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Tanise da Silva Pês

EFEITO PROTETOR DA QUERCETINA SOBRE A TOXICIDADE INDUZIDA PELA OXITETRACICLINA EM *Rhamdia quelen*

Santa Maria, RS, Brasil

2018

Tanise da Silva Pês

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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Farmacologia, Área de Farmacologia Aplicada à Produção Animal, da Universidade Federal de Santa Maria (UFSM-RS), como requisito parcial para obtenção do grau de Doutor em Farmacologia.

Orientadora: Profª. Drª. Maria Amália Pavanato

Santa Maria, RS, Brasil

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LISTA DE ABREVIATURAS E SIGLAS

AA	Ácido ascórbico (*ascorbic acid*)
ACTH	Hormônio adrenocorticotrófico (*adrenocorticotropic hormone*)
CAT	Catalase (*catalase*)
CHO	Colesterol total (*total cholesterol*)
CRH	Hormônio liberador de corticotrofina (*corticotropin-releasing hormone*)
EROs	Espécies reativas de oxigênio (*reactive oxygen species - ROS*)
FBP	Frutose-bifosfatase (*fructose-biphosphatase*)
GDH	Glutamato desidrogenase (*glutamate dehydrogenase*)
GH	Hormônio do crescimento (*growth hormone*)
GLU	Glicose (*glucose*)
GPx	Glutationa peroxidase (*glutathione peroxidase*)
GR	Glutationa redutase (*glutathione reductase*)
GSH	Glutationa reduzida (*glutathione reduced*)
GSSG	Glutationa oxidada (*glutathione oxidised*)
GST	Glutationa S-transferase (*glutathione S-transferase*)
G3PDH	Glicerol-3-fosfato desidrogenase (*glycerol-3-phosphato dehydrogenase*)
G6PDH	Glicose-6-fosfato desidrogenase (*glucose-6-phosphate dehydrogenase*)
HB	Hemoglobina (*hemoglobin*)
HCT	Hematócrito (*hematocrit*)
HDL	Lipoproteína de alta densidade (*high-density lipoprotein cholesterol*)
HK	Hexocinase (*hexokinase*)
H_2O_2	Peróxido de hidrogênio (*hydrogen peroxide*)
LDH	Lactato desidrogenase (*lactate dehydrogenase*)
LDL	Lipoproteína de baixa densidade (*low-density lipoprotein cholesterol*)
LOOH	Hidroperóxidos lipídicos (*lipid hydroperoxide*)
$LOO^{\bullet}$	Radical peroxila (*peroxyl radical*)
LOQ	Limite de quantificação (*Quantification limit*)
LPO	Lipoperoxidação (*lipid peroxidation*)
MCHC	Concentração celular média de hemoglobina (*mean cell hemoglobin concentration*)
NPSH	Tiois não proteicos (*non-protein thiols*)
O_2	Oxigênio molecular (*molecular oxygen*)
$^1O_2$	Oxigênio singlete (*singlet oxygen*)
$O_2^{\bullet-}$	Ânion radical superóxido (*superoxide radical anion*)
$OH^{\bullet}$	Radical hidroxila (*hydroxyl radical*)
OTC	Oxitetraciclina (*oxytetracycline*)
PK	Piruvato cinase (*pyruvate kinase*)
POMC	Proopiomelanocortina (*proopiomelanocortin*)

PRL	Prolactina (*prolactin*)
RL	Radicais livres (*free radicals*)
SL	Somatolactina (*somatolactin*)
SOD	Superóxido dismutase (*superoxide dismutase*)
TBARS	Substâncias que reagem ao ácido tiobarbitúrico (*thiobarbituric acid reactive substance*)
TNF-α	Fator de necrose tumoral alfa (*tumor necrosis factor alpha*)
TRAP	Potencial antioxidante reativo total (*total reactive antioxidant potential*)
TRI	Triglicerídeos (*triglycerides*)
URE	Ureia (*urea*)

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RESUMO

EFEITO PROTETOR DA QUERCETINA SOBRE A TOXICIDADE INDUZIDA PELA OXITETRACICLINA EM *Rhamdia quelen*

AUTORA: Tanise da Silva Pês

ORIENTADORA: Drª. Maria Amália Pavanato

As condições de cultivo na piscicultura podem levar a situações de estresse, contribuindo para a ocorrência de doenças infecciosas, considerada um grande desafio para o sucesso produtivo dessa atividade. Desse modo, antibióticos têm sido utilizados com fins terapêuticos e para profilaxia, destacando-se a oxitetraciclina (OTC) que apresenta vantagens quanto à eficácia e economia em comparação a outros antibióticos. Porém, há uma grande preocupação no uso intensivo de antibióticos devido ao desenvolvimento de resistência antimicrobiana, além da problemática ambiental causada por esses fármacos. A necessidade por alimentos mais saudáveis tem levado à procura por produtos naturais. A quercetina incorporada na dieta pode ser uma alternativa natural para minimizar as alterações fisiológicas decorrentes do uso de antibióticos durante o cultivo, uma vez que já foi descrita sua atividade antioxidante, além de propriedades anti-inflamatórias, anticancerígenas, antibacterianas, entre outras. Assim, este trabalho teve por objetivo avaliar o possível efeito protetor da quercetina suplementada na dieta de jundiás, bem como verificar se o composto reduz e/ou minimiza a toxicidade induzida pela administração oral de OTC. No primeiro experimento, visamos testar o efeito da quercetina e escolher a melhor concentração para os peixes. Para tanto, os peixes foram divididos em três grupos e alimentados com dieta controle e dieta contendo duas concentrações de quercetina (1,5 e 3,0 g por kg de dieta) pelo período de 21 dias. Os resultados indicaram que a quercetina não promoveu qualquer alteração significativa nos parâmetros hematológicos e bioquímicos. Os peixes que receberam as dietas contendo quercetina apresentaram diminuição da lipoperoxidação (LPO) (medida por hidroperóxidos lipídicos (LOOH) e substâncias reativas ao ácido tiobarbitúrico (TBARS) e aumento na atividade das enzimas antioxidantes, como a superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx) e glutationa S-transferase (GST). Adicionalmente, os níveis de tiois não-proteicos (NPSH), potencial antioxidante reativo total e o ácido ascórbico (AA) foram maiores nos peixes alimentados com as dietas contendo quercetina. Não houveram alterações quanto aos níveis de cortisol e à expressão de mRNA do hormônio do crescimento (GH), prolactina (PRL) e somatolactina (SL) nos peixes alimentados com dieta contendo quercetina, quando comparados com o controle. No segundo experimento, os peixes foram divididos em quatro grupos experimentais e receberam as seguintes dietas experimentais por 14 ou 21 dias: (i)-controle; (ii)-0,1 g OTC por kg de dieta; (iii)-1,5 g de quercetina por kg de dieta; ou (iv)- OTC+quercetina. Investigou-se o efeito da quercetina sobre os indicadores de estresse e metabolismo, a expressão de mRNA do GH, PRL, SL, hormônio liberador de corticotrofina (CRH) e duas diferentes proopiomelanocortinas (POMCa e POMCb), bem como a análise de resíduo de OTC nos músculos dos peixes. Tratando dos indicadores oxidativos, os resultados demonstraram que a OTC induz aumento nos níveis de LOOH, TBARS e proteína carbonil, diminuição nas atividades enzimáticas de SOD, CAT, GST, GPx e nos níveis de NPSH e AA. O co-tratamento com quercetina foi capaz de prevenir esses efeitos no músculo, encéfalo, rim e fígado dos jundiás. No entanto, os valores residuais de OTC no músculo dos peixes foram inferiores ao limite de quantificação. Deste modo, podemos reforçar que a OTC ativa a resposta ao estresse nos jundiás, devido ao aumento do cortisol plasmático e diminuição dos níveis de glicose aos 14 e 21 dias. Além disso, também alterou o *status* metabólico dos peixes, uma vez que aumentou os níveis de triglicerídeos hepáticos, bem como a atividade hepática da frutose-bifosfatase e glutamato desidrogenase aos 14 dias. O tratamento com OTC também estimulou a atividade da Na^+/K^+-ATPase nos 14 dias e alterou a expressão do GH (14 e 21 dias) e PRL (14 dias). Assim, este estudo comprovou que a quercetina restaura os danos causados pela OTC, sugerindo que a administração de quercetina pode ser uma estratégia valiosa para previnir ou atenuar a toxicidade causada por antibióticos em diferentes espécies de peixes de importância comercial.

Palavras-chave: Jundiás. Antibiótico. Flavonoide. Dieta. Metabolismo energético. Perfil oxidativo.

ABSTRACT

PROTECTIVE EFFECT OF QUERCETIN ON TOXICITY INDUCED BY OXYTETRACYCLINE IN *Rhamdia quelen*

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ADVISER: Drª. Maria Amália Pavanato

The culture conditions in the fish culture can lead to stress situations, contributing to the occurrence of infectious diseases, considered a great challenge for the productive success of this activity. Thus, antibiotics have been used for therapeutic purposes and for prophylaxis, especially oxytetracycline (OTC), which has advantages in terms of efficacy and economy compared to other antibiotics. However, there is a great concern in the intensive use of antibiotics due to the development of antimicrobial resistance, besides the environmental problems caused by these drugs. The need for healthier foods has led to the demand for natural products. Quercetin incorporated into the diet may be a natural alternative to minimize the physiological changes caused by the use of antibiotics during cultivation, since its antioxidant activity has already been described, as well as anti-inflammatory, anticancer and antibacterial properties, among others. Thus, this work aimed to evaluate the possible protective effect of quercetin supplemented in the diet of silver catfish, as well as to verify if the compound reduces and/or minimizes the toxicity induced by the oral administration of OTC. In the first experiment, we aimed to test the effect of quercetin and to choose the best concentration for the fish. For this, the fish were divided into three groups and fed a control diet and diet containing two concentrations of quercetin (1.5 and 3.0 g per kg of diet) for a period of 21 days. The results indicated that quercetin did not promote any significant changes in hematological and biochemical parameters. Fish receiving diets containing quercetin showed decreased lipid peroxidation (LPO) (measured by lipid hydroperoxides (LOOH) and thiobarbituric acid reactive substances (TBARS) and increased activity of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione S-transferase (GST). In addition, levels of non-protein thiols (NPSH), total reactive antioxidant potential and ascorbic acid (AA) were higher in fish fed diets containing quercetin. There were no changes in cortisol levels and mRNA expression of growth hormone (GH), prolactin (PRL) and somatolactin (SL) in fish fed a diet containing quercetin when compared to control. In the second experiment, the fish were divided into four experimental groups and received the following experimental diets for 14 or 21 days: (i)- control, (ii)- 0.1 g OTC per kg of diet, (iii)- 1.5 g of quercetin per kg of diet; or (iv)- OTC+quercetin. We investigated the effect of quercetin on the stress and metabolism indicators, mRNA expression of GH, PRL, SL, corticotropin-releasing hormone (CRH), and two different proopiomelanocortins (POMCa and POMCb), as well as the analysis of OTC residue in fish muscles. By treating the oxidative indicators, the results showed that OTC induces an increase in the levels of LOOH, TBARS and carbonyl protein, decrease in the enzymatic activities of SOD, CAT, GST, GPx and NPSH and AA levels. Co-treatment with quercetin was able to prevent these effects on the muscle, brain, kidney and liver of the silver catfish. However, residual OTC values in fish muscle were below the limit of quantification. Finally, we can reinforce that OTC activates the stress response in silver catfish, due to the increase of plasma cortisol and a decrease of glucose levels at 14 and 21 days. In addition, it also altered the metabolic status of fish as it increased liver triglyceride levels as well as the liver activity of fructose-biphosphatase and glutamate dehydrogenase at 14 days. OTC treatment also stimulated Na^+/K^+-ATPase activity at 14 days and changed the expression of GH (at 14 and 21 days) and PRL (at 14 days). Thus, our study has proven that quercetin restores OTC damage by suggesting that the administration of quercetin may be a valuable strategy to prevent or attenuate antibiotic toxicity in different commercially important fish species.

Keywords: Silver catfish. Antibiotic. Flavonoid. Diet. Energetic Metabolism. Oxidative profile.

1 INTRODUÇÃO

1.1 ESTRESSE EM PEIXES

A produção de pescado mundial tem crescido de maneira significativa nas últimas cinco décadas. Segundo dados da *Food and Agriculture Organization* (FAO, 2014), a oferta de alimento proveniente de peixe aumenta a uma taxa média anual de 3,2%, superando o crescimento da população mundial que é de 1,6%. Além disso, a aquicultura vem sendo considerada uma das melhores alternativas para diminuir a pressão da pesca sobre os estoques pesqueiros naturais, reduzindo os impactos negativos que a exploração pesqueira indiscriminada pode causar nos ecossistemas aquáticos (IBAMA, 2007).

A piscicultura, apesar de considerada um setor com grande potencial, ainda é pouco aproveitada para a produção de alimentos (BALDISSEROTTO, 2009). Dentre as técnicas que podem maximizar a produção, está o manejo controlado. Durante o processo produtivo, os peixes são submetidos a diferentes procedimentos, tais como manejo, captura, confinamento, transporte, entre outros, que podem desencadear estresse e resultar em perdas na produção (ROSS; ROSS, 2008).

O termo estresse se refere ao estado no qual se encontra um organismo frente a perturbações que ameaçam a sua homeostase e a resposta ao estresse representa a reação a um estímulo adverso (intrínseco e extrínseco) (BARTON; IWAMA, 1991). Essa resposta se aplica a vários mecanismos fisiológicos, incluindo alterações na expressão de genes, proteínas, metabólicas, imunológicas, endócrinas, neurais e até mesmo mudanças no comportamento. Tais mudanças ocorrem para, primeiramente, tentar superar essa situação e, em seguida, compensar os desequilíbrios produzidos pelo estressor ou as consequências geradas pela primeira matriz de respostas, restaurando o equilíbrio corporal (TORT, 2011).

Dependendo de sua intensidade e duração, o estresse pode ser classificado como agudo ou crônico. O estresse agudo é produzido como resposta a perturbações de curta duração, como por exemplo, mudanças bruscas nos fatores ambientais. Já o estresse crônico, é a consequência de exposições contínuas ou periódicas a níveis baixos de agentes causadores de estresse (WEBER et al., 2011; TORT, 2011). No entanto, qualquer fator adicional ao ambiente de cultivo pode causar um estresse agudo e quando a ação de alguns agentes estressantes é prolongada ou repetida

(situação que ocorre frequentemente nos sistemas intensivos de cultivo) existe uma constante perturbação da homeostase, debilitando o animal, diminuindo a eficiência digestiva e predispondo-o a doenças, com consequente redução na taxa de crescimento (SMALL, 2004; ROSS; ROSS, 2008).

O estresse pode ser gerado por variados fatores como altas densidades de estocagem e até mesmo uma variação brusca da temperatura da água, ocasionando a diminuição na imunidade dos peixes, deixando-os mais suscetíveis ao aparecimento de doenças. Por isso, a associação de uma boa qualidade da água dos tanques de cultivo, manejo, densidade de estocagem e adequado teor nutricional da ração, garantem um ótimo desempenho no crescimento e produção dos peixes. Entretanto, todas estas condições são dificilmente controladas e, muitas vezes, os criadores lançam mão de promotores do crescimento, uso de antibióticos e agroquímicos (SANTOS et al., 2009).

1.2 USO DE ANTIBIÓTICOS NA AQUICULTURA

De modo geral, antibióticos são agentes quimioterápicos que inibem ou suprimem o crescimento de microrganismos, não só bactérias, mas também fungos ou protozoários (KÜMMERER, 2009). Eles podem ser produzidos a partir de microrganismos, como fungos ou bactérias, de forma sintética ou semissintética (GUARDABASSI et al., 2010). Os antibióticos são lipofílicos e persistentes no solo, água e organismos porque são produzidos para induzir um efeito biológico (PEREIRA-MAIA et al., 2010).

Doenças infecciosas são consideradas uma grande preocupação para a piscicultura por representarem potenciais riscos na produção, atraso no crescimento culminando em perdas no estoque. Dentre as principais estratégias para o controle deste problema está o uso de antibióticos como medida terapêutica e/ou preventiva dentro de um sistema de produção (BILA; DEZOTTI, 2003).

No entanto, a produção de peixes possui uma característica particular, o número de agentes antimicrobianos autorizados para uso é limitado. Nos Estados Unidos, os fármacos legalmente utilizados são aprovados pelo órgão governamental ligado à Medicina Veterinária. Por exemplo, a Food and Drug Administration autorizou o uso da oxitetraciclina (OTC), florfenicol e sulfadimetoxina/ormetoprim em ração medicamentosa para peixes (FDA, 2016). Para a aquicultura

brasileira não há legislação específica que regulamente o uso de medicamentos veterinários, o que leva ao seu uso indevido.

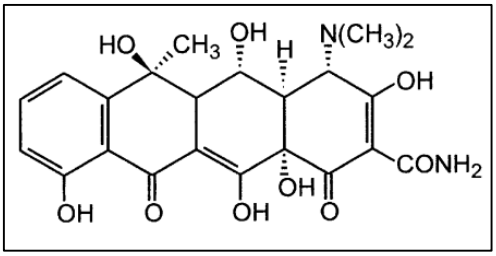
Estudos mostram que antibióticos no meio ambiente têm contribuído para o desenvolvimento de bactérias resistentes, entre outros efeitos (BILA; DEZOTTI, 2003). O uso de fármacos e agroquímicos deve ser evitado devido principalmente à ocorrência de contaminação ambiental e a indução de estresse oxidativo (LUSHCHAK, 2011).

Assim, o uso de antibióticos em excesso, como promotores de crescimento ou até mesmo como meio profilático para prevenir doenças, pode desencadear uma situação de estresse em peixes e obter uma situação inversa da esperada, podendo levar a atrasos no crescimento e perdas na produção (GASKINS et al., 2002).

1.2.1 Oxitetraciclina (OTC)

A OTC é um dos antibióticos mais utilizados em pisciculturas por apresentar vantagens quanto à eficácia e economia em comparação com outros antibióticos (ELIA et al., 2014) (Figura 1).

Figura 1 – Estrutura química da OTC.



Fonte: adaptado de: RABØLLE; SPLIID (2000, p. 716).

É um produto metabólico de *Streptomyces rimosus* e membro da família de antibióticos tetraciclinas. A OTC é utilizada no tratamento de infecções bacterianas sistêmicas, sendo ativa contra uma ampla gama de bactérias Gram-positivas e Gram-negativas, como *Aeromonas hydrophila*, reconhecido como patogênico para peixes desde 1894 (KIRKAN et al., 2003). Exerce seu efeito antimicrobiano por inibir a síntese de proteínas bacterianas no processo de reprodução e

crescimento celular, liga-se à subunidade ribossômica 30S bacteriana impedindo a chegada do complexo aminoacil RNA transportador ao local receptor no complexo RNA mensageiro ribossomal. Essa união é irreversível e impede a incorporação dos aminoácidos que compõem a cadeia peptídica, inibindo a síntese de proteínas (YONAR et al., 2011; GUARDIOLA et al., 2012).

A OTC pode ser administrada por injeção, via oral ou por banho de imersão e tem boa penetração nos tecidos, acumulando-se nas escamas, no tecido ósseo, fígado, músculo, plasma e no pronefro, importante órgão linfoide nos peixes (BJÖRKLUND; BYLUND, 1990; UENO et al., 2004). Em doenças infecciosas, a OTC é geralmente administrada na ração a uma taxa de 50 a 100 mg de OTC por kg de peso corporal por dia, durante 3-21 dias, dependendo da infecção (TREVES-BROWN, 2000). No entanto, a dose recomendada é de 75 mg de OTC por kg de peso corporal por dia, durante 10 dias (LUNDEN; BYLUND, 2000).

A limitada aprovação do uso da OTC deve-se em parte à falta de dados relativos à farmacocinética, eficácia e segurança dos animais-alvo. O uso "*off-label"* de OTC através da alimentação tem sido utilizado para tratar infecções, como flavobacteriose em carpa comum (*Cyprinus carpio*) e carpa capim (*Ctenopharyngodon idella*); furunculose em salmão (*Oncorhynchus kisutch*); estreptococose em truta arco-íris (*Oncorhynchus mykiss*), entre outros tipos (SIDHU et al., 2018).

A OTC não é metabolizada ou biotransformada de forma significativa pelos peixes, desta forma, aproximadamente 60% da OTC é eliminada via filtração glomerular pela urina e os 40% restantes, são eliminados através das fezes no meio ambiente (CRAVEDI et al., 1987; RIVERE; SPOO, 1995; TREVES-BROWN, 2000). Estudos demostram que o principal metabólito da OTC, é a própria molécula de OTC (PASCHOAL et al., 2012). No entanto, Halling-Sørensen et al. (2002) afirmam que ela pode ser degradada em 4-epi-oxitetraciclina (4-EOTC), α-apooxitetraciclina, β-apo-oxitetraciclina e terrinolida, sendo a 4-EOTC um importante produto de degradação do fármaco (LE et al., 2012). Jiao et al. (2008), ressaltam que o resíduo 4-EOTC pode ter a mesma ou maior toxicidade em comparação com a molécula de OTC.

Estudos farmacocinéticos sobre a OTC foram avaliados em *Clarias gariepinus* (GRONDEL et al., 1989), *Salmo salar* (ELEMA et al., 1996), *Oncorhynchus tshawytscha* (ABEDINI et al., 1998; NAMDARI et al., 1998), *Cyprinus carpio* (GRONDEL et al., 1987), *Oncorhynchus mykiss* (BLACK et al., 1991; BJÖRKLUND; BYLUND, 1991; ABEDINI et al., 1998), *Colossoma macropomum* (DIO et al., 1998), *Paralichthys dentatus* (HUGHES, 2003),

Tinca tinca (REJA et al., 1996), *Perca flavescens* (BOWDEN, 2001) e *Megalobrama amblycephala* (LI et al., 2015). Os resultados descritos nesses estudos demonstram que a farmacocinética da OTC é influenciada pela espécie, idade, tamanho, via de administração e condições tais como temperatura da água e salinidade (SIDHU et al., 2018).

Por exemplo, em um estudo com administração oral de 100 mg de OTC por kg de peso corporal na dieta de *Megalobrama amblycephala*, durante 5 dias, a meia vida de absorção foi de 3,62, 7,33, 4,59 e 6,02 horas e a meia vida de eliminação foi de 91,75, 214,87, 126,22 e 135,84 horas no plasma, fígado, rim e músculo, respectivamente. As características farmacocinéticas da OTC também variam conforme o tecido, neste estudo foram encontrados altos níveis de OTC no fígado, devido a ser um órgão altamente vascularizado e bem perfundido (LI et al., 2015).

Vários perigos e efeitos colaterais foram associados com o uso excessivo de drogas antibacterianas para peixes como imunossupressão, nefrotoxicidade, atraso do crescimento, desenvolvimento de cepas bacterianas resistentes, problemas ambientais, tais como resíduos de drogas em sedimentos de piscicultura e resíduos de medicamentos em produtos oriundos de peixes (YONAR et al., 2011). Estudos demostram que drogas como a OTC, o ácido oxolínico e o florfenicol têm sido associadas a efeitos imunossupressores em carpa (*Cyprinus carpio*) e truta arco-íris (*Oncorhynchus mykiss*) (RIJKERS et al., 1980; LUNDEN et al., 1998). Além disso, a administração de OTC em peixes pode causar danos hepáticos (BRUNO, 1989), interferir nos mecanismos imunes, na regulação dos genes imuno-competentes (GUARDIOLA et al., 2012) e causar dano oxidativo (YONAR et al., 2011, YONAR, 2012, RODRIGUES et al., 2016).

Também deve ser levado em consideração o risco de resíduos de fármacos nos alimentos de origem animal, uma vez que resíduos de tetraciclinas em alimentos podem causar reações alérgicas, desequilíbrio da flora intestinal, favorecimento de patógenos e desenvolvimento de resistência dos microrganismos aos antimicrobianos, além de problemas tecnológicos relacionados ao processamento de alimentos (PASCHOAL, 2007).

Antibacterianos e antiparasitários exigem cuidado com o período de carência, que tem como objetivo evitar a presença de resíduos do produto veterinário nos alimentos como carne, leite, ovos, pescado e mel, acima do permitido e considerado prejudicial à saúde humana. Para animais produtores de alimentos, o período de carência deve ser obedecido rigorosamente, atendendo a indicação do fabricante (MAPA, 2008). A fim de evitar a presença de resíduos de medicamentos nos alimentos é estabelecido o Limite Máximo de Resíduos (LMR), definido como a concentração

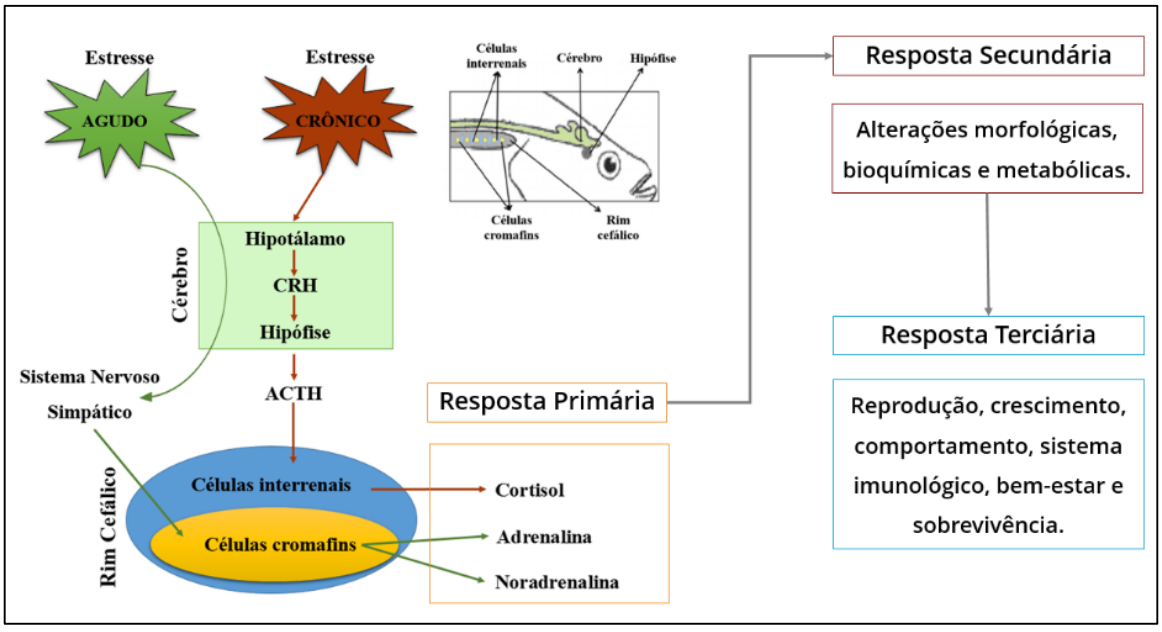
máxima de resíduo tolerável no alimento (MAPA, 2008; EMEA, 2012). Este é baseado no tipo e quantidade de resíduo que não induz efeito adverso à saúde humana, considerando-se a ingestão diária aceitável do composto. O LMR em carne de peixe para a OTC é de 100 ng/g (EMEA, 2012).

