UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS NATURAIS E EXATAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIODIVERSIDADE ANIMAL

Jeane de Lima Costa Gomes

RESPOSTAS BIOQUÍMICAS E HEMATOLÓGICAS DE JUNDIÁS (*Rhamdia quelen*) PRÉ-ALIMENTADOS COM VITAMINA C E EXPOSTOS A ATRAZINA

Santa Maria, RS
2016

Jeane de Lima Costa Gomes

RESPOSTAS BIOQUÍMICAS E HEMATOLÓGICAS DE JUNDIÁS (*Rhamdia quelen*) PRÉ-ALIMENTADOS COM VITAMINA C E EXPOSTOS A ATRAZINA

Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Biodiversidade Animal, Área de Concentração em Bioecologia da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do título de **Mestre em Biodiversidade Animal**

Aprovado em 30 de agosto de 2016:

Vania Lucia Loro

Vania Lucia Loro, Dr. (UFSM)
Presidente/Orientador

Leila Picolli da Silva

Leila Picolli da Silva Drª (UFSM)
Co-orientador

André Passaglia Schuch

André Passaglia Schuch, Dr. (UFSM)

Guendalina

Guendalina Turcato Oliveira, Dr. (PUCRS)

Santa Maria, RS
2016

Ficha catalográfica elaborada através do Programa de Geração Automática
da Biblioteca Central da UFSM, com os dados fornecidos pelo(a) autor(a).

Gomes, Jeane de Lima Costa
Respostas bioquímicas e hematológicas de jundiás (Rhamdia quelen) pré-alimentados com vitamina C e expostos a Atrazina / Jeane de Lima Costa Gomes.- 2016.
52 p.; 30 cm

Orientadora: Vania Lucia Loro
Coorientadora: Leila Picolli da Silva
Dissertação (mestrado) - Universidade Federal de Santa Maria, Centro de Ciências Naturais e Exatas, Programa de Pós-Graduação em Biodiversidade Animal, RS, 2016

1. Exposição a atrazina 2. Estresse oxidativo 3. Parâmetros hematológicos 4. Vitamina C I. Loro, Vania Lucia II. da Silva, Leila Picolli III. Título.

Ao meu esposo, Luis Carlos por estar ao meu lado em todos os momentos da minha vida, bons e ruins. Aos meus pais pelo incentivo e apoio em as minhas decisões.

AGRADECIMENTOS

Agradeço primeiramente a Deus, que faz com que minhas forças sejam infinitas.

Ao meu querido esposo Luis Carlos, sempre ao meu lado, de maneira amorosa e compreensiva. Devido ao seu apoio, paciência e amor este trabalho pôde ser concretizado. Te amo!

Aos meus pais Ana Lucia e Hélio, minha imensa gratidão. Obrigada por tudo! Hoje sou o que sou graças a vocês! Amo vocês!

Aos meus irmãos pelo carinho incentivo e apoio!

À minha orientadora Dra. Vania Loro, obrigada pela oportunidade e pela confiança depositada.

Agradeço as novas amizades feitas no Laboratório de Toxicologia Aquática (LABTAQ), obrigada a todos, cada um de alguma forma contribuiu para a concretização deste trabalho. Em especial à Aline Monique pela força e apoio essencial para o término deste trabalho.

A todos os professores do Programa de Pós-Graduação em Biodiversidade Animal, que de alguma forma contribuíram para minha formação, meu muito obrigada.

Agradeço aos meus amigos e familiares que sempre me incentivaram e me apoiaram nesta jornada.

Enfim a todos àqueles que fazem parte da minha vida e de alguma forma contribuem para que eu cresça e seja um ser humano melhor.

“Talvez não tenha conseguido fazer o melhor, mas lutei para que o melhor fosse feito. Não sou o que deveria ser, mas graças a Deus, não sou o que era antes”

(Marthin Luther King)

RESUMO

RESPOSTAS BIOQUÍMICAS E HEMATOLÓGICAS DE JUNDIÁS (*Rhamdia quelen*) PRÉ-ALIMENTADOS COM VITAMINA C E EXPOSTOS A ATRAZINA

AUTOR: Jeane de Lima Costa Gomes
ORIENTADOR: Vania Lucia Loro

O presente estudo foi realizado no laboratório de Biologia Adaptativa e Toxicologia Aquática, bem como no laboratório de Piscicultura, ambos pertencentes à Universidade Federal de Santa Maria (UFSM). O objetivo geral foi avaliar os efeitos antioxidantes da vitamina C sobre parâmetros bioquímicos e hematológicos de jundiás (*Rhamdia quelen*). O estudo foi dividido em dois experimentos (fases), sendo os resultados do primeiro experimento base para o segundo período experimental. A primeira fase constituiu-se na preparação da ração suplementada com vitamina C, em 3 diferentes concentrações: (1) 0, (2) 500 e (3) 1000 mg/kg. Durante a suplementação foram coletados 10 peixes por dieta, nos dias 0, 7, 14, 21 e 28, com o intuito de realizar uma curva de concentração a fim de conhecer a quantidade mínima de suplementação para que se obtenha os efeitos protetores da vitamina C. Em cada coleta foram amostrados: sangue, fígado e brânquias, para posteriores análises bioquímicas e hematológicas. Nossos resultados mostraram que a atividade da catalase (CAT), em fígado, aumentou apenas no grupo 1, não havendo alterações significativas nos outros grupos. Os níveis de peroxidação lipídica em fígado e brânquias foram reduzidos nos grupos 2 e 3, quando comparado ao grupo 1. A atividade da glutationa-peroxidase (GPx) no fígado aumentou tanto no grupo 2 quanto no grupo 3, quando comparado ao grupo 1. O nível de proteína carbonil, em brânquias, foi elevado apenas no grupo 1, quando comparado com os outros grupos. Em relação as atividade das enzimas glutationa-S-transferase (GST) e superóxido dismutase (SOD), bem como ao nível dos tióis- não proteicos (NPSH), não houve diferença significativa entre os grupos. A contagem total de eritrócitos (RBC), hemoglobina (Hb) e hematócrito (HCT) aumentou em todos os grupos. Não houve diferenças significativas em relação aos valores do volume celular médio (MCV) e da hemoglobina celular média (MCH). A partir dos resultados obtidos observou-se que as quantidades de 500 ou 1000 mg/kg de vitamina C foram suficientes para a melhoria da capacidade antioxidante de *Rhamdia quelen*, bem como foi estabelecido que 28 dias de alimentação seria um período mínimo para se obter os resultados desejados. Por meio destes pressupostos iniciamos a segunda fase do estudo, onde os animais foram alimentados por 30 dias com ração contendo 1000 mg/kg de vitamina C. Os animais foram divididos nos seguintes grupos: (1) controle - vitamina C 0 mg/kg, (2) controle - vitamina C 1000 mg/kg, (3) exposição à atrazina - vitamina C 0 mg/kg e (4) exposição à atrazina - vitamina C 1000 mg/kg. Após o período de alimentação estes animais foram submetidos à exposição de Atrazina, na concentração de 10µg/L, por um período de 96 horas. O objetivo desta etapa foi verificar se essa quantidade de vitamina C seria suficiente para a proteção dos peixes contra o estresse causado pela exposição ao herbicida. Nesta etapa escolhemos realizar as análises apenas em fígado, uma vez que a brânquia mostrou poucas alterações significativas na primeira fase do experimento. Os resultados desta fase mostraram que em todos os grupos não houve diferenças significativas na peroxidação lipídica. Entretanto, houve uma diminuição do conteúdo da

proteína carbonil no grupo 4, quando comparado com os outros grupos. Os níveis de NPSH aumentou apenas nos grupos suplementados com 1000 mg/kg de vitamina C (grupos 2 e 4). Não houve diferença significativa da atividade da enzima GST, em nenhum grupo. A atividade da GPx aumentou significativamente no grupo 4 em comparação com os outros grupos. A atividade da CAT diminuiu, tanto no grupo 3 quanto no grupo 4. Sendo assim, concluímos que a vitamina C mostrou ser capaz de melhorar a proteção do fígado quando os animais foram expostos a Atrazina. Logo, uma dieta suplementada com quantidades adequadas de vitamina C, pode proporcionar tanto uma boa nutrição para os peixes, bem como uma proteção contra fatores de estresse.

PALAVRAS-CHAVE: ácido ascórbico; estresse oxidativo; parâmetros hematológicos, peixe.

ABSTRACT

BIOCHEMICAL AND HEMATOLOGICAL RESPONSES ON JUNDIA (*Rhamdia quelen*) PRE-FED WITH VITAMIN C AND EXPOSED TO ATRAZINE

AUTHOR: Jeane de Lima Costa Gomes
ADVISOR: Vania Lucia Loro

This study was conducted at the laboratory of Biology and Adaptive Aquatic Toxicology, as well as at the Pisciculture Laboratory, both from the Federal University of Santa Maria (UFSM). The overall objective was to evaluate the antioxidant effects of vitamin C on biochemical and hematological parameters of jundiá *Rhamdia quelen*. The study was divided in two experiments (phase), and the results of the first experiment served as the basis for the second period. The first step consisted in preparing food supplemented with vitamin C in 3 different concentrations: (1) 0 (2) and 500 (3) 1000 mg / kg. During supplementation were collected 10 fish per diet on days 0, 7, 14, 21 and 28, for the purpose of performing a concentration curve to ascertain the minimum amount of supplementation it to obtain the protective effect of vitamin C. In each collection, blood, liver and gill samples were collected for subsequent biochemical and hematological analysis. Our results showed that the activity of catalase (CAT) in the liver, increased, only in group 1, with no significant changes in the other groups. The levels of lipid peroxidation in liver and gills were reduced in groups 2 and 3 compared to group 1. The activity of glutathione peroxidase (GPx) in liver increased both in group 2 and in group 3 compared to group 1. The protein carbonyl content in gills was higher in group 1 when compared to others groups. Regarding the activity of glutathione S-transferase enzymes (GST) and superoxide dismutase (SOD) and the level of non-protein thiols (NPSH), there was no significant difference between groups. The total erythrocyte count (RBC), hemoglobin (Hb) and hematocrit (HCT) increased in all groups. There were no significant differences in relation to the mean cell volume (MCV) and mean cell hemoglobin (MCH) values. From the results obtained it was observed that the amount of 500 or 1000 mg/kg vitamin C were sufficient to improve the antioxidant ability to *Rhamdia quelen* and it was established that 28 days of feeding would be a minimum to obtain desired results. Through these assumptions began the second phase of the study, where animals were fed for 30 days with feed containing 1000 mg/kg of vitamin C. The animals were divided into the following groups: (1) control Vitamin C 0 mg/kg (2) control vitamin C 1000 mg/kg, (3) exposure to atrazine vitamin C 0 mg/kg, (4) exposure to atrazine vitamin C 1000 mg/kg. After the feeding period the animals were subjected to exposure atrazine at a concentration of 10µg/L, for a period of 96 hours. The purpose of this step was to determine whether this amount of vitamin C would be sufficient for fish protection against stress caused by exposure to the herbicide. At this stage we choose to perform analysis only in the liver, since the gill showed few significant changes in the first phase of the experiment. The results of this phase showed that in all groups there was no significant difference in lipid peroxidation. However, there was a decrease in protein carbonyl content in group 4 when compared to the other groups. NPSH levels increased only in the groups supplemented with 1000 mg/kg vitamin C (groups 2 and 4). There was no significant difference in the GST activity among the groups. The GPx activity increased significantly in group 4 as compared to the other groups. The CAT activity decreased both in groups 3 and 4. Thus, we conclude that vitamin C improved

liver protection when the animals were exposed to atrazine. Therefore, a diet supplemented with adequate amounts of vitamin C, can provide both good nutrition for the fish as well as a protection against stress factors.

KEYWORDS: ascorbic acid, oxidative stress, hematological parameters, fish.

LISTA DE ILUSTRAÇÕES

Manuscrito 1:

Manuscrito 2:

LISTA DE TABELAS

Manuscrito 1:

Manuscrito 2:

SUMÁRIO

INTRODUÇÃO

Áreas agrícolas muitas vezes se encontram próximo a mananciais, rios e lagos que são utilizados para o abastecimento humano e manutenção de atividades econômicas, além de abrigarem uma diversidade de animais aquáticos. Os herbicidas que são utilizados nessas áreas alcançam os corpos de água, seja por meio de um escoamento superficial ou da lixiviação, causando danos diretos ao meio ambiente, aos seres vivos que nele se encontram e à saúde pública (Ramos et al., 2012; Zhu et al., 2016). Alaclor Nortox, Ametrina Agripec, Atrazina, Bladex 500, Callisto, Sanson 40 SC e Sipazina 800 PM, entre outros, são exemplos dos principais herbicidas recomendados para cultura brasileira de milho no preparo convencional e no sistema plantio direto (Embrapa, 2006).

