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AVALIAÇÃO DE PARÂMETROS BIOQUÍMICOS EM JUNDIÁS EXPOSTOS A TRÊS FORMULAÇÕES COMERCIAIS DE GLIFOSATO

DISSERTAÇÃO DE MESTRADO

Camila Rebellatto Murussi

Santa Maria, RS, Brasil

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AVALIAÇÃO DE PARÂMETROS BIOQUÍMICOS EM JUNDIÁS EXPOSTOS A TRÊS FORMULAÇÕES COMERCIAIS DE GLIFOSATO

Camila Rebellatto Murussi

Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Mestre em Ciências Biológicas: Bioquímica Toxicológica**

Orientadora: Prof[a]. Dr[a]. Vania Lucia Loro

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Camila Rebellatto Murussi

como requisito parcial para obtenção do grau de
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Carlos e Dirce Murussi
a minha tia e avó,
Lurdes e Noeli Murussi,
que sempre me deram força e
acreditaram no meu sonho!

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RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria, RS, Brasil

AVALIAÇÃO DE PARÂMETROS BIOQUÍMICOS EM JUNDIÁS EXPOSTOS A TRÊS FORMULAÇÕES COMERCIAIS DE GLIFOSATO

Autora: Camila Rebellatto Murussi
Orientadora: Vania Lucia Loro
Data e Local da Defesa: Santa Maria, 16 de agosto de 2013.

Devido ao aumento na produção de alimentos nos últimos anos, o uso de pesticidas tem aumentado significativamente. Estes pesticidas contaminam os ecossistemas aquáticos e prejudicam organismos não-alvos, como os peixes. Sendo assim o objetivo deste trabalho foi avaliar possíveis diferenças **em** três formulações comerciais de glifosato (Orium®, Roundup Original® e Biocarb®) sobre alterações em parâmetros bioquímicos de jundiás (*Rhamdia quelen*) expostos a concentrações sub letais (96 h). Os parâmetros analisados foram TBARS (substâncias reativas ao ácido tiobarbitúrico), catalase (CAT), superóxido dismutase (SOD), glutationa-S-transferase (GST), glicose, glicogênio, lactato, proteína, alanina aminotransferase (ALT) e aspartato aminotransferase (AST). Os níveis de TBARS no fígado aumentaram em todas as concentrações e herbicidas testados. No músculo os níveis de TBARS diminuíram em ambas as concentrações de Orium® e aumentaram em 2,5 mg/L de Biocarb®. No cérebro os níveis de TBARS aumentaram em todas as concentrações dos herbicidas testados com exceção de 5,0 mg/L de Roundup Original®. Nas brânquias os níveis de TBARS aumentaram em 2,5 de Orium® e 5,0 mg/L de Roundup Original®, enquanto que em 5,0 mg/L de Orium® foram reduzidos. A atividade da SOD em fígado aumentou em ambas as concentrações de Orium®, 2,5 mg/L de Original® e 5,0 mg/L de Biocarb®. A atividade da CAT em fígado diminuiu em todas as concentrações e herbicidas testados. A atividade da GST em fígado e brânquias diminuiu em 2,5 mg/L de Biocarb®, enquanto que em cérebro a atividade da GST aumentou em ambas as concentrações de Orium®. Em plasma, os níveis de glicose aumentaram em ambas as concentrações de Orium® e Biocarb®. Os níveis de lactato plasmático aumentaram na concentração de 2,5 mg/L em todos os herbicidas testados. ALT plasmática diminuiu em 2,5 mg/L de Biocarb®, enquanto que AST aumentou em todas as concentrações dos herbicidas testados com exceção de 2,5 mg/L de Biocarb® que diminuiu. Em fígado, o glicogênio aumentou em todas as concentrações dos herbicidas testados. A glicose e o lactato hepático diminuíram em todas as concentrações dos três herbicidas testados. Os níveis de proteína do fígado aumentaram em ambas as concentrações de Orium® e diminuíram em 2,5 mg/L de Biocarb®. No músculo os níveis de glicogênio aumentaram em 2,5 mg/L de Orium® e Biocarb® e 5,0 mg/L de Roundup Original®. A glicose no músculo diminuiu em 5,0 mg/L de Orium® e Roundup Original®. Os níveis de lactato no músculo aumentaram em 2,5 mg/L de Orium® e diminuíram em ambas as concentrações de Biocarb®. A proteína no músculo aumentou em ambas às concentrações de Orium® e diminuiu em ambas as concentrações de Roundup Original® e Biocarb®. Os níveis de glicose no cérebro e nas brânquias aumentaram em todas as concentrações dos produtos testados com exceção de 5,0 mg/L de Biocarb® em brânquias. Com estes resultados podemos concluir que apesar de as formulações comerciais possuírem o mesmo princípio ativo estas atuam de forma diferente no organismo do peixe e causam danos oxidativos por diversos mecanismos, afetando de forma diferente os tecidos analisados.

Palavras-chave: Estresse oxidativo. Glifosato. Parâmetros bioquímicos. *Rhamdia quelen*.

ABSTRACT

Dissertation of Master's Degree
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Federal University of Santa Maria, RS, Brazil

EVALUATION OF BIOCHEMICAL PARAMETERS IN SILVER CATFISH EXPOSED TO THREE COMMERCIAL FORMULATIONS OF GLYPHOSATE

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Adviser: Vania Lucia Loro
Date and place of the defense: Santa Maria, August, 16th, 2013.

Due to the increase in food production in the last years, the use of pesticides has also increased significantly. These pesticides pollute aquatic ecosystems and harm non-target organisms, such as fish. Therefore, the aim of this study was to evaluate possible differences of three commercial formulations of glyphosate (Orium®, Roundup Original® and Biocarb®) on changes in biochemical parameters of silver catfish (*Rhamdia quelen*) exposed to sub-lethal concentrations of 2.5 and 5.0 mg/L for 96 hours. The parameters analyzed were the TBARS levels (thiobarbituric acid reactive species), catalase (CAT), superoxide dismutase (SOD), glutathione-S-transferase (GST), glucose, glycogen, lactate, protein, alanine aminotransferase (ALT) and the aspartate aminotransferase (AST). The TBARS levels were increased in the tested concentration of the herbicides. In the muscle, the TBARS levels were decreased in both concentrations of Orium® and higher in 2.5 mg/L of Biocarb®. In the brain, the TBARS levels were higher in all concentrations of the tested herbicides with the exception of 5.0 mg/L of Roundup Original®. The gills TBARS levels were increased in the 2.5 mg/L concentration of Orium® and 5.0 mg/L of Roundup Original®. However in 5.0 mg/L of Orium®, they were decreased. The SOD activity in the liver increased in both concentrations of Orium®, 2.5 mg/L of Roundup Original® and 5.0 mg/L of Biocarb®. CAT activity decreased in all concentrations of the herbicides tested. The GST activity in the liver and gills decreased in 2.5 mg/L of Biocarb®. Yet, in the brain, the GST activity increased in both concentrations of Orium®. In the plasma, the glucose levels increased in both concentrations of Orium® and Biocarb®. The plasma lactate levels increased in 2.5 mg/L of all tested herbicides. Plasma ALT was decreased in 2.5 mg/L of Biocarb®. However, the AST increased in all concentrations of the herbicides tested with the exception of 2.5 mg/L of Biocarb®, which showed decreased levels. In the liver, the glycogen increased and the glucose and hepatic lactate decreased in all concentrations of the tested herbicides. The protein levels in the liver increased in both concentrations of Orium® and decreased in 2.5 mg/L of Biocarb®. In the muscle, the glycogen levels increased in 2.5 mg/L of Orium®, Biocarb® and 5.0 mg/L of Original®. The glucose in the muscle decreased in 5.0 mg/L of Orium® and Roundup Original®. The lactate levels in the muscle increased in 2.5 mg/L of Orium® and decreased in both concentrations of Biocarb®. The protein in the muscle increased in both concentrations of Orium® and decreased in both concentrations of Roundup Original® and Biocarb®. The glucose levels in the brain and gills increased in all concentrations of the products tested with the exception of 5.0 mg/L of Biocarb® in the gills. With these results, we can conclude that although commercial formulations have the same active principle, they act differently in the organism of the fish, causing oxidative damage by various mechanisms, affecting the tissues differently.