1.3 AVALIAÇÕES POR BIOMARCADORES

Diversos estudos utilizam biomarcadores na avaliação dos efeitos de substâncias tóxicas no ambiente aquático. Biomarcadores são definidos como alterações a respostas biológicas, que passam de respostas moleculares, celulares, fisiológicas até mudanças comportamentais, e que podem ser relacionadas à exposição ou efeitos tóxicos de agentes químicos ambientais (PEAKALL, 1994).

Durante as situações que causam estresse, há o desencadeamento de alterações fisiológicas primárias, secundárias e terciárias típicas, que indicam o grau de alteração provocada pelo fator estressor. A ativação da resposta ao estresse, caracterizada pela estimulação do eixo hipotálamo-hipófise-inter-renal (HHI), é uma resposta neuroendócrina em que as catecolaminas (adrenalina e noradrenalina) e o cortisol são liberados das células cromafins e inter-renais, respectivamente, a fim de que o animal se ajuste e mantenha sua homeostase (Figura 2).

Figura 2 - Sistema neuroendócrino de controle da resposta ao estresse e o efeito do estresse crônico e agudo sobre a função do sistema imune em peixes.



Abreviaturas: CRH - hormônio liberador de corticotrofina, ACTH - hormônio adrenocorticotrófico.
Fonte: adaptado de NARDOCCI et al. (2014, p. 535).

Tal resposta é classificada como resposta primária ao estresse (GESTO et al., 2015). A ação dos hormônios corticosteroides e das catecolaminas no organismo do animal provoca os chamados efeitos secundários do estresse, e ocorrem a nível metabólico, hematológico, osmorregulatório e cardiorrespiratório. Em consequência da ação crônica do cortisol, ocorrem prejuízos na resposta imunológica, crescimento e reprodução, caracterizando os efeitos terciários do estresse (BARTON et al., 1998), que podem levar a uma diminuição na sobrevivência.

Os biomarcadores endócrinos são de alta relevância para a avaliação da saúde dos animais, uma vez que a sinalização endócrina controla muitos processos fisiológicos essenciais, como o crescimento e o desenvolvimento, a resposta ao estresse e a reprodução (SCHOLZ; MAYER, 2008). O cortisol é considerado um dos principais biomarcadores de estresse em peixes e, com algumas limitações, pode também ser utilizado para avaliar o bem-estar destes animais (ELLIS et al., 2012). A utilização de cortisol como um marcador de estresse agudo demonstrou ser vantajoso por diferentes razões: a liberação não é tão rápida para impedir a sua utilização como marcador de estresse, como no caso das catecolaminas (MOMMSEN et al., 1999; ELLIS et al., 2012); a resposta do cortisol ao estresse é intensa, se tornando fácil detectar diferenças entre animais estressados e não estressados (MOMMSEN et al., 1999); a resposta ao estresse é relativamente rápida e os níveis observados condizem com os acontecimentos vividos pelo animal e a intensidade da resposta está correlacionada com a intensidade do estressor para uma dada espécie (GESTO et al., 2015).

Situações de estresse podem alterar o metabolismo intermediário, interferindo na atividade de enzimas-chave no metabolismo de carboidratos (hexoquinase-HK, piruvato quinase-PK, glicogênio fosforilase-GP, frutose-1,6-bifosfatase-FBP, glicose-6-fosfato desidrogenase-G6PDH), lipídios (glicerol-3-fosfato desidrogenase-G3PDH) e aminoácidos (glutamato desidrogenase-GDH, transaminase glutâmicooxalacética-TGO e transaminase glutâmico pirúvica-TGP) (MOMMSEN et al., 1999; LAIZ-CARRIÓN et al., 2003). Dessa forma, a análise da atividade dessas enzimas pode dar pistas sobre como o animal reorganiza seu estado energético após deparar-se com um evento de estresse (MENEZES et al., 2015).

Dentre os biomarcadores endócrinos, destaca-se os hormônios pituitários como a prolactina (PRL), o hormônio do crescimento (GH) e a somatolactina (SL). A PRL é responsável pela

regulação do equilíbrio hidromineral (WENDELAAR BONGA et al., 1983) e possui importância na adaptação de teleósteos eurialinos em água doce (BROWN; BROWN, 1987). O GH é responsável pelo estímulo do crescimento somático em peixes e pela regulação da adaptação de teleósteos à água do mar e à água doce (SAKAMOTO; MCCORMICK, 2006), bem como pela aclimatação de espécies eurialinas à água do mar, incluindo salmonídeos e tilápias (*Oreochromis mossambicus*) (MCCORMICK, 1996; SAKAMOTO; MCCORMICK, 2006). Por sua vez, a SL, exclusiva de peixes, tem um papel na maturação sexual (BENEDET et al., 2008). Outros efeitos fisiológicos da SL em teleósteos incluem a função imunológica (CALDUCH-GINER et al., 1998), equilíbrio ácido-base (KAKIZAWA et al., 1996), mobilização de energia (RAND-WEAVER et al., 1995), biossíntese de esteroides gonadais (PLANAS et al., 1992), regulação do fosfato de sódio (ZHU; THOMAS, 1995), e metabolismo do cálcio (KAKIZAWA et al., 1993). Estudos demostram que os níveis plasmáticos desse hormônio estão elevados em resposta ao estresse, e além disso influenciam na regulação do metabolismo lipídico, bem como secreção do cortisol *in vivo* (FUKAMACHI et al., 2005).

Em relação aos biomarcadores hematológicos em peixes, são de efeito potenciais, como enzimas, hematócrito, hemoglobina, proteínas e glicose, que podem ser sensíveis a certos poluentes, fornecendo inferências importantes sobre o organismo em estudo (VAN DER OOST et al., 2003).

Os estudos hematológicos das diferentes espécies de peixe são de interesse ecológico e fisiológico, uma vez que auxiliam na compreensão da relação entre as características sanguíneas, a filogenia, a atividade física, o habitat e a adaptabilidade dos peixes no ambiente. Os valores hematológicos podem ser influenciados por diversas condições como o estado nutricional, sazonalidade, maturação gonadal, sexo e a variação genética (TAVARES-DIAS et al., 2002). As alterações nas variáveis hematológicas são utilizadas para determinação dos efeitos causados por diversos estressores externos (KORI-SIAKPERE et al., 2006).

Lunden et al. (1998) apontaram a redução de leucócitos circulantes, principalmente linfócitos, durante o tratamento com OTC (75 mg/kg) por 3 dias em truta arco-íris, indicando supressão do sistema imunológico. Durante o tratamento com OTC (50 a 75 mg/kg) na dieta de *Sparus auratus* observou-se aumento de células fagocitárias (SEREZLI et al., 2005).

Além disso, alterações nos biomarcadores oxidativos também são importantes para avaliação fisiológica, uma vez que estudos têm demonstrado que o estresse pode desencadear o estresse oxidativo (LUSHCHAK, 2011, 2016; VELISEK et al., 2011).

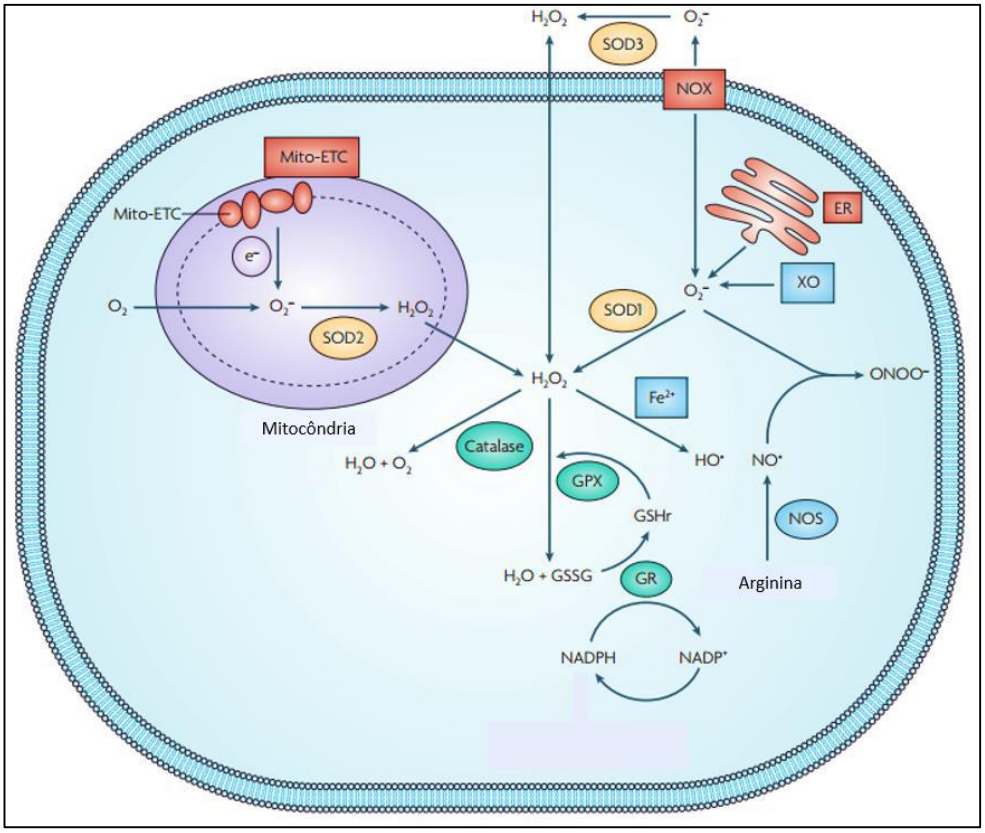
Grande parte do oxigênio molecular consumido pelos animais é destinada para a redução à água ligada à oxidação de alimentos e à produção de energia. Através de reduções parciais do oxigênio são formados intermediários reativos como: o ânion superóxido ($O_2^{\bullet -}$), o peróxido de hidrogênio (H_2O_2), radical hidroxil ($OH^{\bullet}$) e o oxigênio singlete ($^1O_2$). Muitas destas espécies são radicais livres (RL), definidos por Halliwell e Gutteridge (2007) como sendo qualquer espécie química capaz de existir de forma independente e que apresentam um ou mais elétrons desemparelhados na última camada eletrônica. Esses radicais são formados pela perda ou ganho de um elétron de um não-radical.

Os intermediários reativos também são conhecidos como espécies reativas de oxigênio (EROs), sendo encontrados em todos os sistemas biológicos, derivados do metabolismo normal da célula. Entretanto, as mitocôndrias são consideradas como a maior fonte *in vivo* destas espécies. Cerca de 1 a 3% do O_2 consumido é convertido em EROs por diversas fontes e processos endógenos, incluindo certas enzimas, auto oxidação e transporte de elétrons da membrana nuclear, porém, essa taxa de produção pode ser alterada pela presença de diversos xenobióticos (CHANCE et al., 1979).

Como descrito por Sies (1997), altas concentrações das EROs devem ser evitadas pelo organismo, uma vez que a reatividade destes radicais traz consequências celulares deletérias. As EROs podem ocasionar a oxidação de biomoléculas como proteínas e lipídios, levando à lipoperoxidação (LPO), que gera alterações na estrutura e permeabilidade da membrana celular; danos no DNA, ocasionando mutações; e o rompimento da homeostase celular.

Quando há um desequilíbrio entre a concentração das EROs e do sistema de defesa antioxidante, o quadro é reconhecido como estresse oxidativo, levando a uma interrupção na sinalização redox ou danos moleculares (SIES, 2015) (Figura 3). Este estado pode ser resultado de um dos três fatores: (1) aumento na geração das EROs, através do acúmulo de intermediários reativos; (2) prejuízo do sistema de defesa antioxidante (inibição de enzimas antioxidantes, depleção de antioxidantes não enzimáticos); (3) incapacidade para reparar o dano oxidativo (ALY et al., 2010; LUSHCHACK, 2011; 2016).

Figura 3 – Formação das EROs a partir da redução parcial do oxigênio e sua remoção pelas enzimas antioxidantes.

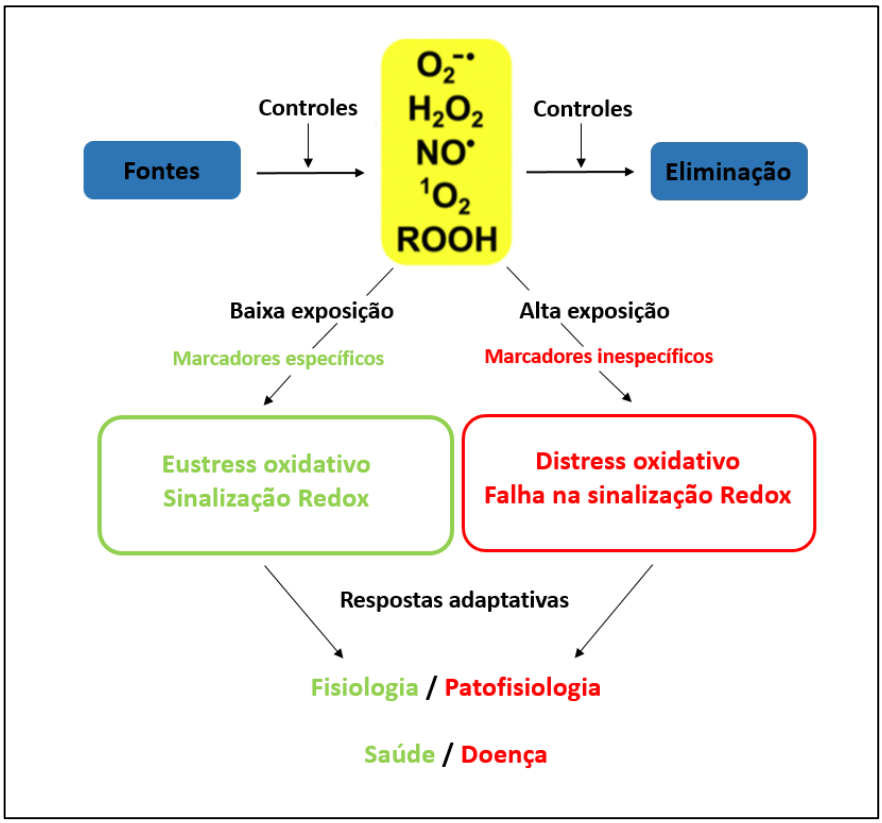


Legenda: Os principais locais de geração das EROs (indicado em vermelho) incluem a cadeia mitocondrial de transporte de elétrons (Mito-ETC), o sistema retículo endoplasmático (ER) e o complexo NADPH oxidase (NOX). Para evitar os efeitos nocivos das EROs, as células regulam seus níveis através da manutenção do equilíbrio entre a produção e a eliminação dessas espécies. As principais enzimas varredoras de EROs são mostradas em verde e laranja. O ânion radical superóxido ($O2^{\bullet-}$) é a principal forma de EROs e pode ser rapidamente convertido em peróxido de hidrogênio (H_2O_2) por atuação da superóxido dismutase (SOD) ou pode formar o peroxinitrito ($ONOO^-$) por meio de reação com o óxido nítrico ($NO^{\bullet}$). Na presença de metais de transição (tais como Fe^{2+}), o H_2O_2 pode ser convertido em radical hidroxila ($HO^{\bullet}$), que, por sua vez, é altamente reativo e pode causar danos aos lipídios, proteínas e DNA, por isso a prevenção da produção deste radical é a melhor maneira de evitar impactos nocivos. A redução de um elétron de H_2O_2 transforma-o em uma espécie menos nociva, seja pela ação da catalase, que catalisa a conversão de H_2O_2 para o oxigênio molecular e água, ou pela glutationa peroxidase (GPx) que utiliza a glutationa como cofator para reduzir o H_2O_2 à água. A restauração da glutationa oxidada (GSSG) para a sua forma reduzida (GSH) é catalisada pela glutationa redutase (GR), utilizando o NADPH como substrato.
Fonte: adaptado de TRACHOOTHAM et al. (2009, p. 581).

No entanto, Sies (2018) descreve uma nova abordagem do termo estresse oxidativo, que pode ser classificado de acordo com a intensidade. As escalas de intensidade variam do estresse oxidativo fisiológico (*eustress*) até a níveis oxidativos tóxicos que danificam as biomoléculas (*distress*). A baixa exposição de células e organismos é utilizado para sinalização redox, abordando

alvos específicos, considerando que a elevada exposição resulta em perturbações na sinalização redox e/ou danos em alvos não específicos (Figura 4).

Figura 4 – Estresse oxidativo e sua relação com a sinalização redox.



Legenda: Vários oxidantes são produzidos por fontes endógenas ou exógenas. Os níveis de seu estado estacionário também são controlados por reações de eliminação. Baixa exposição ao oxidante permite abordar alvos específicos no uso para sinalização redox (oxidação *eustress*), enquanto que a alta exposição leva à sinalização redox interrompida e/ou danos a biomoléculas (oxidação *distress*).
Fonte: adaptado de: SIES (2018 p. 123).

Os animais aquáticos são principalmente afetados pelas EROs, uma vez que estão sujeitos a variações ambientais diárias e acredita-se que o estresse oxidativo seja o responsável pela adaptação desses organismos a uma variedade de estressores ambientais (LUSHCHAK, 2011). Este fato já foi relatado no trabalho de Hermes-Lima e Zenteno-Savín (2002), no qual é evidenciado que os organismos são capazes de se adaptarem a diversas situações crônicas de alta exposição às EROs pelo aumento da expressão de enzimas antioxidantes e muitas outras formas de defesas e respostas para reparar o dano oxidativo. O processo adaptativo de defesa atua na prevenção,

eliminação e no reparo de moléculas modificadas pelas EROs (SIES, 1997), consideradas como pró-oxidantes.

Já os antioxidantes, de acordo com Halliwell e Gutteridge (2007), são quaisquer substâncias que, presentes em baixas concentrações comparadas ao substrato oxidável, retardam ou mesmo impedem a oxidação do substrato. A eliminação das EROs é realizada por mecanismos antioxidantes enzimáticos e não enzimáticos.

O mecanismo antioxidante enzimático envolve as enzimas que fazem a proteção primária e intrínseca do organismo. Estão incluídas as enzimas superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx), glutationa redutase (GR) e glutationa S-transferase (GST). Através da ação destas enzimas, o organismo mantém a concentração das EROs dentro dos limites fisiológicos (RIBEIRO et al., 2005).

O sistema de defesa não enzimático corresponde a moléculas que protegem os alvos biológicos da oxidação, sendo moléculas do próprio organismo, exógenas, sintéticas ou naturais. Este sistema vai atuar na supressão da formação de RL, por quelação de metais ou inibição de enzimas geradoras destes radicais, e na eliminação dos RL. Dentre as principais defesas não enzimáticas estão o α-tocoferol, carotenoides, glutationa e o ácido ascórbico (RIBEIRO et al., 2005).

Martínez-Alvaréz et al. (2005) descrevem que, assim como todos os organismos aeróbicos, os peixes também são suscetíveis ao ataque das EROs e, para isso, desenvolveram defesas antioxidantes, através das enzimas antioxidantes e também de antioxidantes de baixo peso molecular, como os carotenoides, vitaminas, aminoácidos e peptídeos (glutationa). Porém, segundo os mesmos autores, as defesas antioxidantes em peixes dependem de vários fatores, como idade, comportamento alimentar, fatores nutricionais, fatores ambientais, infestações por microrganismos patogênicos, entre outros.

Estudos anteriores demonstram que a OTC leva à situação de estresse oxidativo, aumentando os níveis de LPO e diminuindo a atividade das enzimas antioxidantes em diferentes tecidos de truta-arco-íris (YONAR et al., 2011; YONAR, 2012; RODRIGUES et al., 2016). Dessa forma, acredita-se que um dos mecanismos pelos quais a OTC causa dano oxidativo celular é a inibição da β-oxidação mitocondrial. Esse processo interrompe a cadeia respiratória, causando a produção de $O_2^{\bullet-}$, que por sua vez gera mais EROs e espécies reativas de nitrogênio (PARI e GNANASOUNDARI, 2006; YONAR et al., 2011).

1.4 BENEFÍCIOS DE COMPOSTOS BIOATIVOS NA DIETA DE PEIXES

O controle de uma dieta com as quantidades ideais de nutrientes é essencial para o crescimento, defesas imunológicas e a manutenção das funções vitais dos peixes, como respostas a estressores. Estratégias profiláticas com uso de aditivos naturais na dieta estão sendo testadas para otimização do desempenho das espécies (SANTOS et al., 2009). Estas estratégias podem beneficiar a piscicultura, principalmente nas fases iniciais do cultivo, quando os peixes estão mais suscetíveis a doenças (PORTZ, 2006). Diversos estudos tem relacionado os níveis de vitaminas e aminoácidos na dieta de peixes com o perfil redox nestes organismos (PUANGKAEW et al., 2005; VIJAYAVEL et al., 2006; FENG et al., 2013; FENG et al., 2014).

Em um estudo realizado por nosso grupo foi demonstrado que a suplementação da dieta de jundiás com o flavonoide rutina, reduziu os níveis de cortisol plasmático nos peixes que receberam a 1,5 g de rutina por kg de dieta. Além disso, a adição deste antioxidante não alterou a expressão dos hormônios pituitários, sugerindo que a rutina não atua como um fator estressor e ajuda a manter a homeostase dos peixes (PÊS et al., 2016).

Em truta-arco-íris a suplementação com vitamina E (100 ou 1000 mg de acetato de α-tocoferol/kg de dieta) protege contra o estresse oxidativo causado por altos níveis de ácidos graxos altamente insaturados n-3 (n-3 HUFA, do inglês *n-3 highly unsaturated fatty acids*) (20% ou 40% n-3 HUFA) na dieta. Os resultados mostraram um aumento da atividade das enzimas antioxidantes SOD, CAT e GPx, correspondendo a um mecanismo fisiológico que combate a elevação dos RL sobre situações de estresse oxidativo (PUANGKAEW et al., 2005). A dieta contendo vitaminas C e E (100 mg de ácido ascórbico e α-tocoferol) também foi eficiente na proteção contra o estresse oxidativo causado por exposição ao cobre (4,0, 2,5 e 1 mg/L) em *Terapon jarbua*, sendo verificada uma diminuição da LPO no encéfalo, brânquias, fígado, rim, músculo e baço (VIJAYAVEL et al., 2006).

Em *Cyprinus carpio* a suplementação da dieta com biotina (0,01, 0,028, 0,054, 0,151, 0,330, 1,540 e 2,680 mg de biotina/kg de dieta) por 63 dias diminuiu os níveis de LPO e proteína carbonila (0,151 e 0,330 mg de biotina/kg de dieta), mostrou um aumento na capacidade anti-radical hidroxil e anti-ânion superóxido (0,054 e 1,540 mg de biotina/kg de dieta) no soro, intestino, hepatopâncreas e músculo. A atividade das enzimas antioxidantes, CAT, GPx, GST, GR e o conteúdo de glutationa

estavam aumentados nos tecidos e no soro em todas as dietas testadas, demonstrando que a biotina aumenta o *status* antioxidante desses peixes (FENG et al., 2014). Em outro estudo de Feng e colaboradores (2013), também com *C. carpio*, foi verificado que a dieta contendo altos níveis de histidina (2,3, 4,4, 6,3, 8,6, 10,8 e 12,7 g de histidina/kg de dieta), reduziram os níveis de LPO e aumentaram a atividade das enzimas antioxidantes SOD, CAT, GPx, GR, GST e o conteúdo de glutationa.

Em um estudo de Ndong e Fall (2007), a suplementação da dieta de *Oreochromis niloticus* com extrato de alho (0,5 e 1%) durante 28 dias, melhorou a resposta imune pelo aumento dos leucócitos, da atividade fagocitária e lisossomática dos peixes, quando comparados com o controle. Do mesmo modo, a adição de isoleucina na dieta de *C. carpio* durante 60 dias melhorou a capacidade antioxidante, a resposta imunológica e resistência frente à bactéria *Aeromonas hydrophila* (ZHAO et al., 2013). Em um estudo recente de Mohanty e Samanta, 2018, a adição de spirulina na dieta de *Notopterus notopterus*, protege contra a toxicidade induzida pelo ferro, uma vez que restaurou os níveis de antioxidantes como SOD, CAT, GPx, GST, GR, GSH e NPSH.

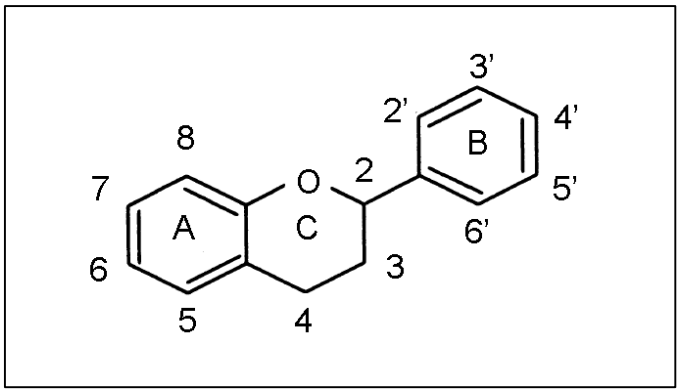
De acordo com estes estudos, fica evidente que a adição de substâncias com propriedades antioxidantes na dieta de peixes pode aumentar a capacidade de eliminação das EROs, inibindo a oxidação de lipídios, estimulando a atividade das enzimas antioxidantes e o conteúdo dos antioxidantes não enzimáticos, contribuindo para o aumento da resposta antioxidante nestes organismos, bem como no perfil imunoestimulante.

1.5 ALTERNATIVAS DE PRODUTOS NATURAIS COM PROPRIEDADES ANTIOXIDANTES: FLAVONOIDES

A crescente demanda por alimentos mais saudáveis, sem resíduos de antibióticos e agroquímicos (SANTOS et al., 2009) e, até mesmo, a redução do efeito poluidor dos mananciais, tem sugerido vias alternativas e naturais para produção animal, principalmente na aquicultura. As propriedades terapêuticas das plantas têm despertado interesse crescente, e por esta razão, vários componentes bioativos dos vegetais têm sido isolados e caracterizados. Dentre estes componentes naturais, encontram-se os flavonoides (VAN DER WATT; PRETORIUS, 2001). Os flavonoides são pigmentos hidrossolúveis presentes nos vacúolos das células vegetais e que representam o maior grupo de compostos fenólicos naturais. Possuem 15 átomos de carbono em seu núcleo

fundamental, que consiste de um esqueleto de propano de difenila (C6-C3-C6), caracterizado por dois anéis aromáticos (A e B) e um anel heterocíclico oxigenado (C) (Figura 5). A sua capacidade antioxidante é determinada pela estrutura, em particular por hidroxilas, que podem doar elétrons (PATEL et al., 2018).

Figura 5- Estrutura química dos flavonoides.



Fonte: adaptado de Martínez-Flórez et al. (2002, p. 272).

Muitos mecanismos antioxidantes têm sido propostos para os flavonoides. Tais mecanismos incluem: inibição da formação de EROs pela inibição do sistema enzimático responsável pela geração de RL (ciclooxigenase, lipoxigenase ou xantina oxidase); quelação de íons metálicos que podem iniciar a produção de $OH^{\bullet}$ pela Reação de Fenton ou Harber-Weis; sequestro de RL; regulação positiva ou proteção das defesas antioxidantes por induzir a fase II de enzimas como GST, que aumenta a excreção de espécies oxidadas ou indução de enzimas antioxidantes como a metalotioneína, que é uma proteína queladora de metais, com propriedades antioxidantes (MIDDLETON et al., 2000; PIETTA, 2000). Essas moléculas são capazes de proteger tecidos contra danos oxidativos devido à propriedade de inativar EROs e outras espécies reativas como aquelas derivadas do nitrogênio (RICE-EVANS et al., 1997; WOJDYLO et al., 2007).

