
22 methionine supplementation. Fish with 48 hours of exposure to predation stress, *SOD*,
23 *CAT* and *GPX* presented higher mRNA expression in the gut. Furthermore, consumption
24 of the diets at 20 days had lower expression of *GPX*, *SOD* and *CAT* in eating animals
25 diets DL and MM. In conclusion, the ability to neutralize oxidant agents is important for

26 growth, intestinal health and animal survival in environments with risk of predation, and
27 a diet with adequate levels of methionine is essential to ensure cell tolerance, providing
28 reducing power for antioxidant systems.

29 ***Keywords:*** *Danio rerio*; essential amino acid; oxidative stress; stress hormone

30

31 **1. Introduction**

32 Zebrafish and most of fish has showed methionine as a limiting amino acid in
33 commercial diets (NRC, 2011), in which the main used source is the DL-Methionine 99%
34 (DLM). Recently, a new industrial product with lower solubility (Niu et al., 2018), which
35 avoid leaching, has been produced, especially for fish and shrimp, a dipeptide named
36 Met-Met (DL-Methionyl-DL-Methionine, 95%). Di and tri-peptides are the major form
37 of intestinal absorption amino acids in fish and other vertebrate animals, in which the
38 main transporter is the PEPT1, while free amino acids have specific form of transporters
39 which could be transported by active transport dependent to sodium (Na) or hydrogen
40 (H). There are evidences that PEPT1 is less affected by damages into the gut due to
41 different aggressions such as micro-organisms, anti-nutritional factors and others stress
42 conditions such as temperature, salinity and predator. All these factors may increase
43 cortisol levels, damage the intestinal absorption area, and change the antioxidant enzymes
44 and PEPT1 expressions.

45 Methionine deficiency itself could be considered as a stress factor (Crespo-Bujosa
46 and Gonzalez, 2018) which reduce the antioxidant system capacity in the animals and
47 increase indicators such as heat shock proteins evidencing abnormalities in animal
48 metabolism. Supplementing methionine in fish diets is important to maintain the optimum
49 amino acid balance, called the ideal protein. The best methionine level in the cells may
50 improve the antioxidant defense, which can have either a direct effect through methionine

51 sulfoxide, where this amino acid side chains in proteins molecule can have effect in
52 scavenging reactive oxygen species (ROS), or by indirect effects, where methionine
53 forms cysteine and glutathione (GSH), helping in controlling ROS, such as hydrogen
54 peroxide (H_2O_2). Another indirect effect of methionine is enhancing the first line defense
55 antioxidant enzymes, which comprehend superoxide dismutase (SOD), catalase (CAT)
56 and glutathione peroxide (GPX) enzymes, responsible for helping tissues, like the gut's
57 tissue, to scavenger ROS (Wu et al., 2017; Ighodaro and Akinloye, 2018).

58 A few studies using high-level protein or amino acids (Wang et al., 2017;
59 Osmanyan et al., 2018) and dipeptides such as lysine-glycine (Kwasek et al., 2012) have
60 showed effect on *PEPT1* expression. Results obtained from those studies showed
61 enhancing of PEPT1 expression when protein, amino acids and dipeptides levels were
62 higher in diets. Nevertheless, methionine or its dipeptide effect on *PEPT1* expression has
63 never been evaluated in zebrafish or other fish species, so far.

64 Although, researches have been showed the effect of either nutrients (Ren et al.,
65 2017) or stress factor like predator (Oliveira et al., 2017) on specifics fish responses
66 (Gorissen and Flik, 2016), we have not seen so far, the effect of both methionine and
67 predator on zebrafish responses, including many parameters such as cortisol. Intestinal
68 histology and gene expression changes, all together, to have a better understanding about
69 the metabolic response of zebrafish.

70 The aim of this study was to verify the effect of methionine and risk of predation
71 on gut histology, cortisol whole body level, and gene expression in zebrafish.

72

73 **2. Materials and methods**

74 This experiment was conducted according to the ethics committee of the State
75 University of Maringa, registered under CEUA nº 9788181117.

76

77 ***2.1 Animals and Experimental Design***

78 For the development of the experiment, 360 one-year-old healthy adults zebrafish
79 (Danio rerio) were used. The experiment was conducted in a completely randomized 3x2
80 factorial design, with three diets: no methionine supplementation (SM); supplemented
81 with DL-methionine 99% amino acid in its free form (DL) and supplemented with
82 methionine in the dipeptide form DL-Methionyl-DL-Methionine 95% (MM), with two
83 systems: with and without direct contact with the predator in the aquarium, with 3
84 replicate per treatment, totaling 18 aquariums. *Astronotus ocellatus* fish were used as
85 predatory stimuli, which were kept under the same experimental conditions as zebrafish.
86 Zebrafish were submitted to a seven-day adaptation period, fed twice daily with
87 feed containing 40% crude protein and 3201 Kcal / kg of digestible energy and adequate
88 levels of amino acids according to the ideal protein. Fish were selected according to their
89 mean weight (0.74 g) and mean total length (39.5 mm). During the experimental period
90 they were kept in aquariums of 32 liters separated by a screen (place where the predator
91 was allocated), with chlorine-free water, constant aeration and controlled temperature,
92 with photo daily period of 14 hours and 10 hours dark.
93 The feeding of the animals used as predatory stimulus was suspended 24 hours
94 before the beginning of the experimental period with the aim of encouraging predatory
95 behavior and then fed once a day. The zebrafish were fed twice a day, according to each
96 treatment with the SM, DL and MM rations. The water quality parameters (Oxygen,
97 temperature, pH, specific conductance and total dissolved solids) were analyzed daily
98 before the first feeding of the day. A partial water exchange (30%) was performed every
99 2 days to ensure that the fish were not stressed due to water quality.

100

101

102 ### *2.2 Experimental diet*

103 The formulation of the experimental diets for zebrafish is presented in Table 1.
104 The control diet is not supplemented with methionine. Two test diets based on the control
105 diet formulation were supplemented with DL-methionine and the other with the dipeptide
106 Met-Met and contain 40 % crude protein and 3201 Kcal / kg digestible energy. The
107 prepared diets were stored in a cold room prior to the start of the experiment.

108

109

Table 1: Experimental diets centesimal composition.

Ingredients	**SM[1]**	**DL[2]**	**MM[3]**
Corn	23.60	23.60	23.60
Soybean flour	27.50	27.50	27.50
Gluten 60	15.00	15.00	15.00
Viscera flour	10.00	10.00	10.00
Feather flour	6.00	6.00	6.00
Fish flour	5.00	5.00	5.00
Rice grits	5.00	5.00	5.00
Wheat gluten	1.00	1.00	1.00
L-Lysine	0.70	0.70	0.70
DL-Methionine	**0.00**	**0.27**	**0.00**
Met-Met	**0.00**	**0.00**	**0.28**
L-Threonine	0.15	0.15	0.15
L-Tryptophan	0.05	0.05	0.05
Colin chloride 60%	0.10	0.10	0.10
Vitamin C	0.10	0.10	0.10
Premix	0.50	0.50	0.50
Phospate	4.35	4.35	4.35
Salt	0.50	0.50	0.50
Antifungal	0.10	0.10	0.10
Antioxidant	0.02	0.02	0.02
Inert (Caolin)	0.30	0.05	0.05
Total	100.0	100.0	100.0

110 [1]SM, without methionine supplementation, [2]DL, supplemented with DL-Methionine 99% -amino acid in
111 its free form; [3]MM, supplemented with methionine in the dipeptide form DL-Methionyl-DL-Methionine
112 95%.