Keywords: Biochemistry parameters. Glyphosate. Oxidative stress. *Rhamdia quelen.*

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LISTA DE ABREVIAÇÕES

ALT - Transaminase Glutâmica Pirúvica

AMPA - Ácido Aminometilfosfônico

ANVISA – Agência Nacional de Vigilância Sanitária

AST – Transaminase Glutâmica Oxalacética

CAT - Catalase

CO_2 – Dióxido de carbono

EPSPs - 5-enolpiruvoilshikimate-3-fosfato sintase

EROS - Espécies Reativas ao Oxigênio

GLY – Glifosato

GSH - Glutationa Reduzida

GST - Glutationa-S-Transferase

H_2O_2 – Peróxido de Hidrogênio

IPA – Sal de Isopropilamina

O^- - Ânion Superóxido

OH – Radical Hidroxila

POEA - Monolaureato de Polioxietileno

SINDAG - Sindicato Nacional da Indústria para Defesa Agrícola

SOD - Superóxido Dismutase

TBARS - Substâncias Reativas ao Ácido Tiobarbitúrico

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1. INTRODUÇÃO

Com a finalidade de aumentar a produção de alimentos, a lucratividade das culturas e a redução no custo de plantio, grande quantidade de pesticidas são liberados diariamente no ambiente, principalmente em lavouras e pastagens (PRIMEL et al., 2005; ÇAVAS; KONEN, 2007). Dessa forma, pesticidas, incluindo os herbicidas que controlam plantas daninhas tiveram seu consumo acentuado. De acordo com dados do SINDAG (2012), o uso de pesticidas cresceu em relação a 2011, sendo que a cultura da soja transgênica absorveu 47,2% do total de pesticidas, sendo que os herbicidas especificamente tiveram 32% deste total de consumo.

Dentro da classe geral de pesticidas, os herbicidas são a maior preocupação devido a sua facilidade de lixiviação até corpos de água. Contudo, estes químicos podem chegar aos ecossistemas aquáticos por deriva, escoamento superficial ou volatilização (Fig. 1) (TOMITA; BEYRUTH, 2002).

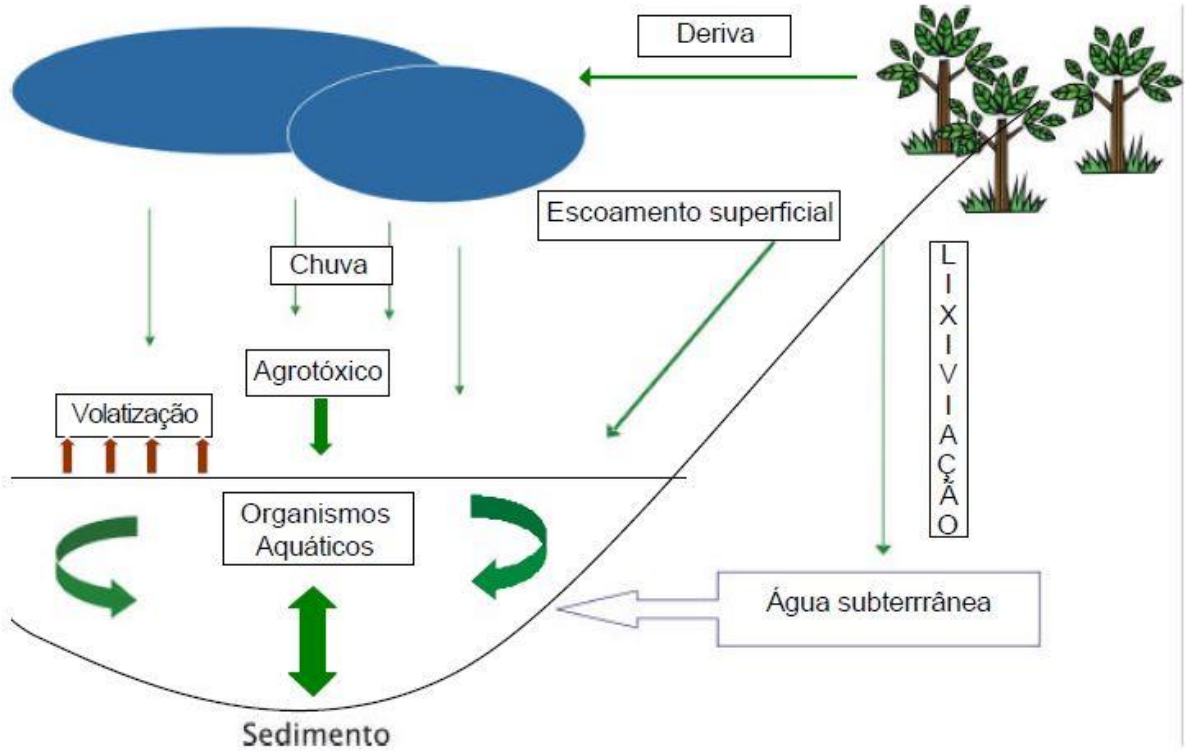

FIGURA 1 – Movimento dos pesticidas em ecossistemas aquáticos. (Adaptado de NIMMO, 1985).

Quando os pesticidas estão em ambientes aquáticos pode-se destacar a presença destes em resíduos no solo, água, ar, nas plantas e animais. A contaminação de ambientes aquáticos tem se tornado um problema de nível global relatado em diversos estudos (HUBER et al., 2000; CEREJEIRA et al., 2003; SPALDING et al., 2003). A crescente utilização de herbicidas nos últimos anos foi atribuída à cultura da soja transgênica, que devido à modificação genética realizada pela Monsanto tornou a soja resistente ao herbicida Roundup®, o qual tem por principio ativo o glifosato **(**LUSHCHAK et al., 2009**).** Contudo, o herbicida glifosato pode ser aplicado em 26 culturas além da soja (MAPA 2010).