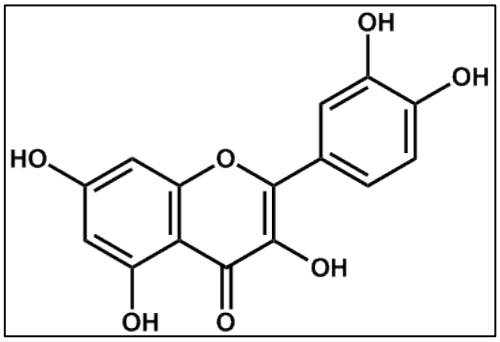
Os flavonoides subdividem-se em 13 subclasses, com mais de 7000 compostos descritos (LILAMAND et al., 2014), de acordo com sua estrutura molecular. As principais subclasses dos flavonoides são: antocianidina (cianidina), flavanol (catequinas, epicatequinas, procianidina), flavanonas (naringina, hesperidina), flavona (apegenina, luteolina, diosmetina), flavonol (quercetina, miricetina, rutina) e isoflavona (genisteína, daizeína) (BRAVO, 1998). São

encontrados em uma ampla variedade de frutas e vegetais como a maçã, feijão, cebola, couve, vagem, tomate, cereja, brócolis, batata, laranja, melão, limão, pera, uva, bem como no chá preto, vinho tinto, cerveja e também em grãos, nozes, sementes e especiarias (LI et al., 2015; PATEL et al., 2018).

1.5.1 Flavonoide quercetina

Entre os flavonoides, a quercetina é um bioflavonoide pertencente à subclasse dos flavonois e tem sido amplamente estudada (Figura 6) (GUO; BRUNO, 2015).

Figura 6- Estrutura química da quercetina.



Fonte: adaptado de Patel et al. (2018, p. 11).

É classificada de acordo com a IUPAC em 3,3′,4′,5,7-pentahidroxiflavanona; e existe primeiramente como glicosídeo na natureza composto por aglicona quercetina conjugado com porções de açúcar, como a glicose ou rutinose (GUO; BRUNO, 2015).

Essa molécula é capaz de inibir o processo de formação de RL em três etapas diferentes, na iniciação (pela interação com $O_2^{\bullet -}$), na formação de $OH^{\bullet}$ (por quelar íons de ferro) e na LPO (por reagir com o radical peroxil de lipídeos) (AFANAS'EV et al., 1989).

A sua ação antioxidante é determinada igualmente por outros efeitos, por exemplo, propriedades anti-inflamatórias. Estudos mostram que a quercetina reduz o nível de EROs possívelmente pela supressão da ativação pró-inflamatória do fator nuclear-kB (NAM, 2006). A quercetina é capaz de reduzir o nível do fator de necrose tumoral-α (TNF-α) no plasma de camundongos. O TNF-α é um fator pró-inflamatório e a quercetina pode atuar contra o estresse

oxidativo induzido pela ação desta citocina (BOESCH-SAADATMANDI et al., 2011). A ação anti-inflamatória da quercetina tem sido também mostrada, por exemplo, em um modelo de inflamação aguda induzida por carragenina em ratos (HEEBA et al., 2012).

No domínio da piscicultura, existem estudos onde a quercetina está sendo utilizada em formulações de dietas para peixes a fim de controlar a diferenciação sexual, selecionando o gênero que tem maior taxa de crescimento para se obter uma população de maior valor no mercado (RODRIGUEZ et al., 2004; TZCHORI et al., 2004). Outro efeito benéfico da suplementação com este flavonoide é o imunoestimulante. Em um estudo realizado por Awad et al. (2013), foi demonstrado um aumento na atividade de lisozima, proteína total, atividade antiprotease e bactericida frente à bactéria *Aeromonas hydrophila* no plasma de truta-arco-íris após suplementação da dieta com diferentes concentrações de quercetina (0,1%, 0,5% e 1%) por 14 dias. A alimentação de tilápias (*Oreochromis niloticus*) com dieta contendo quercetina (200, 400, 800 e 1600 mg/kg), por 49 dias, diminuiu os níveis de triglicerídeos e aumentou os de colesterol HDL no soro (ZHAI; LIU, 2013). Além disso, em estudo recente com peixes da espécie *Channa punctata*, a quercetina na concentração de 0,14 g/L, foi capaz de atenuar os danos causados pelo pesticida piretroide deltametrina a nível macromolecular (proteínas, aminoácidos, carboidrato e glicogênio) e antioxidante (SOD, CAT e GPx) no fígado e brânquias após 21 dias de tratamento (BHATTACHARJEE; DAS, 2017).

1.6 ESPÉCIE UTILIZADA COMO MODELO EXPERIMENTAL: *Rhamdia quelen*

O jundiá, *Rhamdia quelen* (família Heptapteridae, ordem Siluriformes) (Figura 7), é uma das espécies mais cultivadas na região Sul (BALDISSEROTTO, 2009). Nativa da região, essa espécie apresenta uma distribuição neotropical, do sudeste do México ao norte, e centro da Argentina ao sul. Possui hábito alimentar onívoro, com tendência piscívora, alimentando-se de peixes, crustáceos, insetos, restos vegetais e detritos orgânicos (GOMES et al., 2000). Este peixe mostra-se muito resistente ao inverno e com rápido crescimento no verão, sendo importante para aquicultura de clima temperado e subtropical (BARCELLOS et al., 2004).

Figura 7- Exemplar de jundiá, *Rhamdia quelen*.

Fonte: Fish base, disponível em: http://www.fishbase.org.

Mesmo apresentando boa produtividade quando criado em cativeiro e uma boa aceitação no mercado consumidor, devido à sua carne saborosa e ausência de espinhos intramusculares (CARNEIRO; MIKOS, 2005), a produção de jundiá, assim como a de outras espécies, oferece alguns desafios, pois muitos são os problemas enfrentados para o cultivo e a produção eficiente. Estes problemas vão desde o estresse provocado pelo manejo até o estabelecimento de condições propícias ao cultivo e bom desempenho no crescimento (BARCELLOS et al., 2004).

Para espécies nativas do Brasil, como o jundiá, não foram encontrados trabalhos que relacionem o uso de OTC com prejuízos na produção, indicando uma necessidade desses estudos, uma vez que a OTC é bastante utilizada nas pisciculturas da região. Assim, este estudo é importante para obtenção de informações esclarecendo o uso apropriado de antibióticos na aquicultura e a busca por um produto natural que possa minimizar os efeitos deletérios da OTC, já demonstrados em outras espécies de peixes, sem prejudicar o meio ambiente.

Dessa forma, o presente estudo busca verificar o possível efeito protetor da dieta contendo quercetina frente à ação da OTC em jundiás (*Rhamdia quelen*). Essa ideia é baseada nas vantagens que a quercetina proporciona em outras espécies animais (YOUSEF et al., 2010; LUEHRING et al., 2011) e de peixes (AWAD et al., 2013; ZHAI; LIU, 2013, AWAD et al., 2015; BHATTACHARJEE; DAS, 2017) quanto à redução ao estresse, aumento da resposta fisiológica, efeito protetor frente a compostos tóxicos, além do potencial imunoestimulante e antioxidante.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar o possível efeito protetor da quercetina suplementada na dieta de jundiás, bem como verificar se a molécula reduz e/ou minimiza a possível toxicidade induzida pela administração oral de OTC.

2.2 OBJETIVOS ESPECÍFICOS

- Determinar uma concentração de quercetina adequada para a suplementação alimentar dos jundiás de acordo com análises hematológicas, bioquímicas, oxidativas e moleculares;

- Analisar o efeito da dieta contendo quercetina e OTC sobre as vias de ativação da resposta ao estresse nos jundiás, com base em parâmetros metabólicos, moleculares, osmorregulatórios e oxidativos;

- Verificar a presença de resíduos de OTC no músculo de jundiás.

3 DESENVOLVIMENTO

3.1 ARTIGO 1

Quercetin in the diet of silver catfish: Effects on antioxidant status, blood parameters and pituitary hormone expression

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Quercetin in the diet of silver catfish: Effects on antioxidant status, blood parameters and pituitary hormone expression

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ABSTRACT

We analysed the effects of quercetin-containing diet on blood parameters, antioxidant status and pituitary hormone expression in silver catfish. Diets containing three concentrations of quercetin (0, 0.15 and 0.30%) were provided to fish once a day. The results indicated that quercetin did not promote any significant change on the haematological and biochemical parameters measured. Fish that received the diet with quercetin presented decreased lipid peroxidation (LPO) (measured by lipid hydroperoxides and thiobarbituric acid reactive substances) in all tissues evaluated. On the other hand, the activity of antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase and glutathione S-transferase were higher in tissues of fish fed with diets containing quercetin. Additionally, the content of non-protein thiols, total reactive antioxidant potential and ascorbic acid were also higher in tissues of quercetin fed fish. Finally, there was no changes regarding cortisol levels and the expression of growth hormone, prolactin and somatolactin in fish fed with quercetin when compared with the control. Our results suggests that supplementation of silver catfish diet with quercetin is beneficial since it reduced the LPO and increased antioxidant capacity in vital tissues of fish without having any impact on haematological and biochemical parameters, and on pituitary hormone gene expression.

Statement of relevance: We propose quercetin as supplement in fish diets.

1. Introduction

The incorporation of food additives in the diet of fish aims to enhance performance, immunity and quality of fillet, since fish are susceptible to constant stress factors due intensive fish-farming practices that often cause poor health. In general, stressors cause an endocrine response in fish with activation of the hypothalamic–pituitary–interrenal axis, characterised by hypersecretion of catecholamines (epinephrine and norepinephrine) and cortisol. These hormones induce a number of secondary effects including rapid mobilisation of energy reserves (Tort, 2011). Furthermore, the fish pituitary hormones, such as growth hormone, prolactin and somatolactin are involved in stress responses and play a key role in regulating homeostasis of several physiological processes (Kaneko, 1996). Different stressors, such as salinity, confinement and food-deprivation, increased the expression of growth hormone, prolactin and somatolactin in gilthead sea bream (*Sparus aurata*) (Mancera et al., 2002; Laiz-Carrión et al., 2009). Aspects such as water quality, culture density, feeding, nutritional conditions, and handling procedures directly influence these hormonal pathways (Suárez et al., 2015). This stress response might lead to physiological unbalance leading to excessive formation of reactive oxygen species (ROS). Increased ROS levels can potentially damage cellular components, promoting lipid peroxidation (LPO), enzyme inactivation and oxidative DNA damage (Cho and Lee, 2012; Saleh et al., 2015).

The improvement of fish-farming conditions would benefit the welfare of fish as well as increase producers' profit. The use of natural antioxidants in fish diet could reduce production costs and offer an environmentally friendly alternative to synthetic compounds (Zheng et al., 2009; Shin et al., 2010; Awad et al., 2013; Saccol et al., 2013; Zhai and Liu, 2013; Pês et al., 2015).

Quercetin is a flavonol found widely in fruits, vegetables and nuts. In nature it exists primarily as quercetin glycoside and consists of quercetin aglycone conjugated to sugar moieties such as glucose or rutinose (Guo

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and Bruno, 2015). Kawabata et al. (2009) reported that oral administration of quercetin to rats affected the hypothalamic–pituitary–adrenal axis. These effects were associated to reduced stress as a consequence of decreased cortisol levels. This flavonoid is also described as an exceptional scavenger of free radicals such as peroxynitrite and hydroxyl radical (Bors et al., 1994; D'Andrea, 2015). This evidence supports the study of quercetin as a potential natural antioxidant to be incorporated in fish diet.

Silver catfish (*Rhamdia quelen*) has potential for aquaculture in southern Brazil due to its elevated growth rate, good carcass yield and easily controlled reproduction in subtropical climate (Baldisserotto, 2009). The current study aims to examine the effect of quercetin on blood parameters, oxidative biomarkers and pituitary hormone expression in *R. quelen*.

2. Material and methods

2.1. Fish

Silver catfish (215.19 ± 1.04 g, 23.65 ± 0.33 cm) were obtained from a fish culture sector of Federal University of Santa Maria (UFSM), Rio Grande do Sul (RS), Brazil. Experiments were conducted in a recirculating aquaculture system in the Fish Physiology Laboratory at UFSM. Animals were randomly distributed in nine plastic boxes (40 L), four fish per box, and acclimated to the laboratory conditions for two weeks. Water parameters were checked daily (temperature, total ammonia and dissolved oxygen) or weekly (alkalinity, total hardness and pH). The experimental protocol was approved by the Committee on Animal Experimentation of UFSM under registration no. 077/2013.

2.2. Water parameters

Temperature and dissolved oxygen levels were measured with a YSI oxygen metre (Model Y5512; YSI Inc., Yellow Springs, OH, USA). Temperature was maintained at 23.01 ± 0.03 °C and dissolved oxygen levels at 6.81 ± 0.12 mg L^{-1}. pH was verified with a DMPH-2 pH metre and the mean value was 7.32 ± 0.05 (Digimed, São Paulo, SP, Brazil). Nesslerization was used to verify the total ammonia nitrogen levels according to the method of Eaton et al. (2005). Non-ionised ammonia levels were calculated according to the method of Colt (2002). Water hardness was analysed by the EDTA titrimetric method. Alkalinity was determined according to the method of Boyd and Tucker (1992). The mean values of these parameters were as follows: total ammonia (2.51 ± 0.5 mg L^{-1}), non-ionised ammonia (0.09 ± 0.005 mg L^{-1}), hardness (20.7 ± 1.0 mg L^{-1} $CaCO_3$) and alkalinity (24.1 ± 1.3 mg L^{-1} $CaCO_3$).

2.3. Quercetin and reagents

Quercetin ($C_{15}H_{10}O_7$) was obtained from Opção Fênix Petrochemicals Distributor Ltd. (São Paulo, SP, Brazil). All of the other reagent-grade chemicals were obtained from Sigma (St. Louis, Missouri, USA).

2.4. Diets and experimental design

Three diets were formulated based on the study of Pês et al. (2015). The diet consisted of 30% soybean meal, 35% meat and bone meal, 12% rice bran, 15% corn, 3% canola oil, 1% salt, 3% vitamins and minerals (premix) and 1% phosphate dicalcium. The different concentrations of quercetin (0, 0.15 and 0.30%) were added to the mixture with rice bran. Water was added to the diets, and a drying process was performed in a forced air circulation oven for 24 h (45 °C). Such concentrations were chosen based on previous studies using dietary quercetin supplementation for other fish species (Shin et al., 2010; Awad et al., 2013). Fish received the experimental diets until apparent satiation once a day (9 a.m.) for 21 days.

The experimental design included in three groups (in triplicate), one for each quercetin. After 21 days, blood samples were collected from the caudal vein with heparinized sterile syringes and biochemical analysis was performed. Blood was sampled in less than 1 min and no anaesthetic was used for the groups, since previous studies have shown that the use of anaesthetic may affect the stress response (Small, 2003; Velisek et al., 2011; Gressler et al., 2014). Fish were euthanised by sectioning the spinal cord and pituitary, brain, gill, liver, kidney and muscle were removed and immediately frozen in liquid nitrogen. Tissues were stored at −80 °C for further analysis.

2.5. Total phenolic compounds

Total phenolic compounds were determined in the diets according to the Folin-Ciocalteau procedure as described in the study by Finamor et al. (2012). Gallic acid was used as a standard, and the results were expressed as gallic acid equivalents (mg GAE) 100 g of grain (dry weight). The samples analysed showed different concentrations of total phenolic compounds, which were higher in diets with 0.15% (125.97 mg GAE 100 g $diet^{-1}$) and 0.30% (115.29 mg GAE 100 g $diet^{-1}$) of quercetin, than in control (75.11 mg GAE 100 g $diet^{-1}$) ($P < 0.05$).

2.6. Haematological and biochemical analysis

Blood was utilised for different analysis. An aliquot of blood was used to determination of haematocrit (HCT) using microhaematocrit capillary tubes. The haemoglobin concentration (HB) was obtained using the Drabkin reagent (Kamper and Zijlstra, 1964), read spectrophotometrically at 540 nm and expressed as mmol L^{-1} blood. The mean cell haemoglobin concentration (MCHC) was calculated using the equation [Hb] * 100 / Hct and expressed as mmol L^{-1}.

Another aliquot of whole blood was subsequently transferred to microcentrifuge tubes and centrifuged at 3000 × *g* for 10 min (Centrifuge 5804 R) to obtain the plasma for biochemical analysis. Plasma cortisol levels were quantified using an enzyme-linked immunosorbent assay (ELISA) kit (Diagnostics Biochem Canada Inc., Canada), similarly to the study of Gressler et al. (2015). The samples were measured in duplicate, and the absorbance was determined in a spectrophotometer at 450 nm. The inter- and intra-assay variation coefficients were 5.15 ± 0.53 and 4.13 ± 0.67%, respectively. The results are presented as nmol L^{-1}. The levels of glucose (GLU), lactate dehydrogenase (LDH), triglycerides (TRI), cholesterol (CHO), low-density lipoprotein cholesterol (LDL), high-density lipoprotein cholesterol (HDL) and urea (URE) in plasma were determined using commercial kits (Labtest, Minas Gerais, Brazil) and expressed as mmol L^{-1} and LDH as μkat L^{-1}.

2.7. Prooxidants and antioxidants analyses in tissues

For the measurement of oxidative stress biomarkers, each tissue was homogenised in 154 mmol L^{-1} KCl containing 1 mmol L^{-1} phenylmethylsulfonyl fluoride and centrifuged at 700 *g* for 10 min

Table 1
Primers design for amplification of β-actin, growth hormone, prolactin and somatolactin genes based on the sequences of these genes according with Baldisserotto et al. (2014).

Gene		Sequence
β-Actin	Forward	5′-CGA ATG CCA GGG TAC ATG GT-3′
	Reverse	5′-CCA CCT TCA ACT CCA TCA TTGA A-3′
Growth hormone	Forward	5′-TTG ACA GTC TTG GTG CTG CTT T-3′
	Reverse	5′-GAG CGA CTG CGT TGT TGA AG-3′
Prolactin	Forward	5′-ACC AGA GAC AGG AGC TCG TTC T-3′
	Reverse	5′-AGC TCA TGA GAC CGT CCA TGT-3′
Somatolactin	Forward	5′-CGA GGC CAG GAC TTT GTT TG-3′
	Reverse	5′-GAC GCG CAC AAG GTT TGA T-3′

Table 2
Haematological and biochemical parameters of the silver catfish *Rhamdia quelen* fed with diets containing different concentrations of quercetin.

	Control	Q 0.15%	Q 0.30%
Haematocrit (%)	24.12 ± 0.66	24.63 ± 1.39	22.09 ± 1.09
Haemoglobin (mmol L^{-1})	3.40 ± 0.04	3.45 ± 0.14	3.36 ± 0.06
Mean cell haemoglobin concentration (mmol L^{-1})	14.09 ± 0.57	14.00 ± 0.37	15.21 ± 0.33
Cortisol (nmol L^{-1})	593.15 ± 40.15	537.89 ± 54.89	453.21 ± 39.05
Glucose (mmol L^{-1})	2.10 ± 0.14	2.21 ± 0.18	2.14 ± 0.18
Lactate dehydrogenase (μkat L^{-1})	24.60 ± 3.90	21.94 ± 3.87	24.96 ± 3.28
Triglycerides (mmol L^{-1})	2.16 ± 0.35	1.98 ± 0.67	2.20 ± 0.47
Cholesterol (mmol L^{-1})	5.36 ± 0.06	5.54 ± 0.11	5.30 ± 0.05
Low-density lipoprotein cholesterol (mmol L^{-1})	1.03 ± 0.19	0.91 ± 0.22	0.82 ± 0.20
High-density lipoprotein cholesterol (mmol L^{-1})	4.02 ± 0.26	3.28 ± 0.11	3.93 ± 0.25
Urea (mmol L^{-1})	1.40 ± 0.19	1.53 ± 0.25	1.45 ± 0.19

Data are presented as the mean ± SEM (n = 12). Means obtained showed no significant difference (P > 0.05).

at 4 °C to discard the nuclei and cell debris. Supernatant fraction obtained was frozen at −80 °C for further measurements. Protein content was measured using the method of Lowry et al. (1951) and results are reported as mg mL^{-1}. LPO levels were estimated using lipid hydroperoxides (LOOH) and thiobarbituric acid reactive substance (TBARS) assays, similarly to the study of Pês et al. (2015). The first technique can detect the primary products of peroxidation using the oxidation of Fe^{2+} by LOOH in an acidic medium with xylenol orange dye, which forms a complex with Fe^{3+}. The readings were performed using a Thermo Plate reader (Thermo Plate Devices, China) at 560 nm and the results are reported as nmol mg $protein^{-1}$. TBARS was determined by measuring the end products of LPO, including malondialdehyde (MDA). The amount of MDA produced was used as an index of LPO, determined spectrophotometrically at 535 nm. The value is expressed as nmol mg $protein^{-1}$.

Total superoxide dismutase (SOD) activity, expressed as SOD units mg^{-1} protein, was based on the inhibition rate of autocatalytic adenochrome generation at 480 nm (Misra and Fridovich, 1972). Catalase (CAT) activity was evaluated by following the decrease in the 240 nm absorption of H_2O_2 and was reported as pmol min^{-1} mg $protein^{-1}$ (Boveris and Chance, 1973). Glutathione peroxidase (GPx) activity was measured by following NADPH oxidation at 340 nm, as described by Flohé and Gunzler (1984), and results were expressed as nmol min^{-1} mg $protein^{-1}$. Glutathione S-transferase (GST) activity, expressed as μmol min^{-1} mg $protein^{-1}$, was measured by the rate of dinitrophenyl S-glutathione formation at 340 nm (Habig et al., 1974).

The non-protein thiols (NPSH) content, the total reactive antioxidant potential (TRAP) and the ascorbic acid content (AA) was evaluated similarly to the study of Pês et al. (2015). The NPSH content, an indirect measure of GSH, was evaluated at 412 nm after reacting with 5,5′-dithiobis (2-nitrobenzoic acid) and reported as μmol mg $protein^{-1}$. For the measure of AA, the standard curve was prepared by using different concentrations of AA, and the slope was used to express the amount of ascorbic acid as μmol mg $protein^{-1}$. The TRAP was determined by a chemiluminescence assay with 2,2′-azobis (2-aminodipropane) dihydrochloride and luminol, with the results expressed as μmol trolox mg $protein^{-1}$.

2.8. Pituitary hormones expression

Total RNA was extracted from samples using Trizol reagent (Invitrogen) according to manufacture instructions. Total RNA quantity and purity were assessed by NanoDrop (Thermo Scientific, Delaware, USA; Abs 260/280 nm ratio) spectrophotometer. Ratios above 1.7 were used and samples below this threshold were discarded. Total RNA (1 μg) was treated with DNase (Invitrogen) at 37 °C for 5 min to digest any contaminating DNA. The reverse transcriptase reaction was performed with iScript cDNA synthesis kit (Bio-Rad) in a final volume of 20 μL.

mRNA expression was analysed through qRT-PCR, using the StepOnePlus™ RT-PCR system (Applied Biosystems) with Power SYBR Green PCR Master Mix (Applied Biosystems). Primers used for growth hormone were obtained from a complete sequence of *R. quelen* available in GenBank (accession number EF101341), and primers for prolactin (accession number KC195971) and somatolactin (accession number KC195972) were obtained from Baldisserotto et al. (2014). Nucleotide sequences of specific primers are shown in Table 1. Results were normalised to the expression of the constitutive gene β-actin. The calculation of relative expression was performed as recommended by Pfaffl (2001).

2.9. Statistical analysis

Statistical analysis was performed using the software Statistica® 7.0. Levene's test was used to verify whether the data were parametric. One-way analyses of variance followed by Tukey test were performed to assess the differences among the groups. Results are expressed as mean ± standard error (SEM). The minimum significance level was set at 95% (P < 0.05).

3. Results

3.1. Haematological and biochemical analysis

Diets containing different quercetin concentrations did not exert any significant effect on the HCT, HB and MCHC in whole blood. Compared

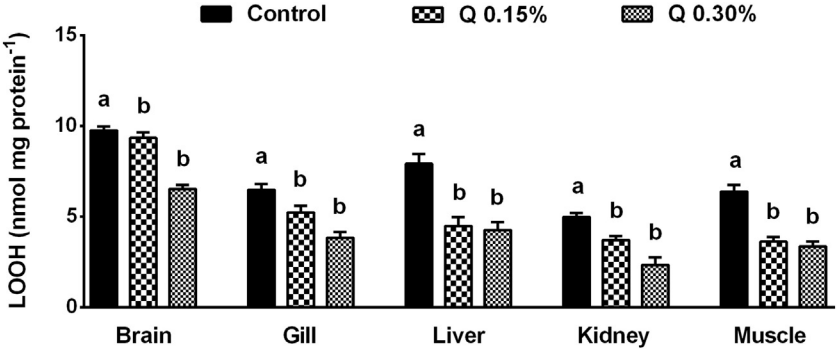

Fig. 1. Levels of lipoperoxidation evaluated by lipid hydroperoxides (LOOH) in silver catfish *Rhamdia quelen* fed with diets containing different concentrations of quercetin. All the values are expressed as mean ± SEM (n = 12). Different letters denote that the data are significantly different from the control (P < 0.05).

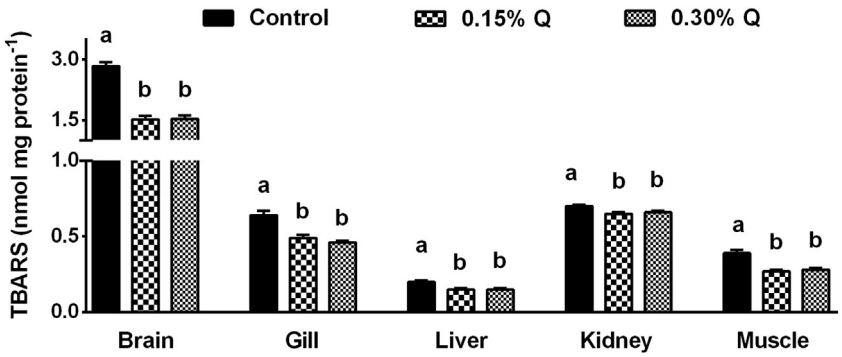

Fig. 2. Levels of lipoperoxidation evaluated by thiobarbituric acid reactive substance (TBARS) in silver catfish *Rhamdia quelen* fed with diets containing different concentrations of quercetin. All the values are expressed as mean ± SEM (n = 12). Different letters denote that the data are significantly different from the control (P < 0.05).

with the control group, plasma levels of cortisol, GLU, LDH, TRI, CHO, LDL, HDL and URE did not differ among the experimental groups (Table 2).

3.2. Prooxidant and antioxidant analysis in tissues

There was a significant decrease of LPO levels measured by LOOH and TBARS in the brain, gill, liver, kidney and muscle of fish fed with diet containing quercetin in both concentrations of diet when compared to the control diet (Figs. 1 and 2).