113

114 *2.3 Sample collection*

115 After the adaptation period, zebrafish were submitted to predation risk stress
116 during 20 days. After 48 hours from the start of the experiment, seven fish from all
117 treatments were selected for cortisol level analysis in the whole body. At 48 hours and on
118 the 20th day of the experiment, 12 animals from all treatments were collected for the
119 analysis of gene expression (6 fish) and histology (6 fish). The gut samples collected for
120 gene expression were kept on ice while being prepared, and stored in a freezer at -80 °C
121 until analyzed.

122

123 *2.4 Histological Analysis*

124 The segment of the gut collected corresponded to the middle third of the gut. After
125 removal, it was sectioned transversely and the ends fixed. The intestinal fragments were
126 isolated and fixed in Bouin solution for 6 hours, dehydrated in increasing series of
127 alcohols, diaphanized in xylol and embedded in paraffin. Semi-serial transverse sections
128 of 3 micrometers were obtained in a microtome and submitted to 1% alcian blue staining
129 at pH 2.5 plus 0.5% Schiff periodic acid (PAS) against stained Hematoxylin-eosin, which
130 allowed the evaluation of the morphometry of the mucosa of the intestinal segments and
131 to identify the goblet cells. They were analyzed by light microscopy (Motic BA310E),
132 Moticam 5.0MP camera, and digital images (50 villi per animal) were analyzed using
133 Image-Pro Plus software.

134

135 *2.5 Whole Body Cortisol Concentrations*

136 The level of cortisol as an indicator of stress was determined on the whole body
137 of the zebrafish. For this, seven fish from each treatment were used. Cortisol extraction
138 was performed according to the method described by Barcellos et al. (2007) and Sink et

139 al. (2007), with some modifications. The fish were captured, euthanized and immediately
140 frozen in a freezer at -80 °C until cortisol extraction. The fish were thawed at room
141 temperature, weighed, smashed, and tissue extracts was placed in a test tube containing 3
142 mL of a sodium phosphate buffer solution with gelatin pH 7.3 (PBSG). Subsequently this
143 content was homogenized in the turrax, and then 1 ml aliquot was withdrawn and
144 transferred to a clean test tube. Onto this homogenate was added 3 mL of ethyl ether and
145 then vortexed. This solution (homogenate + ethyl ether) was frozen in liquid nitrogen,
146 and the non-frozen portion (cortisol containing ethyl ether) was decanted and transferred
147 to a new tube, this step was repeated three times. After this procedure the obtained
148 solution was allowed to rest in a water bath at 37 ºC to completely evaporate the ethyl
149 ether and obtain the lipid extract containing cortisol. After evaporation of ethyl ether, 200
150 µl of the buffer PBSG pH 7.3 was added to the test tube, and sequentially that solution
151 was homogenized in vortex. The resuspended cortisol solution in PBSG pH 7.3 was then
152 stored in a freezer at -20 °C until the time of cortisol levels in the samples.
153 Cortisol levels in the whole body were measured using the CORTISOL ELISA kit
154 (Diagnostic Biochem Canada Inc. Ref: CAN-C-270, version: 5.4). This kit was
155 previously shown to be effective for the analysis of zebrafish fish tissue extracts by Sink
156 et al. (2007). Results of cortisol levels in the whole body are expressed as g/mL.
157

158 ***2.6 Analysis of gene expression***

159 Total RNA was isolated from the gut using the Trizol reagent (Invitrogen,
160 Carlsbad CA, USA) according to the manufacturer's protocol. The absorbance ratio at
161 260 nm and 280 nm, as well as the standard 1% agarose formaldehyde gel bands, was
162 used to verify the RNA quality in each sample. Subsequently, RNA was heated at 65 °C
163 for 10 min. cDNA was synthesized using the SuperScript ™ III First Strand Synthesis

164 Super Mix kit (Invitrogen Corporation, Brazil) and reactions were performed according
165 to the manufacturer's instructions. Shortly after cDNA synthesis, the samples were stored
166 at -20 °C until the time of use. Real-time PCR reactions (polymerase chain reaction - RT-
167 qPCR) were performed using the SYBR GREEN fluorescence compound (SYBR
168 GREEN PCR Master Mix, Applied Biosystems, USA) according to the manufacturer's
169 instructions. The sequences of *SOD2, CAT, GPX1* primers were used according to Sarkar
170 et al. (2014). *PEPT1* and *ß-actin* were designed according to the sequences of the genes
171 for *Danio rerio* (Table 2), deposited at www.ncbi.nlm.nih.gov, using the website
172 www.idtdna.com.
173

Table 2: Primer sequences used for quantitative real-time polymerase chain reaction (real-time PCR - RT-qPCR).

Gene	Primer sequence	Amplicom (bp)	Access number
SOD2	F: 5´-AGCGTGACTTTGGCTCATTT- 3´ R: 5´-ATGAGACCTGTGGTCCCTTG- 3´	166	NM_199976.1
CAT	F: 5´-CTCCTGATGTGGCCCGATAC- 3´ R: 5´-TCAGATGCCCGGCCATATTC- 3´	209	AF170069.1
GPX1	F: 5´-CCCTCTGTTTGCGTTCCTGA- 3´ R: 5´-TCTTGAATGGTTCCCCGTCC- 3´	201	BC164790.1
PEPT1	F: 5´-CTCAAATCAGCCCAAGGAAATG-3´ R: 5´-CCTCCAAAACTACCAACCCTC- 3´	91	NM_198064
ß-actina	F: 5´-ACCCCAAAGCCAACAGA- 3´ R: 5´-CCAGAGTCCATCACAATACC- 3´	136	L08165

174

175 For endogenous control, the *ß-actin* gene was used to eliminate variations in the
176 amount of mRNA and cDNA and quality, each level of mRNA was expressed as its ratio
177 to *ß-actin* mRNA. The following PCR protocol was used in the BIORAD iQ5 apparatus:
178 denaturation for 1 min at 95 °C, followed by 40 cycles of 15 sec at 95 °C, and 1 min at
179 60 °C. All analyzes were performed in a volume of 25 µL and duplicates.
180

181 *2.7 Data analysis*

182 The $2^{-\Delta CT}$ method was used for analyzes of gene expression and the results are
183 presented as arbitrary unit (AU). The results were presented as means and standard
184 deviations. The UNIVARIATE procedure was applied to evaluate the normality of the
185 data. For water quality parameters, weight gain, levels of cortisol and gene expression,
186 the experiment was conducted in a completely randomized factorial design 3 X 2, with
187 three diets (SM, DL and MM) and two systems (with and without predator) evaluated.
188 For histological analysis was performed completely randomized design, with four
189 trataments diet control without predation risk, SM diet with predator, DL diet with
190 predator and MM diet with predator. The means of all analyzes performed were compared
191 using the Tukey test ($P < 0.05$) (SAS Inst. Inc., Cary, NC, USA).

192

193 **3. Results**

194 ***3.1 Water Quality***

195 The parameter of water quality was in the appropriate standard for the studied
196 species and were maintained in the adaptation and in the experimental period. Partial
197 water exchange every two days was sufficient to ensure that the water quality variables
198 did not obtain higher variation and were adequate for fish maintenance (Table 3), thus,
199 oxygen, temperature and pH, hardness did not differ between diets in both environments.
200 The total hardness and total ammonia was performed by test, and there was also no
201 variation between treatments (50 ppm $CaCO_3$ and 0 ppm, respectively).

202

Table 3. Water quality parameters with and without direct contact with the predator in the aquarium.