Herbicidas da família triazina, como a Atrazina, são encontrados em águas superficiais e subterrâneas, e sua ação está ligada com a inibição da fotossíntese das plantas. Estudos apontam que seus compostos atuem como xenoestrógenos, relacionados com a desregulação endócrina em anfíbios, ocasionando a feminização destes animais (Komtchou et al., 2016). Outros estudos relatam que a Atrazina é tóxica tanto para humanos quanto para os animais, sendo um potencial composto carcinogênico (Shirmardi et al., 2016). Além disso, este herbicida possui a habilidade de estimular a produção de espécies reativas de oxigênio (ROS) através de diversos mecanismos diferentes, podendo gerar um desequilíbrio na produção e ou eliminação de ROS, bem como outros radicais livres (Loro et al., 2015).

Quando há um desequilíbrio no balanço redox, onde a geração de ROS e radicais livres superam a capacidade de reparo das defesas antioxidantes do organismo, com a potencialidade de exercer efeitos deletérios, cria-se o estado denominado de estresse oxidativo. Os antioxidantes podem agir enzimaticamente detoxificando o agente oxidante antes que ele cause lesão, por meio da glutationa peroxidase (GPx), catalase (CAT) e da superóxido dismutase (SOD); ou não enzimaticamente como a glutationa (Lushchak, 2011).

Os peixes são sensíveis à contaminação ambiental e, portanto, os poluentes podem interferir de maneira significativa em vários de seus processos fisiológicos e bioquímicos (Ribeiro et al., 2013). Deste modo, os peixes, uma vez expostos a herbicidas são considerados importantes bioindicadores de poluição ambiental, em nível de indivíduo, população e comunidades (Schulz e Martins-Junior, 2001).

Um biomarcador é qualquer substância, ou seu derivado, que demonstram a interação do organismo e um agente ambiental, refletindo possíveis danos no sistema biológico.

Estudos recentes apontam para a validação de biomarcadores que podem ser utilizados para mensurar danos causados aos organismos aquáticos devido à exposição de contaminantes ambientais (Ribeiro et al., 2013; Karami et al., 2016).

A vitamina C é um micronutriente essencial que assume uma importância considerável, uma vez que essa vitamina está envolvida na atuação de diversos processos fisiológicos como reprodução, resposta imune (prevenção da imunossupressão), crescimento, síntese de colágeno e das células vermelhas (Vélez-Alavez et al., 2014). Este micronutriente é amplamente utilizado no tratamento de certas doenças tais como escorbuto, resfriado comum, anemia, distúrbios hemorrágicos, bem como a infertilidade (Singhal et al., 2016).

A vitamina C exerce papel protetor das atividades metabólicas do organismo, uma vez que possui ação antioxidante. Esta função antioxidante é reforçada pela sua capacidade em regenerar a vitamina E (α-tocoferol) a partir do radical α-tocoferilo (Jiménez-Fernández et al., 2015). Alguns sintomas clássicos da deficiência desta vitamina em peixes são: hemorragia interna, imunossupressão, crescimento reduzido, deformidades estruturais, pigmentação anormal e mau desempenho reprodutivo (Vélez-Alavez et al., 2014).

Os peixes teleósteos, assim como macacos, morcegos e humanos perderam, ao longo da evolução, a enzima L-gulonolactona oxidase, e consequentemente a capacidade de produção da vitamina C (ácido ascórbico). Sendo assim, esses animais precisam obter esta vitamina através de uma suplementação alimentar. Frutas e vegetais como laranja, limão, melancia, abacaxi, brócolis, couve-flor e repolho, são ótimas fontes desta vitamina (Jiménez-Fernández et al., 2015). Deste modo torna-se claro a importância de uma ração completa e balanceada, com quantidades suficientes de vitamina C, especialmente para os peixes que são mantidos em confinamento em uma piscicultura.

Estudos recentes apontam para a busca de suplementos, como vitaminas e minerais que adicionados às dietas podem melhorar a capacidade antioxidante e até mesmo resistência a patologias causadas pela exposição a contaminantes (Özkan et al., 2012).

Sendo assim, considerando os efeitos benéficos da vitamina C, no que se refere à melhora no sistema antioxidante do organismo e também aos mecanismos pelo qual o herbicida Atrazina manifesta seus efeitos tóxicos, o presente estudo avaliou os efeitos da vitamina C sobre parâmetros bioquímicos, hematológicos de jundiás expostos ao herbicida atrazina.

Neste contexto o presente estudo teve como objetivos:

Objetivo Geral:

Avaliar os efeitos protetores da vitamina C sobre parâmetros bioquímicos e hematológicos em jundiás (*Rhamdia quelen*) expostos ao herbicida atrazina.

Objetivos Específicos:

a) MANUSCRITO 1 - Investigar os efeitos de diferentes níveis de vitamina C (0, 500 e 1000 mg/kg), em relação aos marcadores de estresse e dano oxidativo e parâmetros hematológicos, em jundiás (*Rhamdia quelen*), afim de verificar o potencial antioxidante desta vitamina para futuros estudos nos quais esta será utilizada contra a toxicidade de pesticidas.

b) MANUSCRITO 2 - Investigar se a quantidade de 1000 mg/kg de vitamina C é suficiente para promover proteção no jundiá (*Rhamdia quelen*) contra o estresse causado pela exposição à atrazina (10µg/L).

REFERÊNCIAS

EMBRAPA. **Documento Online nº 61 - Publicações - Embrapa Trigo**. Passo Fundo, RS. 2006. Disponível em: <http://www.cnpt.embrapa.br/biblio/do/p_do61_13.htm>. Acesso em: 17 de julho. 2016.

JIMÉNEZ-FERNÁNDEZ, E.; PONCE, M.; RODRIGUEZ-RÚA, A.; ZUASTI, E.; MANCHADO, M.; FERNÁNDEZ-DÍAZ, C. Effect of dietary vitamin C level during early larval stages in Senegalese sole (*Solea senegalensis*). **Aquaculture**. v.443, p.65–76, 2015.

KARAMI, A.; OMAR, D.; LAZORCHAK, J. M.; YAP, C. K.; HASHIM, Z.; COURTENAY, S. C. A comparison of biomarker responses in juvenile diploid and triploid African catfish, *Clarias gariepinus*, exposed to the pesticide butachlor. **Environmental Research.** v.151, p.313-320, 2016.

KOMTCHOU, S.; DIRANY, A.; DROGUI, P.; DELEGAN, N.; KHAKANI, M. A. E.; ROBERT, D. LAFRANCE, P. Degradation of atrazine in aqueous solutionwith electrophotocatalytic process using $TiO_{2-}x$ photoanode. **Chemosphere.** v.157, p.79- 88, 2016.

LORO, V. L.; GLUSCZAK, L.; MORAES, B. S.; LEAL, C. A. M.; MENEZES, C.; MURUSSI, C. R.; LEITEMPERGER, J.; SCHETINGER, M. R. C.; MORSCH, V. M. Glyphosate-based herbicide affects biochemical parameters in *Rhamdia quelen* (Quoy & Gaimard, 1824) and *Leporinus obtusidens* (Valenciennes, 1837). **Neotropical Ichthyology**. v.13, n.1, p.229-236, 2015.

LUSHCHAK, V. I. Environmentally induced oxidative stress in aquatic animals. **Aquatic Toxicology**. v.101, p.13-30, 2011.

ÖZKAN, F.; GÜNDÜZ, S. G.; BERKÖZ, M.; HUNT, A. Ö.; YALM, S. The protective role of ascorbic acid (vitamin C) against chlorpyrifos-induced oxidative stress in *Oreochromis niloticus*. **Fish Physiology and Biochemistry**. v.38, p.635–643, 2012.

RAMOS, M. A. G.; YOSHIOKA, S. A. Bioremediation of herbicide velpar K® in vitro in aqueous solution with application of EM-4 (effective microorganisms). **Brazilian Archives of Biology and Technology**. v.55, n.1, p.145-149, 2012.

RIBEIRO, C. A. O.; KATSUMITI, A.; FRANÇA, P.; MASCHIO, J.; ZANDONÁ, E.; CESTARI, M. M.; VICARI, T.; ROCHE, H.; de ASSIS, H. C. S.; NETO F. F. Biomarkers responses in fish (*Atherinella brasiliensis*) of Paranaguá bay, southern Brazil, for assessment of pollutant effects. **Brazilian Journal of Oceanography**. v.61, n.1, p.1-11, 2013

SCHULZ, U. H.; MARTINS-JUNIOR, H. *Astyanax fasciatus* as bioindicator of water pollution of Rio dos Sinos, RS, Brazil. **Brazilian Journal of Biology**. v.61, n.4, p.615-622, 2001.

SINGHAL, C.; MALHOTRA, N.; PUNDIR, C. S.; DEEPSHIKHA; NARANG, J. An enzyme free Vitamin C augmented sensing with different ZnO morphologies on SnO_2/F transparent glass electrode: A comparative study. **Materials Science and Engineering**. v.69, p.769–779, 2016.

SHIRMARDI, M.; ALAVI, N.; LIMA, E. C.; TAKDASTAN, A.; MAHVI, A. H.; BABAEI, A. A. Removal of atrazine as an organic micro-pollutant from aqueous solutions: a comparative study. **Process Safety and Environmental Protection**. v.103, p. 23-35, 2016.

VÉLEZ-ALAVEZ, M.; MÉNDEZ-RODRIGUEZ, L. C.; MONTAÑEZ, J. A. De A.; MEJÍA, C. H.; GALVÁN-MAGAÑA, F.; ZENTENO-SAVÍN, T. Vitamins C and E concentrations in muscle of elasmobranch and teleost fish. **Comparative Biochemistry and Physiology, Part A**. v.170, p.26–30, 2014.

ZHU, X.; SUN, Y.; ZHANG, X.; HENG, H.; NAN, H.; ZHANG, L.; HUANG, Y.; ZHOU, Y. Herbicides interfere with antigrazer defenses in *Scenedesmus Obliquus*. **Chemosphere**. v.162, p. 243-251, 2016.

MANUSCRITO 1

INFLUENCE OF DIET SUPPLEMENTED WITH VITAMIN C ON OXIDATIVE DAMAGE AND BLOOD PARAMETERS OF *Rhamdia quelen*

ABSTRACT

The aim of this study was to investigate the effects of different levels of vitamin C diet on oxidative stress biomarkers and hematological parameters on silver catfish, in order to verify the antioxidant potential of this vitamin, over 28 days of feeding. One hundred and fifty silver catfish juveniles were divided into three groups, fifty silver catfish in each group, were fed with different amounts of vitamin C: (1) 0 mg/kg, (2) 500 mg/kg and (3) 1000 mg/kg. Ten fish per group were collected at 0, 7, 14, 21 and 28 days after the beginning of experiment. In each collection, blood, liver and gills were sampled for further evaluation of the hematological parameters as well the assays of markers of oxidative stress. The activity of catalase (CAT) in liver increased in group 1, when compared to other groups. In groups 2 and 3, this increase was not registered. The levels of lipid peroxidation in liver and gill were reduced in groups 2 and 3 when compared to group 1, there was no such reduction in the group 1. The activity of glutathione peroxidase (GPx), in gills, decrease in all groups, on the other hand, in liver the GPx activity increased in group 2 and 3 when compared to group 1. The level of carbonyl protein in gill was elevated in the group 1, when compared to other groups. There were no statistical difference in NPSH levels between the groups, both in gill and liver. The GST activity, in gills, decrease in all groups and in liver the activity of this enzyme increased, also in all groups. There were no significant changes in SOD activity in relation to the different vitamin C diets. In conclusion, our results indicate that diets with 500 or 1000 mg/kg of vitamin C contributed to improve the antioxidant capacity of *Rhamdia quelen*, suggesting that vitamin C supplementation in the amount of 500 or 1000 mg/kg may be useful in aquaculture, preventing possible damage caused by oxidative stress.

Keywords: ascorbic acid, oxidative stress, hematological parameters.

1.1 INTRODUCTION

The nutrients required by fish for any normal physiological functions are almost the same to those of land animals. On the other hand, feeding fish in their aquatic environment is more complex than feeding land animals because in aquatic environment must be taken into account the nutrient contribution of natural aquatic organisms, the effect of feeding on water quality, and the loss of nutrients not consumed immediately (Lovell, 2013). Therefore, in fish

farming, as the fish are kept confined, natural food becomes scare, thus, animals need a complete and nutritionally balanced diet in order to avoid reduction on resistance to the spread of disease or reduction in growth (Campeche et al., 2009; Garcia et al., 2009; Lovell, 2013). So, the nutritional requirements of fish confined are supplied through organisms or commercial feed.