On the other hand, SOD activity significantly increased in brain, gill, liver and muscle of fish under both concentrations of quercetin supplementation when compared to control (Table 3). The activity of CAT was also significantly increased in liver (only Q 0.15%) and kidney (only Q 0.30%) of fish under the supplementation of quercetin, when compared to control (Table 3). CAT activity did not differ in brain and gill among the experimental groups (Table 3). GPx significantly increased in brain (only Q 0.15%), gill (only Q 0.30%) and liver of fish under the supplementation of quercetin, when compared to control (Table 3). However, GPx did not differ in kidney and muscle among the experimental groups (Table 3). The activity of GST significantly increased in gill, liver, kidney and muscle of fish under both concentrations of quercetin supplementation, when compared to control (Table 3). In contrast, GST activity in brain did not differ among the experimental groups (Table 3).

In the same way, NPSH content significantly increased in brain (only Q 0.15%), gill, liver, kidney (only Q 0.15%) and muscle of fish under quercetin supplementation, when compared to the control ones (Fig. 3). TRAP significantly increased in brain (only Q 0.15%), gill, liver, kidney and muscle of fish under supplementation of quercetin, when compared to control (Fig. 4). The AA content significantly increased in kidney and muscle (only Q 0.15%) of fish under supplementation of quercetin, when compared to control. On the other hand, animals under supplementation of quercetin presented a decrease on AA content in gill and muscle (only Q 0.30%), when compared to control. AA content did not differ in brain and liver among the experimental groups (Fig. 5).

3.3. Pituitary hormone expression

No difference in the expression of growth hormone, prolactin and somatolactin were observed after 21 days of supplementation with quercetin (Table 4).

4. Discussion

Fish routine handling practices can potentially create a stress environment and trigger chemical alterations that would directly affect the animal welfare and ultimately its growth for commercial purposes.

Table 3
Antioxidants enzymes in the brain, gill, liver, kidney and muscle of *Rhamdia quelen* fed with diets containing different concentrations of quercetin.

	Brain	Gill	Liver	Kidney	Muscle
Protein (mg mL^{-1})					
Control	2.30 ± 0.10	12.22 ± 1.13	12.45 ± 0.36	19.41 ± 0.42	9.86 ± 0.29
Q 0.15%	1.99 ± 0.14	10.21 ± 0.39	11.37 ± 0.39	19.49 ± 0.37	9.30 ± 0.47
Q 0.30%	2.08 ± 0.23	11.94 ± 0.45	12.65 ± 0.50	20.35 ± 0.48	10.75 ± 0.47
SOD (units mg protein^{-1})					
Control	1.09 ± 0.09^{a}	0.15 ± 0.03^{a}	0.44 ± 0.05^{a}	–	3.17 ± 0.17^{a}
Q 0.15%	2.34 ± 0.11^{b}	0.55 ± 0.07^{b}	1.10 ± 0.07^{b}	–	6.19 ± 0.21^{b}
Q 0.30%	1.95 ± 0.16^{b}	0.27 ± 0.05^{c}	1.20 ± 0.03^{b}	–	4.43 ± 0.08^{c}
CAT (pmol min^{-1} mg protein^{-1})					
Control	0.25 ± 0.01	0.29 ± 0.02	1.28 ± 0.15^{a}	0.90 ± 0.04^{a}	–
Q 0.15%	0.31 ± 0.02	0.40 ± 0.06	2.65 ± 0.21^{b}	1.20 ± 0.09^{ab}	–
Q 0.30%	0.32 ± 0.04	0.31 ± 0.02	2.25 ± 0.21^{ab}	1.33 ± 0.09^{b}	–
GPx (nmol min^{-1} mg protein^{-1})					
Control	19.83 ± 3.48^{a}	8.00 ± 0.42^{a}	11.43 ± 0.83^{a}	6.60 ± 0.59	8.76 ± 0.86
Q 0.15%	40.22 ± 7.34^{b}	10.15 ± 1.39^{ab}	17.60 ± 0.72^{b}	6.26 ± 0.60	11.19 ± 2.24
Q 0.30%	28.75 ± 3.24^{ab}	14.57 ± 1.96^{b}	27.19 ± 3.48^{b}	6.22 ± 0.80	14.05 ± 0.77
GST (pmol min^{-1} mg protein^{-1})					
Control	0.40 ± 0.02	0.65 ± 0.07^{a}	0.09 ± 0.01^{a}	0.13 ± 0.01^{a}	0.65 ± 0.04^{a}
Q 0.15%	0.34 ± 0.03	0.93 ± 0.05^{b}	0.19 ± 0.01^{b}	0.23 ± 0.01^{b}	1.00 ± 0.06^{b}
Q 0.30%	0.35 ± 0.04	0.97 ± 0.04^{b}	0.22 ± 0.03^{b}	0.16 ± 0.01^{ac}	0.86 ± 0.07^{b}

SOD — superoxide dismutase; CAT — catalase; GPx — glutathione peroxidase; GST — glutathione S-transferase.
Values are mean ± SEM (n = 10). Different lowercase letters indicate significant difference between the treatments using one-way ANOVA and Tukey's test (P < 0.05).
– indicates parameter not measured.

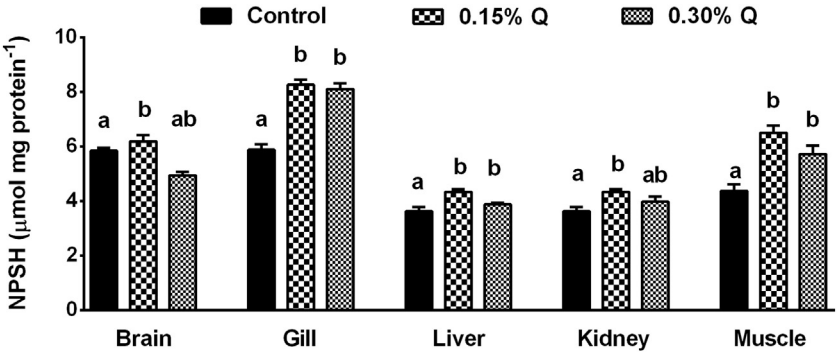

Fig. 3. Content of non-protein thiols (NPSH) in silver catfish *Rhamdia quelen* fed with diets containing different concentrations of quercetin. All the values are expressed as mean ± SEM (n = 12). Different letters denote that the data are significantly different from the control (P < 0.05).

Antioxidant compounds work as protective agents that delay or prevent ROS oxidative damage, playing major roles in the prevention of diseases across different species (Awad et al., 2015).

The evaluation of some parameters in blood and tissues are useful tools for monitoring the physiological condition of the fish and can be used as stress indicators. The haematological and biochemical parameters analysed in this study were similar among all groups. There was no mortality during the 21 days of feeding and there was no apparent loss of appetite in fish receiving the diet with quercetin. Our results are in line with the study of Plakas et al. (1985), which revealed no toxic effects of feeding rainbow trout (*Oncorhynchus mykiss*) with quercetin. Zhai and Liu (2013) observed that tilapia (*Oreochromis niloticus*) fed during 49 days with quercetin displayed decreased serum TRI levels and increased HDL levels. Such changes would be potentially beneficial avoiding fatty liver pathological changes in these animals. After 21 days feeding with quercetin, we did not observe changes on TRI and HDL levels in silver catfish. Such difference could be attributed to the longer feeding (49 days) time used by Zhai and Liu (2013).

Any form of disturbance in fish usually results in production of high cortisol levels. The cortisol is involved in several processes in fish physiology, such as regulation of metabolic pathways, behaviour, growth, reproduction and osmoregulation (Mommsen et al., 1999; Ngugi et al., 2015). Shin et al. (2010) demonstrated that supplementation with 0.25% and 0.5% of quercetin for 30 and 60 days prevented the increase on plasmatic cortisol levels on olive flounder (*Paralichthys olivaceus*) under oxidative stress induced by hypo-osmotic conditions. In our experimental conditions, without a stressful challenge, we did not observe changes on cortisol levels in fish fed with quercetin diets. In addition, it was also observed that the expression of pituitary hormones, growth hormone, prolactin and somatolactin did not change with quercetin supplementation. These results suggest that diet supplementation with this flavonoid did not cause hormonal dysfunctions in the pituitary gland. The dysfunction of these hormones is well characterised when the fish face stress (Laiz-Carrión et al., 2009; Vargas-Chacoff et al., 2009; Baldisserotto et al., 2014). Therefore, according to our findings, we can assume that the addition of quercetin to the diet of silver catfish did not cause a stressful situation.

To verify if quercetin might prevent oxidative damage, we analysed LPO by LOOH and TBARS levels. LOOH are the first stable product of the LPO reaction and TBARS values give a measure of the LPO development in terms of secondary oxidation products (Halliwell and Gutteridge, 1999). The addition of quercetin in the diet of silver catfish resulted in decreased LOOH and TBARS levels in all tissues analysed in this study.

Decreased LPO levels observed in our experiments indicate an important antioxidant effect of quercetin. The quercetin action could be attributed to the chain-breaking action of this compound in the free radical formation and consequent prevention of membrane oxidation (Bischoff, 2008). This effect may be due to the phenolic compounds present in this molecule, since we found higher values of phenolic compounds in the diets containing quercetin in relation to control diet. Moreover, a novel mechanism for quercetin induced cytoprotection has been described. This mechanism involves the sterol regulatory element binding protein-2, which mediates sterol synthesis and decreases LPO by maintaining membrane integrity during oxidative stress (Bischoff, 2008). The significant decrease of LPO in our study was consistent with a previous study (Li et al., 2014), indicating that quercitrin (glycoside formed from the quercetin and the deoxy sugar rhamnose) present of pepper extract showed a lipid-protective effect in salted silver carp (*Hypophthalmichthys molitrix*).

In order to avoid the LPO of biological membranes caused by ROS, enzymatic and non-enzymatic antioxidant defence systems are present in the cell (Halliwell and Gutteridge, 1999). SOD, which catalyses the

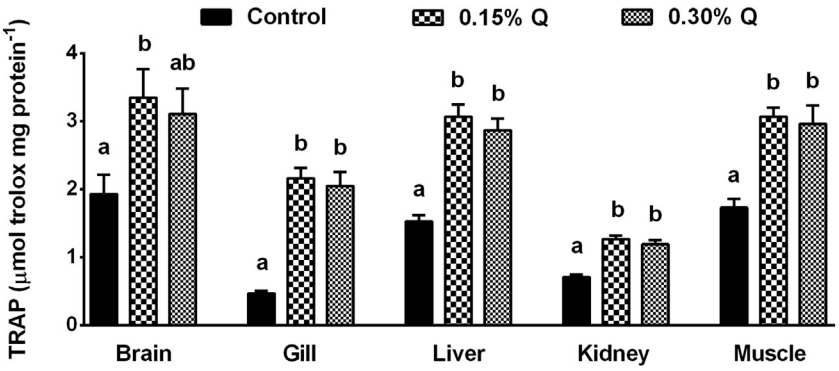

Fig. 4. Total reactive antioxidant potential (TRAP) in silver catfish *Rhamdia quelen* fed with diets containing different concentrations of quercetin. All the values are expressed as mean ± SEM (n = 12). Different letters denote that the data are significantly different from the control (P < 0.05).

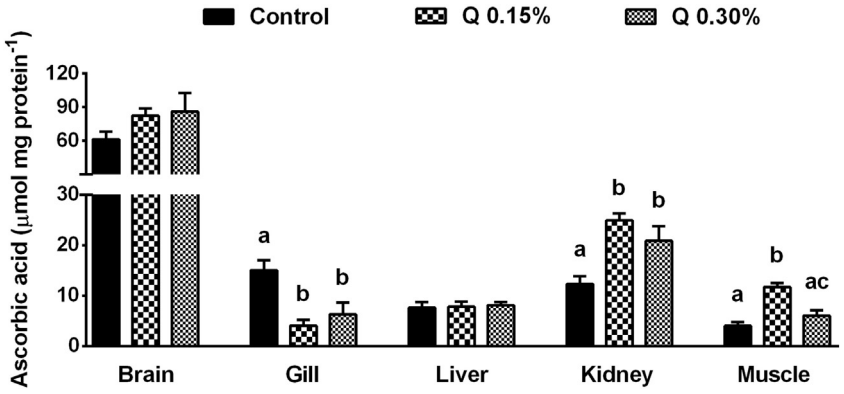

Fig. 5. Content of ascorbic acid (AA) in silver catfish *Rhamdia quelen* fed with diets containing different concentrations of quercetin. All the values are expressed as mean ± SEM (n = 12). Different letters denote that the data are significantly different from the control (P < 0.05).

dismutation of the superoxide anion (O^{2-}) to molecular oxygen and H_2O_2, is the first enzyme responding to the presence of oxygen radicals, preventing the radical chain reaction initiated by O^{2-}. In the present study, SOD activity was higher in brain, gill, liver and muscle of fish fed with quercetin diets, indicating that there was a decrease of O^{2-} generation in fish fed with these diets. In agreement with this finding, SOD mRNA expression level was higher in olive flounder fed with quercetin diet (Shin et al., 2010).

H_2O_2 is detoxified by CAT and GPx. CAT activity was increased in liver and kidney in fish fed with diets containing quercetin. In kidney, there was no difference in GPx activity in fish fed with quercetin enriched diet, suggesting that CAT was the major route for reducing H_2O_2 to molecular oxygen and water in kidney. However, the activity of GPx was increased in brain, gill and liver of fish receiving quercetin. The difference on the two-peroxidation reduction routes might be related to the overall concentration of H_2O_2 generated. It is known that CAT is more active when H_2O_2 production is high, whereas GPx is induced by low H_2O_2 levels (Halliwell and Gutteridge, 2007).

GST is an enzyme that acts in the process of biotransformation, catalysing the conjugation of a variety of metabolites, including xenobiotic and LPO products, with GSH. This pathway converts the toxic compound into an excretable molecule (Lushchak, 2011). In our study, we observed an increase in the activity of GST in gill, liver, kidney and muscle of fish fed with diets containing quercetin. These findings are in agreement with the study of Menezes et al. (2016), where an increase in GST activity in the gill and liver of silver catfish fed with diet containing the diphenyl diselenide antioxidant (1.5, 3.0 and 5.0 mg kg^{-1}) for 30 and 60 days was also observed. Based on the detoxification process, the increase in GST activity induced by quercetin represents an important additional line of defence in fish. This enzyme has an antioxidant function because it protects cells from toxic products resulted from LPO.

Among non-enzymatic antioxidants, glutathione acts as the main antioxidant in the cell and is a co-factor for the action of GST and GPx (Limón-Pacheco and Gonsebatt, 2009). Diets containing quercetin improved the levels of NPSH (indirectly measure of GSH) in all tissues of silver catfish. There was an increase of NPSH content in brain, gill, liver, kidney and muscle of fish receiving diets with quercetin. In the study of Pês et al. (2015) the silver catfish fed with diets containing 0.30% of the flavonoid rutin, during 21 days, also showed an increase in NPSH levels in brain, liver and muscle.

Another non-enzymatic antioxidant, AA is an essential molecule in the overall health of animals, acting on growth, bone formation and reproduction. Results obtained in fish suggest that the kidney is the site of AA biosynthesis, but this is not very clear (Moreau and Dabrowski, 2001). According to this proposition, in our study, the AA content was higher in kidney and muscle of fish fed with diet containing quercetin in both concentrations. The increase in enzymatic antioxidants activity and non-enzymatic antioxidants content is correlated with the increase in antioxidant capacity, measured by TRAP, in all tissues analysed. TRAP was increased in brain, gill, liver, kidney and muscle of fish receiving quercetin. The importance of this type of measurement is because it can provide key information regarding the system capacity to withstand oxidative stress unbalances (Evelson et al., 2001).

Results of our study showed that the addition of quercetin to the diet of silver catfish did not influence blood parameters and hormone pituitary expression. Moreover, quercetin showed important antioxidant properties since this flavonoid prevented LPO and increased the antioxidant status of the evaluated tissues. We suggest that this effect is a result of high levels of phenolic compounds present in quercetin diets. According to these results, quercetin is useful as a dietary supplement of fish at both concentrations. Although accessible, in economic terms we suggest using the lowest concentration tested (0.15%) because it will provide the same benefits at a lower cost to the producer. Additional studies are needed to confirm the mechanism of action of quercetin in the diet of silver catfish.

Table 4
Growth hormone, prolactin and somatolactin expression (mRNA) in the pituitary of *Rhamdia quelen* fed with diets containing different concentrations of quercetin.

	Control	Q 0.15%	Q 0.30%
Growth hormone	245.62 ± 72.45	301.33 ± 104.61	542.20 ± 36.78
Prolactin	400.11 ± 57.00	652.44 ± 239.16	680.43 ± 157.57
Somatolactin	108.80 ± 39.50	107.16 ± 19.26	69.39 ± 14.63

The hormones were presented by relative expression corrected by the amplification of an endogenous control beta actin gene. For better visualisation of results, the data were multiplied by 1000. The data appear as the mean ± SEM (n = 8). Means obtained showed no significant difference (P > 0.05).

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3.2 ARTIGO 2

Protective effect of quercetin against oxidative stress induced by oxytetracycline in muscle of silver catfish

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Protective effect of quercetin against oxidative stress induced by oxytetracycline in muscle of silver catfish

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ABSTRACT

Protective or ameliorative effects of quercetin on the oxytetracycline (OTC) induced oxidative stress and presence of OTC residues in the muscle of silver catfish (*Rhamdia quelen*) were assessed. Fish were divided into four different experimental groups and received the following experimental diets for 14 or 21 days: (i)-control; (ii)-quercetin; (iii)-OTC; or (iv)-quercetin plus OTC. Muscle samples were collected at the end of the experiment and analyzed for the oxidant/antioxidant status and presence of OTC residues. The OTC values in muscle of fish were lower than the quantification limit. There was a significant increase on lipid peroxidation and protein carbonyl content in the muscle of OTC-treated fish at 14 and 21 days. Treatment with quercetin avoided this increase in the groups that received diet with quercetin plus OTC. Superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities decreased in the muscle of OTC-treated fish at 14 days. Quercetin was able to avoid the decrease of GPx activity in the group that fed diet with quercetin plus OTC. After 21 days of treatment there was a decrease of SOD, GPx and glutathione S-transferase activities in the muscle of OTC-treated fish. Quercetin was able prevent the decrease of SOD activity in the group that received diet with quercetin plus OTC. The glutathione reductase activity was higher in quercetin group at 14 and 21 days of treatment. Ascorbic acid (AA) content increased in quercetin and quercetin plus OTC groups after 14 days of treatment. After 21 days of treatment, the AA and non-protein thiols content decreased in the muscle of OTC-treated fish. Thus, the present study demonstrates that quercetin protects the muscle from the OTC-induced oxidative stress, suggesting that it may be a useful compound to minimize the adverse effects in fish requiring treatment with OTC.

1. Introduction

During the production process, fish are subjected to different stressors that need to be given special attention as they may impair the health of animals, triggering losses in production (Segner et al., 2012; Stevens et al., 2017). Stress response is applicable to various physiological mechanisms, including changes in expression of genes and proteins, metabolic, immunological, endocrine, neural disruption, and even changes in behavior. Such changes occur to, first, try to overcome this situation and then offset the imbalances produced by the stressor or the consequences generated by the first array of responses, restoring the body homeostasis (Tort, 2011).

Infectious diseases are of great concern for fish farming because they represent potential risks in production, stunted growth and inventory losses. Among the main strategies to control this problem is the use of antimicrobial substances (Grenni et al., 2017).There is no specific legislation for using veterinary drugs in Brazilian aquaculture, which leads to their misuse. Thus, the excessive use of antibiotics as growth promoters or even as prophylactic measure to prevent disease can trigger a stress situation in fish and result in an undesirable outcome (Gaskins et al., 2002).

Oxytetracycline (OTC) is one of the most commonly used antibiotic in fish farms due to its higher efficiency and economy when compared to other antibiotics (Ren et al., 2017). In infectious diseases, OTC is usually administered as medicament in the feed pellets at a rate of 50 to 100 mg of OTC per kg of body weight per day for 3–21 days depending on the infection (Treves-Brown, 2000; Yonar, 2012).

Besides its beneficial effect as an antibiotic agent, OTC has been shown to induce DNA damage (Rodrigues et al., 2017), to interfere with the immune mechanisms and regulation of immune competent genes

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(Guardiola et al., 2012), to cause liver damage (Bruno, 1989) and lead to oxidative stress, increasing the levels of lipid peroxidation and decreasing the activity of antioxidant enzymes in rainbow trout (*Oncorhynchus mykiss*) (Yonar et al., 2011; Yonar, 2012). Moreover, the overuse of antibacterial drugs in fish can lead to development of resistant bacterial strains, accumulation of antibiotics residues in cultured organisms, causing a potential risk to consumers (Chen et al., 2015) and to the natural environment (Grenni et al., 2017).

In the present study, we tested if dietary supplementation with quercetin avoids the physiological damage caused by oral administration of OTC in silver catfish, *Rhamdia quelen*. Quercetin is a flavonol found widely in fruits, vegetables and nuts. In nature, it exists primarily as quercetin glycoside and consists of quercetin aglycone conjugated to sugar moieties such as glucose or rutinose (Guo and Bruno, 2015). Our previous study demonstrated that quercetin reduces lipid peroxidation and increases antioxidant levels when administrated in diets of silver catfish for 21 days (Pês et al., 2016), which support our hypothesis that quercetin may have a promising effect against the damage caused by OTC.

Silver catfish is the most raised native species in southern Brazil due to its elevated growth rate, good carcass yield and easily controlled reproduction in subtropical climate (Baldisserotto, 2009; Valladão et al., 2016). This study is important for obtaining information to clarify the appropriate use of antibiotics in aquaculture and the search for a natural product that can minimize the undesirable effects of OTC.

The primary aim of this study was to evaluate the protective or ameliorative effects of quercetin on the OTC induced oxidative stress in muscle of silver catfish. The second objective was to determine whether OTC residues remains in the muscle of silver catfish fed the different experimental diets.

2. Materials and methods

2.1. Chemicals

Quercetin ($C_{15}H_{10}O_7$) was obtained from Opção Fênix Petrochemicals Distributor Ltd. (São Paulo, SP, Brazil). Commercial form of OTC (Terramycin®) used in the experiments was obtained from Pfizer (New York, New York, United States). All the other chemicals were obtained from Sigma (St. Louis, Missouri, United States).

2.2. Species and acclimation period

Male and female of silver catfish (*R. quelen*) (weight, 190.0 ± 25.50 g; length, 26.57 ± 1.09 cm) were obtained from a fish culture sector of Federal University of Santa Maria (UFSM). Fish were randomly distributed and acclimated in 250 L tanks (eight fishes per tank) for two weeks. They were maintained in continuously aerated water with a natural photoperiod (12 h light/12 h dark). Water quality parameters were checked daily and were recorded as: temperature 23.0 ± 1.5 °C, pH 6.9 ± 0.30 units, dissolved oxygen 7.2 ± 0.3 mg L^{-1}, hardness 22.3 ± 1.09 mg $CaCO_3$ L^{-1}, non-ionized ammonia 0.12 ± 0.004 mg L^{-1}, nitrite 0.79 ± 0.05 mg L^{-1} and alkalinity 23.1 ± 1.21 mg $CaCO_3$ L^{-1}. All water parameters were determined as described in our previous study (Pês et al., 2016). During acclimation, fish were fed once a day with control diet. Feces and pellet residues were removed by syphon to keep the water quality. The experimental protocol was approved by the Ethics and Animal Welfare of the Federal University of Santa Maria (n° 4,380,290,115) and was performed according to the standards proposed by the same.

2.3. Diets preparation and experimental design

Four diets were formulated based on the study of Pês et al. (2016). The diet consisted of 30% soybean meal, 35% meat and bone meal, 12% rice bran, 15% corn, 3% canola oil, 1% salt, 3% vitamins and minerals (premix) and 1% phosphate dicalcium. The ingredients used to prepare the diets were weighed and then mixed until complete homogenization. After mixing, the diets were moistened, pelleted in a meat grinder and taken to a forced air circulation oven for drying (45 °C) for 24 h. Quercetin (1.5 g kg $diet^{-1}$) and/or oxytetracycline (0.1 g kg $diet^{-1}$) were added to the mixture together with rice bran and subsequently to the other ingredients.

The experimental design included four groups (in triplicate): (i)-control (diet with neither antibiotic nor antioxidant); (ii)-quercetin (1.5 g quercetin kg $diet^{-1}$); (iii)-OTC (0.1 g OTC kg $diet^{-1}$); or (iv)-quercetin plus OTC (1.5 g quercetin kg $diet^{-1}$ plus 0.1 g OTC kg $diet^{-1}$). The amount of feed provided to the animals was fixed in 3% of the biomass of each tank once a day (9 a.m.) for each experimental period, 14 and 21 days. The dosage of quercetin was chosen based on our previous study (Pês et al., 2016) and the dosage of OTC, based on reports found in the literature using OTC in the diet of other fish species (Treves-Brown, 2000; Yonar et al., 2011; Yonar, 2012).

At end of each experimental period (14 and 21 days), ten fish per group were euthanised by sectioning the spinal cord. Muscle were removed, immediately frozen in liquid nitrogen and stored at − 80 °C for further analysis.

2.4. Total phenolic compounds

Total phenolic compounds were determined in the diets according to the Folin-Ciocalteau procedure (Singleton et al., 1999). Gallic acid was used as a standard, and the results were expressed as gallic acid equivalents (mg GAE) 100 g of grain (dry weight)$^{-1}$. The samples analyzed showed different concentrations of total phenolic compounds, which were higher in diets with quercetin (123.52 mg GAE 100 g $diet^{-1}$) and diet with quercetin plus OTC (118.60 mg GAE 100 g $diet^{-1}$), than in control (51.41 mg GAE 100 g $diet^{-1}$) and OTC diet (50.82 mg GAE 100 g $diet^{-1}$) ($p < 0.05$).

2.5. Detection of OTC residues using solid-liquid extraction and UHPLC-MS/MS

Sample preparation, extraction and determination of OTC residues were done using solid-liquid and ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) based on previous work developed by Rizzetti et al. (2017). The extraction was performed in two steps. Three gram of homogenized fish muscle sample were transferred to 15 mL polypropylene (PP) tube and extracted with 10 mL of acetonitrile. The tube was vortexed for 1 min and centrifuged at 2600 × *g* for 8 min. In the second step, 500 μL of upper layer was transferred to an Eppendorf tube containing 500 μL of aqueous solution of TCA 5% (w/v) and the tube was shaken vigorously on the vortex for 1 min and centrifuged at 13300 × *g* for 4 min. The extract was filtered in a 0.2-μm nylon membrane and diluted two times in ultrapure water prior to UHPLC-MS/MS analysis. The validation procedure was performed based on parameters established by INMETRO, 2016.

2.6. Determination of oxidative stress indicators

The muscle was homogenized in 154 mmol L^{-1} KCl containing 1 mmol L^{-1} phenylmethylsulfonyl fluoride and centrifuged at 700 *g* for 10 min at 4 °C to discard the nuclei and cell debris.

Lipid hydroperoxides (LOOH) methodology was performed to detect the primary products of lipid peroxidation using the oxidation of Fe^{2+} by LOOH in an acidic medium with xylenol orange dye, which forms a complex with Fe^{3+}. The readings were performed using a Thermo Plate reader (Thermo Plate Devices, China) at 560 nm and the results were reported as nmol mg protein^{-1} (Södergren et al., 1998). To measure the end products of lipid peroxidation, including malondialdehyde (MDA), we used thiobarbituric acid reactive substance (TBARS) assays.