With predator							
Diet	SM		DL		MM		P<0.05
	Mean	SD	Mean	SD	Mean	SD	
Oxygen	6.99	6.60	6.24	0.69	6.31	0.56	0.4694
Temperature	29.31	1.11	29.24	1.03	29.4	1.09	0.6999
pH	6.44	0.82	6.43	0.79	6.43	0.82	0.9978
No predator							
Diet	SM		DL		MM		P<0.05
	Mean	SD	Mean	SD	Mean	SD	
Oxygen	7.57	6.65	7.52	6.5	6.5	0.52	0.4459
Temperature	28.65	1.16	28.71	1.36	28.83	1.18	0.6898
pH	6.48	0.86	6.45	0.90	6.48	0.83	0.9749

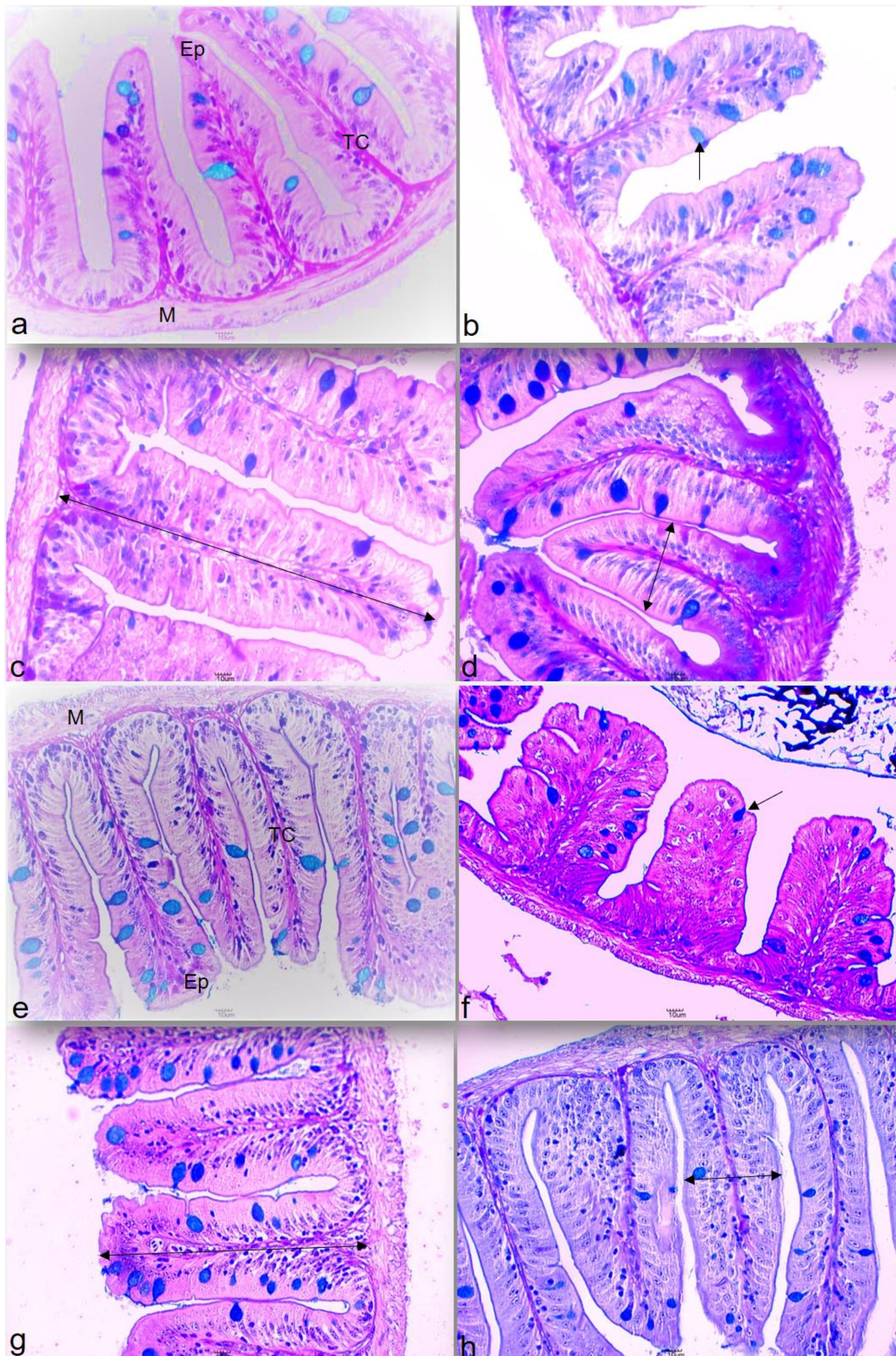
203 [a, b] Means in the same column with different letters are significantly different by Tukey's test ($p < 0.05$).
204 Oxygen (mg/L); temperature (°C); pH (hydrogenation potential); without methionine supplementation
205 (SM); supplemented with DL-methionine 99% -amino acid in its free form (DL) and supplemented with
206 methionine in the dipeptide form DL-Methionyl-DL-Methionine 95% (MM).

207

208 ***3.2 Intestinal Histology***

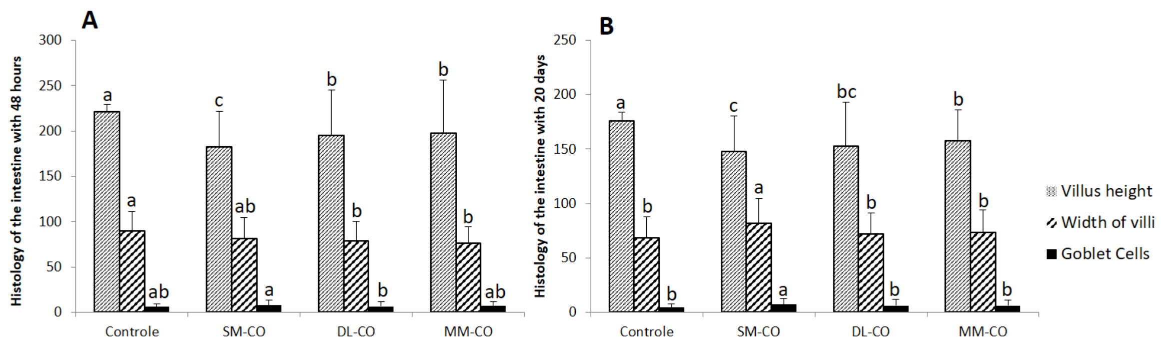
209 In the mucosa, the coating epithelium observed was the simple columnar type,
210 composed by absorbent enterocytes and goblet cells, the last synthesize and secrete mucus
211 (Fig. 1). The microscope slide presented loose connective tissue. The muscular layer was
212 constituted by circular and longitudinal smooth muscle cells. Regardless of the time of
213 collection, the analyzed histological parameters were altered in comparison to the control
214 (Fig. 2).

215



216 **Fig. 1.** Histological sections of the gut of *Danio rerio* underwent 48 hours (a, b, c and d) and 20 days (e, f, 217 g and h) of experimentation stained with HE, Alcian blue and PAS. Animals fed with diet control without 218 predation risk (a and e), SM diet with predator (b and f), DL diet with predator (c and g) and MM diet with 219 predator (d and h). Ep = prismatic simple coat epithelial tissue with striated border and goblet cells; TC = 220 loose connective tissue of the intestinal mucosa; M = smooth muscle layer; Arrows = Goblet cell; Double 221 arrow = villous height (c and g); Double arrow = villous width (d and h). 40X magnification.