Moreover, fish from fish farming have problems with gene pool alterations, competition, and disease transmission due interbreeding and cohabiting within the same environment. This fact, associated with changes in foraging behavior, aggression, individual swimming and group behavior, resulting sometimes in a stress state (Paehlke, 1995; Martins, 2012).

Vitamin C is an important non-proteic antioxidant, and in fact, diets lacking vitamin C can lead to increased cellular oxidation caused by the imbalance between the production of free radicals and the antioxidant response. The production of reactive oxygen species (ROS) occurs innately by cellular metabolism in a normal situation, and organisms are able to control this situation. However, when fish are submitted to environmental pollutants, oxidative damage is frequently recorded (Bloomer et al., 2005; Salaro et al., 2013). In order to inhibit or reduce oxidative damage caused by increased ROS and free radicals, the organism has an efficient antioxidant defense system. Catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) are part of the endogenous enzymatic antioxidant defense system, while reduced glutathione (GSH) and vitamins A, C and E belong to the non-enzymatic system (Paniz et al., 2007).

Vitamin requirements depend on the environmental conditions, the interrelationship with other nutrients present in the diet and fish health (Navarro et al., 2009). Teleost fish are unable to synthesize vitamin C due to the absence of enzyme L-gulonolactone oxidase (GLO), which catalyzes the conversion of L-gulonic acid to ascorbic acid (Wang et al., 2003; De Andrade et al., 2007; Vélez-Alavez et al., 2014). However, vitamin C is an indispensable micronutrient required on diet to maintain the physiological processes of certain animals including most fish. This vitamin is a water-soluble nutrient, essential for the synthesis of red blood cells, blood vessels and tissue repair (Wang et al., 2003; Vélez-Alavez et al., 2014). Many vitamin C deficiency symptoms on fish have been described, including internal bleeding, immunosuppression, increased susceptibility to bacterial infections, reduced growth, skeletal muscle injury, structural deformities, and changes of natural pigmentation, among others (Lee and Dabrowski, 2003; Navarro et al., 2009; Vélez-Alavez et al., 2014).

The silver catfish (*Rhamdia quelen*) is a teleost fish that belongs to the order Siluriformes, family Heptapteridae and subfamily Heptarinae (Borges et al., 2007). It is a promising species for fish cultivation in southern South America and other subtropical climate regions of the world, due to its zootecnic and organoleptic characteristics propitious to its development and due to its tolerance to low temperatures that provide continual growth in winter (Ferreira et al., 2010; Fukushima et al., 2012).

The present study was delineated to investigate the effects of different levels of dietary vitamin C on oxidative stress markers and hematological values of silver catfish in order to verify the antioxidant potential of vitamin C. The results obtained with vitamin addition in food could be used in future studies to improve the antioxidant capacity of *Rhamdia quelen* against toxicity caused by exogenous stressors like pesticides.

1.2 MATERIALS AND METHODS

1.3.1 Fish

One hundred and fifty silver catfish juveniles of both sexes (length 10.61 ± 1cm; weight 9.87 ± 1g) were obtained from fish farming close to Santa Maria (Rio Grande do Sul State, Brazil). The fish were acclimated to the laboratory conditions for 15 days. They were kept in plastic boxes of 170 liters continuously aerated with a biofilter system and a natural photoperiod (12h light/12h dark). Feces and pellet residues were removed by suction. The water parameters averages were: dissolved oxygen 7.22 ± 1.0 mg/L, temperature 23.0 ± 1.0°C, pH 6.9 ± 0.2, ammonia 0.04 ± 0.01µg/L and nitrite 0.05 ± 0.01mg/L. After the acclimation period, the fish were divided into experimental groups.

1.3.2 Diet preparation and experimental design

The experimental diets were prepared at the Pisciculture Laboratory from the Federal University of Santa Maria. Diets were elaborated containing different concentrations of vitamin C: (1) 0 mg/kg, (2) 500 mg/kg and (3) 1000 mg/kg (Table1). All ingredients were

mixed and pellets of approximately 5 mm of diameter were formed by grinding the mixture through a meat grinder. After processing, all the diets were packed into small bags and kept at 4ºC.

The fish were divided randomly into three groups, fifty silver catfish to each diet. During the experiment, the fish were fed with 3% of their body weight per day (Menezes et al., 2014) and daily diet was divided into two equal meals at 09:00 and 16:00 hours. The biofilter system was used to maintain water quality, and the water parameters averages were maintained equal to those of the acclimation period. Feces and pellet residues were also removed by suction.

Table 1 - Control diet composition*

Ingredients	Amount (g)
Fish's flour	60.890
Starch miho	17.000
Celulose	12.580
Vitamin premix	3.000
Dicalcium phosphate	2.000
Calcareous	1.500
Soy oil	1.170
Cod-liver oil	1.000
Salt	0.500
Di methionine	0.360
Vitamin C coated	0.000
Total	1000.000

*The feeds containing 500 to 1000 mg/kg vitamin C were also made according to Table 1, however, cellulose was removed according to the addition of vitamin C (500 or 1000 mg/kg) .

1.3.3 Sample preparation

During the supplementation of vitamin C, ten fish per diet were collected randomly at day 0, 7, 14, 21 and 28. In each collection, the fish were anaesthetized with eugenol (50 µg/L) according to Cunha et al. (2010), and then weight and length were measured. After, the blood from caudal vein was collected using syringes previously heparinized. Then, the fish were euthanized by medullary section. The hematological parameters evaluated were: red blood count (RBC), hemoglobin (HB), hematocrit (HCT), mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH). After the blood collection, samples of tissues (liver and gills) were quickly removed and stored at -80°C for further evaluation of catalase (CAT)

activity, superoxide dismutase (SOD) activity, glutathione S-transferase (GST) activity, glutathione peroxidase (GPx) activity, non-protein thiols (NPSH) levels, lipid peroxidation determination, protein carbonyl assay and ascorbic acid levels. The animal experimentation were according to the National Institute of Health Guide for animal. All experimental protocols were approved by the Ethics Commission on Animal Use of the University Federal of Santa Maria (protocol number: 4302190116).

Hematological parameters

Quantitative determinations of blood cells were performed using a Pentra 80 ABX diagnostics (France).

Protein determination

Protein was determinated by the Comassie Blue Method using bovine serum albumin as standard. Absorbance of samples was measured at 595 nm according Bradford (1976).

Oxidative stress assays

The liver and gills were homogenized with Tris-HCl buffer [50 mM] pH 7.4 and centrifuged at 3000 rpm for 10 min. This homogenate was used for all of the following analyzes.

CAT activity

In liver, the CAT (EC 1.11.1.6) activity was assayed by ultraviolet spectrophotometry according to Nelson and Kiesow (1972). The assay mixture consisted of 2.0 mL potassium phosphate buffer (TFK) [50 mM] pH 7.0, 10 µL homogenate, and 50 µL hydrogen peroxide (H_2O_2) [0.3 mM]. Changes of H_2O_2 were measured in 1 minute; the

reading was performed in kinetic mode at 240 nm. CAT activity was expressed in μmol/min/mg protein.

SOD activity

SOD (EC 1.15.1.1) activity was performed in liver by modification of Misra and Fridovich's (1972). This method was based on the inhibition of the radical superoxide reaction with adrenaline. The SOD present in the sample competes with the detection system for radical superoxide. The oxidation of adrenaline leads to the formation of the colored product, adrenochrome, which is detected using a spectrophotometer. Distinct volumes of homogenate (5, 10, 15 e 20 μL) were pipetted into microplate and 190, 185, 180 and 175 μL of glycine- NaOH [50 mM] pH 10.6 (maintained at 37°C in a water bath) respectively, and 5 μL of the adrenalin [60 mM]. The absorbance was read in kinetic mode at 480 nm for 10 minutes. The SOD activity was expressed in UI SOD/ mg protein. A unit of SOD was defined as the amount of enzyme that inhibits the rate of oxidation of adrenaline by 50%.

GST activity

GST (EC 2.5.1.18) activity was determined in liver and gills by modification of procedure described by Habig et al. (1974). This analysis was performed in microplate spectrophotometer. The assay mixture consisted of 10 μL homogenate, 150 μL potassium phosphate buffer (TFK) [20 mM] pH 6.5 (maintained at 37°C in a water bath), 50 μL reduced glutathione (GSH) and 50 μL 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate. The absorbance was read in kinetic mode at 340 nm for 2 minutes. The specific activity of GST was determined using the extinction coefficient of 9.6 mM/cm and expressed in GS-DNB/ min/mg protein and expressed as μmol/min/mg protein.

GPx activity

GPx (EC 1.11.1.9) activity was determined in liver and gills by modification of the Paglia and Valentine's (1967) method. The analysis was performed in microplate. The assay mixture consisted of 10 μL homogenate and 260 μL of the system containing: NADPH, GSH, GR (Glutathione reductase), azide and potassium phosphate buffer (TFK) [100Mm] pH 7.0. The absorbance was read in kinetic mode at 340 nm for 2 minutes. The specific activity was determined using the extinction coefficient of 6.22 mM/cm and the GPx activity was expressed in μmol/min/mg protein.

NPSH levels

NPSH levels were determined in liver and gills by modification of Ellman's method (1959). 100 µL of the homogenate was mixed with 100 µL 10% trichloroacetic acid (TCA), followed by centrifugation at 3000 rpm for 10 min. 30 µL of the supernatant was pipetted into microplate, 70 µL distilled water, 100 µL potassium phosphate buffer (TFK) [50 mM] pH 7.0, and 10 µL 5,5'-dithio-bis 2-nitrobenzoic acid (DTNB) [10 mM]. The microplate was incubated in the dark for 20 minutes. The absorbance was recorded in endpoint mode at 412 nm. NPSH levels were expressed as µmol SH/g of tissue.

Lipid peroxidation determination

Lipid peroxidation was measured in liver and gills by thiobarbituric acid reactive species (TBARS) production according to Buege and Aust method (1978) with some modifications. 100 µL of the homogenate was mixed with 100 µL 10% TCA, followed by centrifugation at 3000 rpm for 10 min. 100 µL of the supernatant was mixed with 100 µL 2-thiobarbituric acid (TBA) [0.67%]. The mixture was incubated at 100°C, in a water bath, for 30 minutes. 150 µL of the supernatant was pipetted into microplate. The absorbance was read in endpoint mode at 532 nm. The lipid peroxidation was expressed as nmol MDA/mg protein.

Protein carbonyl assay

Protein carbonyl content was assayed in liver and gills by modification the method described by Yan et al. (1995). 200 µL of the homogenate plus 800 µL distilled water were reacted with 150 µL 2,4 dinitrophenylhydrazine (DNPH) [10 nm] in 2N hydrochloric acid. After incubation at room temperature in the dark for 60 minutes, were added sequentially, 125 µL sodium dodecyl sulfate (SDS) 3%, 500 µL ethanol e 500 µL of heptane. This mixture was vortexed for 30 s and centrifuged at 3000 rpm for 15 minutes. Then, the protein isolated from the interface was washed by resuspension in ethanol/ethyl acetate (1:1). After it was added 250µL sodium dodecyl sulfate (SDS) 3% and vortexed again. The mixture was pipetted into a microplate. Assay was performed in duplicate and one blank tube incubated with 2N HCl without DNPH was included for each sample. The absorbance was recorded in endpoint mode at 370 nm. The total carbonylation was calculated using a molar extinction coefficient of 22.000 M/cm. The protein carbonyl content was expressed as nmol carbonyl/mg protein.

Ascorbic acid levels

Ascorbic acid was determined in liver and gills by Roe method (1954). 300 µL homogenate plus 100 µL distilled water, 100 µL TCA [13.3%] and 75 µL 2,4 dinitrophenylhydrazine (DNPH) were incubated at 37° C, in a water bath, for 3 h. Following the incubation, 500µL sulfuric acid (H_2SO_4) 65% was added, to stop the reaction. The absorbance was read at 520 nm. The ascorbic acid content was expressed as µmol/g of tissue.

1.3.4 Statistical analysis

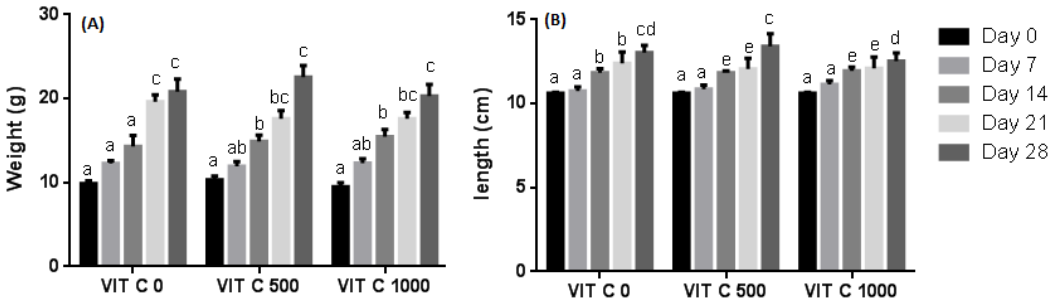
All data were analyzed by Two-way ANOVA to test the effects of the dietary treatments and the different times, followed by Tukey's multiple comparisons test. Homogeneity of variances between groups was tested with Levene test. The values are presented as mean ± S.E.M. Differences were considered to be significant at a probability level of $p < 0.05$ between groups.