The amount of MDA produced was used as an index of lipid peroxidation, determined spectrophotometrically at 535 nm. The value were expressed as nmol mg protein^{-1} (Buege and Aust, 1978).

Protein carbonyl (PC) content was measured by a method based on the reaction of carbonyls with 2,4-dinitrophenylhydrazine forming dinitrophenylhydrazone, a yellow compound. The standard curve was prepared by using different bovine serum albumin concentrations (0.5–1.5 mg mL^{-1}) and the slope was used to express the levels of PC as nmol mg protein^{-1} (Reznick and Packer, 1994).

Total superoxide dismutase (SOD) activity, was determined based on the inhibition rate of autocatalytic adrenochrome generation at 480 nm in a reaction medium containing 1 mM epinephrine and 50 Mm glycine/NaOH (pH 10.2). One SOD unit was defined as the amount of enzyme required for 50% inhibition of the adrenochrome formation and the enzyme activity was expressed as USOD mg protein^{-1} (Misra and Fridovich, 1972).

Glutathione peroxidase (GPx) activity was measured by following NADPH oxidation at 340 nm, as described by Flohé and Gunzler (1984), and results were expressed as nmol min^{-1} mg protein^{-1}. Glutathione reductase (GR) activity was assayed as described by Carlberg and Mannervik (1985). GR is an NADPH-dependent enzyme that regenerates GSH from oxidized glutathione (GSSG). Its activity was expressed as nmol min^{-1} mg protein^{-1} and measured by the rate of NADPH consumption at 340 nm. Glutathione S-transferase (GST) activity was assayed based on the conjugation reaction with reduced glutathione (GSH), using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate (Habig et al., 1974). Sample aliquots (0.05 mL) were added to 0.6 mL of the assay mixture containing 100 mmol L^{-1} phosphate buffer (pH 6.5), GSH and CDNB at a final concentration of 1 mmol L^{-1} each. GST activity was calculated from the changes in absorbance at 340 nm and was expressed as ρmol min^{-1} mg protein^{-1}.

Ascorbic acid (AA) and non-protein thiols (NPSH) contents were determined similarly to the study of Pês et al. (2016). For the measure of AA, the standard curve was prepared by using different concentrations of AA, and the slope was used to express the amount of ascorbic acid as μmol mg protein^{-1}. The NPSH content, an indirect measure of GSH, was evaluated at 412 nm after reacting with 5,5′-dithiobis (2-nitrobenzoic acid) and reported as μmol mg protein^{-1}.

2.6.1. Protein determination

Protein content of the muscle was determinate by the method of Lowry et al. (1951) using bovine serum albumin as standard. Absorbance of samples was measured at 625 nm and results are reported as mg mL^{-1}.

2.7. Statistical analysis

Statistical analysis was performed using the software Statistica® 7.0. Levene's test was used to verify whether the data were parametric. Two-way analyses of variance followed by Tukey test were performed to assess the differences among the groups and times. Results are expressed as mean ± standard error (SEM). The minimum significance level was set at 95% ($p < 0.05$).

3. Results

3.1. Antibiotic residues

At the end of the experimental periods (14 and 21 days), samples from the OTC group presented OTC values lower than the quantification limit (LOQ) (0.03 mg kg^{-1}). After 21 days of treatment, quercetin plus OTC group also had OTC values lower than LOQ. In the other experimental groups, no residues of the OTC antibiotic were detected.

Table 1
Protein content in the muscle of silver catfish fed different diets for 14 and 21 days.

Experimental groups	Days of treatment	
	14	21
Control	8.65 ± 0.18	7.95 ± 0.16
Quercetin	8.62 ± 0.29	8.92 ± 0.17
OTC	8.57 ± 0.25	8.65 ± 0.25
Quercetin plus OTC	8.22 ± 0.25	8.97 ± 0.27

Protein content (mg mL^{-1}). Data are presented as the mean ± SEM (n = 10). Means obtained showed no significant difference ($p > 0.05$).

3.2. Protein determination

Protein contents in the muscle did not differ among the experimental groups (Table 1).

3.3. LOOH and TBARS levels

Lipid peroxidation levels, determined by LOOH, were increased in the OTC group (7.36 ± 0.07) when compared with control (4.80 ± 0.71, $p = 0.00431$), quercetin (3.22 ± 0.44, $p = 0.00016$) and quercetin plus OTC (4.45 ± 0.04, $p = 0.00126$) groups at 14 days of treatment. In 21 days of treatment, LOOH levels were increased in the OTC group (5.96 ± 0.43) when compared with control (3.26 ± 0.20, $p = 0.00266$), quercetin (2.34 ± 0.08, $p = 0.00025$) and quercetin plus OTC (3.73 ± 0.31, $p = 0.00808$) groups. Then, treatment with quercetin avoided the increase on LOOH levels in the group that received diet with quercetin plus OTC in both time of treatment (Fig. 1A). The TBARS values were decreased in quercetin (0.31 ± 0.02) group compared to control (0.44 ± 0.02, $p = 0.00024$), OTC (0.49 ± 0.02, $p = 0.00013$) and quercetin plus OTC (0.47 ± 0.02, $p = 0.00014$) groups at 14 days of treatment (Fig. 1B). Administration of OTC for 21 days resulted in increased TBARS levels (0.55 ± 0.02) when compared with control (0.41 ± 0.02, $p = 0.00220$), quercetin (0.37 ± 0.02, $p = 0.00013$) and quercetin plus OTC (0.38 ± 0.01, $p = 0.00014$) groups. Treatment with quercetin avoided this increase in the group that received diet with quercetin plus OTC (Fig. 1B).

3.4. PC content

After 14 days of treatment, PC content was higher in the OTC (1.40 ± 0.16) group than in the quercetin (1.01 ± 0.08, $p = 0.00907$) and quercetin plus OTC (1.01 ± 0.05, $p = 0.00785$) groups. PC content was increased in the OTC (1.78 ± 0.08) group in relation to the control (1.26 ± 0.08, $p = 0.00028$), quercetin (1.27 ± 0.08, $p = 0.00034$) and quercetin plus OTC (1.09 ± 0.06, $p = 0.00017$) groups, when received the different diets for 21 days. The treatment with quercetin avoided this increase in the group that received diet with quercetin plus OTC for 14 and 21 days. Moreover, the content of PC in OTC group was higher at day 21 (1.78 ± 0.08) than at day 14 (1.40 ± 0.16, $p = 0.00167$) (Fig. 2).

3.5. Antioxidant enzymes activities

Administration of quercetin for 14 days resulted in higher SOD activity (1.87 ± 0.18) compared to control (0.95 ± 0.09, $p = 0.00017$) and OTC (0.97 ± 0.07, $p = 0.00025$) groups. After 21 days of treatment, SOD activity decreased in OTC (1.14 ± 0.07) group in relation to the control (1.81 ± 0.07, $p = 0.00317$), quercetin (1.99 ± 0.07, $p = 0.00026$) and quercetin plus OTC (1.68 ± 0.12, $p = 0.00171$) groups, while the treatment with quercetin avoided this decrease on

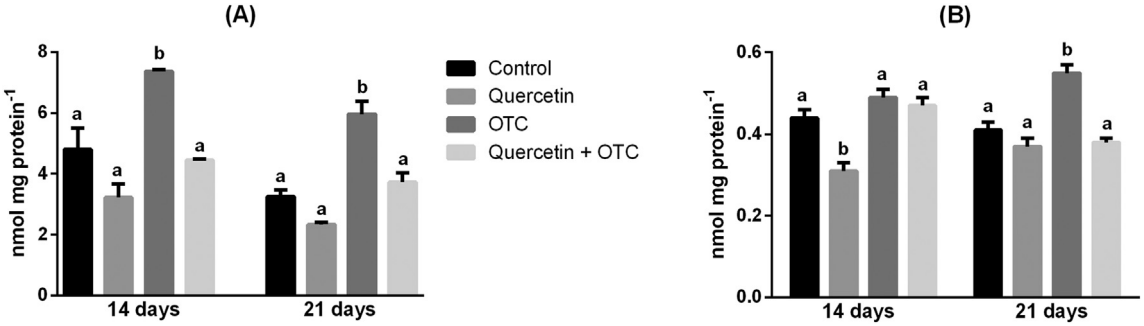

Fig. 1. (A) Levels of lipoperoxidation evaluated by lipid hydroperoxides and (B) thiobarbituric acid reactive substances in the muscle of silver catfish fed different diets for 14 and 21 days. Different letters indicate statistical differences between treatments. All the values are expressed as mean ± SEM (n = 10). Two-way ANOVA and Tukey test ($p < 0.05$).

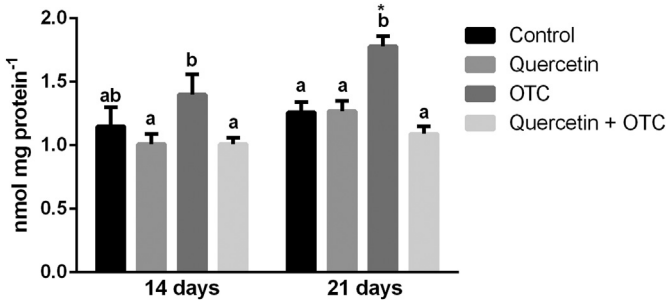

Fig. 2. Protein carbonyl content in the muscle of silver catfish fed different diets for 14 and 21 days. Different letters indicate statistical differences between treatments. (*) indicate significant differences for a treatment at different time. All the values are expressed as mean ± SEM (n = 10). Two-way ANOVA and Tukey test ($p < 0.05$).

SOD activity in the group that received diet with quercetin plus OTC for 21 days. Furthermore, the activity of SOD in control group at 21 days was higher (1.81 ± 0.07) than at 14 days (0.95 ± 0.09, $p = 0.00023$) (Fig. 3A).

GPx activity decreased in OTC (7.43 ± 0.35) group in relation to the control (16.55 ± 1.11, $p = 0.00013$), quercetin (27.27 ± 0.50, $p = 0.00013$) and quercetin plus OTC (16.34 ± 0.67, $p = 0.00013$) groups at 14 days. Administration of quercetin for 14 days was able to increase GPx activity when compared with control and OTC group. The diet with quercetin plus OTC was able to restore GPx activity. After 21 days of treatment, the GPx activity was lower in the OTC (8.96 ± 0.28) and quercetin plus OTC (8.90 ± 0.15) groups than in control (22.06 ± 0.80, $p = 0.00013$) and quercetin (21.40 ± 0.70, $p = 0.00013$) groups. Moreover, the activity of GPx in control group at 21 days was higher (22.06 ± 0.80) than at 14 days (16.55 ± 1.11, $p = 0.00017$) (Fig. 3B).

The highest GR activity was observed in the quercetin (2.70 ± 0.22) group in relation to the control (1.49 ± 0.05, $p = 0.00542$), OTC (1.30 ± 0.25, $p = 0.00144$) and quercetin plus OTC (1.28 ± 0.20, $p = 0.00123$) groups at 14 days of treatment. Administration of quercetin for 21 days resulted in higher GR activity (2.43 ± 0.24) compared to control (1.52 ± 0.20, $p = 0.04090$), OTC (1.17 ± 0.12, $p = 0.00393$) and quercetin plus OTC (1.12 ± 0.10, $p = 0.00271$) groups (Fig. 3C). GST activity was lower in OTC (0.58 ± 0.04) group than in the control (0.77 ± 0.06, $p = 0.00750$) and quercetin (1.01 ± 0.04, $p = 0.02452$) group at 21 days (Fig. 3D).

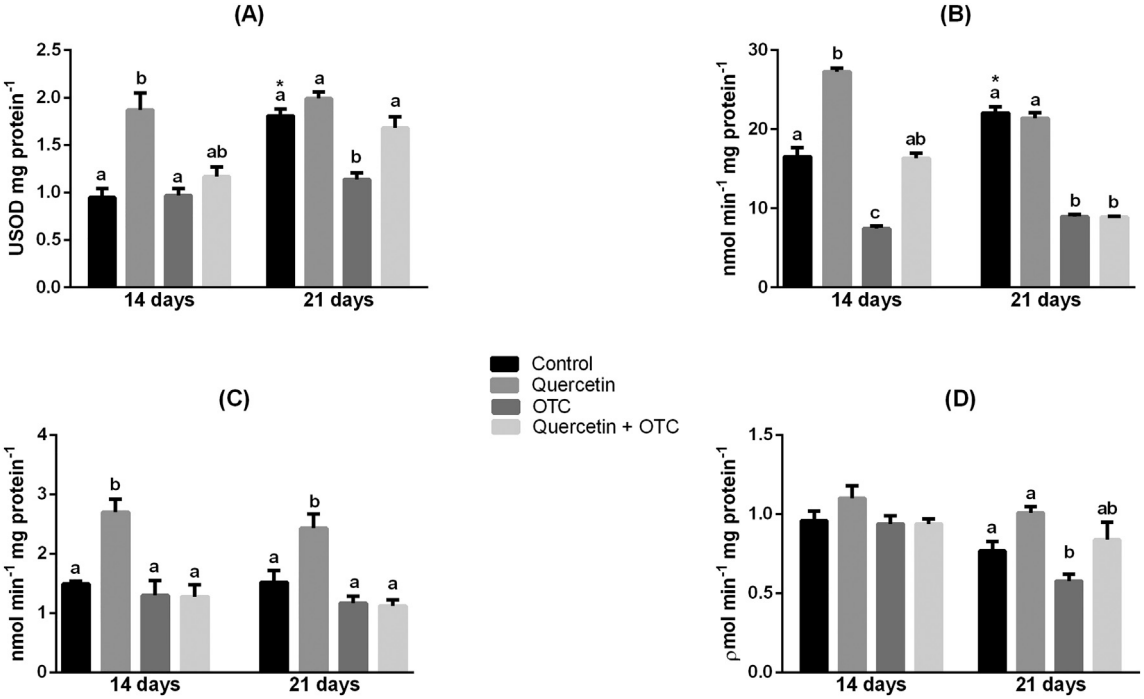

Fig. 3. (A) Superoxide dismutase, (B) glutathione peroxidase, (C) glutathione reductase and (D) glutathione S-transferase activities in the muscle of silver catfish fed different diets for 14 and 21 days. Different letters indicate statistical differences between treatments. (*) indicate significant differences for a treatment at different time. All the values are expressed as mean ± SEM (n = 10). Two-way ANOVA and Tukey test ($p < 0.05$).

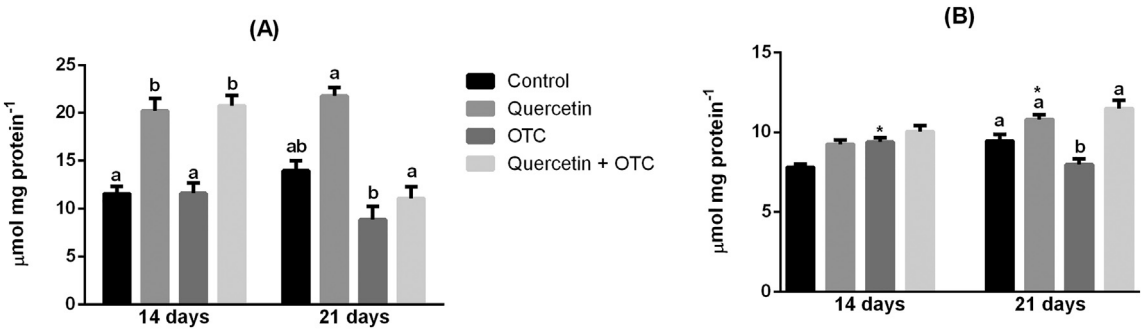

Fig. 4. (A) Ascorbic acid and (B) non-protein thiols content in the muscle of silver catfish fed different diets for 14 and 21 days. Different letters indicate statistical differences between treatments. (*) indicate significant differences for a treatment at different time. All the values are expressed as mean ± SEM (n = 10). Two-way ANOVA and Tukey test ($p < 0.05$).

3.6. Non-enzymatic antioxidants

After 14 days of treatment, the AA content was higher in the quercetin (20.22 ± 1.30) and quercetin plus OTC (20.77 ± 1.04) groups than in the control (11.58 ± 0.79, $p = 0.00715$) and OTC (11.60 ± 1.08, $p = 0.00492$) groups. Administration of OTC for 21 days resulted in lower AA content (8.87 ± 1.37) than quercetin (21.78 ± 0.86, $p = 0.00526$) and quercetin plus OTC (11.10 ± 1.20, $p = 0.02708$) groups (Fig. 4A).

The OTC group showed the lowest NPSH content at 21 days (7.97 ± 0.38) and the treatment with quercetin avoided this decrease in the group that received diet with quercetin plus OTC (11.49 ± 0.51, $p = 0.00029$). Fish fed with quercetin for 21 days presented higher (10.81 ± 0.30) content of NPSH than those fed for 14 days (9.24 ± 0.26, $p = 0.00013$). On the other hand, fish fed with OTC for 21 days had a lower content (7.97 ± 0.38) of NPSH than those receiving the same diet for 14 days (9.38 ± 0.26, $p = 0.00147$) (Fig. 4B).

4. Discussion

Quercetin is quickly absorbed and is then deposited in aglycone form, acting as a powerful antioxidant in fish (Park et al., 2009). Our previous findings have shown that treatment with quercetin alone for 21 days is capable of improving the antioxidant/oxidant status of fish (Pês et al., 2016). The present study revealed that quercetin treatment significantly attenuated the oxidative damage caused by OTC in the muscle of fish due to its antioxidant effect.

Fish from the OTC group showed OTC values in the muscle lower than LOQ (0.03 mg kg^{-1}). The FDA has set a 2 mg kg^{-1} OTC tolerance in fish muscle and a 21-day withdrawal period. Salmonids, sea bream (*Sparus aurata*), and sea bass (*Dicentrarchus labrax*) fed OTC diets accumulated OTC mainly in the stomach, followed by the liver, kidney, skin and muscle (Chen et al., 2004). In the present study, the fish did not go through a period of depletion of OTC residues, but in any case, we did not find excess of OTC in OTC-fed fish muscles at 14 and 21 days of administration. After 14 days of treatment, OTC residues were detected in muscle of fish from the OTC group. In the group that received the diet quercetin plus OTC, no residues of OTC were detected. These results suggest that quercetin was able to prevent the accumulation of OTC in the muscle. More studies should be done to elucidate the mechanisms by which this interaction occurs. After 21 days of treatment, quercetin was not able to prevent the accumulation of OTC, residues in the muscle.

Studies have demonstrated that oral administration of OTC induces the production of reactive oxygen species and DNA damage in different fish species (Yonar et al., 2011; Yonar, 2012; Rodrigues et al., 2017). Lipid peroxidation has a major participation in the loss of cell function under oxidative stress and is usually indicated by LOOH and TBARS in fish (Li et al., 2011). Additionally, reactive oxygen species directly attack protein and catalyze the formation of carbonyl. The formation of carbonyl protein is non-reversible, causing conformational changes, decreasing catalytic activity of enzymes and ultimately resulting, owing to increased susceptibility to protease action, in breakdown of proteins by proteases (Zhang et al., 2008).

Our results showed that oral administration of OTC led to oxidative stress, with higher LOOH, TBARS and PC levels in the muscle of fish at different times of treatment. Furthermore, quercetin was able to protect the tissue from these disorders. The results obtained by the study of Nakano et al. (2013) showed that OTC, at the same concentration as those used on our study, influences stress-related biomarkers and the redox state in *Oncorhynchus kisutch* and results in damage to tissues, especially to the liver.

In general, the activity of antioxidants enzymes of fish fed diet containing quercetin was significantly better in comparison with the OTC treatment, similar or higher to the control. The different treatment times did not significantly influence the enzymatic activities. However, only SOD and GPx were able to return to their levels when administered diet containing quercetin plus OTC for 21 and 14 days, respectively, which might be due to the ability of quercetin to reduce accumulation of free radicals generated during OTC-induced lipid peroxidation. Similar to our results, it was shown that 0.1 g OTC kg $diet^{-1}$ administered orally for 14 days to rainbow trout induced a reduction in SOD, catalase and GPx activities in blood, liver, kidney, spleen and heart tissues (Yonar et al., 2011).

The GR activity of the muscle of silver catfish was not significantly affected by OTC treatment. Diets containing quercetin increased the activity of GR in the muscle at different times of treatment. Another enzyme important in the detoxification process and involved in the glutathione system is GST. In the present study, the oral administration of OTC led to a lower activity of this enzyme after 21 days of treatment, when compared to the control and quercetin group. Considering the detoxification process, the reduction in GST activity induced by OTC is an important factor to consider, because this mechanism represents an important additional line of defense in fish. This enzyme has an antioxidant function because it protects cells from toxic products resulting from lipid peroxidation (Ren et al., 2017). The decreased activities of these antioxidant enzymes in OTC treated fish can be attributed to excess accumulation of free radicals, such as superoxide anion and hydrogen peroxide, in the muscle.

The non-enzyme antioxidant system integrated by AA and NPSH is an important defense against free radicals. In the present study, the AA and NPSH content decreased in silver catfish fed diet supplemented with OTC for 21 days. This decrease may have been caused by the utilization of non-enzymatic antioxidants to control the enormous amount of free radicals that were produced during OTC intoxication. Treatment with quercetin may have increased the NPSH content via increased biosynthesis of glutathione or increased levels of the other antioxidants (Enamorado et al., 2015).

Taken together, these results suggest that quercetin is potentially

useful for the prevention of the oxidative damage induced by OTC, mainly due to its potential to up-regulate NPSH content and cellular antioxidant capacity, preventing oxidative stress.

5. Conclusion

Overall, we conclude that: (i) OTC caused oxidative stress in muscle of *Rhamdia quelen*; (ii) quercetin provided a protective effect against the oxidative stress induced by OTC, acting as a powerful modulator of the enzymatic and non-enzymatic antioxidants; (iii) the OTC values in muscle of fish are below the levels recommended by the FDA; and (iv) this study reinforces that quercetin could be used as an antioxidant in fish. Further investigations are necessary to elucidate the exact mechanism of protection and potential usefulness of quercetin as a protective agent against drug toxicity.

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3.3 MANUSCRITO 1

Diet with quercetin mitigates the oxidative toxicity of oxytetracycline in silver catfish (*Rhamdia quelen*)

Manuscrito submetido ao periódico **Environmental Toxicology and Pharmacology.**

Quercetin mitigates the oxidative toxicity of oxytetracycline in silver catfish (*Rhamdia quelen*)

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Abstract

Oxytetracycline (OTC) is an antibiotic widely used in aquaculture practices. Evidence suggests that its use may be associated with detrimental effects in fish due to oxidative stress. In the current study, we evaluated the protective effect of quercetin on OTC-induced toxicity by assessing biomarkers of oxidative damage (lipid hydroperoxides, LOOH; thiobarbituric acid reactive substances, TBARS; protein carbonyl, PC), antioxidant enzymes (superoxide dismutase, SOD; catalase, CAT; glutathione S-transferase, GST; and glutathione peroxidase, GPx) and non-enzymatic antioxidants (ascorbic acid, AA; and non-protein thiols, NPSH) in brain, kidney and liver of *Rhamdia quelen*. OTC (0.1 g kg diet^{-1}) and quercetin (1.5 g kg diet^{-1}) were administered in the diet for 14 and 21 days. OTC increased LOOH, TBARS and PC levels, decreased SOD, CAT, and GPx activities, and depletion of AA and NPSH levels. Co-treatment with quercetin was able to prevent those effects. Thus, our study has proven that quercetin restores the antioxidant potential and prevents OTC-induced oxidative toxicity, suggesting that quercetin administration might be a valuable strategy to minimize the adverse effects in fish requiring treatment with OTC.

Keywords: antibiotic, oxidative damage, fish, flavonoid, antioxidant

1. Introduction

World aquaculture practices have increased very rapidly due to the high demand for nutritious foods and its economic importance (Ramesh et al., 2018). In addition, pollution and water-borne diseases are a serious problem for aquaculture (Sanawar et al., 2017). In the aquaculture industry, antibiotics, such as oxytetracycline (OTC), play an important role in the growth of fish and in the prevention of infectious diseases. However, these pharmaceutical compounds may persist in the environment and increase the resistance capacity of bacteria, which affects microflora and fauna, leading to immunosuppression and growth retardation of fish (Ramesh et al., 2018). OTC is one of the most widely used drugs to treat bacterial diseases in aquaculture, due to its greater efficiency and low cost when compared to other antibiotics (Guardiola et al., 2012, Ren et al., 2017). However, OTC administration has also been shown to cause toxicity in silver catfish (*Rhamdia quelen*) (Pês et al., 2018) and rainbow trout

(*Oncorhynchus mykiss*) (Yonar et al., 2011; Yonar, 2012), where it induced the formation of reactive oxygen species (ROS) in several tissues.

Fish show the ability to live in contaminated sites, mainly due to their mechanisms of detoxification, excretion, antioxidant protection and stress response (Bard, 2000). However, bioaccumulation of toxic substances triggers redox reactions, generating free radicals and ROS that induce biochemical changes in fish tissues (Narra, 2016, Narra et al., 2017). The response of the organisms to oxidative stress caused by bioaccumulation of water contaminants has been recognized as an important factor to be taken in consideration when designing new aquaculture practices (Livingstone, 2001).

Therefore, the incorporation of natural food additives, mainly antioxidant compounds, in aquaculture fish diets has become widely used practice, since it improves fish performance, immunity, and flesh quality by preventing oxidative stress. However, the search for more efficient antioxidant compounds to be added to fish diets is still a high priority in aquaculture (Saleh et al., 2015). We have recently shown that quercetin can efficiently prevent oxidative stress when combine to fish diet (Pês et al., 2016; 2018). Quercetin is a flavonoid with polyphenolic chemical substructure. In a biological system, it acts as an antioxidant and is known to protect tissue from free radicals (Strugala et al., 2017). This flavonoid is readily absorbed and deposited as aglycone and it has been shown to act as an important antioxidant in tilapia (*Oreochromis niloticus*) (Park et al., 2009). In addition, its use in fish could potentially reduce the production cost and cause less damage to the environment, since it is known to be more biodegradable than synthetic compounds (Awad et al., 2015). Our research group is working in different aspects of stress system in the silver catfish (*Rhamdia quelen*), a native species of great economic importance in South America (Baldisserotto, 2009; Valladão et al., 2016).

In the present study, we aim to investigate whether dietary quercetin is capable to prevent the oxidative stress caused by OTC administration in silver catfish.

2. Material and Methods

2.1 Chemicals

Quercetin ($C_{15}H_{10}O_7$) was obtained from Opção Fênix Petrochemicals Distributor Ltd. (São Paulo, SP, Brazil). The commercial form of OTC (Terramycin®) used in the experiment

was obtained from Pfizer (New York, New York, United States). All other chemicals were obtained from Sigma (St. Louis, Missouri, United States).

2.2 Fish and diet preparation

Juveniles of silver catfish (*R. quelen*) (weight, 190.0 ± 25.5 g; length, 26.6 ± 1.1 cm) were obtained from a fish culture sector of the Federal University of Santa Maria (UFSM). Fish were randomly distributed in 250 L tanks (eight fishes per tank) and acclimated for two weeks. They were maintained in continuously aerated water with a natural photoperiod (12 h light/12 h dark). Water quality parameters as temperature, dissolved oxygen, hardness, non-ionized ammonia, nitrite, and alkalinity were checked daily and are described in our previous study (Pês et al., 2018). During acclimation, fish were fed once a day with the control diet. Feces and pellet residues were removed by a syphon to keep the water quality. The experimental protocol was approved by the Ethics and Animal Welfare of the Federal University of Santa Maria (nº 4380290115) and was performed according to the standards proposed by the same.