Segundo a ANVISA (2010) o glifosato (Fig. 2), pertence ao grupo químico de glicina substituída, pertencendo à classe toxicológica IV (pouco tóxico). A legislação brasileira em âmbito legal não apresenta limites legais da presença do glifosato na água ou solo. Sua aplicação nas culturas geralmente é por pulverização, na qual a planta absorve o herbicida pelas folhas e caulículos novos, de modo sistêmico. Seu modo de ação é por inibição da enzima 5-enolpiruvoilshikimate-3-fosfato sintase (EPSPs) que age na rota de síntese dos aminoácidos aromáticos essenciais (fenilalanina, tirosina e triptofano), os quais são necessários para a síntese de outros compostos posteriores (AMARANTE Jr. et al., 2002). Devido a este modo de ação a planta apresenta aspecto "amarelado" e seca durante dias ou semanas. As concentrações utilizadas em arroz e soja variam de 0,36 a 2,16 mg/L (RODRIGUES; ALMEIDA, 2005).

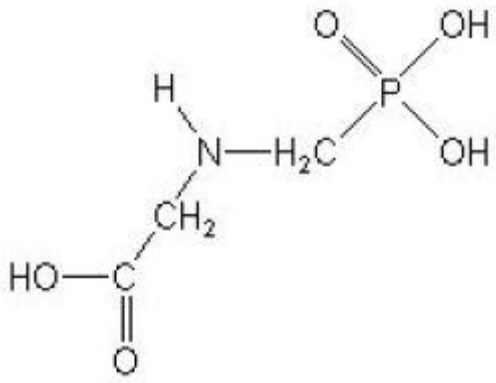

FIGURA 2 – Molécula de glifosato (ANVISA, 2010)

Sua formulação mais conhecida é o Roundup Original®, considerada de amplo espectro e não seletiva para plantas daninhas (ÇAVAS; KONEN, 2007). Sua formulação é composta pelo glifosato (Gly) na forma de sal de isopropilamina (IPA) e um surfactante, o monolaurato

de polioxietileno (POEA), que aumenta a eficácia do herbicida por facilitar a penetração deste pela cutícula da planta (SOLOMON; THOMPSON, 2003). A formulação comercial a base de glifosato é considerada mais tóxica quando comparada ao glifosato puro, devido à interferência do surfactante (POEA) (PEIXOTO, 2005; TSUI; CHU, 2008).

A meia-vida do glifosato em ambientes aquáticos pode variar de 7 dias a 4 meses. Quando dissipado na água ou na microflora do solo, o glifosato é biodegradado em ácido aminometilfosfônico (AMPA) e CO_2 (GIESY et al., 2000; GLUSCZAK et al., 2011). Devido sua alta solubilidade em água (12 g/L a 25ºC), altas concentrações de glifosato foram encontradas em rios próximos de áreas cultivadas no sul do Brasil (AMARANTE Jr. et al., 2002; DA SILVA et al., 2003). Dessa forma, é conhecido que pesticidas em ambientes aquáticos podem afetar a função dos sistemas biológicos, causando impacto em diversos níveis de organização, incluindo os peixes (VAN DER OOST et al., 2003). Vários estudos demonstram os efeitos causados pelo glifosato em diversas espécies de peixes (LUSHCHAK et al., 2009; GUILHERME et al., 2010; MODESTO; MARTINEZ, 2010a; CATTANEO et al., 2011). A formulação completa de cada produto comercial, não é divulgada pelas empresas produtoras, contudo se acredita que a resposta dos peixes também é atribuída aos ingredientes inertes, que variam em cada formulação comercial.

Neste contexto de contaminação ambiental, os peixes teleósteos tem se mostrado bons modelos experimentais devido Ao fato de algumas respostas bioquímicas serem semelhantes as observadas em mamíferos e outros vertebrados (SANCHO et al., 2000). Um exemplo de teleósteo utilizado em experimentação é o jundiá, (*Rhamdia quelen*) (Fig. 3), que tem importante papel no ambiente aquático no sul do Brasil. Esta espécie é nativa e tem grande importância comercial nesta região devido à alta qualidade atribuída aos filés desta espécie. Este peixe suporta o inverno e apresenta rápido crescimento no verão, resultando de um ganho de peso de 600 – 800 g em um período de 8 meses (BARCELLOS et al., 2004).

FIGURA 3 – Exemplar de *Rhamdia quelen* (FISHBASE, 2009)

Vários estudos mostram nesta espécie os efeitos causados por pesticidas (KREUTZ et al., 2008; CERICATO et al., 2009; MENEZES et al., 2011). Com isso, os órgãos dos peixes se tornam o principal alvo de pesquisa na busca de identificar como o peixe exposto está reagindo para eliminar ou se adaptar ao pesticida. O fígado é um órgão essencial para o metabolismo, detoxificação e excreção de substâncias tóxicas no peixe (CRESTANI et al., 2007). Enquanto que, as brânquias são responsáveis pela osmoregulação. Entretanto, as brânquias constituem o primeiro órgão que entra em contato com o agente tóxico (DURMAZ et al., 2006). O sangue dos peixes é extremamente sensível a poluentes, podendo assim ser usado como meio de avaliação de parâmetros de toxicidade a agrotóxicos através de alterações hematológicas e metabólicas (ROCHE; BOGÉ, 2000; SANCHO et al., 2000; CRESTANI et al., 2006). Porém outros órgãos, como cérebro e músculo, são importantes, por expressarem respostas específicas a pesticidas (CRESTANI et al., 2006; FONSECA et al., 2008).

A maioria dos pesticidas podem causar dano oxidativo em peixes e alterar diversas funções metabólicas em função desta exposição (PARVEZ et al., 2006;MENEZES ET AL. 2011). Quando ocorre uma situação de estresse oxidativo, é indicativo que neste organismo ocorreu uma falha no sistema de defesa antioxidante fazendo aumentar a produção de espécies reativas de oxigênio (EROs) (CARDOSO et al., 2006), como peróxido de hidrogênio (H_2O_2), ânion superóxido (O^-) e radicais hidroxila (OH). Devido a alta reatividade destas EROs pode ocorrer danos em lipídeos, proteínas, carboidratos e ácidos nucléicos (HERMES-LIMA, 2004). Na tentativa de neutralizar as EROs, os animais têm um siste6ma de defesa antioxidante enzimático constituído por enzimas como a superóxido dismutase (SOD), catalase (CAT), entre outras e um sistema antioxidante não-enzimático com a glutationa reduzida (GSH) (MODESTO; MARTINEZ, 2010a).

Porém, quando estas defesas são insuficientes, as EROs causam sérios danos na membrana, como a peroxidação lipídica avaliada através das substâncias reativas ao ácido tiobarbitúrico (TBARS) (SCANDALIOS, 2005). A primeira enzima que atua defendendo o organismo contra dano oxidativo é a SOD, responsável por catalisar a conversão do ânion superóxido em peróxido de hidrogênio. Após esta conversão, o peróxido de hidrogênio é transformado em água e hidrogênio molecular, via CAT que está localizada nos peroxissomos das células (GÜL et al., 2004; MODESTO; MARTINEZ, 2010b).