1.4 RESULTS

Body weight and length

No statistical difference was observed between the groups (0, 500 and 1000 mg/kg vitamin C) in relation to body weight (Figure 1A) and length (Figure 1B).

Figure 1 - Relation of body weight (A) and length (B) in silver catfish fed with different vitamin C levels (0, 500 and 1000 mg/kg). Different letters indicate differences between groups ($p < 0.05$). Data are reported as mean ± S.E.M (n= 8).



Blood parameters

There was a significant increase in RBC in the group fed for 7 days, with the diets containing 0 (group 1) and 500 mg/kg (group 2) of vitamin C when compared to day 0 (Table 2). In the group fed with the diet containing 1000 mg/kg (group 3) of vitamin C the increase occurred after 7 and 21 days when compared to day 0. With respect the Hb the data show an increased in all groups of vitamin C after 7 days of feeding and did not change in 14, 21 and 28 days when compared to 0 day (Table 2). The results show an increase of HCT after 7 days of feeding in the groups 1 and 2 when compared to day 0. In the group 3 was observed an increase of HCT after 7 and 21days of feeding when compared to day 0 (Table 2). There were no significant changes in MCV and MCH in relation to the different vitamin C diets (data not shown).

Table 2 - Blood parameters: effect of different amount of vitamin C in relation to blood parameters and the feeding time.

RBC (106 /mm3)					
	Days				
Diets	0	7	14	21	28
0 mg/kg	1.39 ± 0.10^{a}	2.10 ± 0.26^{b}	1.73 ± 0.27^{ab}	1.80 ± 0.19^{ab}	1.53 ± 0.31^{a}
500 mg/kg	1.36 ± 0.08^{a}	2.01 ± 0.21^{b}	1.71 ± 0.27^{ab}	1.73 ± 0.43^{ab}	1.56 ± 0.21^{a}
1000 mg/kg	1.38 ± 0.14^{a}	1.95 ± 0.18^{b}	1.68 ± 0.19^{ab}	1.94 ± 0.32^{b}	1.66 ± 0.22^{ab}
Hb (g/dL)					
	Days				
Diets	0	7	14	21	28
0 mg/kg	5.38 ± 0.31^{a}	7.43 ± 0.81^{b}	7.15 ± 0.67^{b}	7.24 ± 0.81^{b}	6.42 ± 1.39^{b}
500 mg/kg	5.40 ± 0.30^{a}	7.63 ± 0.92^{b}	6.97 ± 0.39^{b}	7.03 ± 1.50^{b}	7.00 ± 1.00^{b}
1000 mg/kg	5.42 ± 0.31^{a}	7.80 ± 0.60^{b}	6.90 ± 0.69^{b}	7.97 ± 1.16^{b}	7.15 ± 0.90^{b}
HCT (%)					
	Days				
Diets	0	7	14	21	28
0 mg/kg	22.12 ± 2.61^{ac}	30.46 ± 2.15^{b}	20.34 ± 5.36^{c}	28.56 ± 4.07^{a}	20.28 ± 5.90^{c}
500 mg/kg	21.12 ± 1.44^{a}	33.03 ± 4.87^{b}	24.03 ± 5.13^{a}	26.49 ± 4.99^{ab}	24.07 ± 5.00^{a}
1000 mg/kg	22.62 ± 2.00^{a}	33.15 ± 5.14^{b}	24.00 ± 2.13^{a}	32.12 ± 3.48^{b}	26.53 ± 5.96^{ab}

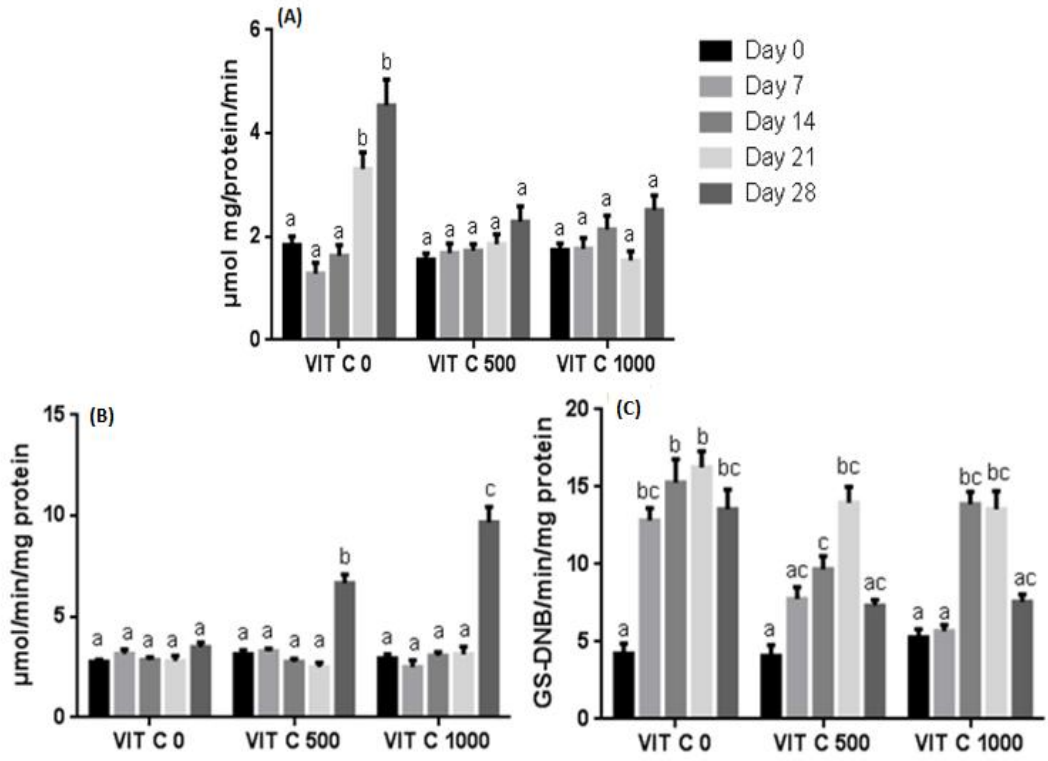
Red blood count (RBC), hemoglobin (Hb) and hematocrit (HCT). Different letters indicate differences between groups ($p < 0.05$). Data are reported as mean ± S.E.M (n=10).

Enzymatic activities in liver: CAT, GPx and GST

A significant increase in CAT activity was observed in the group 1 after 28 days of feeding when compared to day 0 feed and compared with the other groups (2 and 3) (Figure 2A). All concentrations showed no significant changes on the other days. The results indicated a significant increase in the GPx activity in fish fed for 28 days with the diet 2 and 3 when compared to day 0 and compared with the group 1 (Figure 2B). The GST activity (Figure 2C) increased in the group 1 at 7, 14, 21 and 28 days when compared to day 0. An

increase occurred with 14 and 21 days of feeding in the group 2 and 3, however, after 28 days of feeding, the GST activity in these groups proved to be equal to day 0.

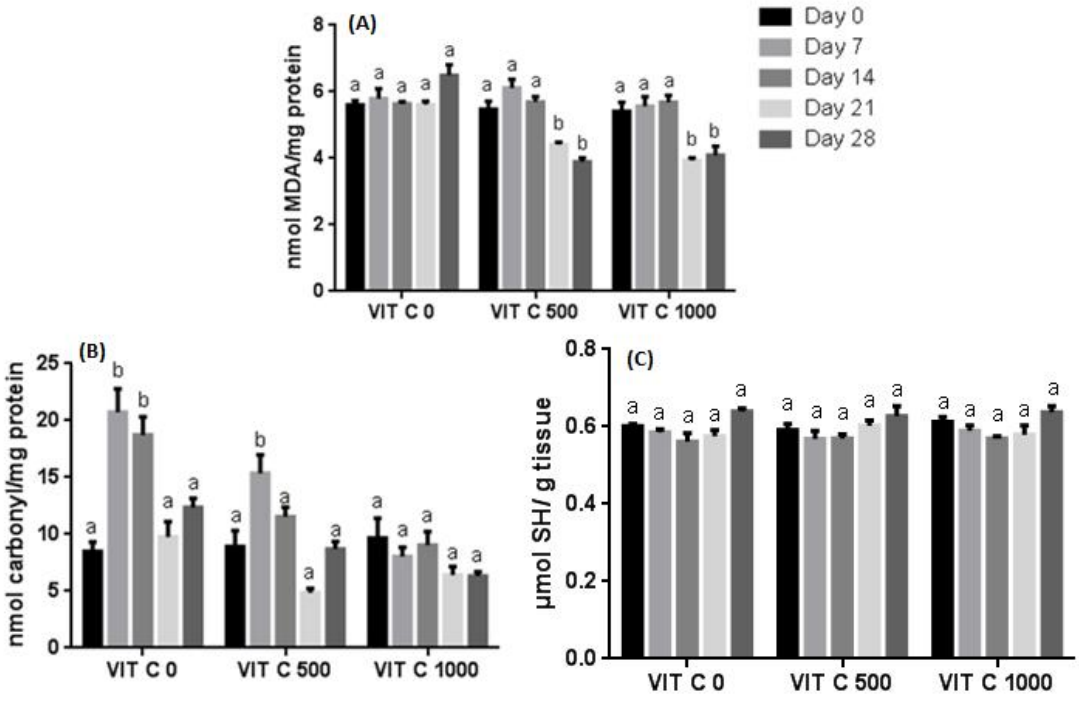
Figure 2 - CAT (A), GPx activity (B) and GST activity in gills (C) in liver of silver catfish fed with different vitamin C levels (0, 500 and 1000 mg/kg). Different letters indicate differences between groups ($p < 0.05$). Data are reported as mean ± S.E.M (n= 8).



Lipid peroxidation determination, protein carbonyl content and NPSH levels in liver

The lipid peroxidation was significantly reduced in liver (Figure 3A) in the groups fed with the diet 2 and 3 only after 21 and 28 days when compared to day 0. The carbonyl protein content increased in liver (Figure 3B), after 7 and 14 days of feeding in the group 1 and only after 7 days of feeding in the group 2. There were no statistical differences in NPSH levels between the groups in liver (Figure 3C).

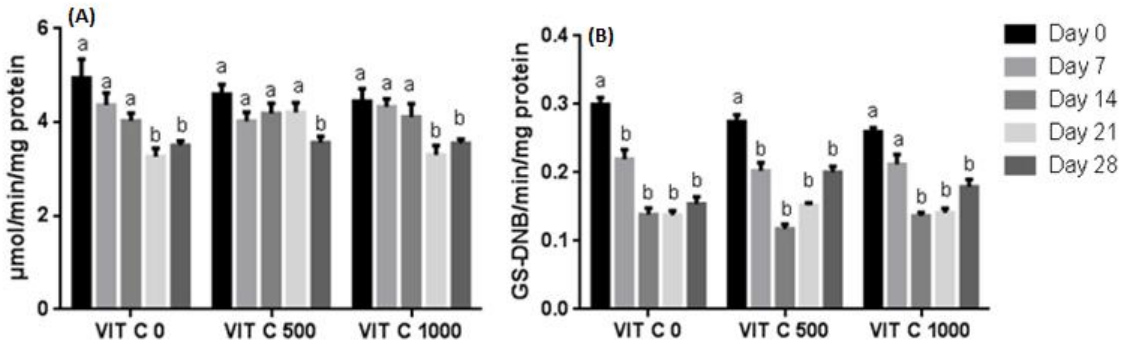
Figure 3 - Lipid peroxidation determination (A), carbonyl protein content (B) and NPSH levels (C) of silver catfish fed with different vitamin C levels (0, 500 and 1000 mg/Kg). Different letters indicate differences between groups ($p < 0.05$). Data are reported as mean ± S.E.M. (n =8).



Enzymatic activities in gills: GPx and GST

The GPx activity in gills (Figure 4A) decreased in all groups after 28 days of feeding when compared to day 0. The GST activity in gills (Figure 4B) decreased after 14 days of feeding in all groups when compared to day 0.