The experimental diets composition was based on previous studies from our group (Pês et al., 2016). Briefly, to obtain the diets containing OTC (0.1 g kg $diet^{-1}$) and/or quercetin (1.5 g kg $diet^{-1}$), the compounds were added to control diet and all the ingredients were completely mixed by adding distilled water before further homogenization. The diet pellets were stored at 4°C until they were used.

2.3 Experimental design

Fish were randomly divided into four experimental groups (in triplicate), composed of ten animals each, and treated as follows:

- Control: received diet with neither OTC nor quercetin;
- OTC: received treatment with 0.1 g OTC kg $diet^{-1}$;
- Quercetin: received treatment with 1.5 g quercetin kg $diet^{-1}$;
- OTC+quercetin: received treatment with 0.1 g OTC kg $diet^{-1}$ and 1.5 g quercetin kg $diet^{-1}$.

The amount of food provided to the animals was fixed in 3% of the biomass of each tank once a day (9 a.m.) for each experimental period, 14 and 21 days. The dosage of quercetin was chosen based on our previous study (Pês et al., 2016) and the dosage of OTC, based on

reports found in the literature using OTC in the diet of other fish species (Treves-Brown, 2000, Yonar et al., 2011, Yonar, 2012).

At end of each experimental period (14 and 21 days), ten fish per group were euthanized by sectioning the spinal cord. Brain, kidney, and liver were removed, immediately frozen in liquid nitrogen, and stored at -80 °C for further analysis.

2.4 Sample preparation

The brain, kidney and liver were homogenized in 154 mmol L^{-1} KCl containing 1 mmol L^{-1} phenylmethylsulfonyl fluoride and centrifuged at 700*g* for 10 min at 4ºC to discard the nuclei and cell debris.

2.5 Estimation of protein

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard. The absorbance of samples was measured at 625 nm and results were reported as mg mL^{-1}.

2.6 Biomarkers of oxidative damage

Lipid hydroperoxides (LOOH) methodology was performed to detect the primary products of lipid peroxidation using the oxidation of Fe^{2+} by LOOH in an acidic medium with xylenol orange dye, which forms a complex with Fe^{3+}. The readings were performed using a Thermo Plate reader (Thermo Plate Devices, China) at 560 nm and the results were reported as nmol mg $protein^{-1}$ (Södergren et al., 1998).

To measure the end products of lipid peroxidation, including malondialdehyde (MDA), we used thiobarbituric acid reactive substance (TBARS) assay. The amount of MDA produced was used as an index of lipid peroxidation, determined spectrophotometrically at 535 nm. The value was expressed as nmol mg $protein^{-1}$ (Buege and Aust, 1978).

Protein carbonyl (PC) content was measured by a method based on the reaction of carbonyls with 2,4-dinitrophenylhydrazine forming dinitrophenylhydrazone, a yellow compound. The standard curve was prepared by using different bovine serum albumin concentrations (0.5-1.5 mg mL^{-1}) and the slope was used to express the levels of PC as nmol mg $protein^{-1}$ (Reznick and Packer, 1994).

2.7 Assay of enzymatic antioxidants

Total superoxide dismutase (SOD) activity, was determined based on the inhibition rate of autocatalytic adrenochrome generation at 480 nm in a reaction medium containing 1 mM epinephrine and 50 mM glycine/NaOH (pH 10.2). One SOD unit was defined as the amount of enzyme required for 50% inhibition of the adrenochrome formation and the enzyme activity was expressed as USOD mg protein^{-1} (Misra and Fridovich, 1972).

Catalase (CAT) activity was evaluated by following the decrease in the 240 nm absorption of H_2O_2 and was reported as ρmol min^{-1} mg protein^{-1} (Boveris and Chance, 1973).

Glutathione S-transferase (GST) activity was assayed based on the conjugation reaction with reduced glutathione (GSH), using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate (Habig et al., 1974). Sample aliquots (0.05 mL) were added to 0.6 mL of the assay mixture containing 100 mmol L^{-1} phosphate buffer (pH 6.5), GSH and CDNB at a final concentration of 1 mmol L^{-1} each. GST activity was calculated from the changes in absorbance at 340 nm and was expressed as ρmol min^{-1} mg protein^{-1}.

Glutathione peroxidase (GPx) activity was measured by following NADPH oxidation at 340 nm, as described by Flohé and Gunzler (1984), and results were expressed as nmol min^{-1} mg protein^{-1}.

2.8 Assay of non-enzymatic antioxidants

For the measure of ascorbic acid (AA) content, the standard curve was prepared by using different concentrations of AA, and the slope was used to express the amount of ascorbic acid as μmol mg protein^{-1} (Roe and Kuether, 1942).

The non-protein thiols (NPSH) content, an indirect measure of GSH, was evaluated at 412 nm after reacting with 5,5'-dithiobis (2-nitrobenzoic acid) and reported as nmol mg protein^{-1} (Ellman, 1959).

2.9 Statistical analysis

Statistical analysis was performed using the software Statistica® 7.0. Levene's test was used to verify whether the data were parametric. Two-way analyses of variance followed by Tukey test were performed to assess the differences among the groups and times. Results are

expressed as mean ± standard error (SEM). The minimum significance level was set at 95% ($p<0.05$).

3. Results

3.1 Quercetin prevents OTC-induced oxidative damage in brain, kidney and liver

OTC induced an increase on TBARS and PC levels in brain after 14 days of treatment, and an increase on LOOH and PC levels after 21 days when compared to the control group ($p<0.05$). Co-treatment with quercetin was able to prevent this increase in both time-points. Within OTC group, LOOH levels were higher after 21 days of treatment when comparing to 14 days (Table 1).

In kidney, OTC induced an increase on PC levels in comparison to the control group after 14 days of treatment ($p<0.05$). After 21 days, we observed an increase on LOOH levels caused by OTC treatment ($p<0.05$). Quercetin was able to prevent those effects, and decreased the levels of LOOH in relation to the control group after 21 days of treatment. In contrast, TBARS levels did not differ among the experimental groups (Table 2).

After 14 days of treatment, OTC induced an increase on LOOH levels in liver, when compared to their control counter-parts ($p<0.05$). On the other hand, after 21 days of OTC treatment, we detected an increase on TBARS and PC levels ($p<0.05$). Co-treatment with quercetin was able to prevent the effects of OTC in both time-points (Table 3).

3.2 Quercetin improves antioxidant potential in brain, kidney and liver of OTC-treated fish

In brain, treatment with OTC induced a decrease on SOD activity in both time-points when comparing to the control group ($p<0.05$). This decrease was prevented by quercetin. After 21 days of treatment, OTC had no effect on AA content, but induced a depletion on NPSH content ($p<0.05$). Quercetin increased AA content in relation to the control group and was able to prevent the depletion of NPSH induced by OTC ($p<0.05$). Moreover, within the quercetin group, AA content was increased after 21 days in relation to 14 days (Figure 1). In contrast, CAT, GPx and GST activities did not differ among the experimental groups (data not shown).

In Kidney, OTC induced a decrease on the activity of the antioxidant enzymes CAT and GPx, and on the content of AA and NPSH after 21 days of treatment ($p<0.05$). Co-treatment with quercetin was able to prevent all those effects, restoring CAT and GPx activities, as well

as AA and NPSH content. Moreover, within OTC group, CAT and GPx activities were higher after 21 days of treatment when comparing to 14 days (Figure 2). In contrast, GST activity did not differ among the experimental groups (data not shown).

The treatment with OTC induced a decrease on hepatic CAT activity in both time-points when comparing to the control group ($p<0.05$). After 21 days of treatment, OTC also caused a decrease on SOD activity and AA content ($p<0.05$). Co-treatment with quercetin did not restore AA content in liver but was able to recover the impairment on CAT and SOD activity induced by OTC (Figure 3). In contrast, GPx and GST activities, and NPSH levels did not differ among the experimental groups (data not shown).

4. Discussion

In this study, we show that quercetin efficiently prevents OTC-mediated oxidative stress in silver catfish. Those findings are of special relevance when consider that OTC is a commonly used antibiotic in aquaculture.

Lipid peroxidation and protein carbonylation are the major culprits behind the loss of cell function observed under oxidative stress conditions, and, for that reason, are the main markers to be measured when evaluating the degree of ROS-mediated toxicity in aquatic animals (Storey, 1996). Considering that the typical reaction during ROS-induced damage involves the peroxidation of unsaturated fatty acids (Li et al., 2011), our results clearly showed that administration of OTC led to oxidative damage, as demonstrated by the increased in lipid peroxidation and protein carbonylation observed in brain, kidney, and liver of silver catfish. The brain is particularly vulnerable to oxidative damage due to its high oxygen consumption and polyunsaturated fatty acids content. In our study, the levels of oxidative damage caused by OTC can potentially cause neurotoxicity in fish, as shown by others in similar fish species (Mieiro et al., 2011). Moreover, it was also shown that OTC-induced nephrotoxicity in fish is associated with an increase in oxidative stress in the kidney (Pari and Gnanasoundari, 2006). Lipid peroxidation was described as the main cause of loss in the cellular membrane bilayer fluidity and consequent liver damage (Ribeiro et al., 2005). The increase in lipid peroxidation and protein damage found in the present study suggests that ROS-induced oxidative damage can be one of the main toxic effects of OTC in fish.

It has been shown that the most common OTC concentrations used in standard aquaculture procedures can significantly modify the antioxidant response in fish (Tapia-Paniagua et al., 2015; Rodrigues et al., 2017a; Rodrigues et al., 2017b; Pês et al., 2018). One of

the mechanisms by which OTC causes cellular oxidative damage is believed to be through the inhibition of mitochondrial β-oxidation. This process would disrupt the respiratory chain, causing superoxide production, which in its turn generates more ROS and nitrogen species (Pari and Gnanasoundari, 2006; Yonar et al., 2011). If these compounds are not effectively removed from the cell, they may react with subcellular structures, resulting in cell death and tissue damage (Wang et al., 2013; Rodrigues et al., 2017b).

Recently, quercetin has attracted attention as a protector agent against the oxidative damage induced by the overuse of OTC, suggesting that dietary supplementation with quercetin has a large potential to be employed in aquaculture to enhance the survival and production of several fish species (Pês et al., 2016; 2018). In the present study, quercetin presented a protective action by normalizing oxidative parameters in fish exposed to OTC.

Biological systems have several mechanisms to counteract the ROS-damage. Antioxidant enzymes such as SOD, CAT, GST, GPx and non-enzymatic antioxidants such as AA and NPSH are the most important antioxidant defences in biological systems (Menezes et al., 2016). Our data clearly demonstrates that quercetin restored SOD activity and NPSH levels in the brain; CAT and GPx activity, AA and NPSH content in kidney; and SOD and CAT activity in liver of fish exposed to OTC, reinforcing the protective role of quercetin against oxidative damage induced by this antibiotic.

The established mechanism of quercetin is free radical scavenging, it is able to protect against *in vivo* oxidation of lipids, proteins, and DNA; and anti-inflammatory action (D´Andrea et al., 2015; Rishitha et al., 2018). However, beyond the antioxidant role of quercetin is also playing a key role in multiple cellular and molecular actions such as regulation of neurotransmitter and hormone modulation (Rishitha et al., 2018).

The present data show that quercetin can stimulate the production of antioxidant compounds in cells, eliminate free radicals, and exert satisfactory protective effects on the oxidative system.

5. Conclusion

The administration of OTC to *Rhamdia quelen* induced lipid peroxidation, protein oxidation, decrease on enzymatic antioxidants activity, and depletion of non-enzymatic antioxidants. The concomitant administration of OTC and quercetin showed that this flavonoid induces a regulating effect of oxidative stress by its antioxidant action. However, further studies should be performed to evaluate the exact mechanism of quercetin protection against drug

toxicity and the benefits of joint administration of antioxidants and antibiotics for industrial aquaculture purposes.

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Legends

Figure 1. Effect of oxytetracycline (OTC) and quercetin on enzymatic and non-enzymatic antioxidants in brain of *Rhamdia quelen*. SOD (superoxide dismutase), AA (ascorbic acid), NPSH (non-protein thiols). Different letters indicate statistical difference between treatments at the same time point. Asterisk (*) indicates significant difference when comparing the same treatment in different time points. All the values are expressed as mean±SEM (n=10).

Figure 2. Effect of oxytetracycline (OTC) and quercetin on enzymatic and non-enzymatic antioxidants in kidney of *Rhamdia quelen*. CAT (catalase), GPx (glutathione peroxidase), AA (ascorbic acid), NPSH (non-protein thiols). Different letters indicate statistical difference between treatments at the same time point. Asterisk (*) indicates significant difference when comparing the same treatment in different time points. All the values are expressed as mean±SEM (n=10).

Figure 3. Effect of oxytetracycline (OTC) and quercetin on enzymatic and non-enzymatic antioxidants in liver of *Rhamdia quelen*. SOD (superoxide dismutase), CAT (catalase), AA (ascorbic acid). Different letters indicate statistical difference between treatments at the same time point. Asterisk. All the values are expressed as mean±SEM (n=10).

Table 1. Effect of oxytetracycline (OTC) and quercetin on biomarkers of oxidative damage in brain of *Rhamdia quelen*.

Parameter	Experimental group	Days of treatment	
		14	21
Protein	Control	12.15 ± 0.34	11.80 ± 0.31
	OTC	10.85 ± 0.30	11.31 ± 0.30
	Quercetin	12.10 ± 0.41	11.65 ± 0.32
	OTC + Quercetin	12.13 ± 0.25	11.60 ± 0.38
LOOH	Control	2.47 ± 0.53	3.17 ± 0.80^{a}
	OTC	2.30 ± 0.38	$5.50 \pm 0.63^{b*}$
	Quercetin	1.90 ± 0.20	2.70 ± 0.24^{a}
	OTC + Quercetin	4.48 ± 0.75	2.48 ± 0.45^{a}
TBARS	Control	1.61 ± 0.15^{a}	1.94 ± 0.21
	OTC	2.63 ± 0.18^{b}	1.85 ± 0.26
	Quercetin	1.94 ± 0.20^{a}	1.76 ± 0.15
	OTC + Quercetin	1.98 ± 0.15^{a}	0.98 ± 0.10
PC	Control	3.15 ± 0.33^{a}	3.31 ± 0.35^{a}
	OTC	6.63 ± 0.21^{b}	6.47 ± 0.48^{b}
	Quercetin	2.91 ± 0.37^{a}	3.51 ± 0.58^{a}
	OTC + Quercetin	3.44 ± 0.25^{a}	2.91 ± 0.64^{a}

Protein: ($mg.mL^{-1}$); LOOH: lipid hydroperoxides ($nmol.mg\ protein^{-1}$); TBARS: thiobarbituric acid reactive substances ($nmol.mg\ protein^{-1}$) and PC: protein carbonyl ($nmol.mg\ protein^{-1}$). Different letters indicate statistical difference between treatments at the same time point. Asterisk (*) indicates significant difference when comparing the same treatment in different time points. All the values are expressed as mean ± SEM (n = 10).

Table 2. Effect of oxytetracycline (OTC) and quercetin on biomarkers of oxidative damage in kidney of *Rhamdia quelen*.

Parameter	Experimental group	Days of treatment	
		14	21
Protein	Control	16.15 ± 0.64	16.81 ± 0.68
	OTC	15.51 ± 0.37	14.97 ± 0.55
	Quercetin	15.17 ± 0.29	15.06 ± 0.58
	OTC + Quercetin	15.47 ± 0.46	14.47 ± 0.40
LOOH	Control	1.97 ± 0.20	1.47 ± 0.10[a]
	OTC	2.06 ± 0.21	2.13 ± 0.40[b]
	Quercetin	1.92 ± 0.15	0.67 ± 0.05[c]
	OTC + Quercetin	1.41 ± 0.06	1.56 ± 0.18[a]
TBARS	Control	1.03 ± 0.02	0.88 ± 0.02
	OTC	1.04 ± 0.02	0.94 ± 0.02
	Quercetin	1.04 ± 0.02	0.92 ± 0.01
	OTC + Quercetin	0.91 ± 0.02	0.94 ± 0.02
PC	Control	0.42 ± 0.03[a]	0.39 ± 0.01
	OTC	0.64 ± 0.07[b]	0.47 ± 0.01
	Quercetin	0.40 ± 0.03[a]	0.32 ± 0.02
	OTC + Quercetin	0.39 ± 0.04[a]	0.34 ± 0.01

Protein: ($mg.mL^{-1}$); LOOH: lipid hydroperoxides ($nmol.mg\ protein^{-1}$); TBARS: thiobarbituric acid reactive substances ($nmol.mg\ protein^{-1}$) and PC: protein carbonyl ($nmol.mg\ protein^{-1}$). Different letters indicate statistical difference between treatments at the same time point. All the values are expressed as mean ± SEM (n = 10).

Table 3. Effect of oxytetracycline (OTC) and quercetin on biomarkers of oxidative damage in liver of *Rhamdia quelen*.

Parameter	Experimental group	Days of treatment	
		14	21
Protein	Control	10.94 ± 0.19	10.20 ± 0.51
	OTC	10.17 ± 0.29	11.76 ± 0.48
	Quercetin	10.49 ± 0.49	11.77 ± 0.48
	OTC + Quercetin	10.35 ± 0.41	11.71 ± 0.45
LOOH	Control	4.13 ± 0.95[a]	5.14 ± 0.99
	OTC	8.03 ± 0.82[b]	3.05 ± 0.56
	Quercetin	2.75 ± 1.15[a]	3.67 ± 0.98
	OTC + Quercetin	3.39 ± 0.77[a]	1.60 ± 0.40
TBARS	Control	0.66 ± 0.14	1.04 ± 0.19[a]
	OTC	0.67 ± 0.14	1.69 ± 0.14[b]
	Quercetin	0.61 ± 0.19	0.63 ± 0.19[a]
	OTC + Quercetin	0.76 ± 0.11	0.76 ± 0.10[a]
PC	Control	2.07 ± 0.26	1.56 ± 0.22[a]
	OTC	2.03 ± 0.42	3.23 ± 0.18[b]
	Quercetin	1.86 ± 0.13	1.57 ± 0.30[a]
	OTC + Quercetin	1.22 ± 0.12	1.86 ± 0.18[a]

Protein: ($mg.mL^{-1}$); LOOH: lipid hydroperoxides ($nmol.mg\ protein^{-1}$); TBARS: thiobarbituric acid reactive substances ($nmol.mg\ protein^{-1}$) and PC: protein carbonyl ($nmol.mg\ protein^{-1}$). Different letters indicate statistical difference between treatments at the same time point. All the values are expressed as mean ± SEM (n = 10).

Figure 1.

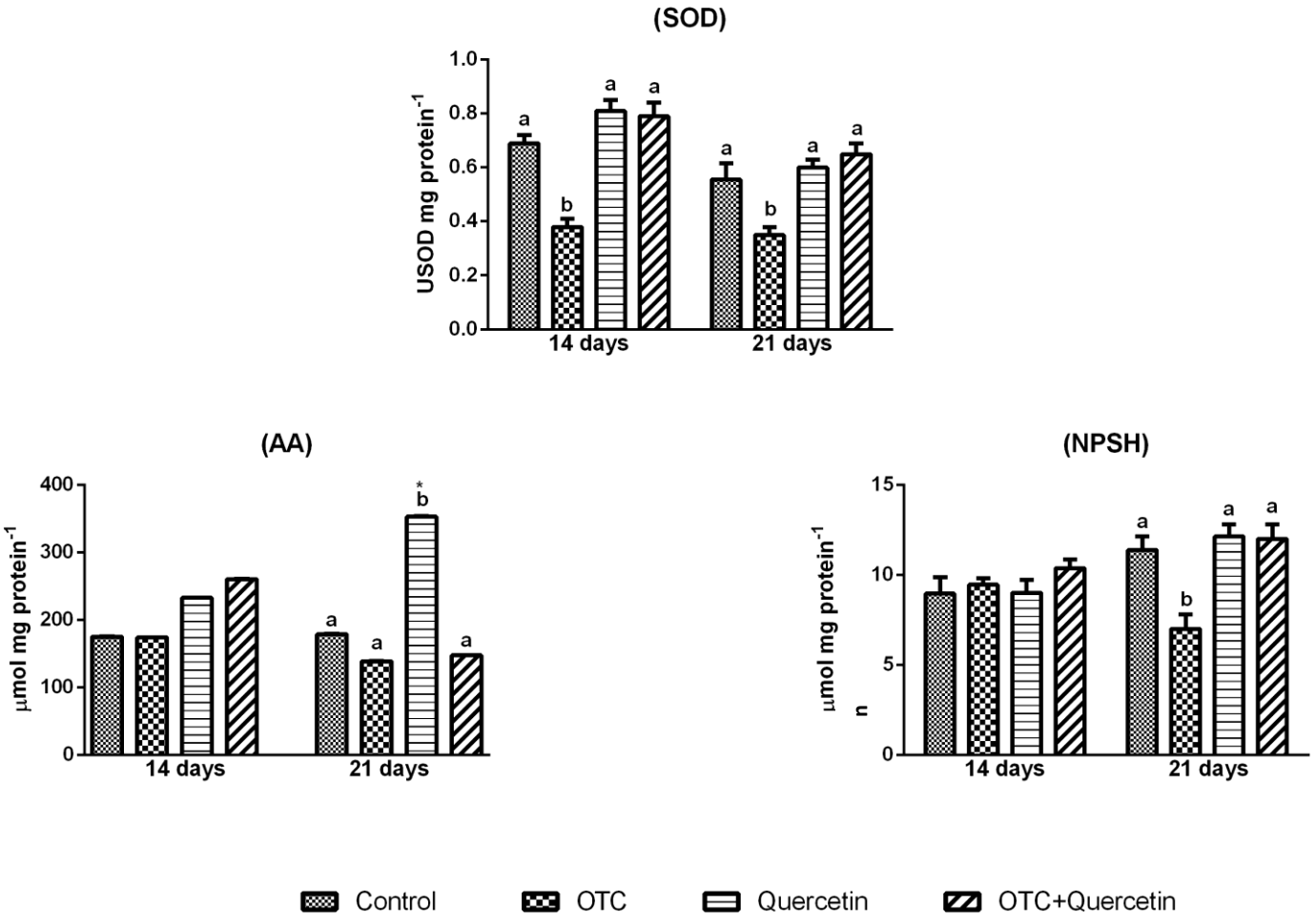

(SOD)
USOD mg protein-1
1.0
0.8
0.6
0.4
0.2
0.0
a
b
a
a
a
b
a
a
14 days
21 days
(AA)
µmol mg protein-1
400
300
200
100
0
a
a
b*
a
14 days
21 days
(NPSH)
µmol mg protein-1
15
10
5
0
a
b
a
a
14 days
21 days
Control
OTC
Quercetin
OTC+Quercetin

Figure 2.

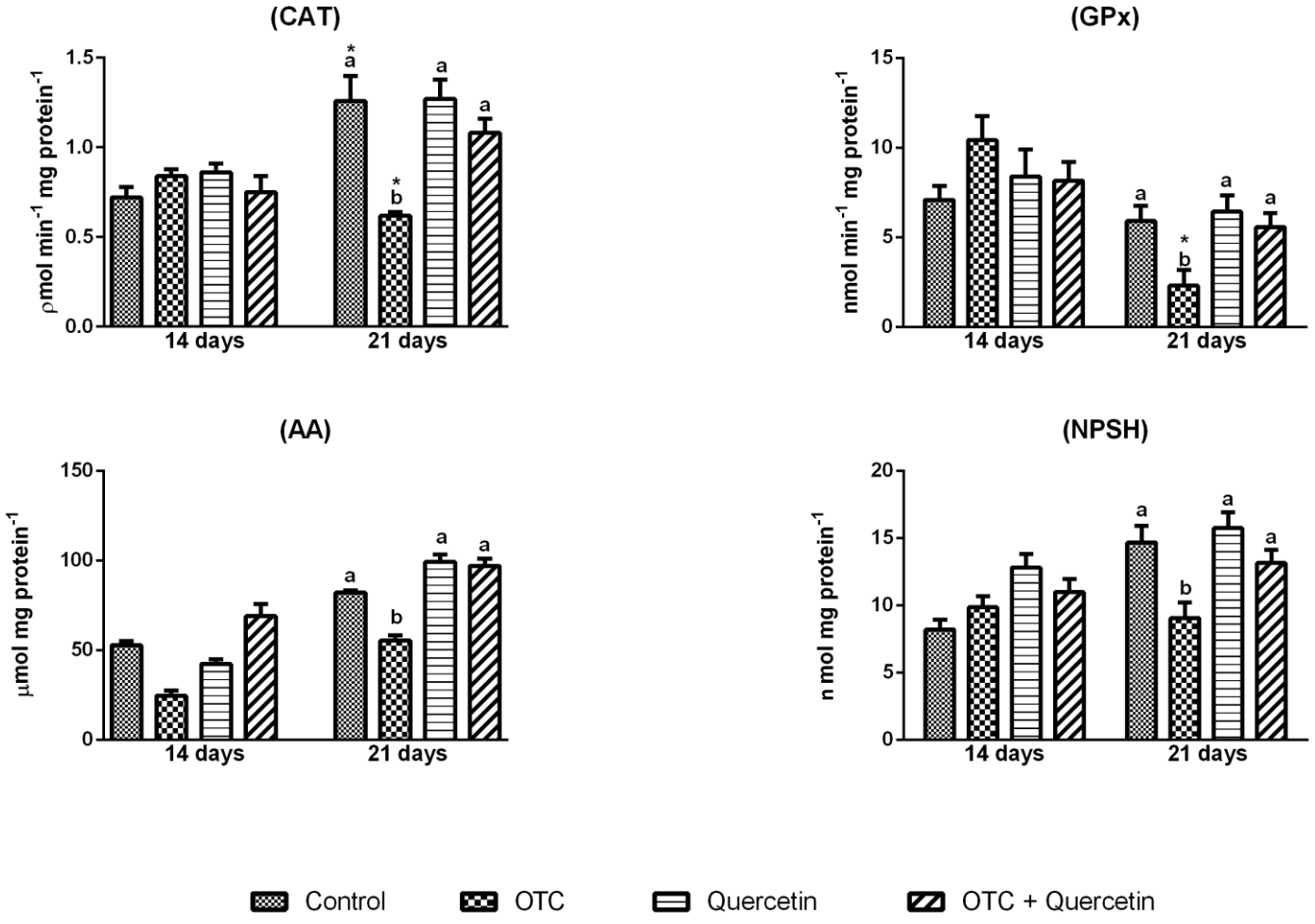
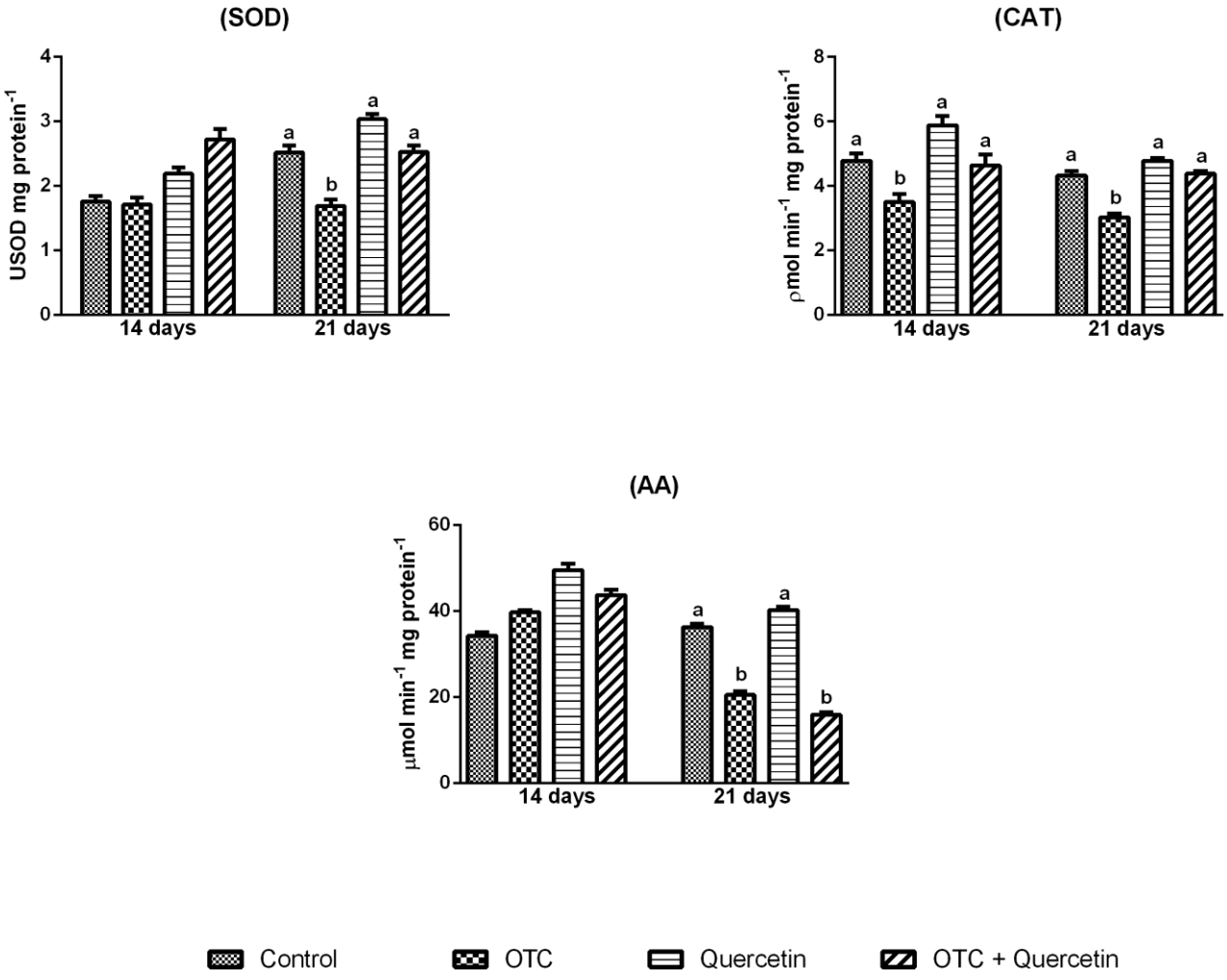

(CAT)
ρmol min-1 mg protein-1
1.5
1.0
0.5
0.0
14 days
21 days
(GPx)
nmol min-1 mg protein-1
15
10
5
0
14 days
21 days
(AA)
µmol mg protein-1
150
100
50
0
14 days
21 days
(NPSH)
n mol mg protein-1
20
15
10
5
0
14 days
21 days
Control
OTC
Quercetin
OTC + Quercetin

Figure 3.