A glutationa-S-transferase é composta por 3 famílias de enzimas (citosólica, mitocondrial e microssomal), que atuam na detoxificação do organismo contra xenobióticos e também na proteção dos tecidos contra situações de estresse oxidativo gerado por poluentes (MASELLA et al., 2005; ZHANG et al., 2005). Além disso, fatores como tempo de exposição e

concentração do pesticida podem influenciar nos níveis metabólicos, enzimáticos e comportamentais. Com isso, podem ser observados diferentes efeitos tóxicos nos peixes expostos (ORUÇ; ÜNER, 1999; FERNANDES-VÉGA et al., 2002; TONI et al., 2013).

Alguns parâmetros metabólicos representam uma boa ferramenta para avaliar os efeitos dos contaminantes e também para o monitoramento ambiental. Dosagens bioquímicas em plasma e órgãos demonstram a situação metabólica do peixe exposto ao estressor. As células dos peixes assim como de mamíferos, apresentam a glicose como principal fonte energética. Quando ingerida acima da necessidade, a glicose é polimerizada e armazenada na forma de glicogênio no músculo e no fígado, sendo controlada pela ação de hormônios e enzimas, ou convertida à gordura (SILVEIRA et al., 2009). Como forma de armazenamento de energia, o glicogênio pode ser gasto em poucos minutos em caso de necessidade, e podendo ser restabelecido após um período de 24 horas. Ainda assim, o glicogênio é muito utilizado como combustível em momentos de estresse ambiental (CYRINO et al., 2000).

As proteínas são essenciais à vida devido a sua importante função estrutural, e por representar a base celular de tecidos e órgãos. Atuam como catalisadores enzimáticos em reações bioquímicas, carreadores de constituintes do plasma e atuam na defesa do organismo, como anticorpos (MELO et al., 2009). Ainda em seu metabolismo, as proteínas podem demonstrar se o organismo está ou não provendo desta fonte como energia no momento de estresse causado pelo pesticida. Dessa forma, o metabolismo proteico é envolvido na produção de energia por via aeróbia. Contudo, quando o organismo necessitar de um aporte maior de energia este poderá utilizar-se da via anaeróbia para dar suprimento energético, formando o lactato. A determinação de lactato no plasma e nos órgãos é um bom indicador de acúmulo de ácido láctico, devido ao aumento do exercício físico ou gerado pela exposição a substâncias estressoras (BARTON et al., 2002).

O plasma pode demonstrar a situação metabólica dos órgãos do peixe. Por meio da determinação de glicose, proteína, lactato, amino ácidos e amônia, além de enzimas é possível detectar alterações no funcionamento e a adaptação do animal diante dos poluentes como os herbicidas. As transaminases aspartato aminotransferase (AST) e alanina aminotransferase (ALT), presentes no plasma, também são usadas como biomarcadores de hepatotoxicidade, e utilizadas como indicadores de danos hepáticos relacionados com alterações no funcionamento deste órgão (CRESTANI et al., 2007).

Desde a implantação do nosso grupo de pesquisa na Universidade Federal de Santa Maria, coordenado pela professora Vania Lucia Loro, veem sendo realizadas pesquisas relacionadas ao estresse oxidativo e determinações bioquímicas em peixes expostos a diferentes

pesticidas. Estes estudos relacionam o dano causado por pesticidas tanto no ambiente como em condições laboratoriais em peixes de várias espécies como jundiá (*R. quelen*), carpa (*Cyprinus carpio*), piava (*Leporinus obtusidens*) entre outras.

Estudos prévios envolvendo o herbicida glifosato, foram realizados em nosso laboratório por GLUSCZAK et al., (2006; 2007; 2011); FERREIRA et al., (2010); SALBEGO et al., (2010); MENEZES et al., (2011); CATTANEO et al., (2011). Analisando estes artigos, nos quais foi utilizado glifosato, surgiu a necessidade da comparação entre as formulações comerciais, por sabermos da existência de mais de 100 formulações contendo este herbicida. A partir deste contexto selecionamos o jundiá, por ser um peixe nativo da região, e delineamos o presente estudo.

Desse modo, considerando o intenso uso do herbicida glifosato em cultivares da nossa região é relevante o conhecimento dos diferentes efeitos que três formulações comerciais contendo glifosato podem causar em peixes nativos. Com base nos resultados, pretendemos estabelecer biomarcadores para estudos futuros de biomonitoramento de rios e ambientes aquáticos contaminados com este herbicida.

2. OBJETIVOS

2.1 Objetivo geral

Avaliar parâmetros bioquímicos após a exposição aguda a três formulações comerciais de glifosato e verificar as alterações causadas por cada formulação observada nos jundiás expostos.

2.2 Objetivos específicos

- Avaliar a possível peroxidação lipídica em órgãos de jundiás após exposição a diferentes concentrações das formulações comerciais de glifosato.

- Determinar a atividade da catalase e superóxido dismutase no fígado e a atividade da glutationa-S-transferase em fígado, cérebro, brânquias e músculo nos peixes expostos.

- Analisar parâmetros metabólicos como glicogênio, glicose, proteínas e lactato em fígado e músculo, além de glicose em cérebro e brânquias dos peixes expostos.

- Verificar as respostas metabólicas no plasma, assim como a medida de indicadores de dano hepático nos peixes expostos as diferentes formulações de glifosato.

3. MANUSCRITO

Avaliação de parâmetros bioquímicos em jundiás expostos a diferentes formulações comerciais contendo glifosato

Evaluation of biochemical parameters in silver catfish exposed the different commercial formulations containing glyphosate

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Evaluation of biochemical parameters in silver catfish exposed the different commercial formulations containing glyphosate

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ABSTRACT

The effects of the three glyphosate-based herbicides (Orium®, Roundup Original® and Biocarb®) were tested in *Rhamdia quelen* for 96 hours. Thiobarbituric acid reactive substances (TBARS), enzymatic activity and metabolic parameters were evaluated in several organs. The liver TBARS increased in all concentrations. Muscle TBARS decreased in both concentrations of Orium® and increased in 2.5 mg/L Biocarb®. Brain TBARS increased in all concentration with the exception of 5.0 mg/L Roundup Original®. Gills TBARS increased in 2.5 mg/L Orium® and 5.0 mg/L Roundup Original® and decreased in 5.0 mg/L Orium®. Superoxide dismutase increased in both concentrations of Orium®, 2.5 mg/L Roundup Original® and in 5.0 mg/L Biocarb®. Catalase decreased in all concentrations. Glutathione-S-transferase in the liver and gills decreased in 2.5 mg/L Biocarb®; in the brain it increased in both concentrations of Orium®. The plasma glucose levels increased in both concentrations of Orium® and Biocarb®. The lactate in plasma increased in 2.5 mg/L of all the tested herbicides. Alanine aminotransferase decreased in 2.5 mg/L Biocarb®. Aspartate aminotransferase increased in all concentrations with the exception of 2.5 mg/L Biocarb®, which decreased. The liver glycogen increased after the exposure. However, the glucose and lactate decreased in all concentrations. The liver protein levels increased in both concentrations of Orium® and decreased in 2.5 mg/L Biocarb®. The muscle glycogen increased in 2.5 mg/L Orium® and Biocarb® and in 5.0 mg/L of Roundup Original®. The glucose in the muscle decreased in 5.0 mg/L Orium® and Roundup Original®. The lactate in the muscle increased in 2.5 mg/L Orium® and decreased in both concentrations of Biocarb®. The muscle protein levels increased in both concentrations of Orium® and decreased in both concentrations of Original® and Biocarb®. The glucose in the brain and gills increased in all tested concentrations with the exception of 5.0 mg/L Biocarb® in the gills. The stress generated by glyphosate showed the different ways of adaptation or elimination of the xenobiotic.