222



223

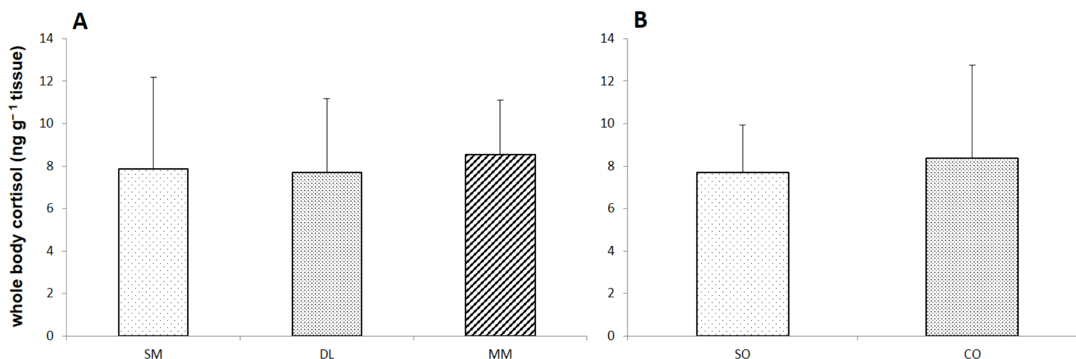
224 **Fig. 2.** Histology of the gut of zebrafish (*Danio rerio*) with 48 hours (A) and 20 days (B). Diet
225 recommended without presence of predation risk in the aquarium (Control); without methionine
226 supplementation with predation risk (SM-CO); supplemented with DL-Methionine 99% - amino acid in
227 free form with predation risk (DL-CO) and supplemented with methionine in the dipeptide form DL-
228 Methionyl-DL-Methionine 95% with predation risk (MM-CO). Error bar is used to represent the standard
229 deviation. The significant differences ($p < 0.05$) among treatments and controls are indicated by the
230 different lowercase letters above the bars.

231

232 *3.4 Cortisol whole body level*

233 In the Fig. 3 the cortisol level in the whole body of zebrafish submitted to SM,
234 DL and MM diets in both environments can be observed. The levels of cortisol,
235 independent of diet and environment, had no significant difference.

236



237

238 **Fig. 3.** Levels of cortisol in the whole body of zebrafish (*Danio rerio*) (ng/g fish) with 48 hours eating the
239 diets: No methionine supplementation (SM); supplemented with DL-methionine 99% -amino acid in free
240 form (DL) and supplemented with methionine in the dipeptide form DL-Methionyl-DL-Methionine 95%

(MM). No predation risk (SO) and with predation risk (CO). Error bar is used to represent the standard deviation.

3.5 SOD2, CAT and GPX1 gene expression

The results of *SOD2*, *CAT* and *GPX1* genes expression in the zebrafish gut with 48 hours and 20 days of experiment, for the three diets, and two environments studied, are presented in figure 5. *SOD2* (Fig. 4A), *CAT* (Fig. 4B) and *GPX1* (Fig. 4C) genes showed lower mRNA expression when fed with SM diet at 48 hours. With 20 days, higher expression of *SOD2* (Fig. 4D), *CAT* (Fig. 4E) and *GPX1* (Fig. 4F) genes was observed in fish fed with SM diet. Animals underwent to stress of 48 hours, increased expression of *SOD2*, *CAT* and *GPX1* genes significantly compared to animals that were not stressed (Fig. 4G, Fig. 4H and Fig. 4I respectively). As for *GPX1* mRNA expression with 20 days, higher values were observed in the animals without the predation risk in relation to with predation risk the opposite was observed for the *CAT* gene (Fig 4L and Fig 4K, respectively).

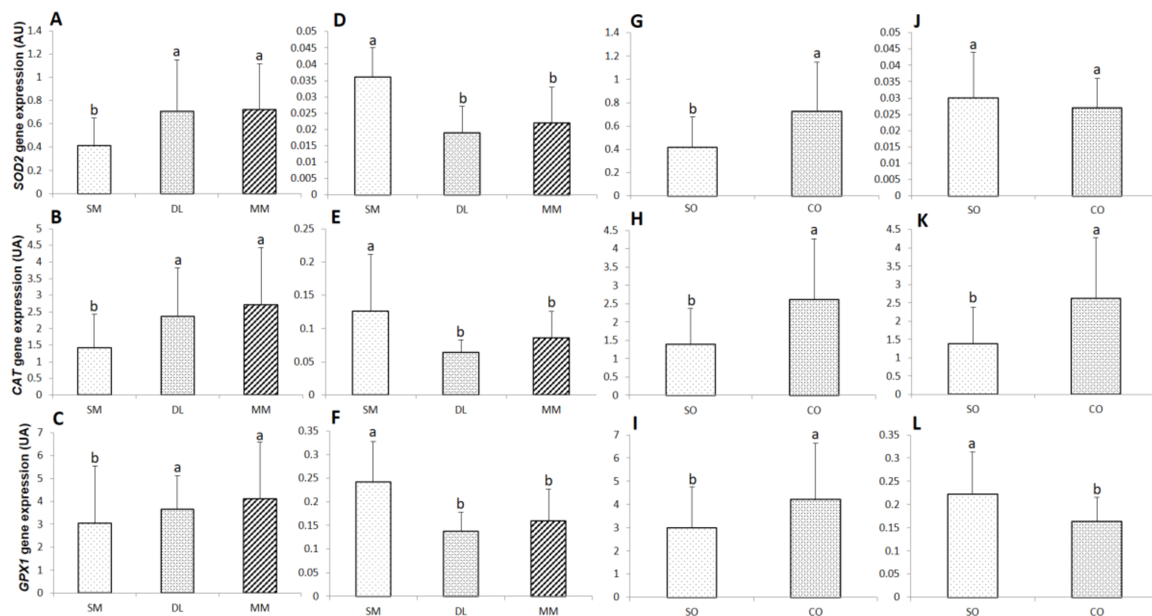


Fig. 4. Expression of superoxide dismutase (*SOD2*), catalase (*CAT*) and glutathione peroxidase (*GPX1*)

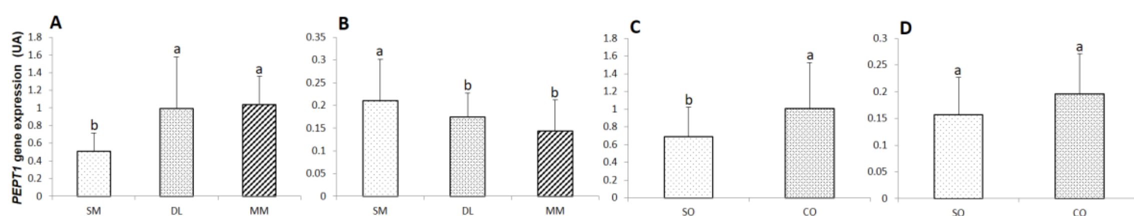
259 genes in the gut of zebrafish with 48 hours (A, B, C, G, H and I) and 20 days (D, E, F, J, K and L) consuming
260 the diets in the environments: without methionine supplementation (SM); supplemented with 99% DL-
261 Methionine-amino acid in its free form (DL); supplemented with methionine in the dipeptide form DL-
262 Methionyl-DL-Methionine 95% (MM); No predation risk (SO) and with predation risk (CO). The
263 significant differences ($p < 0.05$) among treatments and controls are indicated by the different lowercase
264 letters above the bars.

265

266 ### *3.6 PEPT1 mRNA expression.*

267 We did not observe a significant effect of the interaction between the risk of
268 predation and methionine supplementation on the dipeptide transporter mRNA (*PEPT1*).
269 For SM, DL and MM diets with 48 hours, we observed that the *PEPT1* mRNA expression
270 was higher in animals fed with DL and MM diets (Fig. 5), and lower in animals fed with
271 SM. For the same diets with 20 days, we observed higher expression of *PEPT1* for the
272 animals consuming SM diet, and lower for the DL and MM diets. The animals at risk of
273 predation considering 48 hours, the expression of *PEPT1* was higher when compared to
274 animals without risk of predation. There was no difference between the environments
275 with 20 days for *PEPT1* expression.