(SOD)
USOD mg protein-1
0
1
2
3
4
a
b
a
a
14 days
21 days
(CAT)
ρmol min-1 mg protein-1
0
2
4
6
8
a
b
a
a
a
b
a
a
14 days
21 days
(AA)
μmol min-1 mg protein-1
0
20
40
60
a
b
a
b
14 days
21 days
Control
OTC
Quercetin
OTC + Quercetin

3.4 MANUSCRITO 2

Quercetin attenuates endocrine and metabolic responses to oxytetracycline in silver catfish (*Rhamdia quelen*)

Manuscrito a ser submetido ao periódico **Aquaculture.**

Quercetin attenuates endocrine and metabolic responses to oxytetracycline in silver catfish (*Rhamdia quelen*)

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Abstract

Antibiotics, in particular oxytetracycline (OTC), have been therapeutically and prophylactically employed in aquaculture. However, the excessive use of OTC may result in the development of side effects in fish such as alteration on immune mechanisms, DNA damage, and oxidative stress. The incorporation of flavonoid compounds, such as quercetin, to the diet might be a natural alternative to minimize the physiological alterations caused by OTC. Thus, this study aimed to verify whether dietary quercetin protects against the detrimental effects induced by OTC administration in silver catfish (*Rhamdia quelen*) assessing mRNA expression of brain (*corticotropin-releasing hormone* (*crh*), and two different *proopiomelanocortins* (*pomca* and *pomcb*) and pituitary (*growth hormone* (*gh*), *somatolactin*, and *prolactin* (*prl*) hormones related to stress axis, plasma cortisol and metabolites levels (glucose, and lactate), as well metabolites values (glycogen, lactate, and triglycerides), metabolic enzymatic activities (hexokinase, phosphorylase (active GPase), fructose-biphosphatase (FBP), glycerol-3-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase, and glutamate dehydrogenase (GDH) and gill Na^+/K^+-ATPase activity. Fish were divided into four experimental groups and received the following experimental diets for 14 or 21 days: (i)-control; (ii)-OTC; (iii)-quercetin; or (iv)-OTC+quercetin. Plasma, liver, gills, brain, and pituitary samples were collected at the end of each experiment. Results demonstrated that OTC activates the response to stress in silver catfish, since it increased plasma cortisol and decreased glucose levels at 14 and 21 days. In addition, also altered metabolic status of fish since it enhanced hepatic triglyceride levels as well as hepatic FBP and GDH activities at 14 days. Inclusion of OTC also stimulated gill Na^+/K^+-ATPase activity at 14 days. Finally, changed the hypophyseal expression of *gh* (at 14 and 21 days) and *prl* (at 14 days). The co-treatment with quercetin was able to prevent most of the alterations induced by OTC, which strongly suggests that quercetin is a beneficial compound in diets for fish.

Keywords: Antibiotic, Stress, Gene Expression, Metabolism, Flavonoids.

1. Introduction

Infectious diseases are a large preoccupation for fish farming because they represent potential risks in production and inventory losses (Grenni et al., 2018). Different chemicals such as antiseptics, antibiotics, agrochemicals, and parasiticides are frequently used to prevent or to treat diseases in many countries (Elia et al. 2014). Oxytetracycline (OTC) has been widely used as a feed additive for the treatment of systemic bacterial infections in farmed fish (Ambili et al., 2013).

OTC is an antibiotic that belongs to the tetracycline group and is commonly used in aquaculture because of its broad-spectrum efficacy in the treatment of infections caused by gram-positive and gram-negative bacteria, mycoplasma and large viruses. It inhibits protein synthesis by preventing the association of aminoacyl-tRNA with bacterial ribosomes (Nakano et al., 2015). In infectious diseases, OTC is usually administered as medicament in the feed pellets at a rate of 50 to 100 mg of OTC per kg of body weight per day for 3-21 days depending on the infection (Treves-Brown, 2000; Yonar, 2012). There is no specific legislation for using veterinary drugs in Brazilian aquaculture, which leads to their misuse (Gaskins et al., 2002).

Despite the beneficial effect as antibiotic, excessive use of OTC may result in the development of side effects for fish. According to previous studies, OTC led to liver and gill damage (Elia et al., 2014; Rodrigues et al., 2017), alteration on immune mechanisms and regulation of immune competent genes (Guardiola et al., 2012), DNA damage (Rodrigues et al., 2016) and oxidative stress, increasing the levels of lipid and protein oxidation, decreasing the activity of antioxidant enzymes and levels of non-enzymatic antioxidants in silver catfish (*Rhamdia quelen*) (Pês et al., 2018) and rainbow trout (*Oncorhynchus mykiss*) (Yonar et al., 2011; Yonar, 2012).

Stress situations for fish can negatively interfere with biochemical and physiological processes (Schreck and Tort, 2016). Fish neuroendocrine stress pathways rely on the adrenergic system and the hypothalamus-pituitary-interrenal (HPI) axis, whose stimulation culminates in the release of catecholamines and corticosteroids into the bloodstream (Wendelaar Bonga, 1997; Pankhurst, 2011). In the HPI cascade, corticotropin-releasing hormone (Crh) is synthesized in the hypothalamus and acts on pituitary corticotropic cells to stimulate the synthesis of proopiomelanocortin B (Pomc B), resulting in adrenocorticotrophic hormone (Acth) release, which, in turn, will stimulate the production and release of cortisol in interrenal cells (Flik et al., 2006; Bernier et al., 2009). Besides its role in the stress response, cortisol is

involved in several processes in fish physiology, enhances metabolic rate, glycogenolysis, lipolysis, and is associated with degradation of muscle proteins (Mommsen et al., 1999; Laiz-Carrión et al., 2009). In the course of studies on fish fitness in response to stress, stress regulates the expression of genes, such as growth hormone (*gh*), somatolactin (*sl*), and prolactin (*prl*) (Gorissen and Flik, 2016).

Considering that OTC may induce metabolic changes and oxidative damage in fish, the search for potential protective molecules against OTC-damage is of great importance. In this way, several products, including quercetin has been assessed (Yonar et al., 2011; Yonar, 2012; Pês et al., 2018). Quercetin is a flavonoid with polyphenolic chemical substructure. In a biological system, it acts as antioxidant and is known to protect the tissue from free radicals (Strugala el al., 2017). In nature, it exists primarily as quercetin glycoside and consists of quercetin aglycone conjugated to sugar moieties such as glucose or rutinose (Guo and Bruno, 2015). Our previous studies demonstrated that quercetin presents important antioxidant effects in the silver catfish (*Rhamdia quelen*) (Pês et al., 2016) and provides a protective effect against the oxidative stress induced by OTC, acting as a powerful modulator of the enzymatic and non-enzymatic antioxidants (Pês et al., 2018). However, the capacity of this flavonoid to abrogate the OTC-mediated effects on endocrine and metabolic responses was not assessed yet in this species.

Thus, this study aimed to verify whether dietary quercetin protects against the detrimental effects induced by OTC treatment in silver catfish on stress system and metabolic status of *R. quelen* individuals. The results will be interesting in order to develop a strategy to avoid the negative influence of OTC administration on cultured specimens.

2. Material and Methods

2.1 Chemicals

Quercetin ($C_{15}H_{10}O_7$) was obtained from Opção Fênix Petrochemicals Distributor Ltd. (São Paulo, SP, Brazil). Commercial form of OTC (Terramycin®) used in the experiments was obtained from Pfizer (New York, NY, United States America).

2.2. Species and acclimation period

Male and female silver catfish (*R. quelen*) (weight, 190.0 ± 25.5 g; length, 26.6 ± 1.1 cm) were obtained from a fish culture sector at the Federal University of Santa Maria (UFSM). Fish were acclimated in 250 L tanks (stocking densidad: 12 kg m^{-3}) for two weeks (October/2015). They were maintained in continuously aerated water with a natural photoperiod (12 h light/12 h dark). Water quality parameters as well as: temperature, dissolved oxygen, hardness, non-ionized ammonia, nitrite and alkalinity were checked daily and were described in our previous study (Pês et al., 2018). During acclimation, fish were fed once a day with control diet (based on 3 % of the biomass of each tank). Feces and pellet residues were removed by syphoning to keep the water quality. The experimental protocol was approved by the Ethics and Animal Welfare of the Federal University of Santa Maria (protocol nº 4380290115) and was performed according to the standards proposed by the same.

2.3. Diets preparation and experimental design

Four isoenergetic diets were formulated based on the study of Pês et al. (2016). The diet consisted of 30 % soybean meal, 35 % meat and bone meal, 12 % rice bran, 15 % corn, 3 % canola oil, 1 % salt, 3 % vitamins and minerals (premix) and 1 % phosphate dicalcium. The ingredients used to prepare the diets were weighed and then mixed until complete homogenization. After mixing, the diets were moistened, pelleted in a meat grinder and taken to a forced air circulation oven for drying (45 ºC) for 24 hours.

The experimental design included four groups (in triplicate): (i)-control (diet with neither OTC nor quercetin); (ii)-OTC (0.1 g OTC kg $diet^{-1}$); (iii)-quercetin (1.5 g quercetin kg $diet^{-1}$); or (iv)-OTC+quercetin (0.1 g OTC kg $diet^{-1}$ + 1.5 g quercetin kg $diet^{-1}$). The amount of feed provided to the animals was fixed in 3 % of the biomass of each tank once a day (9 a.m.) for each experimental period, 14 and 21 days. The quercetin dosage was chosen based on our previous study (Pês et al., 2016) while the OTC dosage was based on previous studies (Treves-Brown, 2000, Yonar et al., 2011, Yonar, 2012; Pês et al., 2018). Fish were food deprived for 24 h prior to sampling.

At end of each experimental period (14 and 21 days), a blood sample was collected from the caudal peduncle using heparinized sterile syringes. The blood was centrifuged in heparinized vials at 1,200 ×g (Cientec Centrifuge, model CT-15000 R) for 5 min for plasma separation, which was stored at -80 ºC until biochemical analysis. After sampling blood, fish were immediately euthanized by sectioning the spinal cord. Then brains and pituitaries were

collected and placed in tubes with a 1/10 relation w/v of RNAlater™ stabilization solution (Ambion®) for 24 h at 4 ºC and then stored at -20 ºC. Biopsies from liver and gills were taken, frozen in liquid nitrogen and stored at -80 ºC for further analysis.

2.4. Plasma parameters

Plasma cortisol levels were measured by indirect enzyme immunoassay adapted to microplate as described previously by Baldisserotto et al. (2014) for this fish species. The standards and plasma samples were run in duplicate. The standard curve was run from 2.50 ng mL^{-1} to 9.77 pg mL^{-1} ($r^2 = 0.991$). The lower limit of detection (90.80 % of binding, ED 90.80) was 19.55 pg mL^{-1}. The percentage of recovery was 95%. The inter- and intra-assay coefficients of variation (calculated from the duplicate samples) were 2.23 ± 0.24% and 2.88 ± 0.34%, respectively. Cross-reactivity information for specific antibodies with intermediate products involved in steroid synthesis was provided by the supplier [cortexolone (1.6%), 11-deoxycorticosterone (0.23%), 17-hydroxyprogesterone (0.23%), cortisol glucuronide (0.15%), corticosterone (0.14%), cortisone (0.13%), androstenedione (<0.01%), 17-hydroxypregnenolone (<0.01%) and testosterone (<0.01%)].

Glucose and lactate levels were measured using commercial kits from Spinreact (glucose-HK, ref. 1001200; lactate, ref. 10013300; Barcelona, Spain) adapted to microplate. Total proteins were determined using the bicinchoninic acid method with a Pierce BCA protein assay kit (ref. 23225, Thermo Scientific, Rockford, USA) using bovine serum albumin as standard. Spectrophotometric analysis were performed with a PowerWave™ 340 microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA) controlled by KCjunior™ program.

2.5. Hepatic metabolite levels

An aliquot of liver was homogenized using an Ultra-Turrax T25 basic (IKA-Werke) with a 7.5 volume of ice-cooled 0.6 N perchloric acid, neutralized (using 1 M potassium bicarbonate), and centrifuged (30 min at 3,220 g, 4 ºC in an Eppendorf Centrifuge 5810R). The supernatants were stored in different aliquots at -80 °C until use in the different metabolite assays. Tissue glycogen concentration was assessed using the method of Keppler and Decker (1974). Lactate (ref. 10013300) and triglycerides (ref. 10013110) levels were determined using

commercial kits from Spinreact (Sant Esteve d´en Bas, Girona, Spain) adapted to a 96-well microplate.

2.6. Hepatic enzyme activities

An aliquot of liver was homogenized by ultrasonic disruption (Misonix Inc., Microson Ultrasonic liquid processor XL-2000) with 10 volume of ice-cold stopping-buffer containing 50 mM imidazole (Sigma I- 0125) (pH 7.5), 1 mM mercaptoethanol (Sigma M-3148), 50 mM NaF (Merck ref. 1.06449), 4 mM EDTA (Sigma ED2SS), 0.5 mM PMSF (Sigma P-7626) and 250 mM sucrose (Sigma S-9378). The homogenate was centrifuged at 10,000 g for 30 min at 4ºC (Centrifuge 5810R, Eppendorf), and the supernatant was immediately frozen using dry ice and maintained at -80 ºC until enzyme assays.

Enzyme reaction rates were determined by the increase or decrease in absorbance of NAD(P)H at 340 nm. The reactions were started by addition of homogenates (15 μL) in duplicate, at a pre-established protein concentration, omitting the substrate in control wells (final volume of 275-295 μL, depending on the enzyme tested), and allowing the reactions to proceed at 37 ºC. Specific conditions for the enzymes hexokinase (HK, EC 2.7.1.11), glycogen phosphorylase (active GPase, EC 2.4.1.1), fructose-biphosphatase (FBP, EC 3.1.3.11), glycerol-3-phosphate dehydrogenase (G3PDH, EC 1.1.1.8), glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), and glutamate dehydrogenase (GDH, EC 1.4.1.2) were previously described by Sangiao-Alvarellos et al. (2005) and Polakoff et al. (2006). Enzymatic analyses were performed in conditions meeting the requirements for optimal velocities for *R. quelen* (data not shown). Enzyme activities were determined using a spectrophotometer and expressed as U mg protein^{-1}. Protein levels were assayed in triplicate as described in the plasma samples.

2.7. Gill Na^+/K^+-ATPase assay

Na^+/K^+-ATPase activity was measured in gills using a microplate technique developed by McCormick (1993). Gill tissue was homogenized in 125 μL of SEI buffer with 0.1% deoxycholic acid, and then centrifuged at 2,000g for 30 s. Duplicate 10 μL homogenate samples were added to 200 μL assay mixture with and without 0.5mM ouabain in 96-well microplates at 25 ºC and read at 340 nm for 10 min with intermittent mixing. Ouabain-sensitive ATPase activity was detected by enzymatic coupling of ATP dephosphorylation to NADH oxidation

and expressed as μmol ADP mg protein^{-1} h^{-1}. The protein content was determined using the bicinchoninic acid method with a Pierce BCA protein assay kit (ref. 23225, Thermo Scientific, Rockford, USA) using bovine serum albumin as standard. Spectrophotometric analysis were performed with a PowerWave™ 340 microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA) controlled by KCjunior™ program.

2.8. Gene expression of hormones affected by stress

Total RNA was extracted from the pituitary gland and brain using NucleoSpin®RNA XS and RNA II kits (Macherey-Nagel), respectively, together with on-column RNase-free DNase digestion, according to the manufacturer's instructions. RNA concentrations were spectrophotometrically measured at 260 nm with a BioPhotometer Plus (Eppendorf), and its quality was assessed using the Agilent RNA 6000 Nano Assay Kits on an Agilent 2100 Bioanalyzer (Agilent Technologies). Only samples with an RNA Integrity Number (RIN) higher than 8.5 were used for real time PCR.

Firstly, 250 and 500 ng of total RNA form pituitary and brain samples, respectively, were used to synthesize the first strand of cDNA by reverse transcription reaction using a qSCRIPT™ cDNA Synthesis Kit (Quanta BioSciences). In summary, the reaction was performed using qScript Reaction Mix (1× final concentration) and qScript Reverse Transcriptase (2.5× final concentration). The reverse transcription program consisted in 5 min at 22 ºC, 30 min at 42º and 5 min at 85 ºC.

The optimization of qPCR conditions was made on primers annealing temperature (50 to 60 ºC), primers concentration (200, 400, and 600 nM) and template concentration (six different 1:10 dilution series from 10 ng to 100 fg of input RNA). Moreover, two negative controls, with i) 10 ng of RNA per reaction (NRTC, non-reverse transcription control) and ii) sterile water (NTC, non-template control), were performed to detect possible gDNA contamination or primer-dimer artefacts. The resulting curves had their r^2 and amplification efficiencies (E) of 0.992 and 0.94 for *crh* (acc. no. KP795943), 0.999 and 0.92 for *pomca* (acc. no. KP795944), and 0.995 and 0.95 for *pomcb* (acc. no. KP795945), respectively, as previously described by Saccol et al. (2018). Primers for *gh* (r^2: 0.999; E: 0.96; acc. no. EF101341), *prl* (r^2: 0.999; E: 0.97; acc. no. KC195971) and *sl* (r^2: 0.998; E: 0.99; acc. no. KC195972) were used as previously described by Baldisserotto et al. (2014). The nucleotide sequences and concentration of the specific primers used for qPCR are shown in Table 1. To perform qPCR reactions, 4 μL cDNA (10 ng assumed from RNA input), 0.5 μL of specific forward and reverse primers for

each gene at the specified concentrations, and 5 μL PerfeCta™ SYBR® Green Fastmix™ (Quanta BioSciences) were used in twin-tec real-time PCR plates (Eppendorf). qPCR was performed on a Mastercycler®ep*gradient* S Realplex^2 with Realplex software (version 2.2; Eppendorf), and the thermal profile was as follows: 95 ºC for 10 min; [denaturing at 95 ºC for 15 s, annealing and extension at 60 ºC for 45 s] x 40 cycles; melting curve from 60 to 95 ºC for 20 min. The melting curve was used to ensure that a single product was amplified and to check the absence of primer-dimer artefacts. The results were normalized to *β-actin* (r^2: 0.997; E: 0.97; *actb*, acc. no. KC195970) given its abundance and low C_T values variability (less than 0.25 C_T) among tissues and treatments, in our experimental conditions. Relative gene quantification was performed using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001).

2.9. Statistical analysis

Data were presented as the mean ± standard error of the mean (SEM). Levene's test was used to attest whether the data were parametric. Two-way analysis of variance followed by Tukey's post-hoc test was done to evaluate the differences between the groups. The statistical analyses were performed using the software Statistica 7.0 (StatSoft, Inc., Tulsa, Oklahoma, USA) and GraphPad Prism 6.01 (GraphPad Software, Inc.). Differences were considered significant at $p < 0.05$.

3. Results

Plasma cortisol levels, after 14 and 21 days of treatment, increased in OTC group respect to control, while this enhancement were not observed in OTC+quercetin group (Table 2). Plasma glucose values were lower in OTC and OTC+quercetin groups in comparison to control after 14 days of treatment. However, plasma lactate and protein levels were not affected by any dietary treatments (data not shown).

Hepatic triglyceride levels enhanced in OTC group after 14 days of treatment, while OTC+quercetin group presented similar values to control group. These changes were not observed after 21 days (Figure 1). However hepatic lactate and glycogen values were not affected by OTC and/or quercetin administration at any time assessed (data not shown).

Different hepatic enzyme activities related to carbohydrate metabolism (GPactive, G3PDH, G6PDH, and HK) were assessed, but its expression levels were not affected by any dietary treatments (data not shown). Only FPB activity (related to carbohydrate metabolism)

and GDH activity (related to amino acid metabolism) showed changes. Both values enhanced in OTC group at day 14, while inclusion of quercetin in diet avoid the observed increases. However, these changes were not observed after 21 days of dietary treatment (Table 3).

After 14 days of treatment, OTC group increased gill Na^+/K^+-ATPase activity in comparison to control group. However, OTC+quercetin group presented similar values to the control group (Figure 2).

After 14 days of treatment, OTC group presented lower *gh* mRNA expression values than the control group. The OTC+quercetin group presented *gh* mRNA expression similar to the control group. After 21 days of treatment, *gh* expression was lower in OTC group compared to control group. The OTC+quercetin group presented *gh* mRNA expression similar to the control group. Furthermore, the expression of *gh* within the control group increased after 21 days in comparison to 14 days of treatment (Figure 3).

After 14 days of treatment, *prl* mRNA expression values increased in OTC group in comparison to control group. The OTC+quercetin group presented *prl* mRNA expression similar to the control group. Furthermore, *prl* expression of in OTC group after 21 days was lower than after 14 days of treatment (Figure 3).

Finally, no statistical differences were observed among experimental groups for hypothalamic *crh* expression, as well as pituitary *sl*, *pomca* and *pomcb* mRNA expression values (data not shown).

4. Discussion

Quercetin was already been shown to protect silver catfish from the oxidative damage induced by the excessive use of OTC, suggesting that dietary supplementation with quercetin might have economic and beneficial effects to enhance the aquaculture potential of fish species (Pês et al., 2016; 2018). However, until the present study, quercetin capacity to prevent OTC-mediated endocrine and metabolic alterations on fish has not been determined so far. Our results revealed a strongly positive influence of dietary inclusion of quercetin on the physiological biomarkers of silver catfish treated with OTC. Interestingly, more parameters were altered and better responses were observed with quercetin administration on short-term stress induction (14 days of OTC treatment), indicating that there were adaptive responses of fish to long-term of treatment (21 days of OTC treatment).

4.1 Cortisol-Stress System

In the current study, OTC administration lead to increased plasma cortisol levels and quercetin was able to avoid this increase in the group that received diet with OTC+quercetin at both times of treatment. Results with OTC indicated as stimulation of stress axis in the group treated with OTC; however, our results not showed an enhancement of hypothalamic *crh* and adenohypophyseal *pomc b* to important players of this axis.

In general, in the response to stress, ACTH release increases with circulating cortisol concentrations. Thus, an elevation in the ACTH level may be an indicator of an elevated expression of *pomc* mRNA. However, *pomc* gene expression is positively regulated by *crh*. In turn, the effects of cortisol negative feedback on *crh* gene generation are mediated by glucocorticoid receptors, reducing significantly the stress-induced activation of the HPI axis (Palermo et al., 2008, Bernier et al., 2009). Therefore, the suppression of the response in the expression of *crh* and *pomc* mRNA found in our study indicates that a control mechanism for the regulation of these hormones was activated and may be an adaptive response of fish to stress conditions.

In addition, another hypothesis is that OTC may be causing disrupting the complex neurohormonal brain-pituitary-internal organ systems. It is known that OTC inhibits the association of aminoacyl-tRNA and bacterial ribosomes, thus preventing protein synthesis (Reemtsma and Jekel, 2006). In the fish, a study demonstrated that the environmental concentrations of 425 ng L^{-1} and 8000 ng L^{-1} of florfenicol and OTC, respectively, cause damage to the DNA molecule of *Oreochromis niloticus* (Botelho et al., 2015). According to these authors, OTC genotoxicity observed may be explained by the binding of OTC to DNA. Indeed, according to Khan and Musarrat (2003) at a biochemical level, OTC, and other tetracyclines, present a strong affinity to DNA resulting in the formation of TC-DNA binary complex causing alterations in the secondary structure of the native DNA-duplex.

Although the toxic effects of OTC on aquatic organisms have been studied in detail (Botelho et al., 2015, Tapia-Paniagua et al., 2015; Leal et al., 2016; Rodrigues et al., 2016; 2017; Pês et al., 2018), there is limited information on endocrine disruption and exact mechanism underlying the toxicity of OTC to fish. The study of Ji et al. (2010) showed that sulfathiazole, OTC, and chlortetracycline could affect several points of steroid genic pathways at transcriptional level, and influence also enzyme activity and eventually the balance of sex hormones both in human adenocarcinoma (H295R) cells and in male medaka fish (*Oryzias latipes*).