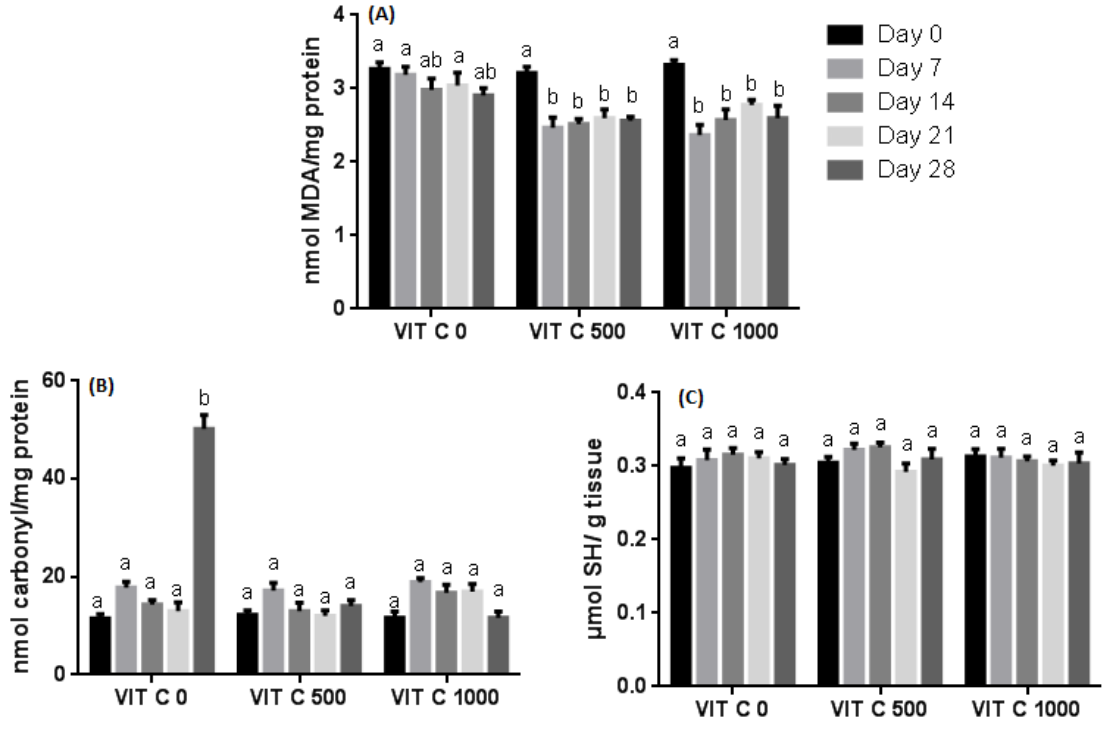
Figure 4 –GPx activity (A) and GST activity in gills of silver catfish fed with different vitamin C levels (0, 500 and 1000 mg/kg). Different letters indicate differences between groups ($p < 0.05$). Data are reported as mean ± S.E.M (n= 8).



Lipid peroxidation determination, protein carbonyl content and NPSH levels in gills

The lipid peroxidation was significantly reduced in gills (Figure 5A) of fish fed for 7, 14, 21 and 28 days with the diets 2 and 3 when compared to day 0. The carbonyl protein content increased in gills (Figure 5B), after 28 days, in the group of fish fed with the diet 1 when compared to day 0 and compared with the others groups. There were no statistical differences in NPSH levels between the groups in gill (Figure 5C).

Figure 5 - Lipid peroxidation determination (A), carbonyl protein content (B) and NPSH levels (C) in gills of silver catfish fed with different vitamin C levels (0, 500 and 1000 mg/Kg). Different letters indicate differences between groups ($p < 0.05$). Data are reported as mean ± S.E.M. (n =8).



Ascorbic acid levels

The level of ascorbic acid in the gills increased significantly after 28 days of feeding in the groups 2 and 3 when compared to day 0 (Table 3). On the other hand, this increase in the liver, when compared to day 0, occurred after 7 days of feeding only in the group 3. However, after this period, the level of ascorbic acid proved to be equal to day 0.

Table 3 - Analyzed dietary concentration of vitamin C (µmol/g tissue) during different days of feeding.

	Gill				
	Days				
Diets	0	7	14	21	28
0 mg/kg	7.78 ± 1.74[a]	7.88 ± 2.16[a]	8.65 ± 2.41[a]	8.71 ± 1.86[a]	8.06 ± 1.92[a]
500mg/kg	8.01 ± 1.58[a]	8.71 ± 2.15[a]	7.87 ± 2.53[a]	7.42 ± 2.41[a]	12.39 ± 2.06[b]
1000mg/kg	9.11 ± 1.88[a]	7.40 ±2.38[a]	8.53 ± 1.83[a]	9.72 ± 2.53[a]	12.61 ± 1.67[b]
	Liver				
	Days				
Diets	0	7	14	21	28
0 mg/kg	10.51 ± 3.27[a]	10.45 ± 1.58[a]	11.35 ± 1.94[a]	9.09 ± 1.23[a]	8.85 ± 1.87[a]
500mg/kg	11.41 ± 2.79[a]	15.24 ± 2.14[ab]	11.46 ± 2.13[a]	11.04 ± 3.36[a]	11.52 ± 2.52[a]
1000mg/kg	11.57 ± 2.62[a]	18.54 ± 2.38[b]	13.60 ± 3.20[a]	11.27 ± 3.12[a]	11.42 ± 1.62[a]

Different letters indicate differences between groups ($p < 0.05$). Data are reported as mean ± S.E.M (n=10).

1.5 DISCUSSION

It is already known the beneficial effects of diphenyl diselenide on growth, oxidative damage and antioxidant response in silver catfish (Menezes et al., 2016), and its protection towards herbicides (Menezes et al., 2014). Therefore, we intend to investigate the antioxidant effects of vitamin C in order to conduct future studies using its protective action against toxicity caused by pesticides.

Vitamin C or 2-oxo-L-threo-hexagon-1,4-lactone-2,3-enediol is an important compound mainly in the maintenance of collagen, which constitutes the main protein of cartilage, heart valves, skin, inter vertebral discs and bones, as well as participating in the synthesis of catecholamines. Moreover, this vitamin is an important dietary antioxidant, capable of neutralizing reactive oxygen species, peroxyl, superoxide anion, hydroperoxyl radicals and reactive nitrogen radicals, besides regenerating other antioxidants such as α-tocopheroxyl, urate and β-carotene. This vitamin mitigates the effect of reactive species such as reactive oxygen species that can cause oxidative damage to lipids, protein and DNA, preventing cancer and neurodegenerative diseases (Naidu, 2003). Unlike most mammals, teleost fish, need to obtain vitamin C from diet, as their bodies can not produce by themselves this vitamin (Schmidt-Nielsen, 1997). Thus the vitamin C becomes essential in the diet of many vertebrates including fish.

CAT is one of the most important antioxidant enzymes involved in the elimination of H_2O_2. This enzyme catalyzes the conversion of H_2O_2 into molecular oxygen and water. The ascorbate (AsA), ionized form of vitamin C, is one of the most important non-enzymatic

antioxidants. The AsA along with GSH participates in the cycle of ascorbate-glutathione which H_2O_2 is eliminated by peroxidation of AsA. Therefore, CAT and ascorbate-glutathione cycle are important in the removal of H_2O_2 and although their properties and requirements are different, they can work effectively in parallel (Barbosa et al., 2014). Therefore, such as catalase and AsA act in parallel in the elimination of H_2O_2, we can deduce that the increased activity of CAT (days 21 and 28) observed in the group 1, may be due the absence of AsA, in the fish reserves, and consequently greater demand of CAT for elimination of H_2O_2 present in the body of the fish. From another point of view, this increase in CAT activity may indicate that group 1 when compared to other groups (2 and 3) and the day zero, may be facing some form of stress, thus causing increased in the activity of this enzyme. In groups 2 and 3, this increase was not registered; this may be due to contribution of vitamin C according to ascorbate-glutathione cycle.

One of the functions of GPx is the reduction of the lipid peroxidation (Özkan et al., 2012). From this point of view, we can deduce that the increase of GPx activity in liver in the groups 2 and 3 (day 28) may be associated with decreased lipid peroxidation in these same groups. However, in the gills, instead of liver, there was no significant changes in GPx activity in relation to the different vitamin C diets. This fact pointed out the tissue specific response of gills as compared to liver. Liver is the central organ of metabolism and detoxification processes, thus in relation to vitamin C the liver could be important to metabolize vitamin C and reduced peroxidation levels, but gills assume another role in fish physiology as osmoregulation control. The GST activity, in gills, decreased in all groups when compared to day 0 feed, and in liver, the activity of this enzyme increased in all groups.

According to literature data, vitamin C acts synergistically with vitamin E, reducing lipid peroxidation (Navarro et al., 2009). The results of lipid peroxidation show a reduction of their levels, in groups fed with 500 to 1000 mg/kg of vitamin C, in gill and liver, when compared to day zero and the group 1. As there was no such reduction in the group 1, we can assume that this reduction in lipid peroxidation levels is associated with vitamin C present in the feed.

Protein carbonyl levels are the most commonly used marker for the presence of protein oxidation. The use of protein carbonyl groups is advantageous because there is a rapid formation of protein carbonyls, besides they present some stability after formation (Dalle-Donne et al., 2003; Dayanand et al., 2012). The results show that there was an increase in protein carbonyl levels in the group 1, compared to day zero and to the other groups. This carbonylation may be associated with the same stress, which has caused also the increase of

CAT. Thus, we can infer that the reduction of carbonyls in the liver occurred possibly due to CAT action. Through these results, we can deduce that the vitamin C in the diet of other groups may have importance in the defense against the oxidation of proteins. Although GSH be the most representative NPSH and be part of the cycle of Vitamin C, there was no difference in their levels between the groups fed with different concentrations of this vitamin, in gill and liver. This result may indicate that due to the lack of a stress induction, such as an exposure to xenobiotic, there was no need to increase the amount of GSH, needed to the reduction of ascorbic acid.

In relation to the hematological parameters, our results showed an increase in number of RBC, HCT and Hb (Table 2) observed in all groups. Therefore, it is difficult to associate the increase of these parameters to vitamin C since this increase occurred in all groups, even the group not supplemented. There was no significant changes in MCV and MCH in relation to the different vitamin C diets. Therefore, we can say that these different concentrations of vitamin C or feeding time were not enough to interfere with the values of MCV and MCH.

Through the above results, we can say that fish fed with diet containing 500 or 1000 mg/kg vitamin C had higher antioxidant capacity compared to the group fed on a diet containing 0 mg/kg of vitamin C. These results were better visualized in the liver than in the gills, which can be explained by the fact that liver is the target organ of metabolism and detoxification.

In conclusion, our results indicate that diets containing 500 or 1000 mg/kg of vitamin C contributed to improve the antioxidant capacity of *Rhamdia quelen*, suggesting that vitamin C supplementation in the amount of 500 or 1000 mg/kg may be useful in aquaculture, preventing possible damage caused by substances or pollutants that cause oxidative damage.

REFERENCES

BARBOSA, M. R.; SILVA, M. M. A.; WILLADINO, L.; ULISSES. C.; CAMARA, T. R. Plant generation and enzymatic detoxification of reactive oxygen species. **Ciência Rural**, Santa Maria. v.44, n.3, p.453-460, 2014.

BLOOMER, R. J.; GOLDFARB, A. H.; WIDEMAN, L.; MCKENZIE, M. J.; CONSITT, L. A. Effects of acute aerobic and anaerobic exercise on blood markers of oxidative stress. **Journal of Strength & Conditioning Research**. v.19, p.245-480, 2005.

BORGES, A.; SCOTTI, L. V.; SIQUEIRA, D. R.; ZANINI, R.; DO AMARAL, F.; JURINITZ, D. F.; WASSERMANN, G. F. Changes in hematological and serum biochemical values in jundiá *Rhamdia quelen* due to sub-lethal toxicity of cypermethrin. **Chemosphere**. v.69, p.920-926, 2007.

BRADFORD, M. M. A. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. **Analytical Biochemistry**. v.72, p.248-254, 1976.

BUEGE, J. A.; AUST, S. D. Microssomal lipid peroxidation. **Methods Enzymol**. v.52, p.302-309, 1978.

CAMPECHE, D. F. B.; CATHARINO, R. R.; GODOY, H. T.; CYRINO, J. E. P.; Vitamin A in diets for Nile Tilapia. **Scientia Agricola**, Piracicaba. v.66, n.6, p.751-756, 2009.

CUNHA, M. A.; ZEPPENFELD, C. C.; GARCIA, L. O.; LORO, V. L.; FONSECA, M. B.; EMANUELLI, T.; VEECK, A. P. L.; COPATTI, C. E.; BALDISSEROTTO, B. Anesthesia of silver catfish with eugenol: time of induction, cortisol response and sensory analysis of fillet. **Ciência Rural**, Santa Maria. v.40, n.10, p. 2107-2114, 2010.