276

277 

278 **Fig. 5.** Expression of the *PEPT1* transporter in the zebrafish gut with 48 hours (A and C) and 20 days (B
279 and D) consuming the diets: without methionine supplementation (SM); supplemented with 99% DL-
280 Methionine-amino acid in its free form (DL); supplemented with methionine in the dipeptide form DL-
281 Methionyl-DL-Methionine 95% (MM), considering the environments: no predation risk (SO) and with
282 predation risk (CO). The significant differences ($p < 0.05$) among treatments and controls are indicated by
283 the different lowercase letters above the bars.

284

285 ***4.0 Discussion***

286 Fish are constantly under the influence of potentially stressful environmental
287 factors such as food shortages, changes in water parameters, toxins and the presence of
288 predators (Liu et al., 2016; Modra et al., 2018; Salin et al., 2018; Swirplies et al., 2019).
289 In the aquatic environment an important source of stress is the presence of individuals of
290 the same species on different sizes competing for food, in addition to the persecution by
291 predators. The non-lethal effects of predation associated with restrictive or unbalanced
292 diets can trigger a series of physiological responses that may interfere in the health,
293 development and animal reproduction (Romero and Wingfield, 2015; Campbell et al.,
294 2016).

295 In this work we evaluated the effect of the predation risk and the methionine
296 supplementation on several physiological parameters in zebrafish. Methionine is an the
297 essential amino acids for aquatic organisms (NRC, 2011) and its deficiency can cause
298 reduced growth and food efficiency (Schwarz et al., 1998; Mukhopadhyay and Ray, 2001)
299 mainly in diets with vegetable protein. Methionine is supplemented in diets in the free
300 form and its requirement for zebrafish is not yet well established (Diogo et al., 2015;
301 Guimarães et al., 2018). Although there is evidence of the synthetic amino acids
302 efficiency for aquatic organisms (Li et al., 2009; Niu et al., 2018) there is still concern
303 about its efficiency and leaching. Thus, we proposed the use of dipeptides which water
304 solubility is lower when compared to other commercially available sources of methionine
305 (DL-methionine, L-methionine and MHA-Met).

306 Under risk of predation, the general behavior of fish can be compromised and
307 there is evidence that glucocorticoid hormones may be directly involved with this
308 phenomenon (Wingfield et al., 1990; Wendelaar Bonga, 1997; Fevolden et al., 2002).

309 Feed cessation in response to stress, associated with the catalytic effects of
310 catecholamines and corticosteroids on energy reserves may reduce fish growth (Barton,
311 2002), once that the organism is kept on alert, which increases the energy expenditure
312 (Romero and Wingfield, 2015). The cortisol, an important fish stress biomarker
313 (Baekelandt et al., 2019) is related not only to energy reserves, but also to the
314 hydromineral balance (Mazeaud et al., 1977), hyperglycemia, depletion of glycogen
315 reserves, lipolysis, inhibition of protein synthesis and suppression of the immune and
316 reproductive system (Buckingham and Fink, 2010). In this study, we did not find
317 statistically significant difference in cortisol levels due to the risk of predation and
318 methionine supplementation (SO = 7.71 and CO = 8.36) (Fig. 3), probably because of the
319 period of evaluated exposure to stress (48hs). It is probable that the greatest differences
320 occurred on the first few hours after exposure of the animals to the predator, once that the
321 plasma cortisol concentration, according with Pickering and Pottinger, 1989, and Brown,
322 1993, reaches the peaks about 115 minutes after the exposure to acute stress, which in
323 this study was provided by the confrontation with the predator, returning to baseline
324 values in approximately 6 hours. In some types of teleost fish, the cortisol peak is reached
325 within 30 min after exposure to stress (Barry et al., 1993; Head and Malison, 2000) and
326 the rate of return of cortisol levels to near-basal levels may suggest a good adaptation to
327 stress (Fevolden et al., 2002; Lima et al., 2006).

328 Physical, chemical or psychological stress can affect the functioning of several
329 organs, including the gut, reducing nutrient absorption, reducing oxygenation, changes in
330 blood flow and reducing the enzymatic activity of this organ (Konturek et al., 2011). All
331 stress-induced effects associated with deficient diets can be harmful to the gut and affect
332 nutrient absorption, especially in the case of chronic stress (Madaro et al., 2015). In our
333 study we did not observe a significant effect of the interaction between the risk of

334 predation and methionine supplementation on the dipeptide transporter mRNA (*PEPT1*).
335 We observed an isolated effect of methionine supplementation with 48 hs and 20 days
336 and under risk of predation only after 48 hours of exposure to the predator (Fig. 5). The
337 highest value of *PEPT1* mRNA was observed in the gut of zebrafish submitted to stress
338 (CO = 1.01) and in diets supplemented with methionine, regardless of the source (DL =
339 1.00 and MM = 1.04) with 48 hs (Fig. 5A and Fig. 5B). However, with 20 days of diets,
340 the higher expression was observed in the SM diet (Fig. 5B). The higher expression of
341 *PEPT1* observed in CO environment (1.96 AU) may have occurred as an attempt by the
342 organism to maintain amino acid homeostasis, since stress caused changes in the
343 intestinal absorption surface of the animals under these conditions (Fig 1). The lower
344 value of *PEPT1* mRNA observed in the SM diet (0.51 AU), associated with the gut
345 histology results (Fig. 2), may suggest that maintenance of gut cell structure as well as
346 expression of the di and tripeptide transporter is related to methionine from the diet.
347 Expression of *PEPT1* can be regulated by the quantity and quality of the diet available in
348 the intestinal lumen (Gilbert et al., 2008) as well as stress situations that may compromise
349 the search for food. High-protein diets have been linked to increases in *PEPT1* mRNA
350 levels (Gilbert et al., 2008), as well as in case of stress caused by the food restriction
351 (Ihara et al., 2000). The increase in *PEPT1* expression observed in animals subjected to
352 stress of predation risk (Fig. 5C and Fig. 5D) may have occurred as a compensation
353 mechanism due to the reduction in the area of absorption and reduction in the size of the
354 intestinal villi (Uni et al., 1998), caused by the presence of the predator and mainly in
355 diets without methionine supplementation. The highest expression of *PEPT1* observed in
356 SM in relation to DL and MM at 20 days (Fig. 6B) may have occurred due to the
357 characteristics of this transporter, which presents low affinity and high capacity (Madsen,
358 2009), being a more efficient and economical mode of assimilating amino acids. It is

359 reasonable to suppose that in restrictive or unbalanced diets, *PEPT1* presented greater
360 expression, similar to what occurs with chickens and mammals when exposed to food
361 restriction (Gilbert et al., 2007; Ostaszewska et al., 2010).