Prl is a pleiotropic hormone involved in various physiological processes such as osmoregulation, stress, and metabolism (Wendelaar Bonga, 1997; Sangiao-Alvarellos et al., 2005; Baldisserotto et al., 2014). This hormone is able to increased plasma cortisol levels in certain teleost species reinforcing the idea that *prl* is involved in their stress pathways (Wendelaar Bonga, 1997). After 14 days of treatment, OTC administration lead to an increase on *prl* expression in silver catfish, while the administration of quercetin avoided this increase in the group that received diet with OTC+quercetin.

OTC administration lead to decreased *gh* levels and the administration of quercetin avoided this decrease in the group that received diet with OTC+quercetin, after 14 and 21 days of treatment. The decrease in *gh* expression caused by OTC, together with the increased levels of plasma cortisol confirm the inhibitory effect of cortisol, at least via the inhibition of *gh* expression. Interestingly in this study, after both time of treatment with OTC, were found lower levels of *gh* compared to control and indicates that fish growth may be compromised to face an adverse situation, once energy is required to restore homeostasis and quercetin was able to return the levels of *gh* close to control (Toni et al., 2015).

Gh has a growth-promoting action in teleost fish known to be mediated by IGF-I (Björnsson, 1997) whose mRNA levels are known to be increased by *gh* treatment in several tissues of rainbow trout (Biga et al., 2004). This hormone has many possible non-somatotropic effects including osmoregulatory, metabolic, insulinotropic, and steroidogenic (Bern and Madsen, 1992). Moreover, *gh* stimulates lipolysis (O'Connor et al., 1993), increases protein synthesis and turnover (Herbert et al., 2001), reduces glycogen synthetase activity, decreased hepatic glycogen levels and induces hyperglycemia in fish species (Leung et al., 1991).

OTC activates stress responses, as cortisol levels rise. Thus, the energy metabolism and other physiological parameters were altered, as was the case of *gh* expression was diminished, using the energy previously spent for growth, now being used to maintain the essential metabolic needs that help the body respond to the stressful situation.

Studies have reported that *R. quelen* under different stressful situations affected *gh* expression in different ways: i) enhancing by food deprivation (Menezes et al., 2015); ii) decreasing when submitted to high ammonia levels (Baldisserotto et al., 2014); or iii) without changes after transport (Saccol et al., 2018). According to Saccol et al. (2018), this response indicates that the role of *gh* in stress response could be stressor-specific for this species, or even depending on the timing and duration in which the stressor is applied.

However, our results did not show changes in the expression of *sl* in any of the groups, which suggests that this hormone did not have an important role (if any) in the stress processes

under these conditions of silver catfish, at least attending on the activation of the molecular pathways to produce higher (or lower) amounts of this protein.

4.2 Metabolism

Any form of disturbance in fish culture units usually results in increased production of cortisol due to mobilization of energy for osmoregulatory purposes and increased glucose levels intermediated by catecholamines that stimulate glycogenolysis in the liver (Wendelaar Bonga, 1997; Mommsen et al., 1999; Laiz-Carrión et al., 2009). This cortisol increase could explain the pattern of changes in glucose observed with an increase of this fuel-metabolite at the first stage of the induced stress and the subsequent decrease in glucose values during the experiment in the OTC-treated group (Baldisserotto et al., 2014). Glucose is a good indicator of physiological disorders resulting from different types of stressors and may be the main source energy used by fish in unfavorable conditions (Brandão et al., 2004). OTC administration led to decreased plasma glucose values in both times of treatment, indicating a decrease in the metabolic capacity of fish, which could not be restored by quercetin. In addition, OTC administration led to increased hepatic triglyceride levels in fish after 14 days of treatment. This suggests that OTC affects the metabolic pathways related to lipid metabolism and the mobilization of triglycerides to cope with increased energy demands imposed by stress (Navarro and Gutiérrez, 1995). In this situation, quercetin was able to return the levels of hepatic triglycerides close to the control.

Additionally, OTC administration led to increased activities of FBP and GDH, which demonstrates that the intermediary metabolism was affected. The administration of quercetin avoided this increase in the group that received diet with OTC+quercetin for 14 days of treatment. The increase of GDH may be related to the removal of excess nitrogen nutrients due to an intense metabolism, aimed at anabolic routes in which new metabolic stores were incorporated, thus leading to the production of numerous nitrogen ammonia residues or a *de novo* synthesis of amino acids using the leftovers of ammonia, thereby wasting energy in this process (Menezes et al., 2015). Assessment of those enzyme activities involved in the metabolism of carbohydrates, lipids, and proteins can give clues on how the animal rearranges its energy status after experiencing a stressful event. The activities of key metabolic enzymes can indicate the metabolic status and its ability to modify their activities against changes in environmental and dietary conditions. Therefore, quercetin reduced energy demand increased by OTC, allowing the organism to rearrange its homeostatic levels.

Gill Na^+/K^+-ATPase contributes to the maintenance of internal homeostasis and participates in important processes including osmoregulation, acid-base regulation and excretion, reasons why this enzyme is considered an indicator of the physiological status of teleost species (Metz et al., 2003). After 14 days of treatment, gill Na^+/K^+-ATPase activity was increased in OTC group. This increased possibly due to a compensation mechanism took place to recover the inhibited enzymes and altered ion balance and quercetin was able to restore its normal activity in the group that received diet with OTC+quercetin. In other organisms, OTC is also known to inhibit many other cellular processes including ATPases which require Na^+/K^+, Mg^{2+} and Ca^{2+} ions for their activity (Kulac et al., 2013; Ramzy, 2014; Rodrigues et al., 2017). Due to the formation of stable complexes between tetracyclines with metal ions that are present as enzyme cofactors, the normal functioning of ATPases in gills may be compromised, consequently threatening ionic homeostasis, enzymatic activity and integrity of the cell membrane. The defense responses will take place at the expense of the respiratory efficiency of the gills and eventually results in the respiratory impairment (Varadarajan et al., 2014; Rodrigues et al., 2017).

5. Conclusion

In this study we conclude that: (i) OTC actives the stress system, indicated by high plasma cortisol, low glucose levels, decreased *gh* expression, and increased *prl* expression; (ii) OTC affects the metabolic pathways related to lipid, carbohydrate, and amino acid metabolism; (iii) OTC increases Na^+/K^+-ATPase activity, decreasing metabolic resource to cope with ion regulation; (iv) quercetin is able to prevent a stress response caused by OTC mostly, once again proving to be a beneficial compound for fish diet.

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Legends

Figure 1. Hepatic triglycerides levels in *R. quelen* specimens fed different diets for 14 and 21 days. Different letters indicate statistical differences between treatments at the same day. All the values are expressed as mean ± SEM (n = 10). Statistical analysis was performed by Two-way ANOVA and Tukey test ($p < 0.05$).

Figure 2. Gill Na^+/K^+-ATPase activity in *R. quelen* specimens fed different diets for 14 and 21 days. Further details as in legend Fig.1.

Figure 3. Pituitary expression of *gh* and *prl* in *R. quelen* specimens fed different diets for 14 and 21 days. (*) indicate significant differences from the same treatment at 14 days. Further details as in legend Fig.1.

Figure 1.

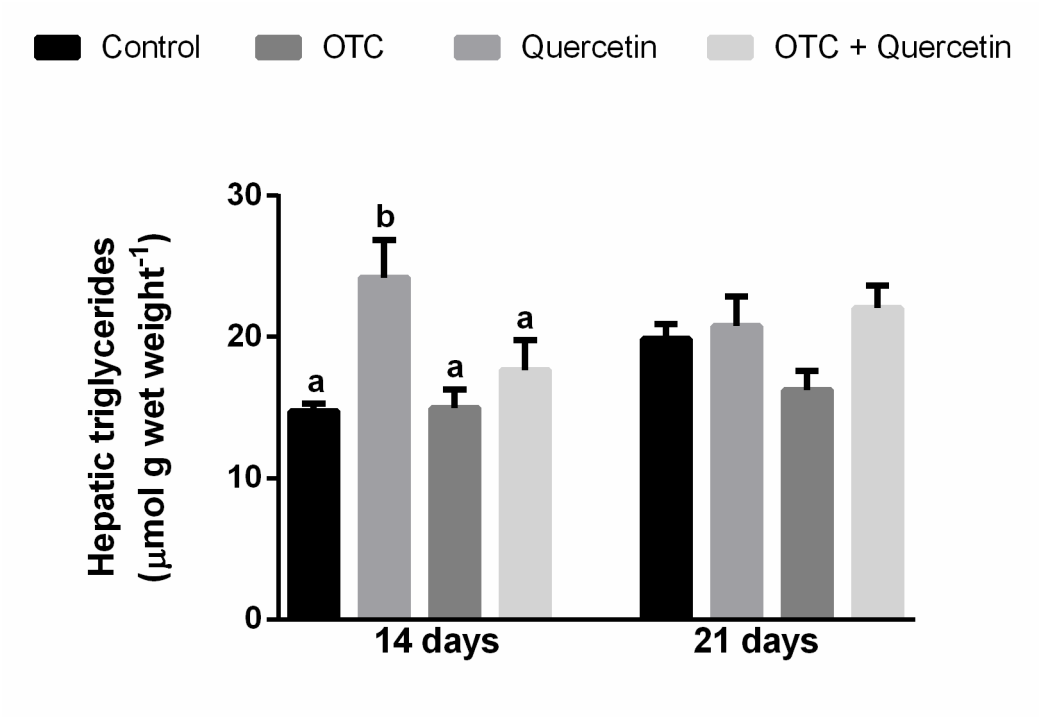
Control
OTC
Quercetin
OTC + Quercetin
Hepatic triglycerides (μmol g wet weight-1)
30
20
10
0
a
b
a
a
14 days
21 days

Figure 2.

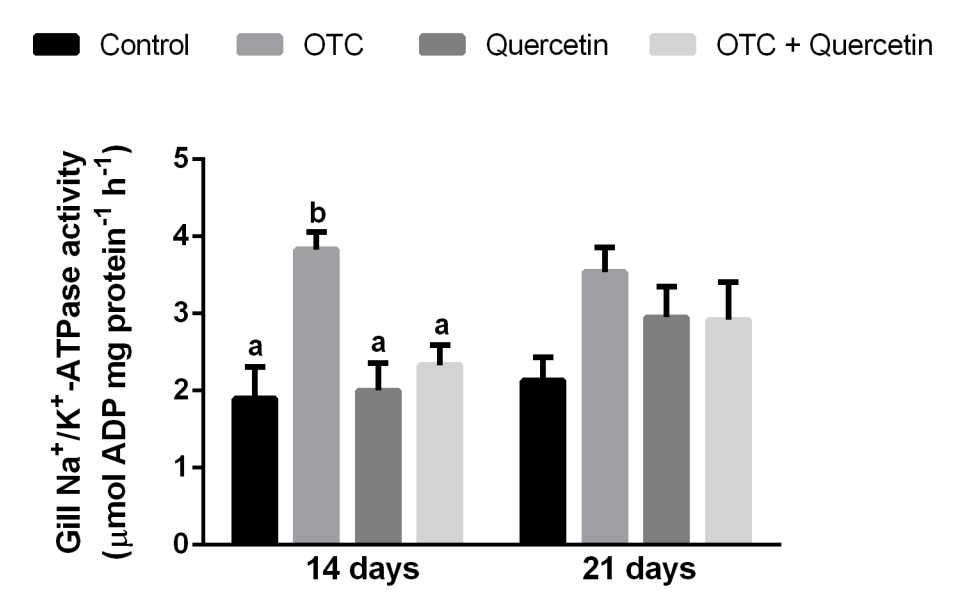
Control
OTC
Quercetin
OTC + Quercetin
Gill Na+/K+-ATPase activity (μmol ADP mg protein-1 h-1)
0
1
2
3
4
5
a
b
a
a
14 days
21 days

Figure 3.

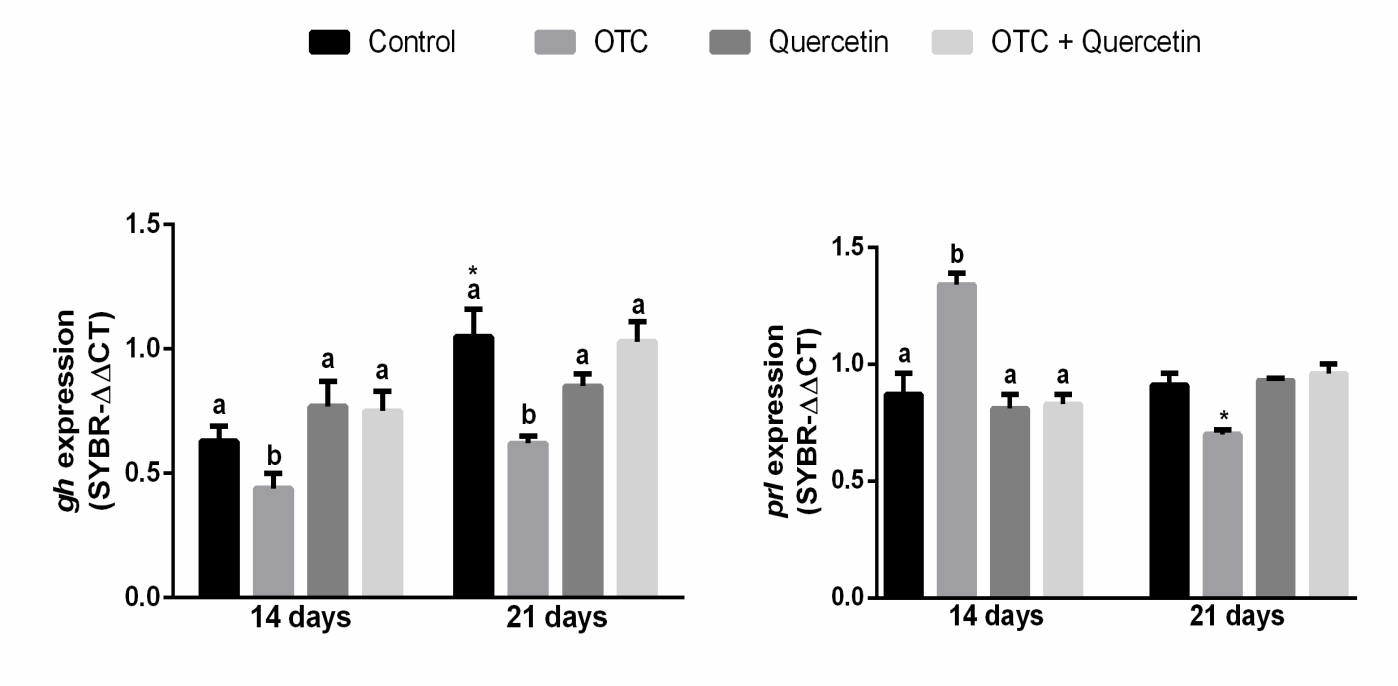
Control
OTC
Quercetin
OTC + Quercetin
gh expression (SYBR-ΔΔCT)
1.5
1.0
0.5
0.0
a
b
a
a
*
a
b
a
a
14 days
21 days
prl expression (SYBR-ΔΔCT)
1.5
1.0
0.5
0.0
a
b
a
a
*
14 days
21 days

Table 1. Sequences and concentrations of the primers for *R. quelen* used in qPCR, and the sizes of the amplicons.

Gene	Primer	Nucleotide sequence	Concentration	Amplicon size
crh	Fw	5'-ATCAGCGCGAACGTGAGTCTG-3'	400 nM	122 bp
	Rv	5'-CTCGGCTACCTCGTCCTCTT-3'		
prl	Fw	5'-CCTGTCTCTGGTTCGCTCTCT-3'	200 nM	127 bp
	Rv	5'-GTCCTGCAGCTCTCTGGTCTT-3'		
sl	Fw	5'-TCCAGCACGCTGAGCTGATCT-3'	200 nM	111 bp
	Rv	5'-AAGAGTTTCCCCCATGACCTT-3'		
gh	Fw	5'-GGACAAACCACCCTAGACGAG-3'	200 nM	116 bp
	Rv	5'-TTCTTGAAGCAGGACAGCAGA-3'		
pomca	Fw	5'-ATGAAGCTCCAGAGTCCGTTC-3'	400 nM	133 bp
	Rv	5'-GATTCTTCCTCCACTCCGTTG-3'		
pomcb	Fw	5'-AGTCCACACCACCTTCTCCAT-3'	400 nM	137 bp
	Rv	5'-TGCTCTTGGCATCTGTGTTCT-3'		
actb	Fw	5'-ATTGGCAATGAGAGGTTCAGG-3'	200 nM	122 bp
	Rv	5'-CGGATATCGACGTCACACTTC-3'		

Table 2. Plasma cortisol and glucose levels in *R. quelen* fed different diets for 14 and 21 days.

Indices	Experimental groups	Days of treatment	
		14	21
Cortisol ($ng\ mL^{-1}$)	Control	36.90 ± 2.08^{a}	44.73 ± 1.26^{a}
	OTC	58.72 ± 2.00^{b}	66.60 ± 3.40^{b}
	Quercetin	35.21 ± 3.04^{a}	$50.42 \pm 4.90^{a*}$
	OTC + Quercetin	43.54 ± 2.35^{a}	48.14 ± 2.62^{a}
Glucose (mM)	Control	4.01 ± 0.44^{a}	4.38 ± 0.72
	OTC	1.85 ± 0.21^{b}	2.47 ± 0.41
	Quercetin	2.91 ± 0.59^{ab}	3.53 ± 0.38
	OTC + Quercetin	1.42 ± 0.15^{b}	2.33 ± 0.44

Different letters indicate statistical differences between treatments at the same day. (*) indicate significant differences from the same treatment at 14 days. All the values are expressed as mean ± SEM (n = 10). Two-way ANOVA and Tukey test ($p < 0.05$).

Table 3. Enzymatic activities (U mg^{-1} protein) in the liver of *R. quelen* fed different diets for 14 and 21 days.

Enzymatic activities	Experimental groups	Days of treatment	
		14	21
Carbohydrate metabolism			
FBP	Control	482.60 ± 51.90[a]	565.61 ± 32.72
	OTC	680.64 ± 18.59[b]	593.60 ± 15.50
	Quercetin	551.67 ± 62.63[ab]	498.61 ± 58.33
	OTC + Quercetin	506.10 ± 37.60[a]	559.58 ± 20.10
Amino acid metabolism			
GDH	Control	4.34 ± 0.17[a]	4.19 ± 0.28
	OTC	5.64 ± 0.19[b]	4.15 ± 0.30
	Quercetin	4.30 ± 0.33[a]	4.94 ± 0.29
	OTC + Quercetin	4.24 ± 0.18[a]	4.47 ± 0.12

FBP: fructose-biphosphatase; GDH: glutamate dehydrogenase. Different letters indicate statistical differences between treatments at the same day. All the values are expressed as mean ± SEM (n = 10). Two-way ANOVA and Tukey test ($p < 0.05$).

4 DISCUSSÃO GERAL

Práticas de aquicultura a nível mundial têm aumentado rapidamente devido à alta demanda para alimentos nutritivos e sua importância econômica (RAMESH et al., 2018). No entanto, a poluição e doenças transmissíveis pela água são um problema grave para a aquicultura (SANAWAR et al., 2017). Antibióticos, como a OTC, desempenham um papel importante no crescimento dos peixes e na prevenção de doenças infecciosas. Entretanto, esses compostos farmacêuticos podem persistir no ambiente e aumentar a resistência bacteriana, que afeta a microflora e a fauna, levam a imunossupressão e atraso no crescimento dos peixes (RAMESH et al., 2018). O uso indiscriminado de antibióticos, como a OTC, resulta em poluição ambiental e risco de toxicidade para organismos não alvos. Dessa forma, torna-se importante encontrar alternativas no sentido de minimizar os efeitos tóxicos desses compostos em ecossistemas aquáticos, incluindo os peixes. Antioxidantes como a quercetina, podem ter funções protetoras contra os danos causados pelos antibióticos.

No **artigo 1** podemos afirmar que a suplementação da dieta com quercetina é benéfica para a saúde dos peixes, uma vez que não altera os parâmetros sanguíneos e expressão de hormônios pituitários; impede a LPO e aumenta o potencial antioxidante nos tecidos avaliados (encéfalo, brânquias, fígado, rim e músculo). A partir desses resultados, no **artigo 2** e **manuscrito 1** podemos avaliar o potencial protetor da quercetina frente a toxicidade oxidativa causada pela OTC. A administração oral de OTC aumentou a LPO e carbonilação de proteínas, bem como diminuiu as defesas antioxidantes no encéfalo, fígado, rim e músculo dos peixes. Assim, verificamos que a suplementação da dieta dos peixes com a quercetina foi efetiva em proteger contra o estresse oxidativo em diferentes tecidos de jundiás tratados com OTC. Além disso, no **artigo 2** analisamos a presença de resíduos de OTC no músculo dos peixes, os quais foram inferiores ao limite de quantificação, dessa forma, não foi encontrado excesso de OTC nos peixes que receberam a dieta com o antibiótico e podemos inferir que a quercetina foi capaz de impedir o acúmulo de OTC no músculo, nos peixes que receberam OTC + quercetina.

No **manuscrito 2** demonstrou-se também o efeito protetor da quercetina contra os distúrbios metabólicos induzidos pela OTC em jundiás. Os resultados mostraram que a OTC ativa a resposta ao estresse nos jundiás, uma vez que aumenta os níveis do cortisol plasmático e diminui os níveis de glicose. Além disso, aumenta os níveis de triglicérideos hepáticos, bem como as atividades hepáticas da FBP e GDH e estimula a atividade da Na^+/K^+-ATPase. Do

mesmo modo, altera a expressão hipofisária de GH e PRL. Assim, sugere-se um papel direto da quercetina na redução à resposta ao estresse.

Estudos anteriores também demonstraram efeitos benéficos da suplementação da dieta de peixes com quercetina. No estudo de Park et al. (2009), é demonstrado que a quercetina é rapidamente absorvida e é depositada em forma de aglicona, atuando como um poderoso antioxidante em peixes. Além disso, a quercetina atua como imunuestimulante, que foi demonstrado pelo aumento na atividade de lisozima, proteína total, atividade antiprotease e bactericida frente à bactéria *A. hydrophila* em truta-arco-íris (AWAD et al., 2013). Em um estudo recente com peixes da espécie *Channa punctata*, a quercetina foi capaz de atenuar os danos causados por pesticidas a nível macromolecular e antioxidante (BHATTACHARJEE; DAS, 2017).

O presente estudo demonstrou maiores modificações nos biomarcadores de estresse oxidativo e defesas antioxidantes após o maior tempo de tratamento com OTC (21 dias). De acordo com os resultados, as respostas antioxidantes induzidas pela OTC podem ser divididas em duas fases: estágio adaptativo (14 dias) e estágio inibitório (21 dias). No primeiro caso, os níveis de LPO e carbonilação de proteínas geralmente permaneceram em valores controle, e os antioxidantes diminuiram ligeiramente, indicando que o sistema antioxidante celular pode eliminar a produção de EROs e manter equilíbrio na maioria das vezes. No caso do tratamento prolongado com OTC (21 dias), a produção de EROs sobrecarregou o balanço das defesas antioxidantes, resultando na acumulação de substâncias oxidativas e inibição do sistema antioxidante, que no qual pode ser restaurado através da ação protetora da quercetina.

No entanto, maiores alterações metabólicas foram observadas após o menor período de tratamento com OTC (14 dias), que no qual ocorreu aumento da demanda de energia imposta pela situação de estresse. Após 21 dias de tratamento podemos inferir que, na maioria das vezes, os parâmetros analisados voltaram a homeostase, não havendo alterações metabólicas nos peixes.

A análise dos 4 trabalhos aqui descritos permite um melhor entendimento sobre os efeitos tóxicos causados pela OTC, bem como a importância dos efeitos da quercetina para diminuir os danos induzidos por essa substância em peixes. Os resultados demonstraram que a administração oral de OTC causa estresse oxidativo, bem como alterações metabólicas em jundiás. A quercetina protegeu os diferentes tecidos contra a toxicidade induzida pela OTC diminuindo a oxidação lipídica e proteica, aumentando as propriedades antioxidantes. Considerando-se que a utilização de antibióticos é cada vez mais frequente e que é a causa de alterações e danos aos peixes, os resultados deste trabalho são de grande importância, uma vez

que a quercetina pode representar uma alternativa para atenuar e/ou minimizar a toxicidade causada por antibióticos.

5 CONCLUSÃO

A partir dos resultados obtidos, pode-se concluir que a quercetina é um composto que possui atividade antioxidante em peixes. Isso pode ser observado primeiramente pelos seus efeitos *per se*, ou seja, a quercetina aumentou *per se* os níveis de antioxidantes enzimáticos e não enzimáticos, mantendo a homeostase dos peixes. Adicionalmente, protegeu contra o dano oxidativo e atenuou a diminuição do sistema de defesa antioxidante causada pela administração oral de OTC em jundiás.

Também podemos verificar que a quercetina foi capaz de evitar a diminuição da expressão do GH e aumento da PRL causada pela OTC, reduziu a síntese ou liberação de cortisol, diminuindo o metabolismo, oferecendo proteção contra uma formação excessiva de EROs induzida pela OTC. Do mesmo modo, a OTC afeta as vias metabólicas relacionadas ao metabolismo de lipídios, carboidratos e aminoácidos; e aumenta a atividade da Na^+/K^+-ATPase, diminuindo o recurso metabólico para lidar com a regulação iônica. Dessa forma, antioxidantes como a quercetina podem ser uma alternativa na suplementação da dieta de peixes para prevenir danos oxidativos e aumentar as defesas antioxidantes desses animais expostos a antibióticos.

Esses resultados corroboram com outros estudos já realizados, os quais demonstram que compostos antioxidantes, como a quercetina, são importantes ferramentas utilizadas como redutores de estresse, moduladores do sistema imunológico e agentes terapêuticos para uso na aquicultura. Destaca-se por ser um produto natural com baixo custo para o produtor, e que pode contribuir para o fortalecimento da produção de espécies nativas como o jundiá, a qual apresenta grande importância econômica na região Sul do Brasil.

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ANEXO A- CARTA DE APROVAÇÃO

Comissão de Ética no Uso de Animais
da
Universidade Federal de Santa Maria

Santa Maria, 15th April 2015

CERTIFIED

We certify that the Research "ANTIBIOTIC AND ANTIOXIDANTS ACTION IN TISSUE BIOMARKERS OF Rhamdia quelen", protocol number CEUA 4380290115, utilizing 600 Fishes (males and females), under the responsibility Maria Amália Pavanato, was approved in the meeting of day 04/10/2015, and agree with Ethical Principles in Animal Research adopted by Ethic Committee on Animal Use of Federal University of Santa Maria.

Certificamos que o Projeto intitulado "AÇÃO DE ANTIBIÓTICO E ANTIOXIDANTES EM BIOMARCADORES TECIDUAIS DE Rhamdia quelen", protocolado sob o CEUA nº 4380290115, utilizando 600 Peixes (machos e fêmeas), sob a responsabilidade de Maria Amália Pavanato, foi aprovado na reunião de 10/04/2015, e está de acordo com os princípios éticos de experimentação animal da Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria.

yours faithfully,

Vânia Lucia Loro
Coordinator of the Ethics Committe on Animal Use
Federal University of Santa Maria

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