DAYANAND, C. D.; VEGI, P. K.; KUTTY, A. V. Protein carbonyl content as a stable Oxidative stress marker in Type II Diabetes. **International Journal of Biological and Medical Research**. v. 3, n. 4, p. 2362-2365, 2012.

DALLE-DONNE, I.; ROSSI, R.; GIUSTARINI, D.; MILZANI, A.; COLOMBO, R. Protein carbonyl groups as biomarkers of oxidative stress. **Clinica Chimia Acta**. v.329, p.23-38, 2003.

DE ANDRADE, J. I. A.; ONO, E. A.; DE MENEZES, G. C.; BRASIL, E. M.; ROUBACH, R.; URBINATI, E. C.; TAVARES-DIAS, M.; MARCON, J. L.; AFFONSO, E. G. Influence of diets supplemented with vitamins C and E on pirarucu (*Arapaima gigas*) blood parameters. **Comparative Biochemistry and Physiology, Part A**. v.146, p.576–580, 2007.

ELLMAN, G. L. Tissue sulfhydryl groups. **Archives of Biochemistry and Biophysics**. v.82, p.70-77, 1959.

FERREIRA, D.; DA MOTTA, A. C.; KREUTZ, L. C.; TONI, C.; LORO, V. L.; BARCELLOS, L. J. G. Assessment of oxidative stress in *Rhamdia quelen* exposed to agrichemicals. **Chemosphere**. v.79, p.914–921, 2010.

FUKUSHIMA, H.; BAILONE, R. L.; WEISS, L. A.; MARTINS, M. L.; ZANIBONI-FILHO, E. Triploidy in the hematology of jundia juveniles (Siluriformes: Heptapteridae). **Brazilian Journal of Biology**. v.72, n.1, p.147-151, 2012.

GARCIA, F.; MORAES, F. R.; MARTINS, M. L. Challenge of pacu (*Piaractus mesopotamicus*) fed diets supplemented with vitamins C and E by *Aeromonas hydrophila* under different temperature. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**. v.61, n.2, p.378-385, 2009.

HABIG, W. H., PABST, M. J., JACOBY, W. B. Glutathione S-transferase, the first enzymatic step in mercapturic acid formation. **Journal of Biological Chemistry**. v.249, p.7130-7139, 1974.

LEE, K.J.; DABROWSKI, K. Interaction between vitamins C and E affects their tissue concentrations, growth, lipid oxidation, and deficiency symptoms in yellow perch (*Perca flavescens*). **British Journal of Nutrition**, v.89, p.589-596, 2003.

LOVELL, T. Nutrition and feeding of fish. Springer. 260 p., 2013.

MARTINS, C. I. M.; GALHARDO, L.; NOBLE, C.; DAMSGARD, B.; SPEDICATO, M. T.; ZUPA, W.; BEAUCHAUD, M.; KULCZYKOWSKA, E.; MASSABUAU, J. C.; CARTER, T.; PLANELLAS, S. R.; KRISTIANSEN, T. Behavioral indicators of welfare in farmed fish. **Fish Physiology and Biochemistry**. v. 38, p.17-41, 2012.

MENEZES, C.; LEITEMPERGER, J.; SANTI, A.; DIAS, G.; PEDRON, F. A.; NETO, J. R.; SALMAN, S. M.; BARBOSA, N. B. V.; LORO, V. L. Evaluation of effects induced by dietary diphenyl diselenide on common carp *Cyprinus carpio*. **Fish Physiology and Biochemistry**. v.40, p.141-149, 2014.

MENEZES, C.; MARINS, A.; MURUSSI, C.; PRETTO, A.; LEITEMPERGER, J.; LORO, V. L. Effects of diphenyl diselenide on growth, oxidative damage, and antioxidant response in silver catfish. **Science of the Total Environment**. v.542, p.231-237, 2016.

MENEZES, C.; RUIZ-JARABO, I.; MARTOS-SITCHA, J. A.; LEITEMPERGER, J.; BALDISSEROTTO, B.; MANCERA, J. M.; ROSEMBERG, D. B.; LORO, V. L. Diet with Diphenyl Diselenide Mitigates Quinclorac Toxicity in Silver Catfish (*Rhamdia quelen*). **Plos One**. v. 9(12), 2014.

MISRA, H. P.; FRIDOVICH, I. The role of superoxide anion in the auto-oxidation of epinephrine and a simple assay for superoxide dismutase. **Journal of Biological Chemistry**. v.247, p.3170-3175, 1972.

NAIDU, K. A. Vitamin C in human health and disease is still a mystery? An overview. **Nutrition Journal**. p.2-7, 2003.

NAVARRO, R. D.; FILHO, O. P. R.; FERREIRA, M. W.; PEREIRA, F. K. S. A importância das vitaminas E, C e A na reprodução de peixes. **Revista Brasileira de Reprodução Animal**, Belo Horizonte. v.33, n.1, p.20-25, 2009.

NELSON, D. P.; KIESOV, L. A. Enthalpy of decomposition of hydrogen peroxide by catalase at 25 degrees C (with molar extinction coefficients of H_2O_2 solutions in the UV). **Analytical Biochemistry**. v.49, p.474-478, 1972.

ÖZKAN, F.; GÜNDÜZ, S. G.; BERKÖZ, M.; HUNT, A. Ö.; YALM, S. The protective role of ascorbic acid (vitamin C) against chlorpyrifos-induced oxidative stress in *Oreochromis niloticus*. **Fish Physiology and Biochemistry**. v.38, p.635–643, 2012.

PAEHLKE, R. **Conservation and environmentalism: an encyclopedia**. Garland publishing, INC. New York & London. 808 p., 1995.

PAGLIA, D. E.; VALENTINE, W. N. Studies on the quantitative and qualitative characterization of erythrocytes glutathione peroxidase. **Journal of Laboratory and Clinical Medicine**. v.70, n.1, p.158-169, 1967.

PANIZ, C.; BAIRROS, A.; VALENTINI, J.; CHARÃO, M.; BULCÃO, R.; MORO, A.; GRUNE, T.; GARCIA, S. C. The influence of the serum vitamin C levels on oxidative stress biomarkers in elderly women. **Clinical Biochemistry**. v.40, p.1367–1372, 2007.

ROE, J. H. Chemical determinations of ascorbic, dehydro-ascorbic, and diketogulonic acids. In: Glick D (ed) **Methods of biochemical analysis**. Interscience, New York. v.1. p.115-139, 1954.

SALARO, A. L.; KASAI, R. Y. D.; CARNEIRO, A. P. S.; SABARENSE, C. M.; HAGE, M. C. F. N. S.; TAVARES, M. M.; ZUANON, J. A. S. Suplementação de vitamina C em dietas para juvenis de trairão. **Pesquisa Agropecuária Brasileira**. v.48, n.8, p.1096-1102, 2013.

SCHMIDT-NIELSEN, K. **Animal physiology: adaptation and environment**. Cambridge University Press. 607 p., 1997.

VÉLEZ-ALAVEZ, M.; MÉNDEZ-RODRIGUEZ, L. C.; MONTAÑEZ, J. A. De A.; MEJÍA, C. H.; GALVÁN-MAGAÑA, F.; ZENTENO-SAVÍN, T. Vitamins C and E concentrations in muscle of elasmobranch and teleost fishes. **Comparative Biochemistry and Physiology, Part A**. v.170, p.26–30, 2014.

WANG, X.; KIMA, K. W.; BAI, S. C.; HUH, M. D.; CHO, B. Y. Effects of the different levels of dietary vitamin C on growth and tissue ascorbic acid changes in parrot fish (*Oplegnathus fasciatus*). **Aquaculture**. v.215, p.203–21, 2003.

YAN, L. J.; TRABER, M. G.; PACKER, L. Spectrophotometric method for determination of carbonyls in oxidatively modified apolipoprotein B of human low-density lipoproteins. **Analytical Biochemistry**. v.228, p.349-351, 1995.

MANUSCRITO 2

VITAMIN C IMPROVES THE ANTIOXIDANT CAPACITY AGAINST TOXICITY OF ATRAZINE IN *Rhamdia quelen*

2.1 ABSTRACT

The aim of this study was to investigate if 1000 mg/kg of vitamin C in diet would protect *Rhamdia quelen* against stress caused by a 96 h exposure to Atrazine (10µg/L). A total of 40 fish were divided into four groups, 10 silver catfish in each group: (1) Control 0 mg/kg, (2) Control 1000 mg/kg, (3) Atrazine 0 mg/kg, (4) Atrazine 1000 mg/kg. After 30 days of feeding, groups 3 and 4 were subjected to exposure to Atrazine (10µg/L) for 96 hours. Samples of liver were collected for assays of biomarkers of oxidative stress. There were no significant differences in lipid peroxidation between all the groups. Protein carbonyl content decrease just in group 4. The NPSH levels increase only in groups supplemented with 1000 mg/kg of vitamin C. GPx activity increased significantly in group 4 compared to the other groups. In conclusion, vitamin C could be able to improve liver antioxidant capacity by increasing NPSH and GPx activity, as well by reducing protein carbonyl levels of fish exposed to Atrazine. Thus, dietary supplementation of vitamin C may be useful in aquaculture in order to mitigate the effects of chemical stress on fish caused by pesticides.

Keywords: ascorbic acid, oxidative stress, herbicide.

2.2 INTRODUCTION

In some regions of the world as well as in South America, the agricultural production system often is located near springs, lakes and rivers. This system is based in the use of pesticides that will eventually reach the aquatic environment, and could have harmful effects on non-target organisms such as fish (Ferreira, et al., 2010). Atrazine is an herbicide which acts by inhibiting the growth of a wide variety of plants such as algae and weeds. This herbicide is prone to leaching, reaching surface water and groundwater.

Currentlly in Brazil, it has been increased interest in fish farming, but an appropriate management of aquaculture species is required to be successful in this sector. Thus, it is necessary to constantly monitor water quality, the stocking density and feeding the animals. However, fish stress is almost inevitable in the routine management of fish farming or during the transport of animals or handling for biometrics. Besides these physics stressors, fish from farming can be exposed to a wide range of chemicals mostly pesticides from nearby

agricultural lands. The herbicide Atrazine may be related to sublethal effects with biochemical and histopathological alteration in fish tissues (Nwani et al., 2010). Some studies show that Atrazine can cause damage in the kidney and in the gill epithelium of carp and rainbow trout, besides reducing the olfactory sensitivity and tolerance to salinity in the Atlantic salmon (Venkatesan et al., 2014). The use of vitamin supplementation is one of the strategies identified in the literature for the mitigation of the effects of stress on fish (Diniz et al., 2012).

Vitamin C is essential micronutrient that works for maintenance of the normal physiological functions of fish, like reproduction, growth and response to stressors. This vitamin also acts as a reducing agent and as an antioxidant. However, teleost fish depends on exogenous source of vitamin C through the diet, since these animals have lost during evolution their ability to synthesize this vitamin due to the lack of the enzyme L-gulonolactone oxidase (Wang et al., 2003; Sarma et al., 2009; Roosta et al., 2014).

In aerobic organisms, reactive oxygen species (ROS), such as superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals, are continuously formed as a natural process. However exposure to xenobiotics or chemical pollutants may increase the production or reduce the elimination of these endogenous and exogenous ROS and can subsequently induce oxidative damage in biomolecules like proteins, lipids and DNA. Fish, like other animals, have protective enzymes and non-enzymes antioxidants which mitigate high levels of ROS (Özkan et al., 2012). Vitamin C as well as reduced glutathione (GSH) and non-protein thiols are part of the non-enzymatic antioxidant defense system. Vitamin C acts capturing free radicals and is related with vitamin E and glutathione cycle. Thus, vitamin C acts protecting tissues, membrane and cytosolic components from cell oxidative damage (Abraham, 2005; Özkan et al., 2012).

Jundiá (*Rhamdia quelen*) commonly called silver catfish (order Siluriformes, family Heptapteridae and subfamily Heptarinae) is a South American teleost fish that occurs from Southern Mexico to Central Argentina. This species is known for its zootecnic and organoleptic characteristics favorable to its development and tolerance to survive in low temperatures providing continual growth in winter, allowing its farming in Southern Brazil (Borges et al., 2007; Ferreira et al., 2010; Fukushima et al., 2012)

In our previous work (unpublished data) it was found that the amount of 1000 mg/kg vitamin C was adequate to improve the antioxidant ability of jundiá (*Rhamdia quelen*). Thus, the aim of this study was to verify if this amount of vitamin C would protect *Rhamdia quelen* against stress caused by exposure to Atrazine (10µg/L).

2.3 MATERIALS AND METHODS

2.3.1 Reagents

The herbicide Atrazine ($C_8H_{14}ClN_5$) was used pure form (99.9%) at a concentration of 10 µg/L.