Keywords: Biochemistry parameters; damage oxidative; glyphosate; *Rhamdia quelen*.

1. INTRODUCTION

Due to the increase in food production in the last years, the use of herbicides also increased considerably. This increase occurred by changes in farming practices and intensive agricultural production. The herbicide glyphosate is widely used around the world due to the cultivation of genetically modified crops that are tolerant to this pesticide; mainly the soybean (Giesy *et al.*, 2000). The soybean and rice culture use concentrations of glyphosate ranging from 0.36 to 2.16 mg/L, which is the recommended levels in southern Brazil (Rodrigues and Almeida, 2005). The glyphosate (*N*-Phosphonomethylglycine) is a non-selective and post-emergence herbicide. Manufactured initially by Monsanto as Roundup®, it contains the isopropylamine salt of glyphosate as active ingredient and polyoxyethylene amine (POEA) as surfactant, which increases the herbicide's efficiency. However, studies suggest that the surfactant is more toxic than the active ingredient (Tsui and Chu, 2003; 2008).

The toxicity of the glyphosate is considered low, according to WHO (1994), for aquatic animals, although different responses were recorded when using several commercial formulations of ghyphosate-based herbicides (Szarek *et al.*, 2003; Tsui and Chu, 2003). Previous studies showed various effects caused by glyphosate exposure in different fish species (Langiano and Martinez, 2008; Cattaneo *et al.*, 2011; Glusczak *et al.*, 2011; Menezes *et al.*, 2011). Despite glyphosate being widely used in Brazil, there are few studies comparing the effects of commercial formulations in native freshwater fish species. The silver catfish (*Rhamdia quelen*) is an endemic fish species in South America that can support cold winters and grows fast during summer. However many sites in which the silver catfish live are located near agricultural areas continuously receiving pesticides from the agricultural activities (Barcellos *et al.*, 2004; Soso *et al.*, 2007).

Glyphosate-based herbicides may induce the formation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and the hydroxyl radical (OH), which may cause an imbalance between pro-oxidant and antioxidant defense mechanisms, resulting in oxidative damage (Modesto and Martinez, 2010; Glusczak *et al.*, 2011). To neutralize the ROS, the fish has an antioxidant defense system that is constituted of antioxidant enzymes as superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST) (Modesto and Martinez, 2010; Menezes *et al.*, 2011). As a result of the oxidative damage, the lipid peroxidation indicates alterations in the cellular membrane, its cohesion, flow, permeability and metabolic function, leading to cellular instability with consequent damage and death. Usually the biochemical parameters are very sensitive to sub-

lethal concentration of glyphosate (Glusczak *et al.*, 2007; Langiano and Martinez, 2008; Menezes *et al.*, 2011).

However, the fish show characteristic response to a stressor that may be measured by the variation of enzyme activities and the metabolic parameters in the blood, liver, gills, brain and muscle (Fonseca *et al.*, 2008). For the present study, the general parameters (glucose, glycogen, lactate, protein, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), (SOD, CAT and GST) were chosen to evaluate a possible stress situation generated by glyphosate-based herbicides. Therefore, we intend to evaluate biochemical parameters after a 96 hour exposure to three commercial glyphosate formulations and verify the changes in silver catfish after such exposure to each formulation.

2. MATERIALS AND METHODS

2.1 Chemicals

Three commercial formulations in the Brazilian market is Orium®, Roundup Original® and Biocarb® of the herbicide glyphosate (N-phosphonomethyl) presented 48% the purity. 5,5′dithio-bis(2-nitrobenzoic acid) (DTNB), 1-chloro 2,4 dinhitrobenzene (CDNB), bovine serum albumin, Triton X-100, hydrogen peroxide (H_2O_2), malondialdehyde (MDA), 2-thiobarbituric acid (TBA), KOH, ethanol and trichloroacetic acid, were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2 Fish

Silver catfish (*Rhamdia quelen*) (12.0 ± 1.0 g and 9.0 ± 1.0 cm) of both sexes were obtained from the fish farm at the Federal University of Santa Maria (UFSM). The fish were acclimated in tanks (250 L) for 10 days in continuously aerated water in a static system and with a natural photoperiod (12 h light – 12 h dark). Water parameters were measured every day and were: temperature 24.5 ± 2.0 ºC, pH 6.8 ± 0.5 units, dissolved oxygen 8.0 ± 0.2 mg/L, nonionized ammonia 0.56 ± 0.02 µg/L, nitrite 0.06 ± 0.01 mg/L, alkalinity 22.0 ± 2.0. During acclimation, the fish were fed once a day with commercial fish pellets (42% crude protein, Supra, Brazil). Feces and pellets residues were removed by suction. The filter systems were used to maintain water quality.

2.3 Experimental design

After acclimation period fish were placed in 45 L boxes (duplicate, n=8 each) and distribute in group control (0.0 mg/L) and two exposure groups of each commercial formulation of glyphosate-based (2.5 and 5.0 mg/L). Acute toxicity assays were according to Antón *et al.,* (1994) for 96 h. The concentrations chosen in this experiment in previous studies of our laboratory (Glusczak *et al.,* 2006; 2011, Cattaneo *et al.,* 2011). The stock solution (300 mg/L) of herbicide was diluted in water to obtain the experimental concentration of 2.5 mg/L and 5.0 mg/L and then added only in the beginning of experiment in the boxes, without herbicide replacement. The concentration of the herbicide in water was monitored in the beginning and end of the experimental period analyzed by liquid chromatography with previous derivatization described by Hidalgo *et al.,* (2004). After experimental period, fish were sampled and blood, liver, brain, gills and muscle organs were collected.

2.4 Lipid peroxidation estimation

Lipid peroxidation was estimated by thiobarbituric acid-reactive substance (TBARS) production, in the liver, muscle, brain and gills, performed by a malondialdehyde (MDA) reaction with 2-thiobarbituric acid (TBA), which was optically measured, according to the method described in Buege and Aust, (1978). The methods were previously tested and the control values were in agreement with recent publication of our research group (Menezes *et al.,* 2011).

2.5 Antioxidant parameters

2.5.1 Superoxide dismutase and catalase

Hepatic SOD activity was performed based on inhibition of the radical superoxide reaction with adrenaline as described by Misra and Fridovich, (1972). Hepatic catalase activity was assayed by ultraviolet spectrophotometry (Nelson and Kiesow, 1972). All the methods were described in Menezes *et al.,* (2011).

2.5.2 Glutathione-S-transferase

The GST activity was assayed in liver, muscle, brain and gills of according Habig *et al.*, (1974), using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate. The method was described in detailed in Menezes *et al.,* (2011).