362 In this work, we expected that the diet with methionine supplementation in the
363 dipeptide form could have an effect on the *PEPT1* mRNA in relation to the other diets,
364 especially under stress conditions, since the absorption of peptides occurs faster, besides
365 being resistant to changes in diet, (Matthews and Adibi, 1976; Ostaszewska et al., 2010)
366 and because they are less affected by problems of intestinal mucosal aggression,
367 compared to the free amino acid transporters. In situations of stress that may lead to an
368 enterocyte-derived decrease in the amount of di- and tripeptides, it could act as a factor
369 in the prevention of protein malnutrition (Artis, 2008). Nevertheless, we did not observe
370 a significant effect of interaction between the factors analyzed and between the diets with
371 supplementation (DL and MM) (Fig. 5A and Fig. 5B). The expression of *PEPT1* in this
372 work may have been diluted as a result of analyzing the entire gut of the zebrafish and
373 not specific segments, since different authors have shown that *PEPT1* expression may be
374 restricted to the proximal segments of the gut in some species of fish, including the
375 zebrafish (Verri et al., 2003; Terova et al., 2009). However, similar results were obtained
376 by Ostaszewska et al. (2010) that working with Rainbow trout, did not observe difference
377 in the expression of *PEPT1* in dipeptide (lys-gly) and amino acid free (lysine + glycine)
378 diets. However, high levels of *PEPT1* expression were observed in lysine deficient diets
379 (first amino acid and fish-vertebrate). Imbalance of diets, as well as dietary restriction,
380 can cause up-regulation of *PEPT1* (Ihara et al., 2000), this fact may be associated with
381 damage to the intestinal epithelium (Ostaszewska et al., 2006) with reduction of the
382 absorption surface, thus acting as a compensatory reaction. According to Gilbert et al.

383 (2008), besides the composition and concentration of the dipeptides, the presence of other
384 components in the intestinal lumen can affect the absorption dynamics.

385 Stress caused by the presence of stressors, as well as predators, in the fish farming
386 may trigger an imbalance between the production and elimination of reactive oxygen
387 species such as H_2O_2, $O_2^{\cdot-}$, singlet oxygen, hydroxyl ions and nitric oxide (Ighodaro and
388 Akinloye, 2018). To decrease the harmful effects of these molecules the body of most
389 vertebrates, triggers the antioxidant systems in order to neutralize them. The main
390 enzymes involved in this process are: superoxide dismutase (SOD), catalase (CAT),
391 glutathione peroxidase (GPX) (Ighodaro and Akinloye, 2018). Stress associated with an
392 imbalanced diet, mainly deficient in methionine, may difficult the action of these
393 enzymes, making the organism subject to oxidative damage in lipids, proteins and DNA
394 (Campbell et al., 2016). We observed that with 48h of exposure to stress by predation,
395 *SOD2*, *CAT* and *GPX1* presented higher values of mRNA in the gut (Fig. 4), suggesting
396 high production of free radicals and activation of antioxidant defenses (Persa et al., 2004;
397 Chen et al., 2012).

398 With 20 days of exposure to the predator, *GPX1* expression decreased
399 significantly, while *CAT* mRNA was higher in this condition (Fig. 4L and Fig. 4K). *CAT*
400 and *GPX1* are enzymes that act in cooperation by sequestering the hydrogen peroxides
401 (Halliwell and Gutteridge, 2015). Reduction of GPX may be related to accumulation of
402 H_2O_2 due to prolonged stress exposure and possible depletion at GSH levels, since GPX
403 acts by catalyzing the conjugation of GSH to H_2O_2 (Chen et al., 2012; Yadav et al., 2015).
404 CAT acts as a primary defense against oxidative stress, acting when H_2O_2 levels are
405 higher (Halliwell and Gutteridge, 2015), therefore it is reasonable to suppose that at 20
406 days under risk of predation there was excessive production of H_2O_2. Under stress
407 conditions caused by chemical agents in fish, researchers observed a reduction in the

408 activity of the enzymes, SOD, CAT and GPX with increased doses applied in different
409 tissues (Dabas et al., 2014; Yang et al., 2014; Yadav et al., 2015), these results suggest
410 the existence of an enzyme action threshold, as well as a limit related to the time of
411 exposure to a stressing agent.

412 In relation to the effects of diet, we observed that methionine supplementation
413 conferred greater availability of substrate for glutathione synthesis, whereas in the MM
414 and DL diet we observed higher values of *GPX1* mRNA in relation to the SM diet (Fig.
415 4C). Methionine is a key component of the glutathione system. It is estimated that about
416 50% of glutathione production is from cysteine origin from homocysteine, through the
417 transsulfuration route, and that under conditions of oxidative stress, in which higher
418 production of glutathione is required and thus higher activity of cystathionine ß-synthase,
419 higher expression of this enzyme occurs (Mosharov et al., 2000). The results of Persa et
420 al. (2004) confirm that free radical presences can induce overexpression of CBS
421 (cystathionine β-synthase) and inhibit methionine synthase, thereby stimulating
422 transsulfuration and increased production of cysteine and glutathione.

423 The abundance of *SOD2* and *CAT* mRNAs was also influenced by the diet, with
424 the highest values observed in fish consuming diets with methionine supplementation,
425 independent of the source with 48hs (Fig. 4A and Fig. 4B).

426 In relation to the prolonged consumption of diets (20 days), we observed lower
427 *GPX1*, *SOD2* and *CAT* expression in animals consuming DL and MM diets (Fig. 4D, Fig.
428 4E and Fig. 4F). The lower expression of the antioxidant enzymes in these treatments
429 may have occurred by the direct interaction of methionine with the free radicals produced,
430 since in the cells, a variety of reactive oxygen species reacts with methionine residues
431 forming methionine sulfoxide that can be restored to methionine in a reaction catalyzed
432 by methionine sulfoxide reductase. Furthermore, methionine could act as antioxidants

433 nutrient (Pamplona and Barja, 2006; Luo and Levine, 2009) and through the glutathione
434 system. The higher expression of *GPX1*, *SOD2* and *CAT* in the SM diet can be attributed
435 to the high production of ROS and the worse condition of the antioxidant systems, due to
436 the nutritional imbalance of the sulfated amino acids deficiency diet for the glutathione
437 system (Antonopoulou et al., 2013).

438 In conclusion, it was observed that methionine supplementation promotes the
439 ability to neutralize oxidizing agents and is important for intestinal health and animal
440 survival in environments at risk of predation, and that a diet with adequate levels of
441 methionine is essential to ensure cell tolerance, providing reducing power for antioxidant
442 systems such as the glutathione and thioredoxin system.

443

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1 *Short communication*

2 IV. Survival of zebrafish (*Danio rerio*) larvae fed diets supplemented 3 with methionine dipeptide and free of live feed at 3 dpf

4

5 (Chemosphere)

6

7 **ABSTRACT**

8 The aim of this study was to evaluate the influence of maternal nutrition on the mouth 9 opening and survival of zebrafish larvae up to 20 days after hatching. Males and females 10 breeding, as well as their offspring, were exclusively fed with formulated fish feed with 11 conventional ingredients and supplemented with dipeptide DL-methionyl-DL- 12 methionine 95% (MM), DL-methionine 99% (DL) and a control treatment (without 13 methionine supplementation SM). A previous study was performed to the continuous 14 monitoring of the animals after hatching, and it was observed that the larvae opened their 15 mouth 3 days post fertilization (dpf). For the experiment, a total of 1200 larvae were 16 selected with 3 dpf, and divided into three treatments related to methionine 17 supplementation: SM, DL and MM, having the same diet as their parents. The animals 18 had 2 periods for the beginning of feeding with 3 or 5 dpf. No significant difference on 19 the survival rate (3 dpf = 72% and 5 dpf = 75%) between the beginning of feeding with 20 3 or 5 dpf was observed. Through the consulted literature we believe that this is the first 21 time that zebrafish consuming diets free of live organism and with the beginning of 22 feeding at 3 dpf was reported. We also observed that, no matter the source of 23 methionine used, the larvae survival was higher (DL + MM = 80.5% and SM = 24 75.8%) with methionine supplementation from parents who consumed the same diets.