2.3.2 Fish

A total of 40 *Rhamdia quelen* juveniles of both sexes (length 13,5 ± 1cm; weight 24 ± 1g) were obtained from a fish farming close to Santa Maria County (Rio Grande do Sul State, Brazil), and kept under laboratory conditions in plastic boxes of 250 liters and continuously aerated water. The fish were acclimated for 15 days, with a natural photoperiod (12h light/12h dark), and the averages of water parameters were: dissolved oxygen 7.0 ± 1.0 mg/L, temperature 23.0 ± 1.0°C, pH 7.1 ± 0.2, ammonia 0.05 ± 0.01µg/L and nitrite 0.04 ± 0.01mg/L. Pellet residues and feces were removed by suction. After the acclimation period, the fish were transferred to 45 liters boxes with constant aeration and stable temperature, and divided into groups.

2.3.3 Diet preparation

The experimental diets were prepared at the Pisciculture Laboratory from the Federal University of Santa Maria. Two diets were produced containing different concentrations of vitamin C (0 and 1000 mg / kg) (Table 1). All dry ingredients were blended, wet ingredients were subsequently added, and pellets of approximately 5 mm diameters were formed by grinding the mixture through a meat grinder. After drying, the diets were packed into small bags and kept at 4ºC until use. During the experiment, the fish were fed with 3% of their body weight per day (Menezes et al., 2014) and daily diet was divided into two equal meals at 09:00 and 16:00 hours.

Table 1 - Composition of experimental diets

	0 mg/kg	1000 mg/kg
Ingredients	Amount (g)	Amount (g)
Fish's flour	60.890	60.890
Starch miho	17.000	17.000
Celulose	12.580	11.580
Vitamin premix	3.000	3.000
Dicalcium phosphate	2.000	2.000
Calcareous	1.500	1.500
Soy oil	1.170	1.170
Cod-liver oil	1.000	1.000
Salt	0.500	0.500
Di methionine	0.360	0.360
Vitamin C coated	0.000	1.000
Total	1000.000	1000.000

2.3.4 Experimental design

Fish were divided randomly into four groups, 10 silver catfish in each group: (1) Control 0 mg/kg, (2) Control 1000 mg/kg, (3) Atrazine 0 mg/kg, (4) Atrazine 1000 mg/kg. After 30 days of feeding, groups 3 and 4 were subjected to exposure to Atrazine (10µg/L) for 96 hours.

The averages of water parameters were maintained equal to those of the acclimation period. Feces and pellet residues were also removed by suction.

2.3.5 Sample preparation

After the 96 hours of the exposure to atrazine, fish from all the groups were anaesthetized with eugenol (50µg/L) according to Cunha et al. (2010), and weight and length were measured. Then, the fish were euthanized by section of spinal cord. Samples of liver was quickly removed and stored at -80°C for further assays of oxidative damage parameters: glutathione S-transferase (GST) activity, glutathione peroxidase (GPx) activity, non-protein thiols (NPSH) levels, lipid peroxidation determination (TBARS), protein carbonyl assay and

ascorbic acid levels. The experiment was approved by Committee on animals care of the Federal University of Santa Maria, protocol number: 4302190116.

Protein determination

Protein was determinated by the Comassie Blue Method using bovine serum albumin as standard. Absorbance of samples was measured at 595 nm according to Bradford (1976).

Oxidative stress assays

The liver was homogenized with Tris-HCl buffer [50 mM] pH 7.4 and centrifuged at 3000 rpm for 10 min. This homogenate was used for all the following analyzes.

GST activity

GST (EC 2.5.1.18) activity was determined in liver by modification of procedure described by Habig et al. (1974). This analysis was performed in microplate spectrophotometer. The assay mixture consisted of 10 µL homogenate, 150 µL potassium phosphate buffer (TFK) [20 mM] pH 6.5 (maintained at 37°C in a water bath), 50 µL reduced glutathione (GSH) and 50 µL 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate. The absorbance was read in kinetic mode at 340 nm for 2 minutes. The GST activity was calculed using the extinction coefficient of 9.6 mM/cm and expressed in GS-DNB/ min/mg protein.

GPx activity

GPx activity was determined in liver by modification of the Paglia and Valentine's (1967) method. The analysis was performed in microplate. The assay mixture consisted of 10 µL homogenate and 260 µL of the system containing: NADPH, GSH, GR (Glutathione reductase), azide and potassium phosphate buffer (TFK) [100Mm] pH 7.0. The absorbance was read in kinetic mode at 340 nm for 2 minutes. The specific activity was determined using the extinction coefficient of 6.22 mM/cm and the GPx activity was expressed in µmol/min/mg protein.

NPSH levels

NPSH levels were determined in liver by modification of Ellman's method (1959). 100 µL of the homogenate was mixed with 100 µL 10% trichloroacetic acid (TCA), followed by centrifugation at 3000 rpm for 10 min. 30 µL of the supernatant was pipetted into microplate, 70 µL distilled water, 100 µL potassium phosphate buffer (TFK) [50 mM] pH 7.0, and 10 µL 5,5'-dithio-bis 2-nitrobenzoic acid (DTNB) [10 mM]. The microplate was incubated in the dark for 20 minutes. The absorbance was read in endpoint mode at 412 nm. NPSH levels were expressed as µmol SH/g of tissue.

Lipid peroxidation determination

Lipid peroxidation was estimated in liver by thiobarbituric acid reactive species (TBARS) production according to Buege and Aust method (1978) with some modifications. 100 µL of the homogenate was mixed with 100 µL 10% TCA, followed by centrifugation at 3000 rpm for 10 min. 100 µL of the supernatant was mixed with 100 µL 2-thiobarbituric acid (TBA) [0.67%]. The mixture was incubated at 100°C, in a water bath, for 30 minutes. 150 µL of the supernatant was pipetted into microplate. The absorbance was read in endpoint mode at 532 nm. The lipid peroxidation was expressed in MDA/mg protein.

Protein carbonyl assay

Protein carbonyl content was assayed in liver by modification of method described by Yan et al. (1995). 200 µL of the homogenate plus 800 µL distilled water were reacted with 150 µL 2,4 dinitrophenylhydrazine (DNPH) [10 nm] in 2N hydrochloric acid. After incubation at room temperature in the dark for 60 minutes, were added sequentially, 125 µL sodium dodecyl sulfate (SDS) 3%, 500 µL ethanol e 500 µL of heptane. This mixture was vortexed for 30 s and centrifuged at 3000 rpm for 15 minutes. Then, the protein isolated from the interface was washed by resuspension in ethanol/ethyl acetate (1:1). After it was added 250µL sodium dodecyl sulfate (SDS) 3% and vortexed again. The mixture was pipetted into a microplate. Assay was performed in duplicate and one blank tube incubated with 2N HCl without DNPH was included for each sample. The absorbance was read in endpoint mode at 370 nm. The total carbonylation was calculated using a molar extinction coefficient of 22.000 M/cm. The protein carbonyl content was expressed as nmol carbonyl/mg protein.

Ascorbic acid levels

Ascorbic acid was determined in liver by Roe's method (1954). 300 µL homogenate plus 100 µL distilled water, 100 µL TCA [13.3%] and 75 µL 2,4 dinitrophenylhydrazine (DNPH) were incubated at 37° C, in a water bath, for 3 h. Following the incubation, 500µL sulfuric acid (H_2SO_4) 65% was added to stop the reaction. The absorbance was read at 520 nm. The ascorbic acid content was expressed as µmol/g of tissue.

2.3.6 Statistical analysis

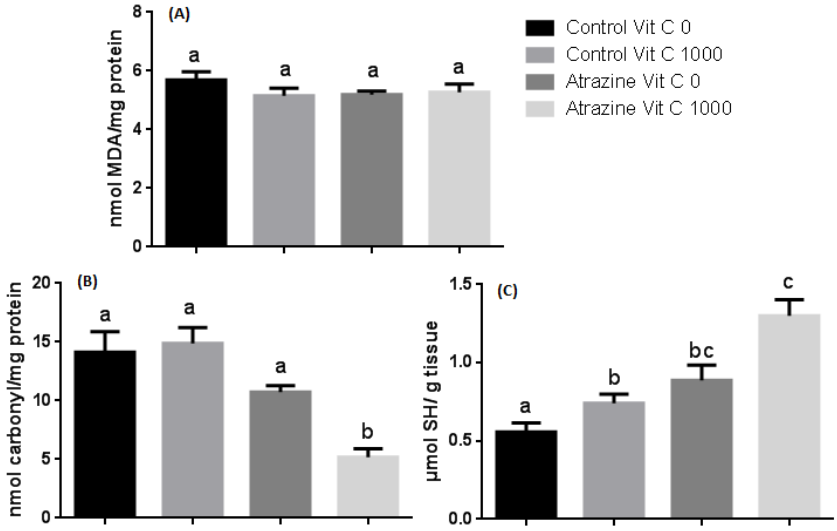
All data were analyzed by One-way ANOVA to test the effects of exposure to Atrazine, followed by Tukey's multiple comparisons test. Homogeneity of variances between groups was tested with Levene test. The values are presented as mean ± S.E.M. Differences were considered to be significant at a probability level of $p < 0.05$ between groups.

2.4 RESULTS

In liver there were no significant differences in lipid peroxidation between all the groups (Figure 4A). In relation to protein carbonyl content, there was a significant decrease just in group 4 exposed to Atrazine and fed with 1000 mg/kg of vitamin C (Figure 4B) compared to the other groups.

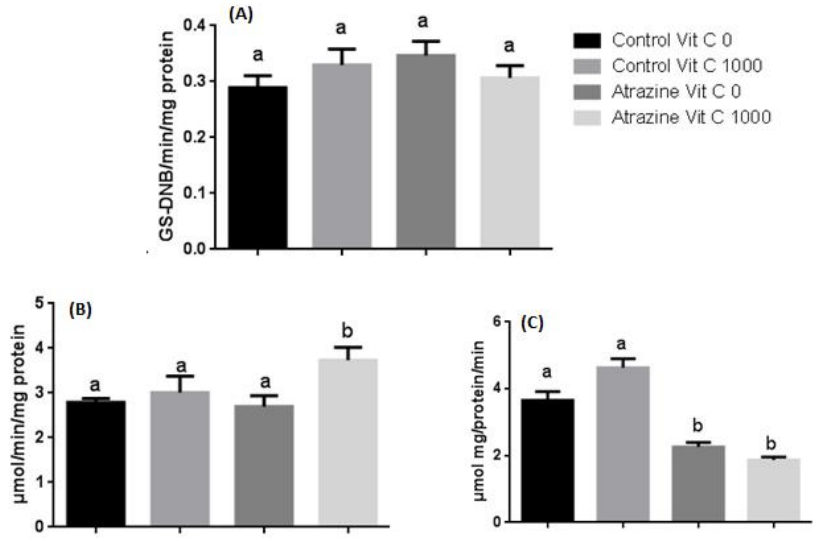
The NPSH (Figure 4C) levels in group 1 were significantly lower than in group 2. However the NPSH levels in group 3 was significantly higher than group 1. In the same way the NPSH levels in group 4 was significantly higher than group 2.

Figure 4 - Lipid peroxidation determination in liver (A), carbonyl protein content in liver (B) and NPSH levels in liver (C) of silver catfish fed with different vitamin C diets (0 and 1000 mg/Kg) and subjected to exposure to atrazine (10µg/L) for 96 hours. Different letters indicate differences between groups ($p<0.05$). Data are reported as mean ± S.E.M. (n =8).



In liver there were no significant changes in GST activity between all the groups (Figure 5A). In relation to GPx activity there was a significant increased in group 4 as compared to the other groups (Figure 5B). Unlike GPx activity, there was a significant decreased in CAT activity in both groups 3 and 4 (Figure 5C).

Figure 5 - GST activity in liver (A) GPx in liver (B) and CAT activity in liver (C), in silver catfish fed with different vitamin C diets (0 and 1000 mg/kg) and subjected to exposure to atrazine (10µg/L) for 96 hours. Different letters indicate differences between groups ($p < 0.05$). Data are reported as mean ± S.E.M (n= 8).



As expected group 2 presented the highest concentration of vitamin C (Table 2). However, the lowest values were shown in group 3, where fish were exposed to Atrazine but there was no supplementation with vitamin C.

Table 2 - Concentration of vitamin C (µmol/g tissue) in liver.

Groups	Vitamin C
(1) Control 0 mg/kg	67.20 ± 10.84 [a]
(2) Control 1000 mg/kg	138.67 ± 43.87 [b]
(3) Atrazine 0 mg/kg	24.43 ± 8.59 [c]
(4)Atrazine 1000 mg/kg	70.30 ± 21.71 [a]

Different letters indicate differences between groups ($p < 0.05$). Data are reported as mean ±S.E.M (n=10).