2.6 Metabolic parameters assays

Plasma glucose, protein, ALT and AST was measured with Bioclin testKit. Plasma was dissolved in 10% trichloroacetic acid (1:30 dilution) and lactate was estimated according to Harrower and Brown, (1972). Liver and muscle glycogen hydrolysis product were quantified using the methodology of Park and Johnson, (1949). Glucose determination in the liver, muscle, brain and gills was according Park and Johnson, (1949) and lactate levels according Harrower and Brown, (1972). The protein analysis in liver and muscle organs followed the method described by Lowry *et al*., (1952). The methods were PREVISOUSLY described in Glusczak *et al.*, (2006; 2007).

2.7 Protein determination

Protein was determined by the Comassie blue method using bovine serum albumin as standard. Absorbance of samples was measured at 595 nm (Bradford, 1976).

2.8 Statistical procedures

Statistical analyses were performed using a one-way analysis of variance (ANOVA). Means of the groups were compared by Tukey's test and expresses as means ± standard error. The value of $p \leq 0.05$ was considered statistically significant for all analyses (n=8).

3. RESULTS

The herbicide levels in the experimental water were measured at the beginning and at the end of the experimental period and demonstrated a decrease of about 15% – 16% of the initial concentration in all concentrations after 96 hours (Table 1). The TBARS levels in the liver of silver catfish exposed for 96 hours increased in all concentrations of glyphosate-based herbicides in comparison to the control group (Fig. 1A). In contrast, the TBARS levels in the

muscle decreased in both concentrations of Orium® and increased in 2.5 mg/L of Biocarb® compared to the control group (Fig. 1B). In the brain, the TBARS levels increased in all concentrations of herbicides tested with the exception of 5.0 mg/L of Roundup Original®, which did not change compared to the control group (Fig. 1C). In the gills, the TBARS levels increased in 2.5 mg/L of Orium® and 5.0 mg/L of Roundup Original®, whereas in 5.0 mg/L of Orium® the levels were decreased when compared to the control group (Fig. 1D). The SOD activity in the liver increased in both concentrations of Orium®, in 2.5 mg/L of Roundup Original® and 5.0 mg/L of Biocarb® compared to the control group (Fig. 2A). In addition, the CAT activity decreased in all concentrations of the tested glyphosate-based compared to the control group (Fig. 2B). The GST activity in the liver and the gills decreased in 2.5 mg/L of Biocarb® compared to the control group. In the muscle, the GST activity did not show significant change results in comparison to the control group. In the brain, an increase of the GST activity was observed in both concentrations of Orium® compared to control group (Table 2). The analyses of metabolic parameters demonstrated that in the plasma, the glucose levels increased in both concentrations of Orium® and Biocarb® when compared to control group. The lactate levels were increase in 2.5 mg/L of Orium®, Roundup Original® and Biocarb®. However, the protein levels in the plasma did not show significant changes compared to the control group. The ALT activity in the plasma decreased only in the 2.5 mg/L concentration of Biocarb® in comparison to the control group. On the other hand, the AST activity increased in all concentrations of glyphosate-based tested with the exception of 2.5 mg/L of Biocarb® which showed decreased of the levels (Table 3). In the liver, the glycogen demonstrated an increase in all concentrations of the three tested herbicides compared to the control group. The glucose and lactate levels decreased in all concentrations of the glyphosate-based when compared to the control group. The protein levels in the liver increased in both concentrations of Orium® and decreased in 2.5 mg/L of Biocarb®. In the muscle, the glycogen increased in the 2.5 mg/L concentration of Orium® and Biocarb® and 5.0 mg/L of Roundup Original® compared to the control group. The glucose levels decreased in 5.0 mg/L of Orium® and Biocarb® compared to the control group. The lactate levels in the muscle increased in 2.5 mg/L of Orium® and decreased in both concentrations of Biocarb® compared to the control group. In addition, the protein levels increased in both concentrations of Orium® and decreased in both concentrations of Roundup Original® and Biocarb®. The glucose levels in the brain and the gills increased in all concentrations of the glyphosate-based tested with the only exception being the gills in the 5.0 mg/L concentration of Biocarb® that did not change significantly when compared to the control group (Table 3).

4. DISCUSSION

Roundup® and/or glyphosate have been studied in fish as an inductor of oxidative stress. Herbicides can induce ROS, causing damage in unsaturated fatty acids leading the lipid peroxidation (Modesto and Martinez, 2010; Cattaneo *et al.*, 2011; Glusczak *et al.*, 2011). In our study, the silver catfish exposed to glyphosate for 96 hours showed an increase in the TBARS levels in the liver and brain with the tested herbicides. The increased TBARS levels in the liver were also observed by Crestani *et al.*, (2007) exposing *R. quelen* to clomazone in a different experimental period and by Glusczak *et al.*, (2011) when exposing *Leporinus obtusidens* to glyphosate for 96 hours. Cattaneo *et al.*, (2011) also showed increased TBARS levels in the brain, as was observed in this study, after exposing *C. carpio* to glyphosate for 96 hours. The liver is the main xenobiotics detoxifying organ and is particularly susceptible to oxidative damage. On the other hand, the brain is susceptible to lipid peroxidation due to low antioxidant defense and high concentration of polyunsaturated fatty acids in the cell membrane (Menezes *et al.*, 2011). The results of this study clearly demonstrated the lipid peroxidation occurring mainly in the liver and brain. However, muscle and gills showed different results in some concentrations, with higher and lower levels in the same tested organ. These results suggest that the fish have different responses to each commercial formulation of the glyphosate-based herbicide in an attempt to adapt to or to eliminate the xenobiotic.

The SOD–CAT system is the first line of defense against oxygen toxicity, due to the inhibitory effects on the formation of oxyradicals and these enzymes are frequently used as biomarkers, indicating the production of reactive oxygen species (ROS) (Pandey *et al.*, 2003). In this study, the SOD activity increased in both concentrations of Orium®, 2.5 mg/L, Roundup Original® and 5.0 mg/ of Biocarb®. The CAT activity was inhibited in all concentrations of the tested herbicides. The reduction in CAT activity may have occurred due to the superoxide ions, which probably were not neutralized efficiently by the SOD. Studies show that CAT activity inhibition occurs in fish exposed to pro-oxidant agents. This is ascribed to the increased SOD activity induced by the ROS and H_2O_2 production, indicating damage due to oxidative stress. In the present experimental model the protection of the organism against ROS by the SOD-CAT in the liver was not efficient. This result may be due to the increased TBARS levels registered in this organ.

The GST is an important enzyme involved in catalyzing the conjugation of a wide variety of electrophilic substrates to reduce glutathione. Furthermore, it protects the cell against the effects of xenobiotics. In the present study, the GST activity decreased in the liver and gills in the 2.5 mg/L concentration of Biocarb®. This decreased GST activity could suggest a failure of detoxification and the occurrence of oxidative damage. Thus the presence of oxidants may lead to the inactivation or inhibition of the enzymatic activity. On the other hand, in the brain, the GST increased in both concentrations of Orium®. The induction of GST in the fish's organs is considered beneficial to handle a stressful condition. These results show that both types of glyphosate-based herbicides can have different effects on the same species.