25 Therefore, our results showed that genetically improved zebrafish and methionine
26 supplementation in the diets, regardless of the source, produced early larvae to the ability
27 of eating food and ensured higher survival of these larvae.

28

29 ***Keywords:*** Artificial diets; methionine; initial development; mortality

30

31 1. Introduction

32 The association of the genetic and embryological characteristics of zebrafish
33 (*Danio rerio*) transformed this fish into an excellent animal model for investigation of the
34 development, physiology and behavior of vertebrates (Gates et al., 1999). Because of a
35 great application in scientific research, its production has generated interest. However,
36 one of the critical phase of this production is the beginning of its development, where
37 high mortality rates of larvae (Sousa, 2016), make it difficult to obtain phenotypically
38 uniform individuals to be used for research.

39 A well-nourished female is more likely to have more able-bodied children in
40 different phenotypes (Fernández-Palacios et al., 1997). Thus, it is possible to supplement
41 the mother's diet with the aim of incorporate nutrients into the yolk sac and make them
42 available to the progeny during initial development (Nowosad et al., 2017). Therefore,
43 the yolk sac composition is important in the initial nutrition of the embryo (Heming and
44 Buddington, 1988).

45 Zebrafish does not present an established feed protocol with commercial diets and
46 mainly formulated exclusively with conventional ingredients, which provides adequate
47 growth and survival compared to live food (Martínez, 2012). Thus, a protocol to initiate
48 feeding and an adequate diet can eliminate or reduce the dependence of live food on fish

49 production, reducing production costs, minimizing mortality, offering a possibility of
50 increased productivity and quality of animals.

51 Providing food for the larvae as soon as they rise to the surface is crucial because
52 they inflate their swimming bladders and are able to get food (Dammski et al., 2011).
53 Some studies showed the beginning of feeding with 6 days post fertilization (Carvalho et
54 al., 2006; Otis and Farber, 2016). Perhaps, this time may be related to mortality due to
55 poor nutrition, the almost complete absorption of the yolk sac, the non-availability of food
56 in the water column or because the fish is not yet conditioned to capture the food
57 (Dammski et al., 2011). The feeding protocols of zebrafish suggest providing live food to
58 the larvae in the first weeks of life (Westerfield, 1995; Matthews et al., 2002). However,
59 the dependence of live food in the production system can be an obstacle, since the
60 production happens to be of two living organisms, the target (zebrafish) and the organism
61 that will be offered as food.

62 For aquatic organisms, methionine is one of the limiting amino acids (NRC, 2011)
63 and its insufficiency, in addition to causing growth limitation, can reduce food efficiency
64 (Schwarz et al., 1998; Mukhopadhyay and Ray, 2001). Methionine supplementation in
65 diets has the main source of DL-Methionine 99%. However, there are studies with the
66 use of synthetic amino acids in the feeding of aquatic organisms (Li et al., 2009), with
67 lower solubility (Niu et al., 2018) which avoids leaching, called Met-Met (DL-Methionyl-
68 DL-Methionine, 95%).

69 The aim of this study was to evaluate the influence of methionine supplementation
70 (free amino acid and dipeptide) in diets formulated with conventional ingredients,
71 supplied to parents and zebrafish larvae (*Danio rerio*), by monitoring survival in the
72 initial phase up to 20 days post fertilization (dpf), fed on the same day of mouth opening
73 (3 dpf) and 5 dpf (end of yolk sac absorption).

74

75 **2. Materials and methods**

76 ***2.1 Animals and experimental design***

77 For the experiment, females and males zebrafish (*Danio rerio*) of the second
78 generation, from the genetic improvement program of the State University of Maringá,
79 were used to compose the generation of parent s that were fed with the experimental diets
80 to obtain the eggs. 60 eight-months-old fish were raised in a controlled environment with
81 a comfort temperature of 28 °C and a cyclic photo-controlled period with 14 hours of
82 light and 10 hours of dark. The breeding animals were kept separated by sex in an
83 aquarium of 50 liters, with constant aeration, and partial water exchange (30%) performed
84 every two days to guarantee water quality parameters. The average temperature was 28 °
85 C, dissolved oxygen 4.00 mg / L and average pH equal to 7.

86 Fish were fed three times a day for one month with the feed: supplementation of
87 DL-Methionine 99% - amino acid in free form (DL); supplemented with methionine in
88 the dipeptide form DL-Methionyl-DL-Methionine 95% (MM) and without
89 supplementation (SM). After this period, couples were selected and transferred to
90 reproductive structures. Eggs were collected, sanitized and kept in plastic structures with
91 a capacity of 2 liters, until the hatching with 3 days post fertilization (dpf). The moment
92 of the larvae mouth opening was monitored hourly and was established that on an
93 average of 3 dpf the animals already presented their mouth opened and were able to ingest
94 food.

95 For the experiment, 1200 larvae of zebrafish (*Danio rerio*) hatched with 3 dpf
96 were used. They were conditioned at the density of 25 larvae per liter in 24 two-liter
97 plastic structures. The experiment was conducted in a completely randomized design in
98 a 3x2 factorial scheme, with three diets: no methionine supplementation (SM);

99 supplemented with DL-Methionine 99% (DL) and supplemented with DL-Methionyl-
100 DL-Methionine dipeptide 95% (MM), following the same diet as their parents and 2
101 feeding periods: 3 dpf and 5 dpf, with 4 repetitions per treatment.
102
103 ***2.2 Larvae data collect***
104 The experimental diets were offered four times a day at the following times: 8, 11,
105 14 and 17 hours. 30% of the water was renewed every two days, and the variables
106 temperature and pH were measured daily.
107 Larvae with 4 dpf of each treatment were photographed and analyzed in a light
108 microscope (Motic BA310E), Moticam 5.0 MP camera, before receiving the first feeding
109 of the day and after feeding, to verify the presence or not of food on the gastrointestinal
110 tract. Mortality within each treatment was counted with 6 dpf and verified on days
111 interspersed up to 20 dpf.
112
113 ***2.3 Data analysis***
114 The data presented as percentage of survival in each diet (SM, DL and MM), and
115 two feeding begin periods (3 dpf and 5 dpf) were analyzed using a 3x2 factorial scheme
116 and linear regression, and the means were compared using the Tukey test ($P < 0.05$)
117 (SAS Inst. Inc., Cary, NC, USA).
118
119 **3.0 Results and discussion**
120 pH and temperature were within the recommended range for zebrafish, and there
121 was no statistical difference between treatments (Table 1).
122

Table 1. pH and temperature in the housing structures of zebrafish larvae (*Danio rerio*).

	SM		DL		MM		3 dpf*		5 dpf*	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
pH	7.13	0.09	7.13	0.09	7.14	0.09	7.12	0.09	7.15	0.09
Temperature	25.39	1.8	25.3	1.94	25.61	1.74	25.37	1.82	25.5	1.83

123 * dpf = days post fertilization; pH (hydrogenation potential); temperature (°C); without
124 methionine supplementation (SM); supplemented with DL-Methionine 99% - amino acid in its
125 free form (DL) and supplemented with methionine in the dipeptide form DL-Methionyl-DL-
126 Methionine 95% (MM).