2.5 DISCUSSION

Fish are usually used as sentinel organisms for toxicological studies, because they play important roles in the accumulation of toxic substances, and respond to low concentrations of xenobiotics. One of the metabolic processes most affected by exposure to contaminants is the tight relationship between ROS production and their elimination, an oxidative stress condition. This situation of stress can induce damage to tissues and cells due to, for example, lipid peroxidation (Ferreira et al., 2010). Therefore, fish oxidative biomarkers are often used to detect, at an early stage, the effects of environmental disturbance, mainly those caused by pesticides (Nwani et al., 2010). Vitamin C is known for its antioxidant function, since this vitamin acts as electron donor in non-enzymatic reactions acting as a reducing agent preventing oxidation of other compounds (Özkan et al., 2012).

Analyses of TBARS are popular and commonly used methods to assess oxidative damage by peroxidation of lipids (Menezes et al., 2016). However, our results indicate that there was no significant difference in the levels of lipid peroxidation neither between the controls groups nor those exposed to Atrazine. Mela et al. (2013) exposed *Rhamdia quelen* to Atrazine, in the same concentration, and similarly to our study, and this herbicide did not cause lipid peroxidation. Özkan et al. (2012), in a study of diet supplemented with vitamin C, followed by exposure to cypermethrin found no significant change in tissues TBARS content. As this absence of lipid peroxidation occurred both in the group fed with vitamin C and in the group without this vitamin in their diet, we can deduce that the protection against lipid

peroxidation has occurred through other antioxidant mechanisms, and not due to protective action of vitamin C.

Proteins are involved in virtually all cellular processes, playing a wide range of functions in the body. The rate of protein carbonyl content serves as protein biomarkers of oxidative damage in various diseases (Dayanand et al., 2012). A decrease in protein carbonyl levels was observed only in group 4. This result may be associated to increased GPx activity in the same group. This reduction of carbonyls in the liver occurred possibly due to GPx action. Through these results we can deduce that the vitamin C in the diet of this group may has helped in the defense against the oxidation of proteins.

Our results demonstrate that the level of NPSH was found to be higher in the animals that were supplemented with vitamin C. So a supplement of 1000 mg/kg of vitamin C, during 30 days, was sufficient to increase the levels of NPSH and consequently increase in these non-enzymatic antioxidant defenses. This increase in NPSH levels may be related to the increase in GPx in group 4, since vitamin C, GPx and GSH are intrinsically related (Foyer and Noctor, 2011).

A significant enzyme in the detoxification process connected to glutathione system is GST (Menezes et al., 2016). Although GST is involved directly with detoxification of xenobiotics via metabolic phase II reaction, in this research, herbicide Atrazine did not affect the activity of GST, which seems to enhance the risk of oxidative stress since it did not increase the cell protection capacity. However, other factors such as the type of herbicide, its concentration, and exposure time, may also have influence in these results.

It is known that vitamin C interacts with vitamin E and selenium in maintaining glutathione peroxidase enzyme activity (GPx). This enzyme is important in eliminating H_2O_2 and lipid peroxides produced in metabolism, protecting tissues from oxidative damage (Navarro et al., 2009). Our results indicate an increase in GPx activity in the group of animals exposed to Atrazine supplemented with 1000 mg/kg of vitamin C. The induction of GPx activity at group 4 represents a valuable defense against the pro-oxidant effect of reactive oxygen species (ROS). Thus, this increase in GPx activity can reinforce the importance of vitamin C in the detoxification capacity against H_2O_2.

In the present study, CAT activity decreased in both groups exposed to Atrazine. This decrease in CAT activity may be a consequence of H_2O_2 overproduction provoked by exposure to Atrazine. Therefore, this decrease of CAT in liver of fish exposed to Atrazine may reflect signs of effective toxicity (Mela et al., 2013). Other studies of fish exposed to agrochemicals also reported decreases in CAT activity (Ferreira et al., 2010).

A higher concentration of ascorbic acid in the liver of animals supplemented with vitamin C was expected when compared to those without this vitamin. However, the amount of ascorbic acid was significantly lower after exposure to Atrazine. This demonstrates that vitamin C was used by these animals in order to mitigate ROS produced from exposure to Atrazine. The antioxidant improvement caused by vitamin C can be related to increasing GPx activities and NPSH levels in group 4 since both participate in the ascorbic acid cycle.

Therefore, the results indicated the potential effects of Atrazine to induce oxidative damage in liver of *Rhamdia quelen*. In the same way, it was demonstrated the capacity of vitamin C to attenuate ROS through increased levels of NPSH and GPx activity which appeared to decrease protein carbonyl content in animals feeding with diet supplemented with this vitamin. The liver was the tissue chosen for this study, since it is the most important target organ of metabolism and detoxification of many toxicants.

In conclusion, vitamin C could be able to improve the protective capability in liver when animals are exposed to agrochemicals, like Atrazine. Thus, a supplemented diet with adequate amounts of vitamin C could provide both fish nutrition as well as a protection against stress factors such as those found in a fish farm. Moreover, this supplementation is economically viable and could enhance the potential of aquaculture fish species, such as *Rhamdia quelen*.

REFERENCES

ABRAHAM, P. Vitamin C may be beneficial in the prevention of paracetamol-induced renal damage. **Clinical and Experimental Nephrology**. v.9, p.24-30, 2005.

BORGES, A.; SCOTTI, L. V.; SIQUEIRA, D. R.; ZANINI, R.; DO AMARAL, F.; JURINITZ, D. F.; WASSERMANN, G. F. Changes in hematological and serum biochemical values in jundiá *Rhamdia quelen* due to sub-lethal toxicity of cypermethrin. **Chemosphere**. v.69, p.920-926, 2007.

BRADFORD, M. M. A. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. **Analytical Biochemistry**. v.72, p.248-254, 1976.

BUEGE, J. A.; AUST, S. D. Microssomal lipid peroxidation. **Methods Enzymol**. v.52, p.302-309, 1978.

CUNHA, M. A.; ZEPPENFELD, C. C.; GARCIA, L. O.; LORO, V. L.; FONSECA, M. B.; EMANUELLI, T.; VEECK, A. P. L.; COPATTI, C. E.; BALDISSEROTTO, B. Anesthesia of silver catfish with eugenol: time of induction, cortisol response and sensory analysis of fillet. **Ciência Rural**, Santa Maria. v.40, n.10, p. 2107-2114, 2010.

DAYANAND, C. D.; VEGI, P. K.; KUTTY, A. V. Protein carbonyl content as a stable Oxidative stress marker in Type II Diabetes. **International Journal of Biological and Medical Research**. v. 3, n. 4, p. 2362-2365, 2012.

DINIZ, N. M.; HONORATO, C. A. Algumas alternativas para diminuir os efeitos do estresse em peixes de cultivo - revisão. **Arquivos de ciências veterinárias e zoologia da UNIPAR**. v.15, n.2, p.149-154, 2012.

ELLMAN, G. L. Tissue sulfhydryl groups. **Archives of Biochemistry and Biophysics**. v.82, p.70-77, 1959.

FERREIRA, D.; DA MOTTA, A. C.; KREUTZ, L. C.; TONI, C.; LORO, V. L.; BARCELLOS, L. J. G. Assessment of oxidative stress in *Rhamdia quelen* exposed to agrichemicals. **Chemosphere**. v.79, p.914–921, 2010.

FOYER, C. H.; NOCTOR, G. Ascorbate and Glutathione: The Heart of the Redox Hub. **Plant Physiology**. v.155, p.2-18, 2011.

FUKUSHIMA, H.; BAILONE, R. L.; WEISS, L. A.; MARTINS, M. L.; ZANIBONI-FILHO, E. Triploidy in the hematology of jundia juveniles (Siluriformes: Heptapteridae). **Brazilian Journal of Biology**. v.72, n.1, p.147-151, 2012.

HABIG, W. H., PABST, M. J., JACOBY, W. B. Glutathione S-transferase, the first enzymatic step in mercapturic acid formation. **Journal of Biological Chemistry**. v.249, p.7130-7139, 1974.

MELA, M.; GUILOSKI, I. C.; DORIA, H. B.; RANDI, M. A. F.; DE OLIVEIRA RIBEIRO, C.A.; PEREIRA, L.; MARASCHI, A. C.; PRODOCIMO, V.; FREIRE, C.A.; SILVA DE ASSIS, H. C. Effects of the herbicide atrazine in neotropical catfish (*Rhamdia quelen*). **Ecotoxicology and Environmental Safety**. v.93, p.13-21, 2013.

MENEZES, C.; MARINS, A.; MURUSSI, C.; PRETTO, A.; LEITEMPERGER, J.; LORO, V. L. Effects of diphenyl diselenide on growth, oxidative damage, and antioxidant response in silver catfish. **Science of the Total Environment**. v.542, p.231-237, 2016.

NAVARRO, R. D.; FILHO, O. P. R.; FERREIRA, M. W.; PEREIRA, F. K. S. A importância das vitaminas E, C e A na reprodução de peixes. **Revista Brasileira de Reprodução Animal**, Belo Horizonte. v.33, n.1, p.20-25, 2009.

NWANI, C. D.; LAKRA, W. S.; NAGPURE, N. S.; KUMAR, R.; KUSHWAHA, B.; SRIVASTAVA, S. K. Toxicity of the herbicide Atrazine: effects on lipid peroxidation and activities of antioxidant enzymes in the freshwater fish *Channa Punctatus* (Bloch). **International Journal of Environmental Research and Public Health**. v.7, p.3298-3312, 2010.

ÖZKAN, F.; GÜNDÜZ, S. G.; BERKÖZ, M.; HUNT, A. Ö.; YALM, S. The protective role of ascorbic acid (vitamin C) against chlorpyrifos-induced oxidative stress in *Oreochromis niloticus*. **Fish Physiology and Biochemistry**. v.38, p.635–643, 2012.

PAGLIA, D. E.; VALENTINE, W. N. Studies on the quantitative and qualitative characterization of erythrocytes glutathione peroxidase. **Journal of Laboratory and Clinical Medicine**. v.70, n.1, p.158-169, 1967.

ROE, J. H. Chemical determinations of ascorbic, dehydro-ascorbic, and diketogulonic acids. In: Glick D (ed) **Methods of biochemical analysis**. Interscience, New York. v.1. p.115-139, 1954.

ROOSTA, Z.; HAJIMORADLOO, A.; GHORBANI, R.; HOSEINIFAR, S. H. The effects of dietary vitamin C on mucosal immune responses and growth performance in Caspian roach (*Rutilus rutilus caspicus*) fry. **Fish Physiology and Biochemistry**. v.40, p.1601-1607, 2014.

SARMA, K.; PAL, A. K.; SAHU, N. P.; AYYAPPAN, S.; BARUAH, K. Dietary high protein and vitamin C mitigates endosulfan toxicity in the spotted murrel, *Channa punctatus* (Bloch, 1793). **Science of the Total Environment**. v.407, p.3668–3673, 2009.

VENKATESAN, S.; BALAMURUGAN, V.; SUSINDREN,P.; SUNDARESAN, A.; RAJKUMAR, K.; VASANTHI, K. Protective efficacy of spirulina against toxicity on GSH-GPx activities in the fresh water fish *Cyprinus carpio* (Linn). **International Journal of Modern Research and Reviews**. v.2, p.511-516, 2014.

WANG, X.; KIMA, K. W.; BAI, S. C.; HUH, M. D.; CHO, B. Y. Effects of the different levels of dietary vitamin C on growth and tissue ascorbic acid changes in parrot fish (*Oplegnathus fasciatus*). **Aquaculture**. v.215, p.203-21, 2003.

YAN, L. J.; TRABER, M. G.; PACKER, L. Spectrophotometric method for determination of carbonyls in oxidatively modified apolipoprotein B of human low-density lipoproteins. **Analytical Biochemistry**. v.228, p.349-351, 1995.

CONCLUSÕES

• Uma ração suplementada com 500 ou 1000 mg/kg de vitamina C, administrada por um período mínimo de 28 dias, contribui para a melhoria da capacidade antioxidante de *Rhamdia quelen*. Sugerindo que rações suplementadas nestas quantidades podem ser úteis na aquacultura, prevenindo possíveis danos provocados pelo estresse oxidativo.

• A quantidade de 10µg/L de Atrazina é suficiente para induzir danos no fígado de jundiás, expostos pelo período de 96 horas.

• A quantidade de 1000 mg/kg de vitamina C, administrada no período de 30 dias, foi suficiente para atenuar os possíveis danos provocados por meio das ROS.

• A quantidade de 1000 mg/kg de vitamina C, administrada no período de 30 dias, é capaz de melhorar a capacidade de proteção do fígado de animais expostos a xenobióticos, como a Atrazina.