In the plasma, an increase in glucose levels was observed in both concentrations of Orium® and Biocarb® and an increase of lactate levels in 2.5 mg/L of the three tested products. This glucose response showed that a disorder in the metabolism of the exposed fish might have occurred. In addition to this, the lactate increase in the 2.5 mg/L concentrations of all tested products may be related to the fermentation metabolism. The results are in agreement with Cattaneo *et al.* (2012), after the exposure of *C. carpio* to clomazone for a different experimental period. The ALT and AST are liver-specific enzymes and an indicative of liver damage or hepatotoxicity. The changes in transaminase activity can be assessed within a shorter time through the plasma (Ferreira *et al.,* 2010). In this study, the ALT activity was decreased only in 2.5 mg/L of Biocarb®. On the other hand, the AST increased in all tested concentrations with the exception of 2.5 mg/L of Biocarb® which was decreased. Similar results were described by Crestani *et al.,* (2006) exposing *R. quelen* to clomazone, demonstrating an elevation in the AST after 96 hours, suggesting damage to the hepatic cells. This elevation of transaminases in plasma may be due to intracellular lesion in the liver that releases enzymes specific to the plasma. However, in 2.5 mg/L of Biocarb® a reduction in ALT and AST was observed. The decrease in the activity of ALT and AST in the plasma may be due to a deficiency of available amino acids, leading to a decrease of alpha keto acids and a consequential decrease in the activity of ALT and AST (Jurss and Bastrop, 1995).

Different classes of environmental pollutants or their metabolites can change the metabolic state of the liver trough the occurrence of the detoxication process. In this study, the liver showed an increase in glycogen and a decrease of the lactate in all concentrations of the tested herbicides. The increased glycogen suggests an elevation of the glycogen stores by increasing the synthesis of these reserves, this affirmation is in accord to Cattaneo *et al.*, (2012) exposing *C. carpio* to clomazone for a different experimental period. Additionally, the lactate reduction in the liver suggests sufficient energy supply where the lactate is consumed

to produce glucose. The protein in the liver showed an increase in both concentrations of Orium® and a decrease in 2.5 mg/L of Biocarb®. However, with this response we can believe that a disorder in the energetic metabolism occurred in the concentrations of Orium®. This affirmation is attributed to the decreased levels of lactate, whereas the glycogen and protein levels are elevated. On the other hand, the protein levels in the liver with Biocarb® suggest that the energy for glycogen synthesis may be due to the catabolism of proteins that occurs in the liver. Analyzing these controversial results, we can observe that the fish exposed to herbicides respond in different ways in order to maintain homeostasis.

In the muscle, the glycogen levels showed an increase in 2.5 mg/L of Orium® and Biocarb® and 5.0 mg/L of Roundup Original®. This indicates that the fish may be synthesizing energy as glycogen. The levels of lactate and protein showed an increase in 2.5 mg/L of Orium® and a decrease in both concentrations of Biocarb®. In 2.5 mg/L of Orium® there is a clear disorder in the metabolism due to the increase of glycogen, lactate and protein which would be three energy sources. The reduction in the protein levels of the exposed fish suggests a hypoproteinemia with a disturbance in the osmoregulation (Glusczak *et al.*, 2006). However in both concentrations of Roundup Original® and Biocarb® the glucose is probably being maintained by the protein catabolism. The glucose in liver and muscle showed a reduction in some concentrations, suggesting that the levels were maintained in organs with intense circulation, such as the gills, and which use glucose as a main energy source to the brain. Thus, this hypothesis is confirmed with the increased glucose in these organs.

From these results, we can conclude that different commercial formulations containing glyphosate cause oxidative damage and metabolism disorder. Furthermore, we can observe that formulations with same active ingredient have various affecting pathways and cause different changes in the organism of the silver catfish as an attempt to maintain homeostasis.

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LEGENDS

Fig. 1 TBARS levels in the liver (A), muscle (B), brain (C) and gills (D), of silver catfish exposed to three commercial herbicide glyphosate-based the 2.5 and 5.0 mg/L. *Significant different to control group ($p \leq 0.05$) ($n = 8$)

Fig. 2 SOD activity (A) and CAT activity (B) in liver of silver catfish exposed to commercial herbicide glyphosate-based the 2.5 and 5.0 mg/L. *Significant different to control group ($p \leq 0.05$) ($n = 8$).

Table 1. Concentrations of the glyphosate-based herbicides (mg/L) in water samples. Indicating the loss of the concentration after 96 h.

mg/L	Orium®	Roundup Original®	Biocarb®
2.5	15.0%	16.85%	16.67%
5.0	16.75%	15.57%	15.55%

Tab. 2 GST in liver, muscle, brain and gills of silver catfish exposed the three formulation commercial glyphosate-based for 96h.

		Orium®		**Roundup Original®**		**Biocarb®**	
	0.0 mg/L	**2.5 mg/L**	**5.0 mg/L**	**2.5 mg/L**	**5.0 mg/L**	**2.5 mg/L**	**5.0 mg/L**
Liver	0.39±0.05	0.57±0.03	0.34±0.02	0.31±0.02	0.29±0.01	0.17±0.01*	0.50±0.07
Muscle	0.18±0.02	0.19±0.02	0.17±0.02	0.20±0.02	0.21±0.04	0.23±0.02	0.19±0.01
Brain	0.14±0.01	0.25±0.01*	0.29±0.03*	0.21±0.01	0.15±0.01	0.17±0.01	0.15±0.01
Gills	0.30±0.01	0.27±0.02	0.25±0.02	0.23±0.01	0.33±0.01	0.10±0.01*	0.23±0.01

GST was expressed in (µmol GS-DNB/min/mg protein). Data represent the mean ± SEM. * Indicate significant different from control ($p \leq 0.05$) (n = 8).

Table 3. Metabolites in plasma, liver, muscle, brain and gills of silver catfish exposed to commercial formulation glyphosate-based by 96 h. Data represent the mean ± SEM. *Indicate significant different from control ($p \leq 0.05$) (n = 8).

		Orium®		Roundup Original®		Biocarb®	
	0.0 mg/L	**2.5 mg/L**	**5.0 mg/L**	**2.5 mg/L**	**5.0 mg/L**	**2.5 mg/L**	**5.0 mg/L**
Plasma							
Glucose	39.13±0.68	49.77±0.26*	51.70±0.21*	39.64±0.80	40.07±1.00	57.66±0.57*	46.90±0.26*
Lactate	1.46±0.05	2.53±0.07*	1.65±0.12	3.54±0.07*	1.51±0.03	3.08±0.13*	1.54±0.11
Protein	2.42±0.17	2.26±0.36	2.41±0.20	1.82±0.14	2.65±0.10	2.98±0.08	2.60±0.08
ALT	26.40±1.32	32.17±1.42	23.91±1.36	31.47±1.05	24.11±0.11	5.71±1.07*	25.60±1.45
AST	75.23±0.78	106.04±1.41*	85.80±0.95*	112.13±1.37*	89.40±0.15*	68.08±1.25*	108.77±0.59*
Liver							
Glycogen	81.89±0.75	95.37±0.99*	92.44±0.88*	100.98±0.88*	98.13±0.95*	105.82±0.77*	97.77±0.81*
Glucose	14.17±0.05	12.29±0.03*	11.36±0.20*	12.86±0.35*	11.74±0.16*	10.43±0.13*	6.97±0.08*
Lactate	5.80±0.02	4.49±0.05*	4.61±0.02*	4.39±0.07*	4.08±0.03*	3.88±0.02*	4.80±0.03*
Protein	108.62±1.71	130.62±2.09*	130.75±2.07*	99.75±1.37	99.62±1.90	45.31±1.95*	116.95±1.11
Muscle							
Glycogen	5.22±0.20	6.73±0.30*	5.12±0.23	5.02±0.31	6.98±0.35*	7.82±0.25*	4.53±0.06
Glucose	1.76±0.04	1.65±0.03	1.38±0.03*	1.44±0.07	1.23±0.07*	1.60±0.08	1.50±0.02
Lactate	12.89±0.16	14.82±0.13*	13.44±0.24	13.99±0.33	12.44±0.41	9.86±0.26*	11.10±0.21*
Protein	276.08±1.82	289.16±0.90*	283.33±1.26*	238.25±1.57*	245.70±0.74*	263.36±0.47*	256.75±0.46*
Brain							
Glucose	18.04±0.21	31.57±0.36*	23.79±0.37*	28.69±0.20*	24.65±0.30*	25.95±0.57*	34.06±0.23*
Gills							
Glucose	17.64±0.38	30.86±0.68*	25.49±0.37*	30.09±0.52*	25.05±0.44*	26.65±0.80*	20.24±0.45