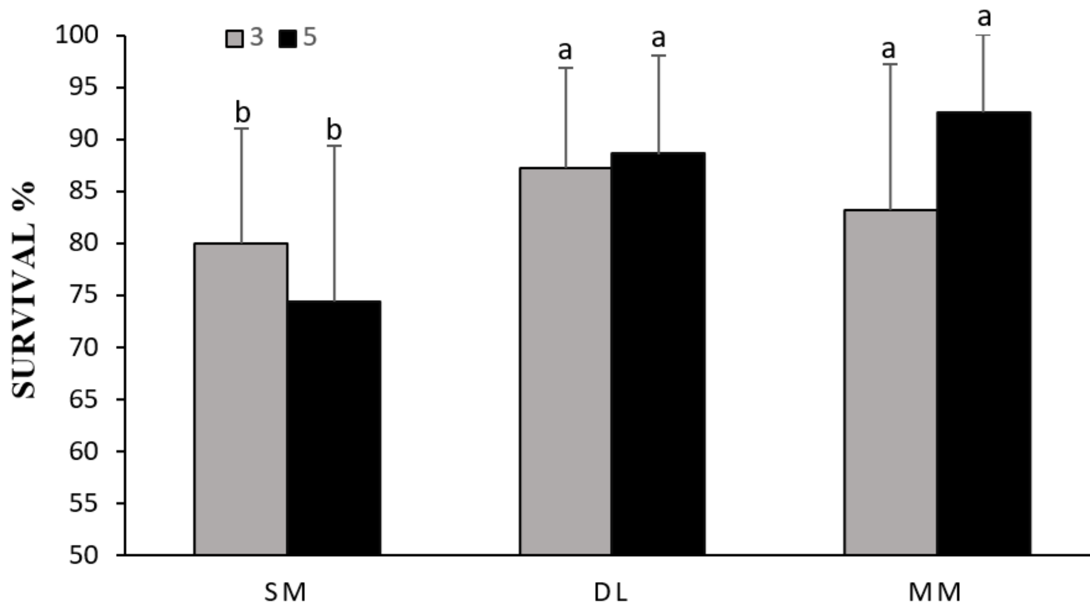
127

128 We observed no effect of interaction between diets and feeding period on the
129 survival of zebrafish larvae ($p > 0.05$) (Fig. 1). With 3 dpf the larva is already able to ingest
130 feed, which can be verified by the presence of food in its gut (Fig.2A), with the majority
131 of the larvae consuming the food within one hour after supplied (Otis and Farber, 2016).
132 The larvae that were not fed presented a clean gut (Fig. 2B).

133 The begin of the exogenous diet with feed (3 or 5 dpf) was not significant ($p >$
134 0.05), considering the final survival. The survival in the period of 20 dpf, found in the
135 animals that had the feeding started with 5 dpf, was 75% and with 3 dpf, was 72%. The
136 methionine supplementation, regardless of DL or MM source in the feed, promoted higher
137 larval survival when compared to larvae fed with no methionine supplementation (SM).

138

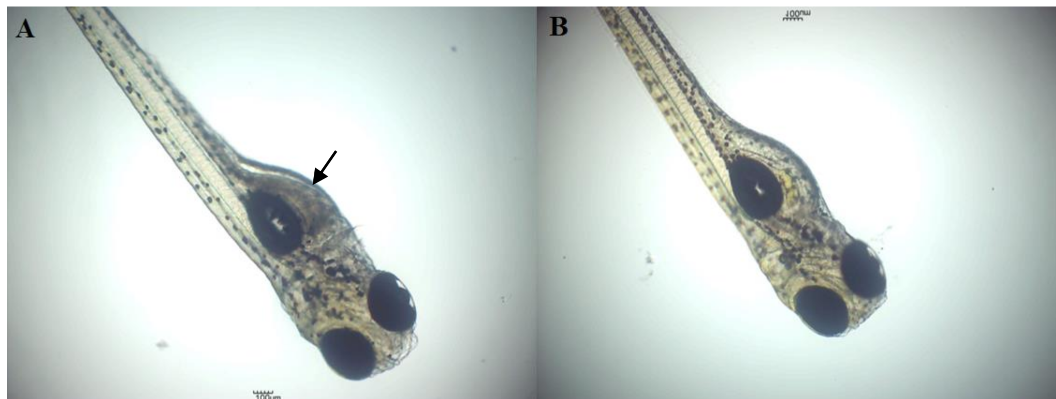
139



140

141 **Fig. 1.** Survival percentage of larvae up to 20 dpf, with feed started with 3 dpf and 5 dpf within
142 diets: DL-Methionine 99% - amino acid in free form (DL); supplemented with methionine in the
143 dipeptide form DL-Methionyl-DL-Methionine 95% (MM) and without supplementation (SM).

144



145

146 **Fig. 2.** Larva of zebrafish (*Danio rerio*) with 4 dpf fed with feed (A) and without feed (B).
147 Arrow indicates gut with feed.

148

149 The beginning of the feed supply with 3 or 5 dpf was enough to guarantee a higher
150 survival of the larvae up to 20 dpf, proving that the digestive system of the larva is already
151 formed, differentiated and functional (Verri et al., 2003). In this work, we can verify that
152 with 3 dpf the larvae of zebrafish had their mouths opened and were able to find the

153 food available in the water column, thus stimulating consumption and learning how to
154 catch the food. We believe that this is the first time that a study describes the larvae
155 beginning of feeding with 3 dpf, exclusively fed diets free from live food. This result may
156 be associated to the genetic improvement of zebrafish conducted by our research group.
157 The larvae parents evaluated belonged to the second generation of individuals selected
158 for total length of the fish, thus the size of the animal could be related to precocity in the
159 opening of the mouth and the capacity of food intake. Studies by Eaton and Farley (1974)
160 and Spence et al. (2007) showed that the growth rates of larvae raised in laboratories was
161 higher when compared to wild fish.

162 The use of free food live diets in the larval stage is not commonly used in diets of
163 zebrafish and other species, due to the immaturity of the digestive system and absence of
164 active digestive enzymes (Portella and Dabrowski, 2008; Holt et al., 2011). However,
165 according to Verri et al. (2003), the peptide transporter 1 (PEPT1), present on the brush
166 border membrane of the intestinal epithelium, accompanies the functional maturation of
167 the gut, preparing for digestion from the first exogenous feeding. Expression of this gene
168 was already identified in 2 dpf larvae, but with low activity, and maximum expression at
169 4 to 7 dpf (Verri et al., 2003). This may be an indication of the capacity of transport and
170 absorption of di and tripeptide already in the first week of larval development, since the
171 PEPT1 performs the transport of di and tripeptides.

172 In this work, it was verified that the diets supplemented with methionine had better
173 survival of the larvae, considering a total period of 20 dpf. We did not observe a
174 significant difference between DL and MM diets during the evaluation period, therefore
175 any source of methionine supplementation can be an option for the larval feeding to
176 increase the survival rate of this species (Zambonino Infante et al., 1997).

177 Carvalho et al. (2006) evaluated the survival of zebrafish larvae in the initial
178 period of development for 21 days (6dpf to 27dpf) and obtained a survival of 56% with
179 feed only. Our study achieved a survival of 80.5% in the period of up to 20 dpf, using
180 feed supplemented with methionine. Higher survival of the larvae can still be obtained
181 due to the feeding of their parents. Parents consumed the same feeding of their offspring
182 a month before egg production, which may have caused the transfer of nutrients to the
183 yolk sac, since the maternal diet has the ability to transfer fatty acids to the yolk sac, if
184 available for the developing embryo (Ram et al., 2008, Nowosad et al., 2017).

185

186 **4.0 Conclusion**

187 In conclusion, our results showed that methionine supplementation in the diets,
188 regardless of source, DL-Methionine 99% - amino acid in its free form (DL) or in the
189 dipeptide form DL-Methionyl-DL-Methionine 95% (MM), produced early larvae to the
190 ability to eat food (3 dpf) and ensured higher survival of these larvae (80.5%).

191

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