Glucose (tissue and plasma), ALT and AST was expressed in mg/dL. Glycogen and lactate in tissue was expressed in µmol/protein. Plasma lactate was expressed in µmol/mL. Data represent the mean ± SEM. * Indicate significant different from control ($p \leq 0.05$) (n = 8).

FIGURES

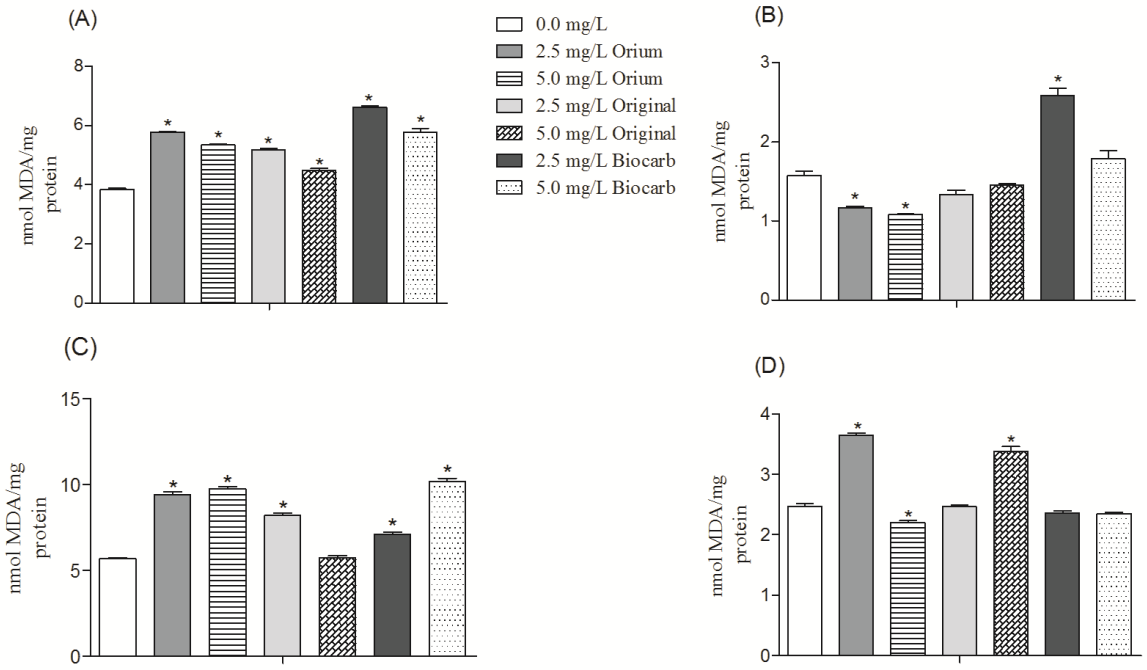

Figure 1

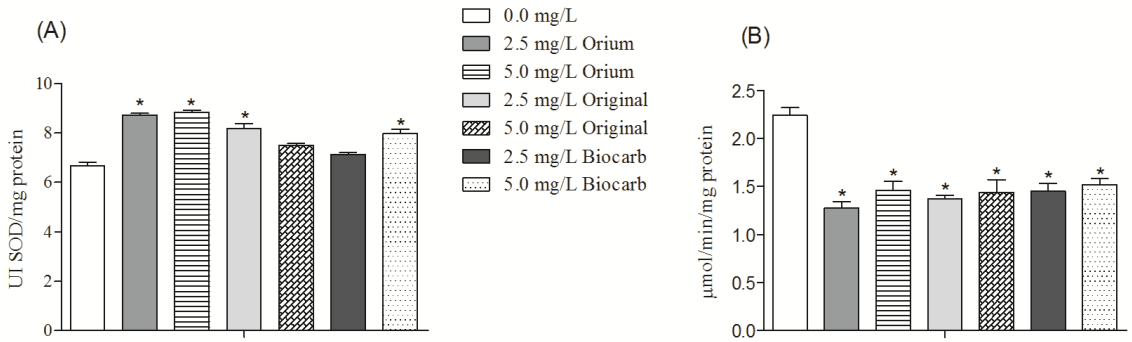
(A)
UI SOD/mg protein
0
2
4
6
8
10
0.0 mg/L
2.5 mg/L Orium
5.0 mg/L Orium
2.5 mg/L Original
5.0 mg/L Original
2.5 mg/L Biocarb
5.0 mg/L Biocarb
(B)
µmol/min/mg protein
0.0
0.5
1.0
1.5
2.0
2.5

Figure 2

4. CONCLUSÕES

- O aumento da peroxidação lipídica e a alteração observada no sistema antioxidante enzimático, dos jundiás expostos as formulações comerciais de glifosato, indicam que estas formulações são prejudiciais aos peixes que entram em contato com estes herbicidas.

- A ativação do sistema da glutationa-S-transferase em ambas as concentrações testadas de Orium® mostrou que os jundiás expostos reagiram de forma positiva na tentativa de eliminar o herbicida no cérebro.

- Os danos hepáticos causados em ambas as concentrações de Orium®, Roundup Original® e em 5,0 mg/L de Biocarb® observados pelas avaliação das transaminases pode ser prejudicial aos jundiás por danificar o fígado. Contudo os peixes expostos à 2,5 mg/L de Biocarb® não demonstraram dano hepático avaliado por estas enzimas.

- A regulação do metabolismo energético no músculo dos peixes expostos a formulação de 2,5 mg/L de Orium® foi danificado pela elevação das fontes energéticas em concomitância como o glicogênio, lactato e proteína.

- Para manter a energia durante a exposição os peixes expostos mantiveram a glicose no plasma, cérebro e brânquias de forma positiva pois estes órgãos utilizam desta como fonte prioritária.

- Considerando os dados metabólicos pode-se concluir que a concentração de 2,5 mg/L de Biocarb® foi a que respondeu de maneira distinta em relação as demais, por demonstrar resultados opostos ou alterados quando as demais se mantiveram constante